



BIOGENIC AMINES AND NEUROMODULATION OF ANIMAL BEHAVIOR, 2nd Edition

EDITED BY: Irina T. Sinakevitch, Gabriella H. Wolff, Hans-Joachim Pflüger
and Brian H. Smith

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BIOGENIC AMINES AND NEUROMODULATION OF ANIMAL BEHAVIOR, 2nd Edition

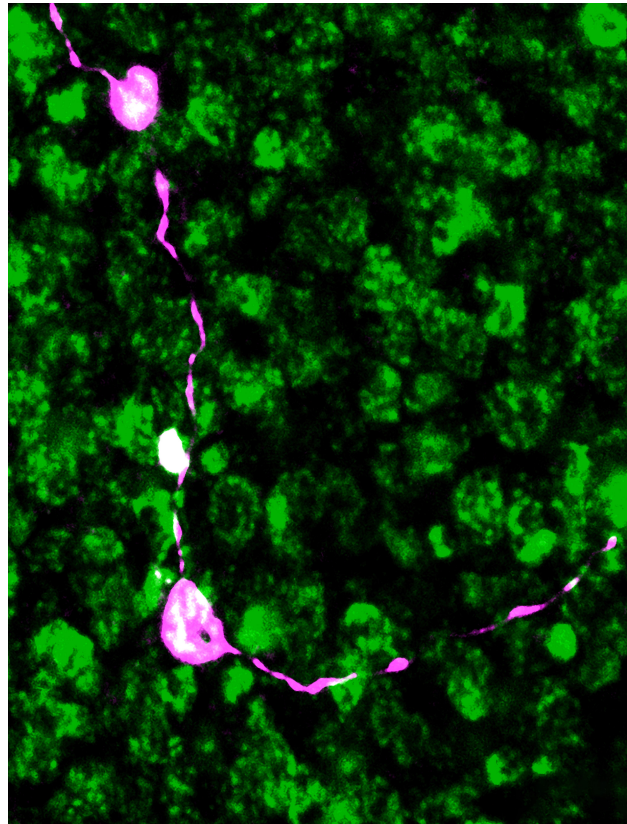
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Confocal image of VUMx neuron synaptic boutons (magenta) in the synaptic microglomeruli (green) of an *Apis mellifera* mushroom body calyx (lip). Anti-synapsin labeled all pre-synaptic sites (green). Contralateral injection of ruby red filled the VUMx axon terminals. The anti-synapsin is co-localized within VUMx bouton terminals (white in the merged image).

Image: Irina T. Sinakevitch

Since Erspamer and Boretti (1951) first described the biogenic amine octopamine in the octopus salivary gland as a molecule with “adrenaline-like” action, decades of extensive studies demonstrated the important role octopamine and its precursor tyramine play in invertebrate physiology and behavior. This book contains the latest original research papers on tyramine/octopamine and their receptors in different neuronal and non-neuronal circuits of insects.

Additionally, this book elucidates in detail the latest research on the function of other biogenic amines and their receptors, such as dopamine and serotonin in insects and mice. The reviews in this book summarize the most recent research on the role of

biogenic amines in insect antennae, synaptic development, and behavioral modulation by spontaneous dopamine release in *Drosophila*. Finally, one perspective paper discusses the evolution of social behavior and biogenic amines.

We recommend this book for all scholars interested in the latest advanced research on the role of biogenic amines in animal behavior.

ITS dedicates the topic to her teacher, Plotnikova Svetlana Ivanovna (1922-2013).

Reference:

Arch Int Pharmacodyn Ther. 1951 Dec;88(3):296-332.

Identification and characterization, by paper chromatography, of enteramine, octopamine, tyramine, histamine and allied substances in extracts of posterior salivary glands of octopoda and in other tissue extracts of vertebrates and invertebrates.

ERSPAMER V, BORETTI G.

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Editorial: Biogenic Amines and Neuromodulation of Animal Behavior

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Editorial on the Research Topic

Biogenic Amines and Neuromodulation of Animal Behavior

Neuropeptides and biogenic amines are important modulators in all nervous systems. Rather than having a specific “point-to-point” function, which is characteristic for classical neurotransmitters released from a presynaptic neuron onto a clearly defined postsynaptic neuron and usually associated with fast transmission in the millisecond range mediated by ionotropic receptor molecules, biogenic amines often act as neuromodulators having long lasting actions up to the second range always mediated by metabotropic receptor molecules (G-proteins) and different cellular signaling pathways. However, a particular chemical substance can either act as a fast neurotransmitter via ionotropic postsynaptic receptors or as a slow but long lasting neuromodulator via metabotropic postsynaptic receptor molecules. Therefore, substances like dopamine, serotonin or even tyramine and octopamine can act as fast neurotransmitters or slow neuromodulators. As such biogenic amines significantly change the efficacy of pre- to post-synaptic connections by affecting the cellular and biochemical properties of neurons. In this respect, neuromodulators are chemical substrate underlying plasticity in all nervous systems. Their range of action goes beyond affecting only the nervous system. Neuromodulators orchestrate a plethora of neuronal and physiological processes that together may serve a particular behavioral context or a specific physiological condition, and reciprocal interactions between the nervous system and metabolic or physiological states in non-nervous tissues are widely accepted as a new research focus. Neuropeptides like biogenic amines are released either in neuropiles of the central nervous system or in the peripheral nervous system.

Total nineteen articles (3 reviews, one perspective, and 15 originals) of this particular issue cover a broad range of topics related to biogenic amines. The first review by Ichinose et al. discusses the role of spontaneously firing dopaminergic neurons in the fruit fly brain and how they reflect the behavioral/internal state of the animal. Dopamine can have both roles, a fast neurotransmitter, a slow neuromodulator, depending on the receptor types of the postsynaptic neurons. Genetic manipulation of the activity of dopamine neurons resulted in changes to the behavioral state of the fly. Behaviors affected were sleep, sexual drive, hunger, and learning and memory. The second review article by Vonhoff and Keshishian discusses how during the development of neuromuscular connections in the fruit fly an interaction between the glutamatergic type I terminals of motoneurons and octopaminergic type II terminals of neuromodulatory neurons may be significant. Also, the growth cones of motoneurons respond to signals from partners via low-frequency calcium waves that may be crucial for regulating temporary and final connections. In the third review, Zhukovskaya and Polyanovsky studied the effects of various amines such as octopamine, tyramine, dopamine, and serotonin on olfactory and gustatory receptor neurons in insect antennae. Many amines are systemically released into haemolymph, an open circulatory system in insects supplies different body compartments, the dorsal or ventral cavity, the muscles,

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the central nervous system, etc. The authors suggest that the antenna may be a partially autonomous haemolymph compartment separated from other body parts.

An important aspect of chemical signaling is that all transmitters, whether they be classical transmitters, neuromodulators or hormones, can only act through their respective receptor molecules. Depending on which receptor type is activated, different signaling cascades can be triggered. In this issue, a number of articles are devoted to receptors:

Blenau et al. report on a fifth serotonin receptor in fruit flies - Dm5-HT2B - in addition to the three Dm5-HT1a, Dm5-HT1B, and Dm5-HT7 coupled to the cAMP signaling cascades and Dm5-HT2A leading to Ca²⁺ signaling through ITP. This fifth receptor is involved in controlling heartbeat and immune system function, and it can be antagonized by metoclopramide and mianserin.

Bridging to vertebrates, Der-Ghazarian et al. describe in mice a 5-HT1B receptor agonist—CP94253—which affects spontaneous and cocaine-induced locomotion and conditioned place preference. Aminergic receptors play key role in the development of addiction, and this study provides evidence that 5-HT1BR agonists may be used for anti-cocaine medications.

Aranda et al. report that in fruit flies an interesting mixed G-protein coupled receptor—DopEcR—which binds both dopamine and ecdysone and mediates ethanol-induced courtship sensitization. DopEcR immunoreactivity was observed in the mushroom body calyx and lobes, and in mutant DopEcR males, the sensitization phenotype was fully rescued by restoring DopEcR expression in mushroom body $\alpha\beta$ and γ neurons.

The distribution of immunoreactivity for tyramine and one of its receptor molecules—AmTyr1—is described by Sinakevitch et al. for the honey bee brain and particular emphasis is placed on neuropils associated with olfactory learning and memory. They focus on two Ventral Unpaired Median neurons of the gnathal (suboesophageal) ganglion whose axons ascend to the brain and innervate the antennal lobe and mushroom body calyx. Interestingly, AmTyr1 expression was found in the presynaptic sites of olfactory receptor neurons and of uniglomerular projection neurons, most likely to exert inhibitory control of neurotransmitter release.

There is accumulating evidence that tyramine and octopamine exert opposite actions in insects. Ryglewski et al. in their study on fruit fly flight behavior, examine the role of tyramine and an enzyme for tyramine catabolism—dehydrogenase/reductase Naz (Nazgul). Naz is found in a particular group of glial cells that are located along the motor neuropil border and with extensions into the flight motor neuropil. If this enzyme is knocked down by RNAi, flight durations are reduced, which is typical for blocked octopamine and high tyramine levels. This article also discusses interesting pathways of tyramine signaling.

Biogenic amines are also involved in orchestrating responses associated with metabolic processes such as starvation. When starved tyrosine- β -hydroxylase mutant fruit flies, which cannot synthesize octopamine, possess higher levels of glucose in their haemolymph than controls, as shown in the study by Damrau

et al.. The article also reviews on the different receptor types of tyramine and octopamine that may be involved in energy homeostasis.

Most neurons that release octopamine belong to the class of dorsal unpaired median neurons, and their electrical properties have been extensively studied in cockroaches by Lapied et al.. They show that pacemaker activity of these neurons is facilitated by a tetrodotoxin-sensitive-low-voltage-activated channel permeable to sodium and calcium and regulated by a cAMP/PKA-cascade. Phospho-DARPP-32 strongly decreased this current and involved in regulating sodium/calcium-currents and contributing to pacemaker activity.

Amine malfunctions are often the causes of severe pathologies, such as Parkinson's disease. Niens et al. show that in the fruit fly, imbalances between dopamine and serotonin can be modeled. Like in rodents, a lack of dopamine leads to increased levels of 5-HT and arborizations in specific brain neuropils. Conversely, increased dopamine levels lead to the reduced connectivity of 5-HT neurons. This suggests that in Parkinson's disease, both dopamine and 5-HT play an important role.

Dopamine signaling is essential for mediating reinforcing properties of unconditioned stimuli during associative learning. Tedjakumala et al. characterize dopaminergic neurons in the honeybee brain by immunoreactivity distribution of the dopamine precursor enzyme, tyrosine-hydroxylase. They also describe new clusters of dopaminergic neurons.

In many social insects, like ants and bees, biogenic amines play functional roles in the control of sociality. How biogenic amines and their receptors in ancestral, solitary species have been co-opted during evolution to control behaviors in socially complex species is addressed by Kamhi et al..

Li et al. studied fat deposition or starvation resistance using flies defective in the expression of receptors for octopamine and tyramine. Their tissue-specific RNAi experiments revealed a very complex interorgan communication leading to the different metabolic phenotypes in octopamine- and tyramine-deficient fruit flies.

Sitaraman et al. described discrete neuronal circuits that mediate aversive reinforcement, escape latencies, and memory levels after place learning in the presence and absence of unexpected aversive events. The results show that two features of learned helplessness depend on the same modulatory system as aversive reinforcement. Moreover, aversive reinforcement and escape latency changes depend on local neural circuit modulation, while memory enhancement requires modulation of multiple behavioral control circuits.

Stocker et al. compared the axon terminals of octopaminergic efferent dorsal and ventral unpaired median neurons in either desert locusts or fruit flies across skeletal muscles, revealing many similarities. These type II terminals are immunopositive for both tyramine and octopamine and, in contrast to the type I terminals, which possess clear synaptic vesicles, they only consist of dense core vesicles. They discovered that starvation changes the morphology of the neuromuscular branches in a time-dependent manner. Besides, the authors provide evidence that the release of octopamine from dendritic

and/or axonal type II terminals uses similar synaptic machinery to glutamate release from type I terminals of excitatory motor neurons.

Scheiner et al. investigated the role of the fat body in modulating gustatory responsiveness through tyramine signaling in different behavioral castes of honeybees. Their work suggests that differential tyramine signaling in the fat body has an essential role in the plasticity of division of labor through changing gustatory responsiveness.

Sun et al. studied startle-induced locomotion and the activity of specific clusters of dopaminergic neurons afferent to the mushroom bodies. Their study contributes to an emerging picture of the brain circuits modulating locomotor reactivity in fruit flies that appear to both overlap and differ from those mediating associative learning and memory, sleep/wake state and stress-induced hyperactivity.

Buckemüller et al. investigated alterations of haemolymph glucose concentration, survival, and feeding behaviors after starvation and examined the impact of octopamine on these processes in pharmacological experiments. Their experiments demonstrated that octopamine in honey bees acts similarly to adrenalin and noradrenalin in mammals in regulating an animal's counter-regulatory response.

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Dm5-HT_{2B}: Pharmacological Characterization of the Fifth Serotonin Receptor Subtype of *Drosophila melanogaster*

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Serotonin (5-hydroxytryptamine, 5-HT) is an important regulator of physiological and behavioral processes in both protostomes (e.g., insects) and deuterostomes (e.g., mammals). In insects, serotonin has been found to modulate the heart rate and to control secretory processes, development, circadian rhythms, aggressive behavior, as well as to contribute to learning and memory. Serotonin exerts its activity by binding to and activating specific membrane receptors. The clear majority of these receptors belong to the superfamily of G-protein-coupled receptors. In *Drosophila melanogaster*, a total of five genes have been identified coding for 5-HT receptors. From this family of proteins, four have been pharmacologically examined in greater detail, so far. While Dm5-HT_{1A}, Dm5-HT_{1B}, and Dm5-HT₇ couple to cAMP signaling cascades, the Dm5-HT_{2A} receptor leads to Ca²⁺ signaling in an inositol-1,4,5-trisphosphate-dependent manner. Based on sequence similarity to homologous genes in other insects, a fifth *D. melanogaster* gene was uncovered coding for a Dm5-HT_{2B} receptor. Knowledge about this receptor's pharmacological properties is very limited. This is quite surprising because Dm5-HT_{2B} has been attributed to distinct physiological functions based on genetic interference with its gene expression. Mutations were described reducing the response of the larval heart to 5-HT, and specific knockdown of Dm5-HT_{2B} mRNA in hemocytes resulted in a higher susceptibility of the flies to bacterial infection. To gain deeper understanding of Dm5-HT_{2B}'s pharmacology, we evaluated the receptor's response to a series of established 5-HT receptor agonists and antagonists in a functional cell-based assay. Metoclopramide and mianserin were identified as two potent antagonists that may allow pharmacological interference with Dm5-HT_{2B} signaling *in vitro* and *in vivo*.

Keywords: biogenic amine, Ca²⁺, cAMP, cellular signaling, insect, G protein-coupled receptor, inositol-1,4,5-trisphosphate, second messenger

INTRODUCTION

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is an ancient neuroactive substance and present throughout the animal kingdom. Serotonin plays a key role in regulating and modulating many physiological and behavioral processes in both protostomes and deuterostomes. In humans, malfunction of the serotonergic system has been associated with several impairments and diseases, such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive-compulsive disorder (for reviews, Green, 2006; Geyer and Vollenweider, 2008; Berger et al., 2009).

To gain insight into serotonergic function(s), insects are highly attractive models. In comparison with vertebrates and especially mammals, they allow assessing the anatomical distribution, development, and neurophysiological properties of serotonergic neurons with unprecedented inter-individual reproducibility and precision. Applying this experimental strategy, the activity of serotonergic neurons has been related to physiological functions and changes in behavior (for reviews, Walz et al., 2006; Blenau and Thamm, 2011; Ellen and Mercer, 2012; Nall and Sehgal, 2014; Vleugels et al., 2015). In fruit flies (*Drosophila melanogaster*), certain behavioral effects have been ascribed to the serotonergic system. In *D. melanogaster* larvae, serotonin modulates the heart rate (Dasari and Cooper, 2006) and is involved in olfactory processing (Python and Stocker, 2002), feeding behavior (Neckameyer et al., 2007; Neckameyer, 2010), locomotion (Majeed et al., 2016), and responses to light (Rodriguez Moncalvo and Campos, 2009). In adult flies, serotonergic neurons participate in the regulation of nutrient balance (Vargas et al., 2010; Ro et al., 2016), insulin signaling and organismal growth (Kaplan et al., 2008; Luo et al., 2012, 2014), locomotion (Neckameyer et al., 2007; Majeed et al., 2016), olfactory processing (Dacks et al., 2009), aggression (Dierick and Greenspan, 2007; Alekseyenko et al., 2010, 2014; Alekseyenko and Kravitz, 2014), circadian rhythm (Yuan et al., 2005), sleep (Yuan et al., 2006), courtship and mating behavior (Becnel et al., 2011), and learning (Sitaraman et al., 2008, 2012; Lee et al., 2011).

The diverse cellular and behavioral effects of serotonin in insects are mediated by a family of G protein-coupled receptors (GPCRs). So far, four 5-HT receptor subtypes have been pharmacologically characterized in *D. melanogaster*. These are Dm5-HT_{1A} and Dm5-HT_{1B} (CG16720 and CG15113; Saudou et al., 1992), Dm5-HT_{2A} (CG1056; Colas et al., 1995), and Dm5-HT₇ (CG12073; Witz et al., 1990). These GPCRs are assumed to be orthologs of mammalian 5-HT_{1A}, 5-HT₂, and 5-HT₇ receptors. Due to sequence homology with a 5-HT₂ receptor in the spiny lobster, *Panulirus interruptus*, a second 5-HT₂ receptor gene (CG42796) has been postulated in *D. melanogaster* (Clark et al., 2004). This observation was corroborated by independent bioinformatics studies (Hauser et al., 2006; Blenau and Thamm, 2011) and was substantiated experimentally by molecular cloning (Gasque et al., 2013). The receptor was named Dm5-HT_{2B}. Although orthologous receptors have been characterized in other insects as well, e.g., the honeybee *Apis mellifera* (Thamm et al., 2013) and the kissing bug *Rhodnius prolixus* (Paluzzi et al., 2015), knowledge about the pharmacological properties of Dm5-HT_{2B}

is rather limited. This is quite surprising because Dm5-HT_{2B} has been attributed to distinct physiological functions. For example, Dm5-HT_{2B} receptor mutations reduce the response of the larval heart to 5-HT (Majeed et al., 2014). Furthermore, knockdown of Dm5-HT_{2B} gene expression by RNAi in hemocytes caused reduced phagocytotic clearance and thus resulted in a higher susceptibility of the flies to bacterial infection (Qi et al., 2016). At the behavioral level, it has been uncovered that reducing the level of Dm5-HT_{2B} expression by either RNAi or transposon insertion into the gene locus leads to a decrease in anxiety-like behavior (Mohammad et al., 2016).

The aim of the current study was to focus on the pharmacological properties of the Dm5-HT_{2B} receptor. The cDNA encoding Dm5-HT_{2B} was amplified on mRNA extracted from *D. melanogaster* heads. A cell line was established constitutively expressing Dm5-HT_{2B}. Since 5-HT_{2B} receptors are known to cause inositol-1,4,5-trisphosphate (IP₃)-mediated Ca²⁺ release from intracellular stores, we examined Dm5-HT_{2B} functionality by Ca²⁺ fluorimetry. The receptor's pharmacological profile was established after applying concentration series of various agonists and antagonists. In addition to serotonin as the native ligand, 5-methoxytryptamine and 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) were very potent agonists. Receptor activity was efficiently blocked by metoclopramide and mianserin. Thus, this study provides important new data regarding the pharmacological characteristics of the fifth 5-HT receptor of the fruit fly.

MATERIALS AND METHODS

Cloning of the *Dm5-HT_{2B}* cDNA

Poly(A)⁺ RNA was prepared from 180 heads of male flies (*D. melanogaster*, *w¹¹¹⁸*) by using the Micro-Fast Track™ 2.0 Kit (Invitrogen, Karlsruhe, Germany). Synthesis of cDNA employed the AccuScript™ High Fidelity First Strand cDNA Synthesis Kit (Stratagene, Amsterdam, Netherlands). For amplification of the entire coding region of *Dm5-HT_{2B}*, specific primers were designed based on available sequence information (Brody and Cravchik, 2000; Clark et al., 2004; Hauser et al., 2006; Gasque et al., 2013): sense primer 5'-CAGAGTAGAGCGCACAATAGG-3' (position -35 to -15); antisense primer 5'-GTTTGCCCGGTTTAACG-3' (position 2724 to 2740; TIB Molbiol, Berlin, Germany). The polymerase chain reaction (PCR) was carried out for 30 s at 98°C (1 cycle) followed by 35 cycles of 10 s at 98°C, 30 s at 62°C, 90 s at 72°C, and a final extension of 10 min at 72°C. The reaction was performed with Phusion® High Fidelity DNA Polymerase (New England Biolabs, Frankfurt am Main, Germany). PCR products were cloned into pGEM-T vector (Promega, Mannheim, Germany) and subsequently sequenced (GATC Biotech AG, Konstanz, Germany).

Multiple Sequence Alignments and Phylogenetic Analysis

Amino-acid sequences used for phylogenetic analysis were identified by protein-protein Basic Local Alignment Search Tool (BLAST) searches of the National Center for Biotechnology

Information (NCBI) database with the deduced amino acid sequence of *Dm5-HT_{2B}* as “bait.” Values for identity (ID) and similarity (S) were calculated by using the BLOSUM62 substitution matrix in BioEdit 7.1.9. Phylogenetic analysis was conducted as described by Reim et al. (2017) using Bayesian analysis (MrBayes v.3.2.6; Ronquist et al., 2012) with the substitution model LG +G, determined by Prottest 3.4.2 (Darriba et al., 2011). Human rhodopsin (HsRHOD) and *D. melanogaster* FMRFamide receptor (DmFMRFaR) sequences were used to root the phylogenetic tree.

Construction of Expression Vectors

An expression-ready construct of *Dm5-HT_{2B}* was generated in pcDNA3.1 vector (Invitrogen/ThermoFisher Scientific, Darmstadt, Germany). PCR was performed with specific primers (sense primer: 5'-AATAAGCTTCCACCATGGAAGAGGATGTGTATGCC-3'; antisense primer first-round PCR: 5'-TGGGACGTCGTATGGGTATCTGCTCGGTCGCCAGG-3'; antisense primer second-round PCR: 5'-TTTTCTAGACTC GAGTTAAGCGTAGTCTGGGACGTCGTATGGGTA-3'). PCR products were digested with HindIII and XhoI, and subcloned into pcDNA3.1(+) vector (Invitrogen). Thus, the resulting construct contained the Kozak consensus motif (CCACC, Kozak, 1984) immediately 5' to the ATG-codon and a hemagglutinin A (HA) epitope tag (amino acid sequence: YPYDVPDYA) at the 3' end of the *Dm5-HT_{2B}* cDNA and was named pcDm5-HT_{2B}-HA. The insert fragment was checked by DNA sequencing.

Functional Expression in Mammalian Cell Lines

Approximately 8 µg of pcDm5-HT_{2B}-HA was transfected into exponentially growing HEK 293 cells (~4 × 10⁵ cells per 5-cm Petri dish) by a modified calcium phosphate method (Chen and Okayama, 1987). Stably transfected cells were selected in the presence of the antibiotic G418 (0.8 mg/ml). Isolated foci were propagated and analyzed for the expression of Dm5-HT_{2B}-HA receptor either by immunocytochemistry, Western blotting or by functional Ca²⁺ imaging upon receptor activation.

Functional and Pharmacological Characterization of Dm5-HT_{2B}

The ability of the Dm5-HT_{2B}-HA receptor (hereafter referred to as Dm5-HT_{2B}) to activate G_q proteins was assessed by monitoring changes in [Ca²⁺]_i with the Ca²⁺-sensitive fluorescent dye Fluo-4 (Invitrogen). Non-transfected HEK 293 cells and cells expressing Dm5-HT_{2B} were grown in minimal essential medium (MEM + GlutaMAXTM I (Gibco/ThermoFisher Scientific, Darmstadt, Germany) containing 2% (w/v) UtroserTM G (Pall, Dreieich, Germany), 1 × non-essential amino acids and 1 × antibiotics/antimycotics) in 96-well plates to a density of ~3 × 10⁴ cells per well. In this format, each vertical row (=8 wells) of the 96-well plate is incubated with the same ligand concentration. Cells were loaded at room temperature with Fluo-4 as described earlier (Thamm et al., 2013; Blankenburg et al., 2015) in extracellular solution [ES = in mM: 120 NaCl, 5 KCl, 2

MgCl₂, 2 CaCl₂, 10 HEPES, 10 Glucose, pH 7.4 (NaOH)]. Plates were transferred into a fluorescence reader (FLUOstar Galaxy/Optima; BMG Labtech, Offenburg, Germany) to monitor Fluo-4 fluorescence. The excitation wavelength was 485 nm, and fluorescence emission was detected at 520 nm. Various concentrations of biogenic amines and synthetic receptor ligands were added, once Fluo-4 fluorescence had reached a stable value in each well. The changes in Fluo-4 fluorescence were recorded automatically. Concentration-response curves for putative agonists/antagonists were established in at least two independent experiments with octuplicate determinations (s.a.) per data point. Data were analyzed and displayed by using PRISM 5.0.4 software (GraphPad, San Diego, CA, USA).

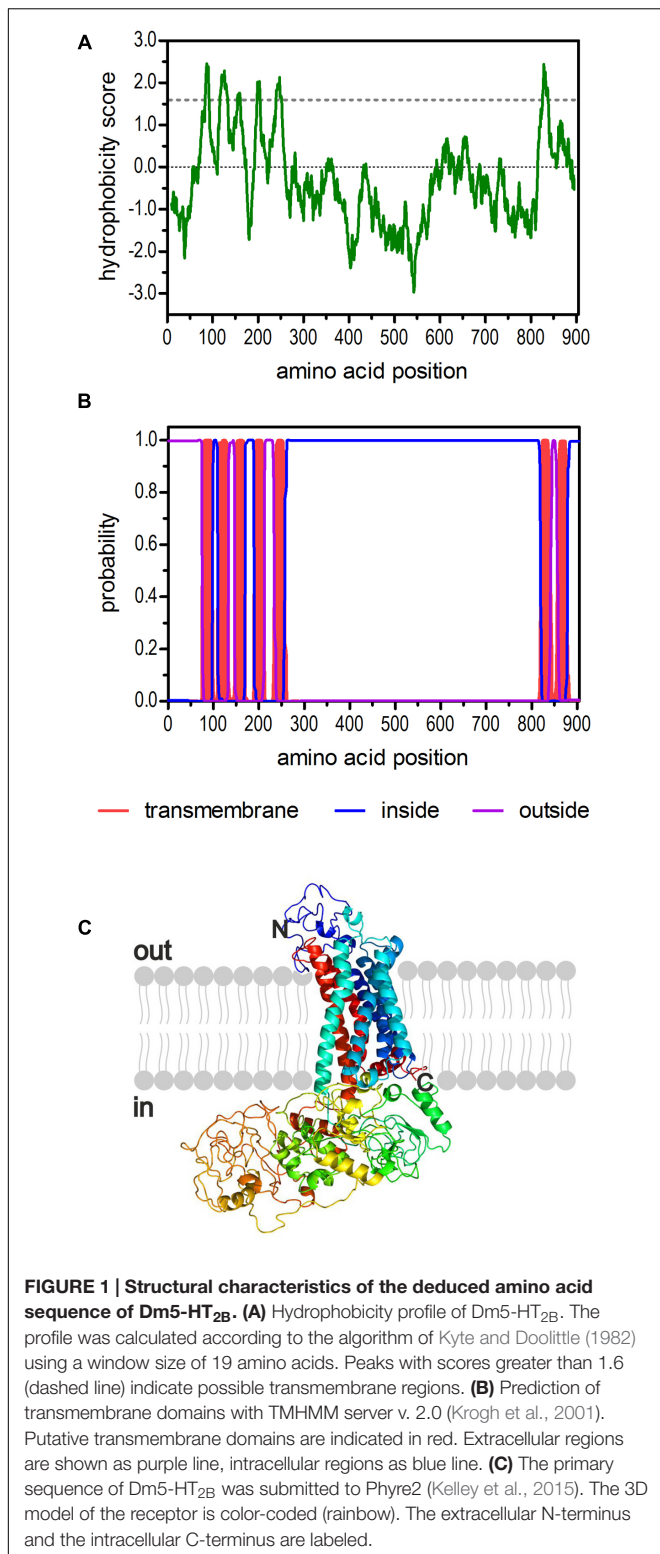
RESULTS

Cloning of *Dm5-HT_{2B}* cDNA and Structural Properties of Dm5-HT_{2B}

The sequence of a second potential 5-HT₂ receptor from *D. melanogaster* had been annotated in previous studies (Brody and Cravchik, 2000; Clark et al., 2004; Hauser et al., 2006). Later, *Dm5-HT_{2B}* (CG42796) was experimentally proven to encode a functional 5-HT receptor (Gasque et al., 2013). Here, we used the available sequence information and applied a PCR-based strategy to amplify the full-length *Dm5-HT_{2B}* cDNA for subsequent detailed pharmacological characterization of this receptor. The *Dm5-HT_{2B}* cDNA contains an open reading frame (ORF) of 2,715 bp and encodes a protein of 904 amino-acid residues (Dm5-HT_{2B}) with a calculated molecular mass of 99.5 kDa. The hydrophobicity profile according to Kyte and Doolittle (1982) and prediction of transmembrane helices using TMHMM Server v. 2.0 (Krogh et al., 2001) suggest seven trans-membrane (TM) domains (Figures 1A,B), which is a characteristic feature of GPCRs. The TM segments are flanked by an extracellular N-terminus of 74 residues and an intracellular C-terminus of 26 residues. The Dm5-HT_{2B} receptor contains an extremely long third cytoplasmic loop (CPL3) of 563 residues. We submitted the Dm5-HT_{2B} sequence to Phyre2 (Kelley et al., 2015) and obtained a three dimensional-model of the receptor (Figure 1C).

Sequence motifs which are essential for three-dimensional structure, ligand binding, and signal transduction of the receptor are well conserved between the various 5-HT_{2B} receptors (Figure 2) and are also present in Dm5-HT_{2B}. Three consensus motifs for potential N-glycosylation (N-X-S/T) are located in the extracellular N-terminus of Dm5-HT_{2B} (Figure 2). A cysteine residue in the C-terminus (Cys₈₉₂) is a possible site for post-translational palmitoylation. Twenty phosphorylation sites for protein kinase A (PKA), 38 phosphorylation sites for protein kinase C (PKC) and nine phosphorylation sites for protein kinase G (PKG) are present within intracellular domains of Dm5-HT_{2B} (Figure 2). N-glycosylation sites were predicted by NetNGlyc 1.0 Server¹ and putative palmitoylation sites were predicted using

¹ <http://www.cbs.dtu.dk/services/NetNGlyc/>



GPS-Lipid². Putative phosphorylation sites were predicted by NetPhos 3.1 Server³ (Blom et al., 2004).

²<http://lipid.biocuckoo.org/>

³<http://www.cbs.dtu.dk/services/NetPhos/>

A comparison of the Dm5-HT_{2B} amino-acid sequence with NCBI databases identified several orthologous protostomian and deuterostomian 5-HT₂ receptors. The highest amino acid identity (ID) and similarity (S) was found with the 5-HT_{2B} receptor of *A. mellifera* (Am5-HT_{2B}; Thamm et al., 2013; ID 48.5%, S 58.0%). Homology was also pronounced to 5-HT_{2B} receptors from the kissing bug *R. prolixus* (Rp5-HT_{2B}; Paluzzi et al., 2015; ID 44.6%, S 54.1%), and the crustaceans *P. interruptus* (Pi5-HT_{2B}; Clark et al., 2004; ID 33.3%, S 45.9%), *Procambarus clarkii* (Pc5-HT_{2B}; Spitzer et al., 2008; ID 34.1%, S 46.0%), and *Macrobrachium rosenbergii* (Mr5-HT_{2B}; Vázquez-Acevedo et al., 2009; ID 32.8%, S 46.0%). In phylogenetic tree analyses (Figure 3), Dm5-HT_{2B} forms a highly supported cluster with other protostomian 5-HT_{2B} receptors. This protostomian 5-HT_{2B} cluster represents the sister group to deuterostomian 5-HT₂ receptors within a monophyletic 5-HT₂ receptor group. However, the basal branching of 5-HT-receptor subgroups is not stable and thus has to be subject of future studies.

Functional and Pharmacological Properties of Dm5-HT_{2B}

In a first set of experiments, Dm5-HT_{2B}-expressing cells and non-transfected HEK 293 cells were incubated with the biogenic amines dopamine, histamine, octopamine, serotonin, and tyramine (1 μ M each, Figure 4A). The application of serotonin led to an increase in the fluorescence signal in Dm5-HT_{2B}-expressing but not in non-transfected cells. Neither dopamine, octopamine nor tyramine evoked responses in transfected or non-transfected cells. Histamine, however, caused a rise in Ca²⁺-dependent Fluo-4 fluorescence in both, Dm5-HT_{2B}-expressing and non-transfected HEK 293 cells. This effect is due to endogenously expressed histamine (H1) receptors in the HEK 293 cell line used in this study (Meisenberg et al., 2015).

To further investigate the pharmacological properties of Dm5-HT_{2B}, concentration-response curves on Dm5-HT_{2B}-expressing and non-transfected HEK 293 cells were established for serotonin. A series of serotonin concentrations was applied ranging from 10⁻⁹ M to 10⁻⁴ M. The concentration-response curve for Dm5-HT_{2B} was sigmoid and saturated at a serotonin concentration of 3×10^{-5} M (Figure 4B). Half-maximal activation of Dm5-HT_{2B} (EC₅₀) was at 2.11×10^{-8} M. In non-transfected HEK 293 cells, a slight increase in the fluorescence signal was observed at the highest ligand concentration applied (10⁻⁴ M).

Two potential agonists were tested for their activity on Dm5-HT_{2B}-expressing cells. For 5-methoxytryptamine and 8-OH-DPAT, concentration series ranging from 10⁻⁹ M to 10⁻⁴ M were applied and Ca²⁺-dependent Fluo-4 fluorescence was monitored (Figure 4B). Both ligands caused specific responses. The EC₅₀ for 5-methoxytryptamine was 1.05×10^{-6} M. In contrast to serotonin and 5-methoxytryptamine, the concentration-response curve for 8-OH-DPAT did not saturate and, therefore, the deduced EC₅₀ of $\cong 6.5 \times 10^{-4}$ M might be taken with some caution.

Next, we examined the ability of potential receptor antagonists for impairing Dm5-HT_{2B} activity. Measurements were

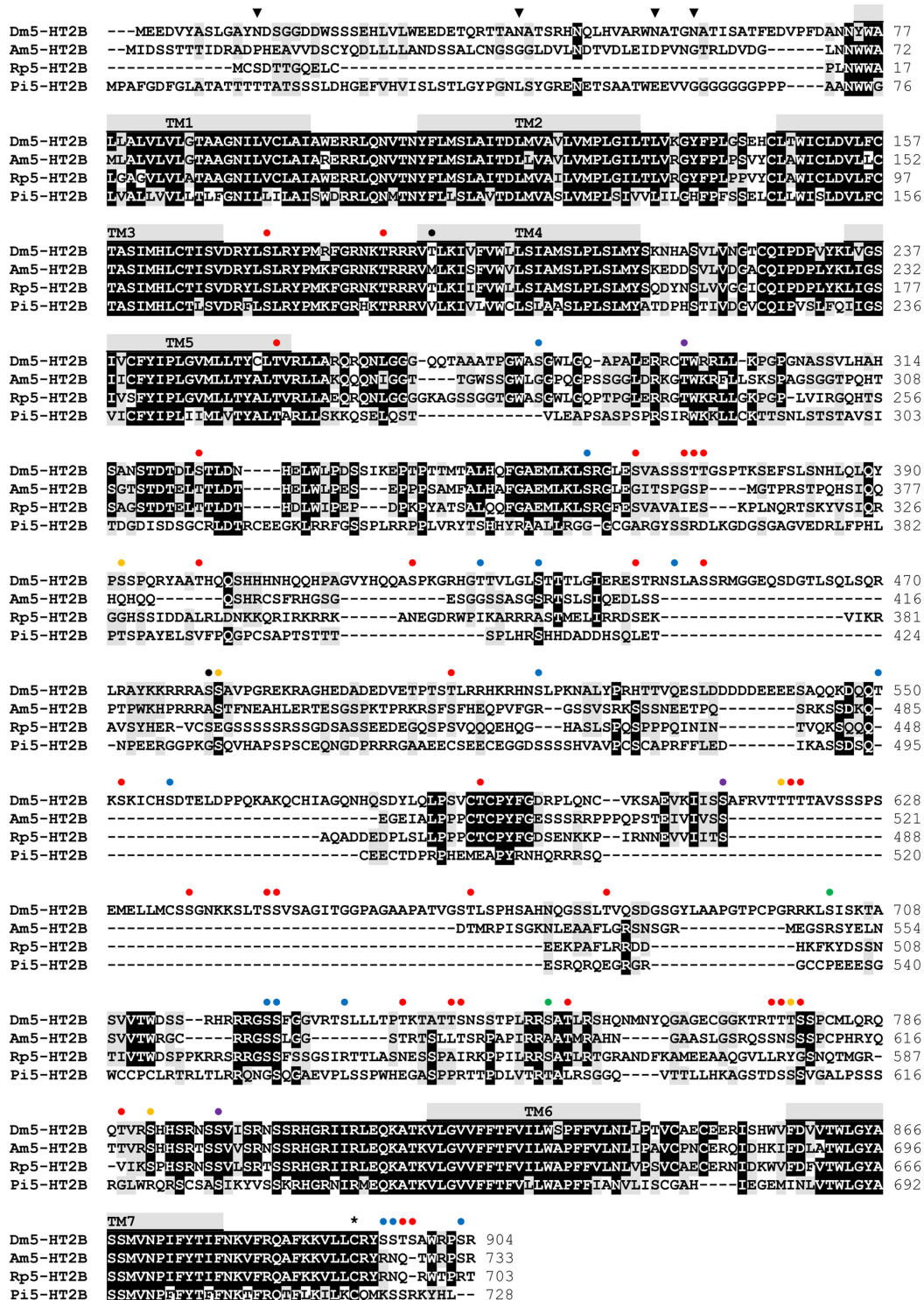


FIGURE 2 | Amino acid sequence alignment of Dm5-HT_{2B} and orthologous receptors from *Apis mellifera* (Am5-HT_{2B}, CBX90121), *Rhodnius prolixus* (Rp5-HT_{2B}, AKQ13312), and *Panulirus interruptus* (Pi5-HT_{2B}, AAS57919). Identical residues (≥75%) are shown as white letters against black, whereas conservatively substituted residues are shaded. Putative transmembrane domains (TM1–TM7) are indicated by gray bars. Potential N-glycosylation sites (▼), PKA phosphorylation sites (●), PKC phosphorylation sites (●), phosphorylation sites for both PKA and PKC (●), PKG phosphorylation sites (●), phosphorylation sites for both PKA and PKG (●), phosphorylation sites for all three kinases (●), and putative palmitoylation sites (*) of Dm5-HT_{2B} are indicated. The amino acid position is given on the right.

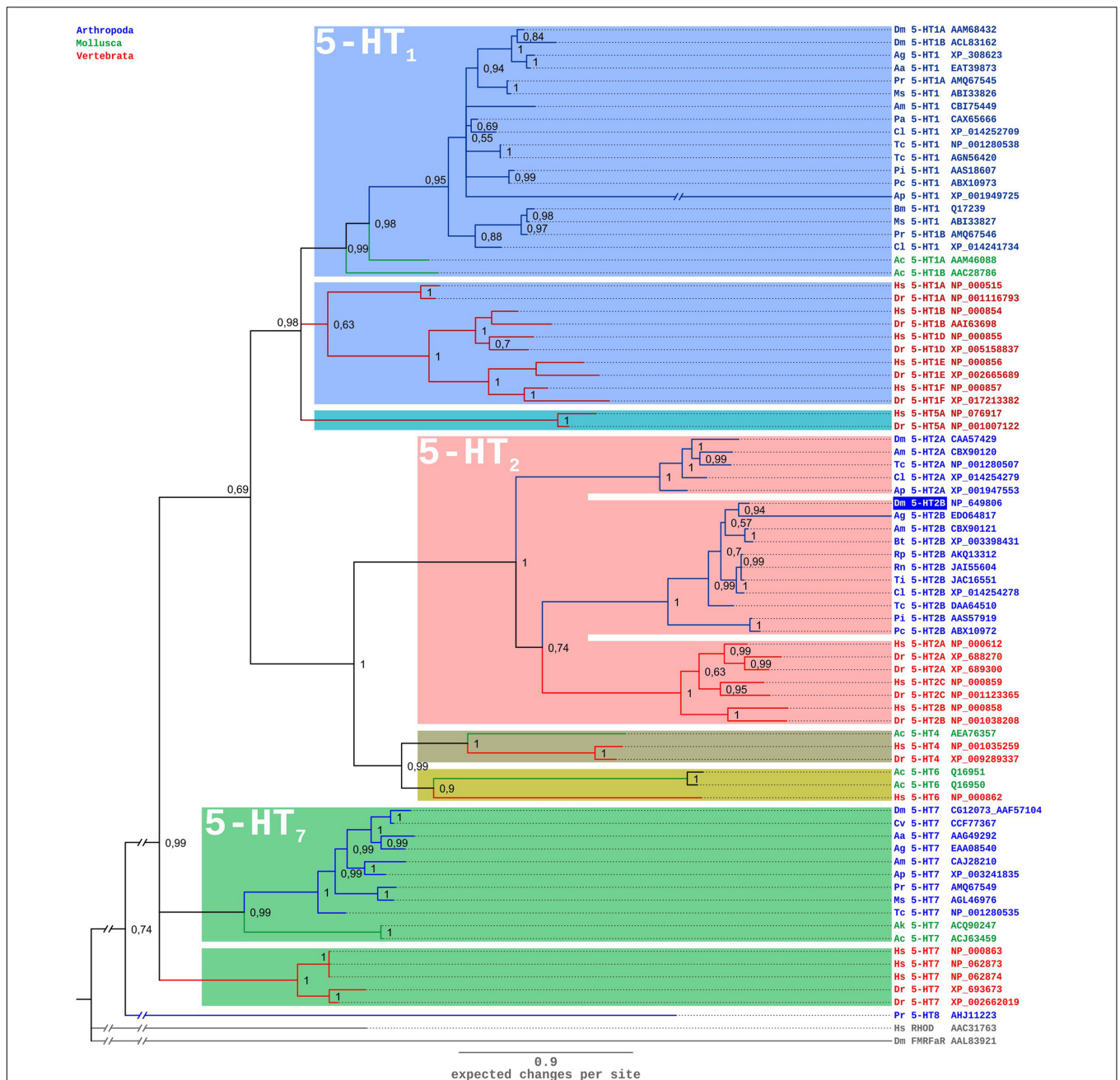
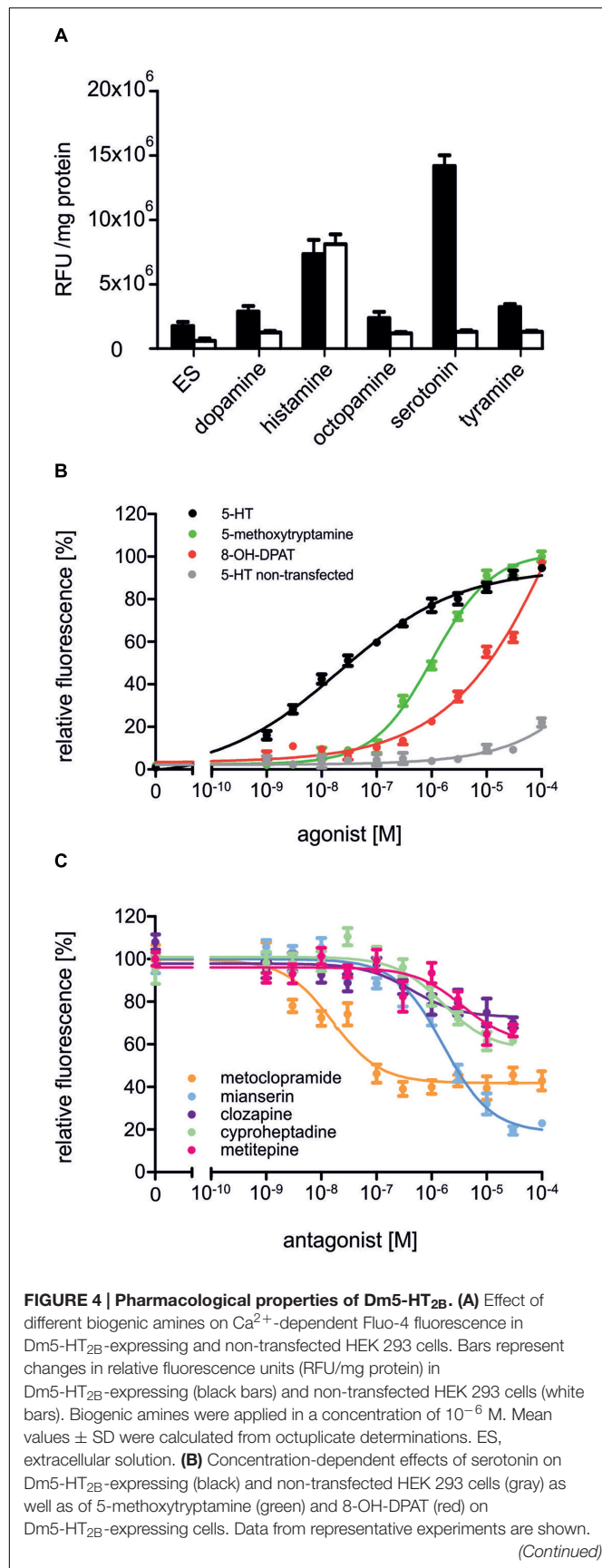


FIGURE 3 | Bayesian phylogeny of 5-HT receptors. Alignments were performed using Clustal W (Thompson et al., 1994) by using the core amino-acid sequences lacking the variable regions of the N- and C-terminus and the third cytoplasmic loop. Human rhodopsin (Hs RHOD) and *D. melanogaster* FMRFaR were used to root the tree. Numbers at branches represent the posterior probabilities. Receptor subclasses are highlighted by distinct colors. Abbreviations of species in alphabetical order: Aa *Aedes aegypti*, Ac *Aplysia californica*, Ag *Anopheles gambiae*, Ak *Aplysia kurodai*, Am *Apis mellifera*, Ap *Acyrtosiphon pisum*, Bm *Bombyx mori*, Bt *Bombus terrestris*, Cv *Cimex lectularius*, Cv *Calliphora vicina*, Dm *Drosophila melanogaster*, Dr *Danio rerio*, Hs *Homo sapiens*, Ms *Manduca sexta*, Pa *Periplaneta americana*, Pc *Procambarus clarkii*, Pi *Panulirus interruptus*, Pr *Pteris rapae*, Rn *Rhodnius neglectus*, Tc *Tribolium castaneum*, Ti *Triatoma infestans*.

performed with increasing concentrations of the antagonists clozapine, cyproheptadine, ketanserin, metitepine (also known as methiothepin), methysergide, metoclopramide, mianserin, prazosin, SB 242084, and spiperone on a background of 10^{-7} M serotonin.

In Dm5-HT_{2B}-expressing cells, many of the antagonists caused a decrease of the serotonin-induced Ca^{2+} -dependent fluorescence signals. Representative data are shown in **Figure 4C**. Ligand concentrations that led to half-maximal inhibition of Dm5-HT_{2B}-induced responses (IC_{50}) were

**FIGURE 4 | Continued**

Each data point represents the mean ± SD of an octuplicate determination. The relative fluorescence signal (%) for measurements with serotonin was normalized to the value measured in the presence of 10⁻⁴ M serotonin in Dm5-HT_{2B}-expressing cells (=100%). The relative fluorescence signals (%) for measurements with 5-methoxytryptamine and 8-OH-DPAT were normalized to the value measured in the presence of 10⁻⁴ M of the respective ligand (=100%). (C) Concentration-dependent effects of potential antagonists on serotonin-stimulated Dm5-HT_{2B}-evoked Ca²⁺ signals. Increasing concentrations (10⁻⁹ M to 10⁻⁴ M) of receptor antagonists were added to the receptor-expressing cell line. The Ca²⁺-dependent Fluo-4 signals were registered and normalized to the fluorescence evoked with 10⁻⁷ M serotonin (=100%). Data from representative experiments are shown. Each data point represents the mean ± SD of an octuplicate determination.

TABLE 1 | IC₅₀ values (potency) and relative efficacy were calculated from concentration-response curves for each drug.

	IC ₅₀ (M)	Log IC ₅₀ ± SD	Maximal inhibition
Clozapine	4.45 × 10 ⁻⁷	-6.35 ± 0.29	40%
Cyproheptadine	1.58 × 10 ⁻⁶	-5.80 ± 0.19	35%
Ketanserin	no effect	—	—
Metitepine	3.56 × 10 ⁻⁶	-5.45 ± 0.24	35%
Methysergide	no effect	—	—
Metoclopramide	1.59 × 10 ⁻⁸	-7.80 ± 0.12	60%
Mianserin	1.64 × 10 ⁻⁶	-5.79 ± 0.08	75%
Prazosin	no saturation	—	50%
SB 242084	no effect	—	—
Sipiperone	no saturation	—	25%

Efficacy is given as the maximal inhibition (%) of Ca²⁺-dependent fluorescence induced by 10⁻⁷ M serotonin in Dm5-HT_{2B}-expressing cells in the absence of antagonist. Values are means of representative experiments in which each data point was obtained from of an octuplicate determination.

determined from the concentration-response curves and are summarized in Table 1. The most effective antagonist on serotonin-stimulated Dm5-HT_{2B} was metoclopramide with an IC₅₀ of 1.78 × 10⁻⁸ M. The order of antagonist efficiency (IC₅₀) on the Dm5-HT_{2B} receptor was: metoclopramide > clozapine > cyproheptadine > mianserin > metitepine (Table 1). Two ligands, prazosin and sipiperone, also caused a reduction of the cellular response. However, the signals did not reach saturation and, due to solubility problems higher concentrations could not be tested (see Supplementary Figure S1). Therefore, IC₅₀ values were not calculated from these concentration-response curves. For three ligands, i.e., ketanserin, methysergide, and SB 242084, we did not observe any effect on serotonin-stimulated Dm5-HT_{2B}-expressing cells.

DISCUSSION

There is ongoing interest to precisely understand the physiological and behavioral roles of serotonergic signaling. To meet this challenge, important steps are to determine the molecular and functional-pharmacological properties of 5-HT receptor subtypes and to address their distribution within the CNS. Based on a rich body of data, a picture emerges that,

e.g., insects and mammals share similar modes of drug action as well as cellular and behavioral responses to serotonergic neurotransmission. Using model insects such as *D. melanogaster* might accelerate the gain of knowledge. Here, we have focused on elucidating the pharmacological properties of a *D. melanogaster* 5-HT receptor, Dm5-HT_{2B}. The pharmacological profile can be used for designing rational *in vitro* and *in vivo* studies to uncover the contribution of Dm5-HT_{2B} to the animal's development, physiology, and behavior.

Molecular Features of the Dm5-HT_{2B} Receptor

Four genes encoding 5-HT receptor subtypes were already cloned from *D. melanogaster* in the 90's of the last century. These were Dm5-HT_{1A} and Dm5-HT_{1B} (CG16720 and CG15113; Saudou et al., 1992), Dm5-HT_{2A} (CG1056; Colas et al., 1995), and Dm5-HT₇ (CG12073; Witz et al., 1990). These GPCRs share cognate properties with mammalian 5-HT_{1A}, 5-HT₂, and 5-HT₇ receptors. Resulting from bioinformatics screening and gene annotation, another GPCR gene (CG42796; Brody and Cravchik, 2000; Hauser et al., 2006; Blenau and Thamm, 2011) was uncovered encoding a protein with pronounced similarity to a 5-HT₂ receptor in the spiny lobster, *P. interruptus* (Clark et al., 2004). The receptor was named Dm5-HT_{2B}. In a recent study in which *D. melanogaster* larvae were used to screen for drugs that mediate food intake, the 5-HT receptor antagonist metitepine was identified as a potent anorectic drug (Gasque et al., 2013). Using cell-based assays, the authors could show that metitepine is an antagonist of all five *D. melanogaster* 5-HT receptors including Dm5-HT_{2B} (Gasque et al., 2013). While Gasque et al. (2013) could identify Dm5-HT_{2A} as the sole molecular target for feeding inhibition by metitepine, they did not establish a full pharmacological profile for Dm5-HT_{2B}. Here, we provide additional information on the molecular and pharmacological properties of this fifth 5-HT receptor subtype of the fruit fly.

With 904 amino acid residues and a calculated molecular weight of 99.5 kDa, the Dm5-HT_{2B} protein is rather large. More than half of the residues (563 amino acids) are present in the third cytoplasmic loop. Interestingly, the Dm5-HT_{2A} receptor is of similar size and contains 869 amino acid residues (Colas et al., 1995). This receptor also harbors a long third cytoplasmic loop of 321 residues but, in addition, Dm5-HT_{2A} has a long N-terminal loop which consists of 286 residues. For this receptor, two variants have been described. Either a point mutation changing Pro₅₂ to Ser in the N-terminus (Schaerlinger et al., 2007) or the complete deletion of the N-terminal domain leads to a significant gain of the receptor's affinity for serotonin (Colas et al., 1997) compared to the wild type protein. Orthologous receptors to Dm5-HT_{2A} and Dm5-HT_{2B} have been characterized from other insects as well. The Am5-HT_{2B} receptor from the honeybee also contains a large third cytoplasmic loop consisting of 399 residues (Thamm et al., 2013). However, with 80.7 kDa (733 amino acid residues) the protein is smaller than Dm5-HT_{2B}. With 653 residues, the honeybee Am5-HT_{2A} receptor is the smallest protein of this foursome. For both honeybee 5-HT₂ receptor subtypes, several splice variants were molecularly

cloned (Thamm et al., 2013). None of these variants gave rise to functional receptors upon heterologous expression of the constructs. This finding, however, does not rule out that full-length and modified variants may assemble in native tissues and thereby potentially expand the repertoire of serotonin binding partners in the honeybee.

Although Dm5-HT_{2B} is set apart by the length of its primary structure from other GPCRs, the protein shares most of the cognate features characterizing this huge gene family. The N-terminus of Dm5-HT_{2B} contains several consensus motifs for post-translational glycosylation (Figure 2). A large number of phosphorylation sites to common protein kinases are spread throughout the intracellular loops (Figure 2). Which of these sites participate in receptor desensitization and/or internalization (Lefkowitz and Shenoy, 2005; Kelly et al., 2008) upon serotonin stimulation awaits independent experimental testing. In addition to site-directed mutagenesis of single or multiple phosphorylation sites, a deletion strategy might be applied to successively reduce the size of the third cytoplasmic loop connecting transmembrane regions (TM) five and six (Figure 2). After heterologous expression of these receptor variants, their signaling properties can be examined and quantified by Ca²⁺ fluorimetry. Finally, residues in the binding site for serotonin that is formed by the transmembrane segments of Dm5-HT_{2B} are well conserved. Notably, the aspartic acid residue (D₁₅₃; D^{3.32}; nomenclature to Ballesteros and Weinstein, 1995) in TM3 is a potential binding partner of the protonated amino group of serotonin. A serine residue (S₂₃₇; S^{5.43}) in TM5 could bind to the 5-hydroxy group of serotonin's phenyl moiety. Phenylalanine and/or tryptophan residues in TM6 and TM7 (Figure 2) might contribute to π - π interaction with delocalized electrons in serotonin and stabilize the receptor ligand interaction.

Although we haven't experimentally addressed the expression pattern of the *Dm5-HT_{2B}* gene in this study, compelling evidence is available from previous studies supporting the general finding that 5-HT receptors are widely expressed in the CNS throughout development of *D. melanogaster* (Yuan et al., 2005, 2006; Nichols, 2007). Since we and others (Gasque et al., 2013) have cloned the cDNA encoding Dm5-HT_{2B} from adult tissue, the previous statement also holds for Dm5-HT_{2B}. Within the brain of adult flies, Dm5-HT_{2B} is expressed in the pars intercerebralis, the ellipsoid body, and photoreceptor cells (Gnerer et al., 2015). Whether the receptor participates in the regulation of heart function in *D. melanogaster* as suggested by recent experiments (Majeed et al., 2014) or is differentially expressed in male and female nervous tissue (Goldman and Arbeitman, 2007), awaits further testing.

Pharmacological Properties of Dm5-HT_{2B}

The Dm5-HT_{2B} receptor was functionally expressed in HEK 293 cells. Coupling of Dm5-HT_{2B} to intracellular signaling cascades was examined via cell-endogenous G-proteins. Like its protostomian and deuterostomian orthologs, Dm5-HT_{2B} caused intracellular Ca²⁺ release after stimulation with serotonin or

synthetic agonists like 5-methoxytryptamine or 8-OH-DPAT. With an EC₅₀ of 2×10^{-8} M, activation of the receptor was much more sensitive to serotonin compared to 5-methoxytryptamine (EC₅₀ $\cong 1 \times 10^{-6}$ M) or 8-OH-DPAT (EC₅₀ $\cong 6.5 \times 10^{-4}$ M). Since the concentration-response curve with 8-OH-DPAT did not saturate, this latter value should be taken with caution. More recently, two 5-HT₂ receptors from the honeybee, Am5-HT_{2A} and Am5-HT_{2B}, have been molecularly and pharmacologically characterized using the same heterologous expression system (Thamm et al., 2013). With EC₅₀ values of 2.57×10^{-8} M and 3.25×10^{-8} M both receptors share similar potencies for serotonin as Dm5-HT_{2B} and the Cv5-HT_{2A} receptor from *Calliphora vicina* (2.4×10^{-8} M; Röser et al., 2012), which was also expressed in HEK 293 cells. With an EC₅₀ of 2.01×10^{-7} M an orthologous receptor cloned from *R. prolixus* (Rp5-HT_{2B}; Paluzzi et al., 2015) was an order of magnitude less sensitive to serotonin. It should be mentioned here, that Rp5-HT_{2B} was not expressed in HEK 293 cells but in a recombinant Chinese hamster ovary cell line (CHOK1-aeq) and that ligand affinity may be influenced by the expression system used. In contrast to Dm5-HT_{2B}, where half-maximal stimulation with 5-methoxytryptamine was at $\cong 1 \times 10^{-6}$ M, both honeybee 5-HT₂ receptors and the *C. vicina* receptor displayed EC₅₀ values in the nanomolar range [Am5-HT_{2A}, 7×10^{-8} M; Am5-HT_{2B}, 6.04×10^{-8} M (Thamm et al., 2013); Cv5-HT_{2A}, 6.7×10^{-8} M (Röser et al., 2012)]. Similar to the results obtained for 5-methoxytryptamine, Dm5-HT_{2B} receptor activation by 8-OH-DPAT (EC₅₀ $\cong 6.5 \times 10^{-4}$ M) was less efficacious than that of Am5-HT_{2A} (EC₅₀ = 5.59×10^{-5} M) and Am5-HT_{2B} receptors (EC₅₀ = 5.6×10^{-7} M; Thamm et al., 2013) or the Cv5-HT_{2A} receptor (EC₅₀ = 6.2×10^{-5} M; Röser et al., 2012). Thus, although active on Dm5-HT_{2B}, both 5-methoxytryptamine and 8-OH-DPAT may not serve as alternatives to serotonin in specifically stimulating the receptor since both are likely to activate additional receptor subtypes at concentrations required for *in vivo* application in *D. melanogaster*.

Inhibition of receptor-mediated Ca²⁺ signaling in the cell line constitutively expressing Dm5-HT_{2B} was examined with a series of synthetic antagonists. In addition to substances that completely lacked inhibitory potential on the receptor (i.e., ketanserin, methysergide, and SB 242084), we observed three distinct types of inhibition profiles on Dm5-HT_{2B}. Two antagonists caused saturating responses and reduced serotonin-evoked Ca²⁺-dependent fluorescence to values $\leq 40\%$ of control measurements. With an IC₅₀ of 1.59×10^{-8} M, metoclopramide was more potent than mianserin (IC₅₀ = 1.64×10^{-6} M). Serotonin-evoked cellular Ca²⁺ responses were reduced to 40 and 25% of control measurements without antagonists by metoclopramide and mianserin, respectively. Responses to clozapine (IC₅₀ = 4.45×10^{-7} M), cyproheptadine (IC₅₀ = 1.58×10^{-6} M), and metitepine (IC₅₀ = 3.56×10^{-6} M) also saturated but all three substances were much less potent inhibitors at the receptor than metoclopramide or mianserin (Figure 4C). A maximal reduction to 60% of the serotonin-evoked signal was achieved with clozapine (Table 1). Finally,

prazosin and spiperone also reduced serotonin-induced Ca²⁺-dependent fluorescence in the cell line but the responses did not saturate. From the whole series of antagonists used in the current study, only metitepine has been tested in an earlier study by Gasque et al. (2013), who expressed Dm5-HT_{2B} in HEK 293T cells to investigate the pharmacology of this drug on *D. melanogaster* 5-HT receptors. Using Ca²⁺ fluorimetry on individual cells expressing Dm5-HT_{2B}, the authors reported an IC₅₀ of 2×10^{-6} M which is very similar to the value determined in the current study. Interestingly, metitepine has been uncovered as a potent anorectic drug in *D. melanogaster* larvae (Gasque et al., 2013). Although active on all five 5-HT receptor subtypes of the fruit fly, metitepine exhibited its anti-feeding activity only by interfering with Dm5-HT_{2A} signaling (Gasque et al., 2013). Some of the antagonists tested on Dm5-HT_{2B} in the current study had been examined previously on honeybee, *C. vicina*, and *R. prolixus* 5-HT receptors, too. Clozapine, cyproheptadine, metitepine, and mianserin inhibited Am5-HT_{2A} receptors in the micromolar range and reduced serotonin-induced Ca²⁺-dependent fluorescence by 44, 36, 39, and 49%, respectively (Thamm et al., 2013). Interestingly, at the Am5-HT_{2B} receptor metitepine did not have any activity at all. In contrast, clozapine, cyproheptadine, and mianserin blocked Ca²⁺-dependent fluorescence to 5, 23, and 24%, respectively, with IC₅₀ values in the low micromolar range (Thamm et al., 2013). Efficient inhibitors acting on the blowfly Cv5-HT_{2A} receptor were metitepine and clozapine which reduced serotonin-induced Ca²⁺ signals to 15 and 25% of control measurements with IC₅₀ values of 1.2×10^{-6} M and 15×10^{-6} M, respectively (Röser et al., 2012). Cyproheptadine, ketanserin, and mianserin reduced activity of the *R. prolixus* Rp5-HT_{2B} receptor by $\geq 50\%$ at the highest from three concentrations tested, i.e., 10^{-7} , 10^{-6} , and 10^{-5} M (Paluzzi et al., 2015).

In the current study, we identified metoclopramide as the most potent antagonist at the Dm5-HT_{2B} receptor. This was surprising, since metoclopramide is an established dopamine D₂ receptor antagonist in vertebrates, where it also inhibits serotonin-gated ion channels (5-HT₃ receptors) and activates 5-HT₄ receptors (Tonini et al., 1995). The latter effects have been therapeutically used to interfere with emesis. Until now, no information is available regarding the pharmacology of metoclopramide on the remaining four *D. melanogaster* 5-HT receptors. Future studies must show whether metoclopramide is really a Dm5-HT_{2B}-specific or a rather non-selective antagonist of 5-HT receptors in the fruit fly. Metoclopramide has also been shown to bind to the tyramine receptor TyrR (CG7431; Arakawa et al., 1990; K_i = 4.6×10^{-6} M) and to block the β -adrenergic-like octopamine receptor Oct β 3R (CG42244; Maqueira et al., 2005) in *D. melanogaster*, although only a high concentration of 10^{-5} M was tested in the latter study. Due to its pronounced sensitivity to Dm5-HT_{2B} (IC₅₀ = 1.59×10^{-8} M), however, the concentration required for *in vivo* experimentation to specifically target this receptor subtype might be kept rather low. In summary, our data may facilitate future behavioral pharmacological studies on the role of Dm5-HT_{2B} in the fruit fly. Such studies would be

desirable, since current knowledge on the role of this 5-HT receptor subtype solely depend on the investigation of flies that have been genetically manipulated.

AUTHOR CONTRIBUTIONS

WB designed and evaluated experiments, wrote the paper; DS conducted experiments and evaluated data; SB conducted experiments and evaluated data; MT conducted experiments; AB designed experiments and wrote the paper.

REFERENCES

- Alekseyenko, O. V., Chan, Y. B., Fernandez, M. P., Bülow, T., Pankratz, M. J., and Kravitz, E. A. (2014). Single serotonergic neurons that modulate aggression in *Drosophila*. *Curr. Biol.* 24, 2700–2707. doi: 10.1016/j.cub.2014.09.051
- Alekseyenko, O. V., and Kravitz, E. A. (2014). Serotonin and the search for the anatomical substrate of aggression. *Fly* 8, 200–205. doi: 10.1080/19336934.2015.1045171
- Alekseyenko, O. V., Lee, C., and Kravitz, E. A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. *PLoS ONE* 5:e10806. doi: 10.1371/journal.pone.0010806
- Arakawa, S., Gocayne, J. D., McCombie, W. R., Urquhart, D. A., Hall, L. M., Fraser, C. M., et al. (1990). Cloning, localization, and permanent expression of a *Drosophila* octopamine receptor. *Neuron* 4, 343–354.
- Ballesteros, J., and Weinstein, H. (1995). Integrated methods for the construction of three-dimensional models of structure-function relations in G protein-coupled receptors. *Methods Neurosci.* 25, 366–428.
- Becnel, J., Johnson, O., Luo, J., Nässel, D. R., and Nichols, C. D. (2011). The serotonin 5-HT7Dro receptor is expressed in the brain of *Drosophila*, and is essential for normal courtship and mating. *PLoS ONE* 6:e20800. doi: 10.1371/journal.pone.0020800
- Berger, M., Gray, J. A., and Roth, B. L. (2009). The expanded biology of serotonin. *Annu. Rev. Med.* 60, 355–366. doi: 10.1146/annurev.med.60.042307.110802
- Blankenburg, S., Balfanz, S., Hayashi, Y., Shigenobu, S., Miura, T., Baumann, O., et al. (2015). Cockroach GABAB receptor subtypes: molecular characterization, pharmacological properties and tissue distribution. *Neuropharmacology* 88, 134–144. doi: 10.1016/j.neuropharm.2014.08.022
- Blenau, W., and Thamm, M. (2011). Distribution of serotonin (5-HT) and its receptors in the insect brain with focus on the mushroom bodies: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arthropod Struct. Dev.* 40, 381–394. doi: 10.1016/j.asd.2011.01.004
- Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4, 1633–1649.
- Brody, T., and Cravchik, A. (2000). *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol.* 150, F83–F88.
- Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell Biol.* 7, 2745–2752.
- Clark, M. C., Dever, T. E., Dever, J. J., Xu, P., Rehder, V., Sosa, M. A., et al. (2004). Arthropod 5-HT2 receptors: a neurohormonal receptor in decapod crustaceans that displays agonist independent activity resulting from an evolutionary alteration to the DRY motif. *J. Neurosci.* 24, 3421–3435.
- Colas, J. F., Choi, D. S., Launay, J. M., and Maroteaux, L. (1997). Evolutionary conservation of the 5-HT_{2B} receptors. *Ann. N. Y. Acad. Sci.* 812, 149–153. doi: 10.1111/j.1749-6632.1997.tb48157.x
- Colas, J. F., Launay, J. M., Kellermann, O., Rosay, P., and Maroteaux, L. (1995). *Drosophila* 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5441–5445.
- Dacks, A. M., Green, D. S., Root, C. M., Nighorn, A. J., and Wang, J. W. (2009). Serotonin modulates olfactory processing in the antennal lobe of *Drosophila*. *J. Neurogenet.* 23, 366–377. doi: 10.3109/01677060903085722
- Darriba, D., Taboada, G. L., Doallo, R., and Posada, D. (2011). ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 27, 1164–1165. doi: 10.1093/bioinformatics/btr088
- Dasari, S., and Cooper, R. L. (2006). Direct influence of serotonin on the larval heart of *Drosophila melanogaster*. *J. Comp. Physiol. B* 176, 349–357.
- Dierick, H. A., and Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat. Genet.* 39, 678–682.
- Ellen, C. W., and Mercer, A. R. (2012). Modulatory actions of dopamine and serotonin on insect antennal lobe neurons: insights from studies in vitro. *J. Mol. Histol.* 43, 401–404. doi: 10.1007/s10735-012-9401-7
- Gasque, G., Conway, S., Huang, J., Rao, Y., and Vosshall, L. B. (2013). Small molecule drug screening in *Drosophila* identifies the 5HT2A receptor as a feeding modulation target. *Sci. Rep.* 3:sre02120. doi: 10.1038/srep02120
- Geyer, M. A., and Vollenweider, F. X. (2008). Serotonin research: contributions to understanding psychoses. *Trends Pharmacol. Sci.* 29, 445–453. doi: 10.1016/j.tips.2008.06.006
- Gnerer, J. P., Venken, K. J., and Dierick, H. A. (2015). Gene-specific cell labeling using MIMIC transposons. *Nucleic Acids Res.* 43:e56. doi: 10.1093/nar/gkv113
- Goldman, T. D., and Arbeitman, M. N. (2007). Genomic and functional studies of *Drosophila* sex hierarchy regulated gene expression in adult head and nervous system tissues. *PLoS Genet.* 3:e216.
- Green, A. R. (2006). Neuropharmacology of 5-hydroxytryptamine. *Br. J. Pharmacol.* 147, S145–S152.
- Hauser, F., Cazzamali, G., Williamson, M., Blenau, W., and Grimmelikhuijzen, C. J. (2006). A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog. Neurobiol.* 80, 1–19.
- Kaplan, D. D., Zimmermann, G., Suyama, K., Meyer, T., and Scott, M. P. (2008). A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size. *Genes Dev.* 22, 1877–1893. doi: 10.1101/gad.1670508
- Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* 10, 845–858. doi: 10.1038/nprot.2015.053
- Kelly, E., Bailey, C. P., and Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* 153, S379–S388.
- Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12, 857–872.
- Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580.
- Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* 157, 105–132.
- Lee, P. T., Lin, H. W., Chang, Y. H., Fu, T. F., Dubnau, J., Hirsh, J., et al. (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13794–13799. doi: 10.1073/pnas.1019483108
- Lefkowitz, R. J., and Shenoy, S. K. (2005). Transduction of receptor signals by β -arrestins. *Science* 308, 512–517.
- Luo, J., Becnel, J., Nichols, C. D., and Nässel, D. R. (2012). Insulin-producing cells in the brain of adult *Drosophila* are regulated by the serotonin 5-HT1A receptor. *Cell. Mol. Life Sci.* 69, 471–484. doi: 10.1007/s00018-011-0789-0
- Luo, J., Lushchak, O. V., Goergen, P., Williams, M. J., and Nässel, D. R. (2014). *Drosophila* insulin-producing cells are differentially modulated by serotonin

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- and octopamine receptors and affect social behavior. *PLoS ONE* 9:e99732. doi: 10.1371/journal.pone.0099732
- Majeed, Z. R., Abdeljaber, E., Soveland, R., Cornwell, K., Bankemper, A., Koch, F., et al. (2016). Modulatory action by the serotonergic system: behavior and neurophysiology in *Drosophila melanogaster*. *Neural Plast.* 2016:7291438. doi: 10.1155/2016/7291438
- Majeed, Z. R., Satsy, A., and Cooper, R. L. (2014). Pharmacological and genetic identification of serotonin receptor subtypes on *Drosophila* larval heart and aorta. *J. Comp. Physiol. B* 184, 205–219. doi: 10.1007/s00360-013-0795-7
- Maquieira, B., Chatwin, H., and Evans, P. D. (2005). Identification and characterization of a novel family of *Drosophila* β -adrenergic-like octopamine G-protein coupled receptors. *J. Neurochem.* 94, 547–560.
- Meisenberg, A., Kaschuba, D., Balfanz, S., Jordan, N., and Baumann, A. (2015). Molecular and functional profiling of histamine receptor-mediated calcium ion signals in different cell lines. *Anal. Biochem.* 486, 96–101. doi: 10.1016/j.ab.2015.06.037
- Mohammad, F., Aryal, S., Ho, J., Stewart, J. C., Norman, N. A., Tan, T. L., et al. (2016). Ancient anxiety pathways influence *Drosophila* defense behaviors. *Curr. Biol.* 26, 981–986. doi: 10.1016/j.cub.2016.02.031
- Nall, A., and Sehgal, A. (2014). Monoamines and sleep in *Drosophila*. *Behav. Neurosci.* 128, 264–272. doi: 10.1037/a0036209
- Neckameyer, W. S. (2010). A trophic role for serotonin in the development of a simple feeding circuit. *Dev. Neurosci.* 32, 217–237. doi: 10.1159/000304888
- Neckameyer, W. S., Coleman, C. M., Eadie, S., and Goodwin, S. F. (2007). Compartmentalization of neuronal and peripheral serotonin synthesis in *Drosophila melanogaster*. *Genes Brain Behav.* 6, 756–769.
- Nichols, C. D. (2007). 5-HT₂ receptors in *Drosophila* are expressed in the brain and modulate aspects of circadian behaviors. *Dev. Neurobiol.* 67, 752–763.
- Paluzzi, J. P., Bhatt, G., Wang, C. H., Zandawala, M., Lange, A. B., and Orchard, I. (2015). Identification, functional characterization, and pharmacological profile of a serotonin type-2b receptor in the medically important insect, *Rhodnius prolixus*. *Front. Neurosci.* 9:175. doi: 10.3389/fnins.2015.00175
- Python, F., and Stocker, R. F. (2002). Immunoreactivity against choline acetyltransferase, gamma-aminobutyric acid, histamine, octopamine, and serotonin in the larval chemosensory system of *Drosophila melanogaster*. *J. Comp. Neurol.* 453, 157–167.
- Qi, Y. X., Huang, J., Li, M. Q., Wu, Y. S., Xia, R. Y., and Ye, G. Y. (2016). Serotonin modulates insect hemocyte phagocytosis via two different serotonin receptors. *Elife* 5:e12241. doi: 10.7554/eLife.12241
- Reim, T., Balfanz, S., Baumann, A., Blenau, W., Thamm, M., and Scheiner, R. (2017). AmTAR2: functional characterization of a honeybee tyramine receptor stimulating adenylyl cyclase activity. *Insect Biochem. Mol. Biol.* 80, 91–100. doi: 10.1016/j.ibmb.2016.12.004
- Ro, J., Pak, G., Malec, P. A., Lyu, Y., Allison, D. B., Kennedy, R. T., et al. (2016). Serotonin signaling mediates protein valuation and aging. *Elife* 5:e16843. doi: 10.7554/eLife.16843
- Rodriguez Moncalvo, V. G., and Campos, A. R. (2009). Role of serotonergic neurons in the *Drosophila* larval response to light. *BMC Neurosci.* 10:66. doi: 10.1186/1471-2202-10-66
- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542. doi: 10.1093/sysbio/sys029
- Röser, C., Jordan, N., Balfanz, S., Baumann, A., Walz, B., Baumann, O., et al. (2012). Molecular and pharmacological characterization of serotonin 5-HT₂ α and 5-HT₇ receptors in the salivary glands of the blowfly *Calliphora vicina*. *PLoS ONE* 7:e49459. doi: 10.1371/journal.pone.0049459
- Saudou, F., Boschert, U., Amlaiki, N., Plassat, J. L., and Hen, R. (1992). A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO J.* 11, 7–17.
- Schaerlinger, B., Launay, J. M., Vonesch, J. L., and Maroteaux, L. (2007). Gain of affinity point mutation in the serotonin receptor gene 5-HT₂Dro accelerates germband extension movements during *Drosophila* gastrulation. *Dev. Dyn.* 236, 991–999.
- Sitaraman, D., LaFerriere, H., Birman, S., and Zars, T. (2012). Serotonin is critical for rewarded olfactory short-term memory in *Drosophila*. *J. Neurogenet.* 26, 238–244. doi: 10.3109/01677063.2012.666298
- Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., et al. (2008). Serotonin is necessary for place memory in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5579–5584. doi: 10.1073/pnas.0710168105
- Spitzer, N., Edwards, D. H., and Baro, D. J. (2008). Conservation of structure, signaling and pharmacology between two serotonin receptor subtypes from decapod crustaceans, *Panulirus interruptus* and *Procambarus clarkii*. *J. Exp. Biol.* 211, 92–105.
- Thamm, M., Rolke, D., Jordan, N., Balfanz, S., Schiffer, C., Baumann, A., et al. (2013). Function and distribution of 5-HT₂ receptors in the honeybee (*Apis mellifera*). *PLoS ONE* 8:e82407. doi: 10.1371/journal.pone.0082407
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Tonini, M., Candura, S. M., Messori, E., and Rizzi, C. A. (1995). Therapeutic potential of drugs with mixed 5-HT₄ agonist/5-HT₃ antagonist action in the control of emesis. *Pharmacol. Res.* 31, 257–260.
- Vargas, M. A., Luo, N., Yamaguchi, A., and Kapahi, P. (2010). A role for S6 kinase and serotonin in postmating dietary switch and balance of nutrients in *D. melanogaster*. *Curr. Biol.* 20, 1006–1011. doi: 10.1016/j.cub.2010.04.009
- Vázquez-Acevedo, N., Reyes-Colón, D., Ruiz-Rodríguez, E. A., Rivera, N. M., Rosenthal, J., Kohn, A. B., et al. (2009). Cloning and immunoreactivity of the 5-HT₁Mac and 5-HT₂Mac receptors in the central nervous system of the freshwater prawn *Macrobrachium rosenbergii*. *J. Comp. Neurol.* 513, 399–416. doi: 10.1002/cne.21979
- Vleugels, R., Verlinden, H., and Vanden Broeck, J. (2015). Serotonin, serotonin receptors and their actions in insects. *Neurotransmitter* 2:e314. doi: 10.14800/nt.314
- Walz, B., Baumann, O., Krach, C., Baumann, A., and Blenau, W. (2006). The aminergic control of cockroach salivary glands. *Arch. Insect Biochem. Physiol.* 62, 141–152.
- Witz, P., Amlaiki, N., Plassat, J. L., Maroteaux, L., Borrelli, E., and Hen, R. (1990). Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 87, 8940–8944.
- Yuan, Q., Joiner, W. J., and Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr. Biol.* 16, 1051–1062.
- Yuan, Q., Lin, F., Zheng, X., and Sehgal, A. (2005). Serotonin modulates circadian entrainment in *Drosophila*. *Neuron* 47, 115–127.

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Effects of a 5-HT_{1B} Receptor Agonist on Locomotion and Reinstatement of Cocaine-Conditioned Place Preference after Abstinence from Repeated Injections in Mice

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5-HT_{1B} receptors (5-HT_{1B}Rs) modulate behavioral effects of cocaine. Here we examined the effects of the 5-HT_{1B}R agonist 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine (CP94253) on spontaneous and cocaine-induced locomotion and on cocaine-primed reinstatement of conditioned place preference (CPP) in male mice given daily repeated injections of either saline or cocaine (15 mg/kg, IP) for 20 days. In the locomotor activity experiment, testing occurred both 1 and 20 days after the final injection. In the CPP experiment, mice underwent conditioning procedures while receiving the last of their daily injections, which were given either during or ≥ 2 h after CPP procedures. The CPP procedural timeline consisted of baseline preference testing (days 12–13 of the chronic regimen), conditioning (days 14–19, 2 daily 30-min sessions separated by 5 h), CPP test (day 21), extinction (days 22–34; no injections), CPP extinction test (day 35), and reinstatement test (day 36). Mice that had not extinguished received additional extinction sessions prior to reinstatement testing on day 42. On test days, mice were pretreated with either saline or CP94253 (10 mg/kg, IP). Testing began 30 min later, immediately after mice were primed with either saline or cocaine (5 mg/kg for locomotion; 15 mg/kg for reinstatement). We found that CP94253 increased spontaneous locomotion in mice receiving repeated injections of either saline or cocaine when tested 1 day after the last injection, but had no effect on spontaneous locomotion after 20 days abstinence from repeated injections. Surprisingly, cocaine-induced locomotion was sensitized regardless of whether the mice had received repeated saline or cocaine. CP94253 attenuated expression of the sensitized locomotion after 20 days abstinence. A control experiment in noninjected, drug-naïve mice showed that CP94253 had no effect on spontaneous or cocaine-induced locomotion. Mice reinstated cocaine-CPP when given a cocaine prime, and CP94253 pretreatment attenuated cocaine reinstatement. The findings suggest that stress from repeated saline injections and/or co-housing with cocaine-injected mice may cross-sensitize with cocaine effects on locomotion and that CP94253 attenuates these effects, as well as reinstatement of cocaine-CPP. This study supports the idea that 5-HT_{1B}R agonists may be useful anti-cocaine medications.

Keywords: serotonin, CP94253, sensitization, withdrawal, addiction, place conditioning

INTRODUCTION

Serotonin plays a role in the reinforcing and incentive motivational effects of cocaine and cocaine-associated cues (Markou et al., 1993; Shaham et al., 2003). One mechanism involved in these effects is the action of serotonin at 5-HT_{1B} receptors (5-HT_{1B}Rs; Clark and Neumaier, 2001; Filip et al., 2010; Miszkiewicz et al., 2011; Neisewander et al., 2014). Parsons et al. (1998) discovered that 5-HT_{1B}R agonists shift the cocaine self-administration (SA) dose-effect function to the left and increase responding on a PR schedule of cocaine reinforcement, suggesting enhanced reinforcing value of cocaine. These 5-HT_{1B}R agonist effects are reversed by a 5-HT_{1B}R antagonist, demonstrating that they are 5-HT_{1B}R-mediated. Furthermore, the agonists do not alter sucrose or food reinforcement or locomotion at doses that enhance the reinforcing value of cocaine (Parsons et al., 1998; Przeglasiński et al., 2007; Pentkowski et al., 2009). Surprisingly, we found that both cue and cocaine-primed reinstatement of cocaine-seeking behaviors are attenuated by 5-HT_{1B}R agonists (Acosta et al., 2005; Pentkowski et al., 2009). These seemingly paradoxical findings led us to discover that 5-HT_{1B}Rs modulate cocaine-related behaviors in opposite directions depending on whether or not animals have undergone an abstinence period prior to testing (Pentkowski et al., 2014). Specifically, either the agonist 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine (CP94253) or viral overexpression of 5-HT_{1B}Rs tested during the maintenance of daily SA sessions *increased* the reinforcing value of cocaine, measured as a leftward shift of the cocaine SA dose-effect function on low ratio schedules of reinforcement and an increase in intake on a progressive ratio schedule (Pentkowski et al., 2012, 2014). In contrast, after a 21-day period of protracted abstinence, the agonist *attenuated* cocaine intake at the same low dose of cocaine (0.075 mg/kg, IV) for which CP94253 had enhanced intake prior to an abstinence period (Pentkowski et al., 2014) and attenuated intake on a progressive ratio schedule of cocaine reinforcement. These findings demonstrate opposite functional effects of 5-HT_{1B}R agonists pre- vs. post-abstinence from cocaine SA.

5-HT_{1B}Rs also modulate spontaneous locomotion and cocaine-induced locomotion under some circumstances. Several studies have found that 5-HT_{1B}R agonists stimulate locomotor activity in drug-naïve rats (Oberlander et al., 1986, 1987; Macor et al., 1990; Koe et al., 1992; Geyer, 1996; Chaouloff et al., 1999), but have no effect on spontaneous locomotion in rats with a history of cocaine SA (Przeglasiński et al., 2007; Pentkowski et al., 2009). 5-HT_{1B}R agonist effects on spontaneous locomotion may be specific to rats since the drugs have no effect in drug-naïve mice (Bannai et al., 2007; Fish et al., 2008; Nasehi et al., 2017). However, in mice that had been stressed by repeated behavior testing, CP94253 increases locomotion (Tatarczyńska et al., 2004, 2005). Additionally, the 5-HT_{1A/1B}R agonist RU24969 dose-dependently increases spontaneous locomotion in wild type mice, but not 5-HT_{1B}R knockout mice (Saudou et al., 1994). CP94253, as well as another 5-HT_{1B}R agonist CP93129, have been shown to potentiate

cocaine-induced locomotion and cocaine sensitization in rats (Przeglasiński et al., 2001, 2002, 2004; Filip et al., 2010). Collectively, these findings suggest that 5-HT_{1B}R stimulation enhances locomotion in rodents given cocaine or with a history of stress.

One goal of the present study was to examine whether the abstinence-induced “switch” in 5-HT_{1B}R functional modulation of cocaine-related behaviors observed in rats previously is also observed in mice. To this end, we investigated whether CP94253 produces opposing effects on spontaneous and cocaine-induced locomotion before and after an abstinence period in C57BL/6 male mice receiving daily injections of either saline or cocaine (15 mg/kg, IP) for 20 days. The second goal was to investigate whether the incentive motivational effects of a cocaine priming injection are attenuated by 5-HT_{1B}R agonist treatment in mice that had undergone abstinence, similar to the decrease in cocaine-primed reinstatement of cocaine-seeking behavior observed previously in rats (Pentkowski et al., 2012, 2014). To this end, we investigated CP94253 effects on cocaine-primed reinstatement of extinguished cocaine-conditioned place preference (CPP).

MATERIALS AND METHODS

Animals

Male C57BL/6 mice arrived at 5 weeks old from Jackson Laboratories (Sacramento, CA, USA) and were group housed 3–4/cage in a climate-controlled facility with a reversed 10 h light/14 h dark cycle (lights off at 6:00 AM). Mice were handled for 2 weeks. For the CPP experiment only, mice were transferred to single housing 1 day prior to the start of behavior testing. Food and water were provided *ad libitum* in the home cage. All behavioral testing occurred between 8 AM and 4 PM. Separate groups of experimentally naïve mice were used for each specific experiment. All husbandry and procedures adhered to the National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011), and all experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Arizona State University.

Drugs

Cocaine hydrochloride (RTI International, Research Triangle Park, NC, USA) and CP94253 (Tocris Bioscience, Minneapolis, MN, USA) were dissolved in bacteriostatic saline. All drugs were injected at a volume of 10 ml/1 kg of body weight. The doses used had been previously reported to produce cocaine- (Tilley et al., 2007; Shuman et al., 2012; Rao et al., 2013) and CP94253-induced hyperlocomotion in mice injected 30 min before testing (Tatarczyńska et al., 2004, 2005; Bannai et al., 2007; Fish et al., 2008).

Apparatus

Locomotor activity tests were conducted in Plexiglas chambers, each measuring 35 × 24 × 31 cm high. The chambers had corn cob bedding on an acrylic floor and alternating

black and white stripes on the walls. CPP experiments were conducted in Plexiglas two-compartment apparatus with each end compartment measuring 35 × 24 × 31 cm high and with a removable partition separating them. One compartment had cedar bedding beneath a wire 1 × 1 cm grid floor and alternating black and white vertical stripes on the walls. The other compartment had pine bedding beneath a parallel bar floor (5 mm diameter) and alternating black and white horizontal stripes on the walls. In order to prevent the mice from escaping from the chambers, while maintaining the ability to record their behavior via an overhanging video camera, a rectangular tower measuring 70 × 24 × 74 cm high of clear Plexiglas was used as an extension of the apparatus. The testing room was dimly lit with two overhead lamps, each containing a 25 Watt light bulb. A camera (Panasonic WV-CP284, color CCTV, Suzhou, China) used to record testing sessions was mounted 101 cm above the center of each apparatus. A WinTV 350 personal video recorder (Hauppauge, NJ, USA) captured live video encoded into MPEG streams. A modified version of TopScan Software (Clever Sys Incorporated, Reston, VA, USA) was used to track the animals' movement. This program uses the orientation of an animal's body parts (e.g., nose, head, center of body, forepaws, base of tail, etc.) to identify the animal's location and specified behaviors.

Experiment 1: Effects of CP94253 on Spontaneous and Cocaine-Induced Locomotion before and after Chronic Daily Injections of Cocaine or Saline

The timeline for Experiment 1 is shown in **Figure 1A**. Adult, male C57BL/6 mice ($n = 91$) were housed four/cage, with two mice in each cage assigned to receive saline and two assigned to receive cocaine (15 mg/kg, IP) at the same time of day for 20 consecutive days. The mice were further assigned to receive two different pretreatments on the test days. The first pretreatment was either vehicle or CP94253 (10 mg/kg, IP) and the second pretreatment was either a saline or cocaine (5 mg/kg, IP) challenge injection. Thus the design of this experiment was a 2 (chronic saline or cocaine) × 2 (vehicle or CP94253 pretreatment) × 2 (saline or cocaine challenge) factorial with eight treatment groups ($n = 8$ –11/group). Test day 1 took place on the day after the last chronic injection. After test 1, the mice underwent a 20-day period of no injections during which they remained in the colony room and their tails were marked twice per week to maintain identification. Test day 2 took place the day after the final abstinence (i.e., no injection) day. On both of the test days, mice were first placed into the test chamber for 1 h to allow for habituation. Immediately following this baseline period, mice were injected with either vehicle or CP94253 and were returned to their home cage for 30 min. Next, mice received the saline or cocaine challenge injection and were returned to the test chamber for an additional 60 min. We used a lower cocaine dose for the challenge (5 mg/kg) on test day than that used during the daily repeated administration (15 mg/kg). This was done in order to avoid potential ceiling effects for detecting

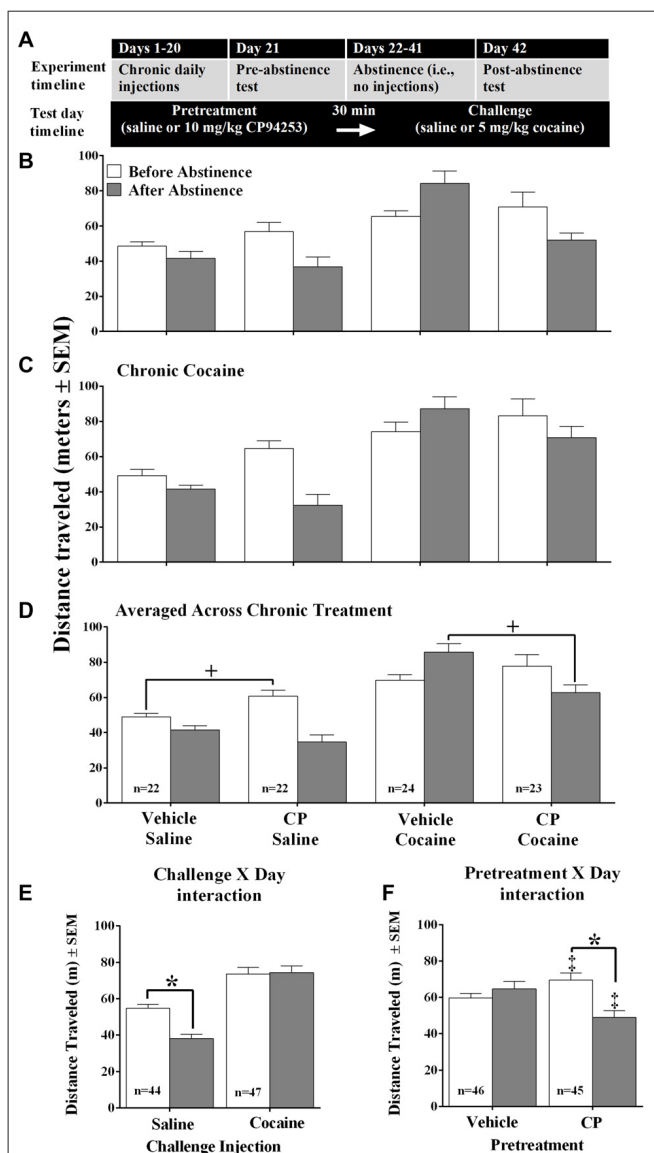


FIGURE 1 | Timeline for Experiments 1–2 (A) and distance traveled (meters ± SEM) by mice that received either chronic daily injections of saline (B) or 10 mg/kg cocaine (C) and were tested both 24 h after the last of 20 injections (i.e., before abstinence, white bars) and 20 days after (i.e., after abstinence, gray bars), $n = 8$ –11/group. Contrary to prediction, there was no effect of chronic treatment conditions nor interactions with cocaine challenge (0 or 5 mg/kg, IP) or 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine (CP94253) (0 or 10 mg/kg, IP), so further analyses were conducted averaged across the chronic treatment variable (D). This analysis yielded a challenge injection by day interaction (E) and a pretreatment by day interaction (F). Asterisk (*) represents a significant *post hoc* comparison, $p < 0.05$; Plus sign (+) represents a significant planned comparison, $p < 0.05$. Double plus (‡) represents a significant difference from respective vehicle condition, Bonferroni *t*-test $p < 0.001$.

sensitization of locomotion, a well-known effect of repeated cocaine administration (Ago et al., 2008; DiRocco et al., 2009; Luo et al., 2010; Thompson et al., 2010; Riday et al., 2012; Robison et al., 2013).

Experiment 2: Effects of CP94253 on Spontaneous and Cocaine-Induced Locomotion in Mice without the Repeated Injection Regimen

In order to assess potential injection stress effects, we repeated Experiment 1 using identical procedures and timeline except that the 5 week old, male C57BL/6 mice ($n = 47$) did not receive any injections during the first 20 days of the experiment. Thus, the four mice/cage were simply handled twice a week to color-mark tails for identification purposes and were otherwise left undisturbed to minimize stress. The design was a 2 (vehicle or CP94253 pretreatment) $\times$ 2 (saline or cocaine challenge) factorial with four treatment groups ($n = 11$ –12/group). Test day procedures were identical to Experiment 1.

Experiment 3: Effects of CP94253 on Reinstatement of Extinguished Cocaine-CPP

The timeline for Experiment 3 is shown in **Figure 3A**. Adult, male C57BL/6 mice received daily injections of cocaine (15 mg/kg, IP) or saline for 11 days in order to keep the same number of cocaine injections prior to testing for effects of CP94253 in this experiment as that given in the previous experiments. Additionally, the mice were housed three/cage and all three mice/cage were assigned to the same chronic drug condition. On day 12 and 13 the mice were allowed free access to both sides of the CPP apparatus for 15 min to habituate them to the novel environments and to assess initial compartment preference. The average of the time spent in the least preferred compartment on days 12 and 13 was used as the baseline preference measure. On both days 12 and 13, mice received their chronic daily injection (saline or cocaine) in their home cage 2–3 h after the preference test. On days 14–19, the mice underwent two daily 30-min conditioning sessions separated by a 5-h period. During the morning session, mice were injected with saline and were placed into their initially preferred side and during the afternoon session mice were injected with cocaine (15 mg/kg, IP) or saline and were placed into their initially non-preferred side. On day 20, mice were not exposed to the apparatus, but did receive either saline or cocaine (15 mg/kg, IP) at the same time of day as all previous injections. On day 21, mice were tested for the expression of cocaine CPP for 15 min. Only 80% of the mice met the CPP expression criterion (spent >450 s in initially non-preferred compartment) and continued in the experiment. These mice next underwent extinction training on Days 22–34. During extinction, the mice received one 30-min exposure to one of the compartments each day, with the particular compartment alternating across the days. On day 35, mice were tested for 15 min to demonstrate that their CPP had extinguished. Mice that extinguished were tested for reinstatement of CPP the following day (day 36). On test day, mice received either saline or CP94253 (10 mg/kg, IP) 30 min prior to the test. Immediately before the test, the mice were primed with either saline or cocaine (15 mg/kg, IP). Mice that did not initially extinguish received four more days of extinction

with two, 30-min sessions per day, one in each compartment. They again received a 15-min preference test to demonstrate that their CPP had extinguished. Mice that extinguished were tested for reinstatement of CPP the following day. Mice that failed to extinguish were removed from the study. The design of the study was a 2 (vehicle or CP94253 pretreatment) $\times$ 2 (saline or cocaine challenge) factorial with four treatment groups ($n = 9$ –11/group). Additionally, a group of mice ($n = 14$) were treated chronically with saline, conditioned with saline during both daily sessions, extinction-trained, and given a saline prime prior to testing (i.e., saline control group).

Statistics

Drug-induced changes in distance traveled (meters) were analyzed and graphed for the first 30 min of each testing session. Only the first 30 min of the testing sessions were analyzed because cocaine is rapidly metabolized in mice (Tilley et al., 2007; Rao et al., 2013) and the difference from baseline calculation controlled for individual differences in baseline activity. The changes in distance traveled measures were analyzed by mixed factor analysis of variances (ANOVAs) with the following between group variables: chronic treatment with cocaine or saline (Experiment 1 only); pretreatment with CP94253 or vehicle; challenge with cocaine or saline prior to test. The ANOVAs also included Test day as a within subjects repeated measure. Interactions were further analyzed by smaller ANOVAs and *t*-test with Bonferroni correction for multiple comparisons where appropriate. In addition, planned comparisons were conducted to test our hypothesis that CP94253 would enhance spontaneous locomotion and cocaine-induced locomotion pre-abstinence, but would have the opposite effect post-abstinence. Mice whose distance traveled score was more than ± 2 standard deviation from the mean were deemed outliers and removed from all analysis. For CPP, time spent in the initially non-preferred side was analyzed by ANOVA with test days as a repeated measure. The test days included the baseline preference test, the CPP test (occurred after six daily pairings with cocaine), and the extinction test (occurred after 18–22 sessions of extinction). This analysis was a manipulation check to demonstrate that cocaine-conditioned rats exhibited CPP and extinction of CPP. To analyze cocaine-primed reinstatement of CPP, time spent in the initially non-preferred compartment of the apparatus (drug-paired compartment) was analyzed by a 2 (Pretreatment: CP94253 and vehicle) $\times$ 2 (Priming injection: Cocaine and saline) ANOVA. Interactions were analyzed by smaller ANOVAs and Tukey *post hoc* tests.

RESULTS

Experiment 1: Effects of CP94253 on Spontaneous and Cocaine-Induced Locomotion before and after Chronic Daily Injections of Cocaine or Saline

We first tested the hypothesis that mice given chronic cocaine treatment would exhibit a “switch” in 5-HT_{1B}R

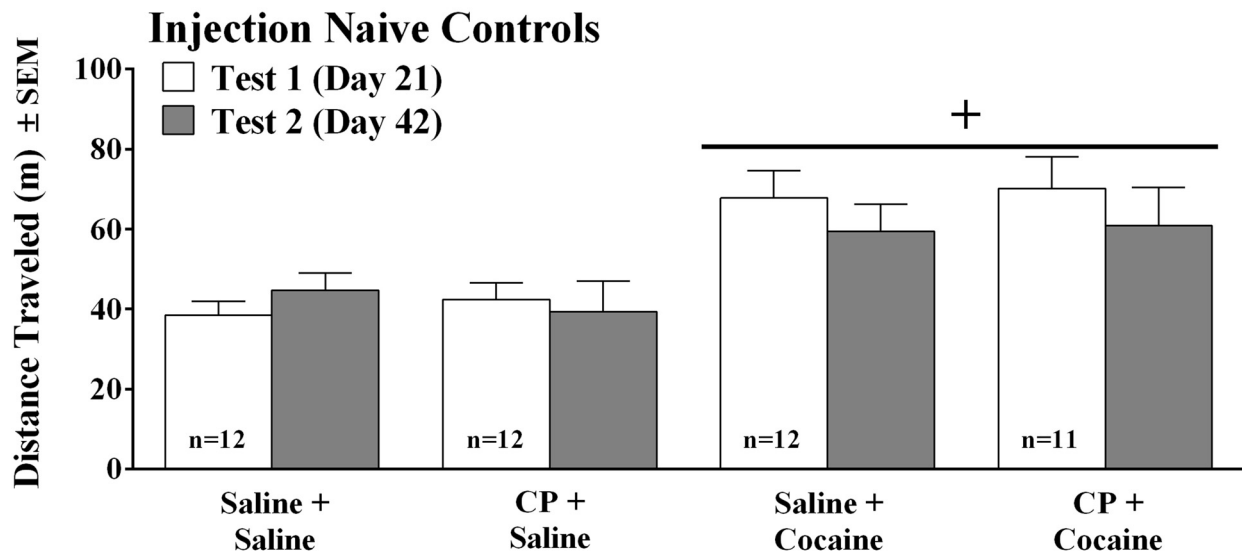


FIGURE 2 | Distance traveled (meters ± SEM) by injection-naïve mice that were treated the same as mice in the previous experiment (see timeline on **Figure 1**) except that they were not given daily injections over the first 20 days of the experiment, but were instead left undisturbed in their home cages except for twice weekly tail marking for identification. On the test days, the mice received an injection of either saline or CP94253 (10 mg/kg, IP) and 30 min later received a saline or cocaine (5 mg/kg, IP) injection ($n = 11$ – 12 /group). Plus sign (+) represents a significant difference from saline-challenged groups, $p < 0.001$.

agonist effects from facilitation of cocaine-induced locomotion during the treatment phase to inhibition of cocaine-induced locomotion after a period of abstinence from chronic cocaine. Surprisingly, the chronic saline group behaved similarly to the chronic cocaine group (**Figures 1B,C**) and the analysis confirmed that there was no main effect nor interactions with chronic treatment (i.e., chronic saline vs. cocaine). Therefore, subsequent analyses were conducted with the data are averaged across chronic condition as shown in **Figure 1D**. This analysis revealed a main effect of Challenge, where the cocaine challenge increased locomotion compared to the saline challenge when averaged across pretreatment with Vehicle or CP94253 ($F_{(1,87)} = 62.28$, $p < 0.001$). However, there was also a Challenge by Day interaction ($F_{(1,87)} = 15.47$, $p < 0.001$) as shown in **Figure 1E**. Subsequent pairwise comparisons with Bonferroni correction indicated that cocaine-challenged mice showed no difference in locomotion across test days, whereas saline challenged mice showed a decrease in locomotion after abstinence compared to before abstinence ($t_{(43)} = 5.8$, $p < 0.001$). There was also a Pretreatment by Day interaction ($F_{(1,87)} = 32.83$, $p < 0.001$) as shown in **Figure 1F**. Subsequent pairwise comparisons indicated that mice pretreated with vehicle showed no difference in locomotion across test days, whereas mice pretreated with CP94253 showed less locomotion after abstinence compared to before abstinence (Bonferroni t -test, $t_{(44)} = 5.8$, $p < 0.001$). In addition to the ANOVAs, planned comparisons were conducted to test the hypothesis that CP94253 pretreatment would facilitate spontaneous and cocaine-induced locomotion before abstinence but inhibit these behaviors after abstinence. The results of these comparisons indicated that there was a significant increase in spontaneous

locomotion after the CP94253 pretreatment compared to vehicle pretreatment in mice challenged with saline before abstinence from repeated injections ($t_{(42)} = 3.0$, $p < 0.01$, **Figure 1D**). In mice challenged with cocaine, there was no difference in cocaine-induced locomotion between vehicle- and CP94253-pretreated mice before abstinence, but the CP94253-pretreated mice showed less cocaine-induced locomotion than vehicle-pretreated mice after abstinence ($t_{(45)} = 3.6$, $p < 0.05$, **Figure 1D**).

Experiment 2: CP94253 Has no Effect in Mice that Have Not Undergone a Repeated Injection Regimen

The finding that chronic cocaine vs. chronic saline treatment did not show differences in locomotion in the previous experiment was puzzling. We reasoned that stress experienced by the saline control group may have cross-sensitized the mice to cocaine such that both groups (i.e., chronic cocaine and chronic saline) showed sensitized responses to cocaine (Sorg, 1992). Indeed, the control mice experienced repeated injections and were housed with cocaine-treated mice, and both of these manipulations are chronic stressors in mice (Ryabinin et al., 1999; Hoplight et al., 2007). Another concern was that rather than CP94253 having opposite effects on cocaine-induced locomotion before and after abstinence from repeated injections, perhaps the agonist simply has opposite effects the first time it is administered compared to the second time it is administered. We examined these possibilities in this experiment. Naïve, non-injected mice arrived at the same age as in the previous experiment and were housed for 20 days during which they were handled twice weekly to color-mark tails for identification purposes and were otherwise left undisturbed. As expected, cocaine increased locomotion to

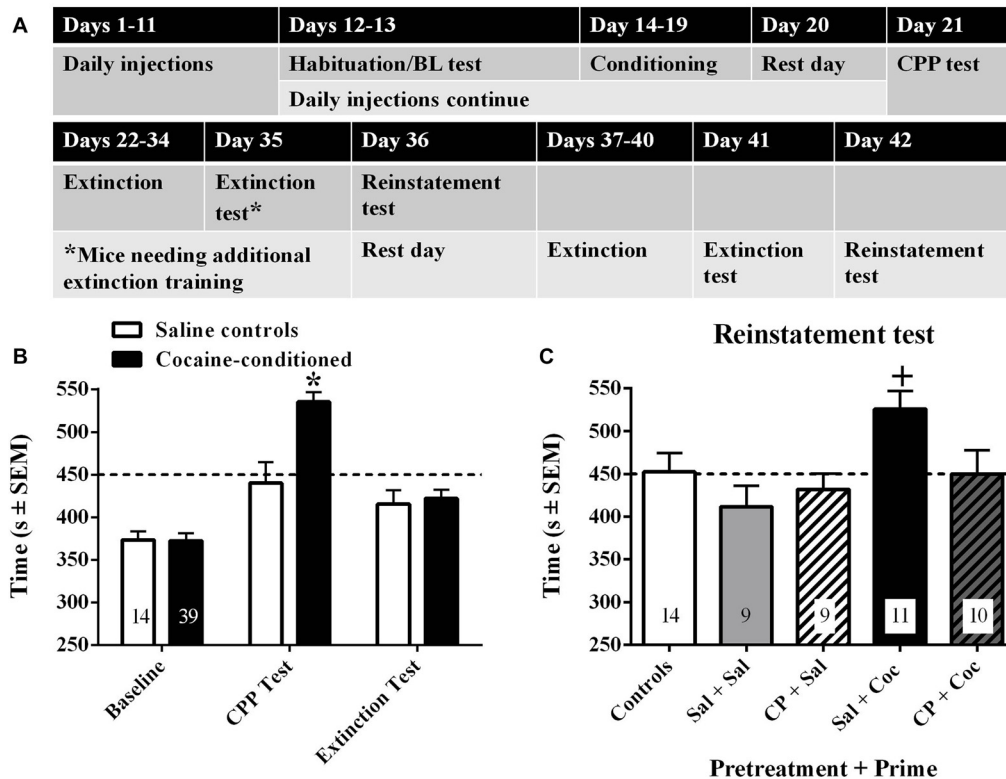


FIGURE 3 | Timeline for Experiment 3 (A) and results of 15-min preference tests to assess baseline preference, cocaine-conditioned place preference (CPP), and extinction of cocaine-CPP (B). Subsequently, mice that had received repeated saline injections (white bars) or repeated cocaine injections (black bars) prior to and during conditioning were tested for reinstatement of CPP (C) following pretreatment with either saline or CP94253 (10 mg/kg, IP) and a priming injection of either saline (Sal) or cocaine (15 mg/kg, IP; Coc) 30 min later and immediately prior to the test ($n = 9-11$ /group). Values are the time ($s \pm SEM$) in the initially non-preferred compartment (i.e., cocaine-paired side for conditioned mice) and dashed line represents 50% of the total test time such that values above the line illustrate a preference switch. Asterisk (*) represents difference from saline group, Bonferroni t -test $p < 0.001$. Plus (+) represents difference from all other groups, Tukey test, $p < 0.05$.

a similar degree on the first (day 21) and second (day 42) test days as there was a main effect of Challenge ($F_{(1,43)} = 15.15$, $p < 0.001$), but no interactions with Pretreatment or Day. In contrast to the effects of CP94253 observed in the repeatedly injected saline controls (Figure 1B), CP94253 had no effects on locomotion in injection-naïve mice (Figure 2). This finding suggests that the saline injections in mice from the previous experiment did indeed produce stress that affected spontaneous and cocaine-induced locomotor activity in a 5-HT_{1B}R-sensitive manner.

Experiment 3: CP94253 Prevents Cocaine-Primed Reinstatement of Extinguished Cocaine CPP

Approximately 40% of the mice preferred the side of the apparatus with horizontal stripes and ~60% preferred the side with vertical stripes, confirming the use of an unbiased apparatus. A repeated measures analysis across the baseline, CPP, and extinction tests showed a significant day by conditioning treatment interaction ($F_{(2,106)} = 13.23$, $p < 0.001$; Figure 3B). Subsequent analyses comparing saline to cocaine

conditioned groups on each test day showed a group difference on the CPP test day but no difference during baseline or extinction (Bonferroni t -test $t_{(51)} = 3.98$, $p < 0.001$). These results indicate that cocaine conditioning produced CPP that was abolished by extinction training. In the cocaine conditioned groups, a 2×2 ANOVA of time spent in the drug-paired side during the reinstatement test revealed a significant Pre-treatment $\times$ Priming injection interaction ($F_{(1,35)} = 4.26$, $p < 0.05$; Figure 3C). Subsequent *post hoc* analyses indicated that the cocaine-primed, saline-pretreated group showed significantly greater CPP than all other groups (Tukey tests, $p < 0.05$). In addition, comparisons of each group to its extinction baseline indicated that only the cocaine-primed group showed a significant increase in time spent in the drug-paired side relative to extinction baseline ($t_{(10)} = 4.1$, $p < 0.005$). Finally, the cocaine-primed, saline-pretreated group also showed a significantly greater amount of time spent in the drug-paired side relative to the saline controls ($t_{(23)} = 2.4$, $p < 0.05$). These results suggest that CP94253 attenuated cocaine-primed reinstatement of cocaine CPP.

DISCUSSION

This study yielded partial support for our hypothesis that mice would show a similar abstinence-dependent change in 5-HT_{1B}R modulation of cocaine effects as observed previously in rats (Pentkowski et al., 2009, 2012, 2014). We predicted that the 5-HT_{1B}R agonist CP94253 would facilitate cocaine-induced locomotion in mice given repeated daily injections of cocaine, but would inhibit this behavior after a 20-day period of abstinence, similar to the “switch” in 5-HT_{1B}R agonist effects observed in rats before and after abstinence from cocaine SA. Surprisingly, we found that CP94253 effects on locomotion were the same regardless of whether or not the mice received repeated injections of saline or cocaine (Figures 1B,C). We then conducted further analyses without the chronic treatment as a factor (Figure 1D). We found that acute administration of CP94253 initially increased spontaneous locomotion in mice tested on the 21st day of their chronic injections as predicted; however, the agonist did not alter spontaneous locomotion after a 21-day abstinence phase. Furthermore, the effects of the agonist on cocaine-induced locomotion only partially supported our predictions because CP94253 failed to alter this behavior initially, but did reverse the cocaine-sensitized hyperlocomotion observed after 20 days abstinence from daily repeated injections. Overall, the results are generally consistent with previous findings in rats of a facilitatory effect on cocaine-induced behavior prior to abstinence and an inhibitory effect after a prolonged period of abstinence.

We had expected that the chronic repeated cocaine injections would sensitize mice to the cocaine challenge given on the first test day and that this effect would be evident as greater locomotor activity in the chronic cocaine-injected group relative to the chronic saline-injected control group. Because there was no difference between these groups, we speculated that our chronic repeated saline injections may have stressed the mice in the experiment resulting in stress-induced cross-sensitization. Previous research has demonstrated cross-sensitization between repeated stress and repeated cocaine injections in both rats and mice (Sorg, 1992; Prasad et al., 1995; Kikusui et al., 2005; Maeda et al., 2006; Boyson et al., 2014), and repeated injections are stressful in both mice and rats (Ryabinin et al., 1999; Ferguson et al., 2009). Another possible stressor was that the control mice were cohoused with the cocaine-treated mice, which may have resulted in chronic social stress. Although we did not notice overt signs of stress such as aggression, Hoplight et al. (2007) have previously shown that saline-injected rats pair housed with cocaine-injected rats have altered 5HT_{1B}R profiles similar to that of cocaine treated rats, but not those housed with saline treated rats. To test this stress cross-sensitization hypothesis, we examined spontaneous and cocaine-induced locomotion in mice that were group housed and left undisturbed for 20 days except for tail-marking twice/week. In these control mice, the second cocaine challenge failed to sensitize locomotion in contrast to the sensitized locomotion observed in mice that were co-housed with cocaine-injected mice and given chronic saline injections. Furthermore, CP94253 failed

to alter either spontaneous or cocaine-induced locomotion on either test day in the noninjected control mice. It is important to note that these control mice were tested on two separate occasions after receiving CP94253 pretreatment, mitigating the idea that CP94253 may simply produce different effects the first vs. second time it is given. The different pattern of behavior across the chronic saline-injected and noninjected mice, coupled with the similar pattern of behavior in the chronic cocaine-injected and chronic saline-injected mice, support the interpretation that stress from repeated injection and living with cocaine-injected mice cross-sensitized the mice to cocaine. CP94253 reversed expression of the sensitized locomotion after a period of abstinence. Although the neural mechanisms underlying the stress cross-sensitization effects will require further investigation, one likely pathway contributing to these effects is the 5-HT_{1B}R-expressing medium spiny neurons projecting from nucleus accumbens (NAc) shell to the VTA. Previous research has shown that 5-HT_{1B}R located on GABAergic projection neurons from the NAc shell to the VTA may mediate stress cross-sensitization with psychostimulant drugs (Furay et al., 2011; Miczek et al., 2011; Nair et al., 2013).

Although we had predicted that CP94253 would attenuate cocaine-sensitized locomotion after a period of abstinence, a previous study by Przeglasiński et al. (2001) showed that while CP94253 dose-dependently enhances hyperlocomotion produced by acute amphetamine administration in mice, it does not affect amphetamine sensitization. The present findings seem discrepant with those of Pentkowski et al. (2009, 2012) however, we suggest that CP94253 may differentially alter locomotion induced by cocaine vs. amphetamines based on recent work from our laboratory demonstrating a different pattern of changes in cocaine vs. methamphetamine SA. Unlike the enhancement of cocaine SA prior to abstinence, CP94253 reduces methamphetamine SA both before and after abstinence (Garcia et al., 2017).

As we had predicted, CP94253 attenuated the cocaine-primed reinstatement of extinguished cocaine-CPP in mice that had a history of chronic cocaine administration followed by protracted abstinence prior to testing. Neither CP94253 pretreatment alone nor a saline prime prior to reinstatement testing altered preference. These control data suggest that reinstatement was specific to cocaine priming and that CP94253 specifically reversed the cocaine priming effect rather than nonspecifically altering preference. The findings are consistent with previous research suggesting that 5-HT_{1B}R agonists attenuate incentive motivational effects of cocaine priming injections in the operant extinction/reinstatement model (Przeglasiński et al., 2002, 2007; Pentkowski et al., 2014). Collectively, the studies suggest that 5-HT_{1B}R modulate the incentive motivational effects of a cocaine prime in both rats and mice (Parsons et al., 1998; Fletcher et al., 2002; Pentkowski et al., 2012, 2014).

Demonstrating effects of 5-HT_{1B}R agonists on psychostimulant-induced and conditioned behaviors in mice is important because transgenic mice are a valuable tool for investigating the neural mechanisms of these behaviors. A

leading hypothesis for the effects of the agonists on cocaine-induced behaviors suggests that 5-HT_{1B}Rs inhibit either GABAergic interneurons in the VTA or GABAergic medium spiny neurons projecting from the NAc to VTA, and this action disinhibits DA neurons (Parsons et al., 1999; Yan and Yan, 2001; Neumaier et al., 2002; O'Dell and Parsons, 2004; Barot et al., 2007; Hoplight et al., 2007). For instance, a microdialysis study suggests that stimulating 5-HT_{1B}Rs in the VTA inhibits GABA release from the neurons that tonically inhibit mesolimbic DA neurons. This leads to disinhibition of the mesolimbic DA neurons, increasing dopaminergic transmission in the NAc (O'Dell and Parsons, 2004). Because viral-mediated overexpression of 5-HT_{1B}Rs in this pathway attenuates cocaine intake after abstinence (Pentkowski et al., 2012), it is likely that cocaine abstinence causes adaptations within the 5-HT_{1B}R→GABAR→DA circuit in the VTA, which may underlie the inhibitory effects of 5-HT_{1B}R agonists on cocaine-induced behaviors that are observed following protracted abstinence. Transgenic mice may be useful in elucidating the neural circuitry involved in 5-HT_{1B}R agonists effects on cocaine-induced behavior.

In conclusion, this study demonstrates that a 5-HT_{1B}R agonist reverses expression of cocaine sensitization and blocks cocaine-primed reinstatement of cocaine-CPP in mice. These findings offer further support for the idea that serotonin inhibits incentive motivational effects of cocaine through an action at 5-HT_{1B}Rs. Furthermore, this research suggests that 5-HT_{1B}Rs may be a useful target for developing medications for cocaine

use disorders and that mice are a useful model for screening the potential anti-cocaine therapeutic effects of 5-HT_{1B}R agonists, as well as for investigating the neural mechanisms involved in 5-HT_{1B}R-mediated inhibition of the incentive motivational effects of cocaine.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. JLN, TSD-G and NSP: study concept and design. TSD-G, TC, SNS, KD, SJB and SNN: acquisition of data. TSD-G, JLN and SNP: analysis and interpretation of data. TSD-G and JLN: drafting of the manuscript. JLN and TSD-G: critical revision of the manuscript for important intellectual content. TSD-G and JLN: statistical analysis. JLN: obtained funding. TSD-G and JLN: study supervision.

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REFERENCES

- Acosta, J. I., Boynton, F. A., Kirschner, K. F., and Neisewander, J. L. (2005). Stimulation of 5-HT_{1B} receptors decreases cocaine- and sucrose-seeking behavior. *Pharmacol. Biochem. Behav.* 80, 297–307. doi: 10.1016/j.pbb.2004.12.001
- Ago, Y., Nakamura, S., Baba, A., and Matsuda, T. (2008). Neuropsychotoxicity of abused drugs: effects of serotonin receptor ligands on methamphetamine- and cocaine-induced behavioral sensitization in mice. *J. Pharmacol. Sci.* 106, 15–21. doi: 10.1254/jphs.fm0070121
- Bannai, M., Fish, E. W., Faccidomo, S., and Miczek, K. A. (2007). Anti-aggressive effects of agonists at 5-HT_{1B} receptors in the dorsal raphe nucleus of mice. *Psychopharmacology* 193, 295–304. doi: 10.1007/s00213-007-0780-5
- Barot, S. K., Ferguson, S. M., and Neumaier, J. F. (2007). 5-HT_{1B} receptors in nucleus accumbens efferents enhance both rewarding and aversive effects of cocaine. *Eur. J. Neurosci.* 25, 3125–3131. doi: 10.1111/j.1460-9568.2007.05568.x
- Boyson, C. O., Holly, E. N., Shimamoto, A., Albrechet-Souza, L., Weiner, L. A., DeBold, J. F., et al. (2014). Social stress and CRF-dopamine interactions in the VTA: role in long-term escalation of cocaine self-administration. *J. Neurosci.* 34, 6659–6667. doi: 10.1523/JNEUROSCI.3942-13.2014
- Chaouloff, F., Courvoisier, H., Moisan, M. P., and Mormède, P. (1999). GR 127935 reduces basal locomotor activity and prevents RU24969-, but not D-amphetamine-induced hyperlocomotion, in the Wistar-Kyoto hyperactive (WKHA) rat. *Psychopharmacology* 141, 326–331. doi: 10.1007/s002130050841
- Clark, M. S., and Neumaier, J. F. (2001). The 5-HT_{1B} receptor: behavioral implications. *Psychopharmacol. Bull.* 35, 170–185.
- DiRocco, D. P., Scheiner, Z. S., Sindreu, C. B., Chan, G. C., and Storm, D. R. (2009). A role for calmodulin-stimulated adenylyl cyclases in cocaine sensitization. *J. Neurosci.* 29, 2393–2403. doi: 10.1523/JNEUROSCI.4356-08.2009
- Ferguson, S. M., Sandygren, N. A., and Neumaier, J. F. (2009). Pairing mild stress with increased serotonin-1B receptor expression in the nucleus accumbens increases susceptibility to amphetamine. *Eur. J. Neurosci.* 30, 1576–1584. doi: 10.1111/j.1460-9568.2009.06933.x
- Filip, M., Alenina, N., Bader, M., and Przeglasiński, E. (2010). Behavioral evidence for the significance of serotonergic (5-HT) receptors in cocaine addiction. *Addict. Biol.* 15, 227–249. doi: 10.1111/j.1369-1600.2010.00214.x
- Fish, E. W., McKenzie-Quirk, S. D., Bannai, M., and Miczek, K. A. (2008). 5-HT_{1B} receptor inhibition of alcohol-heightened aggression in mice: comparison to drinking and running. *Psychopharmacology* 197, 145–156. doi: 10.1007/s00213-007-1017-3
- Fletcher, P. J., Azampanah, A., and Korth, K. M. (2002). Activation of 5-HT_{1B} receptors in the nucleus accumbens reduces self-administration of amphetamine on a progressive ratio schedule. *Pharmacol. Biochem. Behav.* 71, 717–725. doi: 10.1016/s0091-3057(01)00717-1
- Furay, A. R., McDevitt, R. A., Miczek, K. A., and Neumaier, J. F. (2011). 5-HT_{1B} mRNA expression after chronic social stress. *Behav. Brain Res.* 224, 350–357. doi: 10.1016/j.bbr.2011.06.016
- Garcia, R., Cotter, A. R., Leslie, K., Olive, M. F., and Neisewander, J. L. (2017). Preclinical evidence that 5-HT_{1B} receptor agonists show promise as medications for psychostimulant use disorders. *Int. J. Neuropsychopharmacol.* 20, 644–653. doi: 10.1093/ijnp/pyx025
- Geyer, M. A. (1996). Serotonergic function in arousal and motor activity. *Behav. Brain Res.* 73, 31–35. doi: 10.1016/0166-4328(96)00065-4
- Hoplight, B. J., Vincow, E. S., and Neumaier, J. F. (2007). Cocaine increases 5-HT_{1B} mRNA in rat nucleus accumbens shell neurons. *Neuropharmacology* 52, 444–449. doi: 10.1016/j.neuropharm.2006.08.013
- Kikusui, T., Faccidomo, S., and Miczek, K. A. (2005). Repeated maternal separation: differences in cocaine-induced behavioral sensitization in adult male and female mice. *Psychopharmacology* 178, 202–210. doi: 10.1007/s00213-004-1989-1

- Koe, K. B., Nielsen, J. A., Macor, J. E., and Heym, J. (1992). Biochemical and behavioral studies of the 5-HT_{1B} receptor agonist, CP-94253. *Drug Dev. Res.* 26, 241–250. doi: 10.1002/ddr.430260305
- Luo, Y., Good, C. H., Diaz-Ruiz, O., Zhang, Y., Hoffman, A. F., Shan, L., et al. (2010). NMDA receptors on non-dopaminergic neurons in the VTA support cocaine sensitization. *PLoS One* 5:e12141. doi: 10.1371/journal.pone.0012141
- Macor, J. E., Burkhart, C. A., Heym, J. H., Ives, J. L., Lebel, L. A., Newman, M. E., et al. (1990). 3-(1,2,5,6-Tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one: a potent and selective serotonin (5-HT_{1B}) agonist and rotationally restricted phenolic analogue of 5-methoxy-3-(1,2,5,6-tetrahydropyrid-4-yl)indole. *J. Med. Chem.* 33, 2087–2093. doi: 10.1021/jm00170a007
- Maeda, T., Yoshimatsu, T., Hamabe, W., Fukazawa, Y., Kumamoto, K., Ozaki, M., et al. (2006). Involvement of serine/threonine protein phosphatases sensitive to okadaic acid in restraint stress-induced hyperlocomotion in cocaine-sensitized mice. *Br. J. Pharmacol.* 148, 405–412. doi: 10.1038/sj.bjpp.0706769
- Markou, A., Weiss, F., Gold, L. H., Caine, S. B., Schulteis, G., and Koob, G. F. (1993). Animal models of drug craving. *Psychopharmacology* 112, 163–182. doi: 10.1007/bf02244907
- Miczek, K. A., Nikulina, E. M., Shimamoto, A., and Covington, H. E. III (2011). Escalated or suppressed cocaine reward, tegmental, BDNF and accumbal dopamine caused by episodic versus continuous social stress in rats. *J. Neurosci.* 31, 9848–9857. doi: 10.1523/JNEUROSCI.0637-11.2011
- Miszkiel, J., Filip, M., and Przeglinski, E. (2011). Role of serotonin 5-HT_{1B} receptors in psychostimulant addiction. *Pharmacol. Rep.* 63, 1310–1315. doi: 10.1016/S1734-1140(11)70695-8
- Nair, S. G., Furay, A. R., Liu, Y., and Neumaier, J. F. (2013). Differential effect of viral overexpression of nucleus accumbens shell 5-HT_{1B} receptors on stress- and cocaine priming-induced reinstatement of cocaine seeking. *Pharmacol. Biochem. Behav.* 112, 89–95. doi: 10.1016/j.pbb.2013.09.009
- Nasehi, M., Ghadimi, F., Khakpai, F., and Zarrindas, M. R. (2017). Interaction between harmaline, a class of β -carboline alkaloids and the CA1 serotonergic system in modulation of memory acquisition. *Neurosci. Res.* 122, 17–24. doi: 10.1016/j.neures.2017.03.011
- National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). *Guide for the Care and Use of Laboratory Animals*. 8th Edn. Washington, DC: National Academies Press.
- Neisewander, J. L., Cheung, T. H., and Pentkowski, N. S. (2014). Dopamine D3 and 5-HT_{1B} receptor dysregulation as a result of psychostimulant intake and forced abstinence: implications for medications development. *Neuropharmacology* 76, 301–319. doi: 10.1016/j.neuropharm.2013.08.014
- Neumaier, J. F., Vincow, E. S., Arvanitogiannis, A., Wise, R. A., and Carlezon, W. A. Jr. (2002). Elevated expression of 5-HT_{1B} receptors in nucleus accumbens efferents sensitizes animals to cocaine. *J. Neurosci.* 22, 10856–10863.
- Oberlander, C., Blaquiére, B., and Pujol, J. F. (1986). Distinct functions for dopamine and serotonin in locomotor behavior: evidence using the 5HT₁ agonist RU24969 in globus pallidus-lesioned rats. *Neurosci. Lett.* 67, 113–118. doi: 10.1016/0304-3940(86)90382-4
- Oberlander, C., Demasse, Y., Verdu, A., Van de Velde, D., and Bardelay, C. (1987). Tolerance to the serotonin 5-HT₁ agonist RU24969 and effects on dopaminergic behavior. *Eur. J. Pharmacol.* 139, 205–214. doi: 10.1016/0014-2999(87)90253-6
- O'Dell, L. E., and Parsons, L. H. (2004). Serotonin_{1B} receptors in the ventral tegmental area modulate cocaine-induced increases in nucleus accumbens dopamine levels. *J. Pharmacol. Exp. Ther.* 311, 711–719. doi: 10.1124/jpet.104.069278
- Parsons, L. H., Koob, G. F., and Weiss, F. (1999). RU 24969, a 5-HT_{1B/1A} receptor agonist, potentiates cocaine induced increases in nucleus accumbens dopamine. *Synapse* 32, 132–135. doi: 10.1002/(sici)1098-2396(199905)32:2<132::aid-syn6>3.0.co;2-v
- Parsons, L. H., Weiss, F., and Koob, G. F. (1998). Serotonin_{1B} receptor stimulation enhances cocaine reinforcement. *J. Neurosci.* 18, 10078–10089.
- Pentkowski, N. S., Acosta, J. I., Browning, J. R., Hamilton, E. C., and Neisewander, J. L. (2009). Stimulation of 5-HT_{1B} receptors enhance cocaine reinforcement yet reduces cocaine-seeking behavior. *Addict. Biol.* 14, 419–430. doi: 10.1111/j.1369-1600.2009.00162.x
- Pentkowski, N. S., Cheung, T. H., Toy, W. A., Adams, M. D., Neumaier, J. F., and Neisewander, J. L. (2012). Protracted withdrawal from cocaine self-administration flips the switch on 5-HT_{1B} receptor modulation of cocaine abuse-related behaviors. *Biol. Psychiatry* 72, 396–404. doi: 10.1016/j.biopsych.2012.03.024
- Pentkowski, N. S., Harder, B. G., Brunwasser, S. J., Bastle, R. M., Peartree, N. A., Yanamandra, K., et al. (2014). Pharmacological evidence for an abstinence-induced switch in 5-HT_{1B} receptor modulation of cocaine self-administration and cocaine-seeking behavior. *ACS Chem. Neurosci.* 5, 168–176. doi: 10.1021/cn400155t
- Prasad, B. M., Sorg, B. A., Ulibarri, C., and Kalivas, P. W. (1995). Sensitization to stress and psychostimulants. Involvement of dopamine transmission versus the HPA axis. *Ann. N.Y. Acad. Sci.* 771, 617–625. doi: 10.1111/j.1749-6632.1995.tb44714.x
- Przeglinski, E., Filip, M., Papla, I., and Siwanowicz, J. (2001). Effect of serotonin (5-HT_{1B}) receptor ligands on cocaine sensitization in rats. *Behav. Pharmacol.* 12, 109–116. doi: 10.1097/00008877-200104000-00004
- Przeglinski, E., Golda, A., Frankowska, M., Zaniewska, M., and Filip, M. (2007). Effects of serotonin 5-HT_{1B} receptor ligands on the cocaine- and food-maintained self-administration in rats. *Eur. J. Pharmacol.* 559, 165–172. doi: 10.1016/j.ejphar.2006.12.012
- Przeglinski, E., Papla, I., Siwanowicz, J., and Filip, M. (2004). Effects of 5-HT_{1B} receptor ligands microinjected into the ventral tegmental area on the locomotor and sensitizing effects of cocaine in rats. *Eur. Neuropsychopharmacol.* 14, 217–225. doi: 10.1016/s0924-977x(03)00106-8
- Przeglinski, E., Siwanowicz, J., Papla, I., and Filip, M. (2002). Effects of 5-HT_{1B} receptor ligands, microinjected into the accumbal shell or core on the sensitization to cocaine in rats. *Eur. Neuropsychopharmacol.* 12, 387–396. doi: 10.1016/s0924-977x(02)00052-4
- Rao, A., Sorkin, A., and Zahniser, N. R. (2013). Mice expressing markedly reduced striatal dopamine transporters exhibit increased locomotor activity, dopamine uptake turnover rate, and cocaine responsiveness. *Synapse* 67, 668–677. doi: 10.1002/syn.21671
- Riday, T. T., Kosofsky, B. E., and Malanga, C. J. (2012). The rewarding and locomotor-sensitizing effects of repeated cocaine administration are distinct and separable in mice. *Neuropharmacology* 62, 1858–1866. doi: 10.1016/j.neuropharm.2011.12.011
- Robison, A. J., Vialou, V., Mazei-Robison, M., Feng, J., Kourrich, S., Collins, M., et al. (2013). Behavioral and structural responses to chronic cocaine require a feedforward loop involving Δ FosB and calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell. *J. Neurosci.* 33, 4295–4307. doi: 10.1523/JNEUROSCI.5192-12.2013
- Ryabinin, A. E., Wang, Y. M., and Finn, D. A. (1999). Different levels of Fos immunoreactivity after repeated handling and injection stress in two inbred strains of mice. *Pharmacol. Biochem. Behav.* 63, 143–151. doi: 10.1016/s0091-3057(98)00239-1
- Saudou, F., Amara, D. A., Dierich, A., LeMeur, M., Ramboz, S., Segu, L., et al. (1994). Enhanced aggressive behavior in mice lacking 5-HT_{1B} receptor. *Science* 265, 1875–1878. doi: 10.1126/science.8091214
- Shaham, Y., Shalev, U., Lu, L., De Wit, H., and Stewart, J. (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology* 168, 3–20. doi: 10.1007/s00213-002-1224-x
- Shuman, T., Cai, D. J., Sage, J. R., and Anagnostaras, S. G. (2012). Interactions between modafinil and cocaine during the induction of conditioned place preference and locomotor sensitization in mice: implications for addiction. *Behav. Brain Res.* 235, 105–112. doi: 10.1016/j.bbr.2012.07.039

- Sorg, B. A. (1992). Mesocorticolimbic dopamine systems: cross-sensitization between stress and cocaine. *Ann. N Y Acad. Sci.* 28, 136–144. doi: 10.1111/j.1749-6632.1992.tb25962.x
- Tatarczyńska, E., Antkiewicz-Michaluk, L., Kłodzińska, A., Stachowicz, K., and Chojnacka-Wójcik, E. (2005). Antidepressant-like effect of the selective 5-HT_{1B} receptor agonist CP 94253, a possible mechanism of action. *Eur. J. Pharmacol.* 516, 46–50. doi: 10.1016/j.ejphar.2005.04.025
- Tatarczyńska, E., Kłodzińska, A., Stachowicz, K., and Chojnacka-Wójcik, E. (2004). Effects of a selective 5-HT_{1B} receptor agonist and antagonists in animal models of anxiety and depression. *Behav. Pharmacol.* 15, 523–534. doi: 10.1097/00008877-200412000-00001
- Thompson, D., Martini, L., and Whistler, J. L. (2010). Altered ratio of D1 and D2 dopamine receptors in mouse striatum is associated with behavioral sensitization to cocaine. *PLoS One* 5:e11038. doi: 10.1371/journal.pone.0011038
- Tilley, M. R., Cagniard, B., Zhuang, X., Han, D. D., Tiao, N., and Gu, H. H. (2007). Cocaine reward and locomotion stimulation in mice with reduced dopamine transporter expression. *BMC Neurosci.* 8:42. doi: 10.1186/1471-2202-8-42
- Yan, Q. S., and Yan, S. E. (2001). Serotonin-1B receptor-mediated inhibition of [³H]GABA release from rat ventral tegmental area slices. *J. Neurochem.* 79, 914–922. doi: 10.1046/j.1471-4159.2001.00643.x

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Addendum: Effects of a 5-HT_{1B} Receptor Agonist on Locomotion and Reinstatement of Cocaine-Conditioned Place Preference after Abstinence from Repeated Injections in Mice

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DISCREPANCY IN ANIMAL PROTOCOL

The authors have identified a discrepancy between the 15 mg/kg, IP cocaine priming dose used in this study, and the IACUC approved protocol dose of 10 mg/kg, IP. The dose of 15 mg/kg, IP cocaine used for the repeated drug injections in this study is correct and approved in the same IACUC protocol and caused no pain or discomfort to mice.

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Discrete Serotonin Systems Mediate Memory Enhancement and Escape Latencies after Unpredicted Aversive Experience in *Drosophila* Place Memory

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Feedback mechanisms in operant learning are critical for animals to increase reward or reduce punishment. However, not all conditions have a behavior that can readily resolve an event. Animals must then try out different behaviors to better their situation through outcome learning. This form of learning allows for novel solutions and with positive experience can lead to unexpected behavioral routines. Learned helplessness, as a type of outcome learning, manifests in part as increases in escape latency in the face of repeated unpredicted shocks. Little is known about the mechanisms of outcome learning. When fruit fly *Drosophila melanogaster* are exposed to unpredicted high temperatures in a place learning paradigm, flies both increase escape latencies and have a higher memory when given control of a place/temperature contingency. Here we describe discrete serotonin neuronal circuits that mediate aversive reinforcement, escape latencies, and memory levels after place learning in the presence and absence of unexpected aversive events. The results show that two features of learned helplessness depend on the same modulatory system as aversive reinforcement. Moreover, changes in aversive reinforcement and escape latency depend on local neural circuit modulation, while memory enhancement requires larger modulation of multiple behavioral control circuits.

Keywords: serotonin, learning, memory, learned helplessness, *Drosophila melanogaster*

INTRODUCTION

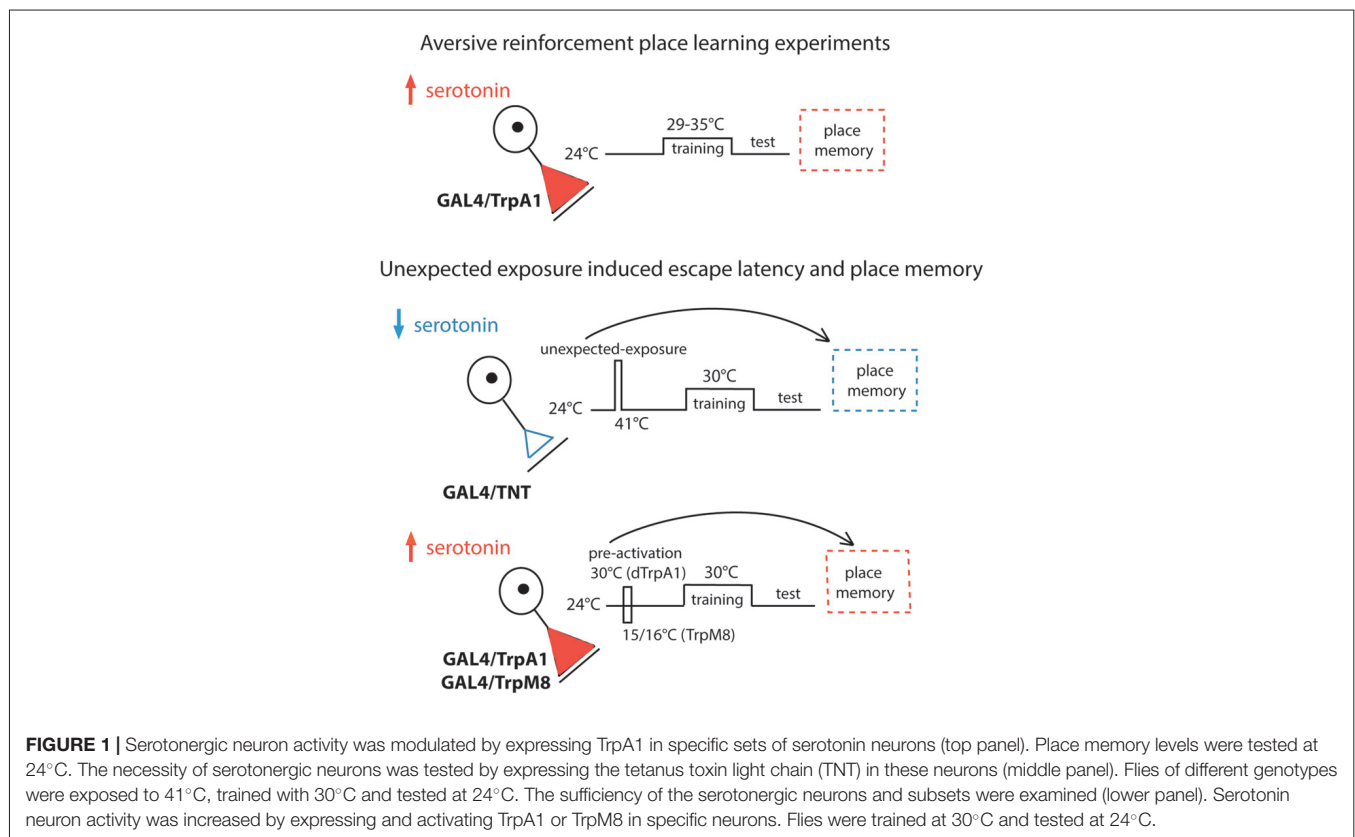
Skinner coined the term operant conditioning to describe a form of associative learning where organisms learn from the consequences of their own behavior (Skinner, 1950). Mechanisms underlying operant learning have been extensively explored in invertebrate and vertebrate animals as these represent an approach to understand the basis of goal directed behaviors. Operant learning is critically dependent on feedback mechanisms that can modify future decision making and action selection processes. This gives an animal the required flexibility to try out different behaviors in an attempt to better their situation through “outcome learning” (Maier and Watkins, 2005; Heisenberg, 2014, 2015). While operant learning allows for generation of novel solutions and with positive experience can lead to selection of unexpected behavioral routines, the underlying neuronal basis remains largely unexplored.

The external conditions an organism faces can often be unpredictable, uncontrollable and dangerous. As the primary example of outcome learning in learned helplessness, dogs took longer to learn to escape foot shocks after they were exposed to uncontrollable electric shocks (Seligman, 1972). This phenomenon has been investigated in other vertebrate and invertebrate animals, but most intensively in rats and mice (e.g., Maier and Watkins, 2005; Yang et al., 2013; Batsching et al., 2016; Kim et al., 2016). While upregulation of serotonin and corticotrophin-release factor systems within the dorsal raphe nucleus is causally related to development of learned helplessness, other neurochemical systems and brain structures have also been implicated (Maier and Watkins, 2005; Kim et al., 2016).

A key feature of learned helplessness in vertebrate and invertebrate animals is a deficit or delay in escaping/avoiding aversive events, but little is known about the neural mechanisms underlying the increase in escape latency (Maier and Watkins, 2005; Yang et al., 2013; Batsching et al., 2016). When *Drosophila* are exposed to unpredicted aversive temperatures in the heat-box place learning paradigm, flies also increase escape latencies (Wustmann et al., 1996; Sitaraman et al., 2007; Sitaraman and Zars, 2010; Yang et al., 2013; Ostrowski and Zars, 2014; Batsching et al., 2016). Moreover, and intriguingly, flies have a robust place memory when given control of a place/temperature contingency. Serotonin is the only biogenic

amine shown to be necessary for *Drosophila* place memory (Sitaraman et al., 2008). It is not clear if the unpredicted exposure induced changes in escape latency and memory require serotonin.

Here we investigated the role of discrete neuronal circuits underlying aversive reinforcement, escape latencies and memory levels in the presence and absence of unexpected aversive events. That is, we asked if serotonin and specific subsets of serotonergic neurons mediate the reinforcing signal for aversive place memory. Furthermore, we explored if specific subsets of serotonergic neurons are necessary and sufficient for the effect of unexpected exposures on increases in escape latency and memory performance. Using an array of genetic tools targeting the serotonin neurons we discovered that aversive reinforcement and escape latency depend on local neural circuit modulation, while memory enhancement requires larger, perhaps bulk, modulation of multiple behavioral control circuits. Thus, two features of learned helplessness, increases in escape latency and changes in memory formation, depend on the same modulatory system as aversive reinforcement. Learned helplessness has been widely cited as a model for anxiety and depression resulting from real or perceived absence of control over the outcome of a situation. In addition to the conserved role of serotonin, our studies in an experimentally tractable system will pave the way for characterizing the precise circuit mechanisms underlying outcome learning.



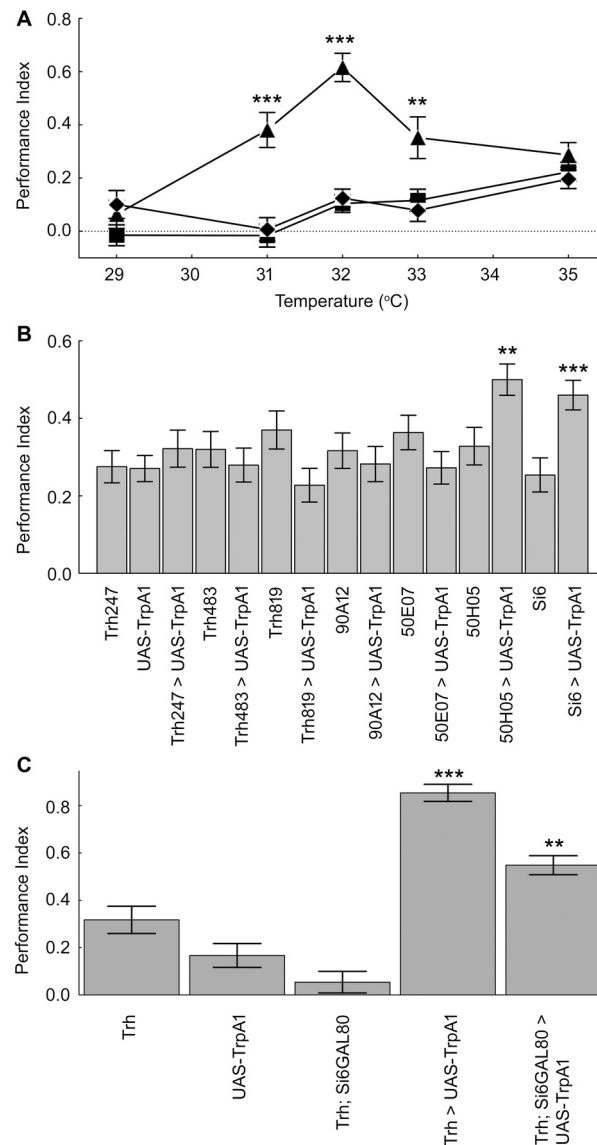


FIGURE 2 | Subsets of the serotonergic system are necessary and sufficient for operant feedback in aversive place memory. **(A)** Activation of serotonergic neurons substitutes for high temperature feedback. Trh-Gal4/TrpA1 (▲), Gal4/+ (◆) and TrpA1/+ (■) flies were conditioned using 29–35°C. Experimental flies had higher place memories than control flies at three temperatures (29°C $H_{(2,N=274)} = 5.14$, $p = 0.08$; 31°C $H_{(2,N=273)} = 27.22$, $p < 0.0001$; 32°C $H_{(2,N=419)} = 70.25$, $p < 0.0001$; 33°C $H_{(2,N=234)} = 12.84$, $p = 0.0016$; 35°C $H_{(2,N=574)} = 2.64$, $p = 0.27$). **(B)** Flies expressing TrpA1 in subsets of serotonergic neurons with the 50H05 and Si6-GAL4 driver had higher memory scores when trained with 32°C compared to genetic control flies ($H_{(14,N=3627)} = 56.3$, $p < 0.00001$; P 's $< 0.01 = **$ and $0.001 = ***$ compared to genetic controls after multiple comparisons). **(C)** A subsystem of serotonergic neurons is necessary for normal place memory. Trh-GAL4; TrpA1 flies showed high place memory when conditioned with 32°C compared to genetic control flies, and Si6-GAL80 reduces the induced place memory ($H_{(4,N=1230)} = 152.7$, $p < 0.0001$; $P < 0.0001 = ***$ for the Trh-GAL4; TrpA1 compared to control genotypes; $P < 0.01 = **$ for Trh-GAL4/Si6-GAL80; TrpA1 compared to Trh-GAL4; TrpA1 after multiple comparisons). Values represent mean and SEMs in all figures.

EXPERIMENTAL PROCEDURES

Behavior

Individual flies were conditioned in the heat-box, a set of long narrow chambers. A single fly is allowed to roam in the chamber, and they usually walk from chamber end to chamber end (Zars et al., 2000; Sitaraman et al., 2008). The chamber dimensions are 34 mm long, 1 mm high and 3 mm wide.

The top and bottom of the chambers are lined with Peltier elements, and temperature is finely controlled within 0.1°C of a called temperature using custom software and thermocouples (Zars et al., 2000). During training, one half of the chamber is associated with rising temperatures with a pre-determined maximum. That is, when a fly moves to the front half of a chamber, the temperature of the whole chamber rises to a maximum temperature. When the fly goes back across the

invisible midline to the rear of the chamber, the whole chamber begins to cool. It takes 3–4 s for the temperature to rise to the maximum or fall again to the cool temperature. Flies typically avoid the chamber-half associated with a high temperature and continue to do so even after the chamber temperature is reset to the preferred 24°C (Wustmann et al., 1996; Kahsai and Zars, 2011). Unexpected exposures were presented as three 1-min exposures to temperatures of 41°C for normal flies (Sitaraman et al., 2007), or other temperatures as indicated for the TrpA1 and TrpM8 experiments. Flies were allowed a rest of 4 min after the unexpected exposure, and then conditioned with a mid-level temperature of 30°C.

Control experiments were done to determine if flies can sense and avoid the temperature used in conditioning. In this case, the temperature in one half of the chamber is raised relative to the control temperature of 24°C. Avoidance of two temperatures, 30 or 41°C, was tested (Zars, 2001). In this case, the rise and fall of temperatures is independent of fly behavior. The response of flies to the temperature gradient is used to test the ability of flies to sense and avoid high temperatures.

The position of each fly is measured every tenth of a second with a spatial resolution of 0.2 mm. Whether a fly is on the punishment associated half of the chamber, or the other side, is used to calculate a Performance Index. The Performance Index is calculated as the time spent on the punishment-associated side minus time spent on the unpunishment associated side divided by total time within a session. There is little ambiguity in where an individual fly is located and when a fly transitions between chamber halves. The maximum error in determining where a fly is located is in the 0.5% range (0.2 mm/34 mm). The maximum error in determining when a fly has transitioned between chamber halves is also small. A fly will typically transition three or four times between chamber halves in a 1 min pre-test phase. This would give a $0.1 \text{ s} \times 4/60 \text{ s}$ calculation of about 0.7%. To avoid a side bias in calculations of a Performance Index, approximately 50% of flies are trained to avoid the front half of the chamber. The other 50% are trained to avoid the back half of the chamber. Largely equal numbers of flies from all genotypes were tested in parallel over several weeks. The number of flies from each experiment is listed in the H-statistics in the figure legends. While the behavioral experiments were not done blind to genotype, data is objectively collected with an automated conditioning apparatus and analytic software.

***Drosophila* Husbandry**

Genetic crosses followed typical methods. The GAL4 and effector lines were introgressed with a cantonized white strain (wCS10) and then the X-chromosomes were replaced with a wild-type version in some lines to prevent *white*-mutant effects on learning behavior (Diegelmann et al., 2006). Flies tested for behavior were 2–7 days old, and raised on cornmeal food in an insectary at 25°C, unless otherwise noted, and 60% humidity.

Immunohistochemistry

Brains from 4–10 day-old females were dissected in $1 \times$ PBS and fixed in 4% paraformaldehyde overnight at 4°C. After 4×10 -min

washing in PAT (0.5% Triton X-100, 0.5% bovine serum albumin in phosphate-buffered saline), tissues were blocked in 3% normal goat serum (NGS) for 90 min, then incubated in primary antibodies diluted in 3% NGS for 12–24 h at 4°C, then washed in PAT, and incubated in secondary antibodies diluted in 3% NGS for 1–2 days at 4°C. Tissues were then washed thoroughly in PAT and mounted using Vectashield (Vector lab, CA, USA) for imaging. Antibodies used were rabbit anti-GFP (Invitrogen A11122) 1:1000, mouse anti-Serotonin (Abcam ab6336) 1:30 and secondary Alexa Fluor 488 and 568 antibodies (1:500). Samples were imaged on a Zeiss 510 confocal microscope (Sitaraman et al., 2008).

Generation of Si6 GAL4 and GAL80 Lines

The potential Si6 enhancer was amplified with the primers: GCT TTATTAAATTCCTCAATCCCA and TTCGGTTAATTAACCTCAAGCA. The cloned fragment was subcloned into Gateway donor and the germline transformation vectors pBPGUW with GAL4 and GAL80 regulators (Pfeiffer et al., 2008). Transgenes were targeted to the 3rd chromosome landing site attP2 by the company Genetic Services, Inc. (Sudbury, MA, USA).

Statistics

Statistical comparisons used non-parametric tests with a Kruskal Wallis ANOVA with multiple comparisons when warranted by significance of the main effect. *P*-values less than 0.05 were considered significant, and marked as $P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$; (Kahsai and Zars, 2011). All data were compared with Statistica software version 8.

RESULTS

General Approach

We investigated the role of serotonergic neurons and serotonergic neuron subsets in regulating place memory and the effects of unexpected exposures to high temperature on escape latencies and memory levels (**Figure 1**). In the first set of behavioral experiments, we increased serotonergic neuron activity by expressing TrpA1 in specific sets of serotonin neurons and trained flies at temperatures that activate TrpA1, but are not otherwise reinforcing (**Figure 1**, top panel). The place memory levels were tested at the baseline temperature of 24°C. In the second set of behavioral experiments, the necessity of serotonergic neurons for changes in escape latencies during training and place memory enhancement after unexpected exposure to high temperature was tested (**Figure 1**, middle panel). The serotonergic neuron activity was blocked by expressing the tetanus toxin light chain (TNT) in these neurons. Flies of different genotypes were exposed to 41°C and trained with the moderate temperature of 30°C. Place memory was tested at 24°C. In the third set of behavioral experiments, the sufficiency of the serotonergic neurons and subsets were examined for changes in escape latencies and place memory (**Figure 1**, lower panel). Serotonin neuron activity was increased by expressing TrpA1 or TrpM8 in specific neurons and exposing flies to temperatures that activate these channels. Flies were then trained at 30°C.

Escape latencies during training and memory at 24°C was tested.

Serotonergic Neurons Are Necessary and Sufficient for Aversive Reinforcement of Place Memory

The serotonergic system is the only biogenic amine system known to be necessary for *Drosophila* place memory (Sitaraman et al., 2008; Kahsai and Zars, 2011; Ostrowski and Zars, 2014). In the *Drosophila* brain the serotonergic system is comprised of ~40 neurons per hemisphere (Sitaraman et al., 2008; Alekseyenko et al., 2010, 2014; Lee et al., 2011), and these neurons broadly innervate the central brain. To manipulate all or nearly all of these neurons, a Trh-GAL4 driver (Sadaf et al., 2012) was used to drive expression of the thermogenetic effector TrpA1, which can increase neuronal activity at specific temperatures with high temporal precision (Hamada et al., 2008). To address the sufficiency of the serotonergic system in providing aversive reinforcement, extrinsic activation of serotonergic neurons was paired with a behavioral routine. That is, the behavior that takes a fly to one end of the chamber was paired with activation of the serotonergic neurons, thus experimentally closing the loop between behavior and activation of this set of neurons. In this case the aversive high temperature feedback was replaced by temperatures that activate TrpA1 in serotonergic neurons. The temperature range of TrpA1 activation (Pulver et al., 2009) is much lower than those used for high temperature reinforcement allowing for a clear dissociation of serotonin activation and aversive reinforcement. The Trh-GAL4/TrpA1 flies conditioned with 31, 32 and 33°C, temperatures that induce TrpA1 activation,

had high memory levels compared to control flies (Figure 2A). Temperatures outside of the activation range of TrpA1 did not support memory formation (Figure 2A). Thus, serotonergic activation can act as an aversive reinforcer (Figure 2).

We next asked if a subset of the serotonergic system can be sufficient for aversive reinforcement in place memory. Seven GAL4 drivers that are expressed in subsets of the serotonergic system were screened for effects on place memory using the TrpA1 effector (Pfeiffer et al., 2008; Lee et al., 2011). These GAL4 lines are from cloned enhancers from genes that are expressed in serotonin neurons (Pfeiffer et al., 2008; Lee et al., 2011), and represent both broad and more restricted expression in the serotonergic neuron set. We had no prediction of which of these lines might influence place memory, but reasoned that this set of GAL4 drivers might identify subsets of critical serotonin neurons since the drivers were expressed in many different serotonin neurons. Of these lines, two were found to have an effect on this direct conditioning. We found that an enhancer from the sixth intron of the SerT gene (Si6-GAL4) when combined with the TrpA1 transgene was sufficient for aversive reinforcement (Figure 2B). When TrpA1 was expressed in the neurons from Si6-GAL4 and flies were trained with 32°C, place memory after training was significantly higher in the experimental flies compared to flies from the control genotypes (Figure 2B). Moreover, a second driver 50H05 when combined with TrpA1 also had significant place memory when conditioned with 32°C.

Flies from all genotypes were tested in control experiments for the ability to sense and avoid a high temperature source. In contrast to conditioning experiments, where temperatures rise

TABLE 1 | Control avoidance behavior in serotonin altered flies.

Genotype	N	30°C	41°C
Trh-GAL4/TrpA1	93	0.14 ± 0.05	0.66 ± 0.05
Trh-GAL4/+	93	0.20 ± 0.05	0.72 ± 0.03
TrpA1/+	99	0.16 ± 0.05	0.71 ± 0.04
Ddc-GAL4, THGAL80/TNT	74	0.13 ± 0.08	0.71 ± 0.05
Ddc-GAL4, THGAL80/+	70	0.14 ± 0.08	0.71 ± 0.03
TNT/+	68	0.16 ± 0.06	0.70 ± 0.06
Trh-GAL4/TNT	81	0.11 ± 0.08	0.54 ± 0.06
Trh-GAL4/+	87	0.14 ± 0.07	0.60 ± 0.06
TNT/+	81	0.19 ± 0.07	0.58 ± 0.06
Trh-GAL4/TrpM8	125	0.16 ± 0.07	0.68 ± 0.06
Trh-GAL4/+	129	0.20 ± 0.06	0.73 ± 0.05
TrpM8/+	133	0.19 ± 0.05	0.65 ± 0.05
TrpA1/+	139	0.21 ± 0.02	0.75 ± 0.02
Trh-GAL4/+	110	0.19 ± 0.04	0.75 ± 0.02
Trh-GAL4/TrpA1	106	0.19 ± 0.04	0.73 ± 0.04
Si6-GAL4/+	100	0.27 ± 0.04	0.80 ± 0.03
Si6-GAL4/TrpA1	93	0.21 ± 0.03	0.70 ± 0.03
Trh-GAL4/Si6-GAL80	140	0.22 ± 0.02	0.76 ± 0.02
Trh-GAL4/Si6GAL80/TrpA1	147	0.18 ± 0.03	0.72 ± 0.03

Statistics: Trh-GAL4/TrpA1: 30°C, $H_{(2, N=285)} = 1.9$, $p = 0.37$; 41°C = 0.28, $p = 0.87$. Ddc-GAL4, TH-GAL80/TNT: 30°C, $H_{(2, N=208)} = 0.66$, $p = 0.72$, 41°C = 2.43, $p = 0.29$. Trh-GAL4/TNT: 30°C, $H_{(2, N=249)} = 0.81$, $p = 0.6$, 41°C = 2.75, $p = 0.25$. Trh-GAL4/TrpM8: 30°C, $H_{(2, N=387)} = 0.107$, $p = 0.95$, 41°C = 3.99, $p = 0.14$. Si6-GAL4/TrpA1: 30°C, $H_{(8, N=835)} = 63.6$, $p < 0.01$, $P < 0.05$ for Si6-GAL4/+ compared to TrpA1/+, other relevant P 's = n.s.; 41°C = 16.6, $p < 0.05$, P 's n.s. after multiple comparisons.

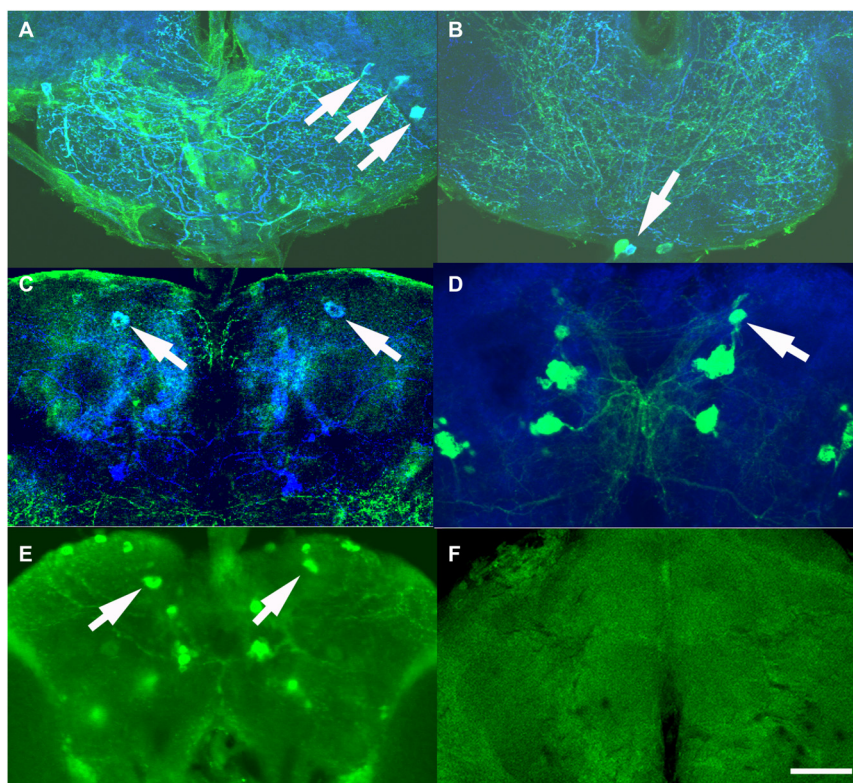


FIGURE 3 | Expression pattern of the serotonin drivers Si6-GAL4, 50H05-GAL4 and Si6-GAL80. **(A–C)** Si6-GAL4 driving UAS-GFP fly brains were co-labeled with anti-GFP (green) and anti-serotonin (blue). **(A)** In an anterior ventral region, three serotonergic neurons are co-labeled (white arrows). A few other small GFP-positive but serotonin negative neurons can also be seen in this region. These SE neurons appear to densely innervate the sub-esophageal ganglion here. **(B)** Multiple GFP neurons are again labeled in this ventral but less anterior section, only one neuron appears to be co-labeled with anti-serotonin (arrow). This serotonergic neuron appears to also innervate the sub-esophageal ganglion. **(C)** In a dorsal posterior section, one pair of large PMP neurons is co-labeled with GFP and anti-serotonin (arrows), termed dorsal PMP neurons (dPMP). **(D)** 50H05-GAL4 driving UAS-GFP brains were co-labeled with anti-GFP (green) and the synapse marker *bruchpilot* (blue). Multiple neurons are labeled in the PMP cluster, including the dPMP neurons. **(E)** Labeling with anti-serotonin in wild-type flies shows multiple PMP neurons, including the dPMP neurons (arrows). **(F)** Addition of a Si6-GAL80 element to the Si6-GAL4 driver suppresses UAS-GFP expression. This is an anterior frontal optical section. Scale bar represents 20 μm in **(A,B)** and 50 μm in **(C–E)**.

and fall depending on where a fly moves in the chamber, the thermosensitivity assay employs a temperature step gradient that is maintained regardless of the behavior of a fly (Zars, 2001). This simpler test asks whether a fly can sense a temperature difference between the preferred 24 and 30 or 41°C. The side of the chamber with the higher temperature was switched when the temperatures changed to force flies to show a temperature preference. Flies from the experimental and control genotypes did not have altered control behaviors (Table 1). Thus, since flies of all genotypes showed that they can sense and avoid high temperatures in the thermosensitivity test, but the experimental flies show an altered memory phenotype indicates that it is memory formation that is specifically altered in these flies.

The Si6-GAL4 neurons are also necessary for normal place memory. We made an Si6-GAL80 line to suppress the potential activity of GAL4 in these neurons. Si6-GAL80 expresses the GAL80 transcription repressor (GAL4 inhibitor) under the control of Si6 enhancer and thereby restricts/eliminates transgene expression in Si6 positive neurons (Lee et al., 2000). Combining the Si6-GAL80 element with TrH-GAL4

and the TrpA1 transgene led to a partial but significant reduction in the place memory that is formed with activation of all of the serotonergic system using the TrH-GAL4 driver (Figure 2C). Again, flies from the different genotypes did not have altered temperature avoidance (Table 1).

We next examined the expression pattern of the Si6- and 50H05 GAL4 drivers. Double labeling experiments (GFP and anti-serotonin) show that Si6-GAL4 drives expression in five serotonergic neurons per brain hemisphere. These include neurons in the SE2 and SE3 clusters and one pair of neuron in the PMP cluster (Figures 3A–C). Based on cell body location, this pair of serotonergic neurons was also identified with the 50H05 driver (Figures 3D,E). 50H05-GAL4 is derived from an intron of the fly serotonin transporter gene and co-labeling with anti-serotonin antibodies revealed that 50H05-GAL4 expresses in 25 serotonergic neurons in each brain hemisphere (Albin et al., 2015). We refer to the neurons that show overlap between the 50H05 and Si6-Gal4 as dorsal (d) PMP neurons, and are different from the DP neurons of Giang et al. (2011)

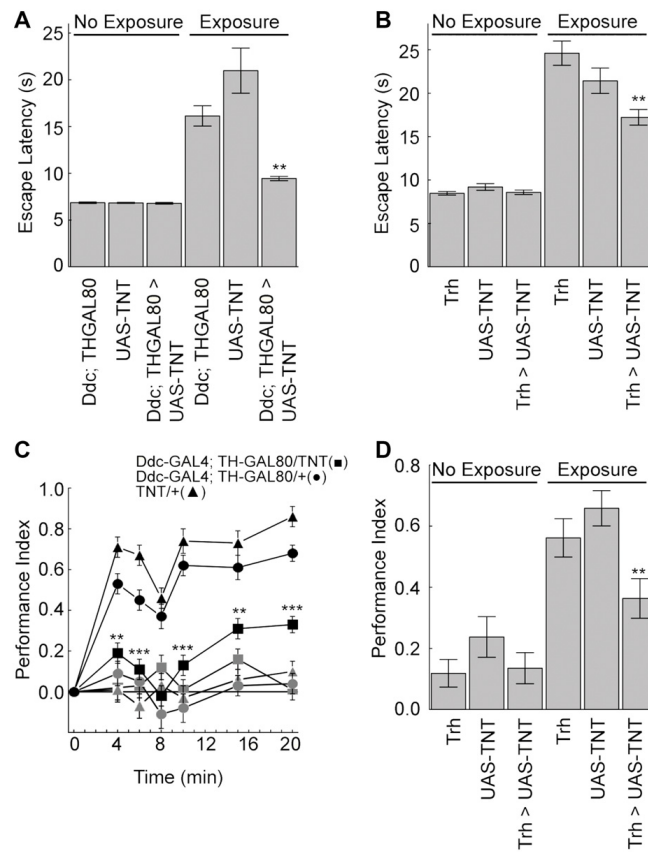


FIGURE 4 | The serotonergic neurons are necessary for the unexpected exposure increases in escape latency and enhancement of place memory.

(A,B) Unexpected exposure to high temperatures increases escape latencies in control flies, but blocking synaptic transmission with UAS-TNT expression in the serotonergic neurons decreases this effect (Ddc-GAL4; TH-GAL80 experiments ($H_{(5,N=36314)} = 200.7$, $p < 0.0001$; $P < 0.0001$ for exposed control genotypes compared to non-exposed; $P < 0.01 = **$ for exposed experimental flies compared to exposed control genotypes); Trh-GAL experiments ($H_{(5,N=21366)} = 154.2$, $p < 0.0001$; $P < 0.0001$ for exposed control genotypes compared to non-exposed; $P < 0.01 = **$ for exposed experimental flies compared to exposed control genotypes). **(C)** Flies with the tetanus toxin light chain (UAS-TNT) expressed with Ddc-GAL4; TH-GAL80 serotonergic neurons (■) and control genotypes (●) and (▲) were conditioned for 4–20 min at 30°C with (dark symbols) or without (light symbols) unexpected exposure. Expression of TNT in the serotonergic neurons reduces the enhancement of place memory (4 min, $H_{(5,N=553)} = 132.25$, $p < 0.0001$; 6 min, $H_{(5,N=486)} = 124.05$, $p < 0.0001$; 8 min, $H_{(5,N=428)} = 57.85$, $p < 0.0001$; 10 min, $H_{(5,N=433)} = 125.41$, $p < 0.0001$; 15 min, $H_{(5,N=477)} = 103.74$, $p < 0.0001$; 20 min, $H_{(5,N=615)} = 212.66$, $p < 0.0001$; P 's $< 0.01 = **$ and $< 0.001 = ***$ comparing the experimental group to control genotypes after multiple comparisons). **(D)** Unexpected enhancement of place memory requires Trh-GAL4 neurons ($H_{(5,N=734)} = 97.6$, $p < 0.0001$; $P < 0.01 = **$ comparing the experimental group to control genotypes after multiple comparisons).

since the dPMP neuron pair is far posterior to the DP neurons. The Si6- and 50H05- GAL4 serotonergic neurons' innervation pattern includes the sub-esophageal ganglion, the median bundle and discrete parts of the superior medial protocerebrum. When Si6-GAL4 was crossed to Si6-GAL80, all GFP expression in Si6-GAL4-positive neurons was blocked (Figure 3F).

It could be that it is the impact of different numbers of serotonin neurons that influences place memory. The two lines that do influence place memory, Si6-GAL4 and 50H05, label 5 and 25 serotonin neurons per hemisphere, respectively (Albin et al., 2015). The other lines, Trh247-, 483- and 819-GAL4 drivers express in about 15, 12 and 17 serotonin neurons per hemisphere (Lee et al., 2011). The 50E07 drives expression in about 19 serotonin neurons per hemisphere (Jenett et al., 2012). It is not clear how many serotonin neurons are affected

by the 90A12 driver since our attempts at labeling detected very weak expression (not shown). Thus, there is not a clear relationship between serotonin cell number and effect on place memory. Taken together, these data suggest that specific subsets of serotonin neurons and their innervation sites are necessary and sufficient for aversive reinforcement in place memory.

Serotonergic Neurons Are Necessary and Sufficient for Unexpected High Temperature Exposure Effects on Conditioned Behavior

We next explored if serotonin is also important for outcome learning in the heat-box. After unexpected high temperature exposure wild-type flies increase both escape latencies and

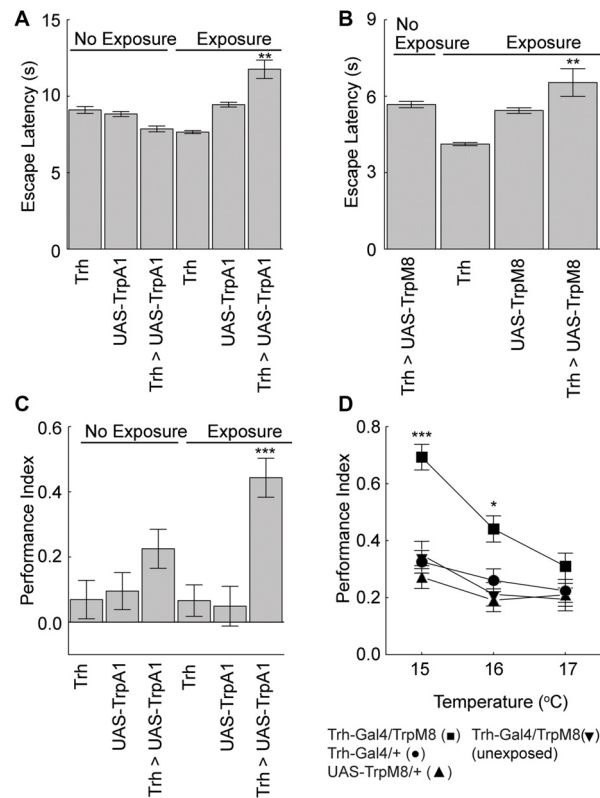


FIGURE 5 | Serotonergic neurons mediate unexpected exposure increases in escape latencies and enhancement of place memory. **(A)** Flies were exposed to 32°C to activate the Trh-GAL4-positive neurons with UAS-TrpA1 expression, which increased escape latencies ($H_{(5,N=35809)} = 1259.8$, $p < 0.0001$; $P < 0.01 = **$ experimental flies with unexpected exposure compared to control genotypes and non-exposed flies after multiple comparisons). **(B)** Flies were exposed to 15°C cool temperatures to activate the Trh-GAL4 neurons with UAS-TrpM8, which increased escape latencies ($H_{(3,N=11681)} = 384.3$, $p < 0.0001$; $P < 0.01 = **$ experimental flies with unexpected exposure compared to control genotypes and non-exposed flies after multiple comparisons). **(C)** Flies expressing the TrpA1 in serotonergic neurons with the Trh-GAL4 driver had an enhanced memory after unexpected exposure to a warm temperature compared to genetic control flies ($H_{(5,N=737)} = 31.9$, $p < 0.0001$; $P < 0.001 = ***$ compared to all other groups after multiple comparisons). **(D)** Flies expressing the cool responsive TrpM8 with the Trh-GAL4 driver (Trh-GAL4/TrpM8 (■)) had enhanced memory levels compared to genetic control flies (Trh-GAL4/+ (●) and UAS-TrpM8/+ (▲)) and GAL4/TrpM8 flies not exposed to a low temperature (▼) (15°C, $H_{(3,N=516)} = 48.1$, $p < 0.0001$; 16°C, $H_{(3,N=536)} = 17.5$, $p = 0.0006$; 17°C, $H_{(3,N=514)} = 3.6$, $p = 0.3$; P 's $< 0.001 = ***$ and $< 0.05 = *$ of the experimental genotype with exposure compared to all other groups with multiple comparisons).

memory levels (Sitaraman et al., 2007; Sitaraman and Zars, 2010; Yang et al., 2013; Batsching et al., 2016). Synaptic output from most or all of the serotonergic neurons was blocked using both a DdcGal4;Th-GAL80 driver combination and the Trh-GAL4 driver with the tetanus toxin light chain (TNT; Scholz et al., 2000). Escape latencies were measured as the time it took for individual flies to escape from the punishment-associated half of the chamber during the training session. Consistent with the idea of learned helplessness, this phase was chosen for measurement as it best measures escape from unfavorable conditions after uncontrollable high temperature exposure.

Flies from the genetic control genotypes strongly increased the escape latency with exposure to high temperatures (Figures 4A,B). Flies with blocked serotonergic synaptic transmission had a significant reduction in the escape latency when exposed to unexpected high temperatures compared to normal flies (Figures 4A,B). Moreover, genetic control flies showed the expected increase in memory levels with unexpected

high temperature exposure, which was dampened when TNT was expressed in the serotonergic neurons (Figures 4C,D). Finally, flies from the tested genotypes had no significant changes in control behaviors (Table 1). Thus, the serotonergic system is necessary for the increases in escape latencies and memory performance after unexpected high-temperature exposure.

We next asked if the serotonergic neurons were also sufficient for the unexpected exposure effects on escape latencies and memory. Flies expressing TrpA1 or the cool responsive TrpM8 (Peabody et al., 2009) in the serotonergic neurons were exposed to activating temperatures. Activation of serotonergic neurons with either effector led to an increase in the escape latency compared to genetic and no-exposure control groups (Figures 5A,B). Moreover, activation of serotonergic neurons with either effector under maximal conditions nearly doubled place memory compared to genetic and conditioning control flies (Figures 5C,D). Flies from the different genotypes did not have

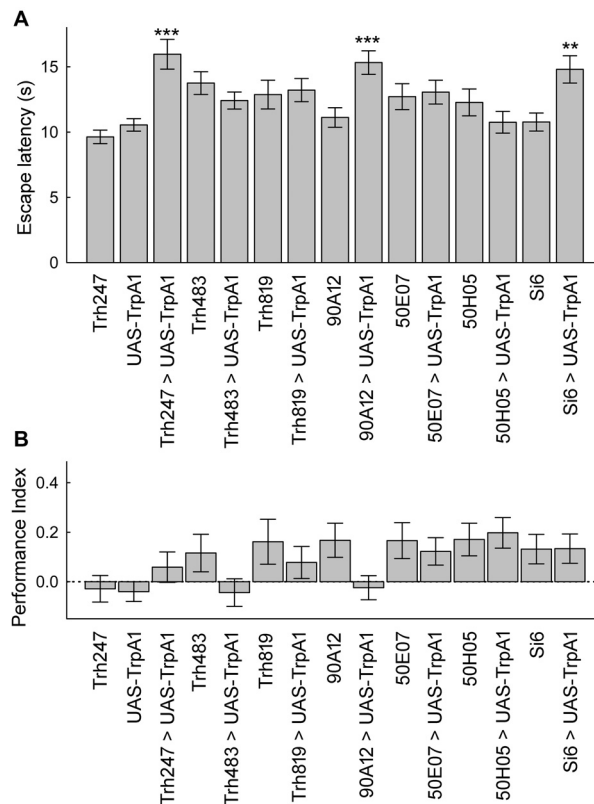


FIGURE 6 | A subset of serotonin drivers can enhance escape latencies, but none can enhance memory with unexpected exposures. **(A)** Flies from three genotypes expressing UAS-TrpA1 had increases in escape latencies after unexpected exposures ($H_{(14,N=17027)} = 355.1$, $p < 0.0001$; P 's $< 0.01 = **$ and $< 0.001 = ***$ compared to control genotypes). **(B)** Memory was not altered in the different genotypes after unexpected exposures ($H_{(14,N=1829)} = 33.0$, $p = 0.003$; P 's = n.s. between all groups after multiple comparisons).

altered control behaviors (Table 1). Thus, extrinsic activation of serotonergic neurons can induce unexpected exposure changes in escape latency and place memory.

Finally, we explored whether or not subsets of serotonergic neurons could alter escape latencies or memory levels with pre-training activation. We tested seven GAL4 lines because of the broad and more restricted expression in the serotonin neuron set. These are the same GAL4 drivers that were previously examined for direct conditioning of place memory. Activation of neurons with three serotonin GAL4 drivers in a pre-test phase significantly increased escape latency (Figure 6A). By contrast, none of the drivers altered place memory after pre-training activation of these neurons (Figure 6B). Thus, while a subset of the serotonin neurons can alter escape latencies upon activation, only activation of large portions of the serotonergic system can induce the memory enhancing effect.

DISCUSSION

Operant learning, where an animal selects one of several potential behaviors to increase reward or reduce punishment plays a key role in development of goal-directed behaviors. Central to this form of learning is feedback that helps an animal select an

appropriate behavior. In our previous work we showed that flies quickly learn to avoid spatial positions associated with aversive high temperature and this avoidance is disrupted by manipulation of the serotonin system (Sitaraman et al., 2008). Although other biogenic amines like dopamine and octopamine play critical roles in other forms of learning in *Drosophila*, they do not influence operant place memory (Sitaraman et al., 2008, 2010). What was unclear was the function of the serotonin neurons in other aspects of operant place learning and memory.

We substituted high temperature punishment in place learning with activation of serotonin neurons and discovered that serotonin release mediates aversive reinforcement in place memory. Furthermore, we find that specific subsets of serotonin neurons labeled by 50H05-GAL4 (expressed in 25 serotonin neurons) and Si6-GAL4 (expressed in 5 serotonin neurons) are sufficient in mediating aversive reinforcement. Since the 5 serotonin neurons labeled by Si6-GAL4 are also found in 50H05 we concluded that these neurons are critical in signaling aversive reinforcement. These neurons innervate the fly brain in several regions, including the sub-esophageal ganglion, the median bundle, and superior medial protocerebrum. This innervation pattern suggests that these neurons can influence multiple

neural sites. A deeper investigation of neurons within these regions that express serotonin receptors will illuminate the circuit pathways by which serotonin mediates aversive reinforcement.

Using the same operant learning paradigm we discovered a novel pre-exposure phenomenon where pre-exposure to unpredicted high temperature enhances place memory formation. Interestingly, the memory enhancement is only observed when conditioning uses low temperature reinforcement of 27–30°C (Sitaraman et al., 2007; Sitaraman and Zars, 2010). Based on these experiments we hypothesized that unpredictable exposures induce a state change in the nervous system that somehow stores the unpredicted high temperature exposure effects until a predictable read-out is determined. When released, this stored information then promotes higher than typical memory levels. The neural identity that stores the unexpected exposure effect was unknown. We asked if the serotonin system mediates the effects of unexpected exposure on memory performance.

Broad manipulation of serotonin system shows that induced serotonin release substitutes the unpredictable high temperature exposure and phenocopies the increase in memory performance. Our analysis of subsets of serotonin neurons reveals that it is only with a large portion of the serotonergic system that a place memory enhancement through unexpected activation can be induced.

Unpredicted aversive events, including high temperature, electric shock and vibration have profound effects on escape latencies and motivated climbing in *Drosophila* (Yang et al., 2013; Batsching et al., 2016; Ries et al., 2017). Results from our experiments confirm that high temperature exposure increases escape latencies. We discovered that the same small set of serotonin neurons that mediate conditioning also mediate the increase in escape latency with exposure to high temperatures. This result is generally in line with the un-signaled vibration induced hesitation of climbing behavior as also requiring the serotonin neurons. Whether or not the same specific set of serotonin neurons are critical for electric shock induced changes in escape latencies awaits future studies (Batsching et al., 2016). It is likely that the vibration induced changes in motivated climbing requires a different set of serotonin neurons since Ries et al. (2017) focus on serotonin neurons that innervate the mushroom bodies. The

mushroom bodies are not required for place memory (Wolf et al., 1998).

Animal models of depression and anxiety have been studied intensively for decades as they might help unravel the mechanistic basis of these conditions and aid development of pharmacological and therapeutic approaches (Abelaira et al., 2013; Logan and McClung, 2016). In most animal models, lack of motivation to perform key behaviors as a result of internal and external stressors has been widely studied in relation to depression. In the first animal model of learned helplessness, dogs lost the motivation to escape/avoid punishment following exposure to unpredictable, uncontrollable electric shocks (Seligman and Maier, 1967). Continued study of this condition in rodents has illuminated several genetic, molecular, and cellular targets (Maier and Watkins, 2005). Drugs targeting serotonin are regularly prescribed to alleviate symptoms associated with stress, anxiety, and depression in human patients. Continued studies from animal models will hopefully point to other drug targets (Hamon and Blier, 2013). Our results highlight the role of serotonin as a modulator of two features of learned helplessness, and provides a promising model to understand neurobiological basis of depression and anxiety.

AUTHOR CONTRIBUTIONS

TZ, DS, EFK, LK and DO: designed approach and experiments; collected data and performed data analysis. DS, EFK, LK and DO: performed experiments. DS and TZ: wrote article with contributions from all authors.

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REFERENCES

- Abelaira, H. M., Reus, G. Z., and Quevedo, J. (2013). Animal models as tools to study the pathophysiology of depression. *Rev. Bras. Psiquiatr.* 35, S112–S120. doi: 10.1590/1516-4446-2013-1098
- Albin, S. D., Kaun, K. R., Knapp, J. M., Chung, P., Heberlein, U., and Simpson, J. H. (2015). A subset of serotonergic neurons evokes hunger in adult *Drosophila*. *Curr. Biol.* 25, 2435–2440. doi: 10.1016/j.cub.2015.08.005
- Alekseyenko, O. V., Chan, Y. B., Fernandez, M. P., Bülow, T., Pankratz, M. J., and Kravitz, E. A. (2014). Single serotonergic neurons that modulate aggression in *Drosophila*. *Curr. Biol.* 24, 2700–2707. doi: 10.1016/j.cub.2014.09.051
- Alekseyenko, O. V., Lee, C., and Kravitz, E. A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. *PLoS One* 5:e10806. doi: 10.1371/journal.pone.0010806
- Batsching, S., Wolf, R., and Heisenberg, M. (2016). Inescapable stress changes walking behavior in flies—learned helplessness revisited. *PLoS One* 11:e0167066. doi: 10.1371/journal.pone.0167066
- Diegelmann, S., Zars, M., and Zars, T. (2006). Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in *Drosophila*. *Learn. Mem.* 13, 72–83. doi: 10.1101/lm.45506
- Giang, T., Ritze, Y., Rauchfuss, S., Ogueta, M., and Scholz, H. (2011). The serotonin transporter expression in *Drosophila melanogaster*. *J. Neurogenet.* 25, 17–26. doi: 10.3109/01677063.2011.553002

- Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J., et al. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature* 454, 217–220. doi: 10.1038/nature07001
- Hamon, M., and Blier, P. (2013). Monoamine neurocircuitry in depression and strategies for new treatments. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 45, 54–63. doi: 10.1016/j.pnpb.2013.04.009
- Heisenberg, M. (2014). The beauty of the network in the brain and the origin of the mind in the control of behavior. *J. Neurogenet.* 28, 389–399. doi: 10.3109/01677063.2014.912279
- Heisenberg, M. (2015). Outcome learning, outcome expectations and intentionality in *Drosophila*. *Learn. Mem.* 22, 294–298. doi: 10.1101/lm.037481.114
- Jenett, A., Rubin, G. M., Ngo, T. T., Shepherd, D., Murphy, C., Dionne, H., et al. (2012). A GAL4-driver line resource for *Drosophila* neurobiology. *Cell Rep.* 2, 991–1001. doi: 10.1016/j.celrep.2012.09.011
- Kahsai, L., and Zars, T. (2011). Learning and memory in *Drosophila*: behavior, genetics, and neural systems. *Int. Rev. Neurobiol.* 99, 139–167. doi: 10.1016/b978-0-12-387003-2.00006-9
- Kim, Y., Perova, Z., Mirrione, M. M., Pradhan, K., Henn, F. A., Shea, S., et al. (2016). Whole-brain mapping of neuronal activity in the learned helplessness model of depression. *Front. Neural Circuits* 10:3. doi: 10.3389/fncir.2016.00003
- Lee, P. T., Lin, H. W., Chang, Y. H., Fu, T. F., Dubnau, J., Hirsh, J., et al. (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 108, 13794–13799. doi: 10.1073/pnas.1019483108
- Lee, T., Winter, C., Marticke, S. S., Lee, A., and Luo, L. (2000). Essential roles of *Drosophila* RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron* 25, 307–316. doi: 10.1016/s0896-6273(00)80896-x
- Logan, R. W., and McClung, C. A. (2016). Animal models of bipolar mania: the past, present and future. *Neuroscience* 321, 163–188. doi: 10.1016/j.neuroscience.2015.08.041
- Maier, S. F., and Watkins, L. R. (2005). Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin and corticotropin-releasing factor. *Neurosci. Biobehav. Rev.* 29, 829–841. doi: 10.1016/j.neubiorev.2005.03.021
- Ostrowski, D., and Zars, T. (2014). “Place memory,” in *Handbook of Behavior Genetics of Drosophila Melanogaster: Behavioral Phenotypes and Models of Neurobehavioral Disorders*, ed. J. Dubnau (Cambridge, MA: Cambridge University Press), 125–134.
- Peabody, N. C., Pohl, J. B., Diao, F., Vreede, A. P., Sandstrom, D. J., Wang, H., et al. (2009). Characterization of the decision network for wing expansion in *Drosophila* using targeted expression of the TRPM8 channel. *J. Neurosci.* 29, 3343–3353. doi: 10.1523/JNEUROSCI.4241-08.2009
- Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T. T., Misra, S., Murphy, C., et al. (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 105, 9715–9720. doi: 10.1073/pnas.0803697105
- Pulver, S. R., Pashkovski, S. L., Hornstein, N. J., Garrity, P. A., and Griffith, L. C. (2009). Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in *Drosophila* larvae. *J. Neurophysiol.* 101, 3075–3088. doi: 10.1152/jn.00071.2009
- Ries, A. S., Hermanns, T., Poeck, B., and Strauss, R. (2017). Serotonin modulates a depression-like state in *Drosophila* responsive to lithium treatment. *Nat. Commun.* 8:15738. doi: 10.1038/ncomms15738
- Sadaf, S., Birman, S., and Hasan, G. (2012). Synaptic activity in serotonergic neurons is required for air-puff stimulated flight in *Drosophila melanogaster*. *PLoS One* 7:e46405. doi: 10.1371/journal.pone.0046405
- Scholz, H., Ramond, J., Singh, C. M., and Heberlein, U. (2000). Functional ethanol tolerance in *Drosophila*. *Neuron* 28, 261–271. doi: 10.1016/s0896-6273(00)00101-x
- Seligman, M. E. (1972). Learned helplessness. *Annu. Rev. Med.* 23, 407–412. doi: 10.1146/annurev.me.23.020172.002203
- Seligman, M. E., and Maier, S. F. (1967). Failure to escape traumatic shock. *J. Exp. Psychol.* 74, 1–9. doi: 10.1037/h0024514
- Sitaraman, D., and Zars, T. (2010). Lack of prediction for high-temperature exposures enhances *Drosophila* place learning. *J. Exp. Biol.* 213, 4018–4022. doi: 10.1242/jeb.050344
- Sitaraman, D., Zars, M., Laferriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., et al. (2008). Serotonin is necessary for place memory in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 105, 5579–5584. doi: 10.1073/pnas.0710168105
- Sitaraman, D., Zars, M., and Zars, T. (2007). Reinforcement pre-exposure enhances spatial memory formation in *Drosophila*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 193, 903–908. doi: 10.1007/s00359-007-0243-9
- Sitaraman, D., Zars, M., and Zars, T. (2010). Place memory formation in *Drosophila* is independent of proper octopamine signaling. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 196, 299–305. doi: 10.1007/s00359-010-0517-5
- Skinner, B. F. (1950). Are theories of learning necessary? *Psychol. Rev.* 57, 193–216. doi: 10.1037/h0054367
- Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eyding, D., and Heisenberg, M. (1998). *Drosophila* mushroom bodies are dispensable for visual, tactile, and motor learning. *Learn. Mem.* 5, 166–178.
- Wustmann, G., Rein, K., Wolf, R., and Heisenberg, M. (1996). A new paradigm for operant conditioning of *Drosophila melanogaster*. *J. Comp. Physiol. A* 179, 429–436. doi: 10.1007/bf00194996
- Yang, Z., Bertolucci, F., Wolf, R., and Heisenberg, M. (2013). Flies cope with uncontrollable stress by learned helplessness. *Curr. Biol.* 23, 799–803. doi: 10.1016/j.cub.2013.03.054
- Zars, T. (2001). Two thermosensors in *Drosophila* have different behavioral functions. *J. Comp. Physiol. A* 187, 235–242. doi: 10.1007/s003590100194
- Zars, T., Wolf, R., Davis, R., and Heisenberg, M. (2000). Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. *Learn. Mem.* 7, 18–31. doi: 10.1101/lm.7.1.18

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Behavioral Sensitization to the Disinhibition Effect of Ethanol Requires the Dopamine/Ecdysone Receptor in *Drosophila*

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Male flies under the influence of ethanol display disinhibited courtship, which is augmented with repeated ethanol exposures. We have previously shown that dopamine is important for this type of ethanol-induced behavioral sensitization but the underlying mechanism is unknown. Here we report that DopEcR, an insect G-protein coupled receptor that binds to dopamine and steroid hormone ecdysone, is a major receptor mediating courtship sensitization. Upon daily ethanol administration, *dumb* and *damb* mutant males defective in D1 (dDA1/DopR1) and D5 (DAMB/DopR2) dopamine receptors, respectively, showed normal courtship sensitization; however, the DopEcR-deficient *der* males exhibited greatly diminished sensitization. *der* mutant males nevertheless developed normal tolerance to the sedative effect of ethanol, indicating a selective function of DopEcR in chronic ethanol-associated behavioral plasticity. DopEcR plays a physiological role in behavioral sensitization since courtship sensitization in *der* males was reinstated when DopEcR expression was induced during adulthood but not during development. When examined for the DopEcR's functional site, the *der* mutant's sensitization phenotype was fully rescued by restored DopEcR expression in the mushroom body (MB) $\alpha\beta$ and γ neurons. Consistently, we observed DopEcR immunoreactivity in the MB calyx and lobes in the wild-type *Canton-S* brain, which was barely detectable in the *der* brain. Behavioral sensitization to the locomotor-stimulant effect has been serving as a model for ethanol abuse and addiction. This is the first report elucidating the mechanism underlying behavioral sensitization to another stimulant effect of ethanol.

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INTRODUCTION

Fruit flies are routinely exposed to ethanol in fermented fruits and food. In a laboratory setting, ethanol causes many behavioral responses that include hyper-locomotor activity, disinhibition, loss of motor control and sedation. Specifically, low doses of ethanol increase walking speed and turning, low to moderate doses induce disinhibited sexual activity and high doses lead to loss of postural control and sedation (Bainton et al., 2000; Lee et al., 2008). Flies develop tolerance to the

sedative effect when repeatedly exposed to ethanol (Scholz et al., 2000; Lee et al., 2008). These observations indicate that ethanol-induced behaviors in flies and intoxicated humans are similar; thus, the knowledge of their neurobiological basis could help not only uncover evolutionarily conserved vs. distinct neural, cellular and molecular pathways but also gain insight into effective intervention of ethanol abuse and addiction. The biogenic amine dopamine is involved in locomotor stimulating and rewarding effects of ethanol in flies, rodents and humans (Deveneni and Heberlein, 2013; Abrahao et al., 2014; Jayaram-Lindström et al., 2016). For example, ethanol intake elevates extracellular dopamine levels in the nucleus accumbens in rodents (Meyer et al., 2009; Vena et al., 2016). Likewise in flies, blockade of dopamine biosynthesis via 3IY that inhibits tyrosine hydroxylase dampens the ethanol's locomotor stimulant effect, which is reversed by L-DOPA feeding (Bainton et al., 2000). D1 and D2 dopamine receptors are involved in the locomotor stimulating and rewarding effects of ethanol in rodents (Lê et al., 1997; Matsuzawa et al., 1999; Arias et al., 2010) while D1 receptor is involved in both effects in flies (Kong et al., 2010; Kaun et al., 2011).

Behavioral sensitization is an escalated response to repeated drug use and underlies drug abuse and addiction (Berridge and Robinson, 2016). Dopamine is also important for behavioral sensitization to the ethanol's locomotor stimulant effect in rodents (Camarini and Pautassi, 2016). Repeated local or global ethanol treatments induce sensitized activity of dopamine neurons in the ventral tegmental area (Brodie, 2002; Ding et al., 2009). Pharmacological and genetic studies show involvement of both D1 and D2 family receptors in sensitization. For example, D1 and D3 knockout mice are defective in sensitization to chronic ethanol exposure (Harrison and Nobrega, 2009). Interestingly, D3 knockout mice develop normal sensitization to amphetamine, indicating the D3's function in the selective sensitization pathway. Observations on D2 knockout mice are conflicting: one study (Harrison and Nobrega, 2009) shows defective sensitization whereas another study (Palmer et al., 2003) reveals enhanced sensitization when the knockout mice in the same genetic background are compared. Thus, only a particular environmental or treatment condition involves D2-mediated sensitization. Together these observations indicate that the dopamine system mediates multiple yet distinct sensitization processes. Similar to rodents, flies develop sensitization to the locomotor stimulant effect of ethanol (Kong et al., 2010) and the mechanism is yet to be determined.

A prominent effect of ethanol in humans is disinhibition. Disinhibited cognition and motor functions lead to risk taking behaviors and impulsivity, which facilitate ethanol or other substance abuse and addiction (Field et al., 2010; Dalley et al., 2011; Morris et al., 2016). However, the mechanism underlying ethanol-induced disinhibition remains poorly understood. We have previously shown that dopamine mediates ethanol-induced courtship disinhibition and behavioral sensitization to this effect in *Drosophila* (Lee et al., 2008). *Drosophila* has three D1 family receptors: dDA1/DopR1 (D1; Sugamori et al., 1995), DAMB/DopR2 (D5; Han et al., 1996) and

DopEcR (Srivastava et al., 2005). When stimulated by dopamine, DopEcR activates an increase in cAMP and the PI3 kinase pathway whereas ecdysone inhibits the effect of dopamine on cAMP and activates the MAP kinase pathway. Here we report that sensitization to the disinhibition effect of ethanol requires DopEcR function in the mushroom body (MB) neurons. The findings reported here provide a framework to unravel the relevant neural circuits and the cellular mechanisms.

MATERIALS AND METHODS

Drosophila Strains and Culture

Flies were maintained on standard cornmeal agar medium at 25°C with 50% relative humidity under the 12 h light/12 h dark illumination condition. *Canton-S* was used as a wild-type strain. The DopEcR mutant used in this study is the insertion mutant *DopEcR*^{c02142} (also known as *DopEcR*^{PB1}; hereafter *der*) generated by the Gene Disruption Project (FlyBase Consortium, 2003; Thibault et al., 2004) and has been previously described (FlyBase Consortium, 2003; Inagaki et al., 2012; Petrucci et al., 2016). *der* was obtained from the Bloomington Stock Center (stock no. 10847) and backcrossed with Cantonized *w*¹¹¹⁸ for six generations, and then the X chromosome was replaced with that of *Canton-S* to remove the *w*¹¹¹⁸ mutation. *elav-GAL4* (stock no. 8765), *c739-GAL4* (stock no. 7362), *c305a-GAL4* (stock no. 30829), *UAS-mCD8-GFP* (stock no. 5137) and *PTRiP.JF03415* (stock no. 31981; FlyBase Consortium, 2003; Perkins et al., 2015) flies were obtained from the Bloomington Stock Center; *NP1131-GAL4* from Dr. Dubnau (Stony Brook University School of Medicine, Stony Brook, NY, USA); *fru*^{NP21}-*GAL4* from Dr. Yamamoto (Tohoku University, Sendai, Japan); *NP225-GAL4* from Dr. Thum (University of Konstanz, Konstanz, Germany); *tub-GS-GAL4* from Dr. Kitamoto (University of Iowa, Iowa City, IA, USA); and *MB-GS-GAL4* from Dr. Roman (University of Houston, Houston, TX, USA). We have previously described *MB247-GAL4* and *MB247-GAL4, GAL80^{ts}* (Kim et al., 2007, 2013). *DopEcR* cDNA containing the open reading frame (Srivastava et al., 2005) was cloned under UAS in the gateway vector pTW (Akbari et al., 2009). The cloned receptor was injected into *w*¹¹¹⁸ embryos, and germ-line transformed lines were outcrossed with Cantonized *w*¹¹¹⁸ for six generations to normalize the genetic background and to remove potential second site mutations. Individual transgenes were placed in the *der* mutant background for rescue experiments. We previously reported the dDA1 (D1) mutant *dumb*¹ and *dumb*² (Kim et al., 2007) and the *damb* mutant defective in DAMB (D5; Cassar et al., 2015). For conditional rescue experiments involving the gene switch lines *MB-GS-GAL4* and *tub-GS-GAL4*, 10 mM RU486 (Mifepristone, M8046, Sigma-Aldrich, Saint Louis, MO, USA) was made in 80% ethanol and added to fly food to the final concentration of 500 μM. Flies were reared on the food containing RU486 for 1 day before and between ethanol exposures. All genotypes used for behavioral analyses including the controls (*Canton-S* and *der* mutants carrying only *GS-GAL4*) were fed with RU486 or vehicle for comparison.

Immunohistochemical Analysis

The polyclonal DopEcR antibody was made commercially in a New Zealand white rabbit against the peptide GEPIHDKEYATALAEN that corresponds to the third cytoplasmic loop of the receptor (Pacific Immunology Corp, Ramona, CA, USA). Immunostaining was performed as previously described (Kim et al., 2013; Lim et al., 2014). Briefly, 4–5 day-old male brains were dissected in phosphate buffered saline (PBS) where the trachea around the brain was removed. Dissected brains were individually fixed with 4% PFA (paraformaldehyde and 0.04 M Lysine in PBS) at 4°C for 3 h and then rinsed three times in PBHT containing 0.5% Triton X-100 for 10 min each. Brains were solubilized in 1% Triton X-100 in PBHT for 1 h, incubated in the blocking solution (5% normal goat serum in PBHT) for 2 h and then incubated with the anti-DopEcR antibody (1:100 diluted in the blocking solution) at room temperature overnight. Brains were washed four times in PBHT for 1 h at room temperature and then overnight at 4°C before incubation with the goat Alexa 488-conjugated anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA) at room temperature for 2 h. After washes in PBHT, PBS and 0.12 M Tris-HCl, pH 7.4 (three times in each solution), brains were mounted in the VECTASHIELD medium (Vector Labs, Burlingame, CA, USA). Images were taken using the Zeiss LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA) and analyzed using the ImageJ software (NIH).

Behavioral Tests

One to two-day-old males were collected under carbon dioxide (CO₂) and aged in food vials for 2–3 days before tests. A group of 33 males was used as one data point in all behavioral tests. Ethanol exposure was performed in the Flypub consisting of a plastic chamber (57 mm D × 103 mm H) with the clear ceiling for videotaping behavior and the open bottom for administering ethanol as previously described (Lee et al., 2008). Flies were acclimated to the chamber for 10 min before ethanol exposure. A small petri dish containing a cotton pad applied with 1 ml of 95% ethanol was inserted to the bottom opening and flies were exposed to ethanol vapor till they were sedated. Four to six Flypubs were recorded together using a HD video camera (Q2F-00013 Microsoft LifeCam Studio, Redmond, WA, USA). The recorded movie files were used to score courtship activity. Flies were exposed to ethanol every 24 h for six consecutive days and were kept in food vials between exposures. The sedative effect of ethanol was measured by counting every 2 min the number of flies lying on their back or immobile for over 10 s. To obtain the mean sedation time (MST), the total sedation time, i.e., $\sum(\text{the number of sedated flies at each time interval} \times \text{each time interval after ethanol administration, e.g., 2, 4, 6 and etc.})$, was divided by the total number of flies (Lee et al., 2008). Courtship activity consisting of singing (unilateral wing vibration), licking or attempted copulation (Baker et al., 2001) was monitored during 30 s (1 block) and the maximum number of flies engaged in courtship at a given time was scored. The average of 10 consecutive blocks (i.e., 5 min) giving the highest value was

used to represent the percentage of males engaged in active intermale courtship per Flypub (Lee et al., 2008). Our earlier study (Lee et al., 2008) has shown that the maximal level of ethanol-induced courtship disinhibition is achieved on the exposure 4 or 5 and then maintained steady. Thus, we focus on exposure 1 for the initial level of ethanol-induced disinhibition, exposure 2 for sensitization induction and exposure 6 for maintenance in this study. The genotypes were blinded to the experimenters conducting ethanol exposure and scoring courtship or sedation.

Data Analysis

Statistical analyses were performed using Minitab 16 (Minitab, State College, PA, USA) and JMP 13 (SAS, Cary, NC, USA). All data are reported as mean + or $\pm$ standard error of means (SEM). Normality was determined by the Anderson Darling goodness-of-fit test. Normally distributed data were analyzed by a two-tailed Student's *t*-test or analysis of variance (ANOVA) with *post hoc* Tukey-Kramer HSD or Dunnett's tests. Non-normally distributed data were analyzed by Kruskal-Wallis and *post hoc* Mann-Whitney tests.

RESULTS

Tolerance to the Sedative Effect of Ethanol

To investigate the roles of D1 family receptors in chronic ethanol effects, we employed the Flypub for mild ethanol delivery (Lee et al., 2008). We first measured the sedative effect of ethanol. Compared to the control *Canton-S* males, it took longer for *der* mutant males to get sedated ($p < 0.0001$; **Figure 1A**), demonstrating that *der* males defective in DopEcR have decreased sensitivity to the sedative effect of ethanol. This corroborates the finding by Petrucci et al. (2016). In contrast, *dumb* and *damb* males defective in dDA1 (D1) and DAMB (D5) receptors, respectively, exhibited normal sensitivity ($p > 0.05$, **Figure 1B**). When MSTs of *dumb*, *damb* and *der* males were examined during daily ethanol exposures, all mutants developed tolerance similar to *Canton-S* (*Canton-S*: $F_{(3,101)} = 35.9762$, $p < 0.0001$; *der*: $F_{(3,90)} = 7.4871$, $p = 0.0002$; *dumb*¹, $p < 0.001$; *dumb*², $p < 0.0001$; $p < 0.0001$, *damb*; **Figures 1C,D**). This indicates that D1 family receptors are not important for tolerance to the sedative effect of ethanol.

Behavioral Sensitization to the Disinhibition Effect of Ethanol

Drosophila males typically court females and rarely court males. Under daily ethanol exposure, however, *Canton-S* males display the escalated levels of intermale courtship ($R^2 = 0.7289$, $F_{(2,48)} = 64.5414$, $p < 0.0001$; **Figure 2A**), which require dopamine neuronal activity (Lee et al., 2008). To explore the mechanism by which dopamine regulates behavioral disinhibition and sensitization, we examined the D1 family receptor mutants' courtship behavior under the influence of ethanol. Both *dumb* and *damb* males developed behavioral sensitization to the disinhibition effect of ethanol

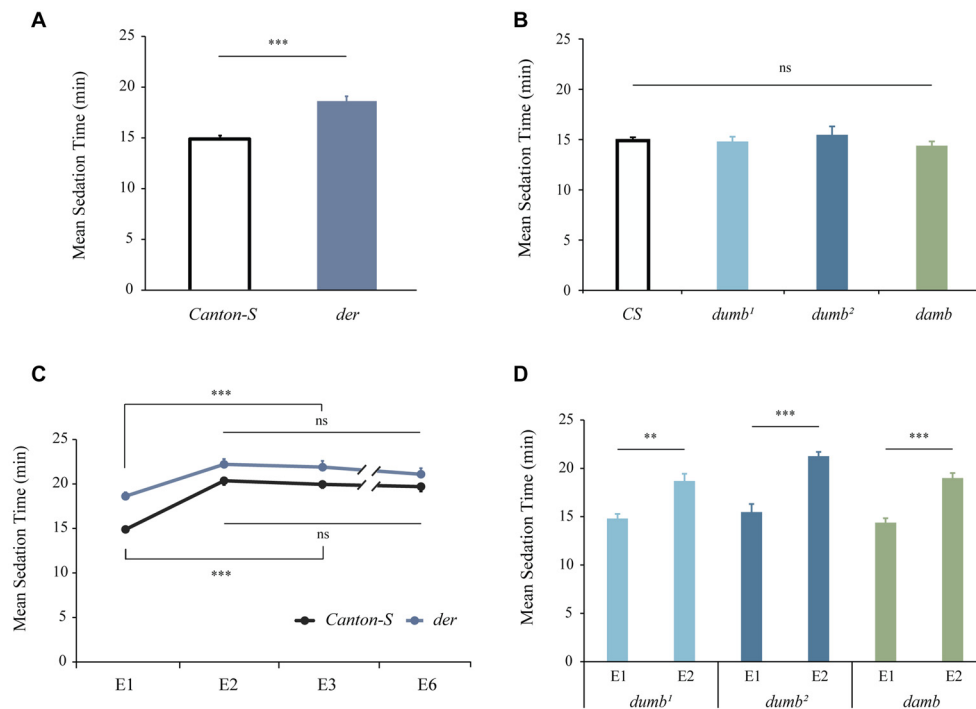


FIGURE 1 | D1 family receptors are dispensable for ethanol tolerance. The wild-type *Canton-S* and D1 receptor mutants were exposed to ethanol and mean sedation time (MST) was measured. **(A)** *der* mutant males defective in DopEcR showed decreased sensitivity to the sedative effect of ethanol (***) $p < 0.0001$ by two-tailed Student's *t*-test; *Canton-S*, $n = 26$; *der*, $n = 22$). **(B)** *dumb¹* and *dumb²* mutant males defective in dDA1 (D1) and *damb* defective in DAMB (D5) exhibited normal sensitivity. Analysis of variance (ANOVA): $p > 0.05$, $n = 8$; ns, not significant. **(C)** *der* males showed normal tolerance development and maintenance to the sedative effect of ethanol. (***) $p < 0.0001$ by ANOVA and *post hoc* Tukey-Kramer HSD tests; *Canton-S*, $n = 26$; *der*, $n = 22$). **(D)** *dumb* and *damb* males displayed normal tolerance. Student *t*-test; ** $p < 0.001$; *** $p < 0.0001$; $n = 8$.

(*dumb¹*: $R^2 = 0.8966$, $F_{(3,24)} = 69.3456$, $p < 0.0001$; *dumb²*: $R^2 = 0.7936$, $F_{(3,24)} = 30.7652$, $p < 0.0001$; *damb*: $R^2 = 0.9316$, $F_{(3,20)} = 90.8291$, $p < 0.0001$; **Figure 2B**). *der* males, on the other hand, exhibited the substantially reduced levels of intermale courtship on all exposures compared to *Canton-S* males ($p < 0.0001$; **Figure 2A**). This suggests that DopEcR is required for behavioral sensitization to the disinhibition effect of ethanol.

Neural Substrate for Behavioral Sensitization

To identify the neural structure where DopEcR regulates behavioral sensitization, we employed the GAL4/UAS binary system and RNA interference (RNAi) for cell type-specific knockdown of DopEcR expression. In this study we used an additional control line carrying *UAS-GFP* and *UAS-DopEcR RNAi* since courtship behavior could be sensitive to the *mw* in a transgenic construct (Lee et al., 2008). To establish effectiveness of DopEcR RNAi, we used the pan-neuronal driver *elav-GAL4* to express double-stranded DopEcR RNA for RNAi in all neurons. Like *der* mutants, the flies with pan neuronal DopEcR knockdown showed severe impairment in behavioral sensitization ($p < 0.0001$; **Figure 3A**). We reasoned that the neural substrate for the DopEcR's function

in behavioral sensitization could be the neurons regulating courtship behavior or high order brain structures mediating learning and memory. Fruitless-expressing neurons control male courtship behavior (Manoli et al., 2005; Stockinger et al., 2005) thus represent a potential neural site for the DopEcR's function. The projection neurons are another candidate for the DopEcR's function because they have dendrites in the antennal lobes and axons at the lateral horn and the MB calyx that are high order brain centers for pheromone information processing, learning and memory (Thum et al., 2007; Grosjean et al., 2011). When DopEcR was knocked down in Fruitless neurons, we did not observe a significant change in behavioral sensitization ($p > 0.05$; *fru-GAL4* in **Figure 3A**) while DopEcR knockdown in the projection neurons resulted in slightly increased sensitization ($p = 0.0186$; *NP225-GAL4*). Above all, we observed markedly reduced sensitization in the flies with DopEcR knockdown in the MB neurons ($p < 0.0001$; *MB247-GAL4* in **Figure 3B**). The MB consists of $\alpha\beta$, $\alpha\beta'$ and γ neurons where *MB247-GAL4* is expressed in $\alpha\beta$ and γ neurons. We next asked whether DopEcR in each MB substructure is sufficient for behavioral sensitization. When DopEcR RNAi was induced only in $\alpha\beta$, $\alpha\beta'$ or γ neurons via the *c739*-, *c305a*- or *NP1131-GAL4* driver, respectively, the flies developed normal behavioral sensitization ($p > 0.05$). This suggests that DopEcR in the $\alpha\beta$ and γ , but not $\alpha\beta'$ or γ alone, is

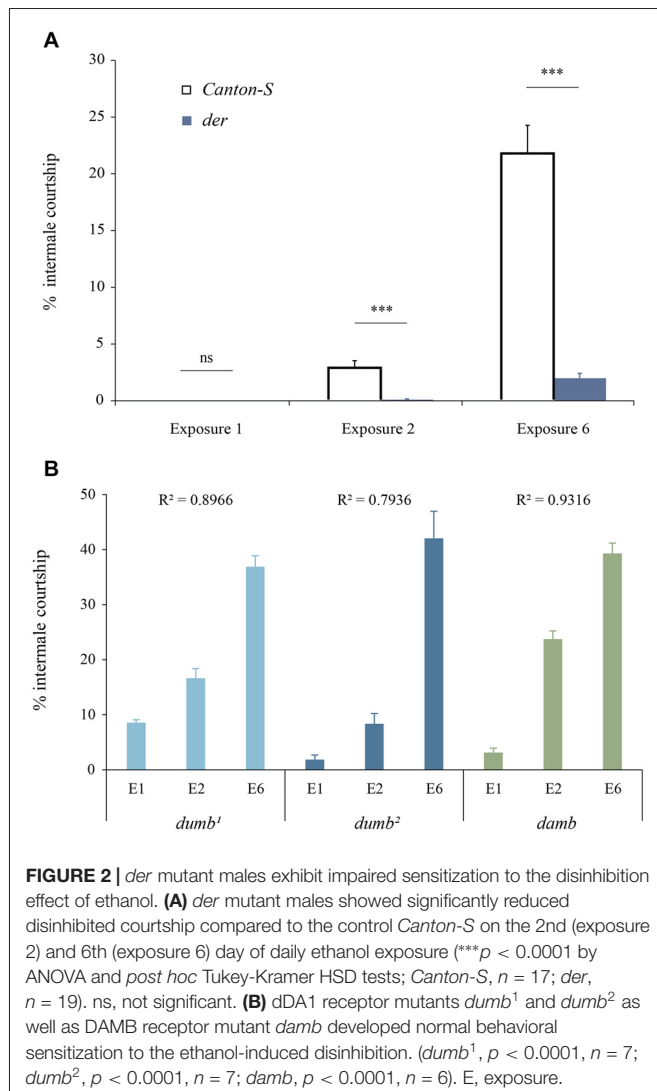


FIGURE 2 | *der* mutant males exhibit impaired sensitization to the disinhibition effect of ethanol. **(A)** *der* mutant males showed significantly reduced disinhibited courtship compared to the control *Canton-S* on the 2nd (exposure 2) and 6th (exposure 6) day of daily ethanol exposure ($***p < 0.0001$ by ANOVA and *post hoc* Tukey-Kramer HSD tests; *Canton-S*, $n = 17$; *der*, $n = 19$). ns, not significant. **(B)** dDA1 receptor mutants *dumb¹* and *dumb²* as well as DAMB receptor mutant *damb* developed normal behavioral sensitization to the ethanol-induced disinhibition. (*dumb¹*, $p < 0.0001$, $n = 7$; *dumb²*, $p < 0.0001$, $n = 7$; *damb*, $p < 0.0001$, $n = 6$). E, exposure.

needed for behavioral sensitization to the disinhibition effect of ethanol.

Temporal Requirement for DopEcR Function

DopEcR is expressed throughout development and adulthood (FlyBase Consortium, 2003; Inagaki et al., 2012; Ishimoto et al., 2013; Petrucci et al., 2016). To test whether the sensitization phenotype is caused by developmental or physiological DopEcR deficiency, we adopted two approaches, TARGET and Gene Switch (GS) for temporally restricted reinstatement of DopEcR expression in the MB neurons of *der* mutants. TARGET (McGuire et al., 2004) is the GAL4/UAS combined with GAL80^{ts} that confers temporally restricted expression of a transgene downstream of UAS, which we used successfully in the study of dDA1 in olfactory memory formation (Kim et al., 2007). Briefly, GAL80^{ts} is active as a GAL4 repressor at 20°C but inactive at 30°C, allowing GAL4 activity thereby UAS activation. The *der* mutants carrying *tub-GAL80^{ts}*, *MB247-GAL4* and *UAS-DopEcR cDNA* were reared at 30°C throughout

development but maintained at 20°C right after eclosion to induce DopEcR expression only during development (Figure 4A). To induce DopEcR only during adulthood, possibly at the time of ethanol exposure, the *der* mutants carrying *tub-GAL80^{ts}*, *MB247-GAL4* and *UAS-DopEcR cDNA* were reared at 20°C throughout development but maintained at 30°C 2 days after eclosion. *Canton-S* and *der* mutant carrying *tub-GAL80^{ts}* and *MB247-GAL4* but not *UAS-DopEcR cDNA* were treated with the same temperature manipulation to serve as controls. As shown in Figure 4A, the *der* males with DopEcR expression only during development exhibited impaired behavioral sensitization thus there was no rescue ($F_{(2,16)} = 23.2$, $p < 0.0001$). In contrast, the *der* males with DopEcR expression only during adulthood fully reinstated behavioral sensitization ($p > 0.05$ compared to *Canton-S*; Figure 4B). This indicates the role of DopEcR during adulthood for disinhibition sensitization.

We observed that the flies with the temperature manipulation displayed highly variable ethanol sensitivity and sensitization. Thus as a complementary approach, we used the GS system in which GAL4 is fused to the progesterone receptor. Only in the presence of the steroid RU486, GAL4 can activate UAS for downstream gene expression (Roman et al., 2001). We tested the *der* mutants carrying *UAS-DopEcR cDNA* and *tub-GS-GAL4* or *MB-GS-GAL4* for ubiquitous or MB expression of DopEcR, respectively, at the time of ethanol exposure. When treated with RU486, the *der* males with DopEcR expression in all cells or MB neurons displayed the level of sensitization substantially higher than that of the *der* males carrying only *tub-GS-GAL4* or *MB-GS-GAL4*, but comparable to the *Canton-S* level ($F_{(6,24)} = 43.2375$, $p < 0.0001$; Figure 4C). The *der* males carrying the same transgenes (i.e., *UAS-DopEcR-cDNA* and *tub-GS-GAL4* or *MB-GS-GAL4*) that were not fed with RU486 exhibited impaired sensitization similar to the *der* mutants carrying *tub-GS-GAL4* or *MB-GS-GAL4* ($p > 0.05$ by *post hoc* Tukey-Kramer HSD test; Figure 4C). These observations together demonstrate that DopEcR expression during adulthood is sufficient for sensitization, supporting the physiological role of DopEcR at the time of ethanol exposure for this behavioral plasticity.

Expression Patterns of DopEcR

The study of *DopEcR enhancer-GAL4* shows that DopEcR is expressed in the MB $\alpha\beta$ and γ neurons (Ishimoto et al., 2013). It is however unclear where DopEcR is localized in the MB. To address this, we used immunohistochemical analysis. We made the fusion construct of Glutathione S-transferase and the third cytoplasmic loop of DopEcR as we have previously characterized the dDA1 and DAMB expression patterns (Han et al., 1996, 1998). We also made the antibody against the peptide corresponding to part of the third cytoplasmic loop. The antibodies made against the fusion protein in rabbits and mice did not provide reliable staining; however, the antibody made against the peptide revealed consistent staining in the MB neuropil. It is worth mentioning that the antibody did not penetrate inside the brain under numerous conditions that we tried and also strongly stained the cell membrane

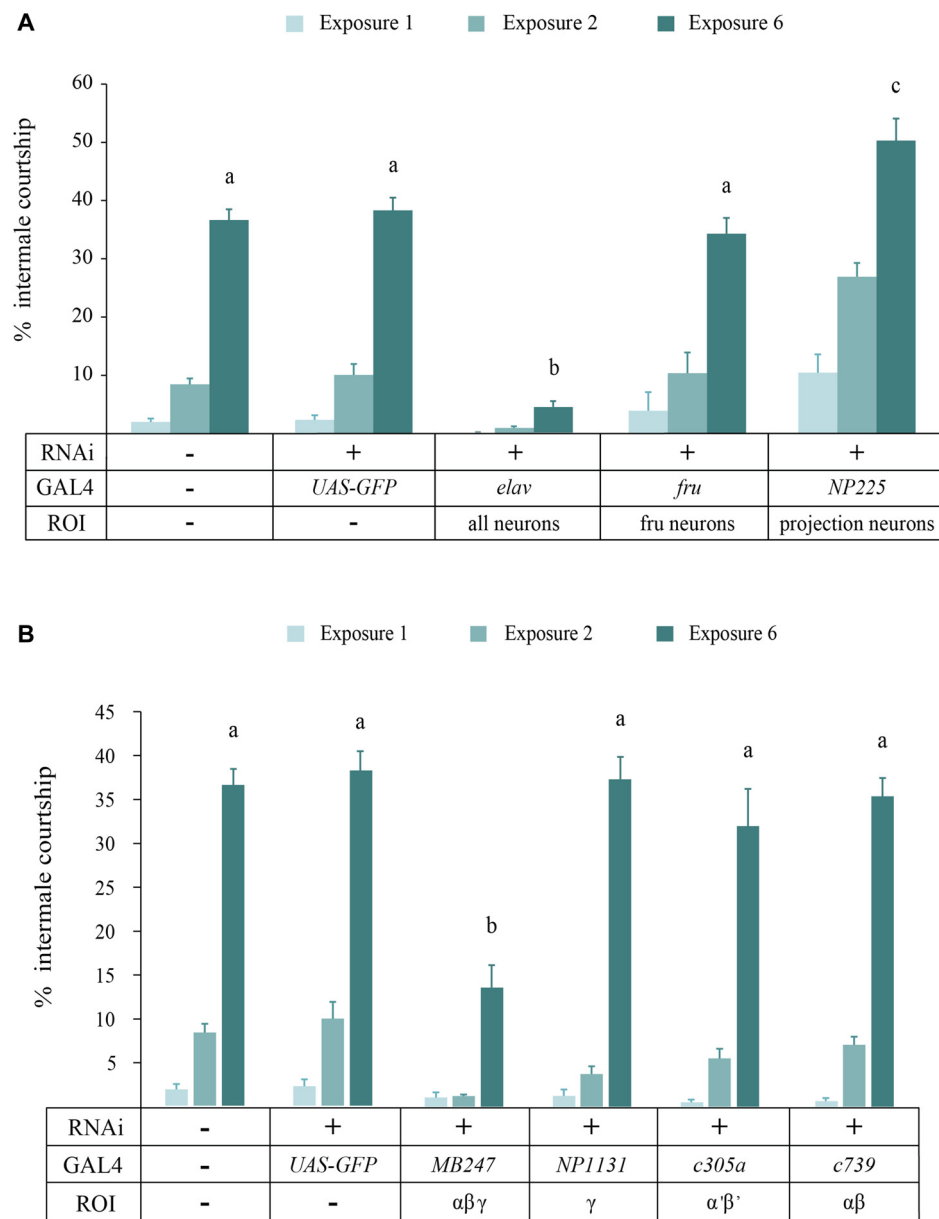


FIGURE 3 | DopEcR knockdown in the mushroom body (MB) $\alpha\beta$ and γ neurons suppresses sensitization. **(A)** Pan-neuronal DopEcR knockdown (*elav-GAL4/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.50$, $n = 6$) led to substantially reduced sensitization compared to *Canton-S* or the transgenic control (*UAS-GFP/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.91$, $n = 6$). Different letters on the bars (i.e., a, b and c) denote significant difference when all genotypes on exposure 6 were compared (ANOVA, $p < 0.0001$). Normal behavioral sensitization was observed when DopEcR was knocked down in the *fruitless* (*fru*) neurons (*fru-GAL4/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.70$, $n = 5$). DopEcR knockdown in the projection neurons resulted in slightly increased sensitization (*NP225-GAL4/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.75$, $n = 7$) (c , $p = 0.0186$ compared to the transgenic control by *post hoc* Dunnett's test). **(B)** DopEcR knockdown in the MB α , β and γ neurons (*MB247-GAL4/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.61$, $n = 7$) led to significant reduction in behavioral sensitization ($p < 0.0001$). DopEcR knockdown in individual MB subsets ($\alpha\beta$, *c739/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.95$, $n = 6$; $\alpha'\beta'$, *c305a/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.81$, $n = 6$; γ , *NP1131/+;UAS-DopEcR-RNAi/+*, $R^2 = 0.93$, $n = 6$) resulted in normal sensitization.

of nearly all neurons and glia in both *Canton-S* and *der* (Figure 5; Supplementary Movie Files). Nonetheless, DopEcR immunoreactivity was clearly visible in the MB calyx (dendritic structure; Figure 5A, Supplementary Figure S1), α lobe core

and β lobe (axonal structure) in the *Canton-S* brain (Figure 5C, Supplementary Movie 1). DopEcR immunoreactivity in the γ lobe was also detectable but at a very low level (Figure 5C and Supplementary Movie 1). On the contrary, DopEcR

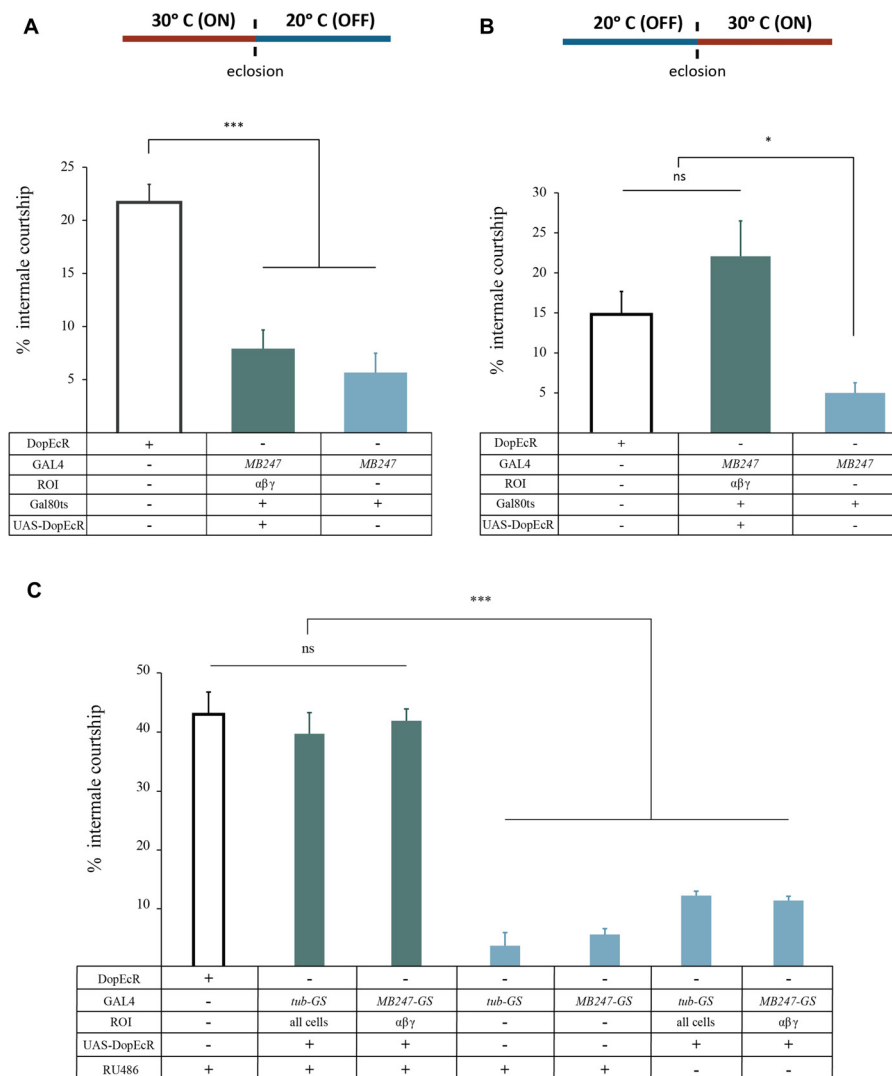


FIGURE 4 | DopEcR is needed during adulthood to mediate sensitization. The *der* mutant males carrying *tub-GAL80^{ts}*, *MB247-GAL4* and *UAS-DopEcR cDNA* were reared at 30°C before eclosion to induce DopEcR expression during development **(A)** or after eclosion to induce DopEcR expression during adulthood **(B)**. **(A)** The *der* males with reinstated DopEcR expression only during development (*MB247, GAL80^{ts}/UAS-DopEcR cDNA;der*, $n = 7$) exhibited behavioral sensitization at the level comparable to that of the *der* transgenic mutant (*MB247, GAL80^{ts}/+;der*; $p > 0.05$, $n = 6$) but lower than that of the *Canton-S* control (** $p < 0.0001$, $n = 6$). **(B)** The *der* males with reinstated DopEcR expression only during adulthood (*MB247, GAL80^{ts}/UAS-DopEcR cDNA;der*, $n = 7$) showed behavioral sensitization comparable to the control (ns, $p > 0.05$, $n = 7$) but higher than the *der* mutant (*MB247, GAL80^{ts}/+;der*; * $p < 0.05$, $n = 4$). **(C)** The *der* males carrying *UAS-DopEcR-cDNA* and either *tub-GS-GAL4* (*UAS-DopEcR cDNA/+;tub-GS-GAL4, der/der*, $R^2 = 0.8486$, $n = 7$) or *MB247-GS-GAL4* (*UAS-DopEcR cDNA/+;MB247-GS-GAL4, der/der*, $R^2 = 0.9834$, $n = 4$) displayed sensitization similar to the control ($R^2 = 0.9113$, $n = 5$) when treated with RU486 (ns, $p > 0.05$), but significantly higher than the *der* mutant controls (*tub-GS-GAL4, der/der*, $n = 4$; *MB247-GS-GAL4, der/der*, $n = 4$) treated with RU486 or the *der* mutants carrying the rescue transgenes without RU486 treatment (** $p < 0.0001$). The percent internale courtship on the exposure 6 are shown. ns, not significant.

immunoreactivity in all MB neuropil was barely detectable in the *der* brain (Figures 5B,D, Supplementary Figure S1 and Supplementary Movie 2). These observations suggest that the site of DopEcR's function for sensitization is the MB dendrites in the calyx or axons in the α , β or γ lobe, or both locations.

DISCUSSION

In this report, we show that DopEcR in the MB $\alpha\beta$ and γ neurons mediates behavioral sensitization to the disinhibition

effect of ethanol. Further, we demonstrate that the DopEcR's function is physiological rather than developmental. As in mammals, dopamine is important for the locomotor activating and rewarding effects of ethanol in flies (Bainton et al., 2000; Kong et al., 2010; Kaun et al., 2011). The D1 receptor dDA1/DopR in the ellipsoid body is involved in the locomotor stimulant effect (Kong et al., 2010) while the dopamine receptor mediating the rewarding effect is unknown. We have noted that the flies deficient in dDA1 or DAMB display augmented disinhibition on all ethanol exposures

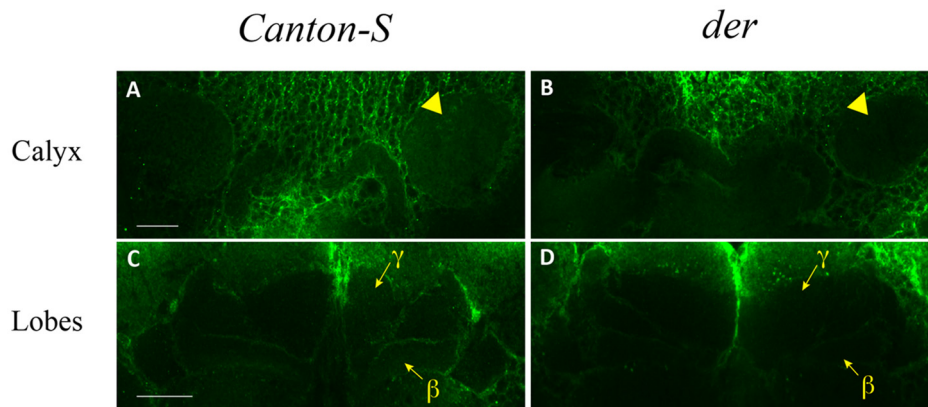


FIGURE 5 | DopEcR expression in the MB neuropil. DopEcR immunoreactivity is evident in the calyx (A, arrowhead) and medial lobes β and γ (C, arrows) in the *Canton-S* brain but barely detectable in the *der* brain (B, arrowhead for calyx; D, arrows for medial lobes). One micron optical sections were made using a 40x (A,B) or 63x (C,D) objective in the confocal microscope and three sections were stacked in all images. Scale bar, 25 micron.

tested, and we are currently following up on this finding. These observations together indicate that dDA1 is involved in diverse effects of ethanol possibly through distinct neural circuits.

Kaun et al. (2011) examined the rewarding property of ethanol using a conditioned preference assay. They have found that all MB subsets are important for conditioned preference to the cue associated with ethanol. It has been postulated that the dopamine signal to the MB $\alpha\beta$ lobe is crucial for preference expression (Kaun et al., 2011). Behavioral sensitization represents a form of learning and memory (Camarini and Pautassi, 2016). The neural substrate that we identified for DopEcR's function in sensitization is consistent with the MB's role in learning and memory as opposed to simple sensory information processing. We have previously shown that the dDA1 receptor in the MB $\alpha\beta$ and γ neurons mediates reward memory of sucrose (Kim et al., 2007) but it is not needed for behavioral sensitization (this study). Thus, the MB $\alpha\beta$ and γ neurons process the reinforcing effects of the natural substance sucrose and the addictive drug ethanol via distinct dopamine receptors dDA1 and DopEcR, respectively.

DopEcR responds to dopamine as well as the steroid hormone ecdysone (Srivastava et al., 2005). For short-term memory in courtship conditioning and the sedative effect of ethanol, ecdysone is as a major ligand for DopEcR (Ishimoto et al., 2013; Petruccioli et al., 2016). Dopamine, on the other hand, activates DopEcR in the gustatory receptor neurons to enhance sensitivity to sugar in hungry flies (Inagaki et al., 2012). In male moths, DopEcR in the antennal lobe regulates behavioral responses to pheromones, which require both dopamine and ecdysone as ligands (Abrieux et al., 2013, 2014). We show that both dopamine neurotransmission blockade (Lee et al., 2008) and DopEcR deficiency (this study) cause severely impaired behavioral sensitization, implicating dopamine as a major ligand for the DopEcR function. This notion is supported by the recent study (Chen et al., 2017) demonstrating that the increased level of dopamine in the PPL2ab neurons enhances intermale

courtship. The PPL2ab neurons innervate the MB calyx (Mao and Davis, 2009) where DopEcR is localized (Figure 5A). It remains to be clarified, nevertheless, whether dopamine or both dopamine and ecdysone together act on DopEcR for behavioral sensitization to the ethanol's effect on courtship disinhibition.

Dopamine is a key neuromodulator mediating not only reward and pleasure associated with natural stimuli and addictive substances but also neuroadaptations underlying abuse and addiction (Clarke and Adermark, 2015; Volkow and Morales, 2015; Camarini and Pautassi, 2016). Behavioral sensitization is widely studied as a model for drug addiction and typically measured to the locomotor-stimulant effect of alcohol and other drugs (Berridge and Robinson, 2016). Enhanced disinhibition and impulsivity induced by ethanol contribute to risky behaviors such as sexual assaults, aggression and drug seeking or abuse (Field et al., 2010; Dalley et al., 2011; Morris et al., 2016), all of which negatively impact our society. However, the underlying mechanism still remains poorly understood. The study reported here may help narrow the knowledge gap. On this line of thought, GPR30/GPER1 represents the membrane G-protein coupled receptor that mediates non-genomic actions of the steroid hormone estrogen in mammals (Maggiolini and Picard, 2010). When tested *in vitro*, GPR30 responds to dopamine in a dose-dependent manner to increase cAMP similar to DopEcR (Evans et al., 2014, 2016). GPR30's function in ethanol-induced behaviors is unknown but it plays a crucial role in sexual motivation of male rats (Hawley et al., 2017). It would be of interest to learn whether GPR30 mediates ethanol-induced disinhibition and sensitization similar to DopEcR.

AUTHOR CONTRIBUTIONS

K-AH conceived and designed the experiments. GPA, SJH, PRS and PDE performed the experiments. K-AH, GPA, SJH, and PRS analyzed the data. GPA and K-AH wrote the article.

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REFERENCES

- Abraham, K. P., Goeldner, F. O., and Souza-Formigoni, M. L. O. (2014). Individual differences in ethanol locomotor sensitization are associated with dopamine D1 receptor intra-cellular signaling of DARPP-32 in the nucleus accumbens. *PLoS One* 9:e98296. doi: 10.1371/journal.pone.0098296
- Abrieux, A., Debernard, S., Maria, A., Gaertner, C., Anton, S., Gadenne, C., et al. (2013). Involvement of the G-protein-coupled dopamine/ecdyseroid receptor DopEcR in the behavioral response to sex pheromone in an insect. *PLoS One* 8:e72785. doi: 10.1371/journal.pone.0072785
- Abrieux, A., Dupontet, L., Debernard, S., Gadenne, C., and Anton, S. (2014). The GPCR membrane receptor, DopEcR, mediates the actions of both dopamine and ecdysone to control sex pheromone perception in an insect. *Front. Behav. Neurosci.* 8:312. doi: 10.3389/fnbeh.2014.00312
- Akbari, O. S., Oliver, D., Eyer, K., and Pai, C. Y. (2009). An Entry/Gateway cloning system for general expression of genes with molecular tags in *Drosophila melanogaster*. *BMC Cell Biol.* 10:8. doi: 10.1186/1471-2121-10-8
- Arias, C., Mlewski, E. C., Hansen, C., Molina, J. C., Paglini, M. G., and Spear, N. E. (2010). Dopamine receptors modulate ethanol's locomotor-activating effects in preweanling rats. *Dev. Psychobiol.* 52, 13–23. doi: 10.1002/dev.20407
- Bainton, R. J., Tsai, L. T., Singh, C. M., Moore, M. S., Neckameyer, W. S., and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol.* 10, 187–194. doi: 10.1016/S0960-9822(00)00336-5
- Baker, B. S., Taylor, B. J., and Hall, J. C. (2001). Are complex behaviors specified by dedicated regulatory genes? Reasoning from *Drosophila*. *Cell* 105, 13–24. doi: 10.1016/S0092-8674(01)00293-8
- Berridge, K. C., and Robinson, T. E. (2016). Liking, wanting, and the incentive-sensitization theory of addiction. *Am. Psychol.* 71, 670–679. doi: 10.1037/amp0000059
- Brodie, M. S. (2002). Increased ethanol excitation of dopaminergic neurons of the ventral tegmental area after chronic ethanol treatment. *Alcohol. Clin. Exp. Res.* 26, 1024–1030. doi: 10.1097/0000374-200207000-00013
- Camarini, R., and Pautassi, R. M. (2016). Behavioral sensitization to ethanol: neural basis and factors that influence its acquisition and expression. *Brain Res. Bull.* 125, 53–78. doi: 10.1016/j.brainresbull.2016.04.006
- Cassar, M., Issa, A.-R., Riemensperger, T., Petitgas, C., Rival, T., Coulom, H., et al. (2015). A dopamine receptor contributes to paraquat-induced neurotoxicity in *Drosophila*. *Hum. Mol. Genet.* 24, 197–212. doi: 10.1093/hmg/ddu430
- Chen, S.-L., Chen, Y.-H., Wang, C.-C., Yu, Y.-W., Tsai, Y.-C., Hsu, H.-W., et al. (2017). Active and passive sexual roles that arise in *Drosophila* male-male courtship are modulated by dopamine levels in PPL2ab neurons. *Sci. Rep.* 7:44595. doi: 10.1038/srep44595

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnsys.2017.00056/full#supplementary-material>

Supplementary Movie 1 (CS) and 2 (der) 1 | The *Canton-S* (CS; Movie 1) and *der* (Movie 2) brains stained with the DopEcR antibody were scanned using the confocal microscope with a 20× objective, and the optical sections made every micron were stacked to create the movie files. Available online at: <http://datarepo.bioinformatics.utep.edu/getdata?acc=SHF53E1DZO VXB01>.

FIGURE S1 | Shown are the posterior areas of the *Canton-S* (top) and *der* (bottom) brains immunostained with the anti-DopEcR antibody. The optical sections were made every micron with a 20X objective and two sections were stacked. The calyx area on the right hemisphere in each brain is marked by arrowheads. Scale bar, 25 micron.

- Clarke, R., and Adermark, L. (2015). Dopaminergic regulation of striatal interneurons in reward and addiction: focus on alcohol. *Neural Plast.* 2015:814567. doi: 10.1155/2015/814567
- Dalley, J. W., Everitt, B. J., and Robbins, T. W. (2011). Impulsivity, compulsivity, and top-down cognitive control. *Neuron* 69, 680–694. doi: 10.1016/j.neuron.2011.01.020
- Devineni, A. V., and Heberlein, U. (2013). The evolution of *Drosophila melanogaster* as a model for alcohol research. *Annu. Rev. Neurosci.* 36, 121–138. doi: 10.1146/annurev-neuro-062012-170256
- Ding, Z. M., Rodd, Z. A., Engleman, E. A., and McBride, W. J. (2009). Sensitization of ventral tegmental area dopamine neurons to the stimulating effects of ethanol. *Alcohol. Clin. Exp. Res.* 33, 1571–1581. doi: 10.1111/j.1530-0277.2009.00985.x
- Evans, P. D., Bayliss, A., and Reale, V. (2014). GPCR-mediated rapid, non-genomic actions of steroids: comparisons between DmDopEcR and GPER1 (GPR30). *Gen. Comp. Endocrinol.* 195, 157–163. doi: 10.1016/j.ygcen.2013.10.015
- Evans, N. J., Bayliss, A. L., Reale, V., and Evans, P. D. (2016). Characterisation of signalling by the endogenous GPER1 (GPR30) receptor in an embryonic mouse hippocampal cell line (mHippoE-18). *PLoS One* 11:e0152138. doi: 10.1371/journal.pone.0152138
- Field, M., Wiers, R. W., Christiansen, P., Fillmore, M. T., and Verster, J. C. (2010). Acute alcohol effects on inhibitory control and implicit cognition: implications for loss of control over drinking. *Alcohol. Clin. Exp. Res.* 34, 1346–1352. doi: 10.1111/j.1530-0277.2010.01218.x
- FlyBase Consortium. (2003). The FlyBase database of the *Drosophila* genome projects and community literature. *Nucleic Acids Res.* 31, 172–175. doi: 10.1093/nar/gkg094
- Grosjean, Y., Rytz, R., Farine, J. P., Abuin, L., Cortot, J., Jefferis, G. S., et al. (2011). An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* 478, 236–240. doi: 10.1038/nature10428
- Han, K.-A., Millar, N. S., and Davis, R. L. (1998). A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* 18, 3650–3658.
- Han, K.-A., Millar, N. S., Grotewiel, M. S., and Davis, R. L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16, 1127–1135. doi: 10.1016/S0896-6273(00)80139-7
- Harrison, S. J., and Nobrega, J. N. (2009). Differential susceptibility to ethanol and amphetamine sensitization in dopamine D3 receptor-deficient mice. *Psychopharmacology* 204, 49–59. doi: 10.1007/s00213-008-1435-x
- Hawley, W. R., Battista, C., Divack, S. R., and Morales Nunez, N. B. (2017). The role of estrogen G-protein coupled receptor 30 (GPR30) and sexual experience in sexual incentive motivation in male rats. *Physiol. Behav.* 177, 176–181. doi: 10.1016/j.physbeh.2017.05.001
- Inagaki, H. K., Ben-Tabou de-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., et al. (2012). Visualizing neuromodulation *in vivo*: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595. doi: 10.1016/j.cell.2011.12.022

- Ishimoto, H., Wang, Z., Rao, Y., Wu, C.-F., and Kitamoto, T. (2013). A novel role for ecdysone in *Drosophila* conditioned behavior: linking GPCR-mediated non-canonical steroid action to cAMP signaling in the adult brain. *PLoS Genet.* 9:e1003843. doi: 10.1371/journal.pgen.1003843
- Jayaram-Lindström, N., Ericson, M., Steensland, P., and Jerlhag, E. (2016). "Dopamine and alcohol dependence: from bench to clinic," in *Recent Advances in Drug Addiction Research and Clinical Applications*, eds M. M. William and L. R. Christina, (East Providence, RI: InTech), 81–114. doi: 10.5772/61445
- Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., and Heberlein, U. (2011). A *Drosophila* model for alcohol reward. *Nat. Neurosci.* 14, 612–619. doi: 10.1038/nn.2805
- Kim, Y.-C., Lee, H.-G., and Han, K.-A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. *J. Neurosci.* 27, 7640–7647. doi: 10.1523/JNEUROSCI.1167-07.2007
- Kim, Y.-C., Lee, H.-G., Lim, J., and Han, K.-A. (2013). Appetitive learning requires the $\alpha 1$ -like octopamine receptor OAMB in the *Drosophila* mushroom body neurons. *J. Neurosci.* 33, 1672–1677. doi: 10.1523/JNEUROSCI.3042-12.2013
- Kong, E. C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M. R., et al. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in *Drosophila*. *PLoS One* 5:e9954. doi: 10.1371/journal.pone.0009954
- Lê, A., Tomkins, D., Higgins, G., Quan, B., and Sellers, E. (1997). Effects of 5-HT 3, D 1 and D 2 receptor antagonists on ethanol- and cocaine-induced locomotion. *Pharmacol. Biochem. Behav.* 57, 325–332. doi: 10.1016/s0091-3057(96)00333-4
- Lee, H.-G., Kim, Y.-C., Dunning, J. S., and Han, K.-A. (2008). Recurring ethanol exposure induces disinhibited courtship in *Drosophila*. *PLoS One* 3:e1391. doi: 10.1371/journal.pone.0001391
- Lim, J., Sabandal, P. R., Fernandez, A., Sabandal, J. M., Lee, H.-G., Evans, P., et al. (2014). The octopamine receptor Oct β 2R regulates ovulation in *Drosophila melanogaster*. *PLoS One* 9:e104441. doi: 10.1371/journal.pone.0104441
- Maggiolini, M., and Picard, D. (2010). The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J. Endocrinol.* 204, 105–114. doi: 10.1677/JOE-09-0242
- Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C., and Baker, B. S. (2005). Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour. *Nature* 436, 395–400. doi: 10.1038/nature03859
- Mao, Z., and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Front. Neural Circuits* 3:5. doi: 10.3389/neuro.04.005.2009
- Matsuzawa, S., Suzuki, T., Misawa, M., and Nagase, H. (1999). Involvement of dopamine D 1 and D 2 receptors in the ethanol-associated place preference in rats exposed to conditioned fear stress. *Brain Res.* 835, 298–305. doi: 10.1016/s0006-8993(99)01606-6
- McGuire, S. E., Mao, Z., and Davis, R. L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. *Sci. STKE* 2004:pl6. doi: 10.1126/stke.2202004pl6
- Meyer, P. J., Meshul, C. K., and Phillips, T. J. (2009). Ethanol- and cocaine-induced locomotion are genetically related to increases in accumbal dopamine. *Genes Brain Behav.* 8, 346–355. doi: 10.1111/j.1601-183X.2009.00481.x
- Morris, L. S., Kundu, P., Baek, K., Irvine, M. A., Mechelmans, D. J., Wood, J., et al. (2016). Jumping the gun: mapping neural correlates of waiting impulsivity and relevance across alcohol misuse. *Biol. Psychiatry* 79, 499–507. doi: 10.1016/j.biopsych.2015.06.009
- Palmer, A. A., Low, M. J., Grandy, D. K., and Phillips, T. J. (2003). Effects of a Drd2 deletion mutation on ethanol-induced locomotor stimulation and sensitization suggest a role for epistasis. *Behav. Genet.* 33, 311–324. doi: 10.1023/A:1023450625826
- Perkins, L. A., Holderbaum, L., Tao, R., Hu, Y., Sopko, R., McCall, K., et al. (2015). The transgenic RNAi project at harvard medical school: resources and validation. *Genetics* 201, 843–852. doi: 10.1534/genetics.115.180208
- Petrucelli, E., Li, Q., Rao, Y., and Kitamoto, T. (2016). The unique dopamine/ecdyseroid receptor modulates ethanol-induced sedation in *Drosophila*. *J. Neurosci.* 36, 4647–4657. doi: 10.1523/JNEUROSCI.3774-15.2016
- Roman, G., Endo, K., Zong, L., and Davis, R. L. (2001). P Switch, a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U S A* 98, 12602–12607. doi: 10.1073/pnas.221303998
- Scholz, H., Ramond, J., Singh, C. M., and Heberlein, U. (2000). Functional ethanol tolerance in *Drosophila*. *Neuron* 28, 261–271. doi: 10.1016/s0896-6273(00)00101-x
- Srivastava, D. P., Yu, E. Y., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., et al. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *J. Neurosci.* 25, 6145–6155. doi: 10.1523/JNEUROSCI.1005-05.2005
- Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., and Dickson, B. J. (2005). Neural circuitry that governs *Drosophila* male courtship behavior. *Cell* 121, 795–807. doi: 10.1016/j.cell.2005.04.026
- Sugamori, K. S., Demchyshyn, L. L., McConkey, F., Forte, M. A., and Niznik, H. B. (1995). A primordial dopamine D1-like adenylyl cyclase-linked receptor from *Drosophila melanogaster* displaying poor affinity for benzazepines. *FEBS Lett.* 362, 131–138. doi: 10.1016/0014-5793(95)00224-w
- Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., et al. (2004). A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat. Genet.* 36, 283–287. doi: 10.1038/ng1314
- Thum, A. S., Jenett, A., Ito, K., Heisenberg, M., and Tanimoto, H. (2007). Multiple memory traces for olfactory reward learning in *Drosophila*. *J. Neurosci.* 27, 11132–11138. doi: 10.1523/JNEUROSCI.2712-07.2007
- Vena, A. A., Mangieri, R., and Gonzales, R. A. (2016). Regional analysis of the pharmacological effects of acute ethanol on extracellular striatal Dopamine activity. *Alcohol. Clin. Exp. Res.* 40, 2528–2536. doi: 10.1111/acer.13246
- Volkow, N. D., and Morales, M. (2015). The brain on drugs: from reward to addiction. *Cell* 162, 712–725. doi: 10.1016/j.cell.2015.07.046

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The Biogenic Amine Tyramine and its Receptor (AmTyr1) in Olfactory Neuropils in the Honey Bee (*Apis mellifera*) Brain

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This article describes the cellular sources for tyramine and the cellular targets of tyramine via the Tyramine Receptor 1 (AmTyr1) in the olfactory learning and memory neuropils of the honey bee brain. Clusters of approximately 160 tyramine immunoreactive neurons are the source of tyraminergeric fibers with small varicosities in the optic lobes, antennal lobes, lateral protocerebrum, mushroom body (calyces and gamma lobes), tritocerebrum and subesophageal ganglion (SEG). Our tyramine mapping study shows that the primary sources of tyramine in the antennal lobe and calyx of the mushroom body are from at least two Ventral Unpaired Median neurons (VUMmd and VUMmx) with cell bodies in the SEG. To reveal AmTyr1 receptors in the brain, we used newly characterized anti-AmTyr1 antibodies. Immunolocalization studies in the antennal lobe with anti-AmTyr1 antibodies showed that the AmTyr1 expression pattern is mostly in the presynaptic sites of olfactory receptor neurons (ORNs). In the mushroom body calyx, anti-AmTyr1 mapped the presynaptic sites of uniglomerular Projection Neurons (PNs) located primarily in the microglomeruli of the lip and basal ring calyx area. Release of tyramine/octopamine from VUM (md and mx) neurons in the antennal lobe and mushroom body calyx would target AmTyr1 expressed on ORN and uniglomerular PN presynaptic terminals. The presynaptic location of AmTyr1, its structural similarity with vertebrate alpha-2 adrenergic receptors, and previous pharmacological evidence suggests that it has an important role in the presynaptic inhibitory control of neurotransmitter release.

Keywords: biogenic amine receptors, G-protein coupled receptors, tyramine, learning and plasticity, olfactory pathways

INTRODUCTION

The biogenic amines tyramine and octopamine are neuroactive compounds that are involved in a large repertoire of invertebrate behaviors, including locomotion, sensory processing, learning and memory (Roeder, 2005; Scheiner et al., 2006; Lange, 2009). Since Erspamer and Boretti (1951a,b) first described octopamine in the salivary gland of the octopus as having “adrenaline-like” action, many studies have demonstrated the important role octopamine and its biosynthetic precursor tyramine play in invertebrate physiology and behavior. The source of octopamine was mostly allocated to the paracrine cells, the so-called dorsal (ventral) unpaired median (DUM/VUM) neurons, first described by Plotnikova (1969). Due to their location on

the midline of the ventral nerve cord and brain, and to their unique morphology, Hoyle (1974) proposed that these octopaminergic neurons are involved in the modulation of many types of behavior.

Tyramine is synthesized from tyrosine by the enzyme tyrosine decarboxylase, and then octopamine is synthesized from tyramine in one step by the action of enzyme tyramine beta-hydroxylase (David and Coulon, 1985). Until recently, tyramine was thought only to be the precursor of octopamine, without playing any other significant role. Studies of tyramine and its receptors in invertebrates clearly indicated that tyramine has sources and functions independent of octopamine (Kutsukake et al., 2000; Roeder et al., 2003; Alkema et al., 2005; Roeder, 2005; Lange, 2009; Bayliss et al., 2013; Scheiner et al., 2014; Ishikawa et al., 2016). In the locust, tyramine is expressed in all neurons that express octopamine as well as in some cells that do not express either the beta-hydroxylase enzyme or octopamine (Kononenko et al., 2009; Homberg et al., 2013). Studies in the fruit fly larval central nervous system also reported the presence of tyramine-containing neurons that are distinct from octopaminergic neurons (Nagaya et al., 2002).

Tyramine and octopamine trigger intracellular signaling pathways by binding with different affinities to a variety of octopamine receptors (OARs) or tyramine receptors (TYRs), most of which are G-protein coupled receptors (GPCRs; Grohmann et al., 2003; Balfanz et al., 2005; Hauser et al., 2006). There are four different classes of invertebrate GPCRs that bind octopamine and/or tyramine (Evans and Maqueira, 2005; Maqueira et al., 2005; Verlinden et al., 2010; Bayliss et al., 2013; Balfanz et al., 2014). Alpha-adrenergic-like OARs (Oct α Rs/OA1), beta-adrenergic-like OARs (Oct β Rs/OA2), octopamine/tyramine (Oct/Tyr/TyrR I) receptors, and TyrR II (Verlinden et al., 2010). When it binds with octopamine, AmOA1 releases calcium from cytosolic stores (Grohmann et al., 2003). The OA2 receptor stimulates adenylyl cyclase activity, which leads to an increase of 3,5-cyclic monophosphate (cAMP; Robb et al., 1994; Roeder, 1999; Maqueira et al., 2005; Balfanz et al., 2014). TyrR I (in the honey bee, AmTyr1 or AmTAR1) preferentially binds to tyramine and inhibits adenylyl cyclase activity. TyrR II (in the honey bee AmTAR2) can mediate calcium signaling and/or affect cAMP levels (Blenau et al., 2000; Verlinden et al., 2010; Ohta and Ozoe, 2014; Reim et al., 2017).

AmTyr1 has been cloned and characterized (Blenau et al., 2000; Blenau and Baumann, 2001, 2003). Earlier localization studies using *in situ* hybridization indicated that AmTyr1 is expressed on cell bodies of mushroom body Kenyon cells (KCs) and in the antennal lobe (Mustard et al., 2005). In the cockroach, PeaTyr1 is expressed in abundance in all brain neuropils as well as in peripheral tissues such as the salivary glands (Rotte et al., 2009). A recent study by Reim et al. (2017) characterized the *Apis mellifera* TYR type 2 (AmTAR2). The authors provide evidence that AmTAR2, when heterologously expressed in flpTM cells, exclusively causes an increase in cAMP.

Here we use immunocytochemistry to describe the localization of tyramine and its receptor AmTyr1 in the

olfactory networks of the antennal lobe and mushroom bodies. We focused on AmTyr1 because it has been implicated in genetic studies of foraging and reproductive behaviors as well as in olfactory learning in honey bees (Chandra et al., 2010; Wang et al., 2012; Scheiner et al., 2014). The antennal lobe of the honey bee is the anatomical and functional analog of the vertebrate olfactory bulb (Hildebrand and Shepherd, 1997). The antennal lobe consists of an aglomerular neuropil that is surrounded by 160 glomeruli, where each glomerulus participates in coding for a subset of odors. The cortex—the outer rind—of each glomerulus receives olfactory receptor inputs from axons of olfactory receptor neurons (ORNs). Each glomerulus contains dendrites of Projection Neurons (PNs), axons from which then connect the antennal lobe to higher order processing centers—the lateral horn (LH) and mushroom body calyx. The PN dendrites in the core of a glomerulus receive synapses from local neurons (Sinakevitch et al., 2013). The mushroom bodies are higher order olfactory processing centers. They contain intrinsic neurons—the KCs—that have cell bodies packed around the mushroom body calyces. The dendrites of KCs in basal ring and lip of the calyces receive olfactory and gustatory afferents (Strausfeld, 2002). The dendrites of KCs in the collar area of the calyx receive visual afferents. KCs axons make up the peduncle and mushroom body lobes: vertical, medial and γ (Strausfeld, 2002).

Tyramine release in the antennal lobe and mushroom body could modulate this network at several points, but the precise anatomical distribution of tyramine and its receptors has not been analyzed in detail, except for a publication contemporary to ours (Thamm et al., 2017). In our study, we used antibodies against conjugated tyramine to show its immunolocalization, and we compared the distribution of anti-tyramine staining to the distribution of octopamine staining published in earlier work (Kreissl et al., 1994; Sinakevitch et al., 2005). We also generated and characterized antibodies against AmTyr1 protein and used them to identify the distribution of the AmTyr1 receptor on neurons that are critical components of the olfactory circuitry. Our study shows how tyramine via AmTyr1 is poised to modulate odor processing at different points in the honey bee brain.

MATERIALS AND METHODS

Animals

Honey bees (*Apis mellifera* L.) were adult New World Carniolan foragers of unknown age obtained from colonies maintained at Arizona State University. The bees were collected at the entrance of the hive when they returned from the field with pollen, usually in the afternoon.

Anti-Tyramine Staining

Tyramine antiserum (AB124; EMD Millipore) was raised in rabbits using p-tyramine conjugated to N-alpha-acetyl-L-lysine-N-methylamide using glutaraldehyde (Geffard et al., 1984b). This antiserum has been used in the locust and fruit fly to describe tyramine-like immunoreactivity in the brain (Nagaya et al., 2002; Kononenko et al., 2009; Homberg et al., 2013).

Antiserum specificity of immunostaining was tested using tyramine/octopamine conjugated to bovine serum albumin (BSA). The conjugates were prepared as described elsewhere (Geffard et al., 1984a; Mons and Geffard, 1987). Brains were dissected out of the head in fixative containing 3% glutaraldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) in 0.1 M cacodylate buffer (EMS, pH 7.0) with 1% sodium metabisulfite (SMB, Sigma-Aldrich, St. Louis, MO, USA). Each dissected brain was then transferred into 1 ml of fresh fixative and left overnight at 4°C. To saturate double bonds, after fixation the brains were treated with 0.5% sodium borohydride (NaBH₄; Sigma-Aldrich) in 0.05 M Tris-HCl buffer containing 0.45% SMB (Tris-HCl-SMB; Sigma), pH 7.4 for 20 min. After washing with Tris-HCl-SMB buffer (4 × 10 min), the brains were embedded in 8% agarose (low melted point A0169, Sigma-Aldrich) in water. Brain sections (70 μm) were made with a Vibratome Leica 1000S (Leica Biosystem, Germany).

Brain sections were washed (6 × 20 min) in Tris-HCl-SMB buffer containing 0.5% Triton-X100 (TX) and were incubated with 1% normal donkey serum (Jackson ImmunoResearch Laboratories) in Tris-HCl-TX for 15 min. Then, tyramine antiserum was added to a final dilution of 1:500 to each brain and left for 48 h. After washing in 0.05 M Tris-HCl-TX, pH 7.5 (6 × 20 min), F(ab')₂ fragments of donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, diluted 1:250 in Tris-HCl-TX) were applied as the secondary antibody overnight. All incubations were at room temperature. After final washing in 0.05 M Tris-HCl pH 7.4 (6 × 20 min) sections were mounted on slides in 80% glycerol in phosphate-buffered saline (PBS) mix.

To test the specificity of tyramine immunostaining, working dilutions of the anti-tyramine antibodies were preincubated overnight without and with tyramine conjugated to BSA, (10⁻⁴ M concentration of tyramine in the conjugate, **Figures 1A,B**). After preincubation of the primary antiserum with tyramine-G-BSA, anti-tyramine staining was absent

(**Figure 1B**). However, after preincubation of the antibodies with octopamine-G-BSA (10⁻⁴ M concentration of octopamine; **Figure 1C**), immunolabeling in the cell body and fine processes was present. Therefore, the working dilution of the tyramine antiserum specifically recognizes tyramine in cell bodies and their processes in fixed honey bee brain sections. Twenty bee brains were processed and analyzed in our studies, eight brains that were fixed and processed on the same days were used to count cell bodies (**Table 1**).

Immunocytochemistry with Anti-Synapsin Antibody

Immunocytochemistry with mouse monoclonal anti-synapsin antibody (SYNORF1; clone 3C11, Developmental Studies Hybridoma Bank, The University of Iowa) was used to label the synaptic neuropil in brain whole-mounts according to the protocol of Brandt et al. (2005). Brains (*n* = 3) were fixed in 4% paraformaldehyde in PBS overnight, washed in PBS containing 1% Triton-X (PBS-TX), preincubated 1 h with normal donkey serum, and then the anti-synapsin antibody 1:1000 was applied for 3 days at room temperature under gentle shaking. After six washes of 1 h each with PBS-TX, the brains were incubated with the secondary antibody (F(ab')₂ fragments of donkey anti-mouse conjugated to Alexa 488, Jackson ImmunoResearch Laboratories) diluted 1:250 in PBS-TX for 2 days at room temperature. After final washing in PBS, the whole-mounts were dehydrated, cleared and embedded in methyl salicylate. The whole-mount brains were embedded in methyl salicylate for observation (*n* = 3). To illustrate the location of the anti-tyramine staining cell bodies, we used the consecutive protocol staining technique on sections. Three brains that were sectioned and labeled with anti-tyramine were post-fixed for 20 min with 4% paraformaldehyde, and then brain sections were processed for anti-synapsin immunostaining as described above to obtain tyramine immunoreactive cell bodies in the sections with the labeled synaptic structure of neuropil (*n* = 3).

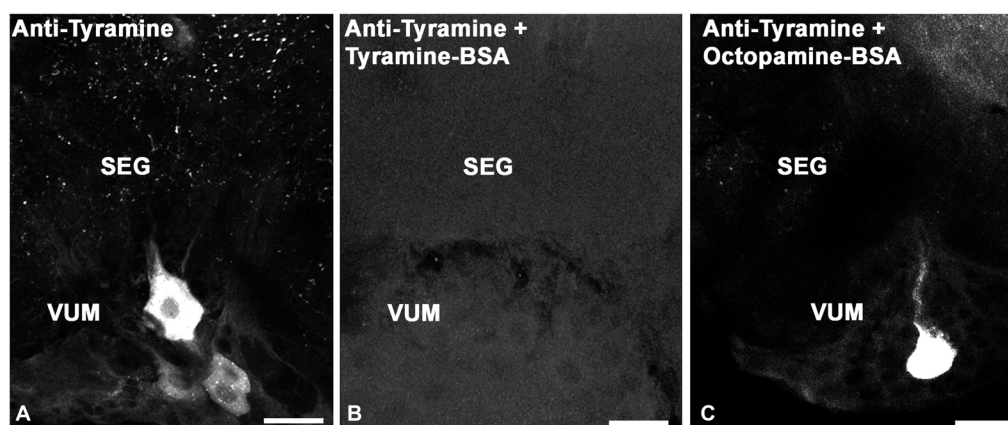


FIGURE 1 | Controls for immunolabeling with tyramine antiserum. We used three sections of the subesophageal ganglion (SEG) that contain ventral median group cells (VUM). The sections were labeled with one of the following: **(A)** tyramine antiserum; **(B)** tyramine antiserum pre-incubated with conjugated tyramine-bovine serum albumin (BSA); **(C)** tyramine antiserum pre-incubated with octopamine-BSA. Scale bar: 25 μm.

TABLE 1 | The number of tyramine-like immunoreactive neurons in clusters of cells in the brain and subesophageal ganglion of *Apis mellifera*^{1,2}.

	Bee N = 8 No. of cells on each side	Neuropils supplied in Apis
Cluster G0	3 (10)	NCC1
Cluster G0b	None	
Cluster G1	None	
Cluster G2a	6 (25)	EB, FB, M Pro (circum Ped, V Lo, α lobe ?
Cluster G2b	5 (10)	V-M Pro
Cluster G3a	6 (25) + 3 (15)	L Pro, Op Lo, Oc N, PB, FB, γ lobe?
Cluster G3b	2 (15)	L Pro
Cluster G4a	2 (10)	PB and EB
Cluster G4b	16 (10) + 1 (25)	PB, EB, D-M Pro
Cluster G4c	2 (15)	L Pro
Cluster G4d	4 (8)	
Cluster G5a	None	
Cluster G5b	5 (25) + 5(8)	L Pro, M Pro, Ant Mech, Ant nerve, sub CB, OpLo
Cluster G6a	3 (25)	Deu, Trito, Ant N? SEG
Cluster G6b	3 (10)	SEG
Ventral unpaired median (VUM) neurons mandibular	8 ³ (25)	SEG Md Neuromere, Trito L Ho, Ca, Ant Lo, Ant Nerve, NCC1?
VUM neurons maxillary	8 ³ (25)	SEG, L Ho, Ca, Ant Lo, L Pro, SEG, Mx nerve
Ventral paired median (VPM) neurons	2 ³ (25)	L Pro, M Pro, NCC1?
VUM neurons labial	8 ³ (25)	SEG Labial Neuromere, NCC1
DUM neurons labial	2 ³ (25)	?

¹The terminology used for cell groups is the same as in Sinakevitch et al. (2005). ²The approximate sizes of cell bodies are shown in parenthesis in μm . ³Total number of cell bodies on midline of SEG. NCC, nervii corpora cardiaci; FB, fan-shaped body; EB, ellipsoid body; PB, protocerebral bridge; D, L, M, or V Pro, dorsal, lateral, medial, or ventral protocerebrum; Ant Lo, antennal lobe; Op Lo, SEG, subesophageal ganglion; Trito, tritocerebrum, Deu, deutocerebrum (other than Ant Lo and Ant Mech); Ant Mech, antennal mechanosensory neuropil; sub CB, neuropil beneath ellipsoid and fan-shaped body; circum Ped, protocerebral neuropil surrounding pedunculus and vertical lobe (V Lo); α and γ division of mushroom body vertical lobe; L Ho, lateral horn of protocerebrum; Ca, calyx; Oc N, ocellar nerve.

Three Dimensional (3D) Model of the AmTyr1 Receptor Structure

The structural model of AmTyr1 (NCBI Reference Sequence: NP_001011594.1) was generated by I-TASSER (Zhang, 2008; Roy et al., 2010; Yang and Zhang, 2015; Yang et al., 2015) based on the top 10 proteins from the PDB that have the closest structural similarity. Besides the threading-based restraints, no additional external restraints were specified. Molecular graphics and localization of the antigenic peptides were performed with the UCSF Chimera package (Pettersen et al., 2004) developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco.

Anti-AmTyr1 Receptor Antibody

Design of Conjugated Peptides and Antibody Production

Anti-AmTyr1 receptor antibodies were produced in two rabbits immunized with two peptides from the N-terminus of AmTyr1 (H₂N-TEDYDMTGCGPPEET-amid (peptide-1, P1) and H₂N-PEELEPGTPCQLTRRQG-amide (peptide-2, P2) conjugated to Keyhole limpet hemocyanin (KLH; **Figure 2A**). After four immunizations, serum from two rabbits was collected and affinity purified. All these procedures were performed by 21st Century Biochemical Incorporation (Marlboro, MA, USA).

Western Blot

The affinity purified anti-AmTyr1 antibody raised against peptide-1 and peptide-2 were further characterized by western blot (**Figure 2B**). The brains were dissected and processed

for membrane protein extraction. Each individual brain was homogenized in 100 μl of lysis buffer (120 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% glycerol, 0.2 mM dithiothreitol, 1% Triton X100 containing 5 $\mu\text{g/ml}$ of each protease inhibitors PMSF (Phenylmethylsulfonylfluoride), Aprotinin, Benzamidin (all from Sigma-Aldrich) pH 6.8). Homogenates were centrifuged at 12,000 g for 20 min at 4°C. Then 30 μl (1/3 of a bee brain) of the supernatant was added with 6 $\times$ Laemmli buffer and loaded on a 7.5% SDS-polyacrylamide Tris-glycine gel to separate the proteins. Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 15% methanol) at 0.45 amp for 1 h 30 min at 4°C. Then the membranes were blocked for 1 h in PBS containing 0.1% Tween-20 (PBS-Tw) and 5% low fat powdered milk. Then they were incubated with anti-AmTyr1 antibody raised against conjugate of peptide-1 and peptide-2, each on separate membrane at 1:1000 in PBS-Tw plus 5% milk for 4 h at room temperature. Following four 15 min washes in PBS-Tw with 5% milk, membranes were incubated with anti-rabbit IgG HRP-conjugated secondary antibodies (Rockland Inc.) at 1:10,000 in PBS-Tw with 5% milk for 2 h. Membranes were washed four times in PBS-Tw and developed using chemiluminescence as described by the manufacturer (Immobilon Western Chemiluminescent HRP Substrate; Millipore Corporation). The preadsorption control was done with the corresponding peptide-conjugated to KLH with each anti-AmTyr1 antibodies where the peptide concentration was approximately 10^{-5} M. In these procedures, anti-AmTyr1 + 1×10^{-5} M peptide-KLH were incubated for

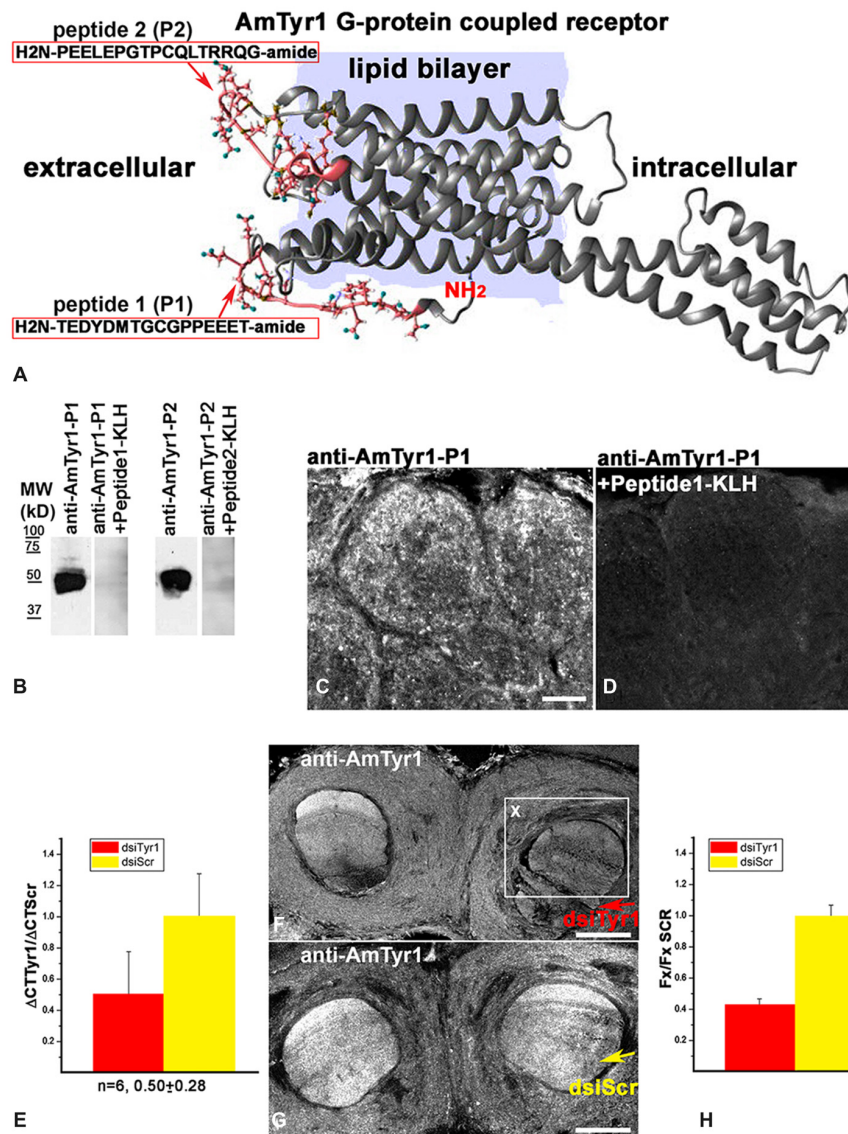


FIGURE 2 | Characterization of anti-AmTyr1 antibodies. **(A)** Predicted structural model of the *Apis mellifera* tyramine receptor (NP_001011594.1). Peptides (P1, P2) from the N-terminal region and the loop between helices 4 and 5 used to generate the antibodies are represented by their amino acid sequence (pink). **(B)** Affinity purified anti-AmTyr1 antibodies against peptide-1 and peptide-2 were tested in western analyses. The relative positions of molecular weight (MW) standards in kDa are indicated. The affinity purified anti-AmTyr1-P1 and anti-AmTyr1-P2 each revealed a band corresponding to an approximate molecular weight of 45 kDa. Preincubation of the anti-AmTyr1 antibodies separately with corresponding peptide conjugates abolished the band. **(C)** Anti-AmTyr1 immunostainings in the antennal lobe revealed processes in the cortex of glomeruli on frontal section of the brain. **(D)** In the next consecutive section, staining in glomeruli was not present when the anti-AmTyr1 antibodies were pre-incubated with Keyhole Limpet Hemocyanin (KLH)-conjugated peptide-1 before immunostaining. **(E)** Expression of *AmTyr1* gene in brains injected with 70 nl of 100 μM dsiAmTyr1 RNA or dsiScramble 14 h after treatments. *AmActin* was used as a reference gene. The relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The data are expressed as mean $\pm$ SE. **(F)** Anti-AmTyr1 staining in the brain section 18 h after injection dsiAmTyr1 RNA **(G)** and dsiScr. Arrows in **(F,G)** indicate injections sites in the frontal sections of the bee brains. **(H)** Quantification of the average fluorescence intensity value in the box X (Fx), outlined in F in the raw images of brains that were injected with dsiTyr1 and dsiScr. Images were collected with a confocal fluorescent microscope with the same gain settings and intensity level. Relative intensity level of fluorescence dropped to $42 \pm 5\%$ (mean $\pm$ SE) in the dsiTyr1 injected brains compared to dsiScr brains in the local area of the injections. Scale bar: **C,D** = 10 μm , **F,G** = 100 μm .

2 h at 37°C with gentle shaking. Then after centrifugation for 10 min at 10,000 g at 4°C, the supernatant was applied on the membrane and processed as described above. This treatment abolished staining on the membrane for both antibodies.

Anti-AmTyr1 Staining

For all immunostaining on bee brain sections, we used the anti-AmTyr1 antibodies that were raised against the peptide-1, and the protocol for testing their immunostaining specificity on bee brain sections is described below.

Honey bee forager brains were removed from the head capsule under fixative containing 4% paraformaldehyde in PBS, then each brain was placed in one milliliter of the same fixative overnight at 4°C. The next morning, brains were washed in PBS and embedded in 8% agarose. Each agarose block was sectioned (70 μ m) and processed for immunostaining. First, brain sections were preincubated with 1% normal donkey serum and then anti-AmTyr1 (affinity purified polyclonal antibodies raised against the peptide-1 in rabbit) at 1:500 dilution in the PBS-0.5%TX solution was added to each brain. To visualize the staining, F(ab)² fragments of donkey anti-rabbit IgG conjugated to Cy3 were used at a dilution of 1:500. After each step, there were at least six washes of 20 min each in PBS-TX (6 $\times$ 20 min). PBS only was used in the final wash before embedding brain sections on a glass slide. Twenty brains were processed with anti-AmTyr1 antibodies in these experiments.

Characterization Specificity of Immunostaining of anti-AmTyr1 (Affinity Purified Antibodies Raised Against Peptide-1) on Bee Brain Sections

To examine the specificity of immunostaining of the anti-AmTyr1 antibodies, sections were incubated with the secondary antibody in the absence of primary antibodies (not shown) or immunoassay with working dilution of anti-AmTyr1 (**Figure 2C**) or anti-AmTyr1 antibody that had been preincubated with KLH conjugated to AmTyr1 peptide-1 via glutaraldehyde (**Figure 2D**). In these procedures, anti-AmTyr1 and anti-AmTyr1 + 2×10^{-4} M peptide-1-KLH were incubated for 2 h at 37°C with gentle shaking. Then after centrifugation for 10 min at 10,000 g at 4°C, the supernatant of each solution was applied on two consecutive sections and processed as described above. All procedures were performed at room temperature unless otherwise noted. Images of sections treated with anti-AmTyr1 antibody preincubated with conjugated peptide were collected at the same level of gain and intensity.

Control for Immunostaining after Knockdown of AmTyr1

To demonstrate that anti-AmTyr1 antibodies specifically recognized the receptor, we used a Dicer-substrate small interfering (dsi) RNA of the *AmTyr1* receptor (NCBI Reference Sequence: NM_001011594.1) to knock down levels of the *AmTyr1* mRNA receptor in the brain (**Figure 2E**). We used the mixture of three dsiAmTyr1 designed by the tool in IDT technology (**Table 2**), and as a control we used scrambled dsiScr. Seventy nanoliters (nL) of 100 μ M mixture of dsiAmTyr1 or dsiScr was injected using a picospritzer into the mushroom body lobe on one side (six bees for each group), and then the brain without optic lobes was dissected and homogenized in 1 ml of TRIzol (Invitrogen) 13–15 h after injections. Then the total mRNA from each injected bee brain part was extracted separately using the manufacturer's protocol for TRIzol method (Invitrogen). Contaminating genomic DNA was removed using DNA-freeTM kit (Ambion, AM1906). RNA quantity and

TABLE 2 | Nucleotide sequences of sense and antisense strands of control DsiSCR and AmTyr1 DsiRNA.

DsiRNA	Sequences
dsiScr	5'-GAGUCCUAAGUUAACCAAGUCACAGCA-3' 3'-CUCAGGAUUAUUGGUUCAGUGUCGU-5'
dsiTyr1_N	5'-AGCGUGACGUUGGAUUGACGAGAGC-3' 3'-CCUCGCACUGCAACCUAACUGCUCUCG-5'
dsiTyr1_T1	5'-CCUGUGCAAAUUGUGGCUAACCCUGC-3' 3'-GUGGACACGUUUAACACCGAUUGGACG-5'
dsiTyr1_C	5'-CAACGCUUUGUUUUAUUGCAUCUAUCG-3' 3'-CCGUUGCGAACAACAAUACGUAGAUAGC-5'

purity was evaluated using a NanoDrop (NanoDrop 2000). Expression of AmTyr1 was quantified using QuantiFAST SYBR Green RT-PCR kit (QIAGEN, 204156) on Applied Biosystem 7500 cyclor with the exact protocol provided by the 96-well kit. We used 20–40 ng of RNA per well. The primers for quantitative real-time PCR assays were characterized and described in Wang et al. (2012): AmTyr1_F 5'-GTTTCGTCGTATGCTGGTTGC-3', AmTyr1_R 5'-GTAGATGAGCGGGTTGAGGG-3' and for reference gene AmActin_F 5'-TGCCAACACTGTCCTTTCTG-3' AmActin_R 5'-AGAATTGACCCACCAATCCA-3'. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

To test immunostaining of the AmTyr1 protein, five dsiAmTyr1 injected brains and five control brains injected with dsiScr were dissected and fixed 18–24 h after injections, then processed for anti-AmTyr1 immunostaining as described in section anti-AmTyr1 staining above (**Figures 2F–H**). To estimate the reduction of protein in fixed brain tissue we used the original raw images of brains injected with dsiTyr1 and dsiScr collected at the same gain. The area of interest X was drawn on each section in the place where dsiRNA was injected (Box X in **Figure 2F**). The average fluorescence intensity value (Fx) was estimated in Adobe Photoshop CC 2015 on each sections on the injected side of the brain. Then statistical analyses and the graph were done in Origin 6.1 software.

Triple Staining with anti-AmTyr1, Anti-Synapsin and Neurobiotin Labeled Neurons

To identify cell types that express AmTyr1 in the antennal lobe, we labeled ORN and PN terminals by injecting neurobiotin into the antenna and antennal lobe. For both procedures, each bee was cooled and placed into a plastic restraining harness, and the head was immobilized with low melting point wax. Then each antenna was gently immobilized with ecosaine, the isomeric hydrocarbons obtained from paraffin wax (Aldrich).

To label ORN endings in the glomeruli, neurobiotin was injected into the antenna, where the ORN axons take up the tracer, which then undergoes anterograde transport to the axon terminals in glomeruli. For neurobiotin injection into the antenna, a small hole was cut in the scape at the base of the antenna, and 50–70 nL of 2% neurobiotin (weight/volume in water) was injected using a picospritzer. To label ORN cell

bodies in the antenna, neurobiotin was injected into the antennal lobe from where the tracer was transported retrogradely to the cell body and dendritic fibers in the sensory receptor pocket. 4',6-diamidino-2-phenylindole (DAPI) was used as a fluorescent marker of cell nuclei in the antenna. Five bees were processed to trace the ORNs and co-labeled with anti-AmTyr1 and anti-synapsin.

To label the PN axon terminals we injected neurobiotin into the antennal lobe, where the PNs take up the tracer and anterogradely transport it to the mushroom body calyx, where axons form the synapses in the KC dendritic field. For this procedure, a small window was cut in the head capsule allowing access for neurobiotin injection into each antennal lobe. The bee then was detached from the holder and placed in a small wooden box with available food (1.5 M sucrose and pollen) and a humidified environment. The bees were sacrificed the next day 16–20 h after dye injections. Brains were dissected and fixed as described above for anti-AmTyr1 staining. Five bees were processed to trace the PNs and co-labeled with anti-AmTyr1 and anti-synapsin.

To label synaptic neuropil, the anti-synapsin antibody (dilution 1:1000) was added together with anti-AmTyr1 (1:500) overnight. Then sections were washed, and secondary antibodies F(ab')₂ fragments donkey anti-rabbit IgG conjugated to Cy3 (1:250) and F(ab')₂ fragments donkey anti-mouse IgG conjugated to 488 (1:250) were added to the sections together with streptavidin conjugated to Cy5 (Jackson ImmunoResearch Laboratories, 1:250) to reveal anti-AmTyr1, anti-synapsin and neurobiotin respectively in the brain sections. Preparations were then thoroughly washed in PBS and embedded in 80% glycerol. To control the specificity of the secondary antibodies, all secondaries were incubated with sections that had only one of the primary antibodies. The staining did not show any cross-reaction between the secondary antibodies and Streptavidin-Cy5. Streptavidin-Cy5 did not interact with any structure in the absence of the neurobiotin in the bee brain.

Confocal Microscopy

Data were collected on a Leica SP5 confocal laser scanning microscope (Leica, Bensheim, Germany) using a Leica HCX PLAPO CS 40_{oil}-immersion objective (numerical aperture: 1.25) with appropriate laser and filter combinations. Stacks of optical sections at 1 μ m spacing were processed using Leica software (1024 $\times$ 1024 pixel resolution) either as a single slice or flattened confocal stacks (maximum intensity projections). Size, resolution, contrast, and brightness of final images were adjusted with Adobe Photoshop software. To generate the general view of a tyramine-containing cell in the whole brain we used whole-mount immunocytochemistry with anti-synapsin antibodies. Serial sections were made at 1 μ m using a Leica $\times$ 10 objective and reconstructed to create 3D images of the brain using AMIRA software (FEI visualization science group). Then the agarose brain sections labeled with both anti-tyramine and anti-synapsin were compared with digital serial sections of the whole-mount brain, and cell bodies were manually added in the appropriate serial digital layer to create the image of tyramine cell body distribution in the whole-mount brain (Figure 3A).

RESULTS

Antibodies and Immunolabeling

The tyramine antiserum used in our studies specifically recognized tyramine, as shown through immunostaining pre-absorption tests (Figures 1A–C). Anti-tyramine immunostainings were repeatable across animals, revealing cell bodies, neurites and varicosities of neurons. The intensity of anti-tyramine staining in the cell bodies was variable, as illustrated in Figure 1A for ventral unpaired neurons: staining ranged from very bright to low intensities. After pre-incubation of working dilutions of anti-tyramine antibodies with tyramine-G-BSA, specific anti-tyramine labeling was abolished, as shown in Figure 1B for SEG frontal sections. When the same working dilution of anti-tyramine antibodies was preincubated with octopamine-G-BSA, the tyramine immunoreactive staining in the cell bodies and processes was still present (Figure 1C). In total, we treated 20 brains with anti-tyramine antibodies, and cell counts in Table 1 were based on eight of the brains.

We created a 3D model of the AmTyr1 receptor protein to show localization of the peptides used for immunization (Figure 2A). We used I-TASSER that employs composite approaches of threading, structural refinement, and ab initio modeling to generate a 3D model of the full-length AmTyr1 receptor. The best predicted model had a C (confidence)-score of -0.89 , and the estimated TM (template modeling)-score and RMSD (root mean square deviation) of 0.60 ± 0.14 Å and 8.8 ± 4.6 Å, respectively. Generally, values of the C-score (typically, in the range of $[-5, 2]$) and the TM-score (range of $[0, 1]$) higher than -1.5 and 0.5 , respectively, are indicative of correct global fold and main chain topology. RMSD is more sensitive to local errors, and it is not unusual to have big values for proteins of considerable length. In the light of the above, the generated model of the AmTyr1 receptor appears to be of reasonably high accuracy/quality.

The AmTyr1 receptor protein has seven transmembrane domains with a large intracellular loop between helices 5 and 6 and a short C-terminus. The peptides used for immunizations are located extracellularly in the N-terminus and between helices 4 and 5 (Figure 2A). After four immunizations, the AmTyr1 antisera were affinity purified and the anti-AmTyr1 antibodies were further characterized. First, we used western blotting to analyze anti-AmTyr1 antibodies for both peptides separately (Figure 2B). We observed one large band in the Western blot of brain homogenate proteins, and it corresponded to the predicted weight of the TYR protein 45 kD (Figure 2B) for both antibodies. Moreover, pre-incubation of anti-AmTyr1-peptide-1 and anti-AmTyr1 peptide-2 with corresponding peptide conjugated to KLH abolished the immunolabeling on Western blot (Figure 2B). In all our AmTyr1 receptor immunolabeling on bee brain sections, we used only the antibody raised against H₂N-TEDYDMTGCGPPEET-amid (peptide-1, P1). Therefore, we used only this antibody in tests for specificity of immunolabeling.

Control immunolabeling on brain sections revealed that anti-AmTyr1 was strongly expressed in the cortex of glomeruli (Figure 2C), and staining was absent after pre-incubation

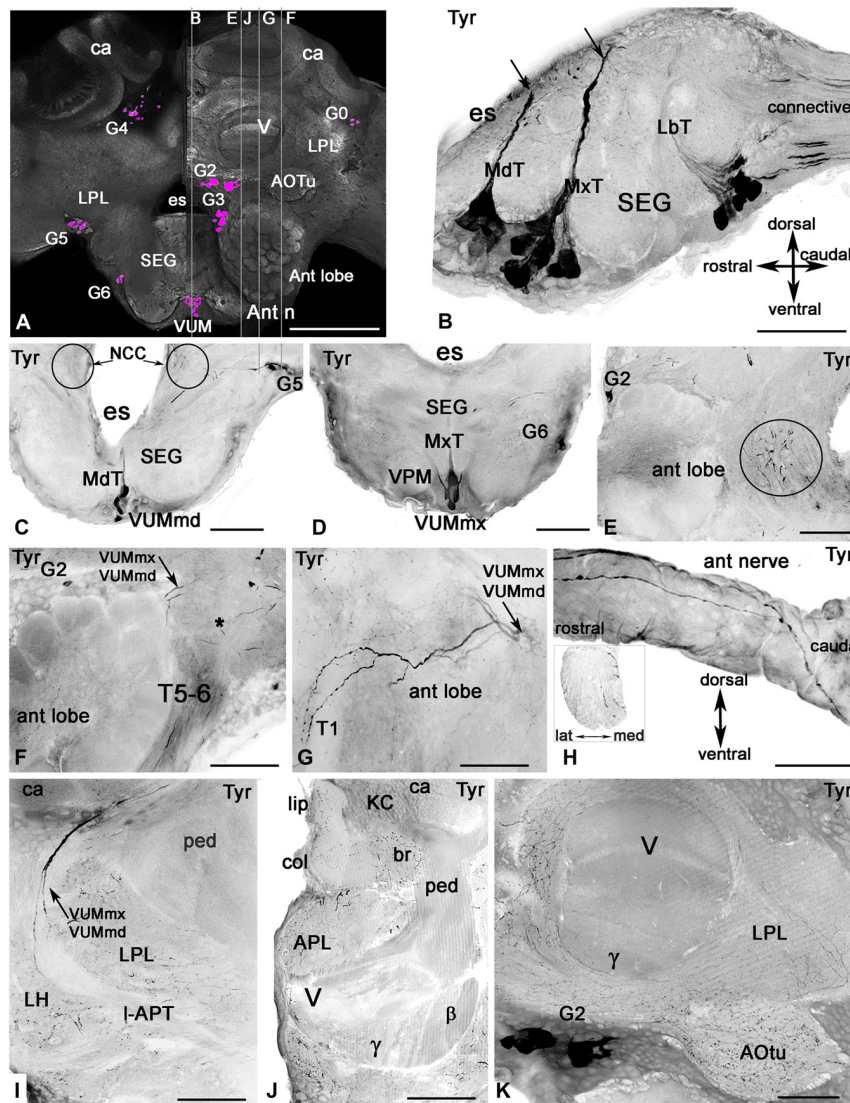


FIGURE 3 | Tyramine-like immunoreactivity in the honey bee brain using inverted fluorescence images. **(A)** Schematic representation of the brain (frontal view) with the groups of cell bodies labeled with anti-tyramine antibodies. The left and right halves of the schematic demonstrate the caudal and rostral planes of the brain. The tyramine containing cell groups (magenta) are G2-G6 and VUM. The plane of the sagittal sections on corresponding images **(B,E,F,G,I,J)** is indicated by the vertical lines. **(B)** The sagittal section through the SEG with anti-tyramine labeled groups of median neurons in mandibular (Md), maxillary (Mx) and labial (Lb) neuromeres with their primary neurites in corresponding Md (MdT), Mx (MxT) and Lb (LbT) tracts. **(C,D)** Frontal sections of SEG made via Md **(C)** and Mx **(D)** neuromeres respectively, show corresponding frontal view of VUMmd **(C)** and VUMmx **(D)** and ventral paired median (VPM) neurons. The VUM neurons send their primary neurites to the corresponding tracts, and the secondary neurites branch in the deutocerebrum (circle in **C,E**). **(F,G)** Two tyramine immunoreactive axons from VUM neurons, one from MdN and one from MxN, innervate the antennal lobe (sagittal sections, front on the left) and give rise to ramifications in glomeruli and aglomerular neuropils of the antennal lobe **(G)**. Asterisk in **(F)** indicates dorsal lobe. **(G)** The tyramine immunoreactivity in tract T5-T6 is from unidentified neurons in the tritocerebrum and SEG. **(H)** These unidentified tyramine immunoreactive neurons enter into the antennal nerve and are running along the top of the antennal nerve in the sagittal view and inside of the nerve (frontal view, insert). **(I)** The secondary neurites from tyramine immunoreactive VUMmd and mx enter in the lateral antenna-protocerebral tract (I-APT) and innervate the lateral horn (LH) and mushroom body calyx (ca). **(J)** In the mushroom body calyx, they innervate the basal ring (br) and lip areas, which receive olfactory afferents from the antennal lobe. The mushroom body pedunculus (ped) and lobe are almost free from tyramine immunoreactive innervation **(I,J,K)** except for a few branches in the γ lobe **(K)** that might originate from LPM neurons from SEG. The arrow in **(C)** indicates tyramine immunoreactive fibers running alongside of the esophageal (es) to the corpora cardiaca nerve (NCC). Ant lobe, antennal lobe; ca, calyx of mushroom body; SEG, subesophageal ganglion; VUM, ventral unpaired median neurons; Ant n, antennal nerve; LPL, lateral protocerebral lobe; APL, anterior protocerebral lobe; AOTu, anterior optic tubercle; V, γ , β , vertical lobe of mushroom bodies, KC, Kenyon cell bodies. Scale bar: **A** = 250 μ m, **B** = 50 μ m, **C-K** = 100 μ m.

of anti-AmTyr1 with peptide conjugated to carrier protein KLH (**Figure 2D**). Furthermore, knockdown experiments using dsiTyr1 RNA injections in an amount that reduced gene

expression by approximately 50% (**Figure 2E**) significantly reduced labeling by anti-AmTyr1 antibodies (**Figure 2F**). In contrast, areas where the scrambled construct (dsiScr) was

injected in the same amount as dsiTyr1 RNA failed to reduce labeling (**Figure 2G**). The average level of fluorescence intensity dropped to 42% in the area close to the dsiTyr1 injection site compared to the same area around the dsSCR injection site (illustrated in **Figure 2H**). **Figures 2F,G** illustrate the frontal sections of the bee brains through the mushroom body vertical lobes embedded into the anterior protocerebrum. From all of these experiments, we conclude that the anti-AmTyr1 antibodies from rabbit specifically recognize the AmTyr1 receptor protein in Western blots and fixed brain sections.

Distribution of Tyramine Immunoreactive Cell Bodies and Processes in the Honey Bee Brain and SEG

The tyramine antiserum labeled clusters of cells in the brain (**Figure 3A**) and on the midline of the SEG (**Figures 3A–D**). Tyramine containing processes are illustrated in the antennal lobe (**Figures 3E–G**) and antennal nerve (**Figure 3H**), the lateral protocerebral lobe, the LH, the mushroom body calyx and lobes (**Figures 3I–K**) and in the anterior optic tubercle (AOTu; **Figure 3K**).

The major tyramine containing fibers in the neuropils of trito-, deuto- and proto-cerebral ganglia arose from the ventral neurons located in the midline of SEG (**Figures 3A–D**). These anti-tyramine labeled median neurons were classified based on the position of their primary neurites in tracts within the three SEG segments corresponding to the mandibular (Md, **Figures 3B,C**), maxillary (Mx, **Figures 3B,D**) and labial (Lb, **Figure 3B**) neuromeres, respectively. These neuromeres receive sensory projections from the nerves of the mouthparts and via the mandibular, maxillary and labial nerves supply muscles of the mouthparts involved in the proboscis-extension reflex (Rehder, 1988; Schröter et al., 2007). The SEG is also the relay station for information in descending and ascending neurons. We focused our study on median ventral neurons from the mandibular and maxillary neuromeres. In particular, we focused on the tyraminerbic/octopaminergic ventral unpaired median (VUM) neurons (VUMmx and VUMmd) that send their symmetrical secondary neurites in each part of the brain to innervate the antennal lobe (**Figures 3E–G**), LH (**Figure 3I**) and mushroom body calyx (**Figures 3I,J**).

The group of anti-tyramine immunostained ventral neurons in the labial neuromere (**Figure 3B**) contains at least two dorsal unpaired neurons (not shown in this illustration). The secondary neurites from some of these neurons branch in the SEG, and there are at least two tyramine containing axons in the labial nerves. The DUM/VUM neurons from the labial neuromere innervate the labial nerves, the labial neuropil of the SEG and the corpora cardiaca (**Figures 3B,C**, **Table 1**). There were also tyramine containing axons in the connectives between the SEG and prothoracic ganglion (**Figure 3B**).

The mandibular and maxillary neuromeres each contain eight anti-tyramine immunoreactive neurons that have a laterally symmetrical morphology in the SEG and branch in the deuto- and proto-cerebrum, the antennal nerve and the lateral nerves (**Figures 3E–H**). At least two anti-tyramine staining branches are

present in each (mandibular and maxillary) lateral nerve on each side of the SEG. The anti-tyramine labeled secondary neurites from some VUMmx and VUMmd neurons travel alongside the esophageal foramen (circle in **Figures 3C,E**) and run through the dorsal lobe and enter to the antennal tracts T5–T6. There were at least five tyramine containing axons in the ventral antennal nerve (**Figure 3F** and insert in **Figure 3H**). The antennal nerve also has large anti-tyramine positive varicose fibers on the surface (**Figure 3H**).

In our study we were particularly interested in VUMmd1 and VUMmx1 (**Figures 3C,D**), because of their importance for octopamine-driven behavioral conditioning (Hammer, 1993; Farooqui et al., 2003). They give rise to primary neurites in the corresponding MdT and MxT tracts and then to secondary neurites in the deutocerebrum caudal to the antennal lobe, as illustrated in the frontal section of the SEG (secondary neurites are circled in **Figure 3C**) and in the sagittal section through the left antennal lobe (**Figure 3E**). These two VUM neurons send branches into the antennal lobe (**Figures 3E,G**). In both **Figures 3E,G**, the anti-tyramine immunolabeled sagittal sections through the antennal lobe illustrate two tyraminerbic branches from VUMx1 and VUMd1 (arrows). In **Figure 3G**, the sections made through the antennal T1 tracts show that each branch from VUMmx1 and VUMmd1 gives rise to additional branches that innervate glomeruli on the dorsal and ventral side of the antennal lobe (**Figure 3G**). The anti-tyramine labeled processes in each glomerulus have varicosities that could be release points for tyramine not only in glomeruli but also in the glomerular neuropil.

The secondary neurites from VUMmx1 and VUMmd1 run through the lateral antenno-protocerebral tract (l-APT) (**Figure 3I**) and give rise to very fine branches in the LH and the calyx of the mushroom body with highest distribution in the lip and basal ring of mushroom body calyx (**Figure 3J**). These neurons arise from VUMmd1 and VUMmx1 and are identical to octopaminergic neurons in the same neuromere described in earlier studies (Schröter et al., 2007). That conclusion is based on our study with anti-tyramine and anti-octopamine labeling on the sections. No additional axons were labeled in the antennal lobe during these procedures [these data are not illustrated here].

The pedunculus and lobe of the mushroom body have little labeling with anti-tyramine staining, except for a few fine branches that were distinguishable in the gamma and vertical lobes (**Figures 3I–K**). In contrast, all protocerebral neuropils surrounding the mushroom bodies and lobes contain abundant tyramine containing varicosities (**Figures 3I–K**). Tyramine immunostaining in the mushroom body calyx has its origin from the VUMmx and VUMmd neurons. However, the mushroom body lobes are scarcely labeled with tyramine immunoreactivity, and staining in the gamma lobe originates from the lateral paired ventral cells (VPM, **Figure 3D**) as well from unidentified neurons in group G2. The tyraminerbic neurons from group G3 also innervate the posterior protocerebrum and optic lobes. The tyramine containing neurons from group G4 innervate the protocerebral bridge and central complex (**Figure 1** for cell bodies group location and **Table 1**). The terminology used for tyramine-

containing cell groups is the same that is described in Sinakevitch et al. (2005).

Distribution of AmTyr1 Receptor in the Antennal Lobes and Mushroom Bodies

We used anti-AmTyr1 antibodies to characterize the distribution of AmTyr1 in the antennal lobe and mushroom bodies (Figure 4). First, we performed single immunofluorescence staining in unknown age forager bee brains to identify the areas in the antennal lobe and mushroom bodies that labeled with anti-AmTyr1 antibodies (Figure 4). In the antennal lobe, anti-AmTyr1 staining is unevenly distributed within each glomerulus as well in the agglomerular neuropil (Figure 4A). High intensity anti-AmTyr1 staining is localized in the cortex area in each glomerulus, with low intensity staining in the core of the glomerulus and in the agglomerular neuropil (Figure 4A). Anti-AmTyr1 staining is absent in the antennal nerve and tracts in the antennal lobe (only tract T1 is shown in Figure 4A). Cell bodies surrounding the antennal lobe also labeled with different levels of intensity: high intensity labeling is in a subset of the medial group of cell bodies (asterisk) in Figure 4A. In contrast, the lateral group of cell bodies is not positive for anti-AmTyr1. Figure 4B illustrates a section through the mushroom body and central complex. Anti-AmTyr1 immunostaining is present in all mushroom body neuropils: calyx, peduncle, and lobes (Figure 4B). The peduncle and lobes have strong anti-AmTyr1 staining compared with KC bodies and calyx (Figure 4B). The anti-AmTyr1 labeling in the calyx and lobes is particularly distinct in the lip, collar and basal ring areas (Figure 4C). The gamma lobe of the mushroom bodies (staining is shown by the arrow in Figure 4C) had the most variable anti-AmTyr1 immunostaining distribution. Note that the ellipsoid body and ocelli also exhibited a high level of anti-AmTyr1 immunolabeling (Figure 4B).

ORN Axons Express AmTyr1 in the Antennal Lobe Terminals

To identify the primary cell types that express AmTyr1 in the antennal lobe we used triple immunofluorescence staining. The images in Figures 5A1–A5, show triple immunofluorescence staining in glomeruli with anti-AmTyr1 (magenta, Figure 5A1), neurobiotin backfills of antennal axons (green, Figure 5A2) and anti-synapsin (blue, Figure 5A4). Close-ups of ORN endings in the box in Figures 5A1–A5 is illustrated in Figures 5B1–B5, respectively. The distribution of anti-AmTyr1 is in the cortex of the glomerulus (magenta, Figures 5A1,B1), where the ORN terminals are located (green, Figures 5A2,B2). The endings of ORNs represent varicosities that synapse on the neuronal processes located in the cortex of the glomerulus. In the merged image (magenta anti-AmTyr1 and green ORNs, Figures 5A3,B3), the white color demonstrates co-labeling of anti-AmTyr1 in the ORN axon endings (Figures 5A3,B3). Therefore, ORNs containing neurobiotin also have anti-AmTyr1 staining (Figures 5A3,B3). Anti-synapsin also co-labeled the neurobiotin containing ORNs (Figures 5A4,B5). An example of staining with anti-AmTyr1 and anti-synapsin in ORNs is shown

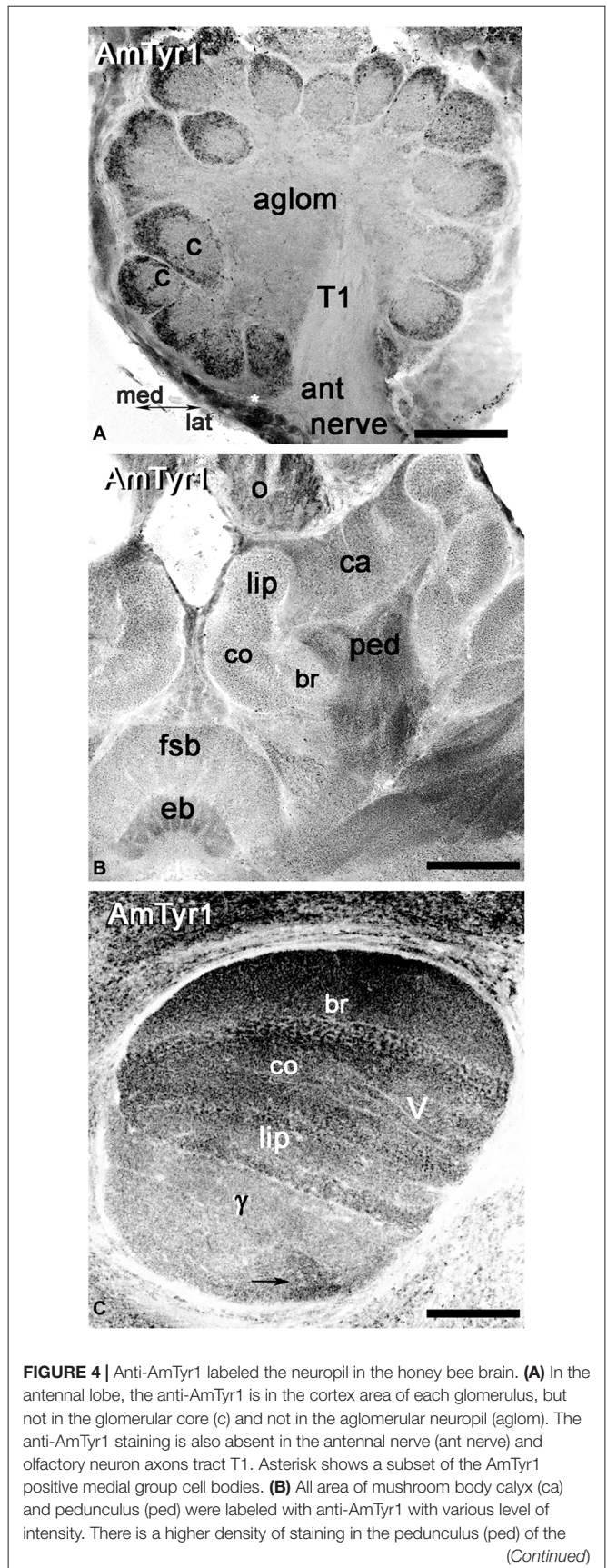


FIGURE 4 | Continued

mushroom body compared to the lip, collar (co) and basal ring (br) area of the calyx. Note: the central complex has anti-AmTyr1 staining in the fan shaped body and ellipsoid body (eb). **(C)** The mushroom body vertical lobe (V) exhibits high-intensity level anti-AmTyr1 staining in a basal ring (br), collar (co) and lip area of the Kenyon cells (KCs) axons that have dendrites in the corresponding area of a calyx. The illustrations in **(A–C)** are inverted fluorescence images.

γ—gamma lobe of mushroom body, o—ocelli. Scale bar: **A** = 50 μm,

B = 75 μm, **C** = 50 μm.

by the white arrows in **Figures 5B1–B5**. However, anti-AmTyr1 labeled processes other than ORN axons. The yellow arrows in **Figure 5B1** demonstrate co-staining of anti-AmTyr1 with anti-synapsin but not with neurobiotin. These data suggest that other cell types could express AmTyr1 in the cortex of glomeruli, or possibly not all ORNs were labeled with neurobiotin in our preparations. Also, due to the limitation of light microscopy we cannot exclude that AmTyr1 is expressed in processes of the glomerular cortex that are not co-labeled with anti-synapsin. More work needs to be done to identify all possible antennal lobe neurons and/or glial cells expressing AmTyr1. To reveal ORN cell bodies and dendrites in sensilla, we injected neurobiotin in the antennal lobe (**Figure 6**). Then the antenna was opened and processed for double immunofluorescence staining to reveal neurobiotin (green) and AmTyr1 (magenta). The neurobiotin labeled ORN cell bodies and processes in sensilla (**Figure 6**). **Figures 6A,B** illustrates a frontal view of antennal subsegments 5 and 6 of a flagellum. This antenna was not cut during immuno-procedures, and the images were taken in a confocal mode with overexposure of all laser channels for illustration of the surface area of an antenna with different types of sensilla. The three olfactory sensilla are identified as sensilla placodea (p in **Figures 6A,B**), sensilla basiconica (b, **Figure 6B**), sensilla trichodea type A (tA, **Figure 6B**) and type B1 (tB1, **Figure 6B**), sensilla trichodea type C (**Figure 6A**,

tC). In the honey bee, sensilla placodea (or poreplate) house between 7 and 30 ORNs (Esslen and Kaissling, 1976). To reveal anti-AmTyr1 and neurobiotin simultaneously in the antennal processes, antennae were cut with a razor into two halves and each half processed for staining with anti-AmTyr1, neurobiotin and DAPI; the latter is the marker for cell nuclei (**Figure 6C**). Neurobiotin processes in the sensilla placodea are not co-labeled with anti-AmTyr1. However, the neurobiotin filled ORN cell bodies (green **Figures 6C,D**) co-labeled with anti AmTyr1 (magenta in **Figures 6C,D**). The example of a cell with co-localization with AmTyr1 is indicated by the arrow in **Figure 6D**. The anti-AmTyr1 labeled processes in the sensillum lymph area (**Figure 6E1**), but they are not co-localized with neurobiotin labeled dendrites in the sensilla placodea (**Figure 6E2**) as demonstrated by the absence of white color in the merged image (**Figure 6E3**).

The uPN Terminals in the Calyx of the Mushroom Body Express AmTyr1

The calyx of the mushroom bodies consists of intrinsic neuron (KC) dendrites and afferent neuronal terminals of uniglomerular PN (uPNs) from the antennal lobe (lip and basal ring), gustatory inputs (area between lip and collar) and visual inputs (collar). Interestingly, the gustatory inputs to mushroom body calyx are from subesophageal neurons, the same brain region where the modulatory octopaminergic/tyramineric VUM neurons originate. In addition, other modulatory neurons and GABAergic inputs are present in the calycal neuropil. The organization of the calyx is microglomerular, which reflects interactions of uPN axons (input) with dendrites of KCs (output) together with the inputs of inhibitory (GABA) and modulatory afferent neurons.

Anti-AmTyr1 antibodies labeled microglomeruli in the calyx of the mushroom bodies (**Figure 7A1**). The higher magnification

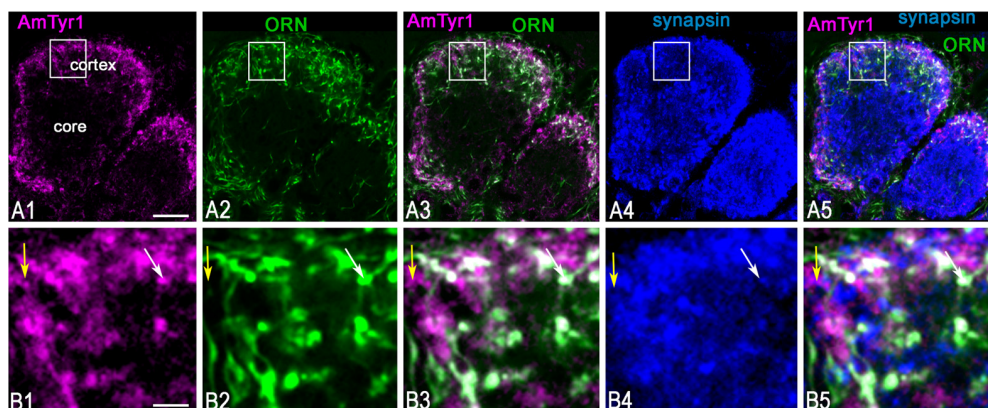


FIGURE 5 | Anti-AmTyr1 labeled synapses of the olfactory receptor neuron (ORN) axons in the antennal lobe glomerulus. **(A,B)** Triple immunofluorescence labeled with anti-AmTyr1 antibodies (magenta), neurobiotin tracer in ORNs (green) and anti-synapsin (blue). **(B)** Images are higher magnifications of details from corresponding squares indicated in **(A)**. **(A1,B1)** Anti-AmTyr1 immunostaining expressed in the cortex of glomeruli (magenta). **(A2,B2)** The ending of the (ORNs, green) revealed by neurobiotin injections into antenna. Anti-AmTyr1 in glomeruli **(A1,A3,A5,B1,B3,B5)**, magenta is in ORN endings **(A2,A3,A5,B2,B3,B5)**, green) co-labeled with anti-synapsin (blue, **A4,A5,B4,B5**). The white color in merged images **(A3,A5,B3,B5)** revealed anti-AmTyr1 co-stained in the ORN together with synapsin. The white arrows in **(B1–B5)** indicate co-localization with ORN endings by both anti-AmTyr1 and anti-synapsin; yellow arrow shows co-localization anti-AmTyr1 with synapsin but not with neurobiotin. Scale bar: **A** = 10 μm; **B** = 2 μm.

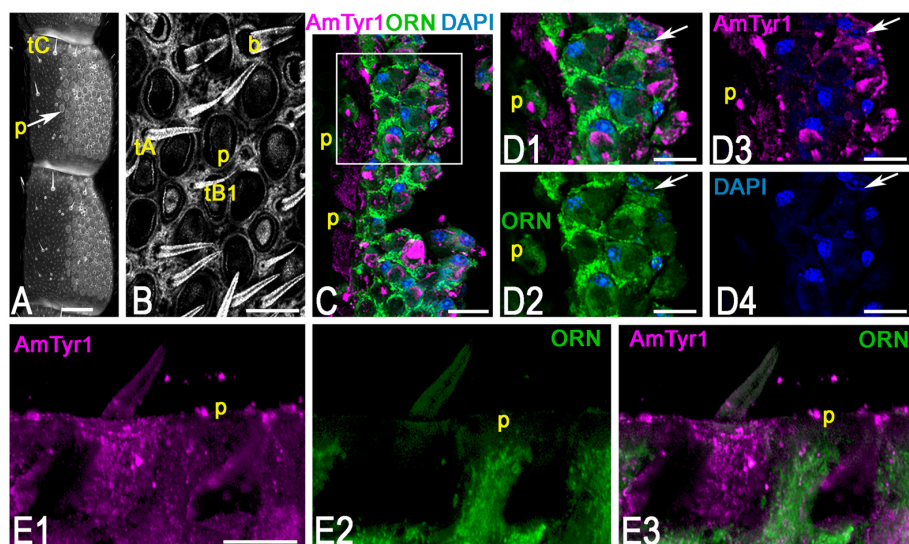


FIGURE 6 | Anti-AmTyr1 immunostaining in the antennal nerve. **(A)** General view of the antennal segments 5 and 6, neurobiotin was injected in the antennal lobe, and the image was obtained by overexposure with the confocal gain to illustrate different types of sensilla (tC—tricoid sensilla type C; Arrow indicates sensilla placodea (p). **(B)** Details of the ventral area of the antenna at higher magnification (tA, tB1, tricoid sensilla type A and B1 respectively, b—basiconic sensilla). **(C)** The section via antenna illustrates merged images of the group of ORN cell bodies and various processes labeled with neurobiotin tracer (green) anti-AmTyr1 (magenta), and 4',6-diamidino-2-phenylindole (DAPI), marking the nucleus, P-indicate the fibers in the sensilla placodea. **(D)** Images present higher magnifications of details from corresponding squares indicated in **(C)**. **(D1)** shows of cell bodies and processes labeled with neurobiotin (green) and anti-AmTyr1 staining (magenta, single staining) and nuclei (blue, DAPI). **(D2)** illustrates only neurobiotin labeled processes (green) and nuclei (DAPI, blue). **(D3)** illustrates only anti-AmTyr1 (magenta) and nuclei (DAPI). **(D4)** shows the nuclei staining. The arrow indicates cell bodies that have co-staining with AmTyr1 and neurobiotin. **(E)** Anti-AmTyr1 (**E1** single image, magenta) is in the area of sensilla placodea (p) with dendrites of ORNs labeled with neurobiotin (green **E2**). The absence of the white staining in merged image (**E3**) demonstrates that AmTyr1 does not co-label dendrites of labeled ORNs. Scale bar: **A** = 100 μ m; **B, E** = 20 μ m; **C** = 25 μ m; **D** = 10 μ m.

in image (**Figure 7B1**) represents the equivalent of an area shown in the square box in **Figure 7A1** and illustrates a few of the microglomeruli. The anti-AmTyr1 is in the cortex of the microglomeruli, but the core is free from immunostaining.

To mark the neuronal structure of uPN terminals in the calyx microglomeruli, neurobiotin was injected in the antennal lobe to reveal uPN boutons in the basal ring and lip areas of the mushroom body calyx (arrows in **Figures 7A2, B2**). The dye revealed uPN boutons (**Figure 7B2**), which are mainly presynaptic to KC dendrites. As demonstrated in **Figures 7A3, B3**, anti-AmTyr1 co-labeled with uPN boutons and was localized in the periphery of the bouton (white color on merged images of **Figures 7A1, A2, B1, B2**). Anti-synapsin labeled the presynaptic sites of the microglomeruli (**Figures 7A4, B4**). When the images (anti-synapsin, anti-AmTyr1 and uPNs terminals) are merged in **Figures 7A5, B5**, the white color demonstrates co-expression of anti-AmTyr1 and synapsin in the periphery of microglomeruli within the same boutons as uPNs. Thus, AmTyr1 is localized in presynaptic sites of uPNs in microglomeruli of mushroom body calyx.

A Subset of Kenyon Cells in the Basal Ring, Lip and Collar Express AmTyr1 in Cell Bodies and Axons but Not in the Dendrites in the Calyx

The mushroom body KC bodies express a low level of AmTyr1 staining compared to staining in the calyx (**Figure 7C1**).

The anti-AmTyr1 marks KC bodies in the basal ring, lip and collar and scattered cell bodies of the gamma lobe with very low intensity, although it is possible to distinguish a different level of staining in subsets of cells with higher expression in the basal ring KCs (**Figure 7C1**). The anti-AmTyr1 staining is strong in the vertical mushroom body lobe, especially in the areas that receive axons from KCs in the basal ring, the collar and the lip areas. In comparison, staining is much lower in the gamma lobe (**Figure 7D1**).

KCs are the intrinsic neurons that make up the mushroom body. They have dendrites in the calyx and axons in the lobe, and different types of afferent (input/output) neurons make their connection to the KCs in the calyx and lobe. To identify localization of the AmTyr1 receptor in KC dendrites and axons, we labeled KCs with neurobiotin before immunostaining procedures with anti-AmTyr1 (**Figures 7C2, C3, D2, D3**) and anti-synapsin (**Figures 7C4, C5, D4, D5**) antibodies. For neurobiotin injected in the calyx of the cell body layer (**Figure 7C**), the area of injection was marked by an ellipsoid in the peduncle of the mushroom body. The dye was taken up by subsets of KC bodies and their dendrites in the calyx as well in the axons in the peduncle (**Figure 7C2**) and lobes (**Figure 7D2**).

The neurobiotin-injected KC dendrites in the calyx were not labeled with anti-AmTyr1. The insert in **Figures 7C1, C5** shows typical AmTyr1 staining in the microglomeruli. In the insert in **Figure 7C2**, fine green fibers of KCs are in close proximity to the AmTyr1 stained PN, but there is no co-labeling as

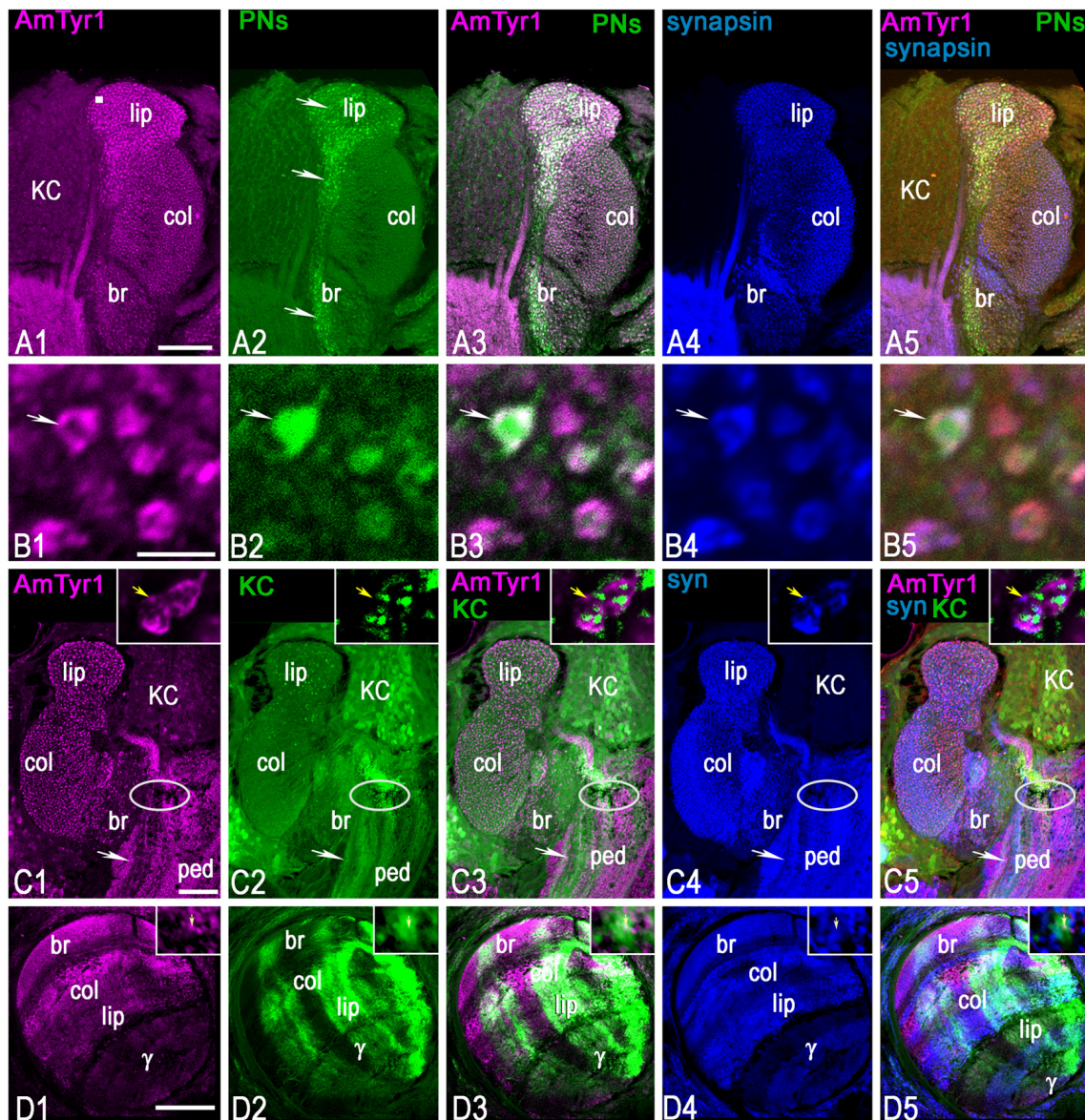


FIGURE 7 | Triple staining with anti-AmTyr1 (magenta) and anti-synapsin (blue) in the mushroom body after neurobiotin injection in uniglomerular projection neurons (uPNs; green, **A,B**) and subsets of KCs (green, **C,D**). (**A**) Anti-AmTyr1 antibodies (magenta, **A1**) co-label the neurobiotin injected uPNs ending in the calyx (green, **A2**). Arrows in (**A2**) show the axon from uPNs entering the basal ring and lip area of the calyx and labeling presynaptic parts of microglomeruli. Images in (**B**) illustrate at higher magnification microglomeruli indicated by the square in (**A1**). Arrows in (**B**) show that single microglomeruli label with anti-AmTyr1 (magenta, **B1**) in a uPN terminal bouton (**B2**). The white staining in merged images (**A3,B3**) indicates co-labeling of anti-AmTyr1 with uPN terminal microglomeruli. The microglomeruli labeled with anti-synapsin as a presynaptic marker (**A4,B4**, blue). The white staining in merged triple staining image (**A5,B5**) indicates that anti-AmTyr1 and anti-synapsin are co-labeled in uPN microglomeruli. (**C,D**) Anti-AmTyr1 labeled microglomeruli (magenta, **C1**, insert in **C1**) in the calyx, subsets of KC bodies, the pedunculus (ped, **C1**). Also, areas of the mushroom body vertical lobe that correspond to KCs with dendrites in the basal ring, lip and collar areas of the calyx have a high level of staining intensity (**D1**). The (KCs) were injected with neurobiotin in the area indicated in (**C**) by an ellipse, and in single image staining (green, in **C2,D2**) the neurobiotin revealed in cell bodies, dendrite in calyx (**C2**, insert in **C2**) and in lobe (**D2**). Only subsets of KCs took up the neurobiotin in this preparation. For the injection of neurobiotin in the area shown by the ellipse in (**C**), the subsets of KCs that took up the tracer express it in cell bodies, in dendrites in the lip, basal ring and collar (**C2**, insert in **C2**), and in the axons of the corresponding area of vertical and gamma lobe (**D2**). Merged images (**C3,D3**) show the co-localization of KCs that take up neurobiotin with AmTyr1 in axons but not in the dendrites of the calyx (inserts in **C3**). (**C**) AmTyr1 (magenta **C1**) expression in subsets of mushroom body KC axons (green, **C2**) but not in the dendrites of the calyx (insert in **C**). The merged images in (**C5**) illustrate co-localization of anti-AmTyr1 with anti-synapsin (blue, **C4** single staining) in calyx microglomeruli, but not with neurobiotin labeled KC dendrites in calyx (insert in **C4,C5** respectively). Anti-AmTyr1 in the mushroom body lobe is in KC axons; these KCs have dendrites in the basal ring and collar areas. In insert (**D1**)—AmTyr1 (magenta) is in axons of KC labeled with neurobiotin (**D2**, single image) and co-labeled with synapsin in (**D4**; single image). In the triple staining image (**D5**; insert **D5**) the white color corresponds to co-labeling of synapsin in KC axons and AmTyr1. Note, that not all synapsin labeling processes (single staining image **D4**, insert **D4**) express AmTyr1 (single staining image **D1**, insert **D1**). Arrows in insert (**D1–D5**) shows AmTyr1 in the axon of KC co-labeled with synapsin. Scale bar: **A,C,D** = 50 μ m, **B** = 2 μ m.

demonstrated in **Figure 7C3** and in the insert, where the white color expected for co-labeling in the dendritic area of KCs is absent. The AmTyr1 receptor is co-expressed in the bundles of axons of neurobiotin labeled KC in the pedunculus (white arrow in **Figures 7C2,C3**). The subsets of neurobiotin labeled KCs (green bundles in **Figures 7C2,C3,C5**) could be traced to the mushroom body basal ring, the collar, the lip and gamma lobe areas (green in **Figures 7D2,D3,D5**). KC axons co-express AmTyr1 in the basal ring, collar and lip area of the mushroom body (**Figure 7D3**). The insert of **Figures 7D1,D2** show the area of the lip KC axons in the vertical lobe, where only a few fibers express receptors as indicated by the yellow arrows in inserts. The **Figures 7C4,D4,C5,D5** show anti-synapsin staining in the calyx and lobes and anti-synapsin co-labeled with KCs axons in the pedunculus and lobe, with subsets of KCs that co-express AmTyr1 in the same axons.

DISCUSSION

Tyramine and octopamine play important roles in insect behavior by acting as neurotransmitters, neurohormones and neuromodulators (Roeder, 2005). Their function is analogous to the adrenergic/noradrenergic system in vertebrates. In the honey bee (*Apis mellifera*), the cellular sources of octopamine (Kreissl et al., 1994; Sinakevitch et al., 2005) and the distribution of one receptor—AmOA1—has been previously described in detail (Sinakevitch et al., 2011, 2013). In the current study, we revealed cellular sources for tyramine and the distribution of one TYR—AmTyr1—in the honey bee brain by using immunocytochemistry with tyramine antiserum (Kononenko et al., 2009; Homberg et al., 2013) and a newly characterized anti-AmTyr1 antibody.

Tyramine-Immunostaining in the Honey Bee Brain

A total of approximately 160 tyramine immunoreactive neurons are organized in different clusters in the brain. They are the source of tyraminergetic fibers with small varicosities in the optic lobes, antennal lobes, lateral protocerebrum, mushroom body calyces and gamma lobes, tritocerebrum and SEG. Also, tyramine-like immunoreactive fibers are present in the antennal nerve and in the nerve innervating the corpora cardiaca (NCC1). Since tyramine is a precursor of octopamine, it is not surprising that our studies revealed that tyramine immunostaining is largely in the same clusters of cells as octopamine, as previously described in the honey bee brain (Kreissl et al., 1994; Sinakevitch et al., 2005). We summarized the cell numbers in **Table 1** and used the same nomenclature of cell groups as Sinakevitch et al. (2005). According to our present report and comparison with previous studies on octopamine immunoreactivity in the honey bee (Kreissl et al., 1994; Sinakevitch et al., 2005), tyramine containing neurons described here are largely the same as those that contain octopamine due to the position of their clusters.

We also found differences from earlier studies (Sinakevitch et al., 2005), as reflected in **Table 1**. For example, we did not find tyramine immunoreactive cells in octopamine-positive clusters G0b, G1 and G5a. It could be that those cells convert tyramine

to octopamine rapidly, such that the tyramine titers are below detection levels. We also found that there is a higher total number of cells containing tyramine immunoreactivity in comparison to octopamine immunoreactive cells reported by Kreissl et al. (1994) and Sinakevitch et al. (2005). Previous work on biogenic amine immunostaining reported that handling procedures could significantly alter octopamine/tyramine levels, and possibly make one or both undetectable by immunostaining (Sinakevitch et al., 2005; Kononenko et al., 2009; Homberg et al., 2013). However, the differences in numbers of octopaminergic and tyraminergetic neurons in the honey bee brain might also be due to the presence of neurons that are only tyraminergetic. Therefore, due to the higher number of tyramine-containing cells, and to a large amount of tyramine-containing profiles in neuropils, our data suggest that some cells could release only tyramine, some cells either release only octopamine or co-release both biogenic amines.

Tyramine Immunoreactivity in the Antennal Nerve

We also found that the antennal nerve contains tyramine-like immunoreactivity originating from processes in SEG neurons that enter the antennal nerve via T5-6. However, it is not clear what neurons in the SEG could be the source of these fibers: e.g., the neurons from cluster six or unidentified median neurons from mandibular and maxillary neuromeres. Tyramine in the antenna could be in the axons of neurons innervating the head and antennal muscles, and it could also act as a hormonal release in the antenna to modulate antennal sensory neurons. Furthermore, tyramine in the antennal lobe could also be involved in the regulation and modulation of sucrose responsiveness (Scheiner et al., 2002). It seems clear, however, that tyramine does not modulate ORN cell bodies via AmTyr1, because we did not observe AmTyr1 on the cell bodies of ORNs.

Tyramine Immunoreactivity in the Antennal Lobe and Mushroom Body

The primary sources of tyramine in the antennal lobe and calyx of the mushroom body are from at least two median neurons with cell bodies in the SEG: VUMmd and VUMmx neurons. In the antennal lobe, these neurons innervate the cortex of each glomerulus, where ORN axons terminate. They also branch to the LH and calyx of the mushroom body via the lateral antenno-protocerebral tract. The entire protocerebral neuropil, except for the mushroom body lobes, is penetrated by very fine varicosities of tyramine-containing fibers, and their sources are the VUM neurons from the SEG as well as the cells from clusters G2 and G3. The mushroom body lobe has very few tyramine containing fibers, only a few branches in the gamma lobe are clearly visible, and the source could be the laterally paired neurons from the SEG (Schröter et al., 2007).

Also, the presence of tyramine in NCC1 could indicate that the corpora cardiaca could receive tyramine-containing branches from some VUM neurons located in the SEG. The morphology of VUM neurons that give rise to axons in the NCC1 was previously described in the labial and maxillary neuromeres by

Eichmüller et al. (1991). While the role of tyramine in NCC1 is unclear, it could be that tyramine is released from these neurons to act as a neurohormone, or/and it could control the release of other hormones from the corpora cardiaca.

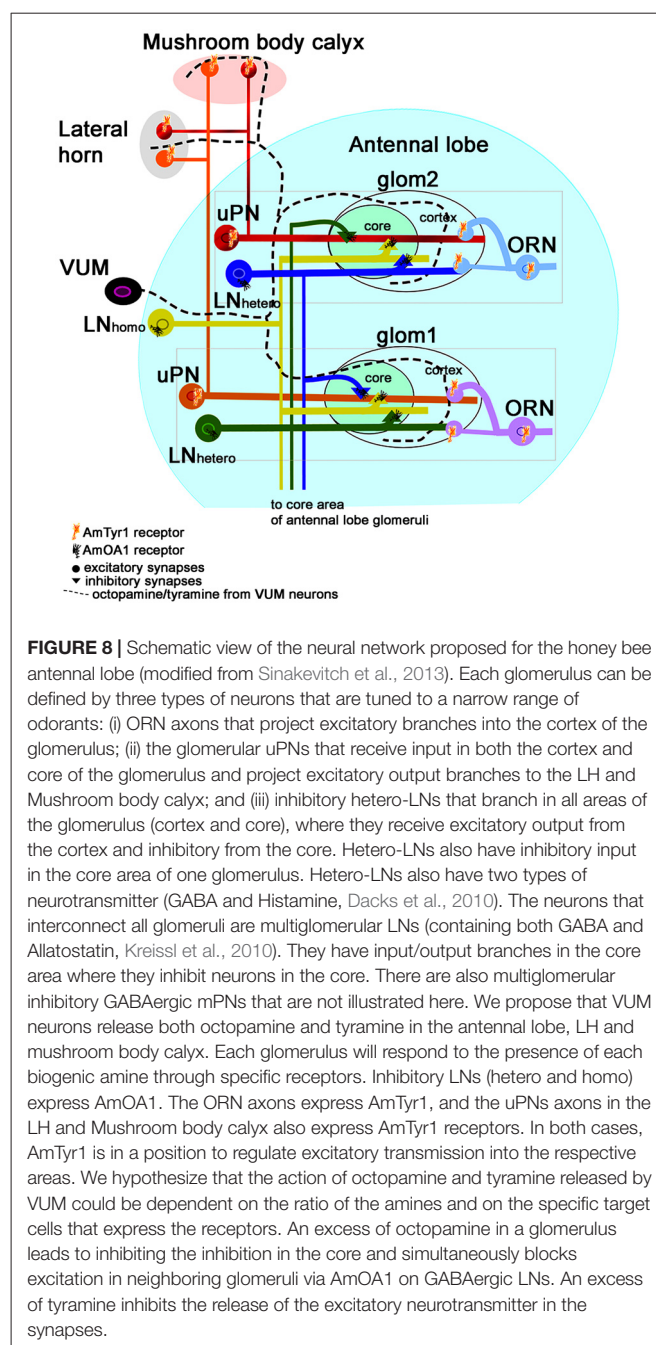
AmTyr1 Receptor Structure

Activation of AmTyr1 in heterologous expression systems leads to reduction of cAMP (Blenau et al., 2000; Reim et al., 2017). Both tyramine and octopamine reduce cAMP when bound to AmTyr1 (Blenau and Baumann, 2016), but tyramine is more potent than octopamine. Moreover, AmTyr1 binds yohimbine, which is an antagonist of the AmTyr1 receptor (Blenau and Baumann, 2001, 2016; Reim et al., 2017). In our 3D model, AmTyr1 has a long intracellular loop 3 and a short C-terminus. Those properties are similar to other GPCRs linked to inhibition of adenylyl cyclase activity; for example, the alpha 2-adrenergic receptors also have a long intracellular loop 3 and a short C-terminal tails (Kuhar et al., 1999; Rosenbaum et al., 2009). Based on the structural and pharmacological properties, AmTyr1 is similar to the vertebrate type alpha 2-adrenergic receptors, which when activated also reduce cAMP, and yohimbine has also high affinity of this vertebrate receptor type. The function of vertebrate-type alpha 2-adrenergic receptors is primarily for inhibitory presynaptic control of the release of norepinephrine, ATP and acetylcholine from the nerve (Rosenbaum et al., 2009).

AmTyr1 Receptors Are Expressed in the Presynaptic Sites of ORN and uPN Axons

The anti-AmTyr1 antibodies we used specifically recognize the AmTyr1 protein, and staining mapped with high intensity to honey bee brain neuropil areas. Some cell bodies immunolabeled with low intensity compared to the neuropil, which could reflect that the AmTyr1 receptor was translated in the cell body and transported to axonal terminals. The present localization studies on anti-AmTyr1 receptor protein distribution provide a further confirmation of the previous work of Mustard et al. (2005), where *in situ* hybridization of the AmTyr1 mRNA was reported to be expressed in the mushroom body and antennal lobe neurons.

Anti-AmTyr1 immunolocalization studies revealed that in the antennal lobe AmTyr1 is expressed in the presynaptic sites of ORN axons as they innervate the cortex of glomeruli. Similarly, in the mushroom body calyx, AmTyr1 is expressed in the presynaptic sites of uPN axons located primarily in the microglomeruli of the lip and basal ring calyx areas. AmTyr1 is expressed in areas innervated by VUM (md and mx) neurons. Therefore, release of tyramine from VUM (md and mx) neurons in the antennal lobe and mushroom body could target presynaptic sites of ORNs and uPNs (Figure 8). Because the AmTyr1 receptor is similar in structure and function to the vertebrate alpha-adrenergic receptor type 2 (Kuhar et al., 1999), we hypothesize that the release of tyramine from tyramine containing VUM neurons could inhibit excitatory neurotransmitter release in the presynaptic axons of ORNs and uPNs. The main excitatory neurotransmitter in ORNs and uPNs is acetylcholine, which plays important roles in olfaction and memory in bees (Gauthier, 2010). AmTyr1 in these neurons might be involved in regulation of the release of acetylcholine.



At the same time the release of octopamine from VUM neurons could coordinate excitation via AmOA1 in inhibitory neurons in the antennal lobe and mushroom bodies (Sinakevitch et al., 2013, Figure 8).

AmTyr1 Receptors Are Expressed in Axons of Kenyon Cells in the Lobe but Not in Dendrites

Only subsets of KCs express the AmTyr1 receptor in the axons and cell bodies, with a higher level of expression in the axons compared to cell bodies. We did not find

any detectable staining in the dendrites of KCs located in calyx. In the KCs axons, AmTyr1 co-localized with synapsin, the presynaptic marker of the neurons. In the mushroom body lobe, the anti-AmTyr1 positive subsets of KCs axons are in the pedunculus, lip, collar and basal ring area of the lobe. The branching pattern of the KCs in the mushroom body lobes was described in detail by Strausfeld (2002).

Tyramine Receptor Distribution in Comparison to Other Insects

Homolog of AmTyr1 have been studied in other insects: fruit fly (TyrR, Oct/TyrR, TYR, Tar1, CG7485; Saudou et al., 1990; El-Kholy et al., 2015); locust (Vanden Broeck et al., 1995); silkworm (Ohta et al., 2003); and cockroach (PeaTyr1, Rotte et al., 2009). Similar to AmTyr1, when investigated in expression systems, these Tyr1 receptors were also negatively coupled to adenylyl cyclase via Gi protein (Uzzan and Dudai, 1982; Aoyama et al., 2001; Blenau and Baumann, 2003). However, activation of locust and fruit fly Tyr1 receptors have also been shown to mobilize intracellular calcium (Ohta and Ozoe, 2014). The brain distribution of PeaTyr1 in the cockroach *Periplaneta americana* is similar to our results with AmTyr1, but that report did not identify specific neurons such as the presynaptic sites we show here (Rotte et al., 2009). Additionally, they reported expression in glial cells and peripherals organs. In our study, we also report the possible expression of AmTyr1 in glial cells and peripheral organs, but we did not illustrate it here. Expression of the moth (*Agrotis ipsilon*) OA/TYR (AipsOAR/TAR) was also reported in the antennae, the antennal lobes and the brain and in the antennal lobes was shown to be regulated with age (Dupont et al., 2010). In the fruit fly *Drosophila melanogaster*, the expression pattern of TyrRs was studied by utilizing the presumptive promotor regions of the TYR and the Gal4/UAS system (El-Kholy et al., 2015). The fruit fly TyrRs are expressed in the tracheal system, in salivary glands and in the mushroom body and ellipsoid body, glial cells, fat body and muscles. However, the fruit fly has three TYRs, one of which does not have an ortholog in the honey bee (Tyr3), which makes it difficult to compare our results on localization with the fruit fly (El-Kholy et al., 2015).

Tyramine and AmTyr1 Receptors in the Mushroom Body Lobe

It was striking in our study that the mushroom body α - and γ -lobes do not have a high amount of tyraminerpic (present studies) or octopaminergic fibers (Sinakevitch et al., 2005) in contrast to the calyx of the mushroom body and the protocerebral area that surrounds mushroom body lobes. However, the mushroom body lobes express both the AmTyr1 (present study) and AmOA1 receptors (Sinakevitch et al., 2011, 2013). We propose that the source of tyramine in the mushroom body lobe could be from the hemolymph or from the release of tyramine in the protocerebrum followed by diffusion into the lobes. The presence of tyramine in the hemolymph was reported in locust, and the level of tyramine is lower than octopamine in brain tissue as well in the hemolymph.

It may be important for behavioral studies to consider the ratio of tyramine and octopamine in the tissue rather than focusing exclusively on a single amine, since they have potentially antagonistic effects (Roeder, 2005). Fussnecker et al. (2006) showed that honey bee flight behavior was affected inversely by octopamine and tyramine treatment. Octopamine increased flight behavior, while tyramine treatment decreased it. Fussnecker et al. (2006) suggested that both biogenic amines affect central pattern generators or interact with sensory perception. Tyramine and octopamine have opposite effects on locomotion in the fruit fly (Saraswati et al., 2004), and have been shown (Brembs et al., 2007) to reduce fruit fly flight initiation. Tyramine also reduces the stimulatory effect of octopamine in the fly (Uzzan and Dudai, 1982). In the honey bee brain tyramine is involved in habituation of an appetitive reflex (Braun and Bicker, 1992) and in inhibition of the initiation of foraging behaviors (Schulz and Robinson, 2001). High brain tyramine in queenless honey bee workers might inhibit foraging behavior and encourage them to stay in the nest and become reproductive workers (Sasaki and Nagao, 2002).

CONCLUSION

One of the important findings in our studies is that tyramine could originate from VUMmx and VUMmd neurons in the antennal lobe, lateral protocerebrum and mushroom body calyx. Our present tyramine mapping results in the honey bee are consistent with reports on octopamine/tyramine containing cells in locust (Kononenko et al., 2009; Homberg et al., 2013) and fruit fly (Monastirioti et al., 1996; Monastirioti, 1999; Sinakevitch and Strausfeld, 2006; Busch et al., 2009; Busch and Tanimoto, 2010; Selcho et al., 2014). Our findings suggest that tyramine is not only the precursor of octopamine but could also be an independent neurotransmitter. From the AmTyr1 distribution, tyramine targets the excitatory synapses of ORNs in glomeruli and of PNs in the calyces of the mushroom bodies. From the similarity to the vertebrate $\alpha 2$ type adrenergic receptor, we suggest that tyramine could inhibit release of the neurotransmitter from both ORNs and PNs. Since the same neurons (VUMmd and mx) have both tyramine and octopamine, it is possible that the ratio of tyramine/octopamine in proximity to the receptors plays a crucial role in the physiological responses of the cells. Also, tyramine and octopamine could be released in hemolymph, which brings additional complex response in brain circuits as the hemolymph circulates through the brain.

AUTHOR NOTE

During revision of our manuscript a new article about tyramine and the AmTyr1 receptor (in this article the authors call it "AmTAR1") was published (Thamm et al., 2017). The authors developed new antibodies against AmTyr1 raised against a cytoplasmic domain of the receptor, whereas our antibodies targeted an extracellular domain. They described staining in sections of the entire brain including the olfactory neuropils, which was the exclusive focus of our work. The

two studies show similar results in regard to the receptor in the mushroom bodies but differ in regard to antennal lobe staining. In our anti-tyramine staining, we used the same anti-tyramine antibodies as Thamm et al. (2017), but we used a different staining procedure. There were differences between the studies in the number of tyramineric cells and location of tyramine in the mushroom body lobes. These differences in tyramine and AmTyr1 receptor expression will require further study.

AUTHOR CONTRIBUTIONS

ITS and BHS designed experiments and wrote the manuscript, SMD made a western blot, the bioinformatic analyses of the AmTyr1 receptor, ITS executed all experiments and made the illustrations.

REFERENCES

- Alkema, M. J., Hunter-Ensor, M., Ringstad, N., and Horvitz, H. R. (2005). Tyramine Functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron* 46, 247–260. doi: 10.1016/j.neuron.2005.02.024
- Aoyama, M., Nakane, T., Ono, T., Khan, M. A., Ohta, H., and Ozoe, Y. (2001). Substituent-dependent, positive and negative modulation of Bombyx mori adenylate cyclase by synthetic octopamine/tyramine analogues. *Arch. Insect Biochem. Physiol.* 47, 1–7. doi: 10.1002/arch.1029
- Balfanz, S., Jordan, N., Langenstück, T., Breuer, J., Bergmeier, V., and Baumann, A. (2014). Molecular, pharmacological, and signaling properties of octopamine receptors from honeybee (*Apis mellifera*) brain. *J. Neurochem.* 129, 284–296. doi: 10.1111/jnc.12619
- Balfanz, S., Strünker, T., Frings, S., and Baumann, A. (2005). A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca²⁺ release in *Drosophila melanogaster*. *J. Neurochem.* 93, 440–451. doi: 10.1111/j.1471-4159.2005.03207.x
- Bayliss, A., Roselli, G., and Evans, P. D. (2013). A comparison of the signalling properties of two tyramine receptors from *Drosophila*. *J. Neurochem.* 125, 37–48. doi: 10.1111/jnc.12158
- Blenau, W., and Baumann, A. (2001). Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* 48, 13–38. doi: 10.1002/arch.1055
- Blenau, W., and Baumann, A. (2003). Aminergic signal transduction in invertebrates: focus on tyramine and octopamine receptors. *Recent. Res. Devel. Neurochem.* 6, 225–240.
- Blenau, W., and Baumann, A. (2016). “Chapter 14—octopaminergic and tyramineric signaling in the honeybee (*Apis mellifera*) brain: behavioral, pharmacological, and molecular aspects A2,” in *Trace Amines and Neurological Disorders*, ed. A. A. Farooqui (San Diego, CA: Academic Press), 203–219.
- Blenau, W., Balfanz, S., and Baumann, A. (2000). Amtyr1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem.* 74, 900–908. doi: 10.1046/j.1471-4159.2000.0740900.x
- Brandt, R., Rohlfing, T., Rybak, J., Kroficzek, S., Maye, A., Westerhoff, M., et al. (2005). Three-dimensional average-shape atlas of the honeybee brain and its applications. *J. Comp. Neurol.* 492, 1–19. doi: 10.1002/cne.20644
- Braun, G., and Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* 67, 588–598.
- Brembs, B., Christiansen, F., Pflüger, H. J., and Duch, C. (2007). Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. *J. Neurosci.* 27, 11122–11131. doi: 10.1523/JNEUROSCI.2704-07.2007

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- Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the *Drosophila* brain. *J. Comp. Neurol.* 513, 643–667. doi: 10.1002/cne.21966
- Busch, S., and Tanimoto, H. (2010). Cellular configuration of single octopamine neurons in *Drosophila*. *J. Comp. Neurol.* 518, 2355–2364. doi: 10.1002/cne.22337
- Chandra, S. B., Wright, G. A., and Smith, B. H. (2010). Latent inhibition in the honey bee, *Apis mellifera*: is it a unitary phenomenon? *Anim. Cogn.* 13, 805–815. doi: 10.1007/s10071-010-0329-6
- Dacks, A. M., Reisenman, C. E., Paulk, A. C., and Nighorn, A. J. (2010). Histamine-immunoreactive local neurons in the antennal lobes of the hymenoptera. *J. Comp. Neurol.* 518, 2917–2933. doi: 10.1002/cne.22371
- David, J.-C., and Coulon, J.-F. (1985). Octopamine in invertebrates and vertebrates. A review. *Prog. Neurobiol.* 24, 141–185. doi: 10.1016/0301-0082(85)90009-7
- Dupontets, L., Barrozo, R. B., Bozzolan, F., Gaertner, C., Anton, S., Gadenne, C., et al. (2010). Cloning of an octopamine/tyramine receptor and plasticity of its expression as a function of adult sexual maturation in the male moth *Agrotis ipsilon*. *Insect Mol. Biol.* 19, 489–499. doi: 10.1111/j.1365-2583.2010.01009.x
- Eichmüller, S., Hammer, M., and Schäfer, S. (1991). Neurosecretory cells in the honeybee brain and suboesophageal ganglion show FMRFamide-like immunoreactivity. *J. Comp. Neurol.* 312, 164–174. doi: 10.1002/cne.903120112
- El-Kholy, S., Stephano, F., Li, Y., Bhandari, A., Fink, C., and Roeder, T. (2015). Expression analysis of octopamine and tyramine receptors in *Drosophila*. *Cell Tissue Res.* 361, 669–684. doi: 10.1007/s00441-015-2137-4
- Ersperer, V., and Boretti, G. (1951a). Identification and characterization, by paper chromatography, of enteramine, octopamine, tyramine, histamine and allied substances in extracts of posterior salivary glands of octopoda and in other tissue extracts of vertebrates and invertebrates. *Arch. Int. Pharmacodyn. Ther.* 88, 296–332.
- Ersperer, V., and Boretti, G. (1951b). Substances of a phenolic and indolic nature present in acetone extracts of the posterior salivary glands of Octopoda (*Octopus vulgaris*, *Octopus macropus* and *Eledone moschata*). *Experientia* 7, 271–273. doi: 10.1007/bf02154550
- Esslen, J., and Kaissling, K.-E. (1976). Zahl und verteilung antennaler sensillen bei der honigbiene (*Apis mellifera* L.). *Zoomorphologie* 83, 227–251. doi: 10.1007/BF00993511
- Evans, P. D., and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert. Neurosci.* 5, 111–118. doi: 10.1007/s10158-005-0001-z
- Farooqui, T., Robinson, K., Vaessin, H., and Smith, B. H. (2003). Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci.* 23, 5370–5380.
- Fussnecker, B. L., Smith, B. H., and Mustard, J. A. (2006). Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey

- bee (*Apis mellifera*). *J. Insect Physiol.* 52, 1083–1092. doi: 10.1016/j.jinsphys.2006.07.008
- Gauthier, M. (2010). “Chapter 8-State of the art on insect nicotinic acetylcholine receptor function in learning and memory,” in *Insect Nicotinic Acetylcholine Receptor: Advance in Experimental Medicine and Biology*, (Vol. 683) ed. S. H. Thany (New York, NY: Springer-Verlag), 97–115.
- Grohmann, L., Blenau, W., Erber, J., Ebert, P. R., Strunker, T., and Baumann, A. (2003). Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J. Neurochem.* 86, 725–735. doi: 10.1046/j.1471-4159.2003.01876.x
- Geffard, M., Buijs, R. M., Seguela, P., Pool, C. W., and Le Moal, M. (1984a). First demonstration of highly specific and sensitive antibodies against dopamine. *Brain Res.* 294, 161–165. doi: 10.1016/0006-8993(84)91323-4
- Geffard, M., Seguela, P., and Heinrich-Rock, A. M. (1984b). Antisera against catecholamines: specificity studies and physicochemical data for anti-dopamine and anti-p-tyramine antibodies. *Mol. Immunol.* 21, 515–522. doi: 10.1016/0161-5890(84)90067-1
- Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63. doi: 10.1038/366059a0
- Hauser, F., Cazzamali, G., Williamson, M., Blenau, W., and Grimmelikhuijzen, C. J. (2006). A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog. Neurobiol.* 80, 1–19. doi: 10.1016/j.pneurobio.2006.07.005
- Hildebrand, J. G., and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20, 595–631. doi: 10.1146/annurev.neuro.20.1.595
- Hombert, U., Seyfarth, J., Binkle, U., Monastirioti, M., and Alkema, M. J. (2013). Identification of distinct tyramineric and octopaminergic neurons innervating the central complex of the desert locust, *Schistocerca gregaria*. *J. Comp. Neurol.* 521, 2025–2041. doi: 10.1002/cne.23269
- Hoyle, G. (1974). A function for neurons (DUM) neurosecretory on skeletal muscle of insects. *J. Exp. Zool.* 189, 401–406. doi: 10.1002/jez.1401890313
- Ishikawa, Y., Aonuma, H., Sasaki, K., and Miura, T. (2016). Tyramineric and octopaminergic modulation of defensive behavior in termite soldier. *PLoS One* 11:e0154230. doi: 10.1371/journal.pone.0154230
- Kononenko, N. L., Wolfenberger, H., and Pflüger, H. J. (2009). Tyramine as an independent transmitter and a precursor of octopamine in the locust central nervous system: an immunocytochemical study. *J. Comp. Neurol.* 512, 433–452. doi: 10.1002/cne.21911
- Kreissl, S., Eichmüller, S., Bicker, G., Rapus, J., and Eckert, M. (1994). Octopamine-like immunoreactivity in the brain and subesophageal ganglion of the honeybee. *J. Comp. Neurol.* 348, 583–595. doi: 10.1002/cne.903480408
- Kreissl, S., Strasser, C., and Galizia, C. G. (2010). Allatostatin immunoreactivity in the honeybee brain. *J. Comp. Neurol.* 518, 1391–1417. doi: 10.1002/cne.22343
- Kuhar, M. J., Couceyro, P. R., and Lambert, P. D. (1999). α - and β -Adrenergic Receptors. Philadelphia: Lippincott-Raven.
- Kutsukake, M., Komatsu, A., Yamamoto, D., and Ishiwa-Chigusa, S. (2000). A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. *Gene* 245, 31–42. doi: 10.1016/s0378-1119(99)00569-7
- Lange, A. B. (2009). Tyramine: from octopamine precursor to neuroactive chemical in insects. *Gen. Comp. Endocrinol.* 162, 18–26. doi: 10.1016/j.ygcen.2008.05.021
- Maquieira, B., Chatwin, H., and Evans, P. D. (2005). Identification and characterization of a novel family of *Drosophila* β -adrenergic-like octopamine G-protein coupled receptors. *J. Neurochem.* 94, 547–560. doi: 10.1111/j.1471-4159.2005.03251.x
- Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech.* 45, 106–121. doi: 10.1002/(sici)1097-0029(19990415)45:2<106::aid-jemt5>3.3.co;2-v
- Monastirioti, M., Linn, C. E., and White, K. Jr. (1996). Characterization of *Drosophila* tyramine β -hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* 16, 3900–3911.
- Mons, N., and Geffard, M. (1987). Specific antisera against the catecholamines: L-3,4-dihydroxyphenylalanine, dopamine, noradrenaline, and octopamine tested by an enzyme-linked immunosorbent assay. *J. Neurochem.* 48, 1826–1833. doi: 10.1111/j.1471-4159.1987.tb05743.x
- Mustard, J. A., Kurshan, P. T., Hamilton, I. S., Blenau, W., and Mercer, A. R. (2005). Developmental expression of a tyramine receptor gene in the brain of the honey bee, *Apis mellifera*. *J. Comp. Neurol.* 483, 66–75. doi: 10.1002/cne.20420
- Nagaya, Y., Kutsukake, M., Chigusa, S. I., and Komatsu, A. (2002). A trace amine, tyramine, functions as a neuromodulator in *Drosophila melanogaster*. *Neurosci. Lett.* 329, 324–328. doi: 10.1016/s0304-3940(02)00596-7
- Ohta, H., and Ozoe, Y. (2014). Molecular signalling, pharmacology, and physiology of octopamine and tyramine receptors as potential insect pest control targets. *Adv. Insect Phys.* 46, 73–166. doi: 10.1016/B978-0-12-417010-0.00002-1
- Ohta, H., Utsumi, T., and Ozoe, Y. (2003). B96Bom encodes a *Bombyx mori* tyramine receptor negatively coupled to adenylate cyclase. *Insect Mol. Biol.* 12, 217–223. doi: 10.1046/j.1365-2583.2003.00404.x
- Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., et al. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612. doi: 10.1002/jcc.20084
- Plotnikova, S. I. (1969). Effector neurons with several axons in the ventral nerve cord of the Asian grasshopper *Locusta migratoria*. *J. Evol. Biochem. Physiol.* 5, 276–277.
- Rehder, V. (1988). A neuroanatomical map of the subesophageal and prothoracic ganglia of the honey bee (*Apis-Mellifera*). *Proc. R. Soc. Series B Biol. Sci.* 235, 179–202. doi: 10.1098/rspb.1988.0071
- Reim, T., Balfanz, S., Baumann, A., Blenau, W., Thamm, M., and Scheiner, R. (2017). AmTAR2: functional characterization of a honeybee tyramine receptor stimulating adenylyl cyclase activity. *Insect Biochem. Mol. Biol.* 80, 91–100. doi: 10.1016/j.ibmb.2016.12.004
- Robb, S., Cheek, T. R., Hannan, F. L., Hall, L. M., Midgley, J. M., and Evans, P. D. (1994). Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems. *EMBO J.* 13, 1325–1330.
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi: 10.1016/s0301-0082(99)00016-7
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Roeder, T., Seifert, M., Kahler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. *Arch. Insect Biochem. Physiol.* 54, 1–13. doi: 10.1002/arch.10102
- Rosenbaum, D. M., Rasmussen, S. G. F., and Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* 459, 356–363. doi: 10.1038/nature08144
- Rotte, C., Krach, C., Balfanz, S., Baumann, A., Walz, B., and Blenau, W. (2009). Molecular characterization and localization of the first tyramine receptor of the American cockroach (*Periplaneta americana*). *Neuroscience* 162, 1120–1133. doi: 10.1016/j.neuroscience.2009.05.066
- Roy, A., Kucukural, A., and Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725–738. doi: 10.1038/nprot.2010.5
- Saraswati, S., Fox, L. E., Soll, D. R., and Wu, C. F. (2004). Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. *J. Neurobiol.* 58, 425–441. doi: 10.1002/neu.10298
- Sasaki, K., and Nagao, T. (2002). Brain tyramine and reproductive states of workers in honeybees. *J. Insect Physiol.* 48, 1075–1085. doi: 10.1016/s0022-1910(02)00200-7
- Saudou, F., Amlaiky, N., Plassat, J. L., Borrelli, E., and Hen, R. (1990). Cloning and characterization of a *Drosophila* tyramine receptor. *EMBO J.* 9, 3611–3617.
- Scheiner, R., Baumann, A., and Blenau, W. (2006). Aminergic control and modulation of honeybee behaviour. *Curr. Neuropharmacol.* 4, 259–276. doi: 10.2174/157015906778520791
- Scheiner, R., Kulikovskaja, L., and Thamm, M. (2014). The honey bee tyramine receptor AmTYR1 and division of foraging labour. *J. Exp. Biol.* 217, 1215–1217. doi: 10.1124/jeb.098475
- Scheiner, R., Plückhahn, S., Oney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* 136, 545–553. doi: 10.1016/s0166-4328(02)00205-x
- Schröter, U., Malun, D., and Menzel, R. (2007). Innervation pattern of subesophageal ventral unpaired median neurones in the honeybee brain. *Cell Tissue Res.* 327, 647–667. doi: 10.1007/s00441-006-0197-1

- Schulz, D. J., and Robinson, G. E. (2001). Octopamine influences division of labor in honey bee colonies. *J. Comp. Physiol. A* 187, 53–61. doi: 10.1007/s003590000177
- Selcho, M., Pauls, D., Huser, A., Stocker, R. F., and Thum, A. S. (2014). Characterization of the octopaminergic and tyramineric neurons in the central brain of *Drosophila* larvae. *J. Comp. Neurol.* 522, 3485–3500. doi: 10.1002/cne.23616
- Sinakevitch, I., Mustard, J. A., and Smith, B. H. (2011). Distribution of the octopamine receptor AmOA1 in the honey bee brain. *PLoS One* 6:e14536. doi: 10.1371/journal.pone.0014536
- Sinakevitch, I., Niwa, M., and Strausfeld, N. J. (2005). Octopamine-like immunoreactivity in the honey bee and cockroach: comparable organization in the brain and subesophageal ganglion. *J. Comp. Neurol.* 488, 233–254. doi: 10.1002/cne.20572
- Sinakevitch, I. T., Smith, A. N., Locatelli, F., Huerta, R., Bazhenov, M., and Smith, B. H. (2013). *Apis mellifera* octopamine receptor 1 (AmOA1) expression in antennal lobe networks of the honey bee (*Apis mellifera*) and fruit fly (*Drosophila melanogaster*). *Front. Syst. Neurosci.* 7:70. doi: 10.3389/fnsys.2013.00070
- Sinakevitch, I., and Strausfeld, N. J. (2006). Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. *J. Comp. Neurol.* 494, 460–475. doi: 10.1002/cne.20799
- Strausfeld, N. J. (2002). Organization of the honey bee mushroom body: representation of the calyx within the vertical and γ lobes. *J. Comp. Neurol.* 450, 4–33. doi: 10.1002/cne.10285
- Thamm, M., Scholl, C., Reim, T., Gröbel, K., Möller, K., Rössler, W., et al. (2017). Neuronal distribution of tyramine and the tyramine receptor AmTAR1 in the honeybee brain. *J. Comp. Neurol.* 525, 2615–2631. doi: 10.1002/cne.24228
- Uzzan, A., and Dudai, Y. (1982). Aminergic receptors in *Drosophila melanogaster*: responsiveness of adenylate cyclase to putative neurotransmitters. *J. Neurochem.* 38, 1542–1550. doi: 10.1111/j.1471-4159.1982.tb06631.x
- Vanden Broeck, J., Vulsteke, V., Huybrechts, R., and De Loof, A. (1995). Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *J. Neurochem.* 64, 2387–2395. doi: 10.1046/j.1471-4159.1995.64062387.x
- Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H.-J., Blenau, W., et al. (2010). The role of octopamine in locusts and other arthropods. *J. Insect Physiol.* 56, 854–867. doi: 10.1016/j.jinsphys.2010.05.018
- Wang, Y., Kocher, S. D., Linksvayer, T. A., Grozinger, C. M., Page, R. E. Jr., Amdam, G. V., et al. (2012). Regulation of behaviorally associated gene networks in worker honey bee ovaries. *J. Exp. Biol.* 215, 124–134. doi: 10.1242/jeb.060889
- Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., and Zhang, Y. (2015). The I-TASSER Suite: protein structure and function prediction. *Nat. Methods* 12, 7–8. doi: 10.1038/nmeth.3213
- Yang, J., and Zhang, Y. (2015). I-TASSER server: new development for protein structure and function predictions. *Nucleic Acids Res.* 43, W174–W181. doi: 10.1093/nar/gkv342
- Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40. doi: 10.1186/1471-2105-9-40

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The Effects of Fat Body Tyramine Level on Gustatory Responsiveness of Honeybees (*Apis mellifera*) Differ between Behavioral Castes

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Division of labor is a hallmark of social insects. In the honeybee (*Apis mellifera*) each sterile female worker performs a series of social tasks. The most drastic changes in behavior occur when a nurse bee, who takes care of the brood and the queen in the hive, transitions to foraging behavior. Foragers provision the colony with pollen, nectar or water. Nurse bees and foragers differ in numerous behaviors, including responsiveness to gustatory stimuli. Differences in gustatory responsiveness, in turn, might be involved in regulating division of labor through differential sensory response thresholds. Biogenic amines are important modulators of behavior. Tyramine and octopamine have been shown to increase gustatory responsiveness in honeybees when injected into the thorax, thereby possibly triggering social organization. So far, most of the experiments investigating the role of amines on gustatory responsiveness have focused on the brain. The potential role of the fat body in regulating sensory responsiveness and division of labor has large been neglected. We here investigated the role of the fat body in modulating gustatory responsiveness through tyramine signaling in different social roles of honeybees. We quantified levels of tyramine, tyramine receptor gene expression and the effect of elevating fat body tyramine titers on gustatory responsiveness in both nurse bees and foragers. Our data suggest that elevating the tyramine titer in the fat body pharmacologically increases gustatory responsiveness in foragers, but not in nurse bees. This differential effect of tyramine on gustatory responsiveness correlates with a higher natural gustatory responsiveness of foragers, with a higher tyramine receptor (*Amtr1*) mRNA expression in fat bodies of foragers and with lower baseline tyramine titers in fat bodies of foragers compared to those of nurse bees. We suggest that differential tyramine signaling in the fat body has an important role in the plasticity of division of labor through changing gustatory responsiveness.

Keywords: biogenic amines, division of labor, nurse bee, forager, PER, octopamine, insect, behavior

INTRODUCTION

Honeybee colonies display a complex yet highly plastic social organization. Each bee performs a series of social tasks (for review see Johnson, 2010). The most important changes in individual behavior occur when nurse bees switch to foraging tasks. While nurse bees stay inside the hive and provide the young larvae with food, foragers leave the hive daily to

forage for nutrients, i.e., pollen and nectar (Johnson, 2010). Nurse bees are normally between 1 week and 2 weeks of age. Foragers leave the hive at roughly 3 weeks of age. Although division of labor clearly depends on the age of the individual, age *per se* cannot account for the behavioral transitions. Because of the great plasticity in division of labor, the honeybee offers the unique opportunity to dissociate age from social role, which is difficult in most other species. Thus it was shown that under certain conditions nurse bees and foragers can be identical in age (Behrends et al., 2007; Scheiner and Amdam, 2009) and that most changes in physiology and gene expression of differently aged bee groups are associated with the behavior of the bee rather than with her age (Toth and Robinson, 2005; Alaux et al., 2009).

A recent hypothesis (Ament et al., 2010) suggests that nutrition-related signaling cascades are involved in regulating and controlling division of labor in a honeybee colony. In support of this theory, foragers differ from nurse bees in their nutrition-related gustatory response thresholds (Thamm and Scheiner, 2014). Further, nurse bees and foragers differ in the amounts of lipids they store in the fat body, with nurse bees losing their lipid stores during the transition to foraging behavior (Toth and Robinson, 2005). Reducing lipid stores pharmacologically by treating bees with a fatty acid synthesis inhibitor similarly induced precocious foraging behavior (Toth et al., 2005). When colonies were deprived of food, bees began foraging earlier than did bees from well-fed colonies (Schulz et al., 1998). In addition, it was shown that the fat body has an important function in honeybee metabolism (Nilsen et al., 2011; Wang et al., 2012), which differs hugely between nurse bees and foragers. These experiments suggest an important role for the honeybee fat body in regulating and modulating honeybee division of labor, possibly through modulation of sensory response thresholds. However, investigations on the functions of the fat body in controlling honeybee behavior are rare. A direct effect of the fat body on behavior was demonstrated when the gene encoding the egg yolk precursor vitellogenin was downregulated in the fat body of honeybees. Reduction in gene expression significantly enhanced responsiveness to gustatory stimuli (Amdam et al., 2006).

We here investigated the role of fat body tyramine signaling in regulating and modulating gustatory responsiveness in nurse bees and honeybee foragers. Tyramine is the metabolic precursor of octopamine. While the latter has been studied in detail, very little is known about tyramine, although two honeybee tyramine receptors have been cloned and characterized (Blenau et al., 2000; Reim et al., 2017). The tyramine receptor gene *Amtar1* (also known as *Amtyr1*) is a candidate gene in a quantitative trait locus that correlates with different aspects of foraging-related behaviors in two honeybee strains that differ in their gustatory responsiveness (Hunt et al., 2007). Furthermore, tyramine injections into the thorax can increase gustatory responsiveness (Scheiner et al., 2017). We address the relationships between tyramine titer and tyramine receptor gene expression in the fat body and gustatory responsiveness in nurse bees and foragers. Additionally, we studied how injection of tyramine into the abdomen affected tyramine and octopamine titers in the brain and fat body and whether it modulated gustatory responsiveness.

Intriguingly, tyramine application to the abdomen has different effects on gustatory responsiveness in nurse bees and foragers.

MATERIALS AND METHODS

Bees

Nurse bees and foragers were randomly obtained from typical honeybee colonies comprising approximately 40,000 honeybees. Honeybees (*Apis mellifera ligustica*) used for determining biogenic amine titers were sampled from hives maintained at Macquarie University Sydney, Sydney, NSW, Australia. Honeybees (*Apis mellifera carnica*) for behavioral analyses, behavioral pharmacology and gene expression studies were kept at the departmental apiary of the University of Würzburg.

Nurse bees were collected from frames containing open brood cells. Only bees poking their heads into an open brood cell for at least 15 s were regarded as nurse bees. Foragers were collected when returning to their colonies. For behavioral pharmacology of nurse bees, frames with capped broods were kept in an incubator maintained at 34°C and 65% humidity until the bees emerged. Newly emerged bees received paint-marks on their thoraces and were restored to the colony. After 1 week, when most of these bees performed nursing tasks, bees were individually retrieved from the colony (Thamm and Scheiner, 2014).

Gustatory Responsiveness

For testing gustatory responsiveness, each bee was immobilized on ice and subsequently mounted in a small holder with antennae and mouth parts protruding. Tests commenced 60 min after mounting (Scheiner et al., 2013). During this time, the bee rested in a humidified chamber. For determining gustatory responsiveness, each bee was sequentially stimulated by application of a series of sucrose concentrations (0%; 0.1%; 0.3%; 1%; 3%; 10%; and 30% w/v) to her antennae (for details see Scheiner et al., 2013). The sum of proboscis extension responses to stimulations with the seven different sucrose concentrations constitutes the gustatory response score (GRS) of a bee, which is a measure of its gustatory responsiveness (Scheiner et al., 2003, 2013; Scheiner, 2004; Behrends and Scheiner, 2010; Scheiner and Arnold, 2010; Thamm and Scheiner, 2014). For determining GRS of nurse bees and foragers without tyramine treatment, 106 nurse bees and 121 foragers were tested. These bees were also used to study effects of different concentrations of tyramine on gustatory responsiveness.

Behavioral Pharmacology

Different tyramine concentrations (10^{-2} mol/l and 10^{-3} mol/l) were dissolved in phosphate-buffered saline solution (PBS: 140 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 ; pH 7.4). Bees were either injected with 2 μl of a tyramine solution or with 2 μl of the PBS solution ("control") into the abdomen. For this, each bee was punctured between the fourth and fifth tergites and injected with a Hamilton syringe (10- μl syringe, Hamilton Bonaduz AG, Switzerland). To evaluate the effect of tyramine, changes in GRS 30 min after application of tyramine compared to GRS prior to treatment were calculated and compared between groups.

Quantification of mRNA

Individual fat body tissues of nurse bees and foragers that had not been used in behavioral tests were homogenized in 750 μ L of *Isol-RNA lysis reagent* (5PRIME, Hilden, Germany). Afterwards, 150 μ L of chloroform were added. After phase separation, the aqueous phase was transferred into 900 μ L ethanol (75%). Subsequently, the *peqGOLD Total RNA Kit* (Peqlab, Erlangen, Germany) was employed to purify RNA following the standard protocol including DNase I digestion step. From each bee, we transcribed 500 μ g of total fat body RNA using *QuantiTect® Reverse Transcription Kit* (Qiagen, Hilden, Germany). Of each cDNA, we amplified 5 μ L in triplicates in a quantitative real time PCR on a Rotor-Gene Q (Qiagen, Hilden, Germany) using the following protocol: 1 min at 60°C, 5 min at 95°C and 45 cycles consisting of 20 s at 95°C followed by 1 min at 60°C each. One reaction (25 μ L) contained each primer (0.25 μ M), TaqMan® probes (0.1 μ M) and Rotor-Gene Multiplex PCR Master Mix (Qiagen, Hilden, Germany). Sequences of primers and Taqman probes are given in **Table 1**. Relative expression to transcript of *AmEF1 α* (Reim et al., 2013) with the $\Delta\Delta$ Ct method was determined using Rotor Gene Q software (Qiagen, Chatsworth, CA, USA). The same bees were investigated with respect to mRNA expression of the three genes. Nevertheless, direct comparisons between mRNA expression of different genes within individuals were inappropriate due to possible differences in primer and probe efficiencies.

Quantification of Tyramine and Octopamine Titers

To measure natural levels of tyramine and octopamine in the fat bodies of nurse bees and foragers, bee samples were collected, and the abdomens dissected under PBS to remove fat bodies. Dissections were performed as quickly as possible, and fat body samples were then stored at -80°C . To determine whether injection with tyramine into the abdomen would lead to elevated tyramine or octopamine titers in the brain or fat body, individuals were treated by abdominal injection as described above. Thirty minutes after treatment, the head of each bee was quickly cut from the body at the neck and immediately flash

frozen in liquid nitrogen. The abdomen was stored briefly on dry ice, and then dissected under PBS to remove the fat body. Heads were lyophilized at -65°C and 320 mTorr for 50 min to remove some water content. Brains were dissected from head capsules over dry ice while frozen. Dissected brains were then stored at -80°C until further processing.

For extraction of biogenic amines from brains or fat body samples, tissue was first centrifuged at 15,000 g for 2 min at 4°C to induce mechanical disruption of tissue. Samples were then homogenized by sonication in 100 μ L of 0.2 M perchloric acid containing 10 μ g/ μ L dihydroxybenzylamine (DHBA). Homogenized samples were incubated on ice in darkness for 20 min, before centrifugation at 15,000 g for 15 min to pellet cell fragments. The supernatant was collected, and 10 μ L of the supernatant of each sample were analyzed with high-pressure liquid chromatography (HPLC). Content of biogenic amines in the extractant from tissue samples was quantified using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) with an ESA Coulechem III electrochemical detector connected to an ESA 5011A dual electrode analytical cell (ESA, Chelmsford, MA, USA). Samples were separated across a 100 mm Thermo Fisher Scientific Hypersil 5 μ m octadecylsilane packaged column (Thermo Fisher Scientific, Waltham, MA, USA). Biogenic amine amounts were quantified relative to known amounts of biogenic amines (Søvik et al., 2013; Scheiner et al., 2014b). The bees whose natural amine titers were measured were different from those treated with tyramine or PBS.

Statistics

Biogenic amine brain titers, GRSs and changes in GRSs were compared between different groups using two-tailed Mann Whitney *U* tests, since data were not distributed normally. For comparing more than two groups, we employed Kruskal-Wallis *H* tests followed by Dunn's *post hoc* tests. Due to the high individual variability of these data, we show individual data points with super-imposed medians for biogenic amine data. Many GRS values were "0" or "1". For clarification, GRS data are therefore represented by medians and upper and lower quartiles. GRS before and after treatment were compared within each group using two-tailed Wilcoxon tests. Relative amine receptor gene expression was compared between groups using *T*-tests, since these data were distributed normally. All tests were two-tailed. Comparisons were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

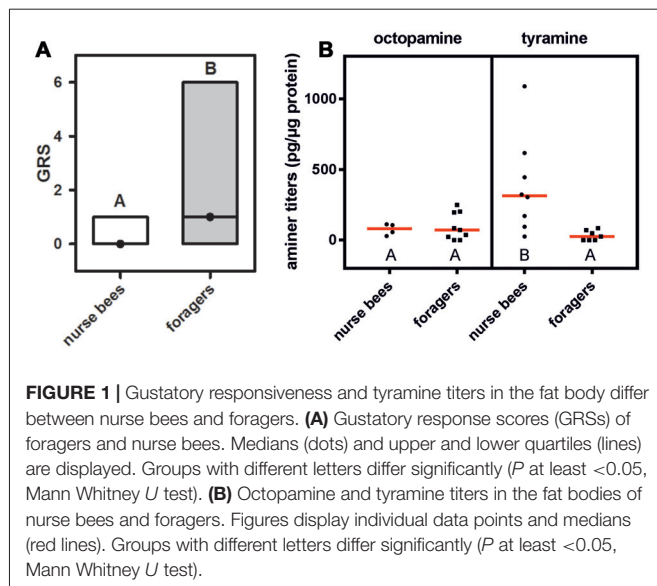
RESULTS

Gustatory Responsiveness of Nurse Bees and Foragers

We first wanted to demonstrate that in our honeybee population we could replicate the finding that foragers have a higher gustatory responsiveness than nurse bees do (Thamm and Scheiner, 2014). We therefore quantified gustatory responsiveness in both social roles. Foragers displayed significantly higher GRSs than did nurse bees (**Figure 1A**: $Z = 4.88$, $n_{\text{nurse bees}} = 106$, $n_{\text{foragers}} = 121$, $P < 0.001$, Mann

TABLE 1 | DNA-Oligos used for quantitative real time PCR.

Gene	Oligo name	Oligo sequence
<i>AmEF1α</i>	AmEF1 α _qF	GAACATTTCGTGTGAAAGAGTTGAGGC
	AmEF1 α _qR	TTTAAAGGTGACACTCTTAATGACGC
	AmEF1 α _TM	6FAM-ACCGAGGAGAATCCGAAGAGCAT CAA-BBQ
<i>Amtar1</i>	AmTar1_F	AGCCGACCGAGGTGACGATAG
	AmTar1_R	CCCATTATCACGCCCAATGTCC
	AmTar1_TM	YAK-AACGAGATCCTCTGCCTCTCCTCGATG AA-BBQ
<i>Amtar2</i>	AmTar2_F	GTTACTAATTGTTTCGTGTCAGCTT
	AmTar2_R	GCAGTACAGAGAAGAATGTCGAGG
	AmTar2_TM	YAK-AGGTACCACCTGTGAGCTGTAACA GCA-BBQ
<i>AmoctaR1</i>	AmOa1_F	GCAGGAGGAACAGCTGCGAG
	AmOa1_R	GCCGCCTTCGTCTCCATTGG
	AmOa1_TM	YAK-TCCCCATCTTCATCACCCTTGGCTTCT CC-BBQ



Whitney U test), demonstrating a higher general responsiveness to all sucrose stimuli offered (Scheiner et al., 2014a).

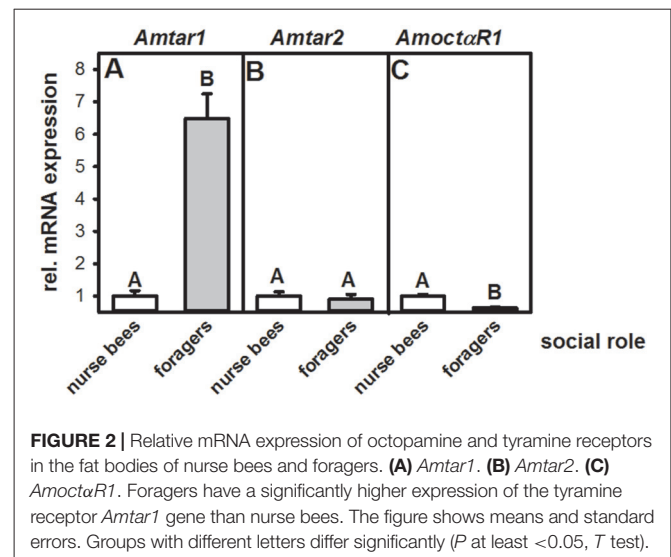
Amine Titters in the Fat Body of Nurse Bees and Foragers

We hypothesized that behavioral differences of nurse bees and foragers could coincide with different amounts of tyramine in their fat bodies. Tyramine is the metabolic precursor of octopamine (Roeder, 2005), and both amines can have similar effects on behavior (Scheiner et al., 2002, 2006). We therefore decided to quantify both amines in the fat bodies of nurse bees and foragers. Octopamine was present in very low amounts in the fat bodies of nurse bees and foragers, and its level did not differ between the two social roles (Figure 1B; $Z = 0.88$, $n_{\text{nurse bees}} = 4$, $n_{\text{foragers}} = 9$, $P > 0.05$). Intriguingly, foragers displayed significantly lower tyramine titers in their fat bodies than nurse bees did (Figure 1B; $Z = 2.90$, $n_{\text{nurse bees}} = 8$, $n_{\text{foragers}} = 7$, $P < 0.01$). These data suggest differential tyramine signaling in nurse bees and foragers, while octopamine titers did not differ between social roles.

Expression of Tyramine and Octopamine Receptors in the Fat Bodies of Nurse Bees and Foragers

This experiment was aimed at finding out whether expression of tyramine receptors in the fat body correlates with social role. We therefore quantified the mRNA of both honeybee tyramine receptor genes, *Amtar1* and *Amtar2*, in the fat bodies of nurse bees and foragers. In addition, we measured expression of the octopamine receptor *Amoctar1*, because mRNA expression of this receptor differs hugely between the brains of nurse bees and those of foragers (Reim and Scheiner, 2014), and tyramine can bind to octopamine receptors at high concentrations (Blenau et al., 2000; Reim et al., 2017).

The mRNA expression of the tyramine receptor *Amtar1* was significantly increased in foragers compared to nurse bees

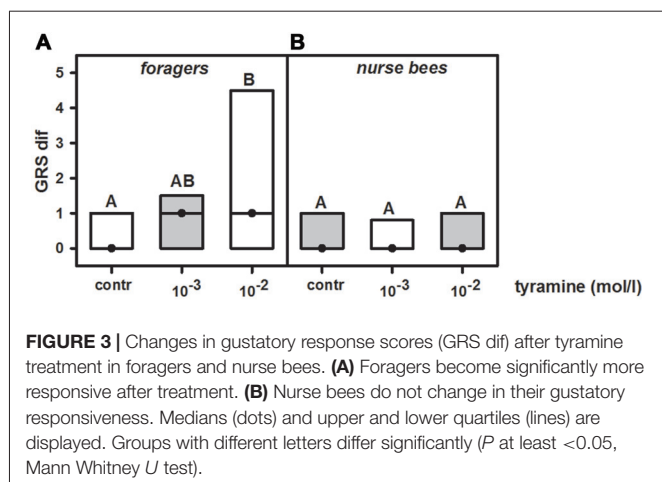


(Figure 2A; $n_{\text{nurse bees}} = 10$, $n_{\text{foragers}} = 8$, $T = 6.95$, $P < 0.001$, T -test). Expression of *Amtar2* mRNA, in contrast, did not differ between fat bodies of foragers and nurse bees (Figure 2B; $n_{\text{nurse bees}} = 7$, $n_{\text{foragers}} = 10$, $T = 0.47$, $P > 0.05$). Further, foragers displayed a significantly reduced mRNA expression of the octopamine receptor *Amoctar1* compared to nurse bees (Figure 2C; $n_{\text{nurse bees}} = 8$, $n_{\text{foragers}} = 8$, $T = 5.91$, $P < 0.001$). These data suggest that differences in the metabolism, physiology or behavior of nurse bees and foragers might be related to tyramine signaling in the fat body through *Amtar1* and possibly also through octopamine signaling through *Amoctar1*.

Tyramine Increases Gustatory Responsiveness in Foragers but Not in Nurse Bees

To test whether changing tyramine titers would increase gustatory responsiveness, we injected tyramine into the fat body of nurse bees and foragers. Preliminary experiments showed that injecting $1 \mu\text{l}$ of tyramine 10^{-2} mol/l into the abdomen did not affect gustatory responsiveness in honeybee foragers ($n_{\text{control}} = 39$, $n_{\text{tyramine}} = 40$, $Z = 1.04$, $P = 0.30$, Mann Whitney U test), although this volume and concentration can effectively increase gustatory responsiveness in foragers when injected into the thorax (Scheiner et al., 2002). We therefore tested whether injection of $2 \mu\text{l}$ of tyramine in the concentrations of 10^{-2} mol/l and 10^{-3} mol/l into the abdomen of foragers and nurse bees would affect their gustatory responsiveness.

Treatment with tyramine had a significant effect on gustatory responsiveness in foragers (KW = 10.01, $P < 0.01$). However, only tyramine at 10^{-2} mol/l significantly increased GRSs in this group (Figure 3A; tyramine 10^{-3} mol/l vs. control: $n_{\text{control}} = 39$, $n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 41$, $P > 0.05$; tyramine 10^{-2} mol/l: $n_{\text{control}} = 39$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 41$, $P < 0.01$). In contrast to foragers, gustatory responsiveness in nurse bees was not affected by tyramine injections (Figure 3B; KW = 0.31, $P > 0.05$). Neither tyramine concentration affected GRSs in this group significantly (tyramine 10^{-3} mol/l: $n_{\text{control}} = 35$,

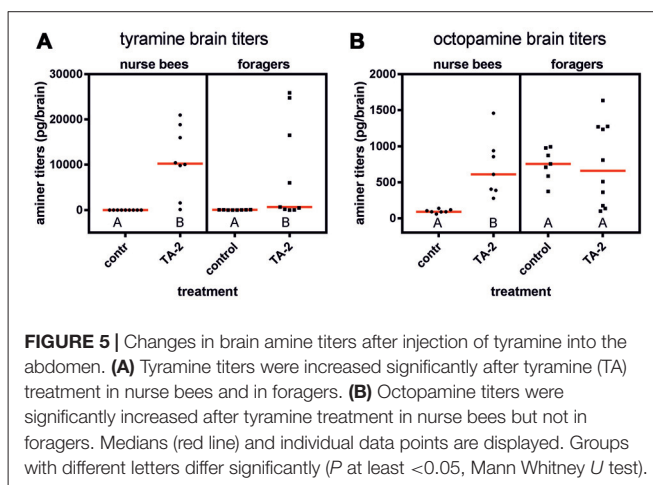
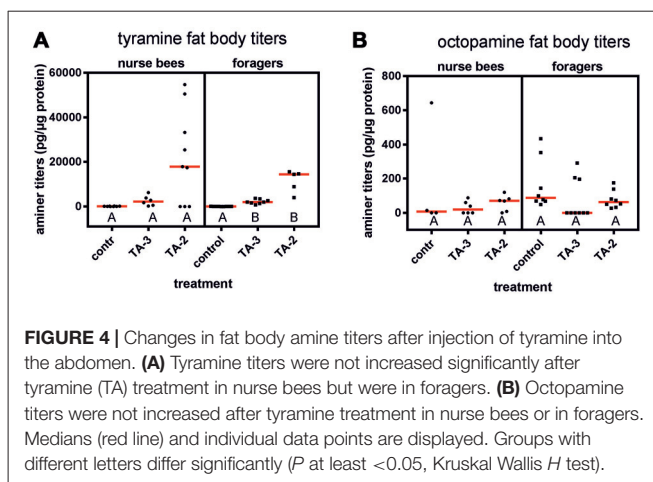


$n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 36$, $P > 0.05$; tyramine 10^{-2} mol/l : $n_{\text{control}} = 35$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 35$, $P > 0.05$). Comparison of GRS within each treatment group showed that only in foragers that were treated with tyramine in the concentrations of 10^{-3} mol/l and 10^{-2} mol/l did GRS significantly increase after treatment (foragers: tyramine 10^{-3} mol/l : $n = 41$, $Z = 2.44$, $P < 0.05$, tyramine 10^{-2} mol/l : $n = 41$, $Z = 4.18$, $P < 0.001$). Treatment of foragers with the control solution did not increase gustatory scores significantly ($n = 39$, $Z = 1.59$, $P < 0.05$). In nurse bees, neither the control group nor the two groups treated with different concentrations of tyramine changed their GRSs significantly after treatment (control: $n = 35$, $Z = 1.16$, $P > 0.05$, tyramine 10^{-3} mol/l : $n = 36$, $Z = 1.40$, $P > 0.05$, tyramine 10^{-2} mol/l : $n = 35$, $Z = 1.58$, $P > 0.05$, Wilcoxon test). These data imply that the action of tyramine on gustatory responsiveness is dependent on social role.

Effects of Tyramine Injections on Amine Titrers in the Fat Body and Brain

To test whether our differential effects of tyramine on gustatory responsiveness in foragers and nurse bees were related to biogenic amine titers, we quantified tyramine and octopamine titers in the fat bodies and in the brains of foragers and nurse bees after injections of tyramine (10^{-2} mol/l) into their abdomens. Since octopamine can have similar effects on gustatory responsiveness as tyramine (Scheiner et al., 2002; Behrends and Scheiner, 2012) and tyramine can be converted into octopamine (Roeder, 2005), we also quantified octopamine titers in the brain and abdomen. This was to ensure that only tyramine levels but not octopamine levels were elevated in the treatment groups.

Tyramine injections into the abdomen significantly increased tyramine titers in the abdomen of foragers (Figure 4A; KW = 18.34, $P < 0.001$). Both tyramine concentrations led to a significant increase in tyramine titers (tyramine 10^{-3} mol/l vs. control: $n_{\text{control}} = 9$, $n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 8$, $P < 0.05$; tyramine 10^{-2} mol/l : $n_{\text{control}} = 9$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 5$, $P < 0.001$). However, the higher tyramine concentration elevated tyramine levels in the fat body more strongly. In contrast, tyramine injections had no effect on octopamine titer in the fat bodies



of foragers (Figure 4B; KW = 4.00, $P > 0.05$, $n_{\text{control}} = 8$, $n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 9$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 8$, Kruskal-Wallis H test).

In contrast to foragers, injection of tyramine into abdomens of nurse bees did not result in increased tyramine titers in their fat bodies, although a similar trend was observable as was demonstrated in foragers (Figure 4A; KW = 5.41, $n_{\text{control}} = 8$, $n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 6$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 9$, $P > 0.05$). Octopamine levels were also not elevated in the fat body of nurse bees after injections of tyramine into the abdomen (Figure 4B; KW = 1.40, $n_{\text{control}} = 4$, $n_{\text{tyramine } 10^{-3} \text{ mol/l}} = 6$, $n_{\text{tyramine } 10^{-2} \text{ mol/l}} = 6$, $P > 0.05$).

Since the behavioral effects observed in foragers after tyramine injection into the abdomen might have been induced by an increased tyramine titer in the brain, we also quantified tyramine levels in the brains of nurse bees and foragers after injection of tyramine into the abdomen. We here only investigated the effect of the higher tyramine concentration (10^{-2} mol/l), since only this concentration had a significant effect on behavior.

After injection of $2 \mu\text{l}$ of tyramine (10^{-2} mol/l) into the abdomen, the tyramine titer was significantly elevated in the brains of forager bees (Figure 5A; $n_{\text{control}} = 8$, $n_{\text{tyramine}} = 9$,

$Z = 2.37$, $P < 0.05$, Mann Whitney U test). The octopamine brain titer was not affected by tyramine injection (**Figure 5B**; $n_{\text{control}} = 7$, $n_{\text{tyramine}} = 10$, $Z = 0.20$, $P > 0.05$). In contrast to foragers, nurse bees displayed both increased tyramine and octopamine titers in their brains (tyramine: **Figure 5A**; $n_{\text{control}} = 9$, $n_{\text{tyramine}} = 8$, $Z = 3.75$, $P < 0.001$; octopamine: **Figure 5B**; $n_{\text{control}} = 7$, $n_{\text{tyramine}} = 7$, $Z = 3.13$, $P < 0.01$).

DISCUSSION

Our data show that foragers were significantly more responsive to sucrose than were nurse bees, confirming the link between social organization and nutrition in a honeybee colony. The higher gustatory responsiveness observed in foragers generally leads to better associative learning performance (Scheiner et al., 1999, 2001a,b, 2003) and correlates with higher visual responsiveness (Erber et al., 2006) and with a higher responsiveness to odors (Scheiner et al., 2004). Whether it is causally related to foraging tasks has yet to be shown.

The differences in gustatory responsiveness of nurse bees and foragers correlated with differences in tyramine physiology. Foragers had higher expression of the tyramine receptor gene *Amtar1* in their fat bodies than nurse bees, while displaying lower tyramine titers. Pharmacological activation of tyramine receptors increased gustatory responsiveness in foragers even further, while no significant effect was observed in nurse bees. Although some of the specifics of the modes of tyramine action presently remain unclear, it seems that tyramine metabolism in the periphery differs between individuals performing different tasks and could thus contribute to the behavioral differences between behavioral states.

The fat body has an important function in honeybee metabolism (Nilsen et al., 2011; Wang et al., 2012), which differs hugely between nurse bees and foragers. Nevertheless, the function of this organ in regulating social organization has hardly been investigated. Our data suggest that the fat body could mediate division of labor through modulating nutrition-related sensory response thresholds via aminergic signaling cascades. The tyramine receptor AmTAR1 is particularly interesting in this respect, since its mRNA expression is more than six-fold elevated in foragers compared to nurse bees. The higher expression of this tyramine receptor, which decreases intracellular cAMP levels upon activation (Blenau et al., 2000; Reim et al., 2017), might be causally related to an increased gustatory responsiveness, as observed in foragers. Future experiments in which the receptor expression is reduced in young bees, for example by RNA interference in the fat body (Amdam et al., 2006; Nilsen et al., 2011; Wang et al., 2012), should lead to a reduced gustatory responsiveness as observed in nurse bees and possibly to a delayed onset of foraging behavior. Reducing tyramine receptor expression in the fat bodies of foragers might even induce nursing behavior. In addition, foragers displayed a significantly lower expression of the octopamine receptor gene *AmoctaR1*. Intriguingly, the lower fat body *AmoctaR1* expression in foragers compared with nurse bees contrasts with the brain, where foragers have higher octopamine titers (Schulz et al., 2002) and higher *AmoctaR1* mRNA expression (Reim and Scheiner, 2014).

Because both tyramine and octopamine can modulate gustatory responsiveness in the same direction, it will be interesting in the future to separate the functions of the different types of octopamine and tyramine receptors in the fat body based on sensory responsiveness and other behaviors.

Our data reveal that injections of tyramine into the abdomen of honeybees are an effective method of increasing tyramine titers in the abdomen of honeybee foragers, but not of nurse bees. In the case of foragers, this method also increases tyramine brain levels, while in nurse bees, both tyramine and octopamine brain titers were increased. These findings are important for behavioral pharmacological experiments with different age groups or behavioral groups in honeybees. Furthermore, it is interesting that nurse bees naturally have lower tyramine brain levels compared to foragers (Reim et al., 2017), which contrasts with their significantly higher tyramine titers in their fat bodies. These findings suggest that nursing behavior and foraging behavior coincide with differential tyramine signaling.

The fact that tyramine acted on gustatory responsiveness in foragers, but not in nurse bees, is challenging. Our data suggest that most likely these differences in behavioral response might have been caused by mechanisms outside the brain through differences in tyraminergergic signaling in the periphery, including the fat body. Foragers showed a significantly higher *Amtar1* mRNA expression than did nurse bees in their fat bodies. Coinciding with this, foragers had a significantly lower tyramine titer in their fat bodies compared to nurse bees. An elevated tyramine titer in the abdomen could therefore effectively modulate behavior through activation of abundant tyramine receptors in foragers. It is unlikely that the effects on behavior were induced by conversion of the injected tyramine into octopamine in the fat bodies, because octopamine titers of foragers were not elevated after tyramine injection. However, we cannot exclude the possibility that the injected tyramine bound to octopamine receptors in fat bodies (Grohmann et al., 2003; Balfanz et al., 2014). In particular, nurse bees had a high expression of the octopamine receptor gene *AmoctaR1* in their fat bodies. If the respective receptor acted in an opposite way compared to the tyramine receptor AmTAR1, the activated receptor might have inhibited the behavioral effects of tyramine on gustatory responsiveness. Since nurse bees showed very low mRNA expression of *Amtar1* coinciding with high natural titer of tyramine in their fat bodies, further elevating tyramine titers in fat bodies might therefore have had little effect on behavior of nurse bees, when controlled by tyraminergergic fat body regulatory mechanisms. Why nurse bees have high tyramine titers is not quite clear. Since they consume large amounts of pollen to produce brood food, they also consume large amounts of tyrosine, which is frequently present in bee-collected plant pollen (Szcześna, 2006). This amino acid can be converted into tyramine by decarboxylation (Roeder, 2005). Also, tyramine itself has been shown to be present in many plants (Smith, 1977). It might therefore be present in the pollen grains of the plants as well. This, however, has not been investigated to the best of our knowledge.

Our results suggest that tyramine can have a decisive function in regulating division of labor through modulating

gustatory responsiveness. They further imply that the fat body of honeybees may have a much more important role in controlling behavior including social organization than believed hitherto. These are important aspects for the function of this organ, which has mainly been placed in the context of metabolism. The new link between fat body tyramine signaling, gustatory responsiveness and division of labor between nurse bees and foragers strengthens the hypothesis proposed by Ament et al. (2010) that nutrition-related mechanisms control social organization in a honeybee colony.

AUTHOR CONTRIBUTIONS

RS designed the experiments, supervised part of the experiments, analyzed data and wrote the first draft of manuscript.

REFERENCES

- Alaux, C., Le Conte, Y., Adams, H. A., Rodriguez-Zas, S., Grozinger, C. M., Sinha, S., et al. (2009). Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav.* 8, 309–319. doi: 10.1111/j.1601-183X.2009.00480.X
- Amdam, G. V., Norberg, K., Page, R. E. Jr., Erber, J., and Scheiner, R. (2006). Downregulation of *vitellogenin* gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behav. Brain Res.* 169, 201–205. doi: 10.1016/j.bbr.2006.01.006
- Ament, S. A., Wang, Y., and Robinson, G. E. (2010). Nutritional regulation of division of labor in honey bees: toward a systems biology perspective. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2, 566–576. doi: 10.1002/wsbm.73
- Balfanz, S., Jordan, N., Langenstück, T., Breuer, J., Bergmeier, V., and Baumann, A. (2014). Molecular, pharmacological, and signaling properties of octopamine receptors from honeybee (*Apis mellifera*) brain. *J. Neurochem.* 129, 284–296. doi: 10.1111/jnc.12619
- Behrends, A., and Scheiner, R. (2010). Learning at old age: a study on winter bees. *Front. Behav. Neurosci.* 4:15. doi: 10.3389/fnbeh.2010.00015
- Behrends, A., and Scheiner, R. (2012). Octopamine improves learning in newly emerged bees but not in old foragers. *J. Exp. Biol.* 215, 1076–1083. doi: 10.1242/jeb.063297
- Behrends, A., Scheiner, R., Baker, N., and Amdam, G. (2007). Cognitive aging is linked to social role in honey bees (*Apis mellifera*). *Exp. Gerontol.* 42, 1146–1153. doi: 10.1016/j.exger.2007.09.003
- Blenau, W., Balfanz, S., and Baumann, A. (2000). *Amyt1*: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem.* 74, 900–908. doi: 10.1046/j.1471-4159.2000.0740900.x
- Erber, J., Hoorman, J., and Scheiner, R. (2006). Phototactic behaviour correlates with gustatory responsiveness in honey bees (*Apis mellifera* L.). *Behav. Brain Res.* 174, 174–180. doi: 10.1016/j.bbr.2006.07.023
- Grohmann, L., Blenau, W., Erber, J., Ebert, P. R., Strünker, T., and Baumann, A. (2003). Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J. Neurochem.* 86, 725–735. doi: 10.1046/j.1471-4159.2003.01876.x
- Hunt, G. J., Amdam, G. V., Schlipalius, D., Emore, C., Sardesai, N., Williams, C. E., et al. (2007). Behavioral genomics of honeybee foraging and nest defense. *Naturwissenschaften* 94, 247–267. doi: 10.1007/s00114-006-0183-1
- Johnson, B. R. (2010). Division of labor in honeybees: form, function, and proximate mechanisms. *Behav. Ecol. Sociobiol.* 64, 305–316. doi: 10.1007/s00265-009-0874-7
- Nilsen, K.-A., Ihle, K. E., Frederick, K., Fondrk, M. K., Smedal, B., Hartfelder, K., et al. (2011). Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. *J. Exp. Biol.* 214, 1488–1497. doi: 10.1242/jeb.050393
- Reim, T., Balfanz, S., Baumann, A., Blenau, W., Thamm, M., and Scheiner, R. (2017). AmTAR2: functional characterization of a honeybee tyramine receptor stimulating adenylyl cyclase activity. *Insect Biochem. Mol. Biol.* 80, 91–100. doi: 10.1016/j.ibmb.2016.12.004
- Reim, T., and Scheiner, R. (2014). Division of labour in honey bees: age- and task-related changes in the expression of octopamine receptor genes. *Insect Mol. Biol.* 23, 833–841. doi: 10.1111/imb.12130
- Reim, T., Thamm, M., Rolke, D., Blenau, W., and Scheiner, R. (2013). Suitability of three common reference genes for quantitative real-time PCR in honey bees. *Apidologie* 44, 342–350. doi: 10.1007/s13592-012-0184-3
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Scheiner, R. (2004). Responsiveness to sucrose and habituation of the proboscis extension response in honey bees. *J. Comp. Physiol.* 190, 727–733. doi: 10.1007/s00359-004-0531-6
- Scheiner, R., Abramson, C. I., Brodschneider, R., Crailsheim, K., Farina, W. M., Fuchs, S., et al. (2013). “Standard methods for behavioral studies of *Apis mellifera*,” in *The COLOSS BEEBOOK: Standard Methodologies for Apis mellifera Research. Journal of Apicultural Research* (Vol. 52), eds V. Dietemann, J. D. Ellis and P. Neumann, 1–58. doi: 10.3896/IBRA.1.52.4.04
- Scheiner, R., and Amdam, G. V. (2009). Impaired tactile learning is related to social role in honey bees. *J. Exp. Biol.* 212, 994–1002. doi: 10.1242/jeb.021188
- Scheiner, R., and Arnold, G. (2010). Effects of patriline on gustatory responsiveness and olfactory learning in honey bees. *Apidologie* 41, 29–37. doi: 10.1051/apido/2009040
- Scheiner, R., Barnert, M., and Erber, J. (2003). Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. *Apidologie* 34, 67–72. doi: 10.1051/apido:2002050
- Scheiner, R., Blenau, W., and Baumann, A. (2006). Aminergic control and modulation of honeybee behaviour. *Curr. Neuropharmacol.* 4, 259–276. doi: 10.2174/157015906778520791
- Scheiner, R., Erber, J., and Page, R. E. Jr. (1999). Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* 185, 1–10. doi: 10.1007/s003590050360
- Scheiner, R., Page, R. E. Jr., and Erber, J. (2001a). Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. *Behav. Brain Res.* 120, 67–73. doi: 10.1016/s0166-4328(00)00359-4
- Scheiner, R., Page, R. E. Jr., and Erber, J. (2001b). The effects of genotype, foraging role, and sucrose perception on the tactile learning performance of honey bees (*Apis mellifera* L.). *Neurobiol. Learn. Mem.* 76, 138–150. doi: 10.1006/nlme.2000.3996
- Scheiner, R., Page, R. E., and Erber, J. (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie* 35, 133–142. doi: 10.1051/apido:2004001

- Scheiner, R., Plückhahn, S., Oney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* 136, 545–553. doi: 10.1016/s0166-4328(02)00205-x
- Scheiner, R., Reim, T., Søvik, E., Entler, B. V., Barron, A. B., and Thamm, M. (2017). Learning, gustatory responsiveness and tyramine differences across nurse and forager honeybees. *J. Exp. Biol.* 220, 1443–1450. doi: 10.1242/jeb.152496
- Scheiner, R., Steinbach, A., Claßen, G., Strudthoff, N., and Scholz, H. (2014a). Octopamine indirectly affects proboscis extension response habituation in *Drosophila melanogaster* by controlling sucrose responsiveness. *J. Insect Physiol.* 69, 107–117. doi: 10.1016/j.jinsphys.2014.03.011
- Scheiner, R., Toteva, A., Reim, T., Søvik, E., and Barron, A. B. (2014b). Differences in the phototaxis of pollen and nectar foraging honey bees are related to their octopamine brain titers. *Front. Physiol.* 5:116. doi: 10.3389/fphys.2014.00116
- Schulz, D. J., Barron, A. B., and Robinson, G. E. (2002). A role for octopamine in honey bee division of labor. *Brain Beh. Evol.* 60, 350–359. doi: 10.1159/000067788
- Schulz, D. J., Huang, Z. Y., and Robinson, G. E. (1998). Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol.* 42, 295–303. doi: 10.1007/s002650050442
- Smith, T. A. (1977). Phenethylamine and related compounds in plants. *Phytochemistry* 16, 9–18. doi: 10.1016/0031-9422(77)83004-5
- Søvik, E., Cornish, J. L., and Barron, A. B. (2013). Cocaine tolerance in honey bees. *PLoS One* 8:e64920. doi: 10.1371/journal.pone.0064920
- Szczęsna, T. (2006). Protein content and amino acid composition of bee-collected pollen from selected botanical origins. *J. Apicult. Science* 50, 81–90.
- Thamm, M., and Scheiner, R. (2014). PKG in honey bees: spatial expression, *Amfor* gene expression, sucrose responsiveness, and division of labor. *J. Comp. Neurol.* 522, 1786–1799. doi: 10.1002/cne.23500
- Toth, A. L., Kantarovich, S., Meisel, A. F., and Robinson, G. E. (2005). Nutritional status influences socially regulated foraging ontogeny in honey bees. *J. Exp. Biol.* 208, 4641–4649. doi: 10.1242/jeb.01956
- Toth, A. L., and Robinson, G. E. (2005). Worker nutrition and division of labour in honeybees. *Anim. Behav.* 69, 427–435. doi: 10.1016/j.anbehav.2004.03.017
- Wang, Y., Brent, C. S., Fennern, E., and Amdam, G. V. (2012). Gustatory perception and fat body energy metabolism are jointly affected by vitellogenin and juvenile hormone in honey bees. *PLoS Genet.* 8:e1002779. doi: 10.1371/journal.pgen.1002779

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Tyramine Actions on *Drosophila* Flight Behavior Are Affected by a Glial Dehydrogenase/Reductase

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The biogenic amines octopamine (OA) and tyramine (TA) modulate insect motor behavior in an antagonistic manner. OA generally enhances locomotor behaviors such as *Drosophila* larval crawling and flight, whereas TA decreases locomotor activity. However, the mechanisms and cellular targets of TA modulation of locomotor activity are incompletely understood. This study combines immunocytochemistry, genetics and flight behavioral assays in the *Drosophila* model system to test the role of a candidate enzyme for TA catabolism, named Nazgul (Naz), in flight motor behavioral control. We hypothesize that the dehydrogenase/reductase Naz represents a critical step in TA catabolism. Immunocytochemistry reveals that Naz is localized to a subset of Repo positive glial cells with cell bodies along the motor neuropil borders and numerous positive Naz arborizations extending into the synaptic flight motor neuropil. RNAi knock down of Naz in Repo positive glial cells reduces Naz protein level below detection level by Western blotting. The resulting consequence is a reduction in flight durations, thus mimicking known motor behavioral phenotypes as resulting from increased TA levels. In accord with the interpretation that reduced TA degradation by Naz results in increased TA levels in the flight motor neuropil, the motor behavioral phenotype can be rescued by blocking TA receptors. Our findings indicate that TA modulates flight motor behavior by acting on central circuitry and that TA is normally taken up from the central motor neuropil by Repo-positive glial cells, desaminated and further degraded by Naz.

Keywords: *Drosophila*, biogenic amine, tyramine, flight, modulation, glia

INTRODUCTION

Neuromodulatory substances shape central pattern generator (CPG) network and motoneuronal (MN) activity into many different forms, thus lending flexibility of the motor output to different behavioral requirements or to different internal states (Harris-Warrick and Marder, 1991). Modulators can act on many different levels of the motor system, ranging from brain circuitry and the motor-network, to actions on sensory neurons, neuromuscular transmission and muscle properties. In many cases, several different modulators affect motor output, and vice versa, the same modulator may act on multiple different levels (Marder et al., 2005).

The highest level of modulatory monoamine input occurs during “fight or flight” behavioral situations (Cannon, 1932). Analogous to noradrenaline (NA) in vertebrates, in insects “fight or flight” reactions are often attributed to the biogenic amine octopamine (OA; Stevenson and Rillich, 2012). Both *Drosophila* larval crawling (Fox et al., 2006) and adult flight motor behaviors (Brembs et al., 2007) are facilitated by OA. Moreover, in invertebrates the biogenic amine tyramine (TA) regulates motor behaviors in an antagonistic manner to OA (Pflüger and Duch, 2011).

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Larval *Drosophila* crawling (Saraswati et al., 2004; Fox et al., 2006) and adult flight (Brembs et al., 2007) initiation and maintenance are augmented by OA but reduced by TA signaling.

However, for both OA and TA the cellular site(s) of action that underlie the modulation of motor behavior remain largely unknown. With regard to insect flight the biogenic amine OA has been reported to affect central pattern generating circuits (Sombati and Hoyle, 1984), sensory sensitivity (Büschges et al., 1993; Ramirez et al., 1993; Matheson, 1997), flight muscle contraction properties, hormone release (Orchard et al., 1993) and muscle metabolism (Mentel et al., 2003). Although the effects of TA are less well described, TA is known to act also on insect central pattern generating circuits (Rillich et al., 2013), muscle contraction properties (Ormerod et al., 2013), metabolism (Downer, 1979) and likely also on sensory systems (Kutsukake et al., 2000). Accordingly, it is difficult to pinpoint whether peripheral or central actions of OA and TA mediate their known effects on flight behavior. Ideally, OA and TA actions have to be selectively manipulated at either site of action to test the resulting effects on motor behavior separately.

In neurons, TA is synthesized from the amino acid tyrosine by the enzyme tyrosine decarboxylase (TDC2, Roeder, 2005). TA can then be further processed into OA by the tyramine beta hydroxylase (T β h, Monastirioti et al., 1996). Therefore, insect octopaminergic neurons also contain TA. Accordingly *Drosophila* *t β h* null mutants lack OA but have strongly increased TA levels (Monastirioti et al., 1996). The results are reduced flight durations and fewer flight initiations, and these behavioral phenotypes can be partially rescued by feeding OA or by blocking TA receptors, thus demonstrating that both amines affect flight motor behavior in an antagonistic manner (Brembs et al., 2007).

This study aims to providing some insight as to whether TA affects flight motor behavior by modulatory actions in the central nervous system (CNS). In an effort to selectively manipulate TA levels in the CNS we employ the genetic power of *Drosophila* to interfere with the putative degradation pathway of TA in glial cells. In general, monoamines are degraded by desamination by monoamine oxidases (MAOs) to aldehydes, which are further processed by dehydrogenases/reductases. Recently, in *Drosophila* a respective candidate dehydrogenase/reductase (accession number CG31235) for TA degradation has been identified and named Nazgul (Naz; de Visser, 2016).

We find that Naz localizes to a specific set of glial cells in the CNS. Furthermore, targeted RNAi knock down of *nazgul* in glial cells phenocopies decreased flight durations as induced by increased TA levels. These data indicate that Nazgul indeed takes place in TA degradation and that TA modulates flight motor behavior at least in part by modulatory actions in the CNS.

MATERIALS AND METHODS

Animals

Drosophila melanogaster were reared in standard 68 ml plastic vials with foam stoppers on a yeast-cornmeal-syrup-agar diet at 25°C and 55% humidity with a 12-h light/dark regimen. Fly food contained the following ingredients (per 1000 ml): 116 g glucose (Carl Roth, Germany, HN06.04), 55 g cornmeal (Rapunzel,

Germany, Demeter standard 420505), 11 g agar (Roth 5210.5), 29 g active dry yeast (Huber Mühle, Germany), 0.6 g ascorbic acid (Roth 3525.3) and 12.2 ml of 10% tegosept (Apex BioResearch Products 20–258) in 100% ethanol. All experiments were carried out with 2–3 days old adult male flies. To drive expression of UAS-transgenes in glial cells we used *repo-GAL4* (*w*¹¹¹⁸; P{GAL4}repo/TM3, Sb¹; Bloomington # 7415). To knock down Naz we used a UAS-*naz*-RNAi flystrain (VDRC RNAi fly stock center # 107974). To enhance RNAi knock down efficacy we included an extra copy of dicer 2 (UAS-Dcr2, Bloomington Stock 24650, Dietzl et al., 2007). To drive expression of UAS transgenes in muscle cells we used Mef2a-GAL4 (Myocyte enhancer factor 2; *y*[1] *w*[*]; P{w[+mC] = GAL4-Mef2.R}3; Bloomington # 27390).

Behavioral Testing

Two to three days old male flies were immobilized by cooling for 30 s in an empty 68 ml plastic vial on ice and then immediately transferred onto a cold plate at 2°C. Then animals were glued (clear glass adhesive (Duro; Pacer Technology, Rancho Cucamonga, CA, USA)) with head and thorax to a triangle-shaped copper hook (0.02 mm diameter). Adhesion was achieved by exposure to UV light for 10 s. The animals were then immediately returned to room temperature and kept individually in small chambers containing a few grains of sucrose on filter paper until testing (2–3 h). Flies, glued to copper hooks were attached to the experimental setup via a clamp to accomplish stationary flight. For observation, the fly was illuminated from behind and above 150 W (15 V; Schott, Elmsford, NY, USA) and fixed in front of a paper panel with horizontal white and black stripe patterns. Tarsal contact with a bead of polystyrene prevented flight initiation before the experiment started. For flight initiation, the polystyrene bead was removed, and the fly was gently aspirated by mouth to produce an air current of about 0.8 m per second. The time until the fly first stopped flying was measured. After each flight bout the fly was again gently aspirated as a stimulation to fly. The time was recorded for each flight bout. The experiment was completed when no flight reaction was initiated in response to three consecutive air stimuli. The person recording the flight times was unaware of the genotype of the animal.

Western Blotting

Brains of 2–3 day-old male adult *Drosophila* were dissected according to Wu and Luo (2006). For SDS-PAGE Western blot 10 brains per lane (40 ml Hoefer Western blot chamber with 1.5 mm spacer and comb with 15 × 100 μ l pockets) were dissected and mechanically homogenized with a clean micro-pestle in 85 μ l ice cold 2× concentrated sample buffer (7.5 ml 4× Tris-HCl/SDS, pH 6.8, 6 ml glycerol, 1.5 g SDS, 0.3 g dithiothreitol (DTT), ~1 mg bromophenol blue in 30 ml with ddH₂O). 4× Tris-HCl was prepared with 40 ml ddH₂O, 6.05 g Tris base, 0.4 g SDS, pH was adjusted to 6.8 with HCl, then filled up to 100 ml with ddH₂O and filtered through a 0.45 μ m sterile filter. Homogenized samples were boiled for 3 min and then loaded into the gel chambers. We used

a 5% polyacrylamide stacking gel (10 ml, 1.7 ml of 30% bis-acrylamide, 1.25 ml 4× Tris/SDS pH 8.8, 6.8 ml ddH₂O, 100 µl 10% ammoniumpersulfate (APS), 10 µl TEMED) and let the samples run through a 10% polyacrylamide running gel (30 ml, 13.3 ml 30% bis-acrylamide, 10 ml 4× Tris/SDS pH 8.8, 16 ml ddH₂O, 400 µl 10% APS, 16 µl TEMED). APS and TEMED were added just prior to pouring the gel. Samples were run through the stacking gel at 20 mA and then through the running gel at 30 mA constant current at room temperature. As protein marker 20 µl Precision Plus ProteinTM WesternCTM Blotting Standard 10–250 kDa was used (Bio Rad #161-0376). Proteins were then transferred onto nitrocellulose membrane (Bio Rad) overnight at 4°C at 35 V constant voltage in a 5 L 42E Hoefer blotting chamber using transfer buffer (18.2 g Tris base, 86.5 g glycine, 900 ml methanol, fill up to 6 L with ddH₂O). Nitrocellulose membrane was incubated in blocking solution (10% milk powder in electrophoresis buffer with Tween-20 (TBST) for 2 h at room temperature. Before antibody incubation, nitrocellulose membrane was cut horizontally because both primary antibodies were made in rabbit. Primary antibodies were prepared in 2.5% milk powder in TBST and the nitrocellulose membrane incubated overnight: rabbit α -hsp90 (New England Biolabs or Cell Signaling Technology, #4872S) as loading control 1:1000 and rabbit α -Naz 1:250 (von Hilchen et al., 2013) were used. Secondary antibody incubation was done together for both pieces of the nitrocellulose membrane. Secondary antibody was applied for 2 h at RT: goat α -rabbit (H + L) HRP conjugate 1:5000 (Millipore #AP106P) was used. Protein bands were detected with luminol-based Immobilon Western Chemiluminescence detection kit (Millipore #WBKLS01 00). Hsp90 band was expected at 84 kDa, Naz (CG31235) was expected at 46 kDa. Pictures were taken and saved as .tiff image files.

Immunocytochemistry

Adult flies were dissected in standard saline along the dorsal midline and the gut was removed to expose the ventral nerve cord (VNC) as described previously (Boerner and Duch, 2010). Next preparations were fixed for 60 min in 4% PFA in 0.1 M PBS, washed 6 × 20 min 0.1 M PBS and 6 × 30 min in PBS Triton-X 0.5%. This was followed by primary antibody incubation overnight at 4°C in 0.1 M PBS with 0.2% BSA and 0.3% Triton-X on a shaker. Rabbit α -Naz and guinea pig α -Repo (von Hilchen et al., 2013) were both used at 1:1000 in 0.1 M PBS Triton-X 0.1%. Specificity for both primary antibodies has previously been reported (von Hilchen et al., 2013). Specificity for α -Naz has been further confirmed in this study by the absence of immunopositive signal in Western blots after *Naz*-RNAi knock down.

Following primary AB incubation preparations were rinsed in 0.1 M PBS and washed in 0.1 M PBS 6 × 30 min. For detection of Naz immunolabel donkey α -rabbit secondary antibody coupled to Alexa 568 (Invitrogen A10042) was used. For detection of Repo immunolabel goat α -guinea pig secondary antibody coupled to Cy5 (Dianova 106-605-003) was used.

Both secondary antibodies were incubated at a concentration of 1:500 in 0.1 M PBS overnight at 4°C on a shaker. Next preparations were rinsed for 6 × 30 min with PBS followed by an ascending ethanol series (50, 70, 90, 100%), and then mounted in methylsalicylate. Immunolabel was visualized using a Leica SP8 confocal laser-scanning microscope (Leica, Germany). Alexa 568 was excited with a 568 solid state laser and detection was set between 580 nm and 610 nm. Cy5 was excited with a 633 Helium Neon laser and detection was set between 640 nm and 690 nm. Images were processed with Amira 5.3.3 (Mercury Systems) and Corel X7 (Corel Corporation) software.

Thoracic neuromeres, lateral nerves and neuropil borders were labeled and defined as previously described (Boerner and Duch, 2010). The thoracic neuropils are characterized by the absence of any cell bodies and a glial lining that separates nervous tissue densely packed with synapses from a VNC cortex packed with cell bodies. The demarcation line can be visualized in single optical sections through the VNC.

Statistical Analysis

All flight behavioral data are presented as box blots with median, 25, and 75 percentiles. Error bars represent the 10 and the 90 percentiles. Statistical differences were tested for with Kruskal Wallis ANOVA. Following a statistical significant Kruskal-Wallis *p* value (significance level was set <0.05) Dunn-Bonferroni *post hoc* method was used for between groups *post hoc* comparisons. For between groups comparisons * indicates *p* < 0.05 and ** indicates *p* < 0.01, whereas ns indicates *p* > 0.1. All statistical testing was conducted with SPSS Statistics 22 software.

RESULTS

We first analyzed the localization of the dehydrogenase/reductase, Naz, in the adult *Drosophila* VNC by immunohistochemistry and confocal laser scanning microscopy (Figure 1). Maximum projection views from optical sections through the entire VNC revealed many Naz positive cells in all three thoracic and all abdominal neuromeres (Figure 1A, magenta). The nuclei of glial cells in the CNS were co-labeled with the glial cell marker Reversed polarity (Figure 1A, green), Repo, which is required for glial cell differentiation (Halter et al., 1995). This indicated that Naz was localized to glial cells. Single optical sections (Figure 1B) at different depths (34, 69 and 88 µm) indicated that Naz was predominantly localized to glial cells which demarked the borders of the neuropil regions.

Selective enlargement of the mesothoracic neuropil showed Repo positive glial cells around the neuropil border (Figures 2A,Ai, green). Many of the Repo positive glial cells were also Naz positive (Figures 2A,Aii, magenta). Similarly, Naz has previously been used as a marker for longitudinal glial cells during embryonic development (von Hilchen et al., 2010). Please note that Repo labeled the nuclei of these glia cells, but by contrast, Naz was localized cytosolically and labeled the soma and glial cell processes that extended into the flight motor

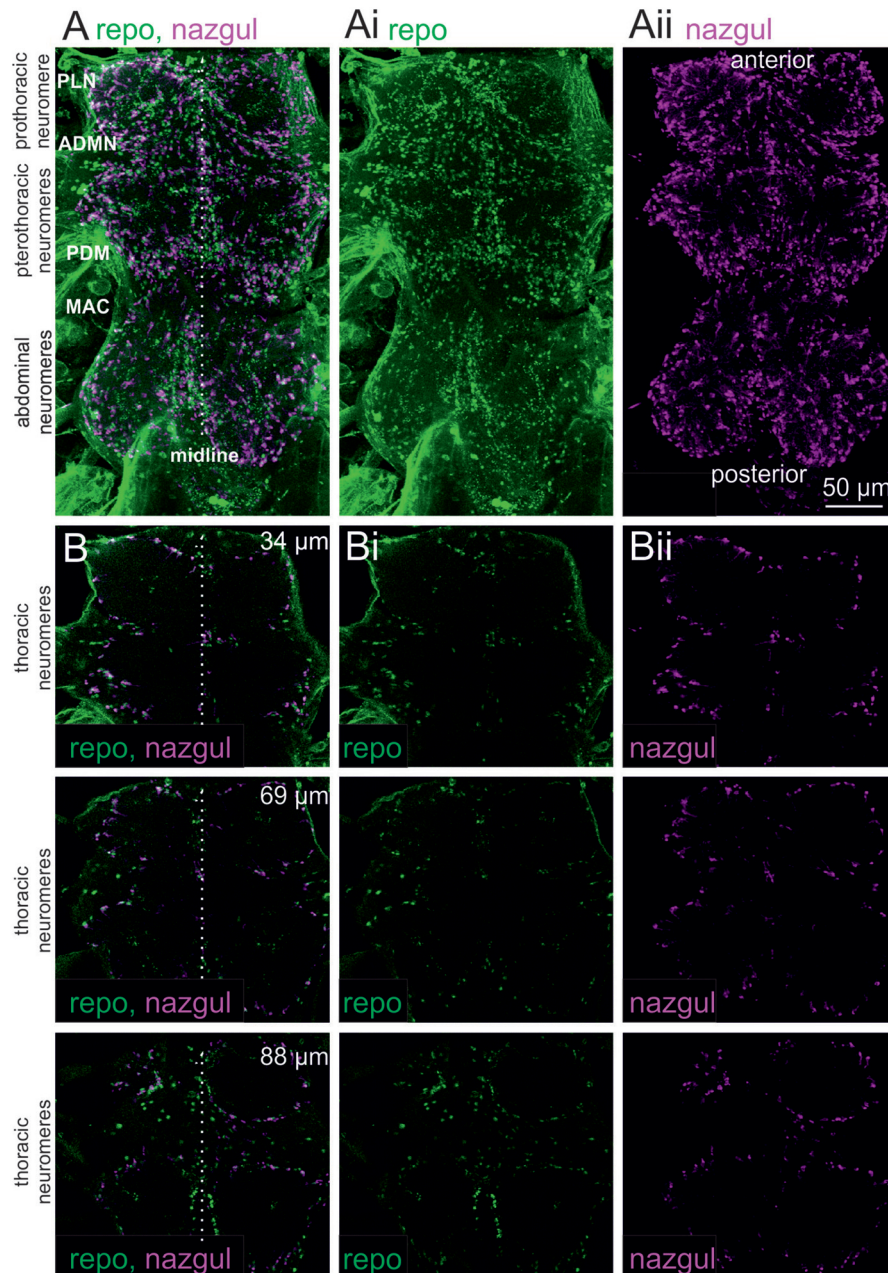
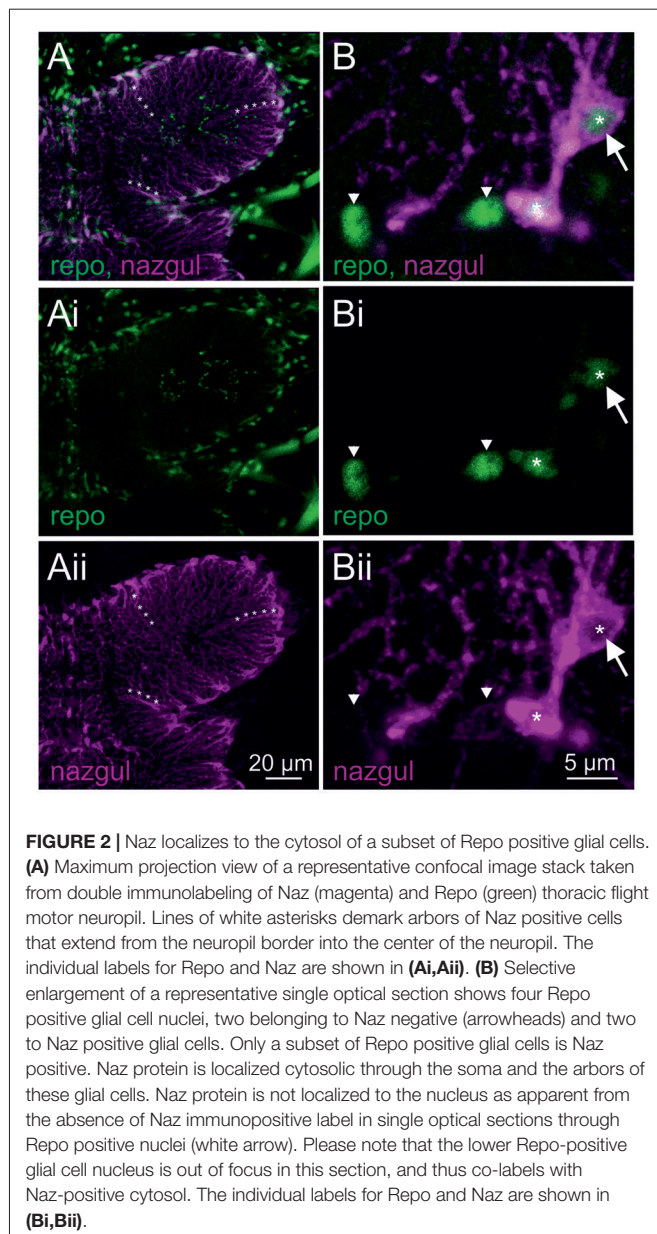


FIGURE 1 | Nazgul (Naz) localizes in similar patterns as Repo positive glial cells in the ventral nerve cord (VNC). **(A)** Maximum projection views of a representative confocal image stack taken from double immunolabeling of Naz (magenta) and Repo (green) in the VNC. The locations of the prothoracic, pterothoracic (meso- and metathoracic) and abdominal neuromeres are indicated. The midline is indicated by a dotted white line and prominent nerves are labeled (PLN, prothoracic leg nerve; ADMN, anterior dorsal mesothoracic nerve; PDM, posterior dorsal mesothoracic nerve; MAC, mesothoracic accessory nerve). The individual labels for Repo and Naz are shown in **(Ai,Aii)**. **(B)** Single optical sections of Naz (magenta) and Repo (green) label at 34 μm (upper row), 69 μm (middle row) and 88 μm (lower row) imaging depth show that Naz and Repo positive cells localize to the borders of the motor neuropils. The individual labels for Repo and Naz are shown in **(Bi,Bii)**.

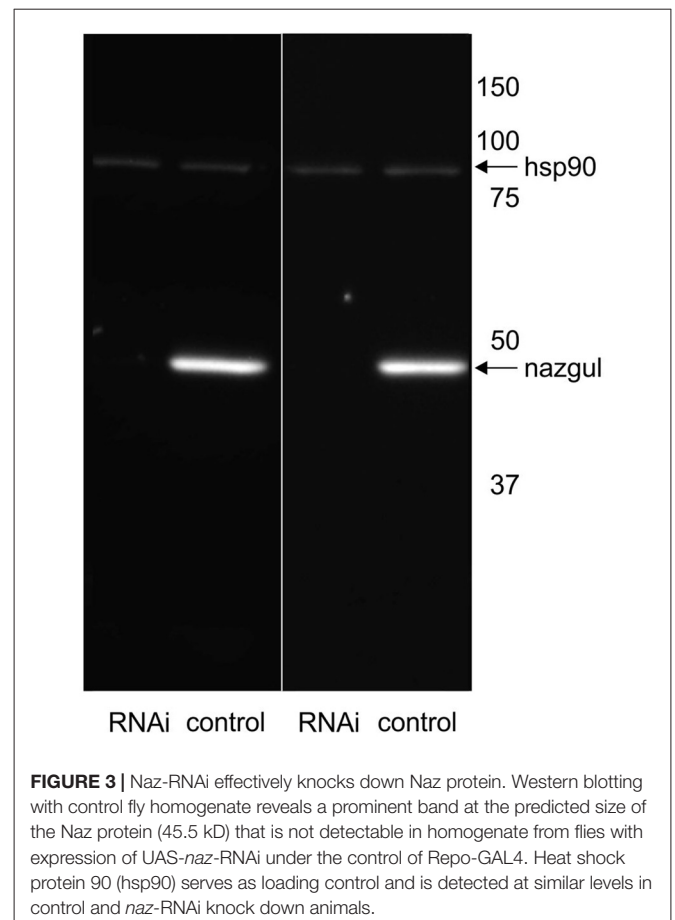
neuropil (as examples three such processes are labeled with white asterisks in **Figures 2A,Aii**). Careful inspection of image stacks from five animals revealed that every Naz positive cell in the VNC was also Repo positive. Representative selective enlargements of few cells revealed two things: first, Naz was always localized to the cytosol but not to the Repo positive

nucleus of the glial cells (**Figure 2B** white arrow, single optical section through the glial cell nucleus). Second, all Naz positive cells contained also a Repo positive nucleus, but not all Repo positive glial cells were also Naz positive (**Figure 2B**). For visualization, in **Figures 2B,Bi,Bii** Naz negative glial cells are labeled by white arrowheads, whereas Naz positive glial cells are



labeled by white asterisks. Therefore, Naz localized to a subset of glial cells that align the flight motor neuropil borders and project extensions into the central neuropil regions.

We next tested whether Naz expression could be eliminated by targeted RNAi knock down in Repo positive glial cells under the control of *repo*-GAL4. RNAi knock down efficacy was enhanced by inclusion of extra Dicer-2 (UAS-Dcr2, Bloomington Stock 24650, Dietzl et al., 2007). We have previously reported that this effectively enhances knock down strength (Ryglewski et al., 2012; Hutchinson et al., 2014). As controls we crossed UAS-Dcr2 to *repo*-GAL4 but did not include UAS-*naz*-RNAi. In controls Western blotting revealed one prominent band at the predicted size for the Naz protein at 45.5 kD (**Figure 3**). By contrast, following targeted RNAi knock down VDRC (107974) of *naz* in *repo* expressing glial cells no Naz protein was detectable



by Western blotting (**Figure 3**). Therefore, RNAi effectively knocked down Naz protein below detection threshold. This confirmed that Naz was exclusively expressed in *repo* positive glial cells because RNAi knock down was targeted selectively to glial cells. It also further confirmed Naz antibody specificity because antibody detection was eliminated by protein knock down with an RNAi that has no reported off-target effects. Given that we found no Naz immunopositive signal in the periphery and that Repo positive glial cells are located in the adult CNS we next utilized *naz* RNAi knock down to test possible effects on flight motor behavior (see “Materials and Methods” Section for flight behavioral assay).

Flies with *naz* RNAi knock down in glial cells (**Figure 4A**, red bar) showed significantly shorter total flight durations as compared to two different controls (**Figure 4A**, white and gray bars, $p = 0.0004$ for control 1 vs. Naz-RNAi, $p = 0.0027$ for control 2 vs. *naz*-RNAi). As controls we used UAS-Dcr2 expressed under the control of *repo*-GAL4 (control 1, white bar) and expression of UAS-Dcr2 and UAS-*naz*-RNAi in muscle under the control of Mef2-GAL4 (control 2, gray bar). A control with RNAi expression in muscle was used to control for possible leak expression of the UAS-RNAi construct in the absence of GAL4. Given that *naz* knock down in the CNS reduced flight durations as previously reported for *tβh* mutant flies that lack OA but have increased TA levels (Brembs et al., 2007), we hypothesized that a reduction of Naz function caused increased TA levels in the flight

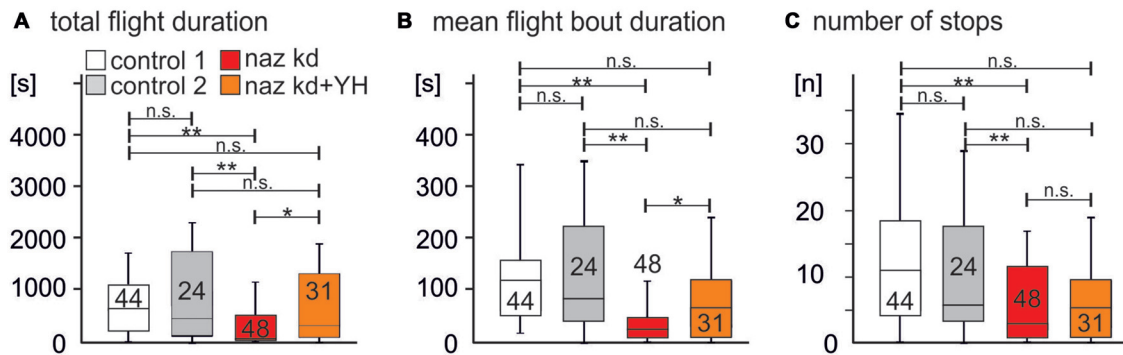


FIGURE 4 | Knock down of Naz reduces flight durations. **(A)** Total flight duration is significantly reduced in animals with knock down of Naz in Repo positive glial cells (Repo-GAL4 × UAS-*naz*-RNAi; UAS-Drc2, red bar; *naz* kd) as separately compared to two different controls (control 1, white bar; expression of UAS-drc2 under the control of Repo-GAL4; control 2, gray bar; expression of UAS-drc2 and UAS-*naz*-RNAi under the control of Mef2A). Total flight duration of animals with knock down of Naz in Repo positive glial cells is significantly increased by feeding of the TAR blocker Yohimbine (YH; *naz* kd + YH, orange bar). Feeding YH to animals with *naz*-RNAi knock down yields flight durations that are not significantly different from those of control 1 or of control 2. **(B)** Similarly, mean flight bout duration is significantly reduced in animals with knock down of Naz in Repo positive glial cells as compared to each control, and this phenotype is also rescued by feeding of YH. **(C)** The number of flight bouts is also significantly reduced in animals with knock down of Naz in Repo positive glial cells as compared to each control. Feeding of YH increase the mean of flight bouts slightly but not significantly. Kruskal Wallis ANOVA with Dunn's *post hoc* testing, ** $p < 0.01$, * $p < 0.05$, n.s. $p > 0.1$.

motor neuropil. If this was correct, pharmacological blockade of TA signaling should provide a rescue. Feeding of the competitive $\alpha 2$ adrenergic receptor antagonist Yohimbine (YH) restored total flight durations to levels that were not statistically significantly different from either of the two controls (Figure 4, orange bar, $p = 0.14$ for comparison to control 1, $p = 0.53$ for comparison to control 2), but differed significantly from the *naz*-RNAi knock down group ($p = 0.02$). YH has been demonstrated to selectively block TA receptors in *Drosophila* (Arakawa et al., 1990; Saudou et al., 1990), and we have previously used it to rescue flight durations in *tβh* mutant flies (Brembs et al., 2007). Similarly to total flight durations the mean duration of individual flight bouts (Figure 4B) and the number of flight bouts (Figure 4C) were significantly reduced by RNAi knock down of *naz* ($p < 0.01$ for both separate comparisons of the RNAi group with each of the two control groups). Mean flight bout durations could also be rescued by feeding YH (Figure 4B, $P > 0.1$ for both separate comparisons of the YH rescue group with each of the two control groups, $p = 0.018$ for the comparison of the *naz*-RNAi group with the YH fed rescue group). The number of flight bouts was slightly but not significantly increased by feeding YH to *naz* knock down animals ($p = 0.12$).

DISCUSSION

Naz Is Likely Involved in Reducing Biologically Active TA Levels in the Flight Motor Neuropil

Our data provide indirect evidence on the behavioral level that Naz normally functions to reduce biologically active TA levels in the flight motor neuropil, because knock down of Naz causes similar flight behavioral changes as observed with genetically increased TA levels (Brembs et al., 2007). Similarly to a reduction in flight durations in adult flies,

increased TA levels reduce *Drosophila* larval crawling distances (Saraswati et al., 2004; Fox et al., 2006), and this phenotype is also recapitulated by knock down of Naz (de Visser, 2016). The hypothesis that Naz functions in the TA degradation pathway is supported by the finding that motor behavioral phenotypes as induced by *naz* knock down can be rescued by feeding the TAR blocker YH. Therefore, our data provide first hints to the mechanisms that might remove TA from the synaptic cleft. The most parsimonious explanation is that TA is taken up into Repo positive glial cells which are located at the neuropil borders and extend extensive processes into the flight motor neuropil. As typical for monoamines, TA is then likely desaminated by a MAO, and thus converted to p-hydroxyphenyl-acetaldehyde. Naz might then convert p-hydroxyphenyl-acetaldehyde further into p-hydroxyphenyl acetic acid (de Visser, 2016). We speculate that *naz* knock down causes accumulation of p-hydroxyphenyl-acetaldehyde, which in turn slows desamination of TA, and thus, transport of TA into glial cells. The resulting consequence would be higher extracellular TA levels in the flight motor neuropil. This scenario is consistent with our findings that *naz* knock down causes similar phenotypes as feeding TA or genetic upregulation of TA signaling (Brembs et al., 2007), and that pharmacological blockade TARs rescues the behavioral phenotype. This would mean that Naz function is rate limiting for TA uptake into glial cells. It would be instructive to test whether overexpression of Naz causes opposite effects on flight performance as compared to *naz*-RNAi, but we have so far not succeeded to produce a UAS-*naz* fly strain. However, it is widely accepted that biologically active monoamine levels in the synaptic cleft can be increased by blocking intracellular degradation. In Parkinson's disease, for instance, pharmacological interventions with MAO function are utilized to enhance dopamine signaling (Unzeta and Sanz, 2011; Pathak et al., 2016). However, we have no direct evidence for the proposed function of Naz, and the

transporter for TA into glial cells has also not yet been identified.

TA Modulates Flight Motor Behavioral Likely by Actions in the CNS

It has long been known in multiple species that the biogenic amines OA and TA modulate insect motor behavior in an antagonistic manner (Roeder, 2005; Pflüger and Duch, 2011). In addition, numerous sites of OA and TA action have been identified, ranging from the modulation of central circuitry, neuromuscular transmission and muscle contraction properties, to sensory sensitivity, hormone release and muscle metabolism (Pflüger and Duch, 2011). However, at present the relative contributions of these different sites of action to the motor behavioral changes that are observed upon altered TA and/or OA signaling remain largely unknown. Our data indicate that central actions of TA might play a prominent role in the modulation of flight motor performance. We found prominent Naz localization in glial cells with arborizations in the central motor neuropils, but almost no Naz immunopositive cells in the periphery. Accordingly, knock down of Naz in glial cells restricts the manipulation mostly to the CNS. This has not been possible with genetic manipulations of the OA and TA synthesizing enzymes (*TDC2* and *Tβh*), the OA and TA receptors, or with drug feeding approaches. Given that we find significant reductions of flight durations and flight bout numbers, we suggest that the tyraminergeric modulation of flight motor behavior is mediated to a large extent by central TA actions. In a next step it will be important to identify the cellular targets of TA action in the CNS. Given the localization of Naz positive glial arbors in the flight motor neuropil we suggest that premotor flight interneurons, flight motoneurons, or synapses between these cells might be promising targets for TA action. Accordingly, we have previously reported spatial overlap between the central arborizations of TDC2 positive OA/TA containing neurons and flight motoneuron dendrites in the VNC flight motor neuropil (Boerner and Duch, 2010). Alternative TA might act on brain circuitry that regulates the motivation to fly, but this is currently unknown. However, in vertebrate

spinal cord, motoneurons are direct targets of monoaminergic modulation. There motoneuron excitability is strongly increased in the course of fight or flight reactions by the OA analog norepinephrine (Heckman et al., 2003). It will be interesting to test whether invertebrate motoneurons are also direct cellular targets of aminergic modulation and whether OA and TA exert opposite effects on motoneuron membrane excitability. Please note that our study refers to the modulation of flight motor behavior, but it is well known from larger insects that walking is also under octopaminergic/tyraminergeric control (Mentel et al., 2008; Rillich et al., 2013). Although flight and leg motoneuron dendrites cover spatially separate area of the thoracic motor neuropils in both larger insects (Ramirez and Pearson, 1988) and *Drosophila* (Baek and Mann, 2009; Brierley et al., 2012), Naz positive glial arbors and central arbors of OA/TA containing neurons are present in both neuropil areas. Therefore, from a sole anatomical point of view similar central actions of TA and OA are possible for the modulation of walking motor behavior.

Given that OA and TA modulate not only motor behavior, but also learning and memory (Burke et al., 2012; Waddell, 2013; Wu et al., 2013) as well as states of motivation, aggression and addiction (McClung and Hirsh, 1999; Scholz et al., 2005; Hoyer et al., 2008; Zhou et al., 2008), identification of the molecular mechanisms of degradation and the cellular sites of action are likely of broad interest.

AUTHOR CONTRIBUTIONS

SR and CD helped conceiving the study, conducted experiments, analyzed data and helped writing the manuscript. BA was the driving force to conceive the study, contributed reagents and helped writing the manuscript.

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REFERENCES

- Arakawa, S., Gocayne, J. D., McCombie, W. R., Urquhart, D. A., Hall, L. M., Fraser, C. M., et al. (1990). Cloning, localization and permanent expression of a *Drosophila* octopamine receptor. *Neuron* 4, 343–354. doi: 10.1016/0896-6273(90)90047-j
- Baek, M., and Mann, R. S. (2009). Lineage and birth data specific motor neuron targeting and dendritic architecture in adult *Drosophila*. *J. Neurosci.* 29, 6904–6916. doi: 10.1523/JNEUROSCI.1585-09.2009
- Boerner, J., and Duch, C. (2010). Average shape standard atlas for the adult *Drosophila* ventral nerve cord. *J. Comp. Neurol.* 518, 2437–2455. doi: 10.1002/cne.22346
- Brembs, B., Christiansen, F., Pflüger, H. J., and Duch, C. (2007). Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. *J. Neurosci.* 27, 11122–11131. doi: 10.1523/JNEUROSCI.2704-07.2007
- Brierley, D. J., Rathore, K., VijayRaghavan, K., and Williams, D. W. (2012). Developmental origins and architecture of *Drosophila* leg motoneurons. *J. Comp. Neurol.* 520, 1629–1649. doi: 10.1002/cne.23003
- Büsches, A., Kittmann, R., and Ramirez, J. M. (1993). Octopamine effects mimic state-dependent changes in a proprioceptive feedback system. *J. Neurobiol.* 24, 598–610. doi: 10.1002/neu.480240506
- Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614
- Cannon, W. B. (1932). *Wisdom of the Body*. New York, NY: W.W. Norton & Company.
- de Visser, A. (2016). *Eine zellspezifische Hypothese zum Tyramin-Abbau im Nervensystem von Drosophila melanogaster*. Mainz: Dissertation at the Faculty of Biology, Johannes Gutenberg-Universität Mainz.
- Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448, 151–156. doi: 10.1038/nature05954
- Downer, R. G. (1979). Trehalose production in isolated fat body of the American cockroach *Periplaneta americana*. *Comp. Biochem. Physiol.* 62C, 31–34.
- Fox, L. E., Soll, D. R., and Wu, C. F. (2006). Coordination and modulation of locomotion in *Drosophila* larvae: effects of altered biogenic amine

- levels by the tyramine β hydroxylase mutation. *J. Neurosci.* 26, 1486–1498. doi: 10.1523/JNEUROSCI.4749-05.2006
- Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A., et al. (1995). The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* 121, 317–332.
- Harris-Warrick, R. M., and Marder, E. (1991). Modulation of neural networks for behavior. *Annu. Rev. Neurosci.* 14, 39–57. doi: 10.1146/annurev.ne.14.030191.000351
- Heckman, C. J., Lee, R. H., and Brownstone, R. M. (2003). Hyperexcitable dendrites in motoneurons and their neuromodulatory control during motor behavior. *Trends Neurosci.* 26, 688–695. doi: 10.1016/j.tins.2003.10.002
- Hoyer, S. C., Eckart, A., Herrel, A., Zars, T., Fischer, S. A., Hardie, S. L., et al. (2008). Octopamine in male aggression of *Drosophila*. *Curr. Biol.* 18, 159–167. doi: 10.1016/j.cub.2007.12.052
- Hutchinson, K. M., Vonhoff, F., and Duch, C. (2014). Dscam1 is required for normal dendrite growth and branching but not for dendritic spacing in *Drosophila* motoneurons. *J. Neurosci.* 34, 1924–1931. doi: 10.1523/JNEUROSCI.3448-13.2014
- Kutsukake, M., Komatsu, A., Yamamoto, D., and Ishiwa-Chigusa, S. (2000). A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. *Gene* 245, 31–42. doi: 10.1016/S0378-1119(99)00569-7
- Marder, E., Bucher, D., Schulz, D., and Taylor, A. L. (2005). Invertebrate central pattern generation moves along. *Curr. Biol.* 15, R685–R699. doi: 10.1016/j.cub.2005.08.022
- Matheson, T. (1997). Octopamine modulates the responses and presynaptic inhibition of proprioceptive sensory neurons in the locust, *Schistocerca gregaria*. *J. Exp. Biol.* 200, 1317–1325.
- McClung, C., and Hirsh, J. (1999). The trace amine tyramine is essential for sensitization to cocaine in *Drosophila*. *Curr. Biol.* 9, 853–860. doi: 10.1016/S0960-9822(99)80389-3
- Mentel, T., Duch, C., Stypa, H., Wegener, G., Müller, U., and Pflüger, H. J. (2003). Central modulatory neurons control fuel selection in flight muscle of migratory locust. *J. Neurosci.* 23, 1109–1113.
- Mentel, T., Weiler, V., Büschges, A., and Pflüger, H. J. (2008). Activity of neuromodulatory neurones during stepping of a single insect leg. *J. Insect Physiol.* 54, 51–61. doi: 10.1016/j.jinsphys.2007.08.010
- Monastriotti, M., Linn, C. E. Jr., and White, K. (1996). Characterization of *Drosophila* tyramine β -hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* 16, 3900–3911.
- Orchard, I., Ramires, J.-M., and Lange, A. B. (1993). A multifunctional role for octopamine in locust flight. *Annu. Rev. Entomol.* 38, 227–249. doi: 10.1146/annurev.en.38.010193.001303
- Ormerod, K. G., Hadden, J. K., Deady, L. D., Mercier, A. J., and Krans, J. L. (2013). Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae. *J. Neurophysiol.* 110, 1984–1996. doi: 10.1152/jn.00431.2013
- Pathak, A., Srivastava, A. K., Singour, P. K., and Gouda, P. (2016). Synthetic and natural monoamine oxidase inhibitors as potential lead compounds for effective therapeutics. *Cent. Nerv. Syst. Agents Med. Chem.* 16, 81–97. doi: 10.2174/1871524915666150624120516
- Pflüger, H. J., and Duch, C. (2011). Dynamic neural control of insect muscle metabolism related to motor behavior. *Physiology* 26, 293–303. doi: 10.1152/physiol.00002.2011
- Ramirez, J.-M., Büschges, A., and Kittmann, R. (1993). Octopaminergic modulation of the femoral chordotonal organ in the stick insect. *J. Comp. Physiol.* 173, 209–219.
- Ramirez, J. M., and Pearson, K. G. (1988). Generation of motor patterns for walking and flight in motoneurons supplying bifunctional muscles in the locust. *J. Neurobiol.* 19, 257–282. doi: 10.1002/neu.480190307
- Rillich, J., Setvona, P. A., and Pflüger, H. J. (2013). Flight and walking in locusts: cholinergic co-activation, temporal coupling and its modulation by biogenic amines. *PLoS One* 8:e62899. doi: 10.1371/journal.pone.0062899
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Ryglewski, S., Lance, K., Levine, R. B., and Duch, C. (2012). Ca_v2 channels mediate low and high voltage-activated calcium currents in *Drosophila* motoneurons. *J. Physiol. Lond.* 590, 809–825. doi: 10.1113/jphysiol.2011.222836
- Saraswati, S., Fox, L. E., Soll, D. R., and Wu, C. F. (2004). Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. *J. Neurobiol.* 58, 425–441. doi: 10.1002/neu.10298
- Saudou, F., Amlaiki, N., Plassat, J. L., Borrelli, E., and Hen, R. (1990). Cloning and characterization of a *Drosophila* tyramine receptor. *EMBO J.* 9, 3611–3617.
- Scholz, H., Franz, M., and Heberlein, U. (2005). The hangover gene defines a stress pathway required for ethanol tolerance development. *Nature* 436, 845–847. doi: 10.1038/nature03864
- Sombati, S., and Hoyle, G. (1984). Generation of specific behaviors in a locust by local release into the neuropil of the natural neuromodulator octopamine. *J. Neurobiol.* 15, 481–506. doi: 10.1002/neu.480150607
- Stevenson, P. A., and Rillich, J. (2012). The decision to fight or flee—insights into underlying mechanism in crickets. *Front. Neurosci.* 6:118. doi: 10.3389/fnins.2012.00118
- Unzeta, M., and Sanz, E. (2011). Novel MAO-B inhibitors: potential therapeutic use of the selective MAO-B inhibitor PF9601N in Parkinson's disease. *Int. Rev. Neurobiol.* 100, 217–236. doi: 10.1016/B978-0-12-386467-3.00011-X
- von Hilchen, C. M., Bustos, A. E., Giangrande, A., Technau, G. M., and Altenhein, B. (2013). Predetermined embryonic glial cells form the distinct glial sheaths of the *Drosophila* peripheral nervous system. *Development* 140, 3657–3668. doi: 10.1242/dev.093245
- von Hilchen, C. M., Hein, I., Technau, G. M., and Altenhein, B. (2010). Netrins guide migration of distinct glial cells in the *Drosophila* embryo. *Development* 137, 1251–1262. doi: 10.1242/dev.042853
- Waddell, S. (2013). Reinforcement signaling in *Drosophila*; dopamine does it all after all. *Curr. Opin. Neurobiol.* 23, 324–329. doi: 10.1016/j.conb.2013.01.005
- Wu, J. S., and Luo, L. (2006). A protocol for dissecting *Drosophila melanogaster* brains for live imaging or immunostaining. *Nat. Protoc.* 1, 2110–2115. doi: 10.1038/nprot.2006.336
- Wu, C. L., Shih, M. F., Lee, P. T., and Chiang, A. S. (2013). An octopamine-mushroom body circuit modulates the formation of anesthesia-resistant memory in *Drosophila*. *Curr. Biol.* 23, 2346–2354. doi: 10.1016/j.cub.2013.09.056
- Zhou, C., Rao, Y., and Rao, Y. (2008). A subset of octopaminergic neurons are important for *Drosophila* aggression. *Nat. Neurosci.* 11, 1059–1067. doi: 10.1038/nn.2164

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Octopamine and Tyramine Contribute Separately to the Counter-Regulatory Response to Sugar Deficit in *Drosophila*

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All animals constantly negotiate external with internal demands before and during action selection. Energy homeostasis is a major internal factor biasing action selection. For instance, in addition to physiologically regulating carbohydrate mobilization, starvation-induced sugar shortage also biases action selection toward food-seeking and food consumption behaviors (the counter-regulatory response). Biogenic amines are often involved when such widespread behavioral biases need to be orchestrated. In mammals, norepinephrine (noradrenalin) is involved in the counterregulatory response to starvation-induced drops in glucose levels. The invertebrate homolog of noradrenalin, octopamine (OA) and its precursor tyramine (TA) are neuromodulators operating in many different neuronal and physiological processes. Tyrosine- β -hydroxylase (*t β h*) mutants are unable to convert TA into OA. We hypothesized that *t β h* mutant flies may be aberrant in some or all of the counter-regulatory responses to starvation and that techniques restoring gene function or amine signaling may elucidate potential mechanisms and sites of action. Corroborating our hypothesis, starved mutants show a reduced sugar response and their hemolymph sugar concentration is elevated compared to control flies. When starved, they survive longer. Temporally controlled rescue experiments revealed an action of the OA/TA-system during the sugar response, while spatially controlled rescue experiments suggest actions also outside of the nervous system. Additionally, the analysis of two OA- and four TA-receptor mutants suggests an involvement of both receptor types in the animals' physiological and neuronal response to starvation. These results complement the investigations in *Apis mellifera* described in our companion paper (Buckemüller et al., 2017).

Keywords: biogenic amines, starvation, starvation resistance, insects, proboscis extension response

INTRODUCTION

There may be more than just cultural value to the old German saying “grain tastes bitter for a satiated mouse” (La Sala et al., 2013). Indeed, it is the state of an organism which determines what, if any, effect external sensory stimuli will have on the nervous system. Whether this is the satiation state of the mouse influencing taste receptors, or the feeding state of the leech which gates

mechanosensory stimuli (Gaudry and Kristan, 2009, 2010), or the locomotor state of flies which adjusts the gain in visual interneurons (Longden and Krapp, 2009; Chiappe et al., 2010; Maimon et al., 2010; Suver et al., 2012; Tuthill et al., 2014; van Breugel et al., 2014), sensory stimuli are rarely, if ever, directly transformed into motor outputs. Instead, all nervous systems seem to constantly balance external and internal demands before they arrive at any given action (Heisenberg, 2009; Brembs, 2013, 2017; Pezzulo and Cisek, 2016). Biogenic amines and neuropeptides have been shown to be crucially involved in orchestrating the processes needed to find this balance.

Starvation and satiation are obvious and experimentally accessible states with immediate and easily recorded behavioral consequences. In both mammals and insects, peptides (glucagon and adipokinetic hormone, respectively) and catecholamines (adrenaline and octopamine, respectively) have been shown to mediate related roles in the counterregulatory response to starvation (Bolli and Fanelli, 1999; Kim and Rulifson, 2004; Grönke et al., 2007; Bharucha et al., 2008; Li et al., 2016; Yu et al., 2016). Apparently, either similar mechanisms evolved in response to similar challenges, or both systems evolved from a common ancestor. This response includes various physiological and metabolic modifications, which are orchestrated via the different neuropeptides and biogenic amines.

Feeding-related behaviors constitute the behavioral aspect of the counterregulatory response to starvation. In flies, general activity and arousal is enhanced (Connolly, 1966; Bell et al., 1985; Lee, 2004; Yang et al., 2015; Yu et al., 2016), arguably to facilitate food discovery. Along the same veins, food sensitivity is also increased (Moss and Dethier, 1983; Colomb et al., 2009), correlated with an increase in sugar receptor neuron sensitivity and gene expression (Amakawa, 2001; Meunier et al., 2007; Nishimura et al., 2012). Several involved neuropeptides have been identified (for a review see: Nässel and Winther, 2010). In addition to neuropeptides, also here the catecholamines are contributing to the processes triggered by starvation. Dopamine (DA) is involved in mediating motivation signals (Krashes et al., 2009) and modulating the starvation-induced sugar response after short starvation periods (Inagaki et al., 2012), while octopamine (OA) or its precursor tyramine (TA) have been reported to promote feeding behaviors (Long and Murdock, 1983; Nisimura, 2005). Starvation may be conceived as a stressor triggering catecholaminergic action. Indeed, different stressors have been shown in different insects to modify the OA/TA-system by enhancing Tβh expression (Châtel et al., 2012), subsequently increasing OA levels (Kononenko et al., 2009), which, in turn, releases triglycerides and carbohydrates into the hemolymph (Woodring et al., 1989).

The study of the role of biogenic amines in the counterregulatory response to starvation is complicated by the amines' broad involvement in many physiological processes. This promiscuity impedes the attribution of an aminergic manipulation to a specific phenotype. In invertebrates, OA and TA act as neurotransmitters, -hormones, and -modulators on many, if not all, physiological processes (reviews: Roeder, 2005; Farooqui, 2012). These processes include, but are not

limited to, locomotion regulation (Saraswati et al., 2004; Brembs et al., 2007), aggression (Baier et al., 2002; Hoyer et al., 2008; Zhou et al., 2008), reaction to light (Gorostiza et al., 2016), feeding behavior (Long and Murdock, 1983; Nisimura, 2005), mobilization of energy metabolites (Mentel et al., 2003) and, upstream of DA, appetitive olfactory learning (Hammer, 1993; Schwaerzel et al., 2003; Burke et al., 2012; Liu et al., 2012).

Thus, while feeding behaviors and their interactions with the state of the animal provide a technically accessible model to study decision-making and action selection, the interrelation between the consequences of starvation on motor control, motivation, stress, and the metabolic state of the animal pose a formidable experimental challenge, in particular in the interpretation of the different phenotypes linked with biogenic amine disruption. Leveraging the neurogenetic tools in *Drosophila*, we attempted to understand how starvation influences the animal's decision-making with regard to feeding-related stimuli. Specifically, we investigated the involvement of the OA/TA-system on starvation-dependent modulation of sugar responsiveness and metabolism. We asked whether the OA/TA-system was involved in the physiological response to starvation or the neuronal changes following starvation, and whether its neuronal action was peripheral or central. Our results corroborate and extend the previous findings on the promiscuous effects of these biogenic amines and suggest that both OA and TA are involved in most of the counterregulatory processes, which occur in parallel.

METHODS

External Depositories

A formatted table of most reagents used in this study, including fly stocks, is available at: <https://doi.org/10.6084/m9.figshare.5398600>. The data and code for this paper are available at <https://doi.org/10.6084/m9.figshare.4663666>. Protocol for carbohydrate measurement is available on protocols.io: <https://doi.org/10.17504/protocols.io.dkn4vd>.

Fly Stocks and Culture

tβh^{nM18} (Monastirioti et al., 1996; FBal0061578), *oamb* (Han et al., 1996; *oamb²⁸⁶* FBal0152344, *oamb⁵⁸⁴* FBal0152335), *honoka* (Kutsukake et al., 2000; *Oct-TyrR*, FBal0104701), *hsp-tβh* (Schwaerzel et al., 2003; FBal0152162), and *w+;;UAS-tβh* (Monastirioti, 2003; FBti0038601) and their control lines were obtained from Henrike Scholz, Cologne; Hiromu Tanimoto, Martinsried; Andreas Thum, Konstanz; and Amita Seghal, Chevy Chase. *TyrR^{f05682}* (CG7431f05682, FBal0184987), *TyrRII^{Δ29}* (CG16766, FBgn0038541) and *TyrRII-TyrR^{Δ124}* were kindly provided before publication by Edward Blumenthal, Milwaukee (Zhang and Blumenthal, 2017). Receptor mutants (and the respective control lines we obtained from the different labs) were outcrossed for at least six generations into a CS background. Flies were kept on standard cornmeal/molasses-food in a 12/12 h light/dark cycle (light on at 8:00 h) at 60% relative humidity and 25°C except for *hsp-tβh*, which were raised at 18°C without humidity control and except for flies used in electrophysiological experiments (see Electrophysiological recordings).

Starvation Procedure

Newly hatched to 1-day-old flies were collected and transferred to fresh food vials. The following day (between 16:00 and 19:00 h), 20 to 30 flies of mixed sexes were transferred into starvation vials (68 ml, Greiner bio-one, Frickenhausen, Germany) by a fly aspirator. The starvation vial contained a cotton pad moistened with 2.5 to 3 ml of Evian® water. If not otherwise indicated, starvation was performed at 25°C and 60% relative humidity and lasted for 20 h. Note that starvation time at 18°C was performed for much longer time.

Survival Experiments

Newly hatched to 1 day-old flies were collected and transferred to fresh food vials. The following day, flies were briefly CO₂-anesthetized and sorted by sex and genotype. At 17:00 h, around 35 female flies were transferred into a starvation vial (see Starvation procedure). Dead flies were counted every 3 h and not removed. Daily counting sessions were repeated from 9:00 to 18:00 h, until all flies were found dead.

Sugar Response Test

Newly hatched to 1 day-old flies were collected and transferred to fresh food vials. The following day, they were starved as described (see Starvation procedure). Four hours before the end of the starvation period, female flies (if not stated otherwise) were briefly immobilized by cold-anesthesia. Their head and thorax were glued to a triangle-shaped copper hook (0.05 mm in diameter) using a UV sensitive glue (3M ESPE, Sinfony Indirect Lab Composite, Minneapolis, USA). Animals were then kept individually in small chambers [14 mm in diameter × 28 mm in height, custom-made, (Brembs, 2008)] with *ad libitum* access to water until the test.

Tests were performed between 12:00 and 16:00 h. Using forceps, we transferred flies by their hook and fixed them to a magnetic clamp, which was then attached to a rack. This treatment established free movement of the flies' tarsi and proboscis and was a modification from a previously described PER assay (Scheiner et al., 2004, 2014) derived from assays used in other insects (Dethier, 1952; Page et al., 1998). A group of six to eight flies was tested in parallel. A filter paper soaked with sucrose solution was presented for 5 s to all six tarsi but not the proboscis. Seven different, increasing concentrations (0, 0.1, 0.3, 0.6, 1, 3, and 30%, i.e., g per 100 ml water) were presented in series with an inter-stimulus interval of 80 s. The proboscis extension response was recorded. Finally, the proboscis was stimulated by 30% sucrose solution. Flies not responding to the proboscis stimulation or responding to the first stimulation (water only) were discarded from the analysis.

For the first sugar response rescue attempt (Figure 4A), flies were raised and starved at 18°C and put into an incubator without humidity control and heated up to 37°C for 30 to 45 min. After the heat shock, flies were kept in a 25°C incubator with humidity control for 3 h until testing. For the second rescue attempt (Figure 4B), the first heat shock was given with the beginning of starvation every 23 h for 45 min until 1 day before testing. Temperature between heat shocks was 18°C.

Carbohydrate Measurement

Newly hatched to 1 day-old flies were collected and transferred to fresh food vials. The following day at 17:00 h, 20 flies of mixed sex were either transferred into starvation vials (see Starvation procedure) or kept in the food vials. After 20 h, approximately 40 female flies per group were cold-anesthetized, pierced through the thorax by the tip of a dissecting needle (0.5 mm in diameter), and collected on ice within a sieve composed of two tubes. The hemolymph was centrifuged out of the fly into the bottom tube at 4°C. 0.5 µl of the extracted hemolymph was transferred by a capillary (0.5 µl, Hirschmann Laborgeraete, Eberstadt, Germany) into 19.5 µl PBS (see <https://doi.org/10.17504/protocols.io.dkn4vd>).

Trehalose and glucose content in the hemolymph were measured according to the protocols provided by the manufacturer (Sigma Aldrich, Seelze, Germany). Ten microliter of the hemolymph-PBS mixture (or calibration solution) were added to 30 µl citric acid buffer (135 mM, pH 5.7 at 37°C) and 10 µl of a trehalase enzyme solution (Sigma Aldrich, 3% in citric acid buffer). After incubation overnight at 37°C, 50 µl of Tris buffer were added. 80 µl of the resulting solution were added to 156.8 µl Glucose oxidase and 3.2 µl o-Dianisidine (Glucose Assay Kit, Sigma Aldrich) and incubated for 30 min at 37°C. Finally, 160 µl of 33% sulfuric acid were added. Absorbance at 540 nm was measured for the resulting solution using a nanoDrop® (nanoDrop Technologies, Wilmington, USA) spectrophotometer. Five samples were measured per solution.

Electrophysiological Recordings

Flies were raised on cornmeal-yeast-glucose-agar medium under a 12/12 h light/dark cycle (lights on at 06:00 h) at 25°C. Newly hatched to 1 day old flies were collected and transferred into a vial containing Kimwipe paper soaked with 100 mM glucose for 1 to 2 days as previously described (Zhang et al., 2010). Starved flies were kept in a vial containing Kimwipe paper soaked with Evian® water for 20 h before testing.

Electrophysiological recordings from I-type labellar chemosensilla were done by the tip-recording method, as previously described (Hodgson et al., 1955; Hiroi et al., 2002). Briefly, the proboscis was fixed at the base of the labellum. A glass capillary filled with Drosophila Ringer solution served as an indifferent electrode. The 100 mM sucrose solution for stimulation contained 1 mM KCl as electrolyte. The recorded signals were digitized and analyzed using the custom software dbWave (Marion-Poll, 1995, 1996). Action potentials were detected by a visually-adjusted threshold set across the digitally filtered signal. The total number of spikes within 1 s was counted. Note that in the tip-recording assay, recording and stimulation of the sensory neurons starts concomitantly.

Statistics

Figures and statistical analyses were performed in R using different packages (Venables and Ripley, 2002; R Core Team, 2015; Therneau, 2015; Wickham, 2016; Wilke, 2016); data and code are available <https://doi.org/10.6084/m9.figshare.4663666>. If not stated otherwise, data are illustrated as boxplots representing the median (line), the 25 and 75% quartiles (boxes),

the data within 1.5 times the interquartile range (whiskers), and data outside that range (outliers, depicted as points). Colors were chosen to be color-blind friendly, according to <http://jfly.iam.u-tokyo.ac.jp/color/>.

The sugar response score was calculated as the sum of all positive responses over the seven sucrose presentations and therefore ranges from 0 to 7 (Total number of PER). For survival measurement, we used Kaplan-Meier curves and Cox proportional hazards regression model. For hemolymph carbohydrate content, we used a paired Wilcoxon rank sum test on the index of change in sugar content with starvation: $(SG_{starved} - SG_{fed}) / (SG_{starved} + SG_{fed})$. Since one calibration experiment (showing the absorbance of a standard glucose/trehalose solution that was treated identically to hemolymph) was performed each day, a paired test is sound. In the 2 days with two measures per group, values were paired following time of measurement.

The significance level of all statistical tests was set to 0.05, and Bonferroni correction was applied where appropriate.

RESULTS

tβh^{nM18} Mutants in Starvation Induced Phenotypes

We developed a new sugar response assay independent of the flies' locomotion, and which restrains their movements to less than in a pipet tip (Scheiner et al., 2004). Flies were tethered to a hook glued between head and thorax and tested for their proboscis extension response to a serial dilution of sucrose after 20 h of starvation. The assay is quite sensitive, since we were able to record a difference in the response of flies starved for 14 vs. 21 h (Damrau et al., 2014), as in the T-maze assay (Colomb et al., 2009). Fed flies do not respond to tarsal stimulation, in contrast to honeybees.

We tested females *tβh^{nM18}* mutant flies lacking OA and accumulating TA (Monastirioti et al., 1996) in our assay. *tβh^{nM18}* mutant flies responded almost 40% less than their control (called w+ because the mutant and the control lines have a wild-type white gene, in contrast to the original mutant obtained after P element excision, **Figure 1A**). The sum of all positive responses over the 7 sucrose presentations was significantly different (**Figure 1B**).

We then compared the change in carbohydrate contents (trehalose plus glucose) in the hemolymph of starved and fed flies. To this end, the hemolymph was extracted and all glucose and trehalose was enzymatically converted into spectrometrically measurable glucose. Carbohydrate content in fed animals appeared very similar (**Figure 2A**, no statistics performed). Because the variability in the score is partly due to the inevitable differences in the manipulations from 1 day to another, we evaluated the change in carbohydrate level after starvation in a paired fashion. It was significantly smaller in *tβh^{nM18}* mutants compared to wild-type controls (**Figure 2B**).

Finally, we recorded survival rate under starvation conditions with *ad libitum* access to water. As expected from the smaller decrease in their sugar content, *tβh^{nM18}* mutants survived longer

than wild-type controls (**Figure 3**). Our experiments show that *tβh^{nM18}* mutants are less affected by starvation than wild-type animals, suggesting a role for OA and/or TA in starvation resistance and sugar response.

OA/TA Role in Sugar Response

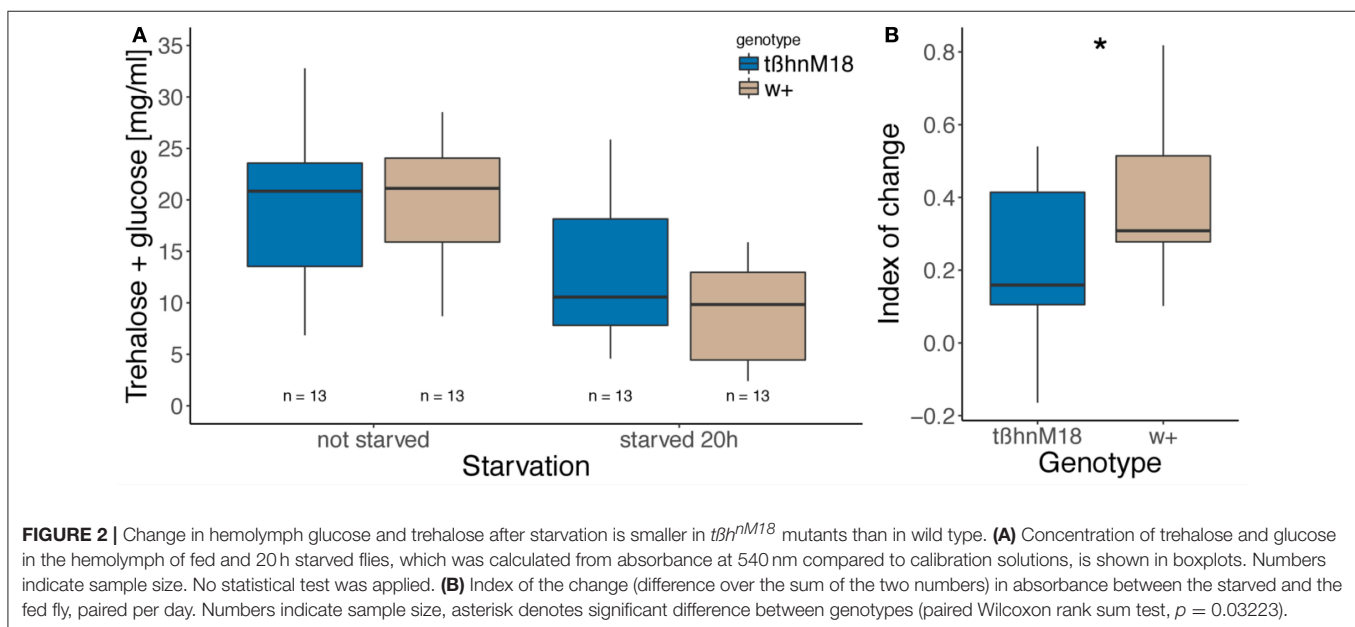
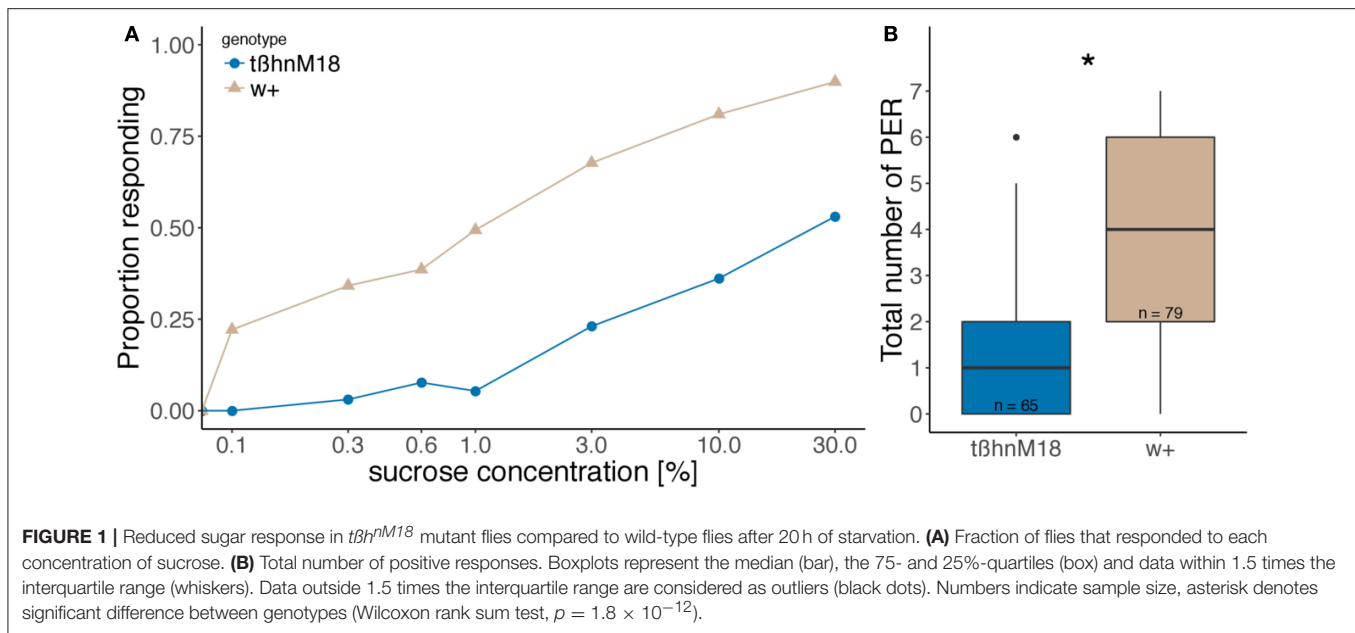
In order to elucidate the temporal requirement of *tβh* activity during starvation or during proboscis extension, we induced ubiquitous, but temporally controlled, *tβh* expression in the mutant background using the heat-inducible *hsp-tβh* construct. To prevent *tβh* expression, flies were kept at 18°C, and the starvation time was increased to until the wild-type flies responded to sugar stimulation in a similar way as after 25°C starvation (see materials and Methods and **Figure 1**). Driving expression 3 h before the test partially rescued the mutant phenotype (**Figure 4A**). In contrast, heat shocks throughout the starvation period did not rescue the sugar response phenotype (**Figure 4B**), suggesting an acute role of OA during the sugar response test, independent of any OA/TA role in starvation resistance.

Since OA is known to modulate different kinds of sensory receptors in insects (Kass et al., 1988; Ramirez and Orchard, 1990; Pophof, 2000), we tested a potential role of OA on gustatory receptor sensitivity. We recorded the response of labellar sensilla to 100 mM sucrose in fed and starved flies by the tip-recording method (Hodgson et al., 1955; Hiroi et al., 2002). The wild-type strain serving as a control for our mutant does not show the increase of spiking rate after starvation (**Figure 5A**), which we see in other wild-type strains (**Figure 5B**) as previously reported (Meunier et al., 2007; Inagaki et al., 2012; Nishimura et al., 2012); and we found a decreased sensillar response to sucrose stimulation after starvation in *tβh^{nM18}* mutants, compared to starved wild-type controls and fed mutants (**Figure 5A**).

OA and TA can act both inside and outside of the nervous system, functioning as either a neurotransmitter or a neurohormone in insects (Cole et al., 2005). Thus, we explored whether the sugar response phenotype of *tβh* mutants was a result of alterations in neurons inside or outside of the brain or in non-neuronal cells. To this end, we expressed Tβh in *tβh^{nM18}* mutant males using different GAL4-lines. We found a significant increase in sugar response compared to the respective mutant control when we used the ubiquitous Actin-promoter to drive Gal4 in all cells, the pan-neuronal nSyb-promoter, or the non-neuronal Tdc1-GAL4 driver (**Figure 6**). In contrast, Tβh expression in subsets of OA/TA-neurons, using either Tdc2- or NP7088-GAL4 did not significantly affect the mutants' response (**Figure 6**), in contrast to a previous report [NP7088-Gal4, (Scheiner et al., 2014)]. These last two results also show that the UAS construct alone is not sufficient to bring a rescue. These results indicate that Tβh expression induced in neurons in the central nervous system or in non-neuronal cells, respectively, is sufficient to enhance the sugar responsiveness of *tβh^{nM18}* mutant flies.

OA/TA-Receptor Manipulations on Survival and Sugar Responsiveness

Because the *tβh* mutation leads to increased TA and decreased OA levels (Monastirioti et al., 1996), we performed additional



experiments to disentangle the relative importance of each amine in the regulation of survival and sugar response. We tested mutants for several OA- and TA-receptors in our PER and survival under starvation condition assays (Figure 7, Table 1).

The two TA-receptor mutants *TyrR*^{f05682} and *honoka* showed a decreased sugar response and an increased survival comparable to *tβhnM18* mutants. In contrast, the double mutant *TyrII-TyrR*^{Δ124} showed an increase in survival but a normal sugar response, while *TyrR*^{Δ29} shows normal survival but a decrease in sugar response. Finally, *oamb*²⁸⁶ mutants lived longer than their control, in contrast to a previously published report (Schwaerzel et al., 2003; Erion et al., 2012), while the *oamb*⁵⁸⁴ allele showed no phenotype. The receptor mutant data suggest that flies can exhibit a wild-type survival simultaneously with

a lower sugar response (*TyrR*^{Δ29}), or a higher survival simultaneously with a wild-type sugar response (*oamb*²⁸⁶, double mutant *TyrII-TyrR*^{Δ124}), suggesting that starvation affects sugar responsiveness and survival via different but amine-dependent pathways.

DISCUSSION

We have used genetic alterations of OA and TA action to elucidate the role of these amines in survival and sugar responsiveness of fruit flies. Our data suggest complex, central and peripheral actions of these amines on physiology and behavior.

We have shown that the *tβh* gene is involved in starvation-induced survival and an increase in sugar response. The phenotype was reported in females (**Figure 1**) and males

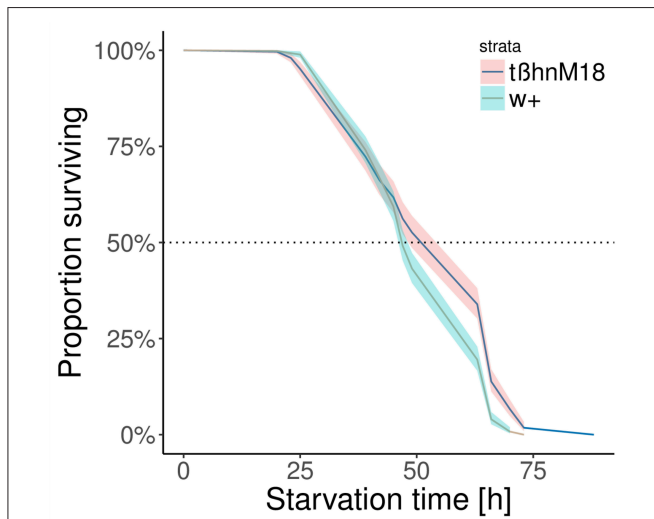


FIGURE 3 | Longer survival of *tβh^{nM18}* mutants under starvation conditions. **(A)** Kaplan Meier survival curve for the two genotypes. We ran 16 experiments with about 35 flies per vial. The difference between the curves was statistically significant, while there was also an effect of the different trials (Cox proportional hazards regression model, $p = 0.029$ for trials, $p = 2 \times 10^{-9}$ for genotypes).

(**Figure 6**), in three different genetic backgrounds (*w+* and *w-;tβh^{nM18}*; *hs-tβh* and *w-;tβh^{nM18}*, *UAS-tβh*) and is independent of the egg-retention phenotype (Partridge et al., 1987), which is rescued in *w-;tβh^{nM18};UAS-tβh* control mutant flies (**Figure 6**). It is interesting to see that the sugar response phenotype appears to vanish with longer starvation periods (Yang et al., 2015). The phenotype was not found in previous reports focused on the learning phenotype of these flies (Schwaerzel et al., 2003), possibly because the assay used was dependent on locomotion, which is also affected in *tβh^{nM18}* mutants (Saraswati et al., 2004; Fox, 2006; Koon et al., 2010). Complementary results were obtained using a different approach in *Drosophila* (Scheiner et al., 2014) and also in *Apis mellifera* (companion paper).

OA/TA and Starvation Resistance

Since sugar response is dependent on starvation (Colomb et al., 2009), a decreased sugar response as found in *tβh^{nM18}* mutants can be understood as resistance to the starvation treatment, an hypothesis that our results appeared to confirm. Indeed, we found that the levels of carbohydrates in the hemolymph of *tβh^{nM18}* mutant flies are higher after starvation than in control flies (**Figure 2**). Since trehalose constitutes the energy store of a fly and its hemolymph concentration reflects starvation level (Thompson, 2003; Isabel, 2004), it is reasonable to argue that the mutant flies were affected less by the starvation treatment than the controls, even though they were deprived of food for the same amount of time. This interpretation is also supported by

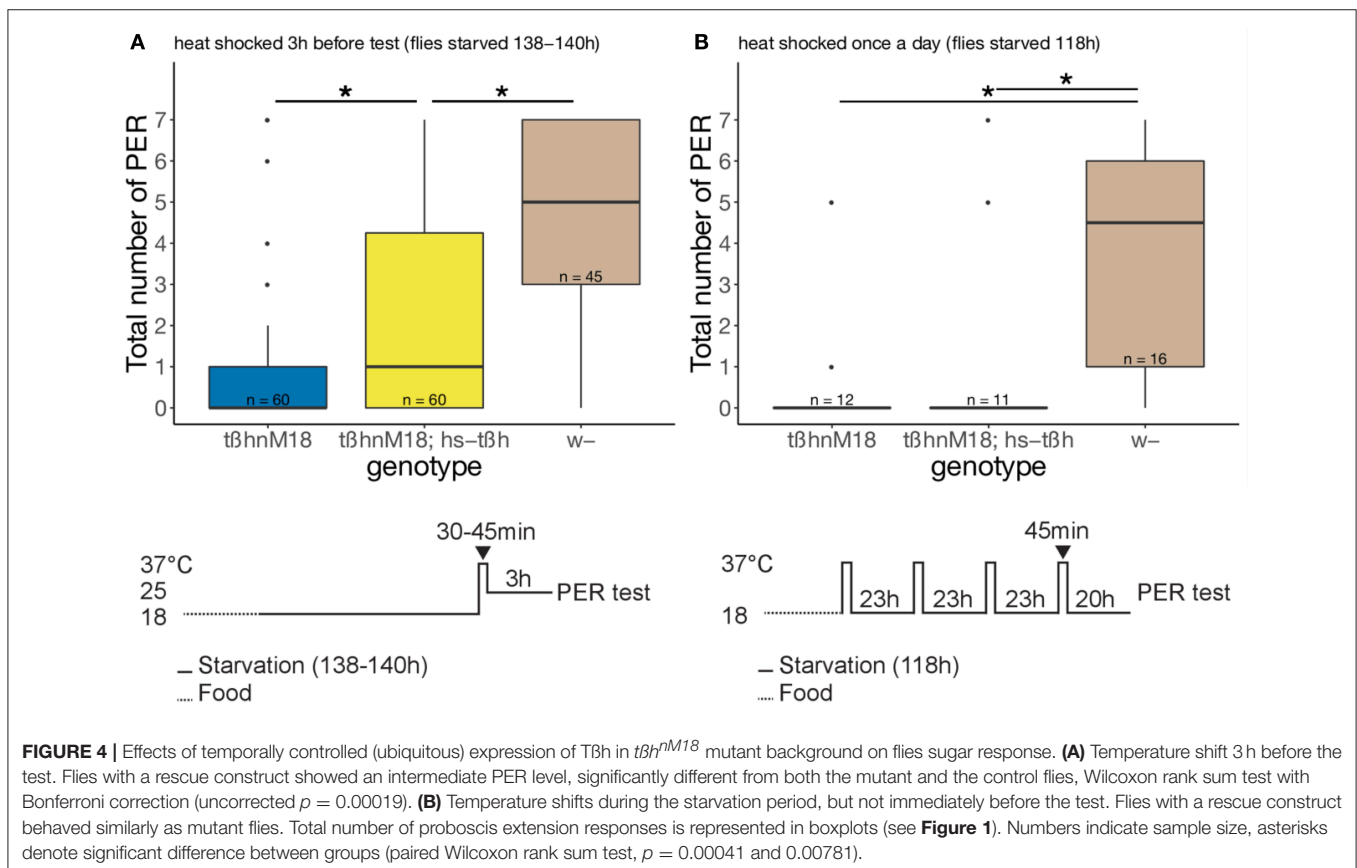
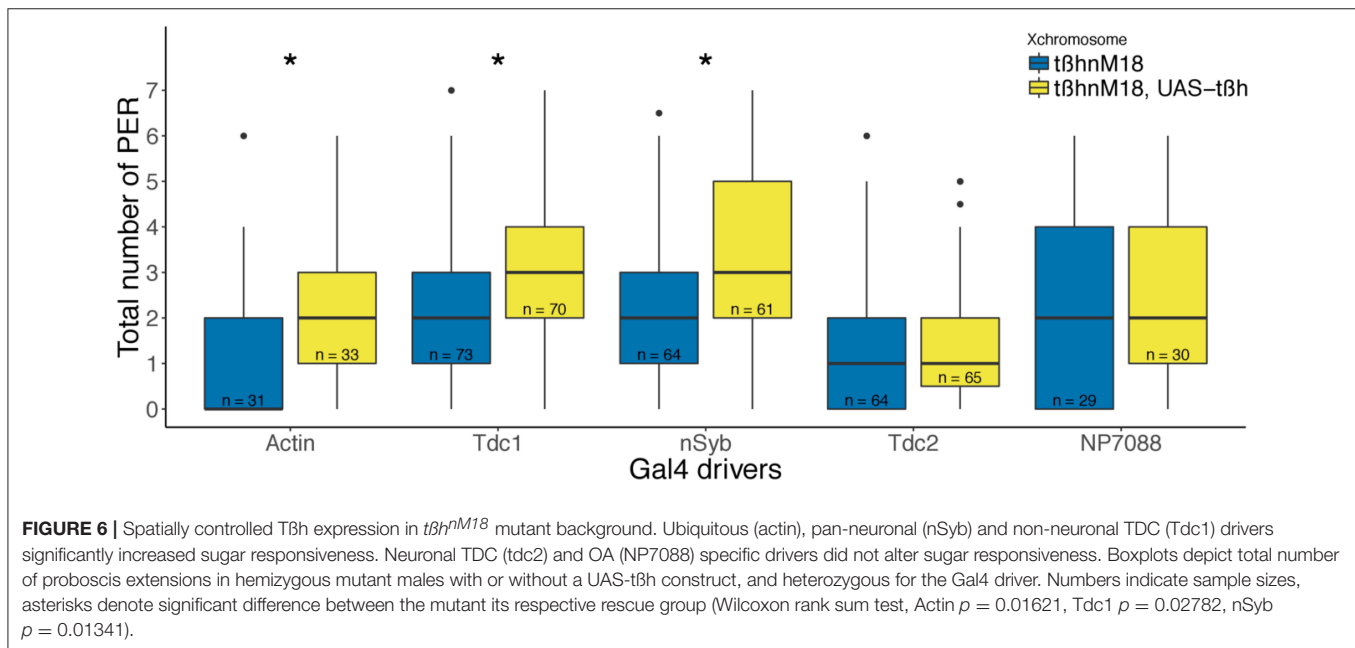
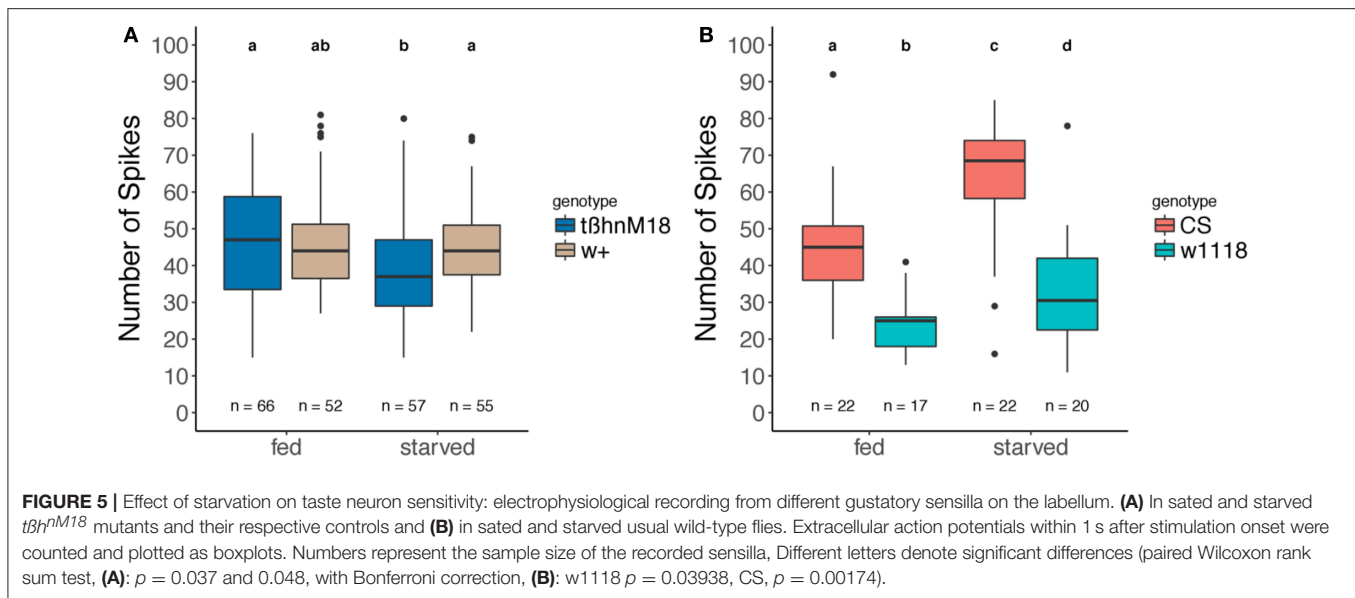


FIGURE 4 | Effects of temporally controlled (ubiquitous) expression of TBh in *tβh^{nM18}* mutant background on flies sugar response. **(A)** Temperature shift 3h before the test. Flies with a rescue construct showed an intermediate PER level, significantly different from both the mutant and the control flies, Wilcoxon rank sum test with Bonferroni correction (uncorrected $p = 0.00019$). **(B)** Temperature shifts during the starvation period, but not immediately before the test. Flies with a rescue construct behaved similarly as mutant flies. Total number of proboscis extension responses is represented in boxplots (see **Figure 1**). Numbers indicate sample size, asterisks denote significant difference between groups (paired Wilcoxon rank sum test, $p = 0.00041$ and 0.00781).



longer survival of *tβh^{M18}* mutants under starvation conditions [Figure 3, a result which was independently replicated (Scheiner et al., 2014; Li et al., 2016): our experiments were carried out before the ones cited]. Complementing our analysis in flies, injection of the OA-receptor antagonist epinastine in honey bees also prolonged survival (companion paper). Taken together, these results suggest that the absence of OA-signaling saves the mutant animal's energy, making the animals less sensitive to starvation, a conclusion in line with previous reports on the role of OA in triglyceride (Woodring et al., 1989; Erion et al., 2012) and carbohydrate (Blau et al., 1994; Park and Keeley, 1998) metabolism. One potential explanation for the reduced energy use may be a reduced locomotor activity in the mutant flies. We

have tested flies in Buridan's paradigm (Colomb et al., 2012) and found several alterations to the locomotor pattern of *tβh^{M18}* mutant flies (Damrau et al. in preparation).

OA/TA and Sugar Responsiveness

While *tβh* is affecting starvation resistance, we asked whether the gene could also have a role in the neuronal modifications caused by starvation signals. Our results separate the starvation resistance from the sugar responsiveness phenotype. The sugar responsiveness phenotype is partially rescued by acute *tβh* expression, while expression during the starvation period had no effect (Figure 4). This suggests that the decrease of carbohydrate levels is not the only *tβh*-dependent starvation-induced alteration

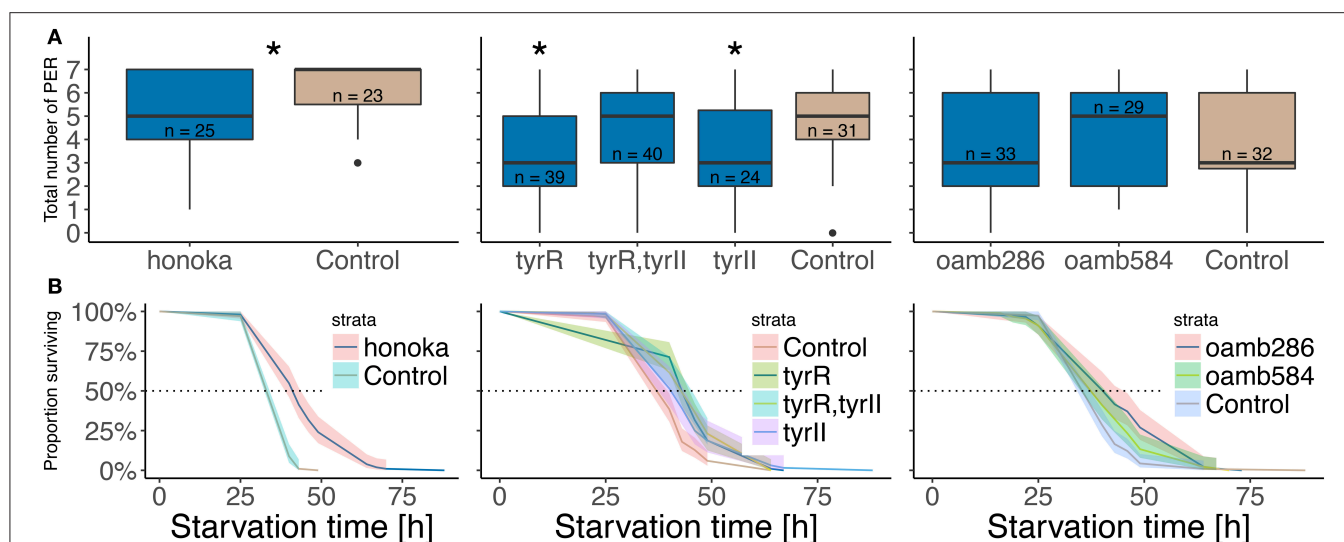


FIGURE 7 | Starvation resistance and sugar responsiveness in TA- and OA-receptor mutants. Total number of proboscis extensions (**A**) and Kaplan Meier survival curve (**B**, see **Figure 3**) in different female mutants and their respective control strains. See text for fly strain labels. The three different groups were tested independently and are therefore statistically treated as different experiments. (**A**) Numbers indicate sample sizes, asterisks denote significant difference between the mutant and its respective control group (Wilcoxon rank sum test, *honoka* $p = 0.0117$, *Tyr* $p = 0.002911$ and 0.007432). (**B**) 5 to 8 experiments were run per genotype, with about 35 flies per vial. Cox proportional hazards regression model was used to test statistical differences between mutants and their control, (different: *honoka* $p = 1.4 \times 10^{-14}$, *TyrR,TyrII* $p = 10^{-5}$, *TyrII* $p = 2.1 \times 10^{-6}$, *oamb²⁸⁶* $p = 0.00029$, not different: *Tyr* $p = 0.067$, *oamb⁵⁸⁴* $p = 0.069$, before bonferroni correction).

TABLE 1 | Summary of TA- and OA-receptor mutant phenotypes.

		Survival	Sugar response
TA-receptors	<i>TyrR⁰⁵⁶⁸²</i>	↑	↓
	<i>TyrR^{II}Δ29</i>	—	↓
	<i>TyrR^{II}-TyrR^{Δ124}</i>	↑	—
	<i>honoka</i>	↑	↓
OA-receptors	<i>oamb²⁸⁶</i>	↑	—
	<i>oamb⁵⁸⁴</i>	—	—

Horizontal lines indicate no effect. Arrows indicate significant difference to respective control and illustrate the trend of the data.

that leads to a normal sugar response. Indeed, the sensitivity of the sugar-sensing neurons is affected by TA/OA imbalance (**Figure 5**), but only after starvation. Interestingly, the control w+ strain did not show the expected (Meunier et al., 2007; Nishimura et al., 2012) increase in sensitivity after starvation (**Figure 5A**), while more common wild-type strains showed the increase in the same experiment (**Figure 5B**). Since the w+ control flies did show an increase in their proboscis extension response to sugar (**Figure 1**), there must be a modulatory mechanism downstream of taste receptor activity. Taken together, these data suggest that in addition to the internal state that is altered by starvation, both sensory transduction and the likelihood to extend the proboscis to the same sensory information are modified by starvation.

Where Is the Site of OA/TA-Action?

In order to identify the cells contributing to starvation resistance and sugar responsiveness, we expressed Tβh in different cells

inside or outside the nervous system in the mutant flies, using the UAS/Gal4 system (**Figure 6**). The expected effect of this manipulation is a production of OA and a decrease in the concentration of TA in the affected cells. Ubiquitous expression of Tβh with the actin-Gal4 driver does increase the PER of starved flies. The non-neuronal Tdc1-GAL4-driver drives expression in crop and hind gut tyramineric cells (Cole et al., 2005; Chintapalli et al., 2007; Blumenthal, 2009), that normally do not produce OA, but only TA (Monastirioti et al., 1995). Ectopic production of OA in these cells rescues the sugar responsiveness phenotypes (**Figure 6**). Because ectopic OA would lack necessary receptors, we tentatively interpret this result as an effect of presumably reduced TA levels. However, the OA produced might also be released into the hemolymph and taken up by neurons, as is proposed to happen when feeding OA (Schwaerzel et al., 2003; Scheiner et al., 2014). Interestingly, pan-neuronal Tβh expression with nsyb-Gal4, but not expression with drivers specifically labeling OA/TA neurons (*tdc2-Gal4* and *NP7088-Gal4*), rescues the phenotype. These results suggest that both neuronal and non-neuronal tissues are affecting the starvation-induced increase in sugar responsiveness (and that the two most commonly used OA/TA drivers remain suboptimal tools to study OA action).

OA and TA Specificity

The Tβh enzyme converts TA into OA such that *tβh^{M18}* mutants not only lack OA but also accumulate TA. To disentangle the roles of the two amines, we tested OA- or TA-receptor mutants in two experiments: starvation resistance and sugar responsiveness (**Figure 7**). Perhaps not surprisingly, given that several processes appear to mediate both starvation-induced

effects, we found the sugar responsiveness and the starvation resistance phenotypes of the tested mutants to be separable: some mutants exhibit a phenotype in none (*oamb*⁵⁸⁴), both (*tβh*^{nM18}, *honoka*), or in individual assays: only in starvation resistance (*oamb*²⁸⁶, *TyrII-TyrR*^{Δ124}) or only in sugar responsiveness (*TyrR*^{Δ29}). These results reinforce our previous conclusion that starvation resistance and sugar responsiveness are not mediated by the same OA/TA-cells and receptors, but by different subpopulations. In addition, the data indicate that both OA and TA play a role in starvation-induced sugar responsiveness. OA- and TA-receptor mutants tend to perform similarly, suggesting they may not be counteracting each other in this behavior, as previously suggested for crawling behavior (Saraswati et al., 2004) or for flight (Brembs et al., 2007).

CONCLUSIONS

Taken together with the experiments from our accompanying paper (Buckemüller et al., 2017), our results suggest that the OA/TA-system is involved in both the physiological and the behavioral changes that follow starvation, and that these changes are regulated independently. They also show that the behavioral change is due not only to a modulation of the taste neuron activity and to action of TA-specific cells

in peripheral, non-neuronal organs, but that a more central effect is probably at play. Finally, these data as well as others (in prep.) suggest that some aminergic pathways operate in a dose-dependent manner and are therefore difficult to dissect using standard transgenic or pharmacological rescue approaches.

AUTHOR CONTRIBUTIONS

CD and JC: Design of experiments, collection and analysis of data, writing and editing of the manuscript. NT and TT: Performed electrophysiology experiments, analyzed data, edited manuscript. BB: Design of experiments, analysis of data, writing and editing of the manuscript.

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REFERENCES

- Amakawa, T. (2001). Effects of age and blood sugar levels on the proboscis extension of the blow fly *Phormia regina*. *J. Insect Physiol.* 47, 195–203. doi: 10.1016/S0022-1910(00)00105-0
- Baier, A., Wittek, B., and Brembs, B. (2002). *Drosophila* as a new model organism for the neurobiology of aggression? *J. Exp. Biol.* 205, 1233–1240.
- Bell, W. J., Cathy, T., Roggero, R. J., Kipp, L. R., and Tobin, T. R. (1985). Sucrose-stimulated searching behaviour of *Drosophila melanogaster* in a uniform habitat: modulation by period of deprivation. *Anim. Behav.* 33, 436–448. doi: 10.1016/S0003-3472(85)80068-3
- Bharucha, K. N., Tarr, P., and Zipursky, S. L. (2008). A glucagon-like endocrine pathway in *Drosophila* modulates both lipid and carbohydrate homeostasis. *J. Exp. Biol.* 211, 3103–3110. doi: 10.1242/jeb.016451
- Blau, C., Wegener, G., and Candy, D. J. (1994). The effect of octopamine on the glycolytic activator fructose 2,6-bisphosphate in perfused locust flight muscle. *Insect Biochem. Mol. Biol.* 24, 677–683. doi: 10.1016/0965-1748(94)90055-8
- Blumenthal, E. M. (2009). Isoform- and cell-specific function of tyrosine decarboxylase in the *Drosophila* malpighian tubule. *J. Exp. Biol.* 212, 3802–3809. doi: 10.1242/jeb.035782
- Bolli, G. B., and Fanelli, C. G. (1999). Physiology of glucose counterregulation to hypoglycemia. *Endocrinol. Metab. Clin. North Am.* 28, 467–493. doi: 10.1016/S0889-8529(05)70083-9
- Brembs, B. (2008). Operant learning of *Drosophila* at the torque meter. *J. Vis. Exp.* 16:731. doi: 10.3791/731
- Brembs, B. (2013). Invertebrate behavior actions or responses? *Front. Neurosci.* 7:221. doi: 10.3389/fnins.2013.00221
- Brembs, B. (2017). “Operant behavior in model systems,” in *Reference Module in Neuroscience and Biobehavioral Psychology* (Elsevier). doi: 10.1016/B978-0-12-809324-5.21032-8
- Brembs, B., Christiansen, F., Pflüger, H. J., and Duch, C. (2007). Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. *J. Neurosci.* 27, 11122–11131. doi: 10.1523/JNEUROSCI.2704-07.2007
- Buckemüller, C., Siehler, O., Göbel, J., Zeumer, R., Ölschläger, A., and Eisenhardt, D. (2017). Octopamine underlies the counter-regulatory response to a glucose deficit in honeybees (*Apis mellifera*). *Front. Syst. Neurosci.* 11:63. doi: 10.3389/fnsys.2017.00063
- Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614
- Châtel, A., Murillo, L., Bourdin, C. M., Quinchard, S., Picard, D., and Legros, C. (2012). Characterization of tyramine β-hydroxylase an enzyme upregulated by stress in periplaneta americana. *J. Mol. Endocrinol.* 50, 91–102. doi: 10.1530/JME-12-0202
- Chiappe, M. E., Seelig, J. D., Reiser, M. B., and Jayaraman, V. (2010). Walking modulates speed sensitivity in *Drosophila* motion vision. *Curr. Biol.* 20, 470–475. doi: 10.1016/j.cub.2010.06.072
- Chintapalli, V. R., Wang, J., and Dow, J. A. (2007). Using flyatlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* 39, 715–720. doi: 10.1038/ng2049
- Cole, S. H., Carney, G. E., McClung, C. A., Willard, S. S., Taylor, B. J., and Hirsh, J. (2005). Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. *J. Biol. Chem.* 280, 14948–14955.
- Colomb, J., Kaiser, L., Chabaud, M.-A., and Preat, T. (2009). Parametric and genetic analysis of *Drosophila* appetitive long-term memory and sugar motivation. *Genes Brain Behav.* 8, 407–415. doi: 10.1111/j.1601-183X.2009.00482.x
- Colomb, J., Reiter, L., Blaszkiewicz, J., Wessnitzer, J., and Brembs, B. (2012). Open source tracking and analysis of adult *Drosophila* locomotion in buridans paradigm with and without visual targets. *PLoS ONE* 7:e42247. doi: 10.1371/journal.pone.0042247
- Connolly, K. J. (1966). Locomotor activity in *Drosophila* as a function of food deprivation. *Nature* 209, 224–224. doi: 10.1038/209224a0
- Damrau, C., Brembs, B., and Colomb, J. (2014). *Drosophila* Sugar Response Is Increased by Starvation Duration and Sucrose Concentration in a Locomotion-Independent Proboscis Extension Test. (Figshare).
- Dethier, V. G. (1952). Adaptation to chemical stimulation of the tarsal receptors of the blowfly. *Biol. Bull.* 103, 178–189.
- Erion, R., DiAngelo, J. R., Crocker, A., and Seghal, A. (2012). Interaction between sleep and metabolism in *Drosophila* with altered octopamine signaling. *J. Biol. Chem.* 287, 32406–32414. doi: 10.1074/jbc.M112.360875
- Farooqui, T. (2012). Review of octopamine in insect nervous systems. *Open Access Insect Physiol.* 4, 1–17. doi: 10.2147/OAIP.S20911

- Fox, L. E. (2006). Coordination and modulation of locomotion pattern generators in *Drosophila larvae*: effects of altered biogenic amine levels by the tyramine beta hydroxylase mutation. *J. Neurosci.* 26, 1486–1498. doi: 10.1523/JNEUROSCI.4749-05.2006
- Gaudry, Q., and Kristan, W. B. (2009). Behavioral choice by presynaptic inhibition of tactile sensory terminals. *Nat. Neurosci.* 12, 1450–1457. doi: 10.1038/nn.2400
- Gaudry, Q., and Kristan, W. B. (2010). Feeding-mediated distention inhibits swimming in the medicinal leech. *J. Neurosci.* 30, 9753–9761. doi: 10.1523/JNEUROSCI.1487-10.2010
- Gorostiza, E. A., Colomb, J., and Brembs, B. (2016). A decision underlies phototaxis in an insect. *Open Biol.* 6:160229. doi: 10.1098/rsob.160229
- Grönke, S., Müller, G., Hirsch, J., Fellert, S., Andreou, A., Haase, T., et al. (2007). Dual lipolytic control of body fat storage and mobilization in *Drosophila*. *PLoS Biol.* 5:e137. doi: 10.1371/journal.pbio.0050137
- Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63.
- Han, K. A., Millar, N. S., Grotewiel, M. S., and Davis, R. L. (1996). DAMB a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16, 1127–1135. doi: 10.1016/S0896-6273(00)80139-7
- Heisenberg, M. (2009). Is free will an illusion? *Nature* 459, 164–165. doi: 10.1038/459164a
- Hiroi, M., Marion-Poll, F., and Tanimura, T. (2002). Differentiated response to sugars among *Labellar chemosensilla* in *Drosophila*. *Zoo. Sci.* 19, 1009–1018. doi: 10.2108/zsj.19.1009
- Hodgson, E. S., Lettvin, J. Y., and Roeder, K. D. (1955). Physiology of a primary chemoreceptor unit. *Science* 122, 417–418. doi: 10.1126/science.122.3166.417-a
- Hoyer, S. C., Eckart, A., Herrel, A., Zars, T., Fischer, S. A., Hardie, S. L., et al. (2008). Octopamine in male aggression of *Drosophila*. *Curr. Biol.* 18, 159–167. doi: 10.1016/j.cub.2007.12.052
- Inagaki, H. K., Ben-Tabou de-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., and Barnea, G. (2012). Visualizing neuromodulation *in vivo*: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595. doi: 10.1016/j.cell.2011.12.022
- Isabel, G. (2004). AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in *Drosophila*. *Regul. Integr. Comp. Physiol.* 288, R531–R538. doi: 10.1152/ajpregu.00158.2004
- Kass, L., Pelletier, J. L., Renninger, G. H., and Barlow, R. B. Jr. (1988). Efferent neurotransmission of circadian rhythms in the lateral eye. *J. Compar. Physiol. A* 164, 95–105. doi: 10.1007/BF00612723
- Kim, S. K., and Rulifson, E. J. (2004). Conserved mechanisms of glucose sensing and regulation by *Drosophila corpora cardiaca* cells. *Nature* 431, 316–320. doi: 10.1038/nature02897
- Kononenko, N. L., Wolfenberger, H., and Pflüger, H. J. (2009). Tyramine as an independent transmitter and a precursor of octopamine in the locust central nervous system: an immunocytochemical study. *J. Comp. Neurol.* 512, 433–452. doi: 10.1002/cne.21911
- Koon, A. C., Ashley, J., Barria, R., DasGupta, S., Brain, R., Waddell, S., et al. (2010). Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. *Nat. Neurosci.* 14, 190–199. doi: 10.1038/nn.2716
- Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. *Cell* 139, 416–427. doi: 10.1016/j.cell.2009.08.035
- Kutsukake, M., Komatsu, A., Yamamoto, D., and Ishiwa-Chigusa, S. (2000). A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. *Gene* 245, 31–42. doi: 10.1016/S0378-1119(99)00569-7
- La Sala, M. S., Hurtado, M. D., Brown, A. R., Bohórquez, D. V., Liddle, R. A., Herzog, H., et al. (2013). Modulation of taste responsiveness by the satiation hormone peptide YY. *FASEB J.* 27, 5022–5033. doi: 10.1096/fj.13-228064
- Lee, G. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. *Genetics* 167, 311–323.
- Li, Y., Hoffmann, J., Li, Y., Stephano, F., Bruchhaus, I., Fink, C., et al. (2016). Octopamine controls starvation resistance life span and metabolic traits in *Drosophila*. *Sci. Rep.* 6:35359. doi: 10.1038/srep35359
- Liu, C., Plaçais, P. Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., et al. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* 488, 512–516. doi: 10.1038/nature11304
- Long, T. F., and Murdock, L. L. (1983). Stimulation of blowfly feeding behavior by octopaminergic drugs. *Proc. Natl. Acad. Sci. U.S.A.* 80, 4159–4163.
- Longden, K. D., and Krapp, H. G. (2009). State-dependent performance of optic-flow processing interneurons. *J. Neurophysiol.* 102, 3606–3618. doi: 10.1152/jn.00395.2009
- Maimon, G., Straw, A. D., and Dickinson, M. H. (2010). Active flight increases the gain of visual motion processing in *Drosophila*. *Nat. Neurosci.* 13, 393–399. doi: 10.1038/nn.2492
- Marion-Poll, F. (1995). Object-oriented approach to fast display of electrophysiological data under MS-windows. *J. Neurosci. Methods* 63, 197–204. doi: 10.1016/0165-0270(95)00110-7
- Marion-Poll, F. (1996). “Display and Analysis of Electrophysiological Data under Windows TM,” in *Proceedings of the 9th International Symposium on Insect-Plant Relationships, Series Entomologica, Vol. 53*, eds E. Städler, M. Rowell-Rahier, and R. Bauer (Dordrecht: Springer), 116–119.
- Mentel, T., Duch, C., Stypa, H., Wegener, G., Müller, U., and Pflüger, H. J. (2003). Central modulatory neurons control fuel selection in flight muscle of migratory locust. *J. Neurosci.* 23, 1109–1113.
- Meunier, N., Belgacem, Y. H., and Martin, J.-R. (2007). Regulation of feeding behaviour and locomotor activity by takeout in *Drosophila*. *J. Exp. Biol.* 210, 1424–1434. doi: 10.1242/jeb.02755
- Monastirioti, M. (2003). Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in *Drosophila melanogaster*. *Dev. Biol.* 264, 38–49. doi: 10.1016/j.ydbio.2003.07.019
- Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. *J. Comp. Neurol.* 356, 275–287. doi: 10.1002/cne.903560210
- Monastirioti, M., Linn, C. E. Jr., and White, K. (1996). Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* 16, 3900–3911.
- Moss, C. F., and Dethier, V. G. (1983). Central nervous system regulation of finicky feeding by the blowfly. *Behav. Neurosci.* 97, 541–548.
- Nässel, D. R., and Winther, Å. M. E. (2010). *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog. Neurobiol.* 92, 42–104. doi: 10.1016/j.pneurobio.2010.04.010
- Nishimura, A., Ishida, Y., Takahashi, A., Okamoto, H., Sakabe, M., Itoh, M., et al. (2012). Starvation-induced elevation of taste responsiveness and expression of a sugar taste receptor gene in *Drosophila melanogaster*. *J. Neurogenet.* 26, 206–215. doi: 10.3109/01677063.2012.694931
- Nisimura, T. (2005). Experiential effects of appetitive and nonappetitive odors on feeding behavior in the blowfly *Phormia regina*: a putative role for tyramine in appetite regulation. *J. Neurosci.* 25, 7507–7516. doi: 10.1523/JNEUROSCI.1862-05.2005
- Page, R. E. Jr., Erber, J., and Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera*, L.). *J. Comp. Physiol. A* 182, 489–500.
- Park, J. H., and Keeley, L. L. (1998). The effect of biogenic amines and their analogs on carbohydrate metabolism in the fat body of the Cockroach *Blaberus discoidalis*. *Gen. Comp. Endocrinol.* 110, 88–95. doi: 10.1006/gcen.1997.7053
- Partridge, L., Green, A., and Fowler, K. (1987). Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *J. Insect Physiol.* 33, 745–749. doi: 10.1016/0022-1910(87)90060-6
- Pezzuolo, G., and Cisek, P. (2016). Navigating the affordance landscape: feedback control as a process model of behavior and cognition. *Trends Cogn. Sci.* 20, 414–424. doi: 10.1016/j.tics.2016.03.013
- Pophof, B. (2000). Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J. Compar. Physiol.* 186, 307–313. doi: 10.1007/s003590050431
- Ramirez, J.-M., and Orchard, I. (1990). Octopaminergic modulation of the forewing stretch receptor in the locust *Locusta migratoria*. *J. Exp. Biol.* 149, 255–279.
- R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at <https://www.R-project.org/>

- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Ann. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Saraswati, S., Fox, L. E., Soll, D. R., and Wu, C. F. (2004). Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. *J. Neurobiol.* 58, 425–441. doi: 10.1002/neu.10298
- Scheiner, R., Sokolowski, M. B., and Erber, J. (2004). Activity of CGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learn. Mem.* 11, 303–311.
- Scheiner, R., Steinbach, A., Claßen, G., Strudthoff, N., and Scholz, H. (2014). Octopamine indirectly affects proboscis extension response habituation in *Drosophila melanogaster* by controlling sucrose responsiveness. *J. Insect Physiol.* 69, 107–117. doi: 10.1016/j.jinsphys.2014.03.011
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Martin Heisenberg, S. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–10502.
- Suver, M. P., Mamiya, A., and Dickinson, M. H. (2012). Octopamine neurons mediate flight-induced modulation of visual processing in *Drosophila*. *Curr. Biol.* 22, 2294–2302. doi: 10.1016/j.cub.2012.10.034
- Therneau, T. M. (2015). *A Package for Survival Analysis in S*. Available online at: <https://CRAN.R-project.org/package=survival>
- Thompson, S. N. (2003). Trehalose – the insect ‘blood’ sugar. *Adv. Insect Physiol.* 31, 205–285.
- Tuthill, J. C., Nern, A., Rubin, G. M., and Reiser, M. B. (2014). Wide-field feedback neurons dynamically tune early visual processing. *Neuron* 82, 887–895. doi: 10.1016/j.neuron.2014.04.023
- van Breugel, F., Suver, M. P., and Dickinson, M. H. (2014). Octopaminergic modulation of the visual flight speed regulator of *Drosophila*. *J. Exp. Biol.* 217, 1737–1744. doi: 10.1242/jeb.098665
- Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics with S*. Fourth. New York, NY: Springer.
- Wickham, H. (2016). *Tidyverse: Easily Install and Load ‘Tidyverse’ Packages*. Available online at: <http://CRAN.R-project.org/package=tidyverse>
- Wilke, C. O. (2016). *Cowplot: Streamlined Plot Theme and Plot Annotations for ‘ggplot2’*. Available online at: <http://CRAN.R-project.org/package=cowplot>
- Woodring, J. P., McBride, L. A., and Fields, P. (1989). The role of octopamine in handling and exercise-induced hyperglycaemia and hyperlipaemia in *Acheta Domesticus*. *J. Insect Physiol.* 35, 613–617. doi: 10.1016/0022-1910(89)90123-6
- Yang, Z., Yu, Y., Zhang, V., Tian, Y., Qi, W., and Wang, L. (2015). Octopamine mediates starvation-induced hyperactivity in adult *drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5219–5224. doi: 10.1073/pnas.1417838112
- Yu, Y., Huang, R., Ye, J., Zhang, V., Wu, C., Cheng, G., et al. (2016). Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in adult *drosophila*. *eLife* 5:e15693. doi: 10.7554/eLife.15693
- Zhang, B., Freeman, M. R., and Waddell, S. (2010). *Drosophila Neurobiology: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Zhang, H., and Blumenthal, E. M. (2017). Identification of multiple functional receptors for tyramine on an insect secretory epithelium. *Sci. Rep.* 7:160. doi: 10.1038/s41598-017-00120-z
- Zhou, C., Rao, Y., and Rao, Y. (2008). A subset of octopaminergic neurons are important for *Drosophila* aggression. *Nat. Neurosci.* 11, 1059–1067. doi: 10.1038/nn.2164

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The Role of Monoaminergic Neurotransmission for Metabolic Control in the Fruit Fly *Drosophila Melanogaster*

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Hormones control various metabolic traits comprising fat deposition or starvation resistance. Here we show that two invertebrate neurohormones, octopamine (OA) and tyramine (TA) as well as their associated receptors, had a major impact on these metabolic traits. Animals devoid of the monoamine OA develop a severe obesity phenotype. Using flies defective in the expression of receptors for OA and TA, we aimed to decipher the contributions of single receptors for these metabolic phenotypes. Whereas those animals impaired in *octβ1r*, *octβ2r* and *tar1* share the obesity phenotype of OA-deficient (*tβh*-deficient) animals, the *octβ1r*, *octβ2r* deficient flies showed reduced insulin release, which is opposed to the situation found in *tβh*-deficient animals. On the other hand, OAMB deficient flies were leaner than controls, implying that the regulation of this phenotype is more complex than anticipated. Other phenotypes seen in *tβh*-deficient animals, such as the reduced ability to perform complex movements tasks can mainly be attributed to the *octβ2r*. Tissue-specific RNAi experiments revealed a very complex interorgan communication leading to the different metabolic phenotypes observed in OA or OA and TA-deficient flies.

Keywords: octopamine receptor, tyramine receptor, insulin, body fat distribution, insulin release

INTRODUCTION

Hormones are known to have a major impact on various metabolic traits. Among these hormones biogenic amines take a special position as they modulate these metabolic traits at different levels. Two of these amines, octopamine (OA) and tyramine (TA) are specifically relevant in invertebrates (Roeder, 1999, 2005). They act as functional equivalents of the vertebrate hormones/transmitters epinephrine and norepinephrine; similar to their roles in vertebrates, in which epinephrine- or norepinephrine-mediated signaling leads to a variety of metabolic changes (Debuyser et al., 1991; Bachman et al., 2002), OA and TA appear to be similarly potent in order to control metabolic traits in invertebrates (Lange, 2009; Li et al., 2016). Although TA and OA have been shown to act as independent neuroactive compounds, they share a large number of similarities (Roeder et al., 2003; Saraswati et al., 2004; Lange, 2009). Most importantly, OA producing cells always contain TA, as the latter one serves as a biological precursor for OA (Roeder, 2002, 2005; Cole et al., 2005). On the other hand, only very few neurons in the insect brain produce TA but no OA, making it hard

to examine how the different actions of both compounds are disentangled under physiological conditions (Monastirioti et al., 1995; Busch et al., 2009; Selcho et al., 2014). Beside the countless modulatory actions in invertebrates that can be attributed to these monoamines (Roeder, 1999, 2002), they have also been shown to regulate various metabolic traits. OA in particular appears to take a central position in regulating metabolism associated traits. It was shown that OA signaling is highly relevant in controlling behaviors with a direct impact on energy expenditure comprising the regulation of physical activity or the timing of sleep. Moreover, it was shown recently that OA directly affects the metabolic resting rate, therewith directly influencing fat storage (Li et al., 2016).

Although this role of both monoamines has mainly been studied in fruit flies, it appears also to apply to other insect and even to other invertebrates such as nematodes (Suo et al., 2006). As already mentioned, the vertebrate counterparts epinephrine and norepinephrine act in very similar ways as OA and TA do. In both systems, the corresponding hormones are released in times of stress and act as major transducers that orchestrate the organism's stress reaction (Atgié et al., 1998; Adamo and Baker, 2011; Even et al., 2012). Release of these compounds should thus increase physical activity and resting metabolic rates reducing body fat stores. Diminished release of these compounds has exactly the opposite effects; it reduces activity and the metabolic rate, which leads, long-term, to more body fat.

Release of OA and TA modulates various behaviors and metabolic traits in a well-coordinated manner in order to shift the animal's physiology to a high performance, high energy-expenditure state (Li et al., 2016). Thus, they appear to take a central position in the regulatory network responsible for inter-organ communication (Rajan and Perrimon, 2011). This comprises both types of behaviors, those that are associated with energy intake as well as those associated with energy expenditure. Food intake as the only energy source is also under the control of OA-mediated signaling (Zhang et al., 2013). Directly associated with this effects is the enhanced physical activity that is seen during periods of starvation, which appears to be devoted to enable efficient searches for novel food sources (Yang et al., 2015). OA and TA control movement activity and movement performance at different levels. In larval muscles, both compounds act antagonistically to each other. Whereas OA enhances the contraction properties of skeletal muscles, TA has the opposite effect (Saraswati et al., 2004; Selcho et al., 2012; Ormerod et al., 2013). Insect flight, which is the most energy-demanding physical activity, is also tightly controlled by OA signaling (Blau et al., 1991; Brembs et al., 2007), further showing the central role of monoaminergic neurotransmission for energy-demanding behaviors in general. Another behavior with a major impact on the balance between energy intake and expenditure is sleep, which thus takes a central position for energy homeostasis. In *Drosophila*, it was shown that the amount of sleep is directly correlated with starvation resistance (Slocumb et al., 2015). OA acts as a wake-promoting agent and impairments in the biosynthesis of OA are associated with enhanced daily sleep (Crocker and Sehgal, 2008; Crocker et al., 2010). At least in part,

these effects of OA and/or TA are mediated through their modulatory action on insulin release from insulin producing neurons, which is thought to be mediated through the OAMB receptor located on these cells (Erion et al., 2012; Luo et al., 2014; Li et al., 2016). Recently, we could show that OA has a direct impact on energy expenditure-related metabolic traits, namely, it enhances the resting metabolic rate, thus reducing body fat. Consequently, reduced OA signaling leads to lowered metabolic rates and increased body fat with all its downstream consequences such as reduced life span and increased starvation resistance (Li et al., 2016). Reproduction, which critically depends on matching metabolic parameters, is also tightly controlled by OA signaling (Lee et al., 2003, 2009; Li et al., 2015), further demonstrating the role of OA to orchestrate numerous physiological actions within the organism.

Despite this body of information, we know little about the molecular mechanisms that are responsible for transducing the effects of either of these two monoamines into a suitable physiological reaction. Most importantly, the specific roles of the four OA and three TA receptors in this process remains to be elucidated (El-Kholy et al., 2015). Thus, we analyzed a set of transgenic animals impaired in expression of one of these different receptors each and employed RNAi experiments with the most relevant receptor genes targeted to major metabolic organs (brain, fat body and oenocytes).

MATERIALS AND METHODS

Fly Stocks and Maintenance

The fly stocks used in this study were as follows: *TDC2^{Ro54}* flies were generously provided by Jay Hirsh (University of Virginia, Charlottesville, VA, USA; Cole et al., 2005), *TβH^{M18}* flies were generously provided by Henrike Scholz (University of Cologne, Köln, Germany) and OAMB-defective flies by Kyung-An Han (University of Texas, El Paso, TX, USA; Lee et al., 2003). The PromE(800)-Gal4 (oenocyte-Gal4) line was obtained from Joel Levine (University of Toronto, Toronto, ON, Canada; Billeter et al., 2009). The *octβ1R^{f02819}*, *octβ2R^{f05679}*, *octβ3R^{MB04794}*, *TAR1^{PL00408}*, *TAR2^{MB03028}* and *TAR3^{MB09692}* mutant lines used in this study were generated by the Gene Disruption Project (Bloomington Stock Center, Indiana, Bloomington, USA). The UAS-dsRNAi lines of *octβ1R* (#47895), *octβ2R* (#104050), *octβ3R* (#6099), *TAR1* (#26876), *TAR2* (#2857) were obtained from the Vienna *Drosophila* Resource Center. Other transgenic strains including *nsyb-GAL4* (#51635), *ppl-GAL4* (#58768), were obtained from the Bloomington *Drosophila* Stock Center. All flies, unless otherwise stated, were raised on standard yeast/cornmeal/agar medium at 25°C with about 50%–60% relative humidity under a 12 h/12 h light/dark cycle as described previously (Rahn et al., 2013; Li et al., 2015). RNAi-mediated knockdown of OAR/TARs genes in different tissues was achieved by crossing UAS-receptor RNAi line to the tissue-specific promoter GAL4 line and the F1 generation flies were kept at 29°C to enhance the RNA interference, the parental lines crossed to *w¹¹¹⁸* were used as controls.

RT-PCR Analysis

Total RNA was extracted from the brains of 15 females kept on normal food. RT-PCR was essentially performed following recently described methods (Li et al., 2015). The following primers were used: *Rpl32* forward (5'-CCG CTT CAA GGG ACA GTA TC-3'), *Rpl32* reverse (5'-GAC AAT CTC CTT GCG CTT CT-3'), *Dilp2* forward (5'-CTG AGT ATG GTG TGC GAG GA-3'), *Dilp2* reverse (5'-ACA AAC TGC AGG GGA TTG AG-3'), *OAMB-F* (5'-CGG TTA ACG CCA GCA AGT G-3'), *OAMB-R* (5'-AAG CTG CAC GAA ATA GCT GC-3'), *Octβ1R-F* (5'-GGC AAC GAG TAA CGG TTT GG-3'), *Octβ1R-R* (5'-TCA TGG TAA TGG TCA CGG GC-3'), *Octβ2R-F* (5'-TCC TGT GGT ACA CAC TCT CCA-3'), *Octβ2R-R* (5'-CCA CCA ATT GCA GAA CAG GC-3'), *Octβ3R-F* (5'-TGT GGT CAA CAA GGC CTA CG-3'), *Octβ3R-R* (5'-GTG TTC GGC GCT GTT AAG GA-3'), *TAR1-F* (5'-AGA CGA GGT GCA AGG TGT TG-3'), *TAR1-R* (5'-TTC CCC GAC TTC TTT GAC TGC-3'), *TAR2-F* (5'-TGC AGT CTT TGC CAC CTT CA-3'), *TAR2-R* (5'-GTT GCC ACG AGC CTA TGA GA-3'), *TAR3-F* (5'-GAA CTT GGC CAT CAC CGA CT-3'), *TAR3-R* (5'-GTG ACG GCG AGA TAC CTG TC-3').

Starvation Resistance Assays

The starvation resistance assays were performed on constant conditions mentioned above. Four to five-day-old adult flies were placed in vials containing 1% agar, and dead flies were recorded every 2–3 h until all flies died. For each genotype, at least 100 flies were used in this assay.

BODIPY Staining and Body Fat Determination

The whole fly bodies were collected and fixed in 4% paraformaldehyde for 30 min at room temperature. After washing with phosphate-buffered saline, the flies were repeatedly frozen in liquid nitrogen and thawed on ice three times, followed by staining with a solution containing 1 µg/ml BODIPY dye (Invitrogen, Darmstadt, Germany) for 1 h in the darkness before observation by epifluorescence microscopy (Olympus, Hamburg, Germany).

Total body triacylglycerols (TAGs) in flies were determined using a coupled colorimetric assay method as described previously (Hildebrandt et al., 2011; Hoffmann et al., 2013; Li et al., 2016). Briefly, eight males (or five females) per group were weighed and homogenized in 1 ml 0.05% Tween-20 using a Bead Ruptor 24 (BioLab Products, Beverly, MA, USA). Homogenates were heat-inactivated for 5 min at 70°C and incubated with triglyceride solution (Fisher Scientific, Waltham, MA, USA) at 37°C for 30 min with mild shaking. The absorbance was read at 562 nm and glyceryl trioleate served as a TAG standard.

Locomotor Activity Assay

For the negative geotaxis assay, groups of 20 flies were transferred into a 20 cm-tall glass tube without CO₂ anesthesia and allowed to recover for 1 h. The tube was tapped three times to initiate flies to the bottom and the climbing height was photographed after 5 s. The average distance climbed in cm for each fly from five replicates was measured.

Glucose and Trehalose Measurement

The hemolymph glucose and trehalose measurement were performed using Glucose (HK) Assay Kit (Sigma, Steinheim, Germany) with minor modifications as described previously (Li et al., 2016). The hemolymph sample was pooled from 15 flies per genotype and added to 50 µl of glucose assay reagent. After incubation for 15 min at room temperature, the glucose levels were calculated according to the standard curve established by measuring absorbance at 340 nm. For trehalose measurement, 0.25 µl of porcine kidney trehalase (Sigma, Steinheim, Germany) was added to convert trehalose to glucose. After incubation at 37°C overnight, the absorbance was measured again, and the amount of trehalose was calculated.

Immunohistochemistry for dILP2 Measurements

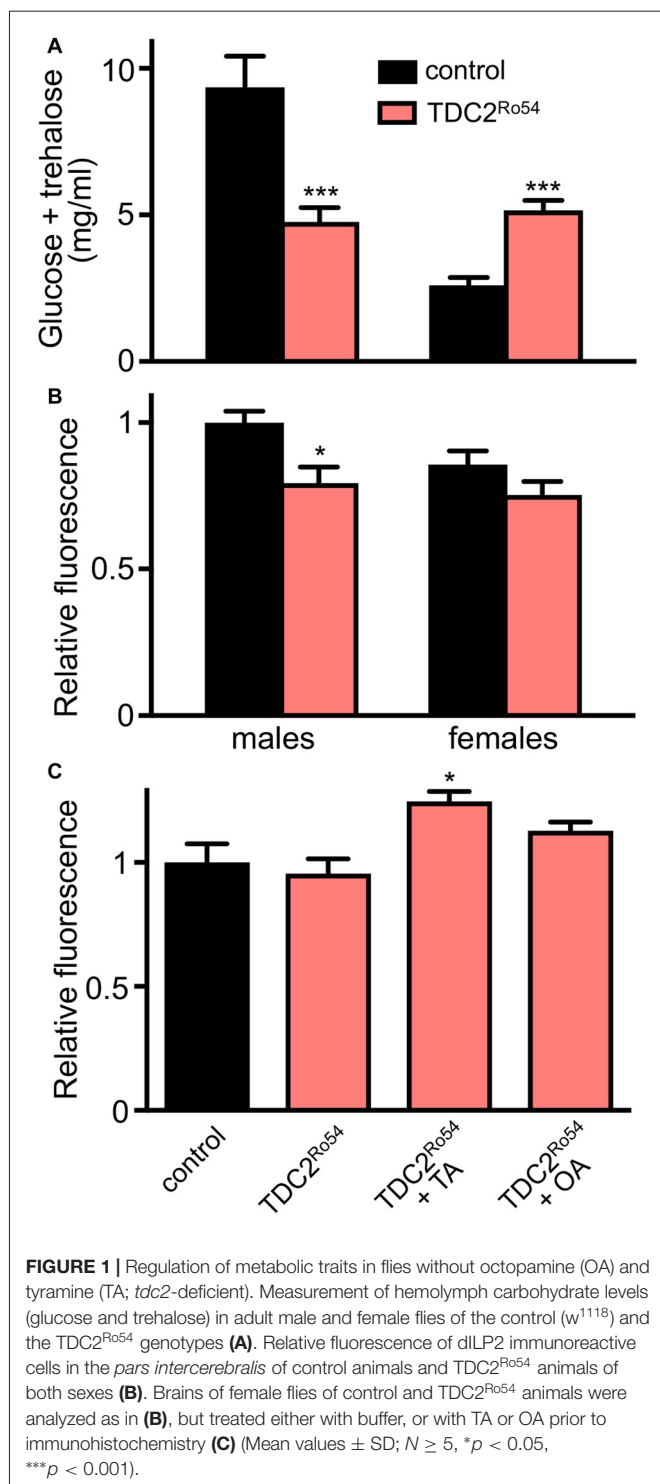
Immunohistochemistry was performed as previously described (Li et al., 2016). The brains were dissected in *Drosophila* Ringer's solution and immediately fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Subsequently, the samples were washed with PBST (0.3% Triton X-100 in PBS) and blocked in blocking-buffer (10% goat serum in PBST) for 30 min at room temperature, followed by incubation with the primary antibody (1:200 rabbit anti-dILP2, a gift from Eric Rulifson, UCSF, USA) overnight at 4°C with subsequent application of the secondary antibody (1:500 donkey anti-rabbit IgG, Jackson ImmunoLabs, Suffolk, UK) for 3 h at room temperature. After three washings, the brains were mounted on slides and images were obtained using a fluorescent microscope equipped with an apotome (Carl Zeiss Image AxioVision, Göttingen, Germany). To facilitate the quantification of dILP2 fluorescence intensities in the region of *pars intercerebralis*, series of sections were gathered under identical thickness, exposure time and all other relevant settings. Fluorescence intensity was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses

All statistical analyses were accomplished using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Starvation survivorship was analyzed by log-rank (Mantel-Cox) assays. Other parameters were evaluated using the unpaired two-tailed Student's *t* test and one-way ANOVA. All data were presented as mean values ± SD.

RESULTS

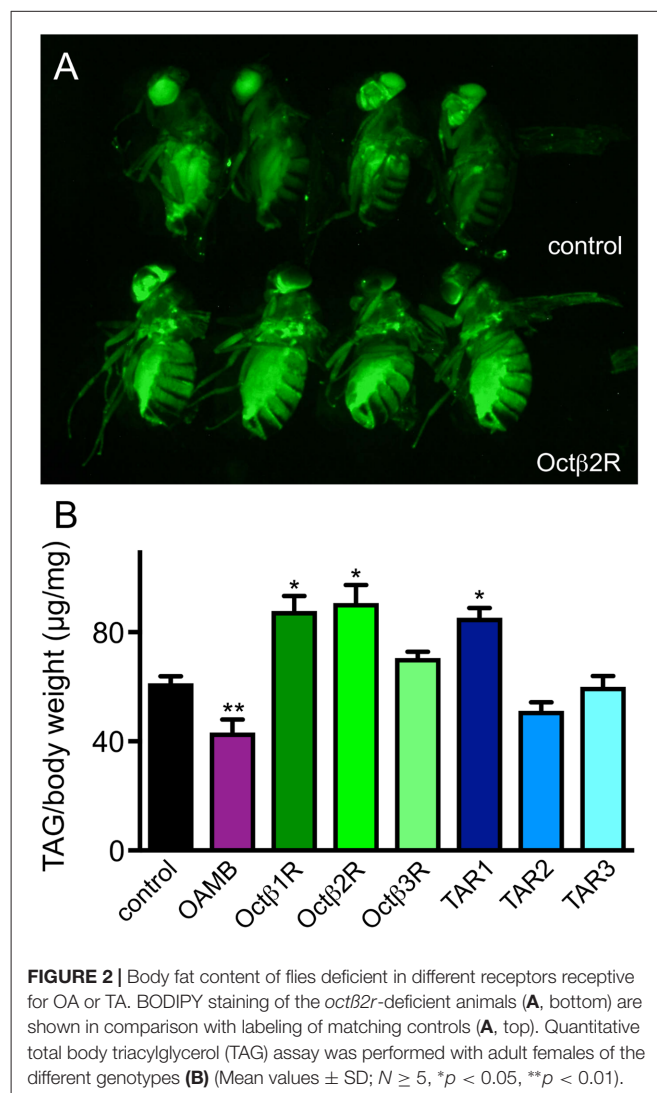
Although TA is the biological precursor of OA, both monoamines act as independent neuroactive compounds in a wide variety of behavioral paradigms. Recently, we could show that differences between animals defective in *tβh* (TA, but no OA) and *tdc2* (no OA, no TA) could be observed regarding their body fat storage (Li et al., 2016). We analyzed these phenotypic peculiarities in more detail and could show in the current work that other metabolically relevant traits also differ between both types of animals. Whereas hemolymph carbohydrate levels are lower in both sexes of the *tβh*-defective animals, we could observe sex-specific differences in *tdc2*-defective animals where



males show the same phenotype as *tβh*-defective animals did, whereas females show the opposite phenotype (Figure 1A). This sex-specific discrepancy was also observed for food intake by *tdc2*-deficient females, which show reduced food intake while males did not show these alterations. Most impressive were the differences in metabolic rates, where only OA-deficient animals show a substantial reduction, while *tdc2*-deficient

ones have an unaltered metabolic resting rate (Li et al., 2016). Regarding insulin secretion, *tdc2*-deficient animals showed a slight reduction in the dILP2 content of the IPCs, which is equivalent to the situation under OA deficiency (Figure 1B). Feeding OA and TA to these animals led to a slight reduction in dILP2 release (Figure 1C).

In order to learn more about their relevance for various metabolic traits, we choose a series of *Drosophila* lines carrying insertions in the respective genes coding for OA and TA receptors that should effectively impair expression of functional proteins. OA and TA transmit their effects via a total of seven G-protein coupled receptors, with four being specifically tuned to react to OA and three to TA. They can be further subgrouped regarding their primary structures into a more alpha-adrenergic subtype (OAMB), those sharing similarities with β-adrenergic receptors (Octβ1R-Octβ3R) and two classes of TA receptors (Maqueira et al., 2005; El-Kholy et al., 2015). The TAR1 (also known as TyrR, Oct/TyrR) and the other two TARs (TAR2, also known as TyrR1 and TAR3) do not cluster together (El-Kholy et al., 2015). A recent analysis utilizing promoter reporter



lines revealed the spatial distribution of the different receptors in the different tissues of the fly. Flies without OA (*tβh*-deficient animals) show an impressive metabolic phenotype, they are obese with fat deposits increased by more than 30% compared with matching controls (Li et al., 2016). We measured the triglyceride levels in different fly lines defective in expression of the corresponding receptors and observed that flies impaired in expression of the Octβ1R, the Octβ2R and the TAR1 showed a significantly enhanced fat deposition as observed by BODIPY staining of the corresponding animals, while the OAMB-deficient ones had reduced fat levels. As an example, we show the staining of the *octβ2r*-deficient flies (Figure 2A). In order to quantify this effect, we measured triglyceride levels of these animals and obtained a similar result (Figure 2B). Results from this quantitative approach were almost congruent with that obtained using the fat staining approach. As the OAMB has already been described in greater detail (Erion et al., 2012), we excluded the OAMB from all downstream studies.

Differential fat deposition is assumed to directly influence important metabolic traits such as starvation resistance (Ballard et al., 2008). Thus, we analyzed starvation resistance in these animals and identified significant differences to matching control populations (Figure 3). Whereas

the Octβ1R, Octβ2R and TAR1 (Figures 3A,B) show statistically significantly increased starvation resistances, the TAR2 had a lower resistance, whereas the resistances of the Octβ3R and TAR3 were not different from the controls (Figures 3A,B).

One component that has a major effect on the fat content of flies is the level of insulin release from cells in the *pars intercerebralis*. We first analyzed which of the corresponding receptors are expressed in this peculiar brain region that contains different neurosecretory cells including those that produce and release the most important insulins in the fly (*dilp2*, *dilp3*, *dilp5*), but also those that produce, e.g., DH44, DH31 or SIFamide. Thus, we isolated the *pars intercerebralis* region manually and used the resulting material as a template for RT-PCR. From the receptors tested, the OAMB, the Octβ1R, the Octβ2R and the TAR1 showed specific signals, which implied that they are indeed expressed in neurosecretory cells of the brain (Figure 4A). We reanalyzed some of the doubtful candidates genes using promoter-Gal4 lines and showed expression in lateral parts of the *pars intercerebralis* for some of them, which implies that most of the OA and TA receptors are present in the *pars intercerebralis*, presumably to modulate hormone release from the corresponding cells in this highly specialized brain region (Figures 4B–E).

Based on this information, we analyzed the *dilp2* content in insulin-producing cells of the *pars intercerebralis* of female

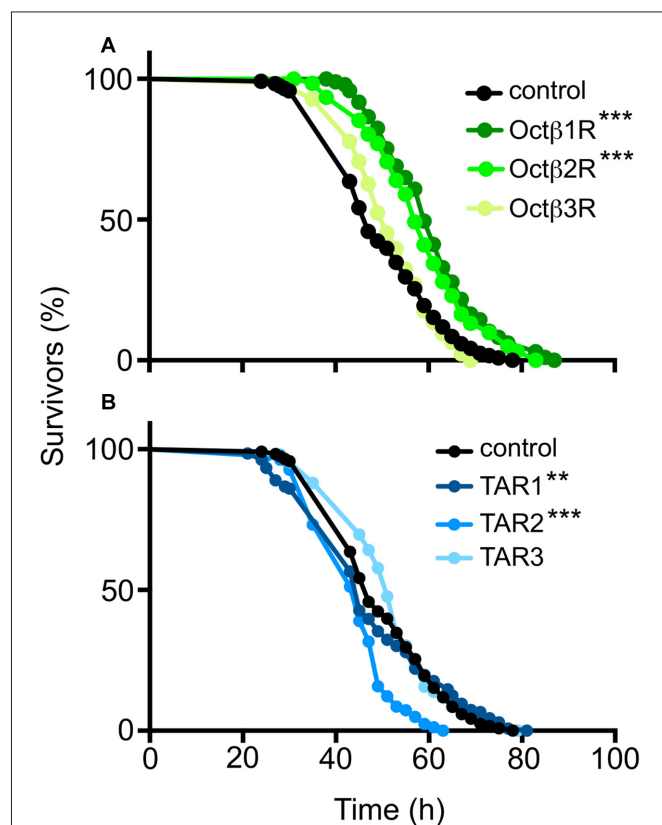


FIGURE 3 | Starvation resistance of flies defective in OA or TA receptor gene expression. Those flies deficient in the expression of OA receptors (A) or TA receptors (B) were starved and the number of dead flies counted every 2 h. *w¹¹¹⁸* flies served as a control. *N* = 100, ***p* < 0.01, ****p* < 0.001.

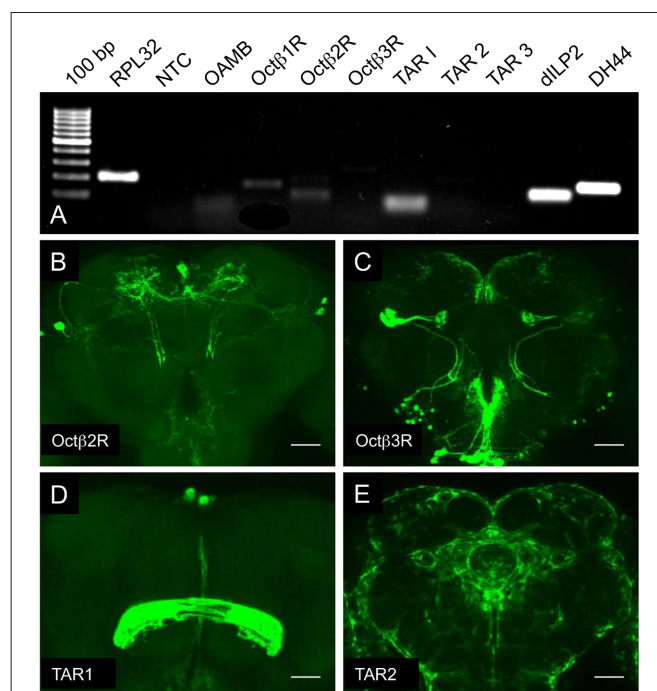
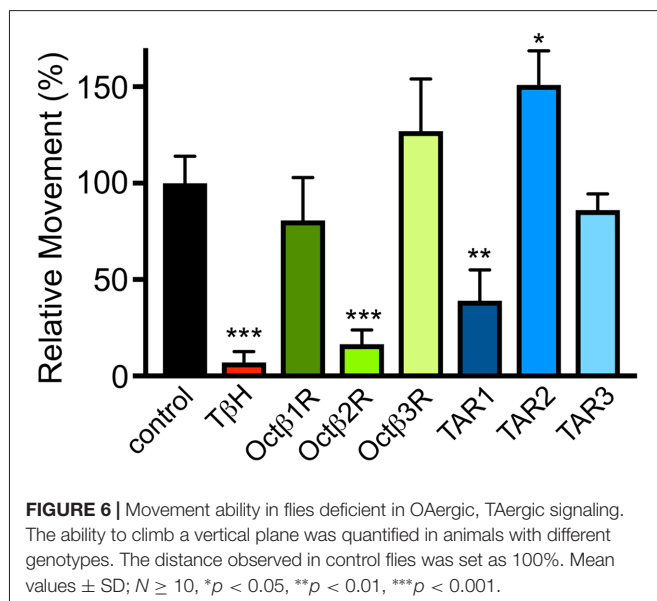
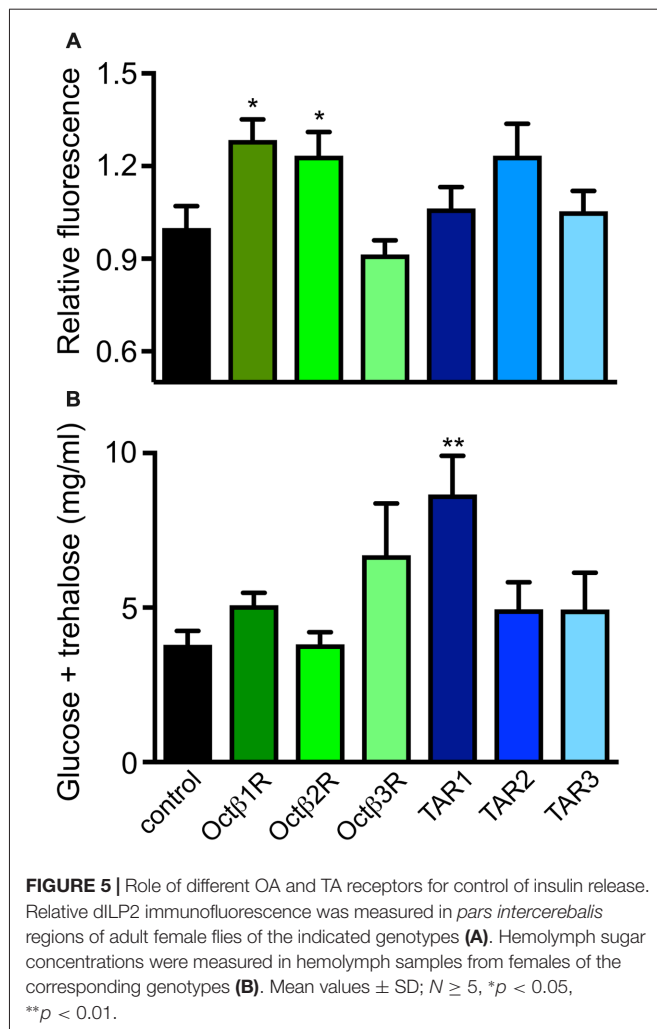
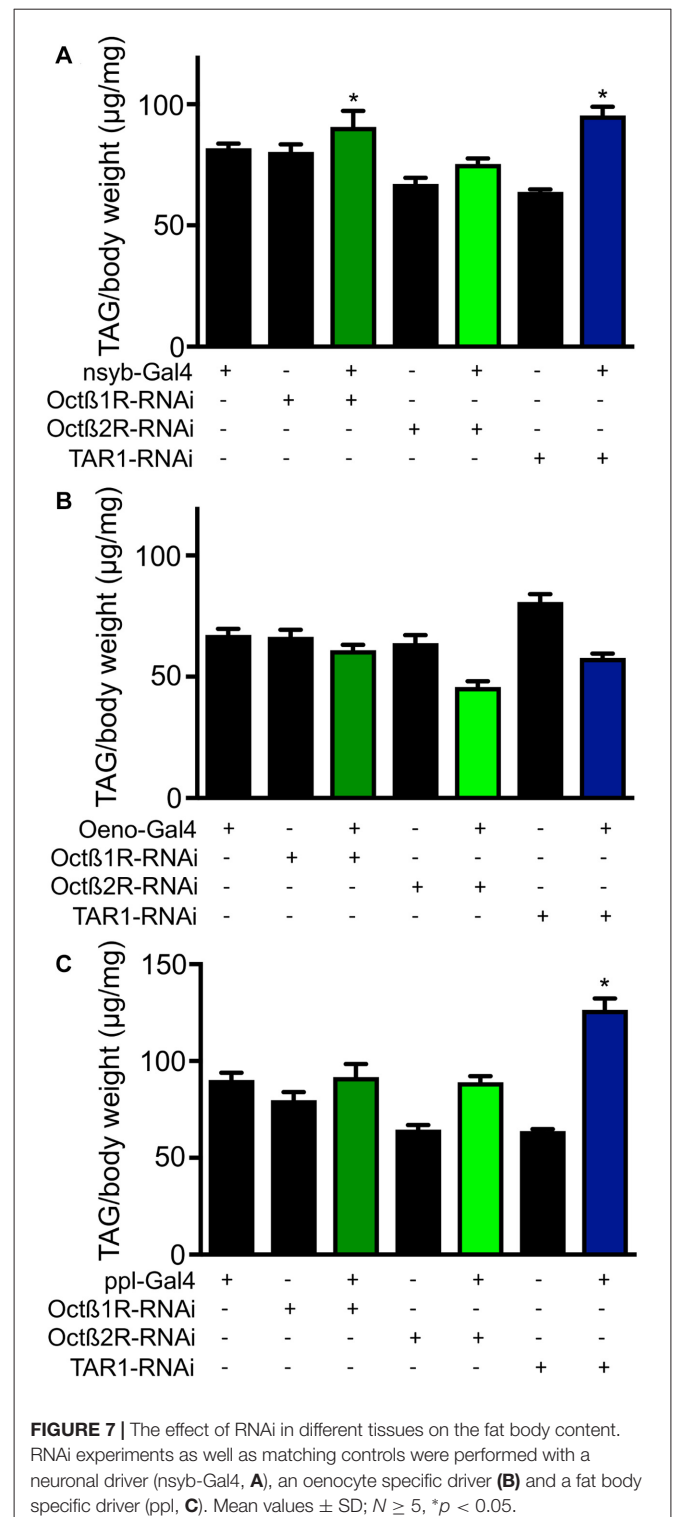


FIGURE 4 | Expression analysis of OA and TA receptor genes in the *pars intercerebralis*. RT-PCR analysis of manually isolated *pars intercerebralis* areas with oligonucleotides specific for the listed genes (A), NTC, no template control. Analysis of the expression patterns of fourth deficient different promoter-Gal4 lines (B–E), specifically labeling cells positive for *octβ2r* (B), *octβ3r* (C), *tar1* (D), *tar2* (E). Scale bars = 50 μm.



flies of the corresponding lines (Figure 5A). Only flies with insertions in the *oct β 1R* or *oct β 2R* genes had statistically



significantly different dILP2 levels in their insulin-producing cells. Directly associated with the insulin release is usually the hemolymph sugar level. We measured the combined sugar levels (glucose + trehalose) in the hemolymph of the corresponding flies and identified higher glucose levels for *oct β 1R*-insertion-carrying flies (Figure 5B). The control of traits that are

directly associated with energy expenditure is obviously highly relevant in this context. The ability to perform complex movement tasks, such as climbing vertical planes, was addressed (Pfeiffenberger et al., 2010; Li et al., 2016). For this, we used a simple negative geotaxis assay. OA (*tβh*-defective) deficient animals showed a massively reduced ability to perform this task. While flies impaired in the *tar2* receptor showed an increased climbing activity, the *tar1*-defective lines showed reduced abilities. Most impressive were the impairments seen in lines with impaired *octβ2r* expression, as they showed movement impairments that almost matched those seen in animals devoid of OA (Figure 6).

In order to elucidate the mechanisms underlying differential fat contents of the different receptor-deficient lines, we performed RNAi experiments with selected receptor lines. Silencing expression in the CNS using the *nsyb* driver line revealed slightly increase body fat especially for those animals with reduced *octβ1r* and *tar1* expression in the nervous system (Figure 7A). Targeting this intervention to oenocytes led to slightly reduced body fat in *octβ2r*-deficient flies (Figure 7B). Silencing expression in the fat body

(*ppl*-Gal4, Figure 7C) increased body fat only in case of *tar1* silencing (Figure 7C). Moreover, we analyzed the effects of RNAi-mediated silencing in the oenocytes (Figure 8A) and the fat body (Figure 8B) on the release of dILP2 from insulin-producing cells in the *pars intercerebralis*. If we analyzed the dILP2 concentration in insulin producing cells in response to RNAi-mediated gene-silencing of the corresponding receptor genes, we observed no changes in response to manipulation in the oenocytes (Figure 8A), but a profound reduction in response to manipulation in the fat body for *octβ1r*, *octβ2r* and *tar1* (Figure 8B).

DISCUSSION

To allow for a suitable organismal reaction in response to different internal or external situations, behavioral and metabolic traits have to be well-orchestrated. The monoamines OA and TA are central mediators of this complex interorgan communication system. Thus, they occupy the same position that epinephrine and norepinephrine take in vertebrates. Consequently, impairing their signaling properties incurs a great variety of metabolic alterations. Among these modifications, those associated with body fat stores are most conspicuous. Despite the structural similarities between OA and TA, they obviously act differentially in the regulation of major metabolic traits. Whereas animals devoid of OA (*tβh*-deficient) are obese, those without OA and TA (*tdc2*-deficient) are not, implying that the effects of both monoamines on this major metabolic trait are opposed. In order to learn more about the underlying mechanisms, we focused on the corresponding receptors, as results obtained with those animals defective in synthesis of either OA (*TβH*) or OA + TA (*TDC2*) are not easy to interpret. *Tβh*-deficient animals produce no OA, but contain higher amounts of TA, *tdc2*-defective animals, on the other hand, have neither OA nor TA, which complicates direct assignments of specific phenotypes to either OA or TA.

It was our assumption that the major metabolic phenotype of *Tβh*-deficient animals, the high fat content, is mediated via interaction with only one specific receptor, which turned out not to be the case. A total of two out of four different line defective in OA receptors (*Octβ1R*, *Octβ2R*) and one line defective in TA receptors (*TAR1*) showed an increased body fat content, thus phenocopying OA-deficient animals (see Table 1). Moreover, animals defective in expression of the *oamb* receptor gene are leaner than matching controls are. As expected, the increased body fat observed in some of the flies came with enhanced starvation resistances. This observation implies that mechanisms required to modulate body fat content are more complex than anticipated, thus mirroring the situation found in vertebrates, where regulation of body fat is mediated by sets of α - and β -adrenergic receptors rather than by only single representatives of this family.

It is not completely understood how signaling through these receptors controls body fat content. Different mechanisms have to be taken into account, comprising behaviors that are directly

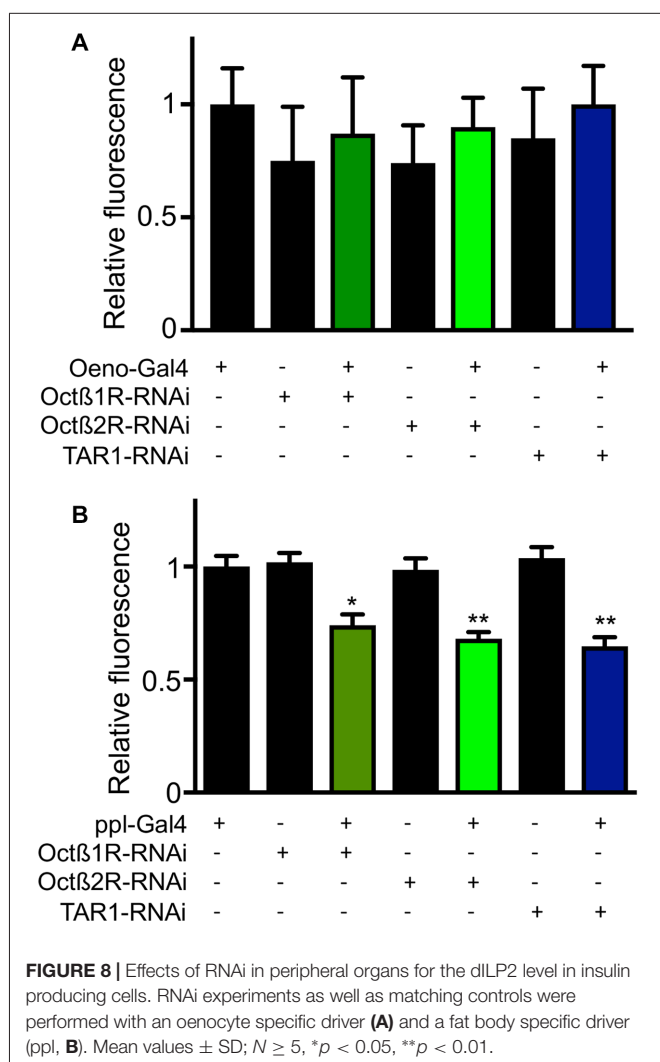


TABLE 1 | Summary of the effects seen in fly lines defective in expression of the corresponding receptors.

	OAMB	Octβ1R	Octβ2R	Octβ3R	Tar1	Tar2	Tar3
Body fat	down	up	up	-	up	-	-
Starvation resist	n.a.	up	up	-	down	down	-
Insulin signaling	n.a.	up	up	-	-	-	-
Glucose level	n.a.	-	-	-	up	-	-
Movement	n.a.	-	down	-	down	up	-

Significant changes in comparison to matching controls are listed as up or down. n.a. means not analyzed.

linked with energy intake or energy expenditure (Crocker and Sehgal, 2008; Li et al., 2016). Moreover, controlling release rates of neurohormones or conveying direct effects on peripheral organs such as the fat body or skeletal muscles may also be relevant in this context (Crocker et al., 2010; Nässel et al., 2015). Regarding the expression profiles of the different OA and TA receptors, all options listed above have to be taken into account. Among these possible actions of OA and TA, the control of insulin release is most interesting, as it would add another mechanism to the list of almost identical functions shared by OA/TA and epinephrine/norepinephrine. It has been proposed that the OAMB receptor mediates the effects of OA on insulin release via direct control of release rates (Luo et al., 2014; Nässel et al., 2015). Apparently, the situation is more complex, as the *oamb*-deficient flies are lean, although they should convey OA's action on the IPCs. Moreover, the body fat phenotype observed in other receptor defective lines did not correlate neither with insulin release rates nor with hemolymph glucose levels. The anticipated role of OAMB as the major OA receptor operative in IPCs is still not fully supported. On the other hand, the other lines defective in expression of other OA (Octβ1R and Octβ2R) receptors that show enhanced body fat deposition, exhibit reduced insulin release rates, which is counterintuitive (Luo et al., 2014; Nässel et al., 2015; Li et al., 2016). The TAR1, which also is involved in body fat control has no obvious effects on insulin release, which shows that controlling insulin release by octopaminergic neurotransmission is primarily (and eventually exclusively) mediated via OA receptors.

If we take a look at the reduced ability to move in the vertical direction, the substantially reduced ability apparent in animals without OA (*TβH*-defective animals) was also observed in animals defective in expression of the *octβ2r* receptor gene. Although some of the other lines defective in one of the various OA and TA receptors showed also reduced abilities to perform this behavioral task, this was in no case as severe as for the OA deficient animals. This phenotype nicely correlates with the massive expression of the *octβ2r* receptor gene in skeletal muscles of larval and adult *Drosophila* (El-Kholy et al., 2015), which thus might be due to the peripheral actions of OA for controlling movement activities mediated via the Octβ2R receptor.

A very complex inter-organ communication was revealed through use of tissue-specific RNAi to analyze contributions of specific OA and TA receptors for various metabolic traits. Silencing expression of specific receptor genes in neurons only (driven by *nsyb*-Gal4) revealed increased body fat only for the *oct1βr* gene, whereas the other were almost unaffected.

The lack of phenotypes observed in RNAi experiments is not easy to explain, it can result from the lack of relevance in the targeted tissue, but it can also be due to insufficient silencing that permits to uncover these relevant phenotypes.

Interestingly, silencing of some receptor genes in the fat body revealed relatively strong effects on dILP2 levels in the brain and therewith on insulin release properties. This might be an effect of remote control of insulin release by the fat body, which has already been shown to be operative in this tissue (Géminard et al., 2009; Rajan and Perrimon, 2012). The three receptors under investigation (Octβ1R, Octβ2R und TAR1) are all expressed in the fat body at low levels (El-Kholy et al., 2015), which making a direct interaction possible.

Taken together, we aimed to understand the various facets of OAergic and TAergic control of metabolic traits in more depth using animals with defective expression of peculiar OA or TA receptors. It became apparent that a complex network comprising different receptors in different tissues is responsible for the control of metabolic traits such as body fat content. Whereas some actions of OA and TA can be attributed to specific receptor subtypes, this is not possible for others. The reduced ability to perform complex movement tasks appears to depend on OA signaling mediated via the Octβ2R in skeletal muscles. The regulation of other metabolic traits appear to be much more complex and involve complex remote control effects, which might have been expectable especially as OA as well as TA are thought to take a central role in interorgan communication.

AUTHOR CONTRIBUTIONS

YL, CF and TR conceived the study and wrote the manuscript, YL, LT, JF, SN, SE-K, FS performed experiments, YL, CG and IB evaluated the data.

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REFERENCES

- Adamo, S. A., and Baker, J. L. (2011). Conserved features of chronic stress across phyla: the effects of long-term stress on behavior and the concentration of the neurohormone octopamine in the cricket, *Gryllus texensis*. *Horm. Behav.* 60, 478–483. doi: 10.1016/j.yhbeh.2011.07.015
- Atgié, C., Faintrenie, G., Carpène, C., Bukowiecki, L. J., and Géloën, A. (1998). Effects of chronic treatment with noradrenaline or a specific β_3 -adrenergic agonist, CL 316 243, on energy expenditure and epididymal adipocyte lipolytic activity in rat. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 119, 629–636. doi: 10.1016/s1095-6433(97)00476-5
- Bachman, E. S., Dhillon, H., Zhang, C. Y., Cinti, S., Bianco, A. C., Kobilka, B. K., et al. (2002). β AR signaling required for diet-induced thermogenesis and obesity resistance. *Science* 297, 843–845. doi: 10.1126/science.1073160
- Ballard, J. W., Melvin, R. G., and Simpson, S. J. (2008). Starvation resistance is positively correlated with body lipid proportion in five wild caught *Drosophila* simulans populations. *J. Insect Physiol.* 54, 1371–1376. doi: 10.1016/j.jinsphys.2008.07.009
- Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G., and Levine, J. D. (2009). Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature* 461, 987–991. doi: 10.1038/nature08495
- Blau, C., Candy, D. J., and Wegener, G. (1991). Regulation of fructose 2,6-bisphosphate in perfused flight muscle of the locust, *Locusta migratoria*: the effect of octopamine. *Biochem. Soc. Trans.* 19:136S. doi: 10.1042/bst019136s
- Brembs, B., Christiansen, F., Pflüger, H. J., and Duch, C. (2007). Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. *J. Neurosci.* 27, 11122–11131. doi: 10.1523/JNEUROSCI.2704-07.2007
- Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the *Drosophila* brain. *J. Comp. Neurol.* 513, 643–667. doi: 10.1002/cne.21966
- Cole, S. H., Carney, G. E., McClung, C. A., Willard, S. S., Taylor, B. J., and Hirsh, J. (2005). Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. *J. Biol. Chem.* 280, 14948–14955. doi: 10.1074/jbc.M414197200
- Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in *Drosophila* through protein kinase A-dependent mechanisms. *J. Neurosci.* 28, 9377–9385. doi: 10.1523/JNEUROSCI.3072-08a.2008
- Crocker, A., Shahidullah, M., Levitan, I. B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep/wake behavior. *Neuron* 65, 670–681. doi: 10.1016/j.neuron.2010.01.032
- Debuyser, A., Drews, G., and Henquin, J. C. (1991). Adrenaline inhibition of insulin release: role of cyclic AMP. *Mol. Cell. Endocrinol.* 78, 179–186. doi: 10.1016/0303-7207(91)90121-8
- El-Kholy, S., Stephano, F., Li, Y., Bhandari, A., Fink, C., and Roeder, T. (2015). Expression analysis of octopamine and tyramine receptors in *Drosophila*. *Cell Tissue Res.* 361, 669–684. doi: 10.1007/s00441-015-2137-4
- Erion, R., DiAngelo, J. R., Crocker, A., and Sehgal, A. (2012). Interaction between sleep and metabolism in *Drosophila* with altered octopamine signaling. *J. Biol. Chem.* 287, 32406–32414. doi: 10.1074/jbc.M112.360875
- Even, N., Devaud, J. M., and Barron, A. B. (2012). General stress responses in the honey bee. *Insects* 3, 1271–1298. doi: 10.3390/insects3041271
- Géminard, C., Rulifson, E. J., and Léopold, P. (2009). Remote control of insulin secretion by fat cells in *Drosophila*. *Cell Metab.* 10, 199–207. doi: 10.1016/j.cmet.2009.08.002
- Hildebrandt, A., Bickmeyer, I., and Kühnlein, R. P. (2011). Reliable *Drosophila* body fat quantification by a coupled colorimetric assay. *PLoS One* 6:e23796. doi: 10.1371/journal.pone.0023796
- Hoffmann, J., Romey, R., Fink, C., Yong, L., and Roeder, T. (2013). Overexpression of *Sir2* in the adult fat body is sufficient to extend lifespan of male and female *Drosophila*. *Aging* 5, 315–327. doi: 10.18632/aging.100553
- Lange, A. B. (2009). Tyramine: from octopamine precursor to neuroactive chemical in insects. *Gen. Comp. Endocrinol.* 162, 18–26. doi: 10.1016/j.ygcen.2008.05.021
- Lee, H. G., Rohila, S., and Han, K. A. (2009). The octopamine receptor OAMB mediates ovulation via Ca^{2+} /calmodulin-dependent protein kinase II in the *Drosophila* oviduct epithelium. *PLoS One* 4:e4716. doi: 10.1371/journal.pone.0004716
- Lee, H. G., Seong, C. S., Kim, Y. C., Davis, R. L., and Han, K. A. (2003). Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. *Dev. Biol.* 264, 179–190. doi: 10.1016/j.ydbio.2003.07.018
- Li, Y., Fink, C., El-Kholy, S., and Roeder, T. (2015). The octopamine receptor oct β 2R is essential for ovulation and fertilization in the fruit fly *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* 88, 168–178. doi: 10.1002/arch.21211
- Li, Y., Hoffmann, J., Li, Y., Stephano, F., Bruchhaus, I., Fink, C., et al. (2016). Octopamine controls starvation resistance, life span and metabolic traits in *Drosophila*. *Sci. Rep.* 6:35359. doi: 10.1038/srep35359
- Luo, J., Lushchak, O. V., Goergen, P., Williams, M. J., and Nässel, D. R. (2014). *Drosophila* insulin-producing cells are differentially modulated by serotonin and octopamine receptors and affect social behavior. *PLoS One* 9:e99732. doi: 10.1371/journal.pone.0099732
- Maqueira, B., Chatwin, H., and Evans, P. D. (2005). Identification and characterization of a novel family of *Drosophila* β -adrenergic-like octopamine G-protein coupled receptors. *J. Neurochem.* 94, 547–560. doi: 10.1111/j.1471-4159.2005.03251.x
- Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. *J. Comp. Neurol.* 356, 275–287. doi: 10.1002/cne.903560210
- Nässel, D. R., Liu, Y., and Luo, J. (2015). Insulin/IGF signaling and its regulation in *Drosophila*. *Gen. Comp. Endocrinol.* 221, 255–266. doi: 10.1016/j.ygcen.2014.11.021
- Ormerod, K. G., Hadden, J. K., Deady, L. D., Mercier, A. J., and Krans, J. L. (2013). Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae. *J. Neurophysiol.* 110, 1984–1996. doi: 10.1152/jn.00431.2013
- Pfeifferberger, C., Lear, B. C., Keegan, K. P., and Allada, R. (2010). Locomotor activity level monitoring using the *Drosophila* activity monitoring (DAM) system. *Cold Spring Harb. Protoc.* 2010:pdb.prot5518. doi: 10.1101/pdb.prot5518
- Rahn, T., Leippe, M., Roeder, T., and Fedders, H. (2013). EGFR signaling in the brain is necessary for olfactory learning in *Drosophila* larvae. *Learn. Mem.* 20, 194–200. doi: 10.1101/lm.029934.112
- Rajan, A., and Perrimon, N. (2011). *Drosophila* as a model for interorgan communication: lessons from studies on energy homeostasis. *Dev. Cell* 21, 29–31. doi: 10.1016/j.devcel.2011.06.034
- Rajan, A., and Perrimon, N. (2012). *Drosophila* cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. *Cell* 151, 123–137. doi: 10.1016/j.cell.2012.08.019
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi: 10.1016/s0301-0082(99)00016-7
- Roeder, T. (2002). Biochemistry and molecular biology of receptors for biogenic amines in locusts. *Microsc. Res. Tech.* 56, 237–247. doi: 10.1002/jemt.10027
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Roeder, T., Seifert, M., Kähler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. *Arch. Insect Biochem. Physiol.* 54, 1–13. doi: 10.1002/arch.10102
- Saraswati, S., Fox, L. E., Soll, D. R., and Wu, C. F. (2004). Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. *J. Neurobiol.* 58, 425–441. doi: 10.1002/neu.10298
- Selcho, M., Pauls, D., El Jundi, B., Stocker, R. F., and Thum, A. S. (2012). The role of octopamine and tyramine in *Drosophila* larval locomotion. *J. Comp. Neurol.* 520, 3764–3785. doi: 10.1002/cne.23152
- Selcho, M., Pauls, D., Huser, A., Stocker, R. F., and Thum, A. S. (2014). Characterization of the octopaminergic and tyramineric neurons in the central brain of *Drosophila* larvae. *J. Comp. Neurol.* 522, 3485–3500. doi: 10.1002/cne.23616

- Slocumb, M. E., Regalado, J. M., Yoshizawa, M., Neely, G. G., Masek, P., Gibbs, A. G., et al. (2015). Enhanced sleep is an evolutionarily adaptive response to starvation stress in *Drosophila*. *PLoS One* 10:e0131275. doi: 10.1371/journal.pone.0131275
- Suo, S., Kimura, Y., and Van Tol, H. H. (2006). Starvation induces cAMP response element-binding protein-dependent gene expression through octopamine-Gq signaling in *Caenorhabditis elegans*. *J. Neurosci.* 26, 10082–10090. doi: 10.1523/JNEUROSCI.0819-06.2006
- Yang, Z., Yu, Y., Zhang, V., Tian, Y., Qi, W., and Wang, L. (2015). Octopamine mediates starvation-induced hyperactivity in adult *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 112, 5219–5224. doi: 10.1073/pnas.1417838112
- Zhang, T., Branch, A., and Shen, P. (2013). Octopamine-mediated circuit mechanism underlying controlled appetite for palatable food in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 110, 15431–15436. doi: 10.1073/pnas.1308816110

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Octopamine Underlies the Counter-Regulatory Response to a Glucose Deficit in Honeybees (*Apis mellifera*)

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An animal's internal state is a critical parameter required for adaptation to a given environment. An important aspect of an animal's internal state is the energy state that is adjusted to the needs of an animal by energy homeostasis. Glucose is one essential source of energy, especially for the brain. A shortage of glucose therefore triggers a complex response to restore the animal's glucose supply. This counter-regulatory response to a glucose deficit includes metabolic responses like the mobilization of glucose from internal glucose stores and behavioral responses like increased foraging and a rapid intake of food. In mammals, the catecholamines adrenalin and noradrenalin take part in mediating these counter-regulatory responses to a glucose deficit. One candidate molecule that might play a role in these processes in insects is octopamine (OA). It is an invertebrate biogenic amine and has been suggested to derive from an ancestral pathway shared with adrenalin and noradrenalin. Thus, it could be hypothesized that OA plays a role in the insect's counter-regulatory response to a glucose deficit. Here we tested this hypothesis in the honeybee (*Apis mellifera*), an insect that, as an adult, mainly feeds on carbohydrates and uses these as its main source of energy. We investigated alterations of the hemolymph glucose concentration, survival, and feeding behavior after starvation and examined the impact of OA on these processes in pharmacological experiments. We demonstrate an involvement of OA in these three processes in honeybees and conclude there is an involvement of OA in regulating a bee's metabolic, physiological, and behavioral response following a phase of prolonged glucose deficit. Thus, OA in honeybees acts similarly to adrenalin and noradrenalin in mammals in regulating an animal's counter-regulatory response.

Keywords: honeybee, octopamine, glucose deficit, feeding state, hunger, hemolymph, survival, PER

INTRODUCTION

An animal's internal state is a critical parameter required for efficient decision-making toward a behavior that satisfies the animal's needs in a given environment (Rangel et al., 2008). An important aspect of an animal's internal state is the energy state that is adjusted to the needs of an animal by energy homeostasis. Glucose is an essential source of energy, especially for the brain, and a

shortage of glucose therefore triggers a complex response to restore the animal's glucose supply. In mammals, this includes metabolic responses like the mobilization of glucose from internal glucose stores in order to guarantee a constant glucose supply for the brain but also behavioral responses like foraging and a rapid food intake (Ritter et al., 2011). Glucose metabolism in mammals is regulated by the autonomic nervous system, consisting of the parasympathetic and the sympathetic nervous system that together orchestrate the interplay between different metabolic organs. The sympathetic nervous system connects to its target organs via noradrenalin and adrenalin. During a glucose-deficit sympathetic activity increases hepatic glucose output, stimulates glucagon release from the pancreas, inhibits pancreatic insulin release, and blocks glucose uptake in skeletal muscles (reviewed in Nonogaki, 2000; Verberne et al., 2014, 2016; Seoane-Collazo et al., 2015; Elliott et al., 2016). Furthermore, central noradrenergic neurons are involved in behavioral responses to a glucose deficit (Ritter et al., 2001, 2011; Li et al., 2014).

Octopamine (OA), an invertebrate biogenic amine, is similar to adrenalin and noradrenalin in its synthesis, its synthesizing enzymes, and the respective receptors; it is therefore suggested that adrenalin, noradrenalin, and OA derive from one ancestral pathway (Gallo et al., 2016). Based on OA's involvement in the fight-or-flight response, motivation, and aggression and its adipokinetic function in insects a similarity of function between OA in insects and the biogenic amines adrenalin and noradrenalin in vertebrates has been suggested (reviewed in Roeder, 2005). Interestingly, in fruit flies, OA plays a role in starvation-induced hyperactivity (Yang et al., 2015; Yu et al., 2016) and regulates insulin-release, hemolymph sugar concentration (Li et al., 2016), and feeding behavior (Zhang et al., 2013). It can therefore be hypothesized that the mechanisms that regulate an animal's response to a glucose deficit might be evolutionary conserved.

We here tested the hypothesis that the response to a glucose deficit is evolutionary conserved in honeybees (*Apis mellifera*). Adult forager bees feed mainly on carbohydrates and use carbohydrates as their main source of energy, but have no substantial carbohydrate, protein, or lipid reserves and only low glycogen stores in their bodies (Blatt and Roces, 2001; Hrassnigg and Crailsheim, 2005; Ihle et al., 2014; Paoli et al., 2014). Accordingly, a tight control of their sugar metabolism as well as their feeding behavior is necessary to avoid starvation. Therefore, we hypothesized that bees would show a counter-regulatory response to a glucose deficit that might be regulated by OA. We here tested this hypothesis and investigated alterations of the hemolymph glucose concentration, survival, and feeding behavior after starvation in pharmacological experiments.

We demonstrated an involvement of OA in regulating the hemolymph glucose concentration, survival, and feeding behavior. Thus, OA in honeybees acts similarly to adrenalin and noradrenalin in mammals in regulating an animal's counter-regulatory response to a glucose deficit.

MATERIALS AND METHODS

General Treatment of Honeybees

Forager bees from the garden of the Neurobiology Institute, Freie Universität Berlin, Germany were caught 1 day before the experiment, cooled on ice until immobilization and harnessed in plastic tubes. In the evening, around 4:00 p.m., bees were fed to satiation with 30% (w/v) sucrose solution (0.88 M). Overnight they were placed in a dark and humid box at room temperature. On the following day, experiments started at 10:00 a.m. (Felsenberg et al., 2011). When experiments took longer than 24 h, bees were fed each subsequent day at 4:00 p.m. four drops (4 μ l each) of 30% (w/v) sucrose solution (0.88 M in tap water) unless otherwise noted.

Drug Injection

Drugs were injected into the flight muscle as has been demonstrated in Felsenberg et al. (2011). A small hole was made in the cuticle above the flight muscle with a hypodermic needle (Sterican, G21, Braun, Melsungen), and with a glass capillary tube (Selzer, Labortechnik, Waghäusel) 1 μ l of the solution was injected through the hole into the flight muscle.

Measurement of Hemolymph Glucose Concentration

Following the protocol of Rether (2012), hemolymph (1–2 μ l) was collected 15 min following drug injection with a microliter syringe (Hamilton) and a hypodermic needle (Sterican G30, Braun) on the lateral abdomen between two (4th and 5th) tergites. The hemolymph was applied to blood glucose test stripes (Accu-Chek Aviva, Roche Diabetes Care) and the glucose-concentration was measured with a blood sugar meter (Accu-Chek Aviva, Roche Diabetes Care).

Survival of Honeybees

Honeybees were caught, harnessed, and fed as indicated above. Two experiments were carried out. Both experiments started the day after capture: 18 h after the bees had been fed to satiation they were divided into three subgroups that were systemically injected in the flight muscle with 1 μ l OA (10 mM), epinastine (40 mM), or PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄). Bees in the two experiments were treated differently following drug injection. In the first experiment bees remained unfed following drug injection until they died. In the second experiment bees were fed with 30% (w/v) sucrose solution to satiation 2 h following drug injection and were left unfed subsequently until they died. In both experiments, bees were inspected every 6 h after injection and survival was noted. The survival score for each bee was calculated from the number of time points the bee was still alive.

Proboscis Extension Response

The proboscis extension response (PER) was released with three solutions: water (H₂O), 0.1% (w/v) sucrose solution, i.e., 2.9 mM sucrose, and 43% (w/v) sucrose solution, i.e., 1.25 M sucrose. The bees' antennae were touched with a toothpick covered with

one of these solutions and the extension of the bees' probosces was noted. Solutions were presented in an ascending order [first water, second 0.1% (w/v) sucrose solution, third 43% (w/v) sucrose solution] after intervals of 2 min.

Statistics

Statistics were carried out with Prism 6.0 (Graph Pad) and Statistica 10.0 (Statsoft).

Ethics Statement

This study involved insects, i.e., honeybees (*Apis mellifera*). The study was carried out in accordance to the Deutsche Tierschutzgesetz.

RESULTS

Octopamine Increases the Hemolymph Glucose Concentration Depending on the Feeding State

First, we tested the hypothesis that OA is involved in the response to a glucose deficit in honeybees. Therefore, we examined whether OA is involved in regulating the honeybee's hemolymph glucose level depending on its feeding state.

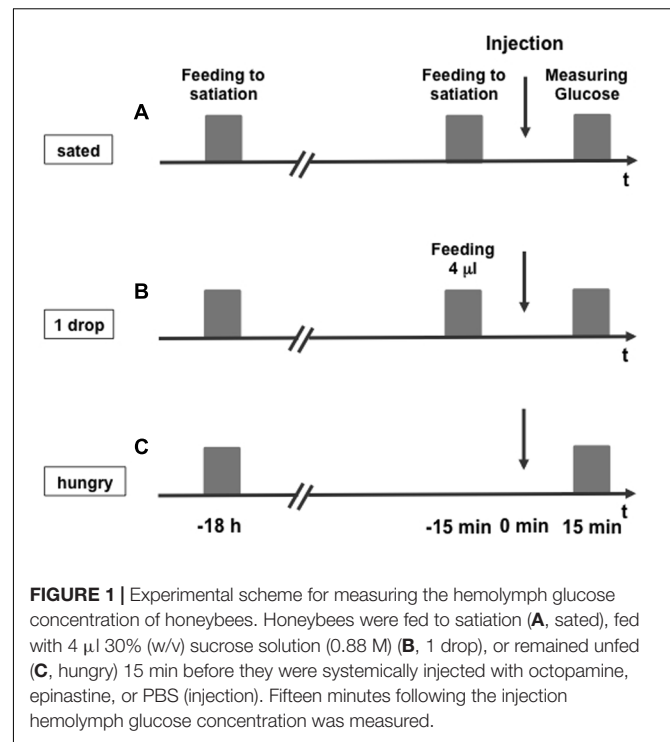
We analyzed three groups of bees: Bees that were fed with 30% (w/v) sucrose solution to satiation, i.e., until they did not extend the proboscis anymore, 15 min before probing the hemolymph glucose level, bees fed with 4 μ l of 30% (w/v) sucrose solution, and bees that were not fed at the same time point (Figure 1).

In two experiments, we tested the impact of OA on the hemolymph glucose level (Figure 2). In the first experiment (Figure 2A), honeybees that were fed to satiation (sated), and honeybees, that were not fed at the same time point (hungry) were compared. In the second experiment (Figure 2B), honeybees that were fed with 4 μ l of 30% (w/v) sucrose solution (1 drop), were compared with honeybees that remained unfed (hungry). Each of the two groups was divided into two subgroups that were systemically injected with either 10 mM OA solved in PBS or with PBS alone. Fifteen minutes later 1–2 μ l of hemolymph were taken from the bees' abdomen, applied to a blood glucose test strip and measured with a blood glucose meter.

Comparison of the PBS-injected groups demonstrated that the hemolymph glucose concentration of hungry bees was significantly lower than the glucose concentration in sated bees was [Figure 2A; Kruskal–Wallis test: $H(2, N = 82) = 54.99$; PBS_{sated}/PBS_{hungry} : $p = 8.0 \times 10^{-9}$] and in bees fed with 4 μ l sucrose (1 drop) [Figure 2B; Kruskal–Wallis test: $H(2, N = 72) = 18.52$; PBS_{1drop}/PBS_{hungry} : $p = 0.0012$].

The hemolymph glucose concentration of bees fed with 4 μ l 30% (w/v) sucrose solution (1 drop) was significantly higher in bees injected with OA than in bees injected with PBS [Figure 2B; Kruskal–Wallis test: $H(2, N = 103) = 33.64$; OA_{1drop} vs. PBS_{1drop} : $p = 0.00054$].

In both experiments, no significant differences between the hemolymph glucose levels of hungry bees that were injected



with OA or PBS were observed [Figure 2A; Kruskal–Wallis test: $H(2, N = 72) = 18.52$; OA_{hungry} vs. PBS_{hungry} : $p = 1$; Figure 2B; Kruskal–Wallis test: $H(2, N = 82) = 54.99$; OA_{hungry} vs. PBS_{hungry} : $p = 1$]. The same holds true for the hemolymph glucose levels of the sated bees [Figure 2A; Kruskal–Wallis test: $H(2, N = 86) = 54.19$; OA_{sated} vs. PBS_{sated} : $p = 1$].

Taken together, these experiments demonstrated an enhancement of the hemolymph glucose level by OA in bees that were fed with a small amount of sucrose, whereas in hungry and in sated bees the effect of OA was not observed.

Epinastine Inhibits the Hemolymph Glucose Concentration Depending on the Feeding State

In order to verify our finding of an effect of OA on the feeding-dependent hemolymph glucose concentration we next examined the effect of epinastine (EPI), an OA-receptor antagonist (Roeder et al., 1998), in the three groups of differently fed bees, i.e., bees that remained hungry, bees that were fed 4 μ l of 30% (w/v) sucrose solution, and bees that were fed to satiation. Again, two different groups of bees were compared in two experiments, sated vs. hungry bees (Figure 3A) and hungry bees vs. bees that were fed with 4 μ l of 30% (w/v) sucrose solution (1 drop) (Figure 3B). In these experiments, 40 mM EPI dissolved in PBS or PBS alone were injected 15 min following feeding and 15 min before probing the hemolymph glucose level.

Comparing the PBS-injected groups revealed a significantly lower concentration of hemolymph glucose in bees that remained

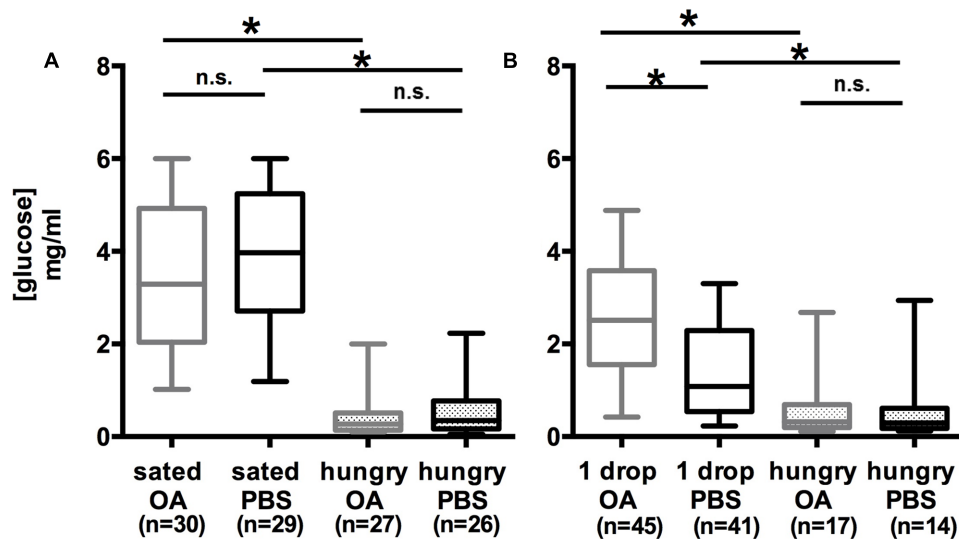


FIGURE 2 | High hemolymph glucose concentration in honeybees following octopamine treatment depends on the feeding state. **(A)** The hemolymph glucose level of bees fed with 30% (w/v) sucrose solution (0.88 M) to satiation (sated PBS) was higher than that of hungry bees that remained unfed (hungry PBS). Octopamine had no effect on the hemolymph glucose concentration of sated (sated PBS vs. sated OA) and hungry (hungry PBS vs. hungry OA) bees. **(B)** The glucose level in the hemolymph of bees fed with 4 μ l 30% (w/v) sucrose solution (0.88 M) (1 drop PBS) was higher than that of hungry bees (hungry PBS). Octopamine increased the hemolymph glucose concentration of bees fed with 4 μ l 30% (w/v) sucrose solution (0.88 M) (1 drop PBS vs. 1 drop OA). * p < 0.05. Number of bees appears in brackets.

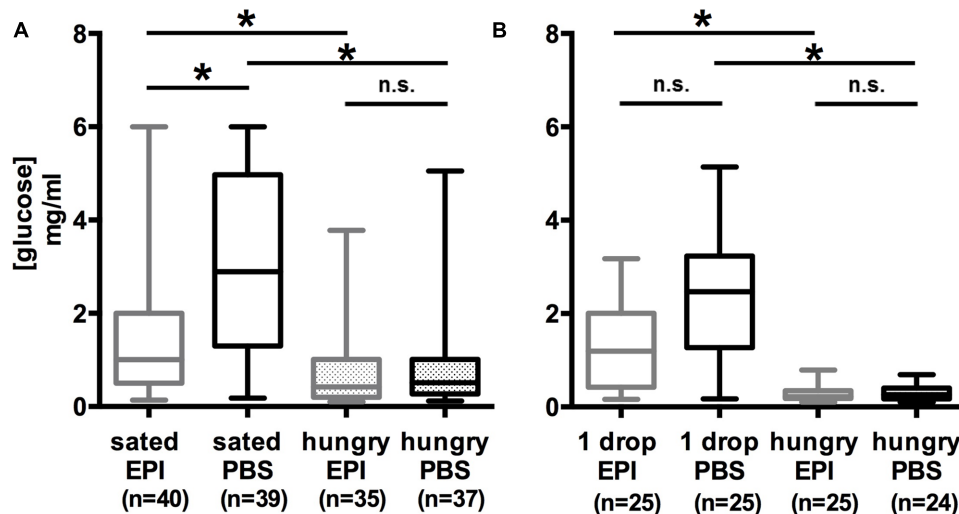
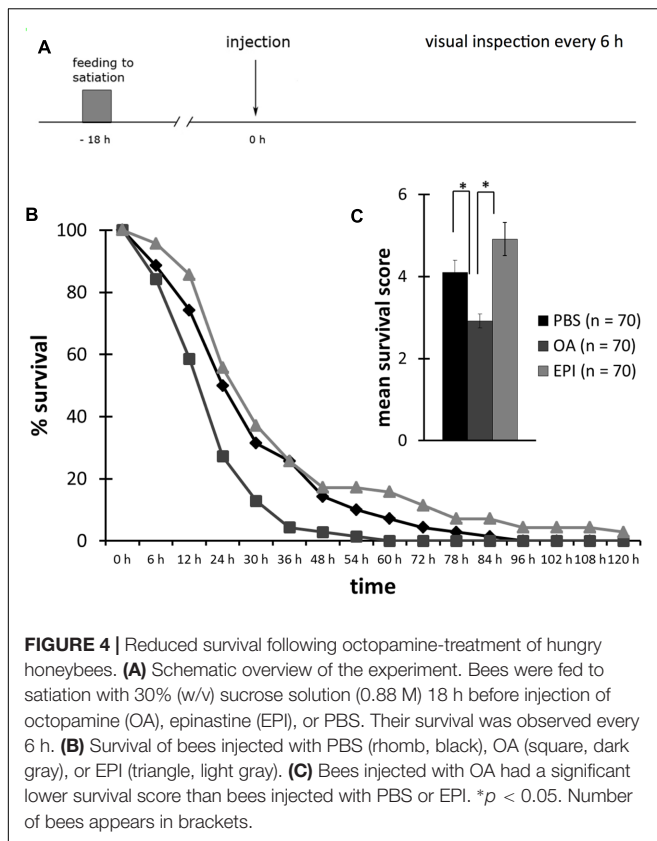


FIGURE 3 | Low hemolymph glucose concentration in honeybees following epinastine treatment depends on the feeding state. **(A)** The hemolymph glucose level of bees fed with 30% (w/v) sucrose solution (0.88 M) to satiation (sated PBS) was higher than that of hungry bees that remained unfed (hungry PBS). Sated and epinastine-treated bees showed a lower hemolymph glucose concentration than sated control animals (sated EPI vs. sated PBS). Epinastine-treatment did not affect hungry bees (hungry EPI vs. hungry PBS). **(B)** The glucose level in the hemolymph of bees fed with 4 μ l 30% (w/v) sucrose solution (0.88 M) (1 drop PBS) was higher than that of hungry bees (hungry PBS). Epinastine-treatment did not affect bees fed with 4 μ l 30% (w/v) sucrose solution (0.88 M) (1 drop EPI vs. 1 drop PBS) and hungry bees (hungry EPI vs. hungry PBS). * p < 0.05. Number of bees appears in brackets.

unfed (hungry) compared to sated bees [Figure 3A; Kruskal–Wallis test: $H(2, N = 111) = 39.43$; $PBS_{\text{hungry}}/PBS_{\text{sated}}$: $p = 1.6 \text{ E-}6$] and hungry bees compared to bees fed with 4 μ l of 30% (w/v) sucrose solution (1 drop) [Figure 3B; Kruskal–Wallis test: $H(2, N = 74) = 42.91$; $PBS_{1\text{drop}}/PBS_{\text{sated}}$: $p = 1.7 \text{ E-}7$].

In sated bees that received an EPI injection the hemolymph glucose concentration was significantly lower than the glucose concentration of bees injected with PBS [Figure 3A; Kruskal–Wallis test: $H(2, N = 114) = 34.40$; $EPI_{\text{sated}}/PBS_{\text{sated}}$: $p = 0.0049$], whereas in bees fed with 4 μ l sucrose (1 drop) the difference



in hemolymph glucose concentration after EPI- and PBS-injection was not significant [Figure 3B; Kruskal–Wallis test: $H(2, N = 75) = 39.56$; EPI_{1drop}/PBS_{1drop} : $p = 0.065$], but the results suggested a less pronounced increase in glucose concentration in bees injected with EPI.

The difference in hemolymph glucose between hungry bees injected with EPI and PBS was not significant [Figure 3A; Kruskal–Wallis test: $H(2, N = 111) = 39.43$; $EPI_{hungry}/PBS_{hungry}$: $p = 1$; Figure 3B; $H(2, N = 74) = 42.91$; $EPI_{hungry}/PBS_{hungry}$: $p = 1$].

Taken together, these experiments demonstrated an inhibitory effect of EPI on the hemolymph glucose level in sated bees but not in bees that were fed with 4 μ l sucrose solution and in hungry bees.

Octopamine Decreases the Survival Rate of Hungry Bees

An appropriate energy supply is essential to maintain an animal during phases with an increased energy demand, for example, when the supply of nutrients is interrupted. Since foragers require a diet high in carbohydrates for survival and glucose is one of the main sugars found in the honeybee's hemolymph (Beutler, 1936; Blatt and Roces, 2001; Ihle et al., 2014; Paoli et al., 2014) we next tested whether OA impacts survival of hungry bees.

We injected bees that were not fed for 18 h (hungry), with 10 mM OA, 40 mM EPI or PBS. We counted the bees that

survived without food 16 times, i.e., every 6 h, until all bees were dead (Figure 4A).

The survival of bees injected with OA was significantly lower than of bees injected with EPI (Figure 4B, rm ANOVA, factor injection: $F_{2,394} = 1.7447$; $p = 0.0206$; Fisher LSD *post hoc* test: $p = 0.0276$). There was no difference between the survival of bees injected with OA or PBS (Fisher LSD *post hoc* test: $p = 0.4064$) and between bees injected with PBS or EPI (Fisher LSD *post hoc* test: $p = 0.1671$).

The mean survival score of OA-injected bees was significantly lower than of PBS- and EPI-injected bees (Figure 4C, one factor ANOVA, factor injection: $F_{2,207} = 10.8305$; $p < 0.001$; Fisher LSD *post hoc* test: PBS vs. OA: $p = 0.0066$; EPI vs. OA: $p < 0.001$). Bees injected with EPI had a higher survival score than did PBS-injected bees, but this difference was not significant (Fisher LSD *post hoc* test: $p = 0.0610$).

Taken together, this experiment demonstrated that OA decreased the survival rate of hungry bees and thus the time span they survive without food.

Feeding Restores the Octopamine-Effect on the Bees Survival

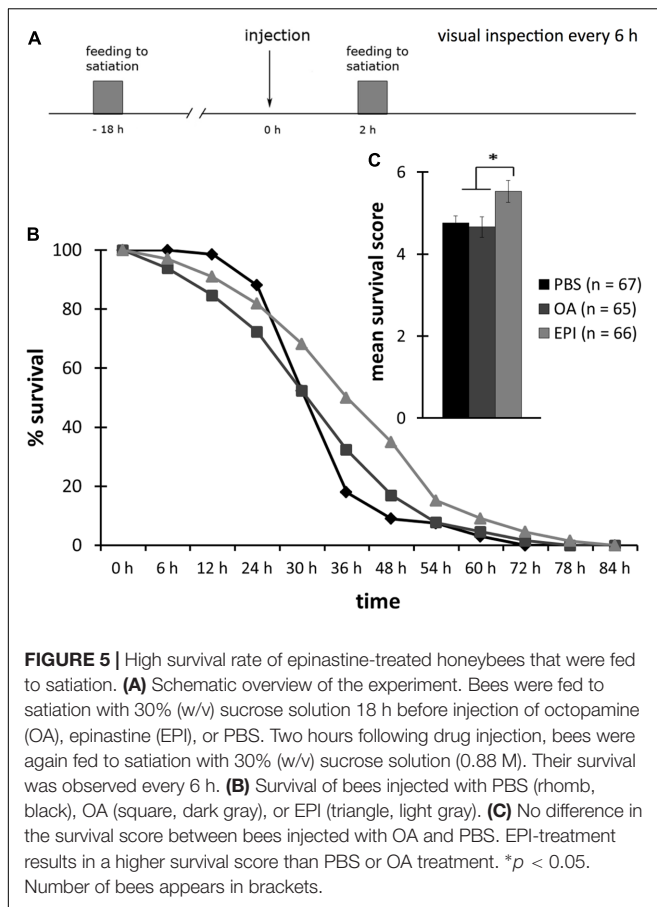
In a second experiment, we considered whether feeding of bees with sucrose following the OA- or EPI-injection restores survival. We again injected bees that were not fed for 18 h (hungry), with 10 mM OA, 40 mM EPI or PBS and fed them to satiation with 30% (w/v) sucrose solution 2 h following drug injection. We counted the number of bees that survived without food 12 times, i.e., every 6 h, until all bees were dead (Figure 5A).

The survival of bees injected with OA or PBS was significantly different (Figure 5B, rm ANOVA, factor injection: $F_{2,364} = 2.0627$; $p = 0.0050$; Fisher LSD *post hoc* test OA vs. PBS: $p = 0.0403$; OA vs. EPI: $p = 0.3070$; PBS vs. EPI: $p = 0.3032$). The survival rate of EPI-treated bees was higher compared to both PBS- and OA-treated bees (one-factor ANOVA, factor injection: $F_{2,195} = 4.118$; $p = 0.0177$; Fisher LSD *post hoc* test EPI vs. PBS: $p = 0.0207$; Fisher LSD *post hoc* test EPI vs. OA: $p = 0.0096$). OA- and PBS-injected bees showed no significant difference in their mean survival rates (Figure 5C, Fisher LSD *post hoc* test OA vs. PBS: $p = 0.7637$).

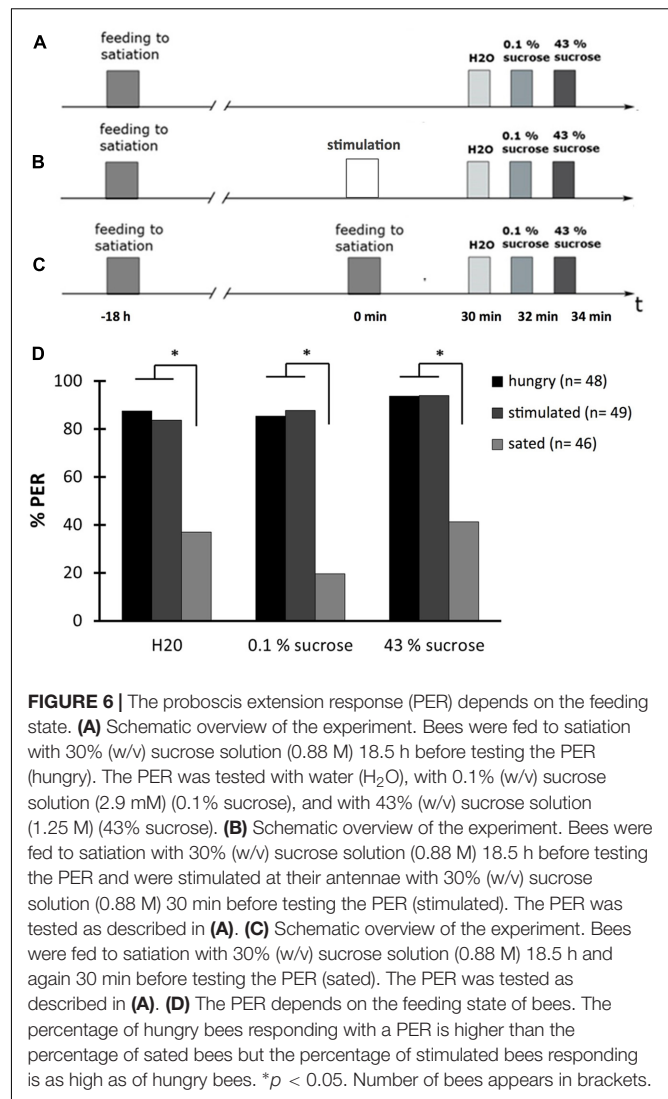
Taken together, this experiment demonstrated that feeding of sucrose 2 h following drug injection restored the OA-dependent decrease of the bees' survival rate, and thus the time span of survival to the level of the PBS-injected control group. Furthermore, feeding resulted in a higher survival rate of bees injected with EPI compared to the PBS-injected control group and thus in an enhancement of the time span of survival following an EPI-treatment.

The Proboscis Extension Response Depends on the Honeybees Feeding State

The response to a glucose deficit is characterized by metabolic and behavioral changes. Therefore, it was prudent to determine whether OA leads to an altered feeding behavior depending on the bees' feeding state. Part of the feeding behavior of honeybees



is the proboscis extension response (PER), which is a reflex-like response to a food stimulus: When the antennae or the proboscis of a honeybee are touched with sucrose solution, the bee extends its proboscis. However, when fed with sucrose, bees decrease this response until it is not elicited anymore. Above we demonstrated that bees that were fed with sucrose until extension could not be elicited anymore showed a higher hemolymph glucose concentration than did bees that were not fed or that were fed with 4 μ l sucrose solution. Thus, it seemed likely that the feeding state impacts the PER. However, this is not entirely clear because multiple stimulations of the antennae with sucrose solution could lead to a decrease of the PER, i.e., habituation. We here tested the hypothesis that the feeding state impacts the PER and examined the PER in bees that were fed to satiation with 30% (w/v) sucrose solution 18.5 h before testing the PER. These bees were divided into three groups. One was not fed again before the PER test (hungry, **Figure 6A**), one group received multiple stimulations with 30% (w/v) sucrose solution to the antennae 30 min before the PER test (stimulated, **Figure 6B**), and one group that was fed again with 30% (w/v) sucrose solution to satiation 30 min before testing the PER (sated, **Figure 6C**). We tested the PER with water, 0.1% (w/v) sucrose solution, and 43% (w/v) sucrose solution 30 min after feeding, respectively stimulation of the antennae.

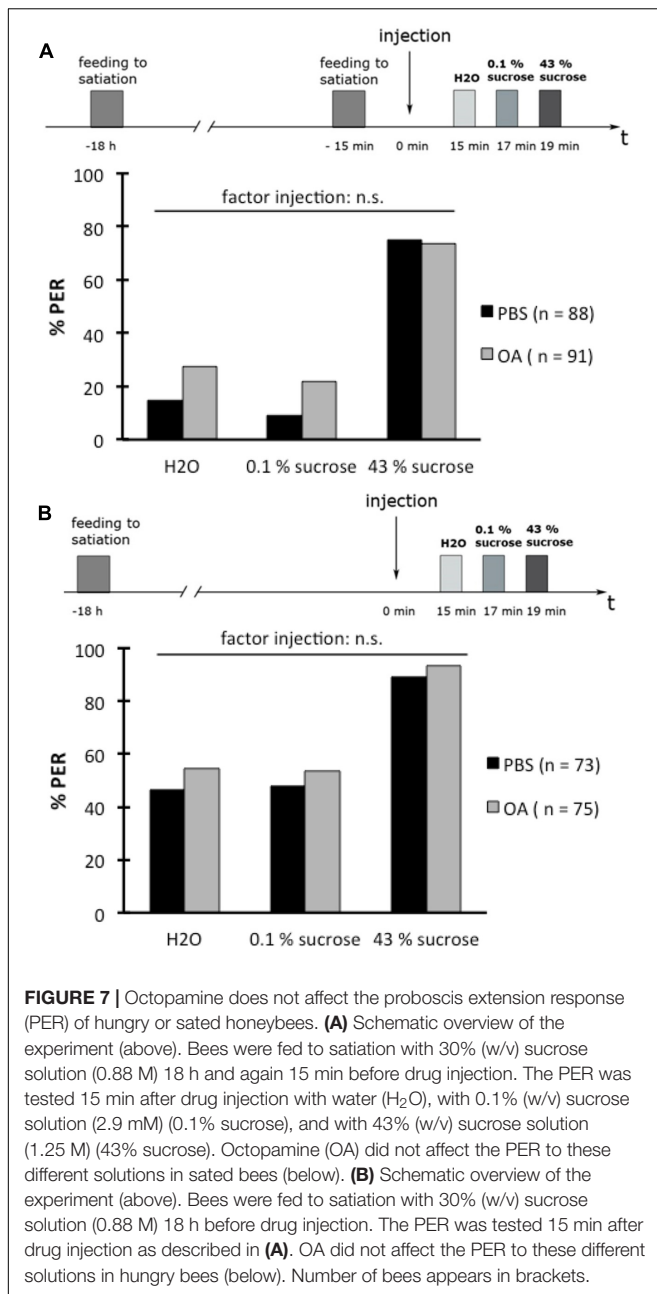


The percentage of bees responding with a PER was not different between the group that was not fed before the PER test and the group that received the sucrose stimulation (**Figure 6D**; rm ANOVA, factor treatment: $F_{2,140} = 51.1503$; $p < 0.001$; Fisher LSD *post hoc* test: hungry bees vs. stimulated bees: $p = 0.9426$). However, a statistically significant difference between these two groups and the fed bees was found: a lower percentage of fed bees responded to all three stimuli with a PER (**Figure 6D**; Fisher LSD *post hoc* test: sated bees vs. hungry bees: $p < 0.001$, stimulated bees vs. sated bees: $p < 0.001$).

Thus, the percentage of bees responding with a PER to water and sucrose stimulation depended on the feeding state of a bee and not on the repetitive stimulation of their antennae during feeding.

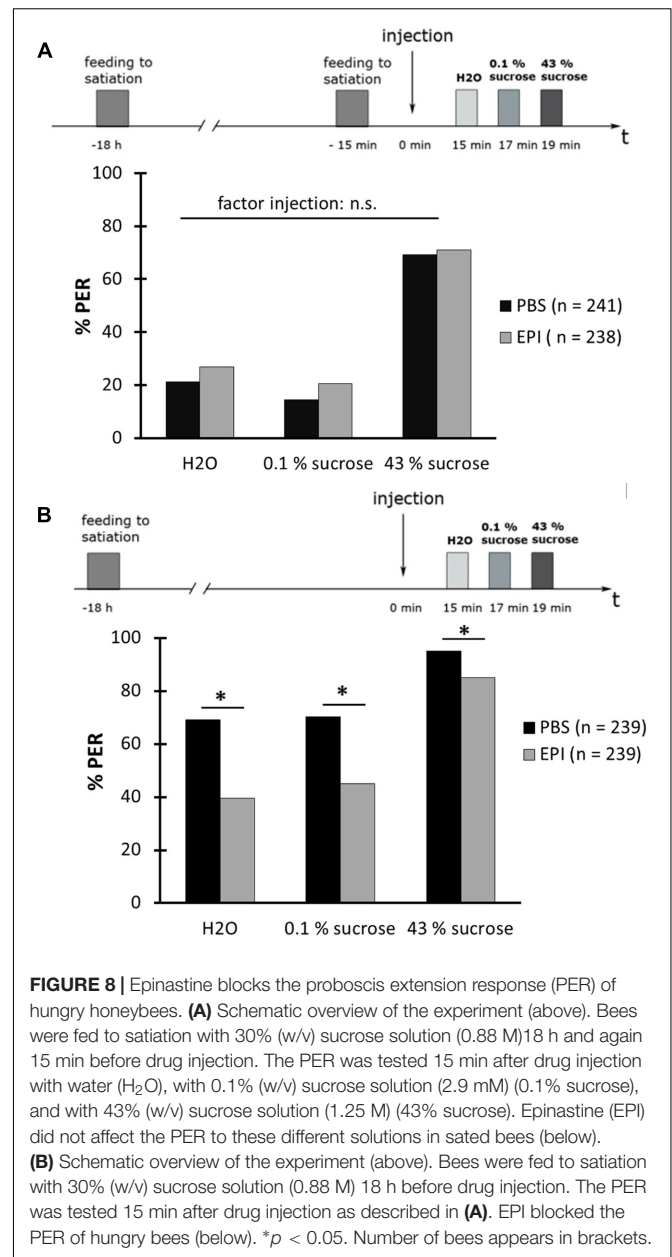
OA-Injection Does Not Affect the PER in Hungry Bees or Sated Bees

Next, we tested whether OA is involved in the PER depending on the bees' feeding state. In two experiments that were done



in parallel, we tested the PER with water, 0.1% (w/v) sucrose solution, and 43% (w/v) sucrose solution. In the first experiment, bees were examined that were fed with 30% (w/v) sucrose solution until the PER was not elicited anymore, 30 min before the PER test (sated) (**Figure 7A**). In the second experiment, bees that were not fed at the same time point (hungry) were tested (**Figure 7B**). In both experiments, bees were divided into two groups, those that received an injection of 10 mM OA or those that received PBS 15 min before the PER test.

OA-injection did not have an effect on the PER rate in both sated bees (**Figure 7A**; rm ANOVA, factor injection: $F_{1;177} = 3.1551$; $p = 0.0774$) and hungry bees (**Figure 7B**; rm



ANOVA, factor injection: $F_{1;146} = 1.1004$; $p = 0.2956$) compared to the PER rate of bees injected with PBS.

Epinastine Reduces the Proboscis Extension Response Rate in Hungry Bees

We next determined whether EPI affects the PER. Again, we carried out two experiments in parallel, one with bees that were fed to satiation 30 min before the PER-test (sated) (**Figure 8A**) and another with bees that were not fed at the same time point (hungry) (**Figure 8B**). Bees in both experiments were divided into two groups: one group that received an injection with EPI (40 mM) while the other group received PBS-injection.

Fifteen minutes following these injections, the PER was tested successively with water, 0.1% (w/v) sucrose solution, and with 43% (w/v) sucrose solution.

In sated bees, the injection of EPI had no effect on the PER rate when compared to the PER rate of PBS-injected bees (**Figure 8A**; rm ANOVA, factor injection: $F_{1,477} = 2.2498$; $p = 0.1343$). Hungry bees showed a significantly lower PER rate after an injection with 40 mM EPI than after an injection with PBS (**Figure 8B**; rm ANOVA, factor injection $\times$ sugar solution: $F_{2,954} = 12.843$; $p < 0.001$).

This result suggested that EPI inhibits the high PER of hungry bees and that OA receptors, and thus OA, might be involved.

α -Methyl-*p*-Tyrosine Inhibits the PER Rate in Hungry Bees and Can Be Rescued by Octopamine But Not Dopamine

Although we could not detect an effect of OA on the PER of honeybees the results of the previous experiment suggested that OA might be involved. Therefore, we next examined whether there is evidence that OA is required to modulate the PER in hungry bees. For this, we utilized the drug α -methyl-*p*-tyrosine (AMT). AMT inhibits synthesis of both OA and dopamine (DA) and therefore reduces the amount of biogenic amines (Stevenson et al., 2000). AMT does not block receptors irreversibly as is the case with the use of receptor antagonists. An injection of OA or DA can therefore restore the amount of these otherwise depleted amines. Accordingly, AMT was used to stop the synthesis of OA and DA to examine the effect of externally added OA and DA on the bees' PER.

In this experiment (**Figure 9A**), bees were fed to satiation 18 h before injection with 30.5 mM AMT. Twenty-four hours later the PER was tested with water, 0.1% (w/v) sucrose solution, and 43% (w/v) sucrose solution. Following this test, bees were injected with 10 mM OA, 10 mM DA, or PBS. Forty-eight hours later the second PER-test was carried out, again using water, 0.1% (w/v) sucrose solution, and 43% (w/v) sucrose solution to elicit the PER.

In the first PER-test, the percentage of AMT-injected bees responding to the three stimuli was significantly lower than that of PBS-injected bees [**Figure 9B**; rm ANOVA, factor injection $\times$ sugar solution: $F_{2,1520} = 6.368$; $p = 0.0012$; Fisher LSD *post hoc* test for H₂O: PBS vs. AMT: $p < 0.001$; for 0.1% (w/v) sucrose solution: PBS vs. AMT: $p < 0.001$; for 43% (w/v) sucrose solution: PBS vs. AMT: $p > 0.001$].

The effect of AMT was still observed 48 h after its injection (**Figure 9C**; rm ANOVA, factor injection: $F_{3,227} = 5.7446$; $p = 0.0009$, Fisher LSD *post hoc* test: AMT–PBS vs. PBS–PBS: $p = 0.0212$; **Figure 9D**; rm ANOVA, factor injection $F_{3,226} = 4.924$; $p = 0.0025$; Fisher LSD *post hoc* test: AMT–PBS vs. PBS–PBS: $p = 0.0117$). After a second injection with OA (AMT–OA) the PER-rate was no longer different from the control group that was injected with PBS at the same time point (PBS–PBS) ($p = 0.2097$). The PER rates of the groups AMT–OA and AMT–PBS differed significantly ($p = 0.0005$). The difference between the groups AMT–OA and PBS–OA was not significant ($p = 0.9304$) (**Figure 9C**).

A second injection with DA 24 h after the AMT injection (AMT–DA) did not increase the PER rate—there was still a significant difference between the groups AMT–DA and PBS–PBS (rm ANOVA, factor injection: $F_{3,226} = 4.9244$, Fisher LSD *post hoc* test: AMT–DA vs. PBS–PBS: $p = 0.0011$) and no significant difference between the groups AMT–DA and AMT–PBS ($p = 0.4816$) (**Figure 9D**).

Taken together, this experiment demonstrated that OA but not DA rescued the inhibiting effect of AMT on the percentage of hungry bees responding with a PER to water, 0.1% (w/v) and 43% (w/v) sucrose solution. Thus, we conclude that OA is involved in enhancing the PER and thus the feeding response of hungry bees.

DISCUSSION

The Hemolymph Glucose Concentration Depends on the Bees' Feeding State and Is Affected by Octopamine

Here we investigated a role for OA in the counter-regulatory response to a glucose deficit and therefore examined the glucose metabolism, survival, and feeding behavior of hungry and sated bees. We demonstrated that the glucose concentration of the bees' hemolymph depends on the bees' feeding state and that the hemolymph glucose concentration is modulated by OA.

We report that OA enhanced the glucose concentration in bees that were fed with 4 μ l of 30% (w/v) sucrose solution but did not affect the hemolymph glucose concentration in hungry bees and bees that were fed to satiation. In hungry bees OA might not have enhanced the glucose concentration because glucose stores were nearly empty. In contrast, in sated bees, a ceiling effect might have been observed, because the hemolymph glucose concentration was as high as possible, and, therefore, no further enhancement following an OA injection was observed. In line with a possible ceiling effect, we demonstrated that the OA receptor antagonist epinastine inhibited the hemolymph glucose concentration in sated bees. We conclude from these data that OA enhances the hemolymph glucose concentration as long as carbohydrate stores were available.

Support for our conclusion comes from an earlier study in the fruit fly *Drosophila melanogaster*, demonstrating a reduced hemolymph concentration of glucose and trehalose in flies, mutant for the tyramine- β -hydroxylase (T β h) gene (T β h^{nM18}) encoding T β h, which converts tyramine to OA (Li et al., 2016). These mutants showed higher insulin release rates than control flies suggesting an increased storage of carbohydrates. In line with this observation, in the nematode *Caenorhabditis elegans* the biosynthesis of OA has been shown to be upregulated upon starvation by upregulation of the t β h-1 gene activity (Tao et al., 2016).

The mechanisms underlying an OA-dependent release of glucose into the honeybees' hemolymph remain unknown. However, hints toward a possible mechanism come from a study by Blatt and Roces (2001). In honeybees, three main sugars are found in the hemolymph: trehalose, glucose, and

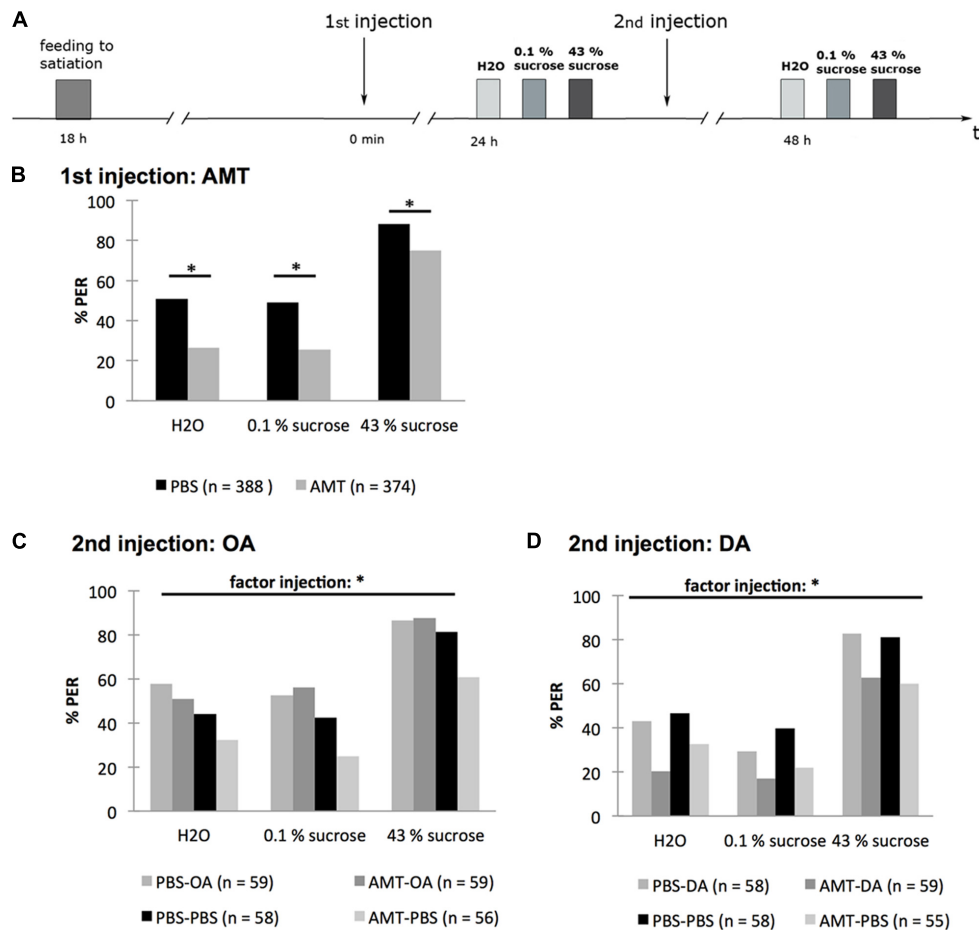


FIGURE 9 | Octopamine but not dopamine rescues the effect of α -methyl-*p*-tyrosine (AMT) on the PER of honeybees. **(A)** Schematic overview of the experiment. Bees were fed to satiation with 30% (w/v) sucrose solution (0.88 M) 18 h before injection of AMT (first injection). Twenty-four hours following AMT injection the PER was tested touching the antennae with water (H₂O), 0.1% (w/v) sucrose solution (2.9 mM) (0.1% sucrose), 43% (w/v) sucrose solution (1.25 M) (43% sucrose). Following this test, subgroups of bees were injected with octopamine (OA), dopamine (DA), or PBS. Another 24 h later the PER was tested again with water (H₂O), 0.1% (w/v) sucrose solution (2.9 mM) (0.1% sucrose), 43% (w/v) sucrose solution (1.25 M) (43% sucrose). **(B)** One day after injection of AMT or PBS significant differences between groups were found. **(C)** Injection of OA rescued the AMT effect. **(D)** Injection of DA did not rescue the AMT effect. * $p < 0.05$. Number of bees appears in brackets.

fructose (Fell, 1990). Blatt and Roces (2001) demonstrated that with an increasing metabolic rate, the hemolymph concentration of glucose and fructose relative to trehalose increased, such that the overall hemolymph sugar levels remained unchanged. Blatt and Roces (2001) concluded that trehalose synthesis was not rapid enough to maintain stable trehalose concentrations at high metabolic rates, i.e., when demand became too great. They suggested that the decreasing trehalose concentration might result in a feedback signal to the proventriculus eliciting release of sucrose into the ventricle. In the ventricle sucrose is cleaved into glucose and fructose, and both sugars are released from the ventricle into the hemolymph (Blatt and Roces, 2001). We demonstrated that OA increased the hemolymph glucose concentration. Thus, OA might enhance the metabolic rate such that the trehalose concentration decreases leading to an increase of hemolymph glucose concentration.

The Effect of Octopamine on the Honeybees' Survival Is Restored by Feeding

We demonstrated that hungry honeybees, which received a systemic injection of OA after 18 h of fasting, survived for a shorter time period afterward (without food) than control bees having received a PBS-injection did. We concluded that OA activates available energy stores during food shortage at the expense of long-term survival. Indeed, when bees were fed once following the OA treatment, survival was restored, indicating that feeding, i.e., energy intake, compensated for the increase in the metabolic rate by OA. We found that the OA receptor antagonist epinastine prolonged survival of the bees fed once, supporting an involvement of OA receptors in regulating the bees' metabolic rate and thus survival. Moreover, this result suggests that blockage of OA-receptors slows down

the mobilization of available energy, such that the bees' survival is prolonged. In line with our observation, $\text{t}\beta\text{h}^{\text{M18}}$ mutant flies died later from starvation than wild-type controls did (Scheiner et al., 2014; Li et al., 2016). Moreover, an ectopic release of OA during starvation reduces survival (Li et al., 2016). These findings in fruit flies again suggested that an increased OA-level mobilizes and empties energy stores, leading to an accelerated starvation (Scheiner et al., 2014; Li et al., 2016). In *C. elegans*, blocking the biosynthesis of OA (by means of RNAi against the $\text{t}\beta\text{h}$ -1 gene activity) has been shown to lead to contrary results, i.e., a reduced survival rate after 3 days of fasting compared to wild-type worms (Tao et al., 2016). This reduced survival rate is rescued by application of OA (Tao et al., 2016). In line with our interpretation, Tao et al. (2016) hypothesized that OA mobilizes energy stores. However, in *C. elegans* mobilization of energy stores seems to enable long-term survival instead of reducing it as has been observed in *D. melanogaster* and *A. mellifera*. The reason for this discrepancy remains unclear. However, it might well be that these differences in OA-dependent long-term survival are due to differences in energy storage and energy metabolism in these three invertebrate species.

We demonstrated that the hemolymph glucose concentration is near zero in bees starved for 18 h. Furthermore, we demonstrated that OA reduced the survival rate of bees that were already starved for 18 h. Since we hypothesized that OA increases the metabolic rate in honeybees, the question remains which energy stores are activated after such a long starvation period. Wang et al. (2016) reported that thorax and abdomen glycogen and triglycerides are decreased 12 h after starvation in honeybees. Thus, it might well be that in our experiments OA triggered the depletion of glycogen and triglyceride stores when applied 12 h after feeding and that this mechanism led to a decreased survival of honeybees. Interestingly, in *C. elegans* it occurred that OA induces the expression of a lipase gene resulting in lipid mobilization (Tao et al., 2016). Furthermore, in the cockroach, *Blaberus discoidalis*, OA has been shown to be a potent activator of fat body glycogen phosphorylase, an enzyme that is needed to mobilize glucose from glycogen stores (Park and Keeley, 1998).

Octopamine Is Involved in Regulating the Honeybees' Feeding Behavior

In addition to a role for OA in regulating the hemolymph glucose concentration, we found an involvement of OA in regulating the bees' feeding behavior. We demonstrated that the PER, which is a component of the bees' feeding behavior, depends on the feeding state of honeybees and not on repeated stimulations of the antennae with sucrose solution, which theoretically could result in habituation. Moreover, we found that systemic application of OA does not affect the PER to different sucrose concentrations in sated and hungry bees. However, applying the OA-receptor antagonist epinastine did reduce the PER in hungry bees, suggesting that in hungry bees a ceiling effect is observed for OA, i.e., that the maximum of OA has been released in hungry bees already, such that additional OA does not affect behavior anymore. Indeed, when we inhibited the biosynthesis of OA and DA using AMT, the PER rate of hungry

bees is reduced and can be rescued by the application of OA but not DA.

Several studies in *Drosophila* fruit flies and the blowfly *Phormia regina* have demonstrated that the hunger state affects the PER via a modulation of the sugar sensitivity (Moss and Dethier, 1983; Inagaki et al., 2012, 2014; Marella et al., 2012; Scheiner et al., 2014; Kain and Dahanukar, 2015; Yapici et al., 2016). Neuropeptide F and DA have been shown to be involved in PER by enhancing the responsiveness of taste sensory neurons (Inagaki et al., 2012, 2014; Marella et al., 2012). Our data indicated that OA modulates the PER as well. In line with this notion, an earlier study in honeybees demonstrated that depleting the nervous system of monoamines by the use of reserpine inhibited the PER, which was restored in reserpinized unresponsive bees by injection of OA (Braun and Bicker, 1992). In fruit flies, a reduced PER in starved $\text{t}\beta\text{h}^{\text{M18}}$ mutant flies has been demonstrated, which is rescued by feeding with OA (Scheiner et al., 2014).

Our data in honeybees and data of Scheiner et al. (2014) in fruit flies clearly indicate that OA modulates the PER depending on the insects' feeding-state. However, the exact mechanism remains unclear. OA could act as a neurotransmitter and/or as a hormone when it is released during starvation.

Is Octopamine Mediating the Stress-Response in Insects?

In mammals, starvation results in an activation of central and sympathetic catecholaminergic neurons, which regulate the release of glucose into the blood and modulate feeding behavior (Nonogaki, 2000; Ritter et al., 2001, 2011; Li et al., 2014; Morton et al., 2014; Verberne et al., 2014, 2016). Our results indicate that OA plays a role in regulating the honeybees' energy state and behavior in response to starvation, supporting the hypothesis that OA is the functional homolog of adrenalin and noradrenalin.

Previous studies in honeybees have demonstrated a role of OA in the context of different physiological processes. In seminal studies on appetitive learning the activation of an octopaminergic Vummx1 neuron or the injection of OA into brain structures critically involved in insect olfactory learning, replaced the unconditioned stimulus, i.e., a sucrose solution (Hammer, 1993; Hammer and Menzel, 1998). Therefore, it has long been hypothesized that OA is the transmitter of the reward system in honeybees and other insects. Lately this hypothesis has seemed controversial, because in the fruit fly OA plays a role in formation of aversive memories as well (Wu et al., 2013), and short-term, but not long-term, memory formation depends on OA (Burke et al., 2012). Thus, the role of OA in learning and memory formation of insects, including the honeybee, remains unclear. Furthermore, OA modulates sensory processes, like vision, olfaction, and gustation (Braun and Bicker, 1992; Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995; Scheiner et al., 2002; Rein et al., 2013), locomotor and heart activity (Fussnecker et al., 2006; Bloch and Meshi, 2007), and the bees' division of labor and dance communication (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999; Barron et al., 2002, 2007;

Schulz et al., 2002; Barron and Robinson, 2005; Giray et al., 2007; Lehmann et al., 2011; Reim and Scheiner, 2014).

Interestingly, noradrenalin and adrenalin modulate taste and olfaction, play a role in cardiovascular regulation and affect memory formation in mammals as well (Herness et al., 2002; Doucette et al., 2007; Roozendaal et al., 2008; Verberne et al., 2014; Tank and Lee Wong, 2015; Ness and Calabrese, 2016; Doyle and Meeks, 2017). Given that OA is a functional homolog of noradrenalin and adrenalin in regulating hunger-stress our results support the notion that OA has similar functions as these two catecholamines in triggering the animal's physiological and behavioral stress-responses (Corbet, 1991; Roeder, 2005; Even et al., 2012). Conceptualizing OA as an insect stress hormone would explain why physiological processes as different as locomotion and learning and memory formation are modulated by OA. However, it would still be an open question how the role of OA in the regulation of the bee's division of labor fits into this concept. Interestingly, it has been demonstrated that stressors like the loss of foragers, starvation, and diseases impact the division of labor, i.e., accelerate the onset of foraging (Schulz et al., 1998; Toth and Robinson, 2005; Higes et al., 2008; Goblirsch et al., 2013). At the same time, it has been shown that the brain OA-level is higher in foragers than in nurse bees (Harris and Woodring, 1992; Schulz et al., 2002; Lehman et al., 2006) and that OA enhances the likelihood to forage (Barron et al., 2002; Schulz et al., 2002; Barron and Robinson, 2005). Thus, an age-dependent increase of OA up to a critical threshold might result in the induction of foraging. OA released as a physiological response to stress might add up to the age-dependent OA-concentration such that the critical OA-threshold

to induce foraging is reached earlier and precocious foraging can be observed.

AUTHOR CONTRIBUTIONS

CB designed experiments, acquired data, analyzed data, interpreted data, and critically revised the manuscript; OS designed experiments, acquired data, analyzed data; JG analyzed data and interpreted data; RZ and AÖ acquired and analyzed data; DE acquired funding, conceptualized and designed experiments, supervised study, interpreted data, and wrote the manuscript.

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REFERENCES

- Barron, A. B., Maleszka, R., Vander Meer, R. K., and Robinson, G. E. (2007). Octopamine modulates honey bee dance behavior. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1703–1707. doi: 10.1073/pnas.0610506104
- Barron, A. B., and Robinson, G. E. (2005). Selective modulation of task performance by octopamine in honey bee (*Apis mellifera*) division of labour. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 191, 659–668. doi: 10.1007/s00359-005-0619-7
- Barron, A. B., Schulz, D. J., and Robinson, G. E. (2002). Octopamine modulates responsiveness to foraging-related stimuli in honey bees (*Apis mellifera*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 188, 603–610. doi: 10.1007/s00359-002-0335-5
- Beutler, R. (1936). Über den Blutzucker der Biene. *Z. Vgl. Physiol.* 24, 71–115. doi: 10.1007/BF00340968
- Blatt, J., and Roces, F. (2001). Haemolymph sugar levels in foraging honeybees (*Apis mellifera* carnica): dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. *J. Exp. Biol.* 204, 2709–2716.
- Bloch, G., and Meshi, A. (2007). Influences of octopamine and juvenile hormone on locomotor behavior and period gene expression in the honeybee, *Apis mellifera*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 193, 181–199. doi: 10.1007/s00359-006-0179-5
- Braun, G., and Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* 67, 588–598.
- Burke, C. J., Huettneroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614
- Corbet, S. A. (1991). A fresh look at the arousal syndrome of insects. *Adv. Insect Physiol.* 23, 81–116. doi: 10.1016/S0065-2806(08)60092-2
- Doucette, W., Milder, J., and Restrepo, D. (2007). Adrenergic modulation of olfactory bulb circuitry affects odor discrimination. *Learn. Mem.* 14, 539–547. doi: 10.1101/lm.606407
- Doyle, W. L., and Meeks, J. P. (2017). Heterogeneous effects of norepinephrine on spontaneous and stimulus-driven activity in the male accessory olfactory bulb. *J. Neurophysiol.* 117, 1342–1351. doi: 10.1152/jn.00871.2016
- Elliott, R. H., Matthews, V. B., Rudnicka, C., and Schlaich, M. P. (2016). Is it time to think about the sodium glucose co-transporter 2 sympathetically? *Nephrology* 21, 286–294. doi: 10.1111/nep.12620
- Erber, J., and Kloppenburg, P. (1995). The modulatory effects of serotonin and octopamine in the visual system of the honey bee (*Apis mellifera* L.). *J. Comp. Physiol. A* 176, 111–118. doi: 10.1007/BF00197757
- Even, N., Devaud, J. M., and Barron, A. B. (2012). General stress responses in the honey bee. *Insects* 3, 1271–1298. doi: 10.3390/insects3041271
- Fell, R. D. (1990). The qualitative and quantitative analysis of insect hemolymph sugars by high performance thin-layer chromatography. *Comp. Biochem. Physiol.* 95A, 539–544. doi: 10.1016/0300-9629(90)90735-B
- Felsenberg, J., Gehring, K. B., Antemann, V., and Eisenhardt, D. (2011). Behavioural pharmacology in classical conditioning of the proboscis extension response in honeybees (*Apis mellifera*). *J. Vis. Exp.* 2011, 2282. doi: 10.3791/2282
- Fussnecker, B. L., Smith, B. H., and Mustard, J. A. (2006). Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (*Apis mellifera*). *J. Insect Physiol.* 52, 1083–1092. doi: 10.1016/j.jinsphys.2006.07.008
- Gallo, V. P., Accordi, F., Chimenti, C., Civinini, A., and Crivellato, E. (2016). Catecholaminergic system of invertebrates: comparative and evolutionary aspects in comparison with the octopaminergic system. *Int. Rev. Cell Mol. Biol.* 322, 363–394. doi: 10.1016/bs.ircmb.2015.12.006

- Giray, T., Galindo-Cardona, A., and Oskay, D. (2007). Octopamine influences honey bee foraging preference. *J. Insect Physiol.* 53, 691–698. doi: 10.1016/j.jinsphys.2007.03.016
- Goblirsch, M., Huang, Z.-Y., and Spivak, M. (2013). Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. *PLoS ONE* 8:e58165. doi: 10.1371/journal.pone.0058165
- Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63. doi: 10.1038/366059a0
- Hammer, M., and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* 5, 146–156.
- Harris, J. W., and Woodring, J. (1992). Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. *J. Insect Physiol.* 38, 29–35. doi: 10.1016/0022-1910(92)90019-A
- Herness, S., Zhao, F. L., Kaya, N., Lu, S. G., Shen, T., and Sun, X. D. (2002). Adrenergic signalling between rat taste receptor cells. *J. Physiol.* 543, 601–614. doi: 10.1113/jphysiol.2002.020438
- Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V., Barrios, L., et al. (2008). How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environ. Microbiol.* 10, 2659–2669. doi: 10.1111/j.1462-2920.2008.01687.x
- Hrassnigg, N., and Crailsheim, K. (2005). Differences in drone and worker physiology in honeybees (*Apis mellifera*). *Apidologie* 36, 255–277. doi: 10.1051/apido:2005015
- Ihle, K. E., Baker, N. A., and Amdam, G. V. (2014). Insulin-like peptide response to nutritional input in honey bee workers. *J. Insect Physiol.* 69, 49–55. doi: 10.1016/j.jinsphys.2014.05.026
- Inagaki, H. K., Ben-Tabou De-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., et al. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595. doi: 10.1016/j.cell.2011.12.022
- Inagaki, H. K., Panse, K. M., and Anderson, D. J. (2014). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in *Drosophila*. *Neuron* 84, 806–820. doi: 10.1016/j.neuron.2014.09.032
- Kain, P., and Dahanukar, A. (2015). Secondary taste neurons that convey sweet taste and starvation in the *Drosophila* brain. *Neuron* 85, 819–832. doi: 10.1016/j.neuron.2015.01.005
- Kloppenborg, P., and Erber, J. (1995). The modulatory effects of serotonin and octopamine in the visual system of the honey bee (*Apis mellifera* L.). *J. Comp. Physiol. A* 176, 119–129. doi: 10.1007/BF00197758
- Lehman, H. K., Schulz, D. J., Barron, A. B., Wraight, L., Hardison, C., Whitney, S., et al. (2006). Division of labor in the honey bee (*Apis mellifera*): the role of tyramine beta-hydroxylase. *J. Exp. Biol.* 209, 2774–2784. doi: 10.1242/jeb.02296
- Lehmann, M., Gustav, D., and Galizia, C. G. (2011). The early bee catches the flower - circadian rhythmicity influences learning performance in honey bees, *Apis mellifera*. *Behav. Ecol. Sociobiol.* 65, 205–215. doi: 10.1007/s00265-010-1026-9
- Li, A. J., Wang, Q., Dinh, T. T., Powers, B. R., and Ritter, S. (2014). Stimulation of feeding by three different glucose-sensing mechanisms requires hindbrain catecholamine neurons. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 306, R257–R264. doi: 10.1152/ajpregu.00451.2013
- Li, Y., Hoffmann, J., Stephano, F., Bruchhaus, I., Fink, C., and Roeder, T. (2016). Octopamine controls starvation resistance, life span and metabolic traits in *Drosophila*. *Sci. Rep.* 6:35359. doi: 10.1038/srep35359
- Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. *Neuron* 73, 941–950. doi: 10.1016/j.neuron.2011.12.032
- Morton, G. J., Meek, T. H., and Schwartz, M. W. (2014). Neurobiology of food intake in health and disease. *Nat. Rev. Neurosci.* 15, 367–378. doi: 10.1038/nrn3745
- Moss, C. F., and Dethier, V. G. (1983). Central nervous system regulation of finicky feeding by the blowfly. *Behav. Neurosci.* 97, 541–548. doi: 10.1037/0735-7044.97.4.541
- Ness, D., and Calabrese, P. (2016). Stress effects on multiple memory system interactions. *Neural Plast* 2016, 20. doi: 10.1155/2016/4932128
- Nonogaki, K. (2000). New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* 43, 533–549. doi: 10.1007/s001250051341
- Paoli, P. P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S. W., Simpson, S. J., et al. (2014). Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids* 46, 1449–1458. doi: 10.1007/s00726-014-1706-2
- Park, J. H., and Keeley, L. L. (1998). The effect of biogenic amines and their analogs on carbohydrate metabolism in the fat body of the cockroach *Blaberus discoidalis*. *Gen. Comp. Endocrinol.* 110, 88–95. doi: 10.1006/gcen.1997.7053
- Rangel, A., Camerer, C., and Montague, P. R. (2008). A framework for studying the neurobiology of value-based decision making. *Nat. Rev. Neurosci.* 9, 545–556. doi: 10.1038/nrn2357
- Reim, T., and Scheiner, R. (2014). Division of labour in honey bees: age- and task-related changes in the expression of octopamine receptor genes. *Insect Mol. Biol.* 23, 833–841. doi: 10.1111/imb.12130
- Rein, J., Mustard, J. A., Strauch, M., Smith, B. H., and Galizia, C. G. (2013). Octopamine modulates activity of neural networks in the honey bee antennal lobe. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 947–962. doi: 10.1007/s00359-013-0805-y
- Rether, K. (2012). *Regulation of Haemolymph Glucose and its Importance in Sensory Sensitivity as Well as in Appetitive Learning and Memory in the Honeybee (Apis mellifera)*. Ph.D. dissertation, Universität des Saarlands, Saarbrücken.
- Ritter, S., Bugarith, K., and Dinh, T. T. (2001). Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of glucoregulatory responses and neuronal activation. *J. Comp. Neurol.* 432, 197–216. doi: 10.1002/cne.1097
- Ritter, S., Li, A. J., Wang, Q., and Dinh, T. T. (2011). Minireview: the value of looking backward: the essential role of the hindbrain in counterregulatory responses to glucose deficit. *Endocrinology* 152, 4019–4032. doi: 10.1210/en.2010-1458
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Roeder, T., Degen, J., and Gewecke, M. (1998). Epinastine, a highly specific antagonist of insect neuronal octopamine receptors. *Eur. J. Pharmacol.* 349, 171–177. doi: 10.1016/S0014-2999(98)00192-7
- Roosendaal, B., Barsegyan, A., and Lee, S. (2008). Adrenal stress hormones, amygdala activation, and memory for emotionally arousing experiences. *Prog. Brain Res.* 167, 79–97. doi: 10.1016/S0079-6123(07)67006-X
- Scheiner, R., Pluckhahn, S., Oney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* 136, 545–553. doi: 10.1016/S0166-4328(02)00205-X
- Scheiner, R., Steinbach, A., Classen, G., Strudthoff, N., and Scholz, H. (2014). Octopamine indirectly affects proboscis extension response habituation in *Drosophila melanogaster* by controlling sucrose responsiveness. *J. Insect Physiol.* 69, 107–117. doi: 10.1016/j.jinsphys.2014.03.011
- Schulz, D. J., Barron, A. B., and Robinson, G. E. (2002). A role for octopamine in honey bee division of labor. *Brain Behav. Evol.* 60, 350–359. doi: 10.1159/000067788
- Schulz, D. J., Huang, Z.-Y., and Robinson, G. E. (1998). Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol.* 42, 295–303. doi: 10.1007/s002650050442
- Schulz, D. J., and Robinson, G. E. (1999). Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J. Comp. Physiol. [A]* 184, 481–488. doi: 10.1007/s003590050348
- Seoane-Collazo, P., Ferno, J., Gonzalez, F., Dieguez, C., Leis, R., Nogueiras, R., et al. (2015). Hypothalamic-autonomic control of energy homeostasis. *Endocrine* 50, 276–291. doi: 10.1007/s12020-015-0658-y
- Stevenson, P. A., Hofmann, H. A., Schoch, K., and Schildberger, K. (2000). The fight and flight responses of crickets depleted of biogenic amines. *J. Neurobiol.* 43, 107–120. doi: 10.1002/(SICI)1097-4695(200005)43:2<107::AID-NEU1>3.0.CO;2-C
- Tank, A. W., and Lee Wong, D. (2015). Peripheral and central effects of circulating catecholamines. *Compr. Physiol.* 5, 1–15. doi: 10.1002/cphy.c140007
- Tao, J., Ma, Y. C., Yang, Z. S., Zou, C. G., and Zhang, K. Q. (2016). Octopamine connects nutrient cues to lipid metabolism upon nutrient deprivation. *Sci. Adv.* 2:e1501372. doi: 10.1126/sciadv.1501372

- Toth, A. L., and Robinson, G. E. (2005). Worker nutrition and division of labour in honeybees. *Anim. Behav.* 69, 427–435.
- Verberne, A. J., Korim, W. S., Sabetghadam, A., and Llewellyn-Smith, I. J. (2016). Adrenaline: insights into its metabolic roles in hypoglycaemia and diabetes. *Br. J. Pharmacol.* 173, 1425–1437. doi: 10.1111/bph.13458
- Verberne, A. J., Sabetghadam, A., and Korim, W. S. (2014). Neural pathways that control the glucose counterregulatory response. *Front. Neurosci.* 8:38. doi: 10.3389/fnins.2014.00038
- Wagener-Hulme, C., Kuehn, J. C., Schulz, D. J., and Robinson, G. E. (1999). Biogenic amines and division of labor in honey bee colonies. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 184, 471–479. doi: 10.1007/s003590050347
- Wang, Y., Campbell, J. B., Kaftanoglu, O., Page, R. E. Jr, Amdam, G. V., and Harrison, J. F. (2016). Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.). *J. Exp. Biol.* 219, 960–968. doi: 10.1242/jeb.136374
- Wu, C. L., Shih, M. F., Lee, P. T., and Chiang, A. S. (2013). An octopamine-mushroom body circuit modulates the formation of anesthesia-resistant memory in *Drosophila*. *Curr. Biol.* 23, 2346–2354. doi: 10.1016/j.cub.2013.09.056
- Yang, Z., Yu, Y., Zhang, V., Tian, Y., Qi, W., and Wang, L. (2015). Octopamine mediates starvation-induced hyperactivity in adult *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5219–5224. doi: 10.1073/pnas.1417838112
- Yapici, N., Cohn, R., Schusterreiter, C., Ruta, V., and Vosshall, L. B. (2016). A taste circuit that regulates ingestion by integrating food and hunger signals. *Cell* 165, 715–729. doi: 10.1016/j.cell.2016.02.061
- Yu, Y., Huang, R., Ye, J., Zhang, V., Wu, C., Cheng, G., et al. (2016). Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in adult *Drosophila*. *Elife* 5:e15693. doi: 10.7554/eLife.15693
- Zhang, T., Branch, A., and Shen, P. (2013). Octopamine-mediated circuit mechanism underlying controlled appetite for palatable food in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15431–15436. doi: 10.1073/pnas.1308816110

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Modulation of Low-Voltage-Activated Inward Current Permeable to Sodium and Calcium by DARPP-32 Drives Spontaneous Firing of Insect Octopaminergic Neurosecretory Cells

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Identification of the different intracellular pathways that control phosphorylation/dephosphorylation process of ionic channels represents an exciting alternative approach for studying the ionic mechanisms underlying neuronal pacemaker activity. In the central nervous system of the cockroach *Periplaneta americana*, octopaminergic neurons, called dorsal unpaired median (DUM; DUM neurons), generate spontaneous repetitive action potentials. Short-term cultured adult DUM neurons isolated from the terminal abdominal ganglion (TAG) of the nerve cord were used to study the regulation of a tetrodotoxin-sensitive low-voltage-activated (LVA) channel permeable to sodium and calcium (Na/Ca), under whole cell voltage- and current-clamp conditions. A bell-shaped curve illustrating the regulation of the amplitude of the maintained current vs. [ATP]i was observed. This suggested the existence of phosphorylation mechanisms. The protein kinase A (PKA) inhibitor, H89 and elevating [cyclic adenosine 3', 5' monophosphate, cAMP]i, increased and decreased the current amplitude, respectively. This indicated a regulation of the current via a cAMP/PKA cascade. Furthermore, intracellular application of PP2B inhibitors, cyclosporine A, FK506 and PP1/2A inhibitor, okadaic acid decreased the current amplitude. From these results and because octopamine (OA) regulates DUM neuron electrical activity via an elevation of [cAMP]i, we wanted to know if, like in vertebrate dopaminergic neurons, OA receptor (OAR) stimulation could indirectly affect the current via PKA-mediated phosphorylation of Dopamine- and cAMP-regulated Phosphoprotein-32 (DARPP-32) known to inhibit PP1/2A. Experiments were performed using intracellular application of phospho-DARPP-32 and non-phospho-DARPP-32. Phospho-DARPP-32 strongly reduced the current amplitude whereas non-phospho-DARPP-32 did not affect the current. All together, these results confirm that DARPP-32-mediated inhibition of PP1/2A regulates the maintained sodium/calcium current, which contributes to the development of the pre-depolarizing phase of the DUM neuron pacemaker activity.

Keywords: DUM neurons, pacemaker activity, DARPP-32, octopamine, low-voltage-activated current

INTRODUCTION

Pacemaker neurons are well characterized by their intrinsic ability to generate spontaneous beating or bursting overshooting action potentials. Generation of spontaneous rhythmic activity involved special class of ionic currents occurring during the interval between spikes (Bean, 2007). Among voltage-gated ion currents underlying the neuronal pacemaker activity, the hyperpolarization-activated cyclic-nucleotide-gated cation non-selective channels, HCN1–4, (Robinson and Siegelbaum, 2003; Santoro and Baram, 2003; He et al., 2014; Cao et al., 2016), activated at subthreshold potentials play crucial roles to establish pacemaker potential.

In addition, T-type channels are known to also shape the firing properties. This low-voltage-activated (LVA) transient calcium current is able to activate from small depolarizations near the resting membrane potential and can generate spontaneous electrical activity (Kostyuk, 1999; Perez-Reyes, 2003; Cueni et al., 2009; Cain and Snutch, 2013; Cheong and Shin, 2013; Lambert et al., 2014; Turner and Zamponi, 2014). Three genes encoding the T-type channel alpha subunit have been identified (Cav 3.1, Cav3.2 and Cav3.3; Perez-Reyes, 2003). The calcium currents generated by Cav3.3 subunit displays slower kinetics that differs from the kinetics observed for Cav3.1 and Cav3.2 (Lacinová et al., 2000). This confirms the existence of a native neuronal sustained calcium current, also considered as member of the LVA calcium channel group. This current, previously described in insect octopaminergic neurons, named the dorsal unpaired median (DUM) neurons is activated with small depolarizations, controls the frequency and pattern of DUM neuron spikes (Avery and Johnston, 1996; Grolleau and Lapied, 1996, 2000; Kostyuk, 1999; Wicher et al., 2001; Heidel and Pflüger, 2006). In addition, another depolarization-activated inward current identified as low-threshold persistent sodium currents also contribute to neuronal excitability in vertebrate as well as in insect neuronal preparations (Lapied et al., 1990; Crill, 1996; Grolleau and Lapied, 2000; Jackson et al., 2004; Yamada-Hanff and Bean, 2013; Deng and Klyachko, 2016; Paul et al., 2016). Although the molecular nature of the channels carrying persistent sodium current seems unclear, the persistent sodium current could be carried by fraction of sodium channels that fails to inactivate (Taverna et al., 1999) or the persistent sodium current could arise from the incomplete inactivation of the fast sodium current (Crill, 1996; Taddese and Bean, 2002). Finally and besides this myriad of LVA currents, another less known LVA maintained inward current permeable to both sodium and calcium (Na/Ca) has been characterized in DUM neurons (Defaix and Lapied, 2005). This mixed conductance is active and does not inactivate at sub-threshold voltages and plays a critical role in setting DUM neuron excitability. Because intracellular signaling pathways are essential in regulating ion channel functions, an essential missing functional consideration emerges linking intracellular signaling mechanisms and electrical signaling where the opening and closing of ion channels control the neuron's firing rate. In insects, one of the most prominent biogenic amine in the nervous system is octopamine (OA), known to act as a neurotransmitter,

neuromodulator and neurohormone (Evans and Maqueira, 2005; Roeder, 2005). Although it is well known that OA is released from a small number of identified neurosecretory cells, the DUM neurons, clustered along the dorsal midline of all ganglia (except from brain) of the ventral nerve cord (Bräunig and Pflüger, 2001), OA is also highly functionally significant, which may strongly influence electrical behaviors and signals produced by DUM neurons (Achenbach et al., 1997; Wicher et al., 2001). However, so far, the OA-induced activation of the complex intracellular signaling pathways involved in such regulation is still elusive. Understanding how intracellular biochemical networks and electrical activity are integrated is an essential ongoing question to go deeper in the DUM neuron physiological functions. Using whole cell patch-clamp technique and immunocytochemistry, we have studied the regulatory role of the biogenic amine, OA on the LVA maintained inward current permeable to both Na/Ca involved in the generation of the pacemaker potential. These findings lead us to propose a novel control of the neuronal pacemaker mechanism.

MATERIALS AND METHODS

Preparation

All experiments were performed on DUM neurons cell bodies isolated from the dorsal midline of the terminal abdominal ganglion (TAG) of the ventral nerve cord of adult male cockroach *Periplaneta americana*, reared under standard conditions (29°C, photocycle of 12 h light/12 h dark). Insects were anesthetized by cold treatment. Animal care and handling procedures were in accordance with French institutional and national health guidelines. Cockroaches were pinned dorsal side up on a dissection dish. The dorsal cuticles were removed to allow access to the ventral nerve cord. The TAG were then carefully dissected under a binocular microscope and placed in normal cockroach saline containing (in mM): NaCl 200, KCl 3.1, CaCl₂ 5, MgCl₂ 4, sucrose 50, HEPES 10; pH was adjusted to 7.4 with NaOH.

Cell Isolation

Isolation of DUM neuron cell bodies was performed under sterile conditions using enzymatic treatment and mechanical dissociation of the median part of the TAG, as previously described (Lapied et al., 1989). DUM neurons were maintained at 29°C for 24 h before electrophysiological experiments were carried out. The DUM neuron cell bodies used in the present study were chosen as indicated previously (Lapied et al., 1989).

Whole Cell Recording and Data Analysis

We used the patch-clamp technique in the whole cell configuration to record spontaneous electrical activity and membrane currents. Patch pipettes were pulled from borosilicate glass capillary tubes (GC 150 T-10, Harvard Apparatus, Edenbridge, UK) with a PP-83 electrode puller (Narishige, Tokyo, Japan). Pipettes had resistances ranging from 0.7 MΩ to 1.3 MΩ when filled with internal solution (see composition

below). The liquid junction potential between the pipette and the superfusing solution was always corrected before formation of a seal ≥ 2 G Ω . Signals were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Electrical commands were generated by a programmable stimulator (SMP 310, Biologic, Claix, France) or an IBM computer (Pentium 100) with software control pClamp 8.0.2 connected to a 16-bit analog-to-digital converter (Digidata 1322A, Axon Instruments). Although leak and capacitive currents were compensated electronically at the beginning of each experiment, subtraction of residual capacitance and leakage currents was performed with an on-line P/4 protocol provided by pClamp. In this procedure, currents elicited by four subpulses from the holding potential with an amplitude one-fourth of the main pulse were added together to compute capacitance and leak-subtracted currents. Series resistance value was obtained by the amplifier for each experiment from the patch-clamp amplifier settings after compensation and varied between 2 M Ω and 3 M Ω . Cells were clamped at a holding potential of -90 mV and 100 ms test pulses (except when otherwise stated) were applied from the holding potential at a frequency of 0.14 Hz. For current-clamp experiments, action potentials were displayed on a digital oscilloscope (310, Nicotlet Instruments, Madison, WI, USA) and stored on a DAT (DTR 1202, Biologic) or on the hard disk of the computer for subsequent off-line analysis.

Immunocytochemistry

For light microscope immunocytochemistry, isolated DUM neuron cell bodies were fixed for 1 h in 4% paraformaldehyde containing 5% (wt/vol) sucrose in phosphate-buffered saline (PBS). To block non specific binding of the primary antibody, isolated DUM neuron cell bodies were incubated with 4% bovine serum albumin (BSA) in PBS containing 0.2% Triton X-100 for 1 h. Primary antiserum (rabbit anti-cyclic AMP, Chemicon International, Temecula, CA, USA) diluted 1/800 in 0.2% Triton X-100 in PBS was applied for 12 h at 4°C. After repeated washing in PBS, the secondary antibody (FITC-labeled goat anti-rabbit IgG, Chemicon International) diluted 1/300 in PBS containing 1% BSA and 0.2% Triton X-100 was applied for 3 h at 20°C in the dark. Isolated DUM neuron cell bodies were then washed in 4% BSA in PBS and mounted on glass slides in glycerol-PBS. Control experiments were performed by omitting primary antibodies. Preparations were viewed and photographed through a Zeiss Axioscope microscope (Germany) with an epifluorescence system. Images were digitized with Axovision software.

Solutions

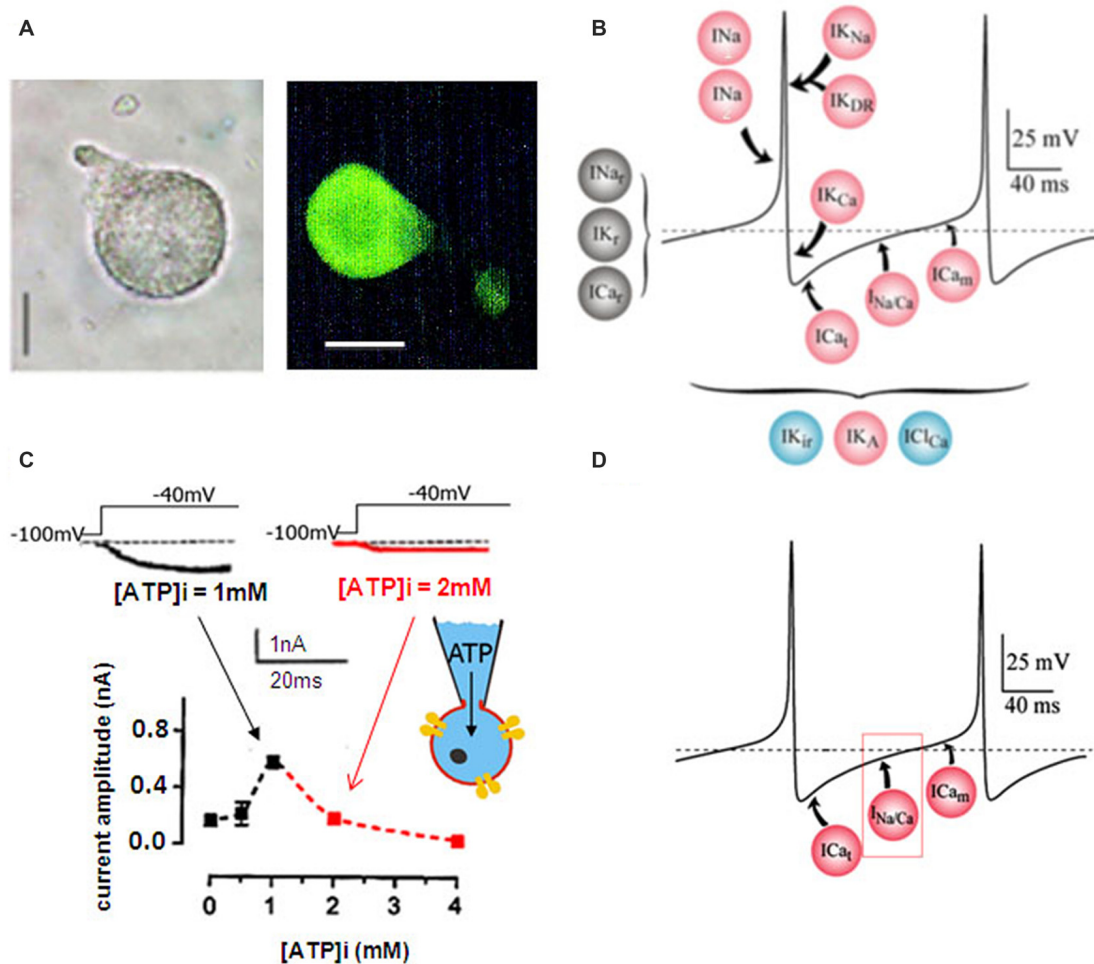
The solutions used to record whole cell inward current were designed to eliminate interference from potassium currents by the combination of external 4-aminopyridine (4-AP) and tetraethylammonium-chloride (TEA-Cl) and by isotonically substituting potassium with cesium in the patch electrode. Inward calcium currents were abolished by adding external 0.5 mM CdCl₂. The extracellular solution superfusing the cell contained (in mM): NaCl 100, TEA-Cl 100, KCl 3.1, CaCl₂ 2, MgCl₂ 7, CdCl₂ 0.5, 4-AP 3, HEPES 10; pH was adjusted

to 7.4 with TEA-OH. For all voltage-clamp experiments, the patch pipette solution contained (in mM): CsCl 90, CsF 80, NaCl 15, MgCl₂ 1, EGTA 5, HEPES 10, ATP-Mg 1 (except when otherwise stated); pH was adjusted to 7.4 with CsOH. For the determination of the physiological role of the current, the bath solution contained (in mM): NaCl 100, TEA-Cl 100, KCl 3.1, CaCl₂ 5, MgCl₂ 4, NiCl₂ 0.1, 4-AP 3, HEPES 10. In this experiment, patch electrodes were filled with an internal solution containing (in mM): CsCl 90, CsF 80, NaCl 15, MgCl₂ 1, EGTA 10, CaCl₂ 0.5, HEPES 20, ATP-Mg 1; pH was adjusted to 7.4 with CsOH. For current-clamp recordings, the patch pipette solution contained (in mM): K aspartate 160, KF 10, NaCl 10, MgCl₂ 1, CaCl₂ 0.5, EGTA 10, HEPES 10; pH was adjusted to 7.4 with KOH. The bathing solution was the normal cockroach saline. Phosphorylated and non-phosphorylated recombinant Dopamine- and cAMP-regulated Phosphoprotein-32 (DARPP-32) were a generous gift of S. N. Schiffmann. Recombinant rat DARPP-32 was expressed in *Escherichia coli* using pEt-3A vector, purified and prepared as previously described (Neyroz et al., 1993; Schiffmann et al., 1998). The two compounds were used at 0.3 mg/mL. All chemical products were purchased from Sigma-Aldrich (L'isle d'Abeau Chesnes, France) except NaCl, KCl, sucrose and MgCl₂ (Merck Eurolab SA, Fontenay sous bois, France), DL OA hydrochloride (Fluka, L'isle d'Abeau Chesnes, France). Experiments were carried out at room temperature (20°C). Data, when quantified, were expressed as mean $\pm$ SEM. Differences between means were tested for statistical significance by Student's *t*-test.

RESULTS

The LVA Maintained Na/Ca Inward Current Is Regulated by Intracellular ATP Concentration

All experiments were performed on isolated adult DUM neuron cell body exhibiting OA-like immunoreactivity (**Figure 1A**) and known to generate endogenous pacemaker activity even in the absence of rhythmic somatic input, which is dependent on multiple different voltage-gated currents and background currents (**Figure 1B**; Grolleau and Lapied, 2000; Wicher et al., 2001). This study was mainly focused on the regulation of a novel LVA maintained inward current permeable to Na/Ca and involved in the generation of the pre-depolarizing phase of the pacemaker activity (**Figure 1D**; Defaix and Lapied, 2005). We previously determined experimental conditions allowing full activation of the maintained Na/Ca current (Defaix and Lapied, 2005). As illustrated in the **Figure 1C**, we used different intracellular solutions with increasing ATP concentration from 0 mM to 4 mM. The amplitude of the maintained current was maximum for 1 mM [ATP]_i and decreased for lower and higher [ATP]_i (**Figure 1C**). Based on the [ATP]_i-induced bell-shaped modulation of the Na/Ca current amplitude, the intracellular ATP concentration of 1 mM was chosen as control conditions for all this study.



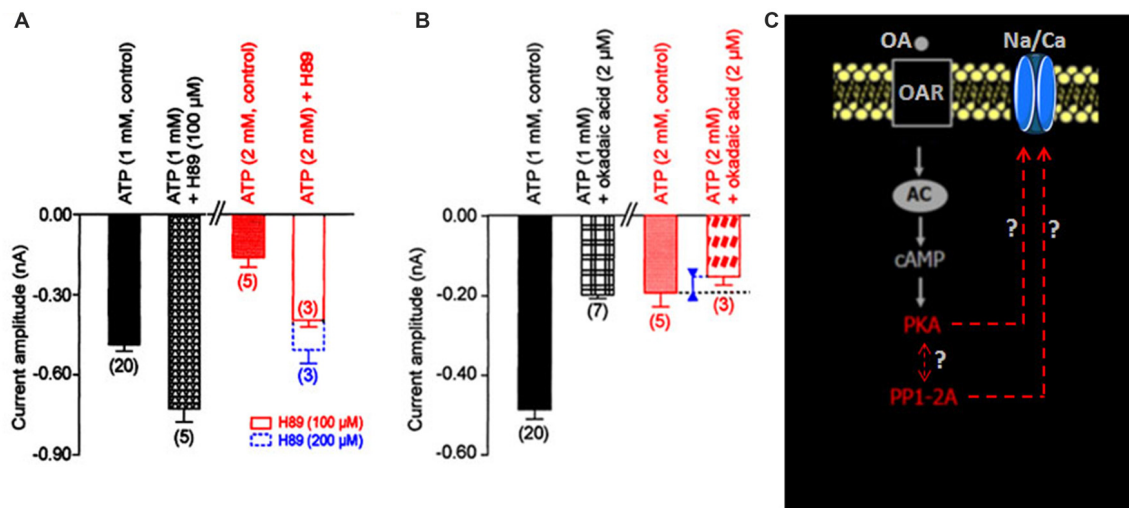


FIGURE 2 | Effects of internal application of H89 and okadaic acid on the LVA maintained Na/Ca inward current amplitude. (A,B) Histograms summarizing the mean current amplitude recorded at -40 mV from a holding potential of -100 mV under different experimental conditions indicated above each bar. Data are means $\pm$ SEM. Values in parentheses indicate number of experiments in each condition. **(C)** Hypothetic model illustrating the participation of the molecular events identified as protein kinase A (PKA) and protein phosphatase (PP1/2A; red characters) in the regulation of the LVA maintained Na/Ca inward current amplitude.

performed with the patch pipette solution containing 1 mM $[ATP]_i$, revealed that okadaic acid (2 μ M) decreased the current amplitude from -0.49 ± 0.03 nA ($n = 20$) to -0.18 ± 0.04 nA ($n = 3$; $p < 0.01$; **Figure 2B**). In the presence of 2 mM $[ATP]_i$, the current amplitude only slightly decreased from -0.19 ± 0.04 nA ($n = 5$) to -0.15 ± 0.02 nA ($n = 3$). These results indicated that the phosphatase PP1/2A was also involved in the regulation of the Na/Ca current and that PP1/2A was obviously inhibited when PKA was activated (i.e., with $[ATP]_i$ 2 mM, see **Figure 2A**). In other words, we revealed that the dephosphorylation mechanism via an okadaic acid-sensitive phosphatase could have important functional consequences on such DUM neuron Na/Ca channels particularly when PKA was activated. According to the hypothetical scheme shown in **Figure 2C**, we proposed that the Na/Ca channel existed either in the phosphorylated or dephosphorylated state. Intracellular ATP concentration regulated the Na/Ca current amplitude by activating PKA, which phosphorylated the molecule and maintained Na/Ca channels in nonfunctional form. Phosphorylation was opposed by a dephosphorylation process, which rendered the channel functional.

The LVA Maintained Inward Na/Ca Current Is Modulated by OA via a cAMP/PKA Cascade

DUM neurons are insect neurosecretory cells whose pacemaker electrical activity is modulated by OA (Achenbach et al., 1997; Wicher et al., 2001). According to these previous findings, we performed additional experiments to study the potential effect of OA on the LVA maintained Na/Ca current, known to play a crucial role in the generation of the DUM neuron pacemaker

activity (**Figure 1D**). When OA (1 μ M) was bath applied onto isolated DUM neuron cell body, an important decrease of the current amplitude was observed (from -0.49 ± 0.03 nA in control, ($n = 20$) to -0.17 ± 0.01 nA ($n = 6$; $p < 0.01$), measured at $t = 14$ min, **Figures 3A,B, 5B**), which was very close to the current amplitude recorded under experimental conditions where PKA was activated (see **Figure 2A**). To confirm whether OA receptors (OARs) were involved in the OA-induced regulatory effect of the Na/Ca current, we applied phentolamine, a well-known OAR antagonist. As illustrated in **Figure 3C**, the effect of OA was completely abolished by phentolamine (10 μ M). As hypothesized in the summarizing scheme shown in **Figure 3D**, the effects of OA are thought to be mainly mediated by interaction with G-protein coupled receptors, which trigger, for instance, activation of the cAMP/PKA cascade (Evans and Maqueira, 2005; Farooqui, 2007). Based on our findings, to study if the action of OA was coupled to increases in intracellular levels of the second messenger cAMP, antibodies raised against cAMP (De Vente et al., 1993) were used. As shown in **Figure 4A**, pretreatment with phentolamine (10 μ M) abolished the intensity of fluorescent cAMP immunostaining produced by OA. These results provided evidence that the action of OA on the Na/Ca current involved the rise in internal cAMP level. To more deeply explore this hypothesis, DUM neurons were dialyzed using an internal solution containing 100 μ M cAMP. When intracellular cAMP (100 μ M) was introduced into cell body by diffusion through the patch pipette, the maximum current amplitude was decreased from -0.49 ± 0.03 nA (control, $n = 20$) to -0.161 ± 0.007 nA (100 μ M cAMP added in the patch pipette, $n = 4$; $p < 0.01$; **Figure 4B**). The effects of regulating PKA phosphorylation were monitored by comparing the amplitude of the Na/Ca current before (standard conditions) and after external application of forskoline.

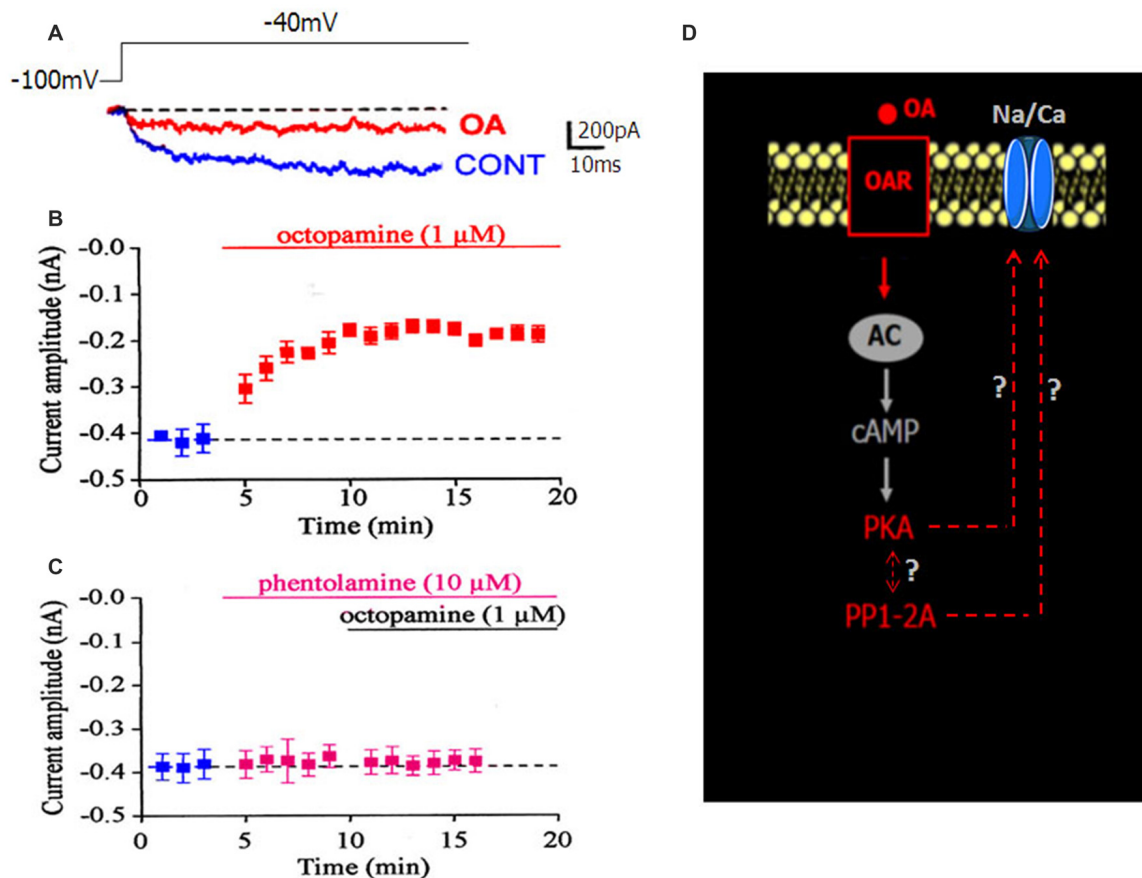


FIGURE 3 | Effect of OA on the LVA maintained Na/Ca inward current. (A) Superimposed current traces generated by depolarizing pulse to -40 mV from a holding potential of -100 mV and recorded in control condition (blue current trace) and in the presence of $1 \mu\text{M}$ OA (red current trace). **(B)** Time course of changed maximum current amplitude induced by bath application of $1 \mu\text{M}$ OA. **(C)** Pretreatment of DUM neuron cell body by the OA receptor (OAR) antagonist, phentolamine ($10 \mu\text{M}$), completely blocked the OA-induced decrease current amplitude. In both cases, data are means $\pm$ SEM. **(D)** Hypothetic scheme illustrating the involvement of OAR activated by OA (red characters) in the modulation of the LVA maintained Na/Ca inward current amplitude.

Figure 4C shows that application of forskoline ($1 \mu\text{M}$), which directly activates adenylyl cyclase (AC), produced a decrease in current amplitude from -0.49 ± 0.03 nA (control, $n = 20$) to -0.13 ± 0.01 nA ($n = 6$; $p < 0.01$). As previously indicated, OA decreased the current amplitude, which was reversed by 300 M H89 (**Figure 5B**). Because this effect was mimicked by a relative high cAMP internal concentration ($100 \mu\text{M}$) and by forskoline and blocked by H89 (**Figure 2A**), we assumed it occurred through cAMP/PKA cascade via the activation of AC (**Figure 4D**).

Modulation of the LVA Maintained Na/Ca Current in DUM Neurons by the Phosphoprotein DARPP-32

DARPP-32 is an important mediator of biogenic amines in vertebrate neurons. It is now assumed that DARPP-32 plays a crucial role as an integrator to diverse neurotransmission inputs in vertebrates (Svenningsson et al., 2004). Based on our results, and because the phosphorylation states of

DARPP-32 are affected by a number of neurotransmitters such as dopamine and serotonin, it is tempting to hypothesize that such phosphoprotein complexes might be involved in the OA-induced modulation of the Na/Ca current in DUM neurons. The phosphorylated and non-phosphorylated forms of DARPP-32 (0.3 mg/mL) were then tested on the amplitude of the Na/Ca current (**Figures 5A,B**). Application of the phosphorylated form of DARPP-32 (DARPP-32-P) decreased the Na/Ca current amplitude, from -0.49 ± 0.03 nA (control, $n = 20$) to -0.16 ± 0.02 nA ($n = 4$; $p < 0.01$) whereas application of the non-phosphorylated form of DARPP-32 had no significant effect on the current amplitude (-0.45 ± 0.01 nA, $n = 7$; $p > 0.05$; **Figure 5A**). OA, which is expected to act via the phosphoprotein DARPP-32, was tested in the presence of the non-phosphorylated form of DARPP-32 (0.3 mg/mL). As indicated above, OA alone strongly decreased the Na/Ca current amplitude (**Figure 5B**). By contrast, application of OA, in the presence of excess DARPP-32 had no significant effect on the current amplitude (-0.43 ± 0.02 nA ($n = 3$; $p > 0.05$) vs. -0.49 ± 0.03 nA ($n = 20$). This effect was very

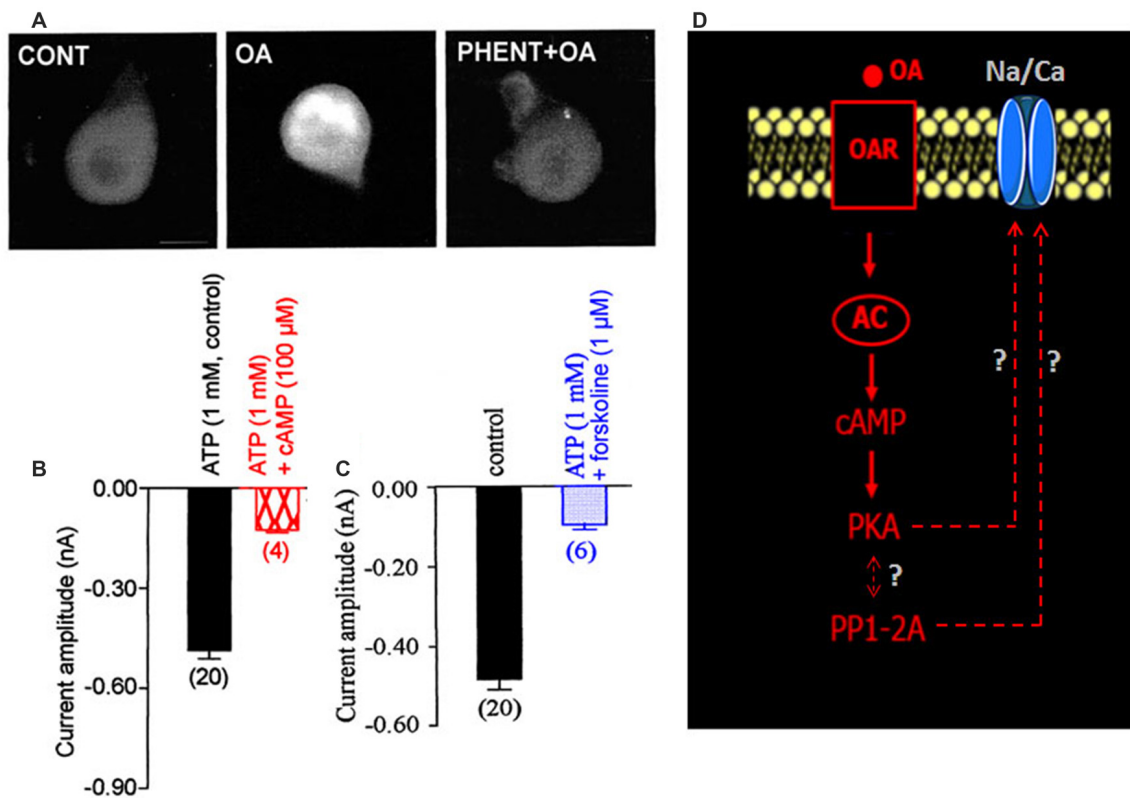


FIGURE 4 | Involvement of adenylyl cyclase (AC) activity in the OA-induced modulation of the LVA maintained Na/Ca inward current. (A) Cyclic adenosine 3',5' monophosphate (cAMP) immunoreactivity of isolated DUM neuron cell bodies. Comparative change in immunofluorescence observed in control and after application of 1 μ M OA. After pretreatment of DUM neuron cell body with 10 μ M phentolamine, the OAR antagonist, cAMP immunostaining induced by OA was completely abolished ($n = 8$, in each experimental conditions). **(B,C)** Comparative histograms illustrating the mean current amplitude recorded at -40 mV from a holding potential of -100 mV under different experimental conditions indicated above each bar. Data are means $\pm$ SEM. Values in parentheses indicate number of experiments in each condition. **(D)** Hypothetic model indicating the participation of the increased cAMP concentration via AC activated by the action of OA on OAR, which modulates the LVA maintained Na/Ca inward current.

similar to that of observed with H89 (Figure 5B). It should be noted that when DUM neuron cell body was pretreated with DARPP-32-P, which already reduced current amplitude (Figure 5A), OA (1 μ M) did not produce any additional effect on the Na/Ca current (Figure 5B). In addition, the physiological role of DARPP-32-P was directly assessed on spontaneously active DUM neurons. As expected, from previous data reporting the involvement of the Na/Ca current in the pre-depolarizing phase of the pacemaker activity (Defaix and Lapied, 2005), the frequency of firing was strongly decreased in the presence of DARPP-32-P (0.3 mg/mL; from 1.4 ± 0.4 Hz to 0.11 ± 0.05 Hz, $n = 6$; Figure 5C). These results indicated that upon activation of OARs DARPP-32 was phosphorylated by PKA, via the cAMP/PKA cascade (Figure 5D). In this case and according to the literature, phosphorylation turned DARPP-32 into a potential potent inhibitor of PP1/2A. This was confirmed by experiments performed with the PP1/2A inhibitor, okadaic acid. Finally, another aspect of the DARPP-32P/PP1-2A cascade was that DARPP-32-P was dephosphorylated by the calcium/calmodulin-dependent protein phosphatase PP2B. To check whether PP2B was also involved in the modulatory

effect of the Na/Ca current, additional set of experiments were performed with intracellular application of BAPTA, a fast efficient calcium chelator and W7, the calmodulin inhibitor. As illustrated in Figure 6A, both W7 (0.5 mM) and BAPTA (10 mM) produced a strong decrease of the current amplitude (from -0.49 ± 0.03 nA ($n = 20$) to -0.19 ± 0.03 nA ($n = 4$) and to -0.11 ± 0.01 nA ($n = 3$), respectively; $p < 0.01$). By contrast, high intracellular calcium concentration (1 μ M) added in the pipette solution slightly increased current amplitude. To substantiate the involvement of PP2B, experiments were also carried out with cyclosporin A (0.1 μ M) and FK506 (5 μ M), two well-know blockers of PP2B. Once again, both compounds reduced the Na/Ca current amplitude from -0.49 ± 0.03 nA ($n = 20$) to -0.17 ± 0.02 nA ($n = 9$) and to -0.09 ± 0.06 nA ($n = 3$), respectively (Figure 6B; $p < 0.01$). It should be mentioned that the amplitude of the Na/Ca current increased following elevation of intracellular calcium concentration (1 μ M). This effect was not observed in the presence of FK506 (5 μ M; not shown) and was only reduced in the presence of excess DARPP-32-P (Figure 6B). Taken all together these results, we proposed the final hypothetical scheme, which summarized the different

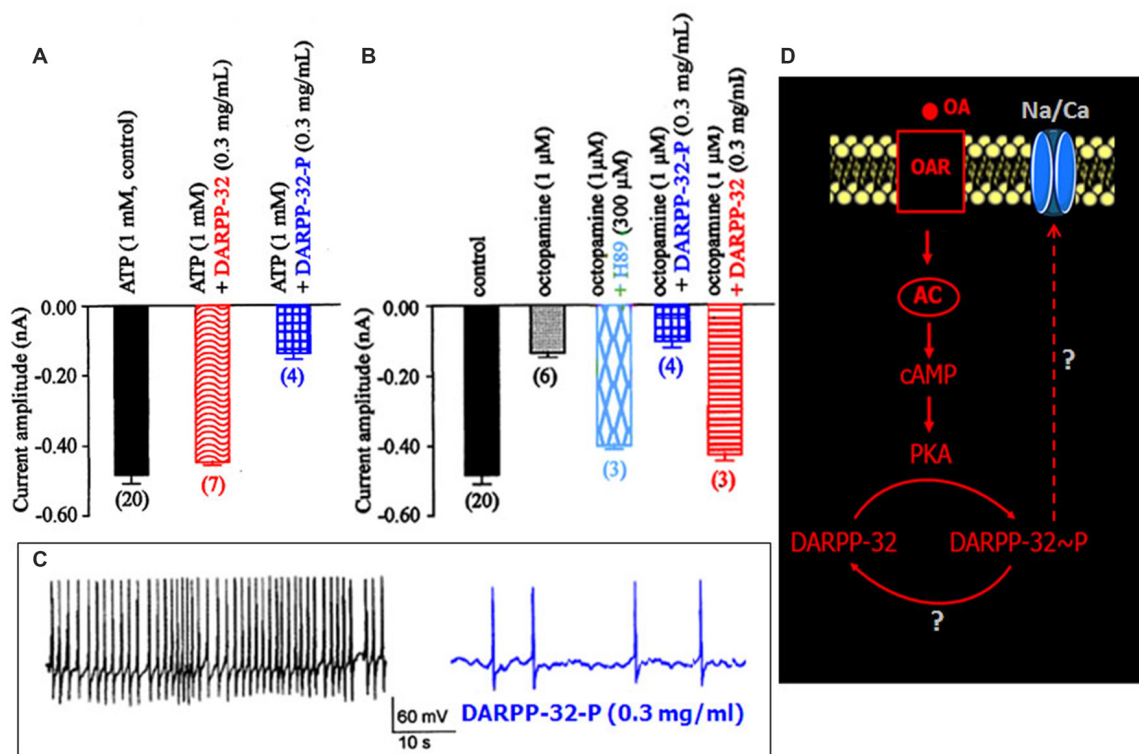


FIGURE 5 | Effects of dephosphorylated and phosphorylated Dopamine- and cAMP-regulated Phosphoprotein-32 (DARPP-32-P) on the LVA maintained Na/Ca inward current. (A,B) Comparative histograms summarizing the mean current amplitude recorded at -40 mV from a holding potential of -100 mV under different experimental conditions indicated above each bar. Data are means $\pm$ SEM. Values in parentheses indicate number of experiments in each condition. **(C)** Effect of DARPP-32-P on spontaneously active DUM neuron. DARPP-32-P (0.3 mg/mL) decreases action potential discharge frequency (similar results were obtained in six different cells). **(D)** Hypothetic model illustrating the implication of DARPP-32 and DARPP-32-P in the modulation of the LVA maintained Na/Ca inward current produced by OA through cAMP/PKA cascade.

molecular events involved in the OA-induced modulation of the Na/Ca inward current occurring through the phosphoprotein DARPP-32 (Figure 6C).

DISCUSSION

Octopaminergic DUM neurons project their axons both centrally, innervating neuropiles of different ganglia but also peripherally to innervate skeletal and visceral muscles and some sense organs. It is also well established that DUM neurons are an important component of different motor networks (Burrows and Pflüger, 1995; Baudoux et al., 1998; Mentel et al., 2008; Vierk et al., 2009). Although DUM neurons may be activated by sensory stimuli (e.g., Baudoux and Burrows, 1998; Field et al., 2008; Pflüger et al., 2011; Rand et al., 2012), they are defined by the absence of common somatic synaptic inputs from presynaptic neurons and by their uncommon intrinsic property allowing adequate beating pacemaker activity (Grolleau and Lapied, 2000; Wicher et al., 2001; Defaix and Lapied, 2005; Heidel and Pflüger, 2006; Lavalie-Defaix et al., 2006; Gautier et al., 2008). One of the most important key determinants of the DUM neuron excitability is the action potential threshold. The threshold

determines when an action potential is initiated, sets the DUM neuron firing rate and shape neuronal computations including, for instance, temporal coding. Pacemaker activity in individual DUM neuron emerges from the concerted action of a complex complement of voltage-gated and background currents (Grolleau and Lapied, 2000; Wicher et al., 2001). However, voltage-gated currents activated near the action potential threshold are considered to be fundamental actors that contribute to controlling excitability. In cockroach isolated DUM neurons, different LVA channels are involved in the generation of the prepolarization, which regulate the firing frequency. In this preparation, two types of LVA calcium currents identified as transient and maintain calcium currents have specialized function in the spontaneous electrical activity. The LVA transient calcium current is involved in the first part of the prepolarization whereas the LVA maintained calcium current participates in the last two-thirds of the prepolarizing phase (Grolleau and Lapied, 1996, 2000; Wicher et al., 2001). Besides these two LVA calcium currents, a third unusual LVA inward current permeable to Na/Ca play an important role in pacemaking of DUM neurons (Defaix and Lapied, 2005). In fact, the activation of the LVA transient calcium current brings the membrane potential to the threshold of the LVA

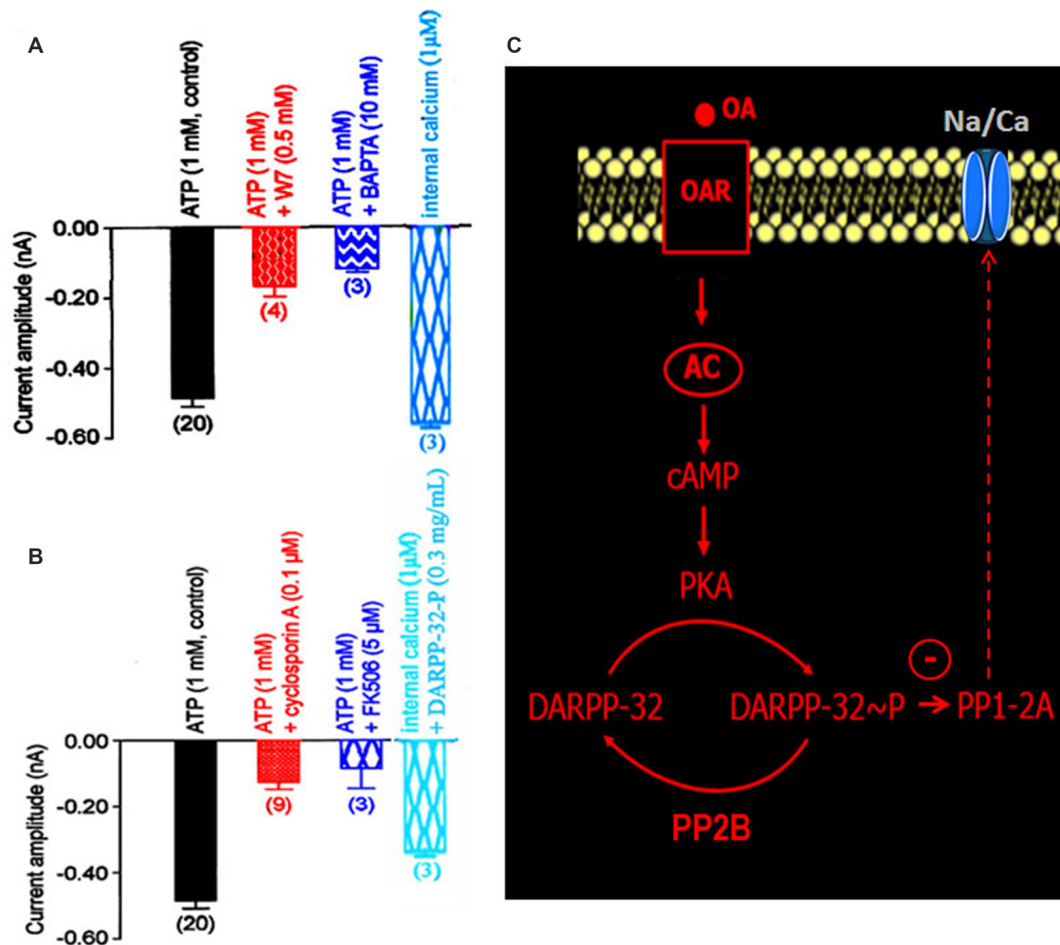


FIGURE 6 | Implication of the calcium/calmodulin-sensitive protein phosphatase PP2B in the DARPP-32/DARPP-32-P-induced modulation of the LVA maintained Na/Ca inward current. (A,B) Comparative histograms illustrating the current amplitude recorded at -40 mV from a holding potential of -100 mV under different experimental conditions indicated above each bar. Data are means $\pm$ SEM. Values in parentheses indicate number of experiments in each condition. **(C)** Summarizing hypothetical model suggesting that PP2B activation dephosphorylated DARPP-32-P. By contrast, the phosphorylation of DARPP-32 via the activation of PKA produced an inhibition of the okadaic acid-sensitive protein phosphatase PP1/2A, which thereby modulated the LVA maintained Na/Ca inward current.

maintained Na/Ca current activation. This current leads to further depolarization, which allows to reach activation threshold of the LVA maintained calcium current. Together, these combined events produce the pre-depolarization (Figure 1C) essential for triggering DUM neuron pacemaker activity. Because the Na/Ca current is activated in an intermediate potential range between LVA transient and maintained calcium currents (i.e., subthreshold potential), it represents the LVA channel, which could be continuously and extensively modulated by a variety of intracellular signaling pathways including octopaminergic neuromodulator receptors, known to modulate spontaneous activity, as previously reported (Achenbach et al., 1997; Wicher et al., 2001). Although OA is known to modulate number of physiological and behavioral processes in invertebrates (Verlinden et al., 2010), there is, however, no data available to explain the modulatory action of OA in DUM neuron firing property.

It has been well established that there are significant similarities between the octopaminergic signaling pathways in invertebrates and the dopaminergic system in vertebrates (Roeder, 1999). The classification profile for OARs is based on the similarities of these receptors to vertebrate adrenergic receptors in terms of amino acid sequence and intracellular signaling pathways. Three classes of OARs have been characterized (Maqueira et al., 2005) and it has been reported, for instance, that activation of α -adrenergic-like OAR by OA results in an increase in intracellular levels of calcium and cAMP whereas β -adrenergic-like receptor activation only elevates cAMP concentrations (Bischof and Enan, 2004; Balfanz et al., 2005; Evans and Maqueira, 2005; Maqueira et al., 2005; Ohtani et al., 2006; Beggs et al., 2011). Based on these data, identifying the intracellular signaling pathway activated by vertebrate dopamine receptor stimulation could contribute to the understanding

of the specific octopaminergic functions in insects. In this context, one of the most interesting features of insect-type octopaminergic receptors is that they could be indirectly coupled to the well-known vertebrate phosphoprotein DARPP-32 (Greengard et al., 1999). Because the phosphorylation of this protein is regulated by dopamine and cAMP, it is named DARPP-32 (Dopamine and cAMP-regulated phosphoprotein Mr 32,000). DARPP-32, expressed in different brain regions in vertebrates but also in peripheral organs such as kidney, adrenal medulla and parathyroid cells, plays a key role in mediating the biochemical, electrophysiological and behavioral of dopamine on dopaminergic neurons (Ouimet et al., 1984; Greengard et al., 1999; Svenningsson et al., 2004). Although DARPP-32 has also been implicated in mediating the actions of other neurotransmitters systems such as glutamate and serotonin, there is no information about the existence of such phosphoprotein in insects, mediating the action of OA via given OARs, which have a close pharmacological relationship with dopamine receptors.

In our study, we have demonstrated that OA modulates DUM neuron firing properties via the regulation of the LVA Na/Ca current through the participation of the phosphoprotein-like DARPP-32. Up to date, this is the first example reporting such physiological function for DARPP-32. Using specific pharmacological agents together with DARPP-32 and DARPP-32-P, we can propose the hypothetical scheme shown in **Figure 6C**. Like dopamine in vertebrates, OA acts on OARs using cAMP as a mediator in the process. Increased cAMP concentration, via AC, activates PKA, which induces DARPP-32 phosphorylation. The PKA-induced DARPP-32 phosphorylation converts this protein into a potent inhibitor of the protein phosphatase PP1/2A. The resulting inhibition of the phosphatase reduces Na/Ca current amplitude, which thereby decreases the DUM neuron pacemaker activity. DARPP-32 phosphorylation is opposed by a dephosphorylation process. For elevated intracellular calcium concentration, the dephosphorylation is catalyzed by a calcium/calmodulin-sensitive protein phosphatase PP2B. In this case, PP2B seems to play a prominent role in the regulation of the DARPP-32

phosphorylation and indirectly in the DUM neuron excitability. Based on the classification of the OARs linked to intracellular signaling pathways (i.e., cAMP and/or calcium; Evans and Maqueira, 2005; Maqueira et al., 2005), the results presented in this study help to understand better why OA, depending on the concentration tested (Achenbach et al., 1997; Wicher et al., 2001), increases or decreases the DUM neuron spontaneous electrical activity. Another interesting point raised is that DARPP-32, which is known to be a key cellular regulator, has been mainly characterized in mammals. Today, there is no comparative analysis of this phosphoprotein complex across other vertebrates and invertebrates. Understanding DARPP-32 function from the evolutionary perspective will help to further our understanding of the phylogenetic origins and evolutionary conservation of this protein. Based on our results, it appears that the phosphoprotein DARPP-32 could represent a more generic signaling motif for different living organisms including insects. On the other hand, the DARPP-32-dependent mechanism proposed here is quite generalizable to various systems. Thus, further exploration of the wider signaling network involved in this process is of interest. An important question, which is currently under investigation, is the unexpected regulation of the LVA maintained Na/Ca current by low internal ATP concentration. Additional experiments are in progress to clarify this point. Finally, it is known that several other crosstalk points exist between the calcium and OA signaling axis at various downstream levels of the synaptic signaling. Thus, phosphoprotein-dependent mechanisms could represent a more general central nervous system-wide signaling motif responsible for the implementation of network coding on sub-cellular signal integration of environmental cues.

AUTHOR CONTRIBUTIONS

BL designed the experiments, made analysis of the results and wrote the manuscript. AD conducted the experiments and made analysis of the results. MS and VR discussed the results and contributed to the text. EM contributed to the text.

REFERENCES

- Achenbach, H., Walther, C., and Wicher, D. (1997). Octopamine modulates ionic currents and spiking in dorsal unpaired median (DUM) neurons. *Neuroreport* 8, 3737–3741. doi: 10.1097/00001756-199712010-00016
- Avery, R. B., and Johnston, D. (1996). Multiple channel types contribute to the low-voltage-activated calcium current in hippocampal CA3 pyramidal neurons. *J. Neurosci.* 16, 5567–5582.
- Balfanz, S., Strunker, T., Frings, S., and Baumann, A. (2005). A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca²⁺ release in *Drosophila melanogaster*. *J. Neurochem.* 93, 440–451. doi: 10.1111/j.1471-4159.2005.03034.x
- Baudoux, S., and Burrows, M. (1998). Synaptic activation of efferent neuromodulatory neurones in the locust *Schistocerca gregaria*. *J. Exp. Biol.* 201, 3339–3354.
- Baudoux, S., Duch, C., and Morris, O. T. (1998). Coupling of efferent neuromodulatory neurones to rhythmical leg motor activity in the locust. *J. Neurophysiol.* 79, 361–370.
- Bean, B. P. (2007). The action potential in mammalian central neurons. *Nat. Rev. Neurosci.* 8, 451–465. doi: 10.1038/nrn2148
- Beggs, K. T., Tyndall, J. D., and Mercer, A. R. (2011). Honey bee dopamine and octopamine receptors linked to intracellular calcium signaling have a close phylogenetic and pharmacological relationship. *PLoS One* 6:e26809. doi: 10.1371/journal.pone.0026809
- Bischof, L. J., and Enan, E. E. (2004). Cloning, expression and functional analysis of an octopamine receptor from *Periplaneta americana*. *Insect Biochem. Mol. Biol.* 34, 511–521. doi: 10.1016/j.ibmb.2004.02.003
- Bräunig, P., and Pflüger, H.-J. (2001). The unpaired median neurons of insects. *Adv. Insect Phys.* 28, 185–266. doi: 10.1016/S0065-2806(01)28011-4
- Burrows, M., and Pflüger, H. J. (1995). Action of locust neuromodulatory neurons is coupled to specific motor patterns. *J. Neurophysiol.* 74, 347–357.
- Cain, S. M., and Snutch, T. P. (2013). T-type calcium channels in burst-firing, network synchrony, and epilepsy. *Biochim. Biophys. Acta* 1828, 1572–1578. doi: 10.1016/j.bbame.2012.07.028
- Cao, Y., Pang, J., and Zhou, P. (2016). HCN channel as therapeutic targets for heart failure and pain. *Curr. Top. Med. Chem.* 16, 1855–1861. doi: 10.2174/1568026616666151215104058

- Cheong, E., and Shin, H. S. (2013). T-type Ca^{2+} channels in normal and abnormal brain functions. *Physiol. Rev.* 93, 961–992. doi: 10.1152/physrev.00010.2012
- Crill, W. E. (1996). Persistent sodium current in mammalian central neurons. *Annu. Rev. Physiol.* 58, 349–362. doi: 10.1146/annurev.ph.58.030196.002025
- Cueni, L., Canepari, M., Adelman, J. P., and Luthi, A. (2009). Ca^{2+} signaling by T-type Ca^{2+} channels in neurons. *Pflugers Arch. Eur. J. Physiol.* 457, 1161–1172. doi: 10.1007/s00424-008-0582-6
- De Vente, J., Schipper, J., and Steinbusch, H. W. (1993). A new approach to the immunocytochemistry of cAMP. Initial characterization of antibodies against acrolein-fixed cAMP. *Histochemistry* 99, 457–462. doi: 10.1007/bf00274098
- Defaix, A., and Lapied, B. (2005). Role of a novel maintained low-voltage-activated inward current permeable to sodium and calcium in pacemaking of insect neurosecretory neurons. *Invert. Neurosci.* 5, 135–146. doi: 10.1007/s10158-005-0003-x
- Deng, P. Y., and Klyachko, V. A. (2016). Increased persistent sodium current causes neuronal hyperexcitability in the entorhinal cortex of Fmr1 knockout mice. *Cell Rep.* 16, 3157–3166. doi: 10.1016/j.celrep.2016.08.046
- Evans, P. D., and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert. Neurosci.* 5, 111–118. doi: 10.1007/s10158-005-0001-z
- Farooqui, T. (2007). Octopamine-mediated neuromodulation of insect senses. *Neurochem. Res.* 32, 1511–1529. doi: 10.1007/s11064-007-9344-7
- Field, L. H., Duch, C., and Pflüger, H. J. (2008). Responses of efferent octopaminergic thoracic unpaired median neurons in the locust to visual and mechanosensory signals. *J. Insect Physiol.* 54, 240–254. doi: 10.1016/j.jinsphys.2007.09.006
- Gautier, H., Auger, J., Legros, C., and Lapied, B. (2008). Calcium-activated potassium channels in insect pacemaker neurons as unexpected target site for the novel fumigant dimethyl disulfide. *J. Pharmacol. Exp. Ther.* 324, 149–159. doi: 10.1124/jpet.107.128694
- Greengard, P., Allen, P. B., and Nairn, A. C. (1999). Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. *Neuron* 23, 435–447. doi: 10.1016/S0896-6273(00)80798-9
- Grolleau, F., and Lapied, B. (1996). Two distinct low-voltage-activated Ca^{2+} currents contribute to the pacemaker mechanism in cockroach dorsal unpaired median neurons. *J. Neurophysiol.* 76, 963–976.
- Grolleau, F., and Lapied, B. (2000). Dorsal unpaired median neurones in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. *J. Exp. Biol.* 203, 1633–1648.
- He, C., Chen, F., Li, B., and Hu, Z. (2014). Neurophysiology of HCN channels: from cellular functions to multiple regulations. *Prog. Neurobiol.* 112, 1–23. doi: 10.1016/j.pneurobio.2013.10.001
- Heidel, E., and Pflüger, H.-J. (2006). Ion currents and spiking properties of identified subtypes of locust octopaminergic dorsal unpaired median neurons. *Eur. J. Neurosci.* 23, 1189–1206. doi: 10.1111/j.1460-9568.2006.04655.x
- Herzig, S., and Neumann, J. (2000). Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. *Physiol. Rev.* 80, 173–210.
- Jackson, A. C., Yao, G. L., and Bean, B. P. (2004). Mechanism of spontaneous firing in dorsomedial suprachiasmatic nucleus neurons. *J. Neurosci.* 24, 7985–7998. doi: 10.1523/JNEUROSCI.2146-04.2004
- Kostyuk, P. G. (1999). Low-voltage activated calcium channels: achievements and problems. *Neuroscience* 92, 1157–1163. doi: 10.1016/s0306-4522(99)00221-3
- Lacinová, L., Klugbauer, N., and Hofmann, F. (2000). Low voltage activated calcium channels: from genes to function. *Gen. Physiol. Biophys.* 19, 121–136.
- Lambert, R. C., Bessaïh, T., Crunelli, V., and Leresche, N. (2014). The many faces of T-type calcium channels. *Pflugers Arch.* 466, 415–423. doi: 10.1007/s00424-013-1353-6
- Lapied, B., Malécot, C. O., and Pelhate, M. (1989). Ionic species involved in the electrical activity of single adult aminergic neurones isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana*. *J. Exp. Biol.* 144, 535–549.
- Lapied, B., Malécot, C. O., and Pelhate, M. (1990). Patch-clamp study of the properties of the sodium current in cockroach single isolated adult aminergic neurones. *J. Exp. Biol.* 151, 387–403.
- Lavialle-Defaix, C., Gautier, H., Defaix, A., Lapied, B., and Grolleau, F. (2006). Differential regulation of two distinct voltage-dependent sodium currents by group III metabotropic glutamate receptor activation in insect pacemaker neurons. *J. Neurophysiol.* 96, 2437–2450. doi: 10.1152/jn.00588.2006
- Maqueira, B., Chatwin, H., and Evans, P. D. (2005). Identification and characterization of a novel family of *Drosophila* beta-adrenergic-like octopamine G-protein coupled receptors. *J. Neurochem.* 94, 547–560. doi: 10.1111/j.1471-4159.2005.03251.x
- Mentel, T., Weiler, V., Büschges, A., and Pflüger, H. J. (2008). Activity of neuromodulatory neurones during stepping of a single insect leg. *J. Insect Physiol.* 54, 51–61. doi: 10.1016/j.jinsphys.2007.08.010
- Neyroz, P., Desdoutis, F., Benfenati, F., Knutson, J. R., Greengard, P., and Girault, J. A. (1993). Study of the conformation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, by fluorescence spectroscopy. *J. Biol. Chem.* 268, 24022–24031.
- Ohtani, A., Arai, Y., Ozoe, F., Ohta, H., Narusuye, K., Huang, J., et al. (2006). Molecular cloning and heterologous expression of an alpha-adrenergic-like octopamine receptor from the silkworm *Bombyx mori*. *Insect Mol. Biol.* 15, 763–772. doi: 10.1111/j.1365-2583.2006.00676.x
- Ouimet, C. C., Miller, P. E., Hemmings, H. C., Walaas, S. I. Jr., and Greengard, P. (1984). DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. immunocytochemical localization. *J. Neurosci.* 4, 111–124.
- Paul, J. R., DeWoskin, D., McMeekin, L. J., Cowell, R. M., Forger, D. B., and Gamble, K. L. (2016). Regulation of persistent sodium currents by glycogen synthase kinase 3 encodes daily rhythms of neuronal excitability. *Nat. Commun.* 7:13470. doi: 10.1038/ncomms13470
- Perez-Reyes, E. (2003). Molecular physiology of low-voltage-activated t-type calcium channels. *Physiol. Rev.* 83, 117–161. doi: 10.1152/physrev.00018.2002
- Pflüger, H.-J., Field, L. H., Nishino, H., and Currie, M. J. (2011). Neuromodulatory unpaired median neurons in the new zealand tree weta, *Hemideina femorata*. *J. Insect Physiol.* 57, 1420–1430. doi: 10.1016/j.jinsphys.2011.07.010
- Rand, D., Knebel, D., and Ayali, A. (2012). The effect of octopamine on the locust stomatogastric nervous system. *Front. Physiol.* 3:288. doi: 10.3389/fphys.2012.00288
- Robinson, R. B., and Siegelbaum, S. A. (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu. Rev. Physiol.* 65, 453–480. doi: 10.1146/annurev.physiol.65.092101.142734
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi: 10.1016/S0301-0082(99)00016-7
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Santoro, B., and Baram, T. Z. (2003). The multiple personalities of h-channels. *Trends Neurosci.* 26, 550–554. doi: 10.1016/j.tins.2003.08.003
- Schiffmann, S. N., Desdoutis, F., Menu, R., Greengard, P., Vincent, J. D., and Vanderhaeghen, J. J. (1998). Modulation of voltage-gated sodium current in rat striatal neurons by DARPP-32, an inhibitor of protein phosphatase. *Eur. J. Neurosci.* 10, 1312–1320. doi: 10.1046/j.1460-9568.1998.00142.x
- Svenningsson, P., Nishi, A., Fisone, G., Girault, J. A., Nairn, A. C., and Greengard, P. (2004). DARPP-32: an integrator of neurotransmission. *Annu. Rev. Pharmacol. Toxicol.* 44, 269–296. doi: 10.1146/annurev.pharmtox.44.1802.121415
- Taddese, A., and Bean, B. P. (2002). Subthreshold sodium current from rapidly inactivating sodium channels drives spontaneous firing of tuberomammillary neurons. *Neuron* 33, 587–600. doi: 10.1016/s0896-6273(02)00574-3
- Taverna, S., Sancini, G., Mantegazza, M., Franceschetti, S., and Avanzini, G. (1999). Inhibition of transient and persistent Na^{+} current fractions

- by the new anticonvulsant topiramate. *J. Pharmacol. Exp. Ther.* 288, 960–968.
- Turner, R. W., and Zamponi, G. W. (2014). T-type channels buddy up. *Pflugers Arch. Eur. J. Physiol.* 466, 661–675. doi: 10.1007/s00424-013-1434-6
- Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H. J., Blenau, W., et al. (2010). The role of octopamine in locusts and other arthropods. *J. Insect Physiol.* 56, 854–867. doi: 10.1016/j.jinsphys.2010.05.018
- Vierk, R., Pflüger, H. J., and Duch, C. (2009). Differential effects of octopamine and tyramine on the central pattern generator for *Manduca* flight. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 195, 265–277. doi: 10.1007/s00359-008-0404-5
- Wicher, D., Walther, C., and Wicher, C. (2001). Non-synaptic ion channels in insects—basic properties of currents and their modulation in neurons and skeletal muscles. *Prog. Neurobiol.* 64, 431–525. doi: 10.1016/s0301-0082(00)00066-6
- Yamada-Hanff, J., and Bean, B. P. (2013). Persistent sodium current drives conditional pacemaking in CA1 pyramidal neurons under muscarinic stimulation. *J. Neurosci.* 33, 15011–15021. doi: 10.1523/JNEUROSCI.0577-13.2013

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Dopamine Modulates Serotonin Innervation in the *Drosophila* Brain

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Parkinson's disease (PD) results from a progressive degeneration of the dopaminergic nigrostriatal system leading to a decline in movement control, with resting tremor, rigidity and postural instability. Several aspects of PD can be modeled in the fruit fly, *Drosophila melanogaster*, including α -synuclein-induced degeneration of dopaminergic neurons, or dopamine (DA) loss by genetic elimination of neural DA synthesis. Defective behaviors in this latter model can be ameliorated by feeding the DA precursor L-DOPA, analogous to the treatment paradigm for PD. Secondary complication from L-DOPA treatment in PD patients are associated with ectopic synthesis of DA in serotonin (5-HT)-releasing neurons, leading to DA/5-HT imbalance. Here we examined the neuro-anatomical adaptations resulting from imbalanced DA/5-HT signaling in *Drosophila* mutants lacking neural DA. We find that, similar to rodent models of PD, lack of DA leads to increased 5-HT levels and arborizations in specific brain regions. Conversely, increased DA levels by L-DOPA feeding leads to reduced connectivity of 5-HT neurons to their target neurons in the mushroom body (MB). The observed alterations of 5-HT neuron plasticity indicate that loss of DA signaling is not solely responsible for the behavioral disorders observed in *Drosophila* models of PD, but rather a combination of the latter with alterations of 5-HT circuitry.

Keywords: Parkinson's disease, dopamine, serotonin, *Drosophila melanogaster*, neuroanatomy, plasticity

INTRODUCTION

Parkinson's Disease (PD) is a progressive degeneration of the dopaminergic nigrostriatal system leading to a decline in movement control associated with resting tremor, rigidity and postural instability. A small minority of PD cases are linked to pathogenic gene mutations responsible for the development of the disease. However, studies of rare Mendelian forms of PD allowed for the identification of more than 20 PD genes and variants that are implicated in its development (Rousseaux et al., 2017). Whereas, about 10–15 percent of Parkinson's patients are thought to suffer from a genetic form of this dystonic movement disorder, most patients suffer from a sporadic form of PD most likely resulting from a combination of environmental factors and undefined individual genetic susceptibility (Obeso et al., 2014; Ascherio and Schwarzschild, 2016). Whether the underlying causes act separately or converge into common pathways remains to be resolved. Both sporadic and hereditary pathogenic events lead to the disease that affects the survival of dopamine (DA) producing neurons in vulnerable brain areas such as the substantia nigra (Westerlund et al., 2010).

Studies in 6-hydroxydopamine (6-OHDA)-treated rats displaying nigrostriatal DA lesions indicate that lack of DA signaling promotes the growth of 5-HT neurons in the striatum (Zhou et al., 1991). The observed hyper-innervation of serotonergic neurons that can be induced by DA neuron denervation imply that it is not exclusively the loss of DA signaling that is responsible for the development of the PD-triggered disorders, but rather a combination of the latter with alterations of 5-HT circuitry.

The powerful genetic tools available in *Drosophila* make it an excellent model system to study the cellular mechanisms underlying neurodegenerative diseases (Feany and Bender, 2000; Marsh and Thompson, 2006; Lu, 2009; Dehay and Bezaud, 2011; Riemensperger et al., 2013; Vanhauwaert and Verstreken, 2015; West et al., 2015; Hewitt and Whitworth, 2017). In recent years, *Drosophila* has proven to be a valuable model system for dopaminergic neurodegeneration under conditions mimicking PD. For instance, ectopic expression of a mutated form of human α -synuclein, α -synA30P, in *Drosophila melanogaster* models the dopaminergic neurodegeneration seen in vertebrates (Feany and Bender, 2000), and the presence of α -synA30P in subsets of the protocerebral anterior medial protocerebrum (AMP) DA neurons leads to gradual loss of their projections onto target neuropils (Riemensperger et al., 2013).

Fruit flies mutant for the enzyme tyrosine hydroxylase (TH), which are unable to produce DA in the central nervous system (CNS), show a variety of behavioral deficits (Hirsh et al., 2010; Riemensperger et al., 2011; Cichewicz et al., 2016), demonstrating the crucial role of DA in the control of diverse behaviors (Friggi-Grelín et al., 2003; Schwaerzel et al., 2003; Andretic et al., 2005; Kume et al., 2005; Ganguly-Fitzgerald et al., 2006; Liu et al., 2008; Lebestky et al., 2009; Riemensperger et al., 2011; Ueno et al., 2012; Oswald and Waddell, 2015; Nall et al., 2016; Fiala and Riemensperger, 2017). However, to understand the development of the wide range of behavioral disorders deriving from a DA dysregulation, it is crucial to understand how neuronal circuits react to the loss of DA signaling in the CNS. Here, we have investigated how long-term or acute changes in DA-signaling affect serotonin-neuron plasticity in *Drosophila*.

MATERIALS AND METHODS

Drosophila Strains

Either Canton S (CS) or w^{1118} , back-crossed for seven generations to CS, were used as wild-type control flies. Brain DA-deficient *Drosophila* (dTH def.) were $DTH^{FS+/-}$ BAC ple^2 and rescue controls (dTH resc.) were DTH BAC ple^2 (as per Cichewicz et al., 2016). Animals used for immunohistochemistry were 3–5 days post eclosion. For reconstitution of splitGFP experiments TrH -Gal4 flies (Cassar et al., 2015) were crossed with flies carrying a combination of UAS:splitGFP1-10 (Pech et al., 2013), DsRed (Riemensperger et al., 2005) and splitGFP11 under the control of the mb247 promoter (Pech et al., 2013). Flies were raised under standard conditions at a 12:12 h light-dark schedule at 25°C and 60% relative humidity.

Immunohistochemistry

If not otherwise indicated, brains of 3- to 5-day old female flies were dissected in ice-cold Ringer's solution containing 5 mM HEPES-NaOH (pH = 7.4), 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 36 mM sucrose, and fixed for 2 h on ice in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS), and subsequently washed three times in PBS containing 0.6% Triton X-100. Samples were incubated overnight at 4°C in PBT containing 2% bovine serum albumin (BSA). If not otherwise indicated, the samples were subsequently incubated with mouse anti-TH (Immunostar, dilution 1:100) with rabbit anti-5-HT (Sigma-Aldrich, dilution 1:500) or for corroboration of the results, with other 5-HT antibody (Supplementary Figure S1; rat anti-5HT, Merck, 1:100) diluted in block solution at 18°C for at least 6 h. After washing the samples at least three times for 20 min each with PBS containing 0.6% Triton X-100, the brains were incubated at 4°C overnight in secondary antibodies diluted in PBS containing 0.6% Triton X-100. Secondary antibodies were anti-mouse Alexa 488-conjugated (Invitrogen, 1:300) or anti-mouse Cy3-conjugated (Jackson, 1:300). Samples were then washed again three times in PBS containing 0.6% Triton X-100, incubated for at least 6 h in PBS, and mounted in Vectashield (Vector Laboratories). Images were taken using a Leica SP8 confocal microscope equipped with a Leica Apochromat 20 $\times$ /0.7 water immersion objective. The brains were scanned at 1.0 μ m steps in the z-axis with a resolution of 1.0 μ m/pixel. Images were analyzed using ImageJ. To determine fluorescence intensities, scans were transformed to z-projections and analyzed as described in Neckameyer and Bhatt (2012).

L-DOPA Treatment

Flies were incubated for 5–10 days at 25°C on standard fly food containing 1 mg/mL L-DOPA (D9628, Sigma-Aldrich). Test and control flies were transferred to fresh food of the according regimen every second day.

RESULTS

Dopamine- and Serotonin-Producing Neurons Joint and Complementary Innervation Patterns in the Brain

To visualize the differential innervation of serotonergic and dopaminergic neurons in the *Drosophila* central brain, we stained with antisera to 5-HT, and to TH, in conjunction with TH -Gal4- (Friggi-Grelín et al., 2003) and TrH -Gal4- (Cassar et al., 2015) driven GFP in DA and 5-HT neurons, respectively (Figures 1A,B). In agreement with previously published observations (Monastirioti, 1999), the anterior dopaminergic system of the adult brain mainly consists of three neuronal clusters, including neurons situated laterally in the anterior protocerebrum (PAL), a small group of neurons located in the lateral and medial parts of the subesophageal zone (SEZ) and about 100 neurons in the medial protocerebrum (PAM). In several aspects, the neurons of the PAM cluster differ from

the other clusters of the DA system. PAM cluster neurons are the last born DA producing neurons and develop only during pupation. They have much smaller somata and they are the only DA producing neurons of the CNS that are for most (~85%) of them not included in the expression pattern of the TH-Gal4 line, indicating that these neurons also differ at the level of gene regulation. Being the only neurons of the anterior DA system innervating the mushroom body (MB), these neurons send their projections towards the tips of the γ -lobes, the β' -lobes and the shaft of the β -lobes (**Figure 1A1**, yellow arrow heads; see also Pech et al., 2013). The posterior DA system of the adult brain consists of the PPL1, PPL2, PPM1/2, PPM3, a group of small neurons located lateral to the SEZ, and a group of large neurons in the medial part of the SEZ. The neurons of the posterior cluster, positioned lateral to the MB calyx (PPL1), densely innervate the heel and the vertical lobes of the MB. The cluster located between the CNS and the optic lobes (OL) innervate the lobula plate at the ipsilateral side and send their projections to the lobula plate of the contralateral OL (**Figure 1A2**).

The dopaminergic system of the adult thoracic nerve cord consists of dense innervations deriving partially from the CNS and projecting to all three segments, as well as from groups of dopaminergic neurons positioned laterally (**Figure 1A3**, ThL) or medially between the first and second (**Figure 1A3**, Th) and between the second and the third segment, as well as between the third thoracic segment and the abdominal ganglion (AG; **Figure 1A3**, ThL). The dopaminergic neurons of the AG are positioned laterally to the ganglion and send their projection to the tip of the AG (**Figure 1A3**, AbL), where a second group of DA producing neuron in the AG is positioned (**Figure 1A3**, AbU). The central DA system of third-instar (LIII) larvae is largely comparable to the one of adult flies, with the exception of the PAM cluster that consists of only four neurons per hemisphere in LIII-larvae and counts about 100 neurons in adults. Despite the much simpler anatomy of the larval DA system, similar functions of these neurons were described for adults and larvae (Rohwedder et al., 2016). In the larval ventral ganglion, lateral DA neurons send long projections to the neuropil where they form lateral, longitudinal bundles and from whence they project towards the medial part of the ventral ganglion, whereas medially positioned DA neurons with short projections form a medial, longitudinal bundle, projecting to the lateral parts of the ventral ganglion (**Figure 1A4**).

The CNS of adult *Drosophila* is densely innervated by ~90 5-HT producing interneurons that can be subdivided in 10 clusters (Vallés and White, 1988; Sitaraman et al., 2008; Alekseyenko et al., 2010; Sadaf et al., 2012; Pech et al., 2013; Pooryasin and Fiala, 2015; **Figures 1B1,B2**). Out of these ~90 5-HT producing neurons about 80 neurons can be targeted by TrH-Gal4 (Sitaraman et al., 2008; Cassar et al., 2015) that however drives ectopic expression in nearly 170 non-serotonergic neurons (**Figure 1B**; see also Pooryasin and Fiala, 2015). Similar to vertebrates, where neuropils that are innervated by DA producing neurons are also innervated by 5-HT neurons (Niederkofler et al., 2015), with the exceptions of the antennal lobes (AL) that are innervated by 5-HT producing

neurons but only sparsely at the outer rim by DA neurons (**Figures 2A1–3**) and the protocerebral bridge (PB) that is innervated by DA neurons but not by 5-HT producing neurons (**Figure 2E1**), both aminergic systems innervate the same target regions, but differ in their density on local innervation patterns. At the anterior side, the serotonergic system can be subdivided in a medial (AMP) and a dorsomedial (ADMP—anterior dorso medial protocerebrum) cluster and a small group of neurons positioned in the anterior lateral protocerebrum (ALP) and a large of group of neurons positioned between the OL and the CNS in the lateral protocerebrum (LP). The SEZ is innervated at the anterior side by a cluster of large lateral neurons (SEL) and a group of small medial neurons (SEM), whereas at the dorsal side a group of small neurons is positioned laterally, and a group of three neurons is located at the medial part (SEM) in direct vicinity to a group of large DA producing neurons (Pooryasin and Fiala, 2015; **Figure 1B1**). At the posterior side, the 5-HT producing neurons can be classified in a medially positioned cluster that can be further subdivided in the posterior medial dorsal (PMPd), posterior medial (PMPm) and posterior ventral cluster (PMPv). In the LP a group of two neurons with strong 5-HT immunoreactivity (IR) forms the posterior lateral protocerebral cluster (LP; Pooryasin and Fiala, 2015; **Figure 1B2**).

The thoracic ganglion (TG) is strongly innervated by 5-HT neurons partially deriving from the CNS and projecting to all three segments as well as from groups of neurons positioned medially between the first and second (**Figure 1B3**, PR) and between the second and the third segment (**Figure 1B3**, MS). The 5-HT neurons of the AG are positioned laterally and medially in the ganglion sending their projection to the tip of the AG (**Figure 1B3**, AB, MT). The 5-HT system of LIII larvae has been described in detail (Vallés and White, 1988; Huser et al., 2012) and consists mainly of four clusters in the brain hemispheres, and four clusters in the SEZ. In the larval ventral ganglion 5-HT neurons with short projections innervate the neuropil at the ipsilateral side and 5-HT neurons with long projections innervate the contralateral side or both sides of the ganglion (**Figure 1B4**; Huser et al., 2012).

Both the AL (**Figures 2A1–3**) and MB (**Figures 2A1–3,B1,B2**) are contacted by fine 5-HT terminals. In agreement with Vallés and White (1988), who used a different antibody, we find that in the CC, a structure composed of the ellipsoid body (**Figures 2B1,B2**, EB), the fan-shaped body (FB; **Figures 2C1,C2**, FB), the noduli (**Figures 2D1,D2**, NO) and the PB (**Figures 2E1,E2**, PB), 5-HT-producing neurons predominantly send their projections to the inner rim of the EB (**Figures 2E1,E2**) and to the superior arch of the FB (**Figures 2D1,D2**), but only sparsely into the noduli (**Figures 2D1,D2**). With the exception of the AL, which is only sparsely innervated by TH immunoreactive neurons at the outer rim and the PB that is exclusively innervated by DA producing neurons (**Figures 2E1,E2**), DA and 5-HT producing neurons project to the same neuropils, but differ in their exact innervation characteristics within their target neuropils. Whereas the MB is sparsely, but homogeneously innervated by the 5-HT producing dorsal pair medial (DPM) neuron, DA

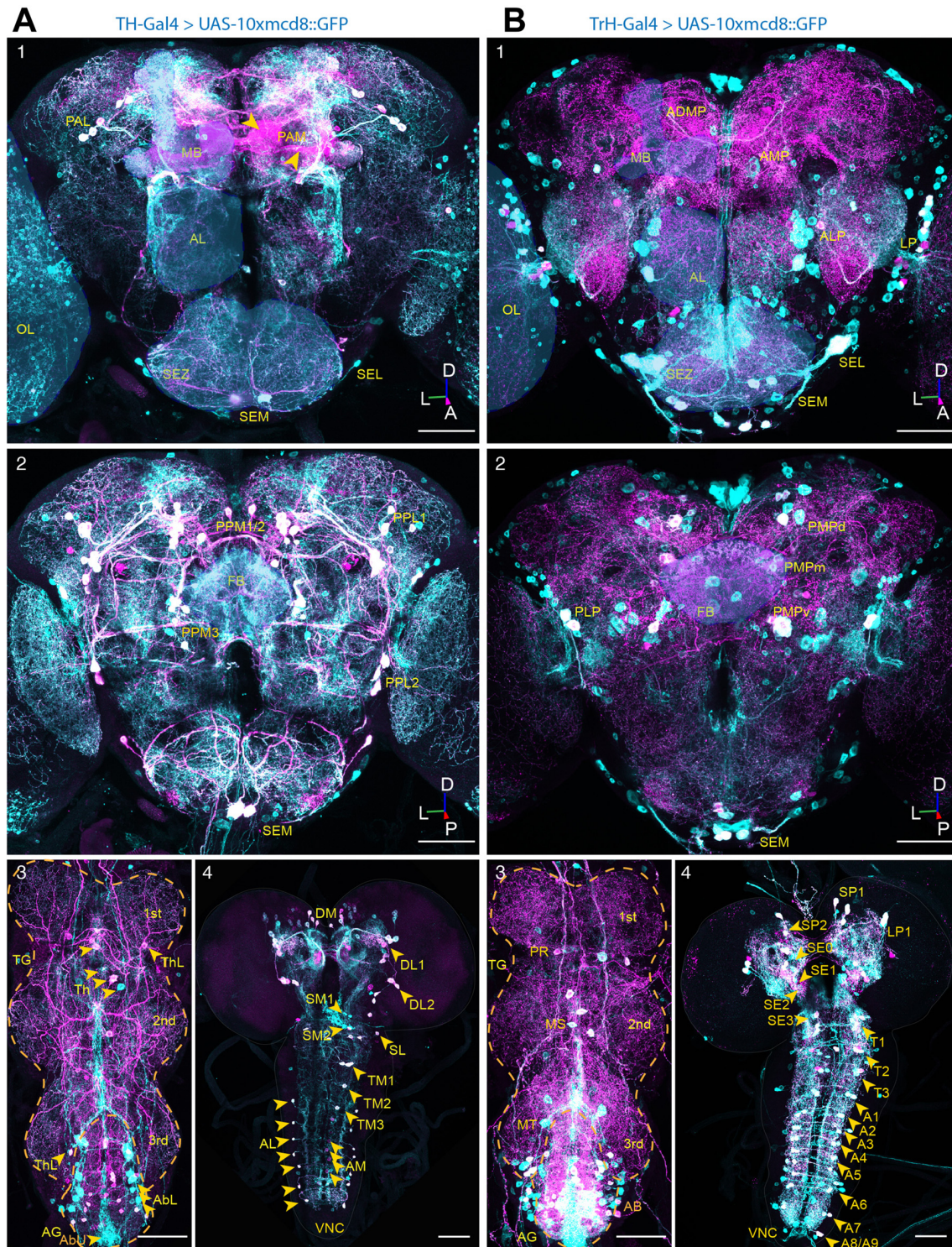


FIGURE 1 | Dopaminergic and serotonergic neurons of the adult *Drosophila* central brain. **(A)** *In situ* co-immunostainings with anti-GFP (green) and anti-tyrosine hydroxylase (TH; magenta) antibodies in whole-mount nervous tissues of *TH-Gal4 > 10xUAS-mCD8<GFP* flies. Co-localizations merge both colors in white, showing driver-targeted somata of the subesophageal medial (SEM), lateral (SEL), the protocerebral medial (PAM) and lateral (PAL) clusters and projections in anterior **(1)** and the posterior lateral (PPL1, PPL2) and medial clusters (PPM1/2, PPM3) in the posterior **(2)** brain, as well as the lateral (ThL) and medial cell cluster (Th) in the thoracic ganglion (TG) and the lateral (AbL) and medial (AbU) situated clusters in the abdominal ganglion (AG) **(3)** of adult wild-type *Drosophila*. In the central nervous system
(Continued)

FIGURE 1 | Continued

(CNS) of LIII-Larvae (**4**) the dopaminergic system consists of one dorso-medially (DM), two dorso-laterally (DL1, DL2) within the hemispheres, three clusters in the medial (SM0, SM1, SM2) and one cluster in lateral (SL) subesophageal zone (SEZ), three medially situated cluster in the thoracic (TM1, TM2, TM3) clusters and an array of neurons in the lateral (AL) and medial (AM) part of the abdominal part of the ventral nerve cord (VNC).

(B) *In situ* co-immunostainings with anti-GFP (green) and anti-5-HT (magenta) antibodies in whole-mount nervous tissues of *TrH-Gal4 > 10xUAS-mCD8 < GFP* flies, showing the clusters in the lateral lateral protocerebrum (LP), the anterior medial protocerebrum (AMP), anterior lateral protocerebrum (ALP) and the anterior dorso-medial protocerebrum (ADMP) and the medially (SEM) and laterally (SEL) situated clusters in the subesophageal subesophageal zone (SEZ) in the anterior (**1**) and the clusters situated in the dorsal (PMPd), medial (PMPm) and ventral posterior protocerebrum as well as the dorsally situated clusters in the medial (SEM) and lateral (SEL) subesophageal ganglion (**2**) and the clusters in the por-, (PR), meso- (MS) and meta- (MT) thoracic neuromere and the AG (AB) (**3**) of an adult fly. The CNS of a LIII-Larva (**4**) of three clusters in the supesophageal ganglion (SP0, SP1, SP2), one in the LP1 and four cell clusters in the SEZ (SE0, SE1, SE2, SE3). The VNC contains three clusters of 5-HT producing neurons in the thoracic (T1, T2, T3) and an array of nine symmetrically organized clusters in the abdominal neuromere (A1–A9). Overlay in white correspond to driver-targeted serotonergic cell bodies (MB, mushroom body; FB, fan-shaped body; OL, optic lobe; SEZ, subesophageal zone; AL, antennal lobe; TG, thoracic ganglion; AG, abdominal ganglion; D, dorsal; L, lateral; P, posterior; A, anterior). Scale bars: 50 μ m.

producing neurons group in different sub-populations that form dense and spatially restricted innervations onto characteristic sub-regions within the MB lobes (**Figures 2B1,B2**; see also Pech et al., 2013), consistent with immunocytochemical pattern observed with an antibody to DA (Cichewicz et al., 2016). The terminal projections of DA-producing neurons into the CC are mainly complementary to those of 5-HT producing neurons. TH immunoreactive neurons innervate densely the outer rim of the EB (**Figures 2C1,C2**) and the medial parts of the FB (**Figures 2D1,D2**), whereas 5-HT projections are concentrated to the inner and anterior part of the EB (**Figures 2C1,C2**) and the dorsal FB (**Figures 2D1,D2**).

In summary, we find in the logic underlying the innervation pattern of the DA and 5-HT system similarities to the innervations characteristics of aminergic systems in vertebrates. DA- and 5-HT-producing neurons innervate large portions of the CNS, with largely overlapping target neuropils. With the exception of the AL and the PB, both 5-HT- and TH-immunoreactive neurons send their projections to the same neuropils, but differ mainly in the densities of their innervation within sub-region of the particular neuropil.

DA-Deficient Flies Show Increased 5-HT Immunoreactivity (IR) in the Posterior Lateral Protocerebral Neurons

To analyze the impact of DA loss on the serotonergic system, we compared the anatomy of flies lacking *dTH* in the CNS, compared to *dTH*-rescue flies and *w¹¹¹⁸* control flies. We find that the overall appearance of the serotonergic system of flies lacking DA is comparable to that of *dTH* rescue flies or *w¹¹¹⁸* controls. However, brains lacking genomic *dTH* show an increased number of 5-HT immunoreactive somata within the posterior lateral protocerebrum (PLP; **Figures 3A–H**, PPL). In

control *w¹¹¹⁸* 5-HT positive neurons in the PLP were described as positioned ventrally to the calyces (**Figures 3A–D**), near the OL (**Figure 3D**; Vallés and White, 1988; Monastirioti, 1999; Sitaraman et al., 2008; Cassar et al., 2015; Pooryasin and Fiala, 2015). Giang et al. (2011) further have identified an additional group neurons with faint IR against the 5-HT transporter positioned laterally to the calyces similarly positioned then the PPL1 dopaminergic neurons. In wild-type brains, a small number of TH IR neurons within the PPL1 cluster show very faint 5-HT IR (**Figures 3A–C**). However, in DA-deficient brains, we find an increased 5-HT IR of neurons juxta-calycal in the PLP (**Figure 3H**, PPL) at the position of the PPL1 DA producing neurons (Nässel and Elekes, 1992; Monastirioti, 1999; Riemensperger et al., 2013). This increased IR we could verify with two different antibodies against 5-HT (**Supplementary Figure S1**). However, it is not clear whether the increased 5-HT IR in DA deficient flies derives from the same neuronal populations. Other clusters, like the PMP clusters (**Figure 3D**), did not reveal any changes in number of visible somata of 5-HT IR. To exclude the possibility that the observed increase in number of these PPL1-like 5-HT neurons observed in DA-deficient brains does not derive from globally increased 5-HT production, we quantified 5-HT IR between DA-deficient flies, rescue flies and *w¹¹¹⁸* control flies in the lateral protocerebrum (LP), PLP, PMPd, PMPm and PMPv 5-HT neuron cluster. With the exception of the LP cluster that showed decreased 5-HT IR in *dTH*-rescue when compared to *w¹¹¹⁸* flies, all three strains showed comparable levels of 5-HT IR in the somata of the PMP neurons (**Figures 4A–F**). It thus appears that increased 5-HT synthesis in the 5-HT/DA PPL1 neurons in the PLP is a selective response occurring in this neuronal cluster that has the capability of increased re-uptake or synthesis of either or both transmitters.

5-HT Neurons Projecting to the MB Show Altered Innervation Densities in DA-Deficient Flies

In vertebrate models of PD, degeneration of DA neurons leads to modifications in 5-HT neurons (Zhou et al., 1991; Rylander et al., 2010; Zeng et al., 2010; Niederkofler et al., 2015). To determine whether the same holds true for *Drosophila*, we analyzed the innervation pattern of 5-HT neurons onto their target region in the vertical MB lobes. The tips of the vertical α - and α' -lobes are densely innervated by both TH- and 5-HT-IR neurons. Whereas TH-IR neurons innervate both regions with similar intensities (**Figures 2E1,E2**), in wild-type brains, 5-HT neurons mainly send projections towards the α' -lobes, but only faintly to the α -lobe (**Figures 2D1,D2**, 5A). However, in the DA-deficient brains, there is a strong increase in IR for 5-HT in the α -lobes, but not in the α' -lobes (**Figure 5B**). This increase in 5-HT-immunoreactive projections onto the α -lobes results in a shift in the proportion of 5-HT positive projections between the two lobe structures (**Figure 5C**). However, it does not affect the overall size in terms of area surface of the innervated neuropils (**Figure 5D**).

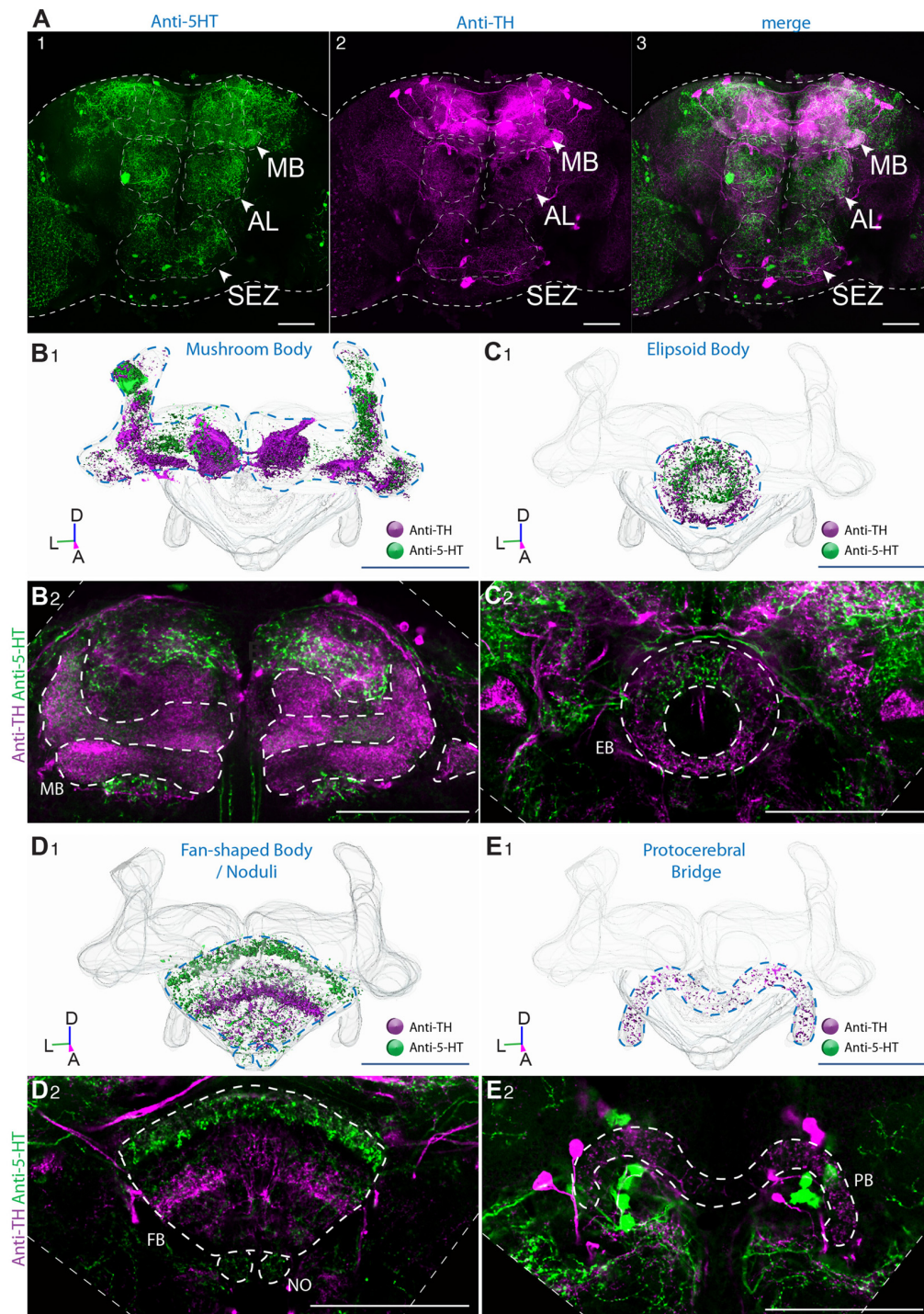


FIGURE 2 | Innervation pattern of dopaminergic and serotonergic neurons in the adult wild-type *Drosophila* central brain. **(A)** Anti-5-HT **(1)** and anti-TH **(2)** immunoreactive neurons show to some degree complementary innervation patterns in the *Drosophila* brain. 5-HT-producing neurons strongly innervate the AL **(A)** and only weakly the MBs **(B1,2)**. TH-positive neurons innervate only the outer rim of the AL **(A2,3)**, but strongly the mushroom body (MB) **(B1,2)**. The tips of the α' lobes are innervated by both populations of aminergic neurons **(B1–2)**. Innervation pattern of 5-HT neurons and dopamine (DA) neurons in the ellipsoid body **(C1,2, EB)** and the fan-shaped body **(D1–2, FB)**. 5-HT-producing neurons innervate strongly the inner rim of the EB **(C1,2)** and the dorsal part of the FB **(D1,2)**. DA-producing neurons innervate strongly the outer rim of the EB **(C1,2)** and the ventral part of the FB **(D1,2)**. The protocerebral bridge (PB) is innervated by DA, but by 5-HT producing neurons **(E1,2)**. 3D reconstruction of the MB **(B1)**, EB **(C1)**, the FB **(D1)** and the PB **(E1)** with DA and 5-HT IR indicated in different colors (D, dorsal; L, lateral; P, posterior; A, anterior). Scale bars: 50 μ m.

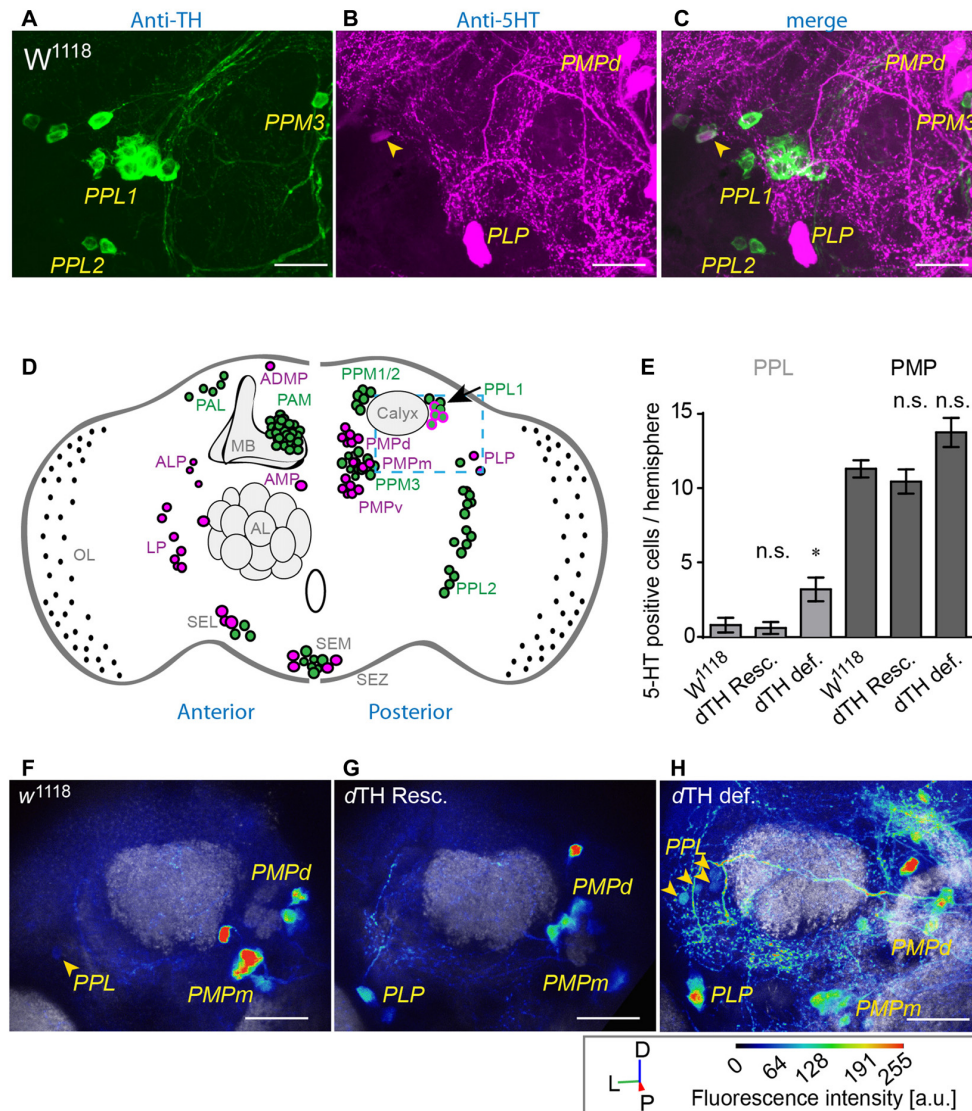


FIGURE 3 | DA-deficient flies show increased 5-HT immunoreactivity (IR) in TH-producing neurons of the posterior lateral protocerebrum (PLP). **(A)** TH- (green) and **(B)** 5-HT- immune reactive neurons (green) in the posterior lateral protocerebrum (PPL) and the posterior medial protocerebrum (PMP) in *w¹¹¹⁸*. Merge is shown in **(C)**. Scale bars: 10 μ m. *w¹¹¹⁸* control flies show a small number of faintly 5-HT-immunoreactive neurons (magenta) among **(A)** DA-producing PPL1 neurons **(C)**, arrow heads). **(D)** Schematic overview of the TH (green) and 5-HT (magenta) immune reactive neurons in the adult *Drosophila* brain. In the PPL (blue dashed line) *w¹¹¹⁸* control flies show a small number of 5-HT-immunoreactive neurons (magenta) among DA-producing neurons (green) in the PPL1 cluster (arrow). **(E)** DA-deficient flies show increased numbers of 5-HT-producing neurons in the PPL when compared to *w¹¹¹⁸* controls and dTH-rescue flies (Dunn's multiple comparison test against *w¹¹¹⁸*, $n > 5$). **(F)** 5-HT immune reactive neurons (green) in the PPL and the PMP in *w¹¹¹⁸*, genomic dTH rescue **(G, dTH Resc.)** and DA-deficient flies **(H, dTH def., arrow head)** (D, dorsal; L, lateral; P, posterior) Scale bars: 20 μ m. n.s.: $p > 0.05$; * $p < 0.05$.

Long-Term L-DOPA Treatment Alters 5-HT Projections to their Target Neuropils in the CNS

We next asked whether enhanced DA levels, attained by feeding wild-type flies L-DOPA, would affect the serotonergic system in the opposite way than lack of CNS DA. To this end we analyzed the IR of 5-HT projections onto their target region in the vertical MB lobes after 10 days L-DOPA treatment. 5-HT IR was significantly decreased in both, α -

(Figures 6A,A1), and α' -lobes (Figures 6A,A2) after L-DOPA treatment when compared to control flies. To determine whether the observed reduction in 5-HT IR was caused by altered 5-HT biosynthesis or altered plasticity we used the splitGFP technique (Gordon and Scott, 2009; Pech et al., 2013) to visualize alteration in connectivity between 5-HT producing neurons and the MB vertical lobes. Adult flies expressing one part of the splitGFP in 5-HT neurons and the counterpart in MB Kenyon neurons were fed for 10 days with L-DOPA, and

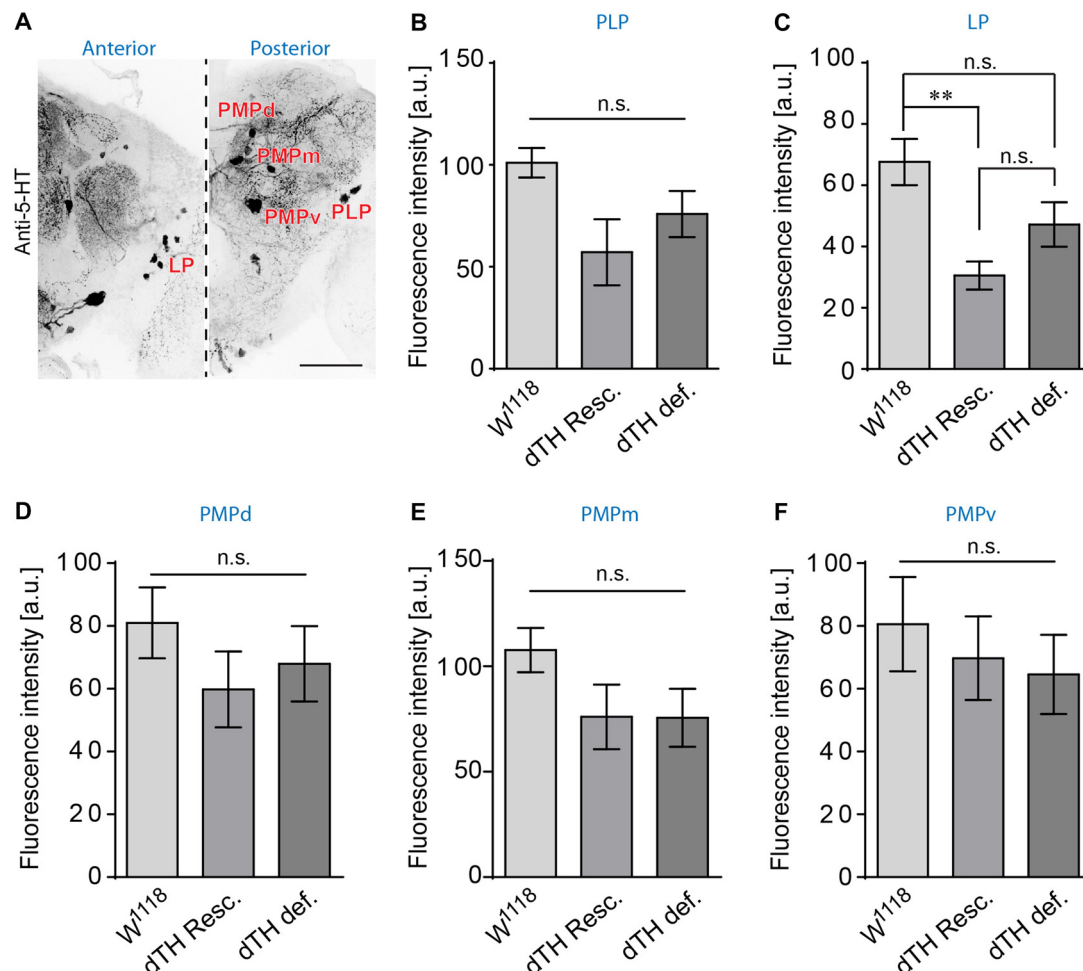


FIGURE 4 | DA deficiency does not increase 5-HT IR in somata of 5-HT-producing neurons. **(A)** 5-HT immunoreactive neurons in the anterior (left, LP) and posterior medial (right, PMPd, PMPm, PMPv) and PLP in *w¹¹¹⁸*. Scale bars: 50 μ m. **(B–F)** DA-deficient flies do not show increased 5-HT somatic IR when compared to *w¹¹¹⁸* or genomic *dTH*-rescue flies (ANOVA with Bonferroni correction, $n < 6$). n.s.: $p > 0.05$; ** $p < 0.01$.

monitored for changes in 5-HT GFP fluorescence. Whereas the general pattern of 5-HT IR appeared largely unchanged and comparable to what has been published before (Pech et al., 2013), we found that certain 5-HT projections were significantly decreased (**Figure 6B**), as can be seen for the intensity of the signal of reconstituted splitGFP between 5-HT terminals and MB lobes at the tip of the α - (**Figures 6B,B1**) and α' -lobes (**Figures 6B,B2**). Thus, 5-HT neurons sharing the same target regions than DA neurons respond with diminished projections with enhanced DA, and enhanced 5-HT IR in the absence of DA. These observations provide evidence of competitive interactions between 5-HT and DA in the *Drosophila* brain.

DISCUSSION

Here we investigated the effects of altered DA signaling on the 5-HT circuitry in the CNS of adult fruit flies. As in

vertebrates (Niederkofler et al., 2015), the DA and 5-HT neurons of *Drosophila* send their projections to many brain areas. Most neuropils that are innervated by TH-immunoreactive neurons are also innervated by 5-HT-producing neurons. Whereas the AL is mainly innervated by 5-HT neurons and only faintly at the outer rim by TH immunoreactive neurons, the PB appears to be innervated exclusively by TH-positive neurons, but not by 5-HT. These innervations resemble to some extent the situation in the vertebrate brain where projections of DA-producing neurons are for the most part accompanied by 5-HT producing neurons (Niederkofler et al., 2015).

We found a previously underappreciated set of neurons that co-express DA and 5-HT in the adult fly brain. These DA neurons, comprising 1–2 neurons of the PPL1 cluster, function in conveying an aversive stimulus when stimulated (Masek et al., 2015). In a normal brain, the PPL1 cluster is positioned in a region that is largely devoid of other 5-HT-immunoreactive cell bodies, but IR against the 5-HT transporter in this region has

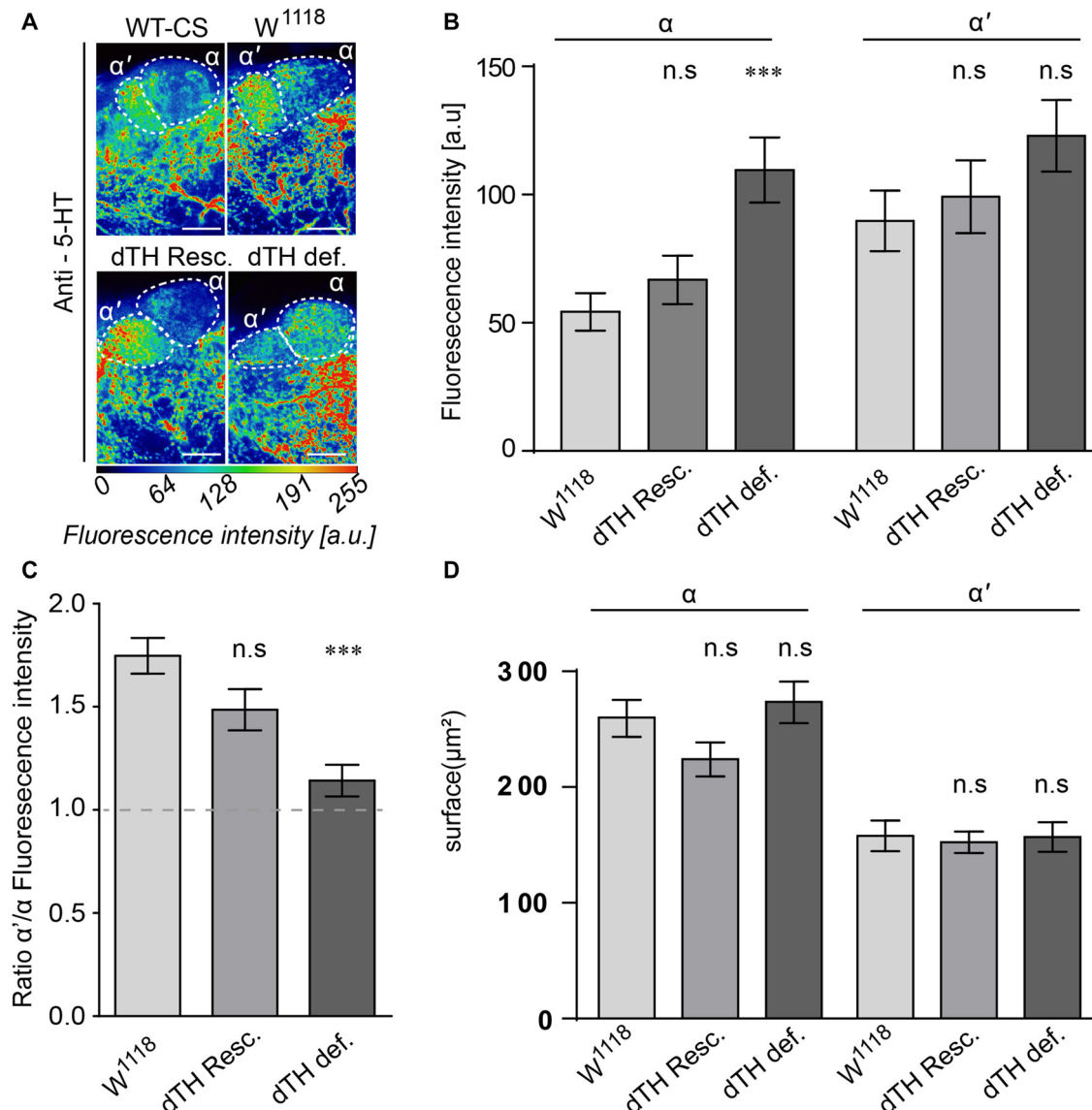


FIGURE 5 | 5-HT neurons projecting to the MB show altered innervation densities in DA-deficient flies. **(A)** Innervation patterns of 5-HT neurons to the α/α' -lobe of the MB. Under wild-type conditions, 5-HT producing neurons innervate strongly the tips of the α' but only faintly the α lobes. In DA-deficient flies, 5-HT innervations to the α lobes are markedly increased. Scale bar: 10 μm **(B)** Fluorescence intensity analysis of the 5-HT IR in α - and α' -lobes. 5-HT innervations to the α -lobes are increased in flies lacking neuronal TH when compared to control flies. **(C)** Ratio of 5-HT innervation density between α' - and α -lobes. Under control conditions, 5-HT neurons innervate α' -lobes stronger than the α lobes. In DA-deficient flies, 5-HT-neuron α -lobe innervations are increased and comparable to the α' -lobe innervations. **(D)** The surface of the α/α' -lobes that are targeted by 5-HT neurons is not altered between DA-deficient and control flies (Dunn's multiple comparison test against w^{1118} , $n < 13$). n.s.: $p > 0.05$; *** $p < 0.001$.

been described beforehand (Giang et al., 2011). However, we find that some of the PPL1 neurons express low levels of 5-HT IR in brains with normal DA synthesis. This 5-HT IR increases significantly in brains lacking DA, both in the cell bodies and in the terminal regions of these neurons, and, therefore, may reflect a potentially compensatory response to DA loss. Whether all of the 5-HT-positive neurons detected indeed correspond to TH-positive PPL1 neurons under wild type conditions remains unknown at the current state. Further investigations on the

nature of these neurons and on the mechanisms of how the presence of dTH or DA may potentially affect the cell fate of other neurons is needed.

We also found enhanced 5-HT IR in DA-deficient brains in the terminal regions of the PPL1 neurons, in the MB α -lobe. This region is strongly innervated by both DPM, 5-HT and the DA PPL1 neurons. These changes may possibly be explained by 5-HT being now expressed or taken up more strongly in these PPL1 neurons or, as observed in vertebrate models for PD, where

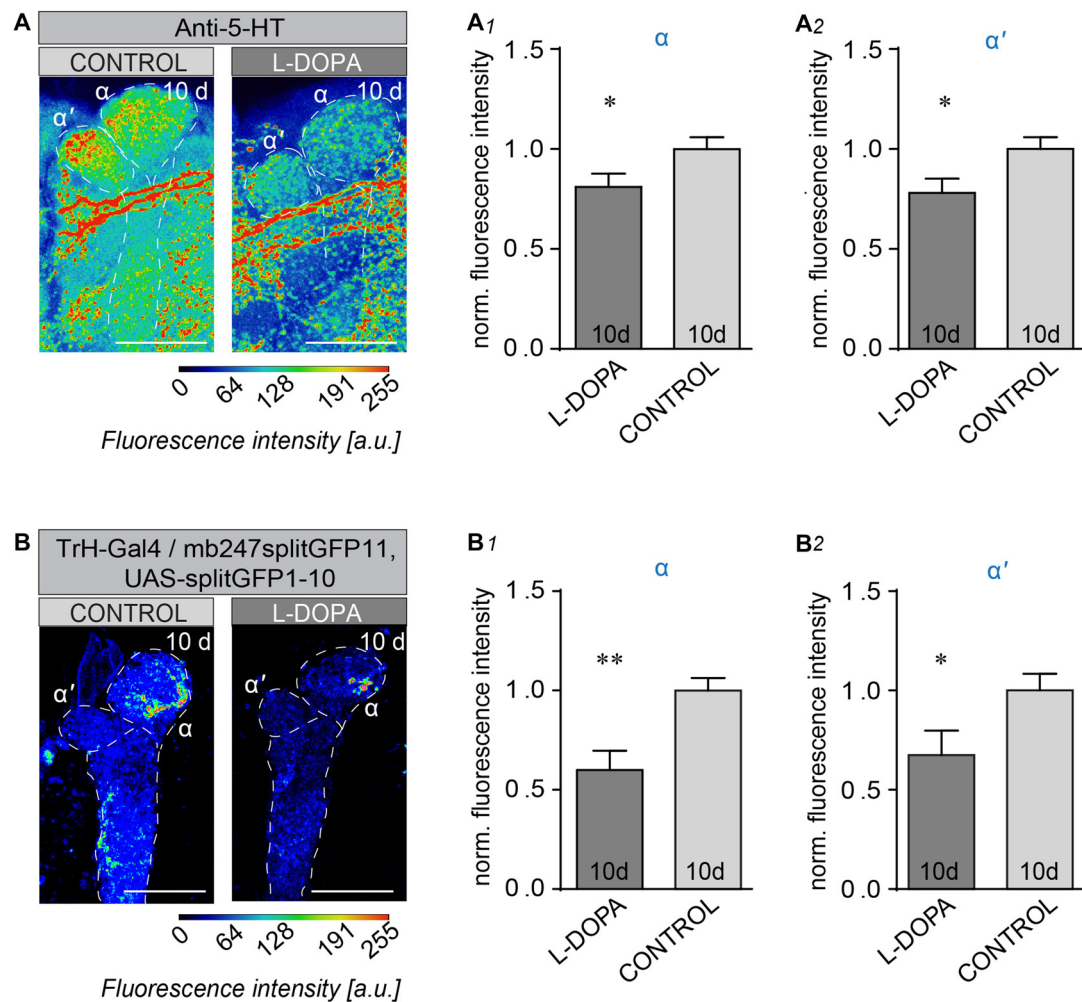


FIGURE 6 | Long-term L-DOPA treatment alters 5-HT neuron innervation to their MB target regions. **(A)** Innervation patterns of 5-HT neurons to the α/α' -lobe of the MB. Projection of 5-HT producing neurons onto α/α' lobes of the MB under control conditions and after 10 days L-DOPA administration. Fluorescence intensity analysis of the 5-HT IR in α -lobes (**A1**) and α' -lobes (**A2**; unpaired students *T*-test, $n < 15$). **(B)** Reconstituted splitGFP between TrH-positive 5-HT producing neurons and the MB vertical lobes in wild-type flies under control conditions (left) and after 10-day L-DOPA treatment (right). Fluorescence intensities are indicated by false colors. Fluorescence intensity analysis of the reconstituted splitGFP signal in the tips of α (**B1**) and α' lobes (**B2**). 5-HT innervations are strongly reduced by L-DOPA treatment compared to non-treated control flies (Unpaired students *T*-test, $n < 11$). Scale bars: 20 μ m. n.s.: $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

denervation of DA neurons was found to potentiate 5-HT IR at neuronal terminals (Zhou et al., 1991; Rylander et al., 2010; Zeng et al., 2010; Niederkofler et al., 2015), derive from altered 5-HT plasticity.

DA deficiency has consequences on a broad variety of behaviors in *Drosophila* (Hirsh et al., 2010; Riemensperger et al., 2011; Cichewicz et al., 2016). The simplest interpretation of the behavioral consequences observed in DA-deficient flies is that the observed phenotypes are solely due to the lack of brain DA. However, quiescence behavior can be induced by reduced DA (Riemensperger et al., 2011; Cichewicz et al., 2016) or increased 5-HT signaling (Pooryasin and Fiala, 2015). Similarly, we have shown that phototactic behavior is strongly decreased in DA deficient flies, whereas increased 5-HT_{1A} receptor signaling has similar effects in honeybees (Thamm et al., 2010). Thus, it

seems likely that 5-HT and DA may antagonize each other, with opposing behavioral effects. Similar interactions of dopaminergic and serotonergic systems occur in the context of arousal in mammals (Wong et al., 1995; Sasaki-Adams and Kelley, 2001; Daw et al., 2002). Consequently, the impact of the 5-HT system in the control of DA neuron activity appears to be a pivotal factor in motor, mood and cognitive effects of DA therapies (reviewed in De Deurwaerdere and Di Giovanni, 2016).

DA neuron denervation strongly alters 5-HT neuron innervation in the rat striatum (Rylander et al., 2010) and increases overall 5-HT IR in the caudate nucleus and globus pallidus (Zeng et al., 2010). Moreover, 5-HT transporters in the putamen are significantly increased in PD patients receiving L-DOPA treatment and suffering from LID, as well as in primates developing dyskinesia from L-DOPA treatment (Rylander et al.,

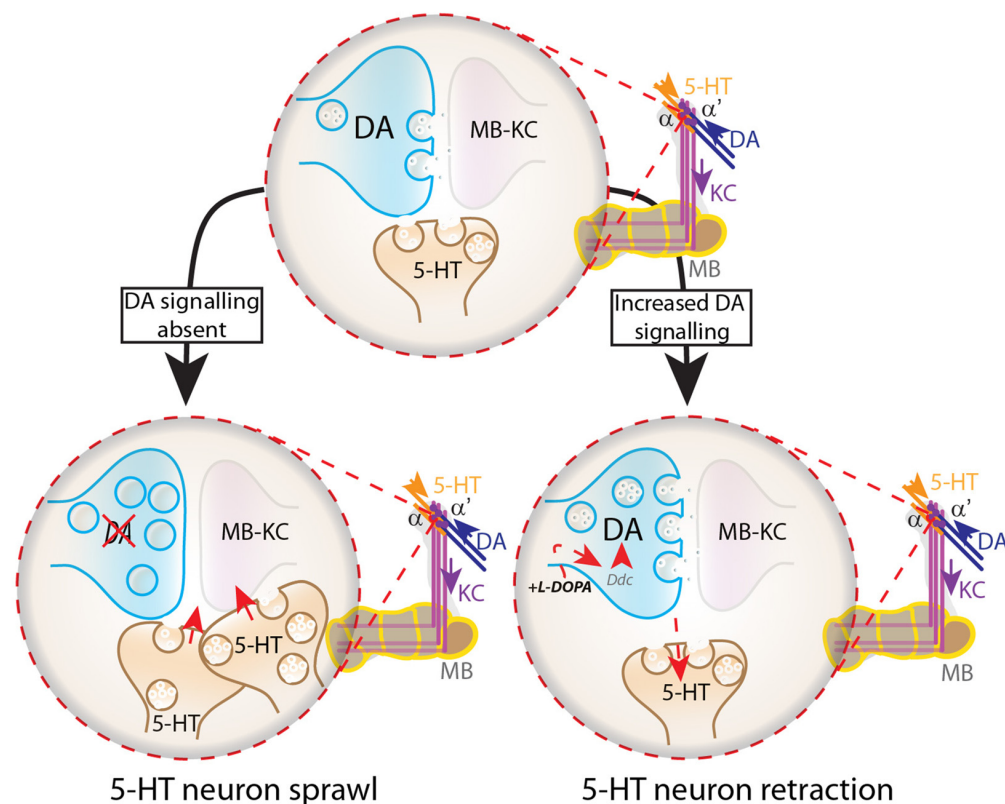


FIGURE 7 | DA modulates serotonin connectivity in the *Drosophila* brain. Under wild-type conditions, dopaminergic neurons predominantly innervate the tips of the α -lobes, whereas 5-HT neurons predominantly innervate the α' -lobes. Lack of DA signaling results in sprawling of 5-HT neurons at the tips of the α -lobes, whereas L-DOPA-induced increase in DA synthesis triggers retraction of 5-HT neurons from their target region on the MB α - and α' -lobes.

2010). As with observations in human patients and in PD vertebrate models (Linazasoro, 2005; Rylander et al., 2010; Zeng et al., 2010) our data show that 5-HT producing neurons respond to DA deficiency with hyper-innervation of some target regions. However, consequences of long-term DA deficiency on the proper development of 5-HT producing neurons cannot be ruled out and a long-term effect may not be directly transferable to PD-like conditions in a brain suffering progressive DA neuron degeneration. Yet even with these different time scales, we see reciprocal effects consistent with competitive interactions between DA and 5-HT.

However, our data show decreases in 5-HT levels in terminal regions and altered 5-HT neuron plasticity in wild type brains with enhanced DA subsequent to L-DOPA treatment. The DPM 5-HT neurons (Lee et al., 2011; Haynes et al., 2015) react to 10 days L-DOPA treatment with decreased 5-HT in their terminal region on the MB vertical lobes, the same region showing enhanced 5-HT subsequent to DA deficiency. This decreased intensity of 5-HT neuron terminals could indicate that pharmacologically increased DA signaling through L-DOPA feeding may have an acute impact on 5-HT neuron plasticity in the fully developed brain and negatively influence outgrowth of 5-HT producing neurons even under wild-type conditions.

However, consequences of L-DOPA treatment on 5-HT neuron functionality cannot be excluded.

In human patients the administration of L-DOPA represents currently the most effective pharmacological treatment for PD, but long-term treatment is hampered by the development of dyskinesia and motor fluctuations, the so-called L-DOPA-induced dyskinesia (LID). The exact cause of LID is unknown, but dysfunctional 5-HT neuron plasticity triggered by the combined effects of DA neuron denervation and pharmacological DA replacement with L-DOPA have been implicated (Calabresi et al., 2000; Hirsch, 2000; Cenci and Lundblad, 2006; Cenci and Lindgren, 2007). Our data indicate that the DA/5-HT competitive interactions can occur in a more normal situation than total DA deficiency (Figure 7). Indeed, these neurons undergo age-dependent plasticity (Tonoki and Davis, 2015). The observed DA/5-HT competitive interactions and the similarities between *Drosophila* and vertebrate models for PD may open novel vistas to better understand the development of LID e.g., through testing how these 5-HT neurons react to L-DOPA treatment under unbalanced DA/5-HT signaling in *dTH* deficient flies, completely devoid of DA signaling in the brain or under neurodegenerative conditions mimicking PD in flies.

AUTHOR CONTRIBUTIONS

TDR, JN, FR, BC, KC, JE, Y-TL and KC performed and analyzed experiments. TDR designed and supervised the study. TDR and JH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | DA-deficient flies show increased 5-HT IR in neurons of the posterior lateral protocerebrum. Quantified 5-HT immune reactivity of neurons in the posterior lateral protocerebrum (PLP) stained with anti-5-HT (rat) in w^{1118} and DA-deficient flies. n.s.: $p > 0.05$; * $p < 0.05$.

REFERENCES

- Alekseyenko, O. V., Lee, C., and Kravitz, E. A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. *PLoS One* 5:e10806. doi: 10.1371/journal.pone.0010806
- Andrejic, R., van Swinderen, B., and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol.* 15, 1165–1175. doi: 10.1016/j.cub.2005.05.025
- Ascherio, A., and Schwarzschild, M. A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. *Lancet Neurol.* 15, 1257–1272. doi: 10.1016/S1474-4422(16)30230-7
- Calabresi, P., Giacomini, P., Centonze, D., and Bernardi, G. (2000). Levodopa-induced dyskinesia: a pathological form of striatal synaptic plasticity? *Ann. Neurol.* 47, S60–S69; discussion S68–S69.
- Cassar, M., Issa, A.-R., Riemensperger, T., Petitgas, C., Rival, T., Coulom, H., et al. (2015). A dopamine receptor contributes to paraquat-induced neurotoxicity in *Drosophila*. *Hum. Mol. Genet.* 24, 197–212. doi: 10.1093/hmg/ddu430
- Cenci, M. A., and Lindgren, H. S. (2007). Advances in understanding L-DOPA-induced dyskinesia. *Curr. Opin. Neurobiol.* 17, 665–671. doi: 10.1016/j.conb.2008.01.004
- Cenci, M. A., and Lundblad, M. (2006). Post- versus presynaptic plasticity in L-DOPA-induced dyskinesia. *J. Neurochem.* 99, 381–392. doi: 10.1111/j.1471-4159.2006.04124.x
- Cichewicz, K., Garren, E. J., Adiele, C., Aso, Y., Wang, Z., Wu, M., et al. (2016). A new brain dopamine-deficient *Drosophila* and its pharmacological and genetic rescue. *Genes Brain Behav.* 16, 394–403. doi: 10.1111/gbb.12353
- Daw, N. D., Kakade, S., and Dayan, P. (2002). Opponent interactions between serotonin and dopamine. *Neural Netw.* 15, 603–616. doi: 10.1016/s0893-6080(02)00052-7
- De Deurwaerdere, P., and Di Giovanni, G. (2016). Serotonergic modulation of the activity of mesencephalic dopaminergic systems: therapeutic implications. *Prog. Neurobiol.* 151, 175–236. doi: 10.1016/j.pneurobio.2016.03.004
- Dehay, B., and Bezard, E. (2011). New animal models of Parkinson's disease. *Mov. Disord.* 26, 1198–1205. doi: 10.1002/mds.23546
- Feany, M. B., and Bender, W. W. (2000). A *Drosophila* model of Parkinson's disease. *Nature* 404, 394–398. doi: 10.1038/35006074
- Fiala, A., and Riemensperger, T. (2017). "Localization of a memory trace: aversive associative olfactory learning and short-term memory in *Drosophila*," in *Learning and Memory: A Comprehensive Reference*, 2nd Edn. eds J. H. Byrne and R. Menzel (Elsevier: Academic Press), 475–482.
- Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic neurons using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* 54, 618–627. doi: 10.1002/neu.10185
- Ganguly-Fitzgerald, I., Donlea, J., and Shaw, P. J. (2006). Waking experience affects sleep need in *Drosophila*. *Science* 313, 1775–1781. doi: 10.1126/science.1130408
- Giang, T., Rauchfuss, S., Ogueta, M., and Scholz, H. (2011). The serotonin transporter expression in *Drosophila melanogaster*. *J. Neurogenet.* 25, 17–26. doi: 10.3109/01677063.2011.553002
- Gordon, M. D., and Scott, K. (2009). Motor control in a *Drosophila* taste circuit. *Neuron* 61, 373–384. doi: 10.1016/j.neuron.2008.12.033
- Haynes, P. R., Cristmann, B. L., and Griffith, L. C. (2015). A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*. *Elife* 4:e03868. doi: 10.7554/eLife.03868
- Hewitt, V. L., and Whitworth, A. J. (2017). Mechanisms of Parkinson's disease: lessons from *Drosophila*. *Curr. Top. Dev. Biol.* 121, 173–200. doi: 10.1016/bs.ctdb.2016.07.005
- Hirsch, E. C. (2000). Nigrostriatal system plasticity in Parkinson's disease: effect of dopaminergic denervation and treatment. *Ann. Neurol.* 47, S115–S121; discussion S120–S121.
- Hirsh, J., Riemensperger, T., Coulom, H., Iché, M., Coupar, J. H., and Birman, S. (2010). Roles of dopamine in circadian rhythmicity and extreme light sensitivity of circadian entrainment. *Curr. Biol.* 20, 209–214. doi: 10.1016/j.cub.2009.11.037
- Huser, A., Rohwedder, A., Apostolopoulou, A. A., Widmann, A., Pfitzenmaier, J. E., Maiolo, E. M., et al. (2012). The serotonergic central nervous system of the *Drosophila* larva: anatomy and behavioral function. *PLoS One* 7:e47518. doi: 10.1371/journal.pone.0047518
- Kume, K., Kume, S., Park, S. K., Hirsh, J., and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25, 7377–7384. doi: 10.1523/JNEUROSCI.2048-05.2005
- Lebestky, T., Chang, J. S., Dankert, H., Zelnik, L., Kim, Y. C., Han, K. A., et al. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* 64, 522–536. doi: 10.1016/j.neuron.2009.09.031
- Lee, P. T., Lin, H. W., Chang, Y. U., Fu, T. F., Dubnau, J., Hirsh, J., et al. (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 108, 13794–13799. doi: 10.1073/pnas.1019483108
- Linazasoro, G. (2005). New ideas on the origin of L-dopa-induced dyskinesias: age, genes and neural plasticity. *Trends Pharmacol. Sci.* 26, 391–397. doi: 10.1016/j.tips.2005.06.007
- Liu, T., Dartevelle, L., Yuan, C., Wei, H., Wang, Y., Ferveur, J. F., et al. (2008). Increased dopamine level enhances male-male courtship in *Drosophila*. *J. Neurosci.* 28, 5539–5546. doi: 10.1523/JNEUROSCI.5290-07.2008
- Lu, B. (2009). Recent advances in using *Drosophila* to model neurodegenerative diseases. *Apoptosis* 14, 1008–1020. doi: 10.1007/s10495-009-0347-5
- Marsh, J. L., and Thompson, L. M. (2006). *Drosophila* in the study of neurodegenerative disease. *Neuron* 52, 169–178. doi: 10.1016/j.neuron.2006.09.025
- Masek, P., Worden, K., Aso, Y., Rubin, G. M., and Keene, A. C. (2015). A dopamine-modulated neural circuit regulating aversive taste memory in *Drosophila*. *Curr. Biol.* 25, 1535–1541. doi: 10.1016/j.cub.2015.04.027

- Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech.* 45, 106–121. doi: 10.1002/(sici)1097-0029(19990415)45:2<106::aid-jemt5>3.3.co;2-v
- Nall, A. H., Shakhmantsir, I., Cichewicz, K., Birman, S., Hirsh, J., and Sehgal, A. (2016). Caffeine promotes wakefulness via dopamine signaling in *Drosophila*. *Sci. Rep.* 6:20938. doi: 10.1038/srep20938
- Nässel, D. R., and Elekes, K. (1992). Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell Tissue Res.* 267, 147–167. doi: 10.1007/bf00318701
- Neckameyer, W. S., and Bhatt, P. (2012). Neurotrophic actions of dopamine on the development of a serotonergic feeding circuit in *Drosophila melanogaster*. *BMC Neurosci.* 13:26. doi: 10.1186/1471-2202-13-26
- Niederkofler, V., Asher, T. E., and Dymeck, S. M. (2015). Functional interplay between dopaminergic and serotonergic neuronal systems during development and adulthood. *ACS Chem. Neurosci.* 6, 1055–1070. doi: 10.1021/acschemneuro.5b00021
- Obeso, J. A., Rodriguez-Oroz, M. C., Stamelou, M., Bhatia, K. P., and Burn, D. J. (2014). The expanding universe of disorders of the basal ganglia. *Lancet* 384, 523–531. doi: 10.1016/s0140-6736(13)62418-6
- Owald, D., and Waddell, S. (2015). Olfactory learning skews mushroom body output pathways to steer behavioral choice in *Drosophila*. *Curr. Opin. Neurobiol.* 35, 178–184. doi: 10.1016/j.conb.2015.10.002
- Pech, U., Pooryasin, A., Birman, S., and Fiala, A. (2013). Localization of the contacts between Kenyon neurons and aminergic neurons in the *Drosophila melanogaster* brain using SplitGFP reconstitution. *J. Comp. Neurol.* 521, 3992–4026. doi: 10.1002/cne.23388
- Pooryasin, A., and Fiala, A. (2015). Identified serotonin-releasing neurons induce behavioral quiescence and suppress mating in *Drosophila*. *J. Neurosci.* 35, 12792–12812. doi: 10.1523/JNEUROSCI.1638-15.2015
- Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., et al. (2011). Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl. Acad. Sci. U S A* 108, 834–839. doi: 10.1073/pnas.1010930108
- Riemensperger, T., Issa, A. R., Pech, U., Coulom, H., Nguyen, M. V., Cassar, M., et al. (2013). A single dopamine pathway underlies progressive locomotor deficits in a *Drosophila* model of Parkinson disease. *Cell Rep.* 5, 952–960. doi: 10.1016/j.celrep.2013.10.032
- Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr. Biol.* 15, 1953–1960. doi: 10.1016/j.cub.2005.09.042
- Rohwedder, A., Wenz, N. L., Stehle, B., Huser, A., Yamagata, N., Zlatić, M., et al. (2016). Four individually identified paired dopamine neurons signal reward in larval *Drosophila*. *Curr. Biol.* 26, 661–669. doi: 10.1016/j.cub.2016.01.012
- Rousseaux, M. W. C., Shulman, J. M., and Jankovic, J. (2017). Progress toward an integrated understanding of Parkinson's disease. *F1000Res.* 6:1121. doi: 10.12688/f1000research.11820.1
- Rylander, D., Parent, M., O'Sullivan, S. S., Dovero, S., Lees, A. J., Bezard, E., et al. (2010). Maladaptive plasticity of serotonin axon terminals in Levodopa-induced dyskinesia. *Ann. Neurol.* 68, 619–628. doi: 10.1002/ana.22097
- Sadaf, S., Birman, S., and Hasan, G. (2012). Synaptic activity in serotonergic neurons is required for air-puff stimulated flight in *Drosophila melanogaster*. *PLoS One* 7:e46405. doi: 10.1371/journal.pone.0046405
- Sasaki-Adams, D. M., and Kelley, A. E. (2001). Serotonin-dopamine interactions in the control of conditioned reinforcement and motor behavior. *Neuropsychopharmacology* 25, 440–452. doi: 10.1016/s0893-133x(01)00240-8
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–10502.
- Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T., et al. (2008). Serotonin is necessary for place memory in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 105, 5579–5584. doi: 10.1073/pnas.0710168105
- Thamm, M., Balfanz, S., Scheiner, R., Baumann, A., and Blenau, W. (2010). Characterization of the 5-HT1A receptor of the honeybee (*Apis mellifera*) and involvement of serotonin in phototactic behavior. *Cell. Mol. Life. Sci.* 67, 2467–2479. doi: 10.1007/s00018-010-0350-6
- Tonoki, A., and Davis, R. L. (2015). Aging impairs protein-synthesis-dependent long-term memory in *J. Neurosci.* 35, 1173–1180. doi: 10.1523/JNEUROSCI.0978-14.2015
- Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., et al. (2012). Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat. Neurosci.* 15, 1516–1523. doi: 10.1038/nn.3238
- Vallés, A. M., and White, K. (1988). Serotonin-containing neurons in *Drosophila melanogaster*: development and distribution. *J. Comp. Neurol.* 268, 414–428. doi: 10.1002/cne.902680310
- Vanhuauwaert, R., and Verstreken, P. (2015). Flies with Parkinson's disease. *Exp. Neurol.* 274, 42–51. doi: 10.1016/j.expneurol.2015.02.020
- West, R. J. H., Furmston, R., Williams, C. A. C., and Elliott, C. J. H. (2015). Neurophysiology of *Drosophila* models of Parkinson's disease. *Parkinsons Dis.* 2015:381281. doi: 10.1155/2015/381281
- Westerlund, M., Hoffer, B., and Olson, L. (2010). Parkinson's disease: exit toxins, enter genetics. *Prog. Neurobiol.* 90, 146–156. doi: 10.1016/j.pneurobio.2009.11.001
- Wong, P. T., Feng, H., and Teo, W. L. (1995). Interaction of the dopaminergic and serotonergic systems in the rat striatum: effects of selective antagonists and uptake inhibitors. *Neurosci. Res.* 23, 115–119. doi: 10.1016/0168-0102(95)90023-3
- Zeng, B. Y., Iravani, M. M., Jackson, M. J., Rose, S., Parent, A., and Jenner, P. (2010). Morphological changes in serotonergic neurites in the striatum and globus pallidus in levodopa primed MPTP treated common marmosets with dyskinesia. *Neurobiol. Dis.* 40, 599–607. doi: 10.1016/j.nbd.2010.08.004
- Zhou, F. C., Bledsoe, S., and Murphy, J. (1991). Serotonergic sprouting is induced by dopamine-lesion in substantia nigra of adult rat brain. *Brain Res.* 556, 108–121. doi: 10.1016/0006-8993(91)90553-8

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A Tyrosine-Hydroxylase Characterization of Dopaminergic Neurons in the Honey Bee Brain

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Dopamine (DA) plays a fundamental role in insect behavior as it acts both as a general modulator of behavior and as a value system in associative learning where it mediates the reinforcing properties of unconditioned stimuli (US). Here we aimed at characterizing the dopaminergic neurons in the central nervous system of the honey bee, an insect that serves as an established model for the study of learning and memory. We used tyrosine hydroxylase (TH) immunoreactivity (ir) to ensure that the neurons detected synthesize DA endogenously. We found three main dopaminergic clusters, C1–C3, which had been previously described; the C1 cluster is located in a small region adjacent to the esophagus (ES) and the antennal lobe (AL); the C2 cluster is situated above the C1 cluster, between the AL and the vertical lobe (VL) of the mushroom body (MB); the C3 cluster is located below the calyces (CA) of the MB. In addition, we found a novel dopaminergic cluster, C4, located above the dorsomedial border of the lobula, which innervates the visual neuropils of the bee brain. Additional smaller processes and clusters were found and are described. The profuse dopaminergic innervation of the entire bee brain and the specific connectivity of DA neurons, with visual, olfactory and gustatory circuits, provide a foundation for a deeper understanding of how these sensory modules are modulated by DA, and the DA-dependent value-based associations that occur during associative learning.

Keywords: *Apis mellifera*, dopamine, dopaminergic signaling, neural circuits, neural clusters

Abbreviations: adpc, anterior dorsal protocerebral commissure; aiot, anterior inferior optic tract; AL, antennal lobe; AMMC, antennal mechanosensory and motor center; AOTU, anterior optic tubercle; asot, anterior superior optic tract; CA, calyx; CB, central body; CX, central complex; DA, dopamine; ES, esophagus; Glo, glomeruli; ir, immunoreactivity; LbDC IV, labial dorsal commissure IV; LbSMC, labial superior median commissure; LbVC II, labial ventral commissure II; l-CA, lateral calyx; LH, lateral horn; MB, mushroom body; MBEN, mushroom-body extrinsic neuron; m-CA, median calyx; ML, medial lobe; MVT, medial ventral tract; MxMT, maxillary midline tract; NO, ventral noduli; OL, optic lobe; PAL cluster, protocerebral anterior lateral cluster; PAM cluster, protocerebral anterior medial cluster; PB, protocerebral bridge; PED, pedunculus; PEDN, pedunculus neck; PPL cluster, protocerebral posterior lateral cluster; PPM cluster, protocerebral posterior medial cluster; SER, sting extension reflex; SEZ, subesophageal zone of the brain; SPZ, supraesophageal zone of the brain; TH, tyrosine hydroxylase; VIT, ventral intermediate tract; VL, vertical lobe; VLT, ventral lateral tract; VMT, ventral medial tract.

INTRODUCTION

Honey bees serve as a well-established model to understand learning and memory (Menzel, 1999, 2001; Giurfa, 2007; Giurfa and Sandoz, 2012), and a number of protocols have been developed to study the behavioral, neural and molecular correlates of such processes (Giurfa, 2007). The olfactory conditioning of the sting extension response (SER) is an important protocol that allows the study of aversive learning and memory under controlled experimental conditions (Vergoz et al., 2007; Carcaud et al., 2009; Giurfa et al., 2009; Roussel et al., 2009; Tedjakumala and Giurfa, 2013). The SER is a defensive behavior elicited in bees by potentially noxious stimuli (Breed et al., 2004). In the laboratory, it can be triggered by an electric shock delivered to a harnessed bee (Burrell and Smith, 1994; Núñez et al., 1997). Bees learn to associate this aversive electric stimulus (the unconditioned stimulus, US) with an odorant (the conditioned stimulus, CS). Furthermore, Dopamine (DA) signaling has been found to be indispensable for SER conditioning, as pharmacological blocking with different DA antagonists suppresses the capacity of bees to learn an odor-shock association through an inhibition of the aversive (US) pathway (Vergoz et al., 2007).

Recent pharmacological experiments have revealed that the role of DA in bees is more complex than just mediating aversive reinforcement (Tedjakumala et al., 2014). These experiments showed that the dopaminergic system can down-regulate the unconditioned responsiveness to electric shocks. This responsiveness is quantified by subjecting harnessed bees to a series of increasing voltages that enhance their tendency to respond with a SER. Pharmacological blockade of the dopaminergic system results in an *increase* of the responsiveness to the aversive US. It has been thus suggested that the dopaminergic system of the bee brain is functionally heterogeneous and includes at least two classes of DA neurons: one controlling global aversive responsiveness through an inhibitory action, and the other mediating aversive US signaling during aversive learning (Tedjakumala et al., 2014).

In the light of this heterogeneity, an accurate neuroanatomical characterization of DA neurons in the bee brain is warranted. This characterization should enable the identification of structures and neural modules of the bee brain that are targeted by DA neurons, thus providing the anatomical bases for associations involved in stimulus-reinforcement and for the modulation of behavioral responsiveness. Previous work performed almost three decades ago has reported the presence of putative dopaminergic neurons in the bee brain by means of immunocytochemical studies using anti-DA antisera (Schürmann et al., 1989; Schäfer and Rehder, 1989). Building on this work, we characterized the dopaminergic neurons in the central nervous system of the honey bee by immunolabeling tyrosine hydroxylase (TH), DA's rate-limiting synthetic enzyme (Fon and Edwards, 2001). TH converts tyrosine into dihydroxyphenylalanine (L-DOPA), which is subsequently converted into DA. Thus by targeting TH we aimed at immunolabeling and analyzing neurons that synthesize DA

endogenously. Our neuroanatomical data were gathered through a combination of immunocytochemistry using fluorescence-conjugated antibodies and 3D-confocal imaging of optical sections captured from whole-mounted bee brains. In this way, it was possible to reconstruct complete dopaminergic networks in the bee brain without the potential for loss of tissue regions. A complete characterization of DA neurons in the protocerebrum of *Drosophila*, at a single cell resolution, has been achieved using TH GAL4-transgene and TH antibody (Mao and Davis, 2009). To facilitate our reconstruction and identification of newly described DA processes in the bee brain, we used this characterization of dopaminergic circuits in the fruit fly brain as a reference. The comprehensive mapping of DA-synthesizing neurons in the honey bee brain sets a strong foundation for understanding the varied roles of DA in learning, memory and other associated behaviors.

MATERIALS AND METHODS

Insects

Honey bees (*Apis mellifera*) were obtained from colonies located in the apiary of the University Paul Sabatier. Only foragers were used for this study as they have significantly higher DA levels than nurses or guards (Taylor et al., 1992). To this end, a feeder filled with 30% (weight/weight) sucrose solution was set at the apiary and true foragers were collected upon feeding.

Bees were brought to the laboratory and chilled on ice for approximately 5 min. Afterwards, they were individually harnessed in metal holders from which only the head capsule protruded. The bees were left for at least 1 h in resting conditions before dissection in order to reduce potential alterations of DA levels due to the prior handling (Chen et al., 2008).

Dissection and Fixation

A window was cut in the upper part of the head capsule, between the compound eyes and the ocelli. The mandibles and the antennae were also removed, thus exposing the whole brain. The compactness of the hypopharyngeal glands was monitored to ensure that the bees were old enough to be considered foragers (Maleszka et al., 2009). The glands were removed to allow the fixative to access the brain optimally. The whole process lasted usually no longer than 30 s. Immediately after this, the bee was decapitated and the whole head capsule was fixed in 1% zinc-formaldehyde (ZnFA) in bee ringer (Ott, 2008) for approximately 20 h (overnight) at room temperature.

The following day, the head capsule was immersed in HEPES-buffered saline (HBS) and the brain was removed. The tracheae covering the brain were also carefully removed. The brain was rinsed three times in HBS, each time during 20 min, to remove the rests of ZnFA. Subsequently, the samples for whole-mounts were de- and rehydrated. The dehydration was done using Dent's fixative (one part of DMSO: four parts of methanol) for 1 h, which was followed by another step in methanol for another hour and finally by rehydration in Tris buffer also for 1 h, all at room temperature.

The samples for microsections were immediately embedded in 5% low melting agarose (in phosphate buffered saline—PBS) after rinsing them three times during 20 min in HBS. Sectioning was done at 80–160 μm using a vibratome (Leica VT1000S). The sections were immediately kept in PBS for further processing.

Immunocytochemistry

Brain slices were permeabilized and blocked in PBS solution containing 0.3% Triton X-100 and 5% normal goat serum (ngs) for 1 h. We used three primary antibodies: (i) a monoclonal antibody α -SYNORF1 raised in mouse against the *Drosophila* synapsin protein (UniProt ID: Q24546; courtesy of Prof. Erich Buchner, Würzburg); (ii) a polyclonal rabbit α -TH antibody (Merck Millipore, AB 152; UniProt ID: P04177); and (iii) a mouse monoclonal α -TH antibody (ImmunoStar, Cat# 22941). The α -SYNORF1 antibody has been used successfully in fruit flies *Drosophila melanogaster* and other invertebrates for synapsin detection (e.g., Klagges et al., 1996; Michels et al., 2005). The rabbit α -TH antibody reacts with most mammalian and many non-mammalian species, including insects. It has been successfully used to stain dopaminergic neurons in *Drosophila melanogaster* and *Caenorhabditis elegans* (e.g., Bou Dib et al., 2014; Lin et al., 2014). The mouse antibody recognizes TH across a wide variety of animal species. It has been shown to label neurons that specifically contain DA and no other amine in both insects and annelids (e.g., Mesce et al., 2001; Crisp et al., 2002).

The rabbit α -TH antibody was used for the main labeling and the mouse α -SYNORF1 for the background. After blocking, we incubated the samples with both antibodies (rabbit α -TH 1:50 and α -SYNORF1 1:50) for 48 h. We then rinsed them multiple times (10–20–30–2 $\times$ 60 min) in 0.3% Triton X-100. The secondary antibodies were Alexa Fluor® 488 α -rabbit (Invitrogen) and DyLight 649 α -mouse (Jackson ImmunoResearch) raised in goat. They were applied 1:100 for 24 h. Afterwards, the samples were again rinsed multiple times (10–20–30–2 $\times$ 60 min) in 0.3% Triton X-100. Whole-mounted samples were dehydrated in increasing alcohol series (50%–70%–90%–95%–2 $\times$ 100%) before clearing them in a benzyl-mixture (two parts of benzyl benzoate: one part of benzyl alcohol). Brain slices were immediately mounted between coverslips in VECTASHIELD® Mounting Medium (Vector Labs).

The mouse α -TH antibody was also used in other preparations for the main labeling with the addition of phalloidin for the background. After fixation and washes, the specimen was incubated for 48 h in a 1:100 dilution of the mouse monoclonal α -TH antibody. After various rinses, the brain was incubated for 24 h in a 1:100 dilution of a DyLight 649 α -mouse (Jackson ImmunoResearch) and prepared as described above.

Confocal Microscopy

Samples were imaged using a confocal laser scanning microscopy (Leica TCS SP5 MP and LSM510 NLO—Carl Zeiss, Jena, Germany), with either a 25 $\times$ oil objective (LCI Plan-Neofluar

25 $\times$ /0.8) or a 20 $\times$ water objective (PL APO 20 $\times$ /0.5 on the Leica microscope and W Plan Apo 20 $\times$ /1.0 on the Zeiss microscope). Ar-Kr and HeNe lasers were used to excite Alexa Fluor® 488, Texas Red® DyLight 649 at 488, 543 and 633 nm, respectively. The emission was detected with a 500–530, 560–615 and 650–680 nm bandpass filter, respectively. Cy3 was excited and detected using the same setting as Texas Red, as they possess similar dye properties and deliver, for our purpose, identical results.

The images were collected as Z stacks with a Z step size between 0.410 μm and 0.709 μm . A visual field of view was registered with a pixel resolution of either 512 $\times$ 512 or 1024 $\times$ 1024 pixels. Each region of interest was captured by moving the visual field of view over the entire region, resulting in a huge and detailed mosaic image. The stacks were rendered for 3D reconstruction with Imaris 7.7 (Bitplane, Zurich, Switzerland). At least five samples were compared for each neuronal cluster considered to confirm the neuroanatomical processes reported.

Nomenclature

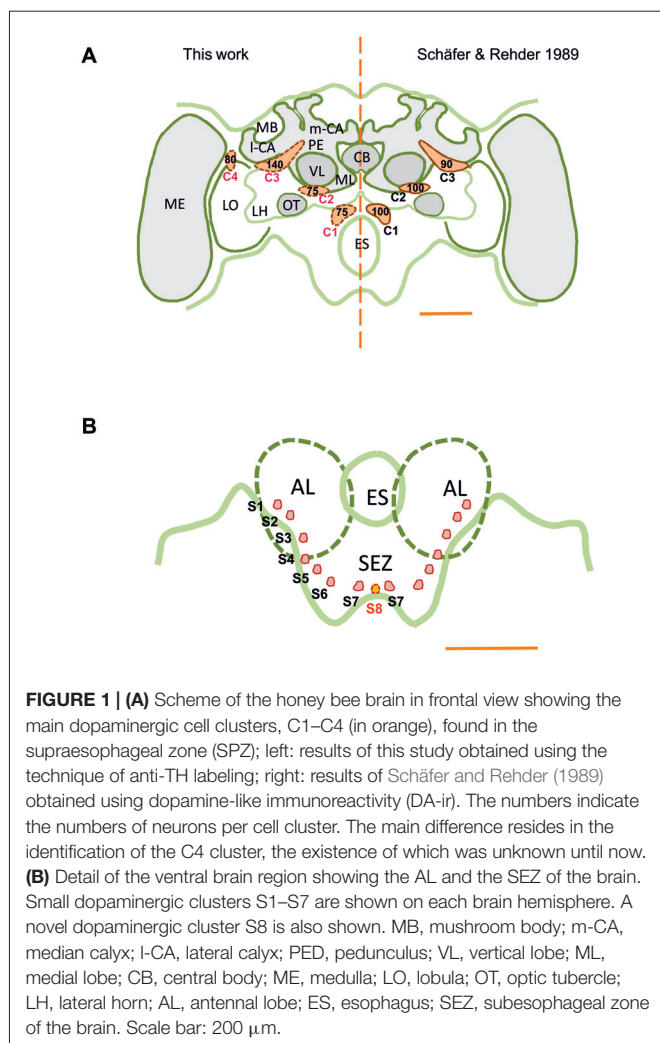
The different spatial axes of orientation used in this study follow the body axes of the honey bee. The nomenclature used for characterizing brain structures and pathways follows that proposed by the Insect Brain Name Working Group (Ito et al., 2014).

RESULTS

Dopaminergic Cell Clusters

TH-ir was detectable throughout the whole brain, i.e., in the brain regions above and below the level of the esophagus (ES), the supraesophageal zone (SPZ) and the subesophageal zone (SEZ). Our results are consistent with previous reports of DA-immunoreactive labeling (Schürmann et al., 1989; Schäfer and Rehder, 1989), as we could identify three main dopaminergic clusters, C1–C3 (**Figure 1A**), in each brain hemisphere (Schürmann et al., 1989; Schäfer and Rehder, 1989). The C1 cluster is located in a small region adjacent to the ES and the antennal lobe (AL), at a depth of ca. 120 μm (see inset in **Figure 2A**). The C2 cluster is more eccentric (**Figures 1, 2C**) and situated above C1; it is located between the AL and the vertical lobe (VL), at a depth of ca. 60 μm . The C3 cluster is located below the calyces (CA) of the mushroom body (MB), from the ventral to the dorsal part of the brain (**Figure 3**).

In addition, we discovered a fourth cluster that we termed C4, which was overlooked in prior studies. This cluster is located above the dorsomedial border of the lobula (**Figures 1, 7**), spanning the anterior part of the brain down to a depth of ca. 120 μm . The discovery of this cluster contradicts prior statements mentioning that the Optic lobes (OLs) are devoid of DA labeling (Schäfer and Rehder, 1989). Several small clusters (S1–S7; **Figures 1B, 11–14**) were identified between the AL and the SEZ, including a novel dopaminergic cluster in the SEZ, which we termed S8 (**Figures 1B, 14**). Other smaller cell clusters were also detected: C3b (**Figure 6**) and S_p



(Figure 8), which are two individual clusters with *ca.* 8 and 15–20 somata, respectively, located in each hemisphere around the protocerebral bridge (PB) and dorsal to the central complex (CX). Further dopaminergic clusters found are the anterior optic tubercle (AOTU) cluster, located below each anterior optic tubercle and presenting 2–3 somata (Figure 9) and the S_L cluster with its 5–8 somata at the border between the lobula and the deutocerebrum (Figure 10).

Dopaminergic Cell Numbers

Our counting of dopaminergic neurons (Figure 1) in the C1 and C2 clusters yielded around 75 somata per cluster; less than the 100 somata (Schäfer and Rehder, 1989) and more than the 40 somata (Schürmann et al., 1989) previously reported for these clusters. Each soma had a diameter of *ca.* 10 μm , similar to the size previously reported. In the C3 cluster, we identified *ca.* 140 somata, which is more than the 80–90 somata (Schäfer and Rehder, 1989) and 50 somata (Schürmann et al., 1989) previously reported. The somata within this cluster had diameters varying between 7 μm and 12 μm . In the C4 cluster, which had not been previously described, *ca.* 80 somata

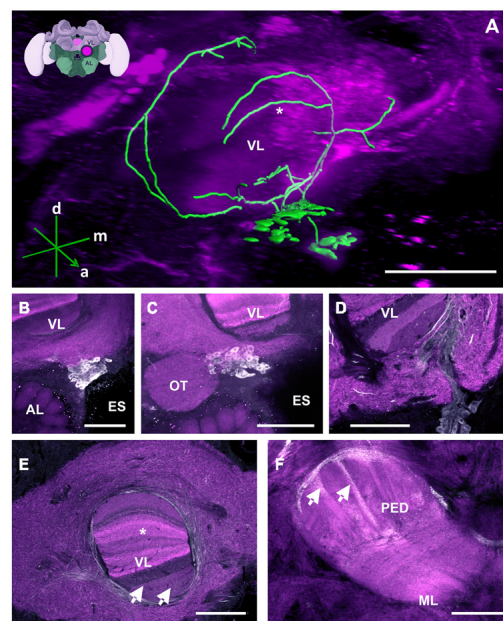
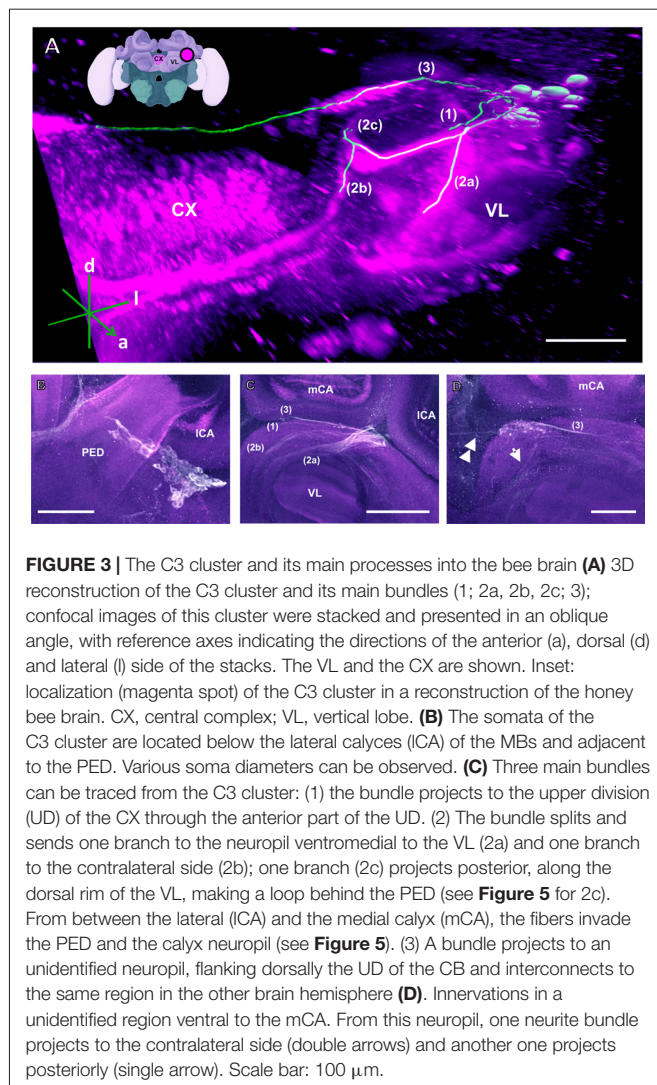


FIGURE 2 | The C1 and C2 clusters and their processes in the bee brain. **(A)** 3D reconstruction of the C1 and C2 clusters. Confocal images of the C1 and C2 clusters were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The reconstructed neurons wrap the VL of the MB. The asterisk highlights an innervation of the VL described in panel **(E)**. Inset: localization (magenta spot) of the C1 and C2 clusters in a 3D reconstruction of the honey bee brain (adapted from Rybak et al., 2010). This reconstruction will be used in the following figures. AL, antennal lobe; VL, vertical lobe; ES, esophagus **(B)** The C1 cluster is located adjacent to the ES and between the VL of the MB and the AL. **(C)** The C2 cluster is located below the ventral part of the VL, above the AL and medial to the optic tuberculum (OT). **(D)** The neurite bundles of the C1 and C2 clusters meet at the ventromedial margin of the VL and then envelop and enter the VL. The lower layers of the VL can be clearly seen. **(E)** Fine fiber-like arborizations in the VL can be clearly observed in various layers of the VL (arrows). The neurite bundles envelop the VL dorsomedially and laterally. Parts of the dorsomedial bundle enter the VL at one of its most dorsal layers (asterisk—see panel **A** for the reconstruction), which presents Kenyon cell axons from the basal ring. Neurites arborize anteriorly to and out of the VL and make ramifications into the neuropils lateral and medial to the VL. **(F)** The PED of the MB is innervated by column-like varicosities (arrows). The main bundle can be traced back to processes running along the lateral border of the VL. ML, medial lobe, visible in this plane of section. Scale bar: 100 μm .

were identified. Their diameters ranged between 8 μm and 10 μm .

Taking into account the various smaller clusters mentioned in the previous section, we counted a total of 400–450 somata per brain hemisphere (range of several samples), an estimation that surpasses the 350 and 120 somata reported by Schäfer and Rehder (1989) and Schürmann et al. (1989), respectively. TH-immunoreactive clusters and their processes were located symmetrically within both brain hemispheres. Signal intensity of the newly identified C4 cluster showed notable variability. It varied across samples and was less robust compared to that of other clusters, a fact that may explain why it was overlooked previously.

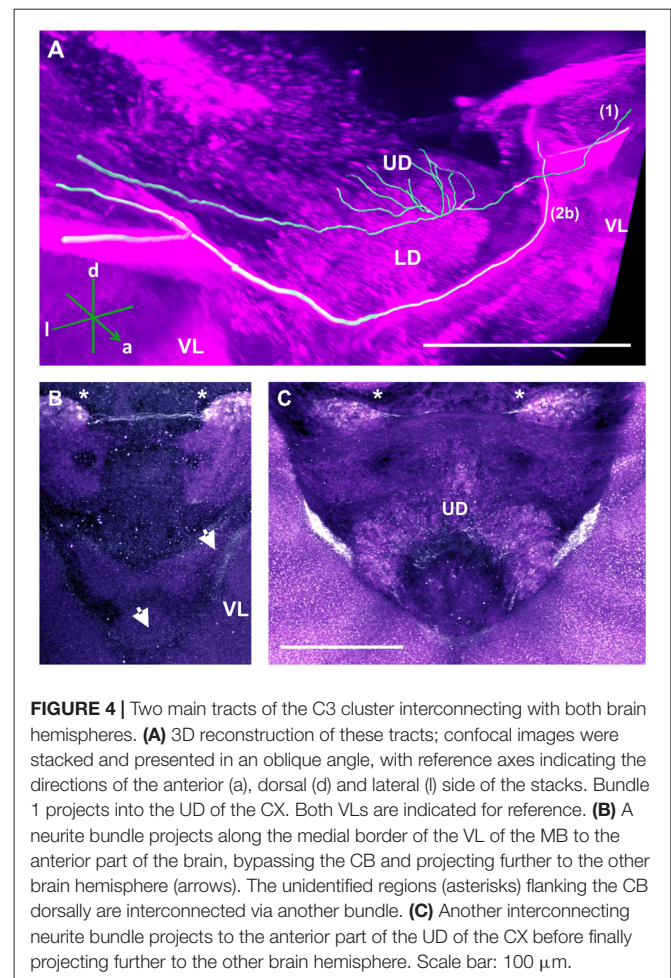


Dopaminergic Innervation of Brain Regions

Dopaminergic Innervation in the Supraesophageal Zone (SPZ)

Mushroom bodies (MBs)

MBs are prominent higher-order integration centers, which receive input from olfactory, visual, gustatory and mechanosensory afferents and from the lateral protocerebrum (LP; Strausfeld, 2002). Each MB is made of approximately 170,000 Kenyon cells (Witthöft, 1967) and has a pair of cup-like neuropils called the calyces, one of which is located in the medial zone and the other in the lateral zone of the brain. Both calyces are connected to a common Pedunculus (PED), which divides into a medial and VL. The VL extends forward to the front surface of the brain where it truncates and lies approximately at 150 μ m above the AL. The cell bodies of the Kenyon cells (class I Kenyon cells) are located in the bowl of each calyx and above its rim. Their dendrites ramify within the cup-shaped neuropil of calyces while their projection fibers pass through the PED, branch at its base, and send one process into the medial lobe



(ML) and another process into the VL. An additional group of cell bodies lies outside each calyx (class II Kenyon cells) and forms a layer around the outer calyx wall. The neurites of these cells penetrate the outer wall to project directly towards the lower part of the VL, which has been identified as the gamma lobe (Strausfeld, 2002).

TH-ir showed that the MB is innervated by the three main clusters C1, C2 and C3, at the level of the vertical and ML, the PED, and the calyces (**Figures 2–5**). Neither the Kenyon cells nor any MB intrinsic neurons were labeled. We have divided our description of MB innervation according to the three main regions of this structure: the lobes, the PED and the calyces.

Medial and vertical lobes. The neurites of the C1 and C2 clusters meet at a point posterior to each cluster (**Figure 2D**). Before meeting, they arborize laterally with intense immunolabeling around the outer medial and lower border of the VL, innervating various regions of the LP (**Figure 2E**). The TH-ir in the MB seems to come from the same bundle of neurites and can be detected in both the vertical and the MLs. In the VL, innervations comprising various layers are observed, with a higher intensity found in the inferior region, corresponding,

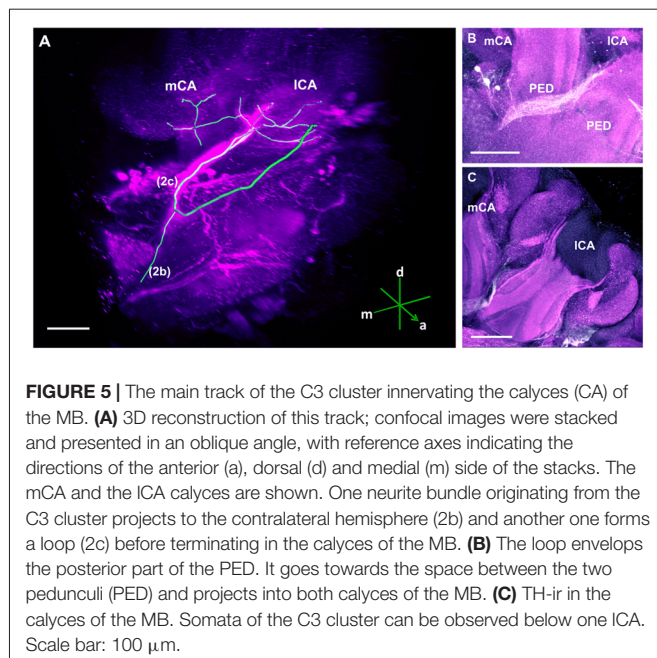


FIGURE 5 | The main track of the C3 cluster innervating the calyces (CA) of the MB. **(A)** 3D reconstruction of this track; confocal images were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The mCA and the ICA calyces are shown. One neurite bundle originating from the C3 cluster projects to the contralateral hemisphere (2b) and another one forms a loop (2c) before terminating in the calyces of the MB. **(B)** The loop envelops the posterior part of the PED. It goes towards the space between the two pedunculi (PED) and projects into both calyces of the MB. **(C)** TH-ir in the calyces of the MB. Somata of the C3 cluster can be observed below one ICA. Scale bar: 100 μm.

in part, to the gamma lobe (**Figure 2E**; Strausfeld, 2002). These projections cannot be distinctly traced as they appear as thin fiber-like arborizations. In the ML, faint and variable signals can be detected, indicating the presence of very fine dopaminergic branches.

The C3 cluster sends one of its neuritic bundles in the direction of the midline of the brain. It splits into three main branches (**Figures 3A,C**; branches termed 2a, 2b and 2c). One branch runs anteriorly and splits medially and laterally at the border of the VL. It projects further ventrally and appears to envelop the VL at its outer dorsal border (**Figures 3A,C**; branch 2a); another branch continues medioventrally to the midline sending projections to the contralateral hemisphere (**Figures 3A,C**; branch 2b). The last branch projects posteriorly along the medial surface of the VL and turns dorsolaterally behind the PED of the medial calyx (mCA; **Figures 3A, 5A**; branch 2c). This thick branch terminates between the medial and lateral calyces (ICA; **Figure 5B**). Furthermore, the lateral projection of the bundle coming from the C3 cluster runs anteriorly where it branches to innervate the PL and then runs further ventrally along the border of the VL. All these branches show strong anti-TH labeling.

Pedunculus (PED). TH-ir originating from the layers of the VL continues further posteriorly and dorsally as fine fibers projecting into the PED where they disperse into columns built by Kenyon cell axons (**Figure 2F**, arrows). An intensely labeled bundle runs along the ventral and lateral border of the VL. It innervates the PED as a net of varicosities at the level where the PED starts to diffuse into the VL. Its origin can be located at the ventral border of this lobe where it diverges from a group of several large processes. Due to the inter-tangled nature of these processes, the neurites could not be traced

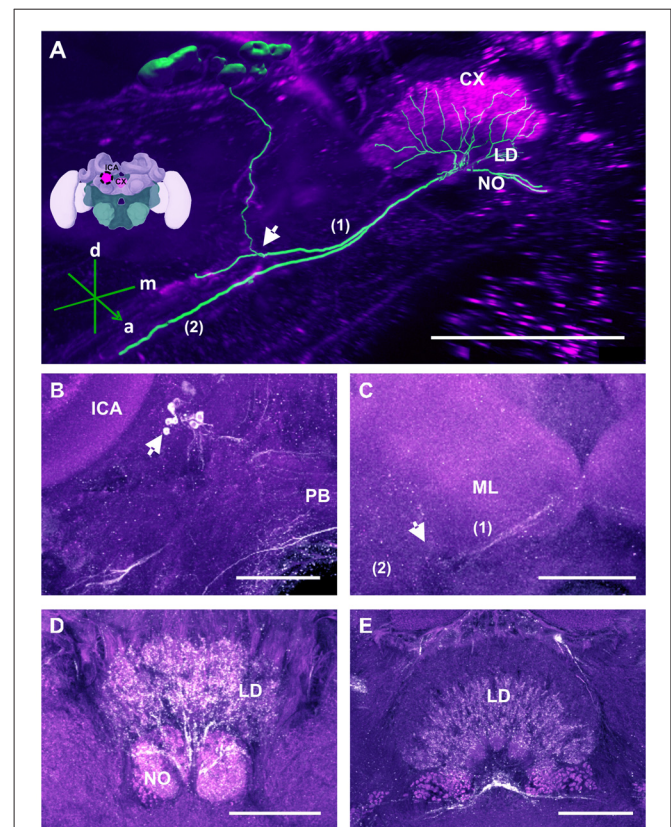


FIGURE 6 | The C3b cluster and its processes in the CX. **(A)** 3D reconstruction of this cluster; confocal images of the C3b cluster were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The innervation of the CX is shown. One branch (1) projects medially toward the ventral border of the CB and enters the lower division (LD) and the noduli (NO). Another branch (2) goes to the ventral border of the ML. Inset: localization (magenta spot) of the C3b cluster in a reconstruction of the honey bee brain. CX, central complex; ICA, lateral calyx. **(B)** The cell cluster (arrow) consists of around eight somata located posterior to the PED and medioventrally to the ICA. The protocerebral bridge (PB) of the CX is shown. **(C)** From the somata, the neurites project ventrally (arrow; see also panel A arrow) where they divide into two branches, innervating (1) the CB, its NO and the LD of the CX—and (2) the ventral border of ML. **(D)** TH-ir in the NO and the posterior part of the LD. **(E)** TH-ir in the LD of the CX. The projections enter the CX ventrally and originate symmetrically from both C3b clusters. Scale bar: 100 μm.

further. The pedunculus neck (PEDN) is innervated by processes terminating as fine varicosities between the lateral and medial calyces (**Figure 2F**).

Calyces. In the calyces, TH-ir exhibits an heterogeneous distribution (**Figure 5C**). The lip and the collar, which receive olfactory and visual input, respectively (Gronenberg, 1999; Ehmer and Gronenberg, 2002; Strausfeld, 2002), present indistinct varicose arborizations whilst the basal ring, which receives olfactory and visual input (Gronenberg, 2001), provides comparatively weaker signals. Unfortunately, it was not possible to determine the location of the somata connected to these arborizations.

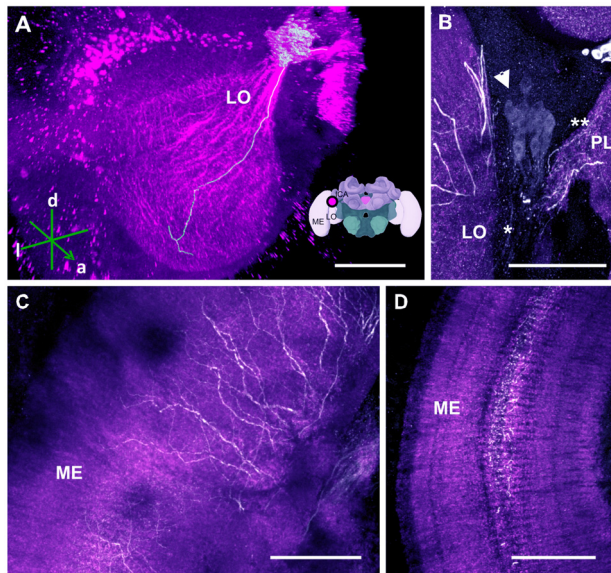


FIGURE 7 | The C4 cluster and its projections to the visual neuropils. **(A)** 3D reconstruction of the C4 cluster and of a single traceable neurite projecting to the LO; confocal images were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and lateral (l) side of the stacks. Inset: localization (magenta spot) of the C4 cluster in a reconstruction of the honey bee brain. ME, medulla; LO, lobula; ICA, lateral calyx. **(B)** Approximately 80 somata (arrow) are found in the C4 cluster. The neurite bundle (asterisk) projects toward the LO and the protocerebral lobe (PL; double asterisk). **(C)** Leaving the LO, the neurites arborize in the ME in a column-like pattern. **(D)** TH-ir in the serpentine layer of the ME. The neurites arborize laterally within the serpentine layer. Scale bar: 100 μm .

Central complex (CX)

TH-ir in the CX revealed that dopaminergic processes could be traced back to at least two origins. The first one contributes to the projections in the anterior part of the upper division (UD) of the CB. It derives from the large neurite bundles coming from the C3 cluster (**Figures 3A,C**; bundle 1). One of these bundles contacts a small region in the PL located superior to the VL and anterior to the PED. There it gives rise to numerous varicosities. Afterwards it projects ventromedially to the midline where it innervates the UD of the CB in the form of densely packed column-like arborizations, invading it in a compartment-wise manner (**Figure 4**; bundle 1). The second bundle contributes to the projections in the posterior part of the UD, the lower division (LD) of the CB, and the noduli (NO). It can be traced back to a set of *ca.* eight labeled somata in each hemisphere that are positioned in a row posterior and lateral to the PB and dorsolaterally to the l-CA (**Figure 6B**, arrow). We call this cluster C3b (**Figure 6**), because its somata also arborize into the CX, similarly to neurons of the C3 cluster. **Figure 6A** shows a 3D reconstruction of the C3b neurons to provide a fuller characterization of their morphology. Their neurites run ventrally and project anteriorly towards the ventral border of the ML (**Figure 6C**). There, the neurites send one branch medially towards the ventral border of the CB from which the labeled fibers enter the lower and UD of the CB and the NO (**Figures 6A,C**, arrow). The posterior

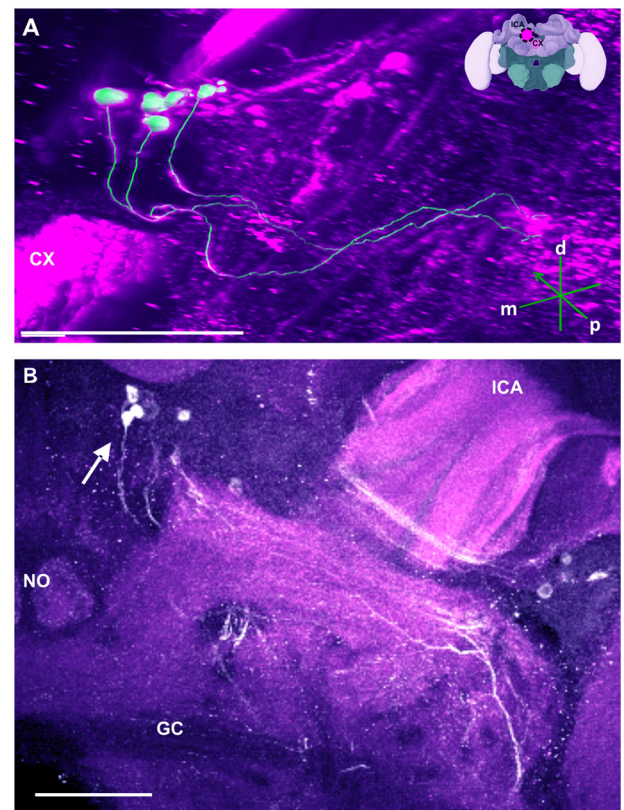
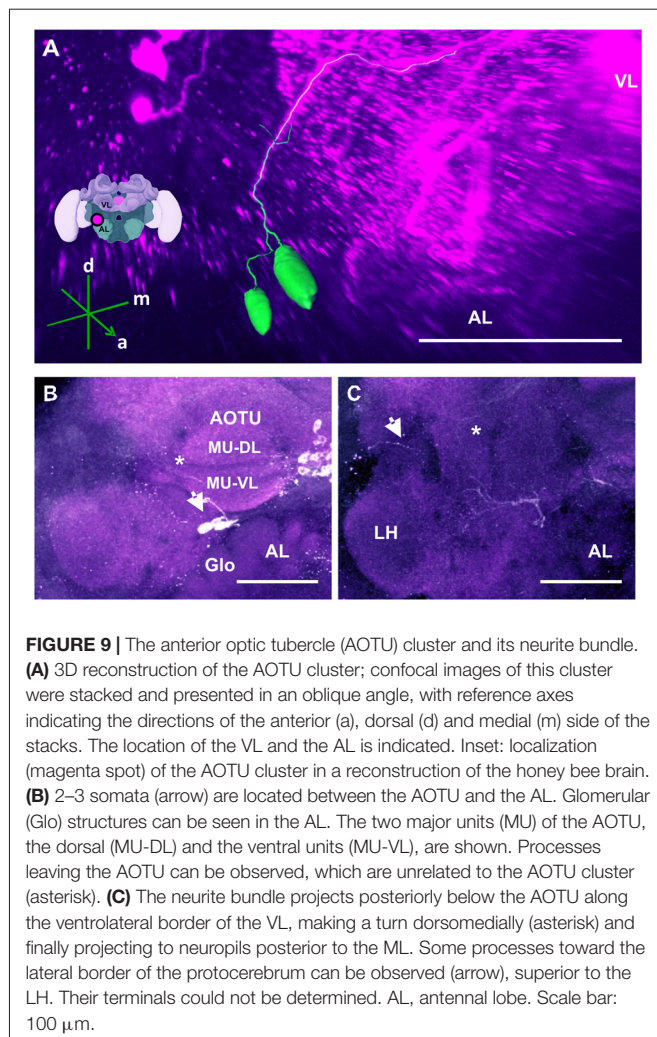


FIGURE 8 | The Sp cluster and its neurite bundle. **(A)** 3D reconstruction of the Sp cluster; confocal images of this cluster were stacked and presented in an oblique angle, with reference axes indicating the directions of the posterior (p), dorsal (d) and medial (m) side of the stacks. CX, central complex. Inset: localization (magenta spot) of the Sp cluster in a reconstruction of the honey bee brain. ICA, lateral calyx. **(B)** Between 15 and 20 somata are found in this cluster (arrow). The neurite bundles form two main tracts projecting to neuropils dorsal to the great commissure (GC). The ICA and a nodulus (NO) are shown. Scale bar: 100 μm .

part of the UD is also innervated at its posterior surface by a number of thin fibers. The PB shows only weak labeling. In general, the intensity of TH-ir in the posterior part of the CX is stronger compared to the anterior part (**Figures 4C, 6D,E**).

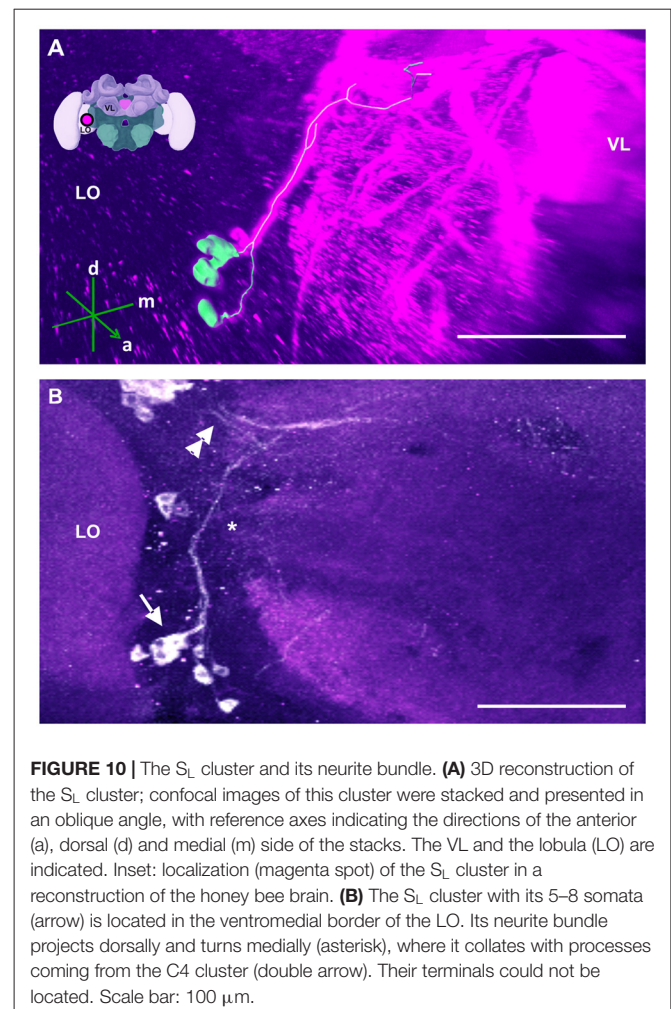
Optic lobes (OLs)

The OLs are responsible for processing visual information acquired via the photoreceptors located within the ommatidia of the compound eyes (Avargues-Weber et al., 2012). They comprise three main regions: the lamina, medulla and lobula. Previous studies using DA-ir did not find dopaminergic innervation in these brain regions (Schürmann et al., 1989; Schäfer and Rehder, 1989). Using anti-TH labeling, however, we detected dopaminergic processes in these neuropils, which were derived from a single cluster located at the dorsomedial border of the lobula (i.e., the C4 cluster; **Figures 1, 7A,B**). This cluster has neurites sending processes both to the OLs (**Figure 7B**, asterisk) and the PL at the level of the PEDN



where the terminals could not be detected (**Figure 7B**, double asterisk).

Two TH antibodies yielded different labeling results at the level of the OLs. Immunolabeling with the TH antiserum raised in rabbit exhibited two subtypes of projections. One subtype consisted of two projections that bypassed the lobula and ran along the dorso- and ventroanterior border of the OL before innervating the medulla. Each bundle comprised large neurites that were intensely labeled and innervated the outer layer of the medulla. They seemed to share the same projection tract running along the dorsal and ventral border of the OL, the anterior superior optic tract (asot), and the anterior inferior optic tract (aiot; Ehmer and Gronenberg, 2002). The projections of the second subtype formed a fan-shaped bundle of relatively large neurites that projected ventrally and entered laterally into the lobula. They innervated this neuropil homogeneously at different depths. Intense labeling was also detected in the medulla's serpentine layer (**Figure 7C**) and in column-like processes of the medulla (**Figure 7D**). Moving towards the outer layer of the medulla, the neurites of the C4 cluster appeared in the form of column-like thin fibers.



Immunolabeling with the TH antibody raised in mouse uncovered only the second projection subtype. Additionally, both antibodies labeled the retina. We therefore reconstructed only the second subtype as its somata were traceable and could be detected by the two antibodies. Our 3D reconstruction was able to trace a process that projected uninterrupted to the lobula (**Figure 7A**).

Other neuropils in the protocerebral lobe

Our anti-TH labeling revealed another projection, which could be traced back to the C3 cluster, and which was not detected in previous reports (Schürmann et al., 1989; Schäfer and Rehder, 1989). This projection reached a small region, which was located posterior to the anterior dorsal protocerebral commissure (adpc) and flanked (dorsally) the UD of the central body (CB; **Figures 3D, 4B,C**, asterisks). The innervations were strong and bleb-like in comparison with the size of its neurites. Two other faint projections were present in this neuropil. The first one (**Figure 3D** double arrows) reached the contralateral hemisphere. The second one (**Figure 3D**, arrow) left posteriorly and turned dorsoposteriorly around the PEDN, where it intertwined with other processes (for example, those originating from the C4 cluster) thus rendering its terminals in the PL untraceable.

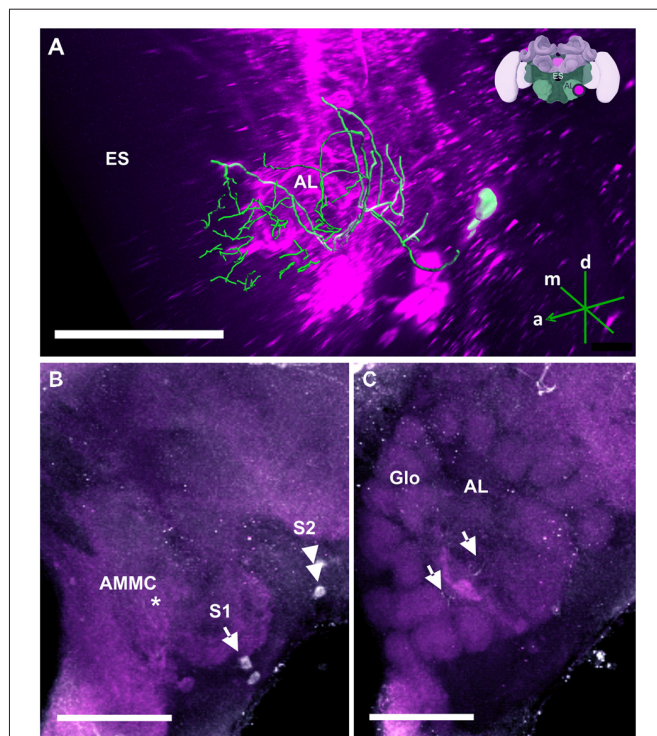


FIGURE 11 | The S1 and S2 clusters and their processes in the AL. **(A)** 3D reconstruction of the S1 and S2 clusters; confocal images were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. Location of the ES is indicated. Inset: localization (magenta spot) of the S1 and S2 clusters in a reconstruction of the honey bee brain (adapted from Rybak et al., 2010). **(B)** The somata of the S1 (arrow) and S2 clusters (double arrow) can be observed at the lateral border of the deutocerebrum. The neurites project medially to a neuropil in the antennal mechanosensory and motor center (AMMC) where they form fine arborizations (asterisk). **(C)** The neurite bundles form delicate arborizations innervating the AL in the center (arrows) and spread into the peripherally arranged Glo. Scale bar: 100 μ m.

Besides this novel projection, some TH-immunoreactive processes were similar to those described before (Schürmann et al., 1989; Schäfer and Rehder, 1989). For example, posterior to the neuropil mentioned above (see Figure 3D) and dorsal to the mCA, and anterior to the PB, there are few somata (Figure 8B, arrow) that project ventrally crossing the neurite of the C3b cluster. This cluster has been termed the S_p cluster (Schäfer and Rehder, 1989). Its projections continued to a neuropil that was dorsal to the great commissure (GC) where they sent very thin side processes into the neuropil at the lateral border of the PB (Figure 8). Figure 8A shows a 3D reconstruction of this cluster and its processes.

The AOTU are small neuropils located in each hemisphere of the insect brain, which are connected by two inter-tubercle tracts (Mota et al., 2011b). They are a major target of visual interneurons from the OL, in particular, from the lobula and the medulla (Mota et al., 2011b). They respond to chromatic information in a spatially and temporally segregated manner and are thought to participate in navigation (Mota et al., 2013). We found that the AOTUs are innervated by dopaminergic

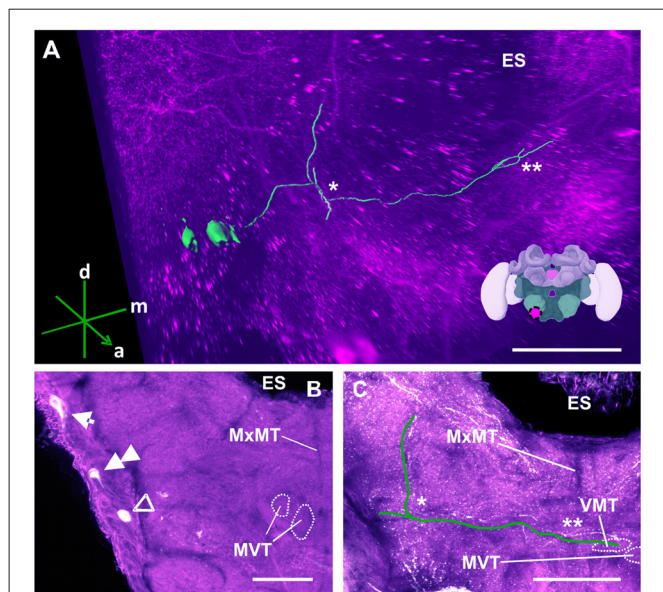
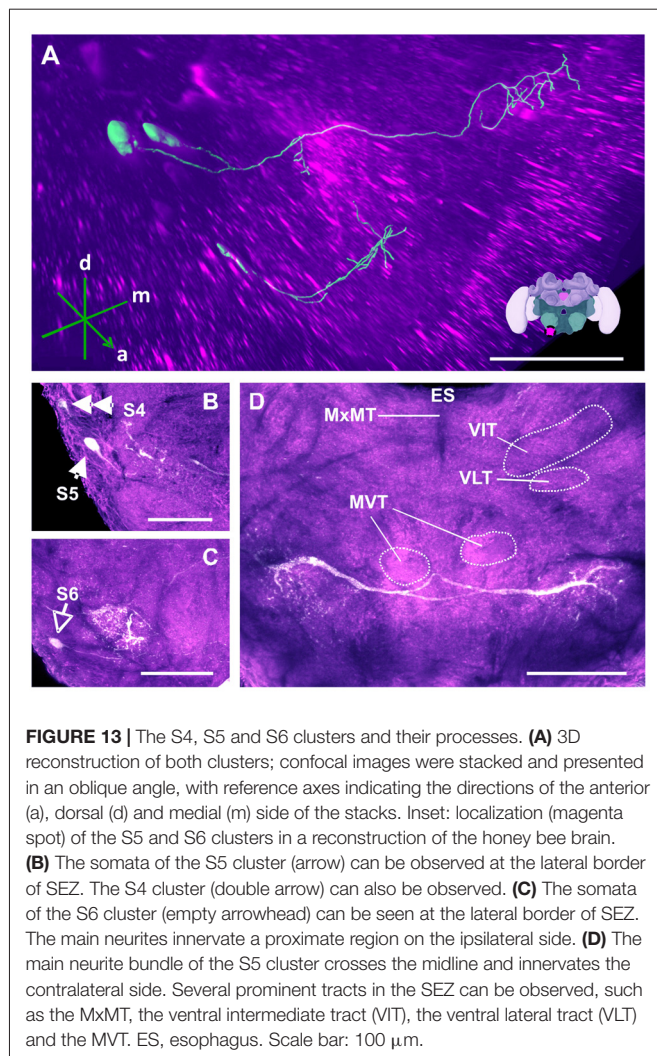


FIGURE 12 | The S3 cluster and its processes. **(A)** 3D reconstruction of the S3 cluster; confocal images were stacked and presented in an oblique angle, with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The first ascending branch to the AMMC and the location where the neurite crosses the midline are indicated by * and **, respectively. The location of ES is also indicated. Inset: localization (magenta spot) of the S3 cluster in a reconstruction of the honey bee brain. **(B)** The somata of the S3 cluster (arrow) can be observed at the lateral somatal ring of the SEZ. Additionally, the S4 (double arrow) and S5 clusters (open arrowhead) are shown. The appearance of the maxillary midline tract (MxMT) and the division of the median ventral tract (MVT) provide the approximate location of these clusters in the SEZ. ES, esophagus. **(C)** The main neurite of the S3 cluster is shown parallel to the green line to allow its tracing on the confocal projection among the extensive dopaminergic network in the SEZ. On the ipsilateral side, it ascends (asterisk) to the AMMC. It also crosses the midline (double asterisk). The terminal appears to end in the ventral median tract (VMT) and the MVT. The MxMT is still observable in this projection. ES, esophagus. Scale bar: 100 μ m.

varicose processes, which, at least in part, can be traced back to a set of about five labeled fibers that run in the inter-tubercle tracts. Due to its location adjacent to the AOTU, this cluster is called the AOTU cluster (Figure 9). The location of its somata, however, could not be determined (Figure 9B, asterisk).

Below each AOTU, 2–3 somata (Figure 9B) with a diameter of 20–25 μ m projected posteriorly to the ventrolateral border of the VL of the MB, ascending and making widespread arborizations in the neuropil lateral to the PED (Figure 9C, star). A few of their processes projected towards the lobula (Figure 9C, arrow), but did not enter the OL.

A further cluster named S_L was located at the ventroposterior border of the lobula (Schäfer and Rehder, 1989; Figure 10A). It consisted of 5–8 somata (Figure 10B, arrow) and gave rise to a thin bundle of neurites that projected dorsally and made a medial turn before reaching the l-CA of the MB. Some of the processes remained in the vicinity of the calyces, while others projected behind the CB across the midline of the brain. The terminals of these fibers could not be detected.

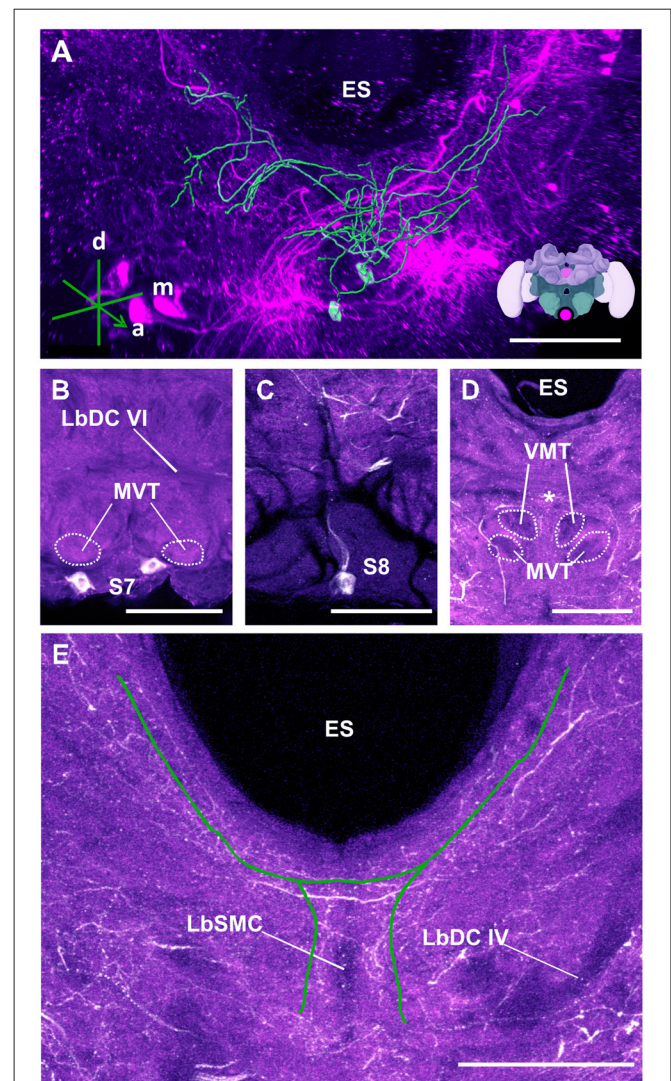


Antennal lobes (AL) and antennal mechanosensory and motor centers (AMMC)

In the AL (Figure 11), TH-ir could be detected in two small clusters of neurons termed S1 (Figure 11B, single arrow) and S2 (Figure 11B, double arrow), which were located in the SEZ. Each cluster contained two somata with a diameter of 10–20 μ m. Both were located in the immediate region posterior to the AL, at the lateral border of the AMMC, with S1 being more ventral and anterior than S2. Their projections shared a similar morphological pattern. Tracing their resolution to the single-cell level was difficult despite their relatively large sizes (Figure 11A). The neurites projected medially to a neuropil in the AMMC where they formed delicate arborizations (Figure 11B, asterisk) before entering the AL. The branches were distributed as fine processes all over the AL, making contacts with fibers across glomeruli (Glo) of the AL (Figure 11C, arrows).

Dopaminergic Innervation in the Subesophageal Zone (SEZ)

TH-ir in the SEZ showed an extensive network of labeled fibers with their projections overlapping some of these tracts. Despite



some minor differences in arborizations, we confirmed the presence of the previously reported dopaminergic clusters S3–S7, which were found in the ventral (S3–S6) and lateral (S7) somatal rind of each SEZ hemisphere. Furthermore, we discovered a new S8 cluster, which was located in the lateral somatal region. In total, we identified the presence of eight paired neurons within each SEZ hemisphere (S3–S7) and two unpaired neurons (S8).

The S3 cluster (**Figure 12**) contained two somata with a diameter between 15 μm and 20 μm (**Figure 12B**, arrow). The neurites of this cluster innervated different regions of the ipsi- and contralateral sides (**Figure 12A**). The main arborizations of the S3 neurons remained ventral to those of the AL neurons S1 and S2 (see above). After leaving the somatal rind, the neurites of the S3 cluster projected ipsilaterally to the antennal mechanosensory and motor center (AMMC; **Figures 12A,C**, asterisk). Before reaching it, however, the signals were mixed with those coming from the S7 and S8 cluster (**Figure 14**), rendering them indistinguishable. The main neurites continued to cross the SEZ midline (**Figures 12A,C**, double asterisk), possibly innervating both the medial ventral tract (MVT) and the ventral medial tract (VMT). This innervation pattern could not be clarified, as there was no clear distinction between these signals and those from the S7 and S8 clusters (**Figure 14E**).

The somata of the S4 cluster were located in the lateral somatal rind of the mandibular neuromere. This cluster was previously reported to contain 6–8 DA-immunoreactive somata with diameters between 8 μm and 11 μm (Schäfer and Rehder, 1989). In our case, we were able to detect only two labeled somata of around 10–15 μm (**Figure 12B**, double arrow), which probably correspond to the two neurons of this cluster. Although Schäfer and Rehder (1989) traced the neurites of these somata, within the SEZ neuropil, our anti-TH labeling yielded faint signals (**Figure 12B**) that disappeared in the extensive network of other labeled fibers.

The S5 cluster (**Figure 13**) was reported to contain two somata, 18–20 μm in diameter, arranged in two bilateral pairs that send their major projections through a labial ventral commissure into the contralateral hemiganglion (Schäfer and Rehder, 1989). Our labeling also showed the presence of two somata, 15–20 μm in diameter, within each SEZ hemisphere. **Figure 13A** shows a 3D-reconstruction of the S5 cluster with its main neurites having bilateral innervation of the SEZ hemispheres. They projected through the labial ventral commissure II (LbVC II) into the contralateral identical region, and also ipsilaterally (**Figure 13D**). The dendrites of the contralateral side were more prominent and bleb-like while the dendrites of the ipsilateral side were more fiber-like. Although the two types of dendrites innervate the same region and occasionally intertwine, their innervation pattern occurs within different depths without any observable overlap.

The S6 cluster (**Figure 13**) was previously described as consisting of two somata, 20–24 μm in diameter, located in the lateral somatal rind of the labial neuromere (Schäfer and Rehder, 1989). Our labeling identified the same two somata; unlike those of neurons in the S3, S4, S5 and S7 clusters, the processes of these somata did not decussate, but remained restricted to the ipsilateral half of the SEZ (**Figure 13A**). The projections of the S6 cluster descended ventrally and innervated the same region as the S5 cluster (**Figure 13C**), wherein they created a dopaminergic network.

The S7 cluster (**Figure 14**) was described as containing two bilaterally arranged somata, 24–30 μm in diameter, in the ventral

somatal rind (Schäfer and Rehder, 1989). We also located these two somata, 20–25 μm in diameter (**Figure 14B**) and identified their neurites, which ascended in a tract lateral to the labial midline tract and branched in the dorsal neuropil of the SEZ (**Figure 14B**).

Finally, the S8 (**Figure 14**) cluster is reported here for the first time. It consisted of a pair of medially-located unpaired neurons with a diameter *ca.* 15–20 μm (**Figure 14C**). The S8 cluster sent its projection dorsally over the labial midline tract (**Figure 14C**). The neurites of this cluster intertwined with those of the S7 cluster close to their somata, making it impossible to delineate their projections (**Figure 14A**). Together, the neurites of the S7 and S8 clusters envelop the MVT and the VMT (**Figure 14D**). At the point where the labeling of both clusters became inseparable, some branches projected posteriorly and formed a bridge while others appeared to cross over dorsally and continue to ascend to the ventral border of the ES, where they further bifurcated and innervated regions along the ES up to the AMMC (**Figure 14E**).

DISCUSSION

In this study, we characterized the distribution pattern of dopaminergic neurons in the central nervous system of the honey bee using TH-ir. Two different commercially available TH antibodies were used, one a polyclonal raised in rabbit and the other a monoclonal raised in mouse; similar results were obtained with each antiserum apart from the expression pattern in the OLs. Our methods also yielded results partially similar to those previously reported (Schürmann et al., 1989; Schäfer and Rehder, 1989), wherein three main dopaminergic clusters, C1–C3 (**Figure 1**), were identified. Some minor clusters previously identified (Schürmann et al., 1989; Schäfer and Rehder, 1989) were also observed. Not previously reported, however, was a novel cluster, C4, located above the dorsomedial border of the lobula, which innervated the visual neuropils of the bee brain (**Figures 1, 7**). A novel eighth cluster, S8, in the ventral somatal rind of the SEZ was also detected for the first time.

Differences inherent to the labeling techniques employed in the prior and present study could account for the discovery of novel dopaminergic clusters. However, at least three studies in insects have shown that the labeling patterns obtained with DA and TH antibodies are not different (Nässel and Elekes, 1992; Hörner et al., 1995; Hamanaka et al., 2016). This suggests that both antisera recognize the same sets of “dopaminergic” neurons. Thus, a likely explanation for the differences between the present and previous studies resides in the fact that 12 μm thick wax sections (which require heating to $>50^\circ\text{C}$) and a conventional light microscope were used in prior studies (Schürmann et al., 1989; Schäfer and Rehder, 1989), while thicker sections (80–160 μm) and confocal and confocal microscopy were used in our work. Given this thickness difference, lower values for cell counts in our study could be hardly attributed to tissue damage or loss during the sectioning process.

The C1, C2 and C3 Clusters

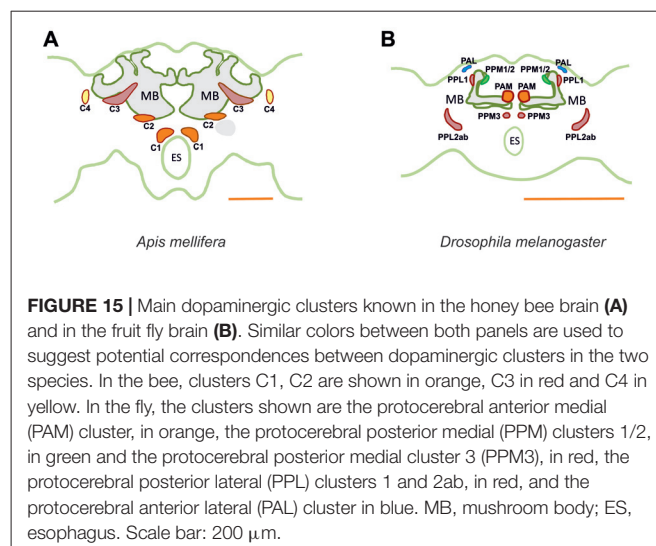
The location and general connectivity of the C1–C3 clusters were consistent with those reported previously (Schürmann et al., 1989; Schäfer and Rehder, 1989). There were, however, slight differences, such as the number of somata and an unreported projection in the small neuropil flanking (dorsally) the CX.

It is important to link the identities of other previously published cell profiles in the honey bee brain to those that are most likely to be dopaminergic. For example, neurons we identified in the C1 and C2 clusters resemble the A1 and A2 MB extrinsic neurons (MBEN) previously described (Rybak and Menzel, 1993). The A1 and A2 MBENs are located anteriorly and in the same depth as the C1 and C2 clusters, projecting in the same manner into the VL of the MB. Their branches envelop the VL and project to the PL unilaterally. Fine varicose fibers are detected in both the vertical and the ML. Rybak and Menzel (1993) counted an average of 50–60 labeled neurons of the A1 and A2 type. This number is close to the 75 somata counted both in the C1 and C2 clusters, thus indicating that these clusters may comprise these two types of MBENs. Possibly, the A1 and A2 neurons described by Rybak and Menzel as MBEN may in fact be the dopaminergic neurons of the C1 and C2 clusters. In *Drosophila*, the homolog of the C1 and C2 clusters may be the protocerebral anterior medial (PAM) cluster with respect to soma location and innervation pattern (Figure 15). Neurons within the PAM cluster also terminate in the MLs of the MB and in neuropils adjacent to them (Mao and Davis, 2009).

Neurons in the C3 cluster resemble the A6 MBENs reported by Rybak and Menzel (1993). Even though neurons in the C3 cluster could not be fully traced, it is possible to sort out a part of their massive projections by way of what is known about the A6 MBEN. The somata of the A6 MBENs are located ventrally to the I-CA of the MB and project to the VL, to neuropils in the contralateral hemisphere, and to the ipsilateral lateral horn (LH). Looking at the number of reported A6 neurons, which ranges between 60 and 80, we can reasonably conclude that they are part of the 140 cells comprising the C3 cluster. The homologs of this cluster in *Drosophila* are likely the protocerebral posterior lateral (PPL) clusters 1 (PPL1) and 2ab (PPL2ab), but also the protocerebral posterior medial cluster 3 (PPM3; Figure 15). First, the somata of the PPL1 cluster are relatively close to the calyx and have terminals in the dorsal part for the fan-shaped body as one of the terminals of the C3 cluster (Mao and Davis, 2009; Liu Q. et al., 2012). Second, the terminals of the PPL2ab cluster are detectable in the calyx of the MB (Mao and Davis, 2009) similar to the C3 cluster. Third, the PPM3 cluster arborizes into the CX like the bee C3 and C3b clusters (Mao and Davis, 2009). It is notable that C3 cluster is big, consisting of different types of neurons or, possibly, sub-clusters.

The C4 Cluster

Our anti-TH labeling uncovered the presence of the C4 cluster, which innervated the OL of the honey bee. In accordance to the nomenclature of previous reports, the name adopted



for this cluster follows the sequential order of the main dopaminergic clusters previously described in the honey bee brain. It is somewhat surprising that the C4 cluster has remained undetected in two parallel neuroanatomical characterizations of dopaminergic neurons (Schürmann et al., 1989; Schäfer and Rehder, 1989), even though Mercer et al. (1983) had first reported faint dopaminergic expression in the OLs. Despite attaining variable levels of DA expression, subsequent reports have confirmed such DA labeling (Taylor et al., 1992; Sasaki and Nagao, 2001).

In worker bees, regardless of their age, the labeling of DA in the OLs is relatively low when compared to that in the protocerebrum (Mercer et al., 1983; Sasaki and Nagao, 2001). An age/caste effect exists and DA levels are higher in bee foragers (Taylor et al., 1992). One cannot rule out, therefore, that in prior studies relatively young bees were used that had levels of DA expression that were below detectability.

The C4 cell morphology partially resembles that of the MBENs that project to the calyces and the OLs (Ehmer and Gronenberg, 2002). The C4 cluster may also contain at least three different cell types: (1) the first one shares the track with the asot along the dorsal border of the OL; (2) the second one shares the track with the aiot along the ventral border; and (3) the third one projects directly into the lobula and then innervates the serpentine layer of the medulla. These three types of neurons share only one single projection into the protocerebrum: it runs to the PEDN where the neurites could not be distinguished from other putative dopaminergic processes.

Even though these neurons share several properties of the MBENs mentioned in prior studies, it is intriguing to see that the cluster is located exclusively at the dorsomedial border of the lobula. Neurons projecting to the asot and aiot have their somata in the dorsomedial edge of the medulla and the base of the I-CA adjacent to the ventral edge of the medulla and lobula, respectively (Ehmer and

Gronenberg, 2002). C4 cluster somata cannot be located in these regions, however, suggesting that they might possess different morphological properties. A corresponding dopaminergic cluster in *Drosophila* cannot be identified. In the fly, the protocerebral anterior lateral (PAL) cluster, which is lateral to the dorsal portion of the VLs (Mao and Davis, 2009), extends its processes to innervate the contralateral optic tubercle and the OL (Figure 15). This contralateral innervation pattern makes it different from the C4 cluster of the bee, although both provide dopaminergic signaling to visual areas of the brain.

The third cell type, but not the first or second, was labeled by both the mono- and polyclonal antibodies we used, and yielded the same labeling pattern. This labeling pattern is notable and is indicative of a dopaminergic modulation of visual circuits and information processing from the lamina to the lobula. It is worth noting that visual forms of aversive learning have been shown in the honey bee, which may depend on dopaminergic signaling in associating the visual chromatic/achromatic stimuli with the aversive electric shock (Mota et al., 2011a). Although the critical coincidence of the visual stimulus and the shock pathways necessary to support visual aversive learning and memory may occur at the level of the MBs and/or CX, such integration could occur at multiple levels upstream of these structures, thus providing multiple substrates for different forms of visual plasticity.

TH-immunoreactivity in the Mushroom Body

The MB is a higher order processing center, which integrates various types of sensory information conveyed by visual, olfactory, mechanosensory and gustatory inputs (Strausfeld, 2002). Dopaminergic processes innervate the calyces, the vertical and MLs and the PED of the MB. TH-ir in the calyces is observable in the lip and the collar regions, known input regions of olfactory and visual afferents, respectively. The vertical and MLs, sites associated with memory retrieval (Cano-Lozano et al., 2001), are variably innervated by dopaminergic processes.

In the fruit fly *Drosophila melanogaster*, studies on olfactory-based aversive and appetitive learning have revealed that several classes of dopaminergic neurons provide distinct forms of reinforcement signals (appetitive, aversive, short-term, long-term), thus resulting in multiple forms of memories (Claridge-Chang et al., 2009; Aso et al., 2010, 2012; Burke et al., 2012; Liu C. et al., 2012). For example, the PAM cluster that has resemblance to the C1 and C2 clusters, mentioned above, mediates the aversive reinforcement properties of the electric shock used as a US in olfactory aversive conditioning (Claridge-Chang et al., 2009; Aso et al., 2010, 2012).

Despite its relatively small number of neurons (Mao and Davis, 2009), the *Drosophila* PPL1 cluster, which resembles the C3 cluster of the honey bee, provides aversive reinforcement signaling and regulates levels of anesthesia-resistant memory (ARM), and gating to stabilized long-term memory (LTM; Claridge-Chang et al., 2009; Aso et al., 2010; Placais et al., 2012). Moreover, neurons in the PAM and PPL1 clusters may interact

at the level of the MBS and tune the stability of aversive memory (Aso et al., 2012). Collectively, at least three DA pathways to the MB can induce aversive (i.e., shock-induced) memory in the fruit fly. The projections arborize in different MB subdomains defined by specific combinations of intrinsic and extrinsic neurons (Aso et al., 2012).

Dopaminergic signaling also mediates appetitive-reinforcement in the fruit fly. It has been recently shown that sucrose reinforcement is mediated by a hierarchical network in which peripheral signaling is mediated by octopaminergic neurons that further convey their signal to dopaminergic neurons within the PAM cluster and on to the MBs (Burke et al., 2012; Liu C. et al., 2012). Thus, a different pathway of dopaminergic signaling indicates the presence of reward in the formation of appetitive memory (Burke et al., 2012; Liu C. et al., 2012).

Results from the fruit fly have underscored the fundamental importance of different subsets of dopaminergic neurons from the PAM and PPL1 clusters, and thus serving as neural correlates of reinforcement signaling in appetitive and aversive olfactory conditioning. In the bee, appetitive reinforcement appears to be independent of dopaminergic signaling as it is mediated by a single octopaminergic neuron, the VUMmx1 neuron, which arborizes in the ALs, LH and MBs, and whose activity substitutes for sucrose reward in appetitive olfactory conditioning (Hammer, 1993). Yet, the dependency of aversive olfactory SER conditioning on dopaminergic signaling has been demonstrated using pharmacological blockade (see “Introduction” Section; Vergoz et al., 2007). The specific neurons mediating the shock signaling in this aversive conditioning paradigm might just be found in the C1, C2 and/or C3 clusters given the apparent homologies with the PAM and PPL1 clusters of the fruit fly.

TH-immunoreactivity in the Central Complex

The CX of the bee is a structure made of four interconnected, midline spanning neuropils: the upper and LDs of the CB, the PB located more posteriorly, and a pair of ventral NO (Kenyon, 1896; Jonescu, 1909). The CX is involved in different functions such as sensory integration, motor control, spatial learning and sensorimotor integration (Pfeiffer and Homberg, 2014). It is particularly important for the processing of visual information (Homberg, 1985; Milde, 1988). We show that dopaminergic innervation of the CX can be attributed to at least two clusters, the C3 cluster that innervates the anterior UD of the CB, and the C3b cluster, which projects to the posterior upper and LDs of the CB and NO. The somata of the C3b cluster were located in the posterior region anterior to the PB. Interestingly, the PPM3 cluster in *Drosophila* is also located in a relatively similar region with a similar number of somata (eight; see Mao and Davis, 2009). In the fly, these projections can be traced to the lower half of the fan-shaped body, the NO (Mao and Davis, 2009; Alekseyenko et al., 2013) and the ellipsoid body (Liu Q. et al., 2012).

The presence of dopaminergic neurons in the CX of *Drosophila* has been associated with sleep, arousal, wakefulness and aggression (Ueno et al., 2012; Alekseyenko et al., 2013). So far, dopaminergic processes in this neuropil have not been associated with reinforcement-signaling functions for appetitive and/or aversive associative learning and memory. This finding may be due to the fact that conditioning protocols in which dopaminergic function has been studied in the fly are mostly olfactory. In contrast, conditioning protocols that involve visual patterns associated with the aversive reinforcement of heat on the thorax (Wolf et al., 1992) do involve groups of horizontal neurons in a substructure of the CX that is required for *Drosophila* visual pattern memory (Liu et al., 2006). In addition, a small set of neurons in the ellipsoid body, another substructure of the CX and connected to the fan-shaped body, is also required for visual pattern memory. Both groups of neurons thus constitute a complex neural circuit in the CX for *Drosophila* visual pattern memory (Pan et al., 2009), which may benefit from a possible association with dopaminergic circuits conveying aversive reinforcement signaling.

TH-immunoreactivity in the Antennal Lobe

The AL and AMMC are prominent neuropils in the bee brain. The AL is the primary olfactory neuropil and, in the honey bee, it comprises *ca.* 160 globular subunits termed Glo. Glo are interaction sites primarily between the afferent projections of olfactory receptors on the antenna, local interneurons connecting glomeruli laterally, and projection neurons conveying olfactory inputs to higher-order centers such as the LH and the MB; efferent modulatory projections are also associated with Glo. The AMMC receive mainly mechanosensory input from the antennae and house antennal motoneurons (Pareto, 1972; Suzuki, 1975).

Our anti-TH labeling revealed dopaminergic projections in the AL, which could be traced back to two small clusters, S1 and S2, located in the SEZ. Because dopaminergic signaling is vital for aversive olfactory conditioning in bees (Vergoz et al., 2007), the presence of TH immunoreactive fibers in the AL may indicate that dopaminergic modulation is important for learning or olfactory plasticity upstream of the MBs and the LH.

TH-immunoreactivity in the Subesophageal Zone

The SEZ is a fused region containing the mandibular, maxillary and labial neuromeres. In the honey bee, as in other insects, the subesophageal ganglion gives rise to motoneurons of the mouthpart muscles and receives sensory (e.g., gustatory) neurons from the mouthparts, mediating the proboscis extension reflex (Rehder, 1988). It processes the gustatory and mechanosensory input from the proboscis and thus, seems to be particularly important for gustatory coding (Rehder, 1988; Marella et al., 2006; de Brito Sanchez et al., 2007). These projections form various tracts: longitudinal, transverse (commissures) and midline; sensory nerve roots are also observed. In the bee, this region is important for associative appetitive learning as it contains the cell body of an important modulatory neuron involved in olfactory appetitive conditioning, the VUMmx1,

which substitutes for sucrose in appetitive olfactory conditioning (Hammer, 1993).

Including the S1 and S2 clusters innervating the AL, we found 18 somata in the SEZ, two of which correspond to the ventral unpaired medial (VUM) neurons. These two dopaminergic VUM neurons belong to the novel cluster, S8, revealed by our work. In two other insect models, *Drosophila* and *Calliphora*, six dopaminergic somata have been identified in the SEZ (Nässel and Elekes, 1992; Friggi-Grelín et al., 2003), two of which are VUM neurons. It is therefore possible that the S8 cluster correspond to these neurons existing in flies.

Unfortunately, we were unable to distinguish clearly the processes coming from the S7 and the S8 clusters. Their arborizations appear to innervate dorsal regions bordering the SPZ where the motoneurons that control the movement of the mouthparts are located (Rehder, 1989). The dopaminergic VUM neurons found in our work might be of particular interest in the context of appetitive learning. Recently, a dopaminergic VUM neuron with extensive branching in the SEZ has been shown to trigger proboscis extension in *Drosophila*, and to have an activity that is altered by satiety state (Marella et al., 2012).

Dopaminergic Neurons as Modulators of Behavior

Besides their role in reinforcement signaling, dopaminergic neurons act as a more global modulatory system, generally depressing several behavioral components. For instance, DA decreases sucrose responsiveness (i.e., PER to increasing sucrose concentrations) when injected into the thorax. Also, injection or feeding of the DA receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduces sucrose responsiveness significantly (Scheiner et al., 2002). In olfactory PER conditioning, injection of DA into the ALs significantly reduces olfactory retention after one and three conditioning trials (Macmillan and Mercer, 1987). In the case of aversive responsiveness (i.e., SER to increasing shock voltages), dopaminergic blockade induces an increase of shock responsiveness, thus reflecting an enhancement of shock sensitivity (Tedjakumala et al., 2014). This result thus indicates that in its default mode, and besides its reinforcement-signaling role, dopaminergic signaling acts as a depressor of sting responsiveness to electric shocks so that when its effect is antagonized, responsiveness increases (Tedjakumala and Giurfa, 2013).

A possible explanation for this dual function is to assume the existence of different classes of dopaminergic neurons mediating different functions: one class acting as a general gain control system, with the specific role of down-regulating responsiveness and another class acting as instructive neurons in aversive associative learning, mediating aversive US signaling. Owing to these different functions, their brain targets could be different. While the first class would exhibit extensive and broad branching within the entire brain in order to modulate different motivational components (appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc.), the second class would exhibit a specific connectivity with respect to CS-processing circuits (e.g., olfactory, visual) in order to facilitate

CS-US associations and provide instructive (i.e., valence) information to the targeted CS circuit (Giurfa, 2006). Although further studies are clearly warranted to address the possible heterogeneity of different dopaminergic clusters in the honey bee brain, in principle, the neural architecture of the dopaminergic circuits we have described in the present work provides a solid foundation for future discovery and identification of these various functions.

ETHICS STATEMENT

Experiments on honey bees are not subject to the approval of ethics committee. All experiments were nevertheless performed taking care of ethic procedures and minimizing the number of animals required for data gathering.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. SRT and MG: study concept and design; drafting of the manuscript. SRT, JR, M-LB and KAM: acquisition of data. LH and IM: support for data acquisition. SRT, JR, M-LB and MG: analysis and interpretation of data. SRT, JR, KAM and

MG: critical revision of the manuscript for important intellectual content. MG: obtained funding; study supervision. SRT, M-LB, JR, IM and LH: administrative, technical and material support.

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REFERENCES

- Alekseyenko, O. V., Chan, Y.-B., Li, R., and Kravitz, E. A. (2013). Single dopaminergic neurons that modulate aggression in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* 110, 6151–6156. doi: 10.1073/pnas.1303446110
- Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., et al. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* 8:e1002768. doi: 10.1371/journal.pgen.1002768
- Aso, Y., Siwanowicz, I., Bracker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. *Curr. Biol.* 20, 1445–1451. doi: 10.1016/j.cub.2010.06.048
- Avargues-Weber, A., Mota, T., and Giurfa, M. (2012). New vistas on honey bee vision. *Apidologie* 43, 244–268. doi: 10.1007/s13592-012-0124-2
- Bou Dib, P., Gnani, B., Daly, F., Sabado, V., Tas, D., Glauser, D. A., et al. (2014). A conserved role for p48 homologs in protecting dopaminergic neurons from oxidative stress. *PLoS Genet.* 10:e1004718. doi: 10.1371/journal.pgen.1004718
- Breed, M. D., Guzman-Novoa, E., and Hunt, G. J. (2004). Defensive behavior of honey bees: organization, genetics, and comparisons with other bees. *Annu. Rev. Entomol.* 49, 271–298. doi: 10.1146/annurev.ento.49.061802.123155
- Burke, C. J., Huettneroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614
- Burrell, B. D., and Smith, B. H. (1994). Age- but not caste-related regulation of abdominal mechanisms underlying the sting reflex of the honey bee, *Apis mellifera*. *J. Comp. Physiol. A* 174, 581–592. doi: 10.1007/bf00217379
- Cano-Lozano, V., Armengaud, C., and Gauthier, M. (2001). Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. *J. Comp. Physiol. A* 187, 249–254. doi: 10.1007/s003590100196
- Carcaud, J., Roussel, E., Giurfa, M., and Sandoz, J. C. (2009). Odour aversion after olfactory conditioning of the sting extension reflex in honeybees. *J. Exp. Biol.* 212, 620–626. doi: 10.1242/jeb.026641
- Chen, Y. L., Hung, Y. S., and Yang, E. C. (2008). Biogenic amine levels in the brains of stressed honeybees. *Arch. Insect Biochem. Physiol.* 68, 241–250. doi: 10.1002/arch.20259
- Claridge-Chang, A., Roorda, R. D., Vrontou, E., Sjulson, L., Li, H. Y., Hirsh, J., et al. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell* 139, 405–415. doi: 10.1016/j.cell.2009.08.034
- Crisp, K. M., Klukas, K. A., Gilchrist, L. S., Nartey, A. J., and Mesce, K. A. (2002). Distribution and development of dopamine- and octopamine-synthesizing neurons in the medicinal leech. *J. Comp. Neurol.* 442, 115–129. doi: 10.1002/cne.10077
- de Brito Sanchez, M. G., Ortigao-Farias, J. R., Gauthier, M., Liu, F. L., and Giurfa, M. (2007). Taste perception in honeybees: just a taste of honey? *Arthropod-Plant Interact.* 1, 69–76. doi: 10.1007/s11829-007-9012-5
- Ehmer, B., and Gronenberg, W. (2002). Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). *J. Comp. Neurol.* 451, 362–373. doi: 10.1002/cne.10355
- Fon, E. A., and Edwards, R. H. (2001). Molecular mechanisms of neurotransmitter release. *Muscle Nerve* 24, 581–601. doi: 10.1002/mus.1044
- Friggi-Grellin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* 54, 618–627. doi: 10.1002/neu.10185
- Giurfa, M. (2006). The instructive function of biogenic amines in insect associative learning. *Curr. Biol.* 16, R892–R895. doi: 10.1016/j.cub.2006.09.021
- Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* 193, 801–824. doi: 10.1007/s00359-007-0235-9
- Giurfa, M., Fabre, E., Flaven-Pouchon, J., Groll, H., Oberwallner, B., Vergoz, V., et al. (2009). Olfactory conditioning of the sting extension reflex in honeybees: memory dependence on trial number, interstimulus interval, intertrial interval and protein synthesis. *Learn. Mem.* 16, 761–765. doi: 10.1101/lm.1603009
- Giurfa, M., and Sandoz, J. C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi: 10.1101/lm.024711.111
- Gronenberg, W. (1999). Modality-specific segregation of input to ant mushroom bodies. *Brain Behav. Evol.* 54, 85–95. doi: 10.1159/000006615
- Gronenberg, W. (2001). Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *J. Comp. Neurol.* 436, 474–489. doi: 10.1002/cne.1045

- Hamanaka, Y., Minoura, R., Nishino, H., Miura, T., and Mizunami, M. (2016). Dopamine- and tyrosine hydroxylase-immunoreactive neurons in the brain of the american cockroach, *Periplaneta americana*. *PLoS One* 11:e0160531. doi: 10.1371/journal.pone.0160531
- Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63. doi: 10.1038/366059a0
- Homberg, U. (1985). Interneurons of the central complex in the bee brain (*Apis mellifera*, L.). *J. Insect Physiol.* 31, 251–264. doi: 10.1016/0022-1910(85)90127-1
- Hörner, M., Spörhase-Eichmann, U., Helle, J., Venus, B., and Schürmann, F.-W. (1995). The distribution of neurones immunoreactive for β -tyrosine hydroxylase, dopamine and serotonin in the ventral nerve cord of the cricket, *Gryllus bimaculatus*. *Cell Tissue Res.* 280, 583–604. doi: 10.1007/s004410050387
- Ito, K., Shinomiya, K., Ito, M., Armstrong, J. D., Boyan, G., Hartenstein, V., et al. (2014). A systematic nomenclature for the insect brain. *Neuron* 81, 755–765. doi: 10.1016/j.neuron.2013.12.017
- Jonescu, C. N. (1909). Vergleichende untersuchungen über das gehirn der honigbiene. *Z. Naturwiss* 45, 111–180.
- Kenyon, F. C. (1896). The brain of the bee. A preliminary contribution to the morphology of the nervous system of the Arthropoda. *J. Comp. Neurol.* 6, 133–210. doi: 10.1002/cne.910060302
- Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pfugfelder, G. O., Reifegerste, R., et al. (1996). Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* 16, 3154–3165.
- Lin, S., Oswald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. *Nat. Neurosci.* 17, 1536–1542. doi: 10.1038/nn.3827
- Liu, Q., Liu, S., Kodama, L., Driscoll Maria, R., and Wu Mark, N. (2012). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. *Curr. Biol.* 22, 2114–2123. doi: 10.1016/j.cub.2012.09.008
- Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., et al. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* 488, 512–516. doi: 10.1038/nature11304
- Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., et al. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* 439, 551–556. doi: 10.1038/nature04381
- Macmillan, C. S., and Mercer, A. R. (1987). An investigation of the role of dopamine in the antennal lobes of the honeybee, *Apis mellifera*. *J. Comp. Physiol.* A 160, 359–366. doi: 10.1007/bf00613025
- Maleszka, J., Barron, A., Helliwell, P., and Maleszka, R. (2009). Effect of age, behaviour and social environment on honey bee brain plasticity. *J. Comp. Physiol.* A 195, 733–740. doi: 10.1007/s00359-009-0449-0
- Mao, Z. M., and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Front. Neural Circuits* 3:5. doi: 10.3389/neuro.04.005.2009
- Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E., and Scott, K. (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. *Neuron* 49, 285–295. doi: 10.1016/j.neuron.2005.11.037
- Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. *Neuron* 73, 941–950. doi: 10.1016/j.neuron.2011.12.032
- Menzel, R. (1999). Memory dynamics in the honeybee. *J. Comp. Physiol.* A 185, 323–340. doi: 10.1007/s003590050392
- Menzel, R. (2001). Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.* 8, 53–62. doi: 10.1101/lm.38801
- Mercer, A. R., Mobbs, P. G., Evans, P. D., and Davenport, A. (1983). Biogenic amines in the brain of the honey bee, *Apis mellifera*. *Cell Tissue Res.* 234, 655–677.
- Mesce, K. A., Delorme, A. W., Brelje, T. C., and Klukas, K. A. (2001). Dopamine-synthesizing neurons include the putative H-cell homologue in the moth *Manduca sexta*. *J. Comp. Neurol.* 430, 501–517. doi: 10.1002/1096-9861(20010219)430:4<501::aid-cne1046>3.0.co;2-u
- Michels, B., Diegelmann, S., Tanimoto, H., Schwenkert, I., Buchner, E., and Gerber, B. (2005). A role for symapsin in associative learning: the *Drosophila* larva as a study case. *Learn. Mem.* 12, 224–231. doi: 10.1101/lm.92805
- Milde, J. J. (1988). Visual responses of interneurons in the posterior median protocerebrum and the central complex of the honeybee *Apis mellifera*. *J. Insect Physiol.* 34, 427–436. doi: 10.1016/0022-1910(88)90113-8
- Mota, T., Gronenberg, W., Giurfa, M., and Sandoz, J. C. (2013). Chromatic processing in the anterior optic tubercle of the honeybee brain. *J. Neurosci.* 33, 4–16. doi: 10.1523/jneurosci.1412-12.2013
- Mota, T., Roussel, E., Sandoz, J. C., and Giurfa, M. (2011a). Visual conditioning of the sting extension reflex in harnessed honeybees. *J. Exp. Biol.* 214, 3577–3587. doi: 10.1242/jeb.062026
- Mota, T., Yamagata, N., Giurfa, M., Gronenberg, W., and Sandoz, J. C. (2011b). Neural organization and visual processing in the anterior optic tubercle of the honeybee brain. *J. Neurosci.* 31, 11443–11456. doi: 10.1523/jneurosci.0995-11.2011
- Nässel, D. R., and Elekes, K. (1992). Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell Tissue Res.* 267, 147–167. doi: 10.1007/bf00318701
- Núñez, J., Almeida, L., Balderrama, N., and Giurfa, M. (1997). Alarm pheromone induces stress analgesia via an opioid system in the honeybee. *Physiol. Behav.* 63, 75–80. doi: 10.1016/s0031-9384(97)00391-0
- Ott, S. R. (2008). Confocal microscopy in large insect brains: zinc-formaldehyde fixation improves synapsin immunostaining and preservation of morphology in whole-mounts. *J. Neurosci. Methods* 172, 220–230. doi: 10.1016/j.jneumeth.2008.04.031
- Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn. Mem.* 16, 289–295. doi: 10.1101/lm.1331809
- Pareto, A. (1972). Die zentrale verteilung der f-hlafferenz bei arbeiterinnen der honigbiene, *Apis mellifera* L. *Z. Zellforschung* 131, 109–140. doi: 10.1007/bf00307204
- Pfeiffer, K., and Homberg, U. (2014). Organization and functional roles of the central complex in the insect brain. *Annu. Rev. Entomol.* 59, 165–184. doi: 10.1146/annurev-ento-011613-162031
- Placais, P.-Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliard-Guerin, G., et al. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat. Neurosci.* 15, 592–599. doi: 10.1038/nn.3055
- Rehder, V. (1988). A neuroanatomical map of the suboesophageal and prothoracic ganglia of the honey bee (*Apis mellifera*). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 235, 179–202. doi: 10.1098/rspb.1988.0071
- Rehder, V. (1989). Sensory pathways and motoneurons of the proboscis reflex in the suboesophageal ganglion of the honey bee. *J. Comp. Neurol.* 279, 499–513. doi: 10.1002/cne.902790313
- Roussel, E., Carcaud, J., Sandoz, J. C., and Giurfa, M. (2009). Reappraising social insect behavior through aversive responsiveness and learning. *PLoS One* 4:e4197. doi: 10.1371/journal.pone.0004197
- Rybák, J., Kuß, A., Lamecker, H. Z. S., Hege, H.-C., Lienhard, M. C., Singer, J. J., et al. (2010). The digital bee brain: integrating and managing neurons in a common 3D reference system. *Front. Syst. Neurosci.* 4:30. doi: 10.3389/fnsys.2010.00030
- Rybák, J., and Menzel, R. (1993). Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. *J. Comp. Neurol.* 334, 444–465. doi: 10.1002/cne.903340309
- Sasaki, K., and Nagao, T. (2001). Distribution and levels of dopamine and its metabolites in brains of reproductive workers in honeybees. *J. Insect Physiol.* 47, 1205–1216. doi: 10.1016/s0022-1910(01)00105-6
- Schäfer, S., and Rehder, V. (1989). Dopamine-like immunoreactivity in the brain and suboesophageal ganglion of the honey bee. *J. Comp. Neurol.* 280, 43–58. doi: 10.1002/cne.902800105
- Scheiner, R., Plückhahn, S., Oney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* 136, 545–553. doi: 10.1016/s0166-4328(02)00205-x

- Schürmann, F. W., Elekes, K., and Geffard, M. (1989). Dopamine-like immunoreactivity in the bee brain. *Cell Tissue Res.* 256, 399–410. doi: 10.1007/bf00218898
- Strausfeld, N. J. (2002). Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. *J. Comp. Neurol.* 450, 4–33. doi: 10.1002/cne.10285
- Suzuki, H. (1975). Convergence of olfactory inputs from both antennae in the brain of the honeybee. *J. Exp. Biol.* 62, 11–26.
- Taylor, D. J., Robinson, G. E., Logan, B. J., Laverty, R., and Mercer, A. R. (1992). Changes in brain amine levels associated with the morphological and behavioural development of the worker honeybee. *J. Comp. Physiol. A* 170, 715–721. doi: 10.1007/bf00198982
- Tedjakumala, S. R. (2014). *Le rôle des Circuits et Signalisations Dopaminergiques dans L'apprentissage Aversif de L'abeille Apis Mellifera*. Toulouse: Paul Sabatier Université.
- Tedjakumala, S. R., Aimable, M., and Giurfa, M. (2014). Pharmacological modulation of aversive responsiveness in honey bees. *Front. Behav. Neurosci.* 7:221. doi: 10.3389/fnbeh.2013.00221
- Tedjakumala, S. R., and Giurfa, M. (2013). Rules and mechanisms of punishment learning in honey bees: the aversive conditioning of the sting extension response. *J. Exp. Biol.* 216, 2985–2997. doi: 10.1242/jeb.086629
- Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., et al. (2012). Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat. Neurosci.* 15, 1516–1523. doi: 10.1038/nn.3238
- Vergoz, V., Roussel, E., Sandoz, J. C., and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS One* 2:e288. doi: 10.1371/journal.pone.0000288
- Witthöft, W. (1967). Absolute anzahl und verteilung der zellen im hirn der honigbiene. *Z. Morphol. Tiere* 61, 160–184. doi: 10.1007/bf00298776
- Wolf, R., Voss, A., Hein, S., and Heisenberg, M. (1992). Can a fly ride a bicycle? *Philos. Trans. R. Soc. Lond B Biol. Sci.* 337, 261–269. doi: 10.1098/rstb.1992.0104

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Behavioral Modulation by Spontaneous Activity of Dopamine Neurons

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Dopamine modulates a variety of animal behaviors that range from sleep and learning to courtship and aggression. Besides its well-known phasic firing to natural reward, a substantial number of dopamine neurons (DANs) are known to exhibit ongoing intrinsic activity in the absence of an external stimulus. While accumulating evidence points at functional implications for these intrinsic “spontaneous activities” of DANs in cognitive processes, a causal link to behavior and its underlying mechanisms has yet to be elucidated. Recent physiological studies in the model organism *Drosophila melanogaster* have uncovered that DANs in the fly brain are also spontaneously active, and that this activity reflects the behavioral/internal states of the animal. Strikingly, genetic manipulation of basal DAN activity resulted in behavioral alterations in the fly, providing critical evidence that links spontaneous DAN activity to behavioral states. Furthermore, circuit-level analyses have started to reveal cellular and molecular mechanisms that mediate or regulate spontaneous DAN activity. Through reviewing recent findings in different animals with the major focus on flies, we will discuss potential roles of this physiological phenomenon in directing animal behaviors.

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INTRODUCTION

Animals need to modify behaviors according not only to the external world but also to their internal states, such as sleep need, hunger, or sexual motivation (Keene et al., 2010; Gorter et al., 2016; Keebaugh et al., 2017). These internal states are encoded in various manners, including ongoing neural activity in the brain. Physiological studies have revealed that these “spontaneously” occurring neural activities often show drastic changes even in the absence of external stimuli (Fox and Raichle, 2007). In this review, we discuss the biological relevance of spontaneous neural activity: how it is regulated and how it modifies behaviors. We define the spontaneous activity as the ongoing neural activity without overt external stimuli, regardless of the properties of the activity, like tonic or burst firing. Activity reflecting self-locomotion is also defined as spontaneous activity in this article.

In mammals, during sleep, electroencephalogram records show characteristic slow waves in the entire cortex (Massimini et al., 2004), which is caused by a spontaneously occurring synchronized neural activity. The slow wave activity is enhanced after sleep deprivation and suppressed after sleep, thereby controlling sleep homeostasis (Tobler and Borbély, 1986; Werth et al., 1996; Finelli et al., 2000; Vyazovskiy et al., 2009). Similar waves also drive rhythmic activity in hippocampus, which is suggested to be critical in memory consolidation (Sirota et al., 2003; Marshall and Born

2007), and a growing number of studies are now revealing how these neural activities occur across various brain regions and how they modify behaviors.

Although many neurotransmitters especially monoamines are reported to control spontaneous activity (Berridge et al., 2012; Dominguez-Lopez et al., 2012; Grace, 2016), we focus on dopaminergic circuits in this review, given converging evidence in identifying spontaneous dopamine signaling as representing states of animals. Dopamine plays a key role in a variety of brain functions such as reward processing, regulation of motivation, or learning and memory (Schultz, 2007). Dopamine functions through both synaptic and volume transmission, thereby enabling it to modulate both intra- and extra-synaptic targets (Rice et al., 2011). Dopamine neurons (DANs) in the midbrain can be characterized by its stimulus-induced phasic firing, the importance of which in behavioral action selection and reward-based learning has been widely acknowledged (Schultz et al., 1997; Tsai et al., 2009; Bromberg-Martin et al., 2010; Cohen et al., 2012; Steinberg et al., 2013; Schultz, 2015). Moreover, a significant number of DANs are known to be spontaneously active (Grace and Bunney, 1984). Studies using microdialysis found that the extracellular dopamine level shows slow fluctuations lasting seconds to minutes without any external stimuli (Schultz, 2007). These dopamine fluctuations are suggested to represent animal states, such as sleep/wake, or motivational state of the animal (Fiorillo et al., 2003; Dahan et al., 2007; Hamid et al., 2016). Dysregulation of dopamine levels causes various mental disorders, suggestive of the crucial role of spontaneously released dopamine in cognition and perception (Krishnan et al., 2007; Cao et al., 2010; Dalley and Roiser, 2012; Chaudhury et al., 2013; Grace, 2016). Although studies in primate and rodent brains have provided us with useful mechanistic insights in spontaneously released dopamine, ultimate behavioral consequences of slow ongoing dopamine activity are less understood due to technical hurdles in achieving non-invasive and precise circuit modulation. In addition, the high-level interconnectivity of the dopaminergic network makes simultaneous manipulation of multiple cells difficult and thus precludes many studies from demonstrating causal relationship.

The brain of the fruit fly *Drosophila melanogaster* provides useful study cases in this respect. Recent studies clearly go beyond correlating physiological DAN activities and animal states, and have succeeded in examining the effect of spontaneous network activities on behaviors. These include specific DAN types that regulate sleep/wake balance, memory processing, feeding motivation or sexual drive (Berry et al., 2012, 2015; Plaçais et al., 2012; Plaçais and Preat, 2013; Cohn et al., 2015; Musso et al., 2015; Yamagata et al., 2016). Thus, this review mainly focuses on recent achievements mainly in flies, and we discuss potential roles of spontaneous DAN activity and its significance in the regulation of a variety of behaviors.

SLEEP AND LOCOMOTION

Historical pharmacological studies proved that dopamine determines the arousal level in many animals. For example,

methylphenidate and amphetamine, which induce dopamine release, promote arousal in humans and have been used for the treatment of narcolepsy since the 1930s (Billiard, 2008). Consistently, mutant mice with disrupted dopamine transporter (DAT) function, which has a critical role in the reuptake of dopamine, show reduced non-rapid eye movement (non-REM) sleep and increased wakefulness (Wisor et al., 2001). Furthermore, the causal nature of DAN activity in regulating behavioral arousal is gradually being revealed by recent optogenetic studies (Taylor et al., 2016; Oishi et al., 2017).

Results of physiological studies in rodents suggest that spontaneously released dopamine underlies the regulation of the wake-sleep balance. Prominent burst firing of DANs in the ventral tegmental area (VTA) was observed when animals are in REM sleep (Dahan et al., 2007). Activity of dopamine neurons in the ventral periaqueductal gray matter, but not in the VTA or the substantia nigra, is enhanced during wakefulness (Lu et al., 2006). These studies imply that the modulation of arousal is region-specific, but precise circuit level understanding remains to be revealed.

Sleep in *Drosophila* is defined by prolonged immobility and shows many common features with sleep in humans. Flies subjected to 12 h: 12 h light/dark cycles exhibit behavioral quiescence in >90% of the dark period. Sleep in flies and mammals share many characteristics. Sleeping flies show an increased threshold for sensory stimuli, and sleep deprivation by mechanical stress causes a “rebound” effect. (Hendricks et al., 2000; Shaw et al., 2000). Notably, some somnolytic drugs known to function through the human dopamine system affect *Drosophila* sleep (McClung and Hirsh, 1998; Bainton et al., 2000; Li et al., 2000; Andretic et al., 2005; Lebestky et al., 2009; Nall et al., 2016). Consistently, a fly strain that was isolated for its short-sleep phenotype was found to have a mutation in DAT, highlighting the importance of dopamine in wake/sleep regulation (Kume et al., 2005).

Until recently, the circuit mechanisms by which dopamine regulates arousal levels have been unknown. Spontaneous activity of specific types of DANs correlates well with the locomotive state of an animal and has a significant role in the regulation of the wake/sleep state (Berry et al., 2015; Cohn et al., 2015). The wake-promoting DANs project their axon terminals to two major neural structures: the dorsal fan-shaped body (dFB) and the mushroom body (MB). Below, we describe recent findings in these neural circuits.

The Dorsal Fan-Shaped Body Circuit

Dopamine released on the dorsal fan-shaped body (dFB) has a central role in the regulation of the sleep-wake balance. A single pair of DANs projecting to the dFB promotes wakefulness, and dopamine receptors are necessary in the dFB neurons to process the waking signal (Liu et al., 2012; Pimentel et al., 2016). Additional physiological experiments revealed that the spontaneous activity of DANs is increased during wakefulness (Liu et al., 2012). In contrast, the downstream dFB neurons are considered sleep-promoting (Donlea et al., 2011, 2014; Ueno et al., 2012), and are inhibited upon dopamine input (Pimentel et al., 2016). Interestingly, the membrane properties of dFB

neurons reflect the animal's sleep need: sleep deprivation by continuous mechanical stress lowers the threshold to spike generation and thus increases sleep-inducing dFB neuron excitability (Donlea et al., 2014). This change is controlled by dopamine: sustained artificial activation of the DANs or sustained dopamine application for several minutes shift the states of these neurons from electrically excitable to quiescence (Pimentel et al., 2016). Therefore, spontaneous activity of these DANs regulates present and future sleep/wake balance, depending on the animal's sleep need.

The Mushroom Body Circuit

Besides the importance of the dFB circuit, the MB also has a critical role in the regulation of sleep (Joiner et al., 2006; Pitman et al., 2006). The MB is primarily composed of ~2,000 Kenyon

cells (KCs) (Aso et al., 2009), which are presynaptic to ~20 types of mushroom body output neurons (MBONs) (Aso et al., 2014a). There are ~20 types of DANs that innervate the MB, each of which projects to a confined region of the MB, thereby controlling specific segments of the MB neurons and MBONs (Aso et al., 2014a; Hige et al., 2015). These DANs originate mainly from two clusters called PAM and PPL1 (Mao and Davis, 2009).

Berry et al. (2015) demonstrated how specific DANs, which innervate the MB- $\gamma 2\alpha'1$ compartment and are also called MB-MV1 (just MV1 hereafter; also known as PPL1- $\gamma 2\alpha'1$, show spontaneous activity that correlates with the locomotive state of the animal; namely, the calcium activity was upregulated during the walking bout (**Figure 1A**). Similarly, Cohn et al. (2015) found that another DAN type innervating the $\gamma 3$ compartment, in addition to MV1, becomes also active during a walking

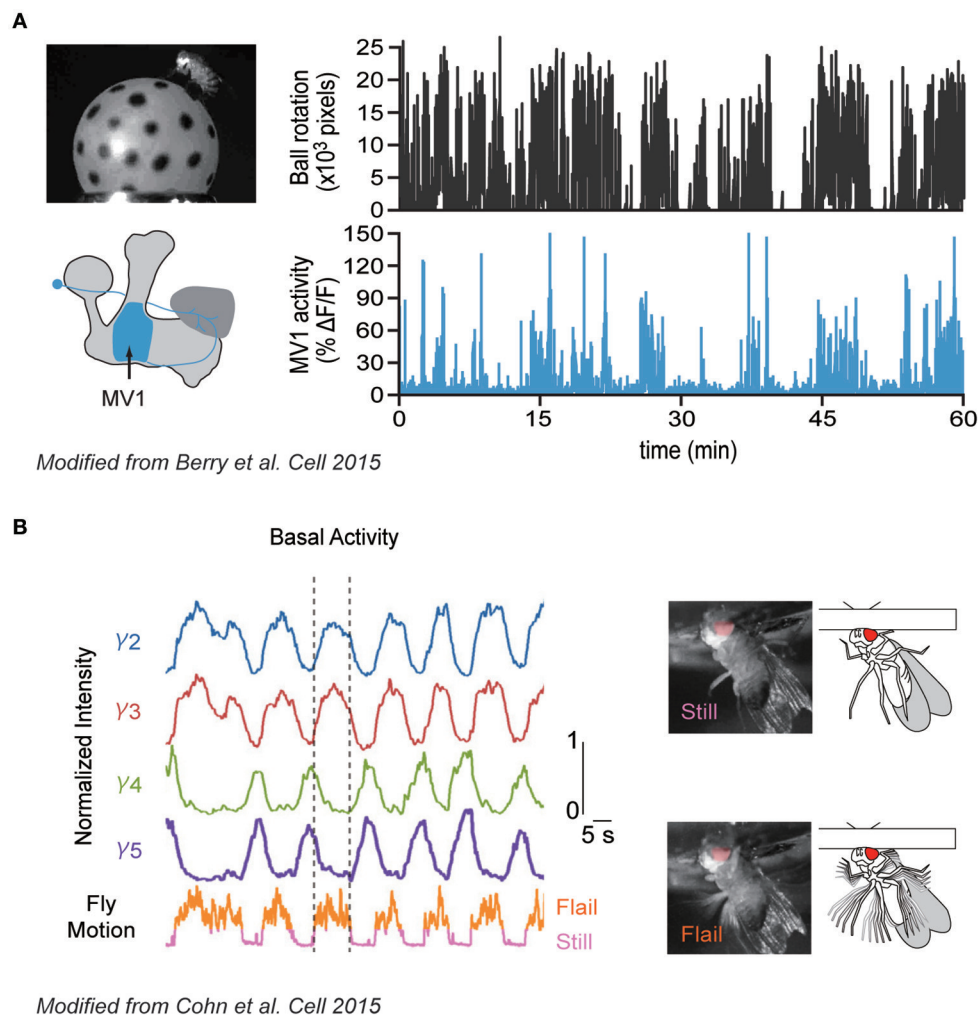


FIGURE 1 | Correlative spontaneous DAN activity with the locomotive state. **(A)** Correlation between locomotive state and DAN activity. Left: Walking activity of a fly was monitored by observing the rotation of the ball during the calcium imaging of defined DAN types (MV1). Right: Walking activity of a fly (top) and calcium responses of MV1 (bottom). MV1 shows strong activity during walking bout. **(B)** Correlative and anti-correlative DAN activities with flailing. Left: Calcium responses of different DAN types innervating different MB compartments ($\gamma 2$ - $\gamma 5$, top), and locomotive activity of a head-fixed fly (bottom). Dashed lines delineate the start and cessation of a single flailing bout. Right: Two activity states of a fly during imaging (still and flail). Modified from Berry et al. (2015) and Cohn et al. (2015) with a permission.

bout (**Figure 1B**). Interestingly, spontaneous activity of DAN types innervating adjacent MB compartments ($\gamma 4$ and $\gamma 5$) was conversely shown to be suppressed during walking. Importantly, these DANs also respond to external stimuli such as sugar reward or electric shock in a cell-type specific manner. Therefore, both the internal locomotive state and external stimuli are integrated by the same DANs, each of which modifies a specific subdomain in the MB.

A series of experiments performed by Sitaraman et al. gives a hint of how the wake-promoting DANs exert their function (Aso et al., 2014b; Sitaraman et al., 2015a,b). Artificial activation of specific MBON types (MBON- $\gamma 5\beta 2$, $\beta 2mp$, and $\gamma 4 > \gamma 1\gamma 2$), which have cell-type specific projection patterns, promotes arousal (Aso et al., 2014b; Sitaraman et al., 2015a). Interestingly, these wake-promoting MBON types receive inputs from and are activated by wake-promoting DANs (Sitaraman et al., 2015b). Note that another MBON type (MBON- $\gamma 2\alpha 1$) in contrast promotes sleep (Aso et al., 2014b; Sitaraman et al., 2015a), although the wake-promoting MV1 project its terminals to the compartment. How MV1 wakes animals has to be answered by future studies. Altogether, DANs seem to control sleep and locomotion by integrating internal states and external stimuli.

LEARNING AND MEMORY

Motivation and Memory-Guided Behavior

It is widely acknowledged that tonic DAN activity is also involved in motivation. Microdialysis studies have demonstrated that dopamine release in slow temporal scales (tens of minutes) strongly correlates with the behavioral activity of rats (Freed and Yamamoto, 1985). Minute-by-minute dopamine levels in the nucleus accumbens correlate with an amount of reward in time and motivational vigor (Hamid et al., 2016). Sustained and ramping dopamine signaling occurs in mice moving toward predictable reward in tasks involving self-paced behavior (Howe et al., 2013). These observations collectively suggest that spontaneous DAN activity subserves motivational control of both innate and memory-guided behaviors.

How does the spontaneous DAN activity regulate learning? Recent findings in *Drosophila* give a hint for the mechanism. In flies, appetitive memory trace is thought to be localized at the synapses between Kenyon cells and MBONs (Heisenberg, 2003). The retrieval of this memory is largely dependent on the hunger state of flies (Krashes et al., 2009; Gruber et al., 2013). Krashes et al. (2009) demonstrated that such a hunger regulation is controlled by the activity of a single class of DAN cell type, called MB-MP1 (just MP1 hereafter; also known as PPL1- $\gamma 1pedc > \alpha/\beta$). An artificial activation of the MP1 in hungry flies during memory retrieval phase blocked the expression of memory. In contrast, a transient blockade of the same neurons restored memory expression in satiated flies. A follow-up study by the same group showed that the activity of MBONs in the corresponding MB compartment (MBON- $\gamma 1pedc > \alpha/\beta$) gates the expression of appetitive memory (Perisse et al., 2016). Therefore the $\gamma 1$ compartment may have a central role in controlling the memory-based behavior, reflecting the hunger motivation. Whether the DAN activity shapes the activity of pre- or post-synapses of the

local circuitry between KC and MBON remains to be clarified in future, as both KCs and MBON can be targeted by DANs in the local circuitry of the MB lobe (Takemura et al., 2017).

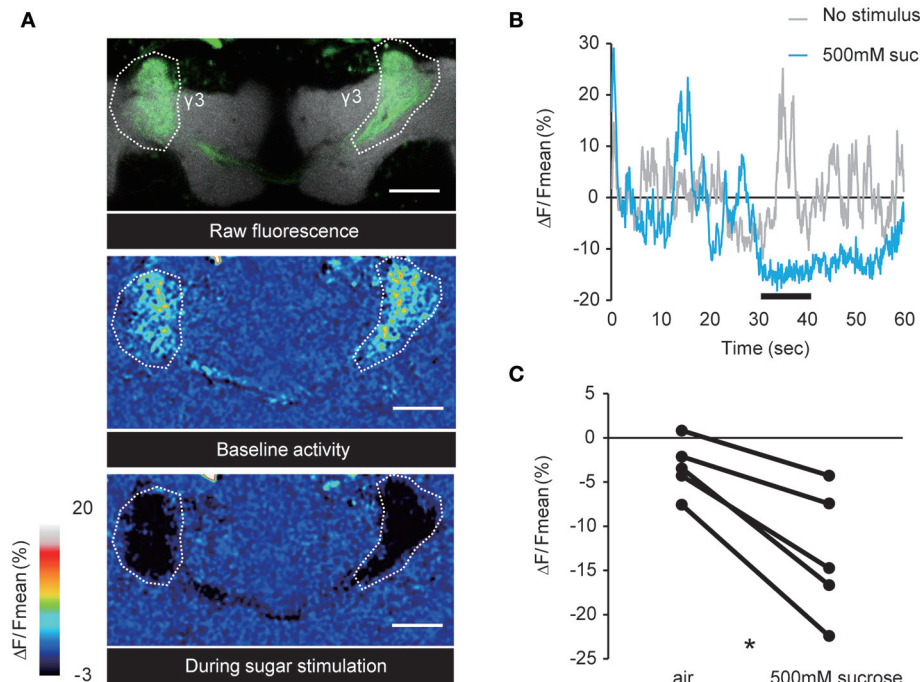
Acquisition

Besides stimulus-induced burst firing, accumulating physiological evidence revealed that spontaneous DAN activity is suppressed by the presentation of reinforcing stimuli (Brischoux et al., 2009; Matsumoto and Hikosaka, 2009; Fiorillo, 2013). This suppression can trigger memory formation: in mice, it has recently been demonstrated that repeated optogenetic silencing of spontaneous VTA DAN activity can induce place aversion (Danjo et al., 2014). Also in rats, brief suppression of spontaneous DAN activity in the VTA can substitute for negative prediction error (Chang et al., 2016), indicative of an importance of spontaneous dopamine release in reinforcement signaling.

One of the major input sources of inhibitory regulation of VTA DANs is afferents from the rostromedial tegmental nucleus (RMTg) (Jhou et al., 2009; Bourdy and Barrot, 2012; Tan et al., 2012; van Zessen et al., 2012). These GABAergic RMTg neurons receive excitatory inputs from structures implicated in aversive processing (Matsumoto and Hikosaka, 2007; Hong et al., 2011). These anatomical and functional studies in mammals provide a basic explanation for how spontaneous DAN activity can be suppressed (Danjo et al., 2014; Chang et al., 2016). However, the reinforcing property of changes in spontaneous activity of defined DANs is largely unclear.

In *Drosophila*, distinct DANs consisting of identified cell types mediate positive or negative valences (Aso et al., 2012; Lin et al., 2014; Huetteroth et al., 2015; Yamagata et al., 2015; Aso and Rubin, 2016). It has recently been demonstrated that these valence-encoding DANs are spontaneously active (Berry et al., 2015; Cohn et al., 2015), and are dynamically tuned by external stimuli as well as the behavioral state of an animal (**Figure 1**). In accordance, PAM- $\gamma 3$, a class of DANs projecting to the third segment of the MB γ lobe, was shown to have fluctuating baseline activity that is suppressed upon sugar ingestion (**Figure 2**; Yamagata et al., 2016). Interestingly, this ingestion-induced suppression of PAM- $\gamma 3$ activity lasted even after the presentation of sugar reward (**Figure 2B**). Furthermore, transient thermogenetic and optogenetic inactivation of the PAM- $\gamma 3$ was sufficient to induce appetitive memory while activation induced aversive memory (Yamagata et al., 2016). Thus, these results suggested that the spontaneous activity of PAM- $\gamma 3$ represents the feeding states and that feeding drives associative memories by changing PAM- $\gamma 3$ activity states regardless of increase or decrease.

Suppression of PAM- $\gamma 3$ was mediated by a satiety-signaling neuropeptide, Allatostatin A (AstA) (Hergarden et al., 2012; Hentze et al., 2015; Chen et al., 2016), which is known to be a potent inhibitory neuromodulator (Birgöl et al., 1999). AstA expressing neurons innervate dendritic region of the PAM- $\gamma 3$, which express the AstA cognate receptor DAR-1 (Lenz et al., 2000; Yamagata et al., 2016). In contrast to aversive memory formation by PAM- $\gamma 3$, activation of AstA neurons induced appetitive memory, suggesting that AstA negatively regulates



Modified from Yamagata et al. *PLoS Biol.* 2016

FIGURE 2 | Sucrose reward suppresses the spontaneous activity of PAM- γ 3. **(A)** Representative images of raw fluorescence of GCaMP expressed in the PAM- γ 3 DANs (top), baseline activity (middle), and calcium responses to sucrose stimulation (bottom). **(B)** Time course of the fluorescence signal. The black bar represents the sucrose presentation. Spontaneous DAN activity is suppressed by the sucrose presentation. **(C)** Average calcium responses to sucrose presentation. Sucrose intake significantly reduces the activity level of PAM- γ 3. Modified from Yamagata et al. (2016).

PAM- γ 3 spontaneous activity (Yamagata et al., 2016). Consistent with this hypothesis, down-regulation of *DAR-1* expression in PAM- γ 3 diminished the feeding-induced suppression of the activity. Altogether, simultaneous recording and genetic manipulation of a DAN population in the fly brain revealed the network dynamics that determines valence (Cohn et al., 2015; Yamagata et al., 2016).

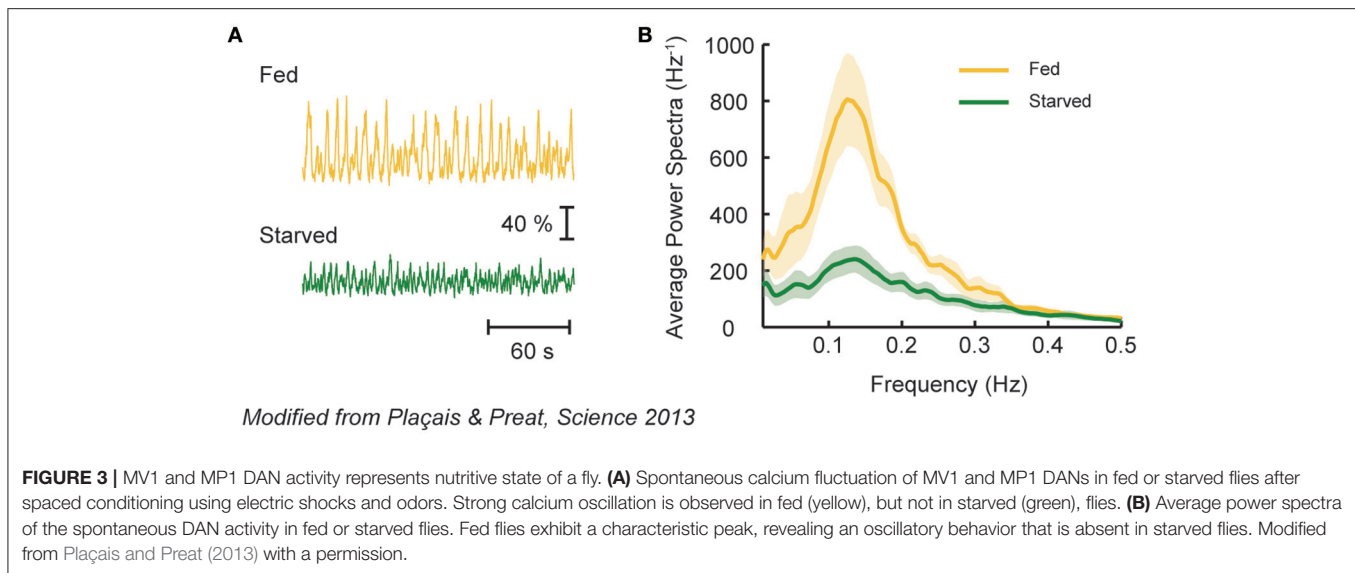
Memory Consolidation

Psychostimulants that augment dopamine signaling are known to facilitate memory consolidation. For example, avoidance learning in rats is enhanced by post-training administration of amphetamine, which increases dopamine signaling (McGaugh and Roozendaal, 2009). Post-training cocaine exposure similarly enhances consolidation of spatial memory in mice (Iniguez et al., 2012). In addition, intrahippocampal application of the D1R agonist at definite post-learning time points converts a rapidly decaying fear LTM into a persistent one (Rossato et al., 2009), suggesting a critical role for dopamine signaling in memory consolidation.

In *Drosophila*, a functional linkage between spontaneous activity of identified DANs and memory consolidation has been demonstrated. Plaçaïs et al. (2012) found that two pairs of DANs, MP1 and MV1, exhibit slow Ca^{2+} oscillations (~ 0.1 Hz) without external stimuli. In flies, repetitive training of paired

presentations of odor cues and electric shocks with intervals (spaced training) is commonly used for the induction of aversive LTM (Tully et al., 1994). The authors found the regularity of slow Ca^{2+} oscillations to be enhanced after spaced training. Strikingly, suppression of the synaptic transmission of MP1 and MV1 after training diminished the formation of LTM, suggestive of a critical role of the spontaneous activity of specific DANs in memory consolidation. Since spontaneous activity of MP1 is also required for the consolidation of appetitive LTM (Musso et al., 2015), a general role may be imposed for the neural class in consolidating a labile memory into a stable, long-lasting one.

Spontaneous activity of MP1 and MV1 reflects the nutrient condition of an animal. In flies, the formation of aversive LTM depends on the animal's post-learning nutrient state (Hirano et al., 2013; Plaçaïs and Preat, 2013). This is because LTM formation is energetically costly and thus its induction is inhibited upon energy shortage (Plaçaïs and Preat, 2013). In accordance, the slow oscillation of MP1 and MV1 occurs only in fed flies after spaced training (Figure 3), which fits with the idea that MP1 mediates hunger motivation (see memory retrieval section). Intriguingly, driving MP1 and MV1 activity in starved flies after learning could still induce aversive LTM at the price of survival duration upon starvation, highlighting an obvious trade-off between survival and LTM formation (Mery and Kawecki,



2005; Plaças and Preat, 2013). Thus, spontaneous activity of these DANs after memory acquisition can act as a homeostatic feedback mechanism to regulate energy state and memory consolidation.

Spontaneous activity of MP1 and MV1 has been implicated also in forgetting of short-lasting aversive memory in flies (Berry et al., 2012, 2015). Artificial activation of those DAN classes after a single training period promoted memory loss (Berry et al., 2012). Conversely, the blockade of these DANs increased the persistence of labile memory. Similar function is also imposed by a neural class belonging to the PAM cluster DANs, called PAM- β^1 . Thermal activation of PAM- β^1 after learning promoted aversive memory loss (Shuai et al., 2015). Note that the identical DANs, MP1 and MV1, on one hand consolidate long-term aversive memory (Plaças et al., 2012), while on the other hand promote forgetting short-lasting memories. Plaças et al. (2012) showed that the memory component affected by post-training dopamine input is anesthesia-resistant memory. Intriguingly, spontaneous activity of MV1 also reflects the wake/sleep state of flies (see above Berry et al., 2015) while sleep prevents memory forgetting by blocking spontaneous activity of MV1. Given such a tight connection between sleep and mnemonic processes (Dissel et al., 2015; Haynes et al., 2015), it is plausible that the spontaneous activity of MV1 acts as a hub to link internal sleep need and memory maintenance processes.

Also in mammals, accumulating physiological evidence points to the importance of spontaneous firing of DANs in memory consolidation. For instance, spontaneous firing of VTA DANs is increased during REM sleep (Dahan et al., 2007), and is coordinated with quiet wakefulness-associated hippocampal sharp wave-ripples (Gomperts et al., 2015), which is believed to be crucial for memory consolidation (Siegel, 2001). A functional loop between the hippocampus and the VTA dopaminergic neurons was thus suggested to be crucial in post-learning DAN activity (Lisman and Grace, 2005; Gruber et al., 2016), although specific neural circuits still remain to be elucidated.

In *Drosophila*, a comprehensive anatomical study identified many feedback connections from the MB to DANs through MBONs (Aso et al., 2014a). Thus, reinforcing DANs projecting to the MB may provide an optimal study case to test the importance of the feedback regulation for memory consolidation. We previously found that a single DAN type innervating the $\alpha 1$ compartment (PAM- $\alpha 1$) has a critical role in signaling reward for appetitive LTM (Yamagata et al., 2015). Interestingly, PAM- $\alpha 1$ undergoes the direct recurrent regulation by MBON- $\alpha 1$, which has dendrites in the $\alpha 1$ compartment of the MB (Aso et al., 2014a; Ichinose et al., 2015). Indeed, transient blockade of neuronal components participating in this $\alpha 1$ feedback circuit during conditioning and early memory consolidation phase impaired appetitive LTM (Ichinose et al., 2015). This demonstrated the necessity of this recurrent circuit for LTM formation and consolidation, and suggests coordinated reverberating activity triggered by associative training (Ichinose et al., 2015). Therefore, further studies on direct measurement of post-training spontaneous activity would provide mechanistic insights to behavioral requirements of this circuit. Similar to the feedback loop between PAM- $\alpha 1$ and MBON- $\alpha 1$ for the formation and consolidation of appetitive memory, MP1 and MV1 mediate punitive reinforcement signals and are required for the consolidation of aversive memory (Claridge-Chang et al., 2009; Aso et al., 2012; Plaças et al., 2012; Aso and Rubin, 2016). Therefore, the functional analysis of analogous recurrent circuits in aversive memory would be informative in examining the importance of this motif of dopaminergic circuits in memory formation/consolidation.

FEEDING

Dopamine is heavily involved in controlling feeding behaviors. For instance, dopamine deficient mice exhibit hypophagia, which is restored by the administration of L-DOPA (Zhou and

Palmiter, 1995) or genetic rescue of dopamine production by the overexpression of tyrosine hydroxylase (Szczytko et al., 2001). The excitatory orexin inputs from the lateral hypothalamus, a neuroanatomical substrate critical for feeding (Stuber and Wise, 2016), regulate activity of VTA DANs (Aston-Jones et al., 2010). Although a causal link between spontaneous activity of VTA DANs and feeding control has been behaviorally demonstrated (van Zessen et al., 2012), mechanisms by which this activity is translated into feeding behavior are still largely unknown.

In *Drosophila*, activity of a specific class of DANs, called TH-VUM, innervating the suboesophageal ganglion (SEG), a gustatory center in the insect brain, is reported to control taste sensitivity to sucrose (Marella et al., 2012). Suppression of TH-VUM activity reduced the sensitivity while activation increased it. Strikingly, TH-VUM exhibits spontaneous activity that is upregulated upon starvation, thereby increasing responsiveness of an animal to sugar. Inagaki et al. (2012) demonstrated that starvation-induced dopamine release alters the sensitivity of sugar-sensing gustatory neurons (GRNs). On the other hand, it has been shown that spontaneous activity of a class of neurons releasing octopamine, which is an invertebrate counterpart of noradrenaline (Roeder, 1999), in the SEG, called OA-VL (Busch et al., 2009), confers bitter taste sensitivity to flies (LeDue et al., 2016). In contrast to TH-VUM, the activity of OA-VL potentiates bitter-sensing GRNs and is downregulated by starvation. In this way, starvation modulates basal dopamine and octopamine levels to control sensitivity to sweet and bitter compounds, respectively. Antagonizing activity of OA-VL and TH-VUM may thus coordinate to set a threshold for the acceptance of foods by flies.

Flies sense amino acids in food. Spontaneous activity of DANs in protocerebral posterior medial 2 (PPM2) cluster encode protein hunger (Liu et al., 2017). The activity of these neurons is upregulated after protein deprivation, and is necessary and sufficient for protein preference. Interestingly, these neurons change not only the spontaneous firing rate but also its morphology, resulting in increased number of connections with the downstream target upon amino acid deprivation. In larvae, brain DANs spanning three clusters (DM1, DM2 and DL1) also detect amino acid imbalance to reject essential amino acid-deficient diet (Bjorndal et al., 2014), though this occurs based on stimulus induced DAN activity. Taken together, feeding motivation of two of the important nutrient factors, sugar and protein, is separately regulated by different DANs that monitor the need of the animal.

SEX DRIVE

Drugs targeting the dopamine system are known to have side effects on human sexual behavior. Spontaneous ejaculations have been reported as a side effect in patients taking Aripiprazole, which is a partial agonist of the D2 receptor (EGILmez et al., 2016). Hypersexuality and excessive masturbation in children and spontaneous erections in adults have been reported by patients taking methylphenidate (also known as Ritalin) (Bilgic et al., 2007), which primarily acts as a norepinephrine-dopamine

reuptake inhibitor. Although these observations suggest a tight relationship existing between dopamine and sexual behavior (Melis and Argiolas, 1995), a functional link between spontaneous DAN activity and sexual drive has yet to be clarified in mammals.

In *Drosophila*, dopamine levels modulate both the mating drive of males as well as sexual receptivity of females: dopamine-deficient males and females respectively court and accept males less, which can be restored by L-DOPA administration (Neckameyer, 1998; Liu et al., 2008). Male flies administered methamphetamine show extremely high courtship activity, yet the latency to copulation is increased (Andretic et al., 2005). Mating drive of males is tightly regulated by their reproductive state: repeated mating progressively reduces his sexual vigor (Zhang et al., 2016). Importantly, spontaneous Ca^{2+} activity in a small subset of DANs that include aSP4 neurons in the PAL cluster are cumulatively decreased by repeated mating (Zhang et al., 2016), indicating that reproductive state is represented by the level of spontaneous activity in them. Another report suggests dopamine production in the yet another class of DANs, called PPL2ab neurons, is critical to maintain courtship activity in aged males (Kuo et al., 2015). Thus, aSP4 and PPL2ab DANs can act cooperatively for the control of sexual vigor in male flies. The target of such a motivational signal can be a group of ~20 Fruitless-expressing neurons per hemisphere called P1 (Zhang et al., 2016). P1 is a male-specific neuronal cluster that has been identified as a putative trigger center for male-type courtship behavior (Kohatsu et al., 2011; Yamamoto and Koganezawa, 2013). In accordance, protocerebral innervations of P1 and aSP4 overlap and form putative synapses (Zhang et al., 2016). Strikingly, knocking down a subtype of dopamine receptor in the P1 significantly attenuates male's courtship behaviors. It is thus conceivable that spontaneous DAN activity modulates the excitability of P1 to control sexual vigor depending on motivational state. Interestingly, this regulatory mechanism of P1 is a reminiscent of sleep/wake control in dFB, activity of which is regulated by spontaneously active DANs that reflect sleep need. Therefore, one of the major functions of spontaneous DAN activity is to represent distinct motivational states and to shape corresponding behavior by modulating the activity of key behavior-executing neurons, such as P1 or dFB.

DETECTION OF SPONTANEOUS DAN ACTIVITY

Spontaneous activity of DANs has to be interpreted by receiving neurons through receptors. Dopamine receptors can be grouped into five classes of the guanine nucleotide-binding protein-coupled receptors (GPCRs): D1- to D5-type receptors (D1R–D5R). It is commonly accepted that D1R and D5R mainly recruit the $\text{G}\alpha_s$ to stimulate cAMP production by adenylyl cyclase, and D2R, D3R, and D4R the $\text{G}\alpha_{i/o}$ to inhibit (Beaulieu and Gainetdinov, 2011). In mammals, the inhibitory receptors show higher affinity to dopamine than the excitatory ones, and thus are suggested to play the main

role in detecting the slow, tonic DAN activity (Grace et al., 2007). These inhibitory receptors are reported to be involved in the detection of wake-promoting dopamine release (Qu et al., 2010) and spontaneous activity of the value coding midbrain DANs (Bromberg-Martin et al., 2010). However, the generality and its intracellular signaling events are largely unknown.

In *Drosophila*, four dopamine receptors, DopR1 (also known as dDA1, DUMB), Dop2R, DopR2 (also known as DAMB, DopR99B) and DopEcR exist in the genome (Adams et al., 2000). Sequence homology with mammalian dopamine receptors suggests that DopR1 and Dop2R are D1- and D2- like, respectively, and the other two are invertebrate-specific (Mustard et al., 2005). Measurement of DopR2 in *Xenopus* oocyte suggested it to be excitatory (Reale et al., 1997), but recent studies suggested that it can be variable among cell types (see below). DopEcR increases cAMP upon binding to dopamine, and binds to insect steroid hormone ecdysone in addition (Srivastava et al., 2005). Affinity of these four receptors to dopamine has been respectively measured *in vitro* but with different cell lines, and never been directly compared. It is thus important to measure the threshold of these receptors for correct interpretation of functional results.

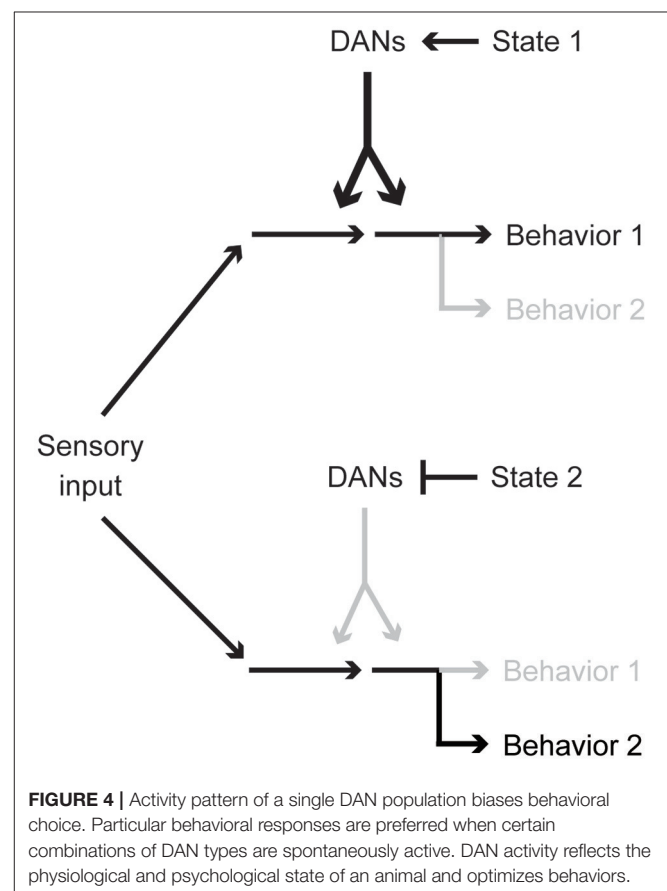
Nonetheless, accumulating behavioral and physiological evidence suggests the critical role of DopR2 in the detection of spontaneous activity of DANs. DopR2 was shown to be critical in regulation of sleep in the dFB (Pimentel et al., 2016), memory maintenance (Berry et al., 2012; Musso et al., 2015; Plačais et al., 2017), and sex drive (Zhang et al., 2016). We will review these cases one by one.

Exquisite *in-vivo* electrophysiology experiments demonstrated that DopR2 in the dFB neurons mediates the wake-promoting dopamine signaling (Pimentel et al., 2016). This study further provided unexpected evidence that DopR2 in the dFB neurons employs $G\alpha_o$ and thereby hyperpolarizes the membrane potential through modulating specific K^+ channels (Pimentel et al., 2016). These results together with biochemistry experiments *in vitro* (Han et al., 1996) suggest that the nature of DopR2—excitatory or inhibitory—can be variable among cell types and imply recruitment of different $G\alpha$ proteins through forming heteromeric receptor complexes.

DopR2 in the MB is also responsible for detecting the spontaneous activity of MV1 or MP1 DANs during memory maintenance (Berry et al., 2012; Musso et al., 2015; Plačais et al., 2017). It is critical to mediate the forgetting signal of aversive short-term memory (Berry et al., 2012). It detects the nutritive value of sugar reward in appetitive conditioning to consolidate memory (Musso et al., 2015). Furthermore, it triggers energy influx to the MB that is critical for aversive LTM formation after spaced conditioning (see also above) (Plačais et al., 2017). Strikingly, this receptor is responsible for subcellular modulation of Kenyon cell outputs in the MB lobes (Cohn et al., 2015). Not only in the MB, but also in the lateral accessory lobe DopR2 mediates increased protein feeding after protein deprivation (Liu et al., 2017). Sex drive is also regulated by DopR2 expressed in P1 neurons, which trigger male courtship behaviors (Kimura et al., 2008; Zhang et al., 2016). These studies collectively highlight

a wide range of functions controlled by this receptor through detection of ongoing activity of DANs.

Two of the recent studies in *Drosophila* addressed intracellular signaling molecules that mediate the effect of spontaneous DAN activity (Cervantes-Sandoval et al., 2016; Pimentel et al., 2016). Pimentel et al. (2016) showed that dopamine/DopR2 signaling switches sleep-promoting dFB neurons from the state of excitability to one of quiescence by mobilizing potassium channels to the plasma membrane. This switch is mediated by heterotrimeric G proteins of the $G\alpha_o$ family (Thambi et al., 1989; Pimentel et al., 2016), which deviates from the measurement in the *Xenopus* oocyte system (Reale et al., 1997). Since individual mammalian GPCRs have been demonstrated to engage multiple G proteins with varying efficacy and kinetics in a cell-specific manner, DopR2 might function as a D2-like receptor under some conditions. Cervantes-Sandoval et al. (2016) described another example of intracellular signaling events in the context of memory forgetting. Upon binding to dopamine, DopR2 activates a small GTPase, Rac1, which had been identified by another group to induce forgetting of memory (Shuai et al., 2010). This activation is mediated by a scaffold protein, Scribbled, which also can activate Pak3 and cofilin, which are key proteins in regulating actin dynamics. Altogether, Rac1, Pak3 and cofilin may thus produce necessary cytoskeletal modifications that underlie neural remodeling and consequential forgetting.



CONCLUSION

In this article, we have reviewed diverse functions of spontaneous activity of DANs, paying special attention to recent *Drosophila* studies. Importantly, different internal animal states, e.g., hunger, sleep need, or sexual drive, are represented by different yet partially overlapping DAN cell types. This combinatorial state coding is reminiscent of various reinforcement signals conveyed by different combinations of DANs (Aso et al., 2012; Lin et al., 2014; Huetteroth et al., 2015; Yamagata et al., 2015; Aso and Rubin, 2016). Therefore, regardless of its spontaneous or stimulus-induced origins, activity patterns of different DAN types might together be a key determinant for state-dependent behavior and action selection (Figure 4). Investigation of circuits influencing DAN activities is thus critical for understanding the cellular basis of behavioral and physiological states.

In some cases, spontaneous DAN activity functions as an information filter to enable animals to respond differently to the same sensory input, such as food, food-associated cues or potential mating partners, depending on the physiological state (Inagaki et al., 2012; Marella et al., 2012; Kuo et al., 2015; Zhang et al., 2016). Similar function of DANs to bias information flow is observed in mammalian systems (Grace et al., 2007). In addition, the spontaneous activity of specific DAN types controls ongoing spontaneous locomotor activity depending on sleep need (Donlea et al., 2011; Liu et al., 2012; Sitaraman et al., 2015b). Importantly, partially overlapping yet different combinations of DANs have an additional role in memory formation, consolidation and forgetting (Berry et al., 2012; Plaçais et al., 2012; Ichinose et al., 2015; Musso et al.,

2015; Shuai et al., 2015; Yamagata et al., 2016), suggestive of the close relationship between sleep and memory formation or maintenance (Berry et al., 2015; Dissel et al., 2015). Taken together, spontaneous DAN activity patterns seem to represent both the past and present states of the animal thereby biasing behavior selection. By exploiting state-of-art genetic techniques, future studies should likely decode how animal behavior is optimized by spontaneous DAN activity.

AUTHOR CONTRIBUTIONS

TI, HT, and NY wrote the manuscript and designed the figures.

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REFERENCES

- Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195. doi: 10.1126/science.287.5461.2185
- Andretic, R., van Swinderen, B., and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol.* 15, 1165–1175. doi: 10.1016/j.cub.2005.05.025
- Aso, Y., Grubel, K., Busch, S., Friedrich, A. B., Siwanowicz, I., and Tanimoto, H. (2009). The mushroom body of adult *Drosophila* characterized by GAL4 drivers. *J. Neurogenet.* 23, 156–172. doi: 10.1080/01677060802471718
- Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T. T., et al. (2014a). The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* 3:e04577. doi: 10.7554/eLife.04577
- Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., et al. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* 8:e1002768. doi: 10.1371/journal.pgen.1002768
- Aso, Y., and Rubin, G. M. (2016). Dopaminergic neurons write and update memories with cell-type-specific rules. *eLife* 5:e16135. doi: 10.7554/eLife.16135
- Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guerin, G., et al. (2014b). Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *eLife* 3:e04580. doi: 10.7554/eLife.04580
- Aston-Jones, G., Smith, R. J., Sartor, G. C., Moorman, D. E., Massi, L., Tahsili-Fahadan, P., et al. (2010). Lateral hypothalamic orexin/hypocretin neurons: a role in reward-seeking and addiction. *Brain Res.* 1314, 74–90. doi: 10.1016/j.brainres.2009.09.106
- Bainton, R. J., Tsai, L. T., Singh, C. M., Moore, M. S., Neckameyer, W. S., and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol.* 10, 187–194. doi: 10.1016/S0960-9822(00)00336-5
- Beaulieu, J. M., and Gainetdinov, R. R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217. doi: 10.1124/pr.110.002642
- Berridge, C. W., Schmeichel, B. E., and Espana, R. A. (2012). Noradrenergic modulation of wakefulness/arousal. *Sleep Med. Rev.* 16, 187–197. doi: 10.1016/j.smrv.2011.12.003
- Berry, J. A., Cervantes-Sandoval, I., Chakraborty, M., and Ronald Davis, L. (2015). Sleep facilitates memory by blocking dopamine neuron-mediated forgetting. *Cell* 161, 1656–1667. doi: 10.1016/j.cell.2015.05.027
- Berry, J. A., Cervantes-Sandoval, I., Nicholas, E. P., and Davis, R. L. (2012). Dopamine is required for learning and forgetting in *Drosophila*. *Neuron* 74, 530–542. doi: 10.1016/j.neuron.2012.04.007
- Bilgic, A., Gurkan, K., and Turkoglu, S. (2007). Excessive masturbation and hypersexual behavior associated with methylphenidate. *J. Am. Acad. Child Adolesc. Psychiatry* 46, 789–790. doi: 10.1097/chi.0b013e318059360a
- Billiard, M. (2008). Narcolepsy: current treatment options and future approaches. *Neuropsychiatr. Dis. Treat.* 4, 557–566. doi: 10.2147/NDT.S484
- Birgöl, N., Weise, C., Kreienkamp, H. J., and Richter, D. (1999). Reverse physiology in *drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J.* 18, 5892–5900. doi: 10.1093/emboj/18.21.5892
- Bjorndal, M., Arquier, N., Kniazeff, J., Jean Pin, P., and Léopold, P. (2014). Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in *Drosophila*. *Cell* 156, 510–521. doi: 10.1016/j.cell.2013.12.024

- Bourdy, R., and Barrot, M. (2012). A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci.* 35, 681–690. doi: 10.1016/j.tins.2012.06.007
- Brischoux, F., Chakraborty, S., Brierley, D. I., and Ungless, M. A. (2009). Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4894–4899. doi: 10.1073/pnas.0811507106
- Bromberg-Martin, E. S., Matsumoto, M., and Hikosaka, O. (2010). Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68, 815–834. doi: 10.1016/j.neuron.2010.11.022
- Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the *Drosophila* brain. *J. Comp. Neurol.* 513, 643–667. doi: 10.1002/cne.21966
- Cao, J. L., Covington, H. E. III, Friedman, A. K., Wilkinson, M. B., Walsh, J. J., Cooper, D. C., et al. (2010). Mesolimbic dopamine neurons in the brain reward circuit mediate susceptibility to social defeat and antidepressant action. *J. Neurosci.* 30, 16453–16458. doi: 10.1523/JNEUROSCI.3177-10.2010
- Cervantes-Sandoval, I., Chakraborty, M., MacMullen, C., and Ronald Davis, L. (2016). Scribble scaffolds a signalosome for active forgetting. *Neuron* 90, 1230–1242. doi: 10.1016/j.neuron.2016.05.010
- Chang, C. Y., Esber, G. R., Marrero-Garcia, Y., H.-Yau, J., Bonci, A., and Schoenbaum, G. (2016). Brief optogenetic inhibition of dopamine neurons mimics endogenous negative reward prediction errors. *Nat. Neurosci.* 19, 111–116. doi: 10.1038/nn.4191
- Chaudhury, D., Walsh, J. J., Friedman, A. K., Juarez, B., Ku, S. M., Koo, J. W., et al. (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature* 493, 532–536. doi: 10.1038/nature11713
- Chen, J., Reiher, W., Hermann-Luibl, C., Sellami, A., Cognigni, P., Kondo, S., et al. (2016). Allatostatin a signalling in *Drosophila* regulates feeding and sleep and is modulated by PDF. *PLoS Genet.* 12:e1006346. doi: 10.1371/journal.pgen.1006346
- Claridge-Chang, A., Roorda, R. D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenbock, G. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell* 139, 405–415. doi: 10.1016/j.cell.2009.08.034
- Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B., and Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature* 482, 85–88. doi: 10.1038/nature10754
- Cohn, R., Morante, I., and Ruta, V. (2015). Coordinated and compartmentalized neuromodulation shapes sensory processing in *Drosophila*. *Cell* 163, 1742–1755. doi: 10.1016/j.cell.2015.11.019
- Dahan, L., Astier, B., Vautrelle, N., Urbain, N., Kocsis, B., and Chouvet, G. (2007). Prominent burst firing of dopaminergic neurons in the ventral tegmental area during paradoxical sleep. *Neuropsychopharmacology* 32, 1232–1241. doi: 10.1038/sj.npp.1301251
- Dalley, J. W., and Roiser, J. P. (2012). Dopamine, serotonin and impulsivity. *Neuroscience* 215, 42–58. doi: 10.1016/j.neuroscience.2012.03.065
- Danjo, T., Yoshimi, K., Funabiki, K., Yawata, S., and Nakanishi, S. (2014). Aversive behavior induced by optogenetic inactivation of ventral tegmental area dopamine neurons is mediated by dopamine D2 receptors in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 111, 6455–6460. doi: 10.1073/pnas.1404323111
- Dissel, S., Angadi, V., Kirszenblat, L., Suzuki, Y., Donlea, J., Klose, M., et al. (2015). Sleep restores behavioral plasticity to *Drosophila* mutants. *Curr. Biol.* 25, 1270–1281. doi: 10.1016/j.cub.2015.03.027
- Dominguez-Lopez, S., Howell, R., and Gobbi, G. (2012). Characterization of serotonin neurotransmission in knockout mice: implications for major depression. *Rev. Neurosci.* 23, 429–443. doi: 10.1515/revneuro-2012-0044
- Donlea, J. M., Pimentel, D., and Miesenbock, G. (2014). Neuronal machinery of sleep homeostasis in *Drosophila*. *Neuron* 81, 860–872. doi: 10.1016/j.neuron.2013.12.013
- Donlea, J. M., Thimman, M. S., Suzuki, Y., Gottschalk, L., and Shaw, P. J. (2011). Inducing sleep by remote control facilitates memory consolidation in *Drosophila*. *Science* 332, 1571–1576. doi: 10.1126/science.1202249
- EGILmez, O., ÇELİK, M., and Kalenderoğlu, A. (2016). Spontaneous ejaculations associated with aripiprazole. *Aripiprazole İlişkili Spontan Ejakülasyonlar*. 53, 85–86. doi: 10.5152/npa.2015.9896
- Finelli, L. A., Baumann, H., Borbely, A. A., and Achermann, P. (2000). Dual electroencephalogram markers of human sleep homeostasis: correlation between theta activity in waking and slow-wave activity in sleep. *Neuroscience* 101, 523–529. doi: 10.1016/S0306-4522(00)00409-7
- Fiorillo, C. D. (2013). Two dimensions of value: dopamine neurons represent reward but not aversiveness. *Science* 341, 546–549. doi: 10.1126/science.1238699
- Fiorillo, C. D., Tobler, P. N., and Schultz, W. (2003). Discrete coding of reward probability and uncertainty by dopamine neurons. *Science* 299, 1898–1902. doi: 10.1126/science.1077349
- Fox, M. D., and Raichle, M. E. (2007). Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. *Nat. Rev. Neurosci.* 8, 700–711. doi: 10.1038/nrn2201
- Freed, C., and Yamamoto, B. (1985). Regional brain dopamine metabolism: a marker for the speed, direction, and posture of moving animals. *Science* 229, 62–65. doi: 10.1126/science.4012312
- Gomperts, S. N., Kloosterman, F., and Wilson, M. A. (2015). VTA neurons coordinate with the hippocampal reactivation of spatial experience. *Elife* 4:e05360. doi: 10.7554/eLife.05360
- Gorter, J. A., Jagadeesh, S., Gahr, C., Boonekamp, J. J., Levine, J. D., and Billeter, J. C. (2016). The nutritional and hedonic value of food modulate sexual receptivity in *Drosophila melanogaster* females. *Sci. Rep.* 6:19441. doi: 10.1038/srep19441
- Grace, A. A. (2016). Dysregulation of the dopamine system in the pathophysiology of schizophrenia and depression. *Nat. Rev. Neurosci.* 17, 524–532. doi: 10.1038/nrn.2016.57
- Grace, A. A., and Bunney, B. (1984). The control of firing pattern in nigral dopamine neurons: single spike firing. *J. Neurosci.* 4, 2866–2876.
- Grace, A. A., Floresco, S. B., Goto, Y., and Lodge, D. J. (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.* 30, 220–227. doi: 10.1016/j.tins.2007.03.003
- Gruber, F., Knappek, S., Fujita, M., Matsuo, K., Bräcker, L., Shinzato, N., et al. (2013). Suppression of conditioned odor approach by feeding is independent of taste and nutritional value in *Drosophila*. *Curr. Biol.* 23, 507–514. doi: 10.1016/j.cub.2013.02.010
- Gruber, M. J., Ritchey, M., Wang, S.-F., Manoj Doss, K., and Ranganath, C. (2016). Post-learning hippocampal dynamics promote preferential retention of rewarding events. *Neuron* 89, 1110–1120. doi: 10.1016/j.neuron.2016.01.017
- Hamid, A. A., Pettibone, J. R., Mabrouk, O. S., Hetrick, V. L., Schmidt, R., Vander Weele, C. M., et al. (2016). Mesolimbic dopamine signals the value of work. *Nat. Neurosci.* 19, 117–126. doi: 10.1038/nn.4173
- Han, K.-A., Millar, N. S., Grotewiel, M. S., and Davis, R. L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16, 1127–1135. doi: 10.1016/S0896-6273(00)80139-7
- Haynes, P. R., Christmann, B. L., and Griffith, L. C. (2015). A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*. *eLife* 7:4. doi: 10.7554/eLife.03868
- Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* 4, 266–275. doi: 10.1038/nrn1074
- Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A., et al. (2000). Rest in *Drosophila* Is a Sleep-like State. *Neuron* 25, 129–138. doi: 10.1016/S0896-6273(00)80877-6
- Hentze, J. L., Carlsson, M. A., Kondo, S., Nässel, D. R., and Rewitz, K. F. (2015). The neuropeptide allatostatin A regulates metabolism and feeding decisions in *Drosophila*. *Sci. Rep.* 5:11680. doi: 10.1038/srep11680
- Hergarden, A. C., Tayler, T. D., and Anderson, D. J. (2012). Allatostatin-A neurons inhibit feeding behavior in adult *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3967–3972. doi: 10.1073/pnas.1200778109
- Hige, T., Aso, Y., Modi, M. N., Rubin, G. M., and Turner, G. C. (2015). Heterosynaptic plasticity underlies aversive olfactory learning in *Drosophila*. *Neuron* 88, 985–998. doi: 10.1016/j.neuron.2015.11.003
- Hirano, Y., Masuda, T., Naganos, S., Matsuno, M., Ueno, K., Miyashita, T., et al. (2013). Fasting launches CRIC to facilitate long-term memory formation in *Drosophila*. *Science* 339, 443–446. doi: 10.1126/science.1227170
- Hong, S., Jhou, T. C., Smith, M., Saleem, K. S., and Hikosaka, O. (2011). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *J. Neurosci.* 31, 11457–11471. doi: 10.1523/JNEUROSCI.1384-11.2011
- Howe, M. W., Tierney, P. L., Sandberg, S. G., Phillips, P. E. M., and Graybiel, A. M. (2013). Prolonged dopamine signalling in striatum signals proximity

- and value of distant rewards. *Nature* 500, 575–579. doi: 10.1038/nature12475
- Huetteroth, W., Perisse, E., Lin, S., Klappenbach, M., Burke, C., and Waddell, S. (2015). Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in *Drosophila*. *Curr. Biol.* 25, 751–758. doi: 10.1016/j.cub.2015.01.036
- Ichinose, T., Aso, Y., Yamagata, N., Abe, A., Rubin, G. M., and Tanimoto, H. (2015). Reward signal in a recurrent circuit drives appetitive long-term memory formation. *Elife* 4:e10719. doi: 10.7554/eLife.10719
- Inagaki, H. K., Ben-Tabou de-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., et al. (2012). Visualizing neuromodulation *in vivo*: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595. doi: 10.1016/j.cell.2011.12.022
- Iniguez, S. D., Charntikov, S., Baella, S. A., Herbert, M. S., Bolanos-Guzman, C. A., and Crawford, C. A. (2012). Post-training cocaine exposure facilitates spatial memory consolidation in C57BL/6 mice. *Hippocampus* 22, 802–813. doi: 10.1002/hipo.20941
- Jhou, T. C., Fields, H. L., Baxter, M. G., Saper, C. B., and Holland, P. C. (2009). The Rostromedial Tegmental Nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* 61, 786–800. doi: 10.1016/j.neuron.2009.02.001
- Joiner, W. J., Crocker, A., White, B. H., and Sehgal, A. (2006). Sleep in *Drosophila* is regulated by adult mushroom bodies. *Nature* 441, 757–760. doi: 10.1038/nature04811
- Keebaugh, E. S., Park, J. H., Su, C., Yamada, R., and Ja, W. W. (2017). Nutrition influences caffeine-mediated sleep loss in *Drosophila*. *Sleep* 40. doi: 10.1093/sleep/zsx146
- Keene, A. C., Duboue, E. R., McDonald, D. M., Dus, M., Suh, G. S., Waddell, S., et al. (2010). Clock and cycle limit starvation-induced sleep loss in *Drosophila*. *Curr. Biol.* 20, 1209–1215. doi: 10.1016/j.cub.2010.05.029
- Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., and Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. *Neuron* 59, 759–769. doi: 10.1016/j.neuron.2008.06.007
- Kohatsu, S., Koganezawa, M., and Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in *Drosophila*. *Neuron* 69, 498–508. doi: 10.1016/j.neuron.2010.12.017
- Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. *Cell* 139, 416–427. doi: 10.1016/j.cell.2009.08.035
- Krishnan, V., Han, M.-H., Graham, D. L., Berton, O., Renthal, W., Nestler, E. J. et al. (2007). Molecular Adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131, 391–404. doi: 10.1016/j.cell.2007.09.018
- Kume, K., Kume, S., Park, S. K., Hirsh, J., and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25, 7377–7384. doi: 10.1523/JNEUROSCI.2048-05.2005
- Kuo, S. Y., Wu, C. L., Hsieh, M. Y., Lin, C. T., Wen, R. K., Chen, L. C., et al. (2015). PPL2ab neurons restore sexual responses in aged *Drosophila* males through dopamine. *Nat. Commun.* 6, 7490. doi: 10.1038/ncomms8490
- Lebestky, T., Chang, J. S., Dankert, H., Zelnik, L., Kim, Y. C., Han, K. A., et al. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* 64, 522–536. doi: 10.1016/j.neuron.2009.09.031
- LeDue, E. E., Mann, K., Koch, E., Chu, B., Dakin, R., and Michael Gordon, D. (2016). Starvation-Induced Depotentiation of Bitter Taste in *Drosophila*. *Curr. Biol.* 26, 2854–2861. doi: 10.1016/j.cub.2016.08.028
- Lenz, C., Sondergaard, L., and Grimmelikhuijzen, C. J. (2000). Molecular cloning and genomic organization of a novel receptor from *Drosophila melanogaster* structurally related to mammalian galanin receptors. *Biochem. Biophys. Res. Commun.* 269, 91–96. doi: 10.1006/bbrc.2000.2251
- Li, H., Chaney, S., Roberts, I. J., Forte, M., and Hirsh, J. (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. *Curr. Biol.* 10, 211–214. doi: 10.1016/S0960-9822(00)00340-7
- Lin, S., Oswald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. *Nat. Neurosci.* 17, 1536–1542. doi: 10.1038/nn.3827
- Lisman, J. E., and Grace, A. A. (2005). The hippocampal-VTA loop: controlling the entry of information into long-term memory. *Neuron* 46, 703–713. doi: 10.1016/j.neuron.2005.05.002
- Liu, Q., Liu, S., Kodama, L., Driscoll, M. R., and Wu, M. N. (2012). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. *Curr. Biol.* 22, 2114–2123. doi: 10.1016/j.cub.2012.09.008
- Liu, Q., Tabuchi, M., Liu, S., Kodama, L., Horiuchi, W., Daniels, J., et al. (2017). Branch-specific plasticity of a bifunctional dopamine circuit encodes protein hunger. *Science* 356, 534–539. doi: 10.1126/science.aal3245
- Liu, T., Dartevelle, L., Yuan, C., Wei, H., Wang, Y., Ferveur, J. F., et al. (2008). Increased dopamine level enhances male-male courtship in *Drosophila*. *J. Neurosci.* 28, 5539–5546. doi: 10.1523/JNEUROSCI.5290-07.2008
- Lu, J., Jhou, T. C., and Saper, C. B. (2006). Identification of wake-active dopaminergic neurons in the ventral periaqueductal gray matter. *J. Neurosci.* 26, 193–202. doi: 10.1523/JNEUROSCI.2244-05.2006
- Mao, Z., and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Front. Neural Circuits* 3:5. doi: 10.3389/neuro.04.005.2009
- Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. *Neuron* 73, 941–950. doi: 10.1016/j.neuron.2011.12.032
- Marshall, L., and Born, J. (2007). The contribution of sleep to hippocampus-dependent memory consolidation. *Trends Cogn. Sci.* 11, 442–450. doi: 10.1016/j.tics.2007.09.001
- Massimini, M., Huber, R., Ferrarelli, F., Hill, S., and Tononi, G. (2004). The sleep slow oscillation as a traveling wave. *J. Neurosci.* 24, 6862–6870. doi: 10.1523/JNEUROSCI.1318-04.2004
- Matsumoto, M., and Hikosaka, O. (2007). Lateral habenula as a source of negative reward signals in dopamine neurons. *Nature* 447, 1111–1115. doi: 10.1038/nature05860
- Matsumoto, M., and Hikosaka, O. (2009). Two types of dopamine neuron distinctly convey positive and negative motivational signals. *Nature* 459, 837–841. doi: 10.1038/nature08028
- McClung, C., and Hirsh, J. (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in *Drosophila*. *Curr. Biol.* 8, 109–112. doi: 10.1016/S0960-9822(98)70041-7
- McGaugh, J. L., and Roozendaal, B. (2009). Drug enhancement of memory consolidation: historical perspective and neurobiological implications. *Psychopharmacology (Berl)* 202, 3–14. doi: 10.1007/s00213-008-1285-6
- Melis, M. R., and Argiolas, A. (1995). Dopamine and sexual behavior. *Neurosci. Biobehav. Rev.* 19, 19–38. doi: 10.1016/0149-7634(94)00020-2
- Mery, F., and Kawecki, T. J. (2005). A cost of long-term memory in *Drosophila*. *Science* 308:1148. doi: 10.1126/science.1111331
- Musso, P. Y., Tchenio, P., and Preat, T. (2015). Delayed dopamine signaling of energy level builds appetitive long-term memory in *Drosophila*. *Cell Rep.* 10, 1023–1031. doi: 10.1016/j.celrep.2015.01.036
- Mustard, J. A., Beggs, K. T., and Mercer, A. R. (2005). Molecular biology of the invertebrate dopamine receptors. *Arch. Insect Biochem. Physiol.* 59, 103–117. doi: 10.1002/arch.20065
- Nall, A. H., Shakhmantsir, I., Cichewicz, K., Birman, S., Hirsh, J., and Sehgal, A. (2016). Caffeine promotes wakefulness via dopamine signaling in *Drosophila*. *Sci. Rep.* 6:20938. doi: 10.1038/srep20938
- Neckameyer, W. S. (1998). Dopamine modulates female sexual receptivity in *Drosophila melanogaster*. *J. Neurogenet.* 12, 101–114. doi: 10.3109/0167706980167259
- Oishi, Y., Suzuki, Y., Takahashi, K., Yonezawa, T., Kanda, T., Takata, Y., et al. (2017). Activation of ventral tegmental area dopamine neurons produces wakefulness through dopamine D2-like receptors in mice. *Brain Struct. Funct.* 222, 2907–2915. doi: 10.1007/s00429-017-1365-7
- Perisse, E., Oswald, D., Barnstedt, O., Talbot, C. B., Huetteroth, W., and Waddell, S. (2016). Aversive learning and appetitive motivation toggle feed-forward inhibition in the *Drosophila* mushroom body. *Neuron* 90, 1086–1099. doi: 10.1016/j.neuron.2016.04.034
- Pimentel, D., Donlea, J. M., Talbot, C. B., Song, S. M. A., Thurston, J. F., and Miesenböck, G. (2016). Operation of a homeostatic sleep switch. *Nature* 536, 333–337. doi: 10.1038/nature19055

- Pitman, J. L., McGill, J. J., Keegan, K. P., and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in *Drosophila*. *Nature* 441, 753–756. doi: 10.1038/nature04739
- Plačais, P. Y., de Tredern, E., Scheunemann, L., Trannoy, S., Goguel, V., Han, K. A., et al. (2017). Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory. *Nat. Commun.* 8:15510. doi: 10.1038/ncomms.15510
- Plačais, P. Y., and Preat, T. (2013). To favor survival under food shortage, the brain disables costly memory. *Science* 339, 440–442. doi: 10.1126/science.1226018
- Plačais, P. Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliart-Guerin, G., et al. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat. Neurosci.* 15, 592–599. doi: 10.1038/nn.3055
- Qu, W. M., Xu, X. H., Yan, M. M., Wang, Y. Q., Urade, Y., and Huang, Z. L. (2010). Essential role of dopamine D2 receptor in the maintenance of wakefulness, but not in homeostatic regulation of sleep, in mice. *J. Neurosci.* 30, 4382–4389. doi: 10.1523/JNEUROSCI.4936-09.2010
- Reale, V., Hannan, F., Hall, L. M., and Evans, P. D. (1997). Agonist-specific coupling of a cloned *Drosophila melanogaster* D1-like dopamine receptor to multiple second messenger pathways by synthetic agonists. *J. Neurosci.* 17, 6545–6553.
- Rice, M. E., Patel, J. C., and Cragg, S. J. (2011). Dopamine release in the basal ganglia. *Neuroscience* 198, 112–137. doi: 10.1016/j.neuroscience.2011.08.066
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi: 10.1016/S0304-0082(99)00016-7
- Rossato, J. I., Bevilacqua, L. R., Izquierdo, I., Medina, J. H., and Cammarota, M. (2009). Dopamine controls persistence of long-term memory storage. *Science* 325, 1017–1020. doi: 10.1126/science.1172545
- Schultz, W. (2007). Multiple dopamine functions at different time courses. *Annu. Rev. Neurosci.* 30, 259–288. doi: 10.1146/annurev.neuro.28.061604.135722
- Schultz, W. (2015). Neuronal reward and decision signals: from theories to data. *Physiol. Rev.* 95, 853–951. doi: 10.1152/physrev.00023.2014
- Schultz, W., Dayan, P., and Montague, P. R. (1997). A neural substrate of prediction and reward. *Science* 275, 1593–1599. doi: 10.1126/science.275.5306.1593
- Shaw, P. J., Cirelli, C., Greenspan, R. J., and Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 287, 1834–1837. doi: 10.1126/science.287.5459.1834
- Shuai, Y., Hirokawa, A., Ai, Y., Zhang, M., Li, W., and Zhong, Y. (2015). Dissecting neural pathways for forgetting in *Drosophila* olfactory aversive memory. *Proc. Natl. Acad. Sci. U.S.A.* 112, E6663–E6672. doi: 10.1073/pnas.1512792112
- Shuai, Y., Lu, B., Hu, Y., Wang, L., Sun, K., and Zhong, Y. (2010). Forgetting is regulated through Rac activity in *Drosophila*. *Cell* 140, 579–589. doi: 10.1016/j.cell.2009.12.044
- Siegel, J. M. (2001). The REM sleep-memory consolidation hypothesis. *Science* 294, 1058–1063. doi: 10.1126/science.1063049
- Sirota, A., Csicsvari, J., Buhl, D., and Buzsáki, G. (2003). Communication between neocortex and hippocampus during sleep in rodents. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2065–2069. doi: 10.1073/pnas.0437938100
- Sitaraman, D., Aso, Y., Jin, X., Chen, N., Felix, M., Rubin, G. M., et al. (2015a). Propagation of Homeostatic Sleep Signals by Segregated Synaptic Microcircuits of the *Drosophila* Mushroom Body. *Curr. Biol.* 25, 2915–2927. doi: 10.1016/j.cub.2015.09.017
- Sitaraman, D., Aso, Y., Rubin, G. M., and Nitabach, M. N. (2015b). Control of sleep by dopaminergic inputs to the *Drosophila* mushroom body. *Front. Neural Circuits* 9:73. doi: 10.3389/fncir.2015.00073
- Srivastava, D. P., Yu, E. J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., et al. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *J. Neurosci.* 25, 6145–6155. doi: 10.1523/JNEUROSCI.1005-05.2005
- Steinberg, E. E., Keiflin, R., Boivin, J. R., Witten, I. B., Deisseroth, K., and Janak, P. H. (2013). A causal link between prediction errors, dopamine neurons and learning. *Nat. Neurosci.* 16, 966–973. doi: 10.1038/nn.3413
- Stuber, G. D., and Wise, R. A. (2016). Lateral hypothalamic circuits for feeding and reward. *Nat. Neurosci.* 19, 198–205. doi: 10.1038/nn.4220
- Szczypka, M. S., Kwok, K., Brot, M. D., Marck, B. T., Matsumoto, A. M., Donahue, B. A., et al. (2001). Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice. *Neuron* 30, 819–828. doi: 10.1016/S0896-6273(01)00319-1
- Takemura, S. Y., Aso, Y., Hige, T., Wong, A., Lu, Z., Xu, C. S., et al. (2017). A connectome of a learning and memory center in the adult *Drosophila* brain. *Elife* 6:e26975. doi: 10.7554/eLife.26975
- Tan, K. R., Yvon, C., Turiault, M., Julie Mirzabekov, J., Doeber, J., Labouèbe, G., et al. (2012). GABA Neurons of the VTA drive conditioned place aversion. *Neuron* 73, 1173–1183. doi: 10.1016/j.neuron.2012.02.015
- Taylor, N. E., Van Dort, C. J., Kenny, J. D., Pei, J., Guidera, J. A., Vlasov, K. Y., et al. (2016). Optogenetic activation of dopamine neurons in the ventral tegmental area induces reanimation from general anesthesia. *Proc. Natl. Acad. Sci. U.S.A.* 113, 12826–12831. doi: 10.1073/pnas.1614340113
- Thambi, N. C., Quan, F., Wolfgang, W. J., Spiegel, A., and Forte, M. (1989). Immunological and molecular characterization of Go alpha-like proteins in the *Drosophila* central nervous system. *J. Biol. Chem.* 264, 18552–18560.
- Tobler, I., and Borbély, A. A. (1986). Sleep EEG in the rat as a function of prior waking. *Electroencephalogr. Clin. Neurophysiol.* 64, 74–76. doi: 10.1016/0013-4694(86)90044-1
- Tsai, H.-C., Zhang, F., Adamantidis, A., Stuber, G. D., Bonci, A., Deisseroth, K., et al. (2009). Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. *Science* 324, 1080–1084. doi: 10.1126/science.1168878
- Tully, T., Preat, T., Boynton, S. C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79, 35–47. doi: 10.1016/0092-8674(94)90398-0
- Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., et al. (2012). Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat. Neurosci.* 15, 1516–1523. doi: 10.1038/nn.3238
- van Zessen, R., Phillips, J. L., Budygin, E. A., and Stuber, G. D. (2012). Activation of VTA GABA neurons disrupts reward consumption. *Neuron* 73, 1184–1194. doi: 10.1016/j.neuron.2012.02.016
- Vyazovskiy, V. V., Olcese, U., Lazimy, Y. M., Faraguna, U., Esser, S. K., Williams, J. C., et al. (2009). Cortical firing and sleep homeostasis. *Neuron* 63, 865–878. doi: 10.1016/j.neuron.2009.08.024
- Werth, E., Dijk, D. J., Achermann, P., and Borbély, A. A. (1996). Dynamics of the sleep EEG after an early evening nap: experimental data and simulations. *Am. J. Physiol.* 271, R501–R510.
- Wisor, J. P., Nishino, S., Sora, I., Uhl, G. H., Mignot, E., and Edgar, D. M. (2001). Dopaminergic role in stimulant-induced wakefulness. *J. Neurosci.* 21, 1787–1794.
- Yamagata, N., Hiroi, M., Kondo, S., Abe, A., and Tanimoto, H. (2016). Suppression of dopamine neurons mediates reward. *PLoS Biol.* 14:e1002586. doi: 10.1371/journal.pbio.1002586
- Yamagata, N., Ichinose, T., Aso, Y., Plačais, P.-Y., Friedrich, A. B., Tanimoto, H., et al. (2015). Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proc. Natl. Acad. Sci. U.S.A.* 112, 578–583. doi: 10.1073/pnas.1421930112
- Yamamoto, D., and Koganezawa, M. (2013). Genes and circuits of courtship behaviour in *Drosophila* males. *Nat. Rev. Neurosci.* 14, 681–692. doi: 10.1038/nrn3567
- Zhang, S. X., Rogulja, D., and Michael Crickmore, A. (2016). Dopaminergic circuitry underlying mating drive. *Neuron* 91, 168–181. doi: 10.1016/j.neuron.2016.05.020
- Zhou, Q. Y., and Palmiter, R. D. (1995). Dopamine-deficient mice are severely hypoactive, adipic, and aphagic. *Cell* 83, 1197–1209. doi: 10.1016/0092-8674(95)90145-0

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Biogenic Amines in Insect Antennae

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Insect antenna is a multisensory organ, each modality of which can be modulated by biogenic amines. Octopamine (OA) and its metabolic precursor tyramine (TA) affect activity of antennal olfactory receptor neurons. There is some evidence that dopamine (DA) modulates gustatory neurons. Serotonin can serve as a neurotransmitter in some afferent mechanosensory neurons and both as a neurotransmitter and neurohormone in efferent fibers targeted at the antennal vessel and mechanosensory organs. As a neurohormone, serotonin affects the generation of the transepithelial potential by sensillar accessory cells. Other possible targets of biogenic amines in insect antennae are hygro- and thermosensory neurons and epithelial cells. We suggest that the insect antenna is partially autonomous in the sense that biologically active substances entering its hemolymph may exert their effects and be cleared from this compartment without affecting other body parts.

Keywords: insect, antenna, sensory plasticity, octopamine, tyramine, serotonin, dopamine

INTRODUCTION

Insect antennae are complex sensory appendages engaged in acquiring information from different mechanical, gustatory and olfactory as well as thermal and humidity cues (Altner et al., 1977). The antenna consists of two basal segments having muscles, which control antennal movements, and flagellum devoid of muscles but bearing sensilla, miniature sensory organs. The antenna is supplied by oxygen through the trachea, originating from the spiracles which are positioned laterally in the thoracic and abdominal segments (Newport, 1836; Yadav, 2003). Haemolymph flows through the antennal vessel pumped by the antennal heart, a circulatory organ found in a handful of insect species (Pass, 2000; Pass et al., 2006). The proximal part of the antennal vessel shows features of the ion-transporting function (Pawlowska, 1895). Hemolymph spills from the vessel into the antennal hemolymphatic space through the openings in the vessel walls, called ostia, and the distal pore (Kapitskii, 1984; Pass et al., 2006; Boppana and Hillyer, 2014). Hormones and other biologically active substances are delivered to the antennal lumen from the two main sources—body hemolymph and secretion from nerve terminals in the wall of the antennal heart (Beattie, 1976; **Figure 1**). No centrifugal axonal processes were found in the antennal flagella other than those coming from tachykinin-reactive cells in the mosquito *Culex salinarius*, which form axo-dendritic synapses with sensory neurons (Meola and Sittertz-Bhatkar, 2002). The neurohemal area in the antennal heart ampulla of the cockroach *Periplaneta americana* releases octopamine (OA) into the antennal hemolymph under control of dorsal unpaired median (DUM) neurons, originating from the subesophageal ganglion (Pass et al., 1988). The possible targets for OA are: (1) sensory receptor organs—sensilla, tuned to olfactory, gustatory and various mechanical cues as well as to humidity and temperature; and (2) non-sensory tissues including the ion-transporting epithelium in the antennal vessel (Pass, 1985) and hypoderm (**Figure 2**). Considerable length of antennae suggests the possibility of mainly local humoral modulation, since active substances contained in

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the hemolymph have enough time to exert their effect and be cleared out before returning to the head and body hemocoel (**Figure 1**).

The tremendous ability of insects to prosper owes, in part, their behavioral plasticity in response to environmental cues. Perception of pheromones and non-pheromone odors by an insect changes depending on various factors, such as age (Takasu and Lewis, 1996; Mechaber et al., 2002; Bohbot et al., 2013), physiological state (Blaney et al., 1986; Anton et al., 2007; Evenden and Gries, 2008), previous experience (Anton et al., 2011; Minoli et al., 2012; Riffell and Hildebrand, 2016), sensory surrounding (Yang et al., 2004; Zhao et al., 2013; Deisig et al., 2014) and circadian rhythmicity (Linn and Roelofs, 1986; Saifullah and Page, 2009; Schendzielorz et al., 2012).

During a long time, plasticity of behavioral responses was attributed to neuromodulation in the central nervous system rather than in antennal sensillae (Roelofs, 1995). However, after the study by von Nickisch-Rosenegk et al. (1996), showing the presence of biogenic amine receptors in the moth antenna, it is accepted now that all levels, from sensory input to motor output, involved in the organization of olfaction-guided behavior are under neurohumoral control (Anton et al., 2007).

All receptors of biogenic amines revealed in insects thus far represent membrane-bound G protein-coupled receptors

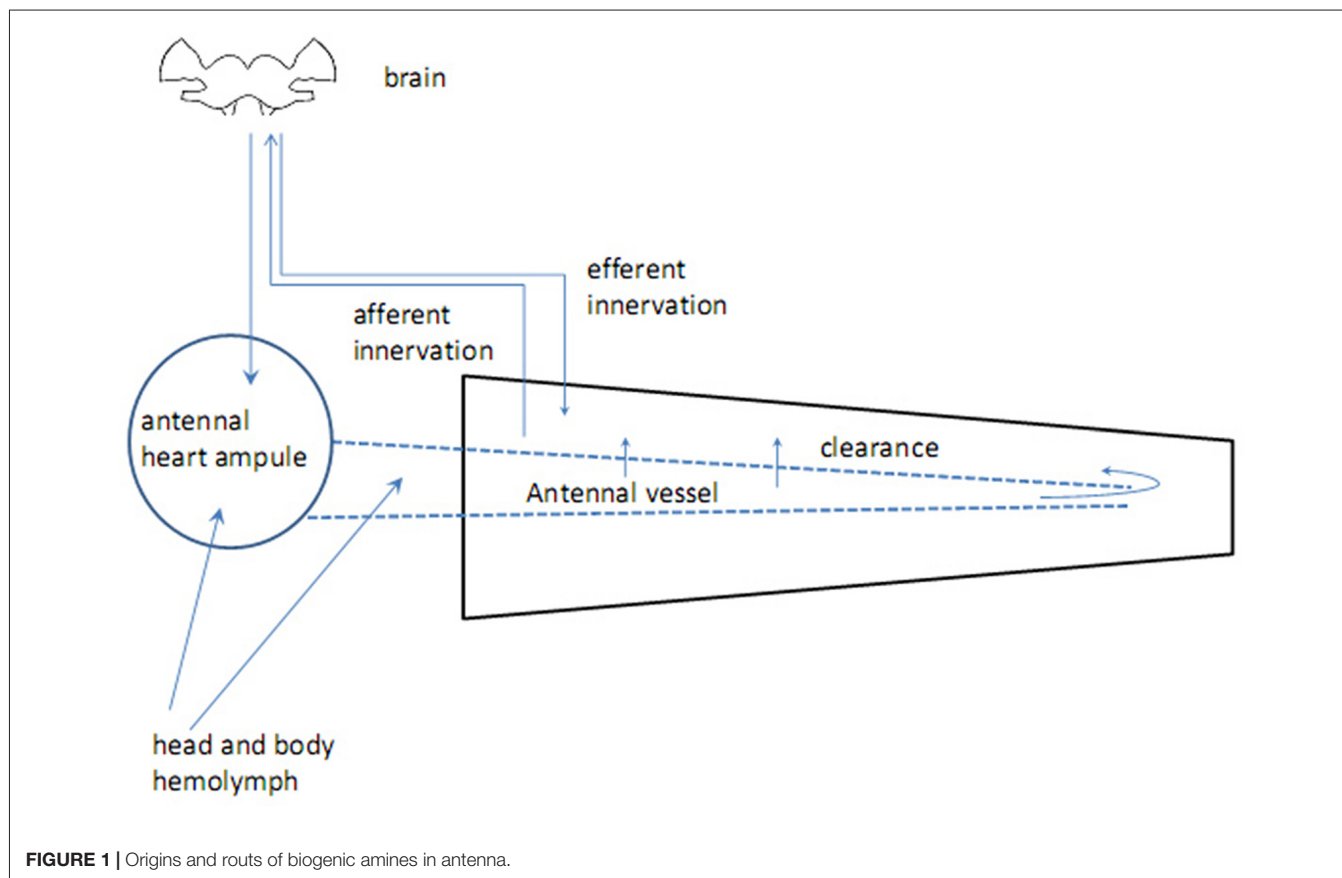
(GPCRs), triggering different signaling cascades, which lead to a rise or fall in the cAMP level and Ca^{2+} release (Blenau and Baumann, 2001; Beggs et al., 2011; Ohta and Ozoe, 2014; Vleugels et al., 2015). The cross-talk between their signaling pathways and the intracellular biochemical machinery in antennal tissues is attracting attention of researchers (Flecke and Stengl, 2009; Flecke et al., 2010; Chen and Luetje, 2014), but is not studied in detail.

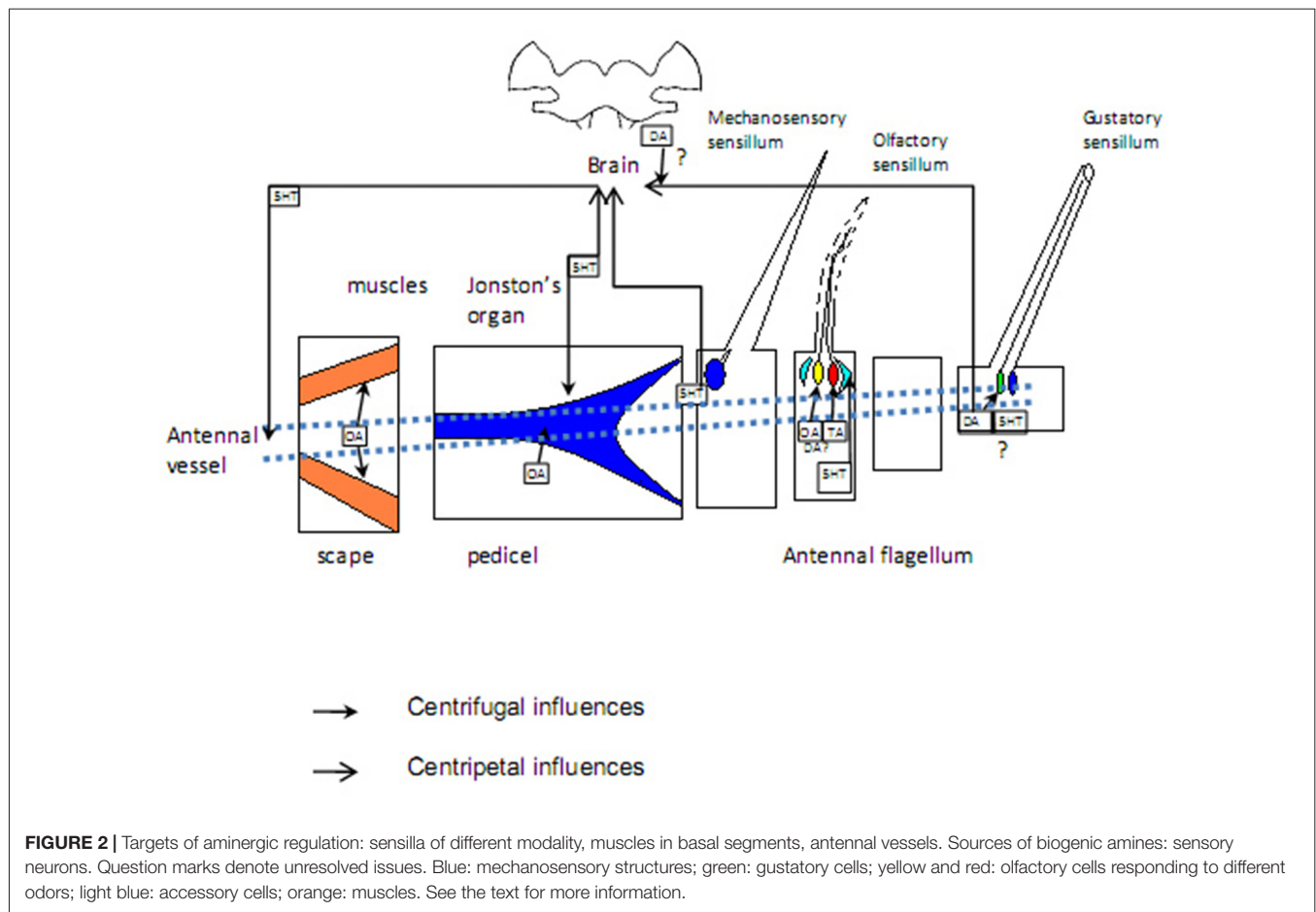
This review addresses those peripheral effects of biogenic amines—OA, tyramine (TA), serotonin (5-HT), and dopamine (DA)—that are confined to the antenna, the major sensory organ in insects.

OCTOPAMINE

Olfactory Reception

OA, topically applied or injected into an insect body, evokes pronounced changes in olfactory responses, both behavioral (Linn and Roelofs, 1986; Linn et al., 1992; Zhukovskaya, 2008) and electrophysiological (Pophof, 2000, 2002; Grosmaître et al., 2001; Kapitsky and Zhukovskaya, 2001; Zhukovskaya and Kapitsky, 2006; Flecke and Stengl, 2009; Zhukovskaya, 2012). Besides, OA increases the spontaneous activity of pheromone-sensitive olfactory receptor neurons (ORNs) in some insect preparations (Grosmaître et al., 2001; Zhukovskaya and Kapitsky, 2006; Flecke and Stengl, 2009;





Stengl, 2010). In general, OA enhances behavioral responses to attractants (Linn and Roelofs, 1986; Zhukovskaya, 2008; Ma et al., 2015), improves nestmate recognition in ants (Vander Meer et al., 2008), and sometimes changes the valence of an odor, making neutral odor attractive and repellent odor neutral, as shown previously (Zhukovskaya, 2012).

Although these data shed some light on the role of octopaminergic regulation in insect olfaction, two issues were left unresolved. First, endogenous OA release can be induced by experimental manipulations. For example, handling alone evokes a 3-fold rise in the OA level in the *P. americana* antennal heart, which supplies the antenna with hemolymph (Möbius and Penzlin, 1993). Besides, injections of agonists as well as antagonists of OA and TA induce a similar rise in displacement grooming suggested to be a deactivating behavior due to stress-induced changes in the OA level (Fussnecker et al., 2006). Second, response modulation, usually attributed to a direct effect of OA, may result from the indirect effect of other biologically active molecules released by OA. Outside the antennae, OA significantly changes the production of juvenile hormone and 20-hydroxyecdysone in cockroaches and flies (Downer et al., 1984; Gruntenko et al., 2007) and adipokinetic hormone

in crickets (Orchard et al., 1983), which affect multiple targets throughout the insect body.

Upregulation of single cell olfactory responses coincides with an OA-induced decrease in the electroantennogram (EAG) amplitude. However, it is noteworthy that changes in EAG under the influence of OA and other humoral factors should be interpreted with caution, since EAG is a complex signal rather than just a voltage drop caused by arithmetical summation of leak currents from ORNs. EAG strongly depends on the electrical resistance of the antennal tissue as well as on the position of recording electrodes relative to the responding sensilla (Nagai, 1981; White, 1991; Kapitskii and Gribakin, 1992). Data reported by Dolzer et al. (2001) showed a dose-dependent decrease in the resistance of the sensillum preparation in the moth *Manduca sexta* under the impact of OA. The simultaneous OA-induced decrease in the EAG amplitude and increase in the action potential frequency led us to suggest that OA causes a depolarization in the ORN resting potential (Kapitsky and Zhukovskaya, 2001). As a result, the reduced ORN membrane potential requires smaller changes to reach the threshold potential level, at which action potentials are triggered, creating smaller currents recorded as EAG after the summation of these miniature leaks from responding ORNs along the antennal length. At the same time, the depolarized membrane is less stable,

triggering more spikes in response to the odor stimulus. Recently, this concept has gained further support (Flecke and Stengl, 2009; Stengl, 2010).

Taking the advantage of the fact that the antennal flagellum is devoid of OA-producing structures, we performed single sensillum recording from the preparations of the isolated cockroach antennal flagellum (Kapitsky and Zhukovskaya, 2001; Zhukovskaya and Kapitsky, 2006) perfused with OA and OA-free saline. This approach allowed us both to administer and wash out OA as well as to control other hemolymph-born substances that bathe proximal parts of ORNs. The increase in the spiking rate in sex-specific male cockroach sensilla in response to pheromone and its background activity proved unequivocally that response modulation in the pheromone-sensitive ORNs results from the direct effect of OA. This finding was later supported in the other cockroach species, *Rhyarobia (Leucophaea) maderae* (Schendzielorz et al., 2012), using perfused sensillum preparations. OA-dependent local release of tachykinin inside the sensillum, as suggested by Jung et al. (2013), provides further downstream regulation of cells in the olfactory sensilla, possibly affecting EAG responses to odors.

Peripheral responses to general odorants may or may not be under OA control (Pophof, 2002; Zhukovskaya, 2012). Our data suggest that some of the hexanol-1-sensitive sensilla, morphologically and physiologically differing from the sex-pheromone sensitive ones (Schaller, 1978; Fujimura et al., 1991), are under OA control in adult *P. americana* males, while other sensilla are not affected (Zhukovskaya, 2012). To ascertain if the olfactory sensillum is modulated by OA via its effect on accessory cells, which partially create a driving force for the receptor current in response to odorants (Kaissling, 1987) and control the composition of the sensillum lymph (Thurm and Küppers, 1980; Keil, 1999), or each ORN is affected independently, we took the advantage of the fact that *P. americana* pheromone-sensitive sensilla houses both pheromone-sensitive and general odorant-sensitive “eucalyptol” cells. OA application enhanced firing responses of this type of sensilla to both pheromone components, periplanones A and B, but did not affect responses to eucalyptol (Zhukovskaya and Kapitsky, 2006; Zhukovskaya, 2012). Thus, in contrast to the cells responding to pheromone components and controlled by OA, the cell responding to general odorant is not OA-controlled. These data provide evidence that receptor cells inside the same sensillum, at least in some cases, are controlled independently via biogenic amine receptors on the ORN membrane. It is important to note that all the tested odorants, namely, pheromone components and plant-derived odorants, eucalyptol and hexanol, showed a decrease in EAG under the effect of OA. We did not detect significant changes in firing responses to eucalyptol, but cannot rule out that other receptor cells in other types of sensilla respond to this odor differently in the presence or absence of OA. Another possible explanation of the uniform EAG decrease under the effect of OA in response to all tested odors is a change in the electrical resistance of non-sensory antennal tissues, such as hemolymph, epithelium or cuticle, which contributes to the cumulative resistance of the antennal preparation. It appears that OA release into antennal hemolymph switches the mode of its

functioning, altering the antennal sensitivity to a particular set of pheromone components and environmental odors in order to better conform the specific needs of the animal.

Coupling of olfactory sensitivity modulation by OA with circadian rhythmicity was initially found in the cabbage looper moth *Trichoplusia ni* (Linn and Roelofs, 1986; Linn et al., 1992). Later, OA modulation of olfactory sensitivity in the antenna of *M. sexta* was found to be linked to the circadian rhythmicity in pheromone reception through cAMP-dependent disadaptation in receptor cells (Flecke and Stengl, 2009; Flecke et al., 2010; Stengl, 2010). The antennal OA receptor cloned in the *M. sexta* shares high sequence similarity with other insect α -adrenergic-like OA receptors and increases both cAMP and Ca^{2+} intracellular concentration in response to an agonist (Dacks et al., 2006). Ca^{2+} and cAMP levels altered due to OA-induced signal transduction are supposed to act through metabotropic activation of Orco, an odorant receptor coreceptor protein, leading to changes in ORN sensitivity (Getahun et al., 2013; Stengl and Funk, 2013).

Octopaminergic modulation in the antenna can be enhanced not only by the elevation of the OA level, but also by upregulation of OA receptors in the antenna of honey bee workers, as shown using the Real-time qPCR technique (McQuillan et al., 2012). Thus, expression of OA receptors (AmOA1) in the antenna was found to be higher in young nurses as compared to pollen foragers of the same age, corresponding to sensitivity to queen mandibular pheromone (QMP). Foragers in the bee colony are not attracted by QMP while the expression level of antennal AmOA1 is low (Vergoz et al., 2009). AmOA1 also belongs to the α -adrenergic-like OA receptor family that induces an oscillatory increase in the intracellular Ca^{2+} concentration under OA stimulation but only a slight elevation of the cAMP level (Grohmann et al., 2003).

Other Targets of Octopamine in Insect Antenna

Muscles situated in the first two antennal segments, scape and pedicel (Chapman, 1998), control movements of the flagellum through motoneurons and modulating neurons, including octopaminergic DUM neurons, descending from the suboesophageal ganglion (Bräunig et al., 1990; Bauer and Gewecke, 1991; Baba and Comer, 2008; **Figure 2**). Stimulation of these cells as well as OA application attenuates slow and enhances fast contractions in cricket preparations (Allgäuer and Honegger, 1993), facilitating fast antennal movements during tracking a target.

The mechanosensory Johnston’s organ, responding to vibrations and low frequency sounds, was recently found to be modulated by OA, which shifts frequency tuning and is likely to allow mosquito males to track females by following their changing flight sound tones due to movement (Andrés et al., 2016). Interestingly, despite the fact that OA plays an important role in arousal and aggression, the threshold for mechanical stimulation of antennae, causing an aggressive response in male crickets, does not depend on OA (Rillich and Stevenson, 2015; Stevenson and Rillich, 2016). Thus, central rather than peripheral

mechanosensory octopaminergic modulation is responsible for adjusting the level of aggression in response to stimulation of antennal mechanosensitive sensilla.

TYRAMINE

TA is, on the one hand, an OA biosynthetic precursor, but on the other hand, it plays a distinctive role in an insect body. Since there were identified some TA-containing neurons devoid of OA, the specific role of TA as a neuroactive compound became evident (Nagaya et al., 2002). In fact, TA and OA are believed to be, in a sense, functionally antagonistic (Roeder et al., 2003; Roeder, 2005; Lange, 2009). For example, in contrast to attractive (pheromone) odors modulated by OA, behavioral responses to aversive (non-pheromone) odors are affected by TA because they were decreased in *hono*, the *Drosophila melanogaster* TA (TA/OA) receptor knockout (Kutsukake et al., 2000). However, it is preliminary to conclude that OA modulates pheromone-sensitive ORNs while TA affects general odorant-sensitive ORNs, because our above data on OA-upregulated responses to the non-pheromone repellent odor of hexanol (Zhukovskaya, 2008, 2012) indicate that responses to general odorants can be regulated by OA. It is also unlikely that there is a strict division of functions, when TA modulates responses to repellents whereas OA modulates attractants, because the valence of a particular odor can be changed by learning and other experience-based effects (McCall and Eaton, 2001; Saleh and Chittka, 2006; Anderson and Anton, 2014). X-gal staining of the *hono* gene product revealed about 10 most probable ORN candidates in the third antennal segment, the main olfactory organ in an adult fly, as well as in the larval dorsal olfactory organ. Although first lepidopteran OAR/TAR, identified in *B. mori* and *H. virescens*, were thought to be OAR (von Nickisch-Roseneck et al., 1996), it has been demonstrated later that at least in *B. mori* OAR/TAR is two orders of magnitude more sensitive to TA than to OA and shows much higher affinity (by about 270 times) to TA than OA, representing, in fact, a TA receptor. TA activation of this receptor leads to G_i protein-mediated inactivation of adenylate cyclase and a reduction in intracellular cAMP levels (Ohta et al., 2003, 2004).

In honey bee antennae, downregulation of OA receptors Amoal and upregulation of TA receptors AmTAR1 were revealed during transition from QMP-sensitive nurses to plant odor-sensitive foragers as detected by a real-time quantitative PCR technique (McQuillan et al., 2012). Another TA receptor from the honey bee, AmTAR2, was shown to increase the intracellular cAMP level in the flpTM heterologous expression system (Reim et al., 2017), but so far is not found in insect antennae.

In the adult *M. brassicae*, TA receptor (MbOAR/TAR) transcripts were detected both in pheromone- and general odor-sensitive antennal sensilla (Brigaud et al., 2009). TA not only affects ORNs in insect antennae, but itself can be synthesized by some of them (Figures 1, 2). Presumably, in the blowfly *Phormia regina* it acts in the antennal lobe neuropile through modulation of responses to aversive odor of d-limonene (Ishida and Ozaki, 2012). Since TA receptors mostly decrease while

OA receptors increase the cAMP level (Ohta and Ozoe, 2014), their effects on cAMP-dependent intracellular events should be mutually opposite, but whether these receptors co-localize in the same cell is an open question.

Tryptamine, produced in plants as their defense reaction against insect herbivores, was found to be antagonistic to olfactory co-receptor Orco in the low micromolar range (Chen and Luetje, 2014), probably interacting with TA or OA binding sites.

SEROTONIN

It is generally accepted that in insect antennal ORNs, the role of neurotransmitter is played by acetylcholine, although there are a few pieces of evidence deviating from this tenet. Serotonin-immunoreactive fibers were identified in the antennal nerve of *P. americana* (Salecker and Distler, 1990), projecting into antennal mechanosensory and contact chemosensory centers mainly in the deutocerebrum. Later, cell bodies of these sensory neurons were found in mechanosensory chaetic and scolopoidal sensilla in the scape, pedicel and first 15 flagellomeres. Moreover, efferent fibers were found within the scape, ramifying along the antennal vessel and inner margin of the epidermal layer without contacting them synaptically (Watanabe et al., 2014). Serotonergic efferent fibers have also been identified in mosquito antennae, where they are targeted at the antennal flagellum and scolopidia of the Johnston's organ (Siju et al., 2008; Andrés et al., 2016). Transcriptomic analysis revealed few putative 5-HT receptor proteins in the antennae of the mosquito *Anopheles gambiae* (Pitts et al., 2011), supporting the role of 5-HT as a neurohormone. 5-HT affects the transepithelial potential, generated by accessory cells in the olfactory sensillum and creating a driving force for the receptor current (Dolzer et al., 2001; Grosmaître et al., 2001).

The direct effect of 5-HT on firing responses in the blowfly *Phormia regina* labellar gustatory receptor neurons during the specific stage of their ovarian maturation period indicates a peripheral modulation of gustatory receptor neurons. Exogenous 5-HT supply specifically increases the chemoreceptor sensitivity to sugar at the mature ovaries and post egg-laying stages (Solari et al., 2015). However, it is not clear whether antennal gustatory neurons are serotonin-modulated or the effect of 5-HT is labellum-specific and this issue should be a matter of future research (Figure 2).

DOPAMINE

Olfactory Reception

There are indications that DA can serve as a neurohormone, modulating odor responses. Expression of the DA receptor Amdop3 in the honey bee antenna was found to correlate with an age-dependent decrease in sensitivity of honey bee workers to the QMP component (Vergoz et al., 2009; McQuillan et al., 2012). The age-dependent decrease in pheromone sensitivity in the male black cutworm *Agrotis ipsilon* is also thought to be associated with DA signaling via the G protein-coupled DA/ecdyteroid receptor AipsDopEc, however, this effect is attributed to the brain

level, since antennal expression was low and age-independent (Abrieux et al., 2013).

Gustatory Reception

Most data on gustatory reception were obtained on flies, which bear short antennae unable to touch the substrate to perform gustatory function, whereas flies taste food using sensilla located on the labella. Gustatory plasticity, similarly to above-described olfactory plasticity, is achieved at different levels of sensory processing. Starvation (nutritional stress) causes changes in the sugar-sensitive gustatory receptor on the fly labellar sensilla due to increased expression of the Gr64a receptor gene (Nishimura et al., 2012). Since responses to nutritional stress in flies are accompanied by changes in biogenic amine levels (Gruntenko et al., 2005), it was logical to look for dopaminergic regulation in gustatory receptor neurons. DA receptors were found to enhance sucrose sensitivity under starvation in *Drosophila* sucrose-sensitive gustatory receptor cells (Inagaki et al., 2012). At the same time, bitter sensitive neurons decrease their output during OA and TA modulation (Inagaki et al., 2014; LeDue et al., 2016). In both cases, however, modulation occurs presynaptically on axonal terminals, projecting from the fly labellum to primary gustatory neuropile of the suboesophageal ganglion.

A majority of insects other than flies bear gustatory sensilla on antennae and are likely to use similar dopaminergic regulatory mechanisms. For example, unpaired H-cells with their bodies located in the suboesophageal ganglion of moths and orthopterans (Mesce et al., 2001) release DA that can be transported to antennae (Galizia and Rössler, 2010).

DA receptors were demonstrated to be expressed in honey bee antennae; moreover, changes in the expression level of one of them, Amdop1, corresponded to the transition from nursing to foraging (McQuillan et al., 2012). Since QMP contains some non-volatile components, DA appears to be a plausible modulator candidate in pheromone-sensing gustatory receptor neurons (Figure 2).

Other Possible Targets of Aminergic Modulation in Antenna

The outer layer of the cuticle bears waxes or liquid cuticular hydrocarbons, the repertoire of which may be body part specific (Oppelt and Heinze, 2009; Bagnères and Blomquist, 2010). Our data suggest that the liquid coating of the cockroach antenna plays an important role in olfaction (Böröczky et al., 2013), providing, in concert with grooming, odorant cleanout from the antennal surface. It can be hypothesized that the cuticular lipid secretion is modulated via the neurohumoral (probably, aminergic) mechanism. Thermosensory neurons may

also be modulated by the hemolymph-born molecules. No direct measurements of thermosensory neuronal responses in the presence of biogenic amines have been found in literature, but some clues on the possibility of the modulation can be found. For example, in the blood-feeding yellow fever mosquito *Aedes aegypti*, long-range perception of CO₂ changes behavioral responses to a short-range thermal signal (McMeniman et al., 2014). The satiety level, interrelated with the 5-HT titer (Lange et al., 1989), influences the response to heat in the bug *Rhodnius prolixus* (Bodin et al., 2009). Previous experience changes behavioral responses to thermal stimulation in the worker ant *Camponotus rufipes* (Weidenmüller et al., 2009) and bumblebee *Bombus terrestris* (Westhus et al., 2013) in a way similar to that described for olfactory reception.

CONCLUSIONS

The insect antenna is a multisensory organ, and each modality can be modulated by biogenic amines. It appears that insect antenna is partially autonomous in the sense that biologically active substances entering its hemolymph may exert their specific effects and be removed predominantly or even totally inside this compartment without affecting other body parts. OA increases activity of pheromone- and some, but not all, non-pheromone-sensitive antennal ORNs. There is some evidence that DA modulates gustatory receptor neurons. TA, a metabolic OA precursor, also modulates ORNs, usually in an antagonistic manner to OA, but it is unclear if TA and OA receptors are co-localized in receptor neurons. Serotonin can serve as a neurotransmitter in some afferent mechanosensory neurons and both as a neurotransmitter and neurohormone in efferent fibers targeted at the antennal vessel and mechanosensory organs. Aminergic modulation of thermo- and hygro-sensory sensilla has not yet been demonstrated, and could potentially be another target for modulation. Functioning of non-sensory antennal tissues in the epithelium, tracheae and hemolymphic vessel may also be under humoral control, including aminergic.

AUTHOR CONTRIBUTIONS

MIZ, ADP: manuscript planning, MIZ: draft writing, ADP: editing.

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REFERENCES

- Abrieux, A., Debernard, S., Maria, A., Gaertner, C., Anton, S., Gadenne, C., et al. (2013). Involvement of the G-protein-coupled dopamine/ecdyteroid receptor DopEcR in the behavioral response to sex pheromone in an insect. *PLoS One* 8:e72785. doi: 10.1371/journal.pone.0072785
- Allgäuer, C., and Honegger, H. W. (1993). The antennal motor system of crickets: modulation of muscle contractions by a common inhibitor, DUM neurons, and proctolin. *J. Comp. Physiol. A* 173, 485–494. doi: 10.1007/bf00193521
- Altner, H., Sass, H., and Altner, I. (1977). Relationship between structure and function of antennal chemoreceptive, hygroreceptive, and thermoreceptive

- sensilla in *Periplaneta americana*. *Cell Tissue Res.* 176, 389–405. doi: 10.1007/bf00221796
- Anderson, P., and Anton, S. (2014). Experience-based modulation of behavioural responses to plant volatiles and other sensory cues in insect herbivores. *Plant Cell Environ.* 37, 1826–1835. doi: 10.1111/pce.12342
- Andrés, M., Seifert, M., Spalthoff, C., Warren, B., Weiss, L., Giraldo, D., et al. (2016). Auditory efferent system modulates mosquito hearing. *Curr. Biol.* 26, 2028–2036. doi: 10.1016/j.cub.2016.05.077
- Anton, S., Dufour, M. C., and Gadenne, C. (2007). Plasticity of olfactored-guided behaviour and its neurobiological basis: lessons from moths and locusts. *Entomol. Exp. Appl.* 123, 1–11. doi: 10.1111/j.1570-7458.2007.00516.x
- Anton, S., Evengård, K., Barrozo, R. B., Anderson, P., and Skals, N. (2011). Brief predator sound exposure elicits behavioral and neuronal long-term sensitization in the olfactory system of an insect. *Proc. Natl. Acad. Sci. U S A* 108, 3401–3405. doi: 10.1073/pnas.1008840108
- Baba, Y., and Comer, C. M. (2008). Antennal motor system of the cockroach, *Periplaneta americana*. *Cell Tissue Res.* 331, 751–762. doi: 10.1007/s00441-007-0545-9
- Bagnères, A.-G., and Blomquist, G. J. (2010). “Site of synthesis, mechanism of transport and selective deposition of hydrocarbons,” in *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*, eds G. J. Blomquist and A.-G. Bagnères (Cambridge, MA: Cambridge University Press), 75–99.
- Bauer, C. K., and Gewecke, M. (1991). Motoneuronal control of antennal muscles in *Locusta migratoria*. *J. Insect Physiol.* 37, 551–562. doi: 10.1016/0022-1910(91)90032-u
- Beattie, T. M. (1976). Autolysis in axon terminals of a new neurohaemal organ in the cockroach *Periplaneta americana*. *Tissue Cell* 8, 305–310. doi: 10.1016/0040-8166(76)90054-9
- Beggs, K. T., Tyndall, J. D. A., and Mercer, A. R. (2011). Honey bee dopamine and octopamine receptors linked to intracellular calcium signaling have a close phylogenetic and pharmacological relationship. *PLoS One* 6:e26809. doi: 10.1371/journal.pone.0026809
- Blaney, W. M., Schoonhoven, L. M., and Simmonds, M. S. J. (1986). Sensitivity variations in insect chemoreceptors; a review. *Experientia* 42, 13–19. doi: 10.1007/bf01975876
- Blenau, W., and Baumann, A. (2001). Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* 48, 13–38. doi: 10.1002/arch.1055
- Bodin, A., Vinauger, C., and Lazzari, C. R. (2009). Behavioural and physiological state dependency of host seeking in the blood-sucking insect *Rhodnius prolixus*. *J. Exp. Biol.* 212, 2386–2393. doi: 10.1242/jeb.030668
- Bohbot, J. D., Durand, N. F., Vinyard, B. T., and Dickens, J. C. (2013). Functional development of the octenol response in *Aedes aegypti*. *Front. Physiol.* 4:39. doi: 10.3389/fphys.2013.00039
- Boppana, S., and Hillyer, J. F. (2014). Hemolymph circulation in insect sensory appendages: functional mechanics of antennal accessory pulsatile organs (auxiliary hearts) in the mosquito *Anopheles gambiae*. *J. Exp. Biol.* 217, 3006–3014. doi: 10.1242/jeb.106708
- Böröczky, K., Wada-Katsumata, A., Batchelor, D., Zhukovskaya, M., and Schal, C. (2013). Insects groom their antennae to enhance olfactory acuity. *Proc. Natl. Acad. Sci. U S A* 110, 3615–3620. doi: 10.1073/pnas.1212466110
- Bräunig, P., Allgäuer, C., and Honegger, H. W. (1990). Suboesophageal DUM neurones are part of the antennal motor system of locusts and crickets. *Experientia* 46, 259–261. doi: 10.1007/bf01951758
- Brigaud, I., Grosmaître, X., François, M. C., and Jacquin-Joly, E. (2009). Cloning and expression pattern of a putative octopamine/tyramine receptor in antennae of the noctuid moth *Mamestra brassicae*. *Cell Tissue Res.* 335, 455–463. doi: 10.1007/s00441-008-0722-5
- Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge, MA: Cambridge University Press.
- Chen, S., and Luetje, C. W. (2014). Trace amines inhibit insect odorant receptor function through antagonism of the co-receptor subunit. *F1000Res.* 3:84. doi: 10.12688/f1000research.3825.1
- Dacks, A. M., Dacks, J. B., Christensen, T. A., and Nighorn, A. J. (2006). The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 36, 741–747. doi: 10.1016/j.ibmb.2006.07.002
- Deisig, N., Dupuy, F., Anton, S., and Renou, M. (2014). Responses to pheromones in a complex odor world: sensory processing and behavior. *Insects* 5, 399–422. doi: 10.3390/insects5020399
- Dolzer, J., Krannich, S., Fischer, K., and Stengl, M. (2001). Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* 204, 2781–2794.
- Downer, R. G. H., Orr, G. L., Gole, J. W. D., and Orchard, I. (1984). The role of octopamine and cyclic AMP in regulating hormone release from corpora cardiaca of the American cockroach. *J. Insect Physiol.* 30, 457–462. doi: 10.1016/0022-1910(84)90025-8
- Evenenden, M. L., and Gries, R. (2008). Plasticity of male response to sex pheromone depends on physiological state in a long-lived moth. *Anim. Behav.* 75, 663–672. doi: 10.1016/j.anbehav.2007.07.020
- Flecke, C., Nolte, A., and Stengl, M. (2010). Perfusion with cAMP analog affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. *J. Exp. Biol.* 213, 842–852. doi: 10.1242/jeb.032839
- Flecke, C., and Stengl, M. (2009). Octopamine and tyramine sensitize pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a daytime-dependent manner. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 195, 529–545. doi: 10.1007/s00359-009-0429-4
- Fujimura, K., Yokohari, F., and Tateda, H. (1991). Classification of antennal olfactory receptors of the cockroach, *Periplaneta americana* L. *Zool. Sci.* 8, 243–255.
- Fussnecker, B. L., Smith, B. H., and Mustard, J. A. (2006). Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (*Apis mellifera*). *J. Insect Physiol.* 52, 1083–1092. doi: 10.1016/j.jinsphys.2006.07.008
- Galizia, C. G., and Rössler, W. (2010). Parallel olfactory systems in insects: anatomy and function. *Annu. Rev. Entomol.* 55, 399–420. doi: 10.1146/annurev-ento-112408-085442
- Getahun, M. N., Olsson, S. B., Lavista-Llanos, S., Hansson, B. S., and Wicher, D. (2013). Insect odorant response sensitivity is tuned by metabotropically autoregulated olfactory receptors. *PLoS One* 8:e58889. doi: 10.1371/journal.pone.0058889
- Grohmann, L., Blenau, W., Erber, J., Ebert, P. R., Strünker, T., and Baumann, A. (2003). Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J. Neurochem.* 86, 725–735. doi: 10.1046/j.1471-4159.2003.01876.x
- Grosmaître, X., Marion-Poll, F., and Renou, M. (2001). Biogenic amines modulate olfactory neurons firing activity in *Mamestra brassicae*. *Chem. Senses* 26, 653–661. doi: 10.1093/chemse/26.6.653
- Gruntenko, N. E., Karpova, E. K., Alekseev, A. A., Chentsova, N. A., Bogomolova, E. V., Bownes, M., et al. (2007). Effects of octopamine on reproduction, juvenile hormone metabolism, dopamine, and 20-hydroxyecdysone contents in *Drosophila*. *Arch. Insect Biochem. Physiol.* 65, 85–94. doi: 10.1002/arch.20187
- Gruntenko, N. E., Karpova, E. K., Adonyeva, N. V., Chentsova, N. A., Faddeeva, N. V., Alekseev, A. A., et al. (2005). Juvenile hormone, 20-hydroxyecdysone and dopamine interaction in *Drosophila virilis* reproduction under normal and nutritional stress conditions. *J. Insect Physiol.* 51, 417–425. doi: 10.1016/j.jinsphys.2005.01.007
- Inagaki, H. K., Ben-Tabou de-Leon, S., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., et al. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595. doi: 10.1016/j.cell.2011.12.022
- Inagaki, H. K., Panse, K. M., and Anderson, D. J. (2014). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in *Drosophila*. *Neuron* 84, 806–820. doi: 10.1016/j.neuron.2014.09.032
- Ishida, Y., and Ozaki, M. (2012). Aversive odorant causing appetite decrease downregulates tyrosine decarboxylase gene expression in the olfactory receptor neuron of the blowfly, *Phormia regina*. *Naturwissenschaften* 99, 71–75. doi: 10.1007/s00114-011-0865-1
- Jung, J. W., Kim, J. H., Pfeiffer, R., Ahn, Y.-J., Page, T. L., and Kwon, H. W. (2013). Neuromodulation of olfactory sensitivity in the peripheral olfactory organs of the American cockroach, *Periplaneta americana*. *PLoS One* 8:e81361. doi: 10.1371/journal.pone.0081361
- Kaissling, K.-E. (1987). *R.H. Wright Lectures on Insect Olfaction*. Burnaby, BC: Simon Fraser University.

- Kapitskii, S. V. (1984). Morphology of the antenna of the male American cockroach *Periplaneta americana*. *Zh. Evol. Biokhim. Fizio.* 20, 79–86.
- Kapitskii, S. V., and Gribakin, F. G. (1992). Electroantennogram of the American cockroach: effect of oxygen and electrical model. *J. Comp. Physiol. A* 170, 651–663. doi: 10.1007/bf00199341
- Kapitsky, S. V., and Zhukovskaya, M. I. (2001). Sensitivity modulation in sex pheromone reception in male American cockroach *Periplaneta americana* L.: octopamine modifies sensillar firing response. *Sens. Syst.* 15, 147–154.
- Keil, T. A. (1999). “Morphology and development of the peripheral olfactory organs,” in *Insect Olfaction*, ed. B. S. Hansson (Heidelberg: Springer), 5–47.
- Kutsukake, M., Komatsu, A., Yamamoto, D., and Ishiwa-Chigusa, S. (2000). A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. *Gene* 245, 31–42. doi: 10.1016/s0378-1119(99)00569-7
- Lange, A. B. (2009). Tyramine: from octopamine precursor to neuroactive chemical in insects. *Gen. Comp. Endocrinol.* 162, 18–26. doi: 10.1016/j.ygcen.2008.05.021
- Lange, A. B., Orchard, L., and Barrett, F. M. (1989). Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, *Rhodnius prolixus*. *J. Insect Physiol.* 35, 393–399. doi: 10.1016/0022-1910(89)90113-3
- LeDue, E. E., Mann, K., Koch, E., Chu, B., Dakin, R., and Gordon, M. D. (2016). Starvation-induced depotentiation of bitter taste in *Drosophila*. *Curr. Biol.* 26, 2854–2861. doi: 10.1016/j.cub.2016.08.028
- Linn, C. E., Campbell, M. G., and Roelofs, W. L. (1992). Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 20, 265–284. doi: 10.1002/arch.940200404
- Linn, C. E., and Roelofs, W. L. (1986). Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 3, 161–171. doi: 10.1002/arch.940030206
- Ma, Z. Y., Guo, X. J., Lei, H., Li, T., Hao, S. G., and Kang, L. (2015). Octopamine and tyramine respectively regulate attractive and repulsive behavior in locust phase changes. *Sci. Rep.* 5:8036. doi: 10.1038/srep08036
- McCall, P. J., and Eaton, G. (2001). Olfactory memory in the mosquito *Culex quinquefasciatus*. *Med. Vet. Entomol.* 15, 197–203. doi: 10.1046/j.0269-283x.2001.00304.x
- McMeniman, C. J., Corfas, R. A., Matthews, B. J., Ritchie, S. A., and Vosshall, L. B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell* 156, 1060–1071. doi: 10.1016/j.cell.2013.12.044
- McQuillan, H. J., Barron, A. B., and Mercer, A. R. (2012). Age- and behaviour-related changes in the expression of biogenic amine receptor genes in the antennae of honey bees (*Apis mellifera*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 198, 753–761. doi: 10.1007/s00359-012-0745-y
- Mechaber, W. L., Capaldo, C. T., and Hildebrand, J. G. (2002). Behavioral responses of adult female tobacco hornworms, *Manduca sexta*, to hostplant volatiles change with age and mating status. *J. Insect Sci.* 2:5. doi: 10.1093/jis/2.1.5
- Meola, S. M., and Sittertz-Bhatkar, H. (2002). Neuroendocrine modulation of olfactory sensory neuron signal reception via axo-dendritic synapses in the antennae of the mosquito, *Aedes aegypti*. *J. Mol. Neurosci.* 18, 239–245. doi: 10.1385/jmn:18:3:239
- Mesce, K. A., DeLorme, A. W., Brelje, T. C., and Klukas, K. A. (2001). Dopamine-synthesizing neurons include the putative H-cell homologue in the moth *Manduca sexta*. *J. Comp. Neurol.* 430, 501–517. doi: 10.1002/1096-9861(20010219)430:4<501::AID-CNE1046>3.0.CO;2-U
- Minoli, S., Kauer, I., Colson, V., Party, V., Renou, M., Anderson, P., et al. (2012). Brief exposure to sensory cues elicits stimulus-nonspecific general sensitization in an insect. *PLoS One* 7:e34141. doi: 10.1371/journal.pone.0034141
- Möbius, P., and Penzlin, H. (1993). Stress-induced release of octopamine in the American cockroach *Periplaneta americana* L. *Acta. Biol. Hung.* 44, 45–50.
- Nagai, T. (1981). Electroantennogram response gradient on the antenna of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 27, 889–894. doi: 10.1016/0022-1910(81)90090-1
- Nagaya, Y., Kutsukake, M., Chigusa, S. I., and Komatsu, A. (2002). A trace amine, tyramine, functions as a neuromodulator in. *Neurosci. Lett.* 329, 324–328. doi: 10.1016/s0304-3940(02)00596-7
- Newport, G. (1836). On the respiration of insects. *Philos. Trans. R. Soc. Lond.* 126, 529–566. doi: 10.1098/rstl.1836.0026
- Nishimura, A., Ishida, Y., Takahashi, A., Okamoto, H., Sakabe, M., Itoh, M., et al. (2012). Starvation-induced elevation of taste responsiveness and expression of a sugar taste receptor gene in *Drosophila melanogaster*. *J. Neurogenet.* 26, 206–215. doi: 10.3109/01677063.2012.694931
- Ohta, H., and Ozoe, Y. (2014). Molecular signalling, pharmacology and physiology of octopamine and tyramine receptors as potential insect pest control target. *Adv. Insect. Physiol.* 46, 73–166. doi: 10.1016/b978-0-12-417010-0.00002-1
- Ohta, H., Utsumi, T., and Ozoe, Y. (2003). B96Bom encodes a *Bombyx mori* tyramine receptor negatively coupled to adenylate cyclase. *Insect Mol. Biol.* 12, 217–223. doi: 10.1046/j.1365-2583.2003.00404.x
- Ohta, H., Utsumi, T., and Ozoe, Y. (2004). Amino acid residues involved in interaction with tyramine in the *Bombyx mori* tyramine receptor. *Insect Mol. Biol.* 13, 531–538. doi: 10.1111/j.0962-1075.2004.00511.x
- Oppelt, A., and Heinze, J. (2009). Mating is associated with immediate changes of the hydrocarbon profile of *Leptothorax gredleri* ant queens. *J. Insect Physiol.* 55, 624–628. doi: 10.1016/j.jinsphys.2009.03.010
- Orchard, L., Gole, J. W. D., and Downer, R. G. H. (1983). Pharmacology of aminergic receptors mediating an elevation in cyclic AMP and release of hormone from locust neurosecretory cells. *Brain Res.* 288, 349–353. doi: 10.1016/0006-8993(83)90116-6
- Pass, G. (1985). Gross and fine structure of the antennal circulatory organ in cockroaches (Blattodea, Insecta). *J. Morphol.* 185, 255–268. doi: 10.1002/jmor.1051850210
- Pass, G. (2000). Accessory pulsatile organs: evolutionary innovations in insects. *Annu. Rev. Entomol.* 45, 495–518. doi: 10.1146/annurev.ento.45.1.495
- Pass, G., Gereben-Krenn, B. A., Merl, M., Plant, J., Szucsich, N. U., and Tögel, M. (2006). Phylogenetic relationships of the orders of Hexapoda: contributions from the circulatory organs for a morphological data matrix. *Arthropod Syst. Phylogeny* 64, 165–203.
- Pass, G., Sperk, G., Agricola, H., Baumann, E., and Penzlin, H. (1988). Octopamine in a neurohemal area within the antennal heart of the American cockroach. *J. Exp. Biol.* 135, 495–498.
- Pawlowa, M. (1895). Über ampullenartige Blutcirculationsorgane im Kopfe verschiedener Orthopteren. *Zool. Anz.* 18, 7–13.
- Pitts, R. J., Rinker, D. C., Jones, P. L., Rokas, A., and Zwiebel, L. J. (2011). Transcriptome profiling of chemosensory appendages in the malaria vector *Anopheles gambiae* reveals tissue- and sex-specific signatures of odor coding. *BMC Genomics* 12:271. doi: 10.1186/1471-2164-12-271
- Pophof, B. (2000). Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J. Comp. Physiol. A* 186, 307–313. doi: 10.1007/s003590050431
- Pophof, B. (2002). Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 188, 659–662. doi: 10.1007/s00359-002-0343-5
- Reim, T., Balfanz, S., Baumann, A., Blenau, W., Thamm, M., and Scheiner, R. (2017). AmTAR2: functional characterization of a honeybee tyramine receptor stimulating adenylyl cyclase activity. *Insect Biochem. Mol. Biol.* 80, 91–100. doi: 10.1016/j.ibmb.2016.12.004
- Riffell, J. A., and Hildebrand, J. G. (2016). “Adaptive processing in the insect olfactory system,” in *The Ecology of Animal Senses*, eds G. von der Emde and E. Warrant (New York, NY: Springer International Publishing), 3–24.
- Rillich, J., and Stevenson, P. A. (2015). Releasing stimuli and aggression in crickets: octopamine promotes escalation and maintenance but not initiation. *Front. Behav. Neurosci.* 9:95. doi: 10.3389/fnbeh.2015.00095
- Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404
- Roeder, T., Seifert, M., Kähler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. *Arch. Insect Biochem. Physiol.* 54, 1–13. doi: 10.1002/arch.10102
- Roelofs, W. L. (1995). Chemistry of sex attraction. *Proc. Natl. Acad. Sci. U S A* 92, 44–49. doi: 10.1073/pnas.92.1.44

- Saifullah, A. S. M., and Page, T. L. (2009). Circadian regulation of olfactory receptor neurons in the cockroach antenna. *J. Biol. Rhythms* 24, 144–152. doi: 10.1177/0748730408331166
- Salecker, I., and Distler, P. (1990). Serotonin-immunoreactive neurons in the antennal lobes of the American cockroach *Periplaneta americana*: light- and electron-microscopic observations. *Histochemistry* 94, 463–473. doi: 10.1007/bf00272608
- Saleh, N., and Chittka, L. (2006). The importance of experience in the interpretation of conspecific chemical signals. *Behav. Ecol. Sociobiol.* 61, 215–220. doi: 10.1007/s00265-006-0252-7
- Schaller, D. (1978). Antennal sensory system of *Periplaneta americana* L. Distribution and frequency of morphologic types of sensilla and their sex-specific changes during postembryonic development. *Cell Tissue Res.* 191, 121–139.
- Schendzielorz, T., Peters, W., Boekhoff, I., and Stengl, M. (2012). Time of day changes in cyclic nucleotides are modified via octopamine and pheromone in antennae of the Madeira cockroach. *J. Biol. Rhythms* 27, 388–397. doi: 10.1177/0748730412456265
- Siju, K. P., Hansson, B. S., and Ignell, R. (2008). Immunocytochemical localization of serotonin in the central and peripheral chemosensory system of mosquitoes. *Arthropod Struct. Dev.* 37, 248–259. doi: 10.1016/j.asd.2007.12.001
- Solari, P., Stoffolano, J. G. Jr., De Rose, F., Barbarossa, I. T., and Liscia, A. (2015). The chemosensitivity of labellar sugar receptor in female *Phormia regina* is paralleled with ovary maturation: effects of serotonin. *J. Insect Physiol.* 82, 38–45. doi: 10.1016/j.jinsphys.2015.08.007
- Stengl, M. (2010). Pheromone transduction in moths. *Front. Cell. Neurosci.* 4:133. doi: 10.3389/fncel.2010.00133
- Stengl, M., and Funk, N. W. (2013). The role of the coreceptor Orco in insect olfactory transduction. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 897–909. doi: 10.1007/s00359-013-0837-3
- Stevenson, P. A., and Rillich, J. (2016). Controlling the decision to fight or flee: the roles of biogenic amines and nitric oxide in the cricket. *Curr. Zool.* 62, 265–275. doi: 10.1093/cz/zow028
- Takasu, K., and Lewis, W. J. (1996). The role of learning in adult food location by the larval parasitoid, *Microplitis croceipes* (Hymenoptera: Braconidae). *J. Insect Behav.* 9, 265–281. doi: 10.1007/bf02213870
- Thurm, U., and Küppers, J. (1980). “Epithelial physiology of insect sensilla,” in *Insect Biology in the Future*, eds M. Locke and D. S. Smith (New York, NY: Academic Press), 735–763.
- Vander Meer, R. K., Preston, C. A., and Hefetz, A. (2008). Queen regulates biogenic amine level and nestmate recognition in workers of the fire ant, *Solenopsis invicta*. *Naturwissenschaften* 95, 1155–1158. doi: 10.1007/s00114-008-0432-6
- Vergoz, V. H., McQuillan, J., Geddes, L. H., Pullar, K., Nicholson, B. J., Paulin, M. G., et al. (2009). Peripheral modulation of worker bee responses to queen mandibular pheromone. *Proc. Natl. Acad. Sci. U S A* 106, 20930–20935. doi: 10.1073/pnas.0907563106
- Vleugels, R., Verlinden, H., and Broeck, J. V. (2015). Serotonin, serotonin receptors and their actions in insects. *Neurotransmitter* 2:e314. doi: 10.14800/nt.314
- von Nickisch-Rosenegk, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., Strotmann, J., et al. (1996). Cloning of biogenic octopamine receptors from moths (*Bombyx mory* and *Heliothis virescens*). *Insect Biochem. Mol. Biol.* 26, 817–827. doi: 10.1016/s0965-1748(96)00031-8
- Watanabe, H., Shimohigashi, M., and Yokohari, F. (2014). Serotonin-immunoreactive sensory neurons in the antenna of the cockroach *Periplaneta americana*. *J. Comp. Neurol.* 522, 414–434. doi: 10.1002/cne.23419
- Weidenmüller, A., Mayr, C., Kleineidam, C. J., and Roces, F. (2009). Preimaginal and adult experience modulates the thermal response behavior of ants. *Curr. Biol.* 19, 1897–1902. doi: 10.1016/j.cub.2009.08.059
- Westhus, C., Kleineidam, C. J., Roces, F., and Weidenmüller, A. (2013). Behavioural plasticity in the fanning response of bumblebee workers: impact of experience and rate of temperature change. *Anim. Behav.* 85, 27–34. doi: 10.1016/j.anbehav.2012.10.003
- White, P. R. (1991). The electroantennogram response: effects of varying sensillum numbers and recording electrode position in a clubbed antenna. *J. Insect Physiol.* 37, 145–152. doi: 10.1016/0022-1910(91)90100-e
- Yadav, M. (2003). *Physiology of Insects*. New Delhi: Discovery Publishing House.
- Yang, Z., Bengtsson, M., and Witzgall, P. (2004). Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. *J. Chem. Ecol.* 30, 619–629. doi: 10.1023/b:joec.0000018633.94002.af
- Zhao, X. C., Pfuhl, G., Surlykke, A., Tro, J., and Berg, B. G. (2013). A multisensory centrifugal neuron in the olfactory pathway of heliothine moths. *J. Comp. Neurol.* 521, 152–168. doi: 10.1002/cne.23166
- Zhukovskaya, M. I. (2008). Selective regulation of sensitivity to odours of different behavioural significance in American cockroach, *Periplaneta americana* L. *Physiol. Entomol.* 33, 162–166. doi: 10.1111/j.1365-3032.2008.00615.x
- Zhukovskaya, M. I. (2012). Modulation by octopamine of olfactory responses to nonpheromone odorants in the cockroach, *Periplaneta americana* L. *Chem. Senses* 37, 421–429. doi: 10.1093/chemse/bjr121
- Zhukovskaya, M. I., and Kapitsky, S. V. (2006). Activity modulation in cockroach sensillum: the role of octopamine. *J. Insect Physiol.* 52, 76–86. doi: 10.1016/j.jinsphys.2005.09.005

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Activity-Dependent Synaptic Refinement: New Insights from *Drosophila*

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During development, neurons establish inappropriate connections as they seek out their synaptic partners, resulting in supernumerary synapses that must be pruned away. The removal of miswired synapses usually involves electrical activity, often through a Hebbian spike-timing mechanism. A novel form of activity-dependent refinement is used by *Drosophila* that may be non-Hebbian, and is critical for generating the precise connectivity observed in that system. In *Drosophila*, motoneurons use both glutamate and the biogenic amine octopamine for neurotransmission, and the muscle fibers receive multiple synaptic inputs. Motoneuron growth cones respond in a time-regulated fashion to multiple chemotropic signals arising from their postsynaptic partners. Central to this mechanism is a very low frequency (<0.03 Hz) oscillation of presynaptic cytoplasmic calcium, that regulates and coordinates the action of multiple downstream effectors involved in the withdrawal from off-target contacts. Low frequency calcium oscillations are widely observed in developing neural circuits in mammals, and have been shown to be critical for normal connectivity in a variety of neural systems. In *Drosophila* these mechanisms allow the growth cone to sample widely among possible synaptic partners, evaluate opponent chemotropic signals, and withdraw from off-target contacts. It is possible that the underlying molecular mechanisms are conserved widely among invertebrates and vertebrates.

Keywords: oscillation, chemorepulsion, neuromuscular junction, non-Hebbian, second messengers

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It is estimated that the nearly 10^{11} neurons of the human nervous system establish over 10^{14} synaptic connections (Azevedo et al., 2009; Kasthuri et al., 2015). To wire up a system of such astonishing complexity requires mechanisms that are highly efficient and flexible. Rather than uniquely specifying each synaptic connection, the developing nervous system can initially establish connections that are characterized by supernumerary synaptic contacts, as widely observed in neural networks. Inappropriate off-target synapses are subsequently pruned away through activity-dependent mechanisms to yield a more precise and functional connectome (reviewed in Katz and Shatz, 1996; Yamamoto and López-Bendito, 2012; Doll and Broadie, 2014; Koropouli and Kolodkin, 2014; Arroyo and Feller, 2016). Errors in synaptic pruning are associated with several neurological disorders, including autism and schizophrenia (Berridge, 2012; Tang et al., 2014; Sekar et al., 2016).

In this review article, we examine synaptic refinement with a focus on the embryonic and larval neuromuscular system of *Drosophila*, where some of the underlying molecular mechanisms have been resolved (Carrillo et al., 2010; Vonhoff and Keshishian, 2017). This simple array of synapses

is established by two distinct classes of motoneurons that use as neurotransmitters either glutamate (Johansen et al., 1989) or the biogenic amine octopamine (Monastirioti, 1999).

ACTIVITY DEPENDENT REFINEMENT

The refinement of neural connections occurs in vertebrates and invertebrates, and has been extensively studied in the developing visual system (reviewed in D'Orazi et al., 2014; Pratt et al., 2016). Although activity-independent synapse elimination has been observed in mouse retinal cells (Morgan et al., 2011; Wei et al., 2011; Yonehara et al., 2011), activity-dependent mechanisms play a crucial role in establishing precise network connectivity (reviewed in Huberman et al., 2008; Cang and Feldheim, 2013). Pioneering work by Hubel and Wiesel showed that visual experience was required for the formation of ocular dominance columns between axons of the lateral geniculate nucleus (LGN) of the thalamus, and layer 4 neurons in primary visual cortex (Wiesel and Hubel, 1963). The requirement for neural activity in the segregation of visual projections was subsequently tested using TTX eye injections in both cold blooded vertebrates (Meyer, 1982), and in mammals (Schatz and Stryker, 1988; Sretavan et al., 1988). Patterned neural activity was also found to be essential for refining retinotopic map projections at other visual centers, such as the superior colliculus (McLaughlin et al., 2003). Activity-dependent refinement is also involved in controlling the balance between excitatory and inhibitory synapses, as found for the *Xenopus* optic tectum (Akerman and Cline, 2007). Elsewhere activity is involved in the elimination of supernumerary contacts at the vertebrate neuromuscular junction (reviewed by Sanes and Lichtman, 2001), and for synapse elimination of climbing fiber inputs to cerebellar Purkinje cells (reviewed by Purves and Lichtman, 1980; Kano and Hashimoto, 2009).

The remodeling that occurs during synaptic refinement suggests that electrical activity influences neurite growth or retraction. The link between activity and growth is a general feature of neural systems. For example, in *Drosophila* altered levels of neural activity in embryonic olfactory projection neurons (Prieto-Godino et al., 2012) and in larval and adult motoneurons (Duch et al., 2008; Hartwig et al., 2008) affects dendrite size and complexity, and thus directly influences synaptic connections. Similarly, in larval motoneurons manipulation of neural activity alters presynaptic NMJ size and arbor complexity, and affects presynaptic bouton morphology (Budnik et al., 1990; Zhong et al., 1992; Lnenicka et al., 2003; Mosca et al., 2005; Berke et al., 2013).

MOLECULAR MECHANISMS UNDERLYING REFINEMENT

How is neural activity linked to the cell biology of neuronal growth and retraction? Depolarization elevates intracellular free calcium (Ca^{2+}) levels through voltage-gated calcium channels (VGCCs). As a result, the mechanisms regulating synaptic connectivity generally involve Ca^{2+} -dependent effectors. Ca^{2+} -dependent signaling can influence early growth events,

such as the motility and exploration of the growth cone (Kater and Shibata, 1994; Zheng and Poo, 2007; Rosenberg and Spitzer, 2011). In some cases this is due to the modulation of the growth cone's response to various exogenous chemotropic factors, such as netrin-1-induced attraction, myelin-associated glycoprotein (MAG)-induced repulsion (Ming et al., 2001), or Ephrin-A induced repulsion of mouse retinal ganglion cells (Nicol et al., 2007).

Within the cytoplasm, Ca^{2+} regulates the activity of various GTPases (Jin et al., 2005), that in turn affect cytoskeletal dynamics within the growing contact. GTPases serve as a key molecular link between changes in free Ca^{2+} levels in the growth cone due to activity, and subsequent responses to chemotropic factors (Lowery and Van Vactor, 2009). One potential mechanism linking neural activity and cytoskeletal dynamics would involve the regulation of actin by the activity of Rho GTPases. Rho is known to regulate ROCK function, which in turn activates LIM Kinase (LIMK; Amano et al., 2010). LIMK inhibits cofilin, an actin severing protein that promotes actin recycling. Consistent with this hypothesis, LIMK is known to regulate synaptic function in mice (Meng et al., 2002) as well as NMJ growth in *Drosophila* (Ang et al., 2006).

A second molecular mechanism regulating activity-dependent refinement involves interactions between Ca^{2+} and cyclic nucleotides such as cAMP and cGMP. Intracellular cyclic nucleotide levels regulate chemotropic growth cone turning (Lohof et al., 1992; Song et al., 1997; Nishiyama et al., 2003), synaptic plasticity (Zhong et al., 1992), and the refinement of axon branches in both retinal cells (Nicol et al., 2006) and *Drosophila* motoneurons (Vonhoff and Keshishian, 2017). Whether cAMP levels are positioned upstream or downstream of Ca^{2+} signaling remains incompletely resolved, as there is evidence in the literature for both scenarios. cAMP levels may act downstream of Ca^{2+} as connectivity defects arise following misregulation of Ca^{2+} -dependent adenylyl cyclases, such as AC1 in mouse retinal neurons (Nicol et al., 2006), ADCY8 in zebrafish retinal neurons (Xu et al., 2010), and Rutabaga in *Drosophila* motoneurons (Vonhoff and Keshishian, 2017). By contrast, cAMP also regulates Ca^{2+} -signaling as it promotes Ca^{2+} -induced Ca^{2+} -release (CICR) from internal stores (Gomez and Zheng, 2006; Zheng and Poo, 2007), modulates the amplitude of growth cone Ca^{2+} -transients (Nicol et al., 2011), and cyclic nucleotide-gated (CNG) ion channels, to allow for Ca^{2+} -influx in growth cones (Togashi et al., 2008).

Intracellular Ca^{2+} activates several pathways that converge on transcription factors that control the expression of activity-regulated genes that may be involved in guidance mechanisms. This was first revealed for the immediate early gene *c-fos*, downstream of Ca^{2+} influx (Greenberg et al., 1986). Fos protein together with Jun family members comprises the AP-1 transcription factor (Curran and Franza, 1988). AP-1 has been involved in synaptic plasticity in mouse hippocampal neurons (Fleischmann et al., 2003) as well as in activity-dependent dendritic growth of *Drosophila* motoneurons (Hartwig et al., 2008; Vonhoff et al., 2013) and synaptic development at the *Drosophila* NMJ (Sanyal et al., 2002).

Finally, there is good evidence from both vertebrates and invertebrates that synaptic refinement requires temporally patterned changes or oscillations in the levels of second messengers. This dynamism has been particularly evident for Ca^{2+} , where spontaneous retinal waves are critical for the refinement of visual maps in the mouse brain (Wong, 1999; Arroyo and Feller, 2016), as well as for the refinement of neuromuscular junctions in *Drosophila* embryos (Carrillo et al., 2010; Vonhoff and Keshishian, 2017). It is intriguing that cAMP levels are also required to oscillate for the refinement of mouse retinal axons (Nicol et al., 2007), or to be dynamically maintained within an optimal level for the refinement of *Drosophila* motoneuron axon branches (Vonhoff and Keshishian, 2017).

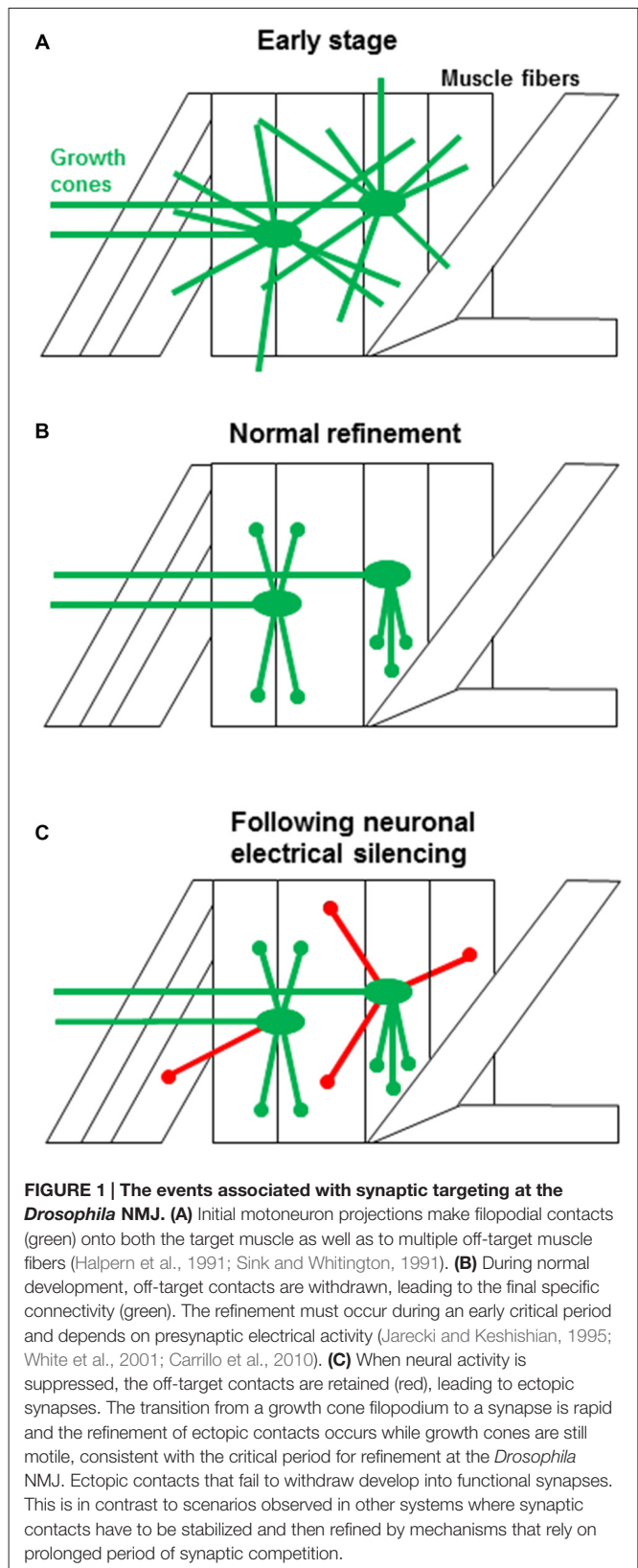
DROSOPHILA NMJ AS A GENETIC MODEL TO STUDY SYNAPTIC REFINEMENT

The *Drosophila* larval bodywall offers an anatomically stereotypic genetic model system for studying many aspects of neuronal connectivity (for reviews see Ruiz-Cañada and Budnik, 2006; Menon et al., 2013). Among its features are singly identifiable glutamatergic motoneurons with very narrow connectivity, innervating only one or two muscle fibers each, and a subset of efferent neuromodulatory neurons that express the biogenic amine octopamine (Monastirioti et al., 1995, 1996; Monastirioti, 1999) that project widely and innervate multiple muscle fibers.

The stereotypic connectivity of the embryonic and larval *Drosophila* NMJ crucially relies on the expression of molecular recognition cues (reviewed in Nose, 2012). Whereas some molecules are expressed by all muscles, the expression pattern of other cues is restricted to individual muscles (Winberg et al., 1998). Examples of muscle-specific cues include Fasciclin III (Halpern et al., 1991), Capricious (Shishido et al., 1998), Connectin (Nose et al., 1992), and NetrinB (Harris et al., 1996). By contrast, other molecules are expressed by numerous muscle fibers, as for example Fasciclin II (Lin and Goodman, 1994), Teneurin-m (Mosca et al., 2012), Dpr11 (Carrillo et al., 2015), and Semaphorin2a (Matthes et al., 1995).

During embryonic development *Drosophila* motoneuron growth cones sample widely among muscle fibers, and inevitably make inappropriate contacts, as shown schematically in **Figure 1A** (Halpern et al., 1991; Sink and Whittington, 1991; Chiba et al., 1993). The off-target contacts are removed during an early critical period (late embryo to early 1st instar; **Figure 1B**), otherwise they mature into functional ectopic synapses (Jarecki and Keshishian, 1995; Carrillo et al., 2010). Ultimately, neural activity refines the motoneuron contacts, so that their connectivity is limited only to their appropriate muscle fiber targets. Silencing electrical activity in the motoneurons during the critical period increases the frequency of ectopic motoneuron contacts throughout the bodywall (**Figure 1C**; Jarecki and Keshishian, 1995; White et al., 2001; Carrillo et al., 2010).

In vivo electrical activity in the embryo is highly patterned, with brief (~15 s) bursts of action potentials spaced every



2–3 min (Pereanu et al., 2007; Crisp et al., 2008; Vonhoff and Keshishian, 2017). Normal synaptic refinement depends on

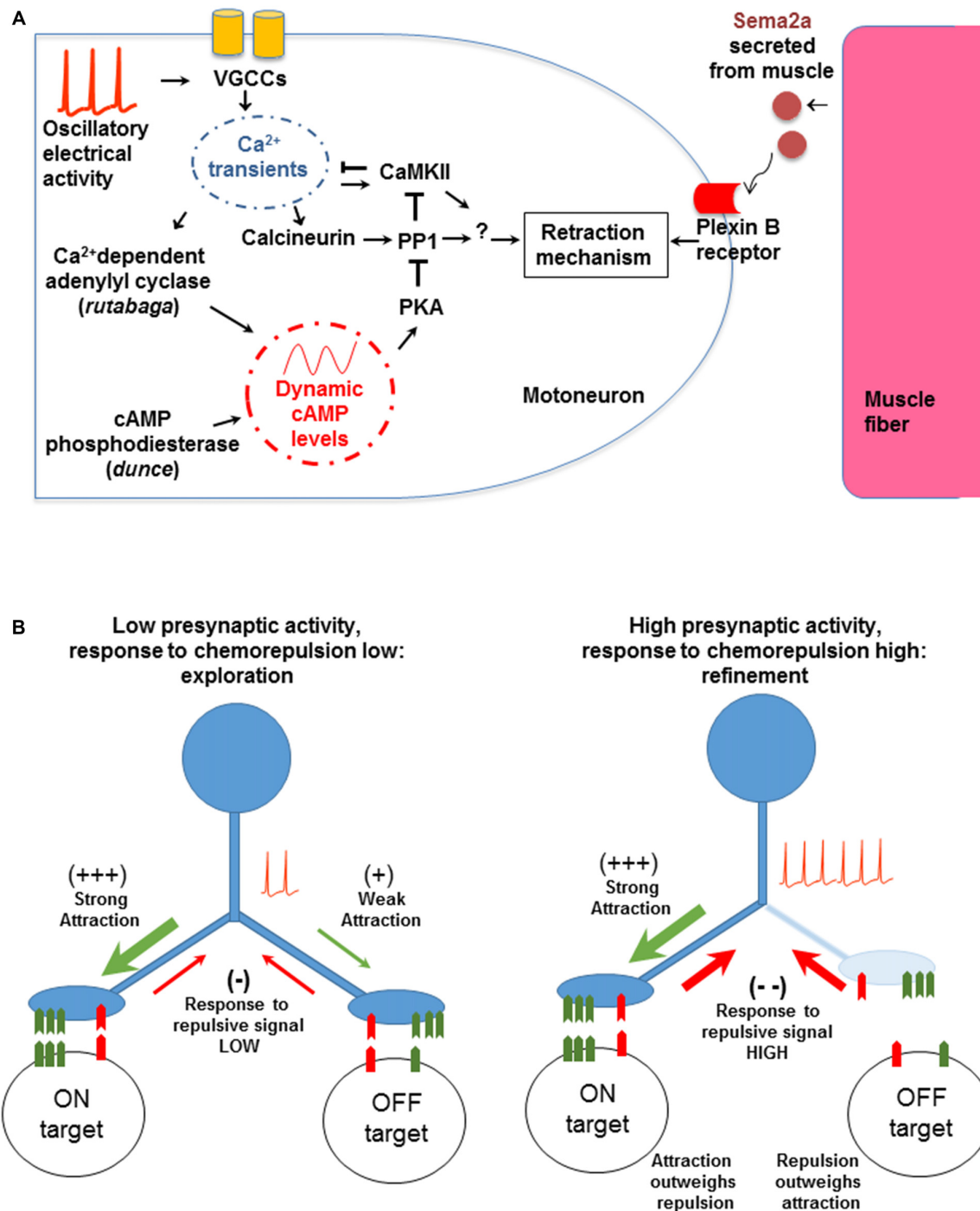


FIGURE 2 | The molecular and cellular mechanisms involved in synaptic refinement. (A) The interactions were identified by genetic tests and transgenic manipulations. A low frequency voltage oscillation activates voltage gated Ca^{2+} channels (VGCCs). The resulting Ca^{2+} entry regulates Ca^{2+} -dependent effectors including Ca^{2+} /calmodulin-dependent serine/threonine kinase II (CaMKII), Calcineurin (CaN), and Rutabaga. The latter increases cAMP levels, which in turn regulate PKA and PP1. The chemorepellant Sema2a is secreted by the muscle and activates the presynaptic PlexinB receptor. The response to Sema2a is gated by the level of presynaptic Ca^{2+} activity (see text for details). Arrows and T-shape lines indicate positive and negative regulation, respectively. The subcellular physical location and region of action of the molecular components have not been determined yet. **(B)** A model for non-Hebbian refinement at the *Drosophila* NMJ. The left panel shows an initial contact made by a motoneuron onto on-target and off-target muscle fibers. The molecular match is stronger with the on-target fiber. When Ca^{2+} levels are low, the response to the retrograde chemorepulsive signal from the muscle is muted, allowing the off-target contact to be retained. With neural activity and elevated presynaptic Ca^{2+} (right panel), the repulsive response is elevated, leading to the withdrawal of the off-target contact. Note that the model does not depend on correlated activity between the synaptic partners, as would be expected in a Hebbian mechanism.

the presence of two voltage-gated Ca^{2+} channels, Cacophony (Cac), the Ca(v)2.1 channel (Carrillo et al., 2010), and Dmca1G , the Ca(v)3 channel (Vanhoff and Keshishian, in preparation). The experimental rescue of the *cac* mutation to restore normal synaptic connectivity requires oscillatory presynaptic Ca^{2+} entry, timed to resemble the native electrical oscillations (Carrillo et al., 2010). This indicates that Ca^{2+} -oscillations at a specific frequency and pattern (in the range of 0.01–0.03 Hz) are required for proper synaptic refinement.

In addition to the activity-dependent entry of Ca^{2+} through Ca^{2+} channels (Figure 2A), refinement also depends on the activity of at least three downstream Ca^{2+} -dependent signaling systems in the presynaptic terminal: the Ca^{2+} /calmodulin-dependent serine/threonine kinase II (CaMKII; Carrillo et al., 2010), the Ca^{2+} /calmodulin-dependent serine/threonine protein phosphatase Calcineurin (CaN; Vanhoff and Keshishian, in preparation), and the Ca^{2+} -dependent adenylyl cyclase Rutabaga (Vanhoff and Keshishian, 2017). Rutabaga elevates intracellular cAMP-levels, which are degraded by the activity of the cAMP-phosphodiesterase Dunce. Similarly, molecules whose activity is typically downstream of cAMP such as PKA and PP1 are also required for synaptic refinement (Vanhoff and Keshishian, 2017). Notably, PKA and CaN are known to interact with PP1 (Blitzer et al., 1998; Oliver and Shenolikar, 1998), which in turn can regulate CaMKII. Collectively, these interactions suggest a complex signaling network to govern synaptic refinement in this system (Figure 2A).

How are off-target contacts withdrawn? There is strong evidence that synaptic pruning depends on an active response by the presynaptic growth cone to *Sema2a*, a chemorepulsive molecule secreted by muscle fibers that acts via the PlexinB receptor in motoneurons (Winberg et al., 1998; Ayoob et al., 2006; Carrillo et al., 2010). We hypothesize that Ca^{2+} entry into the developing motoneuron terminal modulates the cell's chemorepulsive response to *Sema2a*. A similar role for neural activity and Ca^{2+} waves in modulating chemotropic and guidance responses of growth cones has been proposed for vertebrate neurons (Spitzer et al., 2000; Ming et al., 2001; Nicol et al., 2006, 2011; Rosenberg and Spitzer, 2011).

We therefore propose a model where the response of the motoneuron growth cone to muscle-derived *Sema2a* is episodically modulated in an oscillatory fashion (Figure 2B). When Ca^{2+} -levels in growth cones are low, exploratory filopodia are favored to contact and extend on membrane surfaces. By contrast, during activity bouts, Ca^{2+} - and cAMP levels transiently increase, raising the responsiveness of the neuron to the *Sema2a*-chemorepellant and withdrawing the less firmly-associated filopodial contacts from off-target surfaces. Thus, presynaptic electrical activity regulates complex molecular interactions in a time-dependent fashion, to modulate the neuron's responsiveness to chemorepulsion exerted by the muscle fibers. These results provide a coherent picture of the links between neural activity, chemorepulsion, and the refinement of synaptic connectivity.

MOLECULAR CANDIDATES THAT MAY BE INVOLVED IN ACTIVITY-DEPENDENT REFINEMENT

Although a crucial role for Ca^{2+} -influx via VGCCs in the withdrawal of off-target neuromuscular contacts has been observed, a role for CICR in synaptic refinement in *Drosophila* remains untested. CICR is influenced by cAMP (Gomez and Zheng, 2006; Zheng and Poo, 2007) and is required for netrin-1 induced growth cone turning (Hong et al., 2000). Furthermore, filopodial Ca^{2+} transients have been shown to activate the protease calpain to promote growth cone repulsive turning (Robles et al., 2003). Several calpain genes with neural expression have been identified in *Drosophila* (Friedrich et al., 2004), and have been associated with Ca^{2+} -dependent dendrite pruning (Kanamori et al., 2013), offering a potential regulatory mechanism for future examination.

Alternative links between neural activity and CaN for synaptic refinement also remain untested, as for example molecular pathways involving the activity-dependent transcription factor AP1. In murine T-cells, CaN dephosphorylates NFAT, a DNA-binding phosphoprotein that forms a complex with Fos and Jun to activate gene transcription (Jain et al., 1993). In cultured mouse primary neurons, the CaN-NFAT signaling is required to promote the netrin-1 dependent axonal outgrowth (Graef et al., 2003). In *Drosophila* motoneurons, AP1 promotes activity-dependent dendritic growth (Hartwig et al., 2008; Vanhoff et al., 2013) and synaptic plasticity (Sanyal et al., 2002) together with NFAT at the larval NMJ (Freeman et al., 2011). Furthermore, CaN and the GSK-3 β kinase homolog Shaggy have been recently described to regulate bouton stabilization at the larval NMJ by activating or inhibiting the microtubule associated protein-1b fly ortholog futsch/MAP-1b, respectively (Wong et al., 2014). Shaggy activates the CaN-regulator Sra in *Drosophila* eggs (Takeo et al., 2012), and also negatively regulates neuronal AP1 function by inhibiting the JNK pathway, as described in an *in vivo* genetic screen in *Drosophila* (Francisovich et al., 2008). Interestingly, the genes *sema2a* and *fkbp13* (a protein predicted to bind the pharmacological agent FK506, a known inhibitor of CaN) were identified in the same screen among the molecules that regulate AP1 function (Francisovich et al., 2008). Whether these genes play a role in the activity-dependent withdrawal of ectopic contacts or in the modulation of chemorepulsion remains to be tested.

BIOGENIC AMINES AND REFINEMENT

Synaptic connectivity in *Drosophila* can range from precise targeting, as seen for the glutamatergic motoneurons that limit their connections to just one or two bodywall muscle fibers, to efferents that establish broad projections across the musculature, such as those expressing the biogenic amine octopamine. To what extent are the molecular mechanisms governing guidance and synaptic refinement conserved between these two distinct patterns of synaptic connectivity?

The octopaminergic motoneurons are highly plastic and respond to elevated electrical activity by expanding their peripheral arbors on the musculature (Zhong et al., 1992; Budnik, 1996; Koon et al., 2011). Although the octopaminergic projections are made over a broad expanse of the musculature, the wiring is nevertheless subject to activity-dependent refinement. Over half of the activity-dependent ectopic contacts found on muscle fibers are made by the octopaminergic motoneurons and those ectopic contacts are largely eliminated when neuromuscular activity is normal (Jarecki and Keshishian, 1995; Carrillo et al., 2010; Vonhoff and Keshishian, 2017). Thus similar mechanisms are likely at play to refine the connections made by the glutamatergic motoneurons that project to only one or two muscle fibers and the octopaminergic neurons that project to large regions of the musculature.

Octopamine regulates the activity-dependent plasticity of glutamatergic motoneurons in a paracrine fashion, acting through Oct β 2R receptors that regulate cAMP levels at the NMJs (Koon et al., 2011; Koon and Budnik, 2012). It is therefore possible that the octopaminergic efferents are themselves involved in regulating synaptic refinement. *Drosophila* expresses four distinct octopamine receptors (El-Kholy et al., 2015), including multiple forms that are found in neurons and muscles. As the *Drosophila* octopamine GPCRs modulate cAMP levels as well as Ca²⁺ signaling (Balfanz et al., 2005; Evans and Maqueira, 2005; Maqueira et al., 2005; Maiellaro et al., 2016), this raises the possibility that octopamine influences the refinement process by modulating the levels of these second messengers.

CONCLUDING THOUGHTS

The refinement of synaptic connections often involves Hebbian, spike-timing correlation between synaptic partners, with asynchronous inputs removed (an idea first elaborated by Stent, 1973). This ubiquitous mechanism is involved in topographic map development and synaptic refinement throughout the vertebrate CNS. By contrast, the *Drosophila* NMJ apparently does not require postsynaptic depolarization for the removal of off-target contacts (Jarecki and Keshishian, 1995; White et al.,

2001; Carrillo et al., 2010), suggesting a fundamentally different mechanism for synaptic refinement. Moreover, there is no evidence for competition based on correlated synaptic activity at the *Drosophila* NMJ, as is the case for refinement in other systems.

At the *Drosophila* NMJ connectivity is governed by a combinatorial system of recognition molecules expressed by motoneurons and muscles. A correct molecular “match” is needed to stabilize the motoneuronal contact leading to a functional synapse (Furrer and Chiba, 2004; Menon et al., 2013; Carrillo et al., 2015). As noted above, the motoneurons sample among possible synaptic partners, with off-target contacts withdrawn in an activity-dependent fashion. The challenge is to make guidance decisions based on opponent signals that are presented simultaneously: a global chemorepellant signal from all muscles, and a local chemoattractive signal from the target cell. Assuming that the response to the chemorepellant is governed by Ca²⁺ levels, then the growth cone sampling and withdrawal phases would be coordinated by the Ca²⁺ oscillations (Figure 2B). We view this mode of error correction as a form of time-dependent signal multiplexing, where the neuron can respond to distinct chemotropic signals depending on the phase of the Ca²⁺ oscillation. Vital imaging experiments currently underway (Vonhoff and Keshishian, in preparation), are testing whether there is a direct correlation between growth cone motility and the underlying low frequency Ca²⁺ oscillation.

AUTHOR CONTRIBUTIONS

FV and HK wrote the manuscript and designed the figures.

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REFERENCES

- Akerman, C. J., and Cline, H. T. (2007). Refining the roles of GABAergic signaling during neural circuit formation. *Trends Neurosci.* 30, 382–389. doi: 10.1016/j.tins.2007.06.002
- Amano, M., Nakayama, M., and Kaibuchi, K. (2010). Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* 67, 545–554. doi: 10.1002/cm.20472
- Ang, L.-H., Chen, W., Yao, Y., Ozawa, R., Tao, E., Yonekura, J., et al. (2006). Lim kinase regulates the development of olfactory and neuromuscular synapses. *Dev. Biol.* 293, 178–190. doi: 10.1016/j.ydbio.2006.01.030
- Arroyo, D. A., and Feller, M. B. (2016). Spatiotemporal features of retinal waves instruct the wiring of the visual circuitry. *Front. Neural Circuits* 10:54. doi: 10.3389/fncir.2016.00054
- Ayoob, J. C., Terman, J. R., and Kolodkin, A. L. (2006). *Drosophila* Plexin B is a Sema-2a receptor required for axon guidance. *Development* 133, 2125–2135. doi: 10.1242/dev.02380
- Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., et al. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J. Comp. Neurol.* 513, 532–541. doi: 10.1002/cne.21974
- Balfanz, S., Strünker, T., Frings, S., and Baumann, A. (2005). A family of octopamine receptors that specifically induce cyclic AMP production or Ca²⁺ release in *Drosophila melanogaster*. *J. Neurochem.* 93, 440–451. doi: 10.1111/j.1471-4159.2005.03034.x
- Berke, B., Wittnam, J., McNeill, E., Van Vactor, D. L., and Keshishian, H. (2013). Retrograde BMP signaling at the synapse: a permissive signal for synapse maturation and activity-dependent plasticity. *J. Neurosci.* 33, 17937–17950. doi: 10.1523/JNEUROSCI.6075-11.2013
- Berridge, M. J. (2012). Calcium signalling remodelling and disease. *Biochem. Soc. Trans.* 40, 297–309. doi: 10.1042/BST20110766
- Blitzer, R. D., Connor, J. H., Brown, G. P., Wong, T., Shenolikar, S., Iyengar, R., et al. (1998). Gating of CaMKII by cAMP-regulated protein phosphatase

- activity during LTP. *Science* 280, 1940–1943. doi: 10.1126/science.280.5371.1940
- Budnik, V. (1996). Synapse maturation and structural plasticity at *Drosophila* neuromuscular junctions. *Curr. Opin. Neurobiol.* 6, 858–867. doi: 10.1016/s0959-4388(96)80038-9
- Budnik, V., Zhong, Y., and Wu, C. F. (1990). Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci.* 10, 3754–3768.
- Cang, J., and Feldheim, D. A. (2013). Developmental mechanisms of topographic map formation and alignment. *Annu. Rev. Neurosci.* 36, 51–77. doi: 10.1146/annurev-neuro-062012-170341
- Carrillo, R. A., Olsen, D. P., Yoon, K. S., and Keshishian, H. (2010). Presynaptic activity and CaMKII modulate retrograde semaphorin signaling and synaptic refinement. *Neuron* 68, 32–44. doi: 10.1016/j.neuron.2010.09.005
- Carrillo, R. A., Özkan, E., Menon, K. P., Nagarkar-Jaiswal, S., Lee, P. T., Jeon, M., et al. (2015). Control of synaptic connectivity by a network of *Drosophila* IgSF cell surface proteins. *Cell* 163, 1770–1782. doi: 10.1016/j.cell.2015.11.022
- Chiba, A., Hing, H., Cash, S., and Keshishian, H. (1993). Growth cone choices of *Drosophila* motoneurons in response to muscle fiber mismatch. *J. Neurosci.* 13, 714–732.
- Crisp, S., Evers, J. F., Fiala, A., and Bate, M. (2008). The development of motor coordination in *Drosophila* embryos. *Development* 135, 3707–3717. doi: 10.1242/dev.026773
- Curran, T., and Franza, B. R. Jr. (1988). Fos and Jun: the AP-1 connection. *Cell* 55, 395–397. doi: 10.1016/0092-8674(88)90024-4
- Doll, C. A., and Broadie, K. (2014). Impaired activity-dependent neural circuit assembly and refinement in autism spectrum disorder genetic models. *Front. Cell. Neurosci.* 8:30. doi: 10.3389/fncel.2014.00030
- D'Orazi, F. D., Suzuki, S. C., and Wong, R. O. (2014). Neuronal remodeling in retinal circuit assembly, disassembly, and reassembly. *Trends Neurosci.* 37, 594–603. doi: 10.1016/j.tins.2014.07.009
- Duch, C., Vonhoff, F., and Ryglewski, S. (2008). Dendrite elongation and dendritic branching are affected separately by different forms of intrinsic motoneuron excitability. *J. Neurophysiol.* 100, 2525–2536. doi: 10.1152/jn.90758.2008
- El-Kholy, S., Stephano, F., Li, Y., Bhandari, A., Fink, C., and Roeder, T. (2015). Expression analysis of octopamine and tyramine receptors in *Drosophila*. *Cell Tissue Res.* 361, 669–684. doi: 10.1007/s00441-015-2137-4
- Evans, P. D., and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert. Neurosci.* 5, 111–118. doi: 10.1007/s10158-005-0001-z
- Fleischmann, A., Hvalby, O., Jensen, V., Strelakova, T., Zacher, C., Layer, L. E., et al. (2003). Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. *J. Neurosci.* 23, 9116–9122.
- Francisovich, A. L., Mortimer, A. D., Freeman, A. A., Gu, J., and Sanyal, S. (2008). Overexpression screen in *Drosophila* identifies neuronal roles of GSK-3 β /shaggy as a regulator of AP-1-dependent developmental plasticity. *Genetics* 180, 2057–2071. doi: 10.1534/genetics.107.085555
- Freeman, A., Francisovich, A., Bowers, M., Sandstrom, D. J., and Sanyal, S. (2011). NFAT regulates pre-synaptic development and activity-dependent plasticity in *Drosophila*. *Mol. Cell. Neurosci.* 46, 535–547. doi: 10.1016/j.mcn.2010.12.010
- Friedrich, P., Tompa, P., and Farkas, A. (2004). The calpain-system of *Drosophila melanogaster*: coming of age. *Bioessays* 26, 1088–1096. doi: 10.1002/bies.20106
- Furrer, M.-P., and Chiba, A. (2004). Molecular mechanisms for *Drosophila* neuron network formation. *Neurosignals* 13, 37–49. doi: 10.1159/000076157
- Gomez, T. M., and Zheng, J. Q. (2006). The molecular basis for calcium-dependent axon pathfinding. *Nat. Rev. Neurosci.* 7, 115–125. doi: 10.1038/nrn1844
- Graef, I. A., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M., et al. (2003). Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* 113, 657–670. doi: 10.1016/s0092-8674(03)00390-8
- Greenberg, M. E., Ziff, E. B., and Greene, L. A. (1986). Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science* 234, 80–83. doi: 10.1126/science.3749894
- Halpern, M. E., Chiba, A., Johansen, J., and Keshishian, H. (1991). Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. *J. Neurosci.* 11, 3227–3238.
- Harris, R., Sabatelli, L. M., and Seeger, M. A. (1996). Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. *Neuron* 17, 217–228. doi: 10.1016/s0896-6273(00)80154-3
- Hartwig, C. L., Worrell, J., Levine, R. B., Ramaswami, M., and Sanyal, S. (2008). Normal dendrite growth in *Drosophila* motor neurons requires the AP-1 transcription factor. *Dev. Neurobiol.* 68, 1225–1242. doi: 10.1002/dneu.20655
- Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M., and Poo, M. (2000). Calcium signalling in the guidance of nerve growth by netrin-1. *Nature* 403, 93–98. doi: 10.1038/47507
- Huberman, A. D., Feller, M. B., and Chapman, B. (2008). Mechanisms underlying development of visual maps and receptive fields. *Annu. Rev. Neurosci.* 31, 479–509. doi: 10.1146/annurev-neuro.31.060407.125533
- Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., et al. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature* 365, 352–355. doi: 10.1038/365352a0
- Jarecki, J., and Keshishian, H. (1995). Role of neural activity during synaptogenesis in *Drosophila*. *J. Neurosci.* 15, 8177–8190.
- Jin, M., Guan, C.-B., Jiang, Y.-A., Chen, G., Zhao, C.-T., Cui, K., et al. (2005). Ca^{2+} -dependent regulation of rho GTPases triggers turning of nerve growth cones. *J. Neurosci.* 25, 2338–2347. doi: 10.1523/JNEUROSCI.4889-04.2005
- Johansen, J., Halpern, M. E., Johansen, K. M., and Keshishian, H. (1989). Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J. Neurosci.* 9, 710–725.
- Kanamori, T., Kanai, M. I., Dairyo, Y., Yasunaga, K.-I., Morikawa, R. K., and Emoto, K. (2013). Compartmentalized calcium transients trigger dendrite pruning in *Drosophila* sensory neurons. *Science* 340, 1475–1478. doi: 10.1126/science.1234879
- Kano, M., and Hashimoto, K. (2009). Synapse elimination in the central nervous system. *Curr. Opin. Neurobiol.* 19, 154–161. doi: 10.1016/j.conb.2009.05.002
- Kasthuri, N., Hayworth, K. J., Berger, D. R., Schalek, R. L., Conchello, J. A., Knowles-Barley, S., et al. (2015). Saturated reconstruction of a volume of neocortex. *Cell* 162, 648–661. doi: 10.1016/j.cell.2015.06.054
- Kater, S. B., and Shibata, A. (1994). The unique and shared properties of neuronal growth cones that enable navigation and specific pathfinding. *J. Physiol. Paris* 88, 155–163. doi: 10.1016/0928-4257(94)90001-9
- Katz, L. C., and Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. *Science* 274, 1133–1138. doi: 10.1126/science.274.5290.1133
- Koon, A. C., Ashley, J., Barria, R., DasGupta, S., Brain, R., Waddell, S., et al. (2011). Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. *Nat. Neurosci.* 14, 190–199. doi: 10.1038/nn.2716
- Koon, A. C., and Budnik, V. (2012). Inhibitory control of synaptic and behavioral plasticity by octopaminergic signaling. *J. Neurosci.* 32, 6312–6322. doi: 10.1523/JNEUROSCI.6517-11.2012
- Koropoulis, E., and Kolodkin, A. L. (2014). Semaphorins and the dynamic regulation of synapse assembly, refinement and function. *Curr. Opin. Neurobiol.* 27, 1–7. doi: 10.1016/j.conb.2014.02.005
- Lin, D. M., and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* 13, 507–523. doi: 10.1016/0896-6273(94)90022-1
- Lnenicka, G. A., Spencer, G. M., and Keshishian, H. (2003). Effect of reduced impulse activity on the development of identified motor terminals in *Drosophila* larvae. *J. Neurobiol.* 54, 337–345. doi: 10.1002/neu.10133
- Lohof, A. M., Quillan, M., Dan, Y., and Poo, M. M. (1992). Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. *J. Neurosci.* 12, 1253–1261.
- Lowery, L. A., and Van Vactor, D. (2009). The trip of the tip: understanding the growth cone machinery. *Nat. Rev. Mol. Cell Biol.* 10, 332–343. doi: 10.1038/nrm2679
- Maiellaro, L., Lohse, M. J., Kittel, R. J., and Calebiro, D. (2016). cAMP signals in *Drosophila* motor neurons are confined to single synaptic boutons. *Cell Rep.* 17, 1238–1246. doi: 10.1016/j.celrep.2016.09.090
- Maqueira, B., Chatwin, H., and Evans, P. D. (2005). Identification and characterization of a novel family of *Drosophila* β -adrenergic-like octopamine G-protein coupled receptors. *J. Neurochem.* 94, 547–560. doi: 10.1111/j.1471-4159.2005.03251.x

- Matthes, D. J., Sink, H., Kolodkin, A. L., and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell* 81, 631–639. doi: 10.1016/0092-8674(95)90084-5
- McLaughlin, T., Torborg, C. L., Feller, M. B., and O'Leary, D. D. M. (2003). Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development. *Neuron* 40, 1147–1160. doi: 10.1016/s0896-6273(03)00790-6
- Meng, Y., Zhang, Y., Tregubov, V., Janus, C., Cruz, L., Jackson, M., et al. (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 35, 121–133. doi: 10.1016/s0896-6273(02)00758-4
- Menon, K. P., Carrillo, R. A., and Zinn, K. (2013). Development and plasticity of the *Drosophila* larval neuromuscular junction. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 647–670. doi: 10.1002/wdev.108
- Meyer, R. L. (1982). Tetrodotoxin blocks the formation of ocular dominance columns in goldfish. *Science* 218, 589–591. doi: 10.1126/science.7123262
- Ming, G.-L., Henley, J., Tessier-Lavigne, M., Song, H.-J., and Poo, M.-M. (2001). Electrical activity modulates growth cone guidance by diffusible factors. *Neuron* 29, 441–452. doi: 10.1016/s0896-6273(01)00217-3
- Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech.* 45, 106–121. doi: 10.1002/(SICI)1097-0029(19990415)45:2<106::AID-JEMT5>3.3.CO;2-V
- Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. *J. Comp. Neurol.* 356, 275–287. doi: 10.1002/cne.903560210
- Monastirioti, M., Linn, C. E. Jr., and White, K. (1996). Characterization of *Drosophila* tyramine β -hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* 16, 3900–3911.
- Morgan, J. L., Soto, F., Wong, R. O., and Kerschensteiner, D. (2011). Development of cell type-specific connectivity patterns of converging excitatory axons in the retina. *Neuron* 71, 1014–1021. doi: 10.1016/j.neuron.2011.08.025
- Mosca, T. J., Carrillo, R. A., White, B. H., and Keshishian, H. (2005). Dissection of synaptic excitability phenotypes by using a dominant-negative Shaker K⁺ channel subunit. *Proc. Natl. Acad. Sci. U S A* 102, 3477–3482. doi: 10.1073/pnas.0406164102
- Mosca, T. J., Hong, W., Dani, V. S., Favaloro, V., and Luo, L. (2012). Trans-synaptic Teneurin signalling in neuromuscular synapse organization and target choice. *Nature* 484, 237–241. doi: 10.1038/nature10923
- Nicol, X., Hong, K. P., and Spitzer, N. C. (2011). Spatial and temporal second messenger codes for growth cone turning. *Proc. Natl. Acad. Sci. U S A* 108, 13776–13781. doi: 10.1073/pnas.1100247108
- Nicol, X., Muzerelle, A., Rio, J. P., Métin, C., and Gaspar, P. (2006). Requirement of adenylate cyclase 1 for the ephrin-A5-dependent retraction of exuberant retinal axons. *J. Neurosci.* 26, 862–872. doi: 10.1523/JNEUROSCI.3385-05.2006
- Nicol, X., Voyatzis, S., Muzerelle, A., Narboux-Nême, N., Südhof, T. C., Miles, R., et al. (2007). cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map. *Nat. Neurosci.* 10, 340–347. doi: 10.1038/nn1842
- Nishiyama, M., Hoshino, A., Tsai, L., Henley, J. R., Goshima, Y., Tessier-Lavigne, M., et al. (2003). Cyclic AMP/GMP-dependent modulation of Ca²⁺ channels sets the polarity of nerve growth-cone turning. *Nature* 423, 990–995. doi: 10.1038/nature01751
- Nose, A. (2012). Generation of neuromuscular specificity in *Drosophila*: novel mechanisms revealed by new technologies. *Front. Mol. Neurosci.* 5:62. doi: 10.3389/fnmol.2012.00062
- Nose, A., Mahajan, V. B., and Goodman, C. S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in *Drosophila*. *Cell* 70, 553–567. doi: 10.1016/0092-8674(92)90426-d
- Oliver, C. J., and Shenolikar, S. (1998). Physiologic importance of protein phosphatase inhibitors. *Front. Biosci.* 3, D961–D972. doi: 10.2741/a336
- Pereanu, W., Spindler, S., Im, E., Buu, N., and Hartenstein, V. (2007). The emergence of patterned movement during late embryogenesis of *Drosophila*. *Dev. Neurobiol.* 67, 1669–1685. doi: 10.1002/dneu.20538
- Pratt, K. G., Hiramoto, M., and Cline, H. T. (2016). An evolutionarily conserved mechanism for activity-dependent visual circuit development. *Front. Neural Circuits* 10:79. doi: 10.3389/fncir.2016.00079
- Prieto-Godino, L. L., Diegelmann, S., and Bate, M. (2012). Embryonic origin of olfactory circuitry in *Drosophila*: contact and activity-mediated interactions pattern connectivity in the antennal lobe. *PLoS Biol.* 10:e1001400. doi: 10.1371/journal.pbio.1001400
- Purves, D., and Lichtman, J. W. (1980). Elimination of synapses in the developing nervous system. *Science* 210, 153–157. doi: 10.1126/science.7414326
- Robles, E., Huttenlocher, A., and Gomez, T. M. (2003). Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron* 38, 597–609. doi: 10.1016/s0896-6273(03)00260-5
- Rosenberg, S. S., and Spitzer, N. C. (2011). Calcium signaling in neuronal development. *Cold Spring Harb. Perspect. Biol.* 3:a004259. doi: 10.1101/cshperspect.a004259
- Ruiz-Cañada, C., and Budnik, V. (2006). Introduction on the use of the *Drosophila* embryonic/larval neuromuscular junction as a model system to study synapse development and function and a brief summary of pathfinding and target recognition. *Int. Rev. Neurobiol.* 75, 1–31. doi: 10.1016/s0074-7742(06)75001-2
- Sanes, J. R., and Lichtman, J. W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* 2, 791–805. doi: 10.1038/35097557
- Sanyal, S., Sandstrom, D. J., Hoeffler, C. A., and Ramaswami, M. (2002). AP-1 functions upstream of CREB to control synaptic plasticity in *Drosophila*. *Nature* 416, 870–874. doi: 10.1038/416870a
- Sekar, A., Bialas, A. R., de Rivera, H., Davis, A., Hammond, T. R., Kamitaki, N., et al. (2016). Schizophrenia risk from complex variation of complement component 4. *Nature* 530, 177–183. doi: 10.1038/nature16549
- Shatz, C. J., and Stryker, M. P. (1988). Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. *Science* 242, 87–89. doi: 10.1126/science.3175636
- Shishido, E., Takeichi, M., and Nose, A. (1998). *Drosophila* synapse formation: regulation by transmembrane protein with Leu-rich repeats, CAPRICIOUS. *Science* 280, 2118–2121. doi: 10.1126/science.280.5372.2118
- Sink, H., and Whittington, P. M. (1991). Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons. *Development* 112, 307–316.
- Song, H. J., Ming, G. L., and Poo, M. M. (1997). cAMP-induced switching in turning direction of nerve growth cones. *Nature* 388, 275–279. doi: 10.1038/40864
- Spitzer, N. C., Lautermilch, N. J., Smith, R. D., and Gomez, T. M. (2000). Coding of neuronal differentiation by calcium transients. *Bioessays* 22, 811–817. doi: 10.1002/1521-1878(200009)22:9<811::AID-BIES6>3.3.CO;2-7
- Sretavan, D. W., Shatz, C. J., and Stryker, M. P. (1988). Modification of retinal ganglion cell axon morphology by prenatal infusion of tetrodotoxin. *Nature* 336, 468–471. doi: 10.1038/336468a0
- Stent, G. S. (1973). A physiological mechanism for Hebb's postulate of learning. *Proc. Natl. Acad. Sci. U S A* 70, 997–1001. doi: 10.1073/pnas.70.4.997
- Takeo, S., Swanson, S. K., Nandanan, K., Nakai, Y., Aigaki, T., Washburn, M. P., et al. (2012). Shaggy/glycogen synthase kinase 3 β and phosphorylation of Sarah/regulator of calcineurin are essential for completion of *Drosophila* female meiosis. *Proc. Natl. Acad. Sci. U S A* 109, 6382–6389. doi: 10.1073/pnas.1120367109
- Tang, G., Gudsnek, K., Kuo, S. H., Cotrina, M. L., Rosoklija, G., Sosunov, A., et al. (2014). Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. *Neuron* 83, 1131–1143. doi: 10.1016/j.neuron.2014.07.040
- Togashi, K., von Schimmelmann, M. J., Nishiyama, M., Lim, C. S., Yoshida, N., Yun, B., et al. (2008). Cyclic GMP-gated CNG channels function in Semaphorin 3A-induced growth cone repulsion. *Neuron* 58, 694–707. doi: 10.1016/j.neuron.2008.03.017
- Vonhoff, F., and Keshishian, H. (2017). Cyclic nucleotide signaling is required during synaptic refinement at the *Drosophila* neuromuscular junction. *Dev. Neurobiol.* 77, 39–60. doi: 10.1002/dneu.22407
- Vonhoff, F., Kuehn, C., Blumenstock, S., Sanyal, S., and Duch, C. (2013). Temporal coherency between receptor expression, neural activity and AP-1-dependent transcription regulates *Drosophila* motoneuron dendrite development. *Development* 140, 606–616. doi: 10.1242/dev.089235

- Wei, W., Hamby, A. M., Zhou, K., and Feller, M. B. (2011). Development of asymmetric inhibition underlying direction selectivity in the retina. *Nature* 469, 402–406. doi: 10.1038/nature09600
- White, B. H., Osterwalder, T. P., Yoon, K. S., Joiner, W. J., Whim, M. D., Kaczmarek, L. K., et al. (2001). Targeted attenuation of electrical activity in *Drosophila* using a genetically modified K⁺ channel. *Neuron* 31, 699–711. doi: 10.1016/s0896-6273(01)00415-9
- Wiesel, T. N., and Hubel, D. H. (1963). Effects of visual deprivation on morphology and physiology of cells in the cats lateral geniculate body. *J. Neurophysiol.* 26, 978–993.
- Winberg, M. L., Mitchell, K. J., and Goodman, C. S. (1998). Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins and IgCAMs. *Cell* 93, 581–591. doi: 10.1016/s0092-8674(00)81187-3
- Wong, R. O. (1999). Retinal waves and visual system development. *Annu. Rev. Neurosci.* 22, 29–47. doi: 10.1146/annurev.neuro.22.1.29
- Wong, C. O., Chen, K., Lin, Y. Q., Chao, Y., Duraine, L., Lu, Z., et al. (2014). A TRPV channel in *Drosophila* motor neurons regulates presynaptic resting Ca²⁺ levels, synapse growth, and synaptic transmission. *Neuron* 84, 764–777. doi: 10.1016/j.neuron.2014.09.030
- Xu, H., Leinwand, S. G., Dell, A. L., Fried-Cassorla, E., and Raper, J. A. (2010). The calmodulin-stimulated adenylate cyclase ADCY8 sets the sensitivity of zebrafish retinal axons to midline repellents and is required for normal midline crossing. *J. Neurosci.* 30, 7423–7433. doi: 10.1523/JNEUROSCI.0699-10.2010
- Yamamoto, N., and López-Bendito, G. (2012). Shaping brain connections through spontaneous neural activity. *Eur. J. Neurosci.* 35, 1595–1604. doi: 10.1111/j.1460-9568.2012.08101.x
- Yonehara, K., Balint, K., Noda, M., Nagel, G., Bamberg, E., and Roska, B. (2011). Spatially asymmetric reorganization of inhibition establishes a motion-sensitive circuit. *Nature* 469, 407–410. doi: 10.1038/nature09711
- Zheng, J. Q., and Poo, M. M. (2007). Calcium signaling in neuronal motility. *Annu. Rev. Cell Dev. Biol.* 23, 375–404. doi: 10.1146/annurev.cellbio.23.090506.123221
- Zhong, Y., Budnik, V., and Wu, C. F. (1992). Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. *J. Neurosci.* 12, 644–651.

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Neural Control of Startle-Induced Locomotion by the Mushroom Bodies and Associated Neurons in *Drosophila*

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Startle-induced locomotion is commonly used in *Drosophila* research to monitor locomotor reactivity and its progressive decline with age or under various neuropathological conditions. A widely used paradigm is startle-induced negative geotaxis (SING), in which flies entrapped in a narrow column react to a gentle mechanical shock by climbing rapidly upwards. Here we combined *in vivo* manipulation of neuronal activity and splitGFP reconstitution across cells to search for brain neurons and putative circuits that regulate this behavior. We show that the activity of specific clusters of dopaminergic neurons (DANs) afferent to the mushroom bodies (MBs) modulates SING, and that DAN-mediated SING regulation requires expression of the DA receptor Dop1R1/Dumb, but not Dop1R2/Damb, in intrinsic MB Kenyon cells (KCs). We confirmed our previous observation that activating the MB $\alpha'\beta'$, but not $\alpha\beta$, KCs decreased the SING response, and we identified further MB neurons implicated in SING control, including KCs of the γ lobe and two subtypes of MB output neurons (MBONs). We also observed that co-activating the $\alpha\beta$ KCs antagonizes $\alpha'\beta'$ and γ KC-mediated SING modulation, suggesting the existence of subtle regulation mechanisms between the different MB lobes in locomotion control. Overall, this study contributes to an emerging picture of the brain circuits modulating locomotor reactivity in *Drosophila* that appear both to overlap and differ from those underlying associative learning and memory, sleep/wake state and stress-induced hyperactivity.

Keywords: dopamine, mushroom bodies, startle-induced negative geotaxis, neural circuits, *Drosophila melanogaster*

INTRODUCTION

The identification of neural circuits that modulate innate or reflex behaviors is essential to better understand how the brain functions and adapts to a changing environment (LeBeau et al., 2005; Dickinson, 2006; Marder, 2012; Su and Wang, 2014). *Drosophila* is an advantageous organism for studying the neural basis of behavior using genetically-encoded probes that enable *in vivo* control of neuronal activity (White and Peabody, 2009; Griffith, 2012; Yoshihara and Ito, 2012; Kazama, 2015; Oswald et al., 2015b; Riemensperger et al., 2016; Martín and Alcorta, 2017). In this

organism, spontaneous locomotor activity and locomotor reactivity have been described as two separate behavioral systems that are regulated differently (Connolly, 1967; Meehan and Wilson, 1987; O'Dell and Burnet, 1988; Martin et al., 1999a). A sudden external stimulus (startle) usually triggers inhibition or arrest of spontaneous locomotion followed by an appropriate behavioral response, which may itself be a locomotor reaction. Startle-induced reactivity has long been used in *Drosophila* to monitor various behavioral performances, such as phototaxis (Benzer, 1967) or negative geotaxis (Miquel et al., 1972). A widely used paradigm relies on the fast climbing reaction initiated by a gentle mechanical shock of flies entrapped in a vial or a narrow column, an innate reflex called startle-induced negative geotaxis (SING). SING performance progressively declines with age (Ganetzky and Flanagan, 1978; Le Bourg and Lints, 1992; Grotewiel et al., 2005; White et al., 2010; Jones and Grotewiel, 2011; Vaccaro et al., 2017), in contrast to spontaneous locomotion that does not vary during the adult life and even increases in old flies (White et al., 2010). The SING reflex is also progressively altered in various mutant or under neuropathological conditions, as is the case in *Drosophila* models of Parkinson disease (Feany and Bender, 2000; Coulom and Birman, 2004; Chaudhuri et al., 2007; Riemensperger et al., 2013; Bou Dib et al., 2014). It is therefore of particular interest to identify precise neural components underlying the modulation of startle-induced locomotion, in *Drosophila* as in other species (Hale et al., 2016).

The mushroom body (MB) is a paired structure of the insect brain that has important behavioral functions, including the formation of olfactory memory (Heisenberg, 2003; Fiala, 2007; Davis, 2011; Kahsai and Zars, 2011; Waddell, 2013) and the control of sleep (Bushey and Cirelli, 2011; Tomita et al., 2017). The *Drosophila* MB is composed of intrinsic neurons known as Kenyon cells (KCs) and it is innervated by afferent modulatory neurons, in particular subsets of dopaminergic neurons (DANs), as well as efferent MB output neurons (MBONs) (Tanaka et al., 2008; Pech et al., 2013a; Aso et al., 2014a,b). The cell bodies of the KCs form a large cluster in the dorsal posterior brain; their dendritic branches make up the calyx and their axons bundle up in the peduncles. The KCs are named according to the lobes in which they send axonal projections: $\alpha\beta$, $\alpha'\beta'$, and γ (Lee et al., 1999; Tanaka et al., 2008). At the distal end of the peduncles, the axons of the $\alpha\beta$ and $\alpha'\beta'$ KCs bifurcate dorsally and medially to form the vertical (α and α') and horizontal (β and β') lobes, while the γ KCs form only the γ horizontal lobes.

Around 60 years ago, experiments carried out on crickets provided the first evidence that the insect MB contains neurons inhibiting locomotion (Huber, 1960, 1967; Howse, 1975). In

Drosophila, both the *mushroom body miniature* mutation or chemical ablation of the MB increased walking activity when measured over long time intervals, confirming that the MB normally suppresses locomotor behavior (Heisenberg et al., 1985; Martin et al., 1998; Helfrich-Förster et al., 2002), while similar experiments suggested that, by contrast, the MB stimulates initial stages of walking activity (Serway et al., 2009). Neuroanatomical defects in the MB lobes were observed in a set of mutants giving rise to changes in startle-induced locomotion behavior, but without a clear correlation between the two phenotypes (Yamamoto et al., 2008). Furthermore, we previously reported that SING is controlled by the activity of the $\alpha'\beta'$ KCs (Riemensperger et al., 2013). Determining the precise contributions of the various subtypes of MB neurons to startle-induced locomotion required, therefore, further investigations.

Dopamine (DA) is an important neurotransmitter that, in flies, was implicated in the modulation of diverse behaviors including appetitive or aversive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005, 2011; Schroll et al., 2006; Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010; Waddell, 2010; Berry et al., 2012; Burke et al., 2012; Plaçais et al., 2012; Cohn et al., 2015; Musso et al., 2015; Aso and Rubin, 2016; Yamagata et al., 2016) and sleep-wake mechanisms (Van Swinderen and Andretic, 2011; Liu et al., 2012b; Ueno et al., 2012; Berry et al., 2015; Sitaraman et al., 2015b; Pimentel et al., 2016). It is also well established that DA prominently controls locomotor activity in *Drosophila* (Yellman et al., 1997; Bainton et al., 2000; Friggi-Grelin et al., 2003; Kume et al., 2005; Lima and Miesenböck, 2005; Wu et al., 2008; Lebestky et al., 2009; Kong et al., 2010; Riemensperger et al., 2011; Van Swinderen and Andretic, 2011) as it does in vertebrates (Beninger, 1983; Zhou and Palmiter, 1995; Giros et al., 1996; Blum et al., 2014). We have recently reported that the degeneration of DANs of either the protocerebral anterior medial (PAM) or protocerebral posterior lateral 1 (PPL1) clusters afferent to the MBs was associated with an accelerated decline of SING performance in aging flies (Riemensperger et al., 2013; Vaccaro et al., 2017). Further recent studies support a function for the PAM and PPL1 clusters in climbing or flight control (Bou Dib et al., 2014; Agrawal and Hasan, 2015; Pathak et al., 2015). However, the role of these and other DANs in SING modulation has not yet been precisely investigated.

Here we used activation or silencing of synaptic transmission in neuronal subsets targeted with selective drivers in order to identify the MB-associated neurons (KCs, DANs, and MBONs) that control startle-induced locomotion in *Drosophila*. Neuronal activation revealed that several classes of DANs projecting to the MBs have diverse roles in modulatory mechanisms. We show that DANs in the PPL1 cluster act as inhibitory neurons in the SING-modulating circuits, while the PAM cluster appears to contain both inhibitory and excitatory DAN subsets. We also confirm that MB $\alpha'\beta'$ KCs are implicated in SING control and demonstrate that γ KCs are involved in this modulation as well. Interestingly, we find that $\alpha'\beta'$ and γ neuron-mediated SING modulation is antagonized by co-activating the $\alpha\beta$ KCs. Finally, we show that the MBONs M4/M6 and V2 are part of the network, suggesting that they convey SING modulatory information to downstream motor circuits. Overall,

Abbreviations: CRE, crepine; DA, dopamine; DAN, dopaminergic neuron; Dop1R1, Dopamine 1-like receptor 1; Dop1R2, Dopamine 1-like receptor 2; EB, ellipsoid body; dFSB, dorsal fan-shaped body; GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; KC, Kenyon cell; MB, mushroom body; MBON, MB output neuron; msGFP, mCD8::GFP, n-syb::GFP; PAL, protocerebral anterior lateral; PAM, protocerebral anterior medial; PI, performance index; PPL, protocerebral posterior lateral; PPM, protocerebral posterior medial; RNAi, RNA interference; rsGFP, reconstituted splitGFP; SING, startle-induced negative geotaxis; SIP, superior intermediate protocerebrum; SLP, superior lateral protocerebrum; SMP, superior medial protocerebrum; TH, tyrosine hydroxylase.

this work provides a first picture of the brain network and modulatory mechanisms controlling startle-induced locomotion in *Drosophila* that centrally involve a subset of MB-associated neurons.

MATERIALS AND METHODS

Drosophila Culture and Strains

Fly stocks were raised and crossed at 25°C on the standard corn meal/yeast/agar medium supplemented with methyl-4-hydroxybenzoate as a mold protector, under a 12 h/12 h light-dark cycle. The following effector lines were used: *UAS-mCD8::GFP*, *UAS-n-syb::GFP* (here named *UAS-msGFP*) (Riemensperger et al., 2013), *UAS-shi^{ts1}* (Kitamoto, 2001), *UAS-dTrpA1* (Hamada et al., 2008), *UAS-ChR2-XXL* (Dawydow et al., 2014), *LexAop-dTrpA1* (Burke et al., 2012), *UAS-Dumb-RNAi* (Bloomington *Drosophila* Stock Center, line 62193), *UAS-Damb-RNAi* (Vienna *Drosophila* RNAi center, line v3391) (Cassar et al., 2015), *UAS-n-syb::spGFP₁₋₁₀*, *LexAop-CD4::spGFP₁₁/CyO* and *LexAop-n-syb::spGFP₁₋₁₀*, *UAS-CD4::spGFP₁₁* (Bloomington *Drosophila* Stock Center, lines 64314 and 64315) (Macpherson et al., 2015). The driver lines used and their brain expression patterns are described in Table S1. Except for those that were generated in our laboratories, these lines were either obtained from the Bloomington *Drosophila* Stock Center or kindly provided by: Ronald L. Davis (*TH-LexA*, Berry et al., 2015), Thomas Preat and Pierre-Yves Plaçais (*4-59-Gal4*, *238Y-Gal4*, *G0050-Gal4*, *NP2758-Gal4*, *R71D08-Gal4*, *NP2492-Gal4*, *R27G01-Gal4*, *R14C08-LexA*), Hiromu Tanimoto (*R58E02-Gal4*, Liu et al., 2012a) and Mark Wu (*TH-C1-Gal4*, *TH-C'-Gal4*, and *TH-D'-Gal4*) (Liu et al., 2012b).

Locomotion Assay Coupled With Genetic Manipulation of Neuronal Activity

SING assays were generally carried out following thermogenetic inhibition or activation of neuronal activity. Seven- to ten-day-old flies expressing *Shi^{ts1}* or *dTrpA1*, respectively, or *msGFP* as a control, in neuronal subsets, were kept at 19°C overnight. The next day, groups of 10 flies of the same genotype were placed in a vertical column (25 cm long, 1.5 cm diameter) with a conic bottom end, and left for about 20 min at 19°C for habituation. Thermogenetic activation or silencing of neurons was performed by incubating each column for 10 min at 32°C, or at 23°C for control of a potential temperature effect. SING assays were carried out immediately afterwards at 23°C as previously described (Coulom and Birman, 2004; Riemensperger et al., 2013). Briefly, flies were suddenly startled by gently tapping them down. After 1 min, flies having reached the top of the column (above 22 cm) and flies remaining at the bottom end (below 4 cm) were separately counted. Three rounds of test were performed in a row per column. Results are the mean $\pm$ SEM of the scores obtained with ten groups of flies per genotype. The performance index (PI) is defined as $\frac{1}{2}[(n_{\text{tot}} + n_{\text{top}} - n_{\text{bot}})/n_{\text{tot}}]$, where n_{tot} is the total number of flies, and n_{top} and n_{bot} the number of flies at the top and at the bottom, respectively.

In some experiments, optogenetic photostimulation was performed instead on 7 to 10-day-old flies expressing the

channelrhodopsins *ChR2-XXL* (Dawydow et al., 2014) in neuronal subsets. In this case, flies were kept in constant darkness, and all manipulations before the SING assay were done under dim red light. The transparent columns were introduced in a dark box and illuminated during locomotion testing with either blue-light diodes (peak wavelength 468 nm) from two sides (intensity range 6–11 $\times 10^3$ Lux), or red light as a control. Six rounds of tests were performed in a row per column, 3 under red light and 3 under blue light. Further details on the SING assay procedure under optogenetic photostimulation are provided in the legends to Figures S2A,B.

Immunohistochemistry

Adult brains were dissected in ice-cold *Drosophila* Ringer's solution and processed for whole mount immunostaining as previously described (Riemensperger et al., 2011). The primary antibodies were mouse anti-GFP (ThermoFisher Scientific 33-2600, 1:500 for *msGFP* detection or Sigma-Aldrich G6539, 1:200 for reconstituted splitGFP (*rsGFP*) detection) and rabbit anti-TH (Novus Biologicals NB300-109, 1:1,000). The secondary antibodies were goat anti-mouse and anti-rabbit conjugated to Alexa fluor 488 or 555 (Invitrogen Molecular Probes, 1:1,000). The brains were mounted in ProLong Gold Antifade reagent (ThermoFisher Scientific). Images were acquired with a Nikon A1R confocal microscope and processed using the Fiji software (Schindelin et al., 2012).

For the quantification of Gal4 expression patterns in KC subpopulations, the brains of 5–7 day-old female flies expressing *mCD8::GFP* under the control of different Gal4 drivers were dissected in ice cold Ringer's solution, fixed for 2 h on ice in 4% paraformaldehyde and washed 3 $\times$ 20 min in phosphate-buffered saline + 0.6 % Triton X-100 (PBSTx). After a 2 h pre-incubation in PBSTx + 2% bovine serum albumin, brains were incubated overnight at 4°C in the same buffer with mouse monoclonal anti-Bruchpilot antibody (1:10, nc82, Developmental Studies Hybridoma Bank) to visualize synaptic neuropils. After 3 $\times$ 20 min washes in PBSTx, samples were incubated for 2 h with Cy3-conjugated anti-mouse secondary antibody (1:300, Jackson ImmunoResearch), then washed 3 $\times$ 20 min in PBSTx and additionally overnight in PBS. Brains were mounted in Vectashield (Vector Laboratories) and scanned using a Leica SP8 confocal laser scanning microscope equipped with hybrid detectors. Quantification of Gal4-expressing KC somata was conducted by monitoring GFP autofluorescence with the Fiji Cell Counter plugin across the focal planes.

Split-GFP Reconstitution

For the visualization of potential synaptic connectivity with the GRASP method (Feinberg et al., 2008; Gordon and Scott, 2009; Pech et al., 2013a; Macpherson et al., 2015), the *Drosophila* line *LexAop-n-syb::spGFP₁₋₁₀*, *UAS-CD4::spGFP₁₁* was crossed to the recombined driver line *NP2492-Gal4*; *TH-LexA* (MBON-V2 and DANs), and the line *UAS-n-syb::spGFP₁₋₁₀*, *LexAop-CD4::spGFP₁₁* was crossed to the recombined driver lines *R14C08-LexA*; *R58E02-Gal4* (MBON-M4/M6 and PAM DANs) and *NP2492-Gal4*; *R14C08-LexA* (MBON-V2 and MBON-M4/M6). 7–10 day-old female flies were collected for brain

dissection followed by whole-mount brain immunostaining as described in the previous paragraph.

Statistics

All statistical analyses were performed with the GraphPad Prism 6 software. Data from locomotor assays were analyzed using two-way ANOVA with Bonferroni's or Tukey's *post-hoc* tests for multiple comparisons. All data are presented as mean $\pm$ SEM. Significant values in all figures: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RESULTS

Activation of *TH-Gal4*-Targeted DANs Inhibits Fly Locomotor Reactivity to Startle

To determine the effect of DAN inhibition or activation on SING response, we first used *TH-Gal4*, a driver that expresses selectively in brain DANs, except in the PAM cluster where it only labels 12 DANs out of ~ 90 in total (Friggi-Grelin et al., 2003; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010; White et al., 2010; Pech et al., 2013a). We crossed *TH-Gal4* with *UAS-shi^{ts1}* flies to express the thermosensitive variant of *Drosophila* Dynamin *Shi^{ts1}* (Kitamoto, 2001) that blocks neurotransmitter release above 30°C (Kitamoto, 2001), in DANs of the progeny. After a 10-min incubation, these *TH>shi^{ts1}* flies showed no difference in SING performance between the permissive (23°C) and restrictive (32°C) temperatures, indicating that *TH-Gal4*-targeted DANs are not required for the execution of this locomotor response (Figure 1A). We checked that the *UAS-shi^{ts1}* transgene was active by expressing *Shi^{ts1}* in all neurons with *elav-Gal4*, which led to fly paralysis at the restrictive temperature (data not shown). Flies expressing a membrane-associated form of GFP (msGFP, described in section Materials and Methods) in *TH-Gal4* DANs neither showed any difference in SING performance between the two temperatures. This indicates that temperature by itself had no significant effect on the test (Figure 1A). In contrast, expressing the heat-inducible cation channel *dTrpA1* (Hamada et al., 2008) in *TH-Gal4*-targeted DANs (*TH>dTrpA1* flies) led to altered SING performance after activation at 32°C, which was decreased to $\sim 20\%$ of the 23°C control value (Figure 1A). After 10 min of neuronal thermoactivation, *TH>dTrpA1* flies were in fact very active without any inhibition of their spontaneous locomotion (Movie S1). After the startle, most of these thermoactivated flies stayed at the bottom of the column and a few climbed up to the middle and stopped (Movie S2), while in the absence of neuronal thermoactivation, these same flies generally climbed to the top of the column quickly like wild-type flies (Movie S3). This indicates that DANs labeled by *TH-Gal4* inhibit the SING response i.e., locomotor reactivity, but not spontaneous locomotion, when they are stimulated.

In order to better characterize this behavioral modulation, we have monitored the SING performance of *TH>dTrpA1* flies after various times of incubation at 32°C (Figure S1A). We observed that 2 min were required for the temperature inside the column to reach above 30°C. Nevertheless, a decrease in SING performance could be observed after only 1 min of incubation, indicating that this modulation is actually rapid. SING performance continued to decrease until ~ 5 min of

DAN thermoactivation, after what it remained stable at a low value (Figure S1A). Next, we checked whether DAN activation triggered during the climbing test could modulate as well SING behavior. We used for that optogenetic photostimulation in order to activate neurons instantly without the latency of thermoactivation, by expressing in DANs the channelrhodopsin *ChR2-XXL* (Dawydow et al., 2014; Riemensperger et al., 2016; Figures S2A,B). We first tested the efficiency of the system by expressing these optogenetic effectors in all GABAergic neurons with *Gad-Gal4*. As expected, blue light but not red light illumination after startle prevented *Gad>ChR2-XXL* flies from climbing (data not shown). Next we tested optogenetic stimulation of the DANs. We found that illuminating *TH>ChR2-XXL* flies with blue light, but not red light, during the test, i.e., within less than 1 min, was sufficient to reduce significantly their SING performance by $\sim 22\%$ (Figure S2C). These results indicate that DAN-mediated SING modulation is a fast and physiologically-relevant process.

DANs in the PAM Cluster Are Also Involved in SING Modulation

Because the *TH-Gal4* pattern excludes a large part of the PAM clusters, we used the *R58E02-Gal4* driver that labels $\sim 80\%$ of the PAM DANs (Liu et al., 2012a; Pech et al., 2013a) to investigate the role of this cluster in SING modulation. Again, no effect of temperature was detected in control *R58E02>msGFP* flies expressing msGFP in the PAM neurons (Figure 1B). Stimulating PAM DANs activity by *dTrpA1* caused no inhibitory effect on fly locomotion, whereas blocking output from these neurons with *Shi^{ts1}* led to a small but statistically significant increase in SING performance at 32°C compared to 23°C (Figure 1B). This result suggests that the PAM clusters contain neurons that inhibit locomotor reactivity. These neurons appear spontaneously active during the test because their blockade by *Shi^{ts1}* increased SING while their stimulation by *dTrpA1* did not lead to any effect. Indeed, it has recently been shown that some PAM DANs are spontaneously active (Yamagata et al., 2016).

We then constructed a double-driver strain containing both *TH-Gal4* and *R58E02-Gal4*. We checked that this double driver labeled all brain DANs, including the PAM clusters, by expressing msGFP and comparing to the pattern of the *R58E02-Gal4* strain (Figure 1D). Like with *TH-Gal4* alone, *TH, R58E02>shi^{ts1}* and *TH, R58E02>msGFP* flies showed similar SING performance at low and high temperatures. In contrast, *TH, R58E02>dTrpA1* flies showed at 32°C a climbing performance that was reduced to $\sim 33\%$ of the 23°C control value (Figure 1C), an effect that was slightly but significantly lower compared with the decrease observed in a parallel experiment with *TH-Gal4* alone ($19.7 \pm 4.5\%$ vs. $33.1 \pm 4.0\%$ of the 23°C control for *TH>dTrpA1* and *TH, R58E02>dTrpA1* flies, respectively). This result suggests that PAM neuron co-stimulation somewhat offsets the inhibition of locomotor reactivity induced by *TH-Gal4*. It therefore seems that the PAM clusters contain not only neurons that constitutively inhibit locomotor reactivity, but also neurons that, on the other hand, increase SING when stimulated. The PAM clusters are known, indeed, to include functionally heterogeneous subsets of DANs (Liu et al., 2012a; Waddell, 2013).

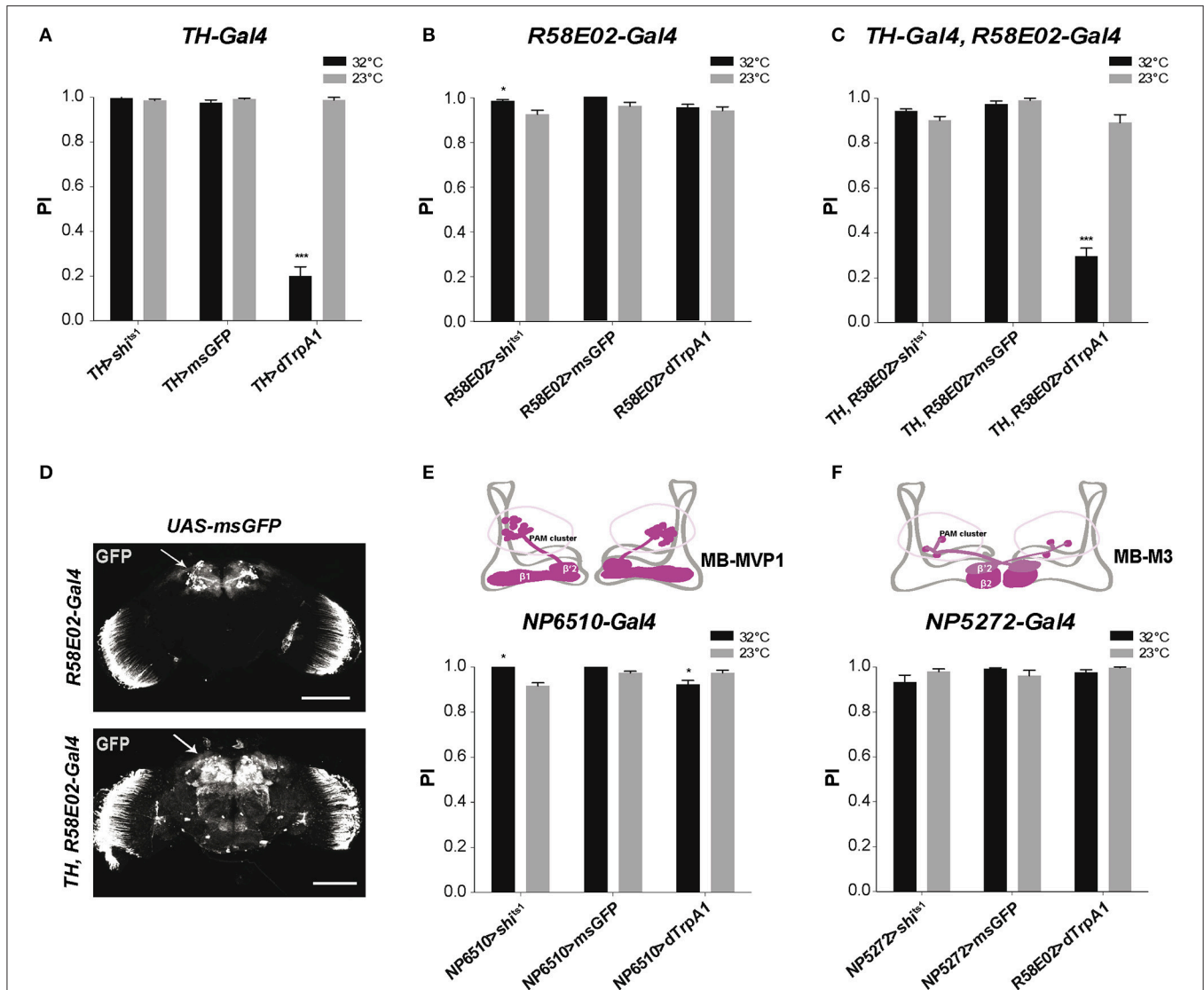


FIGURE 1 | Differential modulation of *Drosophila* locomotor reactivity by brain DANs. **(A)** Thermoactivation of *TH-Gal4*-targeted neurons reduced SING performance of *TH>dTrpA1* flies at 32°C compared to the 23°C control. Expression of *Shi^{ts1}* or membrane-associated GFP (*msGFP*) had no consequence at 32°C, indicating that neither blocking neurotransmitter release in these neurons or temperature rise by itself alters SING. PI: performance index. **(B)** Thermoactivation of PAM neurons targeted by *R58E02-Gal4* (*R58E02>shi^{ts1}* flies) at 32°C increased SING performance compared to the 23°C control, while thermoactivation of these neurons or temperature rise by itself (*R58E02>dTrpA1* and *R58E02>msGFP* flies, respectively) had no significant effects. **(C)** dTrpA1-mediated activation of all brain DANs using the *TH-Gal4*, *R58E02-Gal4* double driver decreased SING performance slightly less than *TH-Gal4* alone (shown in **A**) in parallel experiments ($p < 0.1$). Blocking with *Shi^{ts1}* synaptic output of all DANs at the restrictive temperature did not increase SING performance in contrast to the effect of *R58E02-Gal4* alone (shown in **B**). **(D)** Patterns of *R58E02-Gal4* and of the double driver *TH-Gal4*, *R58E02-Gal4* in the adult brain revealed by the expression of *msGFP*. The double driver labels all DANs including the PAM clusters (arrows). Scale bars represent 100 μm. **(E)** Thermogenetic inhibition or activation of *NP6510-Gal4*-targeted neurons increased and slightly decreased SING, respectively. This driver expresses in 15 PAM DANs including MB-MVP1 that project to the β1 and β'2 compartments in the horizontal lobes of the MBs (inset scheme) plus 3 non-DANs that target the fan-shaped body. **(F)** Inhibition or activation of PAM MB-M3 neurons targeted by *NP5272-Gal4* that project to the MBs in β2 and, more faintly, in β'2 (inset scheme) had no effect on SING performance. **(A–C,E,F)** Two-way ANOVA with Bonferroni's multiple comparisons tests ($*p < 0.05$; $***p < 0.001$).

The driver *NP6510-Gal4* expresses in 15 PAM DANs that are not labeled by *TH-Gal4* and that project to the MB horizontal lobe β1 and β'2 compartments (Figure 1E; Aso et al., 2010; Riemensperger et al., 2013). We previously showed that the degeneration of these 15 DANs induced by mutant α-synuclein accumulation led to progressive SING defects that were as

strong as those observed by expressing mutant α-synuclein in all neurons of the fly (Riemensperger et al., 2013). This suggested that *NP6510-Gal4* DANs could be involved in SING modulation. *NP6510>shi^{ts1}* flies showed indeed a slight increase in SING performance at the restrictive temperature, similar to the effect observed with *R58E02-Gal4*, whereas dTrpA1-induced

thermostimulation of these neurons by contrast led to decreased SING response (**Figure 1E**). No such effects were observed with *NP5272-Gal4* that expresses in three PAM cells involved in aversive odor memory, the MB-M3 neurons, which innervate the tip of the MB horizontal lobes ($\beta 2$ and $\beta'2$ compartments) and are labeled by *TH-Gal4* (Aso et al., 2010; **Figure 1F**). Neither did a *NP6510-Gal4*, *R58E02-Gal80* recombinant driver that expresses only in three *NP6510*-targeted non-DANs have any effect on SING (data not shown). Our results suggest, therefore, that the PAM neurons that inhibit SING correspond to the *NP6510*-targeted DANs or a subset of these cells.

MB-Afferent DANs of the PPL1 Clusters Inhibit the SING Response

We recently reported that the progressive degeneration of DANs in the PPL1 clusters induced by a mutation of the circadian gene *Clock* severely accelerates age-related SING decline (Vaccaro et al., 2017). To identify whether PPL1 plays a direct role in SING modulation, we employed two drivers that label specific neurons in this cluster: *Mz840-Gal4* labeling the MB-V1 neuron that projects to MB dorsal lobes $\alpha 2$, $\alpha'2$ compartments and *NP2758-Gal4* that expresses in the MB-MP1 neuron sending projection to the $\gamma 1$ peduncle (**Figures 2A,B**; Aso et al., 2010, 2012). Whereas, the inhibition of the neurons targeted by each of these drivers had no effect on SING, their thermoactivation significantly decreased performance of the flies to around 41 and 78% of the 23°C control value for *Mz840-Gal4* and *NP2758-Gal4*, respectively (**Figures 2A,B**). We then used the driver *TH-D'-Gal4* (Liu et al., 2012b) that expresses strongly in the PPL1 cluster (**Figure 2C**). SING performance of *TH-D'>dTrpA1* flies at 32°C was markedly reduced to ~16% of the 23°C control (**Figure 2C**), an effect comparable to that of *TH-Gal4* itself (see **Figure 1A**). However, *TH-D'-Gal4* expresses in other DAN clusters than the PPL1 such as PPM2 and PPM3 (Liu et al., 2012b) that could contribute as well to SING modulation.

DANs Localized in Other Clusters Are Also Implicated in SING Regulation

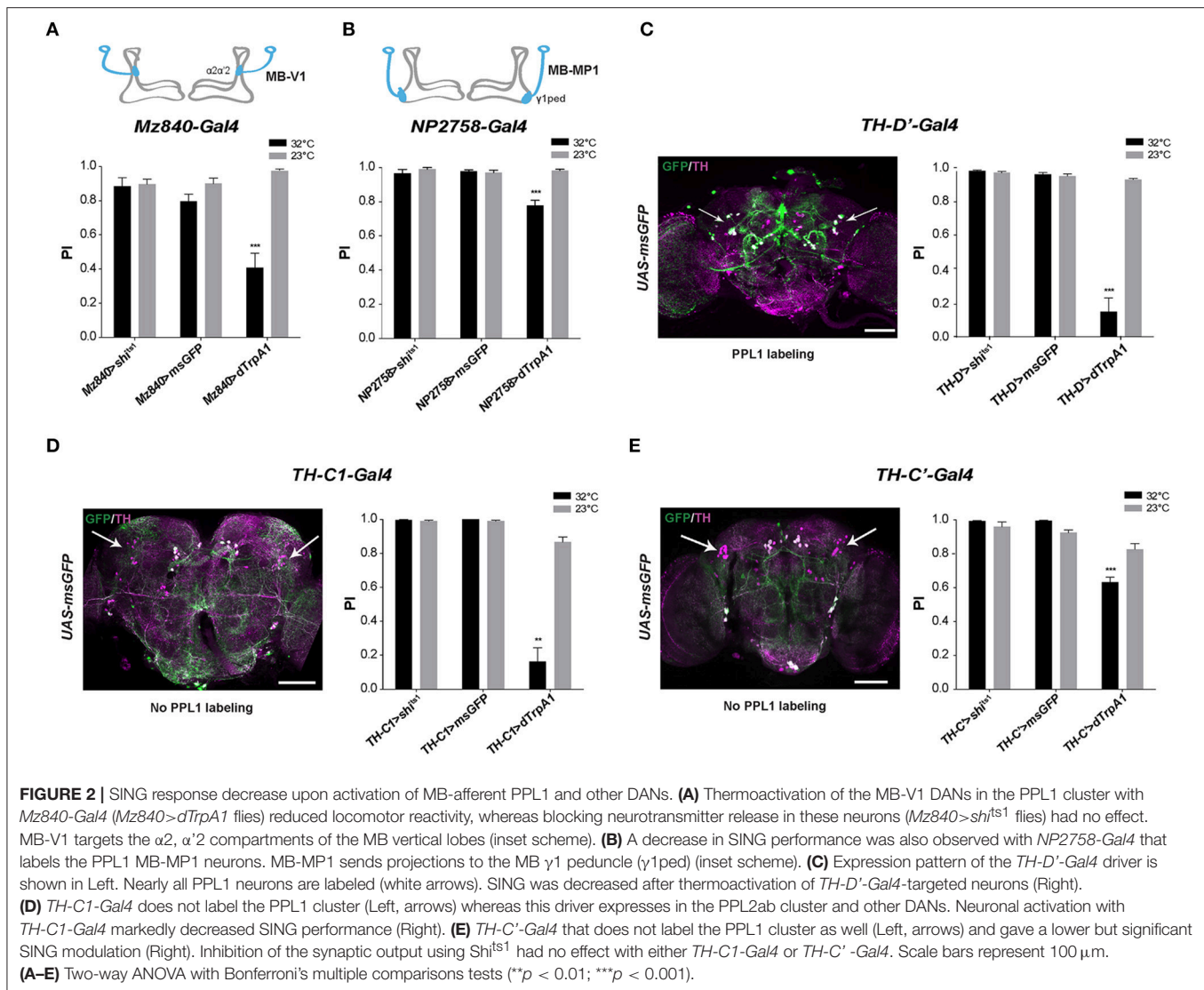
To determine whether other DANs modulate the SING response, we selected two drivers, *TH-C1-Gal4* and *TH-C'-Gal4*, both of which do not express in the PPL1 (Liu et al., 2012b). We first verified that the PPL1 clusters were not labeled by these drivers (**Figures 2D,E**, left). The use of these drivers did not cause any effect on SING upon synaptic blockade with *Shi^{ts1}* but induced down-regulation of SING upon neuronal thermoactivation, which was strong with *TH-C1-Gal4* (18% of the 23°C control) (**Figure 2D**, right) and lower, but still significant, with *TH-C'-Gal4* (77% of the control) (**Figure 2E**, right). Both drivers express similarly in the protocerebral anterior medial (PAL), PPL2 and PPM2 DAN clusters, indicating that some of these clusters, and possibly the PPL2ab neurons that project to the MB calyx (Mao and Davis, 2009), could also be involved in SING modulation. Overall, our results suggest that several brain DAN subsets have the ability to hinder SING behavior when activated or inhibited, indicating that DA-mediated modulation of locomotor reactivity is an important and complex process in the insect brain.

Activation of MB $\alpha'\beta'$ and γ Neurons Decreases SING Performance

We previously reported that SING performance was decreased when synaptic activity in the MB prime ($\alpha'\beta'$) lobes, targeted with *c305a-Gal4*, was either thermogenetically inhibited or stimulated, and that the defect was stronger in the latter case (Riemensperger et al., 2013). We confirmed those results in the present work using either *c305a-Gal4* or *G0050-Gal4*: both drivers did induce SING inhibition at 32°C either with *Shi^{ts1}* or with *dTrpA1* (**Figures 3A,B**). *c305a-Gal4* labels the entire MB $\alpha'\beta'$ lobes and the γ lobes faintly, as well as the antennal lobes, the central complex and other neuropils (Krashes et al., 2007; Pech et al., 2013b), while *G0050-Gal4* selectively labels the $\alpha'\beta'$ lobes in the MB, and also the ellipsoid body and brain glial cells (Lin et al., 2007; Chen et al., 2012). To ascertain the role of the $\alpha'\beta'$ lobes in SING modulation, we used two other drivers, *4-59-Gal4* and *R35B12-Gal4*, that restrictedly express in the MB prime lobes (**Figures 3C,D**, insets). Neuronal activation within *4-59-Gal4*- and *R35B12-Gal4*-labeled KCs decreased SING to around 21 and 54% of the 23°C control value (**Figures 3C,D**), compared to around 12.5% with *c305a-Gal4* and 4% with *G0050-Gal4* (**Figures 3A,B**). In contrast, *Shi^{ts1}* expression with *4-59-Gal4* and *R35B12-Gal4* did not cause any decrease in SING behavior at the restrictive temperature (**Figures 3C,D**). This suggests that the $\alpha'\beta'$ KCs are rather involved in SING inhibition than activation, and that another, still unidentified, targeted neuropile must be responsible for the *Shi^{ts1}*-induced decrease observed with *c305a-Gal4* and *G0050-Gal4* (**Figures 3A,B**).

As mentioned, *c305a-Gal4* expresses in the $\alpha'\beta'$ lobes and in the γ lobes faintly. To further investigate the role of γ lobe KCs, we used the drivers *R16A06-Gal4* and *H24-Gal4* that target selectively γ neurons in the MB. Their expression patterns are shown in **Figure 3G**. We obtained discrepant results. Expressing *dTrpA1* with *R16A06-Gal4* nearly abolished fly locomotor reactivity at 32°C to around 4% of the 23°C control (**Figure 3E**), while the same experiment performed with *H24-Gal4* had no effect on SING (**Figure 3F**). Such a difference prompted us to analyze more precisely the expression patterns of these γ lobe drivers. First, *H24-Gal4* also labels the $\alpha\beta$ lobes slightly in contrast to *R16A06-Gal4* that appears selective for the γ lobes. Second, by counting the labeled MB neurons using two-photon microscopy, we found that *R16A06-Gal4* expresses in around 500 γ lobe KCs per hemisphere while *H24-Gal4* labels around 300 γ neurons only (**Figure 3G**). It is quite possible that *H24-Gal4* does not express in a specific subset of γ KCs involved in SING control that would be in contrast targeted by *R16A06-Gal4*.

The γ lobe driver *R16A06-Gal4* had such a strong effect that we looked more closely at SING modulation in *R16A06>dTrpA1* flies. Kinetics studies showed that the inhibition was fast with this driver indeed, decreasing SING performance to ~10% of controls after only 3 min of thermoactivation (**Figure S1B**). Optogenetic photostimulation of *R16A06>ChR2-XXL* flies during the climbing test was also able to reduce efficiently SING performance by ~30% (**Figure S2D**). Remarkably, at the end of a 10-min thermoactivation period, *R16A06>dTrpA1* flies



were not paralyzed but in contrast very active in the column (Movie S4). After being tapped down, they did not start climbing, possibly because the startle stopped spontaneous locomotion while thermoactivation of the γ lobe prevented their locomotor reactivity (Movie S5). These experiments confirmed that γ lobe activation has a stronger effect on SING than DAN activation.

$\alpha\beta$ Lobe Co-activation Antagonizes SING Modulation by $\alpha\beta'$ and γ KCs

In our previous work, we considered that the $\alpha\beta$ lobe neurons were not involved in SING modulation, because no effect could be seen after synaptic blockade or activation with *mb247-Gal4* that strongly targets the $\alpha\beta$ and γ KCs (Riemensperger et al., 2013). Again, the result with *mb247-Gal4* could be confirmed here (Figure 3H). Similarly, the use of an $\alpha\beta$ -specific driver, *c708a-Gal4*, did not induce any effect on SING (data not shown). Neuronal thermoactivation with *H24-Gal4* did not show any

difference compared to the control, while that of *R16A06-Gal4*-targeted neurons led to a strong SING decrease (Figures 3E–G). Remarkably, both *mb247-Gal4* and *H24-Gal4*, which induce no effect on SING, express both in the $\alpha\beta$ and γ KCs, whereas *R16A06-Gal4* that induce strong effect on SING targets the γ KCs selectively. This led us to the hypothesis that co-activation of $\alpha\beta$ neurons could potentially antagonize SING modulation caused by γ lobe activation.

To test this possibility, a recombined *R16A06-Gal4*, *mb247-Gal4* double driver line was constructed. The pattern of this driver, as characterized by msGFP expression, showed even and strong α , β and γ lobe labeling (Figure 3I). Expressing *dTrpA1* with *R16A06-Gal4* confirmed the decreased fly locomotor reactivity at 32°C (15% of the 23°C control, Figure 3J), while neuronal activation of KCs targeted by the *R16A06-Gal4*, *mb247-Gal4* double driver in a parallel experiment showed remarkably rescued SING response that rose up to 53% of the control in the first round of test (Figure 3J). The response of these flies

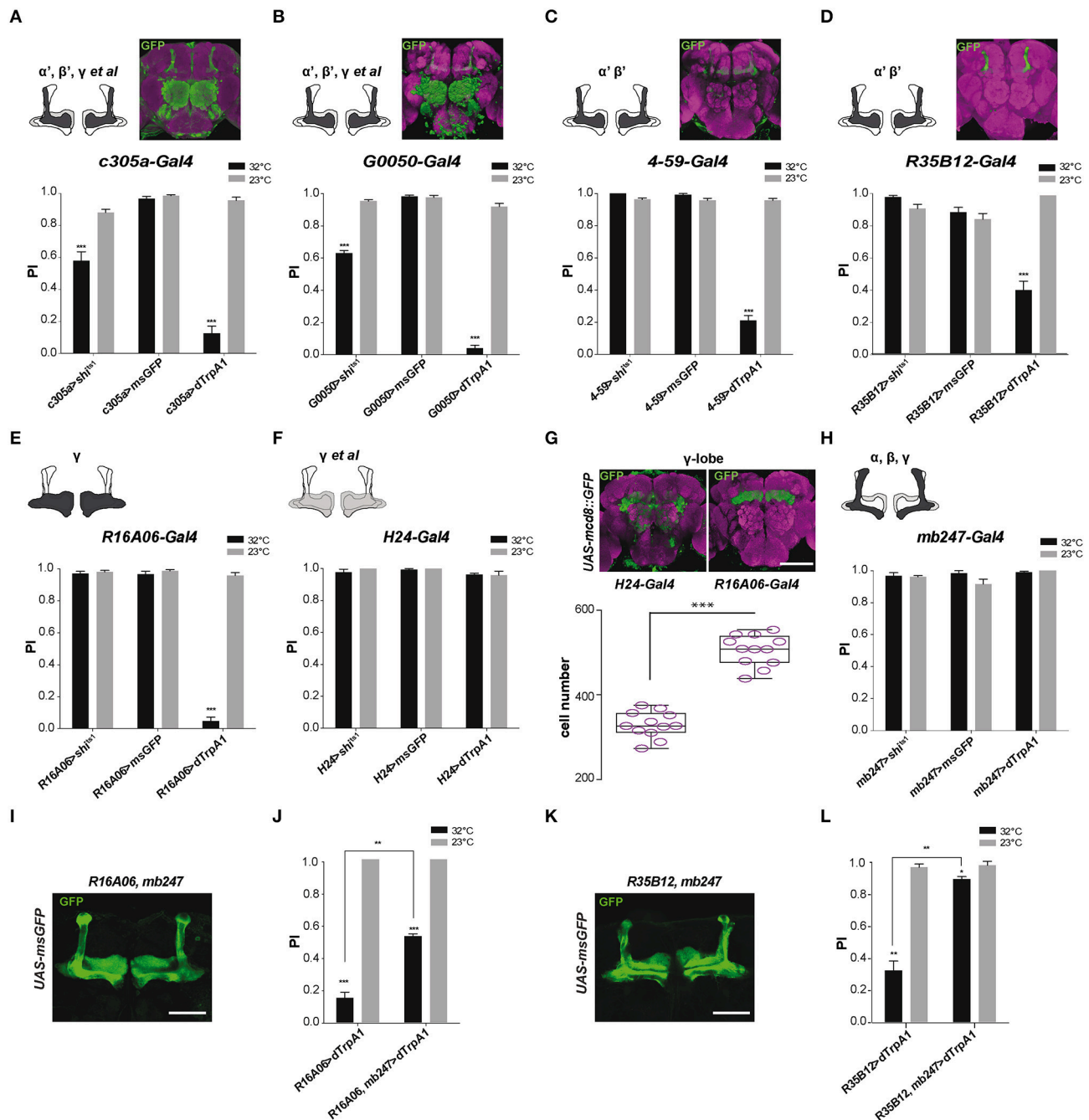


FIGURE 3 | MB $\alpha\beta$ neurons counteract SING modulation induced by $\alpha'\beta'$ and γ neuron activation. (A–D) Effect of drivers targeting $\alpha'\beta'$ lobe KCs. **(A,B)** Thermogenetic activation or synaptic inhibition of neurons labeled with *c305a-Gal4* or *G0050-Gal4* both decreased SING performance, with a stronger effect resulting from their activation. **(C,D)** With *4-59-Gal4* or *R35B12-Gal4*, SING was also reduced upon activation, but not upon block of synaptic output. Note that *c305a-Gal4* and *G0050-Gal4* labels other brain neuropils, whereas *4-59-Gal4* and *R35B12-Gal4* are very specific for the MB prime lobes. **(E,H)** Effect of drivers targeting γ lobe KCs. **(E)** Neuronal activation with the γ driver *R16A06-Gal4* strongly affected SING, while flies had normal response after inhibition of these neurons. **(F)** The use of another γ lobe driver, *H24-Gal4*, did not cause any effect on SING performance. **(G)** Analysis of expressions patterns in the brain indicates that *R16A06-Gal4* is very selective and expresses stronger than *H24-Gal4* in the γ lobe. Scoring the cells showed that *R16A06-Gal4* labels a larger number of γ KCs cells than *H24-Gal4*. **(H)** Neuronal activation or inhibition of MB $\alpha\beta$ and γ lobes with *mb247-Gal4* did not modulate fly locomotor reactivity. **(I)** Expression pattern of the recombined double driver *R16A06-Gal4, mb247-Gal4* as revealed by msGFP expression. **(J)** Parallel experiment were performed to compare the effects on locomotor reactivity resulting from neuronal activation by *R16A06-Gal4* and the double driver *R16A06-Gal4, mb247-Gal4*. The SING decrease induced by *R16A06-Gal4, mb247-Gal4* in the first round of test was significantly mitigated compared to that induced by *R16A06-Gal4* alone. This suggests that γ lobe-induced SING modulation is inhibited by simultaneous $\alpha\beta$ lobe activation. **(K)** Expression pattern of the recombined double driver *R35B12-Gal4, mb247-Gal4* as revealed by msGFP expression.

(Continued)

FIGURE 3 | (L) Parallel experiment was performed to compare the effects on locomotor reactivity resulting from neuronal activation by *R35B12-Gal4* and the double driver *R35B12-Gal4, mb247-Gal4*. Flies with neuronal activation in both $\alpha\beta\gamma$ and $\alpha'\beta'$ with *R35B12-Gal4, mb247-Gal4* showed normal SING performance compared to reduced performance in *R35B12>dTrpA1* flies. This suggests that activation of $\alpha\beta\gamma$ KCs blocked SING modulation induced by $\alpha'\beta'$ KCs. Scale bars represent 100 μm . **(A–F,H)** Two-way ANOVA with Bonferroni's multiple comparisons tests ($***p < 0.001$). **(G)** One-way ANOVA with Tukey's multiple comparisons test ($***p < 0.001$). **(J,L)** Two-way ANOVA with Tukey's multiple comparisons tests ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

then declined in the two subsequent tests, possibly related to a dominant effect of *R16A06-Gal4*-induced neuronal activation. This result indicates that co-activating the $\alpha\beta$ lobes can at least transiently inhibit SING blockade induced by activation of the γ lobe intrinsic neurons.

We then checked if activation of the $\alpha\beta$ KCs could similarly interfere with SING modulation induced by $\alpha'\beta'$ KC activation. A recombined *R35B12-Gal4, mb247-Gal4* double driver line was constructed that strongly expresses in the $\alpha\beta$, γ and $\alpha'\beta'$ KCs, i.e., in all the MB lobes (**Figure 3K**). Strikingly, the significant effect of $\alpha'\beta'$ neuron thermoactivation by *R35B12-Gal4* on SING modulation (reduction of the response to 33% of the 23°C control) was nearly abolished when the double-driver *R35B12-Gal4, mb247-Gal4* was used in a parallel experiment (reduction to 90.5% of the control only) (**Figure 3L**). Therefore, co-activation of the $\alpha\beta$ and γ neurons blocked the inhibitory effect induced by $\alpha'\beta'$ neuron activation. Accordingly, we observed that thermoactivation or synaptic blockade with a driver that expresses specifically in all MB lobes, *VT30559-Gal4*, only had little effects on SING modulation (data not shown). Overall, these results indicate that activity of the $\alpha\beta$ KCs potentially counteracts by an unknown mechanism the behavioral modulation induced by the $\alpha'\beta'$ and γ KCs.

Regulation of Locomotor Reactivity Requires DA Receptor Signaling in the MB

We next investigated whether down-regulation of DA receptor expression in the MB could prevent the decrease in SING caused by thermoactivation of DANs. Two DA receptors, D_1 -like *Dumb/Dop1R1* and $D_{1/5}$ -like *Damb/Dop1R2*, are abundant in the MB lobes where they play key roles in olfactory memory (Kim et al., 2007; Seugnet et al., 2008; Selcho et al., 2009; Berry et al., 2012; Musso et al., 2015; Plaçais et al., 2017). *Dumb* has also been implicated in arousal and grooming (Andreć et al., 2008; Lebestky et al., 2009; Pitmon et al., 2016) and *Damb* in paraquat- and DA-induced neurotoxicity (Cassar et al., 2015). Taking advantage of the *LexA-LexAop* and *Gal4-UAS* expression systems, we expressed *dTrpA1* in DANs using *LexAop-dTrpA1* and the *TH-LexA* driver, whose expression pattern is similar to that of *TH-Gal4* (Berry et al., 2015), while inactivating by targeted RNA interference (RNAi) the genes encoding *Dumb* or *Damb* in all MB lobes with the *238Y-Gal4* driver. As shown in **Figure 4A**, *TH-LexA*-controlled *dTrpA1* expression in the presence of *238Y-Gal4* alone induced a significant decrease in SING performance at 32°C (~48% of the 23°C control value). We observed that adding the *UAS-Dumb-RNAi* construct to allow *Dumb* inactivation in the MB fully restored SING performance to control level despite DAN thermoactivation (**Figure 4A**). In contrast, selective *Damb* inactivation had no such effect

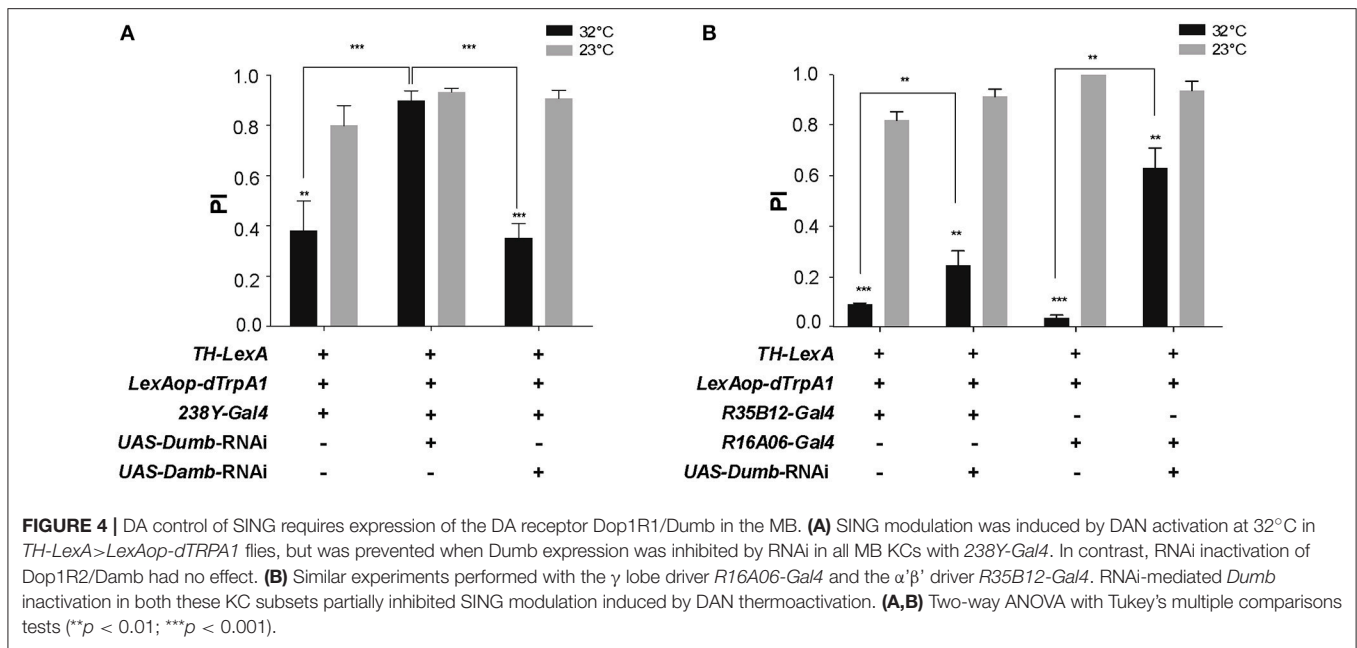
(**Figure 4A**). This experiment suggests that DA modulation of SING requires DA receptor expression in the MB KCs and that this regulation specifically depends on signaling through the *Dumb* receptor.

Next we investigated whether RNAi-mediated inactivation of *Dumb* expression in specific MB lobes could have a similar antagonistic effect on DA modulation of SING. We found that targeting *Dumb* RNAi selectively in the $\alpha'\beta'$ or γ lobes using *R35B12-Gal4* and *R16A06-Gal4*, respectively, in both cases significantly rescued the SING response, in spite of *TH-LexA*-mediated DAN activation (**Figure 4B**). This effect was most prominent with the strong and specific γ driver *R16A06-Gal4* (**Figure 4B**). This indicates a requirement for the DA receptor *Dumb* in the $\alpha'\beta'$ and γ lobes for DAN-mediated SING modulation.

MBON-M4/M6 and MBON-V2 Relay SING Modulation

We then attempted to identify specific MB-output neurons (MBONs) that could transfer MB modulatory information to downstream motor circuits. Since the intrinsic KCs in the MB $\alpha'\beta'$ and γ lobes appear to play a role in SING control, we studied the role of MBONs whose dendrites arborize on these lobes. The glutamatergic MBON-M4 β , M4 β' and M6 (also named MBON- $\beta 2\beta'2$ and MBON- $\beta'2mp$ for M4, and MBON- $\gamma 5\beta'2a$ for M6) arborize on the tip of the β , β' and γ lobes, respectively (Tanaka et al., 2008; Aso et al., 2014b; Oswald et al., 2015a) (**Figure 5A**). These neurons are known to be involved in sleep regulation and the expression of appetitive and aversive memory performance (Aso et al., 2014b; Bouzaiane et al., 2015; Oswald et al., 2015a; Sitaraman et al., 2015a). Using *NP3212-Gal4* and *R27G01-Gal4* that both target the MBON-M4 and M6 neurons (Tanaka et al., 2008; Bouzaiane et al., 2015), we observed that thermogenetic activation of these MB efferent neurons significantly reduced locomotor reactivity, while inhibiting their synaptic output with Shi^{ts1} had no effect (**Figures 5B,C**).

The cholinergic MBON-V2 α and V2 α' (also named MBON- $\alpha 2sc$ and MBON- $\alpha'3$, respectively) have their dendrites in the MB vertical lobes ($\alpha 2$, $\alpha'3$) and are required for retrieval of aversive olfactory memory from the $\alpha\beta$ lobe (Tanaka et al., 2008; Séjourné et al., 2011; Aso et al., 2014b; Bouzaiane et al., 2015; **Figure 5D**). Two specific drivers, *NP2492-Gal4* and *R71D08-Gal4* (Tanaka et al., 2008; Séjourné et al., 2011) were used to test whether V2 neurons are implicated in SING modulation. Activating these neurons with either of these drivers greatly reduced SING performance to around 33 and 21% of the 23°C control value, respectively, and again inhibition of synaptic output had no effect (**Figures 5E,F**). Finally, neither activation nor blocking of the MBON-V3 (alias MBON- $\alpha 3$) output, targeted



by *G0239-Gal4*, had any effect on the SING response (data not shown), indicating that specific MBONs are involved in SING control. Hence, we propose that both MBON-M4/M6 and MBON-V2 participate in the transmission of MB regulatory information to the downstream SING reflex motor circuits.

The Ellipsoid Body Does Not Play a Role in the Modulation of Startle-Induced Locomotion

The *Drosophila* ellipsoid body (EB) is a region of the central complex in the brain that controls locomotor patterns (Strauss and Heisenberg, 1993; Martin et al., 1999b, 2001; Strauss, 2002), as well as spatial orientation and visual pattern memories (Neuser et al., 2008; Pan et al., 2009). Subsets of DANs labeled by *TH-Gal4* heavily innervate the EB (Mao and Davis, 2009; White et al., 2010; Ueno et al., 2012; Riemensperger et al., 2013). Due to the complex structure of the EB, different driver lines have been used which express in various areas of the EB: *c41-Gal4* (all EB neurons), *c105-Gal4* (R1 neurons), *EB1-Gal4* (R2/R4d neurons), and *c232-Gal4* (R3/R4 neurons). Neuronal activation or synaptic inhibition with any of these drivers had no significant effect on the fly's locomotor reactivity, as tested by SING (Figure 6). This suggests that the EB is not involved in the neuronal circuits modulating startle-induced locomotion in *Drosophila*.

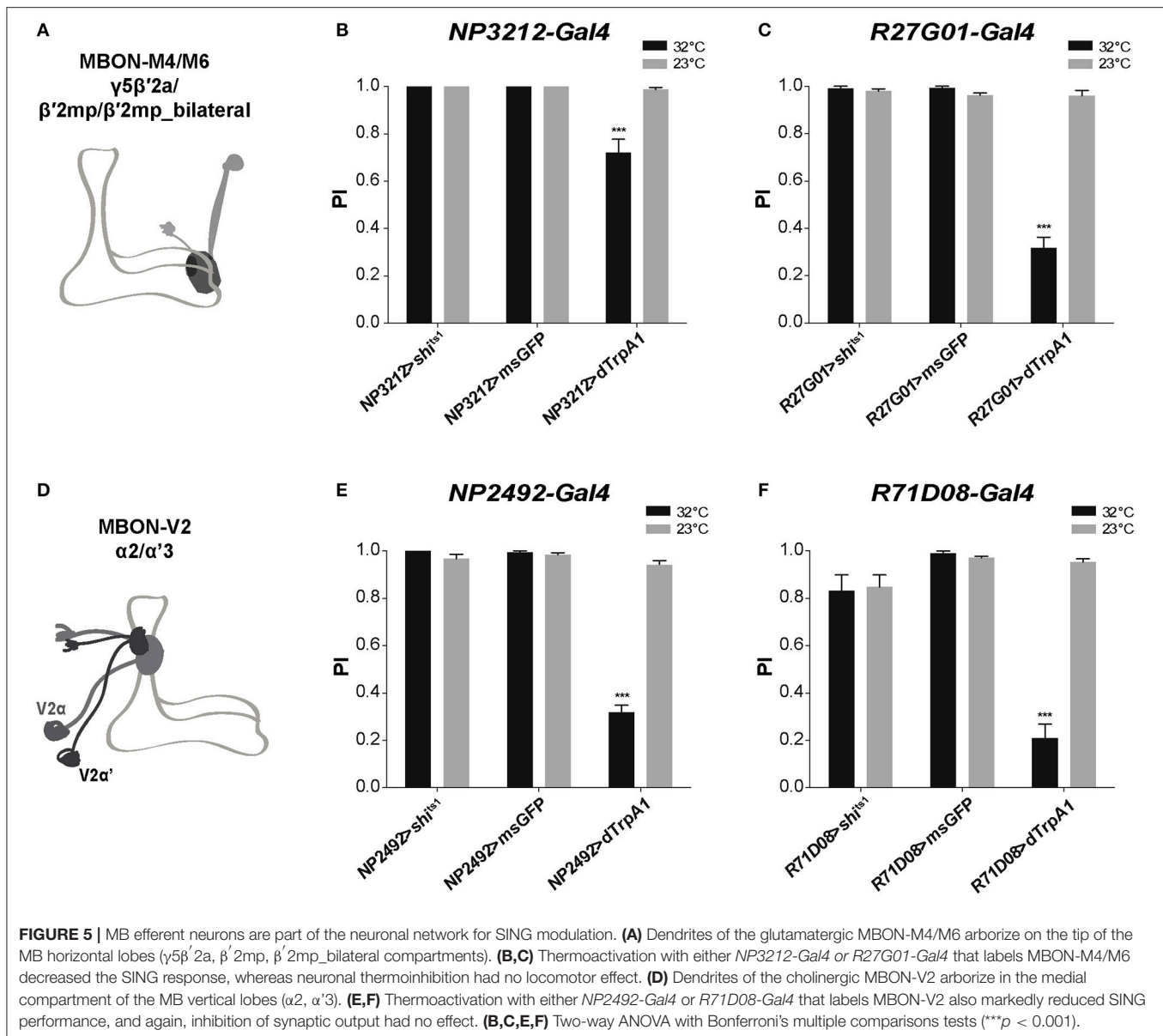
Potential Synaptic Convergence Between DANs and MBONs Controlling SING

According to the MB neuronal architecture reported by Aso et al. (2014a), dendrites from the PAM DANs mainly reside in the crepine (CRE) and superior medial protocerebrum (SMP) brain regions, and slightly also in the superior intermediate protocerebrum (SIP) and superior lateral protocerebrum (SLP). The PPL1 DANs have a large part of their dendrites in the SMP,

which is also where the MBON-M4/M6 and MBON-V2 send axonal projections. In order to detect zones of potential synaptic connections between the afferent and efferent MB neurons, we used the technique of splitGFP reconstitution (also named GFP reconstitution across synaptic partners, GRASP) coupled with the *LexA-LexAop* and *Gal4-UAS* systems (Feinberg et al., 2008; Gordon and Scott, 2009; Pech et al., 2013a; Macpherson et al., 2015).

The PAM DAN projections mainly tile the MB horizontal lobes where the MBON-M4/M6 dendrites arborize (Pech et al., 2013b; Riemensperger et al., 2013; Aso et al., 2014a). Results of splitGFP experiments indicated a potential synaptic convergence between these two groups of neurons in the tips of the MB horizontal lobes ($\gamma 5$, $\beta 2$, and $\beta' 2$ compartments) (Figure 7A1–3) and also in the CRE and SMP neuropiles (Figure 7A2–4). This suggests, in agreement with a previous report (Owald et al., 2015a), that the zones of convergence between PAM and M4/M6 neurons not only localize in the MB horizontal lobes but also in the superior protocerebrum where the M4/M6 neurons appear to project onto the PAM DAN dendrites.

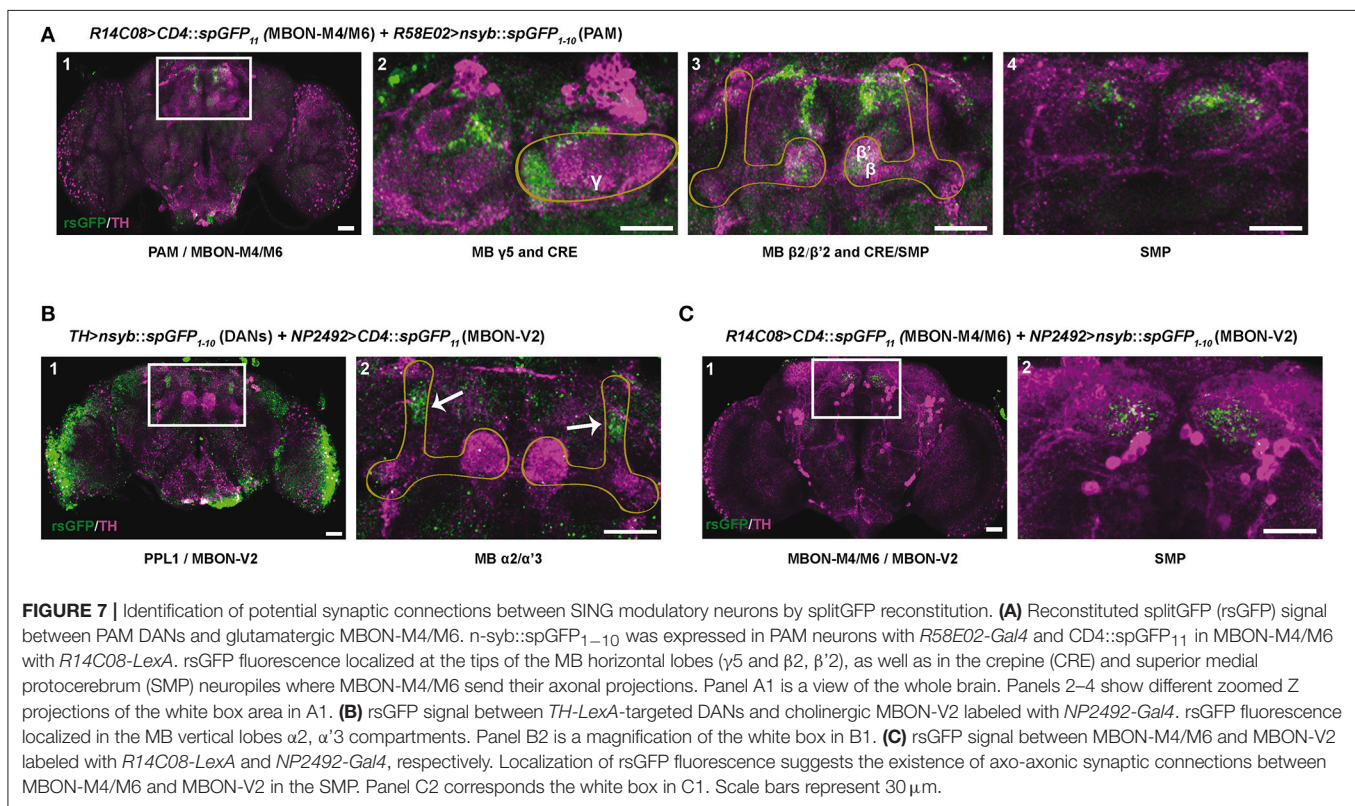
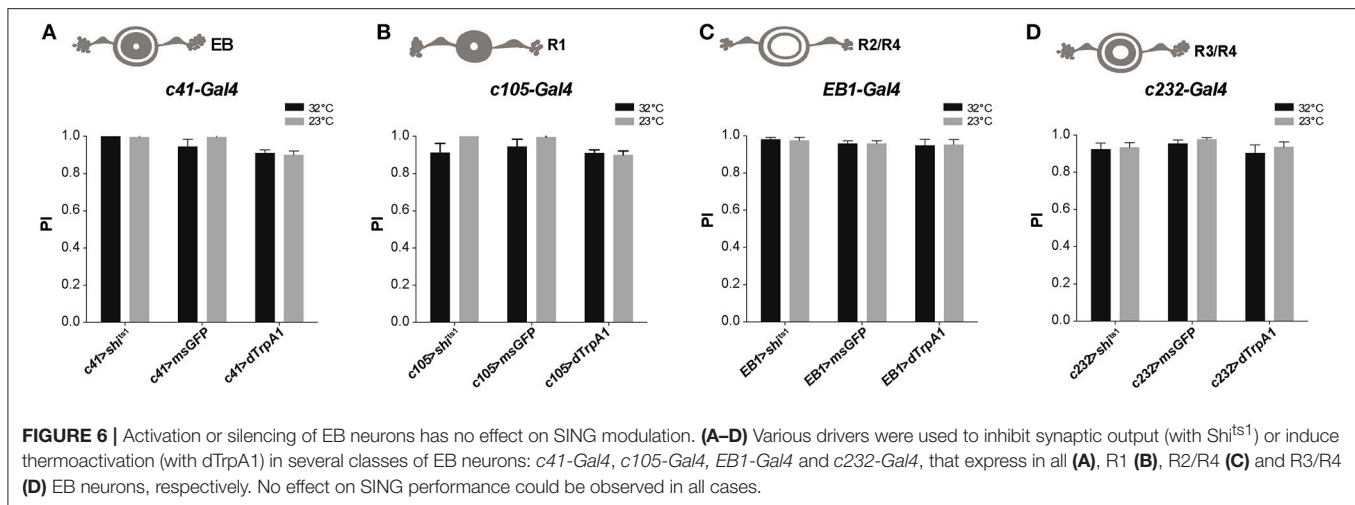
MBON-V2 arborizes on the MB vertical lobes (Tanaka et al., 2008; Séjourné et al., 2011; Aso et al., 2014b). Reconstituted split GFP (rsGFP) signals between MBON-V2 and DANs targeted by *TH-LexA* could be detected in the MB α and α' medial compartments, where the PPL1 MB-V1 neurons send projections (Aso et al., 2010, 2014b), indicating a close proximity between these neurons (Figure 7B1,2). A strong rsGFP signal was only observed when the presynaptic marker *nsyb::spGFP_{1–10}* was driven with *TH-LexA* and *CD4::spGFP₁₁* by the MBON-V2 driver *NP2492-Gal4* (Figures 7B1,2) and not the opposite (not shown), suggesting that DANs project to the MBON-V2 in the MB vertical lobe compartments. The occurrence of DAN>MBON synapses in the MB has recently



been demonstrated in a comprehensive electron microscopy study (Takemura et al., 2017). Furthermore, rsGFP signals were visible between MBON-V2 and MBON-M4/M6 in the SMP region, which suggests that these MBONs may form axo-axonic reciprocal synapses (Figures 7C1,2). It seems that MBON-V2 could be presynaptic and MBON-M4/M6 postsynaptic in these contacts because a rsGFP signal in the SMP was only observed when the V2 driver NP2492-Gal4 expressed the presynaptic marker *nsyb::spGFP₁₋₁₀* and the M4/M6 driver *CD4::spGFP₁₁* (Figures 7C1,2) and not the opposite (not shown). Therefore, there might be feedback signals from the MBON-V2 to MBON-M4/M6 and DANs that could optimize SING modulation, possibly in relation to learning and memory processes, and thus coordinate locomotor behavior with the environment.

DISCUSSION

In this study, we have identified MB afferent, intrinsic and efferent neurons that underlie modulation of startle-induced locomotion in the *Drosophila* brain. Using *in vivo* activation or silencing of synaptic transmission in neuronal subsets, we showed that specific compartments of the MBs are central to this modulation. Implicated neurons include $\alpha'\beta'$ and γ KCs, subsets of PAM and PPL1 DANs, and the MBONs V2 and M4/M6. We have also characterized some of the potential synaptic connections between these elements using splitGFP reconstitution across cells. Although the picture is not complete, these results led us to propose a first scheme of the neuronal circuits underlying the control of locomotor reactivity in an insect brain.



DANs Show Diverse Functions in the Control of Locomotor Reactivity

We previously reported that the degeneration of DANs afferent to the MBs in the PAM and PPL1 clusters is associated with accelerated decline of SING performance in aging flies (Riemensperger et al., 2013; Vaccaro et al., 2017). Here we have specifically addressed the role of these and other DANs in SING modulation. Our initial observation was that thermoactivation of *TH-Gal4*-targeted DANs consistently led to decreased locomotor reactivity, while silencing synaptic output from these neurons

had no effect. This result was verified by rapid optogenetic photostimulation, indicating that indeed DAN activation affects locomotor reactivity during the execution of the behavior. In contrast, blocking selectively synaptic output of the PAM DANs neurons resulted in a slight increase in SING performance, suggesting that a subset of spontaneously active neurons in the PAM inhibits SING. It should be noted, however, that this effect appeared small probably in part because SING performance was already very high for the control flies in our assay condition. This issue may have prevented us from detecting other modulatory

neurons in the course of this study. Interestingly, our data suggest that those PAM neurons that inhibit SING are targeted by *NP6510-Gal4*, a driver that expresses in 15 PAM DANs that project to the MB $\beta 1$ and $\beta 2$ compartments. The degeneration of these neurons also appears to be largely responsible for α -synuclein-induced decline in SING performance in a Parkinson disease model (Riemensperger et al., 2013). Moreover, we provided one observation in this study, using DAN co-activation with *TH-Gal4* and *R58E02-Gal4*, suggesting that other subsets of the PAM cluster may modulate locomotor reactivity with opposite effects, i.e., increase SING when they are stimulated.

Our study further indicated that thermoactivation of two DANs of the PPL1 cluster, either MB-MP1 that projects to the $\gamma 1$ peduncle in the MB horizontal lobes or MB-V1 that projects to the $\alpha 2$ and $\alpha 2'$ compartments of the MB vertical lobes, was sufficient to significantly decrease SING performance. This suggests that the MB-afferent DANs of the PPL1 cluster are also implicated in SING modulation. Other DAN subsets could play a role and are still to be identified. However, inactivation of a DA receptor, Dop1R1/Dumb, in MB KCs precluded DAN-mediated SING modulation, strongly suggesting that DANs afferent to the MBs plays a prominent role in the neuronal network controlling fly's locomotor reactivity. In contrast, inactivating Dop1R2/Damb in KCs did not show any effect on DA-induced SING control.

Therefore, these results suggest that DA input to the MBs can inhibit or increase the reflexive locomotor response to a mechanical startle, allowing the animal to react to an instant, sudden stimulus. In accordance with this interpretation, previous reports have shown that the MB is not only a site for associative olfactory learning, but that it can also regulate innate behaviors (Hige et al., 2015; Lewis et al., 2015; Oswald et al., 2015a). By combining synaptic imaging and electrophysiology, Cohn et al. (2015) have demonstrated that dopaminergic inputs to the MB intrinsic KCs play a central role in this function by exquisitely modulating the synapses that control MB output activity, thereby enabling the activation of different behavioral circuits according to contextual cues.

Interactions Between MB Compartments Contribute to SING Modulation

We previously reported a decrease in SING performance when KCs in the $\alpha\beta'$ lobes, but not in the $\alpha\beta$ and γ lobes, were thermogenetically stimulated or their synaptic output silenced (Riemensperger et al., 2013). Here, using a set of specific drivers, we have more precisely studied the contribution of the various MB lobes in the modulation of this innate reflex. We confirmed that the $\alpha\beta'$ KCs down-regulate SING when they are activated but not when their output is inhibited. Other unidentified neurons, which are targeted by the rather non-selective *c305a-Gal4* and *G0050-Gal4* drivers, trigger a decrease in SING performance when they are inhibited by Shi^{ts1} , and are therefore potential SING-activating neurons. We further found that the MB γ lobes contain KCs that strongly inhibit SING when activated, both by thermogenetic and optogenetic

stimulation, as shown with the γ -lobe specific driver *R16A06-Gal4*. However, thermoactivation of γ neurons with other drivers, like *mb247-Gal4*, which express both in the $\alpha\beta$ and γ lobe, did not decrease SING (Riemensperger et al., 2013 and this study). This could result from an inhibitory effect of $\alpha\beta$ neuron activation on SING modulation by γ neurons. To test this hypothesis, we have generated a double-driver by recombining *mb247-Gal4* with *R16A06-Gal4*. Because both drivers express in the γ lobes, one would expect a stronger effect on SING modulation after thermoactivation with the double-driver than with *R16A06-Gal4* alone. We observed strikingly the opposite, i.e., that SING was decreased to a less extent with the double-driver than with *R16A06-Gal4* alone. Activation of *mb247-Gal4* $\alpha\beta$ neurons therefore likely counterbalanced the effect of γ neuron activation with *R16A06-Gal4* on SING modulation. A similar and even more obvious results was obtained when *mb247-Gal4* was recombined with the $\alpha\beta'$ driver *R35B12-Gal4*: co-activation of the neurons targeted by these two drivers prevented the strong SING modulation normally induced by *R35B12-Gal4* alone. These results suggest the existence of an inter-compartmental communication process for locomotor reactivity control in the *Drosophila* MB. Comparably, it was recently suggested, in the case of memory retrieval, that MB output channels are ultimately pooled such that blockade (or activation) of all the outputs from a given population of KCs may have no apparent effect on odor-driven behavior, while such behavior can be changed by blocking a single output (Oswald et al., 2015a). Such a transfer of information could occur, as was previously reported, through connections involving the MBONs within the lobes or outside the MB (Aso et al., 2014b; Oswald et al., 2015a).

Role of Specific MBONs in Innate Reflex Suppression

Finally, the activation of two sets of MB efferent neurons, cholinergic MBON-V2 and glutamatergic MBON-M4/M6, consistently decreased SING performance of the flies. In contrast, silencing these neurons had no effect on locomotor behavior, as was previously observed (Aso et al., 2014b). The dendrites of these MBONs arborize in the medial part of the vertical lobes ($\alpha 2$, $\alpha 3$) and the tips of the horizontal lobes ($\beta 2$ and $\gamma 5$), respectively, as a further evidence that the prime and γ lobes, and DANs efferent to these compartments, are involved in SING modulation. We also show results from GRASP observations suggesting that the PAM DANs lay very close or make potential synaptic connections with the MBON-M4/M6 neurons in their MB compartments, as well as the M4/M6 with the PAM in the SMP, in agreement with recent evidence from other laboratories (Lewis et al., 2015; Oswald et al., 2015a; Takemura et al., 2017). Our results also provide evidence that the PPL1 DANs and MBON-V2 contact each other in the vertical lobes and that axo-axonic synaptic contacts may occur between the MBON-V2 and M4/M6 neurons in their common projection region in the SMP.

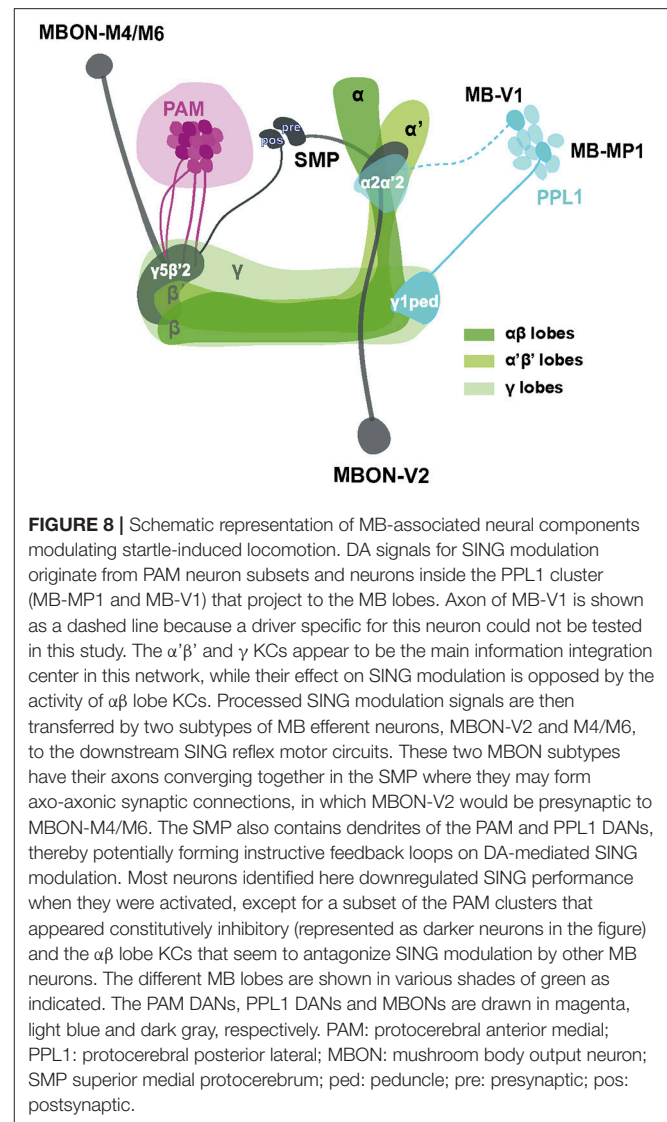
These MBONs are known to be involved in opposite ways in olfactory memory: DAN-induced synaptic repression

of cholinergic or glutamatergic MBONs would result in the expression of aversive or attractive memory, respectively (Aso et al., 2014b). Here we find, in contrast, that the activation of these two sets of MBONs had similar depressing effects on SING behavior. Interestingly, it has been recently reported that the glutamatergic MBONs and PAM neurons that project to the MB β '2 compartment are also required for modulation of another innate reflex, CO₂ avoidance (Lewis et al., 2015). CO₂ exposure, like mechanical startle, represents a potential danger for the flies, thus triggering an avoidance behavior that can be suppressed by silencing these MBONs in specific environmental conditions. However, it is the activation of glutamatergic MBONs that inhibits SING. This apparent discrepancy might be explained if the downstream circuits were different for these two escape behaviors (CO₂ avoidance and fast climbing). Overall, our results further support the hypothesis of a primary role of the MB as a higher brain center for adapting innate sensory-driven reflex to a specific behavioral context (Cohn et al., 2015; Lewis et al., 2015).

Different Neuronal Circuits Control Locomotor Reactivity, Sleep/Wake State and Hyperactivity

Even though the model remains to be confirmed and elaborated, a proposed scheme summarizing our current working hypothesis of the neural components underlying SING control is presented in **Figure 8**. Sensory information from mechanical stimulation triggers an innate climbing reflex (negative geotaxis) that can be regulated by signals transmitted from MB-afferent DANs (in the PAM and PPL1 cluster) to select KCs and two sets of MBONs (V2 and M4/M6) in specific MB compartments. Processing of this information could occur through synergistic or antagonistic interactions between the MB compartments and, finally, the MBON neurons would convey the resulting modulatory signal to downstream motor circuits controlling the climbing reflex. We observed that the axonal projections of these MBONs make synaptic contacts with each other and converge together to the SMP where the dendrites of DANs lie (Aso et al., 2014a), suggesting that they might form feedback loops to control DA signaling in the circuits.

SING performance can be affected by a collection of factors including the arousal threshold of the fly, the ability to sense gravity and also climbing ability. "Arousal" is defined as a state characterized by increased motor activity, sensitivity to sensory stimuli, and certain patterns of brain activity (Coull, 1998; Pfaff and Banavar, 2007). A distinction can be made between endogenous arousal (i.e., wakefulness as opposed to sleep) and exogenous arousal (i.e., behavioral responsiveness) (Van Swinderen and Andretic, 2011). In *Drosophila*, DA level and signaling control all known forms of arousal (Friggi-Grelin et al., 2003; Birman, 2005; Kume et al., 2005; Lebestky et al., 2009; Van Swinderen and Andretic, 2011; Kumar et al., 2012; Liu et al., 2012b; Ueno et al., 2012; Nall et al., 2016). Because the MB plays an important role in sleep regulation (Sitaraman et al., 2015a; Artiushin and Sehgal, 2017; Tomita et al., 2017), sleep- or wake-promoting networks might indeed in part interact or overlap with



those controlling locomotor reactivity. However, we observed that thermoactivation with various drivers had in a number of cases opposite effects on sleep/wake state and SING. First, neuronal thermoactivation with *TH-Gal4* suppresses sleep (Liu et al., 2012b) but decreases the SING response. Second, extensive thermogenetic activation screen revealed that $\alpha'\beta'$ and γ KCs are wake-promoting and γ d KCs are sleep-promoting (Sitaraman et al., 2015a). In our experiments, neuronal activation of $\alpha'\beta'$ or γ KCs both led to strongly decreased locomotor reactivity. Third, stimulating MBON-M4 and M6, which are wake-promoting (Sitaraman et al., 2015a), decreased SING performance.

Another brain structure, the EB, plays important roles in the control of locomotor patterns (Strauss, 2002) and is also sleep-promoting (Liu et al., 2016). Furthermore, the EB is involved in the dopaminergic control of stress- or ethanol-induced hyperactivity (Lebestky et al., 2009; Kong et al., 2010), which can be considered as forms of exogenously-generated arousal. We used several drivers labeling diverse EB neuronal layers

and found no noticeable effects of thermoactivation of these neurons on the SING response. We conclude that the circuits responsible for SING modulation, although they apparently share some similarities, are globally different from those controlling sleep/wake state and environmentally-induced hyperactivity.

Overall, this work identified elements of the neuronal networks controlling startle-induced locomotion in *Drosophila* and confirmed the central role of the MBs in this important function. Future studies are required to complete this scheme and explore the intriguing interactions between the different MB compartments in SING neuromodulation.

ETHICS STATEMENT

Experiments on *Drosophila* are not subject to the approval of ethics committee. All experiments were nevertheless performed in accordance with ethic procedures and by minimizing the number of animals required for data gathering.

AUTHOR CONTRIBUTIONS

JS, TR, AF, and SB: Conceived and designed the experiments; JS, AX, JG, HP, and TR: Performed the experiments; JS, AX, JG, HP,

TR, AF, and SB: Analyzed data; JS, AX, TR, AF, and SB: Wrote the paper; SB: Designed and supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnsys.2018.00006/full#supplementary-material>

REFERENCES

- Agrawal, T., and Hasan, G. (2015). Maturation of a central brain flight circuit in *Drosophila* requires Fz2/Ca²⁺ signaling. *Elife* 4:e07046. doi: 10.7554/eLife.07046
- Andretic, R., Kim, Y.-C., Jones, F. S., Han, K.-A., and Greenspan, R. J. (2008). *Drosophila* D1 dopamine receptor mediates caffeine-induced arousal. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20392–20397. doi: 10.1073/pnas.0806776105
- Artushin, G., and Sehgal, A. (2017). The *Drosophila* circuitry of sleep-wake regulation. *Curr. Opin. Neurobiol.* 44, 243–250. doi: 10.1016/j.conb.2017.03.004
- Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T.-T. B., et al. (2014a). The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife* 3:e04577. doi: 10.7554/eLife.04577
- Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., et al. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* 8:e1002768. doi: 10.1371/journal.pgen.1002768
- Aso, Y., and Rubin, G. M. (2016). Dopaminergic neurons write and update memories with cell-type-specific rules. *Elife* 5:e16135. doi: 10.7554/eLife.16135
- Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., et al. (2014b). Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *Elife* 3:e04580. doi: 10.7554/eLife.04580
- Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. *Curr. Biol.* 20, 1445–1451. doi: 10.1016/j.cub.2010.06.048
- Bainton, R. J., Tsai, L. T., Singh, C. M., Moore, M. S., Neckameyer, W. S., and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol.* 10, 187–194. doi: 10.1016/S0960-9822(00)00336-5
- Beninger, R. J. (1983). The role of dopamine in locomotor activity and learning. *Brain Res.* 287, 173–196. doi: 10.1016/0165-0173(83)90038-3
- Benzer, S. (1967). Behavioral mutants of *Drosophila* isolated by countercurrent distribution. *Proc. Natl. Acad. Sci. U.S.A.* 58, 1112–1119. doi: 10.1073/pnas.58.3.1112
- Berry, J. A., Cervantes-Sandoval, I., Chakraborty, M., and Davis, R. L. (2015). Sleep facilitates memory by blocking dopamine neuron-mediated forgetting. *Cell* 161, 1656–1667. doi: 10.1016/j.cell.2015.05.027
- Berry, J. A., Cervantes-Sandoval, I., Nicholas, E. P., and Davis, R. L. (2012). Dopamine is required for learning and forgetting in *Drosophila*. *Neuron* 74, 530–542. doi: 10.1016/j.neuron.2012.04.007
- Birman, S. (2005). Arousal mechanisms: speedy flies don't sleep at night. *Curr. Biol.* 15, R511–R513. doi: 10.1016/j.cub.2005.06.032
- Blum, I. D., Zhu, L., Moquin, L., Kokoeva, M. V., Gratton, A., Giros, B., et al. (2014). A highly tunable dopaminergic oscillator generates ultradian rhythms of behavioral arousal. *Elife* 3:e05105. doi: 10.7554/eLife.05105
- Bou Dib, P., Gnägi, B., Daly, F., Sabado, V., Tas, D., Glauser, D. A., et al. (2014). A conserved role for p48 homologs in protecting dopaminergic neurons from oxidative stress. *PLoS Genet.* 10:e1004718. doi: 10.1371/journal.pgen.1004718
- Bouzaiane, E., Trannoy, S., Scheunemann, L., Plaçais, P.-Y., and Preat, T. (2015). Two independent mushroom body output circuits retrieve the six discrete components of *Drosophila* aversive memory. *Cell Rep.* 11, 1280–1292. doi: 10.1016/j.celrep.2015.04.044
- Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614
- Bushey, D., and Cirelli, C. (2011). From genetics to structure to function: exploring sleep in *Drosophila*. *Int. Rev. Neurobiol.* 99, 213–244. doi: 10.1016/B978-0-12-387003-2.00009-4
- Cassar, M., Issa, A.-R., Riemensperger, T., Petitgas, C., Rival, T., Coulom, H., et al. (2015). A dopamine receptor contributes to paraquat-induced neurotoxicity in *Drosophila*. *Hum. Mol. Genet.* 24, 197–212. doi: 10.1093/hmg/ddu430
- Chaudhuri, A., Bowling, K., Funderburk, C., Lawal, H., Inamdar, A., Wang, Z., et al. (2007). Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. *J. Neurosci.* 27, 2457–2467. doi: 10.1523/JNEUROSCI.4239-06.2007
- Chen, C.-C., Wu, J.-K., Lin, H.-W., Pai, T.-P., Fu, T.-F., Wu, C.-L., et al. (2012). Visualizing long-term memory formation in two neurons of the *Drosophila* brain. *Science* 335, 678–685. doi: 10.1126/science.1212735
- Claridge-Chang, A., Roorda, R. D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., et al. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell* 139, 405–415. doi: 10.1016/j.cell.2009.08.034

- Cohn, R., Morante, I., and Ruta, V. (2015). Coordinated and compartmentalized neuromodulation shapes sensory processing in *Drosophila*. *Cell* 163, 1742–1755. doi: 10.1016/j.cell.2015.11.019
- Connolly, K. (1967). Locomotor activity in *Drosophila* III. A distinction between activity and reactivity. *Anim. Behav.* 15, 149–152. doi: 10.1016/S0003-3472(67)80026-5
- Coull, J. T. (1998). Neural correlates of attention and arousal: insights from electrophysiology, functional neuroimaging and psychopharmacology. *Prog. Neurobiol.* 55, 343–361. doi: 10.1016/S0304-0082(98)00011-2
- Coulom, H., and Birman, S. (2004). Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. *J. Neurosci.* 24, 10993–10998. doi: 10.1523/JNEUROSCI.2993-04.2004
- Davis, R. L. (2011). Traces of *Drosophila* memory. *Neuron* 70, 8–19. doi: 10.1016/j.neuron.2011.03.012
- Dawydow, A., Gueta, R., Ljaschenko, D., Ullrich, S., Hermann, M., Ehmann, N., et al. (2014). Channelrhodopsin-2-XXL, a powerful optogenetic tool for low-light applications. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13972–13977. doi: 10.1073/pnas.1408269111
- Dickinson, P. S. (2006). Neuromodulation of central pattern generators in invertebrates and vertebrates. *Curr. Opin. Neurobiol.* 16, 604–614. doi: 10.1016/j.conb.2006.10.007
- Feany, M. B., and Bender, W. W. (2000). A *Drosophila* model of Parkinson's disease. *Nature* 404, 394–398. doi: 10.1038/35006074
- Feinberg, E. H., Vanhove, M. K., Bendesky, A., Wang, G., Fetter, R. D., Shen, K., et al. (2008). GFP reconstitution across synaptic partners (GRASP) defines cell contacts and synapses in living nervous systems. *Neuron* 57, 353–363. doi: 10.1016/j.neuron.2007.11.030
- Fiala, A. (2007). Olfaction and olfactory learning in *Drosophila*: recent progress. *Curr. Opin. Neurobiol.* 17, 720–726. doi: 10.1016/j.conb.2007.11.009
- Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* 54, 618–627. doi: 10.1002/neu.10185
- Ganetzky, B., and Flanagan, J. R. (1978). On the relationship between senescence and age-related changes in two wild-type strains of *Drosophila melanogaster*. *Exp. Gerontol.* 13, 189–196. doi: 10.1016/0531-5565(78)90012-8
- Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 379, 606–612. doi: 10.1038/379606a0
- Gordon, M. D., and Scott, K. (2009). Motor control in a *Drosophila* taste circuit. *Neuron* 61, 373–384. doi: 10.1016/j.neuron.2008.12.033
- Griffith, L. C. (2012). Identifying behavioral circuits in *Drosophila melanogaster*: moving targets in a flying insect. *Curr. Opin. Neurobiol.* 22, 609–614. doi: 10.1016/j.conb.2012.01.002
- Grotewiel, M. S., Martin, I., Bhandari, P., and Cook-Wiens, E. (2005). Functional senescence in *Drosophila melanogaster*. *Ageing Res. Rev.* 4, 372–397. doi: 10.1016/j.arr.2005.04.001
- Hale, M. E., Katz, H. R., Peek, M. Y., and Fremont, R. T. (2016). Neural circuits that drive startle behavior, with a focus on the Mauthner cells and spiral fiber neurons of fishes. *J. Neurogenet.* 30, 89–100. doi: 10.1080/01677063.2016.1182526
- Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J., et al. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature* 454, 217–220. doi: 10.1038/nature07001
- Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* 4, 266–275. doi: 10.1038/nrn1074
- Heisenberg, M., Borst, A., Wagner, S., and Byers, D. (1985). *Drosophila* mushroom body mutants are deficient in olfactory learning. *J. Neurogenet.* 2, 1–30. doi: 10.3109/01677068509100140
- Helfrich-Förster, C., Wulf, J., and de Belle, J. S. (2002). Mushroom body influence on locomotor activity and circadian rhythms in *Drosophila melanogaster*. *J. Neurogenet.* 16, 73–109. doi: 10.1080/01677060213158
- Hige, T., Aso, Y., Rubin, G. M., and Turner, G. C. (2015). Plasticity-driven individualization of olfactory coding in mushroom body output neurons. *Nature* 526, 258–262. doi: 10.1038/nature15396
- Howse, P. E. (1975). Brain structure and behavior in insects. *Annu. Rev. Entomol.* 20, 359–379. doi: 10.1146/annurev.en.20.010175.002043
- Huber, F. (1960). Untersuchung über die funktion des zentralnervensystems und insbesondere des gehirns bei der fortbewegung und der lauterzeugung der grillen. *Z. vergl. Physiol.* 44, 60–132. doi: 10.1007/BF00297863
- Huber, F. (1967). “Central control of movements and behavior of invertebrates,” in *Invertebrate Nervous Systems*, ed C. A. G. Wiersma (Chicago, IL: University of Chicago Press), 333–351.
- Jones, M. A., and Grotewiel, M. (2011). *Drosophila* as a model for age-related impairment in locomotor and other behaviors. *Exp. Gerontol.* 46, 320–325. doi: 10.1016/j.exger.2010.08.012
- Kahsai, L., and Zars, T. (2011). Learning and memory in *Drosophila*: behavior, genetics, and neural systems. *Int. Rev. Neurobiol.* 99, 139–167. doi: 10.1016/B978-0-12-387003-2.00006-9
- Kazama, H. (2015). Systems neuroscience in *Drosophila*: conceptual and technical advantages. *Neuroscience* 296, 3–14. doi: 10.1016/j.neuroscience.2014.06.035
- Kim, Y.-C., Lee, H.-G., and Han, K.-A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. *J. Neurosci.* 27, 7640–7647. doi: 10.1523/JNEUROSCI.1167-07.2007
- Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* 47, 81–92. doi: 10.1002/neu.1018
- Kong, E. C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M. R., et al. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in *Drosophila*. *PLoS ONE* 5:e9954. doi: 10.1371/journal.pone.0009954
- Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. *Cell* 139, 416–427. doi: 10.1016/j.cell.2009.08.035
- Krashes, M. J., Keene, A. C., Leung, B., Armstrong, J. D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during *Drosophila* odor memory processing. *Neuron* 53, 103–115. doi: 10.1016/j.neuron.2006.11.021
- Kumar, S., Chen, D., and Sehgal, A. (2012). Dopamine acts through Cryptochrome to promote acute arousal in *Drosophila*. *Genes Dev.* 26, 1224–1234. doi: 10.1101/gad.186338.111
- Kume, K., Kume, S., Park, S. K., Hirsh, J., and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25, 7377–7384. doi: 10.1523/JNEUROSCI.2048-05.2005
- LeBeau, F. E. N., Manira, E. I. A., and Griller, S. (2005). Tuning the network: modulation of neuronal microcircuits in the spinal cord and hippocampus. *Trends Neurosci.* 28, 552–561. doi: 10.1016/j.tins.2005.08.005
- Lebestky, T., Chang, J.-S. C., Dankert, H., Zelnik, L., Kim, Y.-C., Han, K.-A., et al. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* 64, 522–536. doi: 10.1016/j.neuron.2009.09.031
- Le Bourg, E., and Lints, F. A. (1992). Hypergravity and aging in *Drosophila melanogaster*. 4. Climbing activity. *Gerontology* 38, 59–64. doi: 10.1159/000213307
- Lee, T., Lee, A., and Luo, L. (1999). Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* 126, 4065–4076.
- Lewis, L. P. C., Siju, K. P., Aso, Y., Friedrich, A. B., Bulteel, A. J. B., Rubin, G. M., et al. (2015). A higher brain circuit for immediate integration of conflicting sensory information in *Drosophila*. *Curr. Biol.* 25, 2203–2214. doi: 10.1016/j.cub.2015.07.015
- Lima, S. Q., and Miesenböck, G. (2005). Remote control of behavior through genetically targeted photostimulation of neurons. *Cell* 121, 141–152. doi: 10.1016/j.cell.2005.02.004
- Lin, H.-H., Lai, J. S.-Y., Chin, A.-L., Chen, Y.-C., and Chiang, A.-S. (2007). A map of olfactory representation in the *Drosophila* mushroom body. *Cell* 128, 1205–1217. doi: 10.1016/j.cell.2007.03.006
- Liu, C., Plaças, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., et al. (2012a). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* 488, 512–516. doi: 10.1038/nature11304
- Liu, Q., Liu, S., Kodama, L., Driscoll, M. R., and Wu, M. N. (2012b). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. *Curr. Biol.* 22, 2114–2123. doi: 10.1016/j.cub.2012.09.008

- Liu, S., Liu, Q., Tabuchi, M., and Wu, M. N. (2016). Sleep drive is encoded by neural plastic changes in a dedicated circuit. *Cell* 165, 1347–1360. doi: 10.1016/j.cell.2016.04.013
- Macpherson, L. J., Zaharieva, E. E., Kearney, P. J., Alpert, M. H., Lin, T.-Y., Turan, Z., et al. (2015). Dynamic labelling of neural connections in multiple colours by trans-synaptic fluorescence complementation. *Nat. Commun.* 6:ncmms10024. doi: 10.1038/ncomms10024
- Mao, Z., and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Front. Neural Circuits* 3:5. doi: 10.3389/neuro.04.005.2009
- Marder, E. (2012). Neuromodulation of neuronal circuits: back to the future. *Neuron* 76, 1–11. doi: 10.1016/j.neuron.2012.09.010
- Martín, F., and Alcorta, E. (2017). Novel genetic approaches to behavior in *Drosophila*. *J. Neurogenet.* 31, 288–299. doi: 10.1080/01677063.2017.1395875
- Martin, J. R., Ernst, R., and Heisenberg, M. (1998). Mushroom bodies suppress locomotor activity in *Drosophila melanogaster*. *Learn. Mem.* 5, 179–191.
- Martin, J. R., Ernst, R., and Heisenberg, M. (1999a). Temporal pattern of locomotor activity in *Drosophila melanogaster*. *J. Comp. Physiol. A* 184, 73–84. doi: 10.1007/s003590050307
- Martin, J. R., Faure, P., and Ernst, R. (2001). The power law distribution for walking-time intervals correlates with the ellipsoid-body in *Drosophila*. *J. Neurogenet.* 15, 205–219. doi: 10.3109/01677060109167377
- Martin, J. R., Raabe, T., and Heisenberg, M. (1999b). Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*. *J. Comp. Physiol. A* 185, 277–288. doi: 10.1007/s003590050387
- Meehan, M. J., and Wilson, R. (1987). Locomotor activity in the Tyr-1 mutant of *Drosophila melanogaster*. *Behav. Genet.* 17, 503–512. doi: 10.1007/BF01073117
- Miquel, J., Lundgren, P. R., and Binnard, R. (1972). Negative geotaxis and mating behavior in control and gamma-irradiated *Drosophila*. *Drosoph. Inf. Serv.* 48, 60–61.
- Musso, P.-Y., Tchenio, P., and Preat, T. (2015). Delayed dopamine signaling of energy level builds appetitive long-term memory in *Drosophila*. *Cell Rep.* 10, 1023–1031. doi: 10.1016/j.celrep.2015.01.036
- Nall, A. H., Shakhmantsir, I., Cichewicz, K., Birman, S., Hirsh, J., and Sehgal, A. (2016). Caffeine promotes wakefulness via dopamine signaling in *Drosophila*. *Sci. Rep.* 6:20938. doi: 10.1038/srep20938
- Neuser, K., Triphan, T., Mronz, M., Poeck, B., and Strauss, R. (2008). Analysis of a spatial orientation memory in *Drosophila*. *Nature* 453, 1244–1247. doi: 10.1038/nature07003
- O'Dell, K., and Burnet, B. (1988). The effects on locomotor activity and reactivity of the hypoactive and inactive mutations of *Drosophila melanogaster*. *Heredity* 61, 199–207. doi: 10.1038/hdy.1988.106
- Owald, D., Felsenberg, J., Talbot, C. B., Das, G., Perisse, E., Huetteroth, W., et al. (2015a). Activity of defined mushroom body output neurons underlies learned olfactory behavior in *Drosophila*. *Neuron* 86, 417–427. doi: 10.1016/j.neuron.2015.03.025
- Owald, D., Lin, S., and Waddell, S. (2015b). Light, heat, action: neural control of fruit fly behaviour. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370, 20140211. doi: 10.1098/rstb.2014.0211
- Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn. Mem.* 16, 289–295. doi: 10.1101/lm.1331809
- Pathak, T., Agrawal, T., Richhariya, S., Sadaf, S., and Hasan, G. (2015). Store-operated calcium entry through Orai is required for transcriptional maturation of the flight circuit in *Drosophila*. *J. Neurosci.* 35, 13784–13799. doi: 10.1523/JNEUROSCI.1680-15.2015
- Pech, U., Dipt, S., Barth, J., Singh, P., Jauch, M., Thum, A. S., et al. (2013a). Mushroom body miscellanea: transgenic *Drosophila* strains expressing anatomical and physiological sensor proteins in Kenyon cells. *Front. Neural Circuits* 7:147. doi: 10.3389/fncir.2013.00147
- Pech, U., Pooryasin, A., Birman, S., and Fiala, A. (2013b). Localization of the contacts between Kenyon cells and aminergic neurons in the *Drosophila melanogaster* brain using SplitGFP reconstitution. *J. Comp. Neurol.* 521, 3992–4026. doi: 10.1002/cne.23388
- Pfaff, D., and Banavar, J. R. (2007). A theoretical framework for CNS arousal. *Bioessays* 29, 803–810. doi: 10.1002/bies.20611
- Pimentel, D., Donlea, J. M., Talbot, C. B., Song, S. M., Thurston, A. J. F., and Miesenböck, G. (2016). Operation of a homeostatic sleep switch. *Nature* 536, 333–337. doi: 10.1038/nature19055
- Pitmon, E., Stephens, G., Parkhurst, S. J., Wolf, F. W., Kehne, G., Taylor, M., et al. (2016). The D1 family dopamine receptor, DopR, potentiates hind leg grooming behavior in *Drosophila*. *Genes Brain Behav.* 15, 327–334. doi: 10.1111/gbb.12264
- Plaçaïs, P.-Y., de Tredern, É., Scheunemann, L., Trannoy, S., Goguel, V., Han, K.-A., et al. (2017). Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory. *Nat. Commun.* 8:15510. doi: 10.1038/ncomms15510
- Plaçaïs, P.-Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliard-Guérin, G., et al. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat. Neurosci.* 15, 592–599. doi: 10.1038/nn.3055
- Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., et al. (2011). Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 108, 834–839. doi: 10.1073/pnas.1010930108
- Riemensperger, T., Issa, A.-R., Pech, U., Coulom, H., Nguyễn, M.-V., Cassar, M., et al. (2013). A single dopamine pathway underlies progressive locomotor deficits in a *Drosophila* model of Parkinson disease. *Cell Rep.* 5, 952–960. doi: 10.1016/j.celrep.2013.10.032
- Riemensperger, T., Kittel, R. J., and Fiala, A. (2016). Optogenetics in *Drosophila* Neuroscience. *Methods Mol. Biol.* 1408, 167–175. doi: 10.1007/978-1-4939-3512-3_11
- Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr. Biol.* 15, 1953–1960. doi: 10.1016/j.cub.2005.09.042
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019
- Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr. Biol.* 16, 1741–1747. doi: 10.1016/j.cub.2006.07.023
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–10502.
- Séjourné, J., Plaçaïs, P.-Y., Aso, Y., Siwanowicz, I., Trannoy, S., Thoma, V., et al. (2011). Mushroom body efferent neurons responsible for aversive olfactory memory retrieval in *Drosophila*. *Nat. Neurosci.* 14, 903–910. doi: 10.1038/nn.2846
- Selcho, M., Pauls, D., Han, K.-A., Stocker, R. F., and Thum, A. S. (2009). The role of dopamine in *Drosophila* larval classical olfactory conditioning. *PLoS ONE* 4:e5897. doi: 10.1371/journal.pone.0005897
- Serway, C. N., Kaufman, R. R., Strauss, R., and de Belle, J. S. (2009). Mushroom bodies enhance initial motor activity in *Drosophila*. *J. Neurogenet.* 23, 173–184. doi: 10.1080/01677060802572895
- Seugnet, L., Suzuki, Y., Vine, L., Gottschalk, L., and Shaw, P. J. (2008). D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in *Drosophila*. *Curr. Biol.* 18, 1110–1117. doi: 10.1016/j.cub.2008.07.028
- Sitaraman, D., Aso, Y., Jin, X., Chen, N., Felix, M., Rubin, G. M., et al. (2015a). Propagation of homeostatic sleep signals by segregated synaptic microcircuits of the *Drosophila* mushroom body. *Curr. Biol.* 25, 2915–2927. doi: 10.1016/j.cub.2015.09.017
- Sitaraman, D., Aso, Y., Rubin, G. M., and Nitabach, M. N. (2015b). Control of sleep by dopaminergic inputs to the *Drosophila* mushroom body. *Front. Neural Circuits* 9:73. doi: 10.3389/fncir.2015.00073
- Strauss, R. (2002). The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol.* 12, 633–638. doi: 10.1016/S0959-4388(02)00385-9
- Strauss, R., and Heisenberg, M. (1993). A higher control center of locomotor behavior in the *Drosophila* brain. *J. Neurosci.* 13, 1852–1861.
- Su, C.-Y., and Wang, J. W. (2014). Modulation of neural circuits: how stimulus context shapes innate behavior in *Drosophila*.

- Curr. Opin. Neurobiol.* 29, 9–16. doi: 10.1016/j.conb.2014.04.008
- Takemura, S.-Y., Aso, Y., Hige, T., Wong, A., Lu, Z., Xu, C. S., et al. (2017). A connectome of a learning and memory center in the adult *Drosophila* brain. *Elife* 6:e26975. doi: 10.7554/eLife.26975
- Tanaka, N. K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. *J. Comp. Neurol.* 508, 711–755. doi: 10.1002/cne.21692
- Tomita, J., Ban, G., and Kume, K. (2017). Genes and neural circuits for sleep of the fruit fly. *Neurosci. Res.* 118, 82–91. doi: 10.1016/j.neures.2017.04.010
- Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., et al. (2012). Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat. Neurosci.* 15, 1516–1523. doi: 10.1038/nn.3238
- Vaccaro, A., Issa, A.-R., Seugnet, L., Birman, S., and Klarsfeld, A. (2017). *Drosophila* Clock is required in brain pacemaker neurons to prevent premature locomotor aging independently of its circadian function. *PLoS Genet.* 13:e1006507. doi: 10.1371/journal.pgen.1006507
- Van Swinderen, B., and Andretic, R. (2011). Dopamine in *Drosophila*: setting arousal thresholds in a miniature brain. *Proc. Biol. Sci.* 278, 906–913. doi: 10.1098/rspb.2010.2564
- Waddell, S. (2010). Dopamine reveals neural circuit mechanisms of fly memory. *Trends Neurosci.* 33, 457–464. doi: 10.1016/j.tins.2010.07.001
- Waddell, S. (2013). Reinforcement signalling in *Drosophila*; dopamine does it all after all. *Curr. Opin. Neurobiol.* 23, 324–329. doi: 10.1016/j.conb.2013.01.005
- White, B. H., and Peabody, N. C. (2009). Neurotrapping: cellular screens to identify the neural substrates of behavior in *Drosophila*. *Front. Mol. Neurosci.* 2:20. doi: 10.3389/neuro.02.020.2009
- White, K. E., Humphrey, D. M., and Hirth, F. (2010). The dopaminergic system in the aging brain of *Drosophila*. *Front. Neurosci.* 4:205. doi: 10.3389/fnins.2010.00205
- Wu, M. N., Koh, K., Yue, Z., Joiner, W. J., and Sehgal, A. (2008). A genetic screen for sleep and circadian mutants reveals mechanisms underlying regulation of sleep in *Drosophila*. *Sleep* 31, 465–472. doi: 10.1093/sleep/31.4.465
- Yamagata, N., Hiroi, M., Kondo, S., Abe, A., and Tanimoto, H. (2016). Suppression of dopamine neurons mediates reward. *PLoS Biol.* 14:e1002586. doi: 10.1371/journal.pbio.1002586
- Yamamoto, A., Zwarts, L., Callaerts, P., Norga, K., Mackay, T. F. C., and Anholt, R. R. H. (2008). Neurogenetic networks for startle-induced locomotion in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12393–12398. doi: 10.1073/pnas.0804889105
- Yellman, C., Tao, H., He, B., and Hirsh, J. (1997). Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4131–4136. doi: 10.1073/pnas.94.8.4131
- Yoshihara, M., and Ito, K. (2012). Acute genetic manipulation of neuronal activity for the functional dissection of neural circuits—a dream come true for the pioneers of behavioral genetics. *J. Neurogenet.* 26, 43–52. doi: 10.3109/01677063.2012.663429
- Zhou, Q. Y., and Palmiter, R. D. (1995). Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. *Cell* 83, 1197–1209. doi: 10.1016/0092-8674(95)90145-0

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Structural and Molecular Properties of Insect Type II Motor Axon Terminals

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A comparison between the axon terminals of octopaminergic efferent dorsal or ventral unpaired median neurons in either desert locusts (*Schistocerca gregaria*) or fruit flies (*Drosophila melanogaster*) across skeletal muscles reveals many similarities. In both species the octopaminergic axon forms beaded fibers where the boutons or varicosities form type II terminals in contrast to the neuromuscular junction (NMJ) or type I terminals. These type II terminals are immunopositive for both tyramine and octopamine and, in contrast to the type I terminals, which possess clear synaptic vesicles, only contain dense core vesicles. These dense core vesicles contain octopamine as shown by immunogold methods. With respect to the cytomatrix and active zone peptides the type II terminals exhibit active zone-like accumulations of the scaffold protein Bruchpilot (BRP) only sparsely in contrast to the many accumulations of BRP identifying active zones of NMJ type I terminals. In the fruit fly larva marked dynamic changes of octopaminergic fibers have been reported after short starvation which not only affects the formation of new branches (“synaptopods”) but also affects the type I terminals or NMJs via octopamine-signaling (Koon et al., 2011). Our starvation experiments of *Drosophila*-larvae revealed a time-dependency of the formation of additional branches. Whereas after 2 h of starvation we find a decrease in “synaptopods”, the increase is significant after 6 h of starvation. In addition, we provide evidence that the release of octopamine from dendritic and/or axonal type II terminals uses a similar synaptic machinery to glutamate release from type I terminals of excitatory motor neurons. Indeed, blocking this canonical synaptic release machinery via RNAi induced downregulation of BRP in neurons with type II terminals leads to flight performance deficits similar to those observed for octopamine mutants or flies lacking this class of neurons (Brembs et al., 2007).

Keywords: insects, neuromodulation, axonal terminals, biogenic amines, cytomatrix proteins

INTRODUCTION

Vertebrate skeletal muscle is innervated by excitatory cholinergic motor neurons. By contrast, insect skeletal muscle is innervated by glutamatergic excitatory motor neurons, and may receive additional innervation by inhibitory neurons and neuromodulatory neurons (Usherwood, 1975; Wolf and Lang, 1994; Pflüger and Duch, 2011). Among the neuromodulatory neurons which supply insect

muscles those releasing the biogenic amine octopamine are the most prominent. However, peptides such as allatostatin (Kreissl et al., 1999) or insulin-like peptide (Gorczyca et al., 1993) were also described to be released onto a few specialized muscles. As insect muscle fibers do not seem to produce action potentials, the motor axons have to build neuromuscular junctions (NMJs) in regular distances along the muscle fiber in order to ensure sufficient depolarization along the entirety of the fiber, and thus proper contractions (Usherwood, 1967, 1975; Fournier, 1981; Peron et al., 2009). In *Drosophila* larvae, the axon terminals of excitatory glutamatergic motor neurons form NMJs and were named type I terminals (Keshishian et al., 1996; Feeney et al., 1998; Prokop, 2006; Prokop and Meinertzhagen, 2006), and further subdivided into type Ib and Is terminals (Atwood et al., 1993; Jia et al., 1993; Choi et al., 2004; He et al., 2009). In addition, some special peptidergic type III terminals were described (Anderson et al., 1988; Cantera and Nässel, 1992; Gorczyca et al., 1993; Zhong and Peña, 1995; Budnik, 1996), but the majority of axonal terminals of neuromodulatory neurons including octopaminergic neurons (Atwood et al., 1993; Monastirioti et al., 1995) consists of type II terminals. In other insects these aminergic axons were described as “beaded fibers” because of the regular occurrence of varicosities or boutons along their length. With respect to the vesicles found within these different terminals, those of the type I were described as round and clear with a diameter of 40–50 nm (Atwood et al., 1993; Jia et al., 1993), with differentiation into Ib and Is. The type II terminals were described in containing clear elliptical and dense core vesicles of 100–150 nm in diameter (Atwood et al., 1993). **Table 1** provides data on some properties of the different terminals on larval *Drosophila* muscle.

Octopaminergic neurons which form beaded fiber axons and type II like terminals were first recognized by Plotnikova (1969) in locusts and later extensively studied by Hoyle (1974, 1978 for a review, see Bräunig and Pflüger, 2001). These axons arise from a specific class of unpaired median neurons with bilaterally symmetrical axons and either dorsal (DUM-neurons) or ventral (VUM-neurons) cell bodies (Watson, 1984). The

population of DUM/VUM-neurons can be further divided into subpopulations which are differentially recruited in different motor behaviors such as jump, walking or flying (Burrows and Pflüger, 1995; Baudoux et al., 1998; Duch and Pflüger, 1999; Duch et al., 1999; Mentel et al., 2008; Pflüger and Duch, 2011) or crawling (Johnston et al., 1999). The release of octopamine from type II terminals onto insect skeletal muscle has multiple effects: first, it produces a small (up to 10%) increase in twitch tension and, more significantly, an increase in relaxation rate of skeletal muscle (Evans and O’Shea, 1977; O’Shea and Evans, 1979; Ormerod et al., 2013). In contrast, myogenic contractions of specialized skeletal muscle-bundles are inhibited (Evans and O’Shea, 1978). Second, these octopaminergic neurons also have metabolic functions because they are involved in regulating (boosting) glycolysis (Mentel et al., 2003). If locust muscles have to rely on lipid metabolism, for example during flight, these neurons are switched off (Duch and Pflüger, 1999; Mentel et al., 2003). By contrast, shortly before flight octopamine release may prepare the flight power muscles for high glycolytic rates during take-off (Pflüger and Duch, 2011). Accordingly, the octopaminergic system is suggested to prime the whole organism for “soon-to-come dynamic action” and skeletal muscles are, of course, a key target (Orchard et al., 1993; Bräunig and Pflüger, 2001; Pflüger and Duch, 2011).

Octopamine is also known to be an important modulator in the central nervous system, for example in activating the CPG for flight (Wilson, 1961; Stevenson and Kutsch, 1987, 1988), and recruiting motor neurons of other than flight muscles (FMs) to the flight rhythm (Rillich et al., 2013). Correspondingly, octopamine deficient adult fruit flies have severe deficiencies in their flight performance with significantly shorter flight durations than control flies (Brembs et al., 2007). It is, however, still unclear whether this is a peripheral or central effect. Octopamine-release in the thoracic ganglia is either mediated by descending neurons, for example descending DUM/VUM-neurons of the SOG exclusively (Bräunig and Burrows, 2004; Cholewa and Pflüger, 2009), or by the dendritic processes of DUM/VUM-neurons which may also release

TABLE 1 | Properties of types of terminals on larval *Drosophila* muscle based upon the following references: Anderson et al. (1988), Johansen et al. (1989), Budnik and Gorczyca (1992), Cantera and Nässel (1992), Jia and Budnik (1992), Atwood et al. (1993), Gorczyca et al. (1993), Jia et al. (1993), Zhong and Peña (1995) and Prokop et al. (1996).

Terminal type	Bouton size diameter	Vesicle type	Vesicle size	Transmitter
Type I	3–5 μm	Clear vesicles (CV)	40–50 nm; 44 ± 0.3 nm	Glutamate
Type I	3.1 ± 1.6 μm max. 8 μm		30–50 nm	
Type Ib	2–5 μm	CV		
Type Is	1–3 μm	CV		
Type II	1–2 μm	Dense core vesicles (DCV)	100–150 nm	Octopamine
Type II	1.4 ± 0.6 μm			
		Dark DCV	73 ± 2 nm	
		Intermediate and light DCV	108 ± 6 nm; 97 ± 1 nm	
		Small (translucent) CV	33.0 ± 0.5 nm;	
Type III	1–3 μm			Proctolin Insulin-like-peptide PACAP Leucokinin I

octopamine although ultrastructural studies only revealed clear input synapses (Pflüger and Watson, 1995). On the other hand, the dendritic processes of all DUM/VUM-neurons are labeled by tyramine- and octopamine-antibodies (Kononenko et al., 2009) and also bruchpilot (BRP^{NC82}, this study Supplementary Figure S1). In addition, octopamine has also been reported to affect target cell metabolism (Mentel et al., 2003; Pflüger and Duch, 2011). At present it remains unresolved what the relative contributions of the various reported effects of octopamine on central and peripheral excitability, structure, and metabolism are.

There is also a link between hunger and stress and the octopaminergic system (Wicher, 2007; Kononenko et al., 2009). In *Drosophila* larvae, the axons of octopaminergic VUM-neurons form additional *synaptopods*, defined as filipodia-like extensions (Koon et al., 2011) or small axonal sprouts (see also Supplementary Figure S4), after a very short period of starvation and, in addition, the NMJ of excitatory type I terminals itself is also affected by this (Koon et al., 2011). Different octopamine receptors are involved in the fine control of these regulatory processes of type I and type II terminals (Koon and Budnik, 2012). Therefore, it is now clear that the octopaminergic neurons play an important role for adaptive responses in multiple behavioral contexts. The aim of our study was to provide, from a comparative view, a description of structural, molecular, and functional specializations of type II terminals in locusts and fruit flies.

MATERIALS AND METHODS

Locust Care

Individuals of both sexes of adult desert locusts, *Schistocerca gregaria* (Forsk.) were taken from our crowded colony maintained in Berlin. Animals were kept in a constant 12 h light/dark cycle at a temperature of 28°C. If not stated otherwise, the locusts were anesthetized by chilling at 4°C for at least 30 min prior to experiments.

Fruit Fly Care for Anatomical Studies

Fruit flies (*Drosophila melanogaster*) were raised at 24°C and 60% relative humidity with a 14 h:10 h light-dark cycle on cornmeal based food, following the Würzburg recipe (Guo et al., 1996). Genetic crosses were performed according to standard procedures (Sigrist et al., 2003). All experiments were performed with heterozygous 3–5 day old male F1 progeny of homozygous parental lines. Genetic lines used in this study were not outcrossed to a reference strain with a specific genetic background. For some of the experiments transgenic fruit flies TDC2gal4 × UAScd8GFP were used to label all neurons which synthesize tyramine and octopamine from tyrosine (Monastirioti et al., 1995).

Dissections of Animals

Antero-/Retrograde Nerve Stainings in Locusts

Adult locusts ($n = 6$) were mounted laterally in plasticine to expose the thoracic pleura. The motor nerve N4D4 innervating

the pleuroaxillary flight steering muscle (M85/M114) of the meso- or metathorax was exposed by: (i) cutting a small window into the cuticle of the posterior pleuron of the second or third thoracic segment; and then (ii) removing the dorsoventral depressor muscle (M129). The intact nerve was carefully cut and either the distal (for anterograde staining) or the proximal end (for retrograde staining) was placed in a vaseline pool which subsequently was filled with dH₂O. After 10 min the water was replaced by neurobiotin (Neurobiotin tracer, Vector Laboratories) solution (10% in dH₂O). The pool and part of the exposed dissection were sealed with vaseline, and animals were placed in a moist chamber for 10–24 h at 4°C to enable dye diffusion. Prior to fixation, muscles or central ganglia were excised together with their attachment sites at the pleural ridge and the axillary sclerite, and transferred to a Sylgard dish (Silicone elastomere kit, Dow Corning) filled with isotonic locust saline (150 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, pH 7.4). In case of retrograde staining, the thoracic ganglia were isolated and cleansed of connective tissue, fat and trachea, and also pinned to a Sylgard dish. For subsequent tissue fixation and further processing see following paragraphs. The nomenclature of nerves and muscles is used according to Snodgrass (1929) and Campbell (1961).

Dissections of Fruit Fly (*Drosophila*) Larvae and Adults

Drosophila 3rd larval instars were kept on ice, mounted in a Sylgard dish dorsal side up and cut open by a dorsal medial incision. The preparation was carefully outlined by pins ("filet"-preparation), covered by cold *Drosophila*-saline (HL3, pH 7.2; all mM, 70 NaCl, 5 KCl, 20 MgCl₂, 10 NaHCO₃, 5 Trehalose, 115 Sucrose, 5 HEPES), and the gut removed.

Adult flies were also kept on ice, and before mounting all legs and wings were removed. The flies were mounted dorsal side up, covered by *Drosophila*-saline and opened by a dorsal medial incision and carefully pinned to a Sylgard dish. Then guts and reproductive organs together with any loose fat were removed to expose the ventral nerve cord and thoracic muscular system.

Immunocytochemistry of Locust Muscle

Dissected adult locust muscles were fixed in 1% glutaraldehyde (GA) and 2% paraformaldehyde (PFA) in 0.1 M PBS (phosphate buffered saline, pH 7.4) for about 3 h. After rinsing in PBS for 2 h and dehydration by means of an ascending ethanol series (50%, 70%, 90%, 100%; 10 min each) the preparations were clarified in xylene (Merck) and subsequently rehydrated (100%, 90%, 70%, 50%; 10 min each). In order to reduce unspecific staining, 1% sodium borohydride (Merck) in PBS was applied for 10 min followed by incubation in 1% Triton X-100 (TX, Sigma) in PBS (2 h). For 1–2 h the preparations were preincubated in 10% normal goat serum (NGS, ISN Biomedicals) in 1% TX in PBS to block unspecific binding sites. Primary antibodies used were: (i) anti-synapsin I (SynORF, 3C11, kindly provided by Prof. Erich Buchner, Würzburg); and (ii) anti-NC82 (kindly provided by Prof. Stephan Sigrist,

Berlin) both from mouse, applied at a dilution of 1:7 (SynI) and 1:100 (NC82); (iii) a monoclonal octopamine antibody (anti-octopamine from mouse, Bioscience, Jena, see Dacks et al., 2005; and Kononenko et al., 2009), 1:1000; and (iv) a polyclonal antibody to tyramine (anti-tyramine from rabbit, Chemicon; see Kononenko et al., 2009), 1:500. To shield samples from fungal infection, 0.02% sodium acid was added to the 1% TX 1% NGS antiserum solution. Binding of antibodies occurred at 4°C on a shaker for at least 3 days, followed by incubation in 1% TX in PBS for approximately 2 h. Secondary antibodies, Cy2- or Cy3-conjugated goat anti-mouse (Dianova), for synapsin I, NC82 and octopamine, Cy3-conjugated streptavidin (Dianova) for amplification of neurobiotin, and Cy5-conjugated goat anti-rabbit (Dianova) for tyramine (all diluted at 1:200) were added and the preparations were kept overnight at room temperature (RT). After repeated washing in PBS for 2 h the preparations were dehydrated (50%, 70%, 90%, 100%; 10 min each) and finally mounted in methylsalicylate to a microscope slide and sealed with a cover slip (Merck).

Immunocytochemistry of Fruit Fly (*Drosophila*) Muscle

Larval body wall muscles and adult flight muscles (DLMs) in 10 animals each were stained with the following antibodies: anti-GFP, anti-brp (BRP), and anti-HRP (Horseradish peroxidase). In larvae, $n = 3$, body wall muscles were stained with anti-tyramine and anti-octopamine to reveal labeling in type II terminals.

For anti-GFP, anti-brp and anti-HRP the following steps were applied: the dissected larvae were covered for 60 min in 4% PFA in 0.1 M phosphate buffered saline (PBS, pH 7.4) to fix the preparations. The animals were washed overnight in 0.1 M PBS (pH 7.4) while placing the Sylgard dish inside a dark cold room at 4°C. The next day, they were washed in 0.1 M PBS + 0.5% Triton-X (TX) for 160 min while changing the solution every 20 min. For pre-incubation the dissected animals were covered in 10% NGS in 0.1 M PBS + 0.5 TX for 60 min. Then, they were washed in 1960 μ l 0.1 M PBS + 0.3% TX + 20 μ l NGS + 20 μ l of the primary antibody anti-green-fluorescent-protein rabbit (anti-GFP; 1:100) for 2 days in a dark cold room at 4°C. Subsequently, preparations were washed in 0.1 M PBS + 0.3% TX for 120 min and the solution was changed every 15 min. For the secondary antibody staining, the animals were washed in 40 μ l goat anti rabbit Alexa 488 Cy2 (1:100) + 0.2 μ l Atto 565 Phalloidin + 4 μ l anti-HRP Cy5 (1:500) in 1956 μ l 0.1 M PBS + 0.3% TX overnight and protected from light. The next day, specimen were washed for 90 min in 0.1 M PBS with the solution being exchanged every 15 min. After that, the insect pins were carefully removed from the animals and each preparation was placed in Dianova IS mounting medium on a thin microscope slide which was then sealed with a 20 $\times$ 20 mm cover slip and stored at 4°C protected from light until confocal imaging.

For anti-tyramine and anti-octopamine staining of larval body wall muscles the above mentioned recipe for locust muscle was used ($n = 3$, see also Kononenko et al., 2009).

Confocal Microscopy of Locust Muscle

Image Acquisition

Immunofluorescent labels were visualized and scanned with a confocal scanning microscope (TCS SP2, Leica, Germany). Scanning of muscle stainings was performed by using either a HC PL 10 $\times$ /0.5, a HC PL 20 $\times$ /0.7 imm, a HC PL 40 $\times$ /1.4 imm or a HC PL 63 $\times$ /1.52 imm objective at a maximal zoom factor of 3. Signals from fluorophores were detected in serial stacks of appropriate slice numbers, and at an image resolution of 1024 $\times$ 1024 pixels. For sample stack generation of stained muscle tissue three different regions per muscle (proximal, medial, distal) were chosen and stacks of constant dimension and magnification were scanned (x, y 0.15 μ m, step size 1 μ m), at, as far as possible, constant laser intensity. Excitation of fluorescent dyes was enabled by using three different laser lines: an Ar/Kr-Laser at 488 nm and two He/Ne-Lasers at either 543 nm or 633 nm. Images were scanned in a sequential mode to confine cross-excitation of different dyes. For analysis image stacks were processed with the software package Amira 4.1.1 (TGS, Mercury Computer Systems, Mérignac, France).

Ultrastructure and Anti-octopamine Immunogold-Staining of Locust and Fruit Fly Muscle

Electron Microscopy

For the normal ultrastructure of muscles, adult desert locust *Schistocerca gregaria* ($n = 11$) tissue was prefixed in 2.5% GA in 0.1 M Na-cacodylate buffer (pH 7.4), for 3–7 min after dissection of the animal, and then fixed for 1–2 h at RT in the same fixer solution. After fixation, specimen were washed 3 $\times$ 15 min in 0.1 M PBS (pH 7.4) at RT, followed by fixation in 1% OsO₄ in 0.1 M PBS for 1 h at RT. Then specimens were dehydrated in an ascending ethanol series (30%, 50%, 70%, 96%, 100%, 2 $\times$ 10 min each), followed by 2 $\times$ 15 min propylene oxide and 2 $\times$ 15 min acetonitrile. Specimen were then left in a mixture of acetonitrile and epoxy resin (EMbed 812), 2:1, overnight (up to 17 h) and then finally embedded in fresh resin (9 h at RT, over-night at 37°C, 48 h at 60°C) for polymerization.

To investigate the ultrastructure of wild type fruit fly (*Drosophila*) larva body wall ($n = 5$) and adult FMs ($n = 7$), specimen were fixed in 2% GA in 0.1 M PBS + 0.1 M sucrose (pH 7.4), 2 h; washed in the same buffer and post-fixed in 1% OsO₄ in 0.1 M PBS, pH 7.4, 80 min; washed in dH₂O (3 $\times$ 5 min) and contrasted in 4% uranyl acetate in H₂O, 1 h, on a rotor, and rinsed again in dH₂O (3 $\times$ 5 min). Then, specimen were dehydrated in an ascending ethanol series and acetone, and finally embedded in epoxy resin (EMbed 812, ElectronMicroscopy Sciences, Hatfield, PA, USA). Series of ultrathin sections (50–70 nm) were cut on a Leica EM KMR2 ultra-microtome with a diamond knife, mounted on formvar-coated slots, stained with 4% uranyl acetate and 0.4% lead citrate (in dH₂O), and examined/viewed with a Philips 208 transmission

electron microscope, operated at an accelerating voltage of 80 kV.

Immuno-Gold Staining of Locust and Fruit Fly (*Drosophila*) Muscle

For immunogold-studies, muscles of adult locusts (*Schistocerca*, $n = 9$) and larval ($n = 4$) and adult ($n = 5$) fruit flies (*Drosophila*) were examined. For locusts, the same fixation was used as mentioned in the previous chapter. For *Drosophila* we used fixation in 1% GA + 2% PFA in 0.1 M PBS (pH 7.4) for 3 h at RT; after washing in dH₂O (3×5 min) specimen were post-fixed in 1% OsO₄ in the same buffer for 1 h; rinsed again and dehydrated in ascending ethanol series, 30% 50%, 70%, 2×10 min, on a rotor, 96%, 100%, 2×15 min; then specimen were impregnated with resin LRWhite I, 4 h and RWhite II, 3 h, on a rotor at RT and polymerized in fresh LRWhite in gelatin capsules at 58°C for 24 h. The fixation protocol of tissue of adult locusts (*Schistocerca gregaria*) for immunogold-staining was described in Skieba et al. (2006).

Semithin-sections for light microscopy (1.5 μ m) and ultrathin sections (50–70 nm) for electron microscopy were cut on a Leica EM KMR2 ultramicrotome. For ultrastructural immunocytochemistry, the sections were mounted on gold-plated slots. The protocol of etching was the same as described in Skieba et al. (2006) with using 2% periodic acid, saturated Na-metaperiodate and washing in dH₂O. After etching, sections were washed in 1% sodium metabisulfite (SMBS) in dH₂O. 2×10 min, and then in 0.1 M Tris-HCl, pH 7.0, + 0.1% SMBS + 0.05% NaN₃, 10 min, RT.

Pre-incubation was done in 5% NGS in 0.1 M Tris-HCl (pH 7.0) + 0.1% SMBS + 0.05% NaN₃ for 45 min at RT. Subsequently, sections were incubated in the primary monoclonal anti-octopamine antibody developed in mouse (Jena Bioscience), 1:500 in 0.1 M Tris-HCl, pH 7.0, + 0.1% SMBS + 0.05% NaN₃ + 1% NGS for 18 h at 4°C on a shaker. After this sections were washed in 0.1 M Tris-HCl (pH 7.0) + 0.1% SMBS + 0.05% NaN₃ for 3×10 min, then in 0.1 M PBS (pH 7.4) for 3×2 min, and then in 0.1 M PBS + 0.2% bovine serum albumin (BSA) for 3×2 min. Then, the secondary antibody was applied (goat anti-mouse-IgG-gold-conjugate, particle size 15 nm, AURION, Netherlands), 1:20 in 0.1 M PBS (pH 7.4) + 0.2% BSA for 2 h at RT on a shaker. Finally, sections were washed in 0.1 M PBS 5×5 min, then in dH₂O 3×4 min before contrasted in 4% uranyl acetate (in dH₂O) for 10 min at 37°C and in 0.4% lead citrate for 5 min at RT and examined with a Philips 208 transmission electron microscope.

Behavioral Treatments

Starvation Experiments of Fruit Fly (*Drosophila*) Larvae

Wandering, still feeding early third-instar larvae were put on wet filter paper without food for up to 6 h and dissected after 2 h ($n = 8$), 4 h ($n = 8$) and 6 h ($n = 8$). Fed larvae served as the control group ($n = 32$). Each of the flies that were used in this study carried a copy of Tdc2-GAL4 and UAS-CD4-GFP. Larvae were dissected and immuno-stained as described earlier.

Synaptopod Quantification in Fruit Fly Neuromodulatory Axons

Synaptopod numbers of octopaminergic branches were measured manually at two body wall muscle fibers (14, 28) using the open-source software Fiji, a distribution of ImageJ version 1.48b (Schindelin et al., 2012). Branches, other than the “average base (main) branches”, were counted as *synaptopods* if they measured at least 5 μ m in length (see Supplementary Figure S4).

Blocking Release From Fruit Fly Type II Terminals by RNAi-Bruchpilot

Fly Care

Flies were raised at 25°C and 60% relative humidity with a 12 h/12 h light/dark cycle. The Tdc2-GAL4 (II) stock was kindly provided by Henrike Scholz, Cologne, and outcrossed into CantonS background. The w-UAS-brp-RNAi^{B12} (X) stock was kindly provided by Stephan Sigrist, Berlin.

Behavioral Experiments

After briefly immobilizing 2–3 days old female flies by cold-anesthesia, head and thorax were glued (Sinfony Indirect Lab Composite, 3M ESPE, St. Paul, MN, USA) to a triangle-shaped copper hook (0.05 mm diameter). The animals were then kept individually in small moist chambers containing a few grains of sucrose until testing 1 or 5 h later. The hook was clamped magnetically to a stand to accomplish stationary flight. The fly was surrounded by a homogenous panorama under room light conditions. The observer sat behind the setup and removed a polystyrene bead or a piece of filter paper from the fly's tarsi. This initiated spontaneous flight and the experiment was started. The time until the fly stopped flying was recorded for three consecutive flights whereby 600 s was a given maximum of flight duration. When the fly ceased flying it was gently stimulated from the front side using a fly aspirator. The duration of the longest out of the three flights (see **Figure 8A**) or the duration of the first flight (see **Figure 8B**) were listed as a data point for each fly.

Statistical Analysis

For survival analysis of flight duration, we used Kaplan-Meier curves and Cox proportional hazards regression model. For the duration of the first flight, we used a Wilcoxon rank sum test (the data don't show normal distribution since values are truncated at 600 s). Bonferroni corrections were used for multiple comparisons.

RESULTS

Octopaminergic Axonal Terminals in Locusts (*Schistocerca*)

In locusts, a well-studied wing muscle, the mesothoracic pleuroaxillary muscle M85 changes the angle of pronation of a forewing and, thus, is involved in flight steering. Muscle M85 consists of two parts and is innervated by two glutamatergic excitatory motor neurons (Pflüger et al., 1986), one GABAergic common inhibitor neuron (Wolf and Lang, 1994), and by an octopaminergic DUM3,4,5-neuron

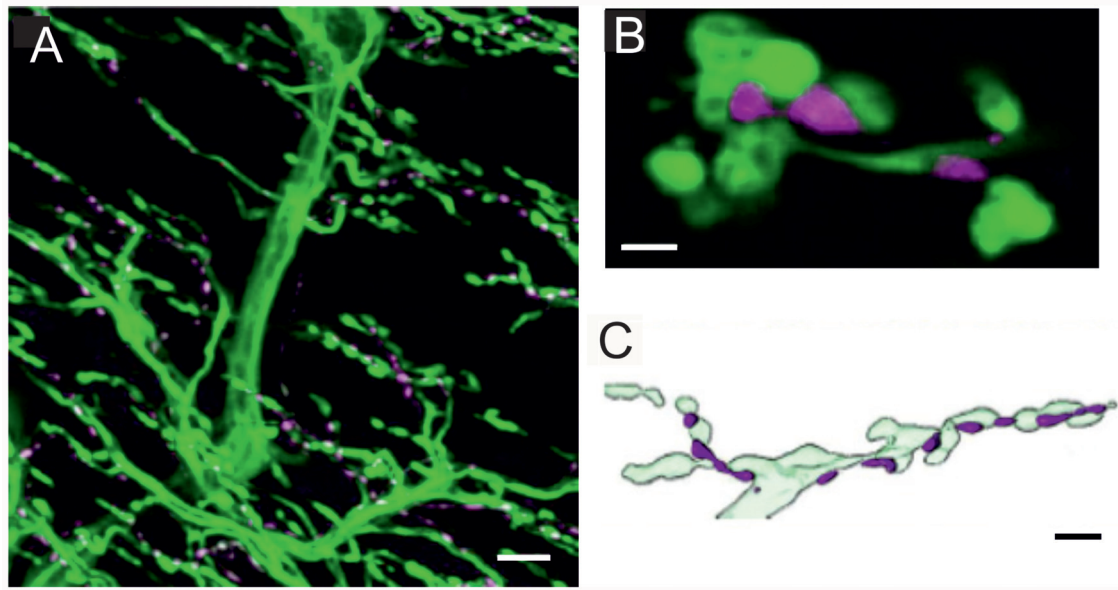


FIGURE 1 | Innervation of locust (*Schistocerca gregaria*) pleuroaxillary muscle M85. **(A)** Neurobiotin-Streptavidine-Fluorochrome (green) labeled anterograde fill of the motor nerve (N4D4) of M85 and anti-octopaminergic labeled varicosities (magenta). Z-dimension of image stack 100 μm , scale bar 10 μm . **(B)** Individual octopaminergic boutons (magenta) lie very close to motor neuron terminals (green); the distance between both structures is less than 300 nm. Single optical slice of 1 μm width, scale bar 2 μm . **(C)** Schematic illustration of the typical arrangement of neuromodulatory octopaminergic varicosities (magenta) and the motor fiber terminals (light green). Scale bar 5 μm .

(Stevenson and Meuser, 1997). **Figure 1A** shows an anterograde neurobiotin fill of the motor nerve N4D4 which labels the axonal paths and terminals of all neurons innervating this muscle (green). The axonal terminals of the octopaminergic DUM3,4,5-neuron are also labeled with an α -octopamine AB (magenta) and appear very similar to the type II terminals of octopaminergic VUM-neurons of *Drosophila* larvae. The small neuromodulatory boutons are in close vicinity to the excitatory glutamatergic motor axons with large terminals corresponding to NMJs and in *Drosophila* terminology to type I terminals. The close spatial relationship between octopaminergic type II terminals and glutamatergic type I terminals is exemplified in a representative single optical section in **Figure 1B**, where the green labeled motor terminals encircle the magenta labeled neuromodulatory terminals of the DUM3,4,5-neuron. **Figure 1C** shows a sketch of the relation between motor and neuromodulatory axons in locust muscle and also schematically reveals the differences between motor axons forming NMJs or type I terminals and the neuromodulatory axons (*beaded fibers*) with their type II terminals.

Labeling of all octopaminergic axonal terminals on a whole locust muscle (M85) is shown in **Figure 2A**. Octopaminergic type II terminals seem to occur at a rather evenly distributed density and to form a dense meshwork through the entire muscle fibers with no obvious spared parts of the muscle. As octopamine is synthesized from tyramine and the dendritic profiles of DUM-neurons were shown to stain with both a tyramine- and octopamine-AB (Kononenko et al., 2009), we also tested whether OA and TA co-existed in the axonal type II terminals. As

for the central profiles of DUM-neurons each axon terminal also expresses tyramine- and octopamine immunoreactivity (**Figures 2B,C**).

Octopaminergic Axonal Terminals in Fruit Fly (*Drosophila*) Muscle

All neurons which synthesize tyramine/octopamine from tyrosine by using the enzyme tyrosine-decarboxylase (TDC2) can be visualized in fruit flies (*Drosophila melanogaster*) by targeted expression of UAS-mcd8GFP under the control of TDC2-gal4. Most larval muscles are supplied by these neuromodulatory neurons which, in *Drosophila*, belong to the class of ventral unpaired median (VUM-) neurons (Bräunig and Pflüger, 2001; Vömel and Wegener, 2008; Busch et al., 2009; Busch and Tanimoto, 2010; Koon et al., 2011; Selcho et al., 2014). In *Drosophila* larvae all VUM-neuron axons also appear as *beaded fibers* with varicosities or boutons in regular distances that are classified as type II terminals (Monastirioti et al., 1995; Sinakevitch and Strausfeld, 2005). **Figure 3** shows that, like in locusts (see **Figures 2B,C**), these axon terminals express both tyramine- and octopamine-ir (see **Figure 3D**).

Octopaminergic Axonal Terminals in Locusts (*Schistocerca*) Contain Major Presynaptic Proteins

Synaptic vesicle exo- and endocytosis and the underlying networks of interacting proteins are particularly well studied at excitatory NMJs in *Drosophila* larvae (Haucke et al., 2011;

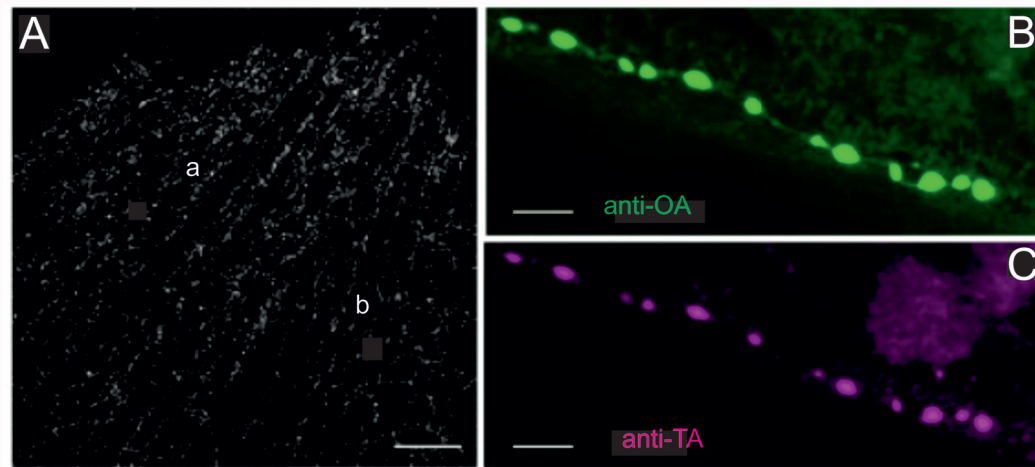


FIGURE 2 | Locust (*Schistocerca gregaria*) pleuroaxillary ("flight steering") muscle (M85) in which terminals are stained by octopamine- and tyramine-immunoreactivity. **(A)** OA-ir reveals evenly distributed chains of varicosities on muscle parts a and b of M85. Z-Dimension of image stack 30 μm , scale bar 100 μm . **(B,C)** Co-localization of OA-ir **(B)** and TA-ir **(C)** in boutons on M85 (same preparations). Z-Dimension of image stacks 11 μm , scale bar 5 μm .

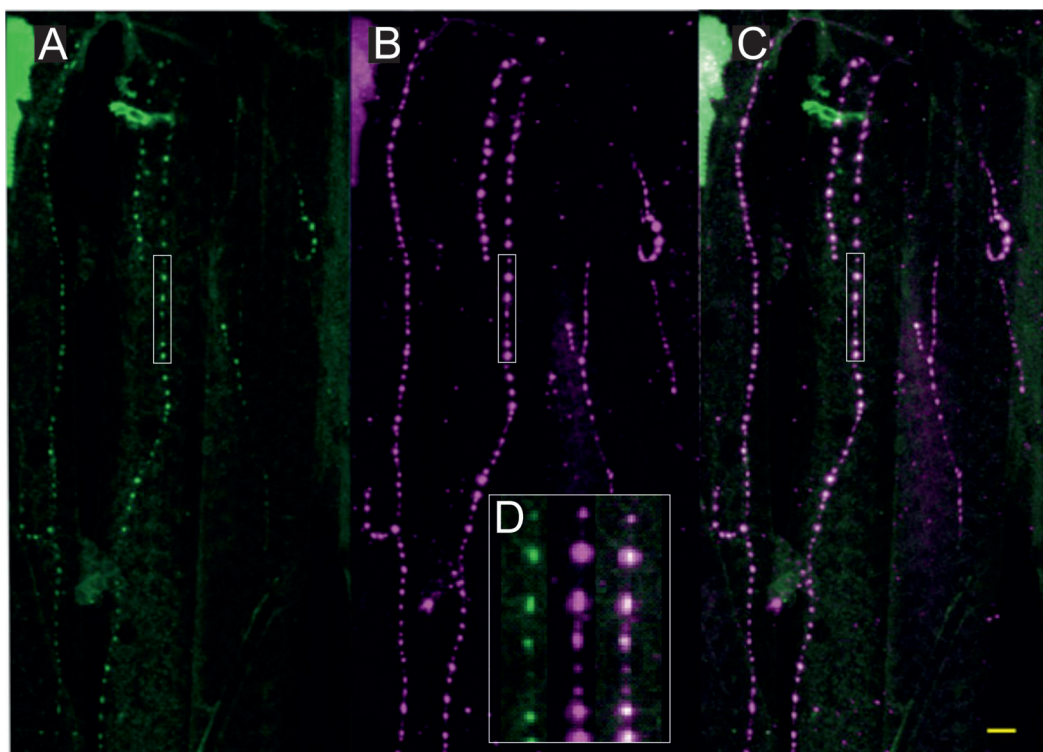


FIGURE 3 | Fruit fly (*Drosophila melanogaster*) larva body wall muscles labeled with anti-tyramine **(A)** and anti-octopamine **(B)** reveals co-labeling in the type II terminals of the beaded fibers (composite **C**). Scale bar: 10 μm . **(D)** Type II terminals at higher magnification. The magnified area is depicted by a white rectangular box in **(A–C)**. Scale bar: 2.5 μm .

Südhof, 2012, 2013; Kononenko and Haucke, 2015) but less so in adult insects including fruit flies and neuromodulatory type II terminals. Therefore, we tested in locust adult muscle

whether some of the key cytomatrix proteins known from type I axon terminals of motor neurons were also present in the axonal type II terminals of neuromodulatory neurons. In

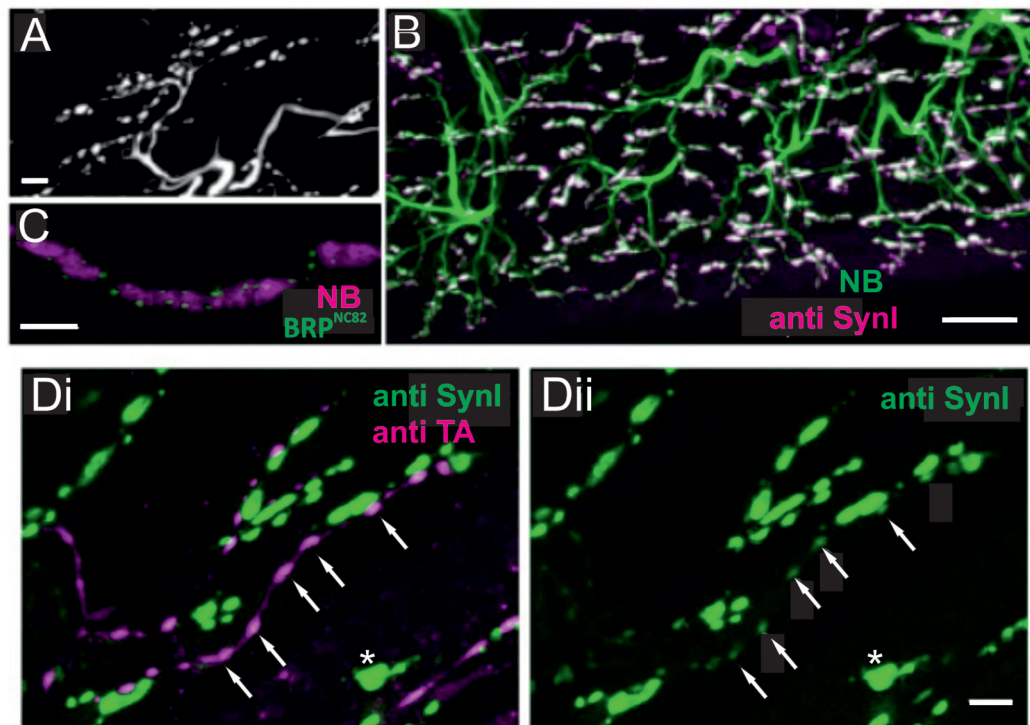


FIGURE 4 | Locust (*Schistocerca gregaria*) motor neuron terminals on M85 stained with antibodies to synaptic proteins. **(A)** Typical appearance of motor neuron terminals on M85 labeled with neurobiotin by anterograde staining of N4D4. Terminals are aligned in parallel to fibers and cover the entire muscle. Z-Dimension of image stack 10 μm , scale bar 10 μm . **(B)** Labeling of M85 with anti-synapsin I (magenta) in addition to the backfilled motor nerve by neurobiotin (green) highlights the presence and distribution of presynaptic sites (white). Z-Dimension of image stack 30 μm , scale bar 20 μm . **(C)** Immunoreactivity to the synaptic protein bruchpilot (BRP), BRP^{NC82} (green), can be located in motor terminals (magenta, NB-labeled) as distinct puncta presumably representing active zones. Z-Dimension of image stack 30 μm , scale bar 5 μm . **(D)** Double-labeling with anti-synapsin I (green) and anti-tyramine (magenta) shows the characteristic beaded octopaminergic/tyramineric fibers in proximity to motor terminals (green, **Di**). In addition to the markedly stained motor terminals by anti-synapsin (green, **Di** and **Dii**), one motor terminal marked by white asterisk weak labeling in O/A/T-A-ir boutons is also revealed (white arrows in **Di** and **Dii**). Z-dimension of image stacks 10 μm , scale bar 5 μm .

Figure 4 labeling with a synapsin-AB (Klagges et al., 1996) and with the active zone marker BRP^{NC82} (BRP; Wagh et al., 2006; Wichmann and Sigrist, 2010), reveals synaptic sites on locust muscle M85. An anterograde fill of the excitatory motor axons to M85 with neurobiotin reveals large axon terminals which run parallel to the muscle fiber length and exhibit the typical morphology of type I terminals (**Figure 4A**). In **Figure 4B** an overlap of such an anterogradely stained motor nerve (green) with an antibody staining against synapsin (magenta) reveals the many presynaptic sites (composite color white) of such an adult insect muscle. Labeling with the NC82-antibody (**Figure 4C**) also shows a “puncta”-distribution (green) on the large axon terminals (magenta), most likely indicating the active zones of presynaptic sites. In **Figures 4Di,Dii** simultaneous immunostainings with synapsin-AB (green) and a tyramine-AB (magenta) are shown. **Figure 4Di** proves that the *beaded fibers* with varicosities are the ones belonging to the neuromodulatory neuron which contains both tyramine and octopamine (white arrows, see also **Figures 2B,C**). In addition to the large motor axon (type I) terminals the varicosities of *beaded*, tyramineric/octopaminergic *fibers* also

express synapsin-ir (white arrows in **Figure 4Dii**). Please note that synapsin positive puncta are considerable smaller and less bright in type II terminals as compared to type I terminals (**Figure 4Dii**).

Octopaminergic Axonal Terminals in Fruit Flies (*Drosophila*) Contain Major Presynaptic Proteins

In addition to type I motor axon terminals, also type II terminals of neuromodulatory VUM-neurons contain the active zone marker BRP (**Figure 5**). **Figure 5A** shows the GFP-labeled axon of a VUM-neuron (green) in the vicinity of NMJs of the motor axons stained in blue/magenta on a larval body-wall muscle. The presynaptic active zones are revealed by BRP^{NC82} (BRP, red) and show their typical circular (“donut”-shaped) arrangement. Magnifications of the VUM-neuron axon (green) shown in **Figures 5B,C** reveal small BRP^{NC82} active zones in type II terminals (see white arrows pointing to red punctae). Again, the close vicinity of the octopaminergic VUM-neuron axons with the much larger terminals of the type I motor axons is obvious, in particular in those preparations in which the motor

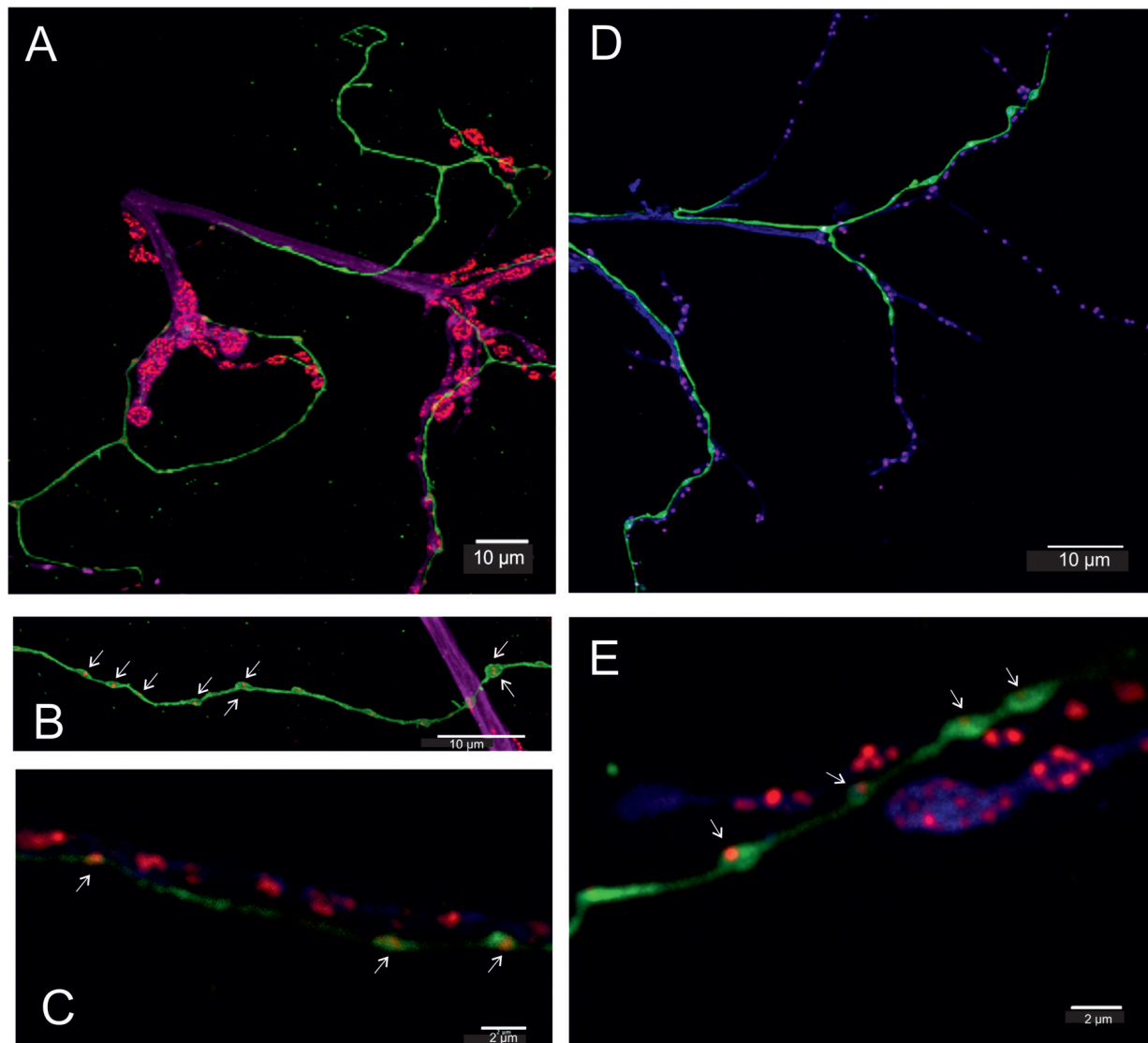


FIGURE 5 | Fruit fly (*Drosophila melanogaster*) larva (**A–C**) and adult (**D,E**). (**A–C**) In transgenic flies ($TDC2\text{-}gal4 \times UAS\text{-}CD8\text{-}GFP$, green) all VUM-neuron axons are labeled in green. Additionally, motor axons of body wall muscles are labeled by anti-Horseradish peroxidase (HRP) in magenta/blue and active zones of synaptic sites of the neuromuscular junction (NMJ) are labeled by BRP^{NC82} (“anti-bruchpilot”) in red and are adjacent to the GFP-labeled (green) octopaminergic axons (**A**). In the GFP-labeled octopaminergic fibers one or two BRP-labels are revealed in each bouton (varicosities in **B,C**, white arrows), and in (**C**) red BRP-labeling is also seen in the motor axon. (**D,E**) In the adult DLM flight muscle (FM) the axons of motor neurons are labeled by anti-HRP (blue) and again show their relationship to the VUM-neuron fiber stained in green and the red labels of the active zones. The structure of the NMJ of adult muscle differs from that of larval muscle, particularly good to see in (**E**). However, the octopaminergic VUM-fiber like in larvae reveals one (or two) BRP-spots (punctae) in each bouton (varicosity). Scale bars: (**A,B,D**): 10 μm , (**C,E**): 2 μm .

axons were additionally stained by using an HRP-antibody. In contrast to the type I terminals with many active zones, the varicosities or boutons of the VUM-neuron axon possess only one or two active zone each (**Figures 5B,C**, see two varicosities with two red punctae in **Figure 5B**, white arrows). In adult muscle (**Figure 5D**), again the GFP-labeled VUM-neuron axon accompanies the motor axons marked in blue by anti-HRP. NMJs in adult muscles are different to their counterparts in larvae as they are much less pronounced and much smaller (compare **Figure 5A** with **Figure 5D**). Nevertheless, they also

show labels of BRP^{NC82} (red) indicating BRP distribution in presynaptic active zones. A magnification in **Figure 5E** clearly shows that also in adult type II terminals one spot (puncta, white arrows) exhibits BRP^{NC82} , in contrast to the larger and more numerous BRP^{NC82} labeling (red) of the motor terminals (blue). In the Supplementary Figure S1 BRP^{NC82} (BRP) labeling is also revealed in the central, dendritic parts of octopaminergic VUM-neurons indicating that VUM-neurons may also possess presynaptic release sites within the ganglia of the ventral nerve cord.

Ultrastructure of Octopaminergic Axon Terminals in Locusts and Fruit Flies Including Immunogold-Stainings

The micrographs in **Figure 6** show the ultrastructure of axon terminals of octopaminergic VUM-neurons in larval (**Figures 6A,B**) and adult (**Figures 6C–E**) fruit flies (*Drosophila melanogaster*). In particular, the micrograph in **Figure 6A** shows the differences in structure of a type I (NMJ, t1) and a type II (octopaminergic, OT, t2) axon terminal. Whereas the type I terminal is characterized by many clear vesicles and the presence of a clear active zone depicted by the electron dense T-bar (black arrow in **Figure 6A**), the type II terminals only contain dense core vesicles (dcv) and no T-bar (**Figures 6A–C**). This was found in all animals tested. Identification of OA positive profiles was accomplished by immuno-gold coupling to an α -octopamine antibody (see black arrows in **Figures 6B,C**). Similar type II terminals full of dense core vesicles are present on the adult flight muscle (FM, **Figure 6D**). The inserts in **Figures 6B,C,E** show anti-octopamine immunogold labeling in dense core vesicles, or in the process of exocytosis from a dense core vesicle (arrow in **Figure 6C**). These ultrastructural studies combined with immunogold-methods unequivocally show that octopamine is stored and released from dense core vesicles (**Figures 6C,E**). In the Supplementary Figure S2 more immunogold-labeling is shown and there are indications based upon the distribution of gold-particles that once octopamine is released into the extracellular space it may be taken up by glial cells (GCs) from where it may be recycled (Ryglewski et al., 2017).

Plasticity of Tyraminergetic/Octopaminergic Axon Terminals in the Fruit Fly Larvae After Starvation

In a series of elegant live imaging experiments of axon terminals of VUM-neurons in *Drosophila* larvae, Koon et al. (2011) found that starvation for 2 h induced an activity dependent growth of octopaminergic *synaptopods* or small axonal sprouts with newly forming output synapses (see also Supplementary Figure S4), and that this is controlled by a cAMP- and CREB-dependent positive-feedback mechanism requiring Octb2R autoreceptors. This autoregulation was necessary for the observed increased locomotor responses after starvation. In order to further investigate the temporal dynamics of this process we examined type II terminals of selected body wall muscles (**Figure 7A**) in transgenic TDC2gal4 $\times$ UASCD8GFP larvae starved for 2, 4 and 6 h (**Figure 6B**) and measured the *synaptopods* per 100 μ m (**Figure 7C**). In addition to the previously reported starvation induced growth of aminergic *synaptopods* after 2 h of starvation (Koon et al., 2011), we observe a temporal dynamic with an initial decrease in the number of *synaptopods* followed by an increase after 6 h of starvation.

Activity-dependent plasticity of octopaminergic type II terminals can also be observed in locust muscle. In the supplementary material more information can be found including Supplementary Figure S3.

The Effect of Blocking Release From VUM-Neuron Terminals on Flight Performance of Adult Fruit Flies (*Drosophila melanogaster*)

This and previous studies demonstrate the existence of similar molecular components of the synaptic vesicle release machinery in type II and type I terminals. However, for type II terminals it has not yet been tested whether molecules, such as the CASK protein BRP, are critically required for synaptic vesicle release as is the case in type I terminals (Wagh et al., 2006). It had been previously shown that flies deficient in octopamine synthesis (Monastirioti et al., 1995) or deficient of octopaminergic/tyraminergetic neurons exhibit deficits in flight performance (Brembs et al., 2007). In order to test whether we could block OA release by disturbing the BRP-dependent vesicle release mechanisms, we expressed a potent UAS-RNAi transgene (brp-RNAi^{B12}) under the control of the tdc2-Gal4 driver. As expected, control flies are flying well in our assay. In contrast, the test flies stopped flying much earlier than their controls (**Figure 8A**). This phenotype was reminiscent of the effect of eliminating these neurons reported in Brembs et al. (2007) compare **Figure 8B** here to their **Figure 5A**). Unfortunately, differences in the performance of the control flies between the two studies, probably due to differences in fly care protocols, make a direct comparison of the two sets of data difficult. But our results strongly suggest that down-regulating of BRP in octopaminergic/tyraminergetic neurons impairs OA release from type II terminals.

DISCUSSION

The evolutionarily old hemimetabolous orthopteran desert locust, *Schistocerca gregaria*, and the evolutionarily more recent holometabolous dipteran insect, *Drosophila melanogaster*, possess octopaminergic/tyraminergetic neurons with highly conserved features. In both species OA/TA neurons have unpaired cell bodies along the dorsal (DUM neurons, locust) or ventral (VUM-neurons, fruit fly) midline and bilaterally symmetrical axons that project through efferent nerves (Bräunig and Pflüger, 2001; Pflüger and Stevenson, 2005). In addition to morphological similarities these neurons share multiple physiological and functional features (see Bräunig and Pflüger, 2001).

In the thoracic ganglia these VUM- or DUM-neurons are efferent cells that send their axons to a variety of target tissues including skeletal and visceral muscles, glands and sense organs. Clearly, in both insects the axon terminals of octopaminergic neurons share many commonalities: (i) they form boutons or varicosities in regular distances across the axon and, thus, give them a “beaded appearance” (*beaded fibers*). These characteristic axon terminals have been named type II terminals in *Drosophila*. In contrast to the type I terminals that represent the NMJ formed by excitatory motor neurons, the synaptic contacts made by the octopaminergic neurons are those of “en-passant-synapses” lacking clear pre- and postsynaptic specializations at an ultrastructural level. The idea from this study is that

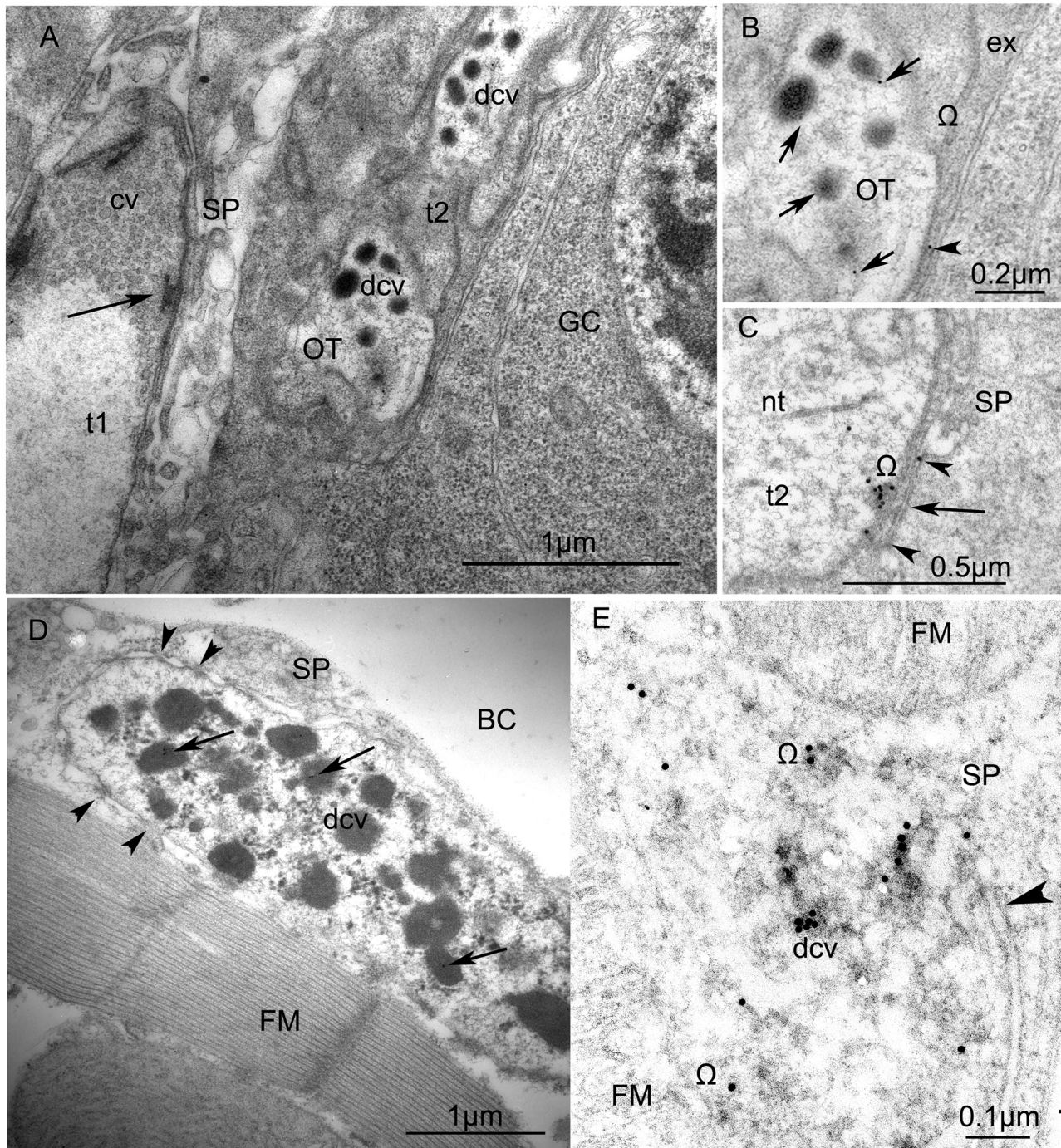


FIGURE 6 | Ultrastructure of VUM-neuron terminals in larval (**A,B**) and adult fruit flies, *Drosophila melanogaster* (**C,D**). (**A**) Arrangement of two terminals on the surface of a larval body-wall muscle: one synaptic terminal (t1) with clear vesicles (cv) has an “electron dense T-bar” (arrow) indicating the active zone, contacts with thin sarcoplasmic processes (SP) and, thus, corresponds to the NMJ (or type I-terminal); the other type (t2) includes dense-core vesicles (dcv) with anti-octopamine Immuno-Gold labels, 10 nm, and corresponds to type II terminals. Other abbreviations: Glial cell (GC) with nucleus; OT, octopaminergic type II terminal (Scale = 1 μ m). The immunogold labels are clearly depicted in (**B**) (arrows) which is a higher magnification of a part of the type II terminal shown in (**A**). Abbreviation: ex, extracellular matrix; Ω , omega-profile on membrane of release-site. (**C**) shows a dense core vesicle in the process of exocytosis (arrow, see accumulated gold particles) and gold particles in the synaptic cleft (arrowheads). (**D**) An octopaminergic type II axon terminal with many dense-core-vesicles (dcv) on an adult *Drosophila* dorsal longitudinal FM labeled by 12 nm Immunogold-gold particles (arrows). The terminal makes desmosome-like contacts (arrowheads) with SP. Other abbreviation: body cavity (BC) (Scale = 1 μ m). (**E**) A higher magnification of anti-OT-immunogold labeling in the dense core vesicles and the release site (arrowhead) (Scale = 1 μ m). This tissue was not contrasted by heavy metals to reveal 15 nm-Immuno-Gold labels.

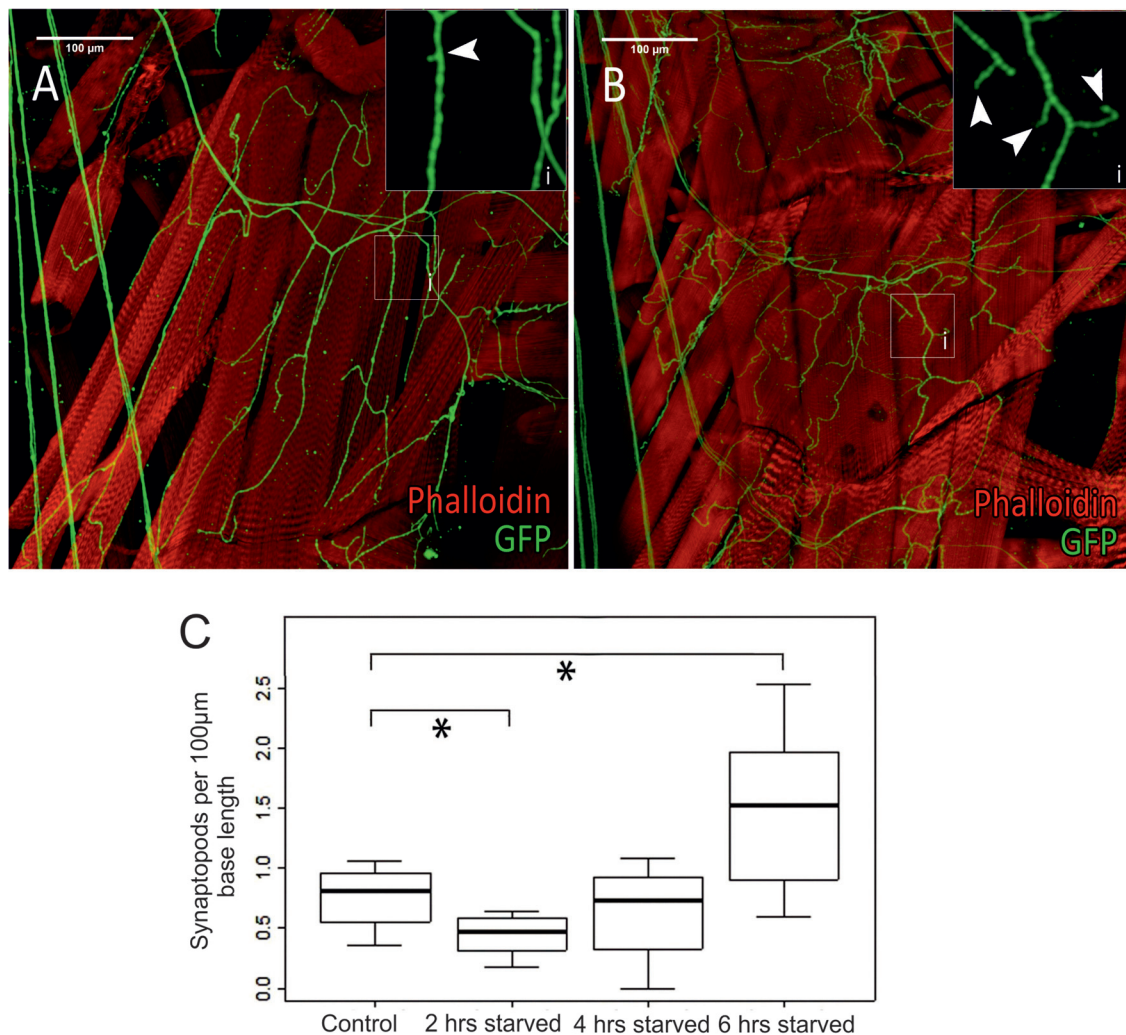


FIGURE 7 | Differences in octopaminergic innervation by VUM-neuron axon (green) of *Drosophila* larval body muscle (14 and 28, below muscle fibers 6 and 7) between a control animal (A) and a larva that was starved for 6 h (B). More octopaminergic branches are found in the starved animal compared to the control. (Ai,Bi) show magnified sections of areas marked by white squares in (A,B), respectively. (C) Within 2 h of food deprivation the number of synaptopods decreases significantly. If starvation continues an increase in the number of synaptopods can be observed. After a total of 6 h of starvation the larvae have a significantly increased number of synaptopods ($n = 32$; $*p < 0.05$).

neuromodulators are released into a “volume space” whose borders may be depicted by GCs, trachea, extracellular matrix or other, more specialized barriers, similar to what is discussed for the efficacy of NO (Münch et al., 2010).

Type I- and Type II-Terminals Share Common Release Mechanisms

In locusts, the vesicular calcium sensor synaptotagmin is present in both the excitatory NMJ (type I-terminal) and the neuromodulatory (octopaminergic) beaded fibers (type II terminals), a finding which is also supported by studies of the octopaminergic unpaired median neurons in the tobacco hornworm (*Manduca sexta*; Consoulas et al., 1999). In larval and adult *Drosophila* muscles, the active zone protein BRP is also present in both type I and type II terminals although the density

of active zones is very different. Whereas the type I terminals or NMJs reveal many active zones, the type II terminals only reveal one or two, maximally three sites of active zones that may easily be overlooked in preparations. Similarly, at the ultrastructural level a classical T-bar is found in the active zone of type I terminals but not in type II terminals. However, the presence of two of the many synaptic proteins in both types of terminals may indicate similar molecular mechanisms for synaptic vesicle release at type I and type II terminals. But note the fact that the NMJ of excitatory glutamatergic motor neurons only contains clear vesicles, whereas we found only dense core vesicles in type II terminals. This is in line with the previous, most elegant study of Koon et al. (2011) in which they also show similarities in release mechanisms of type I and type II terminals. In addition to BRP and synaptotagmin, the synaptic reserve pool protein synapsin

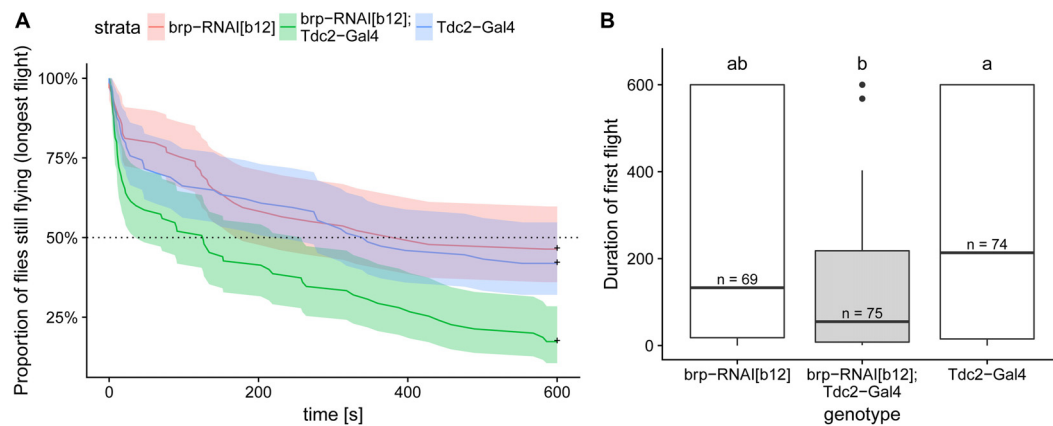


FIGURE 8 | Expression of BRP-RNAi in octopaminergic and tyraminerpic cells led to a decreased flight duration compared to the two genetic controls. **(A)** Kaplan Meier survival curve of the longest of the three first flights. This analysis, which is the most suited to this type of right-censored data, detect the difference between test and control flies (Cox proportional hazards regression model, corrected $p < 0.004$ with Bonferroni corrections). **(B)** Duration of the first flight is shown in order to compare the values with the one reported in Brembs et al. (2007). Boxplots represent the median (bar), the 75%- and 25%-quartiles (box) and all the residual data points (whiskers). Letters indicate statistically significant difference between groups (Wilcoxon rank sum test with bonferroni corrections for multiple comparisons, corrected $p < 0.05$). N-values are the number of animals examined. Data availability: behavioral data and its analysis are available at: <https://doi.org/10.5281/zenodo.1160648>.

is also present in both type I and type II terminals, for example also nicely illustrated in the locust antennal heart (Antemann et al., 2018). This may indicate the existence of reserve and release pools in axonal terminals which only contain dense core vesicles, similar to what is described for terminals with clear vesicles.

Interesting observations can be made by combining ultrastructural studies with immunogold-staining (anti-octopamine). Octopamine is indicated by the distribution of gold-particles which were found clustered in dense core vesicles, less dense in extracellular space close to what is regarded the “synaptic cleft” and a release site (Ω profile) of a type II terminal and also scattered within GCs (see also Supplementary Figure S2). A classification of terminals other than into type I and type II was used by Jia et al. (1993) who distinguished clear vesicle boutons, dense core vesicle boutons and “mixed” vesicle boutons. They report that dense core vesicle boutons also contain small translucent vesicles (33 ± 0.5 nm) which we did not find in our study of octopaminergic type II terminals. In agreement to our results, they also report that release sites in the dense cores vesicle boutons look very different to those of the clear vesicle boutons (which correspond to the NMJ).

Neuromodulatory Type II—Terminals Are Very Dynamic

The morphology of type II axon terminals are not static, but by contrast can be adaptively altered in response to changing conditions. It has been shown that starvation for 2 h causes the formation of additional small branches which were called “synaptopods” (Koon et al., 2011, and Figure 7). Our data further confirmed that type II terminals undergo plastic morphological changes. However, we gathered slightly different results with regard to the timing and the net effect of starvation induced changes of type II terminal morphology. In our study, an initial

decrease in the number of *synaptopods* after 2 h of starvation was followed by a marked increase after 6 h of starvation. This discrepancy could be caused by slightly different ages of the animals at the onset of starvation (for example early vs. late third-instar wandering larvae), differences in diet, or different modulatory backgrounds. Our data indicate a more dynamic regulation process, probably involving positive- as well as negative-feedback mechanisms (Mathejczyk, 2013). The decrease in *synaptopod* numbers within the first 2 h of starvation may be due to activation of Oct β 1R autoreceptors during that time (Koon and Budnik, 2012). Such a negative-feedback system may serve as a natural buffer for avoiding spontaneous stress responses and *synaptopod* formation every time the larva is without food for a short amount of time.

Dynamic changes after stressing stimuli can also be observed in locust octopaminergic terminals as depicted in the supplementary information including Supplementary Figure S3.

Octopamine Release From Type II Terminals Is Necessary for a Normal Performance of Flight Behavior in Adult Fruit Flies

In a previous study (Brembs et al., 2007), fruit flies mutant for octopamine showed significant deficits in their flight performance. As we could show that type II terminals also contain the active zone protein BRP, we tested fruit flies in which the release from octopaminergic type II terminals was blocked by an RNAi-construct. The two main hypotheses explaining the presence of the canonical vesicle release mechanisms proteins at the VUM-neuron synapse are that either OA is released via this mechanism or that other neurotransmitters are present in these neurons. In order to test these hypotheses, we chose

an OA-dependent Behavioral trait, flight performance. We tested the effect of a perturbation of BRP in OA neurons on that behavior. We know that RNAi perturbation blocks the common synaptic release (Wagh et al., 2006) and that ablating OA neurons perturbs fly flight. Our data show that RNAi induction in tyraminerpic/octopaminergic neurons affects flight performance to a similar level like killing the neurons or preventing the production of OA. We therefore conclude that OA is indeed released via the common, BRP dependent, vesicle release mechanism. However, at present we cannot distinguish between release mechanisms centrally from dendrites or peripherally from axonal type II terminals of OA neurons as also the dendritic release sites of VUM-neurons contain BRP (see Supplementary Figure S1) and also label with both the tyramine- and octopamine-antibodies (Kononenko et al., 2009). In addition, ultrastructural studies of octopaminergic DUM-neurons in locusts show that the dendrites also seem to have release sites and that presynaptic electron-dense structures which clearly differ from the T-bar of NMJs are present (Watson, 1984; Pflüger and Watson, 1995). Thus, it cannot be excluded that DUM- or VUM-neurons also release octopamine and/or tyramine in the central neuropile.

CONCLUSION

The results of this study show that the axons of the octopaminergic VUM-neurons in evolutionarily far apart insects such as locusts and fruit flies form *beaded fibers* with type II terminals which are closely associated with the motor axons forming “classical” NMJs or type I terminals. The type II terminals with their dense core vesicles also possess BRP as an important presynaptic cytomatrix protein of the Active Zone although the spatial arrangement must be different. The structure of the cytomatrix and the protein-protein interactions in type II terminals necessary for transmitter release from dense core vesicles has yet to be revealed. In addition, as far as we know all VUM-neurons in *Drosophila* persist through metamorphosis, and subsequently innervate adult muscle. There are indications that interactions between motor and neuromodulatory neurons occur during development (Vonhoff and Keshishian, 2017) and that these interactions are also important for forms of

peripheral plasticity, for example after starvation (Koon et al., 2011) including octopamine signaling via different octopamine receptors (Koon and Budnik, 2012). However, the precise interactions between motor (type I) and neuromodulatory (type II) terminals during normal development and during metamorphosis in the pupal stages are less well studied. Last but not least many neuromodulatory neurons, for example those releasing serotonin and dopamine, possess similar type II terminals and, therefore, the study of octopaminergic type II terminals in *Drosophila* may yield important, more general insights into their release mechanisms.

ETHICS STATEMENT

All experiments comply to the respective German laws on experiments on insects.

AUTHOR CONTRIBUTIONS

JC, CDuch, SS and H-JP designed the experiments and BS, CB, CDamrau, TM, CW and NR carried out parts of the experiments. NMB carried out all ultrastructural work and wrote the respective sections. HW was involved in experimental work and provided expert technical assistance. Some of the results presented here were parts of a PhD-thesis by BS and CDamrau, a bachelor and master thesis by TM, and a bachelor thesis by CW at Freie Universität. All authors contributed to parts of the manuscript which was written by CDuch and H-JP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnsys.2018.00005/full#supplementary-material>

REFERENCES

- Anderson, D. S., Halpern, M. E., and Keshishian, H. (1988). Identification of the neuropeptide transmitter proctolin in *Drosophila* larvae: characterization of muscle fiber-specific neuromuscular endings. *J. Neurosci.* 8, 242–255.
- Antemann, V., Pass, G., and Pflüger, H.-J. (2018). Octopaminergic innervation and a neurohaemal release site in the antennal heart of the locust *Schistocerca gregaria*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 204, 131–143. doi: 10.1007/s00359-017-1213-5
- Atwood, H. L., Govind, C. K., and Wu, C. F. (1993). Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *J. Neurobiol.* 24, 1008–1024. doi: 10.1002/neu.480240803
- Baudoux, S., Duch, C., and Morris, O. T. (1998). Coupling of efferent neuromodulatory neurons to rhythmic leg motor activity in the locust. *J. Neurophysiol.* 79, 361–370. doi: 10.1152/jn.1998.79.1.361
- Bräunig, P., and Burrows, M. (2004). Projection patterns of posterior dorsal unpaired median neurons of the locust subesophageal ganglion. *J. Comp. Neurol.* 478, 164–175. doi: 10.1002/cne.20287
- Bräunig, P., and Pflüger, H.-J. (2001). The unpaired median neurons of insects. *Adv. Insect Physiol.* 28, 185–266. doi: 10.1016/s0065-2806(01)28011-4
- Brembs, B., Christiansen, F., Pflüger, H.-J., and Duch, C. (2007). Flight motor performance deficits in flies with genetically altered biogenic amine levels. *J. Neurosci.* 27, 11122–11131. doi: 10.1523/JNEUROSCI.2704-07.2007
- Budnik, V. (1996). Synapse maturation and structural plasticity at *Drosophila* neuromuscular junctions. *Curr. Opin. Neurobiol.* 6, 858–867. doi: 10.1016/s0959-4388(96)80038-9
- Budnik, V., and Gorczyca, M. (1992). SSB, an antigen that selectively labels morphologically distinct synaptic boutons at the *Drosophila* larval neuromuscular junction. *J. Neurobiol.* 23, 1054–1066. doi: 10.1002/neu.480230810

- Burrows, M., and Pflüger, H.-J. (1995). The action of locust neuromodulatory neurons is coupled to specific motor patterns. *J. Neurophysiol.* 74, 347–357. doi: 10.1152/jn.1995.74.1.347
- Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the *Drosophila* brain. *J. Comp. Neurol.* 513, 643–667. doi: 10.1002/cne.21966
- Busch, S., and Tanimoto, H. (2010). Cellular configuration of single octopamine neurons in *Drosophila*. *J. Comp. Neurol.* 518, 2355–2364. doi: 10.1002/cne.22337
- Campbell, J. I. (1961). The anatomy of the nervous system of the mesothorax of *Locusta migratoria migratorioides* (R. & F.). *Proc. R. Zool. Soc. Lond.* 137, 403–432. doi: 10.1111/j.1469-7998.1961.tb05911.x
- Cantera, R., and Nässel, D. R. (1992). Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. *Cell Tiss. Res.* 269, 459–471. doi: 10.1007/bf00353901
- Choi, J. C., Park, D., and Griffith, L. C. (2004). Electrophysiological and morphological characterization of identified motor neurons in the *Drosophila* third instar larva central nervous system. *J. Neurophysiol.* 91, 2353–2365. doi: 10.1152/jn.01115.2003
- Cholewa, J., and Pflüger, H.-J. (2009). Descending unpaired median neurons with bilaterally symmetrical axons in the subesophageal ganglion of *Manduca sexta* larvae. *Zoology* 112, 251–262. doi: 10.1016/j.zool.2008.10.004
- Consoulas, C., Johnston, R. M., Pflüger, H.-J., and Levine, R. B. (1999). Peripheral distribution of presynaptic sites of abdominal motor and modulatory neurons in *Manduca sexta* larvae. *J. Comp. Neurol.* 410, 4–19. doi: 10.1002/(sici)1096-9861(19990719)410:1<4::aid-cne2>3.0.co;2-w
- Dacks, A. M., Christensen, T. A., Agricola, H. J., Wollweber, L., and Hildebrand, J. (2005). Octopamine-immunoreactive neurons in the brain and subesophageal ganglion of the hawkmoth *Manduca sexta*. *J. Comp. Neurol.* 488, 255–268. doi: 10.1002/cne.20556
- Duch, C., Mentel, T., and Pflüger, H.-J. (1999). Distribution and activation of different types of octopaminergic DUM neurons in the locust. *J. Comp. Neurol.* 403, 119–134. doi: 10.1002/(sici)1096-9861(19990105)403:1<119::aid-cne9>3.0.co;2-f
- Duch, C., and Pflüger, H.-J. (1999). DUM neurons in locust flight: a model system for amine mediated peripheral adjustments to the requirements of a central motor program. *J. Comp. Physiol. A* 184, 489–499. doi: 10.1007/s003590050349
- Evans, P. D., and O'Shea, M. (1977). An octopaminergic neurone modulates neuromuscular transmission in the locust. *Nature* 270, 257–259. doi: 10.1038/270257a0
- Evans, P. D., and O'Shea, M. (1978). The identification of an octopaminergic neurone and the modulation of a myogenic rhythm in the locust. *J. Exp. Biol.* 73, 235–260.
- Feeney, C. J., Karunanithi, S., Pearce, J., Govind, C. K., and Atwood, H. L. (1998). Motor nerve terminals on abdominal muscles in larval flesh flies, *Sarcophaga bullata*: comparisons with *Drosophila*. *J. Comp. Neurol.* 402, 197–209. doi: 10.1002/(sici)1096-9861(19981214)402:2<197::aid-cne5>3.3.co;2-h
- Fourtner, C. R. (1981). "The role of muscle in insect posture and locomotion," in *Locomotion and Energetics in Arthropods*, eds C. F. Herreid and C. R. Fourtner (Boston, MA: Springer), 195–213.
- Gorczyca, M., Augart, C., and Budnik, V. J. (1993). Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in *Drosophila*. *J. Neurosci.* 13, 3692–3704.
- Guo, A., Li, L., Xia, S. Z., Feng, C. H., Wolf, R., and Heisenberg, M. (1996). Conditioned visual flight orientation in *Drosophila*: dependence on age, practice, and diet. *Learn. Mem.* 3, 49–59. doi: 10.1101/lm.3.1.49
- Hauke, V., Neher, E., and Sigrist, S. J. (2011). Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. *Nat. Rev. Neurosci.* 12, 127–138. doi: 10.1038/nrn2948
- He, T., Singh, V., Rumpal, N., and Lnenicka, G. A. (2009). Differences in Ca^{2+} regulation for high-output I_s and low-output I_b motor terminals in *Drosophila* larvae. *Neuroscience* 159, 1283–1291. doi: 10.1016/j.neuroscience.2009.01.074
- Hoyle, G. (1974). A function for neurons (DUM) neurosecretory on skeletal muscle of insects. *J. Exp. Zool.* 189, 401–406. doi: 10.1002/jez.1401890313
- Hoyle, G. (1978). The dorsal, unpaired, median neurones of the locust metathoracic ganglion. *J. Neurobiol.* 9, 43–57. doi: 10.1002/neu.480090105
- Jia, X.-X., and Budnik, V. (1992). Ultrastructural characterization of neuromuscular junctions at *Drosophila* larval body-wall muscles. *Soc. Neurosci. Abstr.* 18:39.
- Jia, X. X., Gorczyca, M., and Budnik, V. (1993). Ultrastructure of neuromuscular junctions in *Drosophila*: comparison of wild type and mutants with increased excitability. *J. Neurobiol.* 24, 1025–1044. doi: 10.1002/neu.480240804
- Johansen, J., Halpern, M. E., Johansen, K. M., and Keshishian, H. (1989). Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J. Neurosci.* 9, 710–725.
- Johnston, R. M., Consoulas, C., Pflüger, H. J., and Levine, R. B. (1999). Patterned activation of unpaired median neurons during fictive crawling in *Manduca* larvae. *J. Exp. Biol.* 202, 103–113.
- Keshishian, H., Broadie, K., Chiba, A., and Bate, M. (1996). The *drosophila* neuromuscular junction: a model system for studying synaptic development and function. *Annu. Rev. Neurosci.* 19, 545–575. doi: 10.1146/annurev.neuro.19.1.545
- Klages, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., et al. (1996). Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* 16, 3154–3165.
- Kononenko, N. L., and Hauke, V. (2015). Molecular mechanisms of presynaptic membrane retrieval and synaptic vesicle reformation. *Neuron* 85, 484–496. doi: 10.1016/j.neuron.2014.12.016
- Kononenko, N. L., Wolfenberger, H., and Pflüger, H.-J. (2009). Tyramine as an independent transmitter and a precursor of octopamine in the locust central nervous system: an immunocytochemical study. *J. Comp. Neurol.* 512, 433–452. doi: 10.1002/cne.21911
- Koon, A. C., Ashley, J., Barria, R., DasGupta, S., Brain, R., Waddell, S., et al. (2011). Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. *Nat. Neurosci.* 14, 190–199. doi: 10.1038/nn.2716
- Koon, A. C., and Budnik, V. (2012). See comment in PubMed Commons below Inhibitory control of synaptic and behavioral plasticity by octopaminergic signaling. *J. Neurosci.* 32, 6312–6322. doi: 10.1523/JNEUROSCI.6517-11.2012
- Kreissl, S., Schulte, C. C., Agricola, H. J., and Rathmayer, W. (1999). A single allatostatin-immunoreactive neuron innervates skeletal muscles of several segments in the locust. *J. Comp. Neurol.* 413, 507–519. doi: 10.1002/(sici)1096-9861(19991101)413:4<507::aid-cne2>3.0.co;2-w
- Mathejczyk, T. (2013). *The Effect of Hunger on Muscle Innervation by Octopaminergic Neurons in Larvae of Drosophila Melanogaster*. Masterthesis. Berlin: Free University of Berlin.
- Mentel, T., Duch, C., Stypa, H., Müller, U., Wegener, G., and Pflüger, H.-J. (2003). Central modulatory neurons control fuel selection in flight muscle of migratory locust. *J. Neurosci.* 23, 1109–1113.
- Mentel, T., Weiler, V., Büschges, A., and Pflüger, H.-J. (2008). Activity of neuromodulatory neurons during stepping of a single insect leg. *J. Insect Physiol.* 54, 51–61. doi: 10.1016/j.jinsphys.2007.08.010
- Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. *J. Comp. Neurol.* 356, 275–287. doi: 10.1002/cne.903560210
- Münch, D., Ott, S. R., and Pflüger, H.-J. (2010). The three dimensional distribution of NO sources in a primary mechanosensory integration centre in the Locust. *J. Comp. Neurol.* 518, 2903–2916. doi: 10.1002/cne.22438
- Orchard, I., Ramirez, J.-M., and Lange, A. B. (1993). A multifunctional role for octopamine in locust flight. *Annu. Rev. Entomol.* 38, 227–249. doi: 10.1146/annurev.ento.38.1.227
- Ormerod, K. G., Hadden, J. K., Deady, L. D., Mercier, A. J., and Krans, J. L. (2013). Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae. *J. Neurophysiol.* 110, 1984–1996. doi: 10.1152/jn.00431.2013
- O'Shea, M., and Evans, P. D. (1979). Potentiation of neuromuscular transmission by an octopaminergic neurone in the locust. *J. Exp. Biol.* 79, 169–190.
- Peron, S., Zordan, M. A., Magnabosco, A., Reggiani, C., and Megighian, A. (2009). From action potential to contraction: neural control and excitation-contraction coupling in larval muscles of *Drosophila*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 154, 173–183. doi: 10.1016/j.cbpa.2009.04.626
- Pflüger, H.-J., and Duch, C. (2011). Dynamic neural control of muscle metabolism related to motor behavior. *Physiology* 26, 293–303. doi: 10.1152/physiol.002.2011

- Pflüger, H.-J., Elson, R., Binkle, U., and Schneider, H. (1986). The central nervous organization of the motor neurones to a steering muscle in locusts. *J. Exp. Biol.* 120, 403–420.
- Pflüger, H.-J., and Stevenson, P. A. (2005). Evolutionary aspects of octopaminergic systems with emphasis on arthropods. *Arthropod Struct. Dev.* 34, 379–396. doi: 10.1016/j.asd.2005.04.004
- Pflüger, H.-J., and Watson, A. H. D. (1995). GABA and glutamate-like immunoreactivity at synapses received by dorsal unpaired median neurones in the abdominal nerve cord of the locust. *Cell Tiss. Res.* 280, 325–333. doi: 10.1007/s004410050360
- Plotnikova, S. I. (1969). Effector neurons with several axons in the ventral nerve cord of the asian grasshopper *Locusta migratoria*. *J. Evol. Biochem. Physiol.* 5, 276–277.
- Prokop, A. (2006). Organization of the efferent system and structure of neuromuscular junctions in *Drosophila*. *Int. Rev. Neurobiol.* 75, 71–90. doi: 10.1016/s0074-7742(06)75004-8
- Prokop, A., Landgraf, M., Rushton, E., Broadie, K., and Bate, M. (1996). Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. *Neuron* 17, 617–626. doi: 10.1016/s0896-6273(00)80195-6
- Prokop, A., and Meinertzhagen, I. A. (2006). Development and structure of synaptic contacts in *Drosophila*. *Semin. Cell. Dev. Biol.* 17, 20–30. doi: 10.1016/j.semcdb.2005.11.010
- Rillich, J., Stevenson, P. A., and Pflüger, H.-J. (2013). Flight and walking in locusts—cholinergic co-activation, temporal coupling and its modulation by biogenic amines. *PLoS One* 8:e62899. doi: 10.1371/journal.pone.0062899
- Ryglewski, S., Duch, C., and Altenhein, B. (2017). Tyramine actions on *Drosophila* flight behavior are affected by a glial dehydrogenase/reductase. *Front. Syst. Neurosci.* 11:68. doi: 10.3389/fnsys.2017.00068
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019
- Selcho, M., Pauls, D., Huser, A., Stocker, R. F., and Thum, A. S. (2014). Characterization of the octopaminergic and tyraminergergic neurons in the central brain of *Drosophila* larvae. *J. Comp. Neurol.* 522, 3485–3500. doi: 10.1002/cne.23616
- Sigrist, S. J., Reiff, D. F., Thiel, P. R., Steinert, J. R., and Schuster, C. M. (2003). Experience-dependent strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci.* 23, 6546–6556.
- Sinakevitch, I., and Strausfeld, N. (2005). Comparison of Octopamine-like Immunoreactivity in the brains of the fruit fly and blow fly. *J. Comp. Neurol.* 494, 460–475. doi: 10.1002/cne.20799
- Skiebe, P., Biserova, N. M., Vedenina, V., Börner, J., and Pflüger, H.-J. (2006). Allatostatin-like immunoreactivity in the abdomen of the locust *Schistocerca gregaria*. *Cell Tissue Res.* 325, 163–174. doi: 10.1007/s00441-006-0169-5
- Snodgrass, R. E. (1929). The thoracic mechanism of a grasshopper and its antecedents. *Smithson. Misc. Collns.* 82, 1–111.
- Stevenson, P. A., and Kutsch, W. (1987). A reconsideration of the central pattern generator concept for locust flight. *J. Comp. Physiol. A.* 161, 115–129. doi: 10.1007/bf00609460
- Stevenson, P. A., and Kutsch, W. (1988). Demonstration of functional connectivity of the flight motor system in all stages of the locust. *J. Comp. Physiol. A.* 162, 247–259. doi: 10.1007/bf00606089
- Stevenson, P. A., and Meuser, S. (1997). Octopaminergic innervation and modulation of a locust flight steering muscle. *J. Exp. Biol.* 200, 633–642.
- Südhof, T. C. (2012). The presynaptic active zone. *Neuron* 75, 11–25. doi: 10.1016/j.neuron.2012.06.012
- Südhof, T. C. (2013). Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron* 80, 675–690. doi: 10.1016/j.neuron.2013.10.022
- Usherwood, P. N. R. (1967). Insect neuromuscular mechanisms. *Am. Zool.* 7, 553–582. doi: 10.1093/icb/7.3.553
- Usherwood, P. N. R. (1975). *Insect Muscle*. London: Academic Press.
- Vömel, M., and Wegener, C. (2008). Neuroarchitecture of aminergic systems in the larval ventral ganglion of *Drosophila melanogaster*. *PLoS One* 3:e1848. doi: 10.1371/journal.pone.0001848
- Vonhoff, F., and Keshishian, H. (2017). Activity-dependent synaptic refinement: new insights from *Drosophila*. *Front. Syst. Neurosci.* 11:23. doi: 10.3389/fnsys.2017.00023
- Wagh, D. A., Rasse, T. M., Asan, E., Hofbauer, A., Schwenkert, I., Dürrbeck, H., et al. (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron* 49, 833–844. doi: 10.1016/j.neuron.2006.02.008
- Watson, A. H. D. (1984). The dorsal unpaired median neurons of the locust metathoracic ganglion: neuronal structure and diversity and synapse distribution. *J. Neurocytol.* 13, 303–327. doi: 10.1007/bf01148121
- Wicher, D. (2007). Metabolic regulation and behavior: how hunger produces arousal—an insect study. *Endocr. Metab. Immune Disord. Drug Targets* 7, 304–310. doi: 10.2174/187153007782794290
- Wichmann, C., and Sigrist, S. J. (2010). The active zone T-bar—a plasticity module? *J. Neurogenet.* 24, 133–145. doi: 10.3109/01677063.2010.489626
- Wilson, D. M. (1961). The central nervous control of flight in a locust. *J. Exp. Biol.* 38, 471–490.
- Wolf, H., and Lang, D. M. (1994). Origin and clonal relationship of common inhibitory motoneurons CII and CI3 in the locust CNS. *J. Neurobiol.* 25, 846–864. doi: 10.1002/neu.480250709
- Zhong, Y., and Peña, L. A. (1995). A novel synaptic transmission mediated by a PACAP-like neuropeptide in *Drosophila*. *Neuron* 14, 527–536. doi: 10.1016/0896-6273(95)90309-7

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Origins of Aminergic Regulation of Behavior in Complex Insect Social Systems

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Neuromodulators are conserved across insect taxa, but how biogenic amines and their receptors in ancestral solitary forms have been co-opted to control behaviors in derived socially complex species is largely unknown. Here we explore patterns associated with the functions of octopamine (OA), serotonin (5-HT) and dopamine (DA) in solitary ancestral insects and their derived functions in eusocial ants, bees, wasps and termites. Synthesizing current findings that reveal potential ancestral roles of monoamines in insects, we identify physiological processes and conserved behaviors under aminergic control, consider how biogenic amines may have evolved to modulate complex social behavior, and present focal research areas that warrant further study.

Keywords: neuromodulation, biogenic amines, eusocial, social brain evolution, collective intelligence

INTRODUCTION

The ubiquitous biogenic amines octopamine (OA), serotonin (5-HT) and dopamine (DA) activate neural circuitry to regulate behavior (Libersat and Pflueger, 2004; Bergan, 2015). The phylogenetic distribution of these neuromodulators suggests a deep evolutionary history predating the origin of the nervous system (Gallo et al., 2016). With few structural modifications, monoamines are functionally diverse in insects (Roeder, 1999; Mustard et al., 2005; Blenau and Thamm, 2011). Conserved aminergic circuits (Kravitz and Huber, 2003; Barron et al., 2010; Perry et al., 2016) and patterns of receptor expression (Roeder, 1999; Blenau and Thamm, 2011) control behavior in diverse species across insect orders. However, how monoamine neurotransmitter systems served as preadaptations for the evolution of derived behaviors associated with the transition from solitary life to sociality in insects is poorly understood. Insect colonies show remarkable variation in structure and degree of integration of worker actions that could underscore complex social behavior. Using well-resolved insect molecular phylogenies (Wiegmann et al., 2011; Song et al., 2012, 2015; Moreau and Bell, 2013; Regier et al., 2013; Schmidt, 2013; Misof et al., 2014; Wang et al., 2014), we explore the evolution of neuromodulation of social behavior (Supplementary Table S1) by analyzing patterns of monoamine function in solitary and social taxa (Figure 1; Supplementary Table S2).

SOCIAL DECISION-MAKING SYSTEMS AND BEHAVIORAL DIVERSITY IN INSECTS

Two neural circuits regulate vertebrate decision-making: the social behavior network, controlled by neuropeptides and gonadal steroids, and the mesolimbic reward system, activated primarily by DA (O'Connell and Hofmann, 2011a,b). These circuits act in concert to regulate social interactions and evaluate stimulus valence, respectively, forming the social decision-making

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network (O'Connell and Hofmann, 2011b, 2012). Insect social decision-making systems are poorly understood in comparison, although behavioral influences of neuromodulators are well known (Supplementary Table S2).

Neurochemical and neuroendocrine analyses of complex social behavior in insects have largely been limited to the species-rich Hymenoptera (>150,000 species), which includes ants, bees and wasps with solitary, presocial and eusocial life histories. Solitary species are composed of individuals that live and forage alone and interact with conspecifics primarily during mating or territorial disputes. Presocial describes life histories that are intermediate between solitary and eusocial (Eickwort, 1981). Eusociality is defined by: (1) reproductive division of labor (the differentiation of fertile [queens and males] and sterile [workers] castes); (2) allomaternal care (cooperative care of immatures by workers); and (3) overlapping generations of reproductive and worker castes (queen longevity allowing coexistence with offspring). Varying degrees of sociality are found in a number of clades. Phase transitions from solitary to gregarious behavior occur in desert locusts (Order Orthoptera; Anstey et al., 2009; Ott and Rogers, 2010), beetles (Order Coleoptera) show multiple occurrences of the evolution of familial sociality, including biparental care (Costa, 2006; Cunningham et al., 2015; Panaitof et al., 2016), and one species of weevil is eusocial (Kent and Simpson, 1992). Solitary life histories predated eusociality in both the Hymenoptera (Wilson, 1971) and Isoptera, which diverged from cockroaches into entirely eusocial forms (Bourguignon et al., 2015). The evolution of a reproductive caste occurred once in ants and multiply in bees and wasps; diversification of workers, particularly in ants, has many independent origins (Oster and Wilson, 1978; Tribble and Kronauer, 2017). Eusociality independently evolved in the Order Isoptera (termites, >3000 species; Thorne and Traniello, 2003).

Parental behavior, reproductive competition and foraging and defense strategies in solitary (Field et al., 2006, 2015; Thompson et al., 2014) and eusocial (Tibbetts, 2013) hymenopteran species reflect social decision-making, although neurochemical and neuroanatomical correlates of such systems are poorly understood (Ilies et al., 2015). For example, neural mechanisms underscoring vertebrate-like cognitive abilities, such as individual facial feature recognition in some eusocial wasps, are not known (Gronenberg et al., 2008; Sheehan and Tibbetts, 2011). Decision-making at the colony level is seen in collective (swarm) intelligence (Seeley, 2010; Jeanson et al., 2012; Sasaki and Pratt, 2012; Reid et al., 2015) and in part concerns worker interactions (Greene and Gordon, 2003; Greene et al., 2013) that may be causally related to brain neurotransmitter levels (Muscedere et al., 2012; Kamhi and Traniello, 2013; Kamhi et al., 2015; Hoover et al., 2016). Studies have focused on the aminergic control of worker interactions that contribute to social organization, including responsiveness to social signals and cues that regulate alloparental care, food exchange, nest construction, defensive behavior and foraging (reviewed in Kamhi and Traniello, 2013; Simpson and Stevenson, 2015; Hamilton et al., 2017). Studies have begun to explore genetic and epigenetic underpinnings of task performance and plasticity

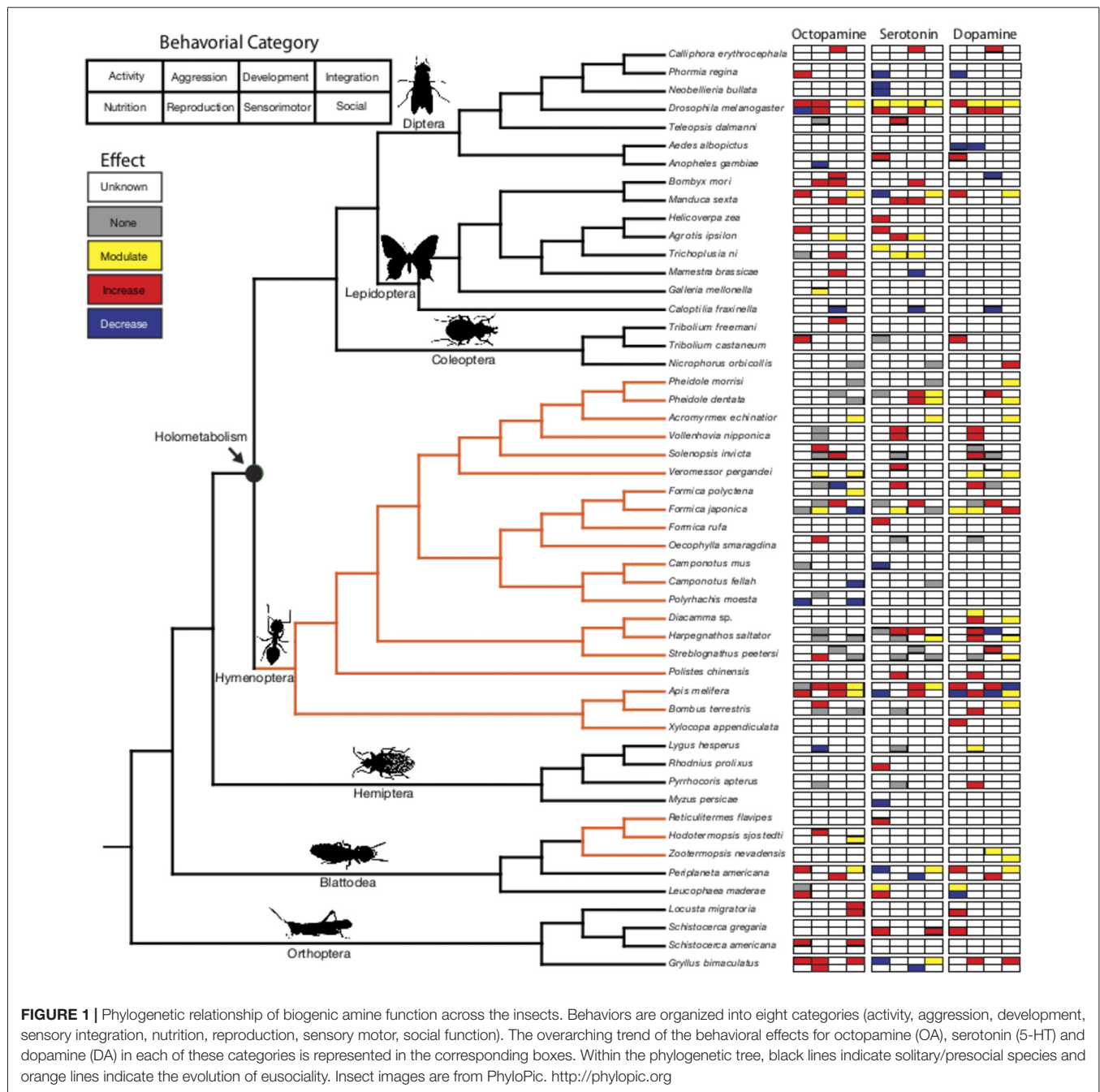
through state changes in behavior (Lucas and Sokolowski, 2009; Simola et al., 2016) that may involve neuromodulators.

BEHAVIOR AND BIOGENIC AMINE FUNCTIONS IN INSECTS

Genes controlling behavior in solitary insects regulate social behavior in eusocial species (LeBoeuf et al., 2013) and affect sensory receptor evolution (Baldwin et al., 2014). Monoamine functions in solitary insects likely reflect this conservation, and appear to have been preadaptive for eusociality. To understand the evolution of neuromodulatory systems in insects, we organized available data on aminergic control into eight behavioral categories: activity, aggression, development, higher-order sensory integration, nutrition, reproduction, sensorimotor functions and social functions (defined in Supplementary Table S1). Behaviors may span multiple categories, such as parental care and mate selection involving reproduction and derived social functions. Statistical tests showed similar patterns of monoamine function in solitary and eusocial species (Supplementary Figure S1), although small sample sizes constrain inferences. While data on biogenic amine regulation is variable and fragmentary, some patterns emerge suggesting that aminergic circuitry has shifted in function during the transition from solitary to social life. Monoamines have been co-opted for social functions through receptor and circuitry evolution and have gained novel functions to regulate social behaviors. For example, 5-HT (Alekseyenko et al., 2010, 2014; Bubak et al., 2014) and OA (Stevenson et al., 2000; Hoyer et al., 2008; Zhou et al., 2008; Stevenson and Rillich, 2017) increase aggression in solitary insects. In social insects, aggression is associated with the ability to pheromonally distinguish nestmates from non-nestmates (Stroeymeyt et al., 2010; Sturgis and Gordon, 2012), and OA is implicated in improved nestmate recognition (Robinson et al., 1999; Vander Meer et al., 2008; Kamhi et al., 2015). OA may thus enhance sensitivity to pheromonal cues and regulate social interactions similarly in both solitary and social insects.

DA, 5-HT and OA are involved in regulating metamorphosis in solitary insects (Nässel and Laxmyr, 1983; Hirashima et al., 1999). In social insects, monoamines are associated with age-related behavioral changes and collateral physiological and neural development (Schulz et al., 2002; Seid and Traniello, 2005; Cuvillier-Hot and Lenoir, 2006; Wnuk et al., 2010; Giraldo et al., 2016). OA increases with age and is causally related to the transition from nursing to foraging in honey bees (Schulz et al., 2002). In ants, 5-HT, DA (Seid and Traniello, 2005; Cuvillier-Hot and Lenoir, 2006), and OA (Wnuk et al., 2010) increase with age; 5-HT, similar to OA in bees, is correlated with age-related initiation of foraging (Seid and Traniello, 2005) and sensitivity to pheromonal signals underscoring trail communication (Muscedere et al., 2012).

In respect to other behaviors, suppressing DA neurons in *Drosophila melanogaster* consistently inhibits aversive but not appetitive learning, whereas manipulating OA action produces the opposite pattern (Schwaerzel et al., 2003;



Claridge-Chang et al., 2009; Aso et al., 2010). Similar patterns have been found in honey bees (Mercer and Menzel, 1982; Hammer and Menzel, 1998). However, appetitive learning in social insects must be considered in respect to the social context, where foraging is dependent on the nutritional state of the colony rather than the individual (Traniello, 1977; Seeley, 1989). OA increases the likelihood of successful foragers waggles dancing, which communicates information about food location and quality to nestmates; this demonstrates that an amine may be adapted to serve a colony-level function in food collection rather than benefit individual nutrition (Barron et al., 2007).

Biogenic amines appear to have gained new functions associated with the regulation of social organization. DA correlates with increased receptivity and mating in solitary insects (Pastor et al., 1991; Neckameyer, 1998; Chvalova et al., 2014; Brent et al., 2016), and reproductive state in many hymenopterans (e.g., Sasaki et al., 2007). Honey bee and some ant workers are reproductively capable; however, both ant and honey bee queens release a pheromone, queen mandibular pheromone (QMP), that inhibits worker reproduction (Fletcher and Blum, 1981; Hoover et al., 2003) by acting through DA circuitry (Harris and Woodring, 1995; Boulay et al., 2001; Beggs et al., 2007).

Aggressive interactions between workers to control reproductive dominance also affect DA levels (Shimoji et al., 2017). These studies suggest that in both solitary and eusocial insects DA regulates reproductive state, and DA additionally may be integral to the maintenance of reproductive division of labor and the resolution of reproductive competition in eusocial species.

FOCAL QUESTIONS

We identify four research areas, among several others, that are significant in the study of the neuromodulation of complex eusocial behavior.

Altruism, Genes and Neuromodulators

Altruism is evident in the sterility of workers and their fatal self-sacrificing behavior. Developmental programming controls ovarian function, feeding the queen and alloparental care, and likely regulates defensive responses that decrease the survival of altruistic workers. Correlations among DA, OA, their receptors, ovarian development and honey bee worker responsiveness to social signals of fertility have been identified (reviewed in Simpson and Stevenson, 2015; Hamilton et al., 2017). As discussed above, worker fertility is controlled by QMP, which also causes workers to feed and groom the queen and activates brain genes associated with alloparenting (Grozinger et al., 2003). Workers showing higher ovarian activity are less likely to show queen-directed behaviors (Galbraith et al., 2015). Honey bee ovarian development is associated with the expression of a tyramine receptor gene (Thompson et al., 2007) and brain levels of the OA receptor *Oa1* (Cardoen et al., 2011; Galbraith et al., 2015; Sobotka et al., 2016). QMP also modulates DA receptor gene expression, decreases brain DA levels, and reduces activity possibly by inhibiting DA function in young workers (Beggs et al., 2007). Homologous systems appear to control reproduction in ants: QMP inhibits reproduction and DA may increase fertility (Boulay et al., 2001; Penick et al., 2014; Okada et al., 2015).

Together, these studies suggest that in eusocial insects DA regulates reproductive state and related social behaviors, which are key to altruism. Thompson et al. (2013) noted that “genes underlying altruism should coevolve with, or depend on, genes for kin recognition”; such genes specify recipients of altruistic actions. The regulation of polygyny (multiple queens) in ants and the direction of lethal aggression toward queens of a certain genotype, is under the control of the *Gp-9* gene, which codes for an odorant-binding protein (Gotzek and Ross, 2007). This indicates that chemical communication underscores strategies associated with inclusive fitness. Nestmate recognition may be causally related to monoamine levels (Kamhi and Traniello, 2013; Kamhi et al., 2015; Hoover et al., 2016) and altruistic defense. Self-sacrifice is associated with defensive specializations of “soldiers,” and may concern serotonergic circuits (Giraldo et al., 2013). Soldiers are more tolerant of risk; elevated monoamine levels or subcaste-specific receptor profiles may underscore their self-sacrificing behavior.

Orchestration of Individual and Colony-Level Behavior

Social decision-making networks in vertebrates and eusocial insects function in different contexts and favor, respectively, individual reproduction and inclusive fitness. Concepts such as social brain theory (Dunbar, 1998), developed for vertebrates, may vary in its applicability to eusocial insects (Lihoreau et al., 2012). Similarly, neuromodulators play a key role in the “orchestration of behavior” (Sombati and Hoyle, 1984; Hoyle, 1985), but analyses of organizational mechanisms should distinguish between the regulation of individual behavior by monoamines and the control of emergent colony properties by pheromones to determine whether the orchestration hypothesis can explain the control of these two systems (Kamhi and Traniello, 2013). The circuitry of social networks underscoring division of labor and collective action may concern interactions of communicating workers, which have been considered to be functionally similar to neurons (Couzin, 2009; Feinerman and Korman, 2017). Similarly, pheromones are behavioral releasers that may parallel neurotransmitter functions in circuits. The role of the “colony brain” in emergent group behavior is therefore in part constructed from the neurochemistry of individual worker brains that modulate responsiveness to social cues and signals as well as social interactions and pheromonal communication systems that modulate group decision-making. Kamhi and Traniello (2013) hypothesized that worker interactions may cause neuromodulatory and behavioral synchronization in collective action, and that monoamine titers could regulate cyclical activity. Control processes analogous to neural synchronization in vertebrate brains may underscore colony-level behavior.

An emergent action that holds promise for such an analysis is cooperative foraging, a goal-oriented system in which chemical signals control colony behavior (Czaczkes et al., 2015). Foraging effort is modified by the responses of individual workers to pheromones that induce and terminate foraging activity by affecting individual and group decisions. The ability of workers to render decisions that modify colony-level responses may be related to worker physical caste or age. OA underscores subcaste-specific behavior in ants (Kamhi et al., 2015), and 5-HT in ants (Seid and Traniello, 2005; Seid et al., 2008) and OA in honey bees (Schulz et al., 2002) modulate age-related task transitions that involve striking shifts in stimulus environments within and outside of the nest. Biogenic amines may thus influence division of labor and collective action through changes in olfactory responsiveness.

Nutrition and Biogenic Amines

Nutrition has diverse effects on social behavior, from group aggregation to brain physiology (Simpson and Raubenheimer, 2012; Lihoreau et al., 2015). Diet influences levels of brain monoamines, which are derived from amino acids such as tryptophan and tyrosine (Crockett et al., 2009; Wada-Katsumata et al., 2011; Fernstrom, 2013). In insects, 5-HT, DA and OA modulate feeding behavior (Braun and Bicker, 1992; Falibene et al., 2012) through regulatory mechanisms that may be conserved between solitary and social species (Dacks et al., 2003;

Haselton et al., 2009; Neckameyer, 2010). Serotonergic fibers innervate the insect digestive system in species-specific patterns of distribution (e.g., Klemm et al., 1986; Molaei and Lange, 2003; Falibene et al., 2012; French et al., 2014). In eusocial insects, food is exchanged among colony members through trophallaxis. In the foregut, the proventriculus controls the transfer of food to the midgut (for individual worker metabolism) and its retention in the crop (to be shared with colony members). In solitary insects, 5-HT increases crop contractions (Liscia et al., 2012), enabling regurgitation (Stoffolano et al., 2008). In honey bees (French et al., 2014) and some ants (Falibene et al., 2012), serotonergic fibers innervate both organs; in honey bees, 5-HT antagonists affect crop and proventriculus contractions (French et al., 2014). In eusocial insects, 5-HT may thus have been co-opted for food sharing, reducing individual feeding behavior and enabling trophallaxis when the crop is full.

In ants, nutrient requirements differ among colony members: workers mainly feed on carbohydrates for energy, whereas larvae require protein for development. Colonies with larvae collect food with higher protein content (Abril et al., 2007; Dussutour and Simpson, 2008, 2009); communication of nutritional needs (Farina and Grüter, 2009; LeBoeuf et al., 2016) may thus modify food choices of foragers. Adjusting protein and carbohydrate intake in ants may affect nestmate recognition (Liang and Silverman, 2000; Buczkowski et al., 2005), social immunity (Kay et al., 2014), and colony behavior (Kay et al., 2010, 2012). However, we do not know how nutritional interactions affect forager monoamine levels and behavior. 5-HT underlies a dietary switch toward foods with higher protein content in fruit flies (Vargas et al., 2010), and OA and DA levels influence individual and social control of feeding in some ants (Wada-Katsumata et al., 2011). Nutritional ecology varies across social insect clades and may significantly impact monoamine levels and trophic behavior.

Ligand and Receptor Coevolution

Biogenic amine receptor distribution in insect brains has been characterized primarily in fruit flies and honey bees (Blenau et al., 1998; Monastirioti, 1999; Blenau and Thamm, 2011; Sinakevitch et al., 2011). Receptor duplication has occurred throughout evolution and the same small number of monoamines appear to have been co-opted for use as ligands for duplicated receptors (Hauser et al., 2006). There are typically several types of receptors for each monoamine, which may lead to different regulatory mechanisms. For example, knocking out the 5-HT receptor d5-HT1A influences sleep in fruit flies (Yuan et al., 2006), whereas overexpression of receptor d5-HT1B reduces the ability to phase-shift in response to light cues (Yuan et al., 2005).

Receptor duplication and adaptation appears to have evolved before the divergence of fruit flies and honey bees, suggesting that solitary and social insects share common monoamine receptors (Hauser et al., 2006; Bauknecht and Jékely, 2017). If ligands, receptors, and downstream regulatory mechanisms are highly conserved across species, how have biogenic amine circuits evolved to control derived social behaviors? Monoamines may have species-specific effects on neural circuits, giving rise to different downstream regulatory effects and thus variable roles

in modulating behavior. Activation of the DA receptor DopR1 increased stress-induced hyperactivity and modulated circadian-dependent activity through different neural circuits in fruit flies (Lebestky et al., 2009). Social insects may have evolved distinct neural circuits to regulate social behaviors using the same signaling molecules as solitary species. Exploring biogenic amine receptors and downstream regulatory pathways involved in insect behavior and derived social functions will advance our understanding of how the eusocial insect brain evolved perceptual and cognitive capacities in association with sociality.

CONCLUSION

Broader sampling is required to gain phylogenetic insight into the evolution of aminergic control systems. Determining patterns of conservation and/or diversification of aminergic regulatory mechanisms of social behavior will benefit from studies of insect genera that include solitary and eusocial species. Despite the widespread activity of biogenic amines, functional patterns appear. 5-HT may control energy expenditure through feeding behavior and circadian rhythms, DA regulates fertility, thus modulating task performance in eusocial species, and OA modulates appetitive learning associated with feeding and nestmate recognition. Advances in epigenetics (Libbrecht et al., 2016), neurogenetics (Friedman and Gordon, 2016), and the integration of sociobiology and neurochemistry (Kamhi and Traniello, 2013) will aid in future research.

AUTHOR CONTRIBUTIONS

JFK, SA and JFAT compiled literature. SA performed statistical analyses and created associated figures, and CSM created the phylogenetic presentation of aminergic control of behavior. JFK, SA and JFAT prepared drafts of the manuscript. All authors contributed to the conception of the perspective, analysis and synthesis of material, manuscript revision and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnsys.2017.00074/full#supplementary-material>

REFERENCES

- Abril, S., Oliveras, J., and Gómez, C. (2007). Foraging activity and dietary spectrum of the Argentine ant (Hymenoptera: Formicidae) in invaded natural areas of the northeast Iberian Peninsula. *Environ. Entomol.* 36, 1166–1173. doi: 10.1603/0046-225x(2007)36[1166:faadso]2.0.co;2
- Alekseyenko, O. V., Chan, Y. B., Fernandez, M. L., Bülow, T., Pankratz, M. J., and Kravitz, E. A. (2014). Single serotonergic neurons that modulate aggression in *Drosophila*. *Curr. Biol.* 24, 2700–2707. doi: 10.1016/j.cub.2014.09.051
- Alekseyenko, O. V., Lee, C., and Kravitz, E. A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. *PLoS One* 5:e10806. doi: 10.1371/journal.pone.0010806
- Anstey, M. L., Rogers, S. M., Ott, S. R., Burrows, M., and Simpson, S. J. (2009). Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts. *Science* 323, 627–630. doi: 10.1126/science.1165939
- Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. *Curr. Biol.* 20, 1445–1451. doi: 10.1016/j.cub.2010.06.048
- Baldwin, M. W., Toda, Y., Nakagita, T., O'Connell, M. J., Klasing, K. C., Misaka, T., et al. (2014). Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor. *Science* 345, 929–933. doi: 10.1126/science.1255097
- Barron, A. B., Maleszka, R., Vander Meer, R. K., and Robinson, G. E. (2007). Octopamine modulates honey bee dance behavior. *Proc. Natl. Acad. Sci. U S A* 104, 1703–1707. doi: 10.1073/pnas.0610506104
- Barron, A. B., Sövik, E., and Cornish, J. L. (2010). The roles of dopamine and related compounds in reward-seeking behavior across animal phyla. *Front. Behav. Neurosci.* 4:163. doi: 10.3389/fnbeh.2010.00163
- Bauknecht, P., and Jékely, G. (2017). Ancient coexistence of norepinephrine, tyramine and octopamine signaling in bilaterians. *BMC Biol.* 15:6. doi: 10.1186/s12915-016-0341-7
- Beggs, K. T., Glendinning, K. A., Marechal, N. M., Vergoz, V., Nakamura, I., Slesoor, K. N., et al. (2007). Queen pheromone modulates brain dopamine function in worker honey bees. *Proc. Natl. Acad. Sci. U S A* 104, 2460–2464. doi: 10.1073/pnas.0608224104
- Bergan, J. F. (2015). Neural computation and neuromodulation underlying social behavior. *Integr. Comp. Biol.* 55, 268–280. doi: 10.1093/icb/icv061
- Blenau, W., Erber, J., and Baumann, A. (1998). Characterization of a dopamine D1 receptor from *Apis mellifera*: cloning, functional expression, pharmacology, and mRNA localization in the brain. *J. Neurochem.* 70, 15–23. doi: 10.1046/j.1471-4159.1998.70010015.x
- Blenau, W., and Thamm, M. (2011). Distribution of serotonin (5-HT) and its receptors in the insect brain with focus on the mushroom bodies. Lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arthropod Struct. Dev.* 40, 381–394. doi: 10.1016/j.asd.2011.01.004
- Boulay, R., Hooper-Bui, L. M., and Woodring, J. (2001). Oviposition and oogenesis in virgin fire ant females *Solenopsis invicta* are associated with a high level of dopamine in the brain. *Physiol. Entomol.* 26, 294–299. doi: 10.1046/j.0307-6962.2001.00250.x
- Bourguignon, T., Lo, N., Cameron, S. L., Šobotník, J., Hayashi, Y., Shigenobu, S., et al. (2015). The evolutionary history of termites as inferred from 66 mitochondrial genomes. *Mol. Biol. Evol.* 32, 406–421. doi: 10.1093/molbev/msu308
- Braun, G., and Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* 67, 588–598.
- Brent, C. S., Miyasaki, K., Vuong, C., Miranda, B., Steele, B., Brent, K. G., et al. (2016). Regulatory roles of biogenic amines and juvenile hormone in the reproductive behavior of the western tarnished plant bug (*Lygus hesperus*). *J. Comp. Physiol. B* 186, 169–179. doi: 10.1007/s00360-015-0953-1
- Bubak, A. N., Renner, K. J., and Swallow, J. G. (2014). Heightened serotonin influences contest outcome and enhances expression of high-intensity aggressive behaviors. *Behav. Brain Res.* 259, 137–142. doi: 10.1016/j.bbr.2013.10.050
- Buczowski, G., Kumar, R., Suib, S. L., and Silverman, J. (2005). Diet-related modification of cuticular hydrocarbon profiles of the Argentine ant, *Linepithema humile*, diminishes intercolony aggression. *J. Chem. Ecol.* 31, 829–843. doi: 10.1007/s10886-005-3547-7
- Cardoen, D., Wenseleers, T., Ernst, U. R., Danneels, E. L., Laget, D., DE Graaf, D. C., et al. (2011). Genome-wide analysis of alternative reproductive phenotypes in honeybee workers. *Mol. Ecol.* 20, 4070–4084. doi: 10.1111/j.1365-294X.2011.05254.x
- Chvalova, D., Zdechovanova, L., Vaneckova, H., and Hodkova, M. (2014). Brain norepinephrine identified by mass spectrometry is associated with reproductive status of females of the linden bug *Pyrrhocoris apterus*. *Comp. Biochem. Phys. B Biochem. Mol. Biol.* 168, 70–75. doi: 10.1016/j.cbpb.2013.11.003
- Claridge-Chang, A., Roorda, R. D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., et al. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell* 139, 405–415. doi: 10.1016/j.cell.2009.08.034
- Costa, J. T. (2006). *The Other Insect Societies*. Cambridge, MA: Harvard University Press.
- Couzin, I. D. (2009). Collective cognition in animal groups. *Trends Cogn. Sci.* 13, 36–43. doi: 10.1016/j.tics.2008.10.002
- Crockett, M. J., Clark, L., and Robbins, T. W. (2009). Reconciling the role of serotonin in behavioral inhibition and aversion: acute tryptophan depletion abolishes punishment-induced inhibition in humans. *J. Neurosci.* 29, 11993–11999. doi: 10.1523/JNEUROSCI.2513-09.2009
- Cunningham, C. B., Ji, L., Wiberg, R. A., Shelton, J., McKinney, E. C., Parker, D. J., et al. (2015). The genome and methylome of a beetle with complex social behavior, *Nicrophorus vespilloides* (Coleoptera: Silphidae). *Genome Biol. Evol.* 7, 3383–3396. doi: 10.1093/gbe/evv194
- Cuvillier-Hot, V., and Lenoir, A. (2006). Biogenic amine levels, reproduction and social dominance in the queenless ant *Streblognathus peetersi*. *Naturwissenschaften* 93, 149–153. doi: 10.1007/s00114-006-0086-1
- Czaczes, T. J., Grüter, C., and Ratnieks, F. L. W. (2015). Trail pheromones: an integrative view of their role in social insect colony organization. *Annu. Rev. Entomol.* 60, 581–599. doi: 10.1146/annurev-ento-010814-020627
- Dacks, A. M., Nickel, T., and Mitchell, B. K. (2003). An examination of serotonin and feeding in the flesh fly *Neobellieria bullata* (Sarcophagidae: Diptera). *J. Insect Behav.* 16, 1–21. doi: 10.1023/A:1022817610378
- Dunbar, R. I. M. (1998). The social brain hypothesis. *Evol. Anthropol.* 6, 178–190. doi: 10.1002/(sici)1520-6505(1998)6:5<178::aid-evan5>3.3.co;2-p
- Dussutour, A., and Simpson, S. J. (2008). Carbohydrate regulation in relation to colony growth in ants. *J. Exp. Biol.* 211, 2224–2232. doi: 10.1242/jeb.017509
- Dussutour, A., and Simpson, S. J. (2009). Communal nutrition in ants. *Curr. Biol.* 19, 740–744. doi: 10.1016/j.cub.2009.03.015
- Eickwort, G. C. (1981). “Presocial insects,” in *Social Insects*, ed. H. R. Hermann, (New York, NY: Academic Press), 199–281.
- Falibene, A., Rössler, W., and Josens, R. (2012). Serotonin depresses feeding behaviour in ants. *J. Insect Physiol.* 58, 7–17. doi: 10.1016/j.jinsphys.2011.08.015
- Farina, W. M., and Grüter, C. (2009). “Trophallaxis: a mechanism of information transfer,” in *Contemp Top Entomol*, eds S. Jarau and M. Hrnir (New York, NY: CRC Press), 173–187.
- Feinerman, O., and Korman, A. (2017). Individual versus collective cognition in social insects. *J. Exp. Biol.* 220, 73–82. doi: 10.1242/jeb.143891
- Fernstrom, J. D. (2013). Large neutral amino acids: dietary effects on brain neurochemistry and function. *Amino Acids* 45, 419–430. doi: 10.1007/s00726-012-1330-y
- Field, J., Cronin, A., and Bridge, C. (2006). Future fitness and helping in social queues. *Nature* 441, 214–217. doi: 10.1038/nature04560
- Field, J., Shreeves, G., Kennedy, M., Brace, S., and Gilbert, J. D. J. (2015). Sex-biased parental care and sexual size dimorphism in a provisioning arthropod. *Behav. Ecol. Sociobiol.* 69, 1897–1906. doi: 10.1007/s00265-015-2002-1
- Fletcher, D. J., and Blum, M. S. (1981). Pheromonal control of dealation and oogenesis in virgin queen fire ants. *Science* 212, 73–75. doi: 10.1126/science.212.4490.73
- French, A. S., Simcock, K. L., Rolke, D., Gartside, S. E., Blenau, W., and Wright, G. A. (2014). The role of serotonin in feeding and gut contractions in the honeybee. *J. Insect Physiol.* 61, 8–15. doi: 10.1016/j.jinsphys.2013.12.005
- Friedman, D. A., and Gordon, D. M. (2016). Ant genetics: reproductive physiology, worker morphology, and behavior. *Annu. Rev. Neurosci.* 39, 41–56. doi: 10.1146/annurev-neuro-070815-013927
- Galbraith, D. A., Wang, Y., Amdam, G. V., Page, R. E., and Grozinger, C. M. (2015). Reproductive physiology mediates honey bee (*Apis mellifera*)

- worker responses to social cues. *Behav. Ecol. Sociobiol.* 69, 1511–1518. doi: 10.1007/s00265-015-1963-4
- Gallo, V. P., Accordi, F., Chimenti, C., Civinini, A., and Crivellato, E. (2016). “Catecholaminergic system of invertebrates: comparative and evolutionary aspects in comparison with the octopamine system,” in *International Review of Cell and Molecular Biology*, ed. K. W. Jeon (London: Elsevier Inc.), 363–394.
- Girardo, Y. M., Kamhi, J. F., Fourcassé, V., Moreau, M., Robson, S. K., Rusakov, A., et al. (2016). Lifespan behavioural and neural resilience in a social insect. *Proc. Biol. Sci.* 283:20152603. doi: 10.1098/rspb.2015.2603
- Girardo, Y. M., Patel, E., Gronenberg, W., and Traniello, J. F. A. (2013). Division of labor and structural plasticity in an extrinsic serotonergic mushroom body neuron in the ant *Pheidole dentata*. *Neurosci. Lett.* 534, 107–111. doi: 10.1016/j.neulet.2012.11.057
- Gotzek, D., and Ross, K. G. (2007). Genetic regulation of colony social organization in fire ants: an integrative overview. *Q. Rev. Biol.* 82, 201–226. doi: 10.1086/519965
- Greene, M. J., and Gordon, D. M. (2003). Social insects: cuticular hydrocarbons inform task decisions. *Nature* 423:32. doi: 10.1038/423032a
- Greene, M. J., Pinter-Wollman, N., and Gordon, D. M. (2013). Interactions with combined chemical cues inform harvester ant foragers' decisions to leave the nest in search of food. *PLoS One* 8:e52219. doi: 10.1371/journal.pone.0052219
- Gronenberg, W., Ash, L. E., and Tibbetts, E. A. (2008). Correlation between facial pattern recognition and brain composition in paper wasps. *Brain Behav. Evol.* 71, 1–14. doi: 10.1159/000108607
- Grozing, C. M., Sharabash, N. M., Whitfield, C. W., and Robinson, G. E. (2003). Pheromone-mediated gene expression in the honey bee brain. *Proc. Natl. Acad. Sci. U S A* 100, 14519–14525. doi: 10.1073/pnas.2335884100
- Hamilton, A. R., Shpigler, H., Bloch, G., Wheeler, D. E., and Robinson, G. E. (2017). “Endocrine influences on insect societies,” in *Hormones, Brain and Behavior*, eds D. W. Pfaff and M. Joëls (Oxford: Academic Press), 421–452.
- Hammer, M., and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* 5, 146–156.
- Harris, J. W., and Woodring, J. (1995). Elevated brain dopamine levels associated with ovary development in queenless worker honey bees (*Apis mellifera* L.). *Comp. Biochem. Physiol.* 3, 271–279. doi: 10.1016/0742-8413(95)00048-s
- Haselton, A. T., Downer, K. E., Zylstra, J., and Stoffolano, J. G. (2009). Serotonin inhibits protein feeding in the blow fly, *Phormia regina* (Meigen). *J. Insect Behav.* 22, 452–463. doi: 10.1007/s10905-009-9184-1
- Hauser, F., Cazzamali, G., Williamson, M., Blenau, W., and Grimmelikhuijzen, C. J. P. (2006). A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog. Neurobiol.* 80, 1–19. doi: 10.1016/j.pneurobio.2006.07.005
- Hirashima, A., Suetsugu, E., Hirokado, S., Kuwano, E., Taniguchi, E., and Eto, M. (1999). Effect of octopamine on the activity of juvenile-hormone esterase in the silkworm *Bombyx mori* and the red flour beetle *Tribolium freemani*. *Gen. Comp. Endocrinol.* 116, 373–381. doi: 10.1006/gcen.1999.7378
- Hoover, K. M., Bubak, A. N., Law, I. J., Yaeger, J. D. W., Renner, K. J., Swallow, J. G., et al. (2016). The organization of societal conflicts by pavement ants *Tetramorium caespitum*: an agent-based model of amine-mediated decision making. *Curr. Zool.* 62, 277–284. doi: 10.1093/cz/zow041
- Hoover, S. E., Keeling, C. I., Winston, M. L., and Slessor, K. N. (2003). The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* 90, 477–480. doi: 10.1007/s00114-003-0462-z
- Hoyer, S. C., Eckart, A., Herrel, A., Zars, T., Fischer, S. A., Hardie, S. L., et al. (2008). Octopamine in male aggression of *Drosophila*. *Curr. Biol.* 18, 159–167. doi: 10.1016/j.cub.2007.12.052
- Hoyle, G. (1985). “Generation of motor activity and control of behaviour: the role of the neuromodulator octopamine and the orchestration hypothesis,” in *Comparative Insect Physiology, Biochemistry and Pharmacology*, eds G. A. Kerkut and L. Gilbert (Toronto: Pergamon Press), 607–621.
- Ilie, I., Muscedere, M. L., and Traniello, J. F. (2015). Neuroanatomical and morphological trait clusters in the ant genus *Pheidole*: evidence for modularity and integration in brain structure. *Brain Behav. Evol.* 85, 63–76. doi: 10.1159/000370100
- Jeanson, R., Dussutour, A., and Fourcassie, V. (2012). Key factors for the emergence of collective decision in invertebrates. *Front. Neurosci.* 6:121. doi: 10.3389/fnins.2012.00121
- Kamhi, J. F., Nunn, K., Robson, S. K. A., and Traniello, J. F. A. (2015). Polymorphism and division of labour in a socially complex ant: neuromodulation of aggression in the Australian weaver ant, *Oecophylla smaragdina*. *Proc. Biol. Sci.* 282:20150704. doi: 10.1098/rspb.2015.0704
- Kamhi, J. F., and Traniello, J. F. A. (2013). Biogenic amines and collective organization in a superorganism: neuromodulation of social behavior in ants. *Brain Behav. Evol.* 82, 220–236. doi: 10.1159/000356091
- Kay, A. D., Bruning, A. J., van Alst, A., Abrahamson, T. T., Hughes, W. O., and Kaspari, M. (2014). A carbohydrate-rich diet increases social immunity in ants. *Proc. Biol. Sci.* 281:20132374. doi: 10.1098/rspb.2013.2374
- Kay, A. D., Shik, J. Z., Van Alst, A., Miller, K. A., and Kaspari, M. (2012). Diet composition does not affect ant colony tempo. *Funct. Ecol.* 26, 317–323. doi: 10.1111/j.1365-2435.2011.01944.x
- Kay, A. D., Zumbusch, T., Heinen, J. L., Marsh, T. C., and Holway, D. A. (2010). Nutrition and interference competition have interactive effects on the behavior and performance of Argentine ants. *Ecology* 91, 57–64. doi: 10.1890/09-0908.1
- Kent, D. S., and Simpson, J. A. (1992). Eusociality in the beetle *Austroplatypus incompertus* (Coleoptera: Curculionidae). *Naturwissenschaften* 79, 86–87. doi: 10.1007/bf01131810
- Klemm, N., Hustert, R., Cantera, R., and Nässel, D. R. (1986). Neurons reactive to antibodies against serotonin in the stomatogastric nervous system and in the alimentary canal of locust and crickets (Orthoptera, Insecta). *Neuroscience* 17, 247–261. doi: 10.1016/0306-4522(86)90240-x
- Kravitz, E. A., and Huber, R. (2003). Aggression in invertebrates. *Curr. Opin. Neurobiol.* 13, 736–743. doi: 10.1016/j.conb.2003.10.003
- Lebestky, T., Chang, J. S., Dankert, H., Zelnik, L., Kim, Y. C., Han, K. A., et al. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* 64, 522–536. doi: 10.1016/j.neuron.2009.09.031
- LeBoeuf, A. C., Benton, R., and Keller, L. (2013). The molecular basis of social behavior: models, methods and advances. *Curr. Opin. Neurobiol.* 23, 3–10. doi: 10.1016/j.conb.2012.08.008
- LeBoeuf, A. C., Waridel, P., Brent, C. S., Goncalves, A. N., Menin, L., Ortiz, D., et al. (2016). Oral transfer of chemical cues, growth proteins and hormones in social insects. *Elife* 5:e20375. doi: 10.7554/eLife.20375
- Liang, D., and Silverman, J. (2000). “You are what you eat”: diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. *Naturwissenschaften* 87, 412–416. doi: 10.1007/s001140050752
- Libbrecht, R., Oxley, P. R., Keller, L., and Kronauer, D. J. (2016). Robust DNA methylation in the clonal raider ant brain. *Curr. Biol.* 26, 391–395. doi: 10.1016/j.cub.2015.12.040
- Libersat, F., and Plueger, H. J. (2004). Monoamines and the orchestration of behavior. *Biosci.* 54, 17–25. doi: 10.1641/0006-3568(2004)054[0017:matoob]2.0.co;2
- Lihoreau, M., Buhl, J., Charleston, M. A., Sword, G. A., Raubenheimer, D., and Simpson, S. J. (2015). Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. *Ecol. Lett.* 18, 273–286. doi: 10.1111/ele.12406
- Lihoreau, M., Latty, T., and Chittka, L. (2012). An exploration of the social brain hypothesis in insects. *Front. Physiol.* 3:442. doi: 10.3389/fphys.2012.00442
- Liscia, A., Solari, P., Gibbons, S. T., Gelperin, A., and Stoffolano, J. G. Jr. (2012). Effect of serotonin and calcium on the supercontractile muscles of the adult blowfly crop. *J. Insect Physiol.* 58, 356–366. doi: 10.1016/j.jinsphys.2011.12.010
- Lucas, C., and Sokolowski, M. B. (2009). Molecular basis for changes in behavioral state in ant social behaviors. *Proc. Natl. Acad. Sci. U S A* 106, 6351–6356. doi: 10.1073/pnas.0809463106
- Mercer, A. R., and Menzel, R. (1982). The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera*. *J. Comp. Physiol.* 145, 363–368. doi: 10.1007/bf00619340
- Misof, B., Liu, S. L., Meusemann, K., Peters, R. S., Donath, A., Mayer, C., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767. doi: 10.1126/science.1257570

- Molaei, G., and Lange, A. B. (2003). The association of serotonin with the alimentary canal of the African migratory locust, *Locusta migratoria*: distribution, physiology and pharmacological profile. *J. Insect. Physiol.* 49, 1073–1082. doi: 10.1016/j.jinsphys.2003.08.004
- Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech.* 45, 106–121. doi: 10.1002/(sici)1097-0029(19990415)45:2<106::aid-jemt5>3.3.co;2-v
- Moreau, C. S., and Bell, C. D. (2013). Testing the museum versus cradle tropical biological diversity hypothesis: phylogeny, diversification, and ancestral biogeographic range evolution of the ants. *Evolution* 67, 2240–2257. doi: 10.1111/evo.12105
- Muscadere, M. L., Johnson, N., Gillis, B. C., Kamhi, J. F., and Traniello, J. F. A. (2012). Serotonin modulates worker responsiveness to trail pheromone in the ant *Pheidole dentata*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 198, 219–227. doi: 10.1007/s00359-011-0701-2
- Mustard, J. A., Beggs, K. T., and Mercer, A. R. (2005). Molecular biology of the invertebrate dopamine receptors. *Arch. Insect Biochem. Physiol.* 59, 103–117. doi: 10.1002/arch.20065
- Nässel, D. R., and Laxmyr, L. (1983). Quantitative determination of biogenic amines and DOPA in the CNS of adult and larval blowflies, *Calliphora erythrocephala*. *Comp. Biochem. Physiol. C* 75, 259–265. doi: 10.1016/0742-8413(83)90190-1
- Neckameyer, W. S. (1998). Dopamine modulates female sexual receptivity in *Drosophila melanogaster*. *J. Neurogenet.* 12, 101–114. doi: 10.3109/01677069809167259
- Neckameyer, W. S. (2010). A trophic role for serotonin in the development of a simple feeding circuit. *Dev. Neurosci.* 32, 217–237. doi: 10.1159/000304888
- O'Connell, L. A., and Hofmann, H. A. (2011a). Genes, hormones, and circuits: an integrative approach to study the evolution of social behavior. *Front. Neuroendocrinol.* 32, 320–335. doi: 10.1016/j.yfrne.2010.12.004
- O'Connell, L. A., and Hofmann, H. A. (2011b). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi: 10.1002/cne.22735
- O'Connell, L. A., and Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science* 336, 1154–1157. doi: 10.1126/science.1218889
- Okada, Y., Sasaki, K., Miyazaki, S., Shimoji, H., Tsuji, K., and Miura, T. (2015). Social dominance and reproductive differentiation mediated by dopaminergic signaling in a queenless ant. *J. Exp. Biol.* 218, 1091–1098. doi: 10.1242/jeb.118414
- Oster, G. F., and Wilson, E. O. (1978). *Caste and Ecology in the Social Insects*. Princeton, NJ: Princeton University Press.
- Ott, S. R., and Rogers, S. M. (2010). Gregarious desert locusts have substantially larger brains with altered proportions compared with the solitary phase. *Proc. Biol. Sci.* 277, 3087–3096. doi: 10.1098/rspb.2010.0694
- Panaitof, S. C., Yaeger, J. D. W., Speer, J. P., and Renner, K. J. (2016). Biparental behavior in the burying beetle *Necrophorus orbicollis*: a role for dopamine? *Curr. Zool.* 62, 285–291. doi: 10.1093/cz/zow032
- Pastor, D., Piulachs, M. D., Cassier, P., André, M., and Bellés, X. (1991). *In vivo* and *in vitro* study of the action of dopamine on oocyte growth and juvenile hormone production in *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *C. R. Acad. Sci. III* 313, 207–212.
- Penick, C. A., Brent, C. S., Dolezal, K., and Liebig, J. (2014). Neurohormonal changes associated with ritualized combat and the formation of a reproductive hierarchy in the ant *Harpegnathos saltator*. *J. Exp. Biol.* 217, 1496–1503. doi: 10.1242/jeb.098301
- Perry, C. J., Baciadonna, L., and Chittka, L. (2016). Unexpected rewards induce dopamine-dependent positive emotion-like state changes in bumblebees. *Science* 353, 1529–1531. doi: 10.1126/science.aaf4454
- Regier, J. C., Mitter, C., Zwick, A., Bazinet, A. L., Cummings, M. P., Kawahara, A. Y., et al. (2013). A large-scale, higher-level, molecular phylogenetic study of the insect order Lepidoptera (moths and butterflies). *PLoS One* 8:e58568. doi: 10.1371/journal.pone.0058568
- Reid, C. R., Lutz, M. J., Powell, S., Kao, A. B., Couzine, I. D., and Garnier, S. (2015). Army ants dynamically adjust living bridges in response to a cost-benefit trade-off. *Proc. Natl. Acad. Sci. U S A* 112, 15113–15118. doi: 10.1073/pnas.1512241112
- Robinson, G. E., Heuser, L. M., Le Conte, Y., Lenquette, F., and Hollingworth, R. M. (1999). Neurochemicals aid bee nestmate recognition. *Nature* 399, 534–535. doi: 10.1038/21095
- Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* 59, 533–561. doi: 10.1016/S0301-0082(99)00016-7
- Sasaki, T., and Pratt, S. C. (2012). Groups have a larger cognitive capacity than individuals. *Curr. Biol.* 22, R827–R829. doi: 10.1016/j.cub.2012.07.058
- Sasaki, K., Yamasaki, K., and Nagao, T. (2007). Neuro-endocrine correlates of ovarian development and egg-laying behaviors in the primitively eusocial wasp (*Polistes chinensis*). *J. Insect Physiol.* 53, 940–949. doi: 10.1016/j.jinsphys.2007.03.006
- Schmidt, C. (2013). Molecular phylogenetics of ponerine ants (Hymenoptera: Formicidae: Ponerinae). *Zootaxa* 3647, 201–250. doi: 10.11646/zootaxa.3647.2.1
- Schulz, D. J., Barron, A. B., and Robinson, G. E. (2002). A role for octopamine in honey bee division of labor. *Brain Behav. Evol.* 60, 350–359. doi: 10.1159/000067788
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–10502.
- Seeley, T. D. (1989). Social foraging in honey bees: how nectar foragers assess their colony's nutritional status. *Behav. Ecol. Sociobiol.* 24, 181–199. doi: 10.1007/bf00292101
- Seeley, T. D. (2010). *Honeybee Democracy*. Princeton, NJ: Princeton University Press.
- Seid, M. A., Goode, K., Li, C., and Traniello, J. F. A. (2008). Age- and subcaste-related patterns of serotonergic immunoreactivity in the optic lobes of the ant *Pheidole dentata*. *Dev. Neurobiol.* 68, 1325–1333. doi: 10.1002/dneu.20663
- Seid, M. A., and Traniello, J. F. A. (2005). Age-related changes in biogenic amines in individual brains of the ant *Pheidole dentata*. *Naturwissenschaften* 92, 198–201. doi: 10.1007/s00114-005-0610-8
- Sheehan, M. J., and Tibbetts, E. A. (2011). Specialized face learning is associated with individual recognition in paper wasps. *Science* 334, 1272–1275. doi: 10.1126/science.1211334
- Shimoji, H., Aonuma, H., Miura, T., Tsuji, K., Sasaki, K., and Okada, Y. (2017). Queen contact and among-worker interactions dually suppress worker brain dopamine as a potential regulator of reproduction in an ant. *Behav. Ecol. Sociobiol.* 71:35. doi: 10.1007/s00265-016-2263-3
- Simola, D. F., Graham, R. J., Brady, C. M., Enzmann, B. L., Desplan, C., Ray, A., et al. (2016). Epigenetic (re)programming of caste-specific behavior in the ant *Camponotus floridanus*. *Science* 351:aac6633. doi: 10.1126/science.aac6633
- Simpson, S. J., and Raubenheimer, D. (2012). *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity*. Princeton, NJ: Princeton University Press.
- Simpson, S. J., and Stevenson, P. A. (2015). “Neuromodulation of social behavior in insects,” in *The Oxford Handbook of Molecular Psychology*, ed. T. Canli (New York, NY: Oxford University Press), 27–51.
- Sinakevitch, I., Mustard, J. A., and Smith, B. H. (2011). Distribution of the octopamine receptor AmOA1 in the honey bee brain. *PLoS One* 6:e14536. doi: 10.1371/journal.pone.0014536
- Sobotka, J. A., Daley, M., Chandrasekaran, S., Rubin, B. D., and Thompson, G. J. (2016). Structure and function of gene regulatory networks associated with worker sterility in honeybees. *Ecol. Evol.* 6, 1692–1701. doi: 10.1002/ece3.1997
- Sombati, S., and Hoyle, G. (1984). Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. *J. Neurobiol.* 15, 481–506. doi: 10.1002/neu.480150607
- Song, H. J., Amédégato, C., Cigliano, M. M., Desutter-Grandcolas, L., Heads, S. W., Huang, Y., et al. (2015). 300 million years of diversification: elucidating the patterns of orthopteran evolution based on comprehensive taxon and gene sampling. *Cladistics* 31, 621–651. doi: 10.1111/cla.12116
- Song, N., Liang, A. P., and Bu, C. P. (2012). A molecular phylogeny of Hemiptera inferred from mitochondrial genome sequences. *PLoS One* 7:e48778. doi: 10.1371/journal.pone.0048778
- Stevenson, P. A., Hofmann, H. A., Schoch, K., and Schildberger, K. (2000). The fight and flight responses of crickets depleted of biogenic amines. *J. Neurobiol.* 43, 107–120. doi: 10.1002/(sici)1097-4695(200005)43:2<107::aid-neu1>3.3.co;2-3

- Stevenson, P. A., and Rillich, J. (2017). Neuromodulators and the control of aggression in crickets. in *The Cricket as a Model Organism: Development, Regeneration, and Behavior*, eds H. Wilson Horch, T. Mito, A. Popadic, H. Ohuchi and S. Noji (Tokyo, Japan: Springer), 169–195.
- Stoffolano, J. G., Acaron, A., and Conway, M. (2008). “Bubbling” or droplet regurgitation in both sexes of adult *Phormia regina* (Diptera: Calliphoridae) fed various concentrations of sugar and protein solutions. *Ann. Entomol. Soc. Am.* 101, 964–970. doi: 10.1603/0013-8746(2008)101[964:bodrib]2.0.co;2
- Stroeymeyt, N., Guerrieri, F. J., van Zweden, J. S., and d’Ettorre, P. (2010). Rapid decision-making with side-specific perceptual discrimination in ants. *PLoS One* 5:e12377. doi: 10.1371/journal.pone.0012377
- Sturgis, S. J., and Gordon, D. M. (2012). Nestmate recognition in ants (Hymenoptera: Formicidae): a review. *Myrm. News* 16, 101–110.
- Thompson, F. J., Donaldson, L., Johnstone, R. A., Field, J., and Cant, M. A. (2014). Dominant aggression as a deterrent signal in paper wasps. *Behav. Ecol.* 25, 706–715. doi: 10.1093/beheco/aru063
- Thompson, G. J., Hurd, P. L., and Crespi, B. J. (2013). Genes underlying altruism. *Biol. Lett.* 9:20130395. doi: 10.1098/rsbl.2013.0395
- Thompson, G. J., Yockey, H., Lim, J., and Oldroyd, B. P. (2007). Experimental manipulation of ovary activation and gene expression in honey bee (*Apis mellifera*) queens and workers: testing hypotheses of reproductive regulation. *J. Exp. Zool. A Ecol. Genet. Physiol.* 307, 600–610. doi: 10.1002/jez.415
- Thorne, B. L., and Traniello, J. F. (2003). Comparative social biology of basal taxa of ants and termites. *Annu. Rev. Entomol.* 48, 283–306. doi: 10.1146/annurev.ento.48.091801.112611
- Tibbetts, E. A. (2013). The function, development, and evolutionary stability of conventional signals of fighting ability. *Adv. Study Behav.* 45, 49–80. doi: 10.1016/b978-0-12-407186-5.00002-1
- Traniello, J. F. A. (1977). Recruitment behavior, orientation, and the organization of foraging in the carpenter ant *Camponotus pennsylvanicus* degeer (Hymenoptera: Formicidae). *Behav. Ecol. Sociobiol.* 2, 61–79. doi: 10.1007/bf00299289
- Trible, W., and Kronauer, D. J. (2017). Caste development and evolution in ants: it’s all about size. *J. Exp. Biol.* 220, 53–62. doi: 10.1242/jeb.145292
- Vander Meer, R. K., Preston, C. A., and Hefetz, A. (2008). Queen regulates biogenic amine level and nestmate recognition in workers of the fire ant, *Solenopsis invicta*. *Naturwissenschaften* 95, 1155–1158. doi: 10.1007/s00114-008-0432-6
- Vargas, M. A., Luo, N., Yamaguchi, A., and Kapahi, P. (2010). A role for S6 kinase and serotonin in postmating dietary switch and balance of nutrients in *D. melanogaster*. *Curr. Biol.* 20, 1006–1011. doi: 10.1016/j.cub.2010.04.009
- Wada-Katsumata, A., Yamaoka, R., and Aonuma, H. (2011). Social interactions influence dopamine and octopamine homeostasis in the brain of the ant *Formica Japonica*. *J. Exp. Biol.* 214, 1707–1713. doi: 10.1242/jeb.051565
- Wang, Y., Li, H., Wang, P., Song, F., and Cai, W. Z. (2014). Comparative mitogenomics of plant bugs (Hemiptera: Miridae): identifying the AGG codon reassignments between serine and lysine. *PLoS One* 9:e101375. doi: 10.1371/journal.pone.0101375
- Wiegmann, B. M., Trautwein, M. D., Winkler, I. S., Barr, N. B., Kim, J. W., Lambkin, C., et al. (2011). Episodic radiations in the fly tree of life. *Proc. Natl. Acad. Sci. U S A* 108, 5690–5695. doi: 10.1073/pnas.1012675108
- Wilson, E. O. (1971). *The Insect Societies*. Cambridge, MA: Belknap Press of Harvard University.
- Wnuk, A., Wiater, M., and Godzinska, E. J. (2010). Effect of past and present behavioural specialization on brain levels of biogenic amines in workers of the red wood ant *Formica polycetna*. *Physiol. Entomol.* 36, 54–61. doi: 10.1111/j.1365-3032.2010.00762.x
- Yuan, Q., Joiner, W. J., and Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr. Biol.* 16, 1051–1062. doi: 10.1016/j.cub.2006.04.032
- Yuan, Q., Lin, F., Zheng, X., and Sehgal, A. (2005). Serotonin modulates circadian entrainment in *Drosophila*. *Neuron* 47, 115–127. doi: 10.1016/j.neuron.2005.05.027
- Zhou, C., Rao, Y., and Rao, Y. (2008). A subset of octopaminergic neurons are important for *Drosophila* aggression. *Nat. Neurosci.* 11, 1059–1067. doi: 10.1038/nn.2164

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The reviewer VM declared a past collaboration with one of the authors CSM to the handling Editor.

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