INVERTEBRATE NEUROSCIENCE: CONTRIBUTIONS FROM MODEL AND NON-MODEL SPECIES

EDITED BY: Jimena A. Sierralta, Maria de la Paz Fernandez and Clare C. Rittschof

PUBLISHED IN: Frontiers in Behavioral Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-320-2
DOI 10.3389/978-2-88971-320-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

INVERTEBRATE NEUROSCIENCE: CONTRIBUTIONS FROM MODEL AND NON-MODEL SPECIES

Topic Editors:
Jimena A. Sierralta, University of Chile, Chile
Maria de la Paz Fernandez, Columbia University, United States
Clare C. Rittschof, University of Kentucky, United States

Citation: Sierralta, J. A., de la Paz Fernandez, M., Rittschof, C. C., eds. (2021). Invertebrate Neuroscience: Contributions from Model and Non-Model Species. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-320-2

Table of Contents

frontiers in Behavioral Neuroscience

EDITORIAL
published: 14 July 2021
doi: 10.3389/fnbeh.2021.726295

Editorial: Invertebrate Neuroscience: Contributions From Model and Non-model Species

Maria P. Fernandez[1], *Clare C. Rittschof*[2] *and Jimena A. Sierralta*[3]*

[1] Department of Neuroscience and Behavior, Barnard College of Columbia University, New York, NY, United States, [2] Department of Entomology, College of Agriculture, Food, and the Environment, University of Kentucky, Lexington, KY, United States, [3] Department of Neuroscience, Faculty of Medicine, Universidad de Chile, Santiago, Chile

Keywords: neurological diseases, aggression, addiction, social evolution, nutrition, *Drosophila*, bees

Editorial on the Research Topic

Invertebrate Neuroscience: Contributions From Model and Non-model Species

What is an animal model? Traditionally, models are used to investigate how humans develop, how our tissues and cells function, and how diseases take hold and progress. However, beyond these medical applications, many fields of biology, from evolution to neuroscience, also use model organisms to both identify foundational principles that unify species and to explore the extensive morphological and functional diversity among species. Although some vertebrates are powerful models due to their relevance to human physiology, they are of slow grow, expensive to breed and keep, and sometimes difficult to work with for *in vivo* experiments. On the other hand, invertebrates' forms, behaviors, sensory capabilities, and body organization are wonderfully diverse and unique, and yet these species also show surprising commonalities with other members of the animal tree of life. Neuroscientists are amazed that tiny brains can produce sophisticated behaviors with a limited number of neurons, that a butterfly can travel thousands of kilometers to rest for the winter, that even a tiny fly sleeps and wakes with a rhythm and that bees can communicate with a dance. This issue combines insights from traditional models such as *Drosophila melanogaster* and *Apis Mellifera* and organisms such as the crab *Neholice* and the cricket *Gryllus bimaculatus* to provide a glimpse into the power of invertebrate diversity to address fundamental and emerging questions in behavioral and comparative neuroscience.

The most widely used invertebrate model system in laboratory studies is *Drosophila melanogaster,* largely due to its unparalleled genetic tools that allow tissue or even cell specific manipulations of gene expression and neuronal activity, as well as a remarkably large collection of mutant and transgenic lines. *D. melanogaster* is used in a wide range of areas and applications in neuroscience, from molecular neuroscience to neurodevelopmental disorders and learning and memory studies. In this issue, several articles explore the utility of *D. melanogaster* in the context of human brain function and disease. McMullen et al. investigate the mechanism of sugar transport into the brain through the blood-brain barrier, a structure that protects the brain from harmful substances while allowing adequate nutrients to enter. The authors identify two previously unknown glucose transporters and perform functional analyses to demonstrate their critical role in survival. These findings are broadly relevant because of the extensive similarities between insect and mammalian brain nutrient transport. Carvajal-Oliveros and Campusano draw an even more direct link between *D. melanogaster* and human disease as they discuss the role of serotonin in neurodevelopmental disorders such as schizophrenia, autism, and attentional deficits; they describe the potential use of *D. melanogaster* to search for novel chemicals that could alleviate these diseases.

Edited and reviewed by:
Thomas Behnisch,
Fudan University, China

***Correspondence:**
Jimena A. Sierralta
jsierral@uchile.cl

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *16 June 2021*
Accepted: *24 June 2021*
Published: *14 July 2021*

Citation:
Fernandez MP, Rittschof CC and Sierralta JA (2021) Editorial: Invertebrate Neuroscience: Contributions From Model and Non-model Species. Front. Behav. Neurosci. 15:726295. doi: 10.3389/fnbeh.2021.726295

A final area that highlights the links between *D. melanogaster* and human health is the neurobiology of addiction, reviewed in this issue by Chvilicek et al. The authors discuss recent progress in our understanding of the role of small-molecule neurotransmitters in alcohol response. They choose seven specific molecules that show functional conservation between mammals and other animal models, highlighting the use of *D. melanogaster* as a model for alcohol abuse.

Beyond human-oriented behavioral neuroscience, *D. melanogaster* research has significantly deepened our understanding of how genes and neuronal circuits control sexually dimorphic, innate behaviors such as courtship and aggression. Ryvkin et al. describe the transcriptome of neurons that express the fly homolog of the neuropeptide Y receptor (known as the neuropeptide F receptor), which plays a role in male courtship and social group interactions. The ultimate goal of this work is to understand how cell type specific gene expression shapes male behaviors. Sato and Yamamoto examine how changes in pheromone signaling and the sensory circuits underlying pheromone detection contribute to mate choice and reproductive isolation across *Drosophila* species. They focus on gustatory and non-volatile signals, as this type of chemosensory communication is key for species and sex recognition in *Drosophila.* Dietary restriction is a common environmental input that induces behavioral variation in animal species. Legros et al. show that *D. melanogaster* males raised on sugary diets attack rivals more frequently to establish dominance, employing fewer threat displays. *Drosophila*, and invertebrate species more generally, provide opportunities to examine both rapid and persistent environmental effects on behavior (Westwick and Rittschof).

Genetically tractable model systems such as *Drosophila* have proven extremely valuable for studying the underlying neuronal circuitry and the genetic architecture of complex behaviors under laboratory conditions. However, genetic models also have limitations, such as the lack of an ethological or ecological context. Pandolfi et al. compare genetic model systems and discuss their advantages and limitations for studying aggression. The authors discuss behavioral patterns and strategies observed in species such as *Homarus americanus* (lobsters) and *Gryllus bimaculatus* (crickets) for the study of aggressive behavior. The authors highlight a more general bias in the study of aggression, a focus on males, despite the importance of female aggressive behavior. Similarly, though *D. melanogaster* and *Apis mellifera* (honey bees) are very powerful models for the study of circadian rhythms, Beer and Helfrich-Förster argue that there are some aspects of chronobiology for which they are insufficient, such as investigating the role of the clock in photoperiodism and diapause. These authors advocate for the development of genetic tools in non-classical models, for example organism that exhibit a real photoperiodic diapause (such as the fruit fly *Drosophila triauraria* or the silkworm *Bombyx mori*), to enable studies of the diversity of biological clocks in insects, especially with respect to the timing of seasonal activity.

Several other contributors highlight interesting ways in which diverse invertebrate species, particularly insects, have the potential to contribute to outstanding questions in behavioral neuroscience. Though their biology can be strikingly different from vertebrates, contributors illustrate how these unique species can be leveraged to investigate questions that are broadly relevant. For example, Cámera et al. use the visually guided escape response in the crab *Neohelice granulata* to examine how distinct neurons act in concert to regulate a single behavior. They introduce a novel extracellular multi-electrode recording methodology that allows them to functionally distinguish previously described neurons, but also to identify novel neurons that do not fit in the known patterns, such as units sensitive to optic flow with directional preference. Muratore and Traniello explore the promise of using ants, specifically fungus-growing ants, to map the relationship between cognitive demand and brain structural evolution. Some species in this group contain several types of highly morphologically and behaviorally distinct sterile workers which show limited behavioral flexibility. Other related species exhibit less elaborate specialization with broad and flexible individual behavioral repertoires. Thus, it is possible through comparative study to examine the types of brain structural features that accompany behavioral specialization, and conversely, the features that are required to maintain cognitive flexibility. Westwick and Rittschof explore the potential for insects to contribute to our understanding of the link between early-life experience, neurobiology, and adult behavior. They highlight both the simple and complex environmental cues that give rise to adult behavioral variation in insects, and the diverse ways in which environmentally induced neurobiological changes are shared across insects and vertebrates. It could be debated to what extent unique insect systems are considered true "models;" however, it is clear that there is opportunity to use insects to study long-standing questions in behavioral neuroscience, some of which are less tractable in vertebrates.

In addition to their use as models and as the subjects of unique comparative studies, invertebrates present opportunities to tie behavioral neuroscience to broader scientific aims, for example conservation biology. Monarch butterfly seasonal migration presents a behavioral phenotype that is both unusual and generally relevant: monarchs are icons of pollinator conservation in the United States, and individuals are subject to unique challenges as they navigate over thousands of miles of mixed habitat. However, long-distance migration is a phenotype that is observed in diverse species including vertebrates. Guerra examines how monarchs integrate sensory cues from the environment to guide their long-distance navigation, a common challenge for migrating species, highlighting ways in which insights from the monarch may apply more broadly to animal migration and navigation. As with the monarch, the extent to which an invertebrate serves as a model for behavioral neuroscience is up to the willingness of the researcher to extend a comparative lens across a broad phylogenetic space.

The use of model and non-model invertebrates bring tremendous opportunity to explore behavioral neuroscience in a variety of laboratory and ecologically relevant contexts. The broad range of topics described in this issue, from the use of *Drosophila* for biomedical research to the fungus-growing ants to study changes in the brain associated to specific behaviors are examples of this.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Millennium Initiative ICN09_015 and FONDECYT 1210586 to JS, Barnard College start-up funds and a National Institutes of Health grant NINDS R01 NS118012 to MF, and National Institute of Food and Agriculture, U.S. Department of Agriculture Hatch Program 1012993, and the Foundation for Food and Agriculture Research Pollinator Health Fund 549049, to CR.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 09 November 2020
doi: 10.3389/fnbeh.2020.592309

Multielectrode Recordings From Identified Neurons Involved in Visually Elicited Escape Behavior

Alejandro Cámera[1], *Mariano Andres Belluscio*[2,3] *and Daniel Tomsic*[1,4]*

[1]Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), UBA-CONICET, Buenos Aires, Argentina, [2]Instituto de Fisiología y Biofísica Bernardo Houssay, National Council for Scientific and Technical Research (CONICET), Buenos Aires, Argentina, [3]Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina, [4]Departamento de Fisiología, Biología Molecular y Celular Dr. Héctor Maldonado, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

A major challenge in current neuroscience is to understand the concerted functioning of distinct neurons involved in a particular behavior. This goal first requires achieving an adequate characterization of the behavior as well as an identification of the key neuronal elements associated with that action. Such conditions have been considerably attained for the escape response to visual stimuli in the crab *Neohelice*. During the last two decades a combination of *in vivo* intracellular recordings and staining with behavioral experiments and modeling, led us to postulate that a microcircuit formed by four classes of identified lobula giant (LG) neurons operates as a decision-making node for several important visually-guided components of the crab's escape behavior. However, these studies were done by recording LG neurons individually. To investigate the combined operations performed by the group of LG neurons, we began to use multielectrode recordings. Here we describe the methodology and show results of simultaneously recorded activity from different lobula elements. The different LG classes can be distinguished by their differential responses to particular visual stimuli. By comparing the response profiles of extracellular recorded units with intracellular recorded responses to the same stimuli, two of the four LG classes could be faithfully recognized. Additionally, we recorded units with stimulus preferences different from those exhibited by the LG neurons. Among these, we found units sensitive to optic flow with marked directional preference. Units classified within a single group according to their response profiles exhibited similar spike waveforms and similar auto-correlograms, but which, on the other hand, differed from those of groups with different response profiles. Additionally, cross-correlograms revealed excitatory as well as inhibitory relationships between recognizable units. Thus, the extracellular multielectrode methodology allowed us to stably record from previously identified neurons as well as from undescribed elements of the brain of the crab. Moreover, simultaneous multiunit recording allowed beginning to disclose the connections between central elements of the visual circuits. This work provides an entry point into studying the neural networks underlying the control of visually guided behaviors in the crab brain.

Keywords: simultaneous extracellular recording, tetrodes, motion detection, avoidance, crustacean, insect, giant neurons

OPEN ACCESS

Edited by:
Maria de la Paz Fernandez, Columbia University, United States

Reviewed by:
Sarah J. Certel, University of Montana, United States
Nara Ines Muraro, CONICET Instituto de Investigación en Biomedicina de Buenos Aires (IBioBA), Argentina

***Correspondence:**
Daniel Tomsic
tomsic@fbmc.fcen.uba.ar

Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *06 August 2020*
Accepted: *09 October 2020*
Published: *09 November 2020*

Citation:
Cámera A, Belluscio MA and Tomsic D (2020) Multielectrode Recordings From Identified Neurons Involved in Visually Elicited Escape Behavior. Front. Behav. Neurosci. 14:592309. doi: 10.3389/fnbeh.2020.592309

INTRODUCTION

To fulfill its biological function the escape response to an impending threat needs to be executed quickly. This implies that sensory information about danger stimuli must be transformed into avoidance actions with the shortest delay, a purpose that is favorably achieved by large neurons capable of conveying information in terms of action potentials (Herberholz and Marquart, 2012). The origin of the action potential is thought to be related to the high speed of conduction required to effectively evade predator attacks (Monk and Paulin, 2014).

Electrophysiology remains the dominant methodology to investigate neuronal activity in the range of high temporal resolution (milliseconds) that characterizes the transfer of information within the nervous system. Electrophysiological measurements can be achieved by intracellular or extracellular recordings. Intracellular recording with sharp electrodes or whole-cell patch provides very detailed data on neurons (i.e., sub-threshold activity) and allows one to make a morphological identification of the recorded neuron. However, it is usually limited to one cell at a time, requires movement restriction, and can be sustained for a relatively short time. On the other hand, the extracellular recording is more easily performed and can be maintained for hours, but only brings information about action potential activity, without direct knowledge of which neuron originated the recorded spike firing. Therefore, these two techniques bring about complementary information.

In part due to the presence of very large neurons involved in avoidance responses, invertebrates have been suitable models to investigate the neuronal physiology using intracellular recordings (e.g., Kandel, 1976; Edwards et al., 1999; Fotowat and Gabbiani, 2011). In these models, the study of neuronal circuit activity has been mostly satisfied by pooling single-cell data from different individuals. This introduces two types of variability, inter-individual and trial-to-trial variability. Therefore, to analyze information encoded in the activity of neuronal populations it is more appropriate to record the activity of several neurons at the same time in the same individual. With multi-channel electrodes and spike sorting fairly large populations of neurons can be analyzed simultaneously (Gray et al., 1995; Buzsáki, 2004; Brill et al., 2013; Rossant et al., 2016). Indeed, research on invertebrates considering groups or populations of neurons instead of single neurons has increasingly gained attention during the last years (Clemens et al., 2011; Brill et al., 2013; Campbell et al., 2013; Guo and Ritzmann, 2013; Saha et al., 2013; Duer et al., 2015).

The crab *Neohelice granulata* has been extensively used as a model animal in different fields of biology, from ecology to neurobiology (Spivak, 2010). It is a highly visual semiterrestrial crab that inhabits densely populated mudflat environments. In nature, the crab is regularly engaged in social interactions that include burrow defense, courtship, chasing after smaller individuals and being chased by larger ones (Fathala and Maldonado, 2011; Sal Moyano et al., 2016; Tomsic et al., 2017; Gancedo et al., 2020), all activities that are guided by sight. The crab also uses vision to detect and avoid aerial predators (Magani et al., 2016). Accordingly, vision plays a leading role in the behavior of this animal.

Neurobiological studies on *Neohelice* mainly focused on the crab's escape response to visual threats and encompassed different aspects such as visuomotor transformation, response modulation, and learning and memory. The studies were performed with a variety of methodologies that include behavioral analyses, neuroanatomy, pharmacology, molecular biology, electrophysiology, and calcium imaging (for reviews see Tomsic and Romano, 2013; Tomsic, 2016; Tomsic et al., 2017). An important step in the establishment of the crab as an invertebrate model for studying the neural control of behavior has been the identification and characterization of a group of giant neurons from the lobula (3rd optic neuropil of arthropods), which were shown to be key elements for visually-elicited avoidance behaviors. The achievements had been possible due to the unique experimental advantages offered by this crab to perform stable intracellular recordings from brain neurons in the practically intact and awake animal (e.g., Berón de Astrada and Tomsic, 2002; Scarano et al., 2018). Four different classes of lobula giant (LG) neurons had been studied. The different classes exhibit commonalities and differences. Morphologically, they all have wide dendritic trees that extend across tangential layers of the lobula, from where they collect visual information provided by the columnar elements of the retinotopic mosaic (Sztarker et al., 2005; Berón de Astrada et al., 2013) and their axons project through the protocerebral track toward the midbrain (Berón de Astrada and Tomsic, 2002; Medan et al., 2007). A common physiological signature to all LG neurons is their response plasticity on repeated motion stimulation. Such plasticity has been shown to underlie part of the short- and long-term memory traces induced by visual training (Tomsic et al., 2003; Sztarker and Tomsic, 2011). LG neurons also share the ability to integrate binocular information (Sztarker and Tomsic, 2004; Scarano et al., 2018) and three classes integrate visual with mechanosensory information from the animal's legs (Berón de Astrada and Tomsic, 2002; Medan et al., 2007). Beyond these commonalities, the four LG classes show substantial differences. Two classes have dendritic trees extended across a single tangential layer of the lobula and, therefore, had been named Monostratified Lobula Giants type 1 and type 2 (MLG1 and MLG2, respectively), whereas the other two classes have dendritic trees extended over two tangential layers and, hence, were named Bistratified Lobula Giants type 1 and type 2 (BLG1 and BLG2). MLG1s form an ensemble of 16 elements distributed across the lateromedial axis of the lobula, mapping the 360° of azimuthal space. These elements are thought to convey information about objects position and object motion dynamics in terms of population code and activity code, respectively (Oliva and Tomsic, 2014; Medan et al., 2015). Contrasting, MLG2 is likely a unique element, with a receptive field covering the entire visual space (Medan et al., 2007). This neuron has been shown to play a central role in regulating the animal's speed of run according to the visual dynamic of approaching stimuli (Oliva and Tomsic, 2016). The BLG1 class is composed of a discrete number of elements (Medan et al., 2007; Scarano et al., 2018), which might participate in encoding information regarding stimulus

elevation (Tomsic, 2016). The BLG2 is a very large neuron, likely a single element, with an extensive receptive visual field (Medan et al., 2007). Contrasting with the three previous classes, which responses to looming stimuli consist of a firing rate increase that follows the dynamic of image expansion, the BLG2 neuron strongly responds at the very beginning of looming stimulation and inactivates with further image expansions. The time course of the BLG2 activity to looming stimuli approximately coincides with transient freezing observed in the animal before initiating the escape (Oliva, personal communication), suggesting a role of this neuron in that behavioral component (Tomsic et al., 2017).

Considering their complex morphology, multisensory integration, plasticity properties, and the correspondence observed between their activity and the behavioral responses under different circumstances, the group of the LG neurons is thought to operate as a decision-making node for several important aspects of the visually-guided avoidance behavior. Yet, the connectivity among the different LG neurons is still unknown. Here, we began to bridge this gap by performing multielectrode recordings of neurons from the lobula neuropil of the crab.

MATERIALS AND METHODS

Animals

The animals were adult male *Neohelice granulata* crabs 2.7–3.0 cm across the carapace, weighing approximately 17 g, collected in the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina. The crabs were maintained individually in glass jars filled to 2 cm depth with artificial seawater prepared using hw-Marinex (Winex, Hamburg, Germany), salinity 10–14%, at a pH of 7.4–7.6 and maintained within a range of 22–24°C. The holding and experimental rooms were kept on a 12 h light/dark cycle (lights on 7:00 AM to 7:00 PM) and the experiments were run between 8:00 AM and 7:00 PM, two to seven days after the animals' arrival to the laboratory.

Visual Stimuli

This study represents our first approach to using multielectrode recording in the crab. For this reason, we included in our experiments stimuli that proved to be effective both for identifying different lobula neurons (Medan et al., 2015; Tomsic et al., 2017; Scarano et al., 2018, 2020) and for eliciting a variety of behavioral responses in this animal, such as escape response (Oliva and Tomsic, 2012; Scarano and Tomsic, 2014), predatory response (Gancedo et al., 2020) and optomotor response (Barnatan et al., 2019). Computer-generated visual stimuli were projected on a computer screen (Samsung S20C300L) placed at a distance of 20 cm, covering the frontolateral right side of the animal. The screen was housed inside a Faraday cage with opaque covers to prevent outside visual stimuli from reaching the animal. Visual stimuli were of three different types: (a) black squares of three different sizes that moved at three different heights; (b) a grating pattern; and (c) a looming stimulus. The first two types moved rightward or leftward over a white background, covering a translation distance of 37 cm (spanning a visual arc of 85° from the crab point of view), at a speed of 18 cm/s (corresponding to a retinal speed at the center of the screen of near 52°/s). According to their size, square stimuli were named small, medium, and large (1.5 × 1.5 cm, 3 × 3 cm, and 6 × 6 cm, subtending angles at the center of the screen of approximately 6, 12, and 17 square degrees respectively). These stimuli moved at the level of the horizon and 17° above and below the horizon. The grating consisted of a pattern of black and white vertical bars of 6 × 24.5 cm (the retinal subtended angle at the center of the screen 17 × 63°) extended over the whole screen. The remaining stimulus was a looming stimulus, a 2D representation of a black square object approaching the crab at constant velocity with an l/v ratio of 120 ms (expanding from 4° to 60° in 3.36 s). Stimulation consisted of four consecutive rounds of stimuli presentations, each round encompassing the 21 different stimuli (including size and direction variations) delivered in random order. The time between stimuli presentations was no less than 45 s. Visual stimuli were generated using Matlab custom-built software. To assess the timing of the stimuli on the neuronal recordings we used an Arduino, which sent a TTL pulse at the start and the end of the stimulus to the electrophysiological interface board.

Animal Preparation

The crab was firmly held in an adjustable clamp which allowed free movements of the walking legs but reduced movements of the chelae (Berón de Astrada and Tomsic, 2002). The eyestalks were cemented to the carapace at an angle of approximately 50° from the horizontal line, which corresponds to their normal seeing position (Scarano et al., 2018). A small hole on the medial side of the eyestalk cuticle was drilled to introduce the electrode at the level of the lobula (**Figures 1A–D**). After this, the clamp with the crab was mounted inside the recording setup using magnetic holding devices. The multielectrode was then positioned and advanced through the opening in the cuticle. All the recordings were taken from the right eyestalk.

Multielectrode Construction and Recording Devices

We used a custom-made eight-channel multielectrode. It consisted of a pair of tetrodes (four twisted 12 μm tungsten wires each) and a reference (single 50 μm tungsten wire). First, both tetrodes and the reference were slid into a metal capillary which was fixed on a small plexiglass plate controlled by a micromanipulator. Then, the tetrode tips were cut at a 45-degree angle with carbide scissors to improve tissue penetration and the two bundles were glued together using methacrylate, with their tips separated by 50–100 μm. The reference was also glued to the tetrodes approximately 500 μm from their tips, helping to straighten the ensemble (**Figure 1C**). Each electrode impedance at 1 kHz was adjusted to approximately 150 KΩ using gold electroplating. The plexiglass plate contained the plugs for connecting every independent wire, which in turn were connected to the amplifier (Intan RHD2132 16-channel amplifier board). An interface board (RHD2000 USB interface board) allowed to simultaneously acquire neuronal data and the timing of visual stimuli (TTL pulses indicated the start and end of each visual stimulus). Data were acquired at 30 kHz and recorded on a PC using Intan software (RHD2000 Evaluation System Software).

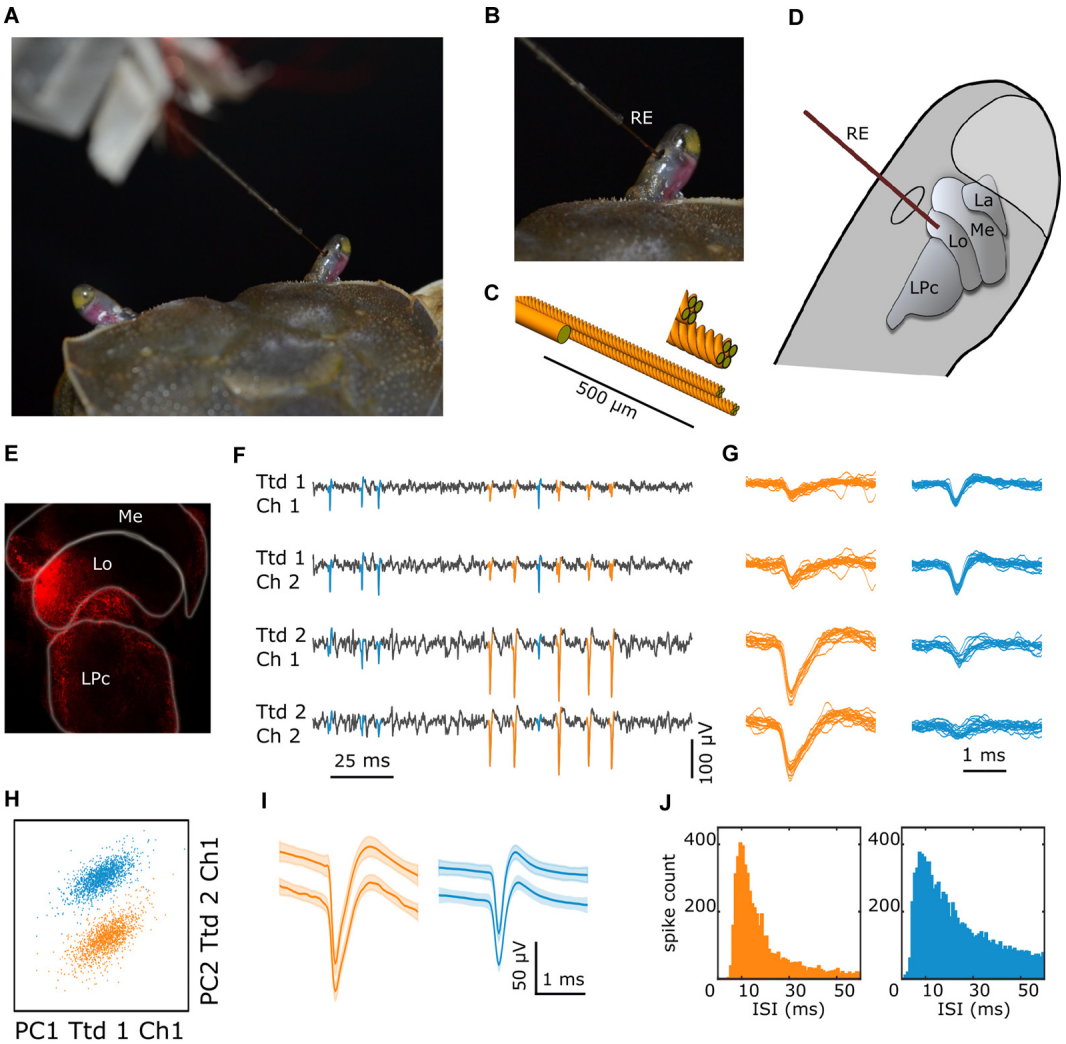

FIGURE 1 | Multielectrode recording procedure and data processing. **(A)** The dorsal part of a crab as viewed from behind with the electrode entering the right eyestalk from its medial side. **(B)** Closer view of the eyestalk with the recording electrode (RE) passing through a small hole cut in the cuticle. **(C)** Detail of the custom-made 8-channel multielectrode, consisting of a pair of tetrodes (four twisted 12 μm tungsten wires each) with their tips separated 50–100 μm and the thicker (50 μm) reference wire 500 μm from the tip. **(D)** Scheme of the eyestalk with the retinotopic neuropils (La: lamina, Me: medulla, Lo: lobula), the lateral protocerebrum (LPc), and the multielectrode targeting the lobula. **(E)** Confocal image showing the spot in the lobula left by the dye at the tip of the electrode. **(F)** Fragment of a recording showing electrical signals obtained in four different channels, with the two upper and two lower traces corresponding to different tetrodes. Signals highlighted in blue and orange identify spikes of two different units. **(G)** Superimposed spikes of each unit were obtained in the four channels. **(H)** Scatter plot of the first two principal components analyses of the waveforms from tetrodes one and two, where cluster membership is indicated by color. **(I)** Mean ± SEM waveforms of the two units from the spikes recorded during 10 min preceding and following the experiment (upper and lower traces, respectively). **(J)** Interspike interval histograms for all spikes of the two sorted units.

Experimental Protocol

Once the multielectrode was inside the eyestalk, it was gently moved forward until clear spike signals to noise ratio were obtained. Then, a rapid preliminary test was performed by presenting a moving stimulus to detect evident neural responses. If satisfactory responses were not observed, the electrode was advanced until a clear-cut response to motion stimulation was achieved. After some practice, we were able to get suitable responses quite easily. However, at this stage, observable responses usually contained the activity of different neuronal units, which could only be separated and distinguished after processing the data off-line. Once the electrode was in a position from which we decided to perform the experiment, we put down the curtain at the front of the Faraday cage and waited for 10 min to start recording. Our experimental protocol included continuous recording during the full sequence of visual motion stimuli presented four times (as described above), plus 10 min of basal activity at both ends of the recording session. These periods of basal activity were used to assure that the signals remained the same across the entire recording.

To confirm that the multielectrode was actually targeting the lobula, in a few experiments we dipped the tips of

the electrodes into a concentrated solution of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) before approaching the tissue. After the recordings, we dissected and prepared the optic ganglia to be observed with the confocal microscope.

Data Processing

There are three main steps involved in spike sorting: spike detection, feature extraction, and spike clustering based on combinations of extracted features (Takekawa et al., 2010). Data were first high pass filtered (a median-based filter with a window half-length of 90 samples), then the spikes (**Figure 1E**) were detected using a voltage threshold of 6 SD, and finally, a principal component analysis (PCA) was performed to each waveform. The waveform time window was set from −0.8 ms before to 1.2 ms after either positive or negative peak amplitude (**Figure 1F**). All these steps were done using NDManager (Hazan et al., 2006). Automated clustering was performed with the program KlustaKwik (version 1.5[1]) and then imported into Klusters (Hazan et al., 2006) for further classification and refinement.

Because spike sorting is sensitive to misclassification (Harris et al., 2000; Joshua et al., 2007; Quiroga et al., 2007), we considered a series of visual tests on the output of automated spike sorting routines that address whether a single cluster of waveforms is self-consistent with a single neuron (Hill et al., 2011). These were as follows.

(a) Inspect the waveforms: for every sorted unit, the spike shapes were superimposed to make false-positive sorting visible. We cleaned false data manually.
(b) Inspect for stationary: for each unit, if the spontaneous activity shifted noticeably during the experiment, data after the shift were excluded from the analysis. We also checked that the mean waveform obtained during a baseline period in the beginning and the end of the experiment was unchanged.
(c) Distribution of interspike intervals (ISIs): very short ISIs (<1–2 ms) are unlikely to occur in a single unit because of its refractory period, so an ISI histogram with a substantial number of occurrences at small ISIs suggests that multiple neurons may be included within a cluster.

After completion of these analyses, clusters were considered to represent spikes of individual neurons.

Analysis of Responses to Visual Stimuli and Classification of Units

Following the identification of the spikes corresponding to individual neurons, the responses to visual stimuli presentations were analyzed. Peri event time histograms (PETH) were computed for each neuron with a bin size of 10 ms. To calculate the instantaneous firing rate, every single raster built on the spike times was smoothed using a Gaussian kernel with a width of 100 ms (10 bins). PETH was constructed with four trials for each stimulus type. In this way, we obtained the response profiles of each unit for all the stimuli.

[1]http://klustakwik.sourceforge.net

Further Analyses

In addition to the response profile to visual stimulation, several features of the extracellularly recorded units were examined. These included spontaneous firing rate, bursting pattern, spike duration, spike asymmetry, the amplitude ratio of the negative and positive peaks and recovery time, as well as features of the auto-correlogram such as the time from peak to peak. Possible interactions between simultaneously recorded units were analyzed using cross-correlograms. Excitatory connections were associated with short-latency and -duration sharp peaks in the cross-correlogram, while short-latency troughs were considered to be due to inhibition (Csicsvari et al., 1998).

RESULTS

General Description

We recorded the neural activity of 93 units from the lobula in 27 animals. The number of reliably identified units per experiment varied between 1 and 8. **Figure 1** shows the method of recording and the general procedure of spike sorting and clustering illustrated on data from a particular experiment. **Figure 1F** shows a recording example where the spike activity of two different units can be observed. The two upper and lower traces correspond to channels of different tetrodes. Note that the blue marked spikes are larger in the two upper channels than in the lower ones, whereas for the orange marked spikes the relation is inverted. This becomes clearer when the waveforms of several individual spikes are superimposed (**Figure 1G**). PCA performed over the entire recording time across all eight channels allowed to distinguish two signal clusters in this particular recording (**Figure 1H**). This result was supported by a series of visual tests that we applied to further address whether a single cluster of waveforms is self-consistent with a single neuron (see "Materials and Methods" section). **Figure 1I** shows the mean ± SEM waveforms of spikes obtained during the first and last 10 min of the experiment (upper and lower traces, respectively) for both units. Despite the samples were taken more than one hour apart, the waveform of each neuron remained unchanged. Also, the distribution of interspike intervals (ISIs) depicted in **Figure 1J** confirms that none of the units reflect refractory period violations (i.e., ISI < 1–2 ms).

Distinct Units Exhibit Differential Response Preferences for Visual Stimuli

Once the spikes of distinct units had been sorted and clustered, we analyzed the responses of each unit to the presentation of the visual stimuli. A first analysis, based on the ratio between the firing rates measured over a 2 s time window immediately before and after the initiation of motion stimulation for all the stimuli, showed that 86% of the recorded units responded with an increase of their firing rate, 4% with a reduction and 10% showed no change. An equivalent result was observed when the different stimuli were analyzed separately.

Responses to square stimuli of different sizes and elevations differed in intensity (being the most effective the large square moving at the level of the horizon), but not in their profiles. Therefore, our description concentrates on the size and elevation that elicited the strongest response. **Figure 2** illustrates the responses of three different units to the large square stimulus (blue) and the looming stimulus (red). Raster plots reflect the responses recorded across four trials and the traces are the mean ± SEM. Each unit exhibits a different response profile. Unit 1 shows a moderate increase of firing rate to the moving square that ends when the stimulus stops moving, whilst it shows a progressive increase of firing rate to the looming stimulus that nearly matches the image growing and suddenly suppresses with the end of the expansion. Unit 2 shows a response to the square lead by a prominent peak of firing rate followed by a steady-state that extends beyond the stimulus end, whereas the response to looming consists of an early substantial increase of firing that progressively decays with the image expansion and is followed by a marked rebound at the end of the expansion. Unit 3 shows no appreciable response to either the square or the looming stimulus. These results demonstrate the feasibility of disclosing and classifying units based on their response preference for distinct visual stimuli, an issue that we further elaborate throughout the next sections.

Identification of LG Neurons

The multielectrode extracellular technique prevents the morphological identification of the recorded neurons. Consequently, in most studies, whether they are carried out in vertebrate or in invertebrate animals, the identity of recorded neurons is essentially unknown. The best approximation for neuronal identification resides on previous knowledge of neurons housed in the area from where the extracellular recording is taken, namely in the possibility of establishing correspondences between patterns of activity recorded extracellularly with those seen in neurons that had been characterized intracellularly. Yet, even in invertebrates, the strategy of identifying neurons by comparing extracellular data with intracellular data proved not to be easy (Bhavsar et al., 2015). We were confident that this could be achieved in the crab because the lobula is an easily targeting neuropil that contains several classes of morphologically identified and physiologically characterized neurons of exceptionally large size, the LG neurons. Results shown in **Figures 3**, **4** substantiate our assumption. **Figure 3** allows comparing the response profile to a looming stimulus obtained by intracellular recording from neurons MLG2 and BLG2 with similar responses from units obtained by extracellular recording. The characterizations performed by intracellular recording followed by cell staining have shown that these two neurons arborize across the whole lobula as well as in several regions of the lateral protocerebrum (**Figures 3A,F**), and their physiological receptive fields cover the entire visual field of the animal (Medan et al., 2007). However, the response of these two cells to looming stimuli was very different. On one hand, the MLG2 neuron increases the firing rate according to the dynamic of image expansion (Oliva et al., 2007; Oliva and Tomsic, 2016). This is illustrated in the intracellularly

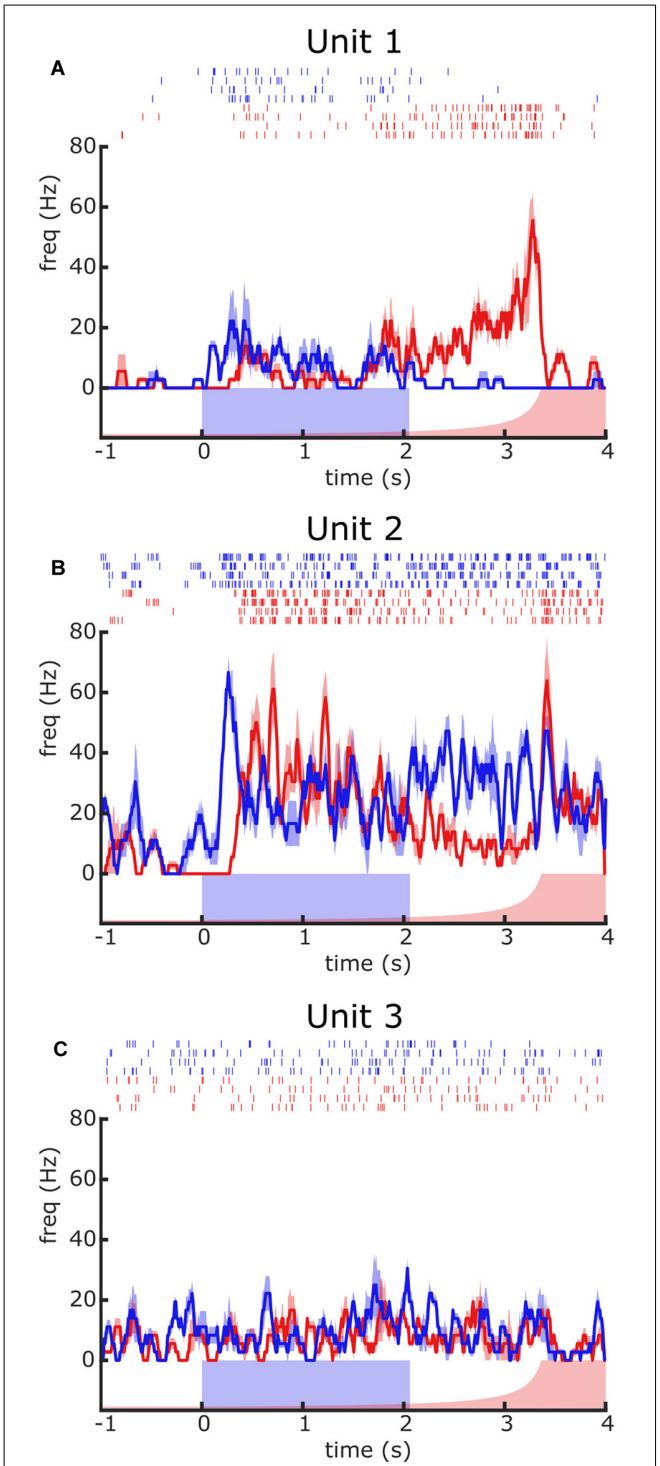

FIGURE 2 | Contrasting responses of different units to visual presentations of a looming (red) or a moving square (blue) stimulus. **(A–C)** Responses of three different units recorded from different crabs. For each unit, the raster plot depicts the temporal course of elicited spikes (response) to four presentations of each stimulus type. The traces correspond to the mean ± SEM firing frequency. The blue rectangle over the X-axis represents the motion duration of the square stimulus. The red curve represents the angular size of the looming stimulus, which remained stationary until time zero when it started to expand. Further details are in the text.

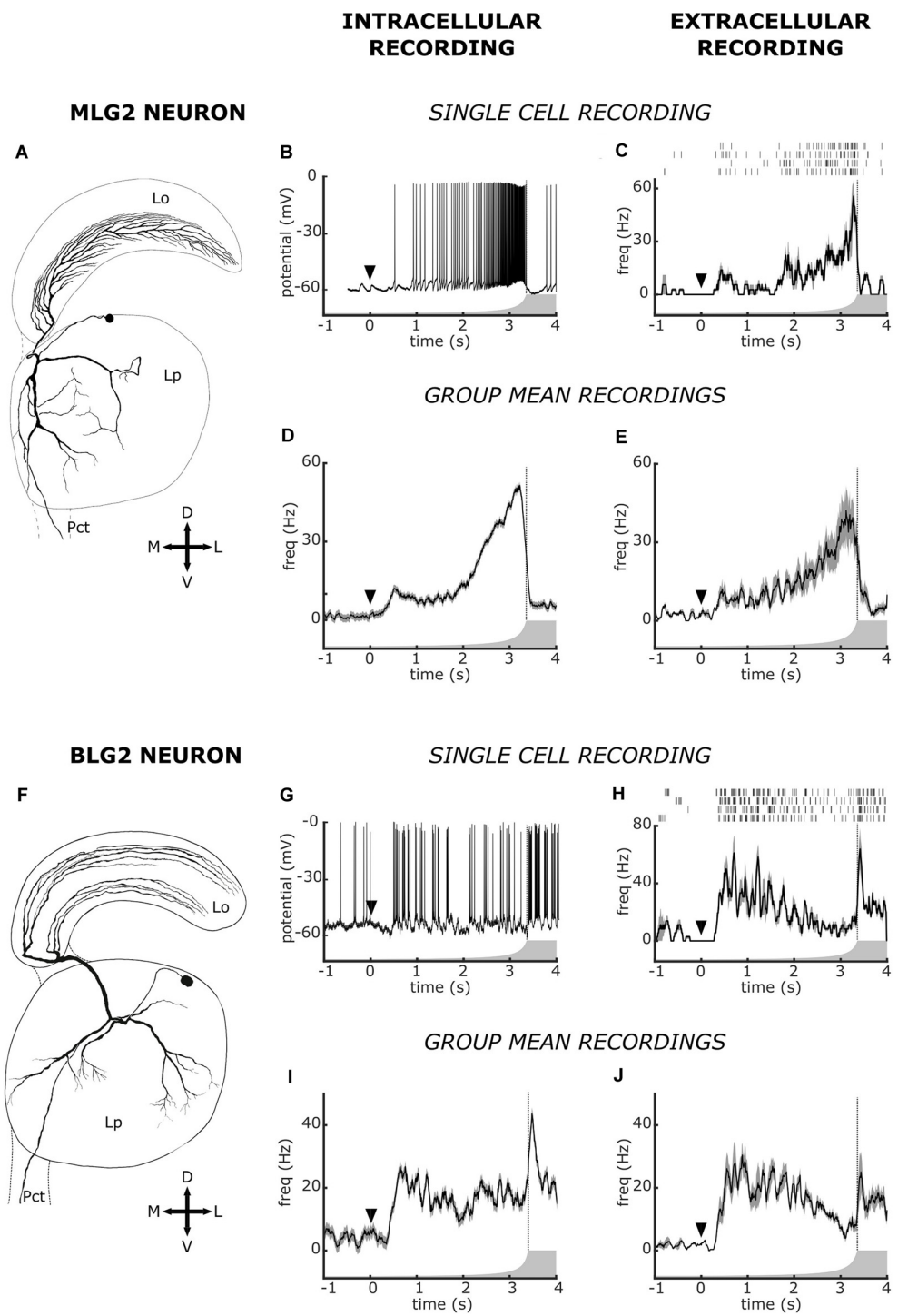

FIGURE 3 | Intracellular and extracellular recorded responses to a looming stimulus of Monostratified Lobula Giant type 2 neuron (MLG2) and of Bistratified Lobula Giant 2 neuron (BLG2). **(A–E)** Data on MLG2. **(F–J)** Data on BLG2. **(A,F)** Morphology of the two neuronal types. **(B,G)** Examples of intracellular recorded responses. **(C,H)** Examples of extracellular recorded responses. For each unit, the raster plot shows responses to four presentations of the looming stimulus and the traces are the mean ± SEM. **(D,I)** Mean ± SEM of intracellularly recorded responses (as those shown in panels **B,G**) obtained from different animals. In panels (**D,I**) the number of averaged animals (one mean response per animal) is 37 and 13, respectively. **(E,J)** Mean ± SEM of extracellularly recorded responses (as those shown in panels **C,H**) obtained from different animals. In panels (**E,J**) the number of animals (one mean response per animal) is five and eight, respectively. The arrowhead at time zero marks the beginning of stimulus expansion, which is represented by the curved profile of the gray form. The vertical dotted line denotes the end of the expansion. The cell morphologies shown in panels (**A,F**) are from Medan et al. (2007). Data shown in panels (**B,D**) have been modified from Oliva and Tomsic (2016). Data in panels (**G,I**) have been modified from Oliva (2010).

recorded trace of **Figure 3B**. A remarkably similar profile of spiking activity was found in some extracellular recorded units, as the one shown in **Figure 3C**. The correspondence between the activity of intracellularly and extracellularly recorded elements becomes more evident and reliable when the mean response from several units with similar responses recorded from different animals are compared (intracellular $n = 37$, extracellular $n = 5$, **Figures 3D,E**, respectively). The BLG2 neuron, on the other hand, has been shown to respond to looming stimuli with an early substantial increase of firing rate, followed by a steady-state or even a gradual suppression during the rapid phase of stimulus expansion and a transient rebound of high-frequency firing just after the end of the expansion. This can be observed in the intracellular recorded response of **Figure 3G**. Again, we found a similar response profile in some of the extracellular recorded units, like the one shown in **Figure 3H**. The resemblance between the response of intracellularly recorded BLG2 neurons and the response of some extracellularly recorded units can be appreciated by comparing the mean response profiles of neurons obtained from different individuals (intracellular $n = 13$, extracellular $n = 8$, **Figures 3I,J**, respectively).

In addition to the response profiles to visual stimulation just described, our knowledge on the activity of LG neurons acquired by intracellular recordings allows comparing other features of the extracellularly recorded units. For example, the MLG2 has been shown to exhibit a spontaneous activity made of individual spikes, whereas the BLG2 was shown to display spontaneous activity characterized by bursts of spikes (tables 1 and 2 in Medan et al., 2007). In the raster plot of **Figure 3C**, the spontaneous activity preceding the start of the looming contains isolated spikes. On the other hand, the raster plot of **Figure 3H** shows a spontaneous activity made of bursts. We then analyzed the firing pattern of all our MLG2 and BLG2 classified units, by calculating the percentage of total spikes that occurred as bursts of three or more spikes with an interspike interval of less than 15 ms (Longden et al., 2017). The mean ± SEM percentage for the MLG2 units ($n = 5$) was 3.3 ± 1, 4 and for the BLG2 units ($n = 8$) was 15.7 ± 2.7, a difference that was statistically significant ($p < 0.01$, Student's t-test). Therefore the pattern of bursting activity provides further confidence for our classification of these extracellularly recorded units as MLG2 and BLG2 neurons.

Identification of Directional Sensitive Neurons

The four LG classes of neurons have scarce or null motion directional preferences (Medan et al., 2007). Recently, we have described a novel group of large neurons of the crab that exhibit a remarkable directional preference for visual stimuli moving along the horizontal plane. Because of their arborizations in the lobula and the lobula plate, we called these cells lobula complex directional cells (LCDC; Scarano et al., 2020). The LCDC response to a moving square is characterized by a

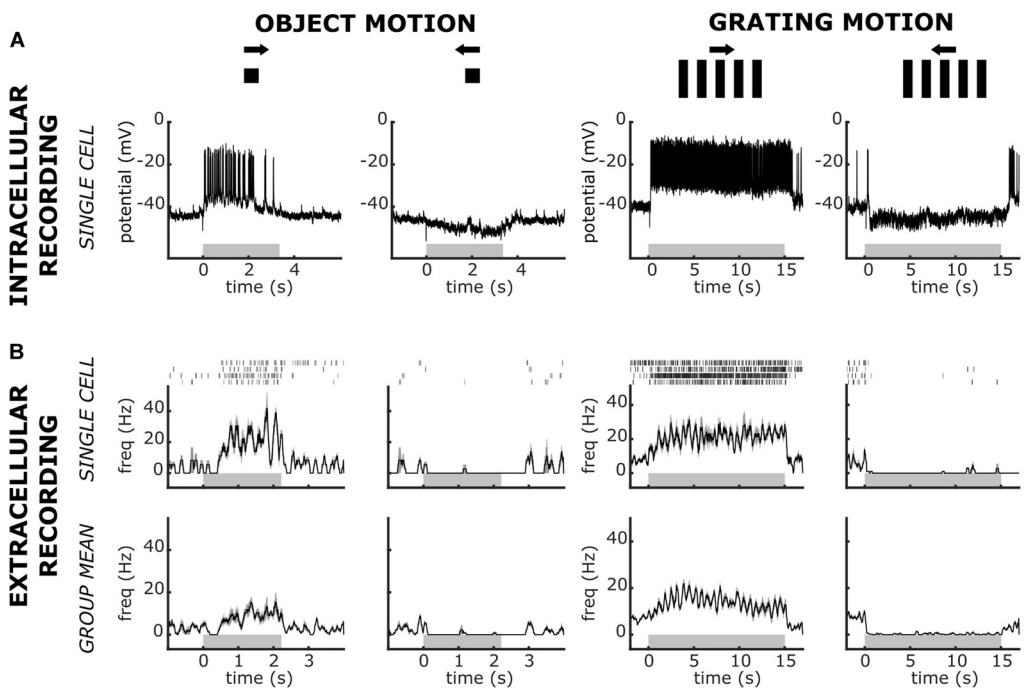

FIGURE 4 | Intracellular and extracellular recorded responses of Lobula Complex Directional Cells (LCDC) to visual presentations of a single moving object or a grating pattern. Responses to the rightward and leftward motion were recorded for both stimulus types. **(A)** Responses from a single intracellularly recorded neuron. **(B)** Responses from extracellular recorded units. Upper panels: responses of a single unit. Raster plots show responses to four presentations of the stimulus and the traces are the mean ± SEM. Lower panels: Mean ± SEM obtained from five animals (one mean response per animal). Gray horizontal rectangles stand for the time of stimulus motion. Note the differences in the scale times among panels. Data in panel **(A)** have been modified from Scarano et al. (2020). See the text for further details.

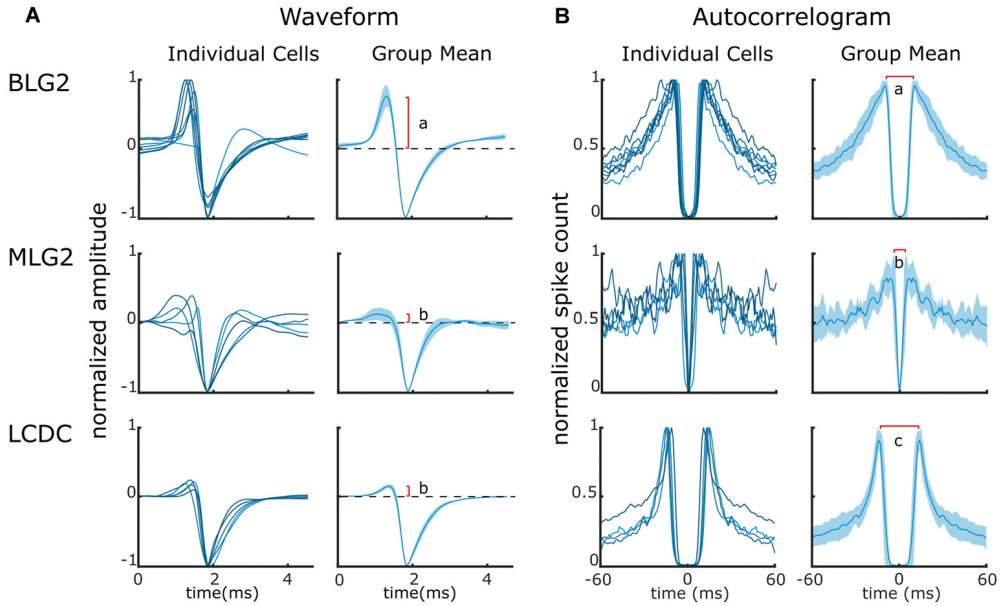

FIGURE 5 | Waveforms and auto-correlograms of BLG2, MLG2, and LCDC neurons. **(A)** Waveforms of individual units (left panels) and Mean ± SEM waveforms (right panels) of the three identified neuronal classes. The red vertical line indicates the mean size of the waveform positive phase. Different letters denote a significant difference. **(B)** Auto-correlograms of individual units (left panels) and Mean ± SEM (right panels). The red horizontal line indicates the mean time between the peeks of the spike count. Different letters denote a significant difference. Further details are in the text.

clear increase of spike discharge in one direction, the so-called preferred direction, and a hyperpolarization with suppression of spontaneous spikes in the opposite direction called the null direction. Some LCDC neurons have been shown to present sustained responses to optic flow in the preferred as well as in the null direction (Scarano et al., 2020). **Figure 4A** shows the intracellularly recorded response of an LCDC to a single object and a grating pattern moved in opposite directions. In our extracellular recordings, we have identified units that responded as LCDC neurons. The performance of one such unit is illustrated in **Figure 4B** (upper row), where the responses to the four trials (raster plot) recorded for each stimulus condition and the averaged response from these trials (trace) are depicted. **Figure 4B** (lower row) shows the mean responses from five recorded units, which responses were similar to those of the unit depicted in the upper panels. The results show the remarkable directional sensitivity of these units, consisting of a sustained increase in the firing rate to one motion direction as well as a sustained suppression of the spontaneous spike activity in the opposite direction. The equivalence between these response profiles and those obtained with intracellular recordings from LCDC neurons strengthens our initial confidence in the feasibility to recognize in extracellular recordings some of the previously identified elements of the crab's lobula neuropil.

Additional Commonalities Within Groups of Identified Units

The identification of extracellularly recorded units as MLG2, BLG2, or LCDC neurons just described was based on the recognition of particular patterns of activity in response to presentations of specific visual stimuli. To further investigate the reliability of this criterion for picking out elements of a particular neuronal class, we analyzed the consistency of the waveforms and the auto-correlograms among the units of each particular group (**Figures 5A,B**, respectively). Contrasting with the analysis of the response profiles during the presentation of visual stimuli, which comprised just a small fraction (4%) of the entire recording time, the mean waveform and the auto-correlogram take into consideration all the spikes sorted for each unit during the whole duration of the recording. **Figure 5A** (left column) shows the mean waveform of each unit, for all those units that were identified by their responses as MLG2, BLG2, or LCDC. A cursory inspection allows seeing that within each group the waveforms have rather similar shapes (with one exception in the BLG2 group) and that the shapes differ among the groups. In particular, BLG2 units exhibit a conspicuous positive phase that is barely observable in MLG2 and LCDC units. The differences become more evident when the mean waveforms of the groups (obtained from the individual means) are compared (**Figure 5A**, right column). A comparison of simple features of the mean waveforms, such as the relative magnitude of the positive phase (red vertical line), reveals statistical differences between groups tagged with different letters (BLG2 vs. MLG2, $p < 0.01$; BLG2 vs. LCDC, $p < 0.01$; MLG2 vs. LCDC, $p > 0.5$, one way ANOVA followed by Tukey's test).

Figure 5B (left column) shows the auto-correlograms of all those units classified as MLG2, BLG2, or LCDC. The auto-correlograms of units within each group are qualitatively more similar between them than to those of units from the other

groups. **Figure 5B** (right column) shows the mean ± SEM of the auto-correlograms of the three cell classes. A simple comparison of the time that separates the peeks of higher probability (red horizontal line) shows statistical differences among all groups (BLG2 vs. MLG2, $p < 0.01$; BLG2 vs. LCDC, $p < 0.05$; MLG2 vs. LCDC, $p < 0.01$, one way ANOVA followed by Tukey's test).

The coherence found in the waveforms and in the auto-correlograms among the units that had been classified by their responses to visual stimulation as belonging to a specific class provides strong support to the use of those responses as a solid criterion for recognizing specific neuronal classes within the lobula of the crab. Moreover, the results show that a proper recognition of a unit as an MLG2, BLG2 or LCDC, should satisfy the three criteria identified here, namely: (i) a particular response profile to visual stimuli; (ii) a predictable waveform shape; and (iii) an expected outline in the auto-correlogram.

Functional Connections Between Units

The simultaneous recording of activity from different units performed with multi electrodes offers the possibility of disclosing functional relationships between them. These interactions are typically visualized through cross-correlation analyses (Barthó et al., 2004). We analyzed cross-correlograms built with the spikes recorded during the whole duration of the experiment, thus comprising periods of spontaneous activity as well as of evoked activity (stimuli presentations). We also examined the cross-correlograms built exclusively with the spikes generated during the stimulation periods but, because the sum of these periods represents only about 4% of the entire recording time, the numbers of events were insufficient for the analyses. Cross-correlograms (and auto-correlograms) built on the total recorded spikes and those built on the periods of spontaneous activity looked very similar.

Of the 155 cell pairs analyzed in our experiments, we found that near 15% showed apparent interactions. **Figure 6** illustrates three different types of interactions found in our recordings. For the cross-correlograms (gray panels) the reference event (time 0) is the spike of the corresponding unit which auto-correlogram is shown in light blue. **Figure 6A** shows the auto-correlograms of a BLG2 cell and a non-identified cell 1 with the corresponding cross-correlogram. The cross-correlogram contains a clear and narrow short-latency peak (<5 ms), indicating that the BLG2 presynaptic neuron was an excitatory cell. **Figure 6B** shows the auto-correlograms of a non-identified cell 2 and a BLG2 cell. The cross-correlogram of these cells exhibits a short-latency suppression (<10 ms), indicating that the non-identified presynaptic neuron was inhibitory on the BLG2 neuron. Finally, **Figure 6C** presents the auto-correlograms corresponding to an MLG2 and a BLG2. In this case, the cross-correlogram shows both a short-latency sustained peak and a delayed trough, suggesting that the elements of the pair were mutually connected. The MLG2 was excitatory on the BLG2, whereas the BLG2 exerted an indirect (delayed) inhibition on the MLG2. The short-latency and long-lasting peak and the delayed inhibition can be better appreciated on the extended timescale shown in the figure inset.

The inhibitory effect of the BLG2 on the MLG2 is in agreement with what is observed in the response profiles to the looming stimulus of these neurons (**Figure 3**), i.e., the time of higher firing frequency of the BLG2 at the beginning and the end of the stimulus expansion coincides with the time of lower spike frequency of the MLG2, which finds its maximal rate when the BLG2 has its trough. Following similar reasoning, it would be expected that the cross-correlogram had revealed an inhibitory effect of MLG2 on the BLG2, but this was not the case. Far more experiments are needed to unravel the complex functional connections existing in the microcircuit formed by the large neurons of the lobula.

DISCUSSION

The crab *Neohelice* is a well established invertebrate model for investigating the neurobiology of visually guided behaviors, including learning and memory processes. Over the last two decades a great deal of knowledge about different lobula giant neurons that play central roles in the crab's escape behavior from visual threats has been acquired (Tomsic et al., 2017). The characterization of these LG neurons has been made by *in vivo* intracellular recording and staining. The present account describes results obtained by multielectrode extracellular recording for the first time. This study aimed to seek out the possibility of identifying LG neurons from the extracellular recorded units based on the similarity of responses to those recorded intracellularly with a variety of visual stimuli. The results show that the expectation was fulfilled. Moreover, by simultaneously recording from multiples units we proved the feasibility of disclosing the interactions between them.

Characteristics of the Multielectrode Extracellular Recording in the Crab

Extracellular recording is the oldest and most common method for recording electrical activity across populations of neurons in awake behaving animals, from invertebrates to human primates. Yet simple criteria for acceptable data, particularly concerning claims of single-unit responses, are largely missing. Such criteria are critical since interpretations of spike trains that are based on inadequately sorted units can lead to erroneous claims on neural coding (Hill et al., 2011). Because this is our first study using this methodology, we adopted conservative spike sorting criteria (see "Materials and Methods" section). This reduced the number of potentially analyzable units per experiment but increased our confidence by relying on units whose signals were most conspicuous. On average, we have considered for analyses 3.4 units per experiment (range 1–8, median 4), which is comparable to the average of 2.7 units (56 units from 21 preparations) recorded from the central complex of the cockroach using a similar pair of 12 μm wire-bundle tetrodes (Guo and Ritzmann, 2013).

Crabs offer the singular advantage of allowing to perform stable intracellular recording in the practically intact animal, which following the experiment remains perfectly healthy (e.g., Tomsic et al., 2003). This holds for multielectrode recording. The stability of these recordings is illustrated in

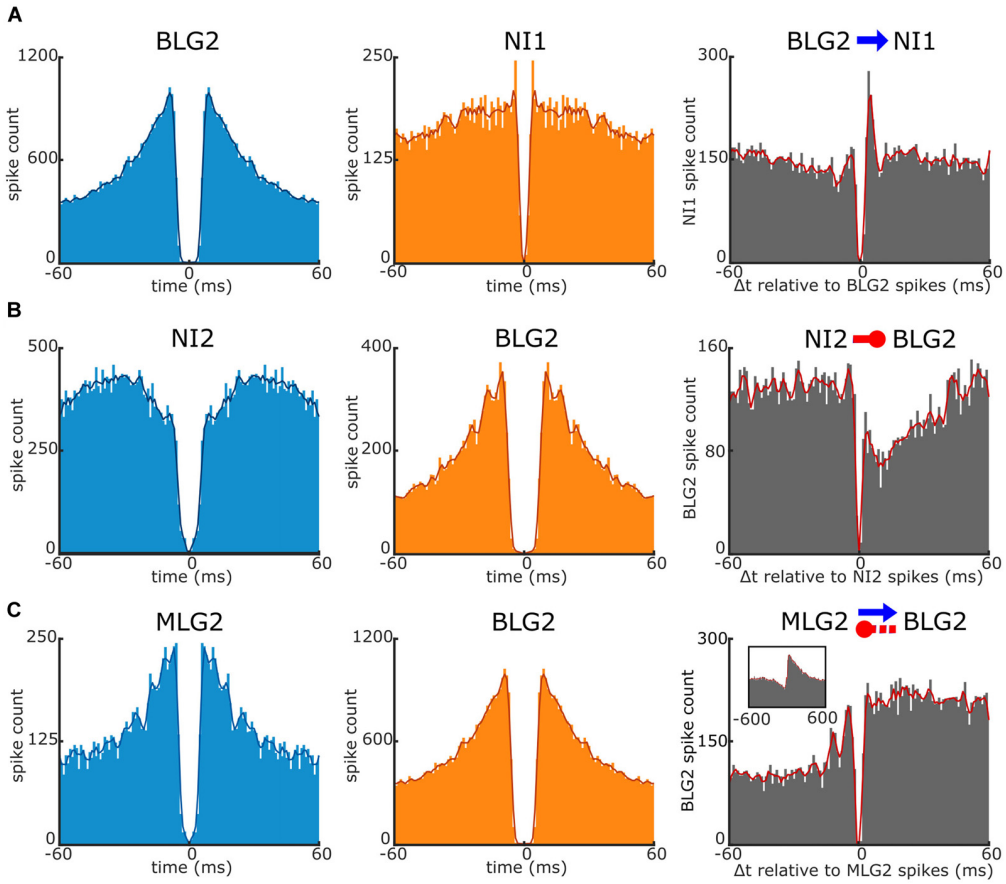

FIGURE 6 | Interaction between a pair of units recorded simultaneously. Examples of interactions revealed by cross-correlogram analyses of three pairs of units. The left and middle panels are the auto-correlograms of the units corresponding to the cross-correlograms shown on the right panels. **(A)** Short-latency monosynaptic excitatory interaction of the BLG2 cell on a non-identified unit 1 (NI1; blue arrow). Note the large, sharp peak at near 4 ms in the cross-correlogram. The reference event (*time 0*) is the spike of the BLG2. **(B)** Short-latency monosynaptic inhibitory interaction of a non-identified unit 2 (NI2) on the BLG2 neuron (red circle-ended line). Note the strong and immediate suppression of target spikes. The reference event is the spike of the NI2 neuron. **(C)** Complex reciprocal interactions between the MLG2 and BLG2 neurons (blue arrow and red circle-ended line). Reference event: the spike of the MLG2 unit. In the inset, data are shown over an extending period. Note the long-lasting excitation of the BLG2 and the delayed suppression of the MLG2 spikes. Further explanations are in the text.

Figure 1I, which shows that the unit's waveforms remained unchanged throughout the experiment, even though the electrode was not affixed to the carapace of the crab and that the animal sporadically moved its legs. The recording stability obtained under such conditions seems to warranty the feasibility of recordings from the freely moving animal. The chances for this are also supported by the fact that after the recording all the animals used in this study remained healthy.

Extracellular Recognition of Previously Identified Neurons

Intracellular recording and staining allow to unequivocally establish fundamental aspects of the cell physiology together with the cell's exact location and morphology. Therefore, the possibility of associating extracellularly recorded units with intracellularly well-characterized elements is of paramount importance. Yet, extracellular multichannel recordings in arthropods have mostly been made from unidentified cells, and attempts to recognize specific neurons based on matching the responses to particular stimuli with those previously obtained from intracellular recordings have largely failed (e.g., Bhavsar et al., 2015). By recording with the duo-tetrode from the lobula we were able to confidently identify two of the four types of LG neurons that have been described so far (e.g., Medan et al., 2007), as well as the recently described directional giant neurons (Scarano et al., 2020). Among the four classes of LG neurons, the MLG2 and BLG2 recognized in the present study are the largest lobula neurons, whose arborizations profusely extend all over the neuropil (Medan et al., 2007). Similarly, the so-called lobula complex directional cells (LCDC) present extensive arborizations within the lobula (Scarano et al., 2020). These characteristics most certainly facilitated recording from these elements. The classification of recorded units as MLG2, BLG2, or LCDC was based on the similarity between the patterns of responses (temporal course and intensity of firing frequency) obtained extracellularly with those previously obtained intracellularly to

identical visual stimuli. Remarkably, the classifications made by this criterion rendered groups of units that could be distinguished by criteria different from the one originally used to separate them. The units gathered as BLG2 have a waveform that allows to distinguish them from units grouped as MLG2 and LCDC. Likewise, an analysis of the auto-correlograms allows us to separate the units of the three groups. The similarity in the waveforms and the auto-correlograms found among units that were ascribed to each one of the three groups based solely on their response profile, and the differences between the units of the separated groups, provide strong validation on our criteria of spike sorting and of neuronal identification.

The highest firing rates reached with extracellular recordings were a bit lower than those obtained with intracellular recordings (**Figure 4**). This may be because looming stimuli are known to induce high frequency firing with similar latency in different LG neurons, especially towards the end of image expansion (Tomsic et al., 2017). Hence, regularly occurring overlapping spikes of different neurons may be interpreted as separate waveforms of a particular neuron. Also, false-negative errors may include misclassification because of a reduction in amplitude and an increase in width for the trailing spikes of a burst. Consequently, at high firing frequency, the spike waveforms of an individual unit become irregular and may not be recognized by the software to be included in the cell cluster (Bhavsar et al., 2015).

The two previously characterized LG classes named MLG1 and BLG1 have been elusive in our experiments. Several reasons may account for their lack. First, both classes are composed of several units whose anatomical and physiological receptive fields are considerably smaller compared to those of the MLG2 and BLG2 classes, which are thought to be represented by one single element per lobula (Medan et al., 2007). In our experiments, the stimulation area was restricted to the screen location, which encompassed a small portion (less than 25%) of the horizontal visual space seen by the crab's eye. This, in combination with the receptive field size of MLG1 and BLG1, could have made these neurons less likely to be activated. Another reason would be related to a low level of spontaneous activity, in particular for MLG1 neurons. Because the reliability of clusters formation depends on the number of detected spikes, neurons with high spontaneous activity are better isolated than neurons that are only activated by the presence of stimuli. In our experiments, the sum of time corresponding to the presentations of all the stimuli comprised less than 4% of the entire duration of the recording. Therefore, for neurons like MLG1s that barely show activity in absence of stimulation, the effectiveness of building reliable clusters is compromised. This being said, we have recorded two units whose response profiles resemble that of MLG1 neurons, but their endorsement is pending until more similar units will be recorded.

Unidentified Recorded Units

Although the present study is focused on the identification of neurons previously characterized by intracellular recordings, a brief discussion on the unidentified units is warranted. Most of the recorded units exhibited response profiles distinct to those that characterize the particular classes of LG neurons or the LCDCs. This is not surprising given that the neurons so far characterized likely represent a fraction of the large tangential elements present in the lobula. Indeed, while attempting to record intracellularly from LG neurons, we often impale neurons that display differential sensitivities for particular visual stimuli. We have not systematically studied these neurons yet. However, comparable response preferences could be observed in some of our extracellularly recorded units. For example, a unit responded with excitation to the large moving square and with inhibition to the small square; another unit responded with similar excitation to all square sizes followed by marked post-stimulus suppression of the spontaneous activity; a unit displayed a stronger response to the grating pattern when it was presented motionless than when it moved; several units responded with transient excitations at the beginning and the end of the square translatory motion. A thorough description of these types of units is pending on further studies.

Functional Neuronal Interactions

Transformation, transmission, and storage of information in the brain are achieved by the cooperative action of neuronal ensembles. The study of population activity of neurons in the crab has been satisfied so far by artificially combining data obtained through intracellular recordings from different individuals (Tomsic et al., 2003; Sztarker and Tomsic, 2011; Oliva and Tomsic, 2014, 2016; Medan et al., 2015). There have been double intracellular recordings performed to study combined responses of different neurons (Scarano et al., 2018), but the success rates for simultaneous recordings in the living crab is usually quite low. Population neural responses have also been studied in the crab by using massive staining and optical recording (Berón de Astrada et al., 2013), however, this methodology does not allow revealing the identity of individual units. Besides, while optical recording methods provide the advantage of spatial information, their temporal resolution does not meet the requirement for assessing the information encoded in the high firing frequency used by neurons (Brill et al., 2013). Thus, simultaneous access to single neurons in the same preparation at high temporal resolution can only be achieved through extracellular multichannel recording. By analyzing the temporal relationship of activity between simultaneously recorded units using cross-correlograms, it is possible to infer different kinds of neuronal interactions (e.g., Barthó et al., 2004; Hill et al., 2011). Nevertheless, the number of detectable interactions is usually low (e.g., Barthó et al., 2004). We recorded different types of interactions. For instance, a likely monosynaptic (short-latency, sharp peak) excitatory synaptic connections of BLG2 on an unidentified unit (**Figure 6A**), a likely mono or disynaptic (<10 ms delay) inhibitory connection of an unidentified unit on the BLG2 (**Figure 6B**), and a reciprocal interaction involving more complex functional relations between the MLG2 and the BLG2 neurons (**Figure 6C**). The connection between this pair entails a short-latency and long-lasting excitatory effect of the MLG2 on the BLG2 and an indirect (>10 ms delay) inhibitory effect of the BLG2 on the MLG2. An inhibitory connection of the

BLG2 on the MLG2, such as the one observed here, has been anticipated by the analyses of the temporal course of response of these neurons to a variety of looming stimuli. Moreover, the interaction has been proposed to be part of the neural mechanism underlying the decision of switching from a freezing response to an escape response (Oliva, 2010). When a crab faces an approaching object, its first strategy is to freeze, but if the object continues to approach the crab runs away. The BLG2 neuron strongly responds to a looming stimulus at the very beginning of its expansion, when the freezing occurs (Tomsic et al., 2017). Thus, the activity of the BLG2 may lead to freezing while contributing to inhibit the MLG2. If the stimulus further approaches the activity of the BLG2 decays, releasing the MLG2 that starts firing and the crab begins to run away (Oliva, 2010). Once the escape has been launched, the response of the MLG2 neuron faithfully encodes the angular velocity of looming stimuli, and thus conveys the information used by the animal to continuously adjust its running speed (Oliva and Tomsic, 2016).

Toward Simultaneous Multiunit Recording in the Freely Moving Crab

Field and laboratory studies have demonstrated that the crab's avoidance behavior is not a stereotyped reflex reaction, but a complex repertoire of strategies that includes freezing, escaping, and confronting. The decision on which strategy should be implemented is based on risk assessment, for which the animal takes into account the stimulus as well as the contextual situation, such as the availability of a near shelter (Hemmi and Tomsic, 2012). When running away from a visual threat the crab continuously adjusts its direction and speed of escape according to changes in the incoming visual information (Oliva and Tomsic, 2016; Medan et al., 2015). Besides, the escape response to a specific stimulus can be rapidly adapted by learning (Tomsic and Maldonado, 2013). By recording intracellularly from immobilized animals we have shown that some of these behavioral attributes are reflected by the activity of the LG neurons (e.g., Sztarker and Tomsic, 2011; Oliva and Tomsic, 2014, 2016; Medan et al., 2015), which lead us to propose that these neurons form a microcircuit that acts as a decision-making node (Tomsic, 2016). The correspondence of the activity of particular LG neurons with a distinct component of the escape response was established by the remarkable matching found between the temporal course of the neuronal and the behavioral responses to a variety of visual stimuli measured separately in different individuals. However, the neural control of elaborated behaviors can hardly be understood by the analysis of single-neuron physiology. Simultaneously recording the individual activity of the foremost neurons of the circuit involved in the avoidance responses of the crab will considerably improve our knowledge on the neural interactions and computations underlying the organization of these behaviors. This goal became more realistic after having confirmed, as we did here, that the identity of LG neurons can be faithfully recognized from extracellular recorded units.

The stability of our recordings in combination with the suitable size and robustness of the crab gives us confidence in the feasibility of recording from the freely moving animal. Besides, the readiness of the crab to behave in the laboratory, where stimulation conditions are well controlled and responses are easy to measure, offers excellent opportunities for evaluating the conjoin activity of lobula neurons in the behaving animal.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

ETHICS STATEMENT

The research was conducted in accordance with the Ethical Reference Frame for Biomedical Investigations of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, equivalent to the standard procedures for animal care and use of the US NIH.

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. DT helped in study concept and design, helped in drafting of the article, and obtained funding. AC performed the experiments and acquired the data. AC, MB, and DT helped in analysis and interpretation of data, and helped in critical revision of the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the following grants to DT: Proyectos de Investigación Científica y Tecnológica (PICT) 2016-1946 from Agencia Nacional de Promoción Científica y Tecnológica and Grant No. 20020170100254BA [Universidad de Buenos Aires Ciencia y Tecnología (UBACYT)] from University of Buenos Aires.

REFERENCES

Barnatan, Y., Tomsic, D., and Sztarker, J. (2019). Unidirectional optomotor responses and eye dominance in two species of crabs. *Front. Physiol.* 10:586. doi: 10.3389/fphys.2019.00586

Barthó, P., Hirase, H., Monconduit, L., Zugaro, M., Harris, K. D., and Buzsáki, G. (2004). Characterization of neocortical principal cells and interneurons by network interactions and extracellular features. *J. Neurophysiol.* 92, 600–608. doi: 10.1152/jn.01170.2003

Berón de Astrada, M., Bengochea, M., Sztarker, J., Delorenzi, A., and Tomsic, D. (2013). Behaviorally related neural plasticity in the arthropod optic lobes. *Curr. Biol.* 23, 1389–1398. doi: 10.1016/j.cub.2013.05.061

Berón de Astrada, M., and Tomsic, D. (2002). Physiology and morphology of visual movement detector neurons in a crab (Decapoda:

Brachyura). *J. Comp. Physiol.* 188, 539–551. doi: 10.1007/s00359-002-0328-4

Bhavsar, M., Heinrich, R., and Stumpner, A. (2015). Multielectrode recordings from auditory neurons in the brain of a small grasshopper. *J. Neurosci. Methods* 256, 63–73. doi: 10.1016/j.jneumeth.2015.08.024

Brill, M. F., Rosenbaum, T., Reus, I., Kleineidam, C. J., Nawrot, M. P., and Rössler, W. (2013). Parallel processing *via* a dual olfactory pathway in the honeybee. *J. Neurosci.* 33, 2443–2456. doi: 10.1523/JNEUROSCI.4268-12.2013

Buzsáki, G. (2004). Large-scale recording of neuronal ensembles. *Nat. Neurosci.* 7, 446–451. doi: 10.1038/nn1233

Campbell, R. A., Honegger, K. S., Hongato, Q., Li, W., Demir, E., and Turner, G. C. (2013). Imaging a population code for odor identity in the *Drosophila* mushroom body. *J. Neurosci.* 33, 10568–10581. doi: 10.1523/JNEUROSCI.0682-12.2013

Clemens, J., Kutzki, O., Ronacher, B., Schreiber, S., and Wohlgemuth, S. (2011). Efficient transformationof an auditory population code in a small sensory system. *Proc. Natl. Acad. Sci. U S A* 108, 13812–13817. doi: 10.1073/pnas.1104506108

Csicsvari, J., Hirase, H., Czurkó, A., and Buzsáki, G. (1998). Reliability and state dependence of pyramidal cell-interneuron synapses in the hippocampus: an ensemble approach in the behaving rat. *Neuron* 21, 179–189. doi: 10.1016/s0896-6273(00)80525-5

Duer, A., Benjamin, P. H., and Menzel, R. (2015). High order neural correlates of social behavior in the honeybee brain. *J. Neurosci. Methods* 254, 1–9. ISSN 0165–0270. doi: 10.1016/j.jneumeth.2015.07.004

Edwards, D. H., Heitler, W. J., and Krasne, F. B. (1999). Fifty years of a command neuron: the neurobiology of escape behavior in the crayfish. *Trends Neurosci.* 22, 153–161. doi: 10.1016/s0166-2236(98)01340-x

Fathala, M. V., and Maldonado, H. (2011). Shelter use during exploratory and escape behaviour of the crab *Chasmagnathus granulatus*: a field study. *J. Ethol.* 29, 263–273. doi: 10.1007/s10164-010-0253-x

Fotowat, H., and Gabbiani, F. (2011). Collision detection as a model for sensory-motor integration. *Annu. Rev. Neurosci.* 34, 1–19. doi: 10.1146/annurev-neuro-061010-113632

Gancedo, B., Salido, C., and Tomsic, D. (2020). Visual determinants of prey chasing behavior in a mudflat crab. *J. Exp. Biol.* 223:jeb217299. doi: 10.1242/jeb.217299

Gray, C. M., Maldonado, P. E., Wilson, M., and McNaughton, B. (1995). Tetrodes markedly improve the reliability and yield of multiple single-unit isolation from multi-unit recordings in cat striate cortex. *J. Neurosci. Methods* 63, 43–54. doi: 10.1016/0165-0270(95)00085-2

Guo, P., and Ritzmann, R. E. (2013). Neural activity in the central complex of the cockroach brain is linked to turning behaviors. *J. Exp. Biol.* 216, 992–1002. doi: 10.1242/jeb.080473

Harris, K. D., Henze, D. A., Csicsvari, J., Hirase, H., and Buzsáki, G. (2000). Accuracy of tetrode spike separation as determined by simultaneous intracellular and extracellular measurements. *J. Neurophysiol.* 84, 401–414. doi: 10.1152/jn.2000.84.1.401

Hazan, L., Zugaro, M., and Buzsáki, G. (2006). Klusters, NeuroScope, NDManager: a free software suite for neurophysiological data processing and visualization. *J. Neurosci. Methods* 155, 207–216. doi: 10.1016/j.jneumeth.2006.01.017

Hemmi, J. M., and Tomsic, D. (2012). The neuroethology of escape in crabs: from sensory ecology to neurons and back. *Curr. Opin. Neurobiol.* 22, 194–200. doi: 10.1016/j.conb.2011.11.012

Herberholz, J., and Marquart, G. (2012). Decision making and behavioral choice during predator avoidance. *Front. Neurosci.* 6:125. doi: 10.3389/fnins.2012.00125

Hill, D. N., Mehta, S. B., and Kleinfeld, D. (2011). Quality metrics to accompany spike sorting of extracellular signals. *J. Neurosci.* 31, 8699–8705. doi: 10.1523/JNEUROSCI.0971-11.2011

Joshua, M., Elias, S., Levine, O., and Bergman, H. (2007). Quantifying the isolation quality of extracellularly recorded action potentials. *J. Neurosci. Methods* 163, 267–282. doi: 10.1016/j.jneumeth.2007.03.012

Kandel, E. R. (1976). *Cellular Basis of Behavior: An Introduction to Behavioral Neurobiology*. San Francisco, CA, W. H. Freeman.

Longden, K. D., Wicklein, M., Hardcastle, B. J., Huston, S. J., and Krapp, H. G. (2017). Spike burst coding of translatory optic flow and depth from motion in the fly visual system. *Curr. Biol.* 27, 3225–3236. doi: 10.1016/j.cub.2017.09.044

Magani, F., Luppi, T., Nuñez, J., and Tomsic, D. (2016). Predation risk modifies behaviour by shaping the response of identified brain neurons. *J. Exp. Biol.* 219, 1172–1177. doi: 10.1242/jeb.136903

Medan, V., De Astrada, M. B., Scarano, F., and Tomsic, D. (2015). A network of visual motion-sensitive neurons for computing object position in an arthropod. *J. Neurosci.* 35, 6654–6666. doi: 10.1523/JNEUROSCI.4667-14.2015

Medan, V., Oliva, D., and Tomsic, D. (2007). Characterization of lobula giant neurons responsive to visual stimuli that elicit escape behaviors in the crab Chasmagnathus. *J. Neurophyol.* 98, 2414–2428. doi: 10.1152/jn.00803.2007

Monk, T., and Paulin, M. G. (2014). Predation and the origin of neurones. *Brain Behav. Evol.* 84, 246–261. doi: 10.1159/000368177

Oliva, D. (2010). *Mecanismos de Detección Visual y Evitación de Colisiones en un nuevo Modelo Experimental, el Cangrejo Chasmagnathus Granulatus*. Buenos Aires: Universidad de Buenos Aires-Facultad de Ciencias Exactas y Naturales. PhD Thesis. Available online at: https://bibliotecadigital.exactas.uba.ar/download/tesis/tesis_n4678_Oliva.pdf.

Oliva, D., Medan, V., and Tomsic, D. (2007). Escape behavior and neuronal responses to looming stimuli in the crab Chasmagnathus granulatus (Decapoda: Grapsidae). *J. Exp. Biol* 210, 865–880. doi: 10.1242/jeb.02707

Oliva, D., and Tomsic, D. (2012). Visuo-motor transformations involved in the escape response to looming stimuli in the crab *Neohelice (=Chasmagnathus) granulata*. *J. Exp. Biol.* 215, 3488–3500. doi: 10.1242/jeb.070755

Oliva, D., and Tomsic, D. (2016). Object approach computation by a giant neuron and its relationship with the speed of escape in the crab neohelice. *J. Exp. Biol.* 219, 3339–3352. doi: 10.1242/jeb.136820

Oliva, D., and Tomsic, D. (2014). Computation of object approach by a system of visual motion-sensitive neurons in the crab neohelice. *J. Neurophysiol.* 112, 1477–1490. doi: 10.1152/jn.00921.2013

Quiroga, R., Reddy, L., Koch, C., and Fried, I. (2007). Decoding visual inputs from multiple neurons in the human temporal lobe. *J. Neurophys.* 98, 1997–2007. doi: 10.1152/jn.00125.2007

Rossant, C., Kadir, S. N., Goodman, D., Schulman, J., Hunter, M., Saleem, A. B., et al. (2016). Spike sorting for large, dense electrode arrays. *Nat. Neurosci.* 19, 634–641. doi: 10.1038/nn.4268

Saha, D., Leong, K., Li, C., Peterson, S., Greogry, S., and Raman, B. (2013). A spatiotemporal coding mechanism for background-invariant odor recognition. *Nat. Neurosci.* 16, 1830–1839. doi: 10.1038/nn.3570

Sal Moyano, M. P., Lorusso, M., Nuñez, J., Ribeiro, P., Gavio, M. A., and Luppi, T. (2016). Male size-dependent dominance for burrow holding in the semiterrestrial crab neohelice granulata: multiple tactics used by intermediate-sized males. *Behav. Ecol. Sociobiol.* 70, 1497–1505. doi: 10.1007/s00265-016-2158-3

Scarano, F., Sztarker, J., Medan, V., Berón de Astrada, M., and Tomsic, D. (2018). Binocular neuronal processing of object motion in an arthropod. *J. Neurosci.* 38, 6933–6948. doi: 10.1523/JNEUROSCI.3641-17.2018

Scarano, F., and Tomsic, D. (2014). Escape response of the crab neohelice to computer generated looming and translational visual danger stimuli. *J. Physiol. Paris* 108, 141–147. doi: 10.1016/j.jphysparis.2014.08.002

Scarano, F., Tomsic, D., and Sztarker, J. (2020). Direction selective neurons responsive to horizontal motion in a crab reflect an adaptation to prevailing movements in flat environments. *J. Neurosci.* 40, 5561–5571. doi: 10.1523/JNEUROSCI.0372-20.2020

Spivak, E. D. (2010). The crab *Neohelice (=Chasmagnathus)* granulata: an emergent animal model from emergent countries. *Helgol. Mar. Res.* 64, 149–154. doi: 10.1007/s10152-010-0198-z

Sztarker, J., Strausfeld, N. J., and Tomsic, D. (2005). Organization of optic lobes that support motion detection in a semiterrestrial crab. *J. Comp. Neurol.* 493, 396–411. doi: 10.1002/cne.20755

Sztarker, J., and Tomsic, D. (2004). Binocular visual integration in the crustacean nervous system. *J. Comp. Physiol.* 190, 951–962. doi: 10.1007/s00359-004-0551-2

Sztarker, J., and Tomsic, D. (2011). Brain modularity in arthropods: individual neurons that support "what" but not "where" memories. *J. Neurosci.* 31, 8175–8180. doi: 10.1523/JNEUROSCI.6029-10.2011

Takekawa, T., Isomura, Y., and Fukai, T. (2010). Accurate spike sorting for multi-unit recordings. *Eur. J. Neurosci.* 31, 263–272. doi: 10.1111/j.1460-9568.2009.07068.x

Tomsic, D. (2016). Visual motion processing subserving behavior in crabs. *Curr. Opin. Neurobiol.* 41, 113–121. doi: 10.1016/j.conb.2016.09.003

Tomsic, D., Berón de Astrada, M., and Sztarker, J. (2003). Identification of individual neurons reflecting short- and long-term visual memory in an arthropodo. *J. Neurosci.* 23, 8539–8546. doi: 10.1523/JNEUROSCI.23-24-08539.2003

Tomsic, D., and Maldonado, H. (2013). "Chapter 19-Neurobiology of learning and memory," in *Crustacean Nervous Systems and Their Control of Behavior. Volume 3 in the Series The Natural History of the Crustacea*, eds C. Derby and M. Thiel (New York: Oxford University Press), 509–553.

Tomsic, D., and Romano, A. (2013). "A multidisciplinary approach to learning and memory in the crab neohelice (Chasmagnathus) granulate," in *The Handbook of Behavioral Neuroscience*, eds R. Menzel and P. R. Benjamin (Amsterdam: Esevier), 337–355.

Tomsic, D., Sztarker, J., Astrada, M., Oliva, D., and Lanza, E. (2017). The predator and prey behaviors of crabs: from ecology to neural adaptations. *J. Exp. Biol.* 220, 2318–2327. doi: 10.1242/jeb.143222

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer NM declared a shared affiliation, with no collaboration, with the authors to the handling editor at the time of review.

frontiers in Behavioral Neuroscience

REVIEW
published: 26 November 2020
doi: 10.3389/fnbeh.2020.601676

Model and Non-model Insects in Chronobiology

Katharina Beer and Charlotte Helfrich-Förster*

Neurobiology and Genetics, Theodor-Boveri Institute, Biocentre, Am Hubland, University of Würzburg, Würzburg, Germany

The fruit fly *Drosophila melanogaster* is an established model organism in chronobiology, because genetic manipulation and breeding in the laboratory are easy. The circadian clock neuroanatomy in *D. melanogaster* is one of the best-known clock networks in insects and basic circadian behavior has been characterized in detail in this insect. Another model in chronobiology is the honey bee *Apis mellifera*, of which diurnal foraging behavior has been described already in the early twentieth century. *A. mellifera* hallmarks the research on the interplay between the clock and sociality and complex behaviors like sun compass navigation and time-place-learning. Nevertheless, there are aspects of clock structure and function, like for example the role of the clock in photoperiodism and diapause, which can be only insufficiently investigated in these two models. Unlike high-latitude flies such as *Chymomyza costata* or *D. ezoana*, cosmopolitan *D. melanogaster* flies do not display a photoperiodic diapause. Similarly, *A. mellifera* bees do not go into "real" diapause, but most solitary bee species exhibit an obligatory diapause. Furthermore, sociality evolved in different Hymenoptera independently, wherefore it might be misleading to study the social clock only in one social insect. Consequently, additional research on non-model insects is required to understand the circadian clock in Diptera and Hymenoptera. In this review, we introduce the two chronobiology model insects *D. melanogaster* and *A. mellifera,* compare them with other insects and show their advantages and limitations as general models for insect circadian clocks.

Keywords: *Drosophila melanogaster*, *Apis mellifera*, circadian clock, complex behavior, diapause, sociality

OPEN ACCESS

Edited by:
Maria de la Paz Fernandez, Columbia University, United States

Reviewed by:
Joanna C. Chiu, University of California, Davis, United States
Charalambos P. Kyriacou, University of Leicester, United Kingdom

****Correspondence:***
Katharina Beer
katharina.beer@uni-wuerzburg.de

Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *02 September 2020*
Accepted: *30 October 2020*
Published: *26 November 2020*

Citation:
Beer K and Helfrich-Förster C (2020) Model and Non-model Insects in Chronobiology. Front. Behav. Neurosci. 14:601676. doi: 10.3389/fnbeh.2020.601676

INTRODUCTION: THE HISTORY OF INSECT MODELS IN CHRONOBIOLOGY

Chronobiology is a field of biology that examines cyclic phenomena in living organisms and their adaptation to solar- and lunar-related rhythms. These cycles are known as biological rhythms and the best known are daily, annual and lunar rhythms. Daily rhythms are controlled by the circadian clock, which has a period of about (circa) a day (dian), but is synchronized to a period of 24 h by the environmental rhythms (= Zeitgeber) on earth. Chronobiologists also say that the circadian clock entrains to the 24 h Zeitgeber. The circadian clock is ubiquitous in living organisms of our planet. Circadian clocks help individual insects and other organisms to anticipate the 24 h environmental cycles and insect populations to synchronize crucial steps in their life (such as eclosion from the pupal case or mating) at the optimal time of the day. In addition, they enable individual insects to measure time, which is important for a time memory. They also provide an internal time reference for insects that orient themselves via a sun compass, which is necessary to compensate for the

sun's predictable daily motion. Furthermore, the circadian clock is needed to measure day length and to prepare in time for the coming season (e.g., to reproduce or to hibernate). Since insects live at different latitudes (from the equator to the poles), at different habitats (conditions of the surrounding environment) and have developed different life styles (e.g., solitary or social), several adaptations of the circadian clock evolved that are just beginning to be investigated.

Different insect models help to elucidate various aspects of circadian clock function. Nevertheless, many concepts in chronobiology, like the interplay of daily and annual time keeping in photoperiodism and hibernation (in insects called diapause) or the influence of inter-individual behavior and social insect communities on the clock, are still not well-understood.

The honey bee was one of the first insect models in chronobiology. Reports of daily foraging behavior in the beginning of the twentieth century inspired research on the biological relevance of clock regulated behavior (Kleber, 1935; Galizia et al., 2011). Studies on the role of the clock in complex behaviors like sun-compass orientation of the honey bee followed (Frisch and Lindauer, 1954; Medugorac and Lindauer, 1967; Lehmann et al., 2011; Cheeseman et al., 2012) and are still an intensely studied topic, since many other insects of different orders, for example the monarch butterfly, desert locusts and the desert ant *Cataglyphis*, use sun- or sky-compass orientation (e.g., Fent and Wehner, 1985; Merlin et al., 2009, 2012; Homberg et al., 2011; Homberg, 2015).

On the search for the location of the circadian clock in the insect brain, first evidence of a circadian pacemaker (= master clock) in the lateral brain was given by surgical removal and transplantation of the optic lobes in cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Page, 1982). Later, more specific tissue transplantation studies identified the accessory medulla, a small neuropil in the optic lobe, as the master clock in cockroaches (Reischig and Stengl, 2003). In comparative studies, master clocks in the lateral and/or dorsal brain could be identified in many different species, for example flies, bugs, bees and some moth species (Siwicki et al., 1988; Nässel et al., 1993; Helfrich-Förster et al., 1998; Wise et al., 2002; Závodská et al., 2003; Vafopoulou et al., 2009; Ikeno et al., 2014; Kobelková et al., 2015; Fuchikawa et al., 2017).

With the isolation of the first clock gene mutants in *Drosophila melanogaster* the molecular basis of the circadian clock was unraveled and subsequently the first functional studies were introduced to insect chronobiology (Konopka and Benzer, 1971). Systematic genetic manipulations of the clock system led to a detailed knowledge about the insect clock in this fly [reviewed in (Hall, 2003)] and therefore the best description of basic concepts of the insect circadian clock so far is found in *Drosophila*.

Lately, new arising methods for genetic manipulation offer the possibility to study circadian clock components and function in detail also in many other insects. RNA interference has been successfully applied in different insects (Moriyama et al., 2008; Lee et al., 2009; for example: Ikeno et al., 2010; Takekata et al., 2012; Kotwica-Rolinska et al., 2017) and genome editing via CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)—Cas (CRISPR associated protein) may provide clock gene manipulation suitable for further insects (Kotwica-Rolinska et al., 2019).

THE MOLECULAR CLOCK—THE CENTRAL NEGATIVE FEEDBACK LOOP

In 2017, the Nobel Prize in Physiology/Medicine was awarded to Jeffrey Hall, Michael Young, and Michael Rosbash for their work that led to the understanding of the molecular basis of circadian rhythms in *D. melanogaster*, a work that was pioneered by Konopka and Benzer in the 70ties of the last century by the isolation of the *period* mutants (Konopka and Benzer, 1971). The *period* gene (*per*) was the first clock gene that was ever isolated and it turned out to be highly conserved in the animal kingdom (**Table 1**). Similarly conserved are the general mechanisms of molecular rhythm generation that involve several other clock genes and proteins that interact in transcriptional/translational feedback loops. Nevertheless, a few features are unique to *D. melanogaster*, or better to say to higher flies (Brachycera) (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015; Chahad-Ehlers, 2017; Bertolini et al., 2018). For example, the second discovered fly clock gene, *timeless1* (*tim1 or dtim*) has a unique function in the first transcriptional/translational feedback loop of higher flies, where its protein product TIM1 dimerizes with PER (the protein product of the *period* gene) and the dimer enters the nucleus (Sehgal et al., 1994; Myers et al., 1996; Saez and Young, 1996) (see **Figure 1**). In other animals, for example the honey bee, *tim1* is substituted by a *cryptochrome* (*cry*) *gene* that codes for a specific light-insensitive form of CRY2 also called mammalian type CRY (mCRY) (Yuan et al., 2007). Another *cry* gene [*Drosophila cry* (*dcry*) or *insect type cry1* (*cry1*)] codes for a light-sensitive CRY1 and usually forms no dimers with PER (Emery et al., 1998), although PER-CRY1 interactions have been found *in vitro* (Rosato et al., 2001; Schlichting et al., 2018). In the fruit fly, CRY1 interacts with TIM1 (insect type TIM1) after it has been activated by light and leads to the degradation of TIM1 in the proteasome (Ceriani et al., 1999; Naidoo, 1999), a feature that makes flies very sensitive to light (see below). TIM1 and CRY1 are also present in for example mosquitoes, aphids, crickets, butter flies and moths (Iwai et al., 2006; Gentile et al., 2009; Cortés et al., 2010; Danbara et al., 2010; Kobelková et al., 2015; Rodriguez-Sanchez et al., 2015; Shaikevich et al., 2016; Barberà et al., 2017). However, in these insects they exist in addition to CRY2 (insect CRY2 or mammalian type CRY) and it is not completely clear, whether and how TIM1 interacts with PER. *Tim1* knock-down studies in crickets gave evidence that indeed *tim1* seems not essential for the central feedback mechanism in crickets, because the knock-down did not destroy rhythmic behavior in the animals (Danbara et al., 2010). Following knock-down studies revealed that circadian behavior of crickets is maintained when either *cry2* or *tim* are rhythmically expressed and that there appear to exist two interconnected negative feedback loops, in which *cry1* and *cry2* apart from *per* and *tim1* are important to maintain clock function (Tokuoka et al., 2017) (see **Figure 1**). Unlike in *Drosophila*, CRY1 does not act as a blue light photoreceptor and light entrainment in the cricket relies

TABLE 1 | Different sets of clock components in insects.

Insect model	PER	CRY1	CRY2	TIM1	TIM2	CLK	CYC	PDP1	VRI	CWO	JET	PDF	References
Drosophila melanogaster	√	√	x	√	√	√	√	√	√	√	√	√	Brown et al., 2012; Tomioka and Matsumoto, 2015
Anopheles gambiae	√	√	√	√	√	√	√	√	√	√	?	√	Janssen et al., 2008; Ingram et al., 2012; Tomioka and Matsumoto, 2015
Danaus plexippus	√	√	√	√	√	√	√	√	√	√	?	√	Zhu et al., 2005, 2008; Reppert et al., 2016; Lam and Chiu, 2019
Gryllus bimaculatus	√	√	√	√	√	√	√	√	√	?	?	√	Singaravel et al., 2003; Moriyama et al., 2008, 2012; Danbara et al., 2010; Hassaneen et al., 2011; Uryu et al., 2013; Tokuoka et al., 2017; Nose et al., 2018; Narasaki-Funo et al., 2020
Acyrthosiphon pisum	√	√	√	√	√	√	√	√	√	√	x	x*	Cortés et al., 2010; Barberà et al., 2017; Lam and Chiu, 2019
Rhyparobia maderae	√	x	√	√	√	√	√	√	√	?	?	√	Petri and Stengl, 1997; Werckenthin et al., 2012, 2020
Tribolium castaneum	√	x	√	√	√	√	√	√	√	√	?	√	Yuan et al., 2007; Ingram et al., 2012; Li C-J et al., 2018; Veenstra, 2019
Apis mellifera	√	x	√	x	√	√	√	√	√	√	√	√	Bloch et al., 2003; Rubin et al., 2006; Sumiyoshi et al., 2011; Beer et al., 2018

Insect models of different orders and their clock genes. PER (PERIOD), CRY (CRYPTOCHROME) 1 and 2, TIM1 (TIMELESS) and TIM2 (TIMEOUT), CLK (CLOCK), CYC (CYCLE), PDP1 (PAR Domain Protein 1), VRI (VRILLE), CWO (CLOCKWORK ORANGE), JET (JETLAG), and the neuropeptide PDF (Pigment Dispersing Factor). Genes encoding these clock components are marked as present (√), absent (x) in genome or unknown (?).
**PDF is found in other Hemiptera (Sato et al., 2002; Závodská et al., 2003).*

purely on photoreception via the compound eyes (Komada et al., 2015; Kutaragi et al., 2018). In other species that lack *cry1* (i.e., beetles and cockroaches), *tim1* may play a different role in the circadian rhythm generation (Werckenthin et al., 2012, 2020; Li C-J et al., 2018), which stresses the complexity in evolution of divers sets of clock genes in different insects. Recently, a model which includes two different pacemakers with different clock gene sets was proposed for the cockroach to explain *per*, *cry2*, and *tim1* function in parallel (Werckenthin et al., 2020) (see **Figure 1**). The cockroach clock may work with three regulatory loops, because knock-down of neither *per*, nor *tim1* nor *cry2* alone was successful to induce arrhythmic behavior in the animals (Werckenthin et al., 2020). Interestingly, Hymenoptera are so far the only insect group that lack both, *cry1* and *tim1* (only *cry2* and *tim2* are present; see also section "***The relevance of Zeitgebers differs between flies and bees***"), and they display a clock gene set that is more similar to the mammalian clock (Rubin et al., 2006; Sandrelli et al., 2008). In the following, we will describe the principal transcriptional/translational feedback loops that lead to circadian oscillations with a focus on Diptera and Hymenoptera (see for more insight: Hardin, 2011; Brown et al., 2012; Özkaya and Rosato, 2012; Hardin and Panda, 2013; Helfrich-Förster, 2017; Top and Young, 2018).

The clock genes *per* and *tim1*/*cry2* and their respective protein products participate in a first negative feedback loop, in which the proteins inhibit the transcription of their own genes. This involves two further clock genes, *cycle* (*cyc*), and *Clock* (*Clk*), and their respective products CYC and CLK. CLK and CYC form heterodimers and bind to E-box regulatory elements in the promoters of *per* and *tim1*/*cry2*, activating their transcription. Consequently, *per* and *tim1*/*cry2* mRNA levels rise and are translated in the cytoplasm, where their products PER and TIM1/CRY2 are subjected to posttranslational modification, dimerize, and after a while enter the nucleus as a complex. In the nucleus, PER-TIM1 or PER-CRY2 complexes bind to CLK-CYC and repress their transcriptional activity. Doing so, they negatively regulate their own expression with a time delay. This delay is provoked by the posttranslational modifications of PER and TIM1/CRY2 and it is essential for provoking stable circadian oscillations. Subsequent PER and TIM1/CRY2 destabilization and degradation stops the repression on CLK-CYC activity, and a new transcriptional-translational cycle restarts.

The basic negative feedback mechanism is very similar in all animals (although gene sets differ), but again there are unique features in higher flies. While CYC (also called BMAL1 in mammals) is the component that binds to the E-boxes and activates transcription of *per* and *tim1*/*cry2* in the great majority of animals (including bees), CLK is the relevant transcriptional activator in higher flies (Bae et al., 1998; Chang et al., 2003; Rubin et al., 2006; Yuan et al., 2007; Sandrelli et al., 2008; Tomioka and Matsumoto, 2015; Chahad-Ehlers, 2017) (see **Figure 1**). Most interestingly, the transcription of the clock factor that possesses the transactivation domain is controlled in a rhythmic manner through a second feedback loop, while the one without transactivation domain is not rhythmically controlled. However, cyclic *Clk* expression has been observed for example in sandflies and in jewel wasps and crickets under certain conditions, although *cyc* encodes the transactivation domain (Meireles-Filho and Kyriacou, 2013; Uryu et al., 2013; Dalla Benetta et al., 2019) (see below).

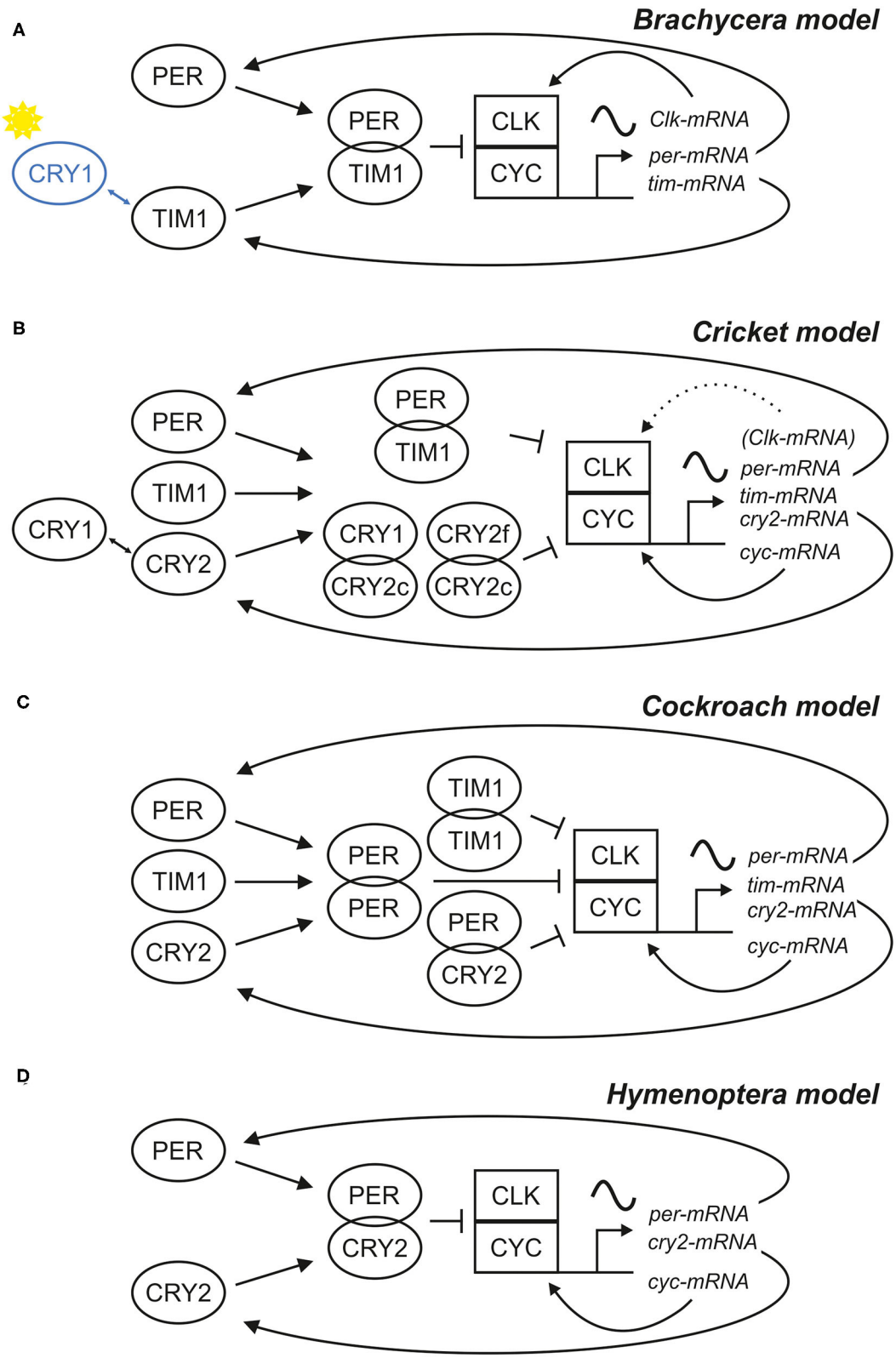

FIGURE 1 | Scheme of four different models for the central negative feedback loop(s). Models based on different clock gene sets have been described for **(A)** *Drosophila* and other **Brachycera**, **(B)** the **cricket** *Gryllus bimaculatus*, **(C)** the **cockroach** *Rhyoparobia maderae* and **(D)** the honey bee *Apis mellifera* and other

(Continued)

FIGURE 1 | Hymenoptera. Different sets of clock proteins (left side of the schemes) build different combinations of dimers, which regulate the cyclic expression of clock gene mRNAs, which in turn provide the basis of clock protein production. In all four systems, the rhythms are generated by a negative feedback of some clock proteins on their transcriptional activators CLK/CYC, but there are differences in the composition of these clock proteins and their properties. In Brachycera, CRY1 acts as a blue-light receptor and can bind TIM1 **(A)**, causing degradation of TIM1 in a light dependent manner. In contrast, the cricket relies purely on photoreception in the compound eye and CRY1 acts together with different isoforms of CRY2 (CRY2c and CRY2f) as part of a second central negative feedback loop **(B)**. In all models, except for the Brachycera model, *cyc* was expressed rhythmically and *Clk* constitutively. Nonetheless, *cyc* knock-down studies showed, that *Clk* expression cycles in the absence of CYC, which implies that *Clk* expression is rhythmically regulated in crickets and rhythms are masked in the natural state (Uryu et al., 2013). It is highly likely that more than four different insect clock models for the central negative feedback loop exist, because in sand flies for example, both *Clk* and *cyc* are rhythmically expressed (Meireles-Filho et al., 2006a,b; Meireles-Filho and Kyriacou, 2013).

THE MOLECULAR CLOCK—FURTHER FEEDBACK LOOPS

There is a second feedback loop that leads to a circadian oscillation in the abundance of CLK in flies and of CYC (BMAL1) in the other animals (Cyran et al., 2003; Meireles-Filho et al., 2006a; Rubin et al., 2006; Sandrelli et al., 2008; Bertolini et al., 2018). This second feedback loop is so far best described in *D. melanogaster*. It involves the clock genes, *cycle* (*cyc*), *Clock* (*Clk*), *vrille* (*vri*), and *PAR domain protein 1* (*pdp1*), and their respective products. *Vri* and *pdp1* carry E-box regulatory elements in their promoters, therefore their expression is also activated by the active CLK-CYC complex. VRI accumulates earlier than PDP1 and it represses the expression of *Clk*, acting at the level of VP-boxes (Emery and Reppert, 2004) present in its promoter region. PDP1 accumulates later than VRI and finally promotes *Clk* expression. The synergistic activity of VRI and PDP1 generates circadian transcription of *clk*. However, different functions of the genes *pdp1* and *vri* of this second feedback loop are still largely undiscovered, because null-mutation studies are limited by the fact that the null-mutants exhibit developmental lethality. Besides their role in development of the fly, *pdp1* and *vri* were proposed to regulate output function of the clock downstream of the central oscillator, because changes in levels of PDP1 and the loss of *vri* in clock cells (*tim*- and *pdf*-expressing cells) caused arrhythmic behavior but did not affect core clock function (Benito et al., 2007; Gunawardhana and Hardin, 2017). The inhibition of the activity of a specific isoform of PDP1 (PDP1ε) and missing *vri* expression in clock cells furthermore displayed a role in regulation of clock neuron morphology and neuropeptide accumulation (Lim et al., 2007a; Gunawardhana and Hardin, 2017). In contrast, Zheng et al. (2009) confirmed *Clk* activation by PDP1ε and suggested that *pdp1* functions in both, core clock and behavioral output pathways.

As already mentioned, in Hymenoptera, *cyc* and not *Clk* appears to be rhythmically controlled, but exact mechanisms remain to be elucidated (Rubin et al., 2006; Ingram et al., 2012; Sadd et al., 2015). For example, in the jewel wasp, *Nasonia vitripennis*, a rhythmic control of *Clk* expression was found additionally to that of *cyc* in one study (Dalla Benetta et al., 2019) and only in *cyc* in another study (Davies and Tauber, 2016). Since *Clk* expression was only cycling in a *Nasonia* strain from Northern regions and only under long day conditions, but cycling was lacking in Southern species, Dalla Benetta et al. (2019) concluded that this may be due to an adaptation mechanism in the clock to photoperiods at higher latitudes. Overall expression levels of Clk and cyc were lower in northern *Nasonia* species (Dalla Benetta et al., 2019). Interestingly, there are parallels to the *cyc* knock-down studies in crickets, which also showed cycling in *Clk* expression when *cyc* levels are diminished (Uryu et al., 2013). This may point to a general mechanism of the circadian clock that promotes rhythmic *Clk* expression in case of low CYC levels.

With *cry1* and *tim1* lacking in the Hymenoptera clock, it may be that another transcription regulator, clockwork *orange* (*cwo*), plays a rather important role for core clock function, which is not yet understood (Ingram et al., 2012; Rodriguez-Zas et al., 2012). In *Drosophila*, *cwo* participates in a third feedback loop that influences CLK-CYC mediated transcription and regulates the amplitude of circadian oscillations in other clock genes (Kadener et al., 2007; Lim et al., 2007b; Matsumoto et al., 2007; Richier et al., 2008). The CWO protein promotes the PER-dependent removal of CLK-CYC complexes from E-boxes, which may be achieved by a binding competition between CWO and CLK-CYC-PER on E-boxes (Zhou et al., 2016). A CWO protein domain that is highly conserved amongst insects but different in the mammalian CWO orthologs DEC1 and DEC2 indicates that *cwo* function may be similar in all insect clocks (Ingram et al., 2012).

There may be more clock regulation factors in the fruit fly, which are still unknown. For example, only recently, a function in circadian clock regulation was postulated for the nuclear receptors ecdysone induced protein 75 (E75) and Unfulfilled (UNF), which may be conserved among different animals (Kumar et al., 2014; Jaumouillé et al., 2015).

The great advantage of *D. melanogaster* as a model in chronobiology is its well-described molecular mechanisms of the circadian clock and these mechanisms start to emerge also in other Diptera (Codd et al., 2007; Gentile et al., 2009; Rund et al., 2011; Meireles-Filho and Kyriacou, 2013; Kyriacou, 2014a; Gesto et al., 2015; Meuti et al., 2015; Kaiser et al., 2016; Bazalova and Dolezel, 2017; Bertolini et al., 2018; Noreen et al., 2018; Rivas et al., 2018). On the other hand, it is not possible to transfer all clock functions to other insect models as we illustrated above. Therefore, studies on various different insect clocks are needed to elucidate the role of the clock in complex behaviors, as we find it in other insects such as Hymenopteran species. In the following section, we will focus on the neuronal network of *D. melanogaster* and *A. mellifera* and show basic similarities and differences in these insect clock networks.

THE CIRCADIAN CLOCK NETWORK OF FRUIT FLIES AND HONEY BEES

In *D. melanogaster,* the central clock is located in dorsal and lateral neurons that express the core clock genes and form

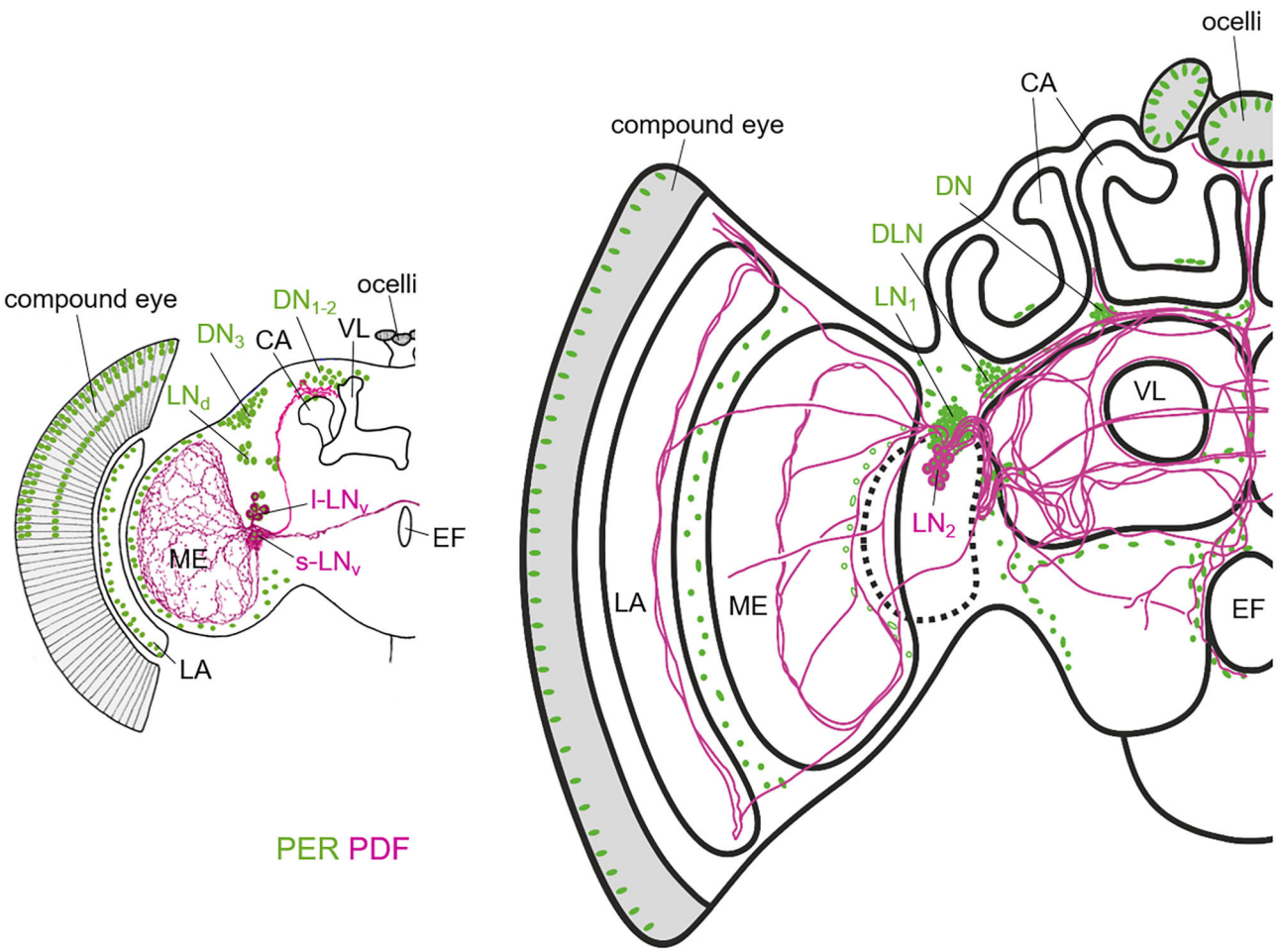

FIGURE 2 | Schematic representation of the PER and PDF expressing neurons in the fruit fly **(left)** and honey bee brain **(right)**. The two brains differ in overall size, but especially in the size of the mushroom bodies of which the Calyces (CA) and the vertical lobes (VL) are highlighted. The vertical lobe is also called α-lobe in *Drosophila*. While the honey bee has two large calyces per brain hemisphere, the fruit fly has only one rather small one. In *Drosophila*, the only PDF-fibers that run into the dorsal brain, terminate anterior, and slightly dorsally of the CA. In the honey bee, the PDF fibers terminate anterior and ventrally of the CAs and they run into many more central brain areas than in *Drosophila*. PER is not only present in the lateral (LN) and dorsal neurons, but also in the photoreceptor cells of the compound eyes and ocelli and in numerous glia cells that are only indicated in the lamina (LA) and the distal medulla (ME) in *Drosophila* and in few other brain regions in the honey bee. Note that the nuclei of the photoreceptor cells are only shown in the dorsal half of the compound eye in *Drosophila*. The honey bee scheme is modified from Beer et al. (2018). For details see text.

an extensive neuropeptidergic network in the brain (**Figure 2**). In addition, the clock ticks in many glia cells (Zerr et al., 1990). The clock neurons are traditionally divided into seven groups–three dorsal ones (DN_{1-3}) in the dorsal brain, three lateral ones (LN_d, l-LN_v, and s-LN_v) in the anterior lateral brain, and one additional lateral group in the posterior brain that is called LPN [reviewed in Helfrich-Förster et al. (2007a), Hermann-Luibl and Helfrich-Förster (2015), Helfrich-Förster (2017), Schubert et al. (2018)]. The clock neurons have two main projection targets: (1) the accessory medulla (aMe), a small neuropil situated between the central brain and the optic lobes and that had been identified as pacemaker center in many insect species and (2) the dorsal brain that houses the hormonal center (pars intercerebralis (PI) of insects and also has connections to most brain areas. The clock neurons form a well-defined fiber network in these two brain areas, putatively allowing considerable crosstalk between them. The aMe is not only invaded by the clock neurons but also by aminergic, glutaminergic, acetylcholinergic, and Glycin- and GABA-ergic inputs from non-clock neurons [reviewed in Helfrich-Förster (2017), Top and Young (2018)]. This emphasizes the role of the aMe in intercellular communication—both among clock neurons and between extrinsic cells and clock neurons. Furthermore, the aMe appears to get light information from the eyes and from the Hofbauer-Buchner eyelets (H-B eyelets), small extra retinal eyelets that are located beneath the compound eyes and are remnants of the larval stemmata [reviewed in Helfrich-Förster (2020)]. The aMe can be regarded as a hub to channel retinal and extra retinal inputs to the central circadian clock entraining it to the periodic environmental cycles (Li M-T et al., 2018). In the

dorsal brain, the clock neurons' fibers terminate close to regions that have been shown to be involved in the control of locomotion, sleep, and metabolism, such as the PI, the mushroom bodies, and the central complex (see below under "***Behavior controlled by the circadian clock of fruit flies and honey bees***").

One of the best conserved and most important neuropeptides in the insect circadian clock is the Pigment-Dispersing Factor (PDF) (Renn et al., 1999; Helfrich-Förster et al., 2000; Helfrich-Förster, 2014; Ikeno et al., 2014; Shafer and Yao, 2014; Wei et al., 2014; Beer et al., 2018). In *D. melanogaster*, PDF is expressed in four small ventro-lateral neurons (s-LN_v) and in four large ventro-lateral neurons (l-LN_v), which have different roles in the clock network. While the l-LN_v are dispensable for rhythmic activity, they signal to the s-LN_v (Klose et al., 2016; Schlichting et al., 2016; Menegazzi et al., 2017) and they are part of the light-input pathway to the clock (Helfrich-Förster, 2020).

The s-LN_v are major pacemaker neurons that are essential for robust rhythmic activity under constant darkness. Although the s-LN_v projections that terminate in the dorsolateral protocerebrum anteriorly of the mushroom body calyces look relatively simple, they appear to signal to different neuropils and downstream neurons via paracrine secretion of PDF. The s-LN_v terminals broaden and PDF accumulates in them in the morning (Park et al., 2000; Fernández et al., 2008) suggesting that it is also released at this time into the dorsolateral brain. PDF-receptors are for example on the ellipsoid body of the central complex (Pírez et al., 2013), which is a higher coordination center in the insect brain responsible for motor control and orientation and recently the ellipsoid body has been established as important output circuit downstream of the circadian clock neurons (Liang et al., 2019) (see also below).

However, PDF is not only a putative output factor of the clock but also the most important communication factor within the clock (Lin et al., 2004; Yoshii et al., 2009a; Helfrich-Förster, 2014; Shafer and Yao, 2014). The PDF receptor is expressed on many clock neurons, including the PDF-positive s-LN_v (Shafer et al., 2008; Im and Taghert, 2010; Choi et al., 2012; Klose et al., 2016). PDF is able to couple the molecular oscillations of individual clock neurons by speeding them up or slowing them down (Lin et al., 2004; Yoshii et al., 2009a). Thus, PDF is the most powerful neuropeptide in the clock network, leading to the hypothesis that the PDF neurons are dominant circadian pacemakers governing the other clock neurons by setting phase and period of their molecular clocks and shaping the activity pattern of the flies (Stoleru et al., 2005; Guo et al., 2014; Chatterjee et al., 2018).

In comparison to the fly, the honey bee brain possesses many more clock neurons (∼400 compared to 150 in the fly brain), which are nevertheless clustered similarly like in the fly brain (Fuchikawa et al., 2017; Beer et al., 2018) (**Figure 2**). There are two rather dorsally located clusters, the DN (dorsal neurons) and the DLN (dorsolateral neurons), and two clusters (LN_1, LN_2) in the lateral brain between the protocerebrum and the optic lobe. The cell bodies of the LN_2 neurons (∼15 per hemisphere) are very closely located to the LN_1, but are a little bit bigger and produce PDF (Fuchikawa et al., 2017; Beer et al., 2018). Like in *Drosophila*, the PDF neurons seem to take on a communication function between different clock cells and brain regions for control of downstream behavior, because injections of artificial PDF peptide have been successful to shift the locomotion rhythms in bees (Beer et al., 2018). The PDF neurons build a highly complex network of arborizations widespread throughout the honey bee brain. Like in other Hymenoptera, classification of PDF neurons in the honey bee into different functional groups, has not been possible so far (Bloch et al., 2003; Weiss et al., 2009; Sumiyoshi et al., 2011; Fuchikawa et al., 2017; Beer et al., 2018; Kay et al., 2018). Nevertheless, a functional subdivision of neurons projecting into different brain areas, like it is the case in *Drosophila* or cockroaches (Reischig et al., 2004; Helfrich-Förster et al., 2007b), is highly likely. Similar to other insects, the PDF neurons in the honey bee brain project into an highly dense fiber hub in the lateral brain close to the optic lobe (Homberg et al., 1991; Helfrich-Förster et al., 1998; Závodská et al., 2003). This "communication center" of the circadian clock may be analog to the aMe in *Drosophila* and other insects, with a small difference in location: it seems rather less associated with the Medulla than with the Lobula (Beer et al., 2018). This was suggested to be related to the fact that Hymenoptera have no stemmata, which are the precursor of the HB-eyelet in *Drosophila* development, and the developing honey bee clock may be consequently less associated with photic inputs (Beer et al., 2018).

Additionally to the clock neurons, numerous glia cells expressing PER (which were similarly observed in *Drosophila* (Siwicki et al., 1988; Helfrich-Förster, 1995) in various brain areas are closely connected via the PDF neuronal network (Fuchikawa et al., 2017; Beer et al., 2018). This fact and evidence from different *per* expression studies in nursing and foraging bees indicates a crucial role of glia cells in the circadian plasticity of the honey bee clock as we will explain later [see section "***Behavior controlled by the circadian clock of fruit flies and honey bees***" and review (Beer and Bloch, 2020)].

THE RELEVANCE OF ZEITGEBERS DIFFERS BETWEEN FLIES AND BEES

Circadian clocks have to be synchronized to the 24-h day of the earth by Zeitgebers. The most reliable Zeitgebers are the daily light-dark (LD) and temperature cycles, but also social interactions, periodic vibration signals and the availability of food can serve as Zeitgeber. For most adult insects, light is the most important Zeitgeber, which is followed by temperature and social interactions, while the impact of food is only studied in some insects (see below).

The effectiveness of Zeitgebers is very different for developing insects that receive no light input at all, because they nest in cavities or mature underground, such as onion flies (Watari and Tanaka, 2010; Miyazaki et al., 2016), tsetse flies (Žďárek and Denlinger, 1995) and solitary bees (Tweedy and Stephen, 1970; Yocum et al., 2016; Bennett et al., 2018; Beer et al., 2019). For these insects the daily temperature cycle is the most important Zeitgeber for emerging rhythmically from their pupal case and at least solitary bees do not entrain to LD cycles when they are present (Tweedy and Stephen, 1970; Beer et al., 2019). Even fruit flies, which can perceive light through their

pupal case and which are nicely entrainable by LD cycles in the lab are very sensitive to temperature cycles (Zimmerman et al., 1968), and under natural conditions, the daily phase of eclosion and the robustness of rhythmicity depends strongly on the environmental temperature conditions (Ruf et al., 2019). This indicates that temperature cycles may be generally more important for entraining the endogenous clock of developing insects than they are for entraining the clock of adult insects. During development the sensitivity to light-dark cycles appears to increase (Watari, 2005; Beer et al., 2019). In some flies, this switch to light sensitivity of the clock may occur earlier than in onion flies or solitary bees [see discussion in Beer et al. (2019)].

Furthermore, although adult insects are all very sensitive to LD cycles, for social insects, such as honey bees, social Zeitgebers can be more important than light (Beer et al., 2016; Fuchikawa et al., 2016). Thereby, different members of a colony can serve as social Zeitgebers: a group of foraging/forager-aged bees or even the single queen was shown to determine the colony rhythm in honey bees (Moritz and Sakofski, 1991; Frisch and Koeniger, 1994; Beer et al., 2016; Fuchikawa et al., 2016). Laboratory experiments with small groups of worker bees showed that the relative number of bees is important for social synchronization and that individual bees preferably adapt the rhythm of larger groups (Moritz and Kryger, 1994). The cues by which social synchronization is mediated between individual honey bees is so far unknown. It appears that pheromones, micro-climate and vibration signals could play a role, while direct tactile and visual contact between con-specifics could be excluded (Eban-Rothschild et al., 2012; Beer et al., 2016; Fuchikawa et al., 2016). Similar factors appear to influence the rhythms of flies. Although, flies are not classified as social, they form groups, interact with each other, adjusting their interactive behavior to group size (Rooke et al., 2020) and their clocks can be entrained by pheromones (Levine et al., 2002; Krupp et al., 2008) and vibrations (Simoni et al., 2014). Clearly, in flies, social synchronization has not the same significance as it has in bees, but studying it might help to unravel the underlying mechanisms.

In the following, we will give an overview about the effectiveness of light and temperature cycles as Zeitgebers in adult flies and bees (**Figure 3**). For a detailed insight into light-input pathways to the circadian clock of adult insects with an emphasis on the fruit fly the reader is referred to a recent review (Helfrich-Förster, 2020).

Adult fruit flies entrain to LD and temperature cycles [reviewed in Helfrich-Förster (2017)]. When both Zeitgebers are combined in a natural-like way with the highest temperature occurring after noon, locomotion rhythms are very precise and the molecular clock cycles with high amplitude in all clock neurons (Currie et al., 2009; Yoshii et al., 2009b). When LD and temperature cycles are completely out of phase with each other, wild-type flies entrain to the LD cycles, while mutants without CRY1 entrain to the temperature cycles, suggesting that light is the predominant Zeitgeber and light entrainment is mediated via CRY1 (Yoshii et al., 2010). However, this conclusion is slightly premature. When the two Zeitgeber cycles are <4 h out of phase, the temperature cycles strongly influence the phase of the activity rhythms, meaning that light and temperature interact in the entrainment of wild-type flies and that light only dominates when the two Zeitgebers are too much out of phase (Harper et al., 2016). That the compound eyes and not only CRY1 contribute to light entrainment under conflicting Zeitgeber cycles was shown by Busza et al. (2007). The compound eyes together with CRY1 actively suppress temperature input to the clock neurons in wild-type flies and by this way make sure that the flies are not too sensitive to sudden temperature fluctuations that can easily happen in nature (Busza et al., 2007; Gentile et al., 2013). Most interestingly, different clock neurons are responsible for mediating light- and temperature entrainment in fruit flies: the Dorsal Neurons are more important for temperature entrainment than the Lateral Neurons, which mediate predominantly light entrainment [reviewed in Helfrich-Förster (2017), Yadlapalli et al. (2018), Lamaze and Stanewsky (2020)].

The light-input pathway to the circadian clock in honey bees is less well-studied. Honey bees miss in comparison to the fruit fly the blue light receptor CRY1 and the HB-eyelet. Similar to CRY1-missing *Drosophila* mutants, the honey bee clock may be less susceptible to light than other environmental cues (and at least for social cues this theory seems to hold true). With *tim1* absent in the molecular clock of honey bees, the light input pathway in bees may utilize another mechanism. *Tim2* (*timeout*) was identified as part of a photo-entrainment mechanism in *Drosophila* (Benna et al., 2010), which only plays an residual role in photoreception compared to *tim1* in *Drosophila*. The relevance of the mammalian ortholog of *tim2* for the circadian system is highly debated until today (Gotter et al., 2000; Barnes, 2003; Gotter, 2006). However, with *cry1* missing, a *tim2* mediated photoreception in the compound eyes may be the major photo-entrainment pathway in Hymenoptera (Benna et al., 2010). Furthermore, a vertebrate-like opsin called pteropsin, may be part of the light-input pathway to the clock in honey bees besides the compound eyes and the ocelli (Velarde et al., 2005). The expression pattern of pteropsin strongly resembles the location of PER expressing clock cells (Fuchikawa et al., 2017; Beer et al., 2018).

BEHAVIOR CONTROLLED BY THE CIRCADIAN CLOCK OF FRUIT FLIES AND HONEY BEES

Basic circadian clock output behavior is similar between fruit flies and honey bees. However, honey bees have an extraordinary rich behavioral repertoire and, due to their age- and caste-dependent differences in behavior, they are perfect models to study circadian clock development and plasticity as well as socially regulated clock output. Therefore, we will first review the general rhythmic behavior of flies and bees and then concentrate on honey bee behavior.

OUTPUT RHYTHMS IN FLIES AND BEES

The best studied daily rhythms in flies and bees are those of locomotion and of sleeping/waking [reviewed for flies by Dubowy and Sehgal (2017), King and Sehgal (2020), and for bees

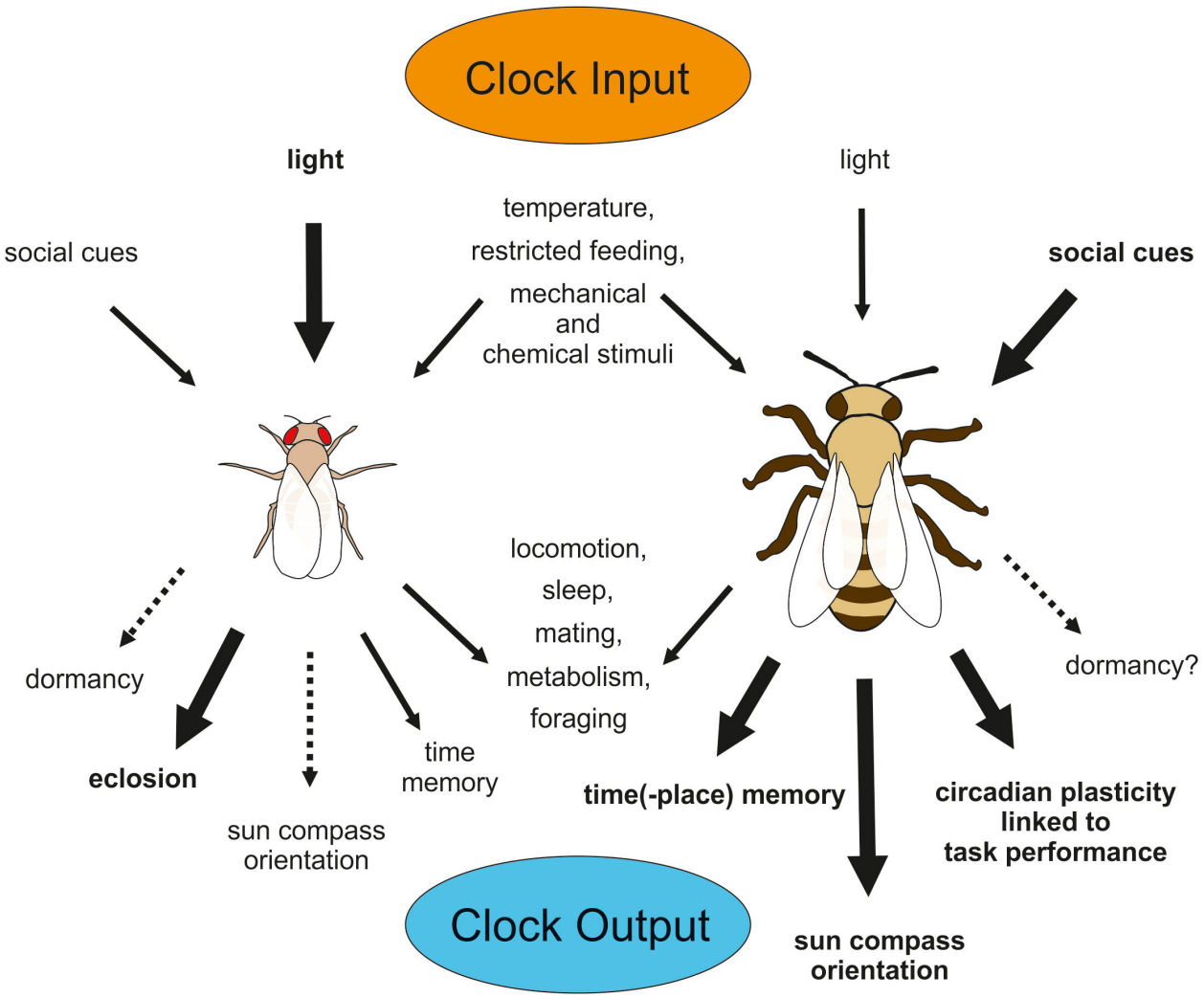

FIGURE 3 | Input and Output of the circadian clock in *D. melanogaster* **(left)** and *A. mellifera* **(right)**. Relevance of input signals to the clocks differs between species, so that light appears to be most important for the fly clock, but social signals out rule light signals in entraining the bee clock. Many output behaviors are common among different insects (e.g., locomotion, sleep, mating, metabolism, and foraging), but others are limited to or more pronounced in some species (e.g., eclosion in *Drosophila* and time-place-memory, sun compass orientation or task-related plasticity in circadian behavior in *Apis*). Although *D. melanogaster* does not exhibit pronounced diapause, basic mechanisms in clock regulation involved in dormancy appear to be shared between this fly and diapausing fly species. Similarly, *A. mellifera* does not go into diapause, but it may be that the honey bee exhibits some residual photoperiodic function in initiating dormancy behavior, because solitary bees display pronounced diapause.

by Moore (2001), Eban-Rothschild and Bloch (2012)]. The honey bee has been found to be a very good model for sleep, because of its detailed description of sleep architecture (Kaiser, 1988; Sauer et al., 2003; Klein et al., 2014), while the fruit fly has been very helpful to unravel the underlying molecular and recently also neuronal mechanism of sleep [reviewed in Helfrich-Förster (2017), Guo et al. (2018)]. In both insects, disturbances of the sleep-wake-rhythm result in reduced learning ability (Hussaini et al., 2009; Toda et al., 2018; Donlea, 2019) and in honey bees additionally in reduced communication ability, which is very similar in humans (Klein et al., 2010), suggesting that sleep is essential to maintain neuronal plasticity, learning and memory in all animals.

The daily rhythm in movement (locomotion), which is best studied in all insects investigated so far, can serve different purposes. Insects may be active for foraging, for seeking mates, for nesting/brood care activity or just because their circadian clock dictates them to be active. In most laboratory systems that record movements of insects, it is impossible to distinguish between these different possibilities. Here, natural studies with honey bee foragers are of great advantage. Indeed, their foraging rhythms are the first behavioral rhythms described in honey bees (Beling, 1929; Wahl, 1933; Kleber, 1935; Frisch and Aschoff, 1987; Moore et al., 1989). These studies showed that honey bees forage throughout the day depending on the available food sources and that they have an excellent memory about time

and location of open flowers. When trained in restricted feeding cycles, honey bee foragers can remember up to nine time-points per day (Koltermann, 1971). Although, entrainment via feeding has been deployed in many studies, the link between the clock and time-place-learning in foraging behavior of bees is largely unknown (Pahl et al., 2007; Moore and Doherty, 2009; Mulder et al., 2013). Only recently, it was shown that restricted feeding indeed can phase shift the molecular clock of honey bees (Jain and Brockmann, 2018).

When recorded in isolation and under controlled conditions in the lab, honey bee foragers show activity throughout the day and few activity during the night (Moore and Rankin, 1985). Fruit flies have a completely different activity pattern under such conditions. They exhibit bimodal activity rhythms with activity bouts in the morning and evening and a pronounced siesta in between, and this is true for both sexes (Helfrich-Förster, 2000). Nevertheless, there are differences between the sexes. Mated females show a greatly reduced siesta, probably because they search for places for depositing their eggs. Oviposition occurs rhythmically in female flies starting in the middle of the day and reaching a maximum in the evening (McCabe and Birley, 1998; Sheeba et al., 2001; Manjunatha et al., 2008), which fits to the high activity of isolated mated females during this time. In contrast, isolated males begin activity significantly earlier in the morning than females, which can be explained by a search for female mating partners (Helfrich-Förster, 2000). Indeed, male sex drive behavior has been shown to be controlled by the circadian clock (Fujii et al., 2007, 2017; Fujii and Amrein, 2010) and mating occurs rhythmically with a maximum in the early morning hours (Sakai and Ishida, 2001; Lin et al., 2014). Nevertheless, sex drive is influenced by the presence of females and is not generally restricted to the morning: male flies that are housed together with females become highly active throughout the night and early morning (Fujii et al., 2007). Similar to activity, feeding occurs at slightly different times of the day in male and female flies. While males feed maximally in the early morning, females do so from the middle of the day until the evening (Seay and Thummel, 2011; Xu et al., 2011; Liu et al., 2020; Schäbler et al., 2020). In summary, general activity, mating and feeding occur at different times of the day and additionally show sex differences in timing. Thus, these three rhythms may be controlled by different neuronal pathways from the clock neurons to the effector organs.

Unlike in the honey bee, no studies under real natural conditions have been performed in fruit flies and only few studies have addressed fly behavior under quasi natural conditions (Vanin et al., 2012; Green et al., 2015). Thus, we don't know yet, which activities are performed by fruit flies in nature and when they occur naturally. Nevertheless, many more rhythms are known from fruit flies studied under laboratory conditions, of which some have been also found in honey bees, while others appear absent in honey bees. For example, fruit flies do not only lay their eggs in a rhythmical manner, but they also eclose rhythmically from their pupal case, with a peak of eclosion in the subjective morning (Pittendrigh, 1954; Lin et al., 2014). Similar rhythms in the queen's oviposition or rhythms in emergence of newly eclosed honey bees could not be detected in honey bees (Free et al., 1992; Harano et al., 2007; Johnson et al., 2010). Most probably, such rhythms have no selective advantage in the protected beehive (but they do so in solitary bees, see above). Nevertheless, mating between drones and queens happens in a rhythmic manner as it does in fruit flies. Mating of bees always occurs in the afternoon (Lensky and Demter, 1985), while the exact timing of mating flights can be altered by selective forces (e.g., the presence of sympatric species within the same location). Generally, honey bees strongly avoid an overlap in flight times between sympatric species, either to avoid interspecific hybrids or a reduction in the efficiency of mating (Koeniger and Koeniger, 2000). We expect that such selective forces will also alter the timing of the different rhythms in fruit flies under natural conditions.

As do bees, flies can also remember the time of day, at least to some degree (Chouhan et al., 2015). Chouhan et al. (2015) showed that flies can remember two time points per day, as long as the two are at least 6 h apart. This memory depended on a functional circadian clock and did neither persist in the absence of the PER protein nor in the absence of the neuropeptide PDF. Time memory in honey bees is much more sophisticated than in flies, but it may relay on the same connections of the circadian clock neurons with the centers of memory, the mushroom bodies (see below). Furthermore, in flies and bees, the ability to learn is modulated by the circadian clock and different at different times of day (Sakai et al., 2004; Lehmann et al., 2011). Again, this requires a functional relationship between the clock neurons and the mushroom bodies.

SUN COMPASS ORIENTATION

Honey bees and other insects are famous for their remarkable spatial orientation, which relies on a time-compensated sun-compass (Lindauer, 1960). Because of the earth's rotation the relative position of the sun changes during the day and the bee has to compensate for the past time during flight. In several studies, in which the honey bee clock was phase shifted, it was shown that the circadian clock is essential for sun-compass orientation (Medugorac and Lindauer, 1967; Cheeseman et al., 2012, Cheeseman et al., 2014). Recently, putative input neurons of the clock to the sky compass orientation pathway in the honey bee brain have been identified (Zeller et al., 2015; Beer et al., 2018). Transmedulla neurons of the sky compass pathway originating at the dorsal rim area of the medulla run in close proximity to PDF neurons (Zeller et al., 2015). PDF neurons, on the other hand may communicate with the central complex, which generally controls orientation and navigation in insects (e.g., Pegel et al., 2019).

Also flies can fly straight over long distances, indicating that they can perceive celestial cues and might even be able to compensate for the movements of the sun although this is not yet proven (Giraldo et al., 2018; Mathejczyk and Wernet, 2019; Warren et al., 2019). If true, research on flies may help to decipher the neuronal pathway from polarization vision to the circadian clock and from there to the brain centers controlling orientation such as the central complex. In any case, good

interdisciplinary communication between fly and bee researchers will strongly support the successful elucidation of sun-compass orientation mechanisms.

TASK RELATED PLASTICITY OF THE CLOCK IN HONEY BEES

Honey bees have an age-related division of labor displayed by worker bees. At the age of 2–10 days bees assume brood care (nursing) functions in the colony, later they take over other duties such as storing and processing food or guarding the hive and around 3 weeks of age they begin to forage pollen or nectar and are now called foragers (Free, 1965). This division of labor is associated with pronounced changes in rhythmic behavior (Crailsheim et al., 1996; Moore et al., 1998; Bloch and Robinson, 2001). Typically, young bees tend the brood without a rhythm in locomotion, which is supposed to be beneficial for optimizing brood care and colony growth. They also display more and less pronounced sleep-bouts scattered over the day (Eban-Rothschild and Bloch, 2008; Klein et al., 2008). Older foraging bees on the other hand, display robust day-night rhythms of activity and sleep. This behavior is highly plastic and bees can start prematurely rhythmic foraging or revert back to nursing without rhythms, all according to the need of the colony (Bloch and Robinson, 2001). The cues establishing arrhythmic behavior in bees are largely unknown, but contact to the brood is essential (Nagari and Bloch, 2012; Nagari et al., 2017a). The plasticity in this behavior is further demonstrated by the fact that nurse bees displayed rhythmic behavior (locomotion) shortly after removing them from the colony (Shemesh et al., 2007, 2010; Eban-Rothschild et al., 2012; Fuchikawa et al., 2016; Nagari et al., 2017b). Moreover, molecular studies were in line with the behavioral experiments and showed attenuation of the circadian rhythm in mRNA levels of the clock genes *per*, *cry2*, *cyc,* and *cwo* in nurses compared to foragers (Bloch et al., 2001; Shemesh et al., 2007, Shemesh et al., 2010; Rodriguez-Zas et al., 2012). This implies a major reorganization of the circadian clock system. But does the clock of nurse bees actually stop? It was puzzling to see that the clock of nurses drove activity rhythms in the laboratory that were in phase with the ambient day-night rhythm of the colony, they had been removed from. Furthermore, PER protein levels were cycling in brains of behaviorally arrhythmic nurse bees (Fuchikawa et al., 2017). That means that the clock in nurse bees keeps ticking even when they are behaviorally arrhythmic! Still, why is it that mRNA oscillation of clock genes is attenuated in comparison to forager bees? As we have already mentioned, the numerous PER expressing glia cells may play an essential role in the honey bee clock. In *Drosophila* and mammals, it was demonstrated that non-neuronal cells are part of the circadian clock and contribute to formation of rhythmic behavior (Ng et al., 2011; Jackson et al., 2015; Brancaccio et al., 2017). Similarly, the PER expressing glia cells could be involved in circadian plasticity in honey bees. A differential coupling of clock cells in nurses and foragers may also be possible in the highly complex circadian clock network of honey bees. Future studies may help to elucidate the regulation mechanism of circadian plasticity in honey bees.

Interestingly, also queens display circadian plasticity in their behavior. In the colony, they were observed to be behaviorally arrhythmic while laying eggs (Free et al., 1992; Johnson et al., 2010). However, when isolated in the laboratory, they show rhythms in locomotion in phase with the ambient day-night cycle (Harano et al., 2007).

NEURONAL OUTPUT PATHWAYS FROM THE CLOCK TO DOWNSTREAM NEURONS

Although circadian clock output behaviors have been studied in numerous insects, the output pathways from the circadian clock in the brain to rhythmic behavior have been revealed only lately and in many cases are not well-understood yet. Here, again *Drosophila* with its manifold genetic tools has been the pioneer model. In the following, we will briefly review the different possible neuronal pathways from the clock to downstream neurons in the brain that may in turn communicate with the body.

Starting with development and eclosion from the pupal case, Selcho et al. (2017) showed that the s-LN_v transfer timing information via the neuropeptide sNPF (small neuropeptide F), which is co-produced with PDF, to neurosecretory cells in the dorso-lateral brain that produce the neuropeptide PTTH (Prothoracotropic hormone). PTTH then forwards time information to the prothoracic gland, which secretes the steroid molting hormone, ecdysone. The titer of ecdysone gates subsequent eclosion. The mating rhythm of adult flies appears to be correlated with pheromone release from the oenocytes and the latter is coupled to the circadian clock in the brain via PDF (Krupp et al., 2013). The precise pathways of this regulation are however not yet known.

Sleep is controlled by the central complex (besides other brain areas) and a connection from the clock neurons to the ellipsoid body of the central complex has been identified by Guo et al. (2018), Lamaze et al. (2018), and Lamaze et al. (2018) [nicely summarized by Hsu and Sehgal (2018)]. These authors show that specific DN_1 clock neurons that get input from the s-LN_v contact so-called tubercular-bulbar (TuBu) neurons that in turn are connected to ellipsoid body ring neurons that promote sleep. Most importantly, these ellipsoid body ring neurons are different from those involved in spatial orientation (see above). They are also different from ring neurons that have been recently shown to be implicated in the control of the flies' bimodal activity in the morning and evening (Liang et al., 2019). Thus, there are several parallel pathways ending in specific neurons of the ellipsoid body. Liang et al. (2019) demonstrated that other specific ring neurons of the ellipsoid body display spontaneous morning and evening neural activity peaks that coincide with the bouts of locomotion and that get inputs from circadian clock neurons that control morning and evening activity, respectively. This input is indirect and occurs via specific dopaminergic neurons that also arborize in the ellipsoid body. The s-LN_v control morning activity and PDF is able to activate the dopaminergic neurons as well as the ellipsoid body ring neurons (Liang et al., 2019). Thus, PDF may be one of the clock factors that signals directly to the ellipsoid

body [see Pírez et al. (2013) and above], while the connection from the evening clock neurons to the ellipsoid body neurons is still unknown [for more information on morning and evening clock neurons see Yoshii et al. (2012)]. Nevertheless, activity is controlled also by other parallel pathways that circumvent the ellipsoid body and run via the hormonal center, the PI in the middle dorsal brain [reviewed in King and Sehgal (2020)]. One important humoral pathway runs via six PI neurons that produce Diuretic Hormone 44 (DH44) a homolog of the mammalian stress hormone releasing factor (Cavanaugh et al., 2014). DH44 neurons receive synaptic inputs from the DN_1 clock neurons and DH44 is important for strong activity rhythms under constant darkness. However DH44 neurons are most active during mid-day (Bai et al., 2018) making it likely that they promote activity for other reasons. Possibly, they elevate activity for searching food or egg-laying places in females. In addition to the DH44 neurons, there are further neurons in the dorsal brain that are contacted by the clock neurons and contribute to shaping activity (Pírez et al., 2019).

Feeding rhythms *per se* are controlled by a different set of PI neurons, the four SIFamide positive neurons (Dreyer et al., 2019). The SIFamide neurons are also contacted by the DN_1 clock neurons and project to the subesophageal ganglion that is involved in gustatory processing and contains feeding-related motor neurons. Indeed, stimulation of the SIFamide neurons increases food intake (Martelli et al., 2017). Feeding is additionally controlled by leucokinin-positive neurons that are downstream of the s-LN_v and other clock neurons (Cavey et al., 2016) and that mediate hunger signals to promote locomotion (Zandawala et al., 2018; Yurgel et al., 2019). Finally, the PI contains 14 Insulin-like-Peptide positive neurons (IPCs) that are contacted by the DN_1 and the s-LN_v clock neurons and control circadian gene expression in the fat body (Barber et al., 2016) and general metabolism (see next chapter).

In summary, there are multiple output pathways from the circadian clock neurons that all originate in the dorsal brain (reaching from lateral to mid-central brain areas) (**Figure 4**). In the honey bee, these output pathways have not been elucidated in detail, but one can easily see in the pattern of the PDF arborizations that these neurons alone can easily establish contacts with the relevant brain areas mentioned for *Drosophila* (**Figure 2**). It will be most interesting to reveal the arborizations of the other clock neurons of the honey bee, especially with regard to the integration of the honey bee complex behaviors in the clock output network.

THE ROLE OF THE CLOCK IN PHOTOPERIODISM AND DIAPAUSE NEEDS NEW INSECT MODELS

A central question in chronobiology is how endogenous clocks changed in order to anticipate vastly different cyclical environmental conditions on earth, especially such that exist close to the poles. Organisms like *D. melanogaster*, and also *Homo sapiens*, are assumed to have developed in tropical regions that are characterized by regular 24 h cycles in irradiance and temperature that remain the same throughout the seasons. However, in northern and southern hemispheres of the earth photoperiods vary throughout the year causing the well-known seasons of spring, summer, autumn and winter. All organisms including insects have to anticipate these seasons in order to be prepared in advance for the coming spring-summer and autumn-winter. Bünning (1936) hypothesized that the main purpose of the circadian clock is to provide the necessary time reference for measuring day length so that organisms can prepare in time for the winter. A failure in such a preparation will ultimately lead to death. Similarly, a failure to predict the coming warm season will lead to the death of the offspring, since a too early or too late reproduction may result in too low temperatures and/or shortages of food. Small animals, such as insects are especially sensitive to seasonal changes and need to be well-prepared.

One strategy of avoiding adverse conditions is reproductive arrest that is also called dormancy or diapause. Dormancy is a generic term covering any state of developmental arrest, which is adaptive and usually accompanied with metabolic suppression (Koštál, 2006). As soon as the adverse environmental conditions disappear, insects can terminate dormancy. Diapause is a specific subtype of dormancy, which is a more profound, endogenously and centrally mediated interruption that routes the developmental program away from direct morphogenesis into an alternative diapause program of succession of several physiological events. The start of diapause usually precedes the advent of adverse conditions. Since temperature alone is not a reliable predictor, most organism use day length (= photoperiod) as a measure for the coming season and diapause is induced as soon as day length falls beyond a certain threshold, the critical day length (Koštál, 2006). The responses to changing photoperiods are called photoperiodic responses. This is different for the obligatory diapause, which is present in insects that complete only one generation each year (Denlinger et al., 2017). Typical examples are solitary bees or *Rhagoletis* fruit flies. Such insects do not need a mechanism to measure day length for diapause induction, because they enter diapause at a fixed developmental stage regardless of prevailing environmental cues. However, environmental cues remain essential for regulating the timing of diapause termination, because the mechanism for terminating diapause at the appropriate time dictates the active window of the insect's life.

Tropical insects are not exposed to seasonal differences in photoperiod and don't have to undergo winter diapause. Thus, they have no need to measure day length. This does not mean that they don't undergo dormancy or even diapause, just other cues such as temperature, moisture, and changes in food quality dictate the induction of dormancy (Denlinger, 1986). Our model organisms, the honey bee, *Apis mellifera*, and the fruit fly, *D. melanogaster,* stem from tropical regions. Honey bees live additionally in hives and can actively produce heat; both protect them from the coldness of winter. Therefore, both species do not exhibit a photoperiodically induced diapause. Nevertheless, they undergo a state of dormancy in response to low temperatures and shortage of available food that can be enhanced by shortening day length (Kefuss, 1978; Saunders et al., 1989). In contrast to real diapause this state does not include a succession of defined

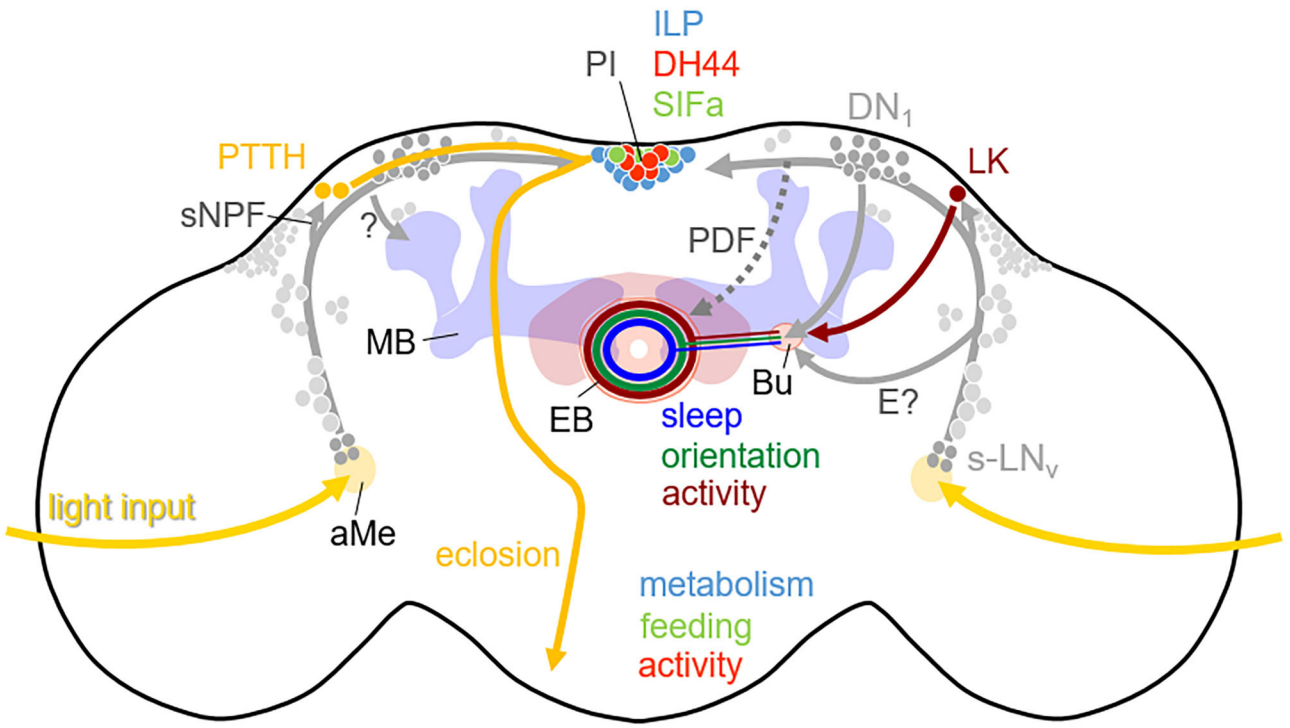

FIGURE 4 | Simplified scheme showing the output pathways of the clock neurons in the brain of *D. melanogaster*. The somata of the clock neurons in the lateral and dorsal brain are depicted in gray with the s-LN_v and DN_1 highlighted. The clock neurons are connected with each other and most of them have neurites in the accessory medulla (aMe), where they receive major light-input, as well as in the dorsal brain where they connect different neuropeptidergic neurons. The prothoracotrophic hormone (PTTH) positive neurons receive rhythmic signals via sNPF from the s-LN_v and control the timing of eclosion by triggering the release of ecdysone from the prothoracotropic glands (shown in the left brain hemisphere). The leukokine (LK) positive neurons receive also rhythmic signals from the s-LNv and perhaps from other lateral neurons and contact via tubercular-bulbar neurons (not shown) the bulb (Bu) where ring neurons of the ellipsoid body (EB) have their dendrites (shown in the right brain hemisphere). The corresponding EB ring neurons contribute to the rhythmic control of activity. In addition, the morning neurons (s-LN_v) and evening neurons (not highlighted) signal via PDF (stippled) and unknown pathways (E?) to dopaminergic neurons (not shown) that arborize in the Bu and the EB and contact putatively the same activity promoting ring neurons in the EB in order to stimulate morning and evening activity. Other ring neurons in the EB receive rhythmic signals from the DN_1 and control sleep, while still others may get rhythmic input from the clock neurons via still unknown pathways and control orientation. Furthermore, activity, feeding and metabolism are controlled by diuretic hormone 44 (DH44), SIFamide (SIFa), and Insulin-like peptide (ILP) expressing neurons in the pars intercerebralis (PI), which receive rhythmic signals from the clock neurons. Finally, the clock neurons control learning and contribute to time memory via still unknown pathways (?) to the mushroom bodies (MB).

physiological events and it can be terminated at any time when the environmental conditions improve.

Can we, nevertheless, learn something about the mechanisms of dormancy from *D. melanogaster*? The adult female flies exhibit a reproductive dormancy manifested by reduced metabolic activity and arrested ovarian development, which is stimulated by low temperatures and can be enhanced by short natural photoperiods and food shortage (Nagy et al., 2018, 2019; Ojima et al., 2018). As in other insects, the insulin-like peptide (ILP) producing cells (IPCs) are key regulators of this process, since they produce and release insulin-like peptides that act as diapause-antagonizing hormones. Although, fruit flies have no photoperiodically induced diapause that needs communication with the endogenous clock to determine day length, it was recently shown that the circadian clock neurons communicate with the IPC cells (Nagy et al., 2019). The s-LN_v clock neurons activate the IPCs via the neuropeptides Pigment-Dispersing Factor (PDF) and short neuropeptide F (sNPF), which in turn release insulin-like peptides, antagonize dormancy and lead to reproductive growth. This result suggests that the secretion of PDF and sNPF is enhanced under long summer days and by this way keep the flies in the reproductive state. PDF is indeed secreted during the day (Park et al., 2000), but it is still unknown whether its secretion is prolonged or enhanced under long days.

That the clock communicates with the dormancy inducing centers in the central brain of *D. melanogaster* flies can also be inferred from the fact that some components of the molecular circadian clock affect dormancy incidence. For example, a long isoform of the clock protein TIM (L-TIM) evolved a few 100–1,000 years ago, after *D. melanogaster* colonized Europe (Sandrelli et al., 2007; Tauber et al., 2007; Zonato et al., 2018); reviewed in Kyriacou (2014b). This long isoform coexists with the original short form (S-TIM), and flies carrying both isoforms ("LS-TIM" flies) gradually invaded Northern Europe and North America (Pegoraro et al., 2017). LS-TIM has a reduced ability to interact with CRY, which makes the flies less light-sensitive and

less likely to become arrhythmic under extreme long days. In addition, the "LS-TIM" flies enter dormancy earlier in autumn than the S-TIM flies. Both properties are advantageous for a life in the north. A very recent study supports the role of TIM in dormancy of *D. melanogaster* (Abrieux et al., 2020). The authors showed that *tim* null mutants exhibit reduced incidence of reproductive dormancy in simulated winter conditions, while flies overexpressing *tim* show an increased incidence of reproductive dormancy even under long photoperiods.

What about insects that exhibit a real photoperiodic diapause? Most interestingly, the *tim1* or *cry1* clock genes plays also a role in the photoperiodic response of such species as for example the Japanese fruit fly *Chymomyza costata* (Stehlík et al., 2008), the fruit fly *Drosophila triauraria* (Yamada and Yamamoto, 2011), the silkworm *Bombyx mori* (Li, 2011), the Asian Tiger mosquito *Aedes albopictus* (Huang et al., 2015), and the Northern house mosquito *Culex pipiens* (Meuti et al., 2015). In photoperiodic species that possess no TIM1 and no light-sensitive CRY1 such as the bean bug *Riptortus pedestris* or the Linden bug *Pyrrhocoris apterus* other clock genes such as *per* or *Clk* are involved in the photoperiodic response (Syrová et al., 2003; Ikeno et al., 2010, 2011a,b).

Analogous to *D. melanogaster*, a knockdown of the neuropeptide PDF caused female *Culex pipiens* that were reared under long day conditions to enter a diapause-like state (Meuti et al., 2015). Furthermore, the ablation of the PDF-positive clock neurons in the blow fly *Protophormia terraenovae* interferes with photoperiodic diapause induction in such a way that the flies could not discriminate long and short days and half of the flies entered diapause at both conditions (Shiga and Numata, 2009). This confirms the importance of PDF as signaling molecule from the circadian clock to the IPC cells.

In most cases, PDF signaling to the IPC cells appears to keep flies in the reproductive state. Most interestingly, several *Drosophila* species such as *D. montana, D. littoralis, D. ezoana,* and *D. virilis* that live in the very north lack PDF in the s-LN_v clock neurons that project to the IPC cells (Bahn et al., 2009; Kauranen et al., 2012; Hermann et al., 2013; Menegazzi et al., 2017; Beauchamp et al., 2018); reviewed in Helfrich-Förster et al. (2018). These species have a high incidence of reproductive arrest already under long-day lengths, which is an adaptation to the low temperatures even under summer photoperiods at these clines. For example, *D. ezoana* enters diapause when day-length falls below 16 h (Vaze and Helfrich-Förster, 2016). The lack of PDF-signaling to the IPCs of these species might facilitate the termination of the reproductive state already at these relatively long days. In addition to lacking PDF in the s-LN_v clock neurons, these high-altitude flies lack CRY in other clock neurons: the l-LN_v [reviewed in Helfrich-Förster et al. (2018)]. This may enhance the flies' ability to enter dormancy earlier in the seasons as true for the less light-sensitive TIM-LS flies of *D. melanogaster*.

Nevertheless, not all fly species that are adapted to high altitudes lack PDF and CRY in certain clock neurons. For example, *C. costata* flies that are distributed in Eastern Siberia, Northern Lapland, Iceland, and from northern Japan to the Artic Cycle (Hackman et al., 1970) possess a *D. melanogaster*-like PDF network (Bertolini et al., 2019). This shows that the circadian clock of *C. costata* flies has found other ways to adapt to high-latitudes. PDF may just promote metabolic and reproductive activity, but there is no prove that it is really necessary for photoperiodic information. On the contrary: most photoperiodic species lack PDF signals to the IPC cells and nevertheless undergo diapause at a critical day length. For example, in the aphid *Acyrthosipon pisum*, which is a classic model for photoperiodism, PDF was even not found at all (Beer, Colizzi and Helfrich-Förster, unpublished). Furthermore, we still lack a detailed pathway leading from photoreception to expression of diapause. Though a functional circadian clock appears essential for the diapause response, it is not at all clear how the circadian clock and the photoperiodic timer are integrated. How are short days distinguished from long days, and how is this critical information stored in the brain to be acted upon at a later stage or even in the following generation?

Although most insects enter dormancy at some point in their life cycle, insects that currently offer the best models for genetic research lack a robust photoperiodic diapause. Further development of genetic tools for non-model species, including both loss and gain-of-function mutations, are urgently needed to advance the exciting field of insect photoperiodism. In addition, laboratory-based experiments can benefit from carefully simulated natural environments under controlled conditions, and whenever possible experiments should also be carried out in the wild. The natural world offers an incredibly rich diversity of biological clocks that can be probed for understanding the timing of seasonal activity.

CONCLUDING REMARKS ON EVOLUTION OF THE CIRCADIAN CLOCK IN INSECTS

As we have elucidated above, the model insects *D. melanogaster* and *A. mellifera*, have remarkable advantages in representing different aspects in chronobiology: Their genomes are sequenced, and many components of the circadian clockwork are already identified. They show a variety of circadian output behaviors with species specific relevance and differing sensitivity to various inputs. Interestingly, some basic concepts may be transferable although the chronobiological function substantially differs between species (e.g., sun compass orientation, time-place-learning, emergence, and diapause). Nevertheless, restricting the research to model organisms is insufficient to understand quite a few aspects in insect clock evolution. We may learn best from insect models, when we investigate the circadian clock in parallel in various insects, which display a modification or more pronounced function of the circadian clock, like diapause in northern flies or other insects with pronounced diapause [e.g., aphids (Barberà and Martínez-Torres, 2017; Barberà et al., 2017), bugs (Kotwica-Rolinska et al., 2017), wasps (Reznik, 2011; Paolucci et al., 2019), or butter flies (Denlinger et al., 2017)], or emergence rhythms in solitary bees. This may provide us furthermore with a better insight into circadian clock evolution. Even task related plasticity in the circadian clock is not restricted to honey bees. Ants were also found to perform arrhythmic brood care and have task related plasticity in clock gene expression

(Ingram et al., 2009; Fujioka et al., 2017). This indicates, that although sociality evolved several times independently in Hymenoptera (in bees, wasps and ants), there seems to be a common ground plan to the social clock of Hymenoptera [reviewed in Bloch (2009), Bloch (2010), Bloch and Grozinger (2011)].

Studying modifications to the circadian clock in social and non-social Hymenopterans may be the key to understand the concept of social clocks. Especially bees (Anthophila) provide a huge range of differently scaled social lifestyles. We find true social (eusocial) bees (e.g., honey bees), primitively social (e.g., bumble bees), facultatively social and solitary bees (Shell and Rehan, 2018). Primitively social bumble bee queens also display circadian plasticity: when founding a new colony they take care of their first brood without behavioral rhythms, but resume rhythmic activity, when the brood is removed (Eban-Rothschild et al., 2011). Contrary to honey bees, brood care of bumble bee workers is rather related to their body size than their age (Yerushalmi et al., 2006).

Regarding the molecular clock, it appears not enough to compare the clockwork of social Hymenoptera with solitary insects from other orders (like for example *D. melanogaster*), because the unique composition of the circadian clock gene set in Hymenoptera indicates a regulatory mechanism that is different from the one in other insect orders. Therefore, exploring the clock genes, neuronal network and clock regulated behavior in solitary bees (and bees of different social grades) appears essential in future studies on the Hymenopteran clock.

Finally, we want to mention one further topic: inter-species interactions and co-evolution in chronobiology. Apart from temporal reproductive barriers (see above: species specific mating flights in honey bees), cohabitating insects establish species specific daytime-dependent foraging activity because of competition for food resources (Krell-Westerwalbesloh et al., 2004; Gottlieb et al., 2005; Wcislo and Tierney, 2009; Bloch et al., 2017; Smith et al., 2017). For example, the solitary bee *Proxylocopa olivieri* forages with a bimodal activity peaking at dusk and dawn and thereby avoids interaction with other bee species like *A. mellifera*, which shows unimodal foraging during the day (Gottlieb et al., 2005). Or in case of different cohabitating dung beetle guilds, the superior competitors are active during the day, while beetle guilds of lower competitive status display a peak in activity around dusk (Krell-Westerwalbesloh et al., 2004). Such inter-species effects in insect interaction networks, just like species specific clock outputs, clearly can only be sufficiently researched by studying the circadian clock of both, model and non-model insects in chronobiology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Our research on the circadian clock of insects was funded by the European Union's Horizon 2020 research and innovation program CINCHRON under the Marie Sklodowska-Curie grant agreement No 765937, by an individual grant of the German Research Foundation DFG to CH-F (FO 207/15-1) and an individual grant of the University of Würzburg to KB (Postdoc researcher funding program SCIENCIA). This publication was supported by the Open Access Publication Fund of the University of Würzburg.

REFERENCES

Abrieux, A., Xue, Y., Cai, Y., Lewald, K. M., Nhu Nguyen, H., Zhang, Y., et al. (2020). EYES ABSENT and TIMELESS integrate photoperiodic and temperature cues to regulate seasonal physiology in *Drosophila*. *BioRxiv* 117, 15293–15304. doi: 10.1101/2020.03.05.979682

Bae, K., Lee, C., Sidote, D., Chuang, K., and Edery, I. (1998). Circadian regulation of a *Drosophila* homolog of the mammalian clock gene: PER and TIM function as positive regulators. *Mol. Cell. Biol.* 18, 6142–6151. doi: 10.1128/MCB.18.10.6142

Bahn, J. H., Lee, G., and Park, J. H. (2009). Comparative analysis of pdf-mediated circadian behaviors between *Drosophila melanogaster* and *D. virilis*. *Genetics* 181, 965–975. doi: 10.1534/genetics.108.099069

Bai, L., Lee, Y., Hsu, C. T., Williams, J. A., Cavanaugh, D., Zheng, X., et al. (2018). A conserved circadian function for the *Neurofibromatosis 1* gene. *Cell Rep.* 22, 3416–3426. doi: 10.1016/j.celrep.2018.03.014

Barber, A. F., Erion, R., Holmes, T. C., and Sehgal, A. (2016). Circadian and feeding cues integrate to drive rhythms of physiology in *Drosophila* insulin-producing cells. *Genes Dev.* 30, 2596–2606. doi: 10.1101/gad.288258.116

Barberà, M., Collantes-Alegre, J. M., and Martínez-Torres, D. (2017). Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. *Insect. Biochem. Mol. Biol.* 83, 54–67. doi: 10.1016/j.ibmb.2017.02.006

Barberà, M., and Martínez-Torres, D. (2017). Identification of the *prothoracicotropic hormone* (*Ptth*) coding gene and localization of its site of expression in the pea aphid *Acyrthosiphon pisum*. *Insect. Mol. Biol.* 26, 654–664. doi: 10.1111/imb.12326

Barnes, J. W. (2003). Requirement of mammalian *timeless* for circadian rhythmicity. *Science* 302, 439–442. doi: 10.1126/science.1086593

Bazalova, O., and Dolezel, D. (2017). Daily activity of the housefly, *Musca domestica*, is influenced by temperature independent of 3′ UTR *period* gene splicing. *G3* 7, 2637–2649. doi: 10.1534/g3.117.042374

Beauchamp, M., Bertolini, E., Deppisch, P., Steubing, J., Menegazzi, P., and Helfrich-Förster, C. (2018). Closely related fruit fly species living at different latitudes diverge in their circadian clock anatomy and rhythmic behavior. *J. Biol. Rhythms* 33, 602–613. doi: 10.1177/0748730418798096

Beer, K., and Bloch, G. (2020). Circadian plasticity in honey bees. *Biochemist* 42, 22–26. doi: 10.1042/BIO04202002

Beer, K., Kolbe, E., Kahana, N. B., Yayon, N., Weiss, R., Menegazzi, P., et al. (2018). Pigment-dispersing factor-expressing neurons convey circadian information in the honey bee brain. *Open Biol.* 8:170224. doi: 10.1098/rsob.170224

Beer, K., Schenk, M., Helfrich-Förster, C., and Holzschuh, A. (2019). The circadian clock uses different environmental time cues to synchronize emergence and locomotion of the solitary bee *Osmia bicornis*. *Sci. Rep.* 9:17748. doi: 10.1038/s41598-019-54111-3

Beer, K., Steffan-Dewenter, I., Härtel, S., and Helfrich-Förster, C. (2016). A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior. *J. Comp. Physiol. A* 202, 555–565. doi: 10.1007/s00359-016-1103-2

Beling, I. (1929). Über das Zeitgedächtnis der Bienen. *Zeitschrift Für Vergleichende Physiologie* 9, 259–338. doi: 10.1007/BF00340159

Benito, J., Zheng, H., and Hardin, P. E. (2007). PDP1 functions downstream of the circadian oscillator to mediate behavioral rhythms. *J. Neurosci.* 27, 2539–2547. doi: 10.1523/JNEUROSCI.4870-06.2007

Benna, C., Bonaccorsi, S., Wülbeck, C., Helfrich-Förster, C., Gatti, M., Kyriacou, C. P., et al. (2010). *Drosophila timeless2* is required for chromosome stability and circadian photoreception. *Curr. Biol.* 20, 346–352. doi: 10.1016/j.cub.2009.12.048

Bennett, M. M., Rinehart, J. P., Yocum, G. D., Doetkott, C., and Greenlee, K. J. (2018). Cues for cavity nesters: investigating relevant zeitgebers for emerging leafcutting bees, *Megachile rotundata*. *J. Exp. Biol.* 221:jeb175406. doi: 10.1242/jeb.175406

Bertolini, E., Kistenpfennig, C., Menegazzi, P., Keller, A., Koukidou, M., and Helfrich-Förster, C. (2018). The characterization of the circadian clock in the olive fly *Bactrocera oleae* (Diptera: Tephritidae) reveals a *Drosophila*-like organization. *Sci. Rep.* 8:816. doi: 10.1038/s41598-018-24850-w

Bertolini, E., Schubert, F. K., Zanini, D., Sehadová, H., Helfrich-Förster, C., and Menegazzi, P. (2019). Life at high latitudes does not require circadian behavioral rhythmicity under constant darkness. *Curr. Biol.* 29, 3928–3936.e3. doi: 10.1016/j.cub.2019.09.032

Bloch, G. (2010). The social clock of the honeybee. *J. Biol. Rhythms* 25, 307–317. doi: 10.1177/0748730410380149

Bloch, G., Bar-Shai, N., Cytter, Y., and Green, R. (2017). Time is honey: circadian clocks of bees and flowers and how their interactions may influence ecological communities. *Phil. Trans. R. Soc. B* 372:20160256. doi: 10.1098/rstb.2016.0256

Bloch, G., and Grozinger, C. M. (2011). Social molecular pathways and the evolution of bee societies. *Philos. Trans. Roy. Soc. B Biol. Sci.* 366, 2155–2170. doi: 10.1098/rstb.2010.0346

Bloch, G., and Robinson, G. E. (2001). Chronobiology: reversal of honey bee behavioural rhythms. *Nat. Brief Commun.* 410:1048. doi: 10.1038/35074183

Bloch, G., Solomon, S. M., Robinson, G. E., and Fahrbach, S. E. (2003). Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*): age- and time-related plasticity. *J. Comp. Neurol.* 464, 269–284. doi: 10.1002/cne.10778

Bloch, G., Toma, D. P., and Robinson, G. E. (2001). Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. *J. Biol. Rhythms* 16, 444–456. doi: 10.1177/074873001129002123

Bloch, G. (2009). "Plasticity in the circadian clock and temporal organization of insect societies," in *Organization of Insect Societies: From Genome to Sociocomplexity,* eds J. Gadau and J. Fewell (Cambridge: Harvard University Press), 402–432.

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron* 93, 1420–1435.e5. doi: 10.1016/j.neuron.2017.02.030

Brown, S. A., Kowalska, E., and Dallmann, R. (2012). (Re)inventing the circadian feedback loop. *Dev. Cell* 22, 477–487. doi: 10.1016/j.devcel.2012.02.007

Bünning, E. (1936). Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion (Endogenous daily rhythms as the basis of photoperiodism). *Berichte Der Deutschen Botanischen Gesellschaft* 54, 590–607.

Busza, A., Murad, A., and Emery, P. (2007). Interactions between circadian neurons control temperature synchronization of *Drosophila* behavior. *J. Neurosci.* 27, 10722–10733. doi: 10.1523/JNEUROSCI.2479-07.2007

Cavanaugh, D. J., Geratowski, J. D., Wooltorton, J. R. A., Spaethling, J. M., Hector, C. E., Zheng, X., et al. (2014). Identification of a circadian output circuit for rest: activity rhythms in *Drosophila*. *Cell* 157, 689–701. doi: 10.1016/j.cell.2014.02.024

Cavey, M., Collins, B., Bertet, C., and Blau, J. (2016). Circadian rhythms in neuronal activity propagate through output circuits. *Nat. Neurosci.* 19, 587–595. doi: 10.1038/nn.4263

Ceriani, M. F., Darlington, T. K., Staknis, D., Más, P., Petti, A. A., Weitz, C. J., et al. (1999). Light-dependent sequestration of timeless by cryptochrome. *Science* 285, 553–556. doi: 10.1126/science.285.5427.553

Chahad-Ehlers, S. (2017). Expanding the view of Clock and cycle gene evolution in Diptera. *Insect. Mol. Biol. v* 26, 317–331. doi: 10.1111/imb.12296

Chang, D. C., McWatters, H. G., Williams, J. A., Gotter, A. L., Levine, J. D., and Reppert, S. M. (2003). Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. *J. Biol. Chem.* 278, 38149–38158. doi: 10.1074/jbc.M306937200

Chatterjee, A., Lamaze, A., De, J., Mena, W., Chélot, E., Martin, B., et al. (2018). Reconfiguration of a multi-oscillator network by light in the *Drosophila* circadian clock. *Curr. Biol.* 28, 2007–2017.e4. doi: 10.1016/j.cub.2018.04.064

Cheeseman, J. F., Millar, C. D., Greggers, U., Lehmann, K., Pawley, M. D., Gallistel, C. R., et al. (2014). Way-finding in displaced clock-shifted bees proves bees use a cognitive map. *Proc. Natl. Acad. Sci. U. S. A.* 111, 8949–8954. doi: 10.1073/pnas.1408039111

Cheeseman, J. F., Winnebeck, E. C., Millar, C. D., Kirkland, L. S., Sleigh, J., Goodwin, M., et al. (2012). General anesthesia alters time perception by phase shifting the circadian clock. *Proc. Natl. Acad. Sci. U. S. A.* 109, 7061–7066. doi: 10.1073/pnas.1201734109

Choi, C., Cao, G., Tanenhaus, A. K., McCarthy, E., v., Jung, M., Schleyer, W., et al. (2012). Autoreceptor control of peptide/neurotransmitter corelease from PDF neurons determines allocation of circadian activity in *Drosophila*. *Cell Rep.* 2, 332–344. doi: 10.1016/j.celrep.2012.06.021

Chouhan, N. S., Wolf, R., Helfrich-Förster, C., and Heisenberg, M. (2015). Flies remember the time of day. *Curr. Biol.* 25, 1619–1624. doi: 10.1016/j.cub.2015.04.032

Codd, V., Dolezel, D., Stehlik, J., Piccin, A., Garner, K. J., Racey, S. N., et al. (2007). Circadian rhythm gene regulation in the housefly *Musca domestica*. *Genetics* 177, 1539–1751. doi: 10.1534/genetics.107.079160

Cortés, T., Ortiz-Rivas, B., and Martínez-Torres, D. (2010). Identification and characterization of circadian clock genes in the pea aphid *Acyrthosiphon pisum*: aphid clock genes. *Insect. Mol. Biol.* 2, 123–139. doi: 10.1111/j.1365-2583.2009.00931.x

Crailsheim, K., Hrassnigg, N., and Stabentheiner, A. (1996). Diurnal behavioural differences in forager and nurse honey bees (*Apis mellifera carnica* Pollm). *Apidologie* 27, 235–244. doi: 10.1051/apido:19960406

Currie, J., Goda, T., and Wijnen, H. (2009). Selective entrainment of the *Drosophila* circadian clock to daily gradients in environmental temperature. *BMC Biol.* 7:49. doi: 10.1186/1741-7007-7-49

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M.-C., Glossop, N. R., Hardin, P. E., et al. (2003). *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell* 112, 329–341. doi: 10.1016/S0092-8674(03)00074-6

Dalla Benetta, E., Beukeboom, L. W., and van de Zande, L. (2019). Adaptive differences in circadian clock gene expression patterns and photoperiodic diapause induction in *Nasonia vitripennis*. *Am. Nat.* 193, 881–896. doi: 10.1086/703159

Danbara, Y., Sakamoto, T., Uryu, O., and Tomioka, K. (2010). RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. *J. Insect. Physiol.* 56, 1738–1745. doi: 10.1016/j.jinsphys.2010.07.002

Davies, N. J., and Tauber, E. (2016). Deep sequencing analysis of the circadian transcriptome of the jewel wasp Nasonia vitripennis. *bioRxiv [Preprint]*. doi: 10.1101/048736

Denlinger, D. L. (1986). Dormancy in tropical insects. *Ann. Rev. Entomol.* 31, 329–364. doi: 10.1146/annurev.en.31.010186.001323

Denlinger, D. L., Hahn, D. A., Merlin, C., Holzapfel, C. M., and Bradshaw, W. E. (2017). Keeping time without a spine: what can the insect clock teach us about seasonal adaptation? *Phil. Trans. R. Soc. B* 372:20160257. doi: 10.1098/rstb.2016.0257

Donlea, J. M. (2019). Roles for sleep in memory: insights from the fly. *Curr. Opin. Neurobiol.* 54, 120–126. doi: 10.1016/j.conb.2018.10.006

Dreyer, A. P., Martin, M. M., Fulgham, C. V., Jabr, D. A., Bai, L., Beshel, J., et al. (2019). A circadian output center controlling feeding:fasting rhythms in *Drosophila*. *PLoS Genet.* 15:e1008478. doi: 10.1371/journal.pgen.1008478

Dubowy, C., and Sehgal, A. (2017). Circadian rhythms and sleep in *Drosophila melanogaster*. *Genetics* 205, 1373–1397. doi: 10.1534/genetics.115.185157

Eban-Rothschild, A., Belluci, S., and Bloch, G. (2011). Maternity-related plasticity in circadian rhythms of bumble-bee queens. *Proc. Royal Soc. B Biol. Sci.* 278, 3510–3516. doi: 10.1098/rspb.2011.0579

Eban-Rothschild, A., and Bloch, G. (2012). "Social influences on circadian rhythms and sleep in insects," in *Advances in Genetics,* eds M. B. Sokolowski and S. F. Goodwin (Elsevier), 1–32. doi: 10.1016/B978-0-12-387687-4.00001-5

Eban-Rothschild, A., Shemesh, Y., and Bloch, G. (2012). The colony environment, but not direct contact with conspecifics, influences the development

of circadian rhythms in honey bees. *J. Biol. Rhythms* 27, 217–225. doi: 10.1177/0748730412440851

Eban-Rothschild, A. D., and Bloch, G. (2008). Differences in the sleep architecture of forager and young honeybees (*Apis mellifera*). *J. Exp. Biol.* 211, 2408–2416. doi: 10.1242/jeb.016915

Emery, P., and Reppert, S. M. (2004). A rhythmic ror. *Neuron* 43, 443–446. doi: 10.1016/j.neuron.2004.08.009

Emery, P., So, W. V., Kaneko, M., Hall, J. C., and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 95, 669–679. doi: 10.1016/S0092-8674(00)81637-2

Fent, K., and Wehner, R. (1985). Ocelli: a celestial compass in the desert ant *Cataglyphis*. *Science* 228, 192–194. doi: 10.1126/science.228.4696.192

Fernández, M. P., Berni, J., and Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol.* 6:e69. doi: 10.1371/journal.pbio.0060069

Free, J. B. (1965). The allocation of duties among worker honeybees. *Symp. Zool.* 14, 39–59.

Free, J. B., Ferguson, A. W., and Simpkins, J. R. (1992). The behaviour of queen honeybees and their attendants. *Physiol. Entomol.* 17, 43–55. doi: 10.1111/j.1365-3032.1992.tb00988.x

Frisch, B., and Aschoff, J. (1987). Circadian rhythms in honeybees: entainment by feeding cycles. *Physiol. Entomol.* 12, 41–49. doi: 10.1111/j.1365-3032.1987.tb00722.x

Frisch, B., and Koeniger, N. (1994). Social synchronization of the activity rhythms of honeybees within a colony. *Behav. Ecol. Sociobiol.* 35, 91–98. doi: 10.1007/BF00171498

Frisch, K. v., and Lindauer, M. (1954). Himmel und erde in konkurrenz bei der orientierung der bienen. *Naturwissenschaften* 41, 245–253. doi: 10.1007/BF00634944

Fuchikawa, T., Beer, K., Linke-Winnebeck, C., Ben-David, R., Kotowoy, A., Tsang, V. W. K., et al. (2017). Neuronal circadian clock protein oscillations are similar in behaviourally rhythmic forager honeybees and in arrhythmic nurses. *Open Biol.* 7:170047. doi: 10.1098/rsob.170047

Fuchikawa, T., Eban-Rothschild, A., Nagari, M., Shemesh, Y., and Bloch, G. (2016). Potent social synchronization can override photic entrainment of circadian rhythms. *Nat. Commun.* 7:11662. doi: 10.1038/ncomms11662

Fujii, S., and Amrein, H. (2010). Ventral lateral and DN1 clock neurons mediate distinct properties of male sex drive rhythm in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 107, 10590–10595. doi: 10.1073/pnas.0912457107

Fujii, S., Emery, P., and Amrein, H. (2017). SIK3–HDAC4 signaling regulates *Drosophila* circadian male sex drive rhythm via modulating the DN1 clock neurons. *Proc. Natl. Acad. Sci. U. S. A.* 114, E6669–E6677. doi: 10.1073/pnas.1620483114

Fujii, S., Krishnan, P., Hardin, P., and Amrein, H. (2007). Nocturnal male sex drive in *Drosophila*. *Curr. Biol.* 17, 244–251. doi: 10.1016/j.cub.2006.11.049

Fujioka, H., Abe, M. S., Fuchikawa, T., Tsuji, K., Shimada, M., and Okada, Y. (2017). Ant circadian activity associated with brood care type. *Biol. Lett.* 13:20160743. doi: 10.1098/rsbl.2016.0743

Galizia, C. G., Eisenhardt, D., and Giurfa, M. (2011). *Honeybee neurobiology and behavior: a tribute to Randolf Menzel* (Dordrecht: Springer). doi: 10.1007/978-94-007-2099-2

Gentile, C., Rivas, G. B. S., Meireles-Filho, A. C. A., Lima, J. B. P., and Peixoto, A. A. (2009). Circadian expression of clock genes in two mosquito disease vectors: *cry2* is different. *J. Biol. Rhythms* 24, 444–451. doi: 10.1177/0748730409349169

Gentile, C., Sehadova, H., Simoni, A., Chen, C., and Stanewsky, R. (2013). Cryptochrome antagonizes synchronization of *Drosophila's* circadian clock to temperature cycles. *Curr. Biol.* 23, 185–195. doi: 10.1016/j.cub.2012.12.023

Gesto, J. S. M., Rivas, G. B., da, S., Pavan, M. G., Meireles-Filho, A. C. A., Amoretty, P.R. de, Souza, N. A., et al. (2015). Clocks do not tick in unison: isolation of *Clock* and *vrille* shed new light on the clockwork model of the sand fly *Lutzomyia longipalpis*. *Parasites Vectors* 8:505. doi: 10.1186/s13071-015-1117-6

Giraldo, Y. M., Leitch, K. J., Ros, I. G., Warren, T. L., Weir, P. T., and Dickinson, M. H. (2018). Sun navigation requires compass neurons in *Drosophila*. *Curr. Biol.* 28, 2845–2852.e4. doi: 10.1016/j.cub.2018.07.002

Gotter, A. L. (2006). A timeless debate: resolving TIM's noncircadian roles with possible clock function. *NeuroReport* 17, 1229–1233. doi: 10.1097/01.wnr.0000233092.90160.92

Gotter, A. L., Manganaro, T., Weaver, D. R., Kolakowski, L. F., Possidente, B., Sriram, S., et al. (2000). A time-less function for mouse *timeless*. *Nat. Neurosci.* 3, 755–756. doi: 10.1038/77653

Gottlieb, D., Keasar, T., Shmida, A., and Motro, U. (2005). Possible foraging benefits of bimodal daily activity in *Proxylocopa olivieri* (Lepeletier) (Hymenoptera: Anthophoridae). *Environ. Entomol.* 34, 417–424. doi: 10.1603/0046-225X-34.2.417

Green, E. W., O'Callaghan, E. K., Hansen, C. N., Bastianello, S., Bhutani, S., Vanin, S., et al. (2015). *Drosophila* circadian rhythms in seminatural environments: summer afternoon component is not an artifact and requires TrpA1 channels. *Proc. Natl. Acad. Sci. U. S. A.* 112, 8702–8707. doi: 10.1073/pnas.1506093112

Gunawardhana, K. L., and Hardin, P. E. (2017). VRILLE controls PDF neuropeptide accumulation and arborization rhythms in small ventrolateral neurons to drive rhythmic behavior in *Drosophila*. *Curr. Biol.* 27, 3442–3453.e4. doi: 10.1016/j.cub.2017.10.010

Guo, F., Cerullo, I., Chen, X., and Rosbash, M. (2014). PDF neuron firing phase-shifts key circadian activity neurons in *Drosophila*. *Elife* 3:e02780. doi: 10.7554/eLife.02780.014

Guo, F., Holla, M., Díaz, M. M., and Rosbash, M. (2018). A circadian output circuit controls sleep-wake arousal in *Drosophila*. *Neuron* 100, 624–635.e4. doi: 10.1016/j.neuron.2018.09.002

Hackman, W., Lakvaara, S., Saura, A., Sorsa, M., and Vepsalainen, K. (1970). On the biology and karyology of *Chymomyza costata* Zetterstedt, with reference to the taxonomy and distribution of various species of *Chymomyza* (Dipt., Drosophilidae). *Ann. Entom. Fenn.* 36, 1–9.

Hall, J. C. (2003). *Genetics and Molecular Biology of Rhythms in Drosophila and Other Insects*, Vol. 48. Waltham, MA: Gulf Professional Publishing; Brandeis University.

Harano, K., Sasaki, M., and Sasaki, K. (2007). Effects of reproductive state on rhythmicity, locomotor activity and body weight in the European honeybee, *Apis mellifera* queens (Hymenoptera, Apini). *Sociobiology* 50, 189–200.

Hardin, P. E., and Panda, S. (2013). Circadian timekeeping and output mechanisms in animals. *Curr. Opin. Neurobiol.* 23, 724–731. doi: 10.1016/j.conb.2013.02.018

Hardin, P. E. (2011). "Chapter 5—Molecular genetic analysis of circadian timekeeping in *Drosophila*," in *The Genetics of Circadian Rhythms,* 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2

Harper, R. E. F., Dayan, P., Albert, J. T., and Stanewsky, R. (2016). Sensory conflict disrupts activity of the *Drosophila* circadian network. *Cell Rep.* 17, 1711–1718. doi: 10.1016/j.celrep.2016.10.029

Hassaneen, E., El-Din Sallam, A., Abo-Ghalia, A., Moriyama, Y., Karpova, S. G., Abdelsalam, S., et al. (2011). Pigment-dispersing factor affects nocturnal activity rhythms, photic entrainment, and the free-running period of the circadian clock in the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms* 26, 3–13. doi: 10.1177/0748730410388746

Helfrich-Förster, C. (1995). The *period* clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 92, 612–616. doi: 10.1073/pnas.92.2.612

Helfrich-Förster, C. (2000). Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*—sex-specific differences suggest a different quality of activity. *J. Biol. Rhythms* 15, 135–154. doi: 10.1177/074873040001500208

Helfrich-Förster, C. (2014). From neurogenetic studies in the fly brain to a concept in circadian biology. *J. Neurogenet.* 28, 329–347. doi: 10.3109/01677063.2014.905556

Helfrich-Förster, C. (2017). "The drosophila clock system," in *Biological Timekeeping: Clocks, Rhythms and Behaviour,* ed V. Kumar (New Delhi: Springer India), 133–176. doi: 10.1007/978-81-322-3688-7_6

Helfrich-Förster, C. (2020). Light input pathways to the circadian clock of insects with an emphasis on the fruit fly *Drosophila melanogaster*. *J. Comp. Physiol. A* 206, 259–272. doi: 10.1007/s00359-019-01379-5

Helfrich-Förster, C., Bertolini, E., and Menegazzi, P. (2018). Flies as models for circadian clock adaptation to environmental challenges. *Eur. J. Neurosci.* 51, 166–181. doi: 10.1111/ejn.14180

Helfrich-Förster, C., Shafer, O. T., Wülbeck, C., Grieshaber, E., Rieger, D., and Taghert, P. (2007b). Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. *J. Comp. Neurol.* 500, 47–70. doi: 10.1002/cne.21146

Helfrich-Förster, C., Stengl, M., and Homberg, U. (1998). Organization of the circadian system in insects. *Chronobiol. Int.* 15, 567–594. doi: 10.3109/07420529808993195

Helfrich-Förster, C., Täuber, M., Park, J. H., Mühlig-Versen, M., Schneuwly, S., and Hofbauer, A. (2000). Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci.* 20, 3339–3353. doi: 10.1523/JNEUROSCI.20-09-03339.2000

Helfrich-Förster, C., Yoshii, T., Wülbeck, C., Grieshaber, E., Rieger, D., Bachleitner, W., et al. (2007a). "The lateral and dorsal neurons of *Drosophila melanogaster*: new insights about their morphology and function," *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. 72 (Cold Spring Harbor Laboratory Press). doi: 10.1101/sqb.2007.72.063

Hermann, C., Saccon, R., Senthilan, P. R., Domnik, L., Dircksen, H., Yoshii, T., et al. (2013). The circadian clock network in the brain of different *Drosophila* species. *J. Comp. Neurol.* 521, 367–388. doi: 10.1002/cne.23178

Hermann-Luibl, C., and Helfrich-Förster, C. (2015). Clock network in *Drosophila*. *Curr. Opin. Insect. Sci.* 7, 65–70. doi: 10.1016/j.cois.2014.11.003

Homberg, U. (2015). Sky compass orientation in desert locusts—evidence from field and laboratory studies. *Front. Behav. Neurosci.* 9:346. doi: 10.3389/fnbeh.2015.00346

Homberg, U., Heinze, S., Pfeiffer, K., Kinoshita, M., and el Jundi, B. (2011). Central neural coding of sky polarization in insects. *Philos. Trans. Roy. Soc. B Biol. Sci.* 366, 680–687. doi: 10.1098/rstb.2010.0199

Homberg, U., Würden, S., Dircksen, H., and Rao, K. R. (1991). Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tissue Res.* 266, 343–357. doi: 10.1007/BF00318190

Hsu, C. T., and Sehgal, A. (2018). Circadian and sleep circuits ring together. *Neuron* 100, 514–516. doi: 10.1016/j.neuron.2018.10.030

Huang, X., Poelchau, M. F., and Armbruster, P. A. (2015). Global transcriptional dynamics of diapause induction in non-blood-fed and blood-fed *Aedes albopictus*. *PLoS Negl. Trop. Dis.* 9, 1–28. doi: 10.1371/journal.pntd.0003724

Hussaini, S. A., Bogusch, L., Landgraf, T., and Menzel, R. (2009). Sleep deprivation affects extinction but not acquisition memory in honeybees. *Learn. Memory* 16, 698–705. doi: 10.1101/lm.1578409

Ikeno, T., Numata, H., and Goto, S. G. (2011a). Circadian clock genes period and cycle regulate photoperiodic diapause in the bean bug *Riptortus pedestris* males. *J. Insect. Physiol.* 57, 935–938. doi: 10.1016/j.jinsphys.2011.04.006

Ikeno, T., Numata, H., and Goto, S. G. (2011b). Photoperiodic response requires mammalian-type cryptochrome in the bean bug *Riptortus pedestris*. *Biochem. Biophys. Res. Commun.* 410, 394–397. doi: 10.1016/j.bbrc.2011.05.142

Ikeno, T., Numata, H., Goto, S. G., and Shiga, S. (2014). Involvement of the brain region containing pigment-dispersing factor-immunoreactive neurons in the photoperiodic response of the bean bug, *Riptortus pedestris*. *J. Exp. Biol.* 217, 453–462. doi: 10.1242/jeb.091801

Ikeno, T., Tanaka, S. I., Numata, H., and Goto, S. G. (2010). Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biol.* 8:116. doi: 10.1186/1741-7007-8-116

Im, S. H., and Taghert, P. H. (2010). PDF receptor expression reveals direct interactions between circadian oscillators in *Drosophila*. *J. Comp. Neurol.* 518, 1925–1945. doi: 10.1002/cne.22311

Ingram, K. K., Krummey, S., and LeRoux, M. (2009). Expression patterns of a circadian clock gene are associated with age-related polyethism in harvester ants, *Pogonomyrmex occidentalis*. *BMC Ecol.* 9:7. doi: 10.1186/1472-6785-9-7

Ingram, K. K., Kutowoi, A., Wurm, Y., Shoemaker, D., Meier, R., and Bloch, G. (2012). The molecular clockwork of the fire ant *Solenopsis invicta*. *PLoS ONE* 7:e45715. doi: 10.1371/journal.pone.0045715

Iwai, S., Fukui, Y., Fujiwara, Y., and Takeda, M. (2006). Structure and expressions of two circadian clock genes, *period* and *timeless* in the commercial silkmoth, *Bombyx mori*. *J. Insect Physiol.* 52, 625–637. doi: 10.1016/j.jinsphys.2006.03.001

Jackson, F. R., Ng, F. S., Sengupta, S., You, S., and Huang, Y. (2015). Glial cell regulation of rhythmic behavior. *Methods Enzymol.* 552, 45–73. doi: 10.1016/bs.mie.2014.10.016

Jain, R., and Brockmann, A. (2018). Time-restricted foraging under natural light/dark condition shifts the molecular clock in the honey bee, *Apis mellifera*. *Chronobiol. Int.* 1723–1734. doi: 10.1080/07420528.2018.1509867

Janssen, T., Husson, S. J., Lindemans, M., Mertens, I., Rademakers, S., Donck, K. V., et al. (2008). Functional characterization of three G protein-coupled receptors for pigment dispersing factors in *Caenorhabditis elegans*. *J. Biol. Chem.* 283, 15241–15249. doi: 10.1074/jbc.M709060200

Jaumouillé, E., Almeida, P. M., Stähli, P., Koch, R., and Nagoshi, E. (2015). Transcriptional regulation via nuclear receptor crosstalk required for the *Drosophila* circadian clock. *Curr. Biol.* 25, 189–200. doi: 10.1016/j.cub.2015.04.017

Johnson, J. N., Hardgrave, E., Gill, C., and Moore, D. (2010). Absence of consistent diel rhythmicity in mated honey bee queen behavior. *J. Insect. Physiol.* 56, 761–773. doi: 10.1016/j.jinsphys.2010.01.004

Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., and Rosbash, M. (2007). *Clockwork Orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. *Genes Dev.* 21, 1675–1686. doi: 10.1101/gad.1552607

Kaiser, T. S., Poehn, B., Szkiba, D., Preussner, M., Sedlazeck, F. J., Zrim, A., et al. (2016). The genomic basis of circadian and circalunar timing adaptations in a midge. *Nature* 540, 69–73. doi: 10.1038/nature20151

Kaiser, W. (1988). Busy bees need rest, too: behavioural and electromyographical sleep signs in honeybees. *J. Comp. Physiol.* 163, 565–584. doi: 10.1007/BF00603841

Kauranen, H., Menegazzi, P., Costa, R., Helfrich-Förster, C., Kankainen, A., and Hoikkala, A. (2012). Flies in the North: locomotor behavior and clock neuron organization of *Drosophila montana*. *J. Biol. Rhythms* 27, 377–387. doi: 10.1177/0748730412455916

Kay, J., Menegazzi, P., Mildner, S., Roces, F., and Helfrich-Förster, C. (2018). The circadian clock of the ant *Camponotus floridanus* is localized in dorsal and lateral neurons of the brain. *J. Biol. Rhythms* 33, 255–271. doi: 10.1177/0748730418764738

Kefuss, J. A. (1978). Influence of photoperiod on the behaviour and brood-rearing activities of honeybees in a flight room. *J. Apicult. Res.* 17, 137–151. doi: 10.1080/00218839.1978.11099919

King, A. N., and Sehgal, A. (2020). Molecular and circuit mechanisms mediating circadian clock output in the *Drosophila* brain. *Eur. J. Neurosci.* 51, 268–281. doi: 10.1111/ejn.14092

Kleber, E. (1935). Hat das Zeitgedächtnis der bienen biologische Bedeutung? *Zeitschrift Für Vergleichende Physiologie* 22, 221–262. doi: 10.1007/BF00586500

Klein, B. A., Klein, A., Wray, M. K., Mueller, U. G., and Seeley, T. D. (2010). Sleep deprivation impairs precision of waggle dance signaling in honey bees. *Proc. Natl. Acad. Sci. U. S. A.* 107, 22705–22709. doi: 10.1073/pnas.1009439108

Klein, B. A., Olzsowy, K. M., Klein, A., Saunders, K. M., and Seeley, T. D. (2008). Caste-dependent sleep of worker honey bees. *J. Exp. Biol.* 211, 3028–3040. doi: 10.1242/jeb.017426

Klein, B. A., Stiegler, M., Klein, A., and Tautz, J. (2014). Mapping sleeping bees within their nest: spatial and temporal analysis of worker honey bee sleep. *PLoS ONE* 9:e102316. doi: 10.1371/journal.pone.0102316

Klose, M., Duvall, L. B., Li, W., Liang, X., Ren, C., Steinbach, J. H., et al. (2016). Functional PDF signaling in the *Drosophila* circadian neural circuit is gated by Ral A-dependent modulation. *Neuron* 90, 781–794. doi: 10.1016/j.neuron.2016.04.002

Kobelková, A., Závodská, R., Sauman, I., Bazalová, O., and Dolezel, D. (2015). Expression of clock genes *period* and *timeless* in the central nervous system of the mediterranean flour moth, *Ephestia kuehniella*. *J. Biol. Rhythms* 30, 104–116. doi: 10.1177/0748730414568430

Koeniger, N., and Koeniger, G. (2000). Reproductive isolation among species of the genus *Apis*. *Apidol.* 31, 313–339. doi: 10.1051/apido:2000125

Koltermann, R. (1971). 24-Std-periodik in der Langzeiterinnerung an Duft- und Farbsignale bei der Honigbiene. *Z. vergl. Physiol.* 75, 49–68. doi: 10.1007/BF00335137

Komada, S., Kamae, Y., Koyanagi, M., Tatewaki, K., Hassaneen, E., Saifullah, A. S. M., et al. (2015). Green-sensitive opsin is the photoreceptor for photic entrainment of an insect circadian clock. *Zool. Lett.* 1:11. doi: 10.1186/s40851-015-0011-6

Konopka, R. J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 68, 2112–2116. doi: 10.1073/pnas.68.9.2112

Koštál, V. (2006). Eco-physiological phases of insect diapause. *J. Insect. Physiol.* 52, 113–127. doi: 10.1016/j.jinsphys.2005.09.008

Kotwica-Rolinska, J., Chodakova, L., Chvalova, D., Kristofova, L., Fenclova, I., Provaznik, J., et al. (2019). CRISPR/Cas9 genome editing introduction and optimization in the non-model insect *Pyrrhocoris apterus*. *Front. Physiol.* 10:891. doi: 10.3389/fphys.2019.00891

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., and Dolezel, D. (2017). The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage: nymphal photoperiodic timer. *Physiol. Entomol.* 42, 266–273. doi: 10.1111/phen.12197

Krell-Westerwalbesloh, S., Krell, F.-T., and Eduard Linsenmair, K. (2004). Diel separation of Afrotropical dung beetle guilds—avoiding competition and neglecting resources (Coleoptera: Scarabaeoidea). *J. Nat. Hist.* 38, 2225–2249. doi: 10.1080/00222930310001618921

Krupp, J. J., Billeter, J.-C., Wong, A., Choi, C., Nitabach, M. N., and Levine, J. D. (2013). Pigment-dispersing factor modulates pheromone production in clock cells that influence mating in *Drosophila*. *Neuron* 79, 54–68. doi: 10.1016/j.neuron.2013.05.019

Krupp, J. J., Kent, C., Billeter, J.-C., Azanchi, R., So, A. K.-C., Schonfeld, J. A., et al. (2008). Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. *Curr. Biol.* 18, 1373–1383. doi: 10.1016/j.cub.2008.07.089

Kumar, S., Chen, D., Jang, C., Nall, A., Zheng, X., and Sehgal, A. (2014). An ecdysone-responsive nuclear receptor regulates circadian rhythms in *Drosophila*. *Nat. Commun.* 5:5697. doi: 10.1038/ncomms6697

Kutaragi, Y., Tokuoka, A., Tomiyama, Y., Nose, M., Watanabe, T., Bando, T., et al. (2018). A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochromes*. *Zool. Lett.* 4:26. doi: 10.1186/s40851-018-0109-8

Kyriacou, C. P. (2014a). Sex and rhythms in sandflies and mosquitoes: an appreciation of the work of alexandre afranio peixoto (1963–2013). *Infect. Genet. Evol.* 28, 662–665. doi: 10.1016/j.meegid.2014.06.016

Kyriacou, C. P. (2014b). "Functional analysis of natural clock gene variation," in *Behavioral Genetics of the Fly*, ed J. Dubnau (New York, NY: Cold Spring Harbor Laboratory; Cambridge University Press), 183–191.

Lam, V. H., and Chiu, J. C. (2019). "Evolution and design of invertebrate circadian clocks," in *The Oxford Handbook of Invertebrate Neurobiology,* ed J.H. Byrne (Oxford University Press), 594–614. doi: 10.1093/oxfordhb/9780190456757.013.25

Lamaze, A., Krätschmer, P., Chen, K.-F., Lowe, S., and Jepson, J. E. C. (2018). A wake-promoting circadian output circuit in *Drosophila*. *Curr. Biol.* 28, 3098–3105.e3. doi: 10.1016/j.cub.2018.07.024

Lamaze, A., and Stanewsky, R. (2020). DN1p or the "fluffy" cerberus of clock outputs. *Front. Physiol.* 10:1540. doi: 10.3389/fphys.2019.01540

Lee, C.-M., Su, M.-T., and Lee, H.-J. (2009). Pigment dispersing factor: an output regulator of the circadian clock in the German cockroach. *J. Biol. Rhythms* 24, 35–43. doi: 10.1177/0748730408327909

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

Lensky, Y., and Demter, M. (1985). Mating flights of the queen honeybee (*Apis mellifera*) in a subtropical climate. *Comp. Physiol. A Physiol.* 81, 229–241. doi: 10.1016/0300-9629(85)90127-6

Levine, J. D., Funes, P., Dowse, H. B., and Hall, J. C. (2002). Resetting the circadian clock by social experience in *Drosophila melanogaster*. *Science* 298, 2010–2012. doi: 10.1126/science.1076008

Li, C.-J., Yun, X.-P., Yu, X.-J., and Li, B. (2018). Functional analysis of the circadian clock gene *timeless* in *Tribolium castaneum*: functions of timeless in *Tribolium castaneum*. *Insect. Science* 25, 418–428. doi: 10.1111/1744-7917.12441

Li, M.-T., Cao, L.-H., Xiao, N., Tang, M., Deng, B., Yang, T., et al. (2018). Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in *Drosophila*. *Nat. Commun.* 9:4247. doi: 10.1038/s41467-018-06506-5

Li, X. (2011). Timeless is a critical gene in the diapause of silkworm, *Bombyx mori*. *Afr. J. Biotechnol.* 10:2522. doi: 10.5897/AJB10.2522

Liang, X., Ho, M. C. W., Zhang, Y., Li, Y., Wu, M. N., Holy, T. E., et al. (2019). Morning and evening circadian pacemakers independently drive premotor centers via a specific dopamine relay. *Neuron* 102, 843–857.e4. doi: 10.1016/j.neuron.2019.03.028

Lim, C., Chung, B. Y., Pitman, J. L., McGill, J. J., Pradhan, S., Lee, J., et al. (2007b). *clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila*. *Curr. Biol.* 17, 1082–1089. doi: 10.1016/j.cub.2007.05.039

Lim, C., Lee, J., Koo, E., and Choe, J. (2007a). Targeted inhibition of Pdp1ε abolishes the circadian behavior of *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 364, 294–300. doi: 10.1016/j.bbrc.2007.10.009

Lin, Q.-C., Zhai, Y.-F., Zhou, C.-G., Li, L.-L., Zhuang, Q.-Y., Zhang, X.-Y., et al. (2014). Behavioral rhythms of *Drosophila suzukii* and *Drosophila melanogaster*. *Florida Entomologist* 97, 1424–1433. doi: 10.1653/024.097.0417

Lin, Y., Stormo, G. D., and Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. *J. Neurosci.* 24, 7951–7957. doi: 10.1523/JNEUROSCI.2370-04.2004

Lindauer, M. (1960). Time-compensated sun orientation in bees. *Cold Spring Harbor Symposia on Quant. Biol.* 25, 371–377. doi: 10.1101/SQB.1960.025.01.039

Liu, X., Blaženović, I., Contreras, A. J., Pham„ T. M., Tabuloc, C. A., Li, Y. H., et al. Hexosamine biosynthetic pathway integrates circadian and metabolic signals to regulate daily rhythms in protein O-linked N-acetylglucosaminylation. *bioRxiv [Preprint]* (2020). doi: 10.1101/2020.08.18.256636

Manjunatha, T., Dass, S. H., and Sharma, V. K. (2008). Egg-laying rhythm in *Drosophila melanogaster*. *J. Genet.* 87, 495–504. doi: 10.1007/s12041-008-0072-9

Martelli, C., Pech, U., Kobbenbring, S., Pauls, D., Bahl, B., Sommer, M. V., et al. (2017). SIFamide translates hunger signals into appetitive and feeding behavior in *Drosophila*. *Cell Rep.* 20, 464–478. doi: 10.1016/j.celrep.2017.06.043

Mathejczyk, T. F., and Wernet, M. F. (2019). Heading choices of flying *Drosophila* under changing angles of polarized light. *Sci. Rep.* 9:16773. doi: 10.1038/s41598-019-53330-y

Matsumoto, A., Ukai-Tadenuma, M., Yamada, R. G., Houl, J., Uno, K. D., Kasukawa, T., et al. (2007). A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the *Drosophila* circadian clock. *Genes Dev.* 21, 1687–1700. doi: 10.1101/gad.1552207

McCabe, C., and Birley, A. (1998). Oviposition in the *Period* genotypes of *Drosophila Melanogaster*. *Chronobiol. Int.* 15, 119–133. doi: 10.3109/07420529808998677

Medugorac, I., and Lindauer, M. (1967). Das Zeitgedächtnis der Bienen unter dem Einfluß von Narkose und von sozialen Zeitgebern. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 55, 450–474. doi: 10.1007/BF00302625

Meireles-Filho, A. C., Amoretty, P. R., Souza, N. A., Kyriacou, C. P., and Peixoto, A. A. (2006a). Rhythmic expression of the *cycle* gene in a hematophagous insect vector. *BMC Mol. Biol.* 7:38. doi: 10.1186/1471-2199-7-38

Meireles-Filho, A. C. A., da, S., Rivas, G. B., Gesto, J. S. M., Machado, R. C., Britto, C., et al. (2006b). The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal. *FEBS Lett.* 580, 2–8. doi: 10.1016/j.febslet.2005.11.031

Meireles-Filho, A. C. A., and Kyriacou, C. P. (2013). Circadian rhythms in insect disease vectors. *Mem. Inst. Oswaldo Cruz.* 108, 48–58. doi: 10.1590/0074-0276130438

Menegazzi, P., Dalla Benetta, E., Beauchamp, M., Schlichting, M., Steffan-Dewenter, I., and Helfrich-Förster, C. (2017). Adaptation of circadian neuronal network to photoperiod in high-latitude European drosophilids. *Curr. Biol.* 27, 833–839. doi: 10.1016/j.cub.2017.01.036

Merlin, C., Gegear, R. J., and Reppert, S. M. (2009). Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. *Science* 325, 1700–1704. doi: 10.1126/science.1176221

Merlin, C., Heinze, S., and Reppert, S. M. (2012). Unraveling navigational strategies in migratory insects. *Curr. Opin. Neurobiol.* 22, 353–361. doi: 10.1016/j.conb.2011.11.009

Meuti, M. E., Stone, M., Ikeno, T., and Denlinger, D. L. (2015). Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens. *J. Exp. Biol.* 218, 412–422. doi: 10.1242/jeb.113233

Miyazaki, Y., Watari, Y., Tanaka, K., and Goto, S. G. (2016). Temperature cycle amplitude alters the adult eclosion time and expression pattern of the circadian clock gene period in the onion fly. *J. Insect. Physiol.* 86, 54–59. doi: 10.1016/j.jinsphys.2016.01.002

Moore, D. (2001). Honey bee circadian clocks: behavioral control from individual workers to whole-colony rhythms. *J. Insect. Physiol.* 47, 843–857. doi: 10.1016/S0022-1910(01)00057-9

Moore, D., Angel, J. E., Cheeseman, I. M., Fahrbach, S. E., and Robinson, G. E. (1998). Timekeeping in the honey bee colony: integration of circadian rhythms and division of labor. *Behav. Ecol. Sociobiol.* 43, 147–160. doi: 10.1007/s002650050476

Moore, D., and Doherty, P. (2009). Acquisition of a time-memory in forager honey bees. *J. Comp. Physiol. A* 195, 741–751. doi: 10.1007/s00359-009-0450-7

Moore, D., and Rankin, M. A. (1985). Circadian locomotor rhythms in individual honeybees. *Physiol. Entomol.* 10, 191–197. doi: 10.1111/j.1365-3032.1985.tb00034.x

Moore, D., Siegfried, D., Wilson, R., and Rankin, M. A. (1989). The influence of time of day on the foraging behavior of the honeybee, *Apis mellifera*. *J. Biol. Rhythms* 4, 305–325. doi: 10.1177/074873048900400301

Moritz, R. F., and Kryger, P. (1994). Self-organization of circadian rhythms in groups of honeybees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* 34, 211–215. doi: 10.1007/BF00167746

Moritz, R. F., and Sakofski, F. (1991). The role of the queen in circadian rhythms of honeybees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* 29, 361–365. doi: 10.1007/BF00165961

Moriyama, Y., Kamae, Y., Uryu, O., and Tomioka, K. (2012). *Gb'Clock* is expressed in the optic lobe and is required for the circadian clock in the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms* 27, 467–477. doi: 10.1177/0748730412462207

Moriyama, Y., Sakamoto, T., Karpova, S. G., Matsumoto, A., Noji, S., and Tomioka, K. (2008). RNA interference of the clock gene *period* disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms* 23, 308–318. doi: 10.1177/0748730408320486

Mulder, C. K., Gerkema, M. P., and Van der Zee, E. A. (2013). Circadian clocks and memory: time-place learning. *Front. Mol. Neurosci.* 6:8. doi: 10.3389/fnmol.2013.00008

Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M. W. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* 271, 1736–1740. doi: 10.1126/science.271.5256.1736

Nagari, M., and Bloch, G. (2012). The involvement of the antennae in mediating the brood influence on circadian rhythms in "nurse" honey bee (*Apis mellifera*) workers. *J. Insect. Physiol.* 58, 1096–1103. doi: 10.1016/j.jinsphys.2012.05.007

Nagari, M., Brenner, Y., and Bloch, G. (2017a). Nurse honeybee workers tend capped-brood, which does not require feeding, around-the-clock. *J. Exp. Biol.* 220, 4130–4140. doi: 10.1242/jeb.166884

Nagari, M., Szyszka, P., Galizia, G., and Bloch, G. (2017b). Task-related phasing of circadian rhythms in antennal responsiveness to odorants and pheromones in honeybees. *J. Biol. Rhythms* 32.6, 593–608. doi: 10.1177/0748730417733573

Nagy, D., Andreatta, G., Bastianello, S., Martín Anduaga, A., Mazzotta, G., Kyriacou, C. P., et al. (2018). A semi-natural approach for studying seasonal diapause in *Drosophila melanogaster* reveals robust photoperiodicity. *J. Biol. Rhythms* 33, 117–125. doi: 10.1177/0748730417754116

Nagy, D., Cusumano, P., Andreatta, G., Anduaga, A. M., Hermann-Luibl, C., Reinhard, N., et al. (2019). Peptidergic signaling from clock neurons regulates reproductive dormancy in *Drosophila melanogaster*. *PLoS Genet* 15, 1–25. doi: 10.1371/journal.pgen.1008158

Naidoo, N. (1999). A role for the proteasome in the light response of the Timeless clock protein. *Science* 285, 1737–1741. doi: 10.1126/science.285.5434.1737

Narasaki-Funo, Y., Tomiyama, Y., Nose, M., Bando, T., and Tomioka, K. (2020). Functional analysis of *Pdp1* and *vrille* in the circadian system of a cricket. *J. Insect. Physiol.* 2020:104156 doi: 10.1016/j.jinsphys.2020.104156

Nässel, D. R., Shiga, S., Mohrherr, C. J., and Rao, K. R. (1993). Pigment-dispersing hormone-like peptide in the nervous system of the flies *Phormia* and *Drosophila*: immunocytochemistry and partial characterization. *J. Comp. Neurol.* 331, 183–198. doi: 10.1002/cne.903310204

Ng, F. S., Tangredi, M. M., and Jackson, F. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Nishiitsutsuji-Uwo, J., and Pittendrigh, C. S. (1968). Central nervous system control of circadian rhythmicity in the cockroach. *J. Comp. Neurol.* 58, 14–46. doi: 10.1007/BF00302434

Noreen, S., Pegoraro, M., Nouroz, F., Tauber, E., and Kyriacou, C. P. (2018). Interspecific studies of circadian genes *period* and *timeless* in *Drosophila*. *Gene* 648, 106–114. doi: 10.1016/j.gene.2018.01.020

Nose, M., Tokuoka, A., Bando, T., and Tomioka, K. (2018). timeless2 plays an important role in reproduction and circadian rhythms in the cricket *Gryllus bimaculatus*. *J. Insect. Physiol.* 105, 9–17. doi: 10.1016/j.jinsphys.2017.12.007

Ojima, N., Hara, Y., Ito, H., and Yamamoto, D. (2018). Genetic dissection of stress-induced reproductive arrest in *Drosophila melanogaster* females. *PLoS Genetics* 14, 1–15. doi: 10.1371/journal.pgen.1007434

Özkaya, Ö., and Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. *Adv. Genet.* 77, 79–123. doi: 10.1016/B978-0-12-387687-4.00004-0

Page, T. L. (1982). Transplantation of the cockroach circadian pacemaker. *Science* 216:73. doi: 10.1126/science.216.4541.73

Pahl, M., Zhu, H., Pix, W., Tautz, J., and Zhang, S. (2007). Circadian timed episodic-like memory a bee knows what to do when, and also where. *J. Exp. Biol.* 210, 3559–3567. doi: 10.1242/jeb.005488

Paolucci, S., Dalla Benetta, E., Salis, L., DoleŽel, D., van de Zande, L., and Beukeboom, L. (2019). Latitudinal variation in circadian rhythmicity in *Nasonia vitripennis*. *Behav. Sci.* 9:115. doi: 10.3390/bs9110115

Park, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3608–3613. doi: 10.1073/pnas.97.7.3608

Pegel, U., Pfeiffer, K., Zittrell, F., Scholtyssek, C., and Homberg, U. (2019). Two compasses in the central complex of the locust brain. *J. Neurosci.* 39, 3070–3080. doi: 10.1523/JNEUROSCI.0940-18.2019

Pegoraro, M., Zonato, V., Tyler, E. R., Fedele, G., Kyriacou, C. P., and Tauber, E. (2017). Geographical analysis of diapause inducibility in European *Drosophila melanogaster* populations. *J. Insect Physiol.* 98, 238–244. doi: 10.1016/j.jinsphys.2017.01.015

Petri, B., and Stengl, M. (1997). Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci.* 17, 4087–4093. doi: 10.1523/JNEUROSCI.17-11-04087.1997

Pírez, N., Bernabei-Cornejo, S. G., Fernandez-Acosta, M., Duhart, J. M., and Ceriani, M. F. (2019). Contribution of non-circadian neurons to the temporal organization of locomotor activity. *Biol. Open* 8:bio039628. doi: 10.1242/bio.039628

Pírez, N., Christmann, B. L., and Griffith, L. C. (2013). Daily rhythms in locomotor circuits in *Drosophila* involve PDF. *J. Neurophysiol.* 110, 700–708. doi: 10.1152/jn.00126.2013

Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 40, 1018–1029. doi: 10.1073/pnas.40.10.1018

Reischig, T., Petri, B., and Stengl, M. (2004). Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. *Cell Tissue Res.* 318, 553–564. doi: 10.1007/s00441-004-0927-1

Reischig, T., and Stengl, M. (2003). Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). *J. Exp. Biol.* 206, 1877–1886. doi: 10.1242/jeb.00373

Renn, S. C. P., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999). A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 99, 791–802. doi: 10.1016/S0092-8674(00)81676-1

Reppert, S. M., Guerra, P. A., and Merlin, C. (2016). Neurobiology of monarch butterfly migration. *Annu. Rev. Entomol.* 61, 25–42. doi: 10.1146/annurev-ento-010814-020855

Reznik, S. (2011). Ecological and evolutionary aspects of photothermal regulation of diapause in Trichogrammatidae. *J Evol Biochem Phys* 47, 512–523. doi: 10.1134/S0022093011060020

Richier, B., Michard-Vanhée, C., Lamouroux, A., Papin, C., and Rouyer, F. (2008). The Clockwork Orange *Drosophila* protein functions as both an activator and a repressor of clock gene expression. *J. Biol. Rhythms* 23, 103–116. doi: 10.1177/0748730407313817

Rivas, G. B. S., Teles-de-Freitas, R., Pavan, M. G., Lima, J. B. P., Peixoto, A. A., and Bruno, R. V. (2018). Effects of light and temperature on daily activity and clock gene expression in two mosquito disease vectors. *J. Biol. Rhythms.* 33, 272–288. doi: 10.1177/0748730418772175

Rodriguez-Sanchez, I. P., Villanueva-Segura, O. K., Gonzalez-Alvarez, R., Flores-Suarez, A. E., Garza-Rodriguez, M., de, L., et al. (2015). Molecular cloning and

characterization of the circadian clock *Timeless* gene in *Culex quinquefasciatus* Say (Diptera: Culicidae). *South. Entomol.* 40, 53–70. doi: 10.3958/059.040.0105

Rodriguez-Zas, S. L., Southey, B. R., Shemesh, Y., Rubin, E. B., Cohen, M., Robinson, G. E., et al. (2012). Microarray analysis of natural socially regulated plasticity in circadian rhythms of honey bees. *J. Biol. Rhythms* 27, 12–24. doi: 10.1177/0748730411431404

Rooke, R., Rasool, A., Schneider, J., and Levine, J. D. (2020). *Drosophila melanogaster* behaviour changes in different social environments based on group size and density. *Commun. Biol.* 3:304. doi: 10.1038/s42003-020-1024-z

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., Costa, R., et al. (2001). Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. *Curr. Biol.* 11, 909–917. doi: 10.1016/S0960-9822(01)00259-7

Rubin, E. B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H. M., and Bloch, G. (2006). Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome Res.* 16, 1352–1365. doi: 10.1101/gr.5094806

Ruf, F., Mitesser, O., Mungwa, S. T., Horn, M., Rieger, D., Hovestadt, T., et al. (2019). Natural Zeitgebers cannot compensate for the loss of a functional circadian clock in timing of a vital behaviour in *Drosophila*. *Anim. Behav. Cogn. bioRxiv [Preprint]*. doi: 10.1101/2019.12.22.886309

Rund, S. S. C., Hou, T. Y., Ward, S. M., Collins, F. H., and Duffield, G. E. (2011). Genome-wide profiling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U. S. A.* 108:E421. doi: 10.1073/pnas.1100584108

Sadd, B. M., Barribeau, S. M., Bloch, G., de Graaf, D. C., Dearden, P., Elsik, C. G., et al. (2015). The genomes of two key bumblebee species with primitive eusocial organization. *Genome Biol.* 16:76. doi: 10.1186/s13059-015-0623-3

Saez, L., and Young, M. W. (1996). Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. *Neuron* 17, 911–920. doi: 10.1016/S0896-6273(00)80222-6

Sakai, T., and Ishida, N. (2001). Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9221–9225. doi: 10.1073/pnas.151443298

Sakai, T., Tamura, T., Kitamoto, T., and Kidokoro, Y. (2004). A clock gene, *period*, plays a key role in long-term memory formation in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16058–16063. doi: 10.1073/pnas.0401472101

Sandrelli, F., Costa, R., Kyriacou, C. P., and Rosato, E. (2008). Comparative analysis of circadian clock genes in insects. *Insect. Mol. Biol.* 17, 447–463. doi: 10.1111/j.1365-2583.2008.00832.x

Sandrelli, F., Tauber, E., Pegoraro, M., Mazzotta, G., Cisotto, P., Landskron, J., et al. (2007). A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster*. *Science* 316, 1898–1900. doi: 10.1126/science.1138426

Sato, S., Chuman, Y., Matsushima, A., Tominaga, Y., Shimohigashi, Y., and Shimohigashi, M. (2002). A circadian neuropeptide, pigment-dispersing factor–PDF, in the last-summer cicada *Meimuna opalifera*: cDNA cloning and immunocytochemistry. *Zool. Sci.* 19, 821–828. doi: 10.2108/zsj.19.821

Sauer, S., Kinkelin, M., Herrmann, E., and Kaiser, W. (2003). The dynamics of sleep-like behaviour in honey bees. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 189, 599–607. doi: 10.1007/s00359-003-0436-9

Saunders, D. S., Vincent, C. H., and Lawrence, I. G. (1989). Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. U. S. A.* 86, 3748–3752. doi: 10.1073/pnas.86.10.3748

Schäbler, S., Amatobi, K. M., Horn, M., Rieger, D., Helfrich-Förster, C., Mueller, M. J., et al. (2020). Loss of function in the *Drosophila* clock gene period results in altered intermediary lipid metabolism and increased susceptibility to starvation. *Cell. Mol. Life Sci.* 77, 1–18. doi: 10.1007/s00018-019-03441-6

Schlichting, M., Menegazzi, P., Lelito, K. R., Yao, Z., Buhl, E., Dalla Benetta, E., et al. (2016). A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. *J. Neurosci.* 36, 9084–9096. doi: 10.1523/JNEUROSCI.0992-16.2016

Schlichting, M., Rieger, D., Cusumano, P., Grebler, R., Costa, R., Mazzotta, G. M., et al. (2018). Cryptochrome interacts with actin and enhances eye-mediated light sensitivity of the circadian clock in *Drosophila melanogaster*. *Front. Mol. Neurosci.* 11:238. doi: 10.3389/fnmol.2018.00238

Schubert, F. K., Hagedorn, N., Yoshii, T., Helfrich-Förster, C., and Rieger, D. (2018). Neuroanatomical details of the lateral neurons of *Drosophila melanogaster* support their functional role in the circadian system. *J. Comp. Neurol.* 526, 1209–1231. doi: 10.1002/cne.24406

Seay, D. J., and Thummel, C. S. (2011). The circadian clock, light, and cryptochrome regulate feeding and metabolism in *Drosophila*. *J. Biol. Rhythms.* 26, 497–506. doi: 10.1177/0748730411420080

Sehgal, A., Price, J., Man, B., and Young, M. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science* 263, 1603–1606. doi: 10.1126/science.8128246

Selcho, M., Millán, C., Palacios-Muñoz, A., Ruf, F., Ubillo, L., Chen, J., et al. (2017). Central and peripheral clocks are coupled by a neuropeptide pathway in *Drosophila*. *Nat. Commun.* 8:15563. doi: 10.1038/ncomms15563

Shafer, O. T., Kim, D. J., Dunbar-Yaffe, R., Nikolaev, V. O., Lohse, M. J., and Taghert, P. H. (2008). Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. *Neuron* 58, 223–237. doi: 10.1016/j.neuron.2008.02.018

Shafer, O. T., and Yao, Z. (2014). Pigment-dispersing factor signaling and circadian rhythms in insect locomotor activity. *Curr. Opin. Insect Sci.* 1, 73–80. doi: 10.1016/j.cois.2014.05.002

Shaikevich, E. V., Zakharov, I. S., and Fedorova, M. V. (2016). Comparative analysis of the circadian rhythm genes *period* and *timeless* in Culex pipiens Linnaeus, 1758 (Diptera, Culicidae). *CCG* 10, 483–504. doi: 10.3897/CompCytogen.v10i4.7582

Sheeba, V., Chandrashekaran, M. K., Joshi, A., and Kumar Sharma, V. (2001). A case for multiple oscillators controlling different circadian rhythms in *Drosophila melanogaster*. *J. Insect. Physiol.* 47, 1217–1225. doi: 10.1016/S0022-1910(01)00107-X

Shell, W. A., and Rehan, S. M. (2018). Behavioral and genetic mechanisms of social evolution: insights from incipiently and facultatively social bees. *Apidologie* 49, 13–30. doi: 10.1007/s13592-017-0527-1

Shemesh, Y., Cohen, M., and Bloch, G. (2007). Natural plasticity in circadian rhythms is mediated by reorganization in the molecular clockwork in honeybees. *FASEB J.* 21, 2304–2311. doi: 10.1096/fj.06-8032com

Shemesh, Y., Eban-Rothschild, A., Cohen, M., and Bloch, G. (2010). Molecular dynamics and social regulation of context-dependent plasticity in the circadian clockwork of the honey bee. *J. Neurosci.* 30, 12517–12525. doi: 10.1523/JNEUROSCI.1490-10.2010

Shiga, S., and Numata, H. (2009). Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, *Protophormia terraenovae*. *J. Exp. Biol.* 212, 867–877. doi: 10.1242/jeb.027003

Simoni, A., Wolfgang, W., Topping, M. P., Kavlie, R. G., Stanewsky, R., and Albert, J. T. (2014). A mechanosensory pathway to the *Drosophila* circadian clock. *Science* 343, 525–528. doi: 10.1126/science.1245710

Singaravel, M., Fujisawa, Y., Hisada, M., Saifullah, A. S. M., and Tomioka, K. (2003). Phase shifts of the circadian locomotor rhythm induced by pigment-dispersing factor in the cricket *Gryllus bimaculatus*. *Zool. Sci.* 20, 1347–1354. doi: 10.2108/zsj.20.1347

Siwicki, K. K., Petersen, G., Rosbash, M., and Hall, J.C. (1988). Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1, 141–150. doi: 10.1016/0896-6273(88)90198-5

Smith, A. R., Kitchen, S. M., Toney, R. M., and Ziegler, C. (2017). Is nocturnal foraging in a tropical bee an escape from interference competition? *J. Insect Sci.* 17:30. doi: 10.1093/jisesa/iex030

Stehlík, J., Závodská, R., Shimada, K., Šauman, I., and Koštál, V. (2008). Photoperiodic induction of diapause requires regulated transcription of timeless in the larval brain of *Chymomyza costata*. *J. Biol. Rhythms* 23, 129–139. doi: 10.1177/0748730407313364

Stoleru, D., Peng, Y., Nawathean, P., and Rosbash, M. (2005). A resetting signal between *Drosophila* pacemakers synchronizes morning and evening activity. *Nature* 438, 238–242. doi: 10.1038/nature04192

Sumiyoshi, M., Sato, S., Takeda, Y., Sumida, K., Koga, K., Itoh, T., et al. (2011). A circadian neuropeptide PDF in the honeybee, *Apis mellifera* : cDNA cloning and expression of mRNA. *Zool. Sci.* 28, 897–909. doi: 10.2108/zsj.28.897

Syrová, Z., DoleŽel, D., Šaumann, I., and Hodková, M. (2003). Photoperiodic regulation of diapause in linden bugs: are *period* and *Clock* genes involved? *Cell. Mol. Life Sci.* 60, 2510–2515. doi: 10.1007/s00018-003-3227-0

Takekata, H., Matsuura, Y., Goto, S. G., Satoh, A., and Numata, H. (2012). RNAi of the circadian clock gene *period* disrupts the circadian rhythm but

not the circatidal rhythm in the mangrove cricket. *Biol. Lett.* 8, 488–491. doi: 10.1098/rsbl.2012.0079

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C., et al. (2007). Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. *Science* 316, 1895–1898. doi: 10.1126/science.1138412

Toda, H., Shi, M., Williams, J. A., and Sehgal, A. (2018). Genetic mechanisms underlying sleep. *Cold. Spring Harb. Symp. Quant. Biol.* 83, 57–61. doi: 10.1101/sqb.2018.83.037705

Tokuoka, A., Itoh, T. Q., Hori, S., Uryu, O., Danbara, Y., Nose, M., et al. (2017). cryptochrome genes form an oscillatory loop independent of the per/tim loop in the circadian clockwork of the cricket *Gryllus bimaculatus*. *Zool. Lett* 3:5. doi: 10.1186/s40851-017-0066-7

Tomioka, K., and Matsumoto, A. (2015). Circadian molecular clockworks in non-model insects. *Curr. Opin. Insect Sci.* 7, 58–64. doi: 10.1016/j.cois.2014.12.006

Top, D., and Young, M. W. (2018). Coordination between differentially regulated circadian clocks generates rhythmic behavior. *Cold Spring Harb. Perspect. Biol.* 10:a033589. doi: 10.1101/cshperspect.a033589

Tweedy, D. G., and Stephen, W. P. (1970). Light refractive emergence rhythm in leafcutter bee, *Megachile rotundata* (F.) (Hymenoptera:Apoidea). *Cell. Mol. Life Sci.* 26, 377–379. doi: 10.1007/BF01896898

Uryu, O., Karpova, S. G., and Tomioka, K. (2013). The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. *J. Insect Physiol.* 59, 697–704. doi: 10.1016/j.jinsphys.2013.04.011

Vafopoulou, X., Terry, K. L., and Steel, C. G. H. (2009). The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J. Comp. Neurol.* 518, 1264–1282. doi: 10.1002/cne.22274

Vanin, S., Bhutani, S., Montelli, S., Menegazzi, P., Green, E. W., Pegoraro, M., et al. (2012). Unexpected features of *Drosophila* circadian behavioural rhythms under natural conditions. *Nature* 484, 371–375. doi: 10.1038/nature10991

Vaze, K. M., and Helfrich-Förster, C. (2016). *Drosophila ezoana* uses an hour-glass or highly damped circadian clock for measuring night length and inducing diapause: Diapause induction in *D. ezoana*. *Physiol. Entomol.* 41, 378–389. doi: 10.1111/phen.12165

Veenstra, J. A. (2019). Coleoptera genome and transcriptome sequences reveal numerous differences in neuropeptide signaling between species. *PeerJ* 7:e7144. doi: 10.7717/peerj.7144

Velarde, R. A., Sauer, C. D. O., Walden, K. K., Fahrbach, S. E., and Robertson, H.M. (2005). Pteropsin: a vertebrate-like non-visual opsin expressed in the honey bee brain. *Insect Biochem. Mol. Biol.* 35, 1367–1377. doi: 10.1016/j.ibmb.2005.09.001

Wahl, O. (1933). Beitrag zur Frage der biologischen Bedeutung des Zeitgedächtnisses der Bienen. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 18, 709–717.

Warren, T. L., Giraldo, Y. M., and Dickinson, M. H. (2019). Celestial navigation in *Drosophila*. *J. Exp. Biol.* 222:jeb186148. doi: 10.1242/jeb.186148

Watari, Y. (2005). Comparison of the circadian eclosion rhythm between non-diapause and diapause pupae in the onion fly, *Delia antiqua*: the change of rhythmicity. *J. Insect Physiol.* 51, 11–16. doi: 10.1016/j.jinsphys.2004.09.013

Watari, Y., and Tanaka, K. (2010). Interacting effect of thermoperiod and photoperiod on the eclosion rhythm in the onion fly, *Delia antiqua* supports the two-oscillator model. *J. Insect. Physiol.* 56, 1192–1197. doi: 10.1016/j.jinsphys.2010.03.022

Wcislo, W. T., and Tierney, S. M. (2009). Behavioural environments and niche construction: the evolution of dim-light foraging in bees. *Biol. Rev.* 84, 19–37. doi: 10.1111/j.1469-185X.2008.00059.x

Wei, H., Yasar, H., Funk, N. W., Giese, M., Baz, E.-S., and Stengl, M. (2014). Signaling of Pigment-Dispersing Factor (PDF) in the Madeira cockroach *Rhyparobia maderae*. *PLoS ONE* 9:e108757. doi: 10.1371/journal.pone.0108757

Weiss, R., Dov, A., Fahrbach, S. E., and Bloch, G. (2009). Body size-related variation in pigment dispersing factor-immunoreactivity in the brain of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). *J. Insect. Physiol.* 55, 479–487. doi: 10.1016/j.jinsphys.2009.01.016

Werckenthin, A., Derst, C., and Stengl, M. (2012). Sequence and expression of *per, tim1* , and *cry2* genes in the madeira cockroach *Rhyparobia maderae*. *J. Biol. Rhythms* 27, 453–466. doi: 10.1177/0748730412462109

Werckenthin, A., Huber, J., Arnold, T., Koziarek, S., Plath, M. J. A., Plath, J. A., et al. (2020). Neither *per*, nor *tim1*, nor *cry2* alone are essential components of the molecular circadian clockwork in the madeira cockroach. *PLoS ONE.* 15:e0235930. doi: 10.1371/journal.pone.0235930

Wise, S., Davis, N. T., Tyndale, E., Noveral, J., Folwell, M. G., Bedian, V., et al. (2002). Neuroanatomical studies of *period* gene expression in the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* 447, 366–380. doi: 10.1002/cne.10242

Xu, K., DiAngelo, J. R., Hughes, M. E., Hogenesch, J. B., and Sehgal, A. (2011). The circadian clock interacts with metabolic physiology to influence reproductive fitness. *Cell Metabol.* 13, 639–654. doi: 10.1016/j.cmet.2011.05.001

Yadlapalli, S., Jiang, C., Bahle, A., Reddy, P., Meyhofer, E., and Shafer, O. T. (2018). Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature* 555, 98–102. doi: 10.1038/nature25740

Yamada, H., and Yamamoto, M.-T. (2011). Association between circadian clock genes and diapause incidence in *Drosophila triauraria*. *PLoS ONE* 6, 1–9. doi: 10.1371/journal.pone.0027493

Yerushalmi, S., Bodenhaimer, S., and Bloch, G. (2006). Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees. *J. Exp. Biol.* 209, 1044–1051. doi: 10.1242/jeb.02125

Yocum, G. D., Rinehart, J. P., Yocum, I. S., Kemp, W. P., and Greenlee, K. J. (2016). Thermoperiodism synchronizes emergence in the alfalfa leafcutting bee (Hymenoptera: Megachilidae). *Environ. Entomol.* 45, 245–251. doi: 10.1093/ee/nvv152

Yoshii, T., Hermann, C., and Helfrich-Förster, C. (2010). Cryptochrome-positive and -negative clock neurons in *Drosophila* entrain differentially to light and temperature. *J. Biol. Rhythms* 25, 387–398. doi: 10.1177/0748730410381962

Yoshii, T., Rieger, D., and Helfrich-F?rster, C. (2012). Two clocks in the brain: an update of the morning and evening oscillator model in *Drosophila*. *Progress Brain Res.* 199, 59–82. doi: 10.1016/B978-0-444-59427-3.00027-7

Yoshii, T., Vanin, S., Costa, R., and Helfrich-Förster, C. (2009b). Synergic entrainment of *Drosophila's* circadian clock by light and temperature. *J. Biol. Rhythms* 24, 452–464. doi: 10.1177/0748730409348551

Yoshii, T., Wulbeck, C., Sehadova, H., Veleri, S., Bichler, D., Stanewsky, R., et al. (2009a). The neuropeptide pigment-dispersing factor adjusts period and phase of *Drosophila's* clock. *J. Neurosci.* 29, 2597–2610. doi: 10.1523/JNEUROSCI.5439-08.2009

Yuan, Q., Metterville, D., Briscoe, A. D., and Reppert, S. M. (2007). Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol. Biol. Evol.* 24, 948–955. doi: 10.1093/molbev/msm011

Yurgel, M. E., Kakad, P., Zandawala, M., Nässel, D. R., Godenschwege, T. A., and Keene, A. C. (2019). A single pair of leucokinin neurons are modulated by feeding state and regulate sleep–metabolism interactions. *PLoS Biol.* 17, 1–26. doi: 10.1371/journal.pbio.2006409

Zandawala, M., Yurgel, M. E., Liao, S., Texada, M. J., Rewitz, K. F., Keene, A. C., et al. (2018). Orchestration of *Drosophila* post-feeding physiology and behavior by the neuropeptide leucokinin. *BioRxiv* 355107. doi: 10.1101/355107

Závodská, R., Šauman, I., and Sehnal, F. (2003). Distribution of PER protein, Pigment-Dispersing Hormone, Prothoracicotropic Hormone, and Eclosion Hormone in the cephalic nervous system of insects. *J. Biol. Rhythms* 18, 106–122. doi: 10.1177/0748730403251711

Ždárek, J., and Denlinger, D.L. (1995). Changes in temperature, not photoperiod control the pattern of adult eclosion in the tsetse, *Glossina morsitans*. *Physiol. Entomol.* 20, 362–366. doi: 10.1111/j.1365-3032.1995.tb00827.x

Zeller, M., Held, M., Bender, J., Berz, A., Heinloth, T., Hellfritz, T., et al. (2015). Transmedulla neurons in the sky compass network of the honeybee (*Apis mellifera*) are a possible site of circadian input. *PLoS ONE* 10:e0143244. doi: 10.1371/journal.pone.0143244

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* 10, 2749–2762. doi: 10.1523/JNEUROSCI.10-08-02749.1990

Zheng, X., Koh, K., Sowcik, M., Smith, C. J., Chen, D., Wu, M. N., et al. (2009). An Isoform-specific mutant reveals a role of PDP1 in the circadian oscillator. *J. Neurosci.* 29, 10920–10927. doi: 10.1523/JNEUROSCI.2133-09.2009

Zhou, J., Yu, W., and Hardin, P. E. (2016). clockwork orange enhances period mediated rhythms in transcriptional repression by antagonizing E-box Binding by clock-cycle. *PLoS Genet* 12:e1006430. doi: 10.1371/journal.pgen.1006430

Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P., et al. (2008). Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biol.*6:e4. doi: 10.1371/journal.pbio.0060004

Zhu, H., Yuan, Q., Froy, O., Casselman, A., and Reppert, S. M. (2005). The two CRYs of the butterfly. *Curr. Biol.* 15:2. doi: 10.1016/j.cub.2005.11.030

Zimmerman, W. F., Pittendrigh, C. S., and Pavlidis, T. (1968). Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. *J. Insect. Physiol.* 14, 669–684. doi: 10.1016/0022-1910(68)90226-6

Zonato, V., Vanin, S., Costa, R., Tauber, E., and Kyriacou, C. P. (2018). Inverse european latitudinal cline at the *timeless* locus of *Drosophila melanogaster* reveals selection on a clock gene: population genetics of *ls-tim*. *J. Biol. Rhythms* 33, 15–23. doi: 10.1177/0748730417742309

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CK declared a past co-authorship with one of the authors CH-F to the handling Editor

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 03 December 2020
doi: 10.3389/fnbeh.2020.600737

The Monarch Butterfly as a Model for Understanding the Role of Environmental Sensory Cues in Long-Distance Migratory Phenomena

*Patrick A. Guerra**

Department of Biological Sciences, College of Arts and Sciences, University of Cincinnati, Cincinnati, OH, United States

The awe-inspiring annual migration of monarch butterflies (*Danaus plexippus*) is an iconic example of long-distance migratory phenomena in which environmental sensory cues help drive successful migration. In this mini-review article, I begin by describing how studies on monarch migration can provide us with generalizable information on how sensory cues can mediate key aspects of animal movement. I describe how environmental sensory cues can trigger the development and progression of the monarch migration, as well as inform sensory-based movement mechanisms in order to travel to and reach their goal destination, despite monarchs being on their maiden voyage. I also describe how sensory cues can trigger season-appropriate changes in migratory direction during the annual cycle. I conclude this mini-review article by discussing how contemporary environmental challenges threaten the persistence of the monarch migration. Environmental challenges such as climate change and shifting land use can significantly alter the sensory environments that monarchs migrate through, as well as degrade or eliminate the sources of sensory cues that are necessary for successful migration.

Keywords: monarch butterfly, animal migration, migratory syndrome, sensory cue degradation, sensory pollution

OPEN ACCESS

Edited by:
Clare C. Rittschof,
University of Kentucky, United States

Reviewed by:
Uwe Homberg,
University of Marburg, Germany
Nathan Freeman Putman,
LGL (United States), United States

***Correspondence:**
Patrick A. Guerra
patrick.guerra@uc.edu

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *31 August 2020*
Accepted: *12 November 2020*
Published: *03 December 2020*

Citation:
Guerra PA (2020) The Monarch Butterfly as a Model for Understanding the Role of Environmental Sensory Cues in Long-Distance Migratory Phenomena. Front. Behav. Neurosci. 14:600737. doi: 10.3389/fnbeh.2020.600737

INTRODUCTION

Sensory Ecology of Long-Distance Animal Migration

In many animal species, individuals can exhibit locomotory behavior and movement patterns across varying temporal (e.g., from seconds to years) and spatial (e.g., from local natal patches to round-the-world journeys) scales. For many of these phenomena, the movement of individuals is goal-driven, such that individuals are moving to travel to specific locations that contain key resources that are often necessary for survival or that can promote individual fitness. Long-distance migration is an example of goal-oriented animal movement phenomena that typically occurs seasonally, with individuals undergoing journeys that can span thousands of miles. Migration can be an adaptive strategy, as individuals travel to take advantage of seasonally available resources found at different locations, such as specific plant hosts, shelters, feeding areas, or breeding grounds. Also, individuals can migrate to escape predictably deteriorating habitats for locations with more hospitable environmental conditions, and then return to their original habitats once conditions have improved or have returned to normal (Dingle, 2014).

Environmental sensory cues can strongly mediate and modulate the goal-directed migratory movement of individuals. For instance, sensory cues that occur with specific timing and that are correlated with the arrival of deteriorating conditions can trigger the development of phenotypic traits in individuals that facilitate migratory movement, as well as initiate the onset of migration. To travel to and reach their destination during migration, individuals will often use or must rely on sensory cues that they also derive from their environment. These sensory cues can vary in both their form and function. For example, individuals might rely on a single cue that can reliably direct their movement towards their goal when still very far away. Once near their destination, individuals might then use sensory cues as guideposts that trigger other behaviors or sensory processes for finding their goal. These sensory cues might also serve as beacons of the destination itself, thereby allowing migrants to recognize, localize, and stop at their goal (Reppert et al., 2010; Mouritsen, 2018). Finally, sensory cues can inform individuals as to if and when they can remigrate back.

Monarch Butterfly Long-Distance Migration

The annual multigenerational migratory cycle of the monarch butterfly (*Danaus plexippus*) is an iconic example of long-distance animal movement phenomena. Found in many different parts of the world, perhaps the most famous population of this species consists of the butterflies that live east of the Rocky Mountains in North America. Each fall, millions of monarchs in Eastern North America leave their summer breeding grounds in Southern Canada and the Northern United States and fly southwards to migrate to their overwintering areas in Central Mexico. These overwintering sites consist of a handful of coniferous fir groves (oyamel) high atop the Transvolcanic Mountains in the state of Michoacán upon which butterflies will aggregate and roost during the winter (Urquhart, 1987). Upon the arrival of spring, these same monarchs leave the overwintering sites, flying northwards to return and start repopulating the southern tier of the United States. The offspring of these spring remigrants, i.e., spring populations of butterflies, continue the migratory cycle by flying northwards. The migratory cycle ends with a summer generation of non-migratory butterflies that repopulates the most northern regions of the monarch habitat range. This migratory cycle begins anew when the next generation of monarchs flies southwards in the fall (Reppert et al., 2016). A similar, albeit smaller scale fall migration occurs with the population of monarchs that live west of the Rocky Mountains. Monarchs of the Pacific Northwest and Northern California fly southwards to overwintering sites along the Pacific Coast in California. In contrast to the overwintering fir groves in Mexico, Western monarchs overwinter on evergreen Monterey Pine and Eucalyptus trees (Reppert and de Roode, 2018). In the spring, remigrants leave the overwintering sites, and successive generations fly northwards to repopulate the habitat range. Fall monarchs from the Southwestern United States also migrate, with monarchs reaching overwintering sites in either California or Mexico (Morris et al., 2015). Outside of North America, monarchs in Eastern Australia can also migrate to seasonally appropriate habitats, in the same manner as their counterparts in the Northern Hemisphere (James and James, 2019; Nail et al., 2019). This group of fall migrants will roost on trees (e.g., native prickly paperbark) that are different from those used by monarchs in either Eastern or Western North America (James, 1993; Nail et al., 2019).

In contrast to these regions with populations of monarchs that display directional flight and migrate, other monarchs can be found in several areas around the world in which they are considered non-migratory, e.g., Florida, Hawaii, and New Zealand. These monarchs can be found as year-round residents or will engage in winter breeding (Reppert and de Roode, 2018; Nail et al., 2019). Although monarchs from these populations have been observed to fly only short distances relative to conspecifics that migrate (e.g., monarchs in New Zealand; Wise, 1980), it remains unknown if these individuals also display oriented flight, especially flight in the seasonally appropriate direction, the hallmark trait of migratory monarchs. It is possible that monarchs from these populations display directional flight, but the distances of their flights are simply limited by geographical constraints, e.g., living on a relatively small island in the middle of the ocean. Although it is possible that traits associated with migration, e.g., oriented flight behavior, can be quickly selected out to produce populations of migratory species that are non-migratory, such traits might remain in the population due to evolutionary inertia (Alerstam, 2006) or exist despite large differences in the movement ecology of populations (Scanlan et al., 2018). For instance, translocated nonanadromous Atlantic salmonids with no recent history of migration, can display similar directed responses to local orientation cues as native Pacific salmonids (Scanlan et al., 2018). Monarchs from populations now considered non-migratory might retain and still be capable of using orientation mechanisms like migratory conspecifics in a similar manner. Behavioral studies assaying the flight orientation of putative non-migratory monarchs at these locations, e.g., flight simulator trials (Mouritsen and Frost, 2002), can address this.

Role of Environmental Sensory Cues in Monarch Butterfly Migration

Research using the monarch as a model system has provided useful and generalizable information on animal migration at different mechanistic levels, from the behavioral, neural, molecular, and genetic substrates of this phenomenon (Reppert et al., 2016; Reppert and de Roode, 2018; Merlin et al., 2020). In particular, previous studies have demonstrated the key role of environmental sensory cues for successful migration, with sensory cues playing a vital function at almost all stages of the monarch migratory cycle (Guerra and Reppert, 2015; **Figure 1**).

Environmental sensory cues are necessary for monarch migration to occur, as the sensing of cues correlated with the arrival of fall, i.e., decreasing photoperiod and cooler and fluctuating temperatures (Goehring and Oberhauser, 2002; Freedman et al., 2018), can help induce the monarch migratory syndrome in individuals. In contrast to summer monarchs that are non-migratory, fly non-directionally, and are

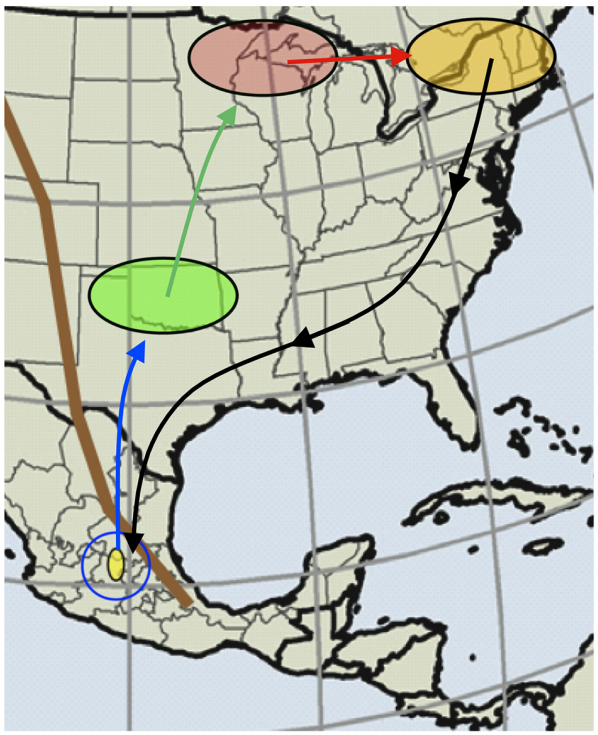

FIGURE 1 | Monarch butterflies use sensory cues to facilitate their annual multigenerational migratory cycle. Shown is the Eastern North American population of butterflies that live east of the Rocky Mountains (brown line). In the late summer and early fall, developing monarchs in the upper regions of the monarch habitat range sense environmental cues that induce the monarch migratory syndrome and that initiate the southwards fall migration (orange oval). These fall migrants use various sensory-based compass mechanisms to guide them southwards during their migratory journey (black line) and potentially use cues once close to their destination (blue circle) that allow them to locate and stop at the overwintering sites in Central Mexico (yellow oval). After receiving a cold trigger while overwintering that recalibrates their compass mechanisms for the return journey, these monarchs remigrate northwards during the spring (blue line). Spring monarchs (green oval), the offspring of spring remigrants, continue the migratory cycle by traveling northwards (green line). These spring monarchs potentially use the same navigational mechanisms as fall conspecifics, but that are calibrated by sensory cues during development for northwards flight instead (green oval). Successive generations of monarchs fly northwards until they repopulate the northern breeding grounds of the monarch range (red oval). The migratory cycle ends, once monarchs experience cues that either signal them to stop or that do not trigger oriented flight behavior (red oval). Summer butterflies repopulate the most northern areas of the monarch range (red oval), and once their offspring experience the necessary cues (orange oval), the migratory cycle begins anew. Figure modified from Guerra and Reppert (2015).

reproductively active (Zhu et al., 2009), monarchs that develop in the late summer and early fall can sense cues that induce the development of morphological (e.g., wings that are redder and have more melanization—Hanley et al., 2013; Satterfield and Davis, 2014; more elongated wings—Satterfield and Davis, 2014; larger forewings—Li et al., 2016), biochemical (e.g., lower juvenile hormone titers—Zhu et al., 2009), reproductive (i.e., diapause—Goehring and Oberhauser, 2002), and sensory traits (e.g., time-compensated sun compass use to maintain directional flight—Zhu et al., 2009) that can facilitate long-distance migration. The onset, timing, and pace of the migration appear to also be regulated by environmental sensory cues. For instance, the timing and pace of the fall migration in Eastern North America are associated with migratory monarchs sensing specific celestial cues (i.e., the sun's position in the sky, specifically the sun angle at solar noon) and environmental parameters (temperature and daylength; Taylor et al., 2019).

Although on their maiden voyage, fall migrants are capable of traveling to their overwintering destinations by using various innate sensory-based orientation mechanisms to guide migratory flight (compass sense—Reppert et al., 2016). Eastern North American fall monarchs can use a time-compensated sun compass, the dominant orientation mechanism of migratory monarchs, to maintain proper southwards flight directionality (Perez et al., 1997; Mouritsen and Frost, 2002; Froy et al., 2003). Monarchs use the sun's position in the daytime sky as a visual cue to maintain a southwards flight orientation. To correct for the apparent movement of the sun across the sky throughout the day, monarchs use timing information derived from antennal circadian clocks that are entrained to local photoperiodic conditions, to compensate for the sun's movement (Merlin et al., 2009; Guerra et al., 2012). Interestingly, recent work has shown that even non-migratory monarchs can use such sun visual cues for orientation during flight (Franzke et al., 2020). On overcast days, a time when the sun's position is occluded, Eastern North American fall migrants can use a magnetic compass as a backup for maintaining southwards directionality. In contrast to the more familiar magnetic compass that distinguishes North from South by measuring the polarity of geomagnetic field lines to compare North vs. South (a "polarity compass"), the monarch magnetic compass utilizes the inclination angle of the geomagnetic field as a cue for directionality (an "inclination compass"). Here, monarchs can sense how geomagnetic field lines intersect the Earth's surface, with field lines ranging from parallel to the Earth's surface at the equator (0° inclination angle), to field lines intersecting the Earth's surface perpendicularly at either pole (90° inclination angle). As the inclination angle of the geomagnetic field predictably covaries with latitude, fall migrants can determine if they are flying either equatorward or polewards (Guerra et al., 2014). In addition to using inclination angle cues for directionality, this can allow migratory animals with a magnetic sense to use these cues as part of a geomagnetic coordinate system that can provide positional or map information during migration (Mouritsen, 2018). To detect magnetic fields, monarchs require exposure to ultraviolet A/B light wavelengths, with the putative magnetosensors located in the antennae (Guerra et al., 2014). Though the flight directionality and compass use of fall migrants in Western North America and Australia have yet to be directly tested, it is highly probable that butterflies in these regions also use the same sensory cues for flight directionality during migration, and employ these compass mechanisms with the same morphological substrates (Merlin et al., 2009; Heinze and Reppert, 2012), neural circuitry (Guerra et al., 2012; Heinze et al., 2013; Shlizerman et al., 2016), and genetic architecture (Zhan et al., 2014).

Compass senses only provide directional information and do not allow monarchs to know where they are relative to their goal. As each generation of fall monarchs is naïve to the location of the overwintering sites, monarchs must possess innate mechanisms that allow them to find and stop at these locations. It remains a great mystery of how monarchs achieve this goal-oriented task each year. A possible mechanism is *via* a map sense that can provide positional information, with one type involving monarchs using their magnetic sense (Guerra et al., 2014) for identifying the specific geomagnetic signatures of the overwintering sites. The recognition and localization of the overwintering grounds by sensing magnetic cues correlated with these locations (a type of beacon cue) can assist monarchs with finding the appropriate groves of trees upon which they aggregate and overwinter (Mouritsen, 2018). Alternatively, monarchs might instead use beacon cues that indicate the overwintering sites independent of a map sense. Like the use of signals by insects for attracting individuals from far away to form large groups (e.g., aggregate male calling song—Guerra and Mason, 2005; aggregation pheromone—Allison and Cardé, 2016), monarchs might use cues emanating from the overwintering sites, e.g., olfactory cues given off by the trees (Reppert and de Roode, 2018), to form their massive overwintering aggregations. Stopping at the overwintering sites might also be a form of habitat selection, in which monarchs are searching for suitable microclimates for overwintering. For example, the microclimate of monarch overwintering sites differs from that outside the tree groves and provides temperatures that are low enough to keep metabolic costs low for overwintering but are not so cold that they lead to freezing and death (Urquhart and Urquhart, 1976). Monarchs might therefore stop at the overwintering sites, by using temperature as an environmental cue once close. It is also possible that monarchs stop at the general area of the overwintering sites since they simply no longer perceive a specific sensory cue that signals to continue migratory flight, such as the sun's angle at solar noon, i.e., loss of cue hypothesis (Taylor et al., 2019). Here, monarchs might then home in on beacon cues for locating the overwintering sites. The cues used to stop at their respective overwintering sites by the different migratory populations might be different and reflect local adaptation, as the geographic locations and trees used for aggregation differ between the groups (see above). In contrast, migrants regardless of region might utilize a common mechanism for stopping based on their shared search for appropriate microclimates for overwintering. Indeed, this might be the case for at least North American monarchs, in which overwintering temperature conditions are similar for both Eastern and Western migrants at the overwintering sites (Guerra and Reppert, 2013).

Finally, sensory cues are also important for the completion of the migratory cycle. Shown with Eastern North American monarchs, migrants need to be exposed to cold temperatures as experienced during overwintering in Mexico, to fly with the appropriate return flight directionality (*via* a recalibrated time-compensated sun compass) for remigration during the spring. Without exposure to such temperatures, monarchs continue to fly with fall flight directionality (Guerra and Reppert, 2013), which can prevent them from remigrating properly. Although still unknown, spring monarchs might use identical compass mechanisms, but with reversed directionality relative to fall monarchs for remigration. Similar to fall monarchs, the remigration directionality of spring monarchs might be induced by sensory cues associated with the season. These cues, however, should display a pattern in spring that is shifted 180° from that in late summer and early fall, such as increasing daylength and warming temperatures. The termination of the migratory cycle with the accompanying loss of directional flight observed in monarchs might also be due to the sensing of environmental cues, e.g., the decrease in the rate of change of increasing daylength that culminates with the summer solstice (Taylor, 2013). Moreover, the longer daylengths and higher temperatures of late spring and summer do not produce butterflies with the migratory syndrome.

DISCUSSION

Although our knowledge on the fundamental role of environmental sensory cues on monarch migration has increased over the past few decades, information on how contemporary changes in the sensory environment of monarchs might affect the migratory cycle remains lacking. This gap in our knowledge on this particular risk to sensory cue usage represents a potential danger to monarchs.

The Effects of Urbanization Threaten Monarch Migration

Major threats to the sensory environment of monarchs are those brought about by human activity (Kelley et al., 2018), such as shifting land usage related to urbanization. For example, human-induced highway noise as experienced by monarch larvae at roadside habitats can be a source of physiological stress (Davis et al., 2018). It is unknown how such physiological stress might affect the development, health, and survivorship of individuals, in particular individuals that will develop into migrants or adult migrants already en route. Urbanization is also a significant source of nighttime light pollution (NLP), such that urban areas with significant levels of NLP can present monarchs that develop and live there, or are just passing through while migrating, with dramatically altered daily light levels and photoperiods (Gaston et al., 2014). As environmental light cues with the appropriate characteristics and proper circadian clock function are important for proper monarch migration, the NLP of urban areas along the migratory routes of monarchs might significantly disrupt the entire migratory cycle. In urban areas, NLP might artificially prolong the subjective daytime hours of monarchs as observed for individuals of other migratory species (Dominoni and Partecke, 2015) or produce constant light conditions with properties (e.g., significant intensity and relevant wavelengths of light) that can significantly disrupt normal circadian clock function. NLP in urbanized areas might significantly alter or obliterate the cue of decreasing photoperiods that induce the migratory syndrome (Goehring and Oberhauser, 2002) and instead lead to the production of non-migratory

individuals even during the fall. Constant light conditions due to NLP can disrupt the development of monarchs, e.g., eclosion behavior (Froy et al., 2003), as well as perturb the antennal circadian clock function of adult migrants, preventing correct flight orientation during migration (Merlin et al., 2009). As many initiatives to conserve the monarch are conducted in urban areas (Baker and Potter, 2019), research on how urban NLP affects monarch migration is now needed to prevent or mitigate any unintended consequences of current and future conservation efforts. Fortunately, it is possible to reduce the negative ecological effects of NLP in urban areas, by using better lighting technologies and altering human behavior and lighting strategies at night (Gaston et al., 2012). Urbanization can also lead to human-induced electromagnetic noise, which can disrupt magnetic compass orientation in migratory animals (Engels et al., 2014). As monarchs can also use a magnetic compass for orientation during migration (Guerra et al., 2014), noise in this sensory modality is another type of sensory pollution that can prevent successful migration.

The Loss of Important Sensory Cues

Habitat loss and degradation are also areas of vulnerability for the persistence of the monarch migration. Central to the monarch migratory cycle are the overwintering sites that provide monarchs with a suitable microclimatic overwintering refuge. As migrating monarchs might use beacon cues provided by the overwintering grounds for locating these sites, the current deforestation and degradation of these areas, such as at the overwintering sites in Mexico of Eastern North American migrants (Vidal et al., 2013; Malcolm, 2018), might significantly reduce the strength, quality, or occurrence of cues emanating from these areas that guide monarchs. Also, as the thermal microclimate of the overwintering sites produces a "cold trigger" cue critical for recalibrating the flight directionality of migrants for proper remigration during the spring (Guerra and Reppert, 2013), worldwide challenges such as global warming and overall global climate change might currently attenuate this coldness cue and imperil the future persistence of this critical sensory cue at the overwintering sites. Without this coldness cue, it is possible that the migratory cycle can be broken, since monarchs may not return to the breeding areas of their habitat range. Unfortunately, previous modeling of the persistence of overwintering sites for migratory monarchs, such as the Monarch Butterfly Biosphere Reserve in Central Mexico, suggests that suitable overwintering habitat for monarchs at current sites might completely disappear by the end of this century (Sáenz-Romero et al., 2012). Therefore, research on the sensory ecology of monarch migration should continue to focus on identifying how monarchs locate the overwintering sites and what cues are used to do so. Once these cues are identified, it might be possible to identify, monitor, and protect new locations that provide these same cues and that monarchs find suitable for overwintering. Similarly, artificial and better-protected overwintering areas could be constructed to attract migrating monarchs. As done with other long-distance migratory animals (e.g., studies delineating the sensory-based orientation and navigational mechanisms of marine species such as sea turtles and salmonid fishes; Putman, 2018), by further studying and understanding the sensory capabilities of monarchs and the cues that they use for migration, we will be better equipped to save this wonder of nature, as well as other animal movement phenomena that face similar challenges.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This research was supported by the University of Cincinnati.

ACKNOWLEDGMENTS

The author thanks his collaborators and colleagues at the University of Cincinnati, the University of Massachusetts Medical School, Emory University, the University of Arkansas, and the University of Kansas for many fruitful discussions that have inspired and facilitated the writing of this article.

REFERENCES

Alerstam, T. (2006). Conflicting evidence about long-distance animal navigation. *Science* 313, 791–794. doi: 10.1126/science.1129048

Allison, J. D., and Cardé, R. T. (2016). *Pheromone Communication in Moths: Evolution, Behavior, and Application*. Oakland, CA: University of California Press.

Baker, A. M., and Potter, D. A. (2019). Configuration and location of small urban gardens affect colonization by monarch butterflies. *Front. Ecol. Evol.* 7:474. doi: 10.3389/fevo.2019.00474

Davis, A. K., Schroeder, H., Yeager, I., and Pierce, J. (2018). Effects of simulated highway noise on heart rates of larval monarch butterflies, *Danaus plexippus*: implications for roadside habitat suitability. *Biol. Let.* 14:20180018. doi: 10.1098/rsbl.2018.0018

Dingle, H. (2014). *Migration: The Biology of Life on the Move*. 2nd Edn. New York, NY: Oxford University Press.

Dominoni, D. H., and Partecke, J. (2015). Does light pollution alter daylength? A test using light loggers on free-ranging European blackbirds (*Turdus merula*). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* B370:20140118. doi: 10.1098/rstb.2014.0118

Engels, S., Schneider, N., Lefeldt, N., Hein, C. M., Zapka, M., Michalik, A., et al. (2014). Anthropogenic electromagnetic noise disrupts magnetic compass orientation in a migratory bird. *Nature* 509, 353–356. doi: 10.1038/nature13290

Franzke, M., Kraus, C., Dreyer, D., Pfeiffer, K., Beetz, J., Stöckl, A. L., et al. (2020). Spatial orientation based on multiple visual cues in non-migratory monarch butterflies. *J. Exp. Biol.* 223:jeb223800. doi: 10.1242/jeb.223800

Freedman, M. G., Dingle, H., Tabuloc, C. A., Chiu, J. C., Yang, Y. H., and Zalucki, M. P. (2018). Non-migratory monarch butterflies, *Danaus plexippus* (L.), retain developmental plasticity and a navigational mechanism associated with migration. *Biol. J. Linn. Soc.* 123, 265–278. doi: 10.1093/biolinnean/blx148

Froy, O., Gotter, A. L., Casselman, A. L., and Reppert, S. M. (2003). Illuminating the circadian clock in monarch butterfly migration. *Science* 300, 1303–1305. doi: 10.1126/science.1084874

Gaston, K. J., Davies, T. W., Bennie, J., and Hopkins, J. (2012). Reducing the ecological consequences of night-time light pollution: options and

developments. *J. Appl. Ecol.* 49, 1256–1266. doi: 10.1111/j.1365-2664.2012.02212.x

Gaston, K. J., Duffy, J. P., Gaston, S., Bennie, J., and Davies, T. W. (2014). Human alteration of natural light cycles: causes and ecological consequences. *Oecologia* 176, 917–931. doi: 10.1007/s00442-014-3088-2

Goehring, L., and Oberhauser, K. S. (2002). Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in *Danaus plexippus*. *Ecol. Entomol.* 27, 674–685. doi: 10.1046/j.1365-2311.2002.00454.x

Guerra, P. A., Gegear, R. J., and Reppert, S. M. (2014). A magnetic compass aids monarch butterfly migration. *Nat. Commun.* 5:4164. doi: 10.1038/ncomms5164

Guerra, P. A., and Mason, A. C. (2005). Male competition and aggregative behaviour are mediated by acoustic cues within a temporally unstructured aggregation. *Behaviour* 142, 71–90. doi: 10.1163/1568539053627730

Guerra, P. A., Merlin, C., Gegear, R. J., and Reppert, S. M. (2012). Discordant timing between antennae disrupts sun compass orientation in migratory monarch butterflies. *Nat. Commun.* 3:958. doi: 10.1038/ncomms1965

Guerra, P. A., and Reppert, S. M. (2013). Coldness triggers northward flight in remigrant monarch butterflies. *Curr. Biol.* 23, 419–423. doi: 10.1016/j.cub.2013.01.052

Guerra, P. A., and Reppert, S. M. (2015). Sensory basis of lepidopteran migration: focus on the monarch butterfly. *Curr. Opin. Neurobiol.* 34, 20–28. doi: 10.1016/j.conb.2015.01.009

Hanley, D., Miller, N. G., Flockhart, D. T. T., and Norris, D. R. (2013). Forewing pigmentation predicts migration distance in wild-caught migratory monarch butterflies. *Behav. Ecol.* 24, 1108–1113. doi: 10.1098/rsbl.2019.0922

Heinze, S., Florman, J., Asokaraj, S., El Jundi, B., and Reppert, S. M. (2013). Anatomical basis of sun compass navigation II: the neuronal composition of the central complex of the monarch butterfly. *J. Comp. Neurol.* 521, 267–298. doi: 10.1002/cne.23214

Heinze, S., and Reppert, S. M. (2012). Anatomical basis of sun compass navigation I: the general layout of the monarch butterfly brain. *J. Comp. Neurol.* 520, 1599–1628. doi: 10.1002/cne.23054

James, D. G. (1993). "Migration biology of the monarch butterfly in Australia," in *Biology and Conservation of the Monarch Butterfly*, eds S. B. Malcolm and M. P. Zalucki (Los Angeles, CA: Natural History Museum), 189–200.

James, D. G., and James, T. A. (2019). Migration and overwintering in Australian monarch butterflies (*Danaus plexippus* (L.) (Lepidoptera: Nymphalidae)): a review with new observations and research needs. *J. Lepid. Soc.* 73, 177–190. doi: 10.18473/lepi.73i3.a7

Kelley, J. L., Chapuis, L., Davies, W. I. L., and Collin, S. P. (2018). Sensory system responses to human-induced environmental change. *Front. Ecol. Evol.* 6:95. doi: 10.3389/fevo.2018.00095

Li, Y., Pierce, A. A., and de Roode, J. C. (2016). Variation in forewing size linked to migratory status in monarch butterflies. *Anim. Migr.* 3, 27–34. doi: 10.1515/ami-2016-0003

Malcolm, S. B. (2018). Anthropogenic impacts on mortality and population viability of the monarch butterfly. *Annu. Rev. Entomol.* 63, 277–302. doi: 10.1146/annurev-ento-020117-043241

Merlin, C., Gegear, R. J., and Reppert, S. M. (2009). Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. *Science* 325, 1700–1704. doi: 10.1126/science.1176221

Merlin, C., Iiams, S. E., and Lugena, A. B. (2020). Monarch butterfly migration moving into the genetic era. *Trends Genet.* 36, 689–701. doi: 10.1016/j.tig.2020.06.011

Morris, G. M., Kline, C., and Morris, S. M. (2015). Status of *Danaus plexippus* population in Arizona. *J. Lepid. Soc.* 69, 91–107. doi: 10.18473/lepi.69i2.a10

Mouritsen, H. (2018). Long-distance navigation and magnetoreception in migratory animals. *Nature* 558, 50–59. doi: 10.1038/s41586-018-0176-1

Mouritsen, H., and Frost, B. J. (2002). Virtual migration in tethered flying monarch butterflies reveals their orientation mechanisms. *Proc. Natl. Acad. Sci. U S A* 99, 10162–10166. doi: 10.1073/pnas.152137299

Nail, K. R., Drizd, L., and Voorhies, K. J. (2019). Butterflies across the globe: a synthesis of the current status and characteristics of monarch (*Danaus plexippus*) populations worldwide. *Front. Ecol. Evol.* 7:362. doi: 10.3389/fevo.2019.00362

Perez, S. M., Taylor, O. R., and Jander, R. (1997). A sun compass in monarch butterflies. *Nature* 387:29. doi: 10.1038/387029a0

Putman, N. F. (2018). Marine migrations. *Curr. Biol.* 28, R972–R976. doi: 10.1016/j.cub.2018.07.036

Reppert, S. M., and de Roode, J. C. (2018). Demystifying monarch butterfly migration. *Curr. Biol.* 28, R1009–R1022. doi: 10.1016/j.cub.2018.02.067

Reppert, S. M., Gegear, R. J., and Merlin, C. (2010). Navigational mechanisms of migrating monarch butterflies. *Trends Neurosci.* 33, 399–406. doi: 10.1016/j.tins.2010.04.004

Reppert, S. M., Guerra, P. A., and Merlin, C. (2016). Neurobiology of monarch butterfly migration. *Annu. Rev. Entomol.* 61, 25–42. doi: 10.1146/annurev-ento-010814-020855

Sáenz-Romero, C., Rehfeldt, G. E., Duval, P., and Lindig-Cisneros, R. A. (2012). *Abies religiosa* habitat prediction in climatic change scenarios and implications for monarch butterfly conservation in Mexico. *Forest Ecol. Manag.* 275, 98–106. doi: 10.1016/j.foreco.2012.03.004

Satterfield, D. A., and Davis, A. K. (2014). Variation in wing characteristics of monarch butterflies during migration: earlier migrants have redder and more elongated wings. *Anim. Migr.* 2, 1–7. doi: 10.2478/ami-2014-0001

Scanlan, M. M., Putman, N. F., Pollock, A. M., and Noakes, D. L. G. (2018). Magnetic map in nonanadromous Atlantic salmon. *Proc. Natl. Acad. Sci. U S A* 115, 10995–10999. doi: 10.1073/pnas.1807705115

Shlizerman, E., Phillips-Portillo, J., Forger, D. B., and Reppert, S. M. (2016). Neural integration underlying a time-compensated sun compass in the migratory monarch butterfly. *Cell Rep.* 15, 683–691. doi: 10.1016/j.celrep.2016.03.057

Taylor, O. R. Jr. (2013). *The Northward Migration: When Does It End?* Available online at: https://monarchwatch.org/blog/page/8/. Accessed August 29, 2020.

Taylor, O. R. Jr., Lovett, J. P., Gibo, D. L., Weiser, E. L., Thogmartin, W. E., Semmens, D. J., et al. (2019). Is the timing, pace, and success of the monarch migration associated with sun angle? *Front. Ecol. Evol.* 7:442. doi: 10.3389/fevo.2019.00442

Urquhart, F. A. (1987). *The Monarch Butterfly: International Traveler*. Chicago, IL: Nelson-Hall.

Urquhart, F. A., and Urquhart, N. R. (1976). The overwintering site of the eastern population of the monarch butterfly (*Danaus p. plexippus*; Danaidae) in Southern Mexico. *J. Lepid. Soc.* 30, 153–158.

Vidal, O., López-García, J., and Rendón-Salinas, E. (2013). Trends in deforestation and forest degradation after a decade of monitoring in the monarch butterfly biosphere reserve in Mexico. *Conserv. Biol.* 28, 177–186. doi: 10.1111/cobi.12138

Wise, K. A. J. (1980). Monarch butterfly dispersal in New Zealand. *Rec. Auckl. Inst. Museum.* 17, 157–173.

Zhan, S., Zhang, W., Niitepõld, K., Hsu, J., Haeger, F., Zalucki, M. P., et al. (2014). The genetics of monarch butterfly migration and warning coloration. *Nature* 514, 317–321. doi: 10.1038/nature13812

Zhu, H. S., Gegear, R. J., Casselman, A., Kanginakudru, S., and Reppert, S. M. (2009). Defining behavioral and molecular differences between summer and migratory monarch butterflies. *BMC Biol.* 7:14. doi: 10.1186/1741-7007-7-14

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 04 December 2020
doi: 10.3389/fnbeh.2020.597428

Contact-Chemosensory Evolution Underlying Reproductive Isolation in *Drosophila* Species

*Kosei Sato and Daisuke Yamamoto**

Neuro-Network Evolution Project, Advanced ICT Research Institute, National Institute of Information and Communications Technology, Kobe, Japan

The main theme of the review is how changes in pheromone biochemistry and the sensory circuits underlying pheromone detection contribute to mate choice and reproductive isolation. The review focuses primarily on gustatory and non-volatile signals in *Drosophila*. Premating isolation is prevalent among closely related species. In *Drosophila*, preference for conspecifics against other species in mate choice underlies premating isolation, and such preference relies on contact chemosensory communications between a female and male along with other biological factors. For example, although *D. simulans* and *D. melanogaster* are sibling species that yield hybrids, their premating isolation is maintained primarily by the contrasting effects of 7,11-heptacosadiene (7,11-HD), a predominant female pheromone in *D. melanogaster*, on males of the two species: it attracts *D. melanogaster* males and repels *D. simulans* males. The contrasting preference for 7,11-HD in males of these two species is mainly ascribed to opposite effects of 7,11-HD on neural activities in the courtship decision-making neurons in the male brain: 7,11-HD provokes both excitatory and inhibitory inputs in these neurons and differences in the balance between the two counteracting inputs result in the contrasting preference for 7,11-HD, i.e., attraction in *D. melanogaster* and repulsion in *D. simulans*. Introduction of two double bonds is a key step in 7,11-HD biosynthesis and is mediated by the desaturase desatF, which is active in *D. melanogaster* females but transcriptionally inactivated in *D. simulans* females. Thus, 7,11-HD biosynthesis diversified in females and 7,11-HD perception diversified in males, yet it remains elusive how concordance of the changes in the two sexes was attained in evolution.

Keywords: premating isolation, pheromones, hybrids, hydrocarbon metabolism, gustatory receptors, central integration, fruitless, doublesex

OPEN ACCESS

Edited by:
Maria de la Paz Fernandez, Columbia University, United States

Reviewed by:
Joanne Yew, University of Hawai'i at Mānoa, United States
Jean-Christophe Billeter, University of Groningen, Netherlands
Yehuda Ben-Shahar, Washington University in St. Louis, United States

***Correspondence:**
Daisuke Yamamoto
daichan@nict.go.jp

Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *21 August 2020*
Accepted: *11 November 2020*
Published: *04 December 2020*

Citation:
Sato K and Yamamoto D (2020) Contact-Chemosensory Evolution Underlying Reproductive Isolation in Drosophila Species. Front. Behav. Neurosci. 14:597428. doi: 10.3389/fnbeh.2020.597428

INTRODUCTION

The lack of gene flow or reproductive isolation is a prerequisite for the persistence of any species inhabiting the same place (Coyne and Orr, 2004). Premating as well as postmating isolation play roles in interfering with free gene flow, although neither would work as a perfect barrier against "interspecific hybridization" between populations that recently diverged. There exist cases where two populations of animals can produce fertile offspring and thus are judged to belong to the same

species, yet mating between two individuals each from an alternative population barely happens in nature, implying that premating isolation could precede the development of postmating isolation (Shumer et al., 2017). The African and cosmopolitan populations of *Drosophila melanogaster* undergo such an incipient speciation that was driven by premating isolation (Wu et al., 1995). Conversely, postmating isolation may occur prior to the development of premating isolation (Sweigart, 2010): the interspecific crosses happen at a high rate between *D. virilis* and *D. americana*, yet fertilization of eggs after mating hardly occurs in such crosses. In contrast to postmating isolation, premating isolation inevitably requires some cognitive process for discriminating a conspecific candidate partner from individuals of closely related species. If premating isolation takes place under the conditions where interspecies hybrids do not suffer from discernible fertility decrement (as expected to be the case for incipient speciation), assortative mating would likely be favored by sexual selection even when the adaptive (or fitness) advantage is limited. Here questions arise as to how the "perceptual shift" to favor a particular sexual trait in a potential mate develops and what genetic and neural mechanisms underlie this shift. *Drosophila* flies offer an ideal platform for addressing these evolutionary questions because of the comprehensive resource for genetic and neurobiological analyses in the model species *D. melanogaster* and because of the rich collection of species in the *Drosophila* phylogeny exhibiting distinct anatomical and behavioral characteristics (Hales et al., 2015).

This review covers mechanistic aspects of mating behavior, because the mechanistic understanding is critical for deciphering how animal behavior diversified thorough evolution. Homologous circuits that underly homologous behaviors need to be compared across species at the level of single cells, in which genes involved in behavioral divergence exert their specific actions. We review the current understanding of contact chemosensory mechanisms by which flies recognize conspecifics and discuss how species-specificity in pheromone perception and mate preference diversified in evolution.

PHEROMONE PRODUCTION

Cuticular hydrocarbons play roles as major sex pheromones in *Drosophila* (Jallon, 1984; Yew and Chung, 2017). These compounds are poorly volatile at room temperature and thus likely to be detected by contact chemoreceptors or gustatory receptors (Kohl et al., 2015). In *D. melanogaster*, 7-tricosene (7-T) is more abundant in males than females and acts as an aphrodisiac for a female, whereas 7,11-heptacosadiene (7,11-HD) and 7,11-non-acosadiene (7,11-ND) are nearly exclusively produced by females and acts as an aphrodisiac for a male (Ferveur, 1997; Bontonou and Wicker-Thomas, 2014; **Figure 1A**). 7-pentacosene (7-P) is present in both sexes at lower levels also stimulates males to court (Ferveur, 1997; Bontonou and Wicker-Thomas, 2014). Conversely, 5-tricosene (5-T), 7-T and the acetylated long-chain hydrocarbon CH503 (Yew et al., 2009) present in *D. melanogaster* inhibits males from courting. Other hydrocarbons may be predominant in *Drosophila* species phylogenetically distant from *D. melanogaster* (Thompkins et al., 1993; Alves et al., 2010); in *D. virilis* females for example, 11-P and 9-T are abundant cuticular hydrocarbons (Fan et al., 2013). Aside from hydrocarbons, cis-vaccenyl acetate (cVA) produced by male ejaculatory bulb functions as a potent suppressor of male courtship (Butterworth, 1969; Antony et al., 1985; Guiraudie-Capraz et al., 2007). There is evidence that 7-T and cVA exert the courtship inhibitory effect only when these two compounds coexist (Billeter et al., 2009; Laturney and Billeter, 2016). The major source of hydrocarbon compounds is oenocytes associated with the epidermis (Ferveur, 1997; Bontonou and Wicker-Thomas, 2014), genetic ablation of which allows one to obtain flies that produce almost no hydrocarbon compounds in their cuticles (Billeter et al., 2009). Unexpectedly, such oenocyte-less flies were highly attractive as a mating partner for both females and males, implying the loss of inhibitory compounds that normally prevent indiscriminate courtship (Billeter et al., 2009). Subsequent studies identified palmitoleic acid and non-esterified versions of the fatty acid methyl esters (Dweck et al., 2015; Lin et al., 2016) as non-sex-specific attractants, potentially accounting for the sexual attractiveness found in oenocyte-less flies. The site of synthesis of these fatty acids has not been determined, but fat bodies are a likely production site (Wicker-Thomas et al., 2009; Bontonou and Wicker-Thomas, 2014; Yew and Chung, 2017). Additionally, in cactus-feeding members of the *Sophophora* subgenus (but not in the subgenus *Drosophila*), the ejaculatory bulb produce male-specific triacylglycerides (TAG) that bear combinations of branched and linear fatty acyl side chains, which act as repellents for males upon transfer to the female mate during copulation (Chin et al., 2014).

The oenocyte-less *D. melanogaster* also provided important insights into the molecular basis for species discrimination in mate choice by males: *D. melanogaster* females without oenocytes were found to provoke strong courtship even from males of other species in the *melanogaster* species subgroup (Billeter et al., 2009). Perfuming oenocyte-less *D. melanogaster* females with female-specific 7,11-HD resumed species specific courtship, i.e., attracting males of *D. melanogaster* while repelling males of other members of the *melanogaster* species subgroup (Billeter et al., 2009). In fact, unlike *D. melanogaster* females, other members of the subgroup including *D. simulans* (**Figure 1A**), barely produce 7,11-HD (Jallon and David, 1987). These results indicate that the contrasting preference for 7,11-HD works as an effective barrier between *D. melanogaster* and other members of the species subgroup that prevents males from engaging in interspecific courtship.

On the other hand, the opposite preference for monoenes, particularly 7-T, constitutes a mating barrier in the partial reproductive isolation between the two strains of *D. melanogaster*, i.e., African (Zimbabwe: Z) vs. cosmopolitan populations (Grillet et al., 2012). As the amount of 7-T relative to 5-T increases in courting males, cosmopolitan females become more receptive to mating, while African-Z females become less receptive (Grillet et al., 2012). These observations reinforce the view that changes in hydrocarbon compositions may be one of the key events that precede reproductive isolation between two populations under

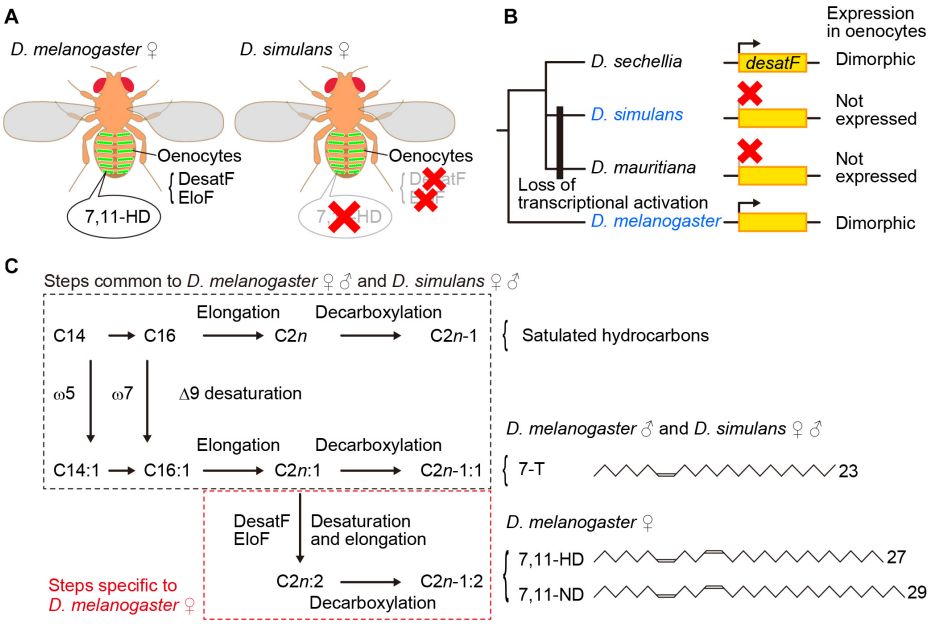

FIGURE 1 | 7,11-HD plays a key role for male mate choice in the *D. melanogaster* species subgroup. **(A)** 7,11-HD is a *D. melanogaster* female-specific pheromone synthesized in oenocytes as mediated by enzymes including DesatF and EloF. **(B)** DesatF expression is female-specific in *D. melanogaster* and *D. sechellia*, whereas it is transcriptionally inactivated in *D. simulans* and *D. mauritiana*. **(C)** Pathways for pheromone synthesis in *D. melanogaster* and *D. simulans*. 7-T is a monoene with a double bond at the 7th carbon whereas 7,11-HD is a diene with two double bonds each at the 7th and 11th carbon as the numbers in the compound names indicate.

incipient speciation, providing a rationale behind the search for evolutionary changes in hydrocarbon synthesis pathways.

Several genes encoding enzymes critical for introducing a double bond have been well-characterized in *D. melanogaster*, i.e., *desaturase1* (*desat1*), *desat2*, *desatF* (also known as *Fad2*), Cyp4G1 (Qiu et al., 2012), and Bond (Ng et al., 2015). *desat1* is a pleiotropic and indispensable gene transcriptionally regulated by 5 promoters each specifying unique spatiotemporal expression (Bousquet et al., 2012): among these, promoter-*RE* functions in oenocytes and is key for pheromone synthesis (Billeter et al., 2009), while promoter-*RC* functions in neurons and is key for female receptivity (Bousquet et al., 2012; see below). *desat2* was discovered as a *desat1* homolog in the genome of an African *D. melanogaster* strain, Tai (African-T), encoding desaturase with Δ9 specificity for omega-7 hydrocarbon precursors (in contrast to desat1 with Δ7 specificity for omega-5 hydrocarbon precursors; Dallerac et al., 2000). Remarkably, *desat2* expression in African-T is female-specific, whereas *desat2* is not expressed at all in the cosmopolitan Canton-special (CS) strain due to a promoter defect. Nucleotide sequence comparisons suggest that the *desat2* gene structure in cosmopolitan populations is a descendant of that in the African counterpart (Takahashi et al., 2001). The presence or absence of functional *desat2* in the African and cosmopolitan *D. melanogaster* nicely explains why females of African *D. melanogaster* preferentially produce 5,9-HD (an omega-7 hydrocarbon) rather than 7,11-HD (an omega-5 hydrocarbon), the latter of which dominates in females of cosmopolitan *D. melanogaster* instead. In contrast, the different cuticular contents of 5-T and 7-T in males from the two populations have been demonstrated to be an important factor for females in choosing a mate, as discussed above. However, the different 5-T vs. 7-T ratio in African and cosmopolitan males cannot be ascribable to the presence or absence of functional *desat2* in the respective genomes, because males do not exhibit *desat2* expression in both populations. Thus, the significance of the discovered genomic changes in the *desat2* gene in incipient speciation in *D. melanogaster* populations has not been fully validated.

desatF was identified as the gene that plays a central role in the synthesis of 7,11-HD and other dienes with two double bonds that are predominant in *D. melanogaster* females (Chertemps et al., 2006): DesatF catalyzes the reaction to introduce the second double bond into fatty acid precursors (**Figures 1B,C**). It was shown that female-specific *desatF* expression relies on a female-determinant, Transformer (Tra), and *desatF* knockdown in females results in a dramatic increase in monoenes (e.g., 7-T) at the expense of dienes (e.g., 7,11-HD). Comprehensive species comparisons of the *desatF* structure and expression unraveled the exceedingly complex evolutionary changes this gene underwent (Shirangi et al., 2009). Although a conserved *desatF* sequence is recognizable in the genomes of 18 out of 24 species examined, it is not functional in 9 species: the *desatF* gene is translationally inactive in 6 species (although *desatF* in some species retains an intact open reading frame, ORF) and it harbors mutations in the coding sequence in 3 species (Shirangi et al., 2009). The *desatF* gene in some species underwent multiple transitions, e.g., once transcriptionally inactivated, it was transcriptionally reactivated and ultimately ORF-disrupted

(Shirangi et al., 2009). Remarkably, female-specific expression as in *D. melanogaster* is not conserved across species that carry an active *desatF* gene: among the species examined, *D. sechellia*, *D. errecta,* and *D. melanogaster* are the only ones that exhibit *desatF* sexually dimorphic expression. The transitions between the monomorphic and dimorphic expression were found to be associated with the loss and gain of distinct biding sites for the sex-determinant transcription factor Doublesex (Dsx) in the *cis*-regulatory region of the *desatF* gene, respectively (Shirangi et al., 2009). In the *D. melanogaster* species subgroup, a common ancestor presumably had dimorphic expression of *desatF* and thus expressed 7,11-HD, which was subsequently lost as a result of transcriptional inactivation of *desatF* in the clade containing *D. simulans* and *D. mauritiana*, while dimorphic expression was sustained in the clade to *D. melanogaster* (**Figure 1B**). It is thus plausible that reproductive isolation between the two sympatric sibling species *D. melanogaster* and *D. simulans* was endowed, in part, by *cis* element mutations in the *desatF* gene, which removed 7,11-HD from females of *D. simulans*, in concordance with changes in the preference for 7,11-HD in males (see below).

Yet another gene of interest is *fatty acid elongase F* (*eloF*), which elongates the DesatF products omega-7,11 fatty acids, the precursors of 7,11-HD and 7,11-ND in *D. melanogaster* females (Chertemps et al., 2007; **Figure 1C**). *eloF* is expressed in a female-biased manner in *D. melanogaster* (Chertemps et al., 2007) and *D. sechellia* (Combs et al., 2018) and is not expressed at all in *D. simulans* (Chertemps et al., 2007; **Figure 1A**). What we see here with *eloF* is exactly the above-described pattern of *desatF* expression in these three species. It remains an open question whether this kind of coordinated evolution of *eloF* and *desatF* can be generalized into other clades of the *Drosophila* phylogeny.

cVA is probably the most studied among pheromones in *Drosophila*, but little is known about its biosynthesis. Unlike major cuticular hydrocarbon pheromones that are produced by oenocytes, cVA is secreted into the lumens of ejaculatory bulb in a male and ejected, together with sperms, into the female genitalia during copulation, reducing the sexual attractiveness of that female for other males (Antony et al., 1985). In addition to such an anti-aphrodisiac effect, cVA enhances aggression among unfamiliar males (Wang and Anderson, 2010; Wang et al., 2011) but reduces aggression among familiar males (Liu et al., 2011), and promotes non-sex-specific aggregation in a context-dependent manner (Bartelt et al., 1985; Wertheim et al., 2002; Lebreton et al., 2014; Cazalé-Debat et al., 2019), and suppresses male courtship toward a virgin female after his exposure to a mated female (Ejima et al., 2007; Keleman et al., 2012). Radioactive tracer labeling of metabolites supported the hypothesis that the male ejaculatory bulb synthesizes cVA from acetate as a starting compound, yet the vaccenyl moiety is of an unknown origin (Guiraudie-Capraz et al., 2007). Notably, in *D. buzzatii*, radiolabeled acetate similarly incubated with male ejaculatory bulb yields two ketone compounds, i.e., (Z)-10-heptadecen-2-one, an aggregation pheromone, and its antagonist, 2-tridecenone (Skiba and Jackson, 1993). A large number of long-chain acetates, alcohols and ketones have been reported as aggregation pheromones in *Drosophila*, and the composition of pheromone blends varies widely across species (Symonds and Wertheim, 2005; Lebreton et al., 2017). It remains to be determined whether these aggregation pheromones also play roles as sex pheromones, and if so, how significant they are in reproductive isolation in speciation events.

SEX PHEROMONE RECEPTION

In the previous section, we saw that a single pheromone may exert contrasting reactions in different species. A favored interpretation for this would be that a receptor for the pheromone responds differently in different species. In this section, we review our current understanding of contact chemoreceptors for pheromones in *Drosophila* and evaluate the above hypothesis.

Electrical recordings of receptor potentials and spiking activities from a receptor cell are the straightforward functional demonstration of ligand-receptor interactions. The female pheromone 7,11-HD was demonstrated to provoke discharges from chemosensory neurons in the foreleg tarsi of *D. melanogaster* males (Toda et al., 2012; **Figure 2A**). When a male fly taps the female abdomen with his foreleg during courtship, these chemosensory neurons will be stimulated by cuticular hydrocarbon compounds on the female abdomen. A fraction of the foreleg chemosensory neurons express the neural masculinizing protein Fruitless (FruM), and these *fru*[+] chemosensory neurons exhibit sex differences in the central projection pattern (Kimura et al., 2019; see below). A subset of such *fru*[+] chemosensory neurons in foreleg tarsi express *ppk23* and related genes that encode Degenerin/Epithelial Na^+ channel (Deg/ENaC) family proteins, which have been implicated in 7,11-HD-dependent male courtship based on behavioral phenotypes upon targeted knockdown and Ca^{2+} neural activity imaging (Liu et al., 2012; Lu et al., 2012; Thistle et al., 2012; Vijayan et al., 2014; see below; **Figure 2B**).

The identity of 7,11-HD responsive cells was further defined by Ca^{2+} activity imaging: the relevant tarsal sensillum houses a pair of *fru*[+]/*ppk23*[+]/*ppk29*[+] cells, each having a complementary function such that one (the F-cell) responds to the female pheromone 7,11-HD but not the male pheromone 7-tricosense (7-T), while the other (the M-cell) responds to 7-T but not 7,11-HD (Thistle et al., 2012; **Figure 2B**). Up- and down-regulation of F-cells promote and repress male courtship activities, respectively, and the converse effects are observed when M-cells are similarly manipulated (Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012). The F-cells are molecularly distinguishable from the M-cells by their expression of *ppk25*, which is required specifically for the 7,11-HD responses of these cells (Vijayan et al., 2014; **Figure 2B**). The F-cells on foreleg tarsi are probably the major sensor for 7,11-HD, although there are other cells that are thought to be additional 7,11-HD sensors (see below). The F-cells and M-cells are present in both sexes, and their sex-specific functions are encoded by sex-specific functions via sexually dimorphic projections (Thistle et al., 2012).

7-T may stimulate additional cells other than the M-cells in the tarsus, including *Gr32a*-expressing bitter responsive cells (Koganezawa et al., 2010; Wang and Anderson, 2010) that are negative for both *ppk23* (Thistle et al., 2012) and *fru*

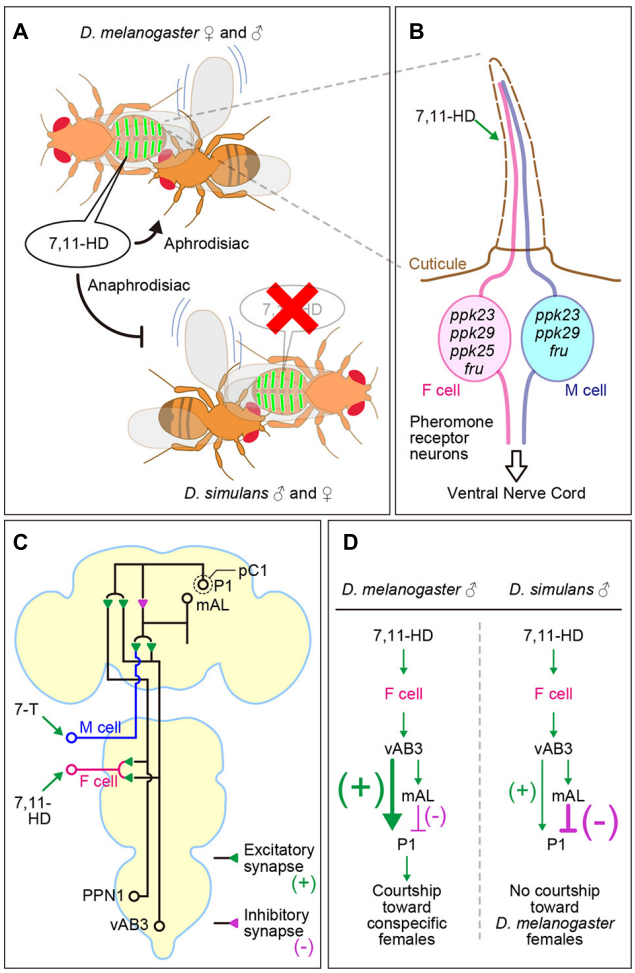

FIGURE 2 | *D. melanogaster* males are attracted and *D. simulans* males are repelled by 7,11-HD. **(A)** Contrasting responses to 7,11-HD underlie conspecific mate choice. **(B)** F-cell and M-cell in the male tarsi sense female pheromones (e.g., 7,11-HD) and male pheromones (e.g., 7-T), respectively. The F-cell and M-cell both express *ppk23*, *ppk29* and *fru*, while *ppk25* expression is F-cell specific. **(C)** Central pathway for 7,11-HD perception in male flies involves ascending excitatory (+) neurons including vAB3 and PPN1, mAL inhibitory (–) interneurons, and courtship triggering P1 excitatory (+) interneurons. **(D)** mAL-mediated inhibition overwhelms vAB3-mediated excitation in P1 neurons in *D. simulans* males but not *D. melanogaster* males, resulting in opposite responses to 7,11-HD in males of these two species. P1 represents a male-specific subset in the pC1 neuron group (circled by a dotted line). Circles, lines, and triangles indicate somata, neurites, and presynaptic terminals of neurons, respectively.

(Koganezawa et al., 2010) expression; *ppk23*-positive cells are located predominantly in the ventral sensory hairs, whereas *Gr32a*-positive cells are located mostly in the dorsal sensory hairs (Ling et al., 2014). Furthermore, Lacaille et al. (2007) showed, using a tungsten electrode inserted into the base of a sensillum, that some bitter-sensitive cells on a labial palp (i.e., mouth) contained sensory neurons responsive to low concentrations of 7-T, a pheromone that inhibits male courtship. Because male flies lick female genitalia during courtship, sensory neurons on the labial palp are likely activated in courting males.

7-T is not the sole ligand for the M-cells: cVA also activates these cells (Thistle et al., 2012). This is rather surprising, because cVA is volatile and known to activate primarily the olfactory receptor neurons expressing *Or67d* (Ha and Smith, 2006; Kurtovic et al., 2007; Datta et al., 2008; Ruta et al., 2010; Liu et al., 2011) and secondarily those expressing *Or65a* (Ejima et al., 2007; Liu et al., 2011; Lebreton et al., 2014) in the antenna. In addition to these tarsal sensory cells, a subset of gustatory cells respond to fly cuticle extracts and promote mating activities in both female and male *D. melanogaster* (Koh et al., 2014; He et al., 2019).

What are the roles of these contact chemosensory cells in sexual isolation among *Drosophila* species? Fan et al. (2013) showed that RNAi-mediated knockdown or genetic ablation of *Gr32a*-expressing neurons in *D. melanogaster* males restores the attractiveness of oenocyte-less *D. melanogaster* females that were perfumed with cuticular extracts from females of other *Drosophila* species (i.e., *D. simulans*, *D. yakuba,* or *D. virilis*) or with synthetic 7-T, 9-T and/or 11-P, the treatments that otherwise abrogate the sex appeal of *D. melanogaster* females (Fan et al., 2013). These results suggest that *Gr32a*-expressing sensory neurons that are responsive to a broad spectrum of

hydrocarbons play a key role in the conspecific preference in *D melanogaster* males. Subsequently, similar behavioral assays were conducted with *Gr32a*-knockout *D. simulans*, which was generated by CRISPR/Cas9-mediated targeted mutagenesis, yielding a contrasting result: *Gr32a* mutant males of *D. simulans* displayed no sign of impairment in discriminating conspecifics from other species, exhibiting a strict preference for females of the same species (Seeholzer et al., 2018; Ahmed et al., 2019). The lack of effect of *Gr32a* knockdown on mating discrimination is intriguing in view of the fact that not only *Gr32a* expression in tarsal sensory neurons but also the function of *Gr32a* in bitterness perception were conserved between *D. melanogaster* and *D. simulans* (Ahmed et al., 2019). *ppk25* knockout in *D. simulans*, on the other hand, diminished male courtship activities toward conspecific females, as in *D. melanogaster* (Ahmed et al., 2019). However, the primary stimulant of *ppk25*-expressing tarsal sensory neurons in *D. melanogaster* is 7,11-HD, which repels *D. simulans* males, implying that *ppk25*-expressing tarsal neurons in *D. simulans* promote male courtship when activated by a pheromone other than 7,11-HD. Alternatively, the pathway initiated by the *ppk25*-expressing sensory neurons is not a simple accelerator of male courtship activity; instead, inputs through this pathway may gain either positive or negative valence upon central integration, which varies depending on the species and context (**Figures 2C,D**). It is an interesting question as to which mechanisms—the peripheral or central mechanisms—are more frequently modified for sexual isolation in incipient speciation.

MOLECULAR IDENTITY OF CONTACT-CHEMICAL PHEROMONE RECEPTORS

It remains an open question as to which proteins function as specific receptors for pheromones. As described above, the reception of major contact-chemosensory pheromones is mediated by cells that express select *ppk* family members (e.g., *ppk23*, *ppk25,* and *ppk29*) or *Gr32a*. *Gr68a* (Bray and Amrein, 2003) and *Gr33a* (Watanabe et al., 2011) have also been suggested to have roles in mating behavior. More recent works have further shown that a subset of the ionotropic glutamate receptor (IR) family contributes to courtship behavior (Koh et al., 2014; He et al., 2019). Are these proteins by themselves function as receptors for pheromones? Are they required for signal transduction downstream of receptors? Liu et al. (2020) argue that *ppk23*, *ppk25,* and *ppk29* form a functional receptor for 7,11-HD based on the observation that the otherwise 7,11-HD-unresponsive M-cells acquire sensitivity to this compound when the *ppk* trio is expressed in the cells. It should be noted that only *ppk25* needs to be overexpressed because *ppk23* and *ppk29* are endogenously expressed in the M-cells. This finding in the M-cells is in line with the aforementioned result in the F-cells that *ppk25* knockdown abrogates their sensitivity to 7,11-HD, which resumes upon *ppk25* overexpression (Vijayan et al., 2014). Nonetheless, these observations do not exclude the possibility that the *ppk* proteins are not receptors that bind 7,11-HD but rather are their effector channels for electrogenesis, amplifying signaling downstream of the receptors (Ng et al., 2019). *ppk25* overexpression might have enhanced the outputs of the receptors that intrinsically respond to a wide spectrum of agonists so that even small responses that might otherwise be overlooked become detectable by the experimental manipulation.

Gr32a is another candidate receptor for hydrocarbon pheromones, particularly 7-T. Gr32a belongs to the insect chemoreceptor superfamily, which is composed of 68 Grs and 62 Ors, which are 7-pass transmembrane proteins that form ion channels on their own without any involvement of additional cytoplasmic factors (i.e., ionotropic receptors), unlike mammalian chemoreceptors, which are typically 7-pass membrane proteins with inverse topology (in comparison with that of insect receptors) that act via a G-protein mediated transduction cascade (i.e., metabotropic receptors; Sato et al., 2008, 2011). A recent cryogenic electron microscopy (cryoEM) study on an Or—namely, the odorant receptor co-receptor (Orco)—in an insect identified a crevice of 10-Å depth and 20 Å length within the extracellular leaflet, along which several residues known to affect ligand sensitivity lie, and which is thus likely to serve as a binding site for ligands (Butterwick et al., 2018). Following the analogy of Orco, Gr32a may have ligand binding activity. However, Gr32a is widely expressed in contact-chemosensory neurons that respond to a wide spectrum of ligands, particularly those known as bitter tastants, raising the question of how the Gr32a protein confers the ligand specificity on the sensory neurons. A recent exhaustive analysis of ligand-receptor-neuron relationships for gustatory responses in the labial palp defined Gr32a, Gr33a, Gr39a.a, Gr66a, Gr89a, and Gr93a as commonly expressed receptors (CERs) in bitter-sensitive receptors, which are equivalent to Orco in olfactory receptors (Dweck and Carlson, 2020; **Figure 3**). Typically, two, three, or four fixed members of CERs need to be coexpressed for normal bitter sensitivity: Gr32a, Gr33a, and Gr66a are the triple constituents and Gr33a, Gr39a, Gr66a, and Gr93a are the quadruple constituents essential for responding to caffeine and some other compounds in a subset of bitter-sensitive chemosensory neurons housed in I-a and I-b sensilla, respectively. When one component of the trio or quartet is lost, the neurons may simply become unresponsive to nearly all bitter tastants to which they normally respond or, alternatively, the neurons may acquire a novel ligand selectivity depending on their neuron type, which would suggest competition among multiple Gr species expressed in the same neuron in forming a functional heteromeric receptor for bitter tastants (Dweck and Carlson, 2020). Thus, the response spectrum of a neuron may change dependent on the combination of Gr species coexpressed and the relative abundance of different Grs. These considerations tempted us to suggest that Gr32a may contribute to the reception of 7-T and other pheromones as one of the CERs.

Then, the question remains as to how the response spectrum of a contact-chemosensory neuron is specified. The I-a and I-b sensilla are morphologically similar, but each respond to mutually exclusive sets of bitter compounds in *D. melanogaster*: for instance, caffeine elicits responses from I-b but not I-a, whereas berberine elicits responses from I-a but not I-b. However,

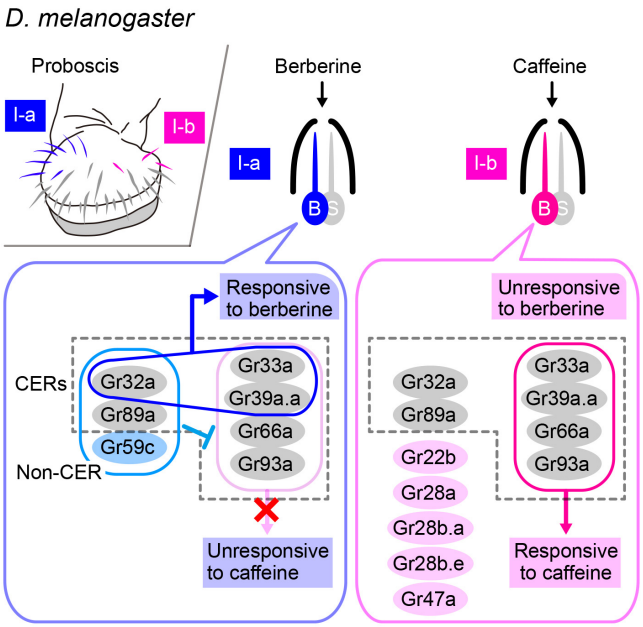

FIGURE 3 | Combination of several GRs expressed in a single neuron determines the response spectrum of the cell. Upper left-side panel: Spatial localization of bitter-responsive I-a (blue) and I-b (red) sensilla on the labellum. Right-side panels: The bitter-responsive neuron (labeled as "B") of each sensillum expresses a different combination of Gustatory receptors (Grs), five of which are referred to as "Commonly Expressed Receptors" (CERs; in dotted rectangles) and are expressed in every bitter-responsive neuron on the labellum. Expression of Gr32a, Gr33a, and Gr39a.a together confers a berberine sensitivity on the I-a sensillum, whereas expression of Gr33a, Gr39a.a, Gr66a, and Gr93a together confers a caffeine sensitivity on the I-b sensillum. However, the latter four Grs are unable to confer a caffeine sensitivity on the I-a sensillum, because Gr32a, Gr59b, and Gr89a are coexpressed in this sensillum.

the I-a sensillum acquires the entire response spectrum of I-b and thus becomes responsive to caffeine when the non-CER Gr59c is lost or when one CER, either Gr32a or Gr89a, is lost (Dweck and Carlson, 2020). Conversely, misexpression of Gr59c in the I-b sensillum confers the I-a type response spectrum on the I-b sensillum, provided that Gr32a and Gr89a are intact (Dweck and Carlson, 2020). This and other experiments demonstrate that the I-a sensillum does not respond to caffeine and other ligands that normally activate the I-b sensillum, because Gr59c in addition to the CER Gr32a and Gr89a coordinately suppress the responses to these substances (Dweck and Carlson, 2020). These observations imply that evolutionary loss or gain of the expression of just one of the Gr-coding genes could produce substantial changes in the ligand specificity of a subset of contact-chemosensory neurons, thereby leading to diversified pheromonal responses that potentially impact speciation events.

CENTRAL PROCESSING OF CONTACT-CHEMICAL PHEROMONE INFORMATION IN EVOLUTION

Contact chemosensory information plays a pivotal role in recognizing potential mating partners, making a decision to court, and initiating the mating motor program in *Drosophila*. One pheromone substance may induce different behavioral responses in recipients of different species, with the differences potentially arising from the different response properties of peripheral receptor cells as discussed above or the different processing of pheromone inputs in the central nervous system (CNS). In this section, we focus on central mechanisms underlying the different behavioral responses to contact chemosensory pheromones among *Drosophila* species in the context of mate preference. Unfortunately, there are no means for systematic labeling of a single neuron along the entity of the cell in non-model species, hampering circuit dissection in these species. Due to this technical difficulty, species differences in the structure and function of central neurons have been least explored. One exception is a study which successfully unraveled the central circuit basis for the biased preference of conspecific over sibling species females by *D. simulans* males (Seeholzer et al., 2018).

The central circuit for mating behavior has been extensively analyzed in *D. melanogaster* (Kohl et al., 2013; Yamamoto and Koganezawa, 2013), in which FruM-positive neurons tend to interconnect in forming the core portion of the circuit (Ruta et al., 2010; Yu et al., 2010). A male-specific interneuron group called P1 (Kimura et al., 2008) or its subpopulation (Ishii et al., 2020) plays a decisive role in initiating courtship behavior (Yamamoto and Koganezawa, 2013; **Figure 2C**). The P1 neuron cluster was first identified as a subset of FruM/Dsx double-positive neurons (20 neurons per hemisphere) that could drive test females to perform male-type courtship behavior toward a target female when those neurons were clonally masculinized in the test female brain by the tra^1 mutation (Kimura et al., 2008); the tra^1 mutation removes the DsxF feminizer protein that otherwise kills

P1 precursor cells during development, thereby allowing male-specific P1 to persist throughout the adult stage in the female brain (Kimura et al., 2008; see also Ren et al., 2016). In a solitary male, artificial activation of P1 neurons via heat-sensitive dTrpA1 channels or the light-activatable channel Channelrhodopsin induces the early steps of courtship, i.e., unilateral wing extension and vibration for singing and tapping with forelegs (Kohatsu et al., 2011; Kohatsu and Yamamoto, 2015). Ca^{2+} imaging of P1 neurons in a tethered male on a treadmill revealed that these neurons are excited when the male touches the female abdomen with his foreleg (Kohatsu et al., 2011). P1 neurons remain continuously and dynamically active throughout the courtship achievements under freely moving (Grover et al., 2016, 2020) as well as tethered (Kohatsu and Yamamoto, 2015) conditions. Contact chemosensory sensation of female cues is crucial for courtship initiation by the male, based on the finding that touch-induced chasing is blocked when the virgin female as a courtship target is perfumed with the hexane extract of male cuticles (Kohatsu et al., 2011). P1 neurons have been shown to be excited when the male foreleg tarsus is touched by a glass rod, provided that it is coated with the hexane-extract of fly cuticles (Kohatsu et al., 2011). Notably, P1 neurons exhibit Ca^{2+} rises upon tarsal stimulation with the extracts of male as well as female cuticles, although female extracts provoke significantly larger responses than male extracts do (Kohatsu et al., 2011). These and other observations support the notion that P1 neurons in the male brain receive contact-chemosensory inputs originating from tarsal pheromone receptors upon the touch of a female and drive persistent courtship toward the female. P1 neuron outputs are relayed by descending interneurons that activate the motor pattern generator for courtship acts (Clyne and Miesenböck, 2008; Kohatsu et al., 2011; von Philipsborn et al., 2011; Kimura et al., 2015; Cande et al., 2018; Clemens et al., 2018; Namiki et al., 2018; McKellar et al., 2019). A subset of P1 neurons provoke not only courtship toward a female but also aggression toward a male (Inagaki et al., 2014; Hoopfer et al., 2015; Koganezawa et al., 2016), presumably dependent on the sensory inputs they receive (Ishii et al., 2020; Wohl et al., 2020), while inhibiting sleep in a manner dependent on the internal state of the fly (Chen et al., 2017; Wu et al., 2019). Note, however, that subpopulations of the P1 cluster and the pC1 cluster to which the P1 cluster belongs need further clarification in terms of functional specialization (see Costa et al., 2016). The internal states, such as the motivational state and sleep/arousal cycle, affect P1 activities via dopaminergic and GABAergic synaptic inputs to promote and inhibit courtship, respectively (Crickmore and Vosshall, 2013; Zhang et al., 2016, 2018).

Given that P1 neurons in the brain trigger the lower center that produce motor outputs for courtship actions, how does the pheromone information received by sensory cells in the legs and mouth reach to the P1 neurons? The pathways through which contact chemical pheromone inputs reach to P1 neurons were revealed in *D. melanogaster* by anatomical detection of putative synaptic contacts in conjunction with Ca^{2+} imaging to monitor the neural activities across synapses (**Figure 2C**). The majority of the *ppk23*-positive tarsal chemosensory neurons responsive to 7,11-HD appear to terminate their axons in the prothoracic ganglion (Mellert et al., 2010; Kimura et al., 2019), and thus direct contact with brain-intrinsic P1 neurons must, if any, be limited. Instead, the ascending interneuron group vAB3 intervene in the communication between the *ppk23*-positive sensory neurons and P1 neurons: vAB3 neurons originate in the abdominal ganglion and terminally project to the lateral protocerebrum, the brain region P1 neurons densely innervate, with *en passant* arbors in the prothoracic and suboesophageal ganglia (Clowney et al., 2015). vAB3 neurons are excited when the male touches the female abdomen with his foreleg, and vAB3 activation by Ach iontophoretically applied to the prothoracic neuropil induces Ca^{2+} elevation in P1 neurons, which is blocked by vAB3 severing (Seeholzer et al., 2018). Thus, vAB3 provides an excitatory pathway that conveys the female pheromone information from leg sensory neurons to P1 neurons that initiate male courtship. Another group of neurons that are likely presynaptic to P1 are the mAL neurons, which are *fru*-positive GABAergic inhibitory interneurons (Koganezawa et al., 2016) that are sexually dimorphic in both structure and cell number (Kimura et al., 2005). A sexually dimorphic neurite of mAL likely contacts, in a male-specific manner, the axon terminals of *Gr32a*-expressing tarsal sensory neurons (Koganezawa et al., 2010). Remarkably, mAL neurons exhibit Ca^{2+} elevation in response to activation of vAB3, whose *en passant* arbors appear to intermingle with mAL arbors in the suboesophageal ganglion. This observation raises the possibility that vAB3 could also deliver an inhibitory input to P1 neurons via mAL neurons. Indeed, P1 activation in response to stimulation of vAB3 is significantly greater after mAL severing, supporting the notion that the reception of aphrodisiac female pheromones by the leg chemosensory receptors ultimately provokes not only excitatory responses but also inhibitory responses in P1 neurons, the decision-making neural center for male courtship behavior. Convergence of these two antagonistic inputs at nearly the same time might create a sensitized condition where additional cues easily bias the activity of P1 neurons that are involved in decision-making to court or not, allowing the male fly to judge whether the confronting target for courtship is truly an appropriate potential mate. There is yet another ascending interneuron group, PPN1, that convey inputs originating from *ppk23/ppk25* double-positive pheromone receptors (female-pheromone sensitive F-cells) to P1 neurons; PPN1 neurons act as excitatory presynaptic fibers for P1 and, at the same time, act as an element in the inhibitory pathway impinging on P1 via mAL interneurons (Kallman et al., 2015). In contrast to F-cell axons, which terminate mostly in the thoracic ganglia, a subset of *ppk23*-positive and *ppk25*-negative M-cells extend their axons beyond the thorax and terminate in the suboesophageal ganglion, where these axons seem to come into contact with an mAL neurite (Kallman et al., 2015). As a consequence, the M-cell activator 7-T primarily inhibits P1 neuron activity and thus represses male courtship, whereas the F-cell activator 7,11-HD elevates P1 neuron activity despite its inhibitory effect through mAL and ultimately promotes male courtship (Kallman et al., 2015). Thus, we find that a common excitatory pheromone input is fed into two pathways, one converts the excitatory signal into an inhibitory signal, while the other conveys the excitatory signal

without inverting its sign, and the two pathways ultimately converge onto the P1 neurons.

This principle would offer a simple means to fine-tune the sensitivity of a decision-making neural center to incoming sensory cues. In fact, different preferences for 7,11-HD in males of the *D. melanogaster* species subgroup are suggested to involve a shift in the excitatory vs. inhibitory balance in contact chemosensory inputs impinging on P1 neurons (**Figure 2D**). As discussed in the preceding sections, males of *D. simulans* avoid 7,11-HD, which is specifically enriched in female cuticles of *D. melanogaster*. The neural pathway through which 7,11-HD-induced activities travel to P1 neurons is, in principle, conserved between *D. melanogaster* and *D. simulans*. As in *D. melanogaster*, *D. simulans ppk23*-positive sensory neurons activate vAB3 ascending interneurons, which in turn produce activities in mAL inhibitory interneurons (Seeholzer et al., 2018). Notably, P1 neurons exhibit no apparent activation when vAB3 is depolarized in *D. simulans*. Upon mAL severing, however, vAB3 activation induces noticeable Ca^{2+} rises in P1 neurons (Seeholzer et al., 2018). These observations suggest that both direct excitatory and indirect inhibitory connections between vAB3 and P1 also exist in *D. simulans*, but in the latter species inhibitory inputs overwhelm excitatory inputs, and, as a consequence, 7,11-HD is unable to activate P1 and thus unable to trigger male courtship behavior in *D. simulans* (Seeholzer et al., 2018; **Figure 2D**). This species difference in the integrative functions of the CNS circuit represents a plausible mechanism for the premating isolation between *D. melanogaster* and *D. simulans*, which involves contrasting preferences for 7,11-HD: attraction in *D. melanogaster* males and avoidance in *D. simulans* males. An intriguing evolutionary scenario is that selective pressure acted on synapses associated with male-specific P1 neurons to shift the balance in favor of excitatory inputs from vAB3 against inhibitory inputs from mAL in an ancestral species of *D. melanogaster*, when females of this species acquired some dienes as new pheromone components, including 7,11-HD on their cuticles. The postulated shift in the balance between excitatory and inhibitory synaptic efficacies needs to be experimentally demonstrated. Also, if a species difference in the synaptic efficacy exists, as expected, it remains to be determined what genetic change is responsible.

CROSSTALK BETWEEN CONTACT-CHEMOSENSORY AND OLFACTORY PATHWAYS

In this article, we focused on the contact-chemosensory signaling that plays a key role in mate choice across *Drosophila* species. However, other sensory modalities also have substantial impacts on partner preference in these flies (Krstic et al., 2009) and the relative contributions of different modalities to mating vary from species to species (Spieth, 1952). Studies in *D. melanogaster* revealed that males rely primarily on visual (Pan et al., 2012; Kohatsu and Yamamoto, 2015) and auditory (von Schilcher, 1976; Ishikawa et al., 2019) cues in tracking a courtship target, while chemosensory cues play major roles in triggering and maintaining courtship actions. In contrast to chemosensory inputs that impinge onto the courtship decision-making P1 neurons (Kohatsu et al., 2011; Clowney et al., 2015; Kallman et al., 2015), auditory and visual inputs seem to be processed by interneurons distinct from P1 neurons, respectively (Ribeiro et al., 2018; Deutsch et al., 2019). It remains to be clarified how the visual and auditory information is integrated with the P1-dependent command in driving courtship behavior.

In many other insects, volatile compounds are commonly used as pheromones, which are processed by olfactory channels in recipient animals and elicit long distance attraction or avoidance (Fleischer and Krieger, 2018). The best-characterized volatile pheromone in *Drosophila* is cVA, which acts through both olfactory and contact-chemosensory pathways (Thistle et al., 2012; Ejima, 2015), and thus these two modalities in fact interact to affect fly mating behavior. Crosstalk between the contact-chemosensory and olfactory systems in controlling mating and other behaviors is probably prevalent (Wang et al., 2011; Laturney and Billeter, 2016), partly reflecting the fact that the same pheromone compound can exist in either the solid/liquid or vapor state at temperatures a fly engages in reproduction. Of note, 7,11-HD is a precursor of Z-4-undecanal, which is known to function as a long range, species-specific, aggregation pheromone detected by odorant receptor Or69a (Lebreton et al., 2017). Alternatively, it might be that Grs can detect volatile compounds and Ors can detect non-volatile compounds.

Crosstalk between contact chemosensory and olfactory pathways also underlies courtship enhancement by food odor. Phenylacetic acid and phenylacetaldehyde are aromatic odors associated with fruit and other plant tissues that feed *Drosophila* flies and provide oviposition sites. These compounds are received by IR84a- and *fru*-expressing olfactory receptor neurons (ORNs) in the antenna. The projection neurons postsynaptic to IR84a ORNs extend their axons into the pheromone-specialist fiber tract even though they convey food odor information (Bates et al., 2020). As a consequence, IR84a-mediated food odor information is sent to a pheromone processing region of the lateral horn, where it is probably integrated together with pheromone information to control mating behavior (Grosjean et al., 2011). Conversely, male-specific cuticular hydrocarbons or cVA deposited onto food promotes landing responses in flying female and male flies, although the neural basis for this effect is not known (Cazalé-Debat et al., 2019; see also Lin et al., 2015; Dumenil et al., 2016). Therefore, crosstalk between contact-chemosensory and olfactory information takes place in both the peripheral and central neural circuitries, and the modes of crosstalk appear to be built in a hardwired connectivity blueprint. Which neurons in the mating circuit receive and process inputs from food-odor interneurons remain unknown. In view of the highly variable feeding habits across species, the circuit bases involved in the integration of food odor and mating signals would also be diversified across species.

A recent comparative study on the olfactory basis for hostplant preference in the *Drosophila melanogaster* subgroup unraveled multilayered modifications at different nodes of olfactory information processing (Auer et al., 2020). *Drosophila sechellia* is a monophagous species specifically associated with noni fruit (*Morinda citrifolia*), whereas the sibling species *D. simulans* is

polyphagous, as are several other members of the group. Long distance attraction to noni fruit in *D. sechellia* depends on at least three modifications of the common design for the olfactory circuitry: specialization in the response spectrum of the olfactory receptor Or22a, an increase in the number of Or22a-harboring sensilla, and acquisition of novel terminal arbors in the lateral horn by the DM2 projection neurons that are postsynaptic to Or22a ORNs (Auer et al., 2020). Yet another study suggested that the odorant binding protein genes *Obp57d* and *Obp57e* were specialized in *D. sechellia* to make this species prefer noni fruit odor, whereas these genes are required for avoiding noni fruit in the sibling species *D. simulans* (Matsuo et al., 2007). This study used species hybrids in conducting unbiased screens for genetic loci that are decisive in contrasting noni fruit preferences between *D. sechellia* and *D. simulans*. A similar and even more thorough approach with species hybrids would be fruitful in identifying a collection of genes that are required for diversified mate preferences.

PERSPECTIVES

The neural mechanism for mating behavior could have accumulated a variety of changes at multiple circuit nodes within the homologous neural pathways across different phylogenetic lineages. Among the members of the *D. melanogaster* species subgroup, species hybrids are relatively easy to obtain, and would offer an ideal platform for studying genome-wide identifications for loci responsible for species differences in mate preference (Castillo and Barbash, 2017). Indeed, genotype-phenotype correlative analyses with whole genome sequencing and behavioral phenotype classification for every hybrid fly is now feasible. Subsequent CRISPR/Cas9-targeted mutagenesis in conjunction with *piggyBac*-based transgenic rescue will be used to assure the causality between the gene and behavior (Tanaka et al., 2016, 2017). The entire brain connectome is near completion in *D. melanogaster*, providing a solid reference map of brain circuitries for the study of neuroanatomy in other members of the *D. melanogaster* species subgroup. We may soon witness the beginning of a new era in the history of evolutionary studies of the neural basis of reproductive isolation and behavioral divergence.

AUTHOR CONTRIBUTIONS

DY: conceptualization, review, and editing. KS and DY: funding acquisition and writing the original draft. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported, in part, by Grants-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology (MEXT) to DY (Grant Nos. 19H04923 and 16H06371) and KS (Grant Nos. 19H04766 and 17K07040), a Life Science Grant from the Takeda Science Foundation to DY and KS, and a Hyogo Science and Technology Association Grant to KS.

ACKNOWLEDGMENTS

We thank current and past members of the Yamamoto laboratory for contributions to the original researches and Y. Takamura for secretarial assistance.

REFERENCES

Ahmed, O. M., Avila-Herrera, A., Tun, K. M., Serpa, P. H., Peng, J., Parthasarathy, S., et al. (2019). Evolution of mechanisms that control mating in *Drosophila* males. *Cell Rep.* 27, 2527–2536.e4. doi: 10.1016/j.celrep.2019.04.104

Alves, H., Rouault, J. D., Kondoh, Y., Nakano, Y., Yamamoto, D., Kim, Y. K., et al. (2010). Evolution of cuticular hydrocarbons of Hawaiian Drosophilidae. *Behav. Genet.* 40, 694–705. doi: 10.1007/s10519-010-9364-y

Antony, C., Davis, T. L., Carlson, D. A., Pechine, J. M., and Jallon, J.-M. (1985). Compared behavioral responses of male *Drosophila melanogaster* (Canton S) to natural and synthetic aphrodisiacs. *J. Chem. Ecol.* 11, 1617–1629. doi: 10.1007/BF01012116

Auer, T. O., Khallaf, M. A., Silbering, A. F., Zappia, G., Ellis, K., Álvarez-Ocaña, R., et al. (2020). Olfactory receptor and circuit evolution promote host specialization. *Nature* 579, 402–408. doi: 10.1038/s41586-020-2073-7

Bartelt, R. J., Jackson, L. L., and Schaner, A. M. (1985). Ester components of aggregation pheromone of *Drosophila virilis* (Diptera: Drosophilidae). *J. Chem. Ecol.* 11, 1197–1208. doi: 10.1007/BF01024108

Bates, A., Schlegel, P., Roberts, R. J. V., Drummond, N., Tamimi, I. F. M., Turnbull, R., et al. (2020). Complete connectomic reconstruction of olfactory projection neurons in the fly brain. *Curr. Biol.* 30, 3183–3199. doi: 10.1016/j.cub.2020.06.042

Billeter, J.-C., Attalah, J., Krupp, 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

Bontonou, G., and Wicker-Thomas, C. (2014). Sexual communication in the *Drosophila* genus. *Insects* 5, 439–458. doi: 10.3390/insects5020439

Bousquet, F., Nojima, T., Houot, B., Chauvel, I., Chaudy, I., Dupas, S., et al. (2012). Expression of a desaturase gene, *desat1*, in neural and nonneural tissues separately affects perception and emission of sex pheromones in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 249–254. doi: 10.1073/pnas.1109166108

Bray, S., and Amrein, H. (2003). A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron* 39, 1019–1029. doi: 10.1016/s0896-6273(03)00542-7

Butterwick, J. A., Mármol, J. D., Kim, K. H., Kahlson, M. A., Rogow, J. A., Walz, T., et al. (2018). Cryo-EM structure of the insect olfactory receptor Orco. *Nature* 560, 447–452. doi: 10.1038/s41586-018-0420-8

Butterworth, F. M. (1969). Lipids of *Drosophila*: a newly detected lipid in the male. *Science* 163, 1356–1357. doi: 10.1126/science.163.3873.1356

Cande, J., Namiki, S., Qiu, J., Korff, W., Card, G. M., Shaevits, J. W., et al. (2018). Optogenetic dissection of descending behavioral control in *Drosophila*. *eLife* 7:e34275. doi: 10.7554/eLife.34275

Castillo, D. M., and Barbash, D. A. (2017). Moving speciation genetics forward: modern techniques build on foundational studies in *Drosophila*. *Genetics* 207, 825–842. doi: 10.1534/genetics.116.187120

Cazalé-Debat, L., Houot, B., Farine, J.-P., Everaerts, C., and Ferveur, J.-F. (2019). Flying *Drosophila* show sex-specific attraction to fly-labelled food. *Sci. Rep.* 9:14947. doi: 10.1038/s41598-019-51351-1

Chen, D., Sitaraman, D., Chen, N., Jin, X., Han, C., Chen, J., et al. (2017). Genetic and neuronal mechanisms governing the sex-specific interaction between sleep and sexual behaviors in *Drosophila*. *Nat. Commun.* 8:154. doi: 10.1038/s41467-017-00087-5

Chertemps, T., Duportets, L., Labeur, C., Ueda, R., Takahashi, K., Saigo, K., et al. (2007). A female-biased expressed elongase involved in long-chain hydrocarbon

biosynthesis and courtship behavior in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4273–4278. doi: 10.1073/pnas.0608142104

Chertemps, T., Duportets, L., Labeur, C., Ueyama, M., and Wicker-Thomas, C. (2006). A female-specific *desaturase* gene responsible for diene hydrocarbon biosynthesis and courtship behaviour in *Drosophila melanogaster*. *Insect Mol. Biol.* 15, 465–473. doi: 10.1111/j.1365-2583.2006.00658.x

Chin, J. S. R., Ellis, S. R., Pham, H. T., Blanksby, S. J., Mori, K., Koh, Q. L., et al. (2014). Sex-specific triacylglycerides are widely conserved in *Drosophila* and mediate mating behavior. *eLife* 3:e01751. doi: 10.7554/eLife.01751

Clemens, J., Coen, P., Roemschied, F. A., Pereira, T. D., Mazumder, D., Aldarondo, D. E., et al. (2018). Discovery of a new song mode in *Drosophila* reveals hidden structure in the sensory and neural drivers of behavior. *Curr. Biol.* 28, 2400–2412.e6. doi: 10.1016/j.cub.2018.06.011

Clowney, E. J., Iguchi, S., Bussell, J. J., Scheer, E., and Ruta, V. (2015). Multimodal chemosensory circuits controlling male courtship in *Drosophila*. *Neuron* 87, 1036–1049. doi: 10.1016/j.neuron.2015.07.025

Clyne, J. D., and Miesenböck, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in *Drosophila*. *Cell* 133, 354–363. doi: 10.1016/j.cell.2008.01.050

Combs, P. A., Krupp, J. J., Khosla, N. M., Bua, D., Petrov, D. A., Levine, J. D., et al. (2018). Tissue-specific *cis*-regulatory divergence implicates *eloF* in inhibiting interspecies mating in *Drosophila*. *Curr. Biol.* 28, 3969–3975.e3. doi: 10.1016/j.cub.2018.10.036

Costa, M., Manton, J., Ostrovsky, A., Prohaska, S., and Jefferis, G. S. X. E. (2016). NBLAST: rapid, sensitive comparison of neuronal structure and construction of neuron family databases. *Neuron* 91, 293–311. doi: 10.1016/j.neuron.2016.06.012

Coyne, J. A., and Orr, H. A. (2004). *Speciation*. (Oxford: Oxford University Press), 545.

Crickmore, M. A., and Vosshall, L. B. (2013). Opposing dopaminergic and GABAergic neurons control the duration and persistence of copulation in *Drosophila*. *Cell* 155, 881–893. doi: 10.1016/j.cell.2013.09.055

Dallerac, R., Labeur, C., Jallon, J.-M., Knipple, D. C., Roelofs, W. L., and Wicker-Thomas, C. (2000). A Δ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9449–9454. doi: 10.1073/pnas.150243997

Datta, S. R., Vasconcelos, M. L., Ruta, V., Luo, S., Wong, A., Demir, E., et al. (2008). The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. *Nature* 452, 473–477. doi: 10.1038/nature06808

Deutsch, D., Clemens, J., Thiberge, S. Y., Guan, G., and Murthy, M. (2019). Shared song detector neurons in *Drosophila* male and female brains drive sex-specific behaviors. *Curr. Biol.* 29, 3200–3215.e5. doi: 10.1016/j.cub.2019.08.008

Dumenil, C., Woud, D., Pinto, F., Alkema, J. T., Jansen, I., Van der Geest, A. M., et al. (2016). Pheromonal cues deposited by mated females convey social information about egg-laying sites in *Drosophila melanogaster*. *J. Chem. Ecol.* 42, 259–269. doi: 10.1007/s10886-016-0681-3

Dweck, H. K. M., and Carlson, J. R. (2020). Molecular logic and evolution of bitter taste in *Drosophila*. *Curr. Biol.* 30, 17–30.e3. doi: 10.1016/j.cub.2019.11.005

Dweck, H. K. M., Ebrahim, S. A., Thoma, M., Mohamed, A. A., Keesey, I. W., Trona, F., et al. (2015). Pheromones mediating copulation and attraction in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 112, E2829–E2835. doi: 10.1073/pnas.1504527112

Ejima, A. (2015). Pleiotropic actions of the male pheromone cis-vaccenyl acetate in *Drosophila melanogaster*. *J. Comp. Physiol. A.* 201, 927–932. doi: 10.1007/s00359-015-1020-9

Ejima, A., Smith, B. P. C., Lucas, C., Naters, W. G., Miller, C. J., Carlson, J. R., et al. (2007). Generalization of courtship learning in *Drosophila* is mediated by cis-vaccenyl acetate. *Curr. Biol.* 17, 599–605. doi: 10.1016/j.cub.2007.01.053

Fan, P., Manoli, D. S., Ahmed, S. M., Chen, Y., Agarwal, N., Kwong, S., et al. (2013). Genetic and neural mechanisms that inhibit *Drosophila* from mating with other species. *Cell* 154, 89–102. doi: 10.1016/j.cell.2013.06.008

Ferveur, J.-F. (1997). The pheromonal role of cuticular hydrocarbons in *Drosophila melanogaster*. *Bioessays* 19, 353–358. doi: 10.1002/bies.950190413

Fleischer, J., and Krieger, J. (2018). Insect pheromone receptors - Key elements in sensing intraspecific chemical signals. *Front. Cell. Neurosci.* 12:425. doi: 10.3389/fncel.2018.00425

Grillet, M., Everaerts, C., Houot, B., Ritchie, M. G., Cobb, M., and Ferveur, J.-F. (2012). Incipient speciation in *Drosophila melanogaster* involves chemical signals. *Sci. Rep.* 2:224. doi: 10.1038/srep00224

Grosjean, Y., Rytz, R., Farine, J.-P., Abuin, L., Cortot, J., Jefferis, G. S. X. E., et al. (2011). An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* 478, 236–240. doi: 10.1038/nature10428

Grover, D., Katsuki, T., and Greenspan, R. (2016). Flyception: imaging brain activity in freely walking fruit flies. *Nat. Methods* 13, 569–572. doi: 10.1038/nmeth.3866

Grover, D., Katsuki, T., Li, J., Dawkins, T. J., and Greenspan, R. (2020). Imaging brain activity during complex social behaviors in *Drosophila* with Flyception2. *Nat. Commun.* 11:623. doi: 10.1038/s41467-020-14487-7

Guiraudie-Capraz, G., Pho, D. B., and Jallon, J.-M. (2007). Role of the ejaculatory bulb in biosynthesis of the male pheromone cis-vaccenyl acetate in *Drosophila melanogaster*. *Integr. Zool.* 2, 89–99. doi: 10.1111/j.1749-4877.2007.00047.x

Ha, T. S., and Smith, D. P. (2006). A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in *Drosophila*. *J. Neurosci.* 26, 8727–8733. doi: 10.1523/JNEUROSCI.0876-06.2006

Hales, K. G., Korey, C. A., Larracuente, A. M., and Roberts, D. M. (2015). Genetics on the fly: a primer on the *Drosophila* model system. *Genetics* 201, 815–842. doi: 10.1534/genetics.115.183392

He, Z., Luo, Y., Shang, X., Sun, J. S., and Carlson, J. R. (2019). Chemosensory sensilla of the *Drosophila* wing express a candidate ionotropic pheromone receptor. *PLoS Biol.* 17:e2006619. doi: 10.1371/journal.pbio.2006619

Hoopfer, E., Jung, Y., Inagaki, H. K., Rubin, G. M., and Anderson, D. J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. *eLife* 4:e11346. doi: 10.7554/eLife.11346

Inagaki, H. K., Jung, Y., Hoopfer, E. D., Wong, A. M., Mishra, N., Lin, J. Y., et al. (2014). Optogenetic control of *Drosophila* using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. *Nat. Methods* 11, 325–332. doi: 10.1038/nmeth.2765

Ishii, K., Wohl, M., DeSouza, A., and Asahina, K. (2020). Sex-determining genes distinctly regulate courtship capability and target preference via sexually dimorphic neurons. *eLife* 9:e52701. doi: 10.7554/eLife.52701

Ishikawa, Y., Okamoto, N., Yoneyama, Y., Maeda, N., and Kamikouchi, A. (2019). A single male auditory response test to quantify auditory behavioral responses in *Drosophila melanogaster*. *J. Neurogenet.* 33, 64–74. doi: 10.1080/01677063.2019.1611805

Jallon, J.-M. (1984). A few chemical words exchanged by *Drosophila* during courtship and mating. *Behav. Genet.* 14, 441–478. doi: 10.1007/BF01065444

Jallon, J.-M., and David, J. R. (1987). Variations in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. *Evolution* 41, 294–302. doi: 10.1111/j.1558-5646.1987.tb05798.x

Kallman, B., Kim, H., and Scott, K. (2015). Excitation and inhibition onto central courtship neurons biases *Drosophila* mate choice. *eLife* 4:e11188. doi: 10.7554/eLife.11188

Keleman, K., Vrontou, E., Krüttner, S., Yu, J. Y., K-Kozaic, A., and Dickson, B. J. (2012). Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. *Nature* 489, 145–149. doi: 10.1038/nature11345

Kimura, K.-I., 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

Kimura, K.-I., Ote, M., Tazawa, T., and Yamamoto, D. (2005). Fruitless specifies sexually dimorphic neural circuitry in the *Drosophila* brain. *Nature* 438, 229–233. doi: 10.1038/nature04229

Kimura, K.-I., Sato, C., Yamamoto, K., and Yamamoto, D. (2015). From the back or front: the courtship position is a matter of smell and sight in *Drosophila melanogaster* males. *J. Neurogenet.* 29, 18–22. doi: 10.3109/01677063.2014.968278

Kimura, K.-I., Urushizaki, A., Sato, C., and Yamamoto, D. (2019). A novel sex difference in *Drosophila* contact chemosensory neurons unveiled using single cell labeling. *J. Neurogenet.* 33, 116–124. doi: 10.1080/01677063.2018.1531858

Koganezawa, M., Haba, D., Matsuo, T., and Yamamoto, D. (2010). The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons. *Curr. Biol.* 20, 1–8. doi: 10.1016/j.cub.2009.11.038

Koganezawa, M., Kimura, K.-I., and Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in *Drosophila* males. *Curr. Biol.* 26, 1395–1403. doi: 10.1016/j.cub.2016.04.017

Koh, R. Q., He, Z., Gorur-Shandilya, S., Menuz, K., Larter, N., Stewart, S., et al. (2014). The *Drosophila* IR20a clade of ionotropic receptors are candidate taste and pheromone receptors. *Neuron* 83, 850–865. doi: 10.1016/j.neuron.2014.07.012

Kohatsu, S., Koganezawa, M., and Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in a *Drosophila* male. *Neuron* 69, 498–508. doi: 10.1016/j.neuron.2010.12.017

Kohatsu, S., and Yamamoto, D. (2015). Visually induced initiation of *Drosophila* innate courtship-like following pursuit is mediated by central excitatory state. *Nat. Commun.* 6:6457. doi: 10.1038/ncomms7457

Kohl, J., Huoviala, P., and Jefferis, G. S. X. E. (2015). Pheromone processing in *Drosophila*. *Curr. Opin. Neurobiol.* 34, 149–157. doi: 10.1016/j.conb.2015.06.009

Kohl, J., Ostrovsky, A. D., Frechter, S., and Jefferis, G. S. X. E. (2013). A bidirectional circuit switch reroutes pheromone signals in male and female brains. *Cell* 155, 1610–1623. doi: 10.1016/j.cell.2013.11.025

Krstic, D., Boll, W., and Noll, M. (2009). Sensory integration regulating male courtship behavior in *Drosophila*. *PLoS One* 4:e4457. doi: 10.1371/journal.pone.0004457

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Lacaille, F., Hiroi, M., Twele, R., Inoshita, T., Umemoto, D., Manière, G., et al. (2007). An inhibitory sex pheromone tastes bitter for *Drosophila* males. *PLoS One* 2:e661. doi: 10.1371/journal.pone.000

Laturney, M., and Billeter, J.-C. (2016). *Drosophila melanogaster* females restore their attractiveness after mating by removing male. *Nat. Commun.* 7:12322. doi: 10.1038/ncomms12322

Lebreton, S., Borrero-Echeverry, F., Gonzalez, F., Solum, M., Wallin, E. A., Hedenström, E., et al. (2017). A *Drosophila* female pheromone elicits species-specific long-range attraction via an olfactory channel with dual specificity for sex and food. *BMC Biol.* 15:88. doi: 10.1186/s12915-017-0427-x

Lebreton, S., Grabe, V., Omondi, A. B., Ignell, R., Becher, P. G., Hansson, B. S., et al. (2014). Love makes smell blind: mating suppresses pheromone attraction in *Drosophila* females via Or65a olfactory neurons. *Science* 4:7119. doi: 10.1038/srep07119

Lin, C. C., Prokop-Prigge, K. A., Preti, G., and Potter, C. J. (2015). Food odors trigger *Drosophila* males to deposit a pheromone that guides aggregation and female oviposition decisions. *eLife* 4:e08688. doi: 10.7554/eLife.08688

Lin, H. H., Cao, D. S., Sethi, S., Zeng, Z., Chin, J. S. R., Chakraborty, T. S., et al. (2016). Hormonal modulation of pheromone detection enhances male courtship success. *Neuron* 90, 1272–1285. doi: 10.1016/j.neuron.2016.05.004

Ling, F., Dahanukar, A., Weiss, L. A., Kwon, J. Y., and Carlson, J. R. (2014). The molecular and cellular basis of taste coding in the legs of *Drosophila*. *J. Neurosci.* 34, 7148–7164. doi: 10.1523/JNEUROSCI.0649-14.2014

Liu, T., Starostina, E., Vijayan, V., and Pikielny, C. W. (2012). Two *Drosophila* DEG/ENaC channel subunits have distinct functions in gustatory neurons that activate male courtship. *J. Neurosci.* 32, 11879–11889. doi: 10.1523/JNEUROSCI.1376-12.2012

Liu, T., Wang, Y., Tian, Y., Zhang, J., Zhao, J., and Guo, A. (2020). The receptor channel formed by *ppk25, ppk29* and *ppk23* can sense the *Drosophila* female pheromone 7,11-heptacosadiene. *Genes Brain Behav.* 19:e12529. doi: 10.1111/gbb.12529

Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y. H., Zhang, J. X., et al. (2011). Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in *Drosophila*. *Nat. Neurosci.* 14, 896–902. doi: 10.1038/nn.2836

Lu, B., LaMora, A., Sun, Y., Welsh, M. J., and Ben-Shahar, Y. (2012). ppk23-dependent chemosensory functions contribute to courtship behavior in *Drosophila melanogaster*. *PLoS Genet.* 8:e1002587. doi: 10.1371/journal.pgen.1002587

Matsuo, T., Sugaya, S., Yasukawa, J., Aigaki, T., and Fuyama, Y. (2007). Odorant-binding proteins OBP57d and OBP57e affect taste perception and host-plant preference in *Drosophila sechellia*. *PLoS Biol.* 5:e118. doi: 10.1371/journal.pbio.0050118

McKellar, C., Lillvis, J. L., Bath, D. E., Fitzgerald, J. E., Cannon, J. G., Simpson, J. H., et al. (2019). Threshold-based ordering of sequential actions during *Drosophila* courtship. *Curr. Biol.* 29, 426–434.e6. doi: 10.1016/j.cub.2018.12.019

Mellert, D. M., Knapp, J. M., Manoli, D. S., Meissner, G. W., and Baker, B. S. (2010). Midline crossing by gustatory receptor neuron axons is regulated by *fruitless, doublesex* and the Roundabout receptors. *Development* 137, 323–332. doi: 10.1242/dev.045047

Namiki, S., Dickinson, M. H., Wong, A. M., Karff, W., and Card, G. M. (2018). The functional organization of descending sensory-motor pathways in *Drosophila*. *eLife* 7:e34272. doi: 10.7554/eLife.34272

Ng, R., Salem, S. S., Wu, S. T., Wu, M., Lin, H. H., Shepherd, A. K., et al. (2019). Amplification of *Drosophila* olfactory responses by a DEG/ENaC channel. *Neuron* 104, 947–959.e5. doi: 10.1016/j.neuron.2019.08.041

Ng, W. C., Chin, J. S., Tan, K. J., and Yew, J. Y. (2015). The fatty acid elongase Bond is essential for *Drosophila* sex pheromone synthesis and male fertility. *Nat. Commun.* 6:8263.

Pan, Y., Meissner, G. W., and Baker, B. S. (2012). Joint control of *Drosophila* male courtship behavior by motion cues and activation of male-specific P1 neurons. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10065–10070. doi: 10.1073/pnas.1207107109

Qiu, Y., Tittiger, C., Wicker-Thomas, C., Le Goff, G., Young, S., Wajnberg, E., et al. (2012). An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14858–14863. doi: 10.1073/pnas.1208650109

Ren, Q., Awasaki, T., Huang, Y. F., Liu, Z., and Lee, T. (2016). Cell class-lineage analysis reveals sexually dimorphic lineage compositions in the *Drosophila* brain. *Curr. Biol.* 26, 2583–2593. doi: 10.1016/j.cub.2016.07.086

Ribeiro, I. M. A., Drews, M., Bahl, A., Machacek, C., Borst, A., and Dickson, B. J. (2018). Visual projection neurons mediating directed courtship in *Drosophila*. *Cell* 174, 607–621.e18. doi: 10.1016/j.cell.2018.06.020

Ruta, V., Datta, S. R., Vasconcelos, L. V., Freeland, J., Looger, L. L., and Axel, R. (2010). A dimorphic pheromone circuit in *Drosophila* from sensory input to descending output. *Nature* 468, 686–690. doi: 10.1038/nature09554

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452, 1002–1006. doi: 10.1038/nature06850

Sato, K., Tanaka, K., and Touhara, K. (2011). Sugar-regulated cation channel formed by an insect gustatory receptor. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11680–11685. doi: 10.1073/pnas.1019622108

Seeholzer, L., Seppi, M., Stern, D., and Ruta, V. (2018). Evolution of a central neural circuit underlies *Drosophila* mate preferences. *Nature* 559, 564–569. doi: 10.1038/s41586-018-0322-9

Shirangi, T. T., Dufour, H. D., Williams, T. M., and Carroll, S. B. (2009). Rapid evolution of sex pheromone-producing enzyme expression in *Drosophila*. *PLoS Biol.* 7:e1000168. doi: 10.1371/journal.pbio.1000168

Shumer, M., Powell, D. L., Delclós, P. J., Squire, M., Cui, R., Andolfatto, P., et al. (2017). Assortative mating and persistent reproductive isolation in hybrids. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10936–10941. doi: 10.1073/pnas.1711238114

Skiba, P. J., and Jackson, L. L. (1993). (Z)-10-heptadecen-2-one and 2-tridecanone biosynthesis from [1-14C]acetate by *Drosophila buzzatii*. *Insect Biochem. Mol. Biol.* 23, 375–380. doi: 10.1016/0965-1748(93)90021-J

Spieth, H. T. (1952). Mating behavior within the genus *Drosophila* (Diptera). *Bull. Am. Mus. Nat.* 99, 399–474.

Starostina, E., Liu, T., Vijayan, V., Zheng, Z., Siwicki, K. K., and Pikielny, C. W. (2012). A *Drosophila* DEG/ENaC subunit functions specifically in gustatory neurons required for male courtship behavior. *J. Neurosci.* 32, 4665–4674. doi: 10.1523/JNEUROSCI.6178-11.2012

Sweigart, A. L. (2010). The genetics of postmating, prezygotic reproductive isolation between *Drosophila virilis* and *D. americana*. *Genetics* 184, 401–410. doi: 10.1534/genetics.109.111245

Symonds, M. R. E., and Wertheim, B. (2005). The mode of evolution of aggregation pheromones in *Drosophila* species. *J. Evol. Biol.* 18, 1253–1263. doi: 10.1111/j.1420-9101.2005.00971.x

Takahashi, A., Tsaur, S. C., Coyne, J. A., and Wu, C. I. (2001). The nucleotide changes governing cuticular hydrocarbon variation and their evolution in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3920–3925. doi: 10.1073/pnas.061465098

Tanaka, R., Higuchi, T., Kohatsu, S., Sato, K., and Yamamoto, D. (2017). Optogenetic activation of the *fruitless*-labeled circuitry in *Drosophila subobscura*

males induces mating motor acts. *J. Neurosci.* 37, 11662–11674. doi: 10.1523/JNEUROSCI.1943-17.2017

Tanaka, R., Murakami, H., Ote, M., and Yamamoto, D. (2016). Clustered regulatory interspaced short palindromic repeats (CRISPR)-mediated mutagenesis and phenotype rescue by *piggyBac* transgenesis in a nonmodel *Drosophila* species. *Insect Mol. Biol.* 25, 355–361. doi: 10.1111/imb.12232

Thistle, R., Camaeron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during *Drosophila* courtship. *Cell* 149, 1140–1151. doi: 10.1016/j.cell.2012.03.045

Thompkins, L., McRobert, S., and Kaneshiro, K. Y. (1993). Chemical communication in Hawaiian *Drosophila*. *Evolution* 47, 1407–1419. doi: 10.1111/j.1558-5646.1993.tb02163.x

Toda, H., Zhao, X., and Dickson, B. J. (2012). The *Drosophila* female aphrodisiac pheromone activates *ppk23*(+) sensory neurons to elicit male courtship behavior. *Cell Rep.* 1, 599–607. doi: 10.1016/j.celrep.2012.05.007

Vijayan, V., Thistle, R., Liu, T., Starostina, E., and Pikielny, C. W. (2014). *Drosophila* pheromone-sensing neurons expressing the Ppk25 ion channel subunit stimulate male courtship and female receptivity. *PLoS Genet.* 10:e1004238. doi: 10.1371/journal.pgen.1004238

von Philipsborn, A. C., Liu, T., Yu, J. Y., Masser, C., Bidaye, S. S., and Dickson, B. J. (2011). Neuronal control of *Drosophila* courtship song. *Neuron* 69, 509–522. doi: 10.1016/j.neuron.2011.01.011

von Schilcher, F. (1976). The role of auditory stimuli in the courtship of *Drosophila melanogaster*. *Anim. Behav.* 24, 18–26. doi: 10.1016/s0003-3472(76)80095-4

Wang, L., and Anderson, D. J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in *Drosophila*. *Nature* 463, 227–231. doi: 10.1038/nature08678

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., et al. (2011). Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*. *Nat. Neurosci.* 14, 757–762. doi: 10.1038/nn.2800

Watanabe, K., Toba, G., Koganezawa, M., and Yamamoto, D. (2011). *Gr39a*, a highly diversified gustatory receptor in *Drosophila*, has a role in sexual behavior. *Behav. Genet.* 41, 746–753. doi: 10.1007/s10519-011-9461-6

Wertheim, B., Dicke, M., and Vet, L. E. M. (2002). Behavioural plasticity in support of a benefit for aggregation pheromone use in *Drosophila melanogaster*. *Entomol. Exp. Appl.* 103, 61–71. doi: 10.1046/j.1570-7458.2002.00954.x

Wicker-Thomas, C., Guenachi, I., and Keita, Y. F. (2009). Contribution of oenocytes and pheromones to courtship behaviour in *Drosophila*. *BMC Biochem.* 10:21. doi: 10.1186/1471-2091-10-21

Wohl, M., Ishii, K., and Asahina, K. (2020). Layered roles of Fruitless isoforms in specification and function of male aggression-promoting neurons in *Drosophila*. *eLife* 9:e52702. doi: 10.7554/eLife.52702

Wu, C. I., Hollocher, H., Gegun, D. J., Aquadro, C. F., Xu, Y., and Wu, M. L. (1995). Sexual isolation in *Drosophila melanogaster*: a possible case of incipient speciation. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2519–2523. doi: 10.1073/pnas.92.7.2519

Wu, S., Guo, C., Zhao, H., Sun, M., Chen, J., Han, C., et al. (2019). Drosulfakinin signaling in *fruitless* circuitry antagonizes P1 neurons to regulate sexual arousal in *Drosophila*. *Nat. Commun.* 10:4770. doi: 10.1038/s41467-019-12758-6

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

Yew, J. Y., and Chung, H. (2017). *Drosophila* as a holistic model for insect pheromone signaling and processing. *Curr. Opin.* 24, 15–20. doi: 10.1016/j.cois.2017.09.003

Yew, J. Y., Dreisewerd, K., Luftmann, H., Müthing, J., Pohlentz, G., and Kravitz, E. A. (2009). A new male sex pheromone and novel cuticular cues for chemical communication in *Drosophila*. *Curr. Biol.* 19, 1245–1254. doi: 10.1016/j.cub.2009.06.037

Yu, J. Y., Kanai, M. I., Demir, E., Jefferis, G. S. X. E., and Dickson, B. J. (2010). Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. *Curr. Biol.* 20, 1602–1614. doi: 10.1016/j.cub.2010.08.025

Zhang, S. X., Miner, L. E., Boutros, C. L., Rogulja, D., and Crickmore, M. A. (2018). Motivation, perception, and chance converge to make a binary decision. *Neuron* 99, 376–388.e6. doi: 10.1016/j.neuron.2018.06.014

Zhang, S. X., Rogulja, D., and Crickmore, M. A. (2016). Dopaminergic circuitry underlying mating drive. *Neuron* 91, 168–181. doi: 10.1016/j.neuron.2016.05.020

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

OPINION
published: 11 December 2020
doi: 10.3389/fnbeh.2020.599234

Fungus-Growing Ants: Models for the Integrative Analysis of Cognition and Brain Evolution

Isabella B. Muratore[1]* *and James F. A. Traniello*[1,2]

[1] Department of Biology, Boston University, Boston, MA, United States, [2] Graduate Program in Neuroscience, Boston University, Boston, MA, United States

Keywords: cognitive ecology, behavior, social brain, ant, division of labor

Agents of selection for behavioral responses to abiotic, biotic, and social environments are described as *cognitive challenges*. Research integrating behavior, ecology, and brain evolution has generated a growing literature—and sometimes controversy—over inferences made from correlating cognitive traits with neural metrics. We propose that our understanding of the role of cognition in brain evolution can be advanced through studies of eusocial insect species differing in agricultural practices and degree of division of labor, and thus social complexity. Fungus-growing ants offer diverse systems to assess the impacts of cognitive challenges on behavioral evolution and its neural and genomic architectures. Workers exhibit variability in social role differentiation in association with diet, morphology, group size, and task efficiency. This suite of covarying traits enables the accurate mapping of cognition, worker repertoire breadth, neuroanatomy, and genomic change in light of social evolution.

OPEN ACCESS

Edited by:
Clare C. Rittschof,
University of Kentucky, United States

Reviewed by:
Christian Rabeling,
Arizona State University, United States
David Baracchi,
University of Florence, Italy

***Correspondence:**
Isabella B. Muratore
imurator@bu.edu

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *26 August 2020*
Accepted: *23 November 2020*
Published: *11 December 2020*

Citation:
Muratore IB and Traniello JFA (2020) Fungus-Growing Ants: Models for the Integrative Analysis of Cognition and Brain Evolution. Front. Behav. Neurosci. 14:599234. doi: 10.3389/fnbeh.2020.599234

HOW DO BRAINS RESPOND TO COGNITIVE CHALLENGES?

Cognition is difficult to universally define (Logan et al., 2018; Bayne et al., 2019) and measure (Rowe and Healy, 2014; Simons and Tibbetts, 2019). However, cognitive ecologists have developed definitions emphasizing divergent demands from behavioral niches and neurobiological capabilities (Balda and Kamil, 1989; Real, 1993; Shettleworth, 2000, 2010; Dukas and Ratcliffe, 2009; Lihoreau et al., 2019). Cognition should be linked to ecological adaptation to understand developmental and evolutionary brain plasticity. Cognitive capability is thus the product of selection for brain organization to adaptively increase computational power and reduce energetic costs. Metrics applied in the study of brain evolution range from genes and cells to nervous system topologies. Correlations between behavioral capabilities and tissue volume have been viewed critically (Herculano-Houzel et al., 2006, 2007; Healy and Rowe, 2007, 2013; Chittka and Niven, 2009; Godfrey and Gronenberg, 2019; Wartel et al., 2019), although in principle quantify brain investment. Functionally specialized brain compartments may develop allometrically (disproportionate scaling) through differential cell and tissue-type trajectories (Barton and Harvey, 2000; Hager et al., 2012), circuitry (Guzowski et al., 2005), neuron structure and function (Quiroga et al., 2005), and genetics (Hibar et al., 2015; Kohno and Kubo, 2019). These patterns provide fine-grain traits for evolutionary analyses.

Social environments can influence brain evolution. Primates distinguish rivals from allies and recall interaction histories. Social brain theory, which posits a positive correlation between brain volume and group size to track social relationships (Dunbar and Shultz, 2017), has been applied to eusocial insects (Lihoreau et al., 2012; Godfrey and Gronenberg, 2019). However, eusocial insect workers typically lack the competing demands of direct reproduction; their brains are functionally dedicated to altruistic labor, and cognitive challenges from specialized behavior can thus be more clearly circumscribed. Diverse social systems enable the functional analysis of mosaic brains and

responsiveness to divergent sensory demands underpinning task specialization (Muscedere and Traniello, 2012; Giraldo et al., 2013; Gordon et al., 2017). Two eusocial insect clades—a tribe of ants and a subfamily of termites—include *ultrasocial* species (Campbell, 1982) that are agriculturalists, producing their own crops of gongylidia—nutritional fungal swellings—and have evolved complex division of labor. These traits are shared with humans (Gowdy and Krall, 2016). Ant societies, as models, can be experimentally dissected (Kennedy et al., 2017), enabling studies of cognitive variation in association with the evolution of division of labor.

Assessing motivated behavior in natural contexts (Rowe and Healy, 2014) and selecting comparative frameworks illustrating divergence in cognitive challenges across related species (Simons and Tibbetts, 2019) are essential to link fitness to behavioral evolution. Therefore, to determine cognitive impacts on brain evolution, a model system should meet the following criteria: (1) the natural behavioral environment can be measured to assess sensory and processing requirements; (2) behavior can be quantified at multiple levels of intraspecies and interspecies biological organization; and (3) the metrics used to identify neural and genomic underpinnings are methodologically and statistically robust. With these points in mind, we identify fungus-growing ants as appropriate and insightful study models for cognitive evolution.

DIVISION OF LABOR AND WORKER COGNITION

The evolution of division of labor in support of agriculture in fungus-growing ants enables societal and individual cognition to be examined. Workers vary morphologically (monomorphism to exceptional polymorphism) and behaviorally (task pluripotency to specialization) across species and within colonies (Mehdiabadi and Schultz, 2010). In highly polymorphic leafcutting ants, colonies are large and may produce size-differentiated workers—for example, minims, medias, and majors in order of increasing size. This variation in body size, colony size, and diet can help disentangle confounding factors that may obscure the linkage of neuroanatomy to behavior. Fungus-growing ants select, harvest, and process plant tissue and other substrates to provide for fungal growth, cultivate fungus, manage waste and control infection, construct and maintain the nest and regulate microclimate, and provide defense. Workers with specialized repertoires are predicted to be more efficient than generalists (Wilson, 1980b). In theory, drivers of worker task performance may differ, but in polymorphic species body size and behavior are integrated and clearly correlate (Beshers and Fewell, 2001). Cognitive needs vary according to role and worksite: tasks performed within the nest by fungal-garden tenders require different stimulus-processing capabilities than foragers or defenders working outside the nest or at multiple worksites. Identifying, cutting, transporting, and mulching leaves forms an assembly line of exterior to interior work where leaf fragments are degraded as they are passed from larger to smaller workers and eventually deposited as fungal mulch. Worker size-related labor therefore requires specific motivation and cognitive abilities.

Minim workers primarily transplant and prune gongylidia. Working in dark underground fungal chambers, they likely rely on sensory inputs other than vision for navigation, which may involve the central complex (Plath and Barron, 2015; Honkanen et al., 2019). They also nurse, recognizing larval needs and discriminating brood stages, and assess humidity and temperature to maintain optimal growth conditions. These tasks involve chemical signals (Schultner and Pulliainen, 2020) processed by the antennal lobes and mushroom bodies, as well as fine motor coordination of the mouthparts, mediated by subesophageal zone circuitry (Paul and Gronenberg, 2002). Minims may deposit pheromones on foraging trails (Howard, 2001; Evison et al., 2008), clean contaminants from incoming leaves and otherwise protect the fungus from microbes (Goes et al., 2020), and defend against parasitic flies (Feener and Moss, 1990).

Media workers engage in diverse tasks. Large-scale agriculture requires evaluating diverse plant chemistries to assess leaf quality and maximize fungal growth (Hubbell et al., 1984; Howard et al., 1988; Saverschek et al., 2010). This discrimination may require learning. Also, the gustatory and olfactory processing abilities of medias should be well developed. Media worker skill in cutting leaves (Wilson, 1980b) requires compass-like coordination of legs and mandibles that determines leaf fragment size, facilitating size-assortative load-bearing for transport (Wilson, 1980a; Burd, 2000; Burd and Howard, 2008). Medias navigate trails between food sources and the nest. In many ants, this process involves recalling landmarks, using odometry and optic flow to measure speed and distance, learning canopy patterns and celestial cues, and decoding chemical recruitment information (Ronacher and Wehner, 1995; Wittlinger et al., 2006; Provecho and Josens, 2009; Basten and Mallot, 2010; Müller and Wehner, 2010; Steck, 2012; Heinze et al., 2018). Media worker foraging thus requires processing multimodal signals through interplay between the antennal and optic lobes, mushroom bodies, and central complex. Behavioral flexibility may be reflected in enlarged mushroom bodies (Farris, 2013), a pattern expected in media brains, but not minims or majors.

Majors defend against army ants and other enemies (Powell and Clark, 2004). Defensive may require close-range vision, mediated by the optic lobes (Via, 1977), and antennal lobe and mushroom body tuning to recruitment and alarm pheromones (López-Riquelme et al., 2006; Mizunami et al., 2010). Differences in task biomechanical demands are evident in subcaste myology (Gronenberg et al., 1997; Paul and Gronenberg, 1999, 2002): larger mandibular muscles provide majors with bite force, controlled in part by the subesophageal zone.

SOCIAL AND PHYLOGENETIC PERSPECTIVES ON COGNITIVE EVOLUTION

Fungus-growing ant species richness (>230 species; Schultz and Brady, 2008) encompasses exceptional heterogeneity

in agricultural practice and social complexity. Behavioral phenotypes evolved greater specialization through developmental divergence in worker morphology (Mehdiabadi and Schultz, 2010; Sosa-Calvo et al., 2018; Solomon et al., 2019). The diversity of worker phenotypes in leafcutting genera such as *Atta* and *Acromyrmex,* which cultivate large quantities of fungus and form colonies of millions of polymorphic workers, is thought to have evolved from an ancestral monomorphic, generalist worker caste (Wilson, 1980a). The ancestral worker phenotype is evident in the paleoattini: these species form small colonies of monomorphic workers that engage in basic agriculture, scavenging insect frass and other materials for fungal substrate. Repertoire breadth is thought to influence brain size: performing more kinds of tasks requires greater processing power (Benson-Amram et al., 2016). A specialist worker of a polymorphic neoattine species would be relatively free of the constraints of maintaining a generalist repertoire and could evolve to prioritize neural capabilities specified by its task set. Size-differentiated workers display disproportionate scaling in morphology and physiology related to social roles that affect task efficiency (Wilson, 1980b). Selection should also be evident in brain structure in both attine clades. In sum, worker morphology, behavior, and brain size and structure are predicted to be integrated.

SOCIETIES, BRAINS, AND GENOMES

Ecological niche differentiation, and thus variability in cognitive needs across attine species and among neoattine worker subcastes, is remarkable. In some socially complex species, brain size (Seid et al., 2011), investment in vision-related compartments (Arganda et al., 2020), and microprocessing circuitry (Groh et al., 2014) vary with worker size. Brain volume decreases and antennal lobe volume increases with social group size in monomorphic species, suggesting decreased selective pressures on brain size coupled with a need for increased olfactory social discrimination (Riveros et al., 2012). Larger *Atta* workers have an antennal lobe macroglomerulus, absent in smaller workers, that likely functions in trail following (Kleineidam et al., 2005). Increased volume in visual processing regions in *A. cephalotes* majors allows greater visual acuity and processing in workers active in light and engaging in close-range defense (Arganda et al., 2020). Neuroanatomical and neurochemical variation (Smith et al., 2013) should integrate with brain gene expression to control behavior (Li et al., 2014; Qiu et al., 2018), enabling neural requirements of specific roles to be met.

Genetic analyses offer mechanistic and evolutionary insight into agriculturally adapted brains. Gene expression regulating attine ant neural phenotypes and behavior (Castillo and Pietrantonio, 2013; Koch et al., 2013) may be influenced by epigenetics, RNA editing, and copy number, as in related systems (Chittka et al., 2012; Scholes et al., 2013; Feldmeyer et al., 2014; Li et al., 2014). Developmental switches mediating size-related differentiation (Rajakumar et al., 2012, 2018) and differentially expressed brain gene modules related to caste determination (Qiu et al., 2018) appear conserved, although some worker-biased genes are more evolutionarily novel (Feldmeyer et al., 2014; Mikheyev and Linksvayer, 2015; Schrader et al., 2017). Deep brain homologies in eusocial insects (Tomer et al., 2010; Shpigler et al., 2017; Trible et al., 2017) provide broadly translatable insights into adaptive brain evolution and development, and their genomic basis.

FUTURE RESEARCH

The ability to identify mechanisms of response to cognitive challenges within phylogenetic context facilitates understanding brain evolution in light of socioecological selective forces. This allows the relative importance of task repertoire breadth and social structure to be examined. Studies that assess the same properties of learning (speed and memory, e.g.,) but consider species-specificity in behavior across size-variable workers in paleo-and neoattine ants can elucidate effects of social complexity on brain evolution. Comparative studies of neuroanatomical scaling and genomics enable variation in task diversity and sensory environments to be mapped onto fungus-growing ant phylogeny to reveal evolutionary patterns. Gene functions influencing neuroanatomy and behavior can reveal the relative importance of metabolism, neurotransmission, growth factors, and other pathways in the evolution of division of labor. The contrast between simple societies of monomorphic fungus-growing ants and complex colonies of leafcutting ants provides opportunities to examine genomic evolution in the brain. With increasingly precise genetic tools available for ant research, components of neural and anatomical phenotypes may be separated and linked to developmental origins. Ultimately, functional manipulations and genomic data will enable the identification of neurogenetic traits associated with cognitive evolution.

AUTHOR CONTRIBUTIONS

IM and JT drafted and edited the manuscript. JT secured funding. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Brenton R. Lutz award to IM and National Science Foundation grants IOS 1354291 and IOS 1953393 to JT.

ACKNOWLEDGMENTS

We gratefully acknowledge the insights of Sara Arganda, Sam Beshers, Zach Coto, and Frank Azorsa in developing the manuscript, and the comments of Clare Rittschof and two reviewers.

REFERENCES

Arganda, S., Hoadley, A. P., Razdan, E. S., Muratore, I. M., and Traniello, J. F. A. (2020). The neuroplasticity of division of labor: worker polymorphism, compound eye structure and brain organization in the leafcutter ant *Atta cephalotes*. *J. Comp. Physiol. A* 206, 651–662. doi: 10.1007/s00359-020-01423-9

Balda, R. P., and Kamil, A. C. (1989). A comparative study of cache recovery by three corvid species. *Anim. Behav.* 38, 486–495. doi: 10.1016/S0003-3472(89)80041-7

Barton, R. A., and Harvey, P. H. (2000). Mosaic evolution of brain structure in mammals. *Nature* 405:1055. doi: 10.1038/35016580

Basten, K., and Mallot, H. A. (2010). Simulated visual homing in desert ant natural environments: efficiency of skyline cues. *Biol. Cybern.* 102, 413–425. doi: 10.1007/s00422-010-0375-9

Bayne, T., Brainard, D., Byrne, R. W., Chittka, L., Clayton, N., Heyes, C., et al. (2019). What is cognition? *Curr. Biol.* 29, R608–R615. doi: 10.1016/j.cub.2019.05.044

Benson-Amram, S., Dantzer, B., Stricker, G., Swanson, E. M., and Holekamp, K. E. (2016). Brain size predicts problem-solving ability in mammalian carnivores. *Proc. Natl. Acad. Sci. U. S. A.* 113, 2532–2537. doi: 10.1073/pnas.1505913113

Beshers, S. N., and Fewell, J. H. (2001). Models of division of labor in social insects. *Annu. Rev. Entomol.* 46, 413–440. doi: 10.1146/annurev.ento.46.1.413

Burd, M. (2000). Body size effects on locomotion and load carriage in the highly polymorphic leaf-cutting ants *Atta colombica* and *Atta cephalotes*. *Behav. Ecol.* 11, 125–131. doi: 10.1093/beheco/11.2.125

Burd, M., and Howard, J. J. (2008). Optimality in a partitioned task performed by social insects. *Biol. Lett.* 4, 627–629. doi: 10.1098/rsbl.2008.0398

Campbell, D. T. (1982). Legal and primary-group social controls. *J. Soc. Biol. Struct.* 5, 431–438. doi: 10.1016/S0140-1750(82)92071-1

Castillo, P., and Pietrantonio, P. V. (2013). Differences in sNPF receptor-expressing neurons in brains of fire ant (*Solenopsis invicta* Buren) worker subcastes: indicators for division of labor and nutritional status? *PLoS ONE* 8:e83966. doi: 10.1371/journal.pone.0083966

Chittka, A., Wurm, Y., and Chittka, L. (2012). Epigenetics: the making of ant castes. *Curr. Biol.* 22, R835–R838. doi: 10.1016/j.cub.2012.07.045

Chittka, L., and Niven, J. (2009). Are bigger brains better? *Curr. Biol.* 19, R995–R1008. doi: 10.1016/j.cub.2009.08.023

Dukas, R., and Ratcliffe, J. M. (eds.). (2009). *Cognitive Ecology II*. Chicago, IL: University of Chicago Press.

Dunbar, R. I. M., and Shultz, S. (2017). Why are there so many explanations for primate brain evolution? *Philos. Trans. R. Soc. B* 372:20160244. doi: 10.1098/rstb.2016.0244

Evison, S. E., Hart, A. G., and Jackson, D. E. (2008). Minor workers have a major role in the maintenance of leafcutter ant pheromone trails. *Anim. Behav.* 75, 963–969. doi: 10.1016/j.anbehav.2007.07.013

Farris, S. M. (2013). Evolution of complex higher brain centers and behaviors: behavioral correlates of mushroom body elaboration in insects. *Brain Behav. Evolut.* 82, 9–18. doi: 10.1159/000352057

Feener, D. H., and Moss, K. A. (1990). Defense against parasites by hitchhikers in leaf-cutting ants: a quantitative assessment. *Behav. Ecol. Sociobiol.* 26, 17–29. doi: 10.1007/BF00174021

Feldmeyer, B., Elsner, D., and Foitzik, S. (2014). Gene expression patterns associated with caste and reproductive status in ants: worker-specific genes are more derived than queen-specific ones. *Mol. Ecol.* 23, 151–161. doi: 10.1111/mec.12490

Giraldo, Y. M., Patel, E., Gronenberg, W., and Traniello, J. F. (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

Godfrey, R. K., and Gronenberg, W. (2019). Brain evolution in social insects: advocating for the comparative approach. *J. Comp. Physiol. A* 205, 13–32. doi: 10.1007/s00359-019-01315-7

Goes, A. C., Barcoto, M. O., Kooij, P. W., Bueno, O. C., and Rodrigues, A. (2020). How do leaf-cutting ants recognize antagonistic microbes in their fungal crops? *Front. Ecol. Evol.* 8:95. doi: 10.3389/fevo.2020.00095

Gordon, D. G., Ilieş, I., and Traniello, J. F. (2017). Behavior, brain, and morphology in a complex insect society: trait integration and social evolution in the exceptionally polymorphic ant *Pheidole rhea*. *Behav. Ecol. Sociobiol.* 71:166. doi: 10.1007/s00265-017-2396-z

Gowdy, J., and Krall, L. (2016). The economic origins of ultrasociality. *Behav. Brain Sci.* 39:e92. doi: 10.1017/S0140525X1500059X

Groh, C., Kelber, C., Grübel, K., and Rössler, W. (2014). Density of mushroom body synaptic complexes limits intraspecies brain miniaturization in highly polymorphic leaf-cutting ant workers. *Proc. R. Soc. B* 281:20140432. doi: 10.1098/rspb.2014.0432

Gronenberg, W., Paul, J., Just, S., and Hölldobler, B. (1997). Mandible muscle fibers in ants: fast or powerful? *Cell Tissue Res.* 289, 347–361. doi: 10.1007/s004410050882

Guzowski, J. F., Timlin, J. A., Roysam, B., McNaughton, B. L., Worley, P. F., and Barnes, C. A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Curr. Opin. Neurobiol.* 15, 599–606. doi: 10.1016/j.conb.2005.08.018

Hager, R., Lu, L., Rosen, G. D., and Williams, R. W. (2012). Genetic architecture supports mosaic brain evolution and independent brain–body size regulation. *Nat. Commun.* 3:1079. doi: 10.1038/ncomms2086

Healy, S. D., and Rowe, C. (2007). A critique of comparative studies of brain size. *Proc. R. Soc. B* 274, 453–464. doi: 10.1098/rspb.2006.3748

Healy, S. D., and Rowe, C. (2013). Costs and benefits of evolving a larger brain: Doubts over the evidence that large brains lead to better cognition. *Anim. Behav.* 4, e1–e3. doi: 10.1016/j.anbehav.2013.05.017

Heinze, S., Narendra, A., and Cheung, A. (2018). Principles of insect path integration. *Curr. Biol.* 28, R1043–R1058. doi: 10.1016/j.cub.2018.04.058

Herculano-Houzel, S., Collins, C. E., Wong, P., and Kaas, J. H. (2007). Cellular scaling rules for primate brains. *Proc. Natl. Acad. Sci. U. S. A.* 104, 3562–3567. doi: 10.1073/pnas.0611396104

Herculano-Houzel, S., Mota, B., and Lent, R. (2006). Cellular scaling rules for rodent brains. *Proc. Natl. Acad. Sci. U. S. A.* 103, 12138–12143. doi: 10.1073/pnas.0604911103

Hibar, D. P., Stein, J. L., Renteria, M. E., Arias-Vasquez, A., Desrivières, S., Jahanshad, N., et al. (2015). Common genetic variants influence human subcortical brain structures. *Nature* 520, 224–229. doi: 10.1038/nature14101

Honkanen, A., Adden, A., da Silva Freitas, J., and Heinze, S. (2019). The insect central complex and the neural basis of navigational strategies. *J. Exp. Biol.* 222(Suppl. 1):jeb188854. doi: 10.1242/jeb.188854

Howard, J. J. (2001). Costs of trail construction and maintenance in the leaf-cutting ant *Atta columbica*. *Behav. Ecol. Sociobiol.* 49, 348–356. doi: 10.1007/s002650000314

Howard, J. J., Cazin, J., and Wiemer, D. F. (1988). Toxicity of terpenoid deterrents to the leafcutting ant *Atta cephalotes* and its mutualistic fungus. *J. Chem. Ecol.* 14, 59–69. doi: 10.1007/BF01022531

Hubbell, S. P., Howard, J. J., and Wiemer, D. F. (1984). Chemical leaf repellency to an attine ant: seasonal distribution among potential host plant species. *Ecology* 65, 1067–1076. doi: 10.2307/1938314

Kennedy, P., Baron, G., Qiu, B., Freitak, D., Helanterä, H., Hunt, E. R., et al. (2017). Deconstructing superorganisms and societies to address big questions in biology. *Trends Ecol. Evol.* 32, 861–872. doi: 10.1016/j.tree.2017.08.004

Kleineidam, C. J., Obermayer, M., Halbich, W., and Rössler, W. (2005). A macroglomerulus in the antennal lobe of leaf-cutting ant workers and its possible functional significance. *Chem. Senses* 30, 383–392. doi: 10.1093/chemse/bji033

Koch, S. I., Groh, K., Vogel, H., Hannson, B. S., Kleineidam, C. J., and Grosse-Wilde, E. (2013). Caste-specific expression patterns of immune response and chemosensory related genes in the leaf-cutting ant, *Atta vollenweideri*. *PLoS ONE* 8:e81518. doi: 10.1371/journal.pone.0081518

Kohno, H., and Kubo, T. (2019). Genetics in the honey bee: Achievements and prospects toward the functional analysis of molecular and neural mechanisms underlying social behaviors. *Insects* 10:348. doi: 10.3390/insects10100348

Li, Q., Wang, Z., Lian, J., Schiøtt, M., Jin, L., Zhang, P., et al. (2014). Caste-specific RNA editomes in the leaf-cutting ant *Acromyrmex echinatior*. *Nat. Commun.* 5:4943. doi: 10.1038/ncomms5943

Lihoreau, M., Dubois, T., Gomez-Moracho, T., Kraus, S., Monchanin, C., and Pasquaretta, C. (2019). Putting the ecology back into insect cognition research. *Adv. Insect Physiol.* 57, 1–25. doi: 10.1016/bs.aiip.2019.08.002

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

Logan, C. J., Avin, S., Boogert, N., Buskell, A., Cross, A., Currie, A., et al. (2018). Beyond brain size: uncovering the neural correlates of behavioral and cognitive specialization. *Comp. Cog. Behav. Rev*. 13, 55–89. doi: 10.3819/CCBR.2018.130008

López-Riquelme, G. O., Malo, E. A., Cruz-López, L., and Fanjul-Moles, M. L. (2006). Antennal olfactory sensitivity in response to task-related odours of three castes of the ant *Atta mexicana* (hymenoptera: formicidae). *Physiol. Entomol.* 31, 353–360. doi: 10.1111/j.1365-3032.2006.00526.x

Mehdiabadi, N. J., and Schultz, T. R. (2010). Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). *Myrmecol. News* 13, 37–55.

Mikheyev, A. S., and Linksvayer, T. A. (2015). Genes associated with ant social behavior show distinct transcriptional and evolutionary patterns. *Elife* 4:e04775. doi: 10.7554/eLife.04775.016

Mizunami, M., Yamagata, N., and Nishino, H. (2010). Alarm pheromone processing in the ant brain: an evolutionary perspective. *Front. Behav. Neurosci.* 4:28. doi: 10.3389/fnbeh.2010.00028

Müller, M., and Wehner, R. (2010). Path integration provides a scaffold for landmark learning in desert ants. *Curr. Biol.* 20, 1368–1371. doi: 10.1016/j.cub.2010.06.035

Muscedere, M. L., and Traniello, J. F. A. (2012). Division of labor in the hyperdiverse ant genus *Pheidole* is associated with distinct subcaste-and age-related patterns of worker brain organization. *PLoS ONE* 7:e31618. doi: 10.1371/journal.pone.0031618

Paul, J., and Gronenberg, W. (1999). Optimizing force and velocity: mandible muscle fibre attachments in ants. *J. Exp. Biol.* 202, 797–808.

Paul, J., and Gronenberg, W. (2002). Motor control of the mandible closer muscle in ants. *J. Insect Physiol.* 48, 255–267. doi: 10.1016/S0022-1910(01)00171-8

Plath, J. A., and Barron, A. B. (2015). Current progress in understanding the functions of the insect central complex. *Curr. Op. Insect Sci.* 12, 11–18. doi: 10.1016/j.cois.2015.08.005

Powell, S., and Clark, E. (2004). Combat between large derived societies: a subterranean army ant established as a predator of mature leaf-cutting ant colonies. *Insectes Soc.* 51, 342–351. doi: 10.1007/s00040-004-0752-2

Provecho, Y., and Josens, R. (2009). Olfactory memory established during trophallaxis affects food search behaviour in ants. *J. Exp. Biol.* 212, 3221–3227. doi: 10.1242/jeb.033506

Qiu, B., Larsen, R. S., Chang, N. C., Wang, J., Boomsma, J. J., and Zhang, G. (2018). Towards reconstructing the ancestral brain gene-network regulating caste differentiation in ants. *Nat. Ecol. Evol.* 2:1782. doi: 10.1038/s41559-018-0689-x

Quiroga, R. Q., Reddy, L., Kreiman, G., Koch, C., and Fried, I. (2005). Invariant visual representation by single neurons in the human brain. *Nature* 435, 1102–1107. doi: 10.1038/nature03687

Rajakumar, R., Koch, S., Couture, M., Favé, M.J., Lillico-Ouachour, A., Chen, T., et al. (2018). Social regulation of a rudimentary organ generates complex worker-caste systems in ants. *Nature* 562, 574–577. doi: 10.1038/s41586-018-0613-1

Rajakumar, R., San Mauro, D., Dijkstra, M. B., Huang, M. H., Wheeler, D. E., Hiou-Tim, F., et al. (2012). Ancestral developmental potential facilitates parallel evolution in ants. *Science* 335, 79–82. doi: 10.1126/science.1211451

Real, L. A. (1993). Toward a cognitive ecology. *Trends Ecol. Evol.* 8, 413–417. doi: 10.1016/0169-5347(93)90044-P

Riveros, A. J., Seid, M. A., and Wcislo, W. T. (2012). Evolution of brain size in class-based societies of fungus-growing ants (Attini). *Anim. Behav.* 83, 1043–1049. doi: 10.1016/j.anbehav.2012.01.032

Ronacher, B., and Wehner, R. (1995). Desert ants *Cataglyphis fortis* use self-induced optic flow to measure distances travelled. *J. Comp. Physiol. A* 177, 21–27. doi: 10.1007/BF00243395

Rowe, C., and Healy, S. D. (2014). Measuring variation in cognition. *Behav. Ecol.* 25, 1287–1292. doi: 10.1093/beheco/aru090

Saverschek, N., Herz, H., Wagner, M., and Roces, F. (2010). Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. *Anim. Behav.* 79, 689–698. doi: 10.1016/j.anbehav.2009.12.021

Scholes, D. R., Suarez, A. V., and Paige, K. N. (2013). Can endopolyploidy explain body size variation within and between castes in ants? *Ecol. Evol.* 3, 2128–2137. doi: 10.1002/ece3.623

Schrader, L., Helanter,ä, H., and Oettler, J. (2017). Accelerated evolution of developmentally biased genes in the tetraphenic ant *Cardiocondyla obscurior*. *Mol. Biol. Evol.* 34, 535–544. doi: 10.1093/molbev/msw240

Schultner, E., and Pulliainen, U. (2020). Brood recognition and discrimination in ants. *Insect. Soc.* 1–24. doi: 10.1007/s00040-019-00747-3

Schultz, T. R., and Brady, S. G. (2008). Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5435–5440. doi: 10.1073/pnas.0711024105

Seid, M. A., Castillo, A., and Wcislo, W. T. (2011). The allometry of brain miniaturization in ants. *Brain Behav. Evolut.* 77, 5–13. doi: 10.1159/000322530

Shettleworth, S. (2010). *Cognition, Evolution, and Behavior*. New York, NY: Oxford University Press.

Shettleworth, S. J. (2000). "Modularity and the evolution of cognition," in *The Evolution of Cognition*, eds C. M. Heyes and L. Huber (Cambridge, MA: MIT Press), 43–60.

Shpigler, H. Y., Saul, M. C., Corona, F., Block, L., Ahmed, A. C., Zhao, S. D., et al. (2017). Deep evolutionary conservation of autism-related genes. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9653–9658. doi: 10.1073/pnas.1708127114

Simons, M., and Tibbetts, E. (2019). Insects as models for studying the evolution of animal cognition. *Curr. Opin. Insect Sci.* 34, 117–122. doi: 10.1016/j.cois.2019.05.009

Smith, A. R., Kapheim, K. M., Pérez-Ortega, B., Brent, C. S., and Wcislo, W. T. (2013). Juvenile hormone levels reflect social opportunities in the facultatively eusocial sweat bee *Megalopta genalis* (Hymenoptera: Halictidae). *Horm. Behav.* 63, 1–4. doi: 10.1016/j.yhbeh.2012.08.012

Solomon, S. E., Rabeling, C., Sosa-Calvo, J., Lopes, C. T., Rodrigues, A., Vasconcelos, H. L., et al. (2019). The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine'ant agriculture. *Syst. Entomol.* 44, 939–956. doi: 10.1111/syen.12370

Sosa-Calvo, J., Schultz, T. R., JeŠovnik, A., Dahan, R. A., and Rabeling, C. (2018). Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. *Syst. Entomol.* 43, 549–567. doi: 10.1111/syen.12289

Steck, K. (2012). Just follow your nose: homing by olfactory cues in ants. *Curr. Opin. Neurobiol.* 22, 231–235. doi: 10.1016/j.conb.2011.10.011

Tomer, R., Denes, A. S., Tessmar-Raible, K., and Arendt, D. (2010). Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium. *Cell* 142, 800–809. doi: 10.1016/j.cell.2010.07.043

Trible, W., Olivos-Cisneros, L., McKenzie, S. K., Saragosti, J., Chang, N. C., Matthews, B. J., et al. (2017). *orco* mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. *Cell* 170, 727–735. doi: 10.1016/j.cell.2017.07.001

Via, S. E. (1977). Visually mediated snapping in the bulldog ant: a perceptual ambiguity between size and distance. *J. Comp. Physiol.* 121, 33–51. doi: 10.1007/BF00614179

Wartel, A., Lindenfors, P., and Lind, J. (2019). Whatever you want: Inconsistent results are the rule, not the exception, in the study of primate brain evolution. *PLoS ONE* 14:e0218655. doi: 10.1371/journal.pone.0218655

Wilson, E. O. (1980a). Caste and division of labor in leaf-cutter ants (Hymenoptera, Formicidae, *Atta*). 1. The overall pattern in *Atta-sexdens*. *Behav. Ecol. Sociobiol. 7*, 143–156. doi: 10.1007/BF00299520

Wilson, E. O. (1980b). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *II: the ergonomic optimization of leaf cutting. Behav. Ecol. Sociobiol.* 7, 157–165 doi: 10.1007/BF00299521

Wittlinger, M., Wehner, R., and Wolf, H. (2006). The ant odometer: stepping on stilts and stumps. *Science* 312, 1965–1967. doi: 10.1126/science.1126912

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 15 December 2020
doi: 10.3389/fnbeh.2020.607700

The Neurotransmitters Involved in *Drosophila* Alcohol-Induced Behaviors

Maggie M. Chvilicek[1,2,3], *Iris Titos*[2] *and Adrian Rothenfluh*[1,2,3,4,5]*

[1] Department of Psychiatry, University of Utah, Salt Lake City, UT, United States, [2] Molecular Medicine Program, University of Utah, Salt Lake City, UT, United States, [3] Neuroscience Graduate Program, University of Utah, Salt Lake City, UT, United States, [4] Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT, United States, [5] Department of Human Genetics, University of Utah, Salt Lake City, UT, United States

Alcohol is a widely used and abused substance with numerous negative consequences for human health and safety. Historically, alcohol's widespread, non-specific neurobiological effects have made it a challenge to study in humans. Therefore, model organisms are a critical tool for unraveling the mechanisms of alcohol action and subsequent effects on behavior. *Drosophila melanogaster* is genetically tractable and displays a vast behavioral repertoire, making it a particularly good candidate for examining the neurobiology of alcohol responses. In addition to being experimentally amenable, *Drosophila* have high face and mechanistic validity: their alcohol-related behaviors are remarkably consistent with humans and other mammalian species, and they share numerous conserved neurotransmitters and signaling pathways. Flies have a long history in alcohol research, which has been enhanced in recent years by the development of tools that allow for manipulating individual *Drosophila* neurotransmitters. Through advancements such as the GAL4/UAS system and CRISPR/Cas9 mutagenesis, investigation of specific neurotransmitters in small subsets of neurons has become ever more achievable. In this review, we describe recent progress in understanding the contribution of seven neurotransmitters to fly behavior, focusing on their roles in alcohol response: dopamine, octopamine, tyramine, serotonin, glutamate, GABA, and acetylcholine. We chose these small-molecule neurotransmitters due to their conservation in mammals and their importance for behavior. While neurotransmitters like dopamine and octopamine have received significant research emphasis regarding their contributions to behavior, others, like glutamate, GABA, and acetylcholine, remain relatively unexplored. Here, we summarize recent genetic and behavioral findings concerning these seven neurotransmitters and their roles in the behavioral response to alcohol, highlighting the fitness of the fly as a model for human alcohol use.

Keywords: *Drosophila*, alcohol behavior, neurotransmitter, alcohol abuse, AUD, genetics

OPEN ACCESS

Edited by:
Maria de la Paz Fernandez, Columbia University, United States

Reviewed by:
Alfredo Ghezzi, University of Puerto Rico, Río Piedras Campus, Puerto Rico
Galit Shohat-Ophir, Bar-Ilan University, Israel

***Correspondence:**
Adrian Rothenfluh
adrian.rothenfluh@hsc.utah.edu

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *17 September 2020*
Accepted: *23 November 2020*
Published: *15 December 2020*

Citation:
Chvilicek MM, Titos I and Rothenfluh A (2020) The Neurotransmitters Involved in Drosophila Alcohol-Induced Behaviors. Front. Behav. Neurosci. 14:607700. doi: 10.3389/fnbeh.2020.607700

INTRODUCTION

Alcohol is one of the most commonly used and abused psychoactive substances. Approximately 86% of American adults have reported drinking alcohol at some point in their lifetimes (Substance Abuse and Mental Health Services Administration, 2019), and, as of 2018, alcohol use disorder (AUD) affected over 14 million adults in the United States (Substance Abuse and Mental Health Services Administration, 2019). AUD is characterized by an impaired ability to control alcohol use despite negative consequences for personal and public health and safety (Substance Abuse and Mental Health Services Administration, 2018). AUD is also frequently correlated with psychological conditions like anxiety (Grant et al., 2004), depression (Hasin et al., 2005), post-traumatic stress disorder (Marshall et al., 2012), and medical history of an anxiety or mood disorder (Martins and Gorelick, 2011). Alcohol-related behaviors are multifaceted, impacted by numerous environmental and individual factors. Due to these complexities, alcohol may cause problematic use and addiction in some people but have minimal consequences in others.

Research established a genetic basis for alcohol use as early as the 1950s (Amark, 1951). Several genes are associated with problematic alcohol use, and twin studies suggest that AUD is ∼50% heritable (Verhulst et al., 2015). Although it is clear that disorders like AUD, which present with behavioral alterations, are influenced by genetics, translating knowledge about genes, cells, and anatomy into a mechanistic understanding of behavior remains one of the biggest challenges in neurobiology. Therefore, the discovery that a genetically tractable organism like *Drosophila melanogaster* (henceforth called *Drosophila* or flies) shows a broad behavioral repertoire facilitated a new chapter of neuroscience research. Flies, like humans and other mammals, modulate their behaviors according to circadian rhythms (Dubowy and Sehgal, 2017), can learn and remember (Cognigni et al., 2018), and show behavioral hallmarks of addiction (Devineni and Heberlein, 2009; Kaun et al., 2012), among other behaviors.

The neurobiological action of alcohol is especially challenging to understand since alcohol does not have a specific target pathway and instead affects pathways intended for other physiological functions (Fadda and Rossetti, 1998). Research is still unraveling how alcohol alters various brain circuits and why some are more susceptible to alcohol than others. Behavior is a useful tool for examining where and how alcohol may be affecting the brain since there are known behavioral outcomes associated with specific circuits and neurotransmitter systems. Given its high rates of use and abuse, understanding the neural and behavioral outcomes of alcohol is critical. Here we will focus on the role of *Drosophila's* neurotransmitter pathways in behavior and how that behavior is affected under the influence of alcohol.

Drosophila AS A MODEL ORGANISM

For over a hundred years, *Drosophila melanogaster,* commonly known as the fruit or vinegar fly, has been a critical model organism for the field of neuroscience (Bellen et al., 2010). Flies have many characteristics that make them an appealing organism in the laboratory: short generation time, low cost, ease of maintenance, and relatively simple genetic and anatomical makeup. *Drosophila* were one of the first organisms for which the genome was fully sequenced (2000), and flies have many genetic similarities to humans, sharing an estimated 62% homology in disease-causing genes (Fortini et al., 2000). The *Drosophila* nervous system consists of ∼300,000 neurons making up the brain and thoracic ganglion, which is the fly equivalent of the spinal cord (Freeman, 2015). The majority of small-molecule neurotransmitters responsible for central nervous system (CNS) function in mammals are conserved in the fly. With the development of ever more sensitive genetic and behavioral tools, utilization of *Drosophila* as a model system has become increasingly prevalent. *Drosophila* models have led, and continue to lead, to advancements in numerous areas of neuroscience. *Drosophila* are an appealing candidate for studying alcohol-related behaviors for a few reasons: face validity, mechanistic validity, and experimental amenability.

Face Validity

Face validity describes how much a model "looks like" the disorder being modeled. In our case, face validity is the degree to which *Drosophila* recapitulate alcohol-induced behaviors seen in humans. Accordingly, *Drosophila* show behavioral and neurobiological responses to alcohol that are very consistent with humans and other mammalian species. These include locomotion changes, development of tolerance, learned preference, withdrawal symptoms, and effects on social behavior (Devineni and Heberlein, 2013). One feature of alcohol's neurobiological activity is the biphasic behavioral response: a period of nervous system stimulation followed by a period of nervous system depression. In the stimulatory phase, blood alcohol content rises, and an individual may experience disinhibition, euphoria, and hyperactivity (Fadda and Rossetti, 1998). Later, during the sedative phase, as blood alcohol content peaks, an individual becomes less active, experiencing motor and cognition impairment, and eventually coma and death (Fadda and Rossetti, 1998; Hendler et al., 2013). This biphasic action likely contributes to the development of alcohol dependence. The association of rising blood alcohol content with elevated mood during the stimulatory phase may positively reinforce alcohol drinking (Addicott et al., 2007). The biphasic alcohol response (see **Figure 1**) is also noted in *Drosophila* (Bainton et al., 2000; Singh and Heberlein, 2000). Upon exposure to ethanol vapor in a video tracking assay, flies show an initial peak of hyperactivity in response to the vapor lasting less than a minute, due to a sensory startle response to ethanol's odor. Following habituation to the odor, flies' locomotion level lessens compared to the startle response. As flies begin to absorb the ethanol and experience intoxication, their locomotion starts to increase again as a consequence of ethanol's pharmacodynamic action on the brain. With further exposure, locomotor activity begins to decline, and sedation takes effect, indicating that flies experience a biphasic ethanol response similar to mammals (Wolf et al., 2002).

Drosophila mirror other characteristics of the mammalian alcohol response, including tolerance, withdrawal, and

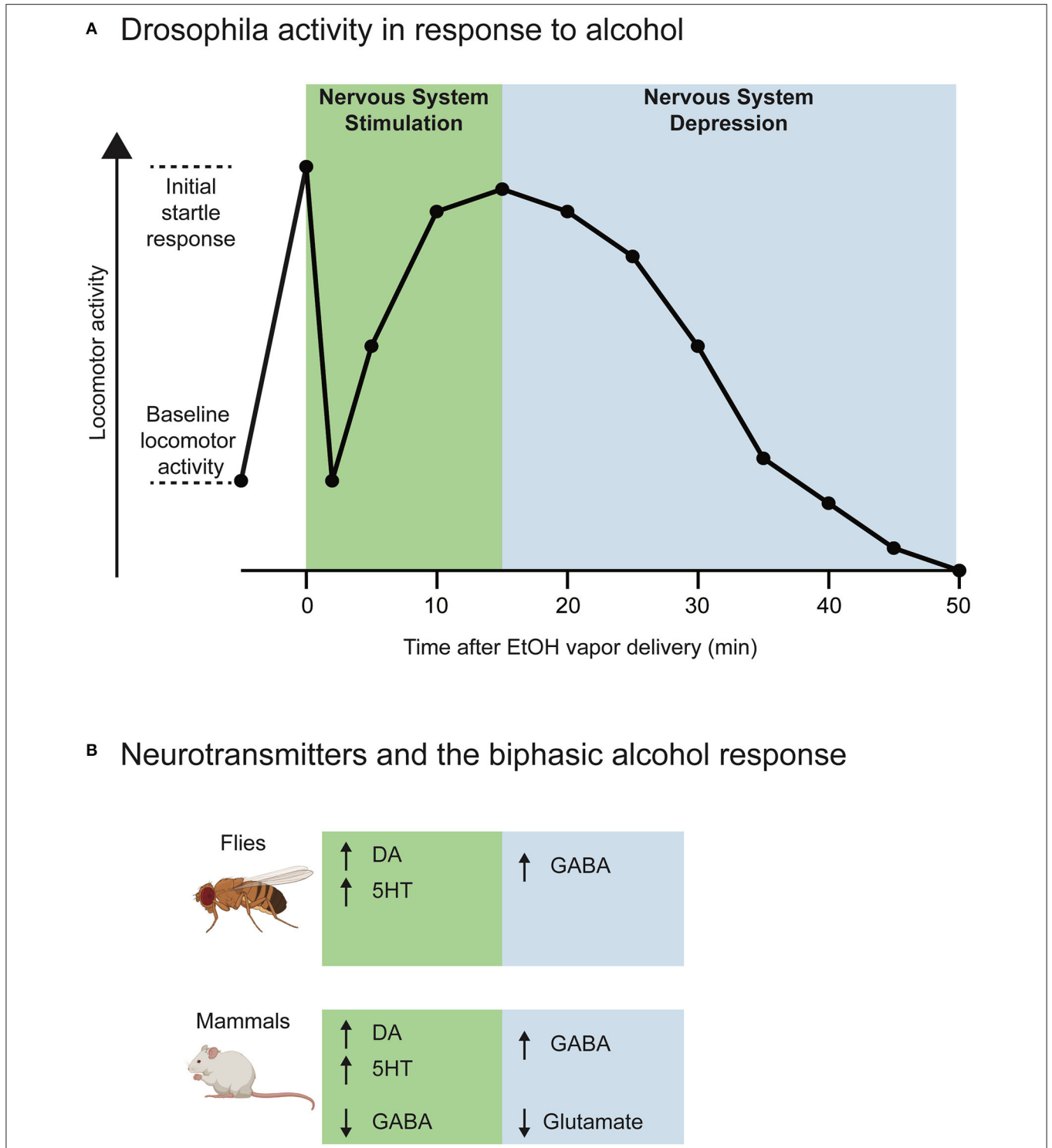

FIGURE 1 | The biphasic alcohol activity response in *Drosophila*, and neurotransmitter involvement in fly and mammalian alcohol responses. **(A)** A sample activity plot shows the change in locomotion of a fly across time following exposure to alcohol vapor. Immediately after ethanol vapor is delivered, the fly has an initial startle response, significantly increasing locomotion from baseline. This startle response quickly drops off and the fly's activity returns to a level close to baseline. As absorption of alcohol takes place, the fly's locomotion gradually increases as nervous system stimulation occurs. Eventually, intoxication peaks, and the fly enters the sedative phase associated with nervous system depression, and activity declines over time until the fly is completely sedated (Bainton et al., 2000; Singh and Heberlein, 2000; Wolf et al., 2002). EtOH = ethanol **(B)** The stimulatory and sedative phases involve distinct neurotransmitter actions. The biphasic alcohol response (nervous system stimulation in green and nervous system depression in blue) is very similar in *Drosophila* and mammals, and some of the same neurotransmitter actions have been implicated in these responses. Arrows indicate increase or decrease in activity for the specified neurotransmitter. See the main text for further details and references.

reinforcing properties leading to learned preference for alcohol. Functional tolerance involves adaptations in neuronal activity following exposure to a psychoactive substance, rather than metabolic tolerance, which depends on changes to enzymatic metabolism of ethanol. As functional tolerance develops, a person (or fly) requires increasing amounts of alcohol to become intoxicated in the future (see **Figure 2**). *Drosophila* demonstrate functional tolerance in as little as 2 h after an initial alcohol exposure (Scholz et al., 2000). Flies and humans also have similarities regarding withdrawal from alcohol. Withdrawal causes a variety of psychological and physiological symptoms, and because drinking alcohol alleviates these symptoms, such attempts to curb withdrawal may contribute to the persistence of AUD (Schuckit, 2009). In *Drosophila* larvae and adults, alcohol withdrawal is associated with neuronal hyperexcitability, which also occurs in humans (Bayard et al., 2004; Cowmeadow et al., 2006; Ghezzi et al., 2014).

Additionally, like humans, flies can develop a learned preference for alcohol (see **Figure 3**). Similar to mammals, *Drosophila* do not have an innate preference for alcohol. Upon a first offer of ethanol for consumption, flies are either indifferent or avoidant, depending on the exact presentation parameters (Devineni and Heberlein, 2009; Peru y Colón de Portugal et al., 2014). However, flies develop persistent, experience-dependent preference following exposure to alcohol (Peru y Colón de Portugal et al., 2014). Flies' acquisition of preference for alcohol is a critical component of their usefulness as an animal model. Humans similarly develop alcohol preference that can drive problematic drinking behavior and lead to AUD (Fadda and Rossetti, 1998). In mammals, preference frequently becomes attached to specific contexts or patterns, a phenomenon examined in a conditioned place preference (CPP) assay, wherein a particular environmental context gains attractive qualities after repeated pairing with a drug (Cunningham et al., 2006). Similar behavioral reinforcement occurs in flies, when they acquire preference for an innocuous odor that has been paired with alcohol vapor (Kaun et al., 2011).

Mechanistic Validity

Mechanistic validity refers to the consistency of neurobiological mechanisms and molecules underlying alcohol response between *Drosophila* and humans. Historically, much of the complexity of studying alcohol lies in its widespread effects throughout the brain. While some drugs, like cocaine, act primarily through a single mechanism (blocking monoamine reuptake into the presynaptic terminal, in cocaine's case) (Hummel and Unterwald, 2002), alcohol's effects on neurotransmission occur in a dose-dependent manner that involves diverse effects across neurotransmitters. Ethanol easily crosses the blood-brain barrier and acts much more globally than other drugs, meaning its mechanisms of action occur quickly and efficiently, contributing to alcohol's propensity for abuse. These diverse mechanisms of alcohol action are consistent from flies to humans.

Many of the genes implicated in mammalian alcohol reactions and human AUD have conserved functions in *Drosophila* (Grotewiel and Bettinger, 2015; Lathen et al., 2020). Some of these involve common molecular pathways for alcohol response, such as cyclic adenosine monophosphate (cAMP) (Moore et al., 1998) and neuropeptide Y (neuropeptide F in flies) (Wen et al., 2005). There is also a high level of conservation in neurotransmitter systems between *Drosophila* and vertebrate species, including humans. While behavioral outcomes may differ, vertebrates and invertebrates share signaling by glutamate, gamma-aminobutyric acid (GABA), acetylcholine (ACh), glycine, dopamine (DA), serotonin (5HT), and numerous neuropeptides (Deng et al., 2019). Although in flies there is no evidence of adrenergic signaling, octopamine (OA), and tyramine (TA) fulfill behavioral roles similar to norepinephrine (NE) and epinephrine in mammals.

In mammals, several neural circuits are involved in behavioral responses to alcohol and the development of AUD [see Abrahao et al. (2017) for a recent review]. For example, in the ventral tegmental area of the mammalian brain, the mesolimbic dopamine pathway is involved in mediating reinforcement and communicating with the nucleus accumbens to drive reward signaling. The fly brain's neuroanatomical structure differs from mammals', but both have circuits implicated in specific behaviors. There are several anatomical regions of interest in the discussion of alcohol-related behaviors. These include the mushroom bodies (MB), a center for associative learning, the antennal lobe (AL), the primary olfactory processing center, and the central complex, which houses the fan-shaped body (FSB), a center of higher integration, and ellipsoid body (EB), a pre-motor structure. Like in mammalian neural circuits, inputs to these brain regions by specific neurotransmitters mediate various alcohol-induced behavioral responses. These are most well-studied for dopamine.

Experimental Amenability

In *Drosophila*, there are many tools available for genetic manipulations, including forward genetics (going from a phenotype to a causative gene), reverse genetics (going from a targeted mutated gene, using a system like CRISPR/Cas9 mutagenesis, to a phenotype), and genomic approaches (Griffiths et al., 2000). One reverse genetics approach is the GAL4/UAS system (Brand and Perrimon, 1993). This approach involves one transgene carrying GAL4 (a transcriptional activator in yeast), which is under the control of a specific promoter determining the spatial and temporal expression of GAL4. This is combined with a second effector transgene under the control of the upstream activating sequence (UAS), where GAL4 binds. A plethora of distinct GAL4 lines exist, including thousands that drive GAL4 expression in different subsets of neurons (Jenett et al., 2012). The effector transgenes include cDNAs for overexpression, RNAi for gene knockdown, or tools to activate and silence neurons under experimenter control. In conjunction with these genetic tools, *Drosophila's* relatively low cost, ease of maintenance, and short generation time make it amenable to a variety of experimental manipulations.

ASSAYING ETHANOL-INDUCED BEHAVIORS

Scientists have studied ethanol responses in *Drosophila* since the 1920s. Early research involved exposing flies to ethanol vapor and measuring time to easily observable behaviors such as sedation

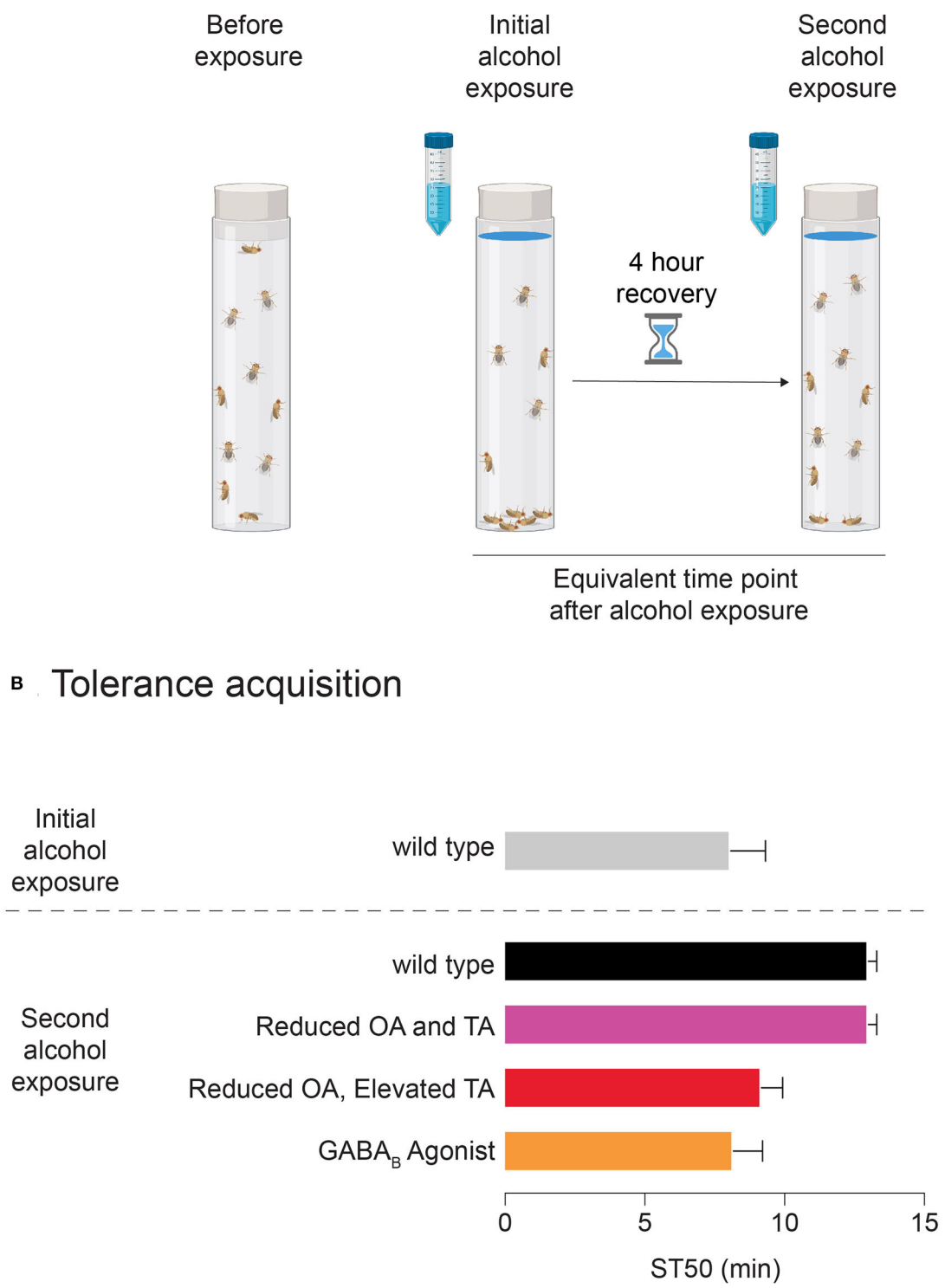

FIGURE 2 | The development of alcohol tolerance in *Drosophila*. **(A)** Schematic of alcohol vapor exposure assay. Blue-dyed alcohol is applied to the vial plug, and following the first exposure, flies recover for 4 hours in fresh air. In the second exposure, at the same time point, fewer flies are sedated that in the first exposure, indicating that tolerance has developed. **(B)** Sample data shows ST50 (time it takes for 50% of the flies to become sedated) for a first and second alcohol vapor exposure for wildtype flies and flies with manipulations of different neurotransmitters. These manipulations are either genetic ("Reduced OA and TA" and "Reduced OA, Elevated TA") or pharmacological via drug feeding (GABA agonist).

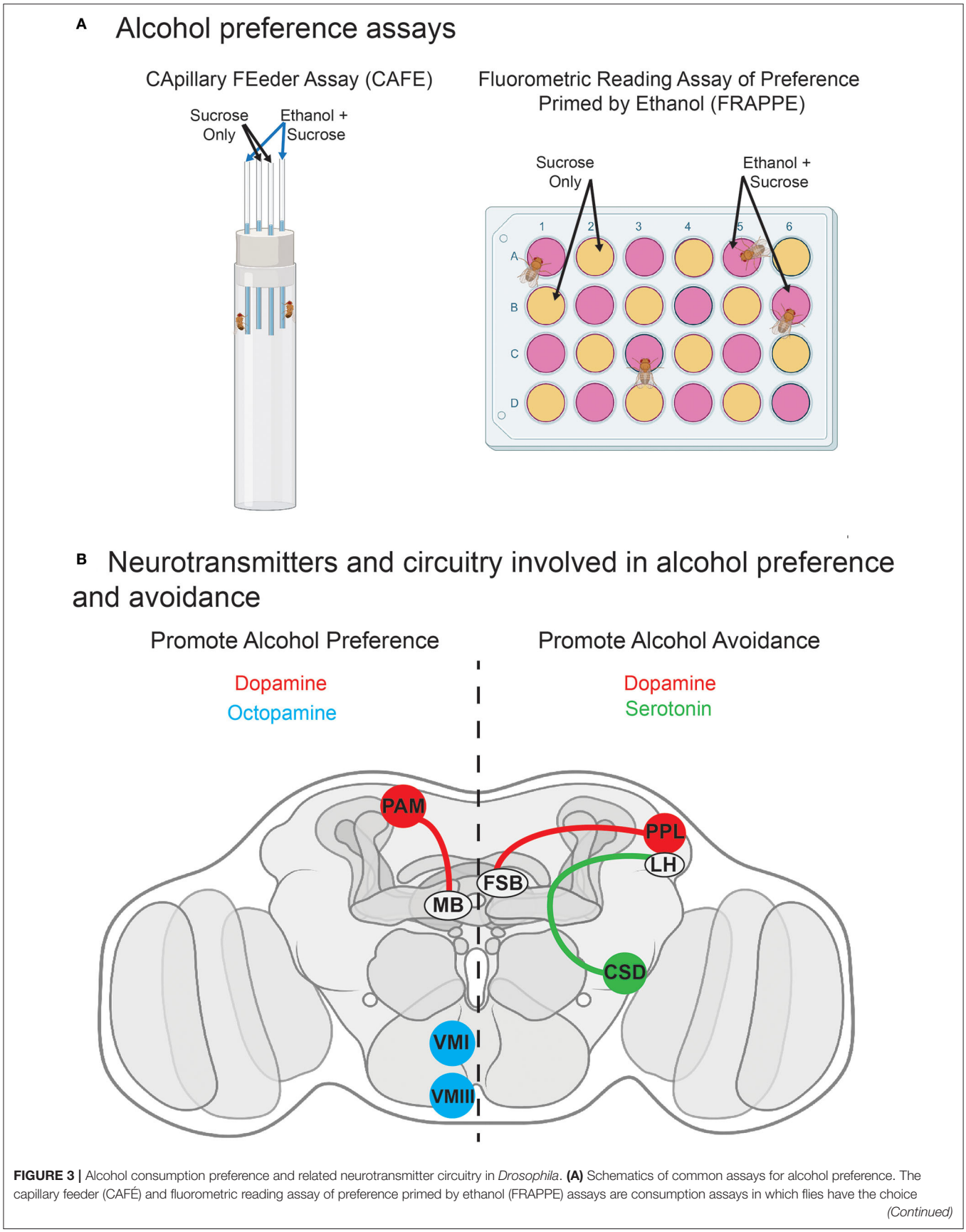

FIGURE 3 | Alcohol consumption preference and related neurotransmitter circuitry in *Drosophila*. **(A)** Schematics of common assays for alcohol preference. The capillary feeder (CAFÉ) and fluorometric reading assay of preference primed by ethanol (FRAPPE) assays are consumption assays in which flies have the choice

(Continued)

FIGURE 3 | between two food sources, one with and one without ethanol. **(B)** Known circuitry for neurotransmitters mediating alcohol preference and avoidance. DAergic projections from the PAM cluster to the mushroom body (Ojelade et al., 2019) and OAergic VMI-VMIII neurons (Schneider et al., 2012) promote alcohol preference. DAergic projections from the PPL cluster to the fan-shaped body (Ojelade et al., 2019) and serotonergic signaling in CSD interneurons (Kasture et al., 2019) promote alcohol avoidance. FSB, fan-shaped body; LH, lateral horn; MB, mushroom body. See the text for further details and additional references.

or death (Pearl et al., 1929; Crozier et al., 1936). Still today, the basis of many assays lies in similar continuous exposure to ethanol vapor. The benefit of assays involving ethanol vapor is that the exposure time is directly proportional to the flies' level of ethanol absorption, giving researchers more control of the level of intoxication of the flies than in assays that require ingestion. Although humans generally drink alcohol rather than inhaling its vapor, both forms of ethanol exposure result in similar behavioral outcomes. In fact, in recent years, alcohol vaporization has become an increasingly common approach for inducing the development of alcohol dependence in rodent models (Avegno and Gilpin, 2019), suggesting the validity of this approach across organisms.

Several systems exist for exposing flies to alcohol and measuring behavioral output. One of these, the inebriometer, assesses fly postural control following exposure to ethanol vapor (Weber, 1988). The inebriometer evaluates alcohol sensitivity and tolerance. Sensitivity is a measure of the effects of intoxication, typically quantified by locomotor changes leading to sedation. As described previously, tolerance is the development of resistance after an intoxicating dose of alcohol, measured in increased time to intoxication following repeated exposure to alcohol. The inebriometer is a vertical cylindrical tube lined with mesh baffles that slope toward the bottom of the tube. Flies are introduced through the top of the tube, and in the presence of fresh air, they naturally tend to stay at the top of the tube. However, when ethanol diffuses through the tube, the flies lose their ability to hold on to the mesh baffles and eventually fall to the bottom of the tube (Weber, 1988). The inebriometer assesses sensitivity to ethanol sedation by measuring the amount of time it takes for flies to elute through the bottom of the tube (Weber, 1988). Through a process of exposure, elution, recovery, then re-exposure, the inebriometer has also shown that flies develop tolerance to alcohol. As flies develop functional tolerance, they will require more alcohol to become intoxicated. When flies were reintroduced to the inebriometer 4 h after the first exposure (in the meantime fully recovering from the initial intoxication), the mean time of elution through the bottom of the inebriometer in the second exposure increased by ∼34% compared to the first exposure, indicating that flies were more resistant to ethanol sedation in the second exposure and that they developed tolerance (Scholz et al., 2000). The contribution of pharmacokinetic changes to this process was ruled out by measuring the ethanol content of prepared fly extracts after exposing naïve and tolerant flies to ethanol vapor. Significantly, the rate of alcohol absorption and metabolism was not significantly different in tolerant flies, suggesting that the development of tolerance is functional rather than metabolic (Scholz et al., 2000).

As described in the previous section, alcohol induces a biphasic behavioral response that impacts activity levels. While the inebriometer essentially indicates whether flies have become sedated or not, other assays can more sensitively quantify locomotor changes. For example, video tracking of walking flies provides a detailed image of locomotion across time. This technique shows that flies have an initial hyperactive startle response to the smell of alcohol, followed by a leveling of the startle response, then gradual increase and decline of activity across time, providing evidence for the biphasic ethanol response in flies (Wolf et al., 2002).

More recently, researchers have developed tools to deliver alcohol to flies in a more translationally relevant way. Although flies will eat alcohol mixed into their food, it has historically been challenging to quantify the amount of food consumed. The capillary feeder (CAFÉ) assay **Figure 3A** has provided a mechanism to overcome this problem by providing food through a glass microcapillary, allowing for precise measurement of consumption by individuals or groups of flies (Ja et al., 2007). Assays such as this one show that *Drosophila* can develop preference for alcohol, choosing ethanol-containing food over standard food (Devineni and Heberlein, 2009). As described in relation to face validity, learned preference is a key feature of human alcohol use, and its recapitulation in *Drosophila* is a critical component of their fitness as an animal model.

Drosophila have a long history of utilization in the study of behavioral responses to alcohol. They have proven especially useful in the discovery and validation of genes affecting alcohol responses. As researchers have developed knowledge of ethanol-related behaviors and tools to assess them, they have asked increasingly complicated questions about the neurobiology underlying these behaviors. The study of specific neurotransmitters is a topic critical to a thorough understanding of behavioral responses to alcohol. As we will discuss in the next section, neurotransmitters are both highly conserved from the mammal to the fly, and they are critically involved in the neurobiological activity of alcohol. With the development of more sensitive genetic tools, the investigation of neurotransmitters has become more attainable.

OVERVIEW OF NEUROTRANSMITTERS

Neurotransmitters can exert excitatory, inhibitory, or modulatory effects. In general, excitatory neurotransmitters increase the likelihood that a neuron will fire an action potential, while inhibitory neurotransmitters decrease the possibility of an action potential firing. Neurotransmitters exert these actions by altering the flow of ions across the

TABLE 1 | *Drosophila* behaviors associated with each neurotransmitter.

Neuro-transmitter	*Drosophila* behaviors *Indicates behaviors impacted by alcohol	References
Dopamine	Aggression	Alekseyenko et al., 2013
	Associative learning*	Tully and Quinn, 1985; Riemensperger et al., 2005
	Aversive association*	Honjo and Furukubo-Tokunaga, 2009
	Circadian rhythms	Allada and Chung, 2010
	Locomotion*	Yellman et al., 1997; Pendleton et al., 2002; Kume et al., 2005; Kong et al., 2010; Strausfeld and Hirth, 2013
	Male courtship behavior*	Liu et al., 2008; Hoopfer et al., 2015; Zhang et al., 2019
	Memory removal	Berry et al., 2012
	Multisensory processing	Wolff and Rubin, 2018
	Olfactory learning and memory*	Cognigni et al., 2018
	Reward signaling*	Liu C. et al., 2012; Yamagata et al., 2015
	Salience-based decision making	Zhang et al., 2007
	Sleep and arousal*	Foltenyi et al., 2007; Van Swinderen and Andretic, 2011; Strausfeld and Hirth, 2013
Octopamine	Aggression	Zhou et al., 2008
	Appetitive and aversive associative learning*	Iliadi et al., 2017
	Egg-laying	Monastirioti et al., 1996
	Locomotion*	Sombati and Hoyle, 1984; Saraswati et al., 2004
	Male and female courtship behavior*	Zhou et al., 2012; Rezával et al., 2014
	Odor processing*	Farooqui et al., 2003
	Positive reinforcement for olfactory learning and memory*	Schwaerzel et al., 2003
	Reward*	Hammer, 1993
	Stress response	Hirashima et al., 2000; Chentsova et al., 2002
Tyramine	Flight behavior	Ryglewski et al., 2017
	Locomotion*	Sombati and Hoyle, 1984; Saraswati et al., 2004
	Stress response	Chentsova et al., 2002
	Male courtship behavior*	Huang et al., 2016
Serotonin	Aggression	Alekseyenko et al., 2010
	Associative learning*	Sitaraman et al., 2008
	Circadian rhythms	Yuan et al., 2005
	Depression-like behaviors	Ries et al., 2017
	Hunger and feeding*	Albin et al., 2015; Majeed et al., 2016
	Locomotion*	Silva et al., 2014; Majeed et al., 2016
	Long-term memory formation*	Sitaraman et al., 2008; Scheunemann et al., 2018
	Odor processing*	Ellen and Mercer, 2012
	Sensory perception	Kaneko et al., 2017; Chakraborty et al., 2019
	Sleep*	Liu et al., 2019
GABA	Associative olfactory learning*	Liu et al., 2007
	Labile memory	Pitman et al., 2011
	Locomotion*	Leal and Neckameyer, 2002; Leal et al., 2004
	Sleep length and onset*	Agosto et al., 2008; Chen et al., 2015
	Sleep and memory consolidation*	Haynes et al., 2015
Acetylcholine	Aversive association*	Silva et al., 2015; Bielopolski et al., 2019
	Olfactory learning*	Barnstedt et al., 2016
	Nicotine-induced locomotor changes	King et al., 2011; Fuenzalida–Uribe et al., 2013; Ren et al., 2015
	Sleep promotion*	Aso et al., 2014b
Glutamate	Olfactory habituation	Das et al., 2011
	Olfactory learning and memory*	Xia et al., 2005
	Olfactory response*	Liu and Wilson, 2013
	Sleep regulation*	Guo et al., 2016
	Wake promotion*	Sitaraman et al., 2015; Zimmerman et al., 2017

Researchers are elucidating which neurotransmitters are involved in regulating the wide behavioral repertoire of Drosophila. Behaviors that could be impacted by or related to alcohol exposure are marked with an asterisk.

cell membrane. Neuromodulators modify the effects of excitatory or inhibitory neurotransmitters and tend to be involved in the slower, longer-lasting activity necessary for higher-order processes.

Despite many conserved similarities, there are some differences between neurotransmitter systems in vertebrates and invertebrates. In mammals, glutamate functions as the primary excitatory neurotransmitter in the CNS, while in *Drosophila,* ACh has this role. Conversely, flies use glutamate at the neuromuscular junction, while in mammals, that neurotransmitter is ACh (Colombo and Francolini, 2019). Although flies do have glutamatergic neurons in the CNS, their role has historically not been well-understood (Liu and Wilson, 2013); but recent advancements will be discussed below. In vertebrates and flies, GABA and glycine both function as inhibitory neurotransmitters (Frenkel et al., 2017), and the two classes of organisms also share many neuromodulators. *Drosophila* neuromodulators include DA, TA, and OA, which come from the common precursor tyrosine (Li et al., 2016b). TA and OA are the functional fly equivalents of mammalian epinephrine and NE, respectively. Both epinephrine and NE are produced from the breakdown of DA, but neither of these chemicals is physiologically relevant for *Drosophila* or other protostomes (Roeder, 2005). 5HT and DA have more known roles in modifying behavior, and these are regulated similarly in vertebrates and invertebrates (Corey et al., 1994; Pörzgen et al., 2001). In flies, neurotransmitters are implicated in a wide variety

of behaviors, which are summarized in **Table 1**. The roles for specific neurotransmitters in alcohol-related behaviors will be discussed in detail later in this review.

Alcohol and Neurotransmitters in Mammals

Much of our knowledge about the effects of alcohol on neurotransmitters comes from studies in mammalian models, particularly rodents. Briefly, we will discuss these findings as a point of comparison with *Drosophila*. As mentioned in the introduction, alcohol exerts action by taking advantage of existing biological pathways, necessitating the study of alcohol in the context of known impacts on these pathways and subsequent behavioral alterations. Alcohol's effects on neurotransmission occur in a dose-dependent manner, differentially impacting neurotransmitter systems (Hummel and Unterwald, 2002). Ethanol acts quickly, efficiently, and globally. As discussed in relation to flies' face validity, alcohol's effects are biphasic: initial low doses produce euphoria and hyperactivity, while over time, higher doses depress activity and eventually lead to sedation (Carlsson et al., 1972; Pohorecky, 1977).

The two phases of the alcohol response involve different neurotransmitter systems **Figure 1B**. At low doses, alcohol acts as a stimulant, causing disinhibition, euphoria, and hyperactivity as blood alcohol content rises (Fadda and Rossetti, 1998). Shortly after ingesting alcohol, mice show a sharp increase in locomotion, attributed to DAergic activation (Carlsson and Lindqvist, 1973). Specifically, these behaviors arise from increased release of DA in the brain's reward system, a mechanism demonstrated in rodents (Yim et al., 1998) as well as humans (Boileau et al., 2003). Due to its involvement in reward processing, DA contributes to both the development and persistence of alcohol dependence (Di Chiara, 1995). In rats, even very small amounts of alcohol administered intravenously increase DA levels in the brain's reward centers and contribute to sustained alcohol self-administration (Lyness and Smith, 1992). Rewarding stimuli are processed via DAergic signaling in the ventral tegmental area (VTA) and nucleus accumbens (NAc). Low doses of alcohol cause dose-dependent activation of DAergic neurons in the rat VTA (Gessa et al., 1985), and alcohol acutely increases synaptic DA levels throughout the reward system, but particularly in the NAc (Di Chiara and Imperato, 1988). Alcohol reduces the activity of GABA in the VTA, thereby disinhibiting DAergic neurons and increasing DAergic activity (Kohl et al., 1998). 5HT is also involved in behavioral regulation, including in brain regions responsible for reward processing, which are implicated in AUD. In humans, 5HT metabolites are more plentiful in blood and urine after drinking alcohol, indicating increased serotonergic transmission, and alcohol consumption increases brain levels of 5HT in animal models (LeMarquand et al., 1994a,b). Additionally, $5HT_{1B}$ (5HT receptor) knockout mice show less ethanol-induced locomotor impairment, indicative of intoxication, across 11 days of ethanol feeding and testing. Therefore, 5HT may have a role in exacerbating the effects of alcohol and in determining alcohol sensitivity (Crabbe et al., 1996). Serotonergic signaling has particular clinical significance due to the comorbidity of AUD with anxiety and mood disorders, which are often treated with selective serotonin reuptake inhibitor (SSRI) drugs (Gimeno et al., 2017).

As alcohol consumption continues, blood alcohol content peaks, and behaviors associated with CNS depression occur. In the sedative phase, alcohol primarily exerts depressant effects by suppressing excitatory neurotransmission and heightening inhibitory neurotransmission. Alcohol activation of $GABA_A$ receptors produces cell hyperpolarization via an influx of chloride ions. Co-administration of ethanol and GABA-mimetic drugs, such as baclofen, enhances the sedative effects of alcohol. Similar experiments with GABA antagonists, such as picrotoxin, reduce alcohol-related incoordination (Martz et al., 1983).

Along with enhancing inhibition, alcohol also suppresses excitation. Beginning in the 1980s, researchers investigated the impact of alcohol on glutamate receptors, showing that even small amounts of alcohol could suppress ion flow through *N*-methyl-*D*-aspartate (NMDA) receptors in cultured rat neurons (Lovinger et al., 1989). Alcohol limits the NMDA-mediated release of neurotransmitters like DA, NE, and ACh, further impairing communication between neurons (Göthert and Fink, 1989; Woodward and Gonzales, 1990). These findings provide a starting point for understanding neurotransmitters' involvement in behavioral responses to alcohol. However, genetic manipulations necessary for greater mechanistic insight are more limited in mammalian models than invertebrates. Therefore, *Drosophila* are an appealing candidate for probing this relationship in greater detail.

Alcohol and Neurotransmitters in *Drosophila*

Drosophila are a useful organism for the study of neurotransmitters because, as described, neurotransmitters are well-conserved from flies to mammals, and they often exert similar effects on behavior. These behavioral effects are particularly useful when considering the effects of alcohol since there is no unique neurobiological pathway for alcohol. However, alcohol has known effects on neurotransmitters that are associated with changes in behavior. See **Table 2** for a summary of neurotransmitter roles in alcohol-related behaviors. Additionally, flies have over 40 neuropeptides and signaling hormones, many of which are shared with vertebrate species (Hewes and Taghert, 2001). The best-studied in the context of alcohol is Neuropeptide F (NPF). NPF has a role in *Drosophila* alcohol-related behaviors such as consumption, conditioned preference (Shohat-Ophir et al., 2012; Bozler et al., 2019), and preference for egg-laying in alcohol-containing food (Kacsoh et al., 2013). However, here we focus on small molecule neurotransmitters.

DOPAMINE

In *Drosophila*, DAergic neurons are distributed throughout the CNS (Budnik and White, 1988) but comprise only about 250 of the ∼100,000 neurons in the fly brain (Mao and Davis, 2009; Aso et al., 2010; Zheng et al., 2018). Despite the relatively small

TABLE 2 | Role of neurotransmitters in alcohol-related phenotypes in *Drosophila*.

Neuro-transmitter	Part of pathway	Activation (+)/Blockage (–) of function	Pharm. (P) or genetic (G) manipulation	Alcohol-related phenotype(s)	Reference
Dopamine	N/A	N/A	N/A	Alcohol potentiates global DA release	Ojelade et al., 2019
	Tyrosine hydroxylase	–	P	Decreased acute hyperactivation	Bainton et al., 2000
	DA neuron synaptic transmission	–	G, P	Decreased acute hyperactivation	Kong et al., 2010
				Increased naïve preference	Ojelade et al., 2019
		–	G	Decreased disinhibition	Lee et al., 2008
		+	G, P	Increased naïve aversion	Ojelade et al., 2019
	DAT	–	G	Decreased acute hyperactivation	Kong et al., 2010
	Central complex neuronal activity	+	G	Increased acute hyperactivation	Kong et al., 2010
	Dop1R1 receptor	–	G	Decreased acute hyperactivation	Kong et al., 2010
				No change in rapid tolerance	Kong et al., 2010
				Increased naïve preference	Ojelade et al., 2019
	Both D1-like receptors	–	G	No change in sensitization to disinhibition	Aranda et al., 2017
	DopEcR	–	G	Acute sedation resistance	Petruccelli et al., 2016
				Increased acute hyperactivation	Petruccelli et al., 2016
				Decreased sensitization to disinhibition	Aranda et al., 2017
	PPL DAergic neurons projecting to FSB	–	G	Decreased naïve aversion	Ojelade et al., 2019
	DA neurons	–	G	Decreased conditioned preference	Kaun et al., 2011
Octopamine and tyramine	Tyrosine decarboxylase	–	G	Decreased acute hyperactivation	Scholz, 2005
				Acute sedation resistance	Chen et al., 2013
	Tyramine beta-hydroxylase	–	G	Increased acute hyperactivation	Scholz, 2005
				Decreased rapid tolerance	Scholz et al., 2000; Scholz, 2005
				Decreased startle response	Scholz, 2005
				Decreased olfactory preference	Schneider et al., 2012
				Decreased olfactory attraction	Claßen and Scholz, 2018
		+	G	Decreased sensitivity	Chen et al., 2013
	Global OA levels	+	P	No change in sensitivity	Chen et al., 2013
				Increased olfactory attraction	Claßen and Scholz, 2018
	OA neurons	+	G	Induced olfactory preference for EtOH	Schneider et al., 2012
	OA receptor	–	G, P	Decreased olfactory attraction	Claßen and Scholz, 2018
	TA receptor	–	G	No change in acute sedation	Scholz, 2005
			P	Decreased sensitivity	Chen et al., 2013
Serotonin	Global 5HT levels	+	P	Decreased olfactory preference	Xu et al., 2016
	SerT	–	G	Decreased olfactory attraction	Xu et al., 2016
				Decreased olfactory preference	Kasture et al., 2019
		–	P	No change in sensitivity	Chen et al., 2010
	SerT in CSD neurons	+	G	Increased olfactory aversion	Kasture et al., 2019
	5-HTP	+	P	Increased sensitivity	Chen et al., 2010
	PKC53E in 5HT neurons	–	G	Reduced activity of 5HT neurons	Chen et al., 2010
				Decreased sensitivity	Chen et al., 2010
GABA	$GABA_BR$	+	P	Increased sensitivity	Ranson et al., 2020
				Increased chronic tolerance	Ranson et al., 2020
				Decreased rapid tolerance	Dzitoyeva et al., 2003

(Continued)

TABLE 2 | Continued

Neuro-transmitter	Part of pathway	Activation (+)/Blockage (–) of function	Pharm. (P) or genetic (G) manipulation	Alcohol-related phenotype(s)	Reference
		–	P	Decreased sensitivity	Ranson et al., 2020
				Decreased alcohol-induced motor impairment	Dzitoyeva et al., 2003
Glutamate	DAergic projections to glutamatergic MBONs	N/A	N/A	Consolidation of alcohol-related memories	Scaplen et al., 2020

For each neurotransmitter, the different manipulations reported are listed (part of the neurotransmitter pathway, whether that component of the pathway is being activated or blocked, and whether the manipulation is pharmacological or genetic) together with their behavioral output in the presence of alcohol. In 2 cases ("N/A" in columns 2–3), no manipulation is indicated and a general behavioral response to alcohol is shown. See the main text for detailed descriptions of the listed behaviors. EtOH = ethanol.

number of DA neurons in the adult brain, DA is involved in many *Drosophila* behaviors (see **Table 1** for a summary). Recent work indicates that many DAergic neurons have distinct functions depending on the specific circuitry in which they are involved (e.g., Azanchi et al., 2013; Ojelade et al., 2019). These recent developments paint an optimistic picture for future advancements regarding neurotransmitters that are currently poorly understood, such as glutamate, GABA, and ACh.

The fly brain does not structurally resemble the mammalian brain, although *Drosophila* have neural circuits fulfilling roles similar to those of the vertebrate brain. Some anatomical regions of interest to the discussion of DA are the central complex, which houses the fan-shaped body (FSB) and ellipsoid body (EB), and the mushroom bodies (MB). DAergic neurons reside in 10 distinct clusters per hemisphere (Xie et al., 2018). Each cluster has stereotyped projections to other brain regions and distinct roles in behavior (Nässel and Elekes, 1992; Mao and Davis, 2009), which we will discuss below.

Dopamine Synthesis, Action, and Metabolism

DA is produced by the metabolism of essential amino acid phenylalanine or its metabolite, non-essential amino acid tyrosine. Dietary ingestion is the primary source of both phenylalanine and tyrosine. Tyrosine is converted by tyrosine hydroxylase (TH) to L-DOPA, which is then converted to DA by dopamine decarboxylase (Cole et al., 2005). There are two classes of DA receptors, classified due to their similarities to mammalian DA receptors: D1-like and D2-like. There are two D1-like receptors, Dop1R1, which signals via Gαs to stimulate cAMP production, and Dop1R2, which couples to Gαq to increase cytosolic calcium (Handler et al., 2019). The D2-like receptor, Dop2R, inhibits the adenylyl cyclase/cAMP pathway (Scholz-Kornehl and Schwärzel, 2016). Both classes are G protein-coupled receptors (GPCRs) with seven transmembrane domains (Karam et al., 2020). Flies also have DopEcR, a G protein-coupled DA/ecdysteroid receptor that can be activated by either DA or the insect hormone ecdysone (Srivastava et al., 2005). Much of the structure of these receptors is conserved between vertebrates and *Drosophila* (Karam et al., 2020). After the presynaptic neuron releases DA into the synapse and DAergic action occurs, the dopamine transporter (DAT) takes DA back up into the neuron.

Dopamine and Ethanol in *Drosophila*

DA has numerous important functions in *Drosophila* behavior, in part due to the widespread projection of DAergic neurons throughout the brain. Circuits responsible for the alcohol response involve anatomical regions such as the MB and central complex, specifically the EB. There is an extensive body of research on DA's roles in fly behavior, so we will focus here on the behaviors and neural circuitry most relevant for alcohol, namely locomotion, which involves the central complex, and learning and memory, punishment, and reward, which involve the MB. DAergic inputs to the central complex mediate motor activity and sleep (Strausfeld and Hirth, 2013), multisensory processing (Wolff and Rubin, 2018), and social behaviors like aggression (Alekseyenko et al., 2013). See **Table 1** for a summary of DA roles in fly behavior.

Like in mammals, alcohol impacts the *Drosophila* DAergic system (Bainton et al., 2000), and many DA-related behaviors are linked to and affected by alcohol. Alcohol affects several DA-mediated behaviors in *Drosophila*, such as locomotion, sedation, and reward. Additionally, DA has an important role in flies' preference for laying eggs in ethanol-containing food. Subsets of competing DA neurons enhance or inhibit this preference (Azanchi et al., 2013), possibly suggesting a DAergic role for flies' innate attraction to alcohol's odor at low concentrations (Ogueta et al., 2010). Significantly, alcohol potentiates the release of DA in the fly brain, which may explain the noted enhancement of locomotion and reinforcing behavioral effects following ethanol exposure (Ojelade et al., 2019).

Locomotion and Sedation: Ellipsoid Body

In general, increased DAergic signaling is associated with increased locomotion, while decreased DAergic signaling is associated with reduced locomotion. In decapitated flies with an exposed nerve cord, application of DA stimulated locomotion and hindleg grooming, while application of a DA antagonist significantly blocked this behavior (Yellman et al., 1997). DA signaling specifically to the EB regulates locomotion (Kong et al., 2010). In studies with live flies, a DAT mutation also increased

locomotive excitability and prolonged response to a mechanical stimulus, suggesting a critical role of DA in the regulation of movement and arousal (Kume et al., 2005). Loss-of-function mutations of the Dop1R DA receptor has been further implicated in elevating startle-induced arousal (the focus for Kume et al., 2005) while decreasing arousal from sleep, suggesting a role for DA activity in independently modulating different forms of arousal (Lebestky et al., 2009).

The role of DA in ethanol-related alterations to locomotion is increasingly well-known in flies. When placed in narrow tubes, flies show a basal activity level that increased for 7–10 min upon exposure to ethanol vapor (Bainton et al., 2000), consistent with the biphasic ethanol response. Certain DAergic perturbations reduce this alcohol-related locomotor activity. For example, in flies fed 3-iodotyrosine (3IY; a competitive antagonist of TH that reduces global DA levels), the shape of the biphasic locomotor response curve was similar to control flies, but the amount of locomotion was significantly blunted, which could be reversed by feeding L-DOPA (Bainton et al., 2000). Therefore, DA has a role in modulating ethanol-induced hyperactivity in flies, like in mammals. Additionally, the tetanus toxin light chain (TeTx), which blocks synaptic transmission (Sweeny et al., 1995), was expressed in a subset of EB-projecting DA neurons using GAL4-UAS (Kong et al., 2010). In TeTx-expressing flies, locomotor activity was significantly reduced compared to controls. However, coordination appeared normal, and the odor-induced startle response to the introduction of ethanol vapor was not affected (Kong et al., 2010). These findings highlight the specificity of *Drosophila* neural circuitry for the modification of unique behaviors. Even within a single neurotransmitter system, individual neurons and neuron subsets have distinct functions depending on the circuitry in which they are involved.

Studies have also attempted to unravel if specific DAergic neurons and receptors are involved in locomotive responses to acute ethanol exposure. In transgenic lines expressing dopamine decarboxylase using GAL4 drivers for subsets of TH-containing neurons, specific DA neurons in the PPM3 cluster and target neurons in the central complex EB promote locomotion (Kong et al., 2010). Additionally, the fly D1-like receptor, Dop1R1, is required for locomotive activation in response to ethanol. None of these neurons was necessary for the olfactory startle response to alcohol, suggesting that alcohol acts on PPM3 DA neurons that signal to the EB through Dop1R1 to evoke a motor response (Kong et al., 2010).

The sedative effects of alcohol have also been an area of investigation concerning DA. Investigations focused on DopEcR showed that flies with mutations on this receptor took over an hour longer to become sedated than control flies. However, DA was not relevant for the DopEcR activation that promoted this particular behavior. The process is likely mediated by ecdysone, as ecdysone-fed flies overexpressing DopEcR were resistant to alcohol sedation (Petruccelli et al., 2016). Although DA does not impact ethanol-induced sedation via DopEcR, DA may act through DopEcR to affect other behaviors. For example, DA action on DopEcR may oppose the ethanol-induced hyperactivity mediated by the two D1-like receptors. Indeed, *DopEcR* mutants show an elevated hyperactive alcohol response, suggesting that wildtype DopEcR is involved in minimizing Dop1R-mediated hyperactivity in response to alcohol (Petruccelli et al., 2016).

Associative Learning and Preference: Mushroom Body

DA is necessary for complex behaviors like learning and memory. In *Drosophila*, blocking DA inhibits the acquisition of aversive memories (Honjo and Furukubo-Tokunaga, 2009), and flies lacking the Dop1R2 receptor, highly expressed in the MBs (Han et al., 1996), have impaired removal of memories (Berry et al., 2012). Additionally, DAergic inputs to the MB are vital for olfactory learning and memory (Cognigni et al., 2018), and DAergic projections from the protocerebral anterior medial (PAM) cluster to the MB are involved in reward signaling (Liu C. et al., 2012). Subsets of DA neurons are also involved in reward signaling for short vs. long-term memory (Yamagata et al., 2015). Alterations to the brain's reward pathways are a critical feature of addiction.

DA has a multifaceted role in mediating alcohol-induced behaviors; it influences both reinforcing and aversive alcohol responses (see **Figure 3B**). Flies are innately indifferent or averse to ingesting alcohol. However, after alcohol exposure, this turns into experience-dependent preference (Peru y Colón de Portugal et al., 2014). DA is involved in both naïve aversion and conditioned preference. Conditioned alcohol preference is the associative learning process by which a fly learns to correlate ethanol with an attractive cue. Although DA was once thought to be involved only in the retrieval of conditioned preference and not acquisition (Kaun et al., 2011), recent evidence suggests a role for DA in preference acquisition (Ojelade et al., 2019). The PAM cluster of DAergic neurons is involved in appetitive olfactory conditioning, and DAergic signaling in these neurons is necessary for experience-dependent alcohol preference (Ojelade et al., 2019). Specifically, PAM DA neuron projections to the MB were necessary for the acquisition of alcohol preference, which is further supported by evidence that knocking down the Dop1R1 receptor in the MB impairs the development of preference (Ojelade et al., 2019). Additionally, recent evidence clarifies the role of DA in the consolidation and retrieval of preference. DAergic activity inhibits specific mushroom body output neurons (MBONs) involved in a circuit for the consolidation of alcohol-related memories. This circuit also converges on the FSB. This inhibition may permit the consolidation of alcohol preference (Scaplen et al., 2020). It is clear that DA plays a dynamic role in the behavioral response to alcohol, and many of these findings have come about in recent years due to advancements in tools for examining the DAergic system. These outcomes are compelling in considering other, less-explored *Drosophila* neurotransmitters, as similar innovations for these neurotransmitters are likely forthcoming.

Acute Aversion to Alcohol: Fan-Shaped Body

As mentioned in the previous section, naïve flies initially show indifference or aversion to alcohol consumption. In the CAFÉ assay, the first preference measurement is generally after 24 h and shows indifference (Devineni and Heberlein, 2009;

Xu et al., 2012). However, Butts et al. (2019) have shown that some flies can acquire preference in <12 h. Indeed, in preference assays that do not use capillaries to offer the food (Park et al., 2018) or that are shorter in duration (Peru y Colón de Portugal et al., 2014; Butts et al., 2019), flies show initial aversion to alcohol. This was further examined by pharmacologically or genetically manipulating DA, showing that flies with increased DA levels have enhanced naïve alcohol aversion. In contrast, flies with decreased DA levels have naïve alcohol preference (Ojelade et al., 2019). These findings indicate that DA is critical for flies' aversion to alcohol. Regarding circuitry, a pair of DAergic protocerebral posterior lateral (PPL) neurons specifically mediates acute aversion: silencing these neurons abolishes aversion **Figure 3B** (Ojelade et al., 2019). The PPL cluster of DA neurons mostly projects to the MB (Aso et al., 2014a) to mediate punishment (Handler et al., 2019) and is activated by aversive stimuli (Mao and Davis, 2009). However, one bilateral PPL neuron projects to the FSB (Liu Q. et al., 2012), and this projection mediates the acute aversion to alcohol (Ojelade et al., 2019). The FSB is therefore emerging as a higher center of integration, where output to learned alcohol responses from the MB (Scaplen et al., 2020) merge with acute sensory processing, and here, alcohol aversion (Ojelade et al., 2019). Similar integrative processes have been found for aversive sensory responses and conditioning by electric shock (Hu et al., 2018).

OCTOPAMINE AND TYRAMINE

In *Drosophila,* OA (the NE homolog) and TA (the epinephrine homolog) are expressed in over 100 neurons (Selcho et al., 2014). OA-immunoreactive neurons, which necessarily contain TA, reside in discrete clusters throughout the fly brain (Sinakevitch and Strausfeld, 2006). Although OA is a metabolite of TA, TA has only recently become an independent target of investigations. OA and TA have often historically been explored together by manipulating metabolic steps upstream of TA. OA has many well-characterized independent impacts on insect physiology and behavior, but these roles are less well-defined for TA (Pauls et al., 2018). It was long thought that while DA mediates the formation of aversive memories, OA has a specific role in appetitive memories (Schwaerzel et al., 2003). However, newer evidence suggests that OA is required for both appetitive and aversive learning and, therefore, associative learning in general (Iliadi et al., 2017).

Octopamine and Tyramine Synthesis, Action, and Metabolism

Like DA, TA and OA are produced by the metabolism of essential amino acid phenylalanine or its metabolite, non-essential amino acid tyrosine, both found in food. Tyrosine is converted to TA by tyrosine decarboxylase (Tdc), and TA is converted to OA by tyramine beta-hydroxylase (Tbh). Manipulating Tbh concentration alters the OA/TA equilibrium: *Tbh*-null flies have increased TA levels and decreased OA levels (Monastirioti et al., 1996). There are 4 GPCRs for OA and 3 for TA. All seven types show high expression in the brain (El-Kholy et al., 2015). Based on parallels with the vertebrate adrenergic system, fly OA receptor classifications include α-adrenergic-like, β-adrenergic-like, and OA/TA or TA receptors (Evans and Maqueira, 2005). These receptors exert a variety of effects. Activation of the α-adrenergic-like receptor leads to elevation of calcium ions, and activation of the three β-adrenergic-like receptors increases intracellular cAMP levels (Balfanz et al., 2005; Maqueira et al., 2005). Interestingly, though researchers have described a specific OA transporter in many insects, one has not been identified in *Drosophila.* OA reuptake in flies may occur via DAT, although this requires further investigation (Arancibia et al., 2019).

Tyramine/Octopamine and Ethanol in *Drosophila*

TA and OA influence a wide variety of behaviors. Both neurotransmitters were initially of interest to researchers because they are critical for insect physiological processes like modulation of organs and muscles, and since vertebrates lack receptors for both, they provided a potential target for insecticides (Roeder, 2005). Although TA was historically thought to function primarily as a precursor of OA and exert few of its own effects, the presence of TA-activated GPCRs suggests that it may function independently as a neurotransmitter (Borowsky et al., 2001), and TA has been independently implicated in some behaviors. See **Table 1** for a summary of OA and TA roles in fly behavior.

Although research has historically focused more on DA, OA, and TA are also involved in behavioral responses to alcohol. OA and TA are implicated in ethanol-related behaviors such as locomotion, sensitivity, tolerance, preference, and olfactory attraction. Investigations often explore TA and OA in conjunction due to their common precursor, and it is not clear whether there are distinct TAergic and OAergic neurons.

Locomotion, Sensitivity, and Tolerance

In *Drosophila* larvae, flies with elevated TA and low OA levels had reduced locomotion compared to wildtype, and flies with reduced levels of both OA and TA showed less severe locomotor impairment (Saraswati et al., 2004). Thus, OA and TA exert opposing effects on larval locomotion, and a balance between both is necessary for normal behavior. As described for DA, in an experiment in which amines were applied to the exposed nerve cord of decapitated flies, OA stimulated hindleg grooming and strong locomotion (Yellman et al., 1997), suggesting an important role for OA and TA in mediating locomotion.

Various genetic mutations impacting the OAergic and TAergic systems are known to have roles in modulating alcohol sensitivity and tolerance, and flies with these mutations are useful for unraveling the impacts of TA and OA on alcohol-related behaviors. Some of these mutations impact synthesis enzymes. For example, in a mutant called *inactive* (*iav*) Tdc activity is reduced, causing reduced TA and OA levels (Chentsova et al., 2002), while *Tbh*-null flies have increased TA levels and decreased OA levels (Monastirioti et al., 1996). Upon first ethanol exposure, *iav* flies' locomotion is reduced compared to controls, while *Tbh* mutants show more locomotion than controls (Scholz, 2005). This suggests opposing roles of TA and OA in regulating the locomotive response to alcohol. In regard to tolerance, mutant *iav* flies are sensitive to sedation in the first alcohol exposure,

but they develop tolerance and are less sensitive during the second exposure (Scholz, 2005; see **Figure 2**). Conversely, *Tbh* mutants showed normal alcohol sensitivity in the first exposure but developed less tolerance during the second exposure (Scholz, 2005). To further explore the role of OA in the development of tolerance, *Tbh*-null flies were tested in the inebriometer. While sensitivity to alcohol did not change, 4 h after the initial exposure, *Tbh* flies showed 50–60% less tolerance than controls (Scholz et al., 2000). This effect was not reversed by 2 days of TA-feeding, suggesting that the effect on tolerance development was due to OA and not elevated TA (Scholz et al., 2000).

Later work on the role of TA in ethanol sensitivity identified the *Bacchus* (*Bacc*) gene. While the molecular function of this gene is not well-understood, a loss-of-function mutation of *Bacc* reduced alcohol sensitivity, likely via heightened Tbh activity converting more TA to OA (Chen et al., 2013). In reducing Tbh activity or orally administering TA, *Bacc* mutant flies show normal ethanol sensitivity (Chen et al., 2013). Additionally, in a GAL4 line in which both OA and TA neurotransmission were blocked, flies were significantly resistant to ethanol sedation compared to controls. This phenotype was restored when flies were fed TA or TA plus OA but not OA alone, indicating that *Bacc* does not impact ethanol sensitivity via increased OA activity (Chen et al., 2013). These findings suggest that TA has independent involvement in regulating ethanol response.

Olfactory Ethanol Attraction

OA has a role in odor processing (Farooqui et al., 2003), extending to ethanol. Based on the theorized OAergic signaling requirement for the positive association of an odor and stimulus (Schwaerzel et al., 2003), Schneider et al. examined OA's role in olfactory ethanol preference (Schneider et al., 2012). In *Tbh* mutants, flies did not show olfactory preference, a phenotype restored by expressing a Tbh cDNA with a Tdc-GAL4 driver line (Schneider et al., 2012). Researchers also assessed Tbh expression patterns to find OAergic neurons and identified 26 neurons specifically involved in olfactory ethanol preference (Schneider et al., 2012; see **Figure 3**). Optogenetic targeting of these neurons determined that activation of OAergic neurons is sufficient for inducing preference and that previously noted alcohol preference in response to OA supplementation in *Tbh* mutants was not simply the result of increased neuronal activity (Schneider et al., 2012).

OA also has a critical role in determining behavior via its role in biasing the fly's decision toward food odors. Pharmacologically increasing OAergic signaling increases ethanol attraction, while blocking OA receptors reduces it (Claßen and Scholz, 2018). *Tbh* mutant flies do not initially show ethanol attraction, but it is rescued upon feeding the flies OA or OA receptor agonists. Convergently, feeding wildtype flies epinastine, an OA receptor antagonist, impairs ethanol attraction similarly to *Tbh* mutants (Claßen and Scholz, 2018). Therefore, OA is required for olfactory ethanol attraction. TA has also been investigated in conjunction with OA to understand olfactory attraction. TA-fed wildtype flies showed a slight but insignificant reduction in attraction to ethanol. However, in *Tbh* mutant flies lacking OA, TA feeding significantly induced ethanol attraction (Claßen and Scholz, 2018). It is possible that TA can act as an agonist for OA receptors at high levels or that elevated activation of TA receptors may induce ethanol attraction. Both TA and OA are likely involved in olfactory attraction to ethanol (Claßen and Scholz, 2018).

SEROTONIN

The serotonin system in *Drosophila* exerts significant behavioral effects despite the very small number (∼80) of serotonergic neurons in the fly brain. These neurons reside in several clusters (Sitaraman et al., 2008). Genetic approaches have shown that regulation of behaviors can stem from individual serotonergic neurons within clusters (Pooryasin and Fiala, 2015). 5HT exerts behavioral and physiological effects on processes such as hunger (Albin et al., 2015), sleep (Liu et al., 2019), and sensory perception (Chakraborty et al., 2019).

Serotonin Synthesis, Action, and Metabolism

Unlike the previously discussed neurotransmitters, 5HT does not originate from the amino acid tyrosine, but tryptophan. The precursor tryptophan, absorbed in the diet, is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. Then, aromatic amino acid decarboxylase converts 5-HTP to 5-hydroxytryptamine (otherwise known as serotonin or 5HT) (Coleman and Neckameyer, 2005). Flies have five different G protein-coupled 5HT receptors. 5-HT1A, 5-HT1B, and 5-HT7 are all coupled to the cAMP signaling cascade, while 5-HT2A and 5-HT2B activation lead to Ca^{2+} signaling (Blenau et al., 2017). Some of these receptor subtypes are involved in specific outcomes, like the role of the 5-HT2B receptor in minimizing anxiety-like behaviors (Mohammad et al., 2016). 5HT is removed from the synapse via reuptake by the *Drosophila* serotonin transporter (SerT) (Demchyshyn et al., 1994). SerT colocalizes with 5HT neurons throughout the brain (Giang et al., 2011), and its mutations provide a useful tool for investigating phenotypic outcomes of serotonergic signaling.

Serotonin and Ethanol in *Drosophila*

5HT affects numerous behaviors in *Drosophila*, and manipulation of the fly serotonergic system has recapitulated symptoms of neuropsychiatric disorders like depression and anxiety (Ries et al., 2017). Importantly for consideration of alcohol-related behaviors, 5HT is critical for memory formation in *Drosophila* (Sitaraman et al., 2008). Memory performance worsened by genetically blocking serotonergic neurotransmission during a task for learned avoidance of high temperatures. A similar result was noted upon the pharmacological blockage of 5HT (Sitaraman et al., 2008). See **Table 1** for a summary of 5HT roles in fly behavior.

Although historically not the subject of intense research efforts, new evidence increasingly supports a role for 5HT in *Drosophila's* ethanol-related behaviors. These behaviors include olfactory attraction, preference, and sensitivity.

Olfactory Attraction

As we discussed in the DA section, *Drosophila* do not have a naïve preference for consuming alcohol. Although there is evidence that they are innately attracted to its odor at low concentrations (Ogueta et al., 2010), it is not clear what the exact role for this attraction is in driving alcohol self-administration. 5HT is involved in odor processing (Ellen and Mercer, 2012), which has made it an appealing candidate for investigating ethanol attraction. In a two-choice assay between a food source with or without ethanol, flies with pharmacologically increased 5HT levels showed significant loss of preference for alcohol's odor (Xu et al., 2016). Also, genetically rendering SerT non-functional, thereby increasing 5HT in the synaptic cleft and prolonging serotonergic signaling, reduced olfactory ethanol attraction. Four serotonergic neurons are implicated in this inhibition (Xu et al., 2016). These researchers went on to explore two neurons distinct from the previously identified four: the contralaterally-projecting, serotonin-immunoreactive deutocerebral (CSD) neurons. The CSD neurons counteract the inhibition of the other four serotonergic neurons (Xu et al., 2016), and they are the only serotonergic neurons innervating the antennal lobes (AL), the fly brain equivalent of the olfactory bulbs (Xu et al., 2016). These are involved with odor detection (Roy et al., 2007). In prolonged exposure to an odor, CSD neurons counteract the inhibition of olfactory attraction by the four previously identified serotonergic neurons and enhance olfactory input via 5HT (Xu et al., 2016). Therefore, 5HT's role in olfactory attraction to ethanol is multifaceted: 5HT generally functions to inhibit olfactory attraction, but the CSD neurons overrule this inhibition in the prolonged presence of an odor (Xu et al., 2016).

In another study of olfactory alcohol preference, researchers generated flies with a non-functional SerT and then placed the flies in a two-choice odor trap with one trap containing ethanol. Flies with a disrupted SerT showed a lower preference for alcohol than wildtype flies, but both groups showed a higher preference for the trap containing ethanol than the one without (Kasture et al., 2019). These effects also have intracellular location-specific characteristics. Restoring SerT in the global mutant in previously described CSD interneurons resulted in olfactory alcohol aversion while restoring SerT function only in the soma and dendrites rescued normal attraction (Kasture et al., 2019; see **Figure 3**). These findings suggest that 5HT transport exerts unique ethanol-related behavioral effects in the somatodendrities vs. axons (Kasture et al., 2019).

Locomotion, Sensitivity, and Sedation

As we have discussed throughout this paper, locomotion is one behavior that is impacted by alcohol. In *Drosophila*, increased 5HT is associated with reduced locomotion. Larvae treated with drugs that increase 5HT signaling [such as fluoxetine and 3,4-Methylenedioxymethamphetamine (MDMA)] decreased their locomotion. Treating larvae with drugs that reduce serotonergic signaling reversed this effect (Silva et al., 2014). This is important in the context of alcohol since locomotion is a behavioral marker for alcohol sensitivity.

Serotonergic signaling is also linked to protein kinase C (PKC), which several studies have shown is involved with alcohol sensitivity (Newton and Ron, 2007). Chen et al. showed that PKC positively regulates 5HT activity to influence ethanol sensitivity. Inhibition of one PKC subtype (PKC53E) in serotonergic neurons reduced the activity of 5HT neurons and reduced sensitivity to ethanol (measured as time to sedation) (Chen et al., 2010). Upon feeding flies an SSRI, ethanol sensitivity was restored to normal, suggesting that PKC53E deficiency influences alcohol-related behaviors via depletion of synaptic 5HT (Chen et al., 2010). 5HT may also be involved in the relationship between diet and ethanol sedation. In general, a high-yeast diet increases 5HT levels in the brain (Ro et al., 2016) and increases flies' resistance to alcohol sedation (Schmitt et al., 2020). Serotonergic neurons can block the sedation resistance caused by a high-yeast diet (Schmitt et al., 2020), suggesting a role for 5HT in mediating the link between diet and ethanol-related behaviors.

In the last several years, there has been an increase in the number of studies investigating the *Drosophila* serotonergic system. However, few of these focus specifically on the role of 5HT in the mediation of ethanol-related behaviors. As genetic and behavioral tools continue to advance, roles for 5HT in behavioral outcomes of alcohol use will continue to be uncovered. An extensive body of research in mammals suggests that increases in serotonergic signaling are associated with decreased alcohol use and vice versa. Additionally, alcohol may elevate 5HT activity to activate DAergic neurons and the reward system (LeMarquand et al., 1994b). Since the *Drosophila* DAergic system is also implicated in the behavioral response to alcohol, flies, and mammals could potentially share mechanisms by which the serotonergic system mediates ethanol-related behaviors.

GABA

In vertebrates and invertebrates alike, GABA functions as the major inhibitory neurotransmitter. In *Drosophila*, although it does not appear at detectable levels until relatively late in development, GABA is distributed throughout the nervous system, and about 20% of neurons show GABA immunoreactivity (Küppers et al., 2003). The olfactory system has been a site for an extensive study of GABAergic signaling, specifically in the fly AL. Two types of GABAergic neurons (projection neurons and local interneurons) project to the AL (Okada et al., 2009), and application of a GABA receptor agonist inhibits AL function (MacLeod and Laurent, 1996; Stopfer et al., 1997; Sachse and Galizia, 2002).

GABA Synthesis, Action, and Metabolism

In flies, GABA is synthesized by glutamic acid decarboxylase (GAD) enzymes, including Gad1 (expressed exclusively in the nervous system) and Gad2 (expressed exclusively in glia) (Manev and Dzitoyeva, 2010). GAD is implicated in the formation of synapses at the neuromuscular junction (NMJ) and may also be involved in local regulation of glutamate at NMJ synapses (Featherstone et al., 2000). Researchers have also mapped the expression of Gad1 and Gad2 within the fly brain and found that while only a few neurons release GABA, most of the neurons in the antennal lobe receive inhibitory signals (Okada et al., 2009). GABA exerts action on both ionotropic receptors and

GPCRs: ligand-gated $GABA_A$-type receptors and metabotropic $GABA_B$-type receptors (Hosie et al., 1997). Flies have subtypes of both of these receptors, which are experimentally useful in their sensitivities to different pharmacological manipulations. For example, RDL receptors ($GABA_A$-type), named for resistance to the insecticide dieldrin (RDL), are highly distributed in the insect CNS and are therefore the target of numerous insecticides (McGonigle and Lummis, 2009). Importantly, fly GABA receptors do not respond to pharmacological agents the same way that mammalian GABA receptors do, so this will be important to consider when evaluating the relationship between alcohol and the fly GABAergic system. GABA action terminates in the synapse through a variety of mechanisms, such as changes in the density of GABA receptors or GABA uptake by astrocytic GABA transporters (GATs) (Muthukumar et al., 2014). The vesicular GABA transporter (VGAT), located pre-synaptically in GABAergic neurons, packages GABA into synaptic vesicles for later release (Enell et al., 2007).

GABA and Ethanol in *Drosophila*

In vertebrates and invertebrates, GABA activity impacts numerous behaviors since it is highly expressed in different types of neurons throughout the brain. In *Drosophila*, these behaviors include locomotion (Leal and Neckameyer, 2002), olfactory learning (Liu et al., 2007), and sleep regulation (Agosto et al., 2008). See **Table 1** for a summary of GABA roles in fly behavior.

GABA is ubiquitously expressed and involved in regulating numerous behaviors, making it a good target for alcohol, which acts in a widespread, non-selective manner throughout the brain. Alcohol increases GABA release in vertebrates, suggesting a possible role in flies (Kelm et al., 2011). Researchers have particularly focused on the role of metabotropic $GABA_B$ receptors in alcohol-related behaviors such as sensitivity, tolerance, and locomotion.

Sensitivity and Sedation

Feeding flies the $GABA_B$ agonist SKF 97541 increases their sensitivity to sedation when exposed to ethanol vapor (Ranson et al., 2020). These effects persisted for 4 days. Additionally, the SKF 97541-fed flies still developed alcohol tolerance and actually became much more tolerant than controls on the fourth day of exposure (Ranson et al., 2020). When repeating these experiments using the $GABA_B$ antagonist CGP 54626, flies became significantly less sensitive to alcohol than controls. However, these manipulations did not affect the development of tolerance, suggesting that $GABA_B$ receptors are just one of several receptor systems contributing to tolerance development (Ranson et al., 2020). These results suggest that $GABA_B$ receptors mediate ethanol sensitivity and the development of tolerance (Ranson et al., 2020; see **Figure 2**).

Locomotion and Tolerance

Early research on *Drosophila* $GABA_B$ receptors involved injecting alcohol into the fly in conjunction with either 3-AMPA, a $GABA_B$ agonist, or CGP 54626. Both ethanol and 3-AMPA caused immobility in the fly when injected initially; however, injecting flies with CGP 54626 before ethanol lessened the effects significantly (Dzitoyeva et al., 2003). This data suggests that $GABA_B$ activation mediates the behavioral outcomes of 3-AMPA and ethanol. Rapid ethanol tolerance was inhibited by pretreatment with the $GABA_B$ agonist, while the antagonist did not impact tolerance (Dzitoyeva et al., 2003). These findings seem to contradict the previously mentioned experiments done by Ranson et al., but may be explained by the length of the study since Ranson et al. did not note significant development of tolerance until the third day of testing and were likely assessing chronic rather than rapid tolerance (Ranson et al., 2020), while Dzitoyeva et al. only noted tolerance for the first 18 h after treatment (2003).

Gamma-hydroxybutyric acid (GHB) also affects ethanol-related behaviors. GHB is a GABA metabolite with medical applications and pharmacological similarities to ethanol. It is also a possible treatment for AUD (Poldrugo and Addolorato, 1999). $GABA_B$ receptors mediate the behavioral effects of GHB in flies, providing helpful information for a better understanding of ethanol-related behaviors. Prior exposure to ethanol reduced GHB-associated effects on alcohol sensitivity. However, this tolerance did not occur in the inverse, suggesting that while both GHB and alcohol involve $GABA_B$ receptors, their sites or mechanisms of action may differ (Dimitrijevic et al., 2005).

ACETYLCHOLINE

In *Drosophila*, ACh is broadly expressed (Buchner, 1991) and is the primary excitatory neurotransmitter, but despite this, little is known about specific outcomes of ACh signaling for fly behavior. In part, this gap in knowledge arises from the highly detrimental nature of systemic manipulation of the fly cholinergic system. Because ACh is so prevalently expressed, perturbations to ACh signaling result in severe behavioral outcomes (like seizures) that are not favorable for survival (e.g., Somers et al., 2018). The further development of genetic tools targeting more specific cell populations will facilitate an increased understanding of the role of ACh. Kenyon cells, the MB intrinsic neurons, contain ACh-processing proteins. Also, cholinergic activity in these cells impacts activity of MB output neurons (MBONs) (Barnstedt et al., 2016). Kenyon cells exclusively use ACh for intercellular communication, supporting ACh's excitatory role in the fly CNS (Shih et al., 2019).

Acetylcholine Synthesis, Action, and Metabolism

ACh is derived from choline, which flies ingest through the diet. Choline acetyltransferase (ChAT) catalyzes ACh biosynthesis, and acetylcholinesterase (Ace) breaks down ACh. There are two categories of ACh receptors: ionotropic (nicotinic) and metabotropic (muscarinic). Nicotinic ACh receptors (nAChRs) in *Drosophila* mediate fast, excitatory synaptic currents (Su and O'Dowd, 2003). Muscarinic ACh receptors (mAChRs) are not as well-understood, although researchers have identified three types (A, B, and C) that signal via activation of different G-protein subunits to initiate various downstream intracellular processes (Collin et al., 2013; Ren et al., 2015). Once synthesized

presynaptically, ACh must be loaded into vesicles by the vesicular ACh transporter (VAChT) (Kitamoto et al., 1998). While AChE normally terminates ACh action in the synaptic cleft, some drugs prevent this process. Inhibiting AChE is lethal, so irreversible AChE inhibitor compounds are extremely toxic and often used as insecticides (Menozzi et al., 2004). Irreversible AChE inhibitors are also lethal to humans, although reversible AChE inhibitors have some therapeutic applications, like as pharmacological treatments for neurodegenerative diseases, including Alzheimer's (Colović et al., 2013).

Behavioral Effects of Acetylcholine

Although we know relatively little about cholinergic effects on *Drosophila* behavior, olfactory associative learning is one area of investigation. Silva et al. examined the role of mAChR-A in aversive learning. Researchers generated fly lines to visualize mAChR-A with GFP, and they confirmed mAChR-A expression in the MBs (Silva et al., 2015). By disrupting mAChR-A pharmacologically or genetically, they significantly impaired the formation of aversive olfactory memory in *Drosophila* larvae and adult flies (Silva et al., 2015). However, when flies received a more intense shock during training (90V compared to 50V), learning was not impacted. Thus, mAChR-A may only reinforce moderate aversion (Bielopolski et al., 2019). These effects were localized to mAChR-A activity in the adult gamma Kenyon cells, showing that aversive olfactory learning and short-term memory require mAChRs (Bielopolski et al., 2019).

Investigators also examined olfactory learning in ionotropic AChRs. Mutant flies with disrupted nAChRs in the MBONs showed a reversal in odor driven behavior. They approached an aversive odor, establishing a role for nAChR subunits in the MBONs in olfactory behaviors (Barnstedt et al., 2016). In another experiment on naïve avoidance utilizing the same aversive odor, knockdown of mAChR-A did not impact naïve avoidance (Bielopolski et al., 2019), suggesting that ionotropic receptors specifically mediate this behavior.

Although little research exists regarding ACh's role in alcohol-related behaviors in *Drosophila*, other drugs of abuse have been examined, such as nicotine. Although nicotine's mechanisms of action and behavioral outcomes differ from those of alcohol, both are associated with a period of elevated mood and increased activity at low doses and aversive effects at higher doses (Little, 2000). Additionally, in humans, use of both substances may arise for similar reasons and share similar patterns of use and abuse (Little, 2000). Nicotine has known effects on neurotransmitter systems. When flies are exposed during development, there are fewer TH-positive neurons in the PPM3 cluster of the adult brain, suggesting dopaminergic impacts (Morris et al., 2018). Nicotine exerts direct effects on the cholinergic system by activating nAChRs and producing fly behavioral responses such as hyperactivity (Ren et al., 2012), disrupted geotaxis (King et al., 2011), and loss of startle response (Fuenzalida–Uribe et al., 2013). These behavioral alterations are similar to those we have discussed in reference to alcohol. Also, in probing these effects at various developmental stages for *Drosophila,* research shows that fly responses to nicotine are comparable to mammals throughout development (Velazquez-Ulloa, 2017). Therefore, nicotine research in *Drosophila* may provide a useful point of reference for future explorations of alcohol and ACh.

Because it is the primary excitatory neurotransmitter, ACh activation drives the downstream release of neuromodulators like DA and OA. nAChR activation with pharmacological agents led to a rapid, dose-dependent release of OA (Fuenzalida–Uribe et al., 2013). In a startle-induced negative geotaxis assay, flies exposed to nicotine do not rapidly climb up the vial following a mechanical disruption that taps them to the bottom of the tube. However, disrupting OA transmission abolished the nicotine-induced impairment of flies' startle response, returning negative geotaxis to normal. Therefore, the behavioral response to drugs like nicotine involves AChR-induced OA release (Fuenzalida–Uribe et al., 2013).

GLUTAMATE

The glutamatergic system in the *Drosophila* brain presents a bit of a mystery. While glutamate is one of the best characterized neurotransmitters in the mammalian brain, it is one of the least understood for the fly. In the mammalian CNS, glutamate is the primary excitatory neurotransmitter, but this is not true for flies. Studies show that there are numerous glutamatergic neurons distributed throughout the adult fly CNS (Daniels et al., 2008; Raghu and Borst, 2011), and glutamate is well-established as the primary excitatory neurotransmitter at the NMJ (Jan and Jan, 1976). However, its role in brain activity remains somewhat enigmatic. Because glutamate exerts excitatory effects at the NMJ and in the vertebrate CNS, investigators have considered its excitatory potential in *Drosophila.* Studies of ionotropic glutamate receptor (iGluR) subunits with homology to vertebrate receptors have not established conclusive excitatory mechanisms in the fly brain, and, in fact, glutamate may be inhibitory in some circuits, like for olfaction (Liu and Wilson, 2013). Specifically, in the olfactory system, glutamatergic inhibition is mediated by the glutamate-gated chloride channel (Liu and Wilson, 2013), and the gene encoding this channel, GluClα, also mediates glutamatergic inhibition in the fly visual system (Molina-Obando et al., 2019).

Glutamate Synthesis, Action, and Metabolism

Glutamate is an amino acid that is produced by neuron-glia interactions in the glutamate-glutamine cycle, which involves the enzymes glutamate dehydrogenase (Gdh) and glutamine synthetase (GS) (Vernizzi et al., 2019). Gdh converts glutamate to alpha-ketoglutarate and ammonia (Plaitakis et al., 2017), and GS is a cytosolic enzyme that produces glutamine (Spodenkiewicz et al., 2016). Cytosolic glutamate is a precursor in the synthesis of GABA (Daniels et al., 2008). The fly genome contains 30 iGluR subunits (Littleton and Ganetzky, 2000), one metabotropic receptor (Mitri et al., 2004), and one glutamate-gated chloride channel (Cully et al., 1996), suggesting that glutamate can exert numerous effects in the fly brain. The vesicular glutamate transporter (VGlut) fills synaptic vesicles with glutamate. There is a single *Drosophila* VGlut found on synaptic vesicles at the

NMJ at synapses on motoneurons and interneurons throughout the CNS (Daniels et al., 2004).

Behavioral Effects of Glutamate

Because glutamate in isolation has historically been challenging to study outside the NMJ, it is not well-understood what behavioral effects glutamate is uniquely involved in regulating. Glutamatergic activity is better characterized in vertebrates, and these findings provide foundations for studying the role of glutamate in behavior for *Drosophila* as well. In vertebrates, much of our understanding of glutamatergic activity comes from understanding the action of the three iGluRs: AMPA, kainate, and NMDA receptors (Traynelis et al., 2010). Although *Drosophila* iGluRs share sequence similarity with the vertebrate receptors, subsequent Investigations have been somewhat limited because invertebrate neurons are small and challenging to access, complicating further characterization of these receptors' functions (Li et al., 2016a).

In mammals, glutamatergic activation likely has circadian fluctuations (Prosser, 2001), which is also true for flies (Zimmerman et al., 2017). Reducing glutamatergic release from glutamatergic neurons decreased wakefulness, and increased glutamatergic activity promoted wakefulness (Zimmerman et al., 2017). These results suggest that glutamate is wake-active in *Drosophila* (Zimmerman et al., 2017). Optogenetic studies indicate that the dorsal population of circadian clock neurons use glutamate as an inhibitory transmitter to promote sleep. This signaling may play a particularly significant role in daytime sleep (Guo et al., 2016). See **Table 1** for a summary of glutamate roles in fly behavior.

Glutamate likely plays an important role in the antennal lobe. The AL is known to contain both ionotropic and metabotropic glutamate receptors, including the fly homolog of the NMDA receptor, Nmdar, which is thought to have a conserved function in synaptic plasticity and, therefore, olfactory learning and memory (Xia et al., 2005). In the fly olfactory circuit, glutamate is a neurotransmitter with inhibitory effects, primarily influencing the response that projection neurons in the AL have to olfactory stimuli (Liu and Wilson, 2013). Interrupting glutamatergic transmission by expressing an RNAi transgene for one of the NMDA receptor subunits, *Nmdar1*, reduces Nmdar1 receptor subunit levels. Reducing Nmdar1 activity in a subset of AL projection neurons responsive to a specific odor blocked short and long-term olfactory habituation to that specified odor without impacting habituation to other odors (Das et al., 2011). Additionally, RNAi knockdown of Gad1 or VGlut in AL local interneurons blocks short and long-term olfactory habituation, suggesting that both GABAergic and glutamatergic activity in local interneurons are important for habituation (Das et al., 2011).

There is little research focused specifically on glutamate's role in ethanol-related behaviors in *Drosophila*. In olfactory receptor neurons of the AL, a single alcohol exposure induces excitotoxic cell death via glycogen synthase kinase-3 beta and NMDA receptors, suggesting a glutamatergic role for alcohol-induced neural deficits in the fly (French and Heberlein, 2009). Some recent evidence suggests that a circuit involving DAergic modulation of glutamatergic MBONs is involved in consolidation and expression for alcohol-associated memories (Scaplen et al., 2020). Two DAergic projections are to glutamatergic MBONs implicated in arousal (Sitaraman et al., 2015; Scaplen et al., 2020). DAergic activity may inhibit these MBONs, permitting consolidation of alcohol preference. These effects provide insight into neural mechanisms for the association of alcohol with context cues and memories, which is a critical feature of the persistence of addiction behaviors (Scaplen et al., 2020). As genetic and behavioral tools become increasingly advanced, this area of research should continue to expand.

Glutamate is unique in that it has a multi-faceted role in the fly brain, exerting both excitatory and inhibitory effects (Jan and Jan, 1976; Liu and Wilson, 2013). Furthermore, the complex action of poorly understood neurotransmitters like glutamate and ACh is complicated by phenomena such as dual neurotransmission. Dual neurotransmission overrides the classical view of "one neuron, one transmitter" and shows that neurons often release two or more neurotransmitters (Vaaga et al., 2014). The majority of OA neurons in the *Drosophila* brain are also glutamatergic, and dual neurotransmission is involved in behaviors like aggression and courtship (Sherer et al., 2020). These discoveries have been made possible via new tools enabling detailed glutamatergic manipulations like RNAi knockout of glutamate in OA neurons (Sherer et al., 2020) and circuit tracing and labeling with trans-Tango (Talay et al., 2017). Other developments include the electrophysiological characterization of neurons (Liu and Wilson, 2013), GAL4/UAS inhibition of specific glutamatergic neurons (Liu and Wilson, 2013), and post-synaptic knockdown of glutamate receptors (Das et al., 2011). Using these glutamatergic advancements as a model, outcomes for other complex and overlapping neurotransmitter systems will continue to be clarified.

CONCLUSION

Twenty years ago, little was known about the role of DA in fly behavior, and it was an area receiving relatively little research focus. However, we have now identified that even though there are only about 250 DA neurons, many small subsets of these impact distinct behaviors (e.g., Kong et al., 2010), and DA is an area of high interest to researchers studying *Drosophila* behavior. These discoveries have been made possible through tools like split-GAL4 lines that enable overexpression or knockdown of genes in specific subsets of neurons, and CRISPR/Cas9 mutagenesis, which allows for the targeted investigation of a mutated gene. Tissue-specific CRISPR has also been applied in *Drosophila* to restrict mutagenesis to a particular subset of cells (Meltzer et al., 2019; Poe et al., 2019; Port et al., 2020). Additionally, researchers recently began to define the *Drosophila* chemoconnectome, which comprises all neurotransmitters, neuromodulators, neuropeptides, and their receptors (Deng et al., 2019). The chemoconnectome has been made possible by advancements in genetic manipulation and neural mapping. As it is expanded upon, it will provide an invaluable resource for describing neurotransmitters anatomically and functionally. As

tools continue to increase in precision and sensitivity, we will further unravel the roles of little-explored neurotransmitters in alcohol-induced behaviors. The future is bright for this area of research, and discoveries are undoubtedly imminent.

Drosophila is genetically tractable and displays a huge behavioral repertoire, making it an extremely useful model organism for neuroscience. Recent years have brought advancements in genetic and behavioral tools that make flies increasingly advantageous. *Drosophila* are an especially suitable candidate for studying behaviors like alcohol response, which is challenging to investigate in mammals due to alcohol's widespread action throughout the brain. No organism has a specific, unique circuit or receptor for alcohol, so it must be explored in reference to its impacts on the various existing biological pathways of which it takes advantage. Alcohol-induced neurotransmitter modifications and associated influence on behavior are one critical tool for unraveling the neurobiological effects of alcohol. In manipulating fly neurotransmitter systems and assessing impacts on ethanol-related behaviors, we further make sense of the complicated relationship between brain and behavior relating to alcohol.

AUTHOR CONTRIBUTIONS

MC wrote the manuscript with input from IT and AR. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Institute of Health, Grant Nos. R01AA019526 and R01AA026818.

ACKNOWLEDGMENTS

We thank members of the Rothenfluh and Rodan Labs for continued discussion.

REFERENCES

Abrahao, K. P., Salinas, A. G., and Lovinger, D. M. (2017). Alcohol and the brain: neuronal molecular targets, synapses, and circuits. *Neuron* 96, 1223–1238. doi: 10.1016/j.neuron.2017.10.032

Addicott, M. A., Marsh-Richard, D. M., Mathias, C. W., and Dougherty, D. M. (2007). The biphasic effects of alcohol: comparisons of subjective and objective measures of stimulation, sedation, and physical activity. *Alcoholism* 31, 1883–1890. doi: 10.1111/j.1530-0277.2007.00518.x

Agosto, J., Choi, J. C., Parisky, K. M., Stilwell, G., Rosbash, M., and Griffith, L. C. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in *Drosophila*. *Nat. Neurosci.* 11, 354–359. doi: 10.1038/nn2046

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., 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

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

Allada, R., and Chung, B. Y. (2010). Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* 72, 605–624. doi: 10.1146/annurev-physiol-021909-135815

Amark, C. (1951). A study in alcoholism; clinical, social-psychiatric and genetic investigations. *Acta Psychiatr. Neurol. Scand. Suppl.* 70, 1–283.

Arancibia, S., Marambio, M., Campusano, J. M., and Fierro, A. (2019). Modeling of the binding of octopamine and dopamine in insect monoamine transporters reveals structural and electrostatic differences. *ACS Chem. Neurosci.* 10, 2310–2317. doi: 10.1021/acschemneuro.8b00631

Aranda, G. P., Hinojos, S. J., Sabandal, P. R., Evans, P. D., and Han, K.-A. (2017). Behavioral sensitization to the disinhibition effect of ethanol requires the dopamine/ecdysone receptor in *Drosophila*. *Front. Syst. Neurosci.* 11:56. doi: 10.3389/fnsys.2017.00056

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., 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

Avegno, E. M., and Gilpin, N. W. (2019). Inducing alcohol dependence in rats using chronic intermittent exposure to alcohol vapor. *Bio Protoc.* 9:e3222. doi: 10.21769/BioProtoc.3222

Azanchi, R., Kaun, K. R., and Heberlein, U. (2013). Competing dopamine neurons drive oviposition choice for ethanol in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 21153–21158. doi: 10.1073/pnas.1320208110

Bainton, R. J., Tsai, L. T.-Y., 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

Balfanz, S., Strünker, T., Frings, S., and Baumann, A. (2005). A family of octapamine 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

Barnstedt, O., Owald, D., Felsenberg, J., Brain, R., Moszynski, J.-P., Talbot, C. B., et al. (2016). Memory-relevant mushroom body output synapses are cholinergic. *Neuron* 89, 1237–1247. doi: 10.1016/j.neuron.2016.02.015

Bayard, M., McIntyre, J., Hill, K. R., and Woodside, J. (2004). Alcohol withdrawal syndrome. *Am. Fam. Phys.* 69, 1443–1450.

Bellen, H. J., Tong, C., and Tsuda, H. (2010). 100 years of *Drosophila* research and its impact on vertebrate neuroscience: a history lesson for the future. *Nat. Rev. Neurosci.* 11, 514–522. doi: 10.1038/nrn2839

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

Bielopolski, N., Amin, H., Apostolopoulou, A. A., Rozenfeld, E., Lerner, H., Huetteroth, W., et al. (2019). Inhibitory muscarinic acetylcholine receptors enhance aversive olfactory learning in adult *Drosophila*. *Elife* 8:e48264. doi: 10.7554/eLife.48264.035

Blenau, W., Daniel, S., Balfanz, S., Thamm, M., and Baumann, A. (2017). Dm5-HT2B: pharmacological characterization of the fifth serotonin receptor subtype of *Drosophila melanogaster*. *Front. Syst. Neurosci.* 11:26. doi: 10.3389/fnsys.2017.00028

Boileau, I., Assaad, J.-M., Pihl, R. O., Benkelfat, C., Leyton, M., Diksic, M., et al. (2003). Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse* 49, 226–231. doi: 10.1002/syn.10226

Borowsky, B., Adham, N., Jones, K. A., Raddatz, R., Artymyshyn, R., Ogozalek, K. L., et al. (2001). Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8966–8971. doi: 10.1073/pnas.151105198

Bozler, J., Kacsoh, B. Z., and Bosco, G. (2019). Transgenerational inheritance of ethanol preference is caused by maternal NPF repression. *Elife* 8:e45391. doi: 10.7554/eLife.45391.027

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415. Available online at: https://dev.biologists.org/content/118/2/401 (accessed June 13, 2020).

Buchner, E. (1991). Genes expressed in the adult brain of *Drosophila* and effects of their mutations on behavior: a survey of transmitter- and second messenger-related genes. *J. Neurogenet.* 7, 153–192. doi: 10.3109/01677069109167432

Budnik, V., and White, K. (1988). Catecholamine-containing neurons in *Drosophila melanogaster*: distribution and development. *J. Comp. Neurol.* 268, 400–413. doi: 10.1002/cne.902680309

Butts, A. R., Ojelade, S. A., Pronovost, E. D., Seguin, A., Merrill, C. B., Rodan, A. R., et al. (2019). Altered actin filament dynamics in the *Drosophila* mushroom bodies lead to fast acquisition of alcohol consumption preference. *J. Neurosci.* 39, 8877–8884. doi: 10.1523/JNEUROSCI.0973-19.2019

Carlsson, A., Engel, J., and Svensson, T. H. (1972). Inhibition of ethanol-induced excitation in mice and rats by a-Methyl-p-tyrosine. *Psychopharmacologia* 26, 307–312. doi: 10.1007/BF00422706

Carlsson, A., and Lindqvist, M. (1973). Effect of ethanol on the hydroxylation of tyrosine and tryptophan in rat brain *in vivo*. *J. Pharm. Pharmacol.* 25, 437–440. doi: 10.1111/j.2042-7158.1973.tb09129.x

Chakraborty, T. S., Gendron, C. M., Lyu, Y., Munneke, A. S., DeMarco, M. N., Hoisington, Z. W., et al. (2019). Sensory perception of dead conspecifics induces aversive cues and modulates lifespan through serotonin in *Drosophila*. *Nat. Commun.* 10:2365. doi: 10.1038/s41467-019-10285-y

Chen, J., Wang, Y., Zhang, Y., and Shen, P. (2013). Mutations in bacchus reveal a tyramine-dependent nuclear regulator for acute ethanol sensitivity in *Drosophila*. *Neuropharmacology* 67, 25–31. doi: 10.1016/j.neuropharm.2012.10.013

Chen, J., Zhang, Y., and Shen, P. (2010). Protein kinase C deficiency-induced alcohol insensitivity and underlying cellular targets in *Drosophila*. *Neuroscience* 166, 34–39. doi: 10.1016/j.neuroscience.2009.12.009

Chen, W.-F., Maguire, S., Sowcik, M., Luo, W., Koh, K., and Sehgal, A. (2015). A neuron-glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Mol. Psychiatry* 20, 240–251. doi: 10.1038/mp.2014.11

Chentsova, N. A., Gruntenko, N. E., Bogomolova, E. V., Adonyeva, N. V., Karpova, E. K., Rauschenbach, I., et al. (2002). Stress response in *Drosophila melanogaster* strain inactive with decreased tyramine and octopamine contents. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 172, 643–650. doi: 10.1007/s00360-002-0293-9

Claßen, G., and Scholz, H. (2018). Octopamine shifts the behavioral response from indecision to approach or aversion in *Drosophila melanogaster*. *Front. Behav. Neurosci.* 12:131. doi: 10.3389/fnbeh.2018.00131

Cognigni, P., Felsenberg, J., and Waddell, S. (2018). Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*. *Curr. Opin. Neurobiol.* 49, 51–58. doi: 10.1016/j.conb.2017.12.002

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. *J. Biol. Chem.* 280, 14948–14955. doi: 10.1074/jbc.M414197200

Coleman, C. M., and Neckameyer, W. S. (2005). Serotonin synthesis by two distinct enzymes in *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* 59, 12–31. doi: 10.1002/arch.20050

Collin, C., Hauser, F., Gonzalez de Valdivia, E., de Valdivia, E. G., Li, S., Reisenberger, J., et al. (2013). Two types of muscarinic acetylcholine receptors in *Drosophila* and other arthropods. *Cell. Mol. Life Sci.* 70, 3231–3242. doi: 10.1007/s00018-013-1334-0

Colombo, M. N., and Francolini, M. (2019). Glutamate at the vertebrate neuromuscular junction: from modulation to neurotransmission. *Cells* 8:996. doi: 10.3390/cells8090996

Colović, M. B., Krstić, D. Z., Lazarević-Pašti, T. D., BondŽić, A. M., and Vasić, V. M. (2013). Acetylcholinesterase inhibitors: pharmacology and toxicology. *Curr. Neuropharmacol.* 11, 315–335. doi: 10.2174/1570159X11311030006

Corey, J. L., Quick, M. W., Davidson, N., Lester, H. A., and Guastella, J. (1994). A cocaine-sensitive *Drosophila* serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1188–1192. doi: 10.1073/pnas.91.3.1188

Cowmeadow, R. B., Krishnan, H. R., Ghezzi, A., Al'Hasan, Y. M., Wang, Y. Z., and Atkinson, N. S. (2006). Ethanol tolerance caused by slowpoke induction in *Drosophila*. *Alcohol. Clin. Exp. Res.* 30, 745–753. doi: 10.1111/j.1530-0277.2006.00087.x

Crabbe, J. C., Phillips, T. J., Feller, D. J., Hen, R., Wenger, C. D., Lessov, C. N., et al. (1996). Elevated alcohol consumption in null mutant mice lacking 5–HT1B serotonin receptors. *Nat. Genet.* 14, 98–101. doi: 10.1038/ng0996-98

Crozier, W. J., Pincus, G., and Zahl, P. A. (1936). The resistance of *Drosophila* to alcohol. *J. Gen. Physiol.* 19, 523–557. doi: 10.1085/jgp.19.3.523

Cully, D. F., Paress, P. S., Liu, K. K., Schaeffer, J. M., and Arena, J. P. (1996). Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. *J. Biol. Chem.* 271, 20187–20191. doi: 10.1074/jbc.271.33.20187

Cunningham, C. L., Gremel, C. M., and Groblewski, P. A. (2006). Drug-induced conditioned place preference and aversion in mice. *Nat. Protoc.* 1, 1662–1670. doi: 10.1038/nprot.2006.279

Daniels, R. W., Collins, C. A., Gelfand, M. V., Dant, J., Brooke, E. S., Krantz, D. E., et al. (2004). Increased expression of the *Drosophila* vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. *J. Neurosci.* 24, 10466–10474. doi: 10.1523/JNEUROSCI.3001-04.2004

Daniels, R. W., Gelfand, M. V., Collins, C. A., and DiAntonio, A. (2008). Visualizing glutamatergic cell bodies and synapses in *Drosophila* larval and adult CNS. *J. Comp. Neurol.* 508, 131–152. doi: 10.1002/cne.21670

Das, S., Sadanandappa, M. K., Dervan, A., Larkin, A., Lee, J. A., Sudhakaran, I. P., et al. (2011). Plasticity of local GABAergic interneurons drives olfactory habituation. *Proc. Natl. Acad. Sci. U.S.A.* 108, E646–E654. doi: 10.1073/pnas.1106411108

Demchyshyn, L. L., Pristupa, Z. B., Sugamori, K. S., Barker, E. L., Blakely, R. D., Wolfgang, W. J., et al. (1994). Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 91, 5158–5162. doi: 10.1073/pnas.91.11.5158

Deng, B., Li, Q., Liu, X., Cao, Y., Li, B., Qian, Y., et al. (2019). Chemoconnectomics: mapping chemical transmission in *Drosophila*. *Neuron 101*, 876–893.e4. doi: 10.1016/j.neuron.2019.01.045

Devineni, A. V., and Heberlein, U. (2009). Preferential ethanol consumption in *Drosophila* models features of addiction. *Curr. Biol.* 19, 2126–2132. doi: 10.1016/j.cub.2009.10.070

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

Di Chiara, G. (1995). The role of dopamine in drug abuse viewed from the perspective of its role in motivation. *Drug Alcohol Depend* 38, 95–137. doi: 10.1016/0376-8716(95)01118-I

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U.S.A.* 85, 5274–5278. doi: 10.1073/pnas.85.14.5274

Dimitrijevic, N., Dzitoyeva, S., Satta, R., Imbesi, M., Yildiz, S., and Manev, H. (2005). *Drosophila* GABAB receptors are involved in behavioral effects of γ-hydroxybutyric acid (GHB). *Eur. J. Pharmacol.* 519, 246–252. doi: 10.1016/j.ejphar.2005.07.016

Dubowy, C., and Sehgal, A. (2017). Circadian rhythms and sleep in *Drosophila melanogaster*. *Genetics* 205, 1373–1397. doi: 10.1534/genetics.115.185157

Dzitoyeva, S., Dimitrijevic, N., and Manev, H. (2003). γ-Aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in *Drosophila*: adult RNA interference and pharmacological evidence. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5485–5490. doi: 10.1073/pnas.0830111100

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

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

Enell, L., Hamasaka, Y., Kolodziejczyk, A., and Nässel, D. R. (2007). γ-Aminobutyric acid (GABA) signaling components in *Drosophila*: immunocytochemical localization of GABAB receptors in relation to the GABAA receptor subunit RDL and a vesicular GABA transporter. *J. Comp. Neurol.* 505, 18–31. doi: 10.1002/cne.21472

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

Fadda, F., and Rossetti, Z. L. (1998). Chronic ethanol consumption: from neuroadaptation to neurodegeneration. *Progr. Neurobiol.* 56, 385–431. doi: 10.1016/S0301-0082(98)00032-X

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. doi: 10.1523/JNEUROSCI.23-12-05370.2003

Featherstone, D. E., Rushton, E. M., Hilderbrand-Chae, M., Phillips, A. M., Jackson, F. R., and Broadie, K. (2000). Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. *Neuron* 27, 71–84. doi: 10.1016/S0896-6273(00)00010-6

Foltenyi, K., Andretic, R., Newport, J. W., and Greenspan, R. J. (2007). Neurohormonal and neuromodulatory control of sleep in *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol.* 72, 565–571. doi: 10.1101/sqb.2007.72.045

Fortini, M. E., Skupski, M. P., Boguski, M. S., and Hariharan, I. K. (2000). A survey of human disease gene counterparts in the *Drosophila* genome. *J. Cell Biol.* 150, 23–30. doi: 10.1083/jcb.150.2.F23

Freeman, M. R. (2015). *Drosophila* central nervous system glia. *Cold Spring Harb. Perspect. Biol.* 7:a020552. doi: 10.1101/cshperspect.a020552

French, R. L., and Heberlein, U. (2009). Glycogen synthase kinase-3/Shaggy mediates ethanol-induced excitotoxic cell death of *Drosophila* olfactory neurons. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20924–20929. doi: 10.1073/pnas.0910813106

Frenkel, L., Muraro, N. I., Beltrán González, A. N., Marcora, M. S., Bernab,ó, G., Hermann-Luibl, C., et al. (2017). Organization of circadian behavior relies on glycinergic transmission. *Cell. Rep.* 19, 72–85. doi: 10.1016/j.celrep.2017.03.034

Fuenzalida–Uribe, N., Meza, R. C., Hoffmann, H. A., Varas, R., and Campusano, J. M. (2013). nAChR-induced octopamine release mediates the effect of nicotine on a startle response in *Drosophila melanogaster*. *J. Neurochem.* 125, 281–290. doi: 10.1111/jnc.12161

Gessa, G. L., Muntoni, F., Collu, M., Vargiu, L., and Mereu, G. (1985). Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. *Brain Res.* 348, 201–203. doi: 10.1016/0006-8993(85)90381-6

Ghezzi, A., Krishnan, H. R., and Atkinson, N. S. (2014). Susceptibility to ethanol withdrawal seizures is produced by BK channel gene expression. *Addict. Biol.* 19, 332–337. doi: 10.1111/j.1369-1600.2012.00465.x

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

Gimeno, C., Dorado, M. L., Roncero, C., Szerman, N., Vega, P., Balanzá-Martínez, V., et al. (2017). Treatment of comorbid alcohol dependence and anxiety disorder: review of the scientific evidence and recommendations for treatment. *Front. Psychiatry* 8:173. doi: 10.3389/fpsyt.2017.00173

Göthert, M., and Fink, K. (1989). Inhibition of N-methyl-d-aspartate (NMDA) and l-glutamate-induced noradrenaline and acetylcholine release in the rat brain by ethanol. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 340, 516–521. doi: 10.1007/BF00260606

Grant, B. F., Stinson, F. S., Dawson, D. A., Chou, S. P., Dufour, M. C., Compton, W., et al. (2004). Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders. *Arch. Gen. Psychiatry* 61, 807–816. doi: 10.1001/archpsyc.61.8.807

Griffiths, A. J., Miller, J. H., Suzuki, D. T., Lewontin, R. C., and Gelbart, W. M. (2000). "Reverse Genetics," in *An Introduction to Genetic Analysis* (New York: W.H. Freeman). Available online at: https://www.ncbi.nlm.nih.gov/books/NBK21843/ (accessed July 13, 2020).

Grotewiel, M., and Bettinger, J. C. (2015). *Drosophila* and *Caenorhabditis elegans* as discovery platforms for genes involved in human alcohol use disorder. *Alcohol. Clin. Exp. Res.* 39, 1292–1311. doi: 10.1111/acer.12785

Guo, F., Yu, J., Jung, H. J., Abruzzi, K. C., Luo, W., Griffith, L. C., et al. (2016). Circadian neuron feedback controls the *Drosophila* sleep-activity profile. *Nature* 536, 292–297. doi: 10.1038/nature19097

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59–63. doi: 10.1038/366059a0

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

Handler, A., Graham, T. G. W., Cohn, R., Morantte, I., Siliciano, A. F., Zeng, J., et al. (2019). Distinct dopamine receptor pathways underlie the temporal sensitivity of associative learning. *Cell 178*, 60–75.e19. doi: 10.1016/j.cell.2019.05.040

Hasin, D. S., Goodwin, R. D., Stinson, F. S., and Grant, B. F. (2005). Epidemiology of major depressive disorder. *Arch. Gen. Psychiatry* 62, 1097–1106. doi: 10.1001/archpsyc.62.10.1097

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* 4:e03868. doi: 10.7554/eLife.03868

Hendler, R. A., Ramchandani, V. A., Gilman, J., and Hommer, D. W. (2013). Stimulant and sedative effects of alcohol. *Curr. Top. Behav. Neurosci.* 13, 489–509. doi: 10.1007/978-3-642-28720-6_135

Hewes, R. S., and Taghert, P. H. (2001). Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res.* 11, 1126–1142. doi: 10.1101/gr.169901

Hirashima, A., Sukhanova, M. J., and Rauschenbach, I. Y. (2000). Genetic control of biogenic-amine systems in *Drosophila* under normal and stress conditions. *Biochem. Genet.* 38, 163–176. doi: 10.1023/A:1001925613951

Honjo, K., and Furukubo-Tokunaga, K. (2009). Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in *Drosophila* larvae. *J. Neurosci.* 29, 852–862. doi: 10.1523/JNEUROSCI.1315-08.2009

Hoopfer, E. D., Jung, Y., Inagaki, H. K., Rubin, G. M., and Anderson, D. J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. *Elife* 4:e11346. doi: 10.7554/eLife.11346.026

Hosie, A., Sattelle, D., Aronstein, K., and ffrench-Constant, R. (1997). Molecular biology of insect neuronal GABA receptors. *Trends Neurosci.* 20, 578–583. doi: 10.1016/S0166-2236(97)01127-2

Hu, W., Peng, Y., Sun, J., Zhang, F., Zhang, X., Wang, L., et al. (2018). Fan-shaped body neurons in the *Drosophila* brain regulate both innate and conditioned nociceptive avoidance. *Cell. Rep.* 24, 1573–1584. doi: 10.1016/j.celrep.2018.07.028

Huang, J., Liu, W., Qi, Y., Luo, J., and Montell, C. (2016). Neuromodulation of courtship drive through tyramine-responsive neurons in the *Drosophila* brain. *Curr. Biol.* 26, 2246–2256. doi: 10.1016/j.cub.2016.06.061

Hummel, M., and Unterwald, E. M. (2002). D1 dopamine receptor: a putative neurochemical and behavioral link to cocaine action. *J. Cell. Physiol.* 191, 17–27. doi: 10.1002/jcp.10078

Iliadi, K. G., Iliadi, N., and Boulianne, G. L. (2017). *Drosophila* mutants lacking octopamine exhibit impairment in aversive olfactory associative learning. *Eur. J. Neurosci.* 46, 2080–2087. doi: 10.1111/ejn.13654

Ja, W. W., Carvalho, G. B., Mak, E. M., de la Rosa, N. N., Fang, A. Y., Liong, J. C., et al. (2007). Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8253–8256. doi: 10.1073/pnas.0702726104

Jan, L. Y., and Jan, Y. N. (1976). L-glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *J. Physiol.* 262, 215–236. doi: 10.1113/jphysiol.1976.sp011593

Jenett, A., Rubin, G. M., Ngo, T.-T. B., 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

Kacsoh, B. Z., Lynch, Z. R., Mortimer, N. T., and Schlenke, T. A. (2013). Fruit flies medicate offspring after seeing parasites. *Science* 339, 947–950. doi: 10.1126/science.1229625

Kaneko, T., Macara, A. M., Li, R., Hu, Y., Iwasaki, K., Dunnings, Z., et al. (2017). Serotonergic modulation enables pathway-specific plasticity in a developing sensory circuit in *Drosophila*. *Neuron 95*, 623–638.e4. doi: 10.1016/j.neuron.2017.06.034

Karam, C. S., Jones, S. K., and Javitch, J. A. (2020). Come fly with me: an overview of dopamine receptors in *Drosophila melanogaster*. *Basic Clin. Pharmacol. Toxicol.* 126, 56–65. doi: 10.1111/bcpt.13277

Kasture, A. S., Bartel, D., Steinkellner, T., Sucic, S., Hummel, T., and Freissmuth, M. (2019). Distinct contribution of axonal and somatodendritic serotonin transporters in *Drosophila* olfaction. *Neuropharmacology* 161:107564. doi: 10.1016/j.neuropharm.2019.03.007

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

Kaun, K. R., Devineni, A. V., and Heberlein, U. (2012). *Drosophila melanogaster* as a model to study drug addiction. *Hum. Genet.* 131, 959–975. doi: 10.1007/s00439-012-1146-6

Kelm, M. K., Criswell, H. E., and Breese, G. R. (2011). Ethanol-enhanced GABA release: a focus on G protein-coupled receptors. *Brain Res. Rev.* 65, 113–123. doi: 10.1016/j.brainresrev.2010.09.003

King, I., Tsai, L. T.-Y., Pflanz, R., Voigt, A., Lee, S., Jäckle, H., et al. (2011). *Drosophila* tao controls mushroom body development and ethanol-stimulated behavior through par-1. *J. Neurosci.* 31, 1139–1148. doi: 10.1523/JNEUROSCI.4416-10.2011

Kitamoto, T., Wang, W., and Salvaterra, P. M. (1998). Structure and organization of the *Drosophila* cholinergic locus. *J. Biol. Chem.* 273, 2706–2713. doi: 10.1074/jbc.273.5.2706

Kohl, R. R., Katner, J. S., Chernet, E., and McBride, W. J. (1998). Ethanol and negative feedback regulation of mesolimbic dopamine release in rats. *Psychopharmacology* 139, 79–85. doi: 10.1007/s002130050692

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:e6654. doi: 10.1371/journal.pone.0009954

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

Küppers, B., Sánchez-Soriano, N., Letzkus, J., Technau, G. M., and Prokop, A. (2003). In developing *Drosophila* neurones the production of γ-amino butyric acid is tightly regulated downstream of glutamate decarboxylase translation and can be influenced by calcium. *J. Neurochem.* 84, 939–951. doi: 10.1046/j.1471-4159.2003.01554.x

Lathen, D. R., Merrill, C. B., and Rothenfluh, A. (2020). Flying together: *Drosophila* as a tool to understand the genetics of human alcoholism. *Int. J. Mol. Sci.* 21:6649. doi: 10.3390/ijms21186649

Leal, S. M., Kumar, N., and Neckameyer, W. S. (2004). GABAergic modulation of motor-driven behaviors in juvenile *Drosophila* and evidence for a nonbehavioral role for GABA transport. *J. Neurobiol.* 61, 189–208. doi: 10.1002/neu.20061

Leal, S. M., and Neckameyer, W. S. (2002). Pharmacological evidence for GABAergic regulation of specific behaviors in *Drosophila melanogaster*. *J. Neurobiol.* 50, 245–261. doi: 10.1002/neu.10030

Lebestky, T. J., 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 independently and oppositely regulated by the dopamine D1 receptor dopr via distinct neural circuits. *Neuron* 64, 522–536. doi: 10.1016/j.neuron.2009.09.031

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

LeMarquand, D., Pihl, R. O., and Benkelfat, C. (1994a). Serotonin and alcohol intake, abuse, and dependence: clinical evidence. *Biol. Psychiatry* 36, 326–337. doi: 10.1016/0006-3223(94)90630-0

LeMarquand, D., Pihl, R. O., and Benkelfat, C. (1994b). Serotonin and alcohol intake, abuse, and dependence: findings of animal studies. *Biol. Psychiatry* 36, 395–421. doi: 10.1016/0006-3223(94)91215-7

Li, Y., Dharkar, P., Han, T.-H., Serpe, M., Lee, C.-H., and Mayer, M. L. (2016a). Novel functional properties of *Drosophila* CNS glutamate receptors. *Neuron* 92, 1036–1048. doi: 10.1016/j.neuron.2016.10.058

Li, Y., Hoffmann, J., Li, Y., Stephano, F., Bruchhaus, I., Fink, C., et al. (2016b). Octopamine controls starvation resistance, life span and metabolic traits in *Drosophila*. *Sci. Rep.* 6:35359. doi: 10.1038/srep35359

Little, H. J. (2000). Behavioral mechanisms underlying the link between smoking and drinking. *Alcohol Res. Health* 24, 215–224.

Littleton, J. T., and Ganetzky, B. (2000). Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* 26, 35–43. doi: 10.1016/S0896-6273(00)81135-6

Liu, C., Meng, Z., Wiggin, T. D., Yu, J., Reed, M. L., Guo, F., et al. (2019). A serotonin-modulated circuit controls sleep architecture to regulate cognitive function independent of total sleep in *Drosophila*. *Curr. Biol. 29*, 3635–3646.e5. doi: 10.1016/j.cub.2019.08.079

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, 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, 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

Liu, W. W., and Wilson, R. I. (2013). Glutamate is an inhibitory neurotransmitter in the *Drosophila* olfactory system. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10294–10299. doi: 10.1073/pnas.1220560110

Liu, X., Krause, W. C., and Davis, R. L. (2007). GABAA receptor RDL inhibits *Drosophila* olfactory associative learning. *Neuron* 56, 1090–1102. doi: 10.1016/j.neuron.2007.10.036

Lovinger, D. M., White, G., and Weight, F. F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science* 243, 1721–1724. doi: 10.1126/science.2467382

Lyness, W. H., and Smith, F. L. (1992). Influence of dopaminergic and serotonergic neurons on intravenous ethanol self-administration in the rat. *Pharmacol. Biochem. Behav.* 42, 187–192. doi: 10.1016/0091-3057(92)90465-R

MacLeod, K., and Laurent, G. (1996). Distinct mechanisms for synchronization and temporal patterning of odor-encoding neural assemblies. *Science* 274, 976–979. doi: 10.1126/science.274.5289.976

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

Manev, H., and Dzitoyeva, S. (2010). GABA-B receptors in *Drosophila*. *Adv. Pharmacol.* 58, 453–464. doi: 10.1016/S1054-3589(10)58017-7

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* 3:2009. doi: 10.3389/neuro.04.005.2009

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

Marshall, B. D. L., Prescott, M. R., Liberzon, I., Tamburrino, M. B., Calabrese, J. R., and Galea, S. (2012). Coincident posttraumatic stress disorder and depression predict alcohol abuse during and after deployment among army national guard soldiers. *Drug Alcohol Depend* 124, 193–199. doi: 10.1016/j.drugalcdep.2011.12.027

Martins, S. S., and Gorelick, D. A. (2011). Conditional substance abuse and dependence by diagnosis of mood or anxiety disorder or schizophrenia in the U.S. *population. Drug Alcohol Depend*. 119, 28–36. doi: 10.1016/j.drugalcdep.2011.05.010

Martz, A., Dietrich, R. A., and Harris, R. A. (1983). Behavioral evidence for the involvement of γ-aminobutyric acid in the actions of ethanol. *Eur. J. Pharmacol.* 89, 53–62. doi: 10.1016/0014-2999(83)90607-6

McGonigle, I., and Lummis, S. C. R. (2009). RDL receptors. *Biochem. Soc. Trans.* 37, 1404–1406. doi: 10.1042/BST0371404

Meltzer, H., Marom, E., Alyagor, I., Mayseless, O., Berkun, V., Segal-Gilboa, N., et al. (2019). Tissue-specific (ts)CRISPR as an efficient strategy for *in vivo* screening in *Drosophila*. *Nat. Commun.* 10:2113. doi: 10.1038/s41467-019-10140-0

Menozzi, P., Shi, M. A., Lougarre, A., Tang, Z. H., and Fournier, D. (2004). Mutations of acetylcholinesterase which confer insecticide

resistance in *Drosophila melanogaster* populations. *BMC Evol. Biol.* 4:4. doi: 10.1186/1471-2148-4-4

Mitri, C., Parmentier, M.-L., Pin, J.-P., Bockaert, J., and Grau, Y. (2004). Divergent evolution in metabotropic glutamate receptors. *J. Biol. Chem.* 279, 9313–9320. doi: 10.1074/jbc.M310878200

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

Molina-Obando, S., Vargas-Fique, J. F., Henning, M., Gür, B., Schladt, T. M., Akhtar, J., et al. (2019). ON selectivity in the *Drosophila* visual system is a multisynaptic process involving both glutamatergic and GABAergic inhibition. *Elife* 8:e49373. doi: 10.7554/eLife.49373.037

Monastirioti, M., Charles, E., Linn, J., and White, K. (1996). Characterization of *Drosophila* tyramine β-hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* 16, 3900–3911. doi: 10.1523/JNEUROSCI.16-12-03900.1996

Moore, M. S., DeZazzo, J., Luk, A. Y., Tully, T., Singh, C. M., and Heberlein, U. (1998). Ethanol intoxication in *Drosophila*: genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell* 93, 997–1007. doi: 10.1016/S0092-8674(00)81205-2

Morris, M., Shaw, A., Lambert, M., Perry, H. H., Lowenstein, E., Valenzuela, D., et al. (2018). Developmental nicotine exposure affects larval brain size and the adult dopaminergic system of *Drosophila melanogaster*. *BMC Dev. Biol.* 18:13. doi: 10.1186/s12861-018-0172-6

Muthukumar, A. K., Stork, T., and Freeman, M. R. (2014). Activity-dependent regulation of astrocyte GAT levels during synaptogenesis. *Nat. Neurosci.* 17, 1340–1350. doi: 10.1038/nn.3791

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

Newton, P. M., and Ron, D. (2007). Protein kinase C and alcohol addiction. *Pharmacol. Res.* 55, 570–577. doi: 10.1016/j.phrs.2007.04.008

Ogueta, M., Cibik, O., Eltrop, R., Schneider, A., and Scholz, H. (2010). The influence of Adh function on ethanol preference and tolerance in adult *Drosophila melanogaster*. *Chem. Senses* 35, 813–822. doi: 10.1093/chemse/bjq084

Ojelade, S. A., Butts, A. R., Merrill, C. B., Champaloux, E. P., Aso, Y., Wolin, D., et al. (2019). Dopaminergic learning and arousal circuits mediate opposing effects on alcohol consumption in *Drosophila*. *bioRxiv [Preprint]*. doi: 10.1101/624833

Okada, R., Awasaki, T., and Ito, K. (2009). Gamma-aminobutyric acid (GABA)-mediated neural connections in the *Drosophila* antennal lobe. *J. Comp. Neurol.* 514, 74–91. doi: 10.1002/cne.21971

Park, A., Tran, T., and Atkinson, N. S. (2018). Monitoring food preference in *Drosophila* by oligonucleotide tagging. *Proc. Natl. Acad. Sci. U.S.A.* 115, 9020–9025. doi: 10.1073/pnas.1716880115

Pauls, D., Blechschmidt, C., Frantzmann, F., El Jundi, B., and Selcho, M. (2018). A comprehensive anatomical map of the peripheral octopaminergic/tyraminergic system of drosophila melanogaster. *Sci. Rep.* 8:15314. doi: 10.1038/s41598-018-33686-3

Pearl, R., White, F. B., and Miner, J. R. (1929). Age changes alcohol tolerance in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 15, 425–429. doi: 10.1073/pnas.15.5.425

Pendleton, R. G., Rasheed, A., Sardina, T., Tully, T., and Hillman, R. (2002). Effects of tyrosine hydroxylase mutants on locomotor activity in drosophila: a study in functional genomics. *Behav. Genet.* 32, 89–94. doi: 10.1023/A:1015279221600

Peru y Colón de Portugal, R. L., Ojelade, S. A., Penninti, P. S., Dove, R. J., Nye, M. J., Acevedo, S. F., et al. (2014). Long-lasting, experience-dependent alcohol preference in *Drosophila*. *Addict. Biol.* 19, 392–401. doi: 10.1111/adb.12105

Petruccelli, E., Li, Q., Rao, Y., and Kitamoto, T. (2016). The unique dopamine/ecdysteroid receptor modulates ethanol-induced sedation in *Drosophila*. *J. Neurosci.* 36, 4647–4657. doi: 10.1523/JNEUROSCI.3774-15.2016

Pitman, J. L., Huetteroth, W., Burke, C. J., Krashes, M. J., Lai, S.-L., Lee, T., et al. (2011). A pair of inhibitory neurons are required to sustain labile memory in the *Drosophila* mushroom body. *Curr. Biol.* 21, 855–861. doi: 10.1016/j.cub.2011.03.069

Plaitakis, A., Kalef-Ezra, E., Kotzamani, D., Zaganas, I., and Spanaki, C. (2017). The glutamate dehydrogenase pathway and its roles in cell and tissue biology in health and disease. *Biology* 6:11. doi: 10.3390/biology6010011

Poe, A. R., Wang, B., Sapar, M. L., Ji, H., Li, K., Onabajo, T., et al. (2019). Robust CRISPR/Cas9-mediated tissue-specific mutagenesis reveals gene redundancy and perdurance in *Drosophila*. *Genetics* 211, 459–472. doi: 10.1534/genetics.118.301736

Pohorecky, L. A. (1977). Biphasic action of ethanol. *Biobehav. Rev.* 1, 231–240. doi: 10.1016/0147-7552(77)90025-0

Poldrugo, F., and Addolorato, G. (1999). The role of gamma-hydroxybutyric acid in the treatment of alcoholism: from animal to clinical studies. *Alcohol Alcohol* 34, 15–24. doi: 10.1093/alcalc/34.1.15

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

Port, F., Strein, C., Stricker, M., Rauscher, B., Heigwer, F., Zhou, J., et al. (2020). A large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*. *Elife* 9:e53865. doi: 10.7554/eLife.53865.sa2

Pörzgen, P., Park, S. K., Hirsh, J., Sonders, M. S., and Amara, S. G. (2001). The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Mol. Pharmacol.* 59, 83–95. doi: 10.1124/mol.59.1.83

Prosser, R. A. (2001). Glutamate blocks serotonergic phase advances of the mammalian circadian pacemaker through AMPA and NMDA receptors. *J. Neurosci.* 21, 7815–7822. doi: 10.1523/JNEUROSCI.21-19-07815.2001

Raghu, S. V., and Borst, A. (2011). Candidate glutamatergic neurons in the visual system of *Drosophila*. *PLoS ONE* 6:e19472. doi: 10.1371/journal.pone.0019472

Ranson, D. C., Ayoub, S. S., Corcoran, O., and Casalotti, S. O. (2020). Pharmacological targeting of the GABAB receptor alters *Drosophila's* behavioural responses to alcohol. *Addict. Biol.* 25:e12725. doi: 10.1111/adb.12725

Ren, G. R., Folke, J., Hauser, F., Li, S., and Grimmelikhuijzen, C. J. P. (2015). The A- and B-type muscarinic acetylcholine receptors from *Drosophila* melanogaster couple to different second messenger pathways. *Biochem. Biophys. Res. Commun.* 462, 358–364. doi: 10.1016/j.bbrc.2015.04.141

Ren, J., Sun, J., Zhang, Y., Liu, T., Ren, Q., Li, Y., et al. (2012). Down-regulation of decapping protein 2 mediates chronic nicotine exposure-induced locomotor hyperactivity in *Drosophila*. *PLoS ONE* 7:e52521. doi: 10.1371/journal.pone.0052521

Rezával, C., Nojima, T., Neville, M. C., Lin, A. C., and Goodwin, S. F. (2014). Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in *Drosophila*. *Curr. Biol.* 24, 725–730. doi: 10.1016/j.cub.2013.12.051

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

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

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.16843.020

Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404

Roy, B., Singh, A. P., Shetty, C., Chaudhary, V., North, A., Landgraf, M., et al. (2007). Metamorphosis of an identified serotonergic neuron in the *Drosophila* olfactory system. *Neural. Dev.* 2:20. doi: 10.1186/1749-8104-2-20

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

Sachse, S., and Galizia, C. G. (2002). Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. *J. Neurophysiol.* 87, 1106–1117. doi: 10.1152/jn.00325.2001

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

Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., et al. (2020). Circuits that encode and guide alcohol-associated preference. *Elife* 9:e48730. doi: 10.7554/eLife.48730.sa2

Scheunemann, L., Plaçais, P.-Y., Dromard, Y., Schwärzel, M., and Preat, T. (2018). Dunce phosphodiesterase acts as a checkpoint for *Drosophila* long-term memory in a pair of serotonergic neurons. *Neuron 98*, 350–365.e5. doi: 10.1016/j.neuron.2018.03.032

Schmitt, R. E., Messick, M. R., Shell, B. C., Dunbar, E. K., Fang, H.-F., Shelton, K. L., et al. (2020). Dietary yeast influences ethanol sedation in *Drosophila* via serotonergic neuron function. *Addict. Biol.* 25:e12779. doi: 10.1111/adb.12779

Schneider, A., Ruppert, M., Hendrich, O., Giang, T., Ogueta, M., Hampel, S., et al. (2012). Neuronal basis of innate olfactory attraction to ethanol in *Drosophila*. *PLoS ONE* 7:e52007. doi: 10.1371/journal.pone.0052007

Scholz, H. (2005). Influence of the biogenic amine tyramine on ethanol-induced behaviors in *Drosophila*. *J. Neurobiol.* 63, 199–214. doi: 10.1002/neu.20127

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

Scholz-Kornehl, S., and Schwärzel, M. (2016). Circuit analysis of a *Drosophila* dopamine type 2 receptor that supports anesthesia-resistant memory. *J. Neurosci.* 36, 7936–7945. doi: 10.1523/JNEUROSCI.4475-15.2016

Schuckit, M. A. (2009). Alcohol-use disorders. *Lancet* 373, 492–501. doi: 10.1016/S0140-6736(09)60009-X

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. doi: 10.1523/JNEUROSCI.23-33-10495.2003

Selcho, M., Pauls, D., Huser, A., Stocker, R. F., and Thum, A. S. (2014). Characterization of the octopaminergic and tyraminergic neurons in the central brain of *Drosophila* larvae. *J. Comp. Neurol.* 522, 3485–3500. doi: 10.1002/cne.23616

Sherer, L. M., Catudio Garrett, E., Morgan, H. R., Brewer, E. D., Sirrs, L. A., Shearin, H. K., et al. (2020). Octopamine neuron dependent aggression requires dVGLUT from dual-transmitting neurons. *PLoS Genet.* 16:e8609. doi: 10.1371/journal.pgen.1008609

Shih, M.-F. M., Davis, F. P., Henry, G. L., and Dubnau, J. (2019). Nuclear transcriptomes of the seven neuronal cell types that constitute the *Drosophila* mushroom bodies. *G3* 9, 81–94. doi: 10.1534/g3.118.200726

Shohat-Ophir, G., Kaun, K. R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual deprivation increases ethanol intake in *Drosophila*. *Science* 335, 1351–1355. doi: 10.1126/science.1215932

Silva, B., Goles, N. I., Varas, R., and Campusano, J. M. (2014). Serotonin receptors expressed in *Drosophila* mushroom bodies differentially modulate larval locomotion. *PLoS ONE* 9:e89641. doi: 10.1371/journal.pone.0089641

Silva, B., Molina-Fernández, C., Ugalde, M. B., Tognarelli, E. I., Angel, C., and Campusano, J. M. (2015). Muscarinic ACh receptors contribute to aversive olfactory learning in *Drosophila*. *Neural Plast* 2015:658918. doi: 10.1155/2015/658918

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

Singh, C. M., and Heberlein, U. (2000). Genetic control of acute ethanol-induced behaviors in *Drosophila*. *Alcoholism* 24, 1127–1136. doi: 10.1111/j.1530-0277.2000.tb02075.x

Sitaraman, D., Aso, Y., Jin, X., Chen, N., Felix, M., Rubin, G. M., et al. (2015). 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., 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

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

Somers, J., Luong, H. N. B., Batterham, P., and Perry, T. (2018). Deletion of the nicotinic acetylcholine receptor subunit gene Dα1 confers insecticide resistance, but at what cost? *Fly (Austin)* 12, 46–54. doi: 10.1080/19336934.2017.1396399

Spodenkiewicz, M., Diez-Fernandez, C., Rüfenacht, V., Gemperle-Britschgi, C., and Häberle, J. (2016). Minireview on glutamine synthetase deficiency, an ultra-rare inborn error of amino acid biosynthesis. *Biology* 5:40. doi: 10.3390/biology5040040

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

Stopfer, M., Bhagavan, S., Smith, B. H., and Laurent, G. (1997). Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature* 390, 70–74. doi: 10.1038/36335

Strausfeld, N. J., and Hirth, F. (2013). Deep homology of arthropod central complex and vertebrate basal ganglia. *Science* 340, 157–161. doi: 10.1126/science.1231828

Su, H., and O'Dowd, D. K. (2003). Fast synaptic currents in drosophila mushroom body Kenyon cells are mediated by alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors and picrotoxin-sensitive GABA receptors. *J. Neurosci.* 23, 9246–9253. doi: 10.1523/JNEUROSCI.23-27-09246.2003

Substance Abuse and Mental Health Services Administration. (2018). *Key Substance Use and Mental Health Indicators in the United States: Results From the 2017 National Survey on Drug Use and Health*. Rockville, MD: Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration.

Substance Abuse and Mental Health Services Administration. (2019). *Key Substance Use and Mental Health Indicators in the United States: Results From the 2018 National Survey on Drug Use and Health*. Rockville, MD: Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration.

Sweeny, S. T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C. J. (1995). Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* 14, 341–351. doi: 10.1016/0896-6273(95)90290-2

Talay, M., Richman, E. B., Snell, N. J., Hartmann, G. G., Fisher, J. D., Sorkaç, A., et al. (2017). Transsynaptic mapping of second-order taste neurons in flies by trans-tango. *Neuron 96*, 783–795.e4. doi: 10.1016/j.neuron.2017.10.011

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., et al. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* 62, 405–496. doi: 10.1124/pr.109.002451

Tully, T., and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol.* 157, 263–277. doi: 10.1007/BF01350033

Vaaga, C. E., Borisovska, M., and Westbrook, G. L. (2014). Dual-transmitter neurons: functional implications of co-release and co-transmission. *Curr. Opin. Neurobiol.* 29, 25–32. doi: 10.1016/j.conb.2014.04.010

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

Velazquez-Ulloa, N. A. (2017). A *Drosophila* model for developmental nicotine exposure. *PLoS ONE* 12:e177710. doi: 10.1371/journal.pone.0177710

Verhulst, B., Neale, M. C., and Kendler, K. S. (2015). The heritability of alcohol use disorders: a meta-analysis of twin and adoption studies. *Psychol. Med.* 45, 1061–1072. doi: 10.1017/S0033291714002165

Vernizzi, L., Paiardi, C., Licata, G., Vitali, T., Santarelli, S., Raneli, M., et al. (2019). Glutamine synthetase-1 induces autophagy-lysosomal degradation of huntingtin aggregates and ameliorates animal motility in a drosophila model for huntington's disease. *bioRxiv [Preprint]*. doi: 10.1101/618629

Weber, K. E. (1988). An apparatus for measurement of resistance to gas-phase agents. *Drosoph. Inf. Serv.* 67, 91–93.

Wen, T., Parrish, C. A., Xu, D., Wu, Q., and Shen, P. (2005). Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2141–2146. doi: 10.1073/pnas.0406814102

Wolf, F. W., Rodan, A. R., Tsai, L. T.-Y., and Heberlein, U. (2002). High-resolution analysis of ethanol-induced locomotor stimulation in *Drosophila*. *J. Neurosci.* 22, 11035–11044. doi: 10.1523/JNEUROSCI.22-24-11035.2002

Wolff, T., and Rubin, G. M. (2018). Neuroarchitecture of the *Drosophila* central complex: a catalog of nodulus and asymmetrical body neurons and a

revision of the protocerebral bridge catalog. *J. Comp. Neurol.* 526, 2585–2611. doi: 10.1002/cne.24512

Woodward, J. J., and Gonzales, R. A. (1990). Ethanol inhibition of N-methyl-D-aspartate-stimulated endogenous dopamine release from rat striatal slices: reversal by glycine. *J. Neurochem.* 54, 712–715. doi: 10.1111/j.1471-4159.1990.tb01931.x

Xia, S., Miyashita, T., Fu, T.-F., Lin, W.-Y., Wu, C.-L., Pyzocha, L., et al. (2005). NMDA receptors mediate olfactory learning and memory in *Drosophila*. *Curr. Biol.* 15, 603–615. doi: 10.1016/j.cub.2005.02.059

Xie, T., Ho, M. C. W., Liu, Q., Horiuchi, W., Lin, C.-C., Task, D., et al. (2018). A genetic toolkit for dissecting dopamine circuit function in *Drosophila*. *Cell. Rep.* 23, 652–665. doi: 10.1016/j.celrep.2018.03.068

Xu, L., He, J., Kaiser, A., Gräber, N., Schläger, L., Ritze, Y., et al. (2016). A single pair of serotonergic neurons counteracts serotonergic inhibition of ethanol attraction in *Drosophila*. *PLoS ONE* 11:e167518. doi: 10.1371/journal.pone.0167518

Xu, S., Chan, T., Shah, V., Zhang, S., Pletcher, S. D., and Roman, G. (2012). The propensity for consuming ethanol in *Drosophila* requires rutabaga adenylyl cyclase expression within mushroom body neurons. *Genes Brain Behav.* 11, 727–739. doi: 10.1111/j.1601-183X.2012.00810.x

Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A. B., Sima, R. J., 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

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

Yim, H. J., Schallert, T., Randall, P. K., and Gonzales, R. A. (1998). Comparison of local and systemic ethanol effects on extracellular dopamine concentration in rat nucleus accumbens by microdialysis. *Alcohol. Clin. Exp. Res.* 22, 367–374. doi: 10.1111/j.1530-0277.1998.tb03662.x

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

Zhang, K., Guo, J. Z., Peng, Y., Xi, W., and Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. *Science* 316, 1901–1904. doi: 10.1126/science.1137357

Zhang, S. X., Rogulja, D., and Crickmore, M. A. (2019). Recurrent circuitry sustains *Drosophila* courtship drive while priming itself for satiety. *Curr. Biol.* 29, 3216–3228.e9. doi: 10.1016/j.cub.2019.08.015

Zheng, Z., Lauritzen, J. S., Perlman, E., Robinson, C. G., Nichols, M., Milkie, D., et al. (2018). A complete electron microscopy volume of the brain of adult *Drosophila melanogaster*. *Cell 174*, 730–743.e22. doi: 10.1016/j.cell.2018.06.019

Zhou, C., Huang, H., Kim, S. M., Lin, H., Meng, X., Han, K.-A., et al. (2012). Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in *Drosophila* courtship conditioning. *J. Neurosci.* 32, 14281–14287. doi: 10.1523/JNEUROSCI.0517-12.2012

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

Zimmerman, J. E., Chan, M. T., Lenz, O. T., Keenan, B. T., Maislin, G., and Pack, A. I. (2017). Glutamate is a wake-active neurotransmitter in *Drosophila melanogaster*. *Sleep* 40:zsw046. doi: 10.1093/sleep/zsw046

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 12 January 2021
doi: 10.3389/fnbeh.2020.601449

Studying the Contribution of Serotonin to Neurodevelopmental Disorders. Can This Fly?

*Angel Carvajal-Oliveros[1] and Jorge M. Campusano[2,3]**

[1] Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico, [2] Laboratorio Neurogenética de la Conducta, Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [3] Centro Interdisciplinario de Neurociencia UC, Pontificia Universidad Católica de Chile, Santiago, Chile

Serotonin is a biogenic amine that acts as neurotransmitter in different brain regions and is involved in complex behaviors, such as aggression or mood regulation. Thus, this amine is found in defined circuits and activates specific receptors in different target regions. Serotonin actions depend on extracellular levels of this amine, which are regulated by its synthetic enzymes and the plasma membrane transporter, SERT. Serotonin acts also as a neurotrophic signal in ontogeny and in the mature brain, controlling cell proliferation, differentiation, neurogenesis, and neural plasticity. Interestingly, early alterations in serotonergic signaling have been linked to a diversity of neurodevelopmental disorders, including autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), or mental illnesses like schizophrenia or depression. It has been proposed that given the complex and numerous actions of serotonin, animal models could better serve to study the complexity of serotonin actions, while providing insights on how hindering serotonergic signaling could contribute to brain disorders. In this mini-review, it will be examined what the general properties of serotonin acting as a neurotransmitter in animals are, and furthermore, whether it is possible that *Drosophila* could be used to study the contribution of this amine to neurodevelopmental and mental disorders.

Keywords: neurodevelopmental disorders, serotonin, SERT, *Drosophila*, ASD

OPEN ACCESS

Edited by:
Clare C. Rittschof, University of Kentucky, United States

Reviewed by:
Therese A. Kosten, University of Houston, United States
Jorge A. Bergado, Universidad del Sinú, Colombia

****Correspondence:***
Jorge M. Campusano
jcampusano@bio.puc.cl

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *01 September 2020*
Accepted: *14 December 2020*
Published: *12 January 2021*

Citation:
Carvajal-Oliveros A and Campusano JM (2021) Studying the Contribution of Serotonin to Neurodevelopmental Disorders. Can This Fly? Front. Behav. Neurosci. 14:601449. doi: 10.3389/fnbeh.2020.601449

SEROTONIN IS A BIOGENIC AMINE ACTING NOT ONLY AS A CLASSICAL NEUROTRANSMITTER, BUT ALSO AS A NEUROTROPHIC FACTOR

Biogenic amines (BAs) are a group of neuroactive molecules that contain one or more amino group, are synthesized from amino acids, and act as classical neurotransmitters, neuromodulators or neurohormones. Among them, serotonin is a BA associated with a number of physiological processes and the control of several behaviors, including sleep regulation, social rank, mood and learning. The alteration of serotonergic neural systems is associated with some neurodevelopmental and mental disorders, including anxiety, depression and ASD. However, we are far from fully understanding the complex cellular and molecular actions of amines and how the alteration of neural systems that store and release them contributes to these disorders.

As with other classical neurotransmitters, serotonin is synthetized in the cytosol in a two-step biosynthetic pathway (**Figure 1**). The enzyme tryptophan hydroxylase (TPH, aka TRH or TrpH) catalyzes the conversion of Tryptophan to 5-hydroxytryptophan. Then, the Dopa decarboxylase enzyme (DDC, aka AADC) catalyzes the final conversion to serotonin (5-hydroxytryptamine). Serotonin is stored in vesicle compartments located mainly in axon terminals (the presynaptic neuron). Upon the arrival of an action potential, these vesicles fuse with the plasma membrane to release their content in the extracellular space (the synaptic cleft), so that the neurotransmitters can reach specific receptors in the postsynaptic neuron to induce cellular responses. These receptors mostly belong to the superfamily of G protein-coupled metabotropic receptors. Finally, the end of the action of the neurotransmitter depends on the reuptake of the chemical from the synapse back into the presynaptic neuron, via SERT. Once inside the cytosol, the neurotransmitter can be reutilized as a neurotransmitter (is transported back into a synaptic vesicle by the vesicle monoamine transporter, VMAT), or it can be metabolized by the MAO enzyme (Daubert and Condron, 2010). The two biosynthetic enzymes and SERT help define the neurochemical identity of a neuron as serotonergic (**Figure 1**).

Interestingly, serotonin also plays a critical role as a signaling/trophic molecule early in development (Gaspar et al., 2003; Daubert and Condron, 2010; Bonnin and Levitt, 2011). Actually, when no cells from the developing organism are able to synthetize or release serotonin, some placental cells transiently acquire the ability to synthetize and release serotonin, which can then reach the embryo (Bonnin and Levitt, 2011). Earlier, the first source for serotonergic information for the rodent embryo is the maternal blood (Koren et al., 1966). In addition, neurons that in the rodent mature brain are not serotonergic (e.g., thalamic neurons), transiently express VMAT and SERT early in development (by E13) in order to accumulate and release serotonin at later times (Lebrand et al., 1996; Cases et al., 1998). All these findings support the idea that this amine is required early in development and that non-embryonic sources of this BA are crucial to fulfill this need. Later in development, the embryonic neurons of the Raphe Nucleus, the main serotonergic nucleus in vertebrates, acquire the ability to synthetize and release serotonin, and the fetus becomes independent of the exogenous supply of the amine. It is possible to find the first serotonergic neurons in 5-week old human embryos, earlier than other aminergic populations (Sundstrom et al., 1993). A similar situation—serotonin neurons established a little earlier than dopaminergic cells—is observed in rodents. Thus, it is possible to propose a dynamic change in serotonin levels in the central nervous system (CNS) over development (**Figure 2**; Suri et al., 2015). Importantly, it has been postulated that interruption or alteration of the serotonergic information reaching the embryo or fetus, is implicated in a higher incidence for several brain disorders including ASD, major depressive disorder (MDD), ADHD, anxiety, and schizophrenia, among others (Schain and Freedman, 1961; Bleich et al., 1988; Caspi et al., 2003; Hranilovic et al., 2007; Olivier et al., 2011). This mini review intends to discuss the contribution of serotonin to the onset and progression of behavioral traits common to several neuropsychiatric diseases. However, particular attention is placed on ASD, given that several evidences support that this amine plays a role in this particular disorder.

ALTERATION IN SEROTONIN LEVELS ASSOCIATED WITH ASD AND OTHER BRAIN DISORDERS

It has been reported that about 30% of children diagnosed with ASD show higher levels of serotonin in the blood as compared to control children (Gabriele et al., 2014). The increased serotonemia seems to be unrelated to any specific genetic alteration linked to this disorder. Actually, only about 15–20% of ASD cases are genetic; among the alterations associated with ASD, it has been reported mutations in specific genes (e.g., Shank or Neuroliguin 3), gene copy number variation, and chromosomal disorders (Miles, 2011). Increase in blood serotonin concentration is also detected in schizophrenic patients, while hyposerotonemia has been described in people diagnosed with depression (Muck-Seler et al., 2004). Most of these disorders are classified as neurodevelopmental disorders, in that genetic or genomic alterations underlying these conditions are present as early as the embryo develops and/or over critical time windows in the development of brain neural circuits (Suri et al., 2015).

Similarly, it has been proposed that environmental factors affecting serotonergic signaling (e.g., exposure to drugs affecting the serotonergic system) could play a role in neurodevelopmental disorders. It has been shown in rodent models that these chemicals could be particularly effective at hindering serotonergic signaling early over development, since the blood–brain barrier (BBB) becomes fully functional only by E15 (Ben-Zvi et al., 2014). Therefore, at earlier time points there is no obstacle that stops these chemicals from acting at targets such as serotonin receptors or SERT, affecting serotonergic signaling both in the periphery and centrally. SERT plays a crucial role as the major regulator of serotonin homeostasis and over recent years this transporter has been the focus of several studies (reviewed in Muller et al., 2016). Importantly, several environmental stimuli modifying SERT operation and some mutations affecting the SERT gene have been associated to brain disorders, including ASD.

GENE AND ENVIRONMENTAL ALTERATIONS AFFECTING SERT, LINKED TO BRAIN DISORDERS

Mutations in SERT Linked to Neurodevelopmental and Mental Disorders

Several modifications in the gene coding for SERT have been associated to different brain disorders, including obsessive-compulsive disorder and Asperger syndrome/ASD (Kilic et al., 2003; Prasad et al., 2009). Some are located in the untranslated

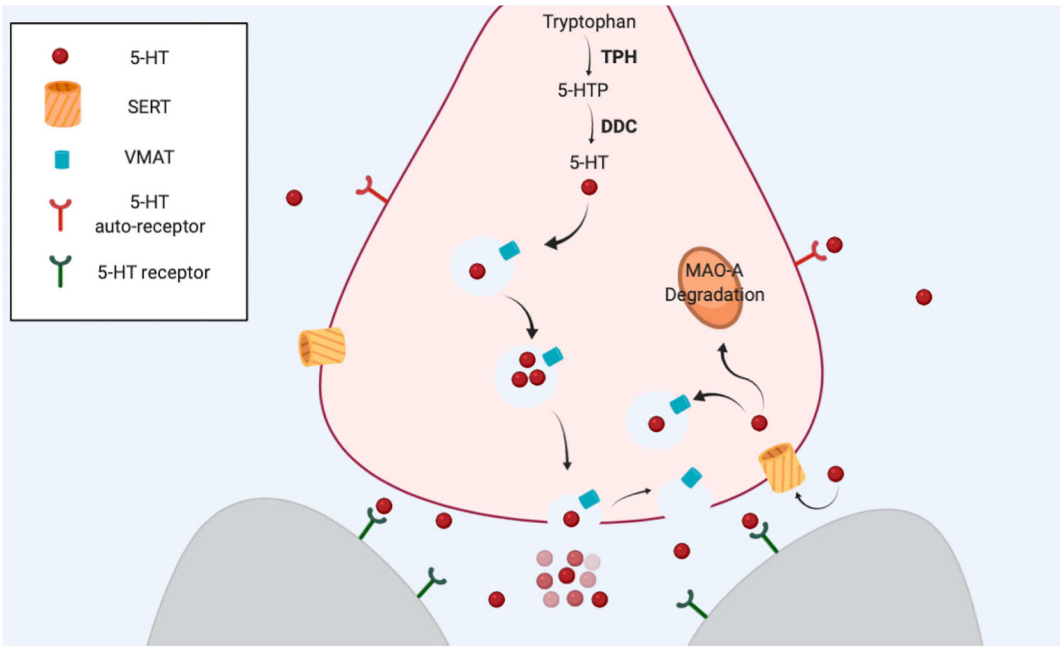

FIGURE 1 | The serotonergic synapse. Serotonin is synthetized from amino acid tryptophan. The first step is the hydroxylation of tryptophan carried out by TPH, the limiting enzyme in this process. DDC catalyzes the final step. VMAT transport serotonin from the cytosol into the vesicles. After an action potential reaches the terminal, serotonin is released from vesicles, and the neurotransmitter is able to bind and activate specific receptors in the same neuron (presynaptic) or in other neurons (postsynaptic). SERT reuptakes serotonin back to the cytosol in the presynaptic neuron. The amine can be used again as a neurotransmitter or alternatively can be degraded by MAO-A.

regions (UTR) of SERT gene, particularly the promoter, resulting in decreased transported expression. Reduced SERT expression could underlie the increased extracellular serotonin levels found in ASD and other disorders. Interestingly, some of the SERT mutations described (e.g., Ile425Leu, Phe465Leu, and Leu550Val) are gain-of-function mutations that increase the activity of the transporter, but hinder SERT insertion in the plasma membrane. Thus, reduced localization of SERT in the membrane would result in reduced serotonin uptake and consequently hyperserotonemia (Prasad et al., 2009), as it is reported in ASD (Gabriele et al., 2014).

Environmental Manipulations Affecting SERT and Linked to Brain Disorders: Clinical Studies

It is estimated that about 10% of pregnant women are prescribed antidepressant drugs for treatment of clinical depression and anxiety (Cooper et al., 2007; Huybrechts et al., 2013). A big proportion of antidepressant drugs are Serotonin Selective Reuptake Inhibitors (SSRIs), whose molecular target is SERT, and include fluoxetine and citalopram (Patil et al., 2011). These chemicals are able to cross cell membranes and tissues like the placenta and the BBB (Heikkinen et al., 2002), which also explains why that they can be found in the breast milk (Hendrick et al., 2001). The focus of this mini-review is on discussing how manipulations affecting SERT result in brain-associated deficits, but several reports have described that prenatal exposure to SSRIs is associated to increased risk for preterm delivery (Davis et al., 2007; Maschi et al., 2008; Reis and Kallen, 2010; Huang et al., 2014), low birth weight (Oberlander et al., 2006; Reis and Kallen, 2010; Huang et al., 2014), and cardiac defects (Kallen, 2007; Pedersen et al., 2009; Kornum et al., 2010). This is consistent with the idea that serotonin plays key roles in the development of the entire organism.

A number of reports have argued that prenatal exposure of SSRIs is associated to higher incidence of ASD (e.g., Boukhris et al., 2016). Similar studies have shown a positive correlation between prenatal SSRI exposure and ADHD (Figueroa, 2010; Clements et al., 2015). Only one study (Malm et al., 2016) has assessed depression and prenatal exposure to SSRIs, and reported a positive correlation, as well. On the other hand, a different set of studies has argued that no association between prenatal exposure to SSRIs and ASD or ADHD exists (e.g., Castro et al., 2016). Interestingly, this and other studies (Mezzacappa et al., 2017) proposed that a relevant factor explaining the higher incidence for ASD is the medical condition of the mother—i.e., maternal depression. However, Croen et al. (2011) determined no increase in the risk of ASD in the offspring of mothers with a history of depression, an effect that did not depend on prenatal consumption of SSRI. All these (and other) studies (Kaplan et al., 2017; Maloney et al., 2018; Halvorsen et al., 2019) show that it is not clear whether the use of SSRIs results in increased incidence for ASD, ADHD or any other disorder. One additional problem with these studies is the difficulty to access and study larger populations (as discussed in Millard et al., 2017). In spite of these

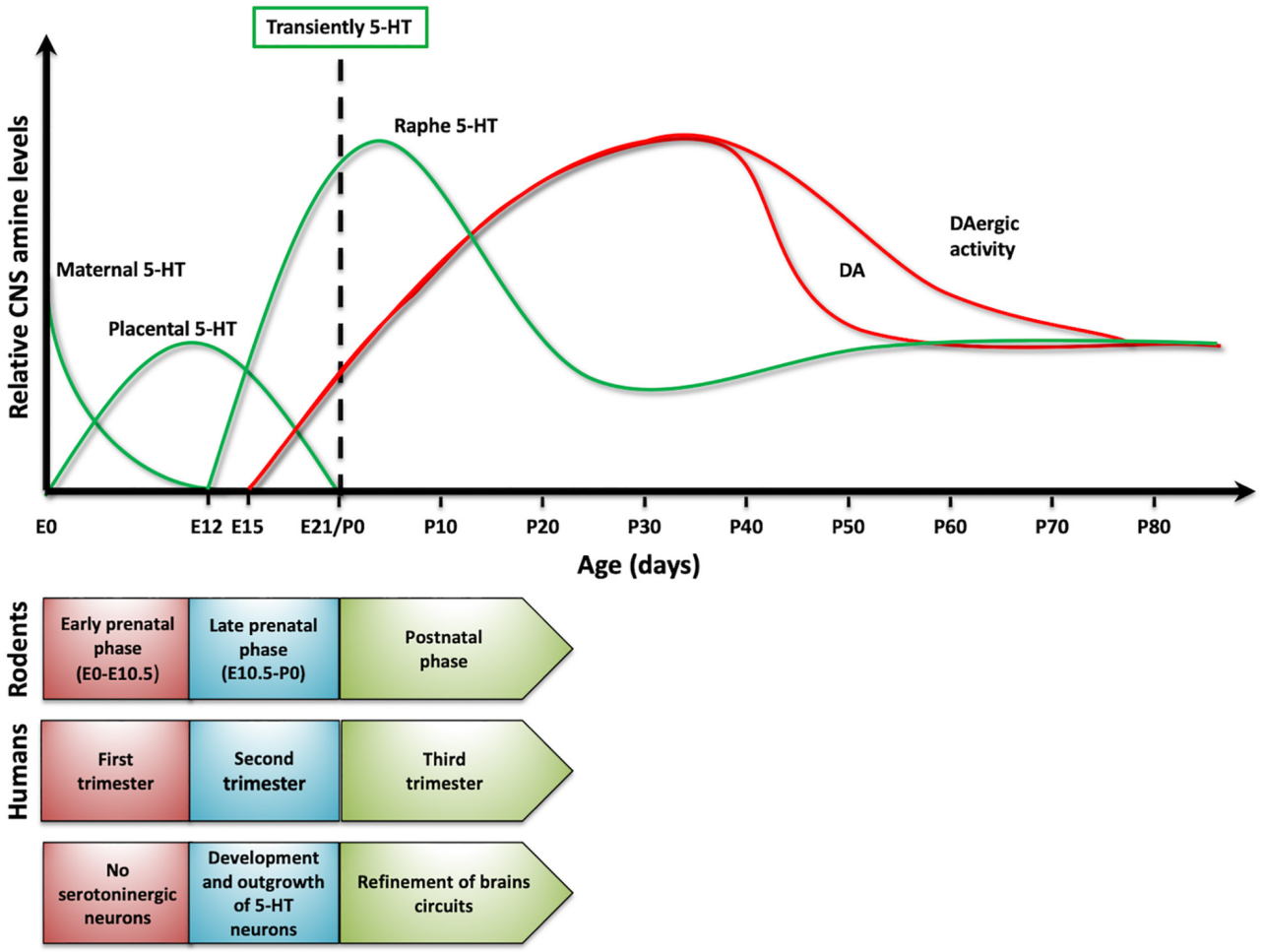

FIGURE 2 | Serotonin dynamics on development. The **upper panel** presents changes in serotonin levels (green traces) in rodents and its sources. As a comparison, it presented dopamine levels (red traces). In **lower panel**, it compared developmental stages in rodents and humans, as serotonin levels change over development.

considerations, it is possible to argue that SSRIs could reach the embryo and neonates and possibly affect the developing central nervous system in a way not fully understood but that could lead to brain disorders. In reviewing the available information on this issue, authors have come to the conclusion that relevant information to further support or discard the contribution of SSRIs to these disorders could be obtained from animal models (Pedersen, 2015).

Environmental Manipulations Affecting SERT and Linked to Brain Disorders: Animal Models

When modeling human disorders in animals, researchers have focused on replicating one or few specific behavioral features, although it has to be considered that some features are difficult to recreate in animal models (e.g., hallucinations or psychosis) (Anderson and Adolphs, 2014). Thus, for instance, ASD animal models usually recreate repetitive behaviors or the impaired social interaction observed in this disorder (Yenkoyan et al., 2017). An additional issue to be considered when modeling these disorders is that it is estimated that postnatal day 7 in rodents is equivalent to time of birth in humans (**Figure 2**). This means that a postnatal manipulation in rodents could be equivalent to a prenatal one in humans (Clancy et al., 2007). In spite of these caveats, the key impact of animal models is on advancing our understanding of these disorders at the cellular, molecular, neurochemical and/or circuit levels.

In particular, very few studies have directly assessed the possibility that antenatal or perinatal exposure to SSRIs affects incidence of ASD-like features in animals. For instance, Sprowles et al. (2016) studied the effects of perinatal exposure to citalopram in rats. Results obtained demonstrate several autistic-like behavioral traits in the offspring (repetitive behaviors, impaired social behavior), which are consistent with the concurrent description of anatomical alterations in Raphe and in cortical structure and physiology (Darling et al., 2011; Simpson et al., 2011). These findings argue in favor of the idea that prenatal SSRI exposure increases the incidence of ASD. A different study reported that perinatal inhibition of MAO, which could

be considered a manipulation that like SSRIs increases the availability of serotonin, results in alteration of serotonin metabolism and hyperserotonemia (Hranilovic et al., 2011), and an increased incidence of ASD features (Davis et al., 2008). There is not much information on how perinatal exposure to SSRIs affects incidence for other disorders, and the little data available is contradictory. For instance, while one report supports the idea that perinatal exposure to SSRIs exacerbates depressive-like behaviors in a strain of rats prone to anxiety and depression (Glover et al., 2015), other works propose that perinatal SSRIs partially reverses some of these behavioral traits (Rayen et al., 2011; Boulle et al., 2016), while another study shows no effect of the treatment (Zohar et al., 2016). Unfortunately, these studies have not been consistent with the SSRI used, doses of these chemicals, the developmental age at which interventions begin, or the gender of the animals studied, among other factors. Thus, it remains an open question whether exposure to SSRIs over ontogeny contributes to brain disorders.

On the other hand, increased serotonin levels observed in SERT knockout mice have been associated with abnormal development of thalamocortical axons and somatosensory cortical barrels (Persico et al., 2001; Gaspar et al., 2003), and also anxiety and depressive-like behaviors (Ansorge et al., 2004).

DROSOPHILA AS ANIMAL MODEL FOR THE STUDY OF NEURODEVELOPMENTAL AND MENTAL DISORDERS

The vinegar fly *Drosophila melanogaster* has served as a workhorse in various fields in biology, in part based on the diverse genetic toolbox available, as discussed elsewhere in this special issue. Remarkably, *Drosophila* exhibits a complex behavioral repertoire. For instance, one of the best-studied social behaviors in flies is courtship behavior: male flies court a female animal in order to mate, a behavior that is decreased after males have experienced rejection by a fertilized female (Siegel and Hall, 1979; Kamyshev et al., 1999). New social paradigms have been described including the study of clustering behavior observed in groups of flies (Simon et al., 2012). Recent studies have also shown that flies may exhibit attention-like processes (van Swinderen and Flores, 2007), goal-driven behavioral adaptations (Pick and Strauss, 2005) and decision making (Zhang et al., 2007). *Drosophila* has been also been used as a model organism to study aggression (Baier et al., 2002) and addiction (Wolf, 1999).

In recent years, it has become evident that it is possible to model neurodevelopmental disorders in *Drosophila*, aiming at reproducing some of the key behavioral traits associated with these illnesses. One of the best-studied models for neurodevelopmental disorders in *Drosophila* is the ASD model based on mutations in the FMR1 gene (van Alphen and van Swinderen, 2013). This is a gene associated with fragile X syndrome, a disorder linked to intellectual disability and where a high incidence for ASD is reported. *Drosophila FMR1* mutants exhibit reduced memory in a courtship social paradigm (McBride et al., 2005) and repetitive grooming, which is reminiscent of recurring behaviors observed in ASD (Tauber et al., 2011). In addition, brain and circuit organization is affected in fly *FMR1* mutants (Siller and Broadie, 2011), consistent with structural changes in axonal and dendritic branches, a feature shared by mice FMR1 mutants (Zhang et al., 2001; Zhang and Broadie, 2005; Callan and Zarnescu, 2011; van Alphen and van Swinderen, 2013).

Drosophila as an Animal Model for Studying the Contribution of Serotonin to Neurodevelopmental and Mental Disorders?

The molecular mechanisms involved in serotonin biosynthesis are evolutionary conserved, and in *Drosophila,* they begin with the hydroxylation of the tryptophan amino acid by TPH (Coleman and Neckameyer, 2005). Likewise, it has been described a *Drosophila* SERT (Giang et al., 2011; Hidalgo et al., 2017) and one VMAT (Greer et al., 2005) that share structural and functional similarities to that of vertebrates. Five serotonin receptors have been described in the *Drosophila* genome, all of them classified as metabotropic. Thus, the *Drosophila* serotonergic system is highly conserved as compared to its mammalian counterpart (Kasture et al., 2018). Importantly, it is already known that serotonin contributes to several behaviors in *Drosophila* including locomotion, feeding behavior, circadian activity, sleep regulation, and aggression (Silva et al., 2014; Kasture et al., 2018; Bacque-Cazenave et al., 2020).

Out of the approximately 100,000 neurons in the fly brain, about 80 cells are identified as serotonergic neurons, organized in 11 clusters (reviewed in Kasture et al., 2018)). Valles and White (1988), by using immunochemistry, described the serotonergic neural system in the larval and adult CNS and also described how serotonin levels change over development. The first detection of serotonin-positive cells is in 16–20 h *Drosophila* embryos. The detection of immunopositive serotonin cells before fly CNS is fully developed and supports the idea that serotonin could play a role as developmental signaling molecule, as in vertebrates (Lundell and Hirsh, 1994). In this regard, it has been suggested that serotonin modulates the development of serotonergic varicosities in the fly CNS (Sykes and Condron, 2005). Consistent with this, mutants in DOPA decarboxylase which are associated with reduced amine levels exhibit alterations in branch spacing (Budnik et al., 1989). Conversely, overexpression of TPH in *Drosophila* promotes higher levels of cytoplasmic serotonin, which is related with abnormalities in neurite morphology in larval and adult fly neuropils (Daubert et al., 2010). Moreover, altering serotonin synthesis in early embryos results in impaired anatomy and functioning of the feeding circuit in larvae, a phenotype that can be reversed as serotonin levels are rescued (Neckameyer, 2010). All these findings suggest that, as in mammals, hindering serotonergic signaling at early developmental stages does have implications for the establishment of mature circuits that underlie behaviors. However, the information on this issue is limited. New research should ask whether pharmacological or

genetic manipulations tampering with serotonergic components (SERT, biosynthetic enzymes or any of the receptors), affect the organization of brain circuits and consequently, result in behavioral features associated with neurodevelopmental or mental disorders.

Although the literature has not thoroughly explored this, there are some reports supporting this idea. For instance, centrophobism, a behavior in which flies avoid the center of an arena, is considered to be an anxiety-like behavior in flies. Different genetic or pharmacological manipulations that affect SERT functioning affect centrophobism in *Drosophila* (Mohammad et al., 2016; Hidalgo et al., 2017). In particular, we showed that feeding flies an amphetamine derivative that stimulates serotonin release decreases centrophobism, a similar result observed in animals mutant for SERT (Hidalgo et al., 2017). In addition, a recent work from our group showed deficits in social behavior and locomotion in a *Drosophila* mutant for the dysbindin gene, an animal model for schizophrenia. Interestingly, the phenotypes observed in the dysbindin mutants seem to depend at least in part on altered serotonergic signaling (Hidalgo et al., 2020).

Demonstrating that serotonin signaling is affected in fly models for brain disorders, is not only relevant for advancing our understanding of the underpinnings of these illnesses, it also opens up the possibility to carry out a high-throughput search for new chemicals that affect specific phenotypes in flies, which could eventually lead to new therapeutic tools for these disorders (Nichols, 2006; Roy et al., 2020).

A better understanding of serotonin dynamics over development and how serotonergic deficiency could be involved in mental disorders could provide insights in the search for new treatments for these disorders, a path in which *Drosophila* could play an important role.

AUTHOR CONTRIBUTIONS

AC-O and JC wrote this manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

AC-O received a doctoral fellowship (446128) from CONACyT (Mexico) and Alianza del Pacífico-AGCID.

ACKNOWLEDGMENTS

We thank the Campusano Lab for comments on the idea of this mini review.

REFERENCES

Anderson, D. J., and Adolphs, R. (2014). A framework for studying emotions across species. *Cell* 157, 187–200. doi: 10.1016/j.cell.2014.03.003

Ansorge, M. S., Zhou, M., Lira, A., Hen, R., and Gingrich, J. A. (2004). Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* 306, 879–881. doi: 10.1126/science.1101678

Bacque-Cazenave, J., Bharatiya, R., Barriere, G., Delbecque, J. P., Bouguiyoud, N., Di Giovanni, G., et al. (2020). Serotonin in animal cognition and behavior. *Int. J. Mol. Sci.* 21, 1649.

Baier, A., Wittek, B., and Brembs, B. (2002). *Drosophila* as a new model organism for the neurobiology of aggression? *J. Exp. Biol.* 205(Pt 9), 1233–1240.

Ben-Zvi, A., Lacoste, B., Kur, E., Andreone, B. J., Mayshar, Y., Yan, H., et al. (2014). Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature* 509, 507–511. doi: 10.1038/nature13324

Bleich, A., Brown, S. L., Kahn, R., and van Praag, H. M. (1988). The role of serotonin in schizophrenia. *Schizophr. Bull.* 14, 297–315. doi: 10.1093/schbul/14.2.297

Bonnin, A., and Levitt, P. (2011). Fetal, maternal, and placental sources of serotonin and new implications for developmental programming of the brain. *Neuroscience* 197, 1–7. doi: 10.1016/j.neuroscience.2011.10.005

Boukhris, T., Sheehy, O., Mottron, L., and Berard, A. (2016). Antidepressant use during pregnancy and the risk of autism spectrum disorder in children. *JAMA Pediatr.* 170, 117–124. doi: 10.1001/jamapediatrics.2015.3356

Boulle, F., Pawluski, J. L., Homberg, J. R., Machiels, B., Kroeze, Y., Kumar, N., et al. (2016). Prenatal stress and early-life exposure to fluoxetine have enduring effects on anxiety and hippocampal BDNF gene expression in adult male offspring. *Dev. Psychobiol.* 58, 427–438. doi: 10.1002/dev.21385

Budnik, V., Wu, C. F., and White, K. (1989). Altered branching of serotonin-containing neurons in *Drosophila* mutants unable to synthesize serotonin and dopamine. *J. Neurosci.* 9, 2866–2877. doi: 10.1523/jneurosci.09-08-02866.1989

Callan, M. A., and Zarnescu, D. C. (2011). Heads-up: new roles for the fragile X mental retardation protein in neural stem and progenitor cells. *Genesis* 49, 424–440. doi: 10.1002/dvg.20745

Cases, O., Lebrand, C., Giros, B., Vitalis, T., De Maeyer, E., Caron, M. G., et al. (1998). Plasma membrane transporters of serotonin, dopamine, and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. *J. Neurosci.* 18, 6914–6927. doi: 10.1523/jneurosci.18-17-06914.1998

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., et al. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301, 386–389. doi: 10.1126/science.1083968

Castro, V. M., Kong, S. W., Clements, C. C., Brady, R., Kaimal, A. J., Doyle, A. E., et al. (2016). Absence of evidence for increase in risk for autism or attention-deficit hyperactivity disorder following antidepressant exposure during pregnancy: a replication study. *Transl. Psychiatry* 6:e708. doi: 10.1038/tp.2015.190

Clancy, B., Finlay, B. L., Darlington, R. B., and Anand, K. J. (2007). Extrapolating brain development from experimental species to humans. *Neurotoxicology* 28, 931–937. doi: 10.1016/j.neuro.2007.01.014

Clements, C. C., Castro, V. M., Blumenthal, S. R., Rosenfield, H. R., Murphy, S. N., Fava, M., et al. (2015). Prenatal antidepressant exposure is associated with risk for attention-deficit hyperactivity disorder but not autism spectrum disorder in a large health system. *Mol. Psychiatry* 20, 727–734. doi: 10.1038/mp.2014.90

Coleman, C. M., and Neckameyer, W. S. (2005). Serotonin synthesis by two distinct enzymes in *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* 59, 12–31. doi: 10.1002/arch.20050

Cooper, W. O., Willy, M. E., Pont, S. J., and Ray, W. A. (2007). Increasing use of antidepressants in pregnancy. *Am. J. Obstet. Gynecol.* 196:544 e1–5.

Croen, L. A., Grether, J. K., Yoshida, C. K., Odouli, R., and Hendrick, V. (2011). Antidepressant use during pregnancy and childhood autism spectrum disorders. *Arch. Gen. Psychiatry* 68, 1104–1112. doi: 10.1001/archgenpsychiatry.2011.73

Darling, R. D., Alzghoul, L., Zhang, J., Khatri, N., Paul, I. A., Simpson, K. L., et al. (2011). Perinatal citalopram exposure selectively increases locus ceruleus circuit function in male rats. *J. Neurosci.* 31, 16709–16715. doi: 10.1523/jneurosci.3736-11.2011

Daubert, E. A., and Condron, B. G. (2010). Serotonin: a regulator of neuronal morphology and circuitry. *Trends Neurosci.* 33, 424–434. doi: 10.1016/j.tins.2010.05.005

Daubert, E. A., Heffron, D. S., Mandell, J. W., and Condron, B. G. (2010). Serotonergic dystrophy induced by excess serotonin. *Mol. Cell. Neurosci.* 44, 297–306. doi: 10.1016/j.mcn.2010.04.001

Davis, L. K., Hazlett, H. C., Librant, A. L., Nopoulos, P., Sheffield, V. C., Piven, J., et al. (2008). Cortical enlargement in autism is associated with a functional VNTR in the monoamine oxidase A gene. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 147B, 1145–1151. doi: 10.1002/ajmg.b.30738

Davis, R. L., Rubanowice, D., McPhillips, H., Raebel, M. A., Andrade, S. E., Smith, D., et al. (2007). Risks of congenital malformations and perinatal events among infants exposed to antidepressant medications during pregnancy. *Pharmacoepidemiol. Drug Saf.* 16, 1086–1094. doi: 10.1002/pds.1462

Figueroa, R. (2010). Use of antidepressants during pregnancy and risk of attention-deficit/hyperactivity disorder in the offspring. *J. Dev. Behav. Pediatr.* 31, 641–648. doi: 10.1097/dbp.0b013e3181e5ac93

Gabriele, S., Sacco, R., and Persico, A. M. (2014). Blood serotonin levels in autism spectrum disorder: a systematic review and meta-analysis. *Eur. Neuropsychopharmacol.* 24, 919–929. doi: 10.1016/j.euroneuro.2014.02.004

Gaspar, P., Cases, O., and Maroteaux, L. (2003). The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci.* 4, 1002–1012. doi: 10.1038/nrn1256

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

Glover, M. E., Pugh, P. C., Jackson, N. L., Cohen, J. L., Fant, A. D., Akil, H., et al. (2015). Early-life exposure to the SSRI paroxetine exacerbates depression-like behavior in anxiety/depression-prone rats. *Neuroscience* 284, 775–797. doi: 10.1016/j.neuroscience.2014.10.044

Greer, C. L., Grygoruk, A., Patton, D. E., Ley, B., Romero-Calderon, R., Chang, H.-Y., et al. (2005). A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. *J. Neurobiol.* 64, 239–258. doi: 10.1002/neu.20146

Halvorsen, A., Hesel, B., Østergaard, S. D., and Danielsen, A. A. (2019). In utero exposure to selective serotonin reuptake inhibitors and development of mental disorders: a systematic review and meta-analysis. *Acta Psychiatr. Scand.* 139, 493–507. doi: 10.1111/acps.13030

Heikkinen, T., Ekblad, U., Kero, P., Ekblad, S., and Laine, K. (2002). Citalopram in pregnancy and lactation. *Clin. Pharmacol. Ther.* 72, 184–191. doi: 10.1067/mcp.2002.126181

Hendrick, V., Stowe, Z. N., Altshuler, L. L., Mintz, J., Hwang, S., Hostetter, A., et al. (2001). Fluoxetine and norfluoxetine concentrations in nursing infants and breast milk. *Biol. Psychiatry* 50, 775–782. doi: 10.1016/s0006-3223(01)01197-0

Hidalgo, S., Castro, C., Zarate, R. V., Valderrama, B. P., Hodge, J. J. L., and Campusano, J. M. (2020). The behavioral and neurochemical characterization of a *Drosophila* dysbindin mutant supports the contribution of serotonin to schizophrenia negative symptoms. *Neurochem. Int.* 138:104753. doi: 10.1016/j.neuint.2020.104753

Hidalgo, S., Molina-Mateo, D., Escobedo, P., Zarate, R. V., Fritz, E., Fierro, A., et al. (2017). Characterization of a novel *Drosophila* SERT mutant: insights on the contribution of the serotonin neural system to behaviors. *ACS Chem. Neurosci.* 8, 2168–2179. doi: 10.1021/acschemneuro.7b00089

Hranilovic, D., Blazevic, S., Ivica, N., Cicin-Sain, L., and Oreskovic, D. (2011). The effects of the perinatal treatment with 5-hydroxytryptophan or tranylcypromine on the peripheral and central serotonin homeostasis in adult rats. *Neurochem. Int.* 59, 202–207. doi: 10.1016/j.neuint.2011.05.003

Hranilovic, D., Bujas-Petkovic, Z., Vragovic, R., Vuk, T., Hock, K., and Jernej, B. (2007). Hyperserotonemia in adults with autistic disorder. *J. Autism Dev. Disord.* 37, 1934–1940. doi: 10.1007/s10803-006-0324-6

Huang, H., Coleman, S., Bridge, J. A., Yonkers, K., and Katon, W. (2014). A meta-analysis of the relationship between antidepressant use in pregnancy and the risk of preterm birth and low birth weight. *Gen. Hosp. Psychiatry* 36, 13–18. doi: 10.1016/j.genhosppsych.2013.08.002

Huybrechts, K. F., Palmsten, K., Mogun, H., Kowal, M., Avorn, J., Setoguchi-Iwata, S., et al. (2013). National trends in antidepressant medication treatment among publicly insured pregnant women. *Gen. Hosp. Psychiatry* 35, 265–271. doi: 10.1016/j.genhosppsych.2012.12.010

Kallen, B. (2007). The safety of antidepressant drugs during pregnancy. *Expert Opin. Drug Saf.* 6, 357–370. doi: 10.1517/14740338.6.4.357

Kamyshev, N. G., Iliadi, K. G., and Bragina, J. V. (1999). *Drosophila* conditioned courtship: two ways of testing memory. *Learn. Mem.* 6, 1–20.

Kaplan, Y. C., Keskin-Arslan, E., Acar, S., and Sozmen, K. (2017). Maternal SSRI discontinuation, use, psychiatric disorder and the risk of autism in children: a meta-analysis of cohort studies. *Br. J. Clin. Pharmacol.* 83, 2798–2806. doi: 10.1111/bcp.13382

Kasture, A. S., Hummel, T., Sucic, S., and Freissmuth, M. (2018). Big lessons from tiny flies: *Drosophila melanogaster* as a model to explore dysfunction of dopaminergic and serotonergic neurotransmitter systems. *Int. J. Mol. Sci.* 19:1788. doi: 10.3390/ijms19061788

Kilic, F., Murphy, D. L., and Rudnick, G. (2003). A human serotonin transporter mutation causes constitutive activation of transport activity. *Mol. Pharmacol.* 64, 440–446. doi: 10.1124/mol.64.2.440

Koren, Z., Pfeifer, Y., and Sulman, F. G. (1966). Distribution and placental transfer of C-14-serotonin in pregnant rats. *Am. J. Obstet. Gynecol.* 95, 290–295. doi: 10.1016/0002-9378(66)90182-7

Kornum, J. B., Nielsen, R. B., Pedersen, L., Mortensen, P. B., and Norgaard, M. (2010). Use of selective serotonin-reuptake inhibitors during early pregnancy and risk of congenital malformations: updated analysis. *Clin. Epidemiol.* 2, 29–36. doi: 10.2147/clep.s9256

Lebrand, C., Cases, O., Adelbrecht, C., Doye, A., Alvarez, C., El Mestikawy, S., et al. (1996). Transient uptake and storage of serotonin in developing thalamic neurons. *Neuron* 17, 823–835. doi: 10.1016/s0896-6273(00)80215-9

Lundell, M. J., and Hirsh, J. (1994). Temporal and spatial development of serotonin and dopamine neurons in the *Drosophila* CNS. *Dev. Biol.* 165, 385–396. doi: 10.1006/dbio.1994.1261

Malm, H., Brown, A. S., Gissler, M., Gyllenberg, D., Hinkka-Yli-Salomaki, S., McKeague, I. W., et al. (2016). Gestational exposure to selective serotonin reuptake inhibitors and offspring psychiatric disorders: a national register-based study. *J. Am. Acad. Child Adolesc. Psychiatry* 55, 359–366. doi: 10.1016/j.jaac.2016.02.013

Maloney, S. E., Akula, S., Rieger, M. A., McCullough, K. B., Chandler, K., Corbett, A. M., et al. (2018). Examining the reversibility of long-term behavioral disruptions in progeny of maternal SSRI exposure. *eNeuro* 5:ENEURO.0120-18.2018.

Maschi, S., Clavenna, A., Campi, R., Schiavetti, B., Bernat, M., and Bonati, M. (2008). Neonatal outcome following pregnancy exposure to antidepressants: a prospective controlled cohort study. *BJOG* 115, 283–289. doi: 10.1111/j.1471-0528.2007.01518.x

McBride, S. M., Choi, C. H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., et al. (2005). Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a *Drosophila* model of fragile X syndrome. *Neuron* 45, 753–764. doi: 10.1016/j.neuron.2005.01.038

Mezzacappa, A., Lasica, P. A., Gianfagna, F., Cazas, O., Hardy, P., Falissard, B., et al. (2017). Risk for autism spectrum disorders according to period of prenatal antidepressant exposure: a systematic review and meta-analysis. *JAMA Pediatr.* 171, 555–563. doi: 10.1001/jamapediatrics.2017.0124

Miles, J. H. (2011). Autism spectrum disorders–a genetics review. *Genet. Med.* 13, 278–294. doi: 10.1097/gim.0b013e3181ff67ba

Millard, S. J., Weston-Green, K., and Newell, K. A. (2017). The effects of maternal antidepressant use on offspring behaviour and brain development: implications for risk of neurodevelopmental disorders. *Neurosci. Biobehav. Rev.* 80, 743–765. doi: 10.1016/j.neubiorev.2017.06.008

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

Muck-Seler, D., Pivac, N., Mustapic, M., Crncevic, Z., Jakovljevic, M., and Sagud, M. (2004). Platelet serotonin and plasma prolactin and cortisol in healthy, depressed and schizophrenic women. *Psychiatry Res.* 127, 217–226. doi: 10.1016/j.psychres.2004.04.001

Muller, C. L., Anacker, A. M., and Veenstra-VanderWeele, J. (2016). The serotonin system in autism spectrum disorder: from biomarker to animal models. *Neuroscience* 321, 24–41. doi: 10.1016/j.neuroscience.2015.11.010

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

Nichols, C. D. (2006). *Drosophila melanogaster* neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. *Pharmacol. Ther.* 112, 677–700. doi: 10.1016/j.pharmthera.2006.05.012

Oberlander, T. F., Warburton, W., Misri, S., Aghajanian, J., and Hertzman, C. (2006). Neonatal outcomes after prenatal exposure to selective serotonin reuptake inhibitor antidepressants and maternal depression using population-based linked health data. *Arch. Gen. Psychiatry* 63, 898–906. doi: 10.1001/archpsyc.63.8.898

Olivier, B., Chan, J. S., Snoeren, E. M., Olivier, J. D., Veening, J. G., Vinkers, C. H., et al. (2011). Differences in sexual behaviour in male and female rodents: role of serotonin. *Curr. Top. Behav. Neurosci.* 8, 15–36. doi: 10.1007/7854_2010_116

Patil, A. S., Kuller, J. A., and Rhee, E. H. (2011). Antidepressants in pregnancy: a review of commonly prescribed medications. *Obstet. Gynecol. Surv.* 66, 777–787. doi: 10.1097/ogx.0b013e31823e0cbf

Pedersen, L. H. (2015). Prenatal antidepressant exposure and childhood autism spectrum disorders: cause for concern? *Paediatr. Drugs* 17, 443–448. doi: 10.1007/s40272-015-0141-5

Pedersen, L. H., Henriksen, T. B., Vestergaard, M., Olsen, J., and Bech, B. H. (2009). Selective serotonin reuptake inhibitors in pregnancy and congenital malformations: population based cohort study. *BMJ* 339:b3569. doi: 10.1136/bmj.b3569

Persico, A. M., Mengual, E., Moessner, R., Hall, F. S., Revay, R. S., Sora, I., et al. (2001). Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. *J. Neurosci.* 21, 6862–6873. doi: 10.1523/jneurosci.21-17-06862.2001

Pick, S., and Strauss, R. (2005). Goal-driven behavioral adaptations in gap-climbing *Drosophila*. *Curr. Biol.* 15, 1473–1478. doi: 10.1016/j.cub.2005.07.022

Prasad, H. C., Steiner, J. A., Sutcliffe, J. S., and Blakely, R. D. (2009). Enhanced activity of human serotonin transporter variants associated with autism. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 163–173. doi: 10.1098/rstb.2008.0143

Rayen, I., van den Hove, D. L., Prickaerts, J., Steinbusch, H. W., and Pawluski, J. L. (2011). Fluoxetine during development reverses the effects of prenatal stress on depressive-like behavior and hippocampal neurogenesis in adolescence. *PLoS One* 6:e24003. doi: 10.1371/journal.pone.0024003

Reis, M., and Kallen, B. (2010). Delivery outcome after maternal use of antidepressant drugs in pregnancy: an update using Swedish data. *Psychol. Med.* 40, 1723–1733. doi: 10.1017/s0033291709992194

Roy, B., Han, J., Hope, K. A., Peters, T. L., Palmer, G., and Reiter, L. T. (2020). An unbiased drug screen for seizure suppressors in duplication 15q syndrome reveals 5-HT1A and dopamine pathway activation as potential therapies. *Biol. Psychiatry* 88, 698–709. doi: 10.1016/j.biopsych.2020.03.021

Schain, R. J., and Freedman, D. X. (1961). Studies on 5-hydroxyindole metabolism in autistic and other mentally retarded children. *J. Pediatr.* 58, 315–320. doi: 10.1016/s0022-3476(61)80261-8

Siegel, R. W., and Hall, J. C. (1979). Conditioned responses in courtship behavior of normal and mutant *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 76, 3430–3434. doi: 10.1073/pnas.76.7.3430

Siller, S. S., and Broadie, K. (2011). Neural circuit architecture defects in a *Drosophila* model of Fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase. *Dis. Model. Mech.* 4, 673–685. doi: 10.1242/dmm.008045

Silva, B., Goles, N. I., Varas, R., and Campusano, J. M. (2014). Serotonin receptors expressed in *Drosophila* mushroom bodies differentially modulate larval locomotion. *PLoS One* 9:e89641. doi: 10.1371/journal.pone.0089641

Simon, A. F., Chou, M. T., Salazar, E. D., Nicholson, T., Saini, N., Metchev, S., et al. (2012). A simple assay to study social behavior in *Drosophila*: measurement of social space within a group. *Genes Brain Behav.* 11, 243–252. doi: 10.1111/j.1601-183x.2011.00740.x

Simpson, K. L., Weaver, K. J., de Villers-Sidani, E., Lu, J. Y., Cai, Z., Pang, Y., et al. (2011). Perinatal antidepressant exposure alters cortical network function in rodents. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18465–18470. doi: 10.1073/pnas.1109353108

Sprowles, J. L., Hufgard, J. R., Gutierrez, A., Bailey, R. A., Jablonski, S. A., Williams, M. T., et al. (2016). Perinatal exposure to the selective serotonin reuptake inhibitor citalopram alters spatial learning and memory, anxiety, depression, and startle in Sprague-Dawley rats. *Int. J. Dev. Neurosci.* 54, 39–52. doi: 10.1016/j.ijdevneu.2016.08.007

Sundstrom, E., Kolare, S., Souverbie, F., Samuelsson, E. B., Pschera, H., Lunell, N. O., et al. (1993). Neurochemical differentiation of human bulbospinal monoaminergic neurons during the first trimester. *Brain Res. Dev. Brain Res.* 75, 1–12. doi: 10.1016/0165-3806(93)90059-j

Suri, D., Teixeira, C. M., Cagliostro, M. K., Mahadevia, D., and Ansorge, M. S. (2015). Monoamine-sensitive developmental periods impacting adult emotional and cognitive behaviors. *Neuropsychopharmacology* 40, 88–112. doi: 10.1038/npp.2014.231

Sykes, P. A., and Condron, B. G. (2005). Development and sensitivity to serotonin of *Drosophila* serotonergic varicosities in the central nervous system. *Dev. Biol.* 286, 207–216. doi: 10.1016/j.ydbio.2005.07.025

Tauber, J. M., Vanlandingham, P. A., and Zhang, B. (2011). Elevated levels of the vesicular monoamine transporter and a novel repetitive behavior in the *Drosophila* model of fragile X syndrome. *PLoS One* 6:e27100. doi: 10.1371/journal.pone.0027100

Valles, 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

van Alphen, B., and van Swinderen, B. (2013). *Drosophila* strategies to study psychiatric disorders. *Brain Res. Bull.* 92, 1–11. doi: 10.1016/j.brainresbull.2011.09.007

van Swinderen, B., and Flores, K. A. (2007). Attention-like processes underlying optomotor performance in a *Drosophila* choice maze. *Dev. Neurobiol.* 67, 129–145. doi: 10.1002/dneu.20334

Wolf, M. E. (1999). Cocaine addiction: clues from *Drosophila* on drugs. *Curr. Biol.* 9, R770–R772.

Yenkoyan, K., Grigoryan, A., Fereshetyan, K., and Yepremyan, D. (2017). Advances in understanding the pathophysiology of autism spectrum disorders. *Behav. Brain Res.* 331, 92–101. doi: 10.1016/j.bbr.2017.04.038

Zhang, K., Guo, J. Z., Peng, Y., Xi, W., and Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. *Science.* 316, 1901–1904. doi: 10.1126/science.1137357

Zhang, Y. Q., Bailey, A. M., Matthies, H. J., Renden, R. B., Smith, M. A., Speese, S. D., et al. (2001). *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell* 107, 591–603. doi: 10.1016/s0092-8674(01)00589-x

Zhang, Y. Q., and Broadie, K. (2005). Fathoming fragile X in fruit flies. *Trends Genet.* 21, 37–45. doi: 10.1016/j.tig.2004.11.003

Zohar, I., Shoham, S., and Weinstock, M. (2016). Perinatal citalopram does not prevent the effect of prenatal stress on anxiety, depressive-like behaviour and serotonergic transmission in adult rat offspring. *Eur. J. Neurosci.* 43, 590–600. doi: 10.1111/ejn.13150

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
published: 14 January 2021
doi: 10.3389/fnbeh.2020.599676

Long-Term Dietary Restriction Leads to Development of Alternative Fighting Strategies

Jeanne Legros[1], *Grace Tang*[2], *Jacques Gautrais*[1], *Maria Paz Fernandez*[2] and *Séverine Trannoy*[1]*

[1] Research Center on Animal Cognition (CRCA), Center for Integrative Biology, Toulouse University, CNRS, UPS, Toulouse, France, [2] Department of Neuroscience and Behavior, Barnard College of Columbia University, New York, NY, United States

In competition for food, mates and territory, most animal species display aggressive behavior through visual threats and/or physical attacks. Such naturally-complex social behaviors have been shaped by evolution. Environmental pressure, such as the one imposed by dietary regimes, forces animals to adapt to specific conditions and ultimately to develop alternative behavioral strategies. The quality of the food resource during contests influence animals' aggression levels. However, little is known regarding the effects of a long-term dietary restriction-based environmental pressure on the development of alternative fighting strategies. To address this, we employed two lines of the wild-type *Drosophila melanogaster* Canton-S (CS) which originated from the same population but raised under two distinct diets for years. One diet contained both proteins and sugar, while the second one was sugar-free. We set up male-male aggression assays using both CS lines and found differences in aggression levels and the fighting strategies employed to establish dominance relationships. CS males raised on a sugar-containing diet started fights with a physical attack and employed a high number of lunges for establishing dominance but displayed few wing threats throughout the fight. In contrast, the sugar-free-raised males favored wing threats as an initial aggressive demonstration and used fewer lunges to establish dominance, but displayed a higher number of wing threats. This study demonstrates that fruit flies that have been raised under different dietary conditions have adapted their patterns of aggressive behavior and developed distinct fighting strategies: one favoring physical attacks, while the other one favoring visual threats.

Keywords: fighting strategies, adaptation, dietary restriction, social rank, *Drosophila melanogaster*, aggression

OPEN ACCESS

Edited by:
Livio Luongo, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Serena Boccella, University of Campania Luigi Vanvitelli, Italy
Flavia Ricciardi, University of Campania Luigi Vanvitelli, Italy

***Correspondence:**
Séverine Trannoy
severine.trannoy@univ-tlse3.fr

Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *27 August 2020*
Accepted: *15 December 2020*
Published: *14 January 2021*

Citation:
Legros J, Tang G, Gautrais J, Fernandez MP and Trannoy S (2021) Long-Term Dietary Restriction Leads to Development of Alternative Fighting Strategies. Front. Behav. Neurosci. 14:599676. doi: 10.3389/fnbeh.2020.599676

INTRODUCTION

Aggression is an innate and complex social behavior observed throughout the animal kingdom that takes different forms: threat displays, physical approaches, chases, and physical attacks. Multiple aggressive interactions with high-intensity physical attacks among members of a social group lead to the formation of hierarchies (Chase and Seitz, 2011). Once established, a stable social hierarchy structures the group, decreasing future aggressive interactions among members. Therefore, animals tend to employ the best fighting strategy to reach a short- or long-lasting social consensus (Holekamp and Strauss, 2016).

Although an innate behavior, aggression contains adaptive features crucial for animals living in constantly changing environments (Reichert and Quinn, 2017). Two types of behavioral plasticity are related to environmental changes: (i) short-term with changes in color, size, or locomotor activity in response to novel but predictable environmental modifications, and (ii) long-term plasticity involving development of alternative and irreversible behavioral phenotypes in response to environmental pressure (Brockmann, 2001). Dietary-restriction is one example of driving force exerted on animals to adapt to limited conditions and ultimately to develop alternative behavioral strategies (Han and Dingemanse, 2015; Zhang et al., 2019). Dietary-restriction is known to induce behavioral changes and reduce the reproductive yield (Adler et al., 2013), affect flight endurance in insects (Nguyen, 2008), and extend lifespan in a wide range of animal species (Nakagawa et al., 2012). Diet is also known to modulate social behaviors, including aggression (Wallner, 2009). In humans, eating disorders enhance the frequency of aggressive behavioral manifestations (Truglia et al., 2006). Regarding the effect of different macronutrients on social behaviors, it has been shown that male rats fed with carbohydrates present a higher rate of fighting behavior and anxiety-like behavior (Hanstock et al., 2004), while Argentinian ants show lower level of aggression when deprived of sucrose (Grover et al., 2007). Moreover, Gottingen minipigs subjected to a high fat/low carbohydrate regime present a decrease in aggressive behavior (Haagensen et al., 2014). However, little is known about how the fighting strategies developed by animals are influenced by dietary regimes.

A variety of studies on invertebrates showed that aggressive behavior is modulated by genetic factors (Dierick and Greenspan, 2006), environmental conditions (Rittschof and Robinson, 2013; Rillich et al., 2019), social influences (Kilgour et al., 2019; Balsam and Stevenson, 2020), sex (Benelli et al., 2015), and previous experiences (Goubault and Decuigniere, 2012; Rose et al., 2017). Indeed, previous victory and defeat induce behavioral plasticity in the form of winner and loser effects (previous victory/defeat increase the probability of winning/losing subsequent fight) (Hsu et al., 2006). *Drosophila melanogaster* represents an attractive model to study the environmental influences on aggressive behaviors, dominance relationships and the development of alternative fighting strategies. In competition for food, mates, and territory, fruit flies exhibit a series of stereotypical sex-specific aggressive patterns, but only males establish dominance between competitors using the male-specific lunge behavior (Chen et al., 2002; Nilsen et al., 2004). For this reason, our study focuses on males' aggressive behaviors. Flies also display visual threats, but their exact function remains debated: are they "bluffs" or "honest" signals? On one hand, wing threats displayed throughout the fight might reinforce the functions of lunges in escalating fights. On the other hand, they might serve independent functions. Yet, wing threats are not always considered a crucial element of the fighting strategy when analyzing Drosophila aggression. Nevertheless, the observation that 3 neurons promote threat displays without interfering with other types of agonistic behavior, supports the notion that lunges and wing threats are independent patterns controlled by distinct sets of neurons (Duistermars et al., 2018).

Studies have shown a correlation between male aggression levels and foraging-related behavior (Wang and Sokolowski, 2017), high fat dietary regimes (Meichtry et al., 2020), and the food value available during aggression assays (Lim et al., 2014). However, it remains to be determined how dietary regimes influence aggressive patterns and the development of fighting strategies. In addition, it is still unclear whether wing threats are an integral part of the fighting strategy used by flies to form and maintain dominance.

Here, using two lines of the wild-type Drosophila Canton-S (CS) that originated from the same population but raised under two distinct diets for about 10 years, we found that flies exhibited behavioral plasticity in response to distinct environmental conditions, leading to two different fighting strategies. Our results indicate that males from the line raised on a sugar-containing diet started fights with lunges and escalated fights quickly. Moreover, dominant individuals used lunges to establish and maintain dominance relationships. On the contrary, males raised in the sugar-free diet started fights either with lunges or wing threats and escalated fights to establish dominance with fewer lunges. In this case, dominants displayed threats to maintain their social rank, avoiding using higher-intensity patterns such as lunges. The differences in aggression levels based on lunges and fighting strategies could not be reversed by switching diets. Our data highlights a potential link between aggression levels, the development of alternative fighting strategies and dietary regimes.

MATERIALS AND METHODS

Flies Stocks

Flies were raised at 25°C under a 12 h:12 h light/dark cycle (LD = 8:30 a.m.−8:30 p.m.). Two populations of *D. melanogaster* were used in this study: CS_A (from Edward Kravitz's laboratory at Harvard Medical School, Boston, USA) and CS_B (from Guillaume Isabel's laboratory at the Research Center of Animal Cognition CRCA, Toulouse, France). These CS lines were raised from 2010 to 2019 on standard medium, respectively: 52% cornmeal, 28% yeast, 121% sugar, 15% agar, 20% Moldex, and 70% corn flour, 70% yeast, 0% sugar, 9% agar, 20% Moldex. Since we started the study on October 2019, CS_A and $_B$ were maintained on a medium labeled the *sugar-containing* diet that was composed of: 74% corn flour, 28%yeast, 40% sugar, 8% agar, 20% Moldex (to match as best as possible the recipe from HMS for raising CS_A), and on a medium called *sugar-free* that was composed of: 70% corn flour, 70% yeast, 0% sugar, 9% agar, 20% Moldex (the same recipe used at the CRCA for raising CS_B) (**Supplementary Table 1** summarizes the composition of the two diets used, and **Supplementary Figure 1** the experimental design).

Experimental Chamber

The experimental setup used in this study to examine social behaviors has already been described (Trannoy et al., 2015). Briefly, a divider was inserted through the top of the arenas (22 mm diameter x 16 mm height) which contained a food cup (13 mm diameter x 6 mm height) separating them into two equal sizes. Flies were then inserted on each side of the arenas by negative geotaxis, so they can acclimate without interacting with each other. Behavioral experiments start once the separator was removed allowing flies to interact together.

Behavioral Assays

On day 0, late stage male pupae were sexed and socially isolated in vials containing 1 ml of either sugar-containing or sugar-free diet, for 7 days, under 25°C 12 h:12 h LD cycles as described above. On day 5, flies were anesthetized with CO_2 to apply a dot of paint on the dorsal thorax of flies for identification purposes. At day 7, behavioral experiments were performed between Zeitgeber time zero (ZT0, right after the lights on transition) and continued for up to 3 h (ZT3). During the maintaining and isolation phases, light conditions were constant (12 h:12 h L/D cycle = 8:30 a.m. to 8:30 p.m.). All behavioral experiments were performed during the first 3 h after the lights on transition (ZT0 to ZT3).

Aggression Assays

Two males from the same CS line were paired in each chamber with a food cup containing fresh fly food (either sugar-containing or sugar-free diet) with a drop of yeast paste on the surface. We scored all aggressive patterns that happened on the food cup and for 10 min after the time of the first lunge. If no lunges were observed for 15 min after t0 (time when the divider was removed from the arenas), we stopped the scoring. The latencies to lunge, wing threat (WT) represent the time between the first meeting and the first lunge and the time between t0 and the first WT. The latency to dominance is the time between the first meeting and the time to dominance. Time to dominance was determined when the putative loser retreats from the food cup three times after having received lunges from the other (Trannoy et al., 2016). Fight outcomes were either (i) no fight: when 0 lunges were observed, (ii) draw: when lunges were observed but were not sufficient to induce dominance or because of retaliation, or (iii) dominance: when dominance has been established between competitors during the 15 min after t0 of observation (**Supplementary Table 2** summarizes the behavioral parameters used to score aggressive behavior).

Courtship Assays

One sexually mature male and one virgin female (both 7-days old) from the same CS line were inserted into each side of a behavioral chamber in the absence of a food resource. Courtship Vigor Index (CVI) was calculated as the fraction of time that males spent courting the female (including tapping, wing extension and vibration, chasing and attempted copulation) during a 10-min period after the first courtship behavior. The latencies to court and to copulate were the times between the first meeting and the first courtship behavior or the initiation of copulation, respectively.

Activity and Sleep Assays

Locomotor activity and sleep profiles were recorded using DAM2 *Drosophila* Activity Monitors (Trikinetics, Waltham, MA). Three-to five-day old males from CS lines were placed individually in Trikinetics capillary tubes containing either their "respective" or "switched" food. Flies were entrained to 12 h:12 h LD cycles for 5 days at a constant temperature of 25°C. Activity counts were collected in 1-min bins that were subsequently summed into 30-min bins for the time-series analysis of locomotor activity. Activity levels were normalized for individual flies by setting the average activity level for all 30-min bins across days 3–5 equal to 1.0. Population profiles were then averaged into a single representative 24-h day, displayed as histograms. For sleep quantifications, beam-crossings were also collected in 1-min bins. A sleep bout was defined as a period of inactivity of at least 5-min (Hendricks et al., 2000). Sleep plots represent averaged population sleep profiles and were obtained by averaging the sleep data over days 3–5 of the LD cycle, displayed as line plots.

Statistical Analysis

GraphPad Prism 8 was used to assess the normality of the distribution with the Shapiro-Wilk test and to identify outlier values with a Grubb's test (alpha = 0.05). R software was used to assess the effects of the factors "CS line" and "Diet" as well as the possible interaction between them, by using distinct statistical methods according to the nature of the behavioral response tested:

- For all latencies (**Figures 1A,F**, **3A,B** and **Supplementary Figure 2**): survival analysis of Kaplan-Meier (survdiff) with a χ^2test.
- For binomial distribution (**Figures 1E**, **3D**): logistic regression (lm) model with a χ^2test to assess the effect of both factors, followed by a Tukey post-test.
- For percentages (**Figure 3C**): logistic regression (lm) with an ANOVA to assess the effect of both factors, followed by a Tukey post-test.
- All numerical measurements (**Figures 1B–D**, **2A,B**, **4B,D,E** and **Supplementary Figures 4, 5**): Generalized linear model (glm) with Quasi-Poisson error distribution was used to overcome the non-Gaussian distribution of the data. The significance of the effect due to the CS line, diet or the interaction, was assessed using an ANOVA followed by a Tukey post-test.
- For data distribution (**Figures 2C,D** and **Supplementary Figure 3**): χ^2test to compare to the expected value of 50%. The tests were done with GraphPad online software.

Differences were considered statistically significant at $p < 0.05$. Data are presented as boxplots including all data points. The lower and upper edges of each box correspond to the 25 and

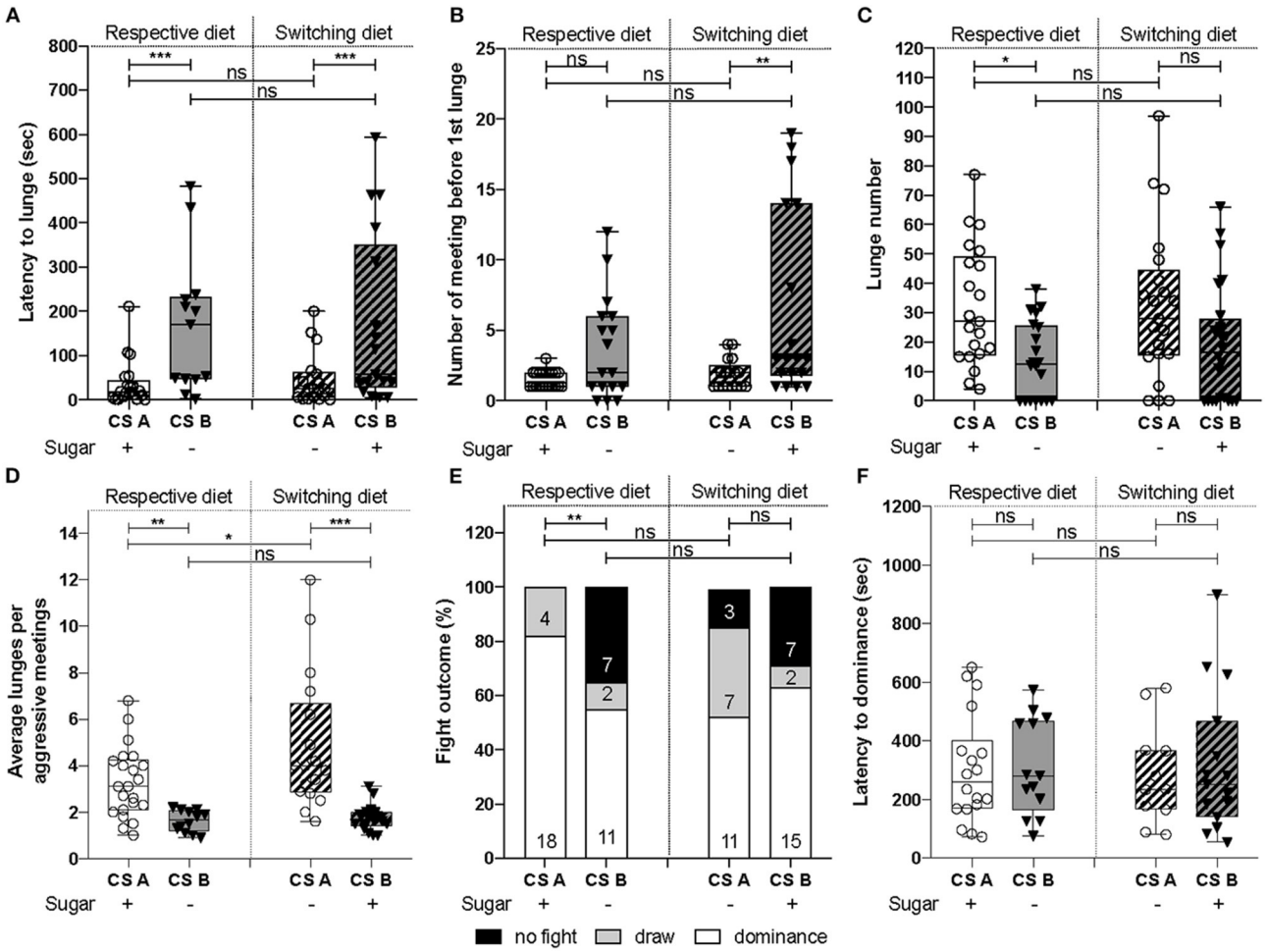

FIGURE 1 | Dietary sugar content leads to different aggression levels. **(A)** CS_A showed a significantly decreased latency to lunge compared to CS_B. Statistics revealed that this decrease can be explained by an effect due to the CS line (χ^2test = 23.3, d.f = 1, p = 1e-06) and not the diet (χ^2test = 1.5, d.f = 1, p = 0.2). **(B)** The number of meetings before the first lunge was different between CS lines [$F_{(1, 71)}$ = 24.53, p = 5e-06), but was not affected by the diet [$F_{(1, 70)}$ = 2.6, p = 0.11]. **(C)** CS_A lunged significantly more than CS_B [$F_{(1, 84)}$ = 12.51, p = 6.6e-04]. The diet did not significantly affect this parameter [$F_{(1, 83)}$ = 0.4, p = 0.53]. **(D)** CS_A line gave higher number of lunges per aggressive meeting than CS_B. Both CS lines [$F_{(1, 66)}$ = 41.94, p = 1.5e-08] and diets [$F_{(1, 65)}$ = 5.63, p = 0.02) affected the average number of lunges per aggressive meetings. **(E)** Fight outcomes were affected by the CS line (χ^2test = 9.14, d.f = 1, p = 0.002), but not by the diet (χ^2test = 1.54, d.f = 1, p = 0.21). **(F)** The latency to dominance was not different between CS line (χ^2test = 0.7, d.f = 1, p = 0.4) and between diet (χ^2test = 2.6, d.f = 1, p = 0.1). All the behavioral experiments were performed between ZT0 and ZT3. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

75% quantiles, respectively. Percentages are presented as stacked bars. All the statistics can be found in **Supplementary Table 3**.

RESULTS

Dietary Sugar Content Leads to Different Aggression Levels

To assess whether dietary regimes might have driven adaptation of aggressive behavior, we collected two CS lines that serve as reference lines in two laboratories: CS_A and $_B$ lines, that were raised for ∼10 years on either sugar-containing or sugar-free diets, respectively. We set up male-male aggression assays and scored behavioral parameters. When comparing the latency to lunge, we observed that CS_A males started fighting significantly sooner (**Figure 1A**, left panel "respective diet"), with a tendency to meet fewer times before the first lunge (**Figure 1B**, left panel), suggesting that CS_A males have a higher motivation to fight compared to CS_B. As the latency to lunge was significantly increased in the CS_B line, we assessed the aggressiveness level of both lines by scoring the number of lunges displayed within 10 min since the first lunge (as opposed to quantifying it for a fixed amount of time after the first meeting). CS_B males showed a significant reduction of the total number of lunges (**Figure 1C**, left panel), as well as of the average number of

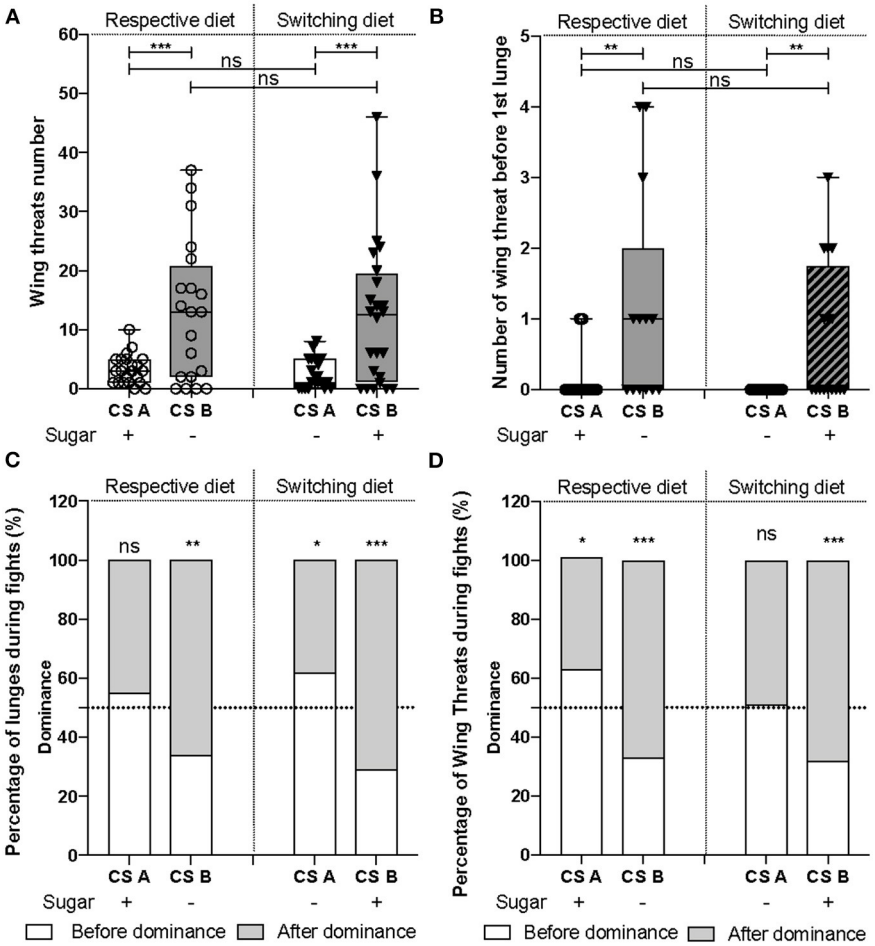

FIGURE 2 | The two CS lines employed different fighting strategies. **(A)** The number of wing threats [$F_{(1, 85)} = 41.98$, $p = 6e\text{-}09$] and **(B)** the number of wing threats before the first lunge [$F_{(1, 66)} = 30.32$, $p = 6.9e\text{-}07$] were significantly higher in CS_B line than CS_A. However, diet does not influence **(A)** the display of wing threats [$F_{(1, 84)} = 0.004$, $p = 0.94$], nor **(B)** the number of wing threats before the first lunge [$F_{(1, 65)} = 0.34$, $p = 0.56$]. **(C)** CS_A lunged equally often before and after dominance was established ($\chi^2test_{CSAsugar+} = 1$, d.f = 1, $p = 0.32$; $\chi^2test_{CSAsugar-} = 5.7$, d.f = 1, $p = 0.02$), while CS_B, preferentially lunged after dominance ($\chi^2test_{CSBsugar+} = 17.64$, d.f = 1, $p = 0.0001$; $\chi^2test_{CSBsugar-} = 10.24$, d.f = 1, $p = 0.0014$). **(D)** In CS_A, wing threats were displayed throughout the fight ($\chi^2test_{CSAsugar+} = 6.18$, d.f = 1, $p = 0.0129$; $\chi^2test_{CSAsugar-} = 0.04$, d.f = 1, $p = 0.84$), while in CS_B, they were observed mostly after dominance ($\chi^2test_{CSBsugar+} = 12.96$, d.f = 1, $p = 0.0003$; $\chi^2test_{CSBsugar-} = 11.56$, d.f = 1, $p = 0.0007$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

lunges per aggressive meetings (**Figure 1D**, left panel), showing that aggressiveness level was reduced in the CS_B line compared to CS_A. In the same way, CS_B fought in only 65% of assays (assays with at least one lunge) compared to 100% for CS_A (**Figure 1E**, left panel). However, no difference was observed in the latency to dominance **Figure 1E**, left panel). Together, these results demonstrate that the two CS populations raised on their respective diets for almost 10 years differed by their motivation to start fighting, their aggressiveness level, and fight outcomes.

Aggression Level Is Negatively Impacted by Long-Term Sugar-Restriction

Next, we asked whether switching diets would affect aggressive behavior of both CS lines. Would raising CS_B on a sugar-containing diet rescue the diminution of their aggressiveness level? On the contrary, would depriving CS_A from sugar negatively impact males' aggression level? To address these questions, we raised the CS_A line on sugar-free diet and CS_B line on sugar-containing diet for 3 months and performed male-male aggression assays – an experimental condition called "switching diet." Behavioral experiments on respective and switching diets were done in parallel to compare aggressive patterns of CS_A and CS_B B when raised on both diets. No significant differences were found (**Figure 1**, entire panel), except for the average number of lunges per aggressive meetings (**Figure 1D**). However, when comparing both CS_A and CS_B raised on switching diets, we still observed that the CS_B line showed a reduction in their motivation to fight, exhibited fewer lunges per aggressive encounter, and fought less often (**Figures 1A–F**, right panels), recapitulating the results observed when raised on their respective diets. This "switching diet" experimental condition indicated that depriving CS_A flies from

sugar did not reduce their aggressiveness. In agreement with this, raising CS_B flies on a diet containing sugar did not either enhance aggressiveness. These results demonstrate that the motivation to fight, aggressiveness, and fight outcomes could not be restored by switching diets for 3 months, rather they suggest that they potentially result from a longer-term influence of diet, and a behavioral adaptation to an environmental condition.

CS Lines Have Developed Distinct Fighting Strategies

Employing high-intensity lunges throughout the fight remains an efficient fighting strategy used by males to establish a stable dominance relationship. However, as CS_B males formed dominance relationships while using fewer lunges than CS_A during fights (**Figure 1**), we asked whether they have developed an alternative fighting strategy to attain and maintain this social consensus between competitors. For this, we scored the number of wing threats and found that CS_B displayed significantly more of these visual threats than did CS_A (**Figure 2A**). Moreover, CS_B displayed more wing threats before the first lunge (**Figure 2B**). Indeed, in 54% (7/13) and 47% (8/17) of assays, CS_B males displayed wing threats as the first aggressive demonstration when raised on their respective and switching diets, respectively, while these observations dropped to 13% (3/22) and 0.05% (1/18) for CS_A. However, the latency to display the first wing threat was not different (**Supplementary Figure 2**). This shows that sugar-free raised-males may have developed an alternative fighting strategy in which dominance could be formed and maintained by using fewer lunges but more threats. To further explore this hypothesis, we compared the percentages of lunges (**Figure 2C**) and wing threats (**Figure 2D**) given before and after dominance, to the random value of 50%. A value near 0% implies that most of the lunges were given before dominance, a value near 50% implies that flies lunged before and after dominance equally, and a value near 100% implies that most lunges occurred after dominance. We observed that CS_A raised on their respective and switching diets, respectively, exhibited 55 and 62% of the lunges (**Figure 2C**) and 63 and 51% of the wing threats (**Figure 2D**) before establishment of dominance, showing that aggressive patterns are almost equally distributed throughout the fight. On the contrary, CS_B males exhibited only 34 and 29% of the lunges (**Figure 2C**) and 33 and 32% of the wing threats (**Figure 2D**) before dominance, showing that they are preferentially distributed after dominance was established. As the majority of lunges and wing threats were displayed by the winners (**Supplementary Figure 3**), they are likely used by dominants to establish and maintain dominance relationships. Altogether, these results demonstrate that males from the two CS lines employ distinct fighting strategies to establish and maintain dominance: CS_A started fights with lunges, while CS_B with either wing threats or lunges. Also, CS_A favored the use of lunges to establish and maintain dominance, while CS_B preferentially used both behavioral patterns to maintain it.

Courtship Performances and Reproductive Capacities Are Not Affected by Diet

We next investigated whether males would also employ distinct reproductive strategies in a male-to-female courtship context. We therefore set up courtship assays involving one CS_A or CS_B male with a female from the same line, and scored courtship behavior. We observed that the latencies to court and to copulate were not statistically different between lines or when lines were raised on either diet (**Figures 3A,B**). In the same way, male courtship performances did not differ significantly between lines and diets (**Figure 3C**). Finally, the copulation success rate was not statistically different between CS lines (**Figure 3D**). These results demonstrate that there were no differences in courtship performances and reproductive abilities between both CS lines.

CS Lines Showed Differences in Their Activity and Sleep Patterns

A reduction of aggression could come from a reduction of locomotor activity. Therefore, we performed activity and sleep experiments with males of both lines using either their respective and switched diet. When restricting the analysis to the first 3 h of the day (Zeitgeber time 0-3, ZT0-ZT03) to match the time when aggression experiments were performed, we observed differences in both parameters (**Figure 4**). Males of the CS_B line exhibited lower levels of activity in this specific time window regardless of the diet (**Figures 4A,B**). Consistently with this observation, we noticed an increase in total sleep for the CS_B line compared to CS_A, in both diets (**Figures 4C,D**). However, for the total number of sleep bouts (5-min period of inactivity), we only noticed a mild increase for CS_B in their switched diet (**Figure 4E**). When analyzing the same parameters during the day (ZT0-ZT12) or night (ZT12-ZT24) phases, CS_B only showed significant decreases in activity relative to CS_A when analyzed in their respective diet (**Supplementary Figure 4**). In the case of sleep, only minutes of day sleep was increased for CS_B in both diets but not sleep bouts (**Supplementary Figures 5A,B**). Night sleep showed no differences between lines (**Supplementary Figures 5C,D**).

DISCUSSION

Dietary regimes play crucial roles in the life history of animals and can affect their behaviors in many ways (Tremmel and Müller, 2013; Han and Dingemanse, 2015). Animals living in changing environments must develop adaptive behavioral responses to withstand dietary challenging situations (Partridge and Brand, 2005; Adler et al., 2013). In Drosophila, manifestation of aggressive behavior is subjected to modifications by environmental and social conditions (Svetec and Ferveur, 2005; Bath et al., 2018; Kilgour et al., 2019). To further follow these studies, we investigated the behavioral plasticity of Drosophila aggressive behaviors in response to two distinct dietary regimes. The long-term diet-related consequences are a modification of the fighting motivational state, aggressiveness level, and fighting strategy employed to reach dominance.

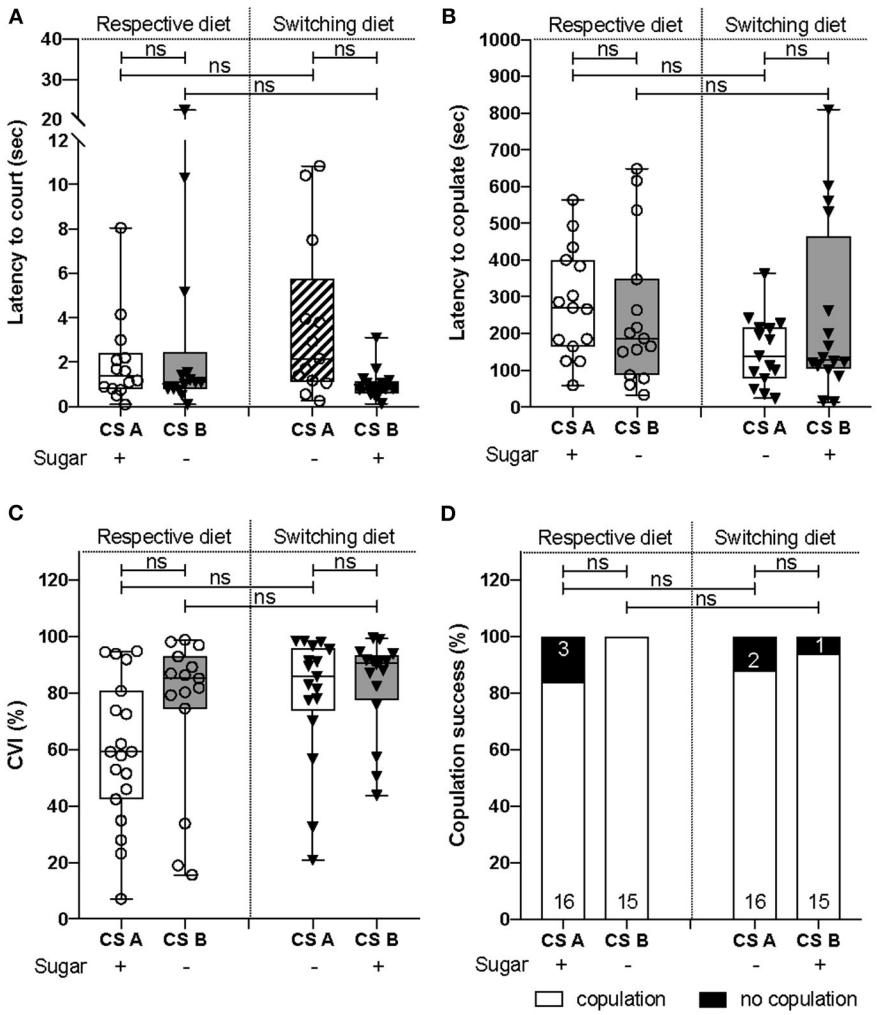

FIGURE 3 | Courtship abilities did not differ between both CS lines. **(A)** There was no difference in the latency to court between CS lines (χ^2test = 1.2, d.f = 1, p = 0.3) and diets (χ^2test = 0.4, d.f = 1, p = 0.6). **(B)** No difference were observed across CS lines (χ^2test = 1.7, d.f = 1, p = 0.2) and diets (χ^2test = 2.5, d.f = 1, p = 0.1) for the latency to copulate. **(C)** CS lines [$F_{(1, 64)}$ = 1.94, p = 0.18) and diet [$F_{(1, 64)}$ = 3.93, p = 0.052] did not influence CVI. **(D)** The copulation success did not vary according to the CS lines (χ^2test = 2.67, d.f = 1, p = 0.1) nor the diets (χ^2test = 0.53, d.f = 1, p = 0.46).

In addition to its essential function of nutrition, diets modulate behavioral expression and ultimately control social interactions, including aggression (Wallner, 2009). Here, we revealed that male flies raised on sugar-containing diet are overall more aggressive than those raised on a sugar-free diet. We also showed that visual threats are another key component of Drosophila fights. Indeed, sugar-free raised-flies showed fewer lunges but more wing threats, which significantly impacted the fight dynamics and modified the fighting strategy to reach and maintain dominance. Based on our results, we propose that, in addition to lunges, wing threats should be considered as an informative behavioral pattern when studying fighting strategies and establishment of dominance relationships in *Drosophila* males. Our results demonstrate that the reduction in lunging behavior and fighting motivational state can't be rescued by switching diets, suggesting that the diet-induced males' aggression phenotype observed results from a long-term behavioral adaptation to diet. However, investigating aggression in a female-female context would provide additional information about how dietary regimes influence fighting strategies in general, and would reinforce our current hypothesis.

Raising flies on two different diets does not interfere with males' courtship performance, nor with their reproduction capacities between males and females from the same CS line. Performing courtship experiments with reciprocal females, however, could affect these parameters. In the same way, performing competitive courtship assays could reveal whether a preference for a non-random mating has emerged in these lines after years of potential experimental evolution, which has been observed when investigating the emergence of behavioral isolation (Belkina et al., 2018).

CS males raised on sugar-free diet also show a reduction in their locomotor activity and an increase in sleep patterns, particularly during the daytime. This could account for some

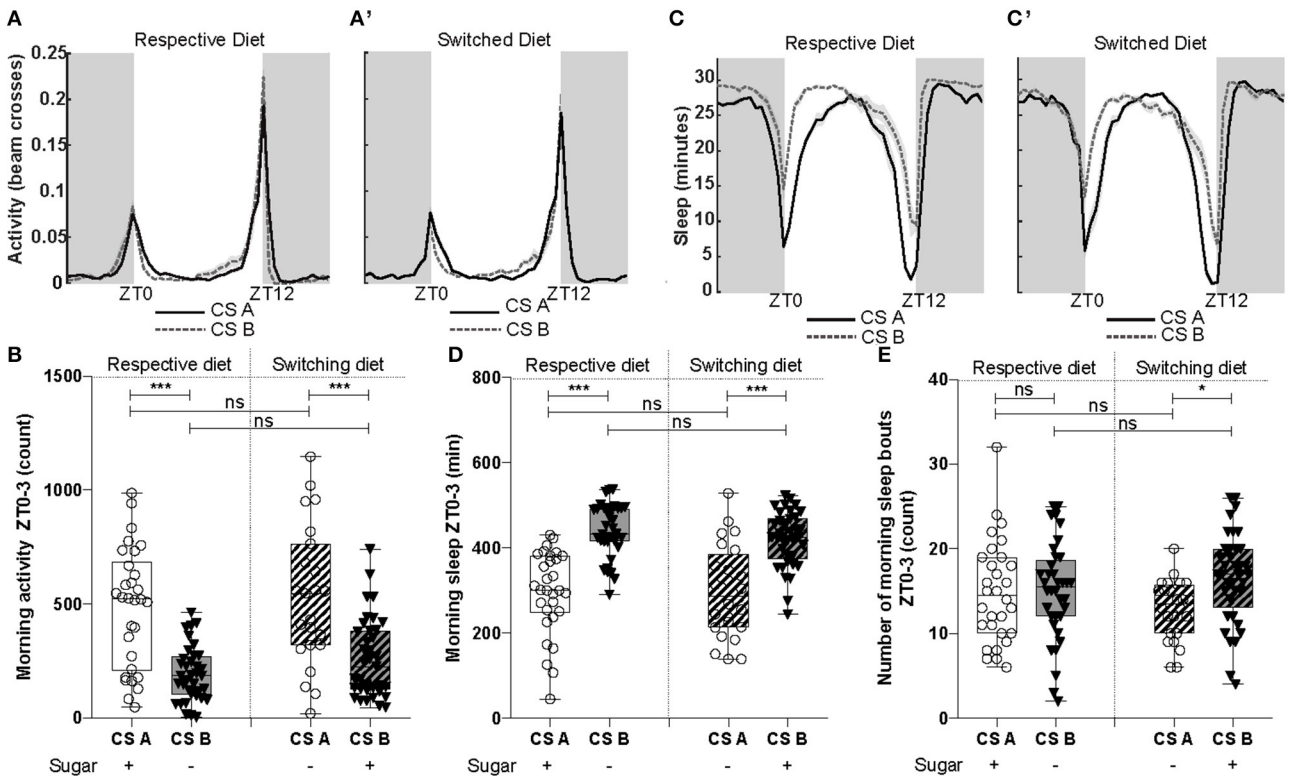

FIGURE 4 | Cs line presented differences in their activity and sleep patterns. **(A)** Representation of activity patterns across day (white) and night (gray) for both lines when raised on their respective or **(A')** switching diet. **(B)** The CS_A line was significantly more active during the morning (ZT0-3) when behavioral experiments were done [$F_{(1,\ 124)} = 55.76$, $p = 1.34e\text{-}11$]. Diet did not affect morning activity [$F_{(1,\ 123)} = 0.29$, $p = 0.58$]. **(C)** Sleep patterns of CS lines when raised on their respective and **(C')** switching diets. **(D)** The line CS_B slept significantly more during the first 3 h of the morning [$F_{(1,\ 125)} = 73{,}88$, $p = 3.16e\text{-}14$]. Diet did not significantly affect morning sleep [$F_{(1,\ 124)} = 0.42$, $p = 0.51$]. **(E)** Number of morning sleep bouts (ZT0-3) were not significantly different between CS lines [$F_{(1,\ 125)} = 3.72$, $p = 0.056$] and diets [$F_{(1,\ 124)} = 3.51$, $p = 0.064$], even though a statistical difference was detected by the post-test between CS lines when raised on switching diet. $^*P < 0.05$, $^{***}P < 0.001$, ns, not significant.

of the described aggression phenotypes, like latency to the first lunge, but not for all. In the latter case, we could expect increases in both latencies to lunge and to dominance, and a reduction in all aggressive patterns, including wing threats. Also, we would expect differences in their latency to court and/or copulate with females, which was not the case. Therefore, differences in activity and/or sleep levels do not explain what we consider the most salient aspects of the behavioral differences: the frequency of wing threat displays leading to modification of fight's dynamics and the development of alternative fighting strategy to reach dominance.

Our findings support previous observations that animals fed with low sucrose diet are less aggressive than those fed with high level of sucrose (Grover et al., 2007; Haagensen et al., 2014; Meichtry et al., 2020). From a physiological point of view, as the production of ATP from the conversion of carbohydrates is a key source of energy for insects, exposure to low sugar or sugar-free diets might have forced animals to develop less energy-consuming fighting strategies, while staying competitive toward others. However, sugar-deprived diets may have additional consequences. Indeed, insects use cuticular hydrocarbons (CHC), acting as pheromones, to drive social behaviors (Yew and Chung, 2017). For example, changes in the amount of 11-cis-vaccenyl acetate (cVa) pheromone modulate male courtship behavior (Ejima, 2015) and aggression by altering the number of lunges (Fernandez et al., 2010; Wang et al., 2011). As diet (Fedina et al., 2012) and circadian rhythm (Krupp et al., 2008) influence the CHC profile of flies, it is possible that CS lines present differences in the amount of some CHC, leading to changes in the expression of lunge behavior. Another explanation would be that diet affects anterior inferior protocerebrum (AIP) neuronal activity, recently described to specifically control threat displays without affecting other types of agonistic behavior (Duistermars et al., 2018). To further follow this work on behavioral adaptation to diets, it would be interesting to perform whole brain RNAi sequencing on these CS lines. This would allow to identify whether genes already known to control social behaviors are differentially expressed between these lines in response to distinct dietary regimes.

In sum, our results show that fruit flies raised for years under different dietary conditions have adapted their aggressive behaviors and developed two distinct fighting strategies: one favoring physical attacks, while the other one employing both

physical attacks and visual threats. This shows the long-term influence of diet-based environmental pressure on aggression and adaptation of animals' fighting strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

ETHICS STATEMENT

Despite the widespread use of invertebrates in research, only few ethical guidelines exist and applied to crustaceans and cephalopods. However, using Drosophila as a model for behavioral research also involve experimental design planification and the use of anesthetic methods for reducing animals' pain and suffering during and after experiments (Drinkwater et al., 2019).

AUTHOR CONTRIBUTIONS

JL performed preliminary experiments on CS_A and $_B$ lines. GT and MF performed and analyzed activity and sleep patterns, and edited the paper. JG and ST wrote the scripts for statistical analysis in R software. ST designed the study, performed, analyzed and scored all behavioral experiments, performed statistical analysis, and wrote the paper. All the authors gave final approval for submission.

FUNDING

This research was supported by Agence Nationale de la Recherche (ANR) Grant (ANR-19-CE37-0018-01 to ST) and Barnard College start-up funds to MF.

ACKNOWLEDGMENTS

We thank members of the IVEP team and Alexandre Roland for their comments on the manuscript. We thank Edward A. Kravitz and Guillaume Isabel for sharing the CS lines A and B used in this study, and Eric LeBourg for helpful scientific discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.599676/full#supplementary-material

REFERENCES

Adler, M. I., Cassidy, E. J., Fricke, C., and Bonduriansky, R. (2013). The lifespan-reproduction trade-off under dietary restriction is sex-specific and context-dependent. *Exp. Gerontol.* 48, 539–548. doi: 10.1016/j.exger.2013.03.007

Balsam, J. S., and Stevenson, P. A. (2020). Pre-adult aggression and its long-term behavioural consequences in crickets. *PLoS ONE* 15:e0230743. doi: 10.1371/journal.pone.0230743

Bath, E., Morimoto, J., and Wigby, S. (2018). The developmental environment modulates mating-induced aggression and fighting success in adult female Drosophila. *Funct. Ecol.* 32, 2542–2552. doi: 10.1111/1365-2435.13214

Belkina, E. G., Naimark, E. B., Gorshkova, A. A., and Markov, A. V. (2018). Does adaptation to different diets result in assortative mating? Ambiguous results from experiments on Drosophila. *J. Evol. Biol.* 31, 1803–1814. doi: 10.1111/jeb.13375

Benelli, G., Romano, D., Desneux, N., Messing, R. H., and Canale, A. (2015). Sex differences in fighting-induced hyperaggression in a fly. *Anim. Behav.* 104, 165–174. doi: 10.1016/j.anbehav.2015.02.026

Brockmann, H. J. (2001). The evolution of alternative strategies and tactics. *Adv. Stud. Behav.* 30, 1–51. doi: 10.1016/S0065-3454(01)80004-8

Chase, I. D., and Seitz, K. (2011). Self-structuring properties of dominance hierarchies a new perspective. *Adv. Genet.* 75, 51–81. doi: 10.1016/B978-0-12-380858-5.00001-0

Chen, S., Lee, A. Y., Bowens, N. M., Huber, R., and Kravitz, E. A. (2002). Fighting fruit flies: a model system for the study of aggression. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5664–5668. doi: 10.1073/pnas.082102599

Dierick, H. A., and Greenspan, R. J. (2006). Molecular analysis of flies selected for aggressive behavior. *Nat. Genet.* 38, 1023–1031. doi: 10.1038/ng1864

Drinkwater, E., Robinson, E. J. H., Hart, A. G., and Ellison, A. (2019). Keeping invertebrate research ethical in a landscape of shifting public opinion. *Methods Ecol. Evol.* 10, 1265–1273. doi: 10.1111/2041-210X.13208

Duistermars, B. J., Pfeiffer, B. D., Hoopfer, E. D., and Anderson, D. J. (2018). A brain module for scalable control of complex, multi-motor threat displays. *Neuron* 100, 1474–1490.e4. doi: 10.1016/j.neuron.2018.10.027

Ejima, A. (2015). Pleiotropic actions of the male pheromone cis-vaccenyl acetate in *Drosophila melanogaster*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 201, 927–932. doi: 10.1007/s00359-015-1020-9

Fedina, T. Y., Kuo, T. H., Dreisewerd, K., Dierick, H. A., Yew, J. Y., and Pletcher, S. D. (2012). Dietary effects on cuticular hydrocarbons and sexual attractiveness in Drosophila. *PLoS ONE* 7:e49799. doi: 10.1371/journal.pone.0049799

Fernandez, M. P., Chan, Y. B., Yew, J. Y., Billeter, J. C., Dreisewerd, K., Levine, J. D., et al. (2010). Pheromonal and behavioral cues trigger male-to-female aggression in Drosophila. *PLoS Biol.* 8:e1000541. doi: 10.1371/journal.pbio.1000541

Goubault, M., and Decuigniere, M. (2012). Previous experience and contest outcome: winner effects persist in absence of evident loser effects in a parasitoid wasp. *Am. Nat.* 180, 364–371. doi: 10.1086/667192

Grover, C. D., Kay, A. D., Monson, J. A., Marsh, T. C., and Holway, D. A. (2007). Linking nutrition and behavioural dominance: carbohydrate scarcity limits aggression and activity in Argentine ants. *Proc. Biol. Sci.* 274, 2951–2957. doi: 10.1098/rspb.2007.1065

Haagensen, A. M., Sorensen, D. B., Sandoe, P., Matthews, L. R., Birck, M. M., Fels, J. J., et al. (2014). High fat, low carbohydrate diet limit fear and aggression in Gottingen minipigs. *PLoS ONE* 9:e93821. doi: 10.1371/journal.pone.0093821

Han, C. S., and Dingemanse, N. J. (2015). Effect of diet on the structure of animal personality. *Front. Zool.* 12:S5. doi: 10.1186/1742-9994-12-S1-S5

Hanstock, T. L., Clayton, E. H., Li, K. M., and Mallet, P. E. (2004). Anxiety and aggression associated with the fermentation of carbohydrates in the hindgut of rats. *Physiol. Behav.* 82, 357–368. doi: 10.1016/j.physbeh.2004.04.002

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

Holekamp, K. E., and Strauss, E. D. (2016). Aggression and dominance: an interdisciplinary overview. *Behav. Sci.* 12, 44–51. doi: 10.1016/j.cobeha.2016.08.005

Hsu, Y., Earley, R. L., and Wolf, L. L. (2006). Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. *Biol. Rev. Camb. Philos. Soc.* 81, 33–74. doi: 10.1017/S146479310500686X

Kilgour, R. J., Norris, D. R., Mcadam, A. G., and Quinn, J. (2019). Carry-over effects of resource competition and social environment on aggression. *Behav. Ecol.* 31, 140–151. doi: 10.1093/beheco/arz170

Krupp, J. J., Kent, C., Billeter, J. C., Azanchi, R., So, A. K., Schonfeld, J. A., et al. (2008). Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. *Curr. Biol.* 18, 1373–1383. doi: 10.1016/j.cub.2008.07.089

Lim, R. S., Eyjolfsdottir, E., Shin, E., Perona, P., and Anderson, D. J. (2014). How food controls aggression in Drosophila. *PLoS ONE* 9:e105626. doi: 10.1371/journal.pone.0105626

Meichtry, L. B., Poetini, M. R., Dahleh, M. M. M., Araujo, S. M., Musachio, E., a,.S., et al. (2020). Addition of saturated and trans-fatty acids to the diet induces depressive and anxiety-like behaviors in *Drosophila melanogaster*. *Neuroscience* 443, 164–175. doi: 10.1016/j.neuroscience.2020.07.042

Nakagawa, S., Lagisz, M., Hector, K. L., and Spencer, H. G. (2012). Comparative and meta-analytic insights into life extension via dietary restriction. *Aging Cell* 11, 401–409. doi: 10.1111/j.1474-9726.2012.00798.x

Nguyen, D. T. (2008). Effects of starvation period on the locomotory response of Rhyzopertha dominica (F.). *J. Stored Prod. Res.* 44, 100–102. doi: 10.1016/j.jspr.2007.06.007

Nilsen, S. P., Chan, Y. B., Huber, R., and Kravitz, E. A. (2004). Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12342–12347. doi: 10.1073/pnas.0404693101

Partridge, L., and Brand, M. (2005). Special issue on dietary restriction: dietary restriction, longevity and ageiná the current state of our knowledge and ignorance. *Mech. Ageing Dev.* 126, 911–912. doi: 10.1016/j.mad.2005.04.003

Reichert, M. S., and Quinn, J. L. (2017). Cognition in contests: mechanisms, ecology, and evolution. *Trends Ecol. Evol.* 32, 773–785. doi: 10.1016/j.tree.2017.07.003

Rillich, J., Rillich, B., and Stevenson, P. A. (2019). Differential modulation of courtship behavior and subsequent aggression by octopamine, dopamine and serotonin in male crickets. *Horm. Behav.* 114:104542. doi: 10.1016/j.yhbeh.2019.06.006

Rittschof, C. C., and Robinson, G. E. (2013). Manipulation of colony environment modulates honey bee aggression and brain gene expression. *Genes Brain Behav.* 12, 802–811. doi: 10.1111/gbb.12087

Rose, J., Cullen, D. A., Simpson, S. J., and Stevenson, P. A. (2017). Born to win or bred to lose: aggressive and submissive behavioural profiles in crickets. *Anim. Behav.* 123, 441–450. doi: 10.1016/j.anbehav.2016.11.021

Svetec, N., and Ferveur, J. F. (2005). Social experience and pheromonal perception can change male-male interactions in *Drosophila melanogaster*. *J. Exp. Biol.* 208, 891–898. doi: 10.1242/jeb.01454

Trannoy, S., Chowdhury, B., and Kravitz, E. A. (2015). A new approach that eliminates handling for studying aggression and the "loser" effect in *Drosophila melanogaster*. *J. Vis. Exp.* 106:e53395. doi: 10.3791/53395

Trannoy, S., Penn, J., Lucey, K., Popovic, D., and Kravitz, E. A. (2016). Short and long-lasting behavioral consequences of agonistic encounters between male *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4818–4823. doi: 10.1073/pnas.1520953113

Tremmel, M., and Müller, C. (2013). Insect personality depends on environmental conditions. *Behav. Ecol.* 24, 386–392. doi: 10.1093/beheco/ars175

Truglia, E., Mannucci, E., Lassi, S., Rotella, C. M., Faravelli, C., and Ricca, V. (2006). Aggressiveness, anger and eating disorders: a review. *Psychopathology* 39, 55–68. doi: 10.1159/000090594

Wallner, B. (2009). Influence of nutrition on aggression. *CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour.* 4, 1–10. doi: 10.1079/PAVSNNR20094075

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J. C., Miyamoto, T., et al. (2011). Hierarchical chemosensory regulation of male-male social interactions in Drosophila. *Nat. Neurosci.* 14, 757–762. doi: 10.1038/nn.2800

Wang, S., and Sokolowski, M. B. (2017). Aggressive behaviours, food deprivation and the foraging gene. *R. Soc. Open Sci.* 4:170042. doi: 10.1098/rsos.170042

Yew, J. Y., and Chung, H. (2017). Drosophila as a holistic model for insect pheromone signaling and processing. *Curr. Opin. Insect Sci.* 24, 15–20. doi: 10.1016/j.cois.2017.09.003

Zhang, D. W., Xiao, Z. J., Zeng, B. P., Li, K., and Tang, Y. L. (2019). Insect behavior and physiological adaptation mechanisms under starvation stress. *Front. Physiol.* 10:163. doi: 10.3389/fphys.2019.00163

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 22 January 2021
doi: 10.3389/fnbeh.2020.612430

Plasticity of Carbohydrate Transport at the Blood-Brain Barrier

Ellen McMullen[1], *Astrid Weiler*[1], *Holger M. Becker*[2] *and Stefanie Schirmeier*[1]*

[1] Department of Biology, Institute of Zoology, Technische Universität Dresden, Dresden, Germany, [2] Division of General Zoology, Department of Biology, University of Kaiserslautern, Kaiserslautern, Germany

Neuronal function is highly energy demanding, requiring efficient transport of nutrients into the central nervous system (CNS). Simultaneously the brain must be protected from the influx of unwanted solutes. Most of the energy is supplied from dietary sugars, delivered from circulation via the blood-brain barrier (BBB). Therefore, selective transporters are required to shuttle metabolites into the nervous system where they can be utilized. The Drosophila BBB is formed by perineural and subperineurial glial cells, which effectively separate the brain from the surrounding hemolymph, maintaining a constant microenvironment. We identified two previously unknown BBB transporters, MFS3 (Major Facilitator Superfamily Transporter 3), located in the perineurial glial cells, and Pippin, found in both the perineurial and subperineurial glial cells. Both transporters facilitate uptake of circulating trehalose and glucose into the BBB-forming glial cells. RNA interference-mediated knockdown of these transporters leads to pupal lethality. However, null mutants reach adulthood, although they do show reduced lifespan and activity. Here, we report that both carbohydrate transport efficiency and resulting lethality found upon loss of MFS3 or Pippin are rescued via compensatory upregulation of Tret1-1, another BBB carbohydrate transporter, in *Mfs3* and *pippin* null mutants, while RNAi-mediated knockdown is not compensated for. This means that the compensatory mechanisms in place upon mRNA degradation following RNA interference can be vastly different from those resulting from a null mutation.

Keywords: blood-brain barrier, carbohydrate transport, compensatory mechanisms, transporter regulation, transport dynamics

OPEN ACCESS

Edited by:
Jimena A. Sierralta, University of Chile, Chile

Reviewed by:
Xavier Decleves, Université Paris Descartes, France
Chul Hoon Kim, Yonsei University, South Korea
Macarena Sánchez-Navarro, Instituto de Parasitología y Biomedicina López-Neyra (IPBLN), Spain

***Correspondence:**
Stefanie Schirmeier
stefanie.schirmeier@tu-dresden.de

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *30 September 2020*
Accepted: *17 December 2020*
Published: *22 January 2021*

Citation:
McMullen E, Weiler A, Becker HM and Schirmeier S (2021) Plasticity of Carbohydrate Transport at the Blood-Brain Barrier. Front. Behav. Neurosci. 14:612430. doi: 10.3389/fnbeh.2020.612430

INTRODUCTION

To allow full functionality the brain requires a lot of energy. Most of the energy used in the nervous system is gained via carbohydrate metabolism. The human adult brain, despite accounting for only 2% of the bodies overall mass, consumes ∼20% of the total oxygen (Mink et al., 1981; Laughlin et al., 1998; Harris et al., 2012). The oxygen is used to metabolize large amounts of glucose. The human brain uses about 90 g of glucose per day; during childhood carbohydrate usage is even higher (Kuzawa et al., 2014). Likewise, the blowfly retina consumes ∼10% of the total ATP produced, which is close to the consumption observed in vertebrates (Laughlin et al., 1998).

Neuronal activity also relies on a tightly regulated extracellular milieu to allow signal conductance. Thus, the brain is shielded from potentially harmful substances, like high, and fluctuating ion concentrations found in circulation, by the blood-brain barrier (BBB). In mammals, the endothelial cells forming brain capillaries build intercellular tight junctions that prevent paracellular diffusion, thereby uncoupling the brain from circulation. In addition, efflux

transporters of the ABC family transport lipophilic, membrane-permeable molecules out of the BBB-forming cells to protect the nervous system form neurotoxic substances (for reviews see Löscher and Potschka, 2005; Koehn, 2020). To allow sufficient nutrient supply, a variety of transport proteins are expressed in the endothelial cells (for a review on metabolite transport at the BBB, see Weiler et al., 2017). In mammals, Glut1 is the main carbohydrate transporter found in the BBB-forming cells. Two differently glycosylated isoforms of Glut1 have been found in the mammalian nervous system, a 45 kDa and a 55 kDa isoform, that show identical transport kinetics (Birnbaum et al., 1986; Sivitz et al., 1989). The 55 kDa isoform is exclusively expressed in the endothelial cells and localizes to the luminal and abluminal membranes, while the 45 kDa isoform is found in astrocytes (Dick et al., 1984; Gerhart et al., 1989; Sivitz et al., 1989; Harik et al., 1990; Farrell and Pardridge, 1991; Maher et al., 1991, 1994; Simpson et al., 2001). In addition to Glut1, sodium glucose cotransporters (SGLTs) are expressed in the BBB upon stress. SGLT1 and SGLT2 have been shown to be expressed upon oxygen deprivation or ischemia, but seem to play a minor role in glucose uptake under normal conditions (Nishizaki et al., 1995; Nishizaki and Matsuoka, 1998; Elfeber et al., 2004; Enerson and Drewes, 2006; Vemula et al., 2009; Yu et al., 2013). Interestingly, the abundance of GLUT1 in the BBB seems to be regulated by hypoglycemia (Boado and Pardridge, 1993; Kumagai et al., 1995; Simpson et al., 1999). However, the regulatory mechanisms that underlie transporter regulation in the mammalian BBB are unknown.

In Drosophila, as in mammals, the brain is shielded from circulation. Here, the BBB is formed by two layers of glial cells, the subperineurial glial cells and the perineurial glial cells that surround the entire nervous system (reviewed in Limmer et al., 2014; Yildirim et al., 2019). Insects possess an open circulatory system, thus all organs, including the brain, are floating in the hemolymph. Therefore, the BBB surrounds the entire nervous system like a sheath. The subperineurial glial cells form intercellular pleated septate junctions that prevent paracellular diffusion (Stork et al., 2008). As in mammals, efflux transporters protect the nervous system from lipid-soluble toxic substances (reviewed in Hindle and Bainton, 2014). To ensure sufficient supply of nutrients and other essential substances to the nervous system a variety of solute carrier family transporter proteins are expressed in the BBB (Desalvo et al., 2014; Weiler et al., 2017). In addition, carbohydrate transporters are required to provide a sufficient supply of carbohydrates to the nervous system. As well as glucose, the non-reducing disaccharide trehalose is found in high quantities in circulation in Drosophila (Wyatt and Kalf, 1957; Lee and Park, 2004; Broughton et al., 2008; Pasco and Léopold, 2012). It has been shown that glucose can be readily taken up into the nervous system (Volkenhoff et al., 2018). Furthermore, the trehalose transporter 1-1 (Tret1-1) is expressed specifically in the perineurial glial cells of the Drosophila BBB (Volkenhoff et al., 2015). Tret1-1 is homologous to mammalian GLUT6 and GLUT8 and has been shown to transport trehalose and glucose (Kanamori et al., 2010; Hertenstein et al., 2020). How carbohydrates are taken up into the subperineurial glial cells of the BBB and the other neural cells in the Drosophila nervous system is currently unknown. There are several homologs of mammalian GLUT1 encoded in the Drosophila genome: the closest homologs are dmGlut1, dmSut1 (sugar transporter 1), dmSut2, dmSut3, and CG7882. dmGlut1 is specifically expressed in neurons and may facilitate carbohydrate uptake there (Volkenhoff et al., 2018). Transcriptomic and *in situ* data for CG7882 and dmSut1-3, indicate very little or no expression in the nervous system, suggesting no major role in neural carbohydrate transport (Weiszmann et al., 2009; Croset et al., 2018; Davie et al., 2018).

Here, we identify two additional carbohydrate transporters expressed in the Drosophila BBB, Major Facilitator Superfamily Transporter 3 (MFS3, CG4726) and Pippin (CG4797). Pippin is expressed in both perineurial and subperineurial glial cells, while MFS3 is expressed in the perineurial glial cells only. Both transporters are able to facilitate uptake of glucose and trehalose when heterologously expressed in *Xenopus laevis* oocytes. Likewise, the simultaneous loss of Pippin and MFS3 in perineural glia, and Pippin alone in subperineurial glia leads to decreased uptake of glucose. Interestingly, loss of either transporter or both transporters does not have any major phenotypic consequences. We demonstrate here that in null mutants compensatory upregulation of Tret1-1 rescues the detrimental effects of acute transporter loss on viability and carbohydrate transport at the BBB, while RNAi-mediated knockdown is not compensated for. In summary, we show that expression of carbohydrate transporters in the Drosophila BBB is highly dynamic and can be adapted to suboptimal circumstances like loss of one transporter. This dynamic adaptation of carbohydrate transport can most likely also be used to spare the nervous system from effects of hypoglycemia or malnutrition.

MATERIALS AND METHODS

Fly Stocks

Flies were kept at room temperature or 25°C. The following fly lines were used w^{-};nrv2-Gal4;nrv2-Gal4, apontic-Gal4, mCherry^{dsRNA} (BL35785), UAS-CD8-GFP, nanos-Cas9^{attP2A} (BL36046) (Bloomington Drosophila stock center), *PBac{681.P.FSVS-1}MFS3*^{CPTI002305} (Kyoto stock center), pippin-dsRNA: w^{1118}; P{GD4548}^{v10598} (VDRC), repo-Gal4; repo-Gal4, alrm-Gal4; alrm-Gal4, gli-Gal4 (Christian Klämbt), moody-Gal4 (Stork et al., 2008), 46F-Gal4 (Hummel et al., 2002), MFS $^{dsRNA4726R-3}$ (Japanese National Institute of Genetics), UAS-FLII^{12}Pglu-700μδ6 (Volkenhoff et al., 2018). The dsRNA-constructs used in the RNAi screen are indicated in **Supplementary Table 1** and were obtained from Bloomington Drosophila stock center, VDRC or the National Institute of Genetics (NIG).

RNA Interference Screen

The RNAi screen was performed as follows: dsRNA lines were crossed to repo-Gal4; repo-Gal4 for panglial dsRNA expression. Crosses were kept at 25°C throughout development. After 2 weeks, viability of the offspring was determined and, if available, 20 female flies were selected, and locomotor capacity was tested in the island assay 1 week later (Schmidt et al., 2012).

RNA Interference Viability Assessment

pippin^{dsRNA10598} and *MFS3* $^{dsRNA4726R-3}$ were first crossed with a panglial driver (repo-Gal4; repo-Gal4). Crosses were performed at 25°C, after 2 weeks the viability of the offspring was determined. This method was repeated using glial subtype specific Gal4 drivers.

Analysis of Sugar Transport Capacity in *Xenopus laevis* Oocytes

Oocytes were isolated from female *Xenopus leavis* frogs as previously described (Becker et al., 2004; Becker, 2014). The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz (23 177-07/A07-2-003 §6). *D. melanogaster pippin* and *Mfs3* were first cloned into a pUASTattBrfa3xHA vector (Rodrigues et al., 2012). Afterwards, the coding sequence with the C-terminal 3xHA-tag was cloned into a pGEM-He-Juel vector. cRNA was synthesized by *in vitro* transcription using the mMESSAGE mMACHINE® T7 Kit (Fisher Scientific). Oocytes of the developmental stages V and VI were injected with 18–20 ng of cRNA. Measurements were conducted 3–6 days after injection. Expression of Pippin-3xHA and MFS3-3xHA on the surface of oocytes was confirmed by immunohistochemistry using an anti HA antibody (Covance).

82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES. Transport capacity for trehalose, glucose and fructose was determined using ^{14}C-labeled sugar in oocyte saline (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.2) at a concentration of 0.15 μCi/100 μl. $^{14}C_{12}$-trehalose was purchased from Hartmann Analytic, Braunschweig (#1249), $^{14}C_6$-glucose and $^{14}C_6$-fructose were purchased from Biotrend, Köln (#MC144-50 and 66 #MC1459-50). For each experiment 95 μl of sugar substrate was added to a batch of 6-8 oocytes and incubated for 60 min. Cells were washed four times with 4 ml of ice-cold oocyte saline. Individual cells were transferred to Pico Prais scintillation vials (Prekin Elmer) and lysed in 200 μl 5% SDS by shaking at 190 rpm for 30 min at 20–28°C. Three milliliters of Rotiszint® eco plus scintillation cocktail (Carl Roth) was added to each vial and scintillation was measured using a Tri-Carb 2810TR scintillation counter (Perkin Elmer).

Transport-mediated substrate uptake was determined by subtracting the uptake in native oocytes from the uptake in Pippin or Mfs3-expressing cells. Significance in difference was calculated using a one-tailed *T*-test or the Mann–Whitney-*U*-test for analysis of non-uniformly distributed samples.

Generation of CRISPR Mutants

Null mutants were generated using CRISPR-mediated homologous recombination. The sgRNA target sequences (*Mfs3*: sgRNA1: GGATATATAGGCCTTACTG, sgRNA2: A ATGAATTCGCTATTCAGGG; *pippin*: sgRNA1: GGTAGCA TATAGTAGGGGC, sgRNA2: CGAGTCTAGGGCGACTAC G) were cloned into a pCFD3-dU6:3gRNA vector (Addgene). To generate the homology construct, the mini-white coding sequence flanked by homology arms (about 1.5 kb upstream and downstream of the coding sequence of either *Mfs3* or *pippin*) was cloned into a pCR-Blunt (Thermofisher) backbone using Golden Gate cloning (Engler et al., 2008) (primers to amplify homology regions from genomic DNA: Mfs3: upstream homology arm: CCACTGCAAATGGGGAAG and CTGCCGAATGCTAAT, downstream homology arm: CCCTGAATAGCGAATTCATTG and GGTCCAAGTGCAGCGTCT; pippin upstream homology arm: TCAATGGCAAAATGACG and CCTATTATCAAGGTG C, downstream homology arm: CGTAGTCGCCCTAGACTC and CCCAAAGCTCAACCAAC). The sgRNA vectors together with the homology construct were injected into nanos-Cas9^{attP2A} embryos to induce homologous recombination.

Generation of Pippin-HA Minigene

The gene locus (including 2.2 kb upstream and 0.5 kb downstream of the coding sequence) of pippin was assembled and C-terminally 3xHA-tagged using Golden Gate cloning (Engler et al., 2008). The assembled locus was inserted into a pUAST attB rfa vector (Stephan et al., 2008) using XbaI and HindIII restriction sites (thereby removing the UAS cassette). The resulting vector was integrated into the fly genome at landing site 86Fb.

Age Matching of Flies for Lifespan and Activity Monitoring

Flies of the deserved genotype were placed in cages with an apple juice agar plate. After 24 h, plates were exchanged and left overnight. Embryos were washed from the plate with PBS and collected using a Pasteur pipette. Embryos were transferred into vials containing standard food. Vials were kept at 18°C for 3 weeks and adult females were collected.

Survival Analysis

Female flies were kept in batches of 20 at 25°C throughout the experiment. Flies were flipped three times a week onto fresh food, deaths were counted. Survival rates were determined using the Kaplan-Meier approach. *P*-values were calculated using Log Rank test.

Analysis of Locomotive Activity (DAM)

Female flies were sorted into vials of 20 and aged at 25°C for 2 or 5 weeks. Single flies were sorted into tubes containing standard food and loaded into a Drosophila activity monitor (DAM). Monitors were placed in an incubator with a 12-h light dark cycle and activity was recorded. The activity over 24 h was determined by the number of beam crosses made by the animal in this time period. *P*-values for significance were determined using Mann–Whitney rank sum test.

Analysis of Escape Response (RING Assay)

Female flies were kept in batches of 20 and aged at 25°C for 2 or 5 weeks. Flies were transferred into negative geotaxis tubes and loaded into the RING apparatus in groups of 10 (Gargano et al., 2005). Tubes were dropped from a height of 30 cm to initiate climbing response. This was repeated five times with a 30 s break between drops to allow flies to recover. The position of the flies in the tubes was captured in digital images and the mean velocity of the flies was determined. *P*-values for significance were determined using Mann–Whitney rank sum test.

Analysis of Circulating Glucose Levels (Glucose GO kit)

Fifteen adult female flies were collected, and a puncture was made in the thorax of each fly using forceps. Flies were then transferred to a 0.5 ml tube (containing a small hole in the base) that was placed in a 1.5 ml Eppendorf tube. Tubes were centrifuged at 13,000 RPM for 5 min at 4°C. The supernatant was collected and transferred to a new Eppendorf tube. Hemolymph was heat-inactivated at 80°C for 10 min to abolish endogenous enzymatic activity, cooled and 25 μl of buffer A (5mM Tris-HCL (pH 6.6), 137 mM NaCl, 2.7 mM KCL) was added. Glucose levels were determined using a Glucose (GO) assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Difference between the control and null mutants was assessed using a one-tailed *t*-test.

Immunohistochemistry

Wandering third instar (L3) larval or adult brains were dissected and stained following standard protocols. Samples were imaged using a Zeiss LSM 880 (Zeiss, Oberkochen, Germany). The following antibodies were used: guinea pig anti-Tret1-1 PA (1:50, Volkenhoff et al., 2015), mouse anti-NC120 (1:2 Hybridoma), rabbit anti-laminin gamma (1:1,000 Abcam A47651), Chicken anti-GFP (1:500, Aves Labs), mouse anti-HA (1:1,000 Covance). Tret1-1 fluorescence was determined by comparing the mean gray values of Tret1-1 staining of null mutants or knockdown animals to the respective control. N is the number of independent experiments; n is the total number of animals analyzed.

Measurement of Glucose Uptake (FRET)

Null mutants or dsRNA lines were crossed with flies expressing *UAS-FLII*^{12}*Pglu-700μδ6* FRET glucose sensor under the control of either apt-Gal4 or moody-Gal4. Larval brains of the desired genotype were dissected in HL3 buffer (70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES; pH 7.2; ca. 350 mOsm) and attached to Poly-D-Lysine-coated coverslips. Samples were then mounted in a custom-made flow through chamber and secured to a Zeiss LSM 880 (Zeiss, Oberkochen, Germany). Buffer exchange was facilitated using a mini-peristaltic pump (MPII, Harvard Apparatus). Fluorescent images were captured using a 20x/1,0 DIC M27 75mm emersion objective (Zeiss, Oberkochen, Germany) directly after dissection. An excitation of 436/25 nm, beam splitter 455 nm, emission 480/40 nm (CFP channel); excitation 436/25 nm, beam splitter 455 nm, emission 535/30 nm (YFP channel) was used. Each brain was imaged in an independent experiment ($n = 8$–12). After 2.5 min HL3 buffer was replaced with 10 mM glucose buffer (HL3 supplemented with glucose; pH 7.2) then exchanged back to HL3 after 9 min. Data analysis was performed by generating a ROI containing the larval brain and calculating the mean gray value, minus background. N is the number of independent experiments; n is the total number of animals analyzed. Statistical regression and analysis was carried out using SigmaPlot software (Jandel). The rate of glucose uptake was calculated by selecting 10 consecutive timepoints at the beginning of the slope. The volume of glucose entering the cell was determined by the mean difference between the baseline and the maximum plateau (10 mM glucose). Statistical differences were calculated using a Mann–Whitney Rank Sum test (pairs). $P < 0.05$ were considered as significant.

RESULTS

CG4797 (Pippin) and Mfs3 Encode Putative Carbohydrate Transporters of the BBB

Previously, we showed that all cell types of the Drosophila nervous system are capable of taking up glucose (Volkenhoff et al., 2018). Since carbohydrates are hydrophilic molecules, they cannot diffuse over the plasma membrane and thus need to be transported. The only two carbohydrate transporters identified in the Drosophila CNS by now, Tret1-1 and Glut1, however, are expressed in the perineurial glial cells or the neurons, respectively. Thus, we set out to identify additional carbohydrate transporters expressed in the Drosophila nervous system. To this end we performed a small, biased RNA interference-based screen, in which we knocked down putative carbohydrate transporters encoded in the Drosophila genome specifically in the glial cells [genes with a predicted sugar transport function according to protein domain annotations from InterPro (http://www.ebi.ac.uk/interpro/) and UniProt (http://www.uniprot.org/), **Supplementary Table 1**]. This screen identified 14 putative carbohydrate transporters required in glial cells, amongst them CG4797 (Pippin) and Major Facilitator Superfamily Transporter 3 (MFS3, CG4762) (**Supplementary Table 1**). We focused our efforts on these two genes. Knockdown of the two genes specifically in glial cells using RNA interference (*pippin*^{dsRNA10598}, *MFS3* $^{dsRNA4726R-3}$) leads to pupal lethality, indicating a function in glial cells (**Figure 1A**). Drosophila MFS3 shows 35% identity to the mouse anion/cation symporter (ACS) Sialin (NCBI protein blast), but the ACS consensus sequence is not fully conserved, indicating that MFS3 does not encode an ACS (Laridon et al., 2008). CG4797 encodes an SLC2 family glucose transporter most homologous to mouse GLUT6 and GLUT8 (NCBI protein blast). This indicates that *CG4797* encodes a carbohydrate transporter; thus, we named the gene *pippin*, after Frodo's friend, whose biggest concern is usually where to get the next meal.

To identify the glial subtype in which the putative transporters are needed, we repeated the knockdown experiments using glial subtype drivers (nrv2-Gal4: cortex glia, ensheathing glia and wrapping glia; alrm-Gal4: astrocyte-like glia cells; Gli-Gal4 or moody-Gal4: subperineurial glial cells; apt-Gal4 or 46F-Gal4: perineurial glial cells). Knockdown of *pippin* in perineurial or subperineurial glial cells led to lethality, while knockdown in any other glial subtype had no phenotypic consequences (**Figure 1A**). In contrast, knockdown of *Mfs3* only led to lethality in perineurial glial cells (**Figure 1A**). This indicates that Pippin is needed in both BBB-forming glial cells, while MFS3 is just essential in the perineurial glial cells. To verify the expression, we took advantage of an existing EYFP protein trap for MFS3 (MFS3^{CPTI002305}). MFS3-EYFP localizes to the perineurial glial cells as seen when co-stained with NC120 (subperineurial glial cells) and laminin (neural lamella) (**Figure 1B**), as suggested from the knockdown experiments. To analyze the localization of *pippin*, we cloned the complete *pippin* locus, including upstream and

A

Glial cell type	Pippin	MFS3
Pan glial	L	L
AG	V	V
CG, EG, WG	V	V
SPG	L	V
PG	L	L *

L: lethal
V: viable
*: some escapers

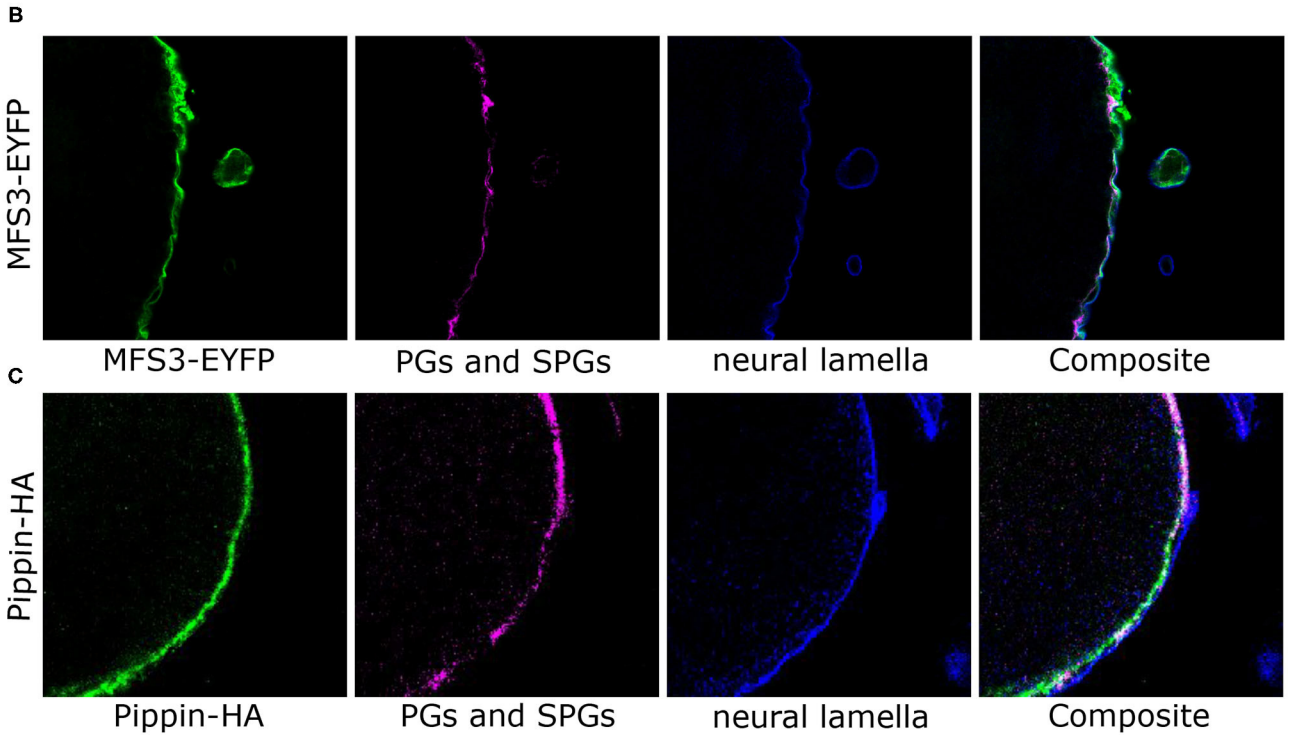

FIGURE 1 | *CG4797* (*pippin*) and *Mfs3* encode putative BBB carbohydrate transporters. **(A)** RNAi interference-mediated silencing of *pippin* (using $CG4797^{dsRNA10598}$) and *Mfs3* $Mfs3^{dsRNA4726R-3}$ in selected glial subtypes using glial subtype-specific Gal4 driver lines (pan glial: repo-Gal4; astrocyte-like glial cells (AG): alrm-Gal4; cortex glia (CG), ensheathing glia (EG), and wrapping glia (WG): nrv2-Gal4; subperineurial glial cells (SPG): Gli-Gal4, moody-Gal4; perineurial glial cells (PG): apt-Gal4, 46F-Gal4). Lethality was observed upon panglial and perineurial glia-specific suppression of *pippin* and *Mfs3*. In addition, lethality also occurred upon subperineurial glia-specific silencing of *pippin*. **(B)** Immunofluorescence staining of a transgenic *Pippin-HA* adult brain [green: Pippin-HA, magenta: NC120 (PGs and SPGs), blue: laminin (neural lamella)]. Pippin is found in subperineurial and perineurial glial cells. **(C)** Immunofluorescence staining of an MFS3-EYFP expressing adult brain [green: MFS3-EYFP, magenta: NC120 (PGs and SPGs), blue: laminin (neural lamella)]. MFS3-EYFP is expressed in the perineurial glial cells.

downstream regions to include all regulatory elements, and fused a C-terminal 3xHA-tag to the coding sequence. Flies carrying this *pippin* minigene construct, show Pippin-HA expression in the perineurial and subperineurial glial cells as assumed from the RNAi-experiments (**Figure 1C**).

Pippin and MFS3 Facilitate Carbohydrate Transport

To analyze whether the two newly identified BBB transporters are indeed able to facilitate carbohydrate uptake into the perineurial and/or subperineurial glial cells, we expressed Drosophila Pippin and MFS3 in *X. laevis* oocytes. To verify expression of the transporters we tagged Pippin and MFS3 with a 3xHA-tag. Both Pippin-HA and MFS3-HA are produced in Xenopus oocytes upon mRNA injection and localize to the membrane (**Figures 2A–C**). To analyze whether the transporters facilitate uptake of carbohydrates found in the Drosophila hemolymph, we incubated the respective oocytes with different concentrations of ^{14}C-labeled glucose, trehalose, or fructose (**Figures 2D,E**). Interestingly, both Pippin and MFS3 facilitate uptake of glucose and trehalose efficiently (**Figures 2D,E**). Fructose, however, is transported at a much lower rate. Since naturally occurring

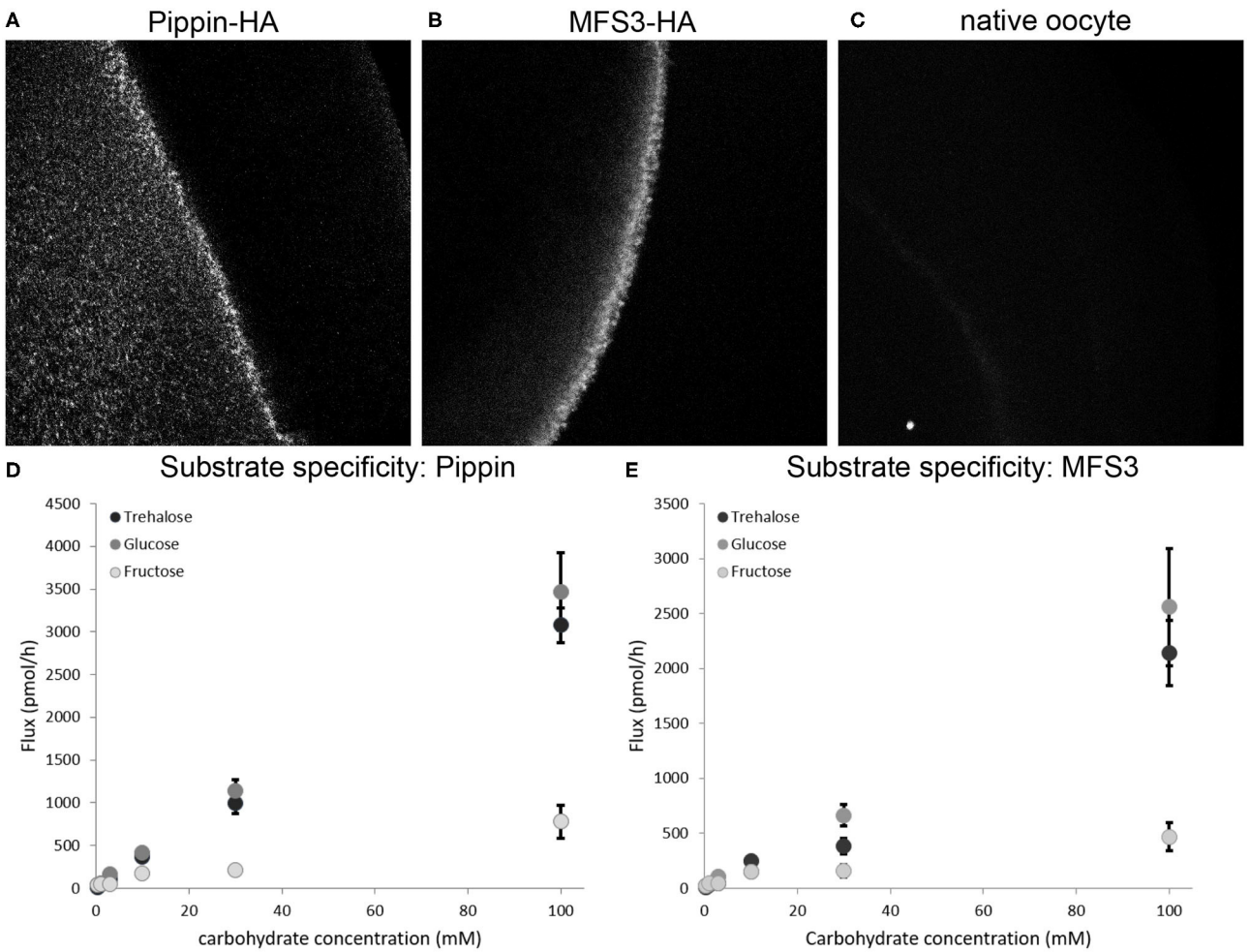

FIGURE 2 | (A,B) Pippin and MFS3 facilitate carbohydrate transport. Heterologous expression of Pippin-HA and MFS3-HA in *Xenopus* oocytes **(A)** Pippin-HA **(B)** MFS3-HA are detectable on the surface of *Xenopus* oocytes (anti-HA immunostaining). **(C)** Native oocytes show no detectable HA staining. **(D,E)** Substrate specific uptake of $^{14}C_{12}$-trehalose, $^{14}C_6$-glucose and $^{14}C_6$-fructose into *Xenopus* oocytes expressing **(D)** Pippin-HA or **(E)** MFS3-HA. Both Pippin and MFS3 show considerable transport capacity for trehalose and glucose, but not for fructose. Shown is the net-flux (flux observed in transporter-expressing oocytes minus flux observed in native oocytes). Values represent means ± standard error, $N = 1$–4.

fructose concentrations in the larva seem to be rather low compared to glucose and trehalose concentrations, it is unlikely that this transport is of physiological relevance (Mishra et al., 2013). These experiments show that the newly identified BBB transporters are indeed carbohydrate transporters. Fitting of the data, shown in **Figures 2D,E**, did not result in reliable K_m or V_{max} values. Therefore, more experiments need be carried out to analyze the transport kinetics of Pippin and MFS3 in Xenopus oocytes.

Pippin and MFS3 Null Mutants Are Viable, but Display Shortened Lifespan and Reduced Locomotor Activity

To further analyze the consequences of loss of Pippin or MFS3, we generated null mutants for both transporters. We used CRISPR-mediated recombination to replace the entire coding sequence of *pippin* or *Mfs3* with a mini-white, thereby creating null mutants (**Supplementary Figure 1**). Interestingly, both $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants are viable and fertile, which contrasts with the phenotype observed upon glia-specific knockdown using RNA interference.

To assess viability of the mutants, we performed lifespan experiments. Indeed, $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants are short-lived compared to control animals (**Figure 3A**). Thus, we analyzed their phenotype in more detail. We assessed the activity of the null mutants after 2 and 5 weeks of age (**Figures 3B,C**). Already at the age of 2 weeks, both $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants are less active than control animals. To distinguish between a reduction in activity to save energy and the incapacity to move, we in addition studied the animals' escape response at the age of 2 and 5 weeks using a rapid iterative negative geotaxis

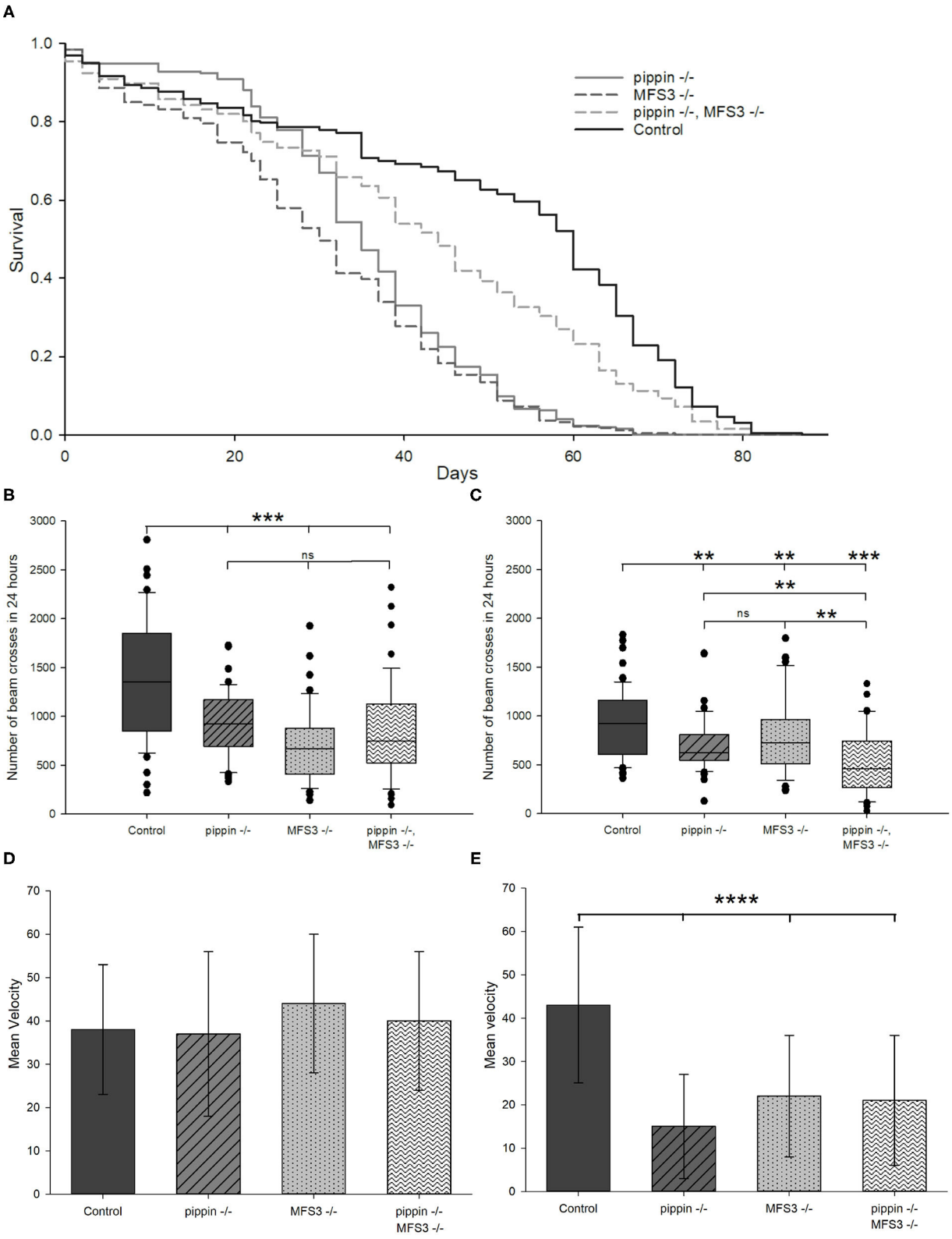

FIGURE 3 | *pippin* and *Mfs3* null mutants are viable, but display shortened lifespan and reduced locomotor activity. **(A)** Survival curves of null mutants and control animals. All mutants show a reduced lifespan compared to control animals, however double *pippin*$^{-/-}$, *Mfs3*$^{-/-}$ mutants live significantly longer than single mutants.

(Continued)

FIGURE 3 | $N = 3$, $n \geq 180$ ($pippin^{-/-}$: $p < 10^{-21}$, $Mfs3^{-/-}$: $p < 10^{-23}$, $pippin^{-/-}$, $Mfs3^{-/-}$: $p < 10^{-4}$; log-rank test). **(B,C)** Activity monitored over 24 h of null mutants and control animals at 2 weeks old **(B)** and 5 weeks **(C)** of age. All mutants show a reduction in activity compared to control animals at 2 weeks of age, however the double mutants show a progressive reduction in activity moving significantly less than the single mutant at 5 weeks of age. $N = 3$; $n \geq 58$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. **(D,E)** Negative geotaxis assay measuring climbing ability as an escape response of 2 **(D)** and 5 **(E)** weeks old flies. Single $pippin^{-/-}$ or $Mfs3^{-/-}$ mutants show no reduction in climbing ability at 2 weeks but show a decrease in activity at 5 weeks of age. $N = 5$; $n \geq 500$; $^{****}p \leq 0.0001$.

(RING) assay (Gargano et al., 2005), in which the flies are put in vials that are tapped on the table. This tapping induces an escape response, where the flies run up the walls of the vial. Depending on their locomotor capabilities the animals climb the walls faster or slower (**Figures 3D,E**). At the age of 2 weeks all genotypes are capable of a fast escape response (**Figure 3D**). Thus, at this age, the animals are able to move as well as control flies, but are nevertheless less active, most likely as a means of saving energy. However, at the age of 5 weeks the velocity of $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants is significantly reduced, indicating progressive loss of locomotor abilities (**Figure 3E**).

Since RNAi-mediated knockdown of *pippin* or *Mfs3* are pupal lethal, but the null mutants are not, we checked for putative compensation of the loss of either transporter. To this end, we created double $pippin^{-/-}$, $Mfs3^{-/-}$ mutants and analyzed their phenotype. Interestingly, $pippin^{-/-}$, $Mfs3^{-/-}$ double mutants are also viable and fertile. Surprisingly, lifespan experiments show that the double mutants live longer than the respective single mutants, albeit not as long as control animals (**Figure 3A**). To establish whether the double mutants move even less than the single mutants to save energy, we analyzed their activity at 2 and 5 weeks of age (**Figures 3B,C**). At 2 weeks of age the double mutant is significantly less active than wildtype control animals but moves as much as either single mutant (**Figure 3B**). In contrast, at the age of 5 weeks, the double mutant animals are significantly less active than either single mutant (**Figure 3C**). To distinguish between an inability to move and an energy-saving reduction of activity, we also analyzed the escape response. Here, the double mutant animals are indistinguishable from single mutant animals at either time point (**Figures 3D,E**). This indicates that the double mutant animals have the ability to move as well as the single mutants. However, they seem to move progressively less over their lifespan, probably to save energy.

Compensatory Increase in Circulating Carbohydrate Levels and Upregulation of Tret1-1 Upon Loss of Pippin or MFS3

To understand why $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants are viable, while glia-specific acute knockdown is lethal, we investigated other possible compensatory mechanisms. Classic carbohydrate transporters, like SLC2 family carbohydrate transporters, are facilitative transporters, which means that they allow uptake of the respective carbohydrate into a cell driven by a concentration gradient. Thus, an increase in the concentration gradient between the extracellular milieu and the cytosol of the respective cell, accelerates carbohydrate uptake into the cell. Therefore, we analyzed circulating glucose levels in $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants to see if deficits in transporter expression might be compensated by elevated circulating sugar levels (**Figure 4A**). Indeed, $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants, as well as the double mutants display elevated hemolymph glucose levels that might facilitate glucose uptake into the brain.

An alternative mode of compensation for the loss of a carbohydrate transporter would be to upregulate an alternative transporter. The only other carbohydrate transporter known to be expressed in the Drosophila BBB, besides Pippin and MFS3, is Tret1-1 (Volkenhoff et al., 2015). As Pippin and MFS3, Tret1-1 facilitates uptake of glucose and trehalose when heterologously expressed in Xenopus oocytes (Kanamori et al., 2010; Hertenstein et al., 2020). To assess if Tret1-1 could compensate for the loss of either Pippin or MFS3 in the null mutants, we stained null mutant L3 brains for Tret1-1 expression in the perineurial glial cells. Interestingly, Tret1-1 expression is strongly increased in the perineurial glial cells of $pippin^{-/-}$ and $Mfs3^{-/-}$ null mutants as well as $pippin^{-/-}$, $Mfs3^{-/-}$ double mutants (**Figures 4B–F**). The increase in Tret1-1 expression is not significantly higher in the double mutants than in the single mutants. Loss of Pippin, however, does not induce compensatory misexpression of Tret1-1 in the subperineurial glial cells (**Supplementary Figure 2**). This increase in Tret1-1 expression in the perineurial glial cells could compensate for a reduction of carbohydrate uptake caused by loss of Pippin and/or MFS3. To understand the difference between RNAi-mediated knockdown of *pippin* and *Mfs3* and the null mutants, we also analyzed Tret1-1 expression in animals with a glia-specific knockdown of either *pippin* or *Mfs3* (**Figures 4G–J**). Indeed, glia-specific knockdown of *pippin* or *Mfs3* does not induce a compensatory upregulation of Tret1-1, potentially explaining the phenotypic differences (**Figures 4G–J**). These findings suggest that null mutations, like a complete loss of the coding region as in the case of our $pippin^{-/-}$ and $Mfs3^{-/-}$ mutants, induce different compensatory mechanisms than constant degradation of the respective mRNAs, as induced by RNA interference. If such differences in compensation are common, this could explain the discrepancies often found between RNAi-mediated knockdown phenotypes and null mutant phenotypes.

Pippin and MFS3 Facilitate Glucose Uptake in the Drosophila BBB

To study if loss of any of the described carbohydrate transporters has an effect on carbohydrate uptake into the BBB-forming glial cells, we analyzed glucose uptake into the respective cells using a genetically-encoded Förster resonance energy transfer (FRET)-based glucose sensor, FLII12Pglu-700μδ6 (Fehr et al., 2003; Takanaga et al., 2008; Volkenhoff et al., 2018). This sensor allows visualizing carbohydrate uptake in living *ex vivo* L3 larval brains (Volkenhoff et al., 2018). To understand the changes in carbohydrate uptake in the different mutants and

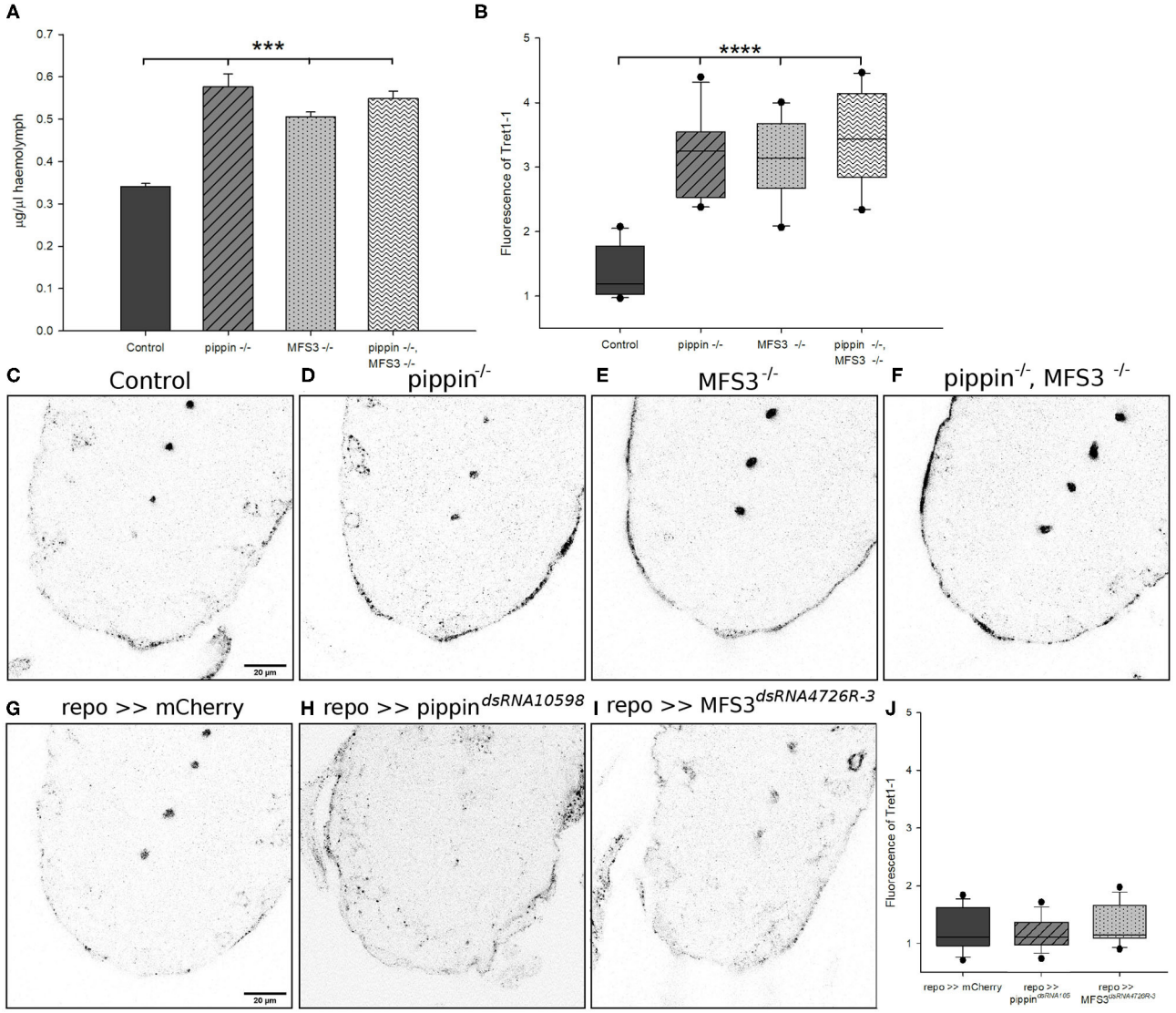

FIGURE 4 | Compensatory increase in circulating carbohydrate levels and upregulation of Tret1-1 upon loss of Pippin or MFS3. **(A)** Glucose levels in the hemolymph of adult flies were analyzed. Both single and double *pippin*$^{-/-}$ and *Mfs3*$^{-/-}$ mutants show an increase in circulating glucose levels. $N = 3$; ***$p \leq 0.001$. **(B–J)** L3 larval brains of null mutants or panglial knockdown of *pippin* and *Mfs3* (*repo-Gal4> CG4797*^{dsRNA10598}; *repo-Gal4>MFS3*$^{dsRNA4726R-3}$) were dissected and stained for Tret1-1 expression. **(B)** Quantification shows the difference in Tret1-1 fluorescence between controls and null mutants. $N \geq 4$; $n = 10–12$; ****$p \leq 0.0001$. **(C)** Control animals show wildtype levels of Tret1-1 in the brain. **(D)** *pippin*$^{-/-}$ mutants **(E)**, *Mfs3*$^{-/-}$ mutants **(F)**, and *pippin*$^{-/-}$, *Mfs3*$^{-/-}$ double mutants show a strong upregulation in Tret1-1 expression in perineurial glial cells. **(G)** *repo>>mCherry*^{dsRNA} serves as a control for panglial knockdown of *pippin* and *Mfs3* by RNA interference. *repo>>mCherry*^{dsRNA} animals show wildtypic levels of Tret1-1. **(H)** Glia-specific knockdown of *pippin* (*repo>>CG4797*^{dsRNA10598}). No increase in Tret1-1 levels can be found. **(I)** Glia-specific knockdown of *Mfs3* (*repo>>MFS3*$^{dsRNA4726R-3}$). No increase in Tret1-1 levels can be seen. **(J)** Quantification of Tret1-1 fluorescence of pan glial knockdown of mCherry, *pippin* or *Mfs3*. $N \geq 4$; $n = 14–15$.

knockdown animals, we expressed the glucose sensor either in the perineurial or subperineurial glial cells of the animals and analyzed glucose uptake capacity (**Figures 5**, **6**). When we analyzed glucose uptake into the perineurial glial cells of animals with a perineurial glia-specific knockdown of *pippin* (using apt-Gal4), we found that the maximum concentration of glucose found in the cells is significantly reduced compared to that found in control animals (expressing mCherry-dsRNA) (**Figures 5A,D**). This indicates that Pippin indeed acts as a carbohydrate transporter in the perineurial glial cells and that loss of Pippin reduces glucose uptake efficiency significantly. Interestingly, the initial glucose uptake rate does not change (**Figure 5C**). Since Pippin is also expressed in the subperineurial glial cells, we also analyzed glucose uptake into those cells. In this case, we expressed the dsRNA-construct as well as the glucose sensor using moody-Gal4. As expected upon loss of a carbohydrate transporter, both the glucose uptake rate as well as the maximal glucose concentration reached in the cells are

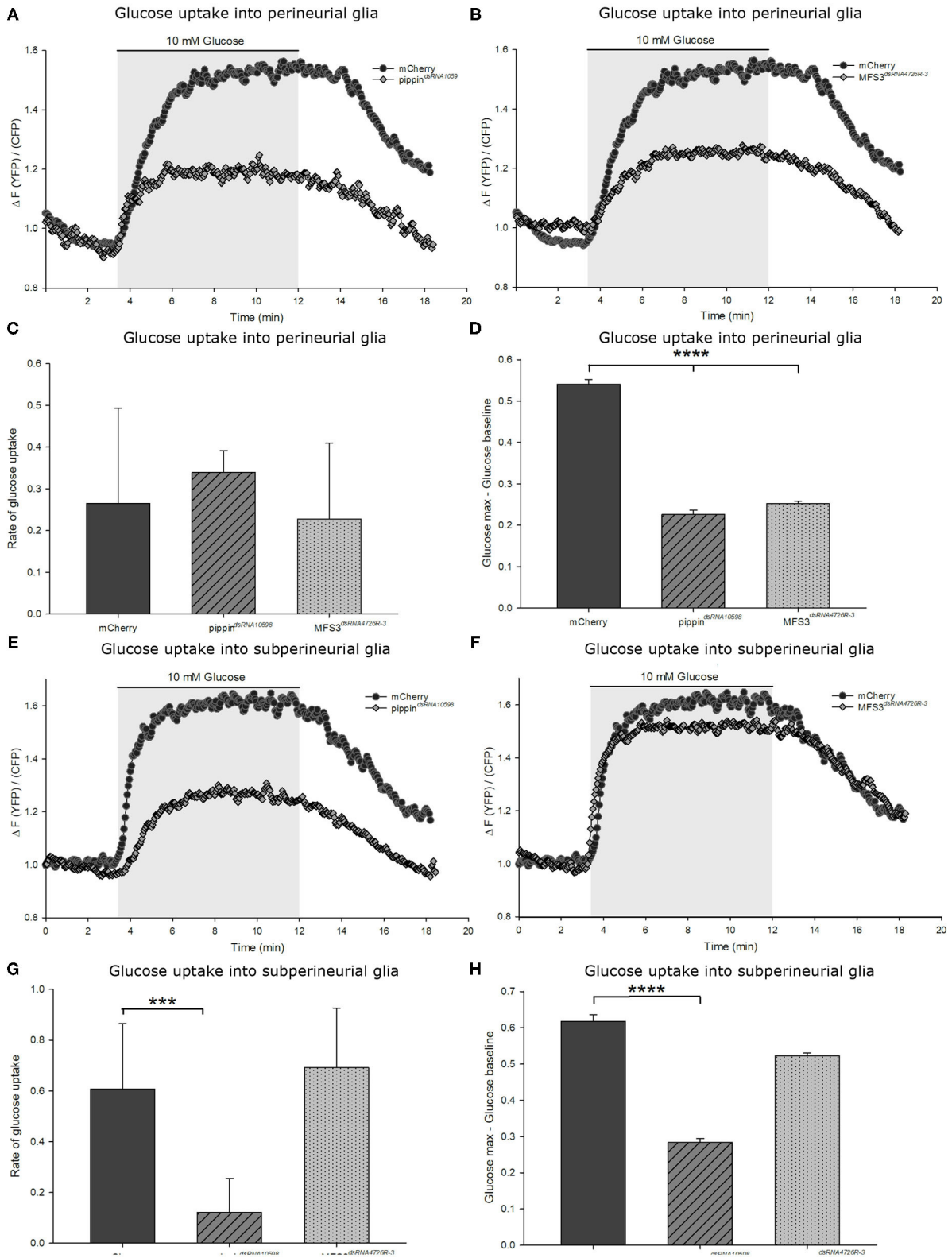

FIGURE 5 | Pippin and MFS3 facilitate glucose uptake into the Drosophila BBB-forming cells. Glucose uptake was measure in *ex vivo* L3 larval brains expressing a genetically encoded glucose sensor (FLII^{12}Pglu-700μδ6). **(A,B)** Example traces of brains with a perineurial knockdown of *pippin* (*apt-Gal4>pippin*^{dsRNA10598}) **(A)** or

(Continued)

FIGURE 5 | *Mfs3* (*apt-Gal4>MFS3$^{dsRNA4726R-3}$*) **(B)**, where the glucose sensor is expressed in the perineural glial cells. **(C)** Quantification of the rate of glucose uptake. The glucose uptake rate as calculated by the steepness of the slope shows no difference between transporter knockdown and control brains. **(D)** Quantification of the maximum glucose concentration in the cells. Shown is the difference between the maximum glucose concentration and the baseline glucose concentration. $N = 8$–12; $^{****}p \leq 0.0001$. **(E–H)** Glucose uptake into the subperineurial glial cells. **(E)** *moody-gal4>pippin^{dsRNA10598}* brains show a reduction in the uptake rate and overall levels of glucose entering the subperineurial glia. **(F)** *moody-Gal4>Mfs3$^{dsRNA4726R-3}$* brains show no difference in glucose uptake rate or maximum glucose levels. **(G,H)** Quantification of the glucose uptake rate and the maximum glucose concentrations reached in the subperineurial glial cells. **(G)** Rate of glucose uptake into the subperineurial glial cells in brains of the different genotypes (subperineurial glial knockdown). Knockdown of *pippin* in the subperineurial glial cells severely reduces glucose uptake rates. **(H)** Maximum glucose levels in subperineurial glial cells expressing FLII^{12}Pglu-700μδ6. Brains in which *pippin* is knocked down in the subperineurial glial cells show a lower maximum glucose level than control brains or *Mfs3* knockdown brains. $n = 8$–12; $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Error bars show standard deviation.

significantly decreased upon loss of Pippin in the subperineurial glial cells (**Figures 5E–H**). Upon RNAi-mediated loss of MFS3, glucose transport is impaired in the perineurial glial cells, but not in the subperineurial glial cells (**Figures 5B–D,F–H**). This fits the expression of MFS3 in the perineurial but not in the subperineurial glial cells and suggests that, indeed, also MFS3 is essential for glucose transport into the perineurial glial cells.

Compensatory Upregulation Rescues Deficits in Carbohydrate Uptake Caused by Loss of Pippin and MFS3

To analyze if compensatory upregulation of Tret1-1 can rescue glucose uptake efficiency in the Drosophila BBB, we analyze glucose uptake into the perineurial and subperineurial glial cells in *pippin*$^{-/-}$ and *Mfs3*$^{-/-}$ null mutant animals. *pippin*$^{-/-}$ and *Mfs3*$^{-/-}$ null mutant animals display wild typic glucose uptake into the perineurial glial cells (**Figures 6A–D**). Thus, compensatory upregulation of Tret1-1 indeed rescues deficits in carbohydrate transport. As expected, glucose uptake into the subperineurial glial cells is indistinguishable from controls in *Mfs3*$^{-/-}$ null mutant animals (**Figure 6G**). In contrast, *pippin*$^{-/-}$ mutant animals show reduced glucose uptake efficiency into the subperineurial glial cells, indicating a lack of compensation in this cell type (**Figures 6F–J**). These results match the expectations, since MFS3 is not expressed in the subperineurial glial cells and subperineurial loss of Pippin is not compensated for by Tret1-1 upregulation.

We also analyzed carbohydrate uptake into the perineurial and subperineurial glial cells of *pippin*$^{-/-}$, *Mfs3*$^{-/-}$ double mutants. Here, we find reduced uptake efficiency in both the perineurial and subperineurial glial cells (**Figures 6C,D,H–J**). The reduction of glucose uptake into the subperineurial glial cells most likely phenocopies the reduction found in *pippin*$^{-/-}$ null mutants, since Pippin is the only transporter expressed in those cells. Interestingly, Tret1-1 upregulation does not seem to be sufficient to rescue glucose transport deficits caused by loss of both Pippin and MFS3 in the perineurial glial cells (**Figures 6C,D**). This finding might explain the differences in lifespan and activity between the single and the double mutant animals.

DISCUSSION

Sufficient nutrient supply to the nervous system is essential for its proper function. Since the main energy source of the brain is sugar, adequate carbohydrate transport over the BBB needs to be ensured. Thus, the vertebrate as well as the insect BBB-forming cells express carbohydrate transporters to facilitate uptake of sugars (Weiler et al., 2017). We report the identification of two additional carbohydrate transporters expressed by the BBB-forming glial cells of Drosophila, Pippin, and MFS3. Both transporters can facilitate uptake of glucose and trehalose (**Figure 2**). RNAi-mediated knockdown of either gene induces pupal lethality, while null mutants are viable and fertile. This discrepancy is found since null mutants show a compensatory upregulation of the carbohydrate transporter Tret1-1. Interestingly, such upregulation cannot be seen in knockdown animals, suggesting that there is a major difference in compensation if the mRNA of a certain gene is produced and then degraded or if there is no mRNA production since the coding sequence has been deleted. Similar discrepancies have been found comparing morpholino-induced knockdown phenotypes vs. mutant phenotypes in zebrafish or siRNA-mediated knockdown phenotypes vs. mutant phenotypes in mice (De Souza et al., 2006; Daude and Westaway, 2012; Kok et al., 2015; Rossi et al., 2015). In zebrafish, for example, Egfl7 null mutants show compensatory upregulation of Emilin genes that rescue Egfl7 loss. Such upregulation is not found in morpholino-knockdowns that thus show a severe vascular defects (Rossi et al., 2015). Interestingly, such compensation might even be conserved in humans. On Iceland individuals with a homozygous loss of Egfl7 were identified, who do not suffer from any symptoms (Sulem et al., 2015). However, the underlying regulatory mechanisms are currently unknown. They are likely to be complex and will probably require much effort to unravel. In any case, such conserved differential compensation should be considered when studying the effects of gene knockdown and null mutations.

The data reported here shows that transporter expression at the BBB can be adapted to suboptimal circumstances, like in this case loss of one transporter. There are two potential mechanisms that could compensate for transporter loss: increase of the concentration gradient at the plasma membrane (circulation vs. cytosol), and compensatory upregulation of another transporter. In case of our null mutant flies we see compensation via both possibilities (**Figure 4**). The animals display higher circulating sugar concentrations that most likely increase the concentration gradient over the plasma membrane and thus make carbohydrate transport via facilitative transporters more efficient, as well as an upregulation of another transporter, Tret1-1. These compensatory mechanisms rescue transport

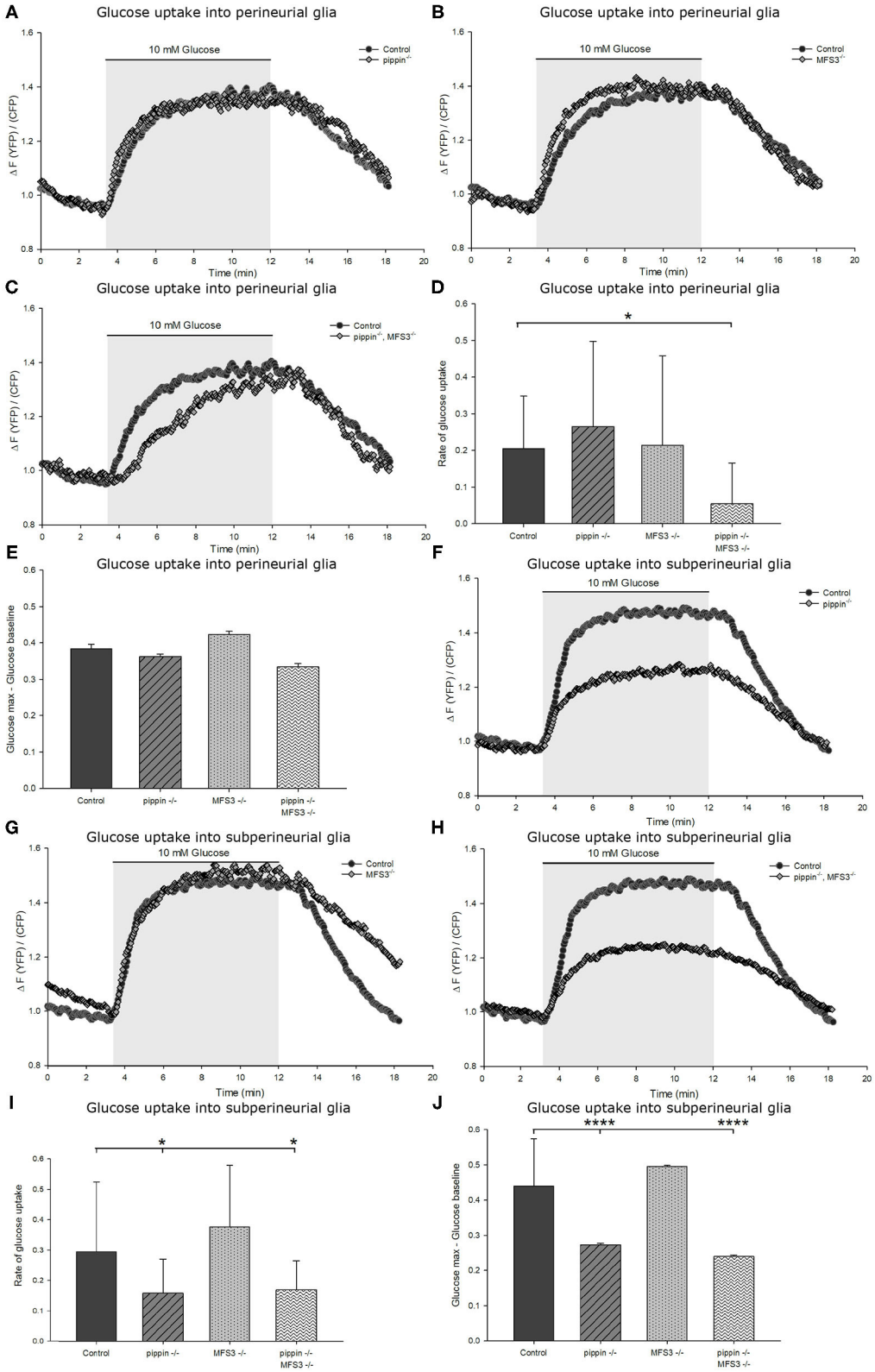

FIGURE 6 | Compensatory upregulation rescues deficits in carbohydrate uptake caused by loss of Pippin and MFS3**. (A–E)** Capacity of glucose uptake in *ex vivo* larval brains of *pippin*$^{-/-}$ or *Mfs3*$^{-/-}$ null single or double mutants expressing FLII^{12}Pglu-700μδ6 in the perineurial glial cells. There is no difference observed in

(Continued)

FIGURE 6 | glucose uptake rate or maximum glucose concentration compared to control brains for *pippin*$^{-/-}$ **(A)** or *Mfs3*$^{-/-}$ **(B)** single mutants. However, the double mutant **(C)** perineurial glial cells take up glucose significantly slower than single mutants or wildtype controls. **(D)** Quantification of the glucose uptake rate into perineurial glial cells in the indicated genotypes. The double mutant perineurial glial cells take up glucose significantly slower than single mutants or wildtype controls. $n = 8–12$; $^{*}p \leq 0.05$. **(E)** Quantification of the difference between maximum glucose concentration and baseline glucose concentration. There is no observable difference between the genotypes. $n = 8–12$. **(F–J)** Glucose uptake and maximum glucose level in the subperineurial glial cells. **(F)** *pippin*$^{-/-}$ mutant brains, but not *Mfs3*$^{-/-}$ mutant brains **(G)** show reduced glucose uptake into the subperineurial glial cells. **(H)** *pippin*$^{-/-}$, *MFS3*$^{-/-}$ double mutant brains show the same phenotype as *pippin*$^{-/-}$ single mutant brains. Both the rate of glucose uptake **(I)** and the maximum glucose concentration **(J)** are reduced in both *pippin*$^{-/-}$ single and *pippin*$^{-/-}$, *MFS3*$^{-/-}$ double mutant brains. $n = 8–12$; $^{*}p \leq 0.05$, $^{****}p \leq 0.0001$. Error bars show standard deviation.

efficiency as seen using a genetically encoded glucose sensor to assess glucose uptake properties (**Figure 6**). The increase in circulating carbohydrates suggests a crosstalk between the nervous system, probably the BBB-forming glial cells, and the periphery to regulate nutrient mobilization most likely form the fat body. That BBB transport defects can regulate systemic metabolism is a very interesting finding that will foster exciting follow up studies to unravel the regulatory mechanisms.

It has been shown previously that Tret1-1 is upregulated upon starvation-induced hypoglycemia (Hertenstein et al., 2020). Together with the data reported here, this suggests that any alteration that leads to insufficient carbohydrate uptake results in compensatory upregulation of transport proteins, most likely to ensure sufficient energy provision to the nervous system. In the case of starvation, Tret1-1 is upregulated via TGF-β signaling (Hertenstein et al., 2020). Since this signaling seems to be induced by hypoglycemia, it is very unlikely that TGF-β signaling is also regulating compensatory upregulation in the case of transporter loss (Hertenstein et al., 2020). Mammalian GLUT1 and SGLT1 and 2 have also been shown to be dynamically upregulated upon hypoglycemia or other insults like oxygen and glucose deprivation as a result of ischemia (Boado and Pardridge, 1993; Kumagai et al., 1995; Nishizaki et al., 1995; Nishizaki and Matsuoka, 1998; Simpson et al., 1999, reviewed in Elfeber et al., 2004; Enerson and Drewes, 2006; Vemula et al., 2009; Yu et al., 2013; Patching, 2016; Rehni and Dave, 2018). Thus, it is very likely that a flexible and dynamic regulation of carbohydrate transporters is an evolutionary conserved mechanism that ensures proper nervous system function even under suboptimal conditions. Since aberrations in carbohydrate availability and transport are thought to be a major cause of severe illnesses, like GLUT1 deficiency syndrome, Alzheimer's disease or epilepsy (Kapogiannis and Mattson, 2011; Arsov et al., 2012; Hoffmann et al., 2013; Koepsell, 2020), it will be very interesting to unravel the regulatory mechanisms that can lead to a compensation of insufficient carbohydrate uptake. Studying these mechanisms might enable us in the future to treat the effects of insufficient carbohydrate uptake at the BBB.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz (23 177-07/A07-2-003 §6).

AUTHOR CONTRIBUTIONS

EM designed and conducted most experiments, helped conceiving the study, and wrote the paper with SS. AW conducted the Xenopus experiments together with HB and did some of the fly experiments. HB designed the Xenopus experiments and helped conducting them. SS conceived the study, assisted in designing and interpreting experiments, and wrote the paper with EM and obtained funding from the DFG. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by grants of the DFG to SS (SFB1009, SCHI 1380/2-1).

ACKNOWLEDGMENTS

We are grateful to C. Klämbt for discussions and critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.612430/full#supplementary-material

REFERENCES

Arsov, T., Mullen, S. A., Damiano, J. A., Lawrence, K. M, Huh, L. L., Nolan, M., et al. (2012). Early onset absence epilepsy: 1 in 10 cases is caused by GLUT1 deficiency. *Epilepsia* 53, e204–e207. doi: 10.1111/epi.12007

Becker, H. M. (2014). "Transport of lactate: characterization of the transporters involved in transport at the plasma membrane by heterologous protein expression in xenopus oocytes," in *Neuromethods*, eds J. Hirrlinger and H. Waagepetersen (New York, NY: Springer), 25–43.

Becker, H. M., Bröer, S., and Deitmer, J. W. (2004). Facilitated lactate transport by MCT1 when coexpressed with the sodium bicarbonate cotransporter (NBC) in xenopus oocytes. *Biophys. J.* 86, 235–247. doi: 10.1016/S0006-3495(04)74099-0

Birnbaum, M. J., Haspel, H. C., and Rosen, O. M. (1986). Cloning and characterization of a cDNA encoding the rat brain glucose-transporter

protein. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5784–5788. doi: 10.1073/pnas.83.16.5784

Boado, R. J., and Pardridge, W. M. (1993). Glucose deprivation causes posttranscriptional enhancement of brain capillary endothelial glucose transporter gene expression via GLUT1 mRNA stabilization. *J. Neurochem.* 60, 2290–2296. doi: 10.1111/j.1471-4159.1993.tb03516.x

Broughton, S., Alic, N., Slack, C., Bass, T., Ikeya, T., Vinti, G., et al. (2008). Reduction of DILP2 in Drosophila triages a metabolic phenotype from lifespan revealing redundancy and compensation among DILPs. *PLoS ONE* 3:e3721. doi: 10.1371/journal.pone.0003721

Croset, V., Treiber, C. D., and Waddell, S. (2018). Cellular diversity in the Drosophila midbrain revealed by single-cell transcriptomics. *Elife* 7:e34550. doi: 10.7554/eLife.34550

Daude, N., and Westaway, D. (2012). Shadoo/PrP (Sprn0/0/Prnp0/0) double knockout mice: more than zeroes. *Prion* 6, 420–424. doi: 10.4161/pri.21867

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, Ł., et al. (2018). A single-cell transcriptome atlas of the aging drosophila brain. *Cell* 174, 982–998.e20. doi: 10.1016/j.cell.2018.05.057

De Souza, A. T., Dai, X., Spencer, A. G., Reppen, T., Menzie, A., Roesch, P. L., et al. (2006). Transcriptional and phenotypic comparisons of Ppara knockout and siRNA knockdown mice. *Nucleic Acids Res.* 34, 4486–4494. doi: 10.1093/nar/gkl609

Desalvo, M. K., Hindle, S. J., Rusan, Z. M., Orng, S., Eddison, M., Halliwill, K., et al. (2014). The Drosophila surface glia transcriptome: evolutionary conserved blood-brain barrier processes. *Front. Neurosci.* 8:346. doi: 10.3389/fnins.2014.00346

Dick, A. P., Harik, S. I., Klip, A., and Walker, D. M. (1984). Identification and characterization of the glucose transporter of the blood-brain barrier by cytochalasin B binding and immunological reactivity. *Proc. Natl. Acad. Sci. U.S.A.* 81, 7233–7237.

Elfeber, K., Köhler, A., Lutzenburg, M., Osswald, C., Galla, H. J., Witte, O. W., et al. (2004). Localization of the Na+-D-glucose cotransporter SGLT1 in the blood-brain barrier. *Histochem. Cell Biol.* 121, 201–207. doi: 10.1007/s00418-004-0633-9

Enerson, B. E., and Drewes, L. R. (2006). The rat blood-brain barrier transcriptome. *J. Cereb. Blood Flow Metab.* 26, 959–973. doi: 10.1038/sj.jcbfm.9600249

Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. *PLoS ONE* 3:e3647. doi: 10.1371/journal.pone.0003647

Farrell, C. L., and Pardridge, W. M. (1991). Blood-brain barrier glucose transporter is asymmetrically distributed on brain capillary endothelial lumenal and ablumenal membranes: an electron microscopic immunogold study. *Proc. Natl. Acad. Sci. U.S.A.* 88, 5779–5783.

Fehr, M., Lalonde, S., Lager, I., Wolff, M. W., and Frommer, W. B. (2003). *In vivo* imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J. Biol. Chem.* 278, 19127–19133. doi: 10.1074/jbc.M301333200

Gargano, J. W., Martin, I., Bhandari, P., and Grotewiel, M. S. (2005). Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in Drosophila. *Exp. Gerontol.* 40, 386–395. doi: 10.1016/j.exger.2005.02.005

Gerhart, D. Z., Levasseur, R. J., Broderius, M. A., and Drewes, L. R. (1989). Glucose transporter localization in brain using light and electron immunocytochemistry. *J. Neurosci. Res.* 22, 464–472. doi: 10.1002/jnr.490220413

Harik, S. I., Kalaria, R. N., Andersson, L., Lundahl, P., and Perry, G. (1990). Immunocytochemical localization of the erythroid glucose transporter: abundance in tissues with barrier functions. *J. Neurosci.* 10, 3862–3872.

Harris, J. J., Jolivet, R., and Attwell, D. (2012). Synaptic energy use and supply. *Neuron* 75, 762–777. doi: 10.1016/j.neuron.2012.08.019

Hertenstein, H., McMullen, E., Weiler, A., Volkenhoff, A., Becker, H. M., and Schirmeier, S. (2020). Starvation-induced regulation of carbohydrate transport at the blood-brain barrier is TGF-β-signaling dependent. *bioRxiv*. doi: 10.1101/2020.09.21.306308

Hindle, S. J., and Bainton, R. J. (2014). Barrier mechanisms in the Drosophila blood-brain barrier. *Front. Neurosci.* 8:414. doi: 10.3389/fnins.2014.00414

Hoffmann, U., Sukhotinsky, I., Eikermann-Haerter, K., and Ayata, C. (2013). Glucose modulation of spreading depression susceptibility. *J. Cereb. Blood Flow Metab.* 33, 191–195. doi: 10.1038/jcbfm.2012.132

Hummel, T., Attix, S., Gunning, D., and Zipursky, S. L. (2002). Temporal control of glial cell migration in the Drosophila eye requires gilgamesh, hedgehog, and eye specification genes. *Neuron.* 33, 193–203. doi: 10.1016/S0896-6273(01)00581-5

Kanamori, Y., Saito, A., Hagiwara-Komoda, Y., Tanaka, D., Mitsumasu, K., Kikuta, S., et al. (2010). The trehalose transporter 1 gene sequence is conserved in insects and encodes proteins with different kinetic properties involved in trehalose import into peripheral tissues. *Insect Biochem. Mol. Biol.* 40, 30–37. doi: 10.1016/j.ibmb.2009.12.006

Kapogiannis, D., and Mattson, M. P. (2011). Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. *Lancet. Neurol.* 10, 187–198. doi: 10.1016/S1474-4422(10)70277-5

Koehn, L. (2020). ABC efflux transporters at blood-central nervous system barriers and their implications for treating spinal cord disorders. *Neural Regen. Res.* 15, 1235–1242. doi: 10.4103/1673-5374.272568

Koepsell, H. (2020). Glucose transporters in brain in health and disease. *Pflügers Arch. Eur. J. Physiol.* 472, 1299–1343. doi: 10.1007/s00424-020-02441-x

Kok, F. O., Shin, M., Ni, C. W., Gupta, A., Grosse, A. S., VanImpel, A., et al. (2015). Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev. Cell.* 32, 97–108. doi: 10.1016/j.devcel.2014.11.018

Kumagai, A. K., Kang, Y.-S., Boado, R. J., and Pardridge, W. M. (1995). Upregulation of blood-brain barrier GLUT1 glucose transporter protein and mRNA in experimental chronic hypoglycemia. *Diabetes* 44, 1399–1404. doi: 10.2337/diab.44.12.1399

Kuzawa, C. W., Chugani, H. T., Grossman, L. I., Lipovich, L, Muzik, O., Hof, P. R., et al. (2014). Metabolic costs and evolutionary implications of human brain development. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13010–13015. doi: 10.1073/pnas.1323099111

Laridon, B., Callaerts, P., and Norga, K. (2008). Embryonic expression patterns of Drosophila ACS family genes related to the human sialin gene. *Gene Expr. Patterns* 8, 275–283. doi: 10.1016/j.gep.2007.12.003

Laughlin, S. B., de Ruyter Van Steveninck, R. R., and Anderson, J. C. (1998). The metabolic cost of neural information. *Nat. Neurosci.* 1, 36–41. doi: 10.1038/236

Lee, G., and Park, J. H. (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. doi: 10.1534/genetics.167.1.311

Limmer, S., Weiler, A., Volkenhoff, A., Babatz, F., and Klämbt, C. (2014). The Drosophila blood-brain barrier: development and function of a glial endothelium. *Front. Neurosci.* 8:365. doi: 10.3389/fnins.2014.00365

Löscher, W., and Potschka, H. (2005). Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx* 2, 86–98. doi: 10.1602/neurorx.2.1.86

Maher, F., Davies-Hill, T. M., Lysko, P. G., Henneberry, R. C., and Simpson, I. A. (1991). Expression of two glucose transporters, GLUT1 and GLUT3, in cultured cerebellar neurons: evidence for neuron-specific expression of GLUT3. *Mol. Cell. Neurosci.* 2, 351–360. doi: 10.1016/1044-7431(91)90066-W

Maher, F., Vannucci, S. J., and Simpson, I. A. (1994). Glucose transporter proteins in brain. *FASEB J.* 8, 1003–1011. doi: 10.1096/fasebj.8.13.7926364

Mink, J. W., Blumenschine, R. J., and Adams, D. B. (1981). Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 241, R203–R212. doi: 10.1152/ajpregu.1981.241.3.r203

Mishra, D., Miyamoto, T., Rezenom, Y. H., Broussard, A., Yavuz, A., Slone, J., et al. (2013). The molecular basis of sugar sensing in drosophila larvae. *Curr. Biol.* 23, 1466–1471. doi: 10.1016/j.cub.2013.06.028

Nishizaki, T., Kammesheidt, A., Sumikawa, K., Asada, T., and Okada, Y. (1995). A sodium- and energy-dependent glucose transporter with similarities to SGLT1-2 is expressed in bovine cortical vessels. *Neurosci. Res.* 22, 13–22. doi: 10.1016/0168-0102(95)00876-U

Nishizaki, T., and Matsuoka, T. (1998). Low glucose enhances Na+/glucose transport in bovine brian artery endothelial cells. *Stroke* 29, 844–849. doi: 10.1161/01.STR.29.4.844

Pasco, M. Y., and Léopold, P. (2012). High sugar-induced insulin resistance in Drosophila relies on the lipocalin Neural Lazarillo. *PLoS ONE* 7:e36583. doi: 10.1371/journal.pone.0036583

Patching, S. G. (2016). Glucose transporters at the blood-brain barrier: function, regulation and gateways for drug delivery. *Mol. Neurobiol.* 54, 1046–1077. doi: 10.1007/s12035-015-9672-6

Rehni, A. K., and Dave, K. R. (2018). Impact of hypoglycemia on brain metabolism during diabetes. *Mol. Neurobiol.* 55, 9075–99088. doi: 10.1007/s12035-018-1044-6

Rodrigues, F., Thuma, L., and Klämbt, C. (2012). The regulation of glial-specific splicing of Neurexin IV requires HOW and Cdk12 activity. *Development* 139, 1765–1776. doi: 10.1242/dev.074070

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., et al. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 524, 230–233. doi: 10.1038/nature14580

Schmidt, I., Thomas, S., Kain, P., Risse, B., Naffin, E., and Klämbt, C. (2012). Kinesin heavy chain function in Drosophila glial cells controls neuronal activity. *J. Neurosci*. 32, 7466–7476. doi: 10.1523/JNEUROSCI.0349-12.2012

Simpson, I. A., Appel, N. M., Hokari, M., Oki, J., Holman, G. D., Maher, F., et al. (1999). Blood-brain barrier glucose transporter: effects of hypo- and hyperglycemia revisited. *J. Neurochem*. 72, 238–247. doi: 10.1046/j.1471-4159.1999.0720238.x

Simpson, I. A., Vannucci, S. J., DeJoseph, M. R., and Hawkins, R. A. (2001). Glucose transporter asymmetries in the bovine blood-brain barrier. *J. Biol. Chem*. 276, 12725–12729. doi: 10.1074/jbc.M010897200

Sivitz, W., DeSautel, S., Walker, P. S., and Pessin, J. E. (1989). Regulation of the glucose transporter in developing rat brain. *Endocrinology* 124, 1875–1880. doi: 10.1210/endo-124-4-1875

Stephan, R., Grevelhörster, A., Wenderdel, S., Klämbt, C., and Bogdan, S. (2008). Abi induces ectopic sensory organ formation by stimulating EGFR signaling. *Mech. Dev*. 125, 183–195 doi: 10.1016/j.mod.2007.12.002

Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R. J., and Klambt, C. (2008). Organization and function of the blood brain barrier in Drosophila. *J. Neurosci.* 28, 587–597. doi: 10.1523/JNEUROSCI.4367-07.2008

Sulem, P., Helgason, H., Oddson, A., Stefansson, H., Gudjonsson, S. A., Zink, F., et al. (2015). Identification of a large set of rare complete human knockouts. *Nat. Genet*. 47,448–452. doi: 10.1038/ng.3243

Takanaga, H., Chaudhuri, B., and Frommer, W. B. (2008). GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim. Biophys. Acta Biomembr*. 1778, 1091–1099. doi: 10.1016/j.bbamem.2007.11.015

Vemula, S., Roder, K. E., Yang, T., Bhat, G. J., Thekkumkara, T. J., and Abbruscato, T. J. (2009). A functional role for sodium-dependent glucose transport across the blood-brain barrier during oxygen glucose deprivation. *J. Pharmacol. Exp. Ther*. 328, 487–495. doi: 10.1124/jpet.108.146589

Volkenhoff, A., Hirrlinger, J., Kappel, J. M., Klämbt, C., and Schirmeier, S. (2018). Live imaging using a FRET glucose sensor reveals glucose delivery to all cell types in the Drosophila brain. *J. Insect Physiol*. 106, 55–64. doi: 10.1016/j.jinsphys.2017.07.010

Volkenhoff, A., Weiler, A., Letzel, M., Stehling, M., Klämbt, C., and Schirmeier, S. (2015). Glial glycolysis is essential for neuronal survival in Drosophila. *Cell Metab*. 22, 437–447. doi: 10.1016/j.cmet.2015.07.006

Weiler, A., Volkenhoff, A., Hertenstein, H., and Schirmeier, S. (2017). Metabolite transport across the mammalian and insect brain diffusion barriers. *Neurobiol. Dis*. 107, 15–31. doi: 10.1016/j.nbd.2017.02.008

Weiszmann, R., Hammonds, A. S., and Celniker, S. E. (2009). Determination of gene expression patterns using high-throughput RNA *in situ* hybridization to whole-mount Drosophila embryos. *Nat. Protoc.* 4, 605–618. doi: 10.1038/nprot.2009.55

Wyatt, G. R., and Kalf, G. F. (1957). The chemistry of insect hemolymph: II. Trehalose and other carbohydrates. *J. Gen. Physiol.* 40, 833–847.

Yildirim, K., Petri, J., Kottmeier, R., and Klämbt, C. (2019). Drosophila glia: few cell types and many conserved functions. *Glia* 67, 5–26. doi: 10.1002/glia.23459

Yu, A. S., Hirayama, B. A., Timbol, G., Liu, J., Diez-Sampedro, A., Kepe, V., et al. (2013). Regional distribution of SGLT activity in rat brain *in vivo*. *Am. J. Physiol. Cell Physiol*. 304, C240–C247. doi: 10.1152/ajpcell.00317.2012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 31 March 2021
doi: 10.3389/fnbeh.2021.628662

Transcriptome Analysis of NPFR Neurons Reveals a Connection Between Proteome Diversity and Social Behavior

Julia Ryvkin[1†], Assa Bentzur[1†], Anat Shmueli[1], Miriam Tannenbaum[1], Omri Shallom[1], Shiran Dokarker[1], Jennifer I. C. Benichou[1], Mali Levi[1] and Galit Shohat-Ophir[1,2‡]*

[1] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel, [2] The Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat Gan, Israel

OPEN ACCESS

Edited by:
Jimena A. Sierralta, University of Chile, Chile

Reviewed by:
Hans A. Hofmann, University of Texas at Austin, United States
Ivan V. Brak, State Scientific Research Institute of Physiology and Basic Medicine, Russia

***Correspondence:**
Galit Shohat-Ophir
galit.ophir@biu.ac.il

[†] These authors have contributed equally to this work

[‡] Lead contact

Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: 12 November 2020
Accepted: 16 February 2021
Published: 31 March 2021

Citation:
Ryvkin J, Bentzur A, Shmueli A, Tannenbaum M, Shallom O, Dokarker S, Benichou JIC, Levi M and Shohat-Ophir G (2021) Transcriptome Analysis of NPFR Neurons Reveals a Connection Between Proteome Diversity and Social Behavior. Front. Behav. Neurosci. 15:628662. doi: 10.3389/fnbeh.2021.628662

Social behaviors are mediated by the activity of highly complex neuronal networks, the function of which is shaped by their transcriptomic and proteomic content. Contemporary advances in neurogenetics, genomics, and tools for automated behavior analysis make it possible to functionally connect the transcriptome profile of candidate neurons to their role in regulating behavior. In this study we used *Drosophila melanogaster* to explore the molecular signature of neurons expressing receptor for neuropeptide F (NPF), the fly homolog of neuropeptide Y (NPY). By comparing the transcription profile of NPFR neurons to those of nine other populations of neurons, we discovered that NPFR neurons exhibit a unique transcriptome, enriched with receptors for various neuropeptides and neuromodulators, as well as with genes known to regulate behavioral processes, such as learning and memory. By manipulating RNA editing and protein ubiquitination programs specifically in NPFR neurons, we demonstrate that the proper expression of their unique transcriptome and proteome is required to suppress male courtship and certain features of social group interaction. Our results highlight the importance of transcriptome and proteome diversity in the regulation of complex behaviors and pave the path for future dissection of the spatiotemporal regulation of genes within highly complex tissues, such as the brain.

Keywords: ***Drosophila melanogaster*, behavior, motivation, reward, social interaction**

INTRODUCTION

Behavior is the result of an orchestrated neuronal activity, where a complex collection of cell types assembled into circuits process external and internal information into a consistent motor output that ultimately promotes survival and reproduction (Bargmann and Marder, 2013; Anderson, 2016; Chen and Hong, 2018; Datta et al., 2019). The immense complexity and heterogeneity of the nervous system results from molecular programs that dictate the range of expressed proteins, including their localization and function, giving rise to cell populations with diverse anatomy, physiology, connectivity, and functional roles (Cabrera, 1992; Franco and Müller, 2013; Mo et al., 2015; Chen et al., 2017; Gray and Spiegel, 2019; Mickelsen et al., 2019; Sapiro et al., 2019; Winnubst et al., 2019; Xu et al., 2020). This diversity poses a challenge when trying to functionally

associate neurons to particular behaviors but can be resolved by genetically dividing the brain into discrete cell types and subsequently study their anatomy, connectivity, molecular architecture and physiology (Henry et al., 2012; Croset et al., 2018; Agrawal et al., 2019; Shih et al., 2019; Davis et al., 2020). Recent advances in targeting increasingly smaller sub populations of neurons, together with tools to manipulate their activity, make it possible to connect the function of neurons to their identity, thus facilitating greater understanding of the molecular underpinning of brain development and mechanisms that regulate complex behaviors (Venken et al., 2011; Yizhar, 2012; Waddell et al., 2015; Abruzzi et al., 2017; Anpilov et al., 2020). This can be useful when studying the function of neurons that control complex behaviors, particularly those that are regulated by motivation such as foraging, food and water consumption, mating and various forms of social interactions (Goodson and Bass, 2001; Desai et al., 2013; Arias-Carrión et al., 2014; Anderson, 2016; LeGates et al., 2018; Parker et al., 2019; Senapati et al., 2019; Sternson, 2020).

The fruit fly *Drosophila melanogaster* is a useful model organism for investigating the genetic underpinnings of motivational behaviors, owing to variety of tools for neuro-genetic manipulations, together with the fact that flies exhibit several forms of behaviors that are shaped by motivation (Wu et al., 2003; Certel et al., 2007; Krashes et al., 2009; Aso et al., 2014; Perisse et al., 2016; Bentzur et al., 2018; Pu et al., 2018; Zer-Krispil et al., 2018; Zhang et al., 2018; Senapati et al., 2019; Wilinski et al., 2019; Thornquist et al., 2020). One of the systems that encodes internal states and dictates motivational drives, and consequently, behavioral choices in *Drosophila* is the Neuropeptide F/Neuropeptide F receptor (NPF/R) (Wen et al., 2005; Wu et al., 2005; Lingo et al., 2007; Xu et al., 2010; Ida et al., 2011; Beshel and Zhong, 2013; He et al., 2013a; Kacsoh et al., 2013; Erion et al., 2016; Kim et al., 2017; Liu et al., 2019; Tsao et al., 2018; Zhang et al., 2019). Similar to its mammalian homolog NPY, *Drosophila* NPF system regulates male sexual behavior (Liu et al., 2019; Zhang et al., 2019), ethanol consumption and sensitivity (Wen et al., 2005; Shohat-Ophir et al., 2012; Kacsoh et al., 2013), feeding behavior (Kim et al., 2017; Tsao et al., 2018), appetitive memory (Krashes et al., 2009), arousal and sleep (He et al., 2013b; Chung et al., 2017). While most studies in the field focused on NPF-producing neurons, less is known about NPF-receptor neurons and the molecular basis for their diverse functions.

In this work, we investigated the transcriptome of NPFR neurons, comparing it to those of nine other neuronal populations, and discovered that NPFR neurons have a unique signature that is enriched in neuropeptide and neuromodulator receptors. We tested the functional relevance of their transcriptome and proteome by disturbing two molecular systems that regulate large number of cellular targets: RNA editing and protein ubiquitination. Adenosine-to-inosine (A-to-I) RNA editing, is a cellular mechanism that generates transcriptomic and proteomic diversity by recoding certain adenosines within pre-mRNA sequences into inosines, leading to a variety of consequences that include amino acid sequence changes in proteins (Keegan et al., 2005; Stapleton et al., 2006; Jepson and Reenan, 2009; Rosenthal and Seeburg, 2012; Maldonado et al., 2013; Li et al., 2014). Protein ubiquitination is a highly regulated post-translational cellular mechanism that shapes protein abundance and function (Schnell and Hicke, 2003; Callis, 2014). Our results show that manipulating the transcriptome and proteome of NPFR neurons enhance certain aspects of male-female and male-male interactions, suggesting a role for NPFR neurons in restraining social and sexual behaviors.

RESULTS

To explore the connection between transcriptional programs in NPFR neurons and behavior, we used a recently generated dataset from our lab, that was used to profile spatial RNA editing across the fly brain (Sapiro et al., 2019). The dataset consists of RNA sequences from several neuronal populations in the brain that were obtained by immunoprecipitation of genetically tagged nuclei (INTACT method) (Sapiro et al., 2019). The dataset comprises of nine neuronal populations that are known to regulate various motivational behaviors: neuromodulatory neurons, including dopaminergic neurons (*TH-Gal4* marking 515 cells), octopaminergic neurons (the fly homolog of mammalian norepinephrine, *Tdc2-Gal4* marking 265 cells), serotonergic neurons (*TRH-Gal4* marking 989 cells), Corazonin neurons (structurally related to mammalian GnRHs, *CRZ-Gal4* marking 300 cells), NPF neurons (*NPF-Gal4* marking 41 cells), Dh44 neurons (*CRF* ortholog, *DH44-Gal4* marking 6 cells) and neurons, which express receptors for NPF (*NPFR-Gal4* marking 100 cells). Two additional population of neurons, that harbor larger number of cells were analyzed; mushroom body neurons involved in learning and memory (*OK107-Gal4* marking 2,000 cells), and fruitless-expressing neurons, that are known to regulate sex specific behavior (*Fru-Gal4* marking 1,454 cells).

Analysis of transcriptomic datasets offers a way to compare the levels of transcription per gene across different cell populations, or within the same cells under different conditions. To explore the transcriptomic landscape of NPFR cells, we took two complementary approaches: pairwise comparison of gene expression profiles between each neuronal population and all neurons (pan-neuronal driver, *Elav-Gal4*); and pairwise comparison of gene expression profiles between each neuronal population and NPFR neurons.

The Transcriptomes of NPFR, Fru, and OK107 Neurons Are Most Similar to Those of the General Neuronal Population

Starting with the first approach, we generated a list of differentially expressed genes (DEGs) for each neuronal population with significantly different expressions than those in all neurons (greater than twofold change compared to the expression in ElaV and have an adjusted *p*-value smaller than 0.05) (**Figures 1A,B** and **Supplementary Table 1**). Since the number of DEGs in each neuronal population represents the difference in transcriptome between this population and all

neurons, we expected that the more specific the transcriptome in a population is, the more unique it will be compared to ElaV. Interestingly, DH44- and NPF-expressing neurons displayed the largest number of DEGs (2,758 and 1,990, respectively), while OK107- and NPFR-expressing neurons presented the smallest number of DEGs (40 and 42, respectively) (**Figure 1A**). Most DEGs in OK107, NPFR, TRH, Tdc2, and TH were found to be over-expressed compared to those in ElaV, while most DEGs in Fru neurons were under-expressed compared to those in ElaV (**Figure 1A**). Hierarchical clustering analysis of average normalized reads for all the DEGs between the different neuronal populations (union of all cell type specific DEGs) confirmed this finding: DH44 cells were clustered apart from all other populations, followed by NPF cells (**Figure 1B**); in addition, OK107 cells clustered closest to ElaV, and NPFR neurons are located next to the OK107-ElaV cluster (**Figure 1B**). Altogether, this suggests that the transcriptomes of DH44- and NPF-expressing cells are the most unique, whereas those of OK107-, and NPFR-expressing neurons resemble the general neuronal population.

Shared DEGs Between Neuronal Populations Reveal a Complex Pattern

Given the partial anatomical overlap between several neuronal populations in our dataset (Certel et al., 2010; Andrews et al., 2014; Shao et al., 2017; Croset et al., 2018; Davie et al., 2018; Liu et al., 2019), we next asked whether some DEGs are shared across different neuronal populations. Enrichment or depletion of the same genes in more than one population suggests that these neuronal populations share differences from the general population, and/or that some of their neurons overlap. Searching for DEGs that are shared by different neuronal populations, we did not document any genes that are shared by all nine populations (**Figure 2A**, **Table 1** and **Supplementary Table 2**). When comparing shared DEGs across 8-3 neuronal populations, only a single gene (CG9466) was found to be shared by eight populations, exhibiting similar pattern of enrichment in all eight populations (**Figure 2A** and **Supplementary Table 2**). The long non-coding RNA CR45456 is another example for a transcript that is enriched in six neuronal populations when compared to its expression in ElaV (**Figure 2A** and **Supplementary Table 2**).

Two neuronal populations shared the largest number of DEGs with all other populations: NPF and DH44 (1,253 genes in 67 comparisons and 1,309 genes in 56 comparisons, respectively, **Figure 2A**, **Table 1**, and **Supplementary Table 2**). The number of DEGs varied across all populations by two orders of magnitude (**Figure 1A**), increasing the odds for shared DEGs in certain populations due to the overall number of DEGs and not because they were expressed within overlapping neurons. To control for this, we normalized the number of shared DEGs by the total number of DEGs in each population and found a reduction in the variation of the numbers of shared DEGs between populations (**Figure 2B**). This finding implies that the probability of sharing a DEGs is similar across different populations, and that the more DEGs a population has, the higher the probability that some will be shared, emphasizing the need to use other criteria to determine whether two populations share similar transcriptional patterns or just mutual neurons.

Interestingly, and although Fru shares neurons with several other populations, such as NPF and Tdc2 (Certel et al., 2010; Andrews et al., 2014; Liu et al., 2019), as evidenced by the enrichment of Tbh (Tyramine β hydroxylase) in Fru and Tdc2 neurons, most DEGs in Fru neurons were depleted compared to their expressions in other populations (**Figure 2A**). Striking examples are Cyp6a20, Glutactin, Tequila, and quasimodo, which support the notion that most Fru neurons are distinct from the rest of the analyzed neuronal populations. In addition, CRZ-, DH44- and NPF-expressing neurons shared similar expression patterns of groups of genes that shape neurophysiology, possibly due to all of them being peptidergic neurons. Examples of these neurophysiology-associated genes include: the shared patterns of ion channels, such as NaCP6OE (Voltage gated Na channel), Teh1 (TipE homolog 1 sodium transport regulation), genes involved in neuronal signaling, such as Neuroligin 3 (synaptic adhesion molecule), beat-1C (beaten path 1C axon guidance), Tehao (Toll signaling); and the shared patterns of receptors, such as nicotinic acetylcholine receptor alpha3 and 6, Toll6 (Toll-like receptor family), IR47a + b (ionotropic receptor a + b), GluR1A (Glutamate receptor 1A), and Oct-beta-3R (Octopamine receptor beta 3).

The shared DEGs between NPFR neurons and other neuronal populations illuminated a complex pattern of 21 genes that are similarly and oppositely expressed (**Figure 2A**). The two most differentially regulated genes were hamlet (ham) and spineless (ss), both highly enriched in NPFR neurons and depleted in all other neuronal populations (**Figures 2C,D**). Examining shared DEGs in comparison to NPF neurons revealed two more genes with opposite expression that are enriched in NPFR (Octopamine-Tyramine Receptor and CG34353) and 11 DEGs with similar expression. NPFR neurons also displayed expression patterns of DEGs different from those in DH44 neurons, with four oppositely expressed DEGs, including ham, ss, CG34353 and CG12344, and similarly expressed genes, like CG9466, CR45456, mt:srRNA, CG10175, CG34189, Listericin, CHKOV1, CG12239, CG8713, CG31705, CG3921, CR43717, and CG33093 (**Figure 2A**). Interestingly, Octopamine-Tyramine Receptor (Oct-TyrR), which is regulated by feeding and mediates appetitive changes in locomotion (Schützler et al., 2019), was enriched in NPFR-expressing neurons and depleted in NPF- and CRZ-expressing neurons (**Figure 2A**). Furthermore, ss, which encodes a transcription factor regulating female receptivity to male courtship (Mcrobert, 1991), was enriched in NPFR neurons. This data suggests that while it is possible that some of the NPF and DH44 neurons share neuronal subpopulations with NPFR, many of the neurons in these populations do not overlap.

Next, we analyzed the relative expression patterns of NPFR neurons using the second pairwise approach, comparing NPFR neurons to each of the neuronal populations (**Figure 3** and **Supplementary Table 3**). The pairwise comparison of NPFR to ElaV expression profiles resulted in the identification of 42 DEGs, but comparing the expression pattern of NPFR neurons to those of all other populations revealed a larger number of differentially expressed genes than when compared to ElaV neurons, with

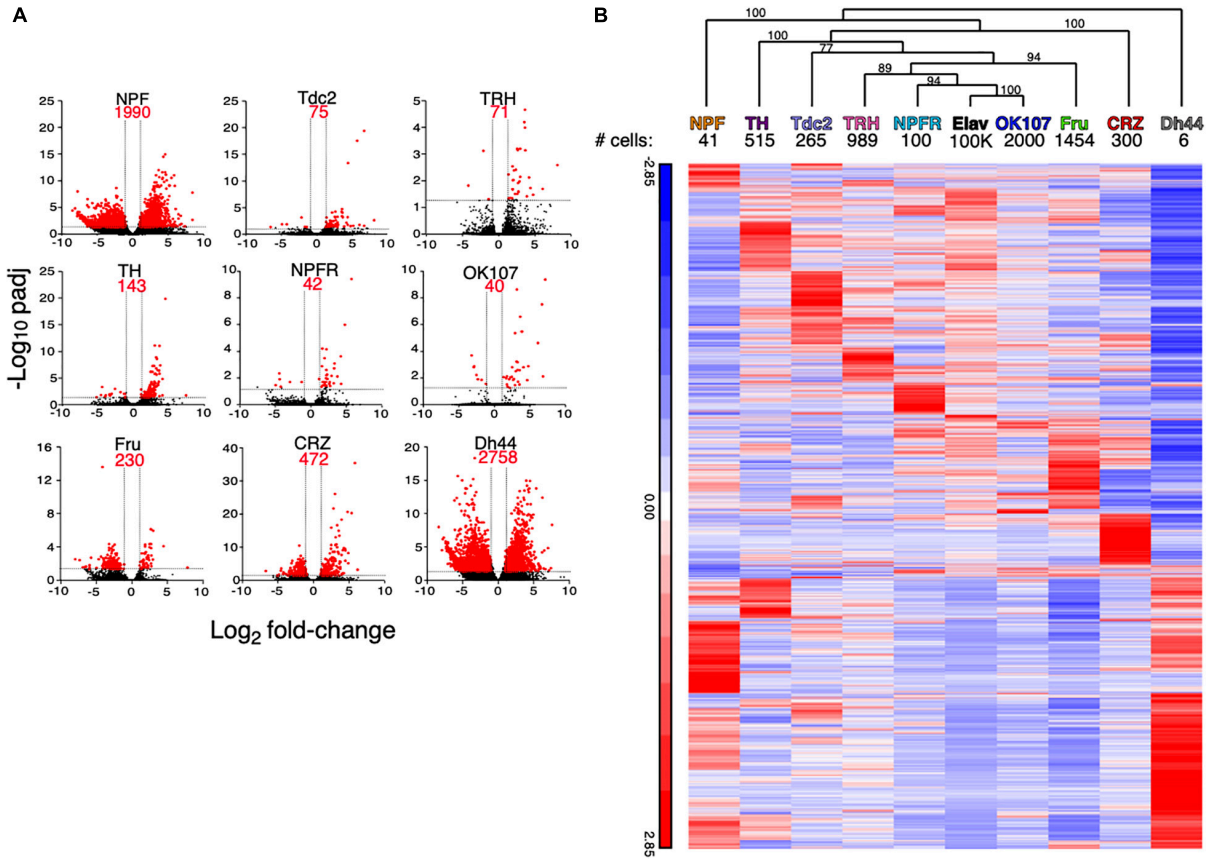

FIGURE 1 | Different neuronal populations exhibit varying number of differentially expressed genes when compared to the general population of neurons. **(A)** Volcano plots of $\log_2$ average fold change per population of all genes (black) and significantly expressed genes (red) compared to ElaV. Dashed lines indicate thresholds for fold change and adjusted *p*-values. **(B)** Hierarchical clustering of average normalized reads for all significantly expressed genes in 9 neuronal populations compared to a pan neuronal driver (ElaV). Clustering analysis was performed using Partek. Hierarchical clustering dendrogram with *p*-values. Values on the edges of the clustering are AU (Approximately Unbiased) *p*-values. Clusters with AU $\geq$ 95% are considered to be strongly supported by data.

the exception of DH44 neurons. Hierarchical clustering of the identified DEGs in each of the comparisons, followed by boot strapping analysis revealed that DH44- and NPF-expressing neurons clustered away from the rest of the populations, while Fru and OK107 neurons were most similar to NPFR neurons (**Figure 3**). In addition, Crz, NPF, and DH44 neurons clustered further from NPFR, while TH, TRH, Tdc2 clustered apart from Ok107, Fru, Elav, NPFR.

To further explore the biological relevance of the identified DEGs, we used a statistical overrepresentation analysis (PANTHER), which highlighted several biological processes, including enrichment of genes associated with regulation of behavior (**Figure 4** and **Supplementary Table 4**). We focused on behavior-associated genes that were enriched or depleted in NPFR vs. CRZ, TH, Fru, and OK107 neurons and found some interesting patterns (**Figure 4**). NPFR neurons displayed enrichment of genes that mediate different forms of learning and memory, such as derailed, 2mit, klingon, CG18769, Oamb, mGluR (metabotropic Glutamate Receptor), eag, Ank2, ss, and Tequila (**Figure 4**). In addition, we identified enrichment of genes involved in sensory perception of sound and touch, such as Ank2, btv, nompC, CG14509, DCX-EMAP, dila, and Rootletin. Interestingly, we documented enrichment of a few genes that participate in insulin signaling, such as dilps 2, 3, and 5 in Dh44 neurons, suggesting an anatomical overlap between some NPFR neurons and insulin-producing cells (IPCs).

Intriguingly, NPFR neurons exhibited enriched levels of various receptors for neuropeptides and neuromodulators like Oamb, mGluR, Dop1R1, Dop2R, CCKLR-17D1, Lestin-46Ca, Ms, TrissinR, CCHa1-R (CCHamide-1 receptor), AstA-R1(Allatostatin A receptor1), rk (rickets), Proc-R (Proctolin receptor), SPR (sex peptide receptor), sNPF-R, and (of course) the receptor for NPF (**Figure 4** and **Supplementary Figure 2**). The enrichment of such diverse types of receptors indicates that NPFR neurons receive multiple inputs from many neuromodulator systems, and/or that they are composed of diverse groups of neurons, with distinct combinations of receptors. In any event, these findings support the hypothesis that NPFR neurons are located at a convergence point of information that is relevant for the integration of internal state and action selection.

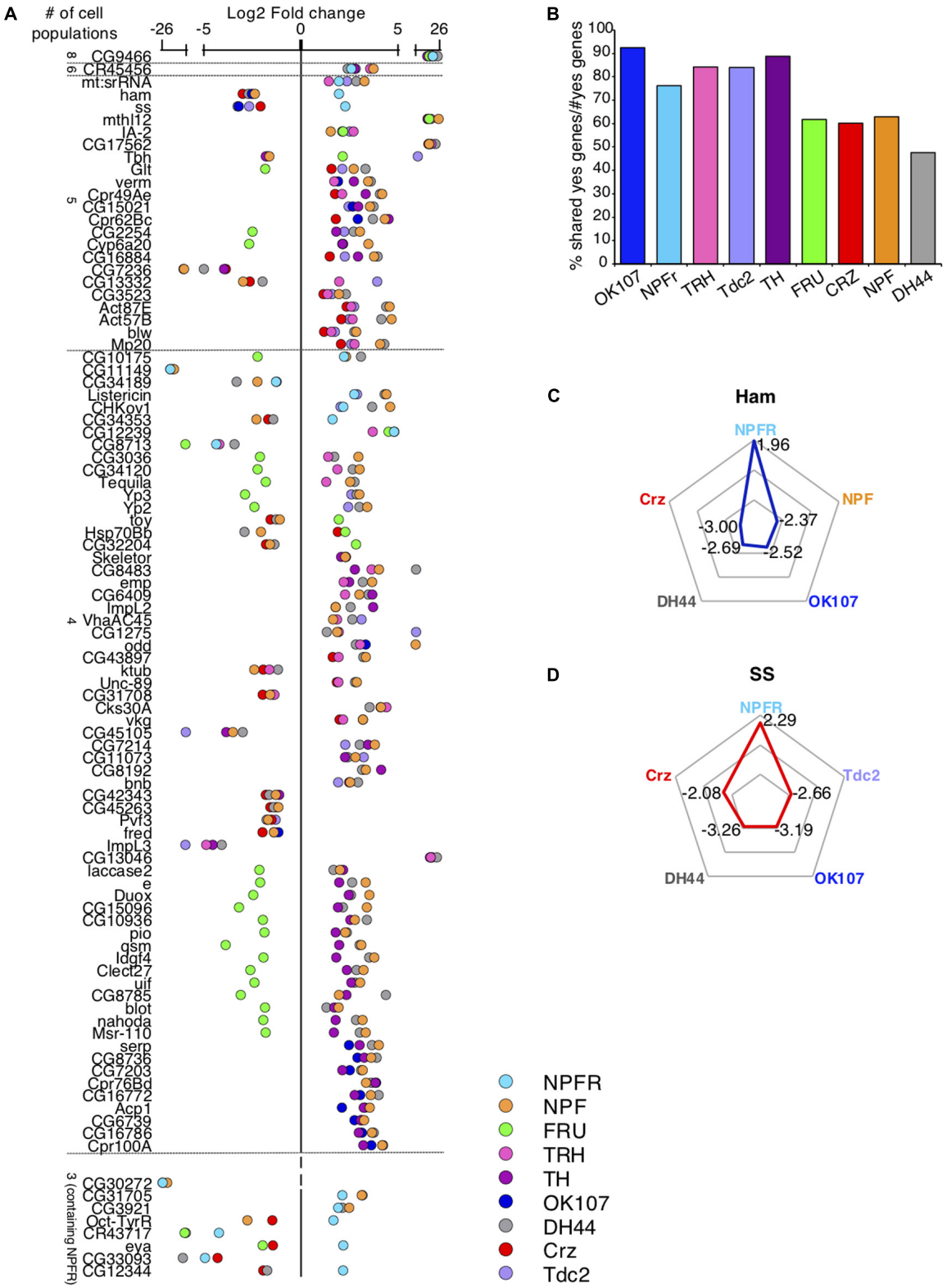

FIGURE 2 | Certain DEGs are shared across many populations. **(A)** Scatter plot representing log_2-fold change of all genes that are differentially expressed compared to ElaV and are shared across 8–4 different cell populations (upper part) and across 3 populations containing NPFR (bottom part). **(B)** Percent of shared DEGs normalized by total number of DEGs in each neuronal population. **(C,D)** Radar plots of two DEGs: ham **(C)** and ss **(D)** both of which were enriched in NPFR cells compared to 4 other cell types.

TABLE 1 | The number of shared DEGs varies across populations.

# shared genes	% of total number of yes genes in each population								
	CRZ	DH44	FRU	NPF	NPFr	OK107	TH	TRH	Tdc2
1	0.21	0.04	0.44	0.05	2.38	2.56	0.70	1.43	
1		0.04		0.05	2.38		0.70	1.43	1.33
1		0.04		0.05	2.38			1.43	1.33
1	0.21	0.04		0.05	2.38	2.56			
1	0.21	0.04		0.05	2.38	2.56			1.33
1	0.21		0.44	0.05			0.70	1.43	
1		0.04	0.44	0.05				1.43	1.33
1	0.21	0.04	0.44	0.05				1.43	
2		0.07	0.88	0.10			1.40		2.67
1	0.21		0.44	0.05			0.70		1.33
1	0.21	0.04	0.44	0.05					1.33
1		0.04		0.05		2.56	0.70	1.43	
1	0.21	0.04		0.05			0.70	1.43	
6	1.27	0.22		0.30				8.57	8.00
1		0.04		0.05		2.56	0.70		1.33
2	0.42	0.07		0.10			1.40		2.67
1	0.21	0.04		0.05		2.56	0.70		
1		0.04	0.44	0.05	2.38				
1				0.05	2.38		0.70		1.33
1		0.04		0.05	2.38		0.70		
2		0.07		0.10	4.76				2.67
1	0.21	0.04		0.05	2.38				
2		0.07	0.88	0.10	4.76			2.86	
3		0.11	1.32	0.15				4.29	
14		0.51	6.14	0.70			9.79		
2		0.07	0.88	0.10					2.67
3	0.64	0.11	1.32	0.15					
5		0.18		0.25			3.50	7.14	
2		0.07		0.10				2.86	2.67
1		0.04		0.05		2.56		1.43	
6	1.27	0.22		0.30				8.57	
5		0.18		0.25			3.50		6.67
9		0.33		0.45		23.08	6.29		
1	0.21	0.04		0.05			0.70		
2	0.42	0.07		0.10					2.67
1	0.21	0.04		0.05		2.56			
1		0.04					0.70	1.43	1.33
1	0.21	0.04					0.70	1.43	
1				0.05	2.38		0.70		
2		0.07		0.10	4.76				
1	0.21			0.05	2.38				
1		0.04	0.44	0.05	2.38				
1	0.21		0.44	0.05	2.38				
2	0.42	0.07		0.10	4.76				
2			0.88	0.10			1.40		

# shared genes	% of total number of yes genes in each population								
	CRZ	DH44	FRU	NPF	NPFr	OK107	TH	TRH	Tdc2
1			0.44	0.05		2.56			
48		1.74	21.05	2.41					
2	0.42		0.88	0.10					
16		0.58		0.80				22.86	
2				0.10			1.40		2.67
48		1.74		2.41			33.57		
10		0.36		0.50					13.33
1	0.21			0.05					1.33
5		0.18		0.25		12.82			
1	0.21			0.05		2.56			
118	25.00	4.28		5.93					
1			0.44					1.43	1.33
2		0.07	0.88				1.40		
1	0.21		0.44			2.56			
1	0.21	0.04	0.44						
1		0.04						1.43	1.33
2	0.42	0.07				5.13			
2				0.10	4.76				
1			0.44	0.05	2.38				
1				0.05	2.38		0.70		
2				0.10	4.76				2.67
2		0.07		0.10	4.76				
3	0.64			0.15	7.14				
11			4.82	0.55					
1				0.05				1.43	
6				0.30			4.20		
6				0.30					8.00
1				0.05		2.56			
850		30.83		42.71					
35	7.42			1.76					
1			0.44					1.43	
4			1.75				2.80		
1			0.44						1.33
2			0.88			5.13			
21		0.76	9.21						
7	1.48		3.07						
3		0.11						4.29	
1	0.21							1.43	
1							0.70		1.33
2						5.13	1.40		
8		0.29					5.59		
6		0.22							8.00
3		0.11				7.69			
1	0.21					2.56			
75	15.89	2.72							

Number of shared DEGs across populations (left column) represented as % from the total number of DEGsin each population (color coded: red-high, blue-low).

Lastly, we found that NPFR neurons possessed a unique mixture of ion channels compared to both Crz and Fru neurons (**Supplementary Figure 3**), with an overrepresentation of seven potassium and sodium ion transmembrane transport subgroups in NPFR neurons (**Supplementary Table 4**). We also documented an overrepresentation of amino acid transmembrane transport proteins with 2 enriched genes in Fru cells (vGAt, CG5549) and 6 enriched in NPFR neurons (Ncc69, CG7888, Eaat1, CG43693, CG8785, CG16700). Interestingly, Orct2 (Organic cation transporter 2), which is a transcriptional target of the insulin receptor pathway (Herranz et al., 2006; **Supplementary Figure 3**) was enriched in NPFR compared to Crz, further supporting the involvement of NPFR neurons in insulin signaling.

Manipulation of the Proteome Profile in NPFR Neurons Affects Social and Sexual Behavior in Flies

The distinct patterns of transcription in each neuronal population gives rise to a specific proteome diversity that shapes the functional output of neurons. To investigate this assumption further, one can modify the expression levels of genes that are enriched or depleted in certain populations or

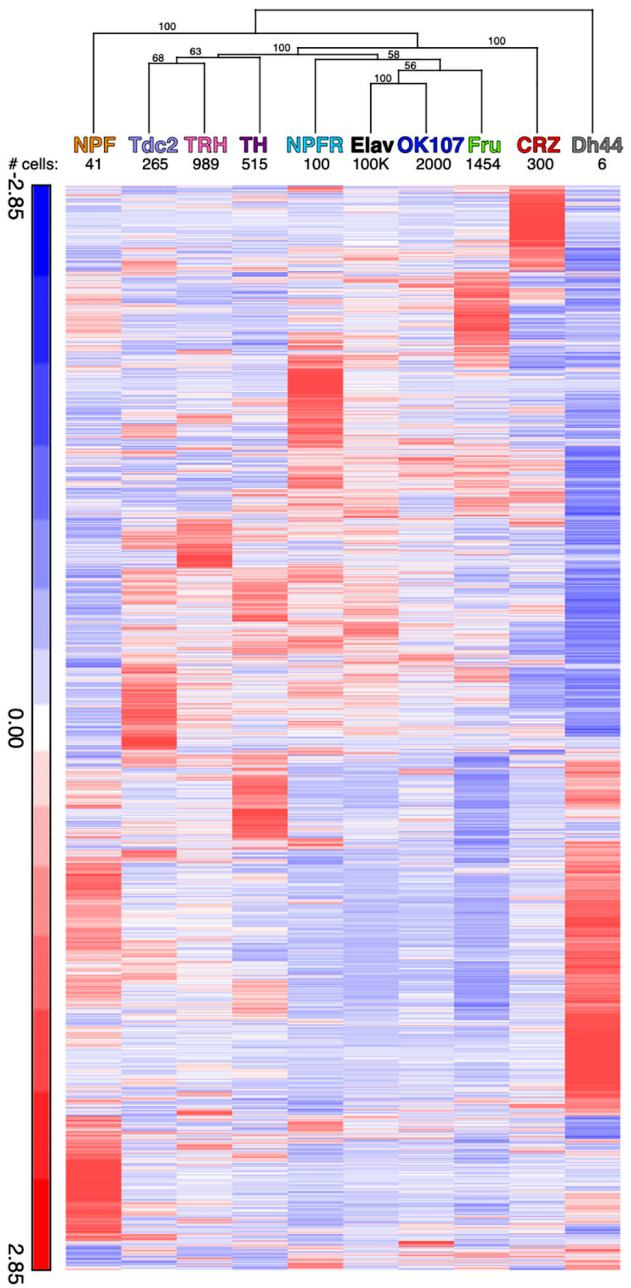

FIGURE 3 | Hierarchical clustering of average reads for all differentially expressed genes compared to the NPFR neurons. Clustering analysis was performed using Partek. Hierarchical clustering dendrogram with *p*-values. Values on the edges of the clustering are AU (Approximately Unbiased) *p*-values. Clusters with AU ≥ 95% are considered to be strongly supported by data.

use a more global approach to disturb the proteomic signature of specific neurons. We chose to perturb the transcriptomic and proteomic signature of NPFR neurons by manipulating the function of two molecular systems that regulate a large number of cellular targets (RNA editing and protein ubiquitination) and to analyze the effects on the social behavior of male flies.

Thousands of RNA editing sites have been discovered in *Drosophila* (Rosenthal and Seeburg, 2012), most of which lead to recoding events in genes that are expressed and function specifically in the neuron (Keegan et al., 2005; Stapleton et al., 2006; Jepson and Reenan, 2009; Rosenthal and Seeburg, 2012; Maldonado et al., 2013; Li et al., 2014). As such, null mutation of ADAR in *Drosophila* leads to strong locomotor phenotypes that become more severe with age, although the underlying mechanisms is still mostly unknown (Palladino et al., 2000). Therefore, we hypothesized that reducing ADAR expression in NPFR neurons would affect the proteomic profile and could result in behavioral phenotypes. To test this, we downregulated the expression of dADAR in NPFR neurons (NPFR > UAS-dicer, UAS-dADAR RNAi) and analyzed behavior in groups of 10 flies, using the "FlyBowl" system, a suite of tracking and behavior analysis software that score plethora of locomotion and social behaviors (Robie et al., 2012; Bentzur et al., 2021). We used the tracking data obtained to generate a comprehensive behavioral representation for experimental flies and genetic controls that included kinetic features and eight complex behaviors. The overall differences between the genotypes are depicted in a scatter plot of normalized differences, divided into four main categories: activity-related features, interaction-related features, coordination between individuals, and social clustering-related features (**Figure 5A** and **Supplementary Figure 1**).

Unlike dADAR null flies and pan-neuronal knockdown (KD) of dADAR flies, which display strong motor impairments, downregulation of dADAR expression in NPFR neurons did not lead to any differences in locomotion and general activity levels. Specifically, the average velocity of experimental flies and the percentage of time they spent walking and performing body turns was similar to those of the genetic controls (NPFR-Gal4/+ and UAS-dicer, UAS-dADAR RNAi/+, **Figure 5A**). We further analyzed several types of social behaviors, including touch (active leg touching between two flies), approach (fly approaching another fly), song (wing extension and vibration to generate male courtship song), chase (fly chasing another fly), and chaining (one fly following a fly while being followed by another fly, in a minimum chain length of three flies). Interestingly, reducing ADAR levels in NPFR neurons resulted in strong elevation in social interaction between male flies, as manifested by increased levels of close touch behavior, increased levels of song display, increased values of active approaches and male-male chase events that resulted in multiple formations of chains (**Figure 5A**). In addition to these behaviors, we analyzed another feature associated with social interaction: the number of flies close-by (nflies-close), representing the number of flies within two body lengths of a focal fly as a measure of sociality (**Figure 5A** and **Supplementary Figure 1**). Flies harboring reduced levels of ADAR in NPFR neurons depict significantly higher value of nflies-close compared to the control groups, suggestive of close distance between flies (**Figure 5A**), altogether indicating that RNA editing in NPFR expressing cells is important for the correct expression of certain social behaviors. A previous study in our lab demonstrated that NPFR and CRZ neurons possess distinct RNA editing profiles (Sapiro et al., 2019). This

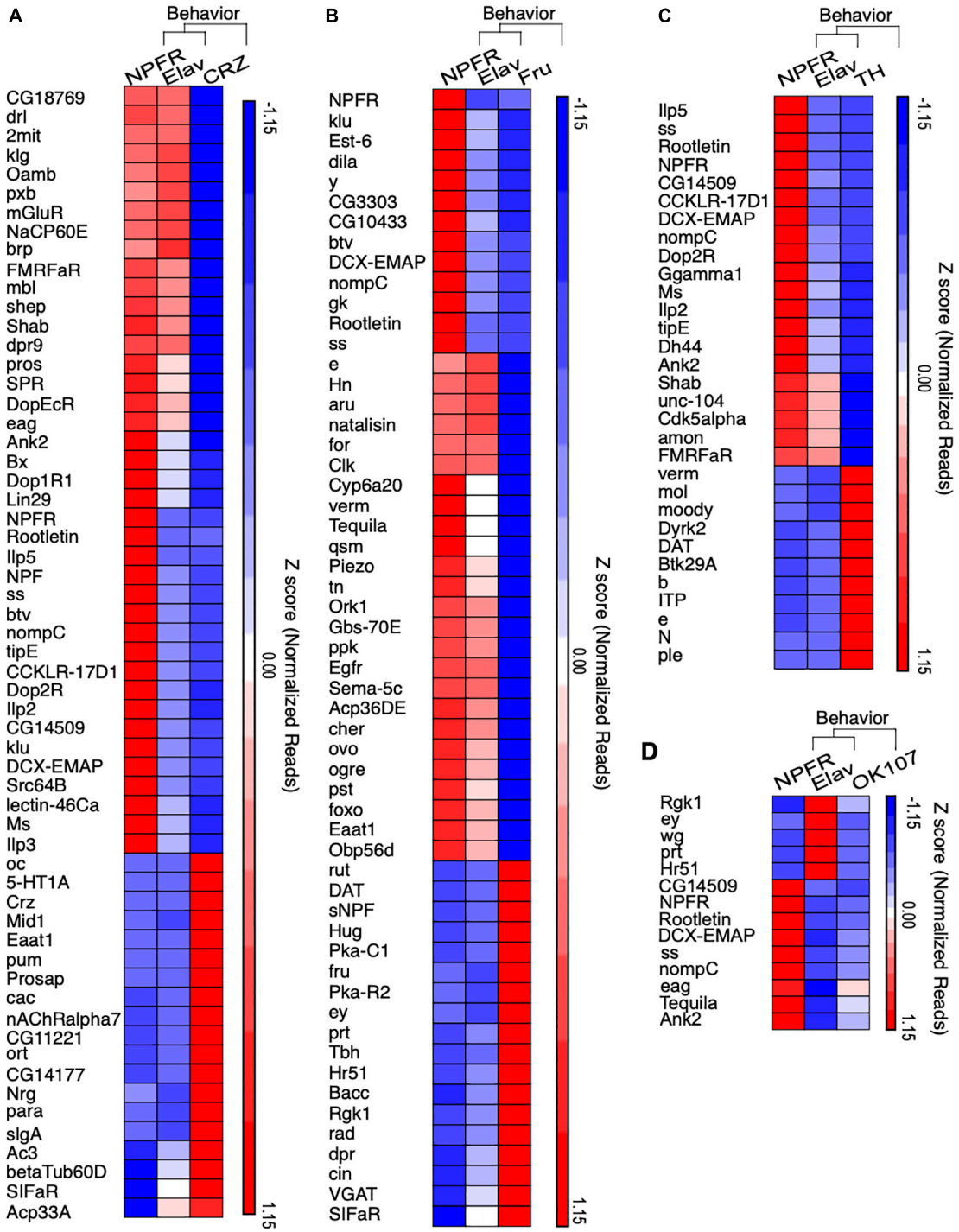

FIGURE 4 | NPFR expressing neurons exhibit enrichment of behavior related genes. **(A–D)** Hierarchical clustering of statistically overrepresented biological processes that are related to behavior in differentially expressed genes of NPFR expressing neurons compared to Corazonin (CRZ, **A**), Fruitless (FRU, **B**), Dopamine producing (TH, **C**) neurons and Mushroom bodies neurons (OK107, **D**). Biological overrepresentation was performed using PANTHER. Clustering analysis was performed using Partek.

prompted us to test the behavioral significance of reducing RNA editing in CRZ neurons as well. However, knocking down ADAR in CRZ neurons only led to moderate effects on the male-male social interactions. Specifically, we documented longer bouts of song, turn, and chase events than in genetic controls (**Figure 5B**).

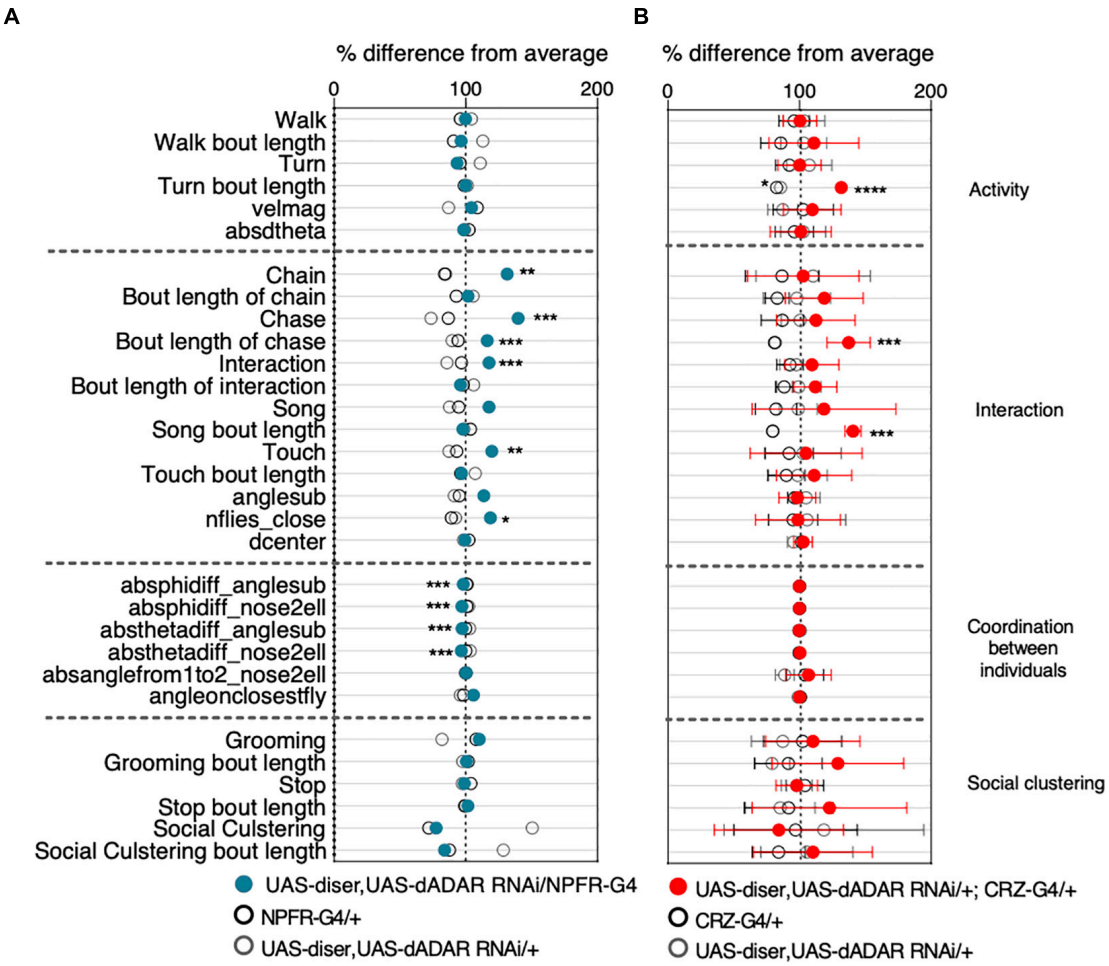

FIGURE 5 | RNA editing is required in NPFR neurons for typical male social behavior. Behavioral signatures of social group interaction. Data is presented as normalized scatter plots depicting% difference from average of 31 behavioral features. **(A)** Behavior profile of male flies harboring NPFR-Gal4/UAS-Dicer, UAS-dADAR RNAi (Blue) compared to genetic controls (UAS-Dicer, UAS-dADAR RNAi/ +, and NPFR-Gal4/ +, gray and black, respectively). $n = 17$. **(B)** Behavior profile of male flies harboring UAS-Dicer, UAS-dADAR RNAi/ +; CRZ-Gal4/ + (red) compared to genetic controls (UAS-Dicer, UAS-dADAR RNAi/ +, CRZ-Gal4/ +, gray and black, respectively). $n = 7$. One-way ANOVA with Tukey's *post hoc* for normally distributed parameters and Kruskal-Wallis with Dunn's test *post hoc* for non-normally distributed parameters. FDR correction was performed for all features. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $^{n.s.}P > 0.05$.

Next, we perturbed the proteome diversity in NPFR cells by targeting the protein ubiquitination machinery. To manipulate this multiplayer system, we targeted the expression of one central player, ubiquitin-conjugating enzyme 7 (Ubc7), orthologous to the human ubiquitin-conjugating enzyme E2 G2. We used the CrispR-Cas9 system to generate tissue specific knockout (KO) of Ubc7 using a combination of Ubc7-specific guide RNAs and specific expression of Cas9 in NPFR neurons (NPFR > UAS Cas9) (Xue et al., 2014; Meltzer et al., 2019; Poe et al., 2019). We generated a pair of guide-RNAs (gRNA) targeting the beginning of the second exon of Ubc7. We validated their efficiency by using a germline deletion within the Ubc7 locus by driving Cas9 expression using a germline-specific driver (Vas-Cas9), which resulted in an 18- to 23-bp deletion at the beginning of the second exon of both Ubc7 isoforms (**Figure 6A** and **Supplementary Figure 4**). To affect Ubc7 in NPFR cells, we crossed *NPFR*-G4; *UAS-Cas9.c* flies with flies carrying our gRNA for Ubc7. Since Ubc7 null mutation was shown to suppress courtship toward females (Orgad et al., 2000), we first analyzed the effects of knocking out Ubc7 in NPFR neurons on male courtship behavior. For that, we introduced experimental male flies (NPFR > UAS Cas9, *gRNAs*) or genetic control male flies (*NPFR-G4/attp1; UAS-Cas9.c*) into courtship arenas with virgin females and recorded and analyzed their behavior (**Figures 6C–E**). Surprisingly, and in contrast with Ubc-7 null mutants, male flies lacking Ubc7 expression in NPFR cells displayed shorter latency to court, shorter latency to first copulation attempt, and shorter time to successful copulation (**Figures 6C–E**), all signs suggestive of higher motivation to court and mate. Next, we analyzed the behavioral responses of male flies when interacting with nine other male flies in a group (**Figure 6F**). Contrary to the previous results obtained after manipulating the proteome of NPFR by

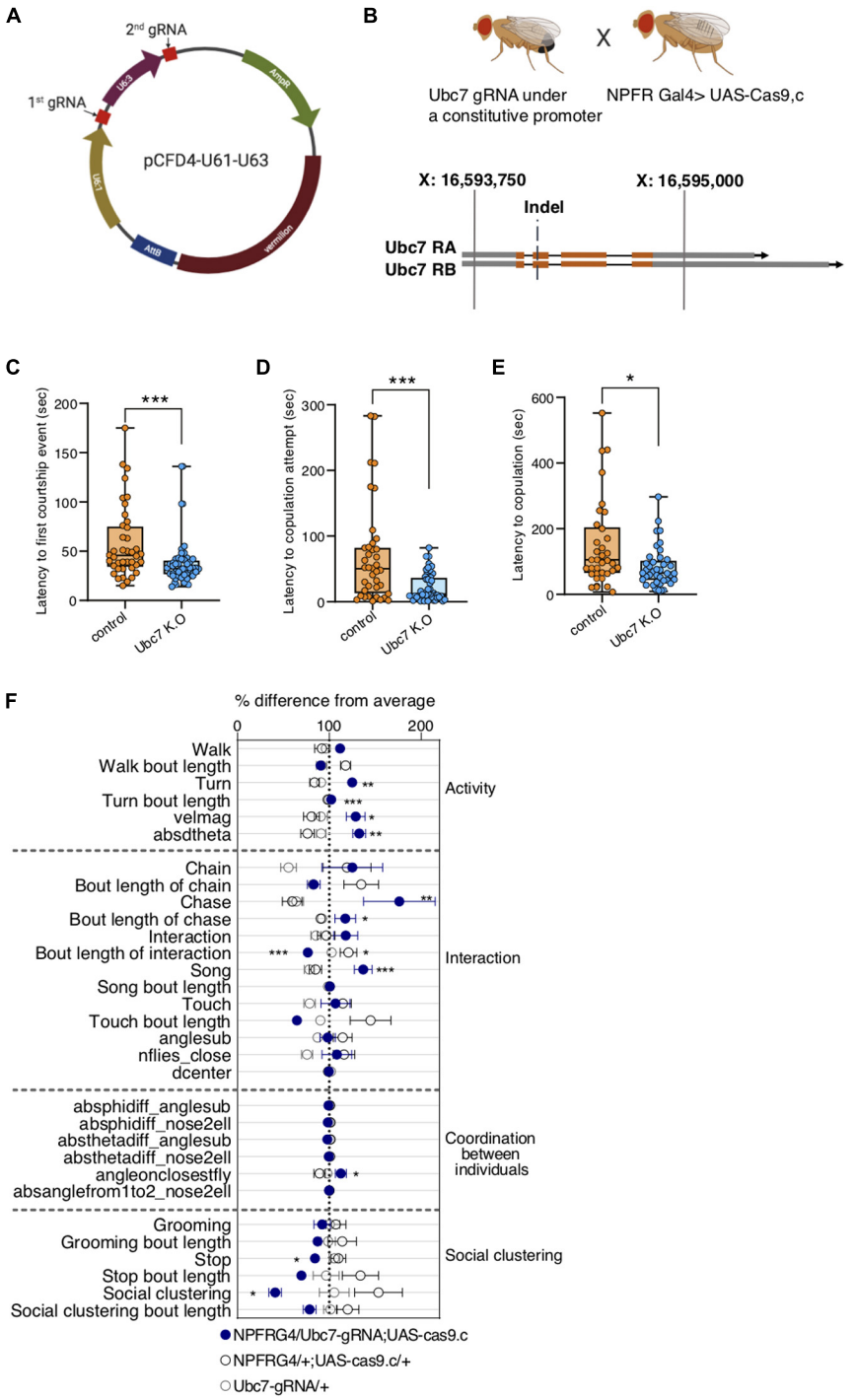

FIGURE 6 | Tissue specific K.O of Ubc7 elevates the motivation to court and enhances male-male social interaction. **(A)** Representation of pCDF4 plasmid containing two gRNAs (red). **(B)** Crossing scheme of flies containing gRNAs with NPFR-Gal4; Cas9.c flies to generate tissue specific Ubc7 K.O flies (upper panel). Lower panel depicts two Ubc7 isoforms (orange and gray blocks representing coding and non-coding exons, respectively, Black lines representing introns). The double strand break occurred at the beginning of the 2nd coding exon. **(C–E)** Male flies containing NPFR-Gal4/Ubc7-gRNA; UAS-Cas9.c/ + (blue) were introduced to naïve females in courtship arenas and were video recorded, their courtship behavior was analyzed for latency to first courtship event **(C)**, latency to first copulation attempt **(D)** and latency to copulation **(E)** compared to genetic controls (NPFRG4/attp1;UAScas9.c, orange). n = 47 and 40 in **(C)**, n = 46 and 39 in **(D)**, n = 39 and 33 in **(E)** for experimental and control groups, respectively. Mann-Whitney test. $^{*}P < 0.05$, $^{***}P < 0.001$. **(F)** Behavioral signatures of social group interaction in male flies harboring NPFR-Gal4/Ubc7-gRNA; UAS-Cas9.c/+ (blue) compared to NPFR-Gal4/+; UAS-Cas9.c/+ and Ubc7 gRNA/+ (gray and black, respectively). Data is presented as normalized scatter plots depicting % difference from average of 31 behavioral features. n = 8, 5, and 11, respectively. One-way ANOVA with Tukey's *post hoc* for normally distributed parameters and Kruskal-Wallis with Dunn's test *post hoc* for non-normally distributed parameters. FDR correction was performed for all features. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, $^{n.s.}P > 0.05$.

disturbing RNA editing programs, which did not affect any of the measured activity-related features, knocking out Ubc7 in NPFR neurons led to a pronounced increase in the number of time flies spent walking and performing turns, and to an overall increase in their average velocity compared to genetic controls (**Figure 6F**), suggestive of increased arousal. Moreover, Ubc7 KO male flies exhibited increased social interactions between males, as shown by the higher levels of chase and song and reduced social clustering (**Figure 6F**). This suggests, that protein ubiquitination in NPFR neurons is important for regulating the intensity of male-female and male-male sexual and social behaviors, and that Ubc7 is necessary to reduce male social interactions.

DISCUSSION

The complex interplay between genes, neurons and behavior started to be deciphered decades ago with the Benzerian revolution in neurogenetics and is still under intense investigation these days using a plethora of tools in various model organisms. This study joins a growing body of studies that use contemporary genomic approaches to dissect the brain into units and illuminate their molecular content, as a step toward understanding the dynamic spatiotemporal environments in which genes function (Croset et al., 2018; Agrawal et al., 2019; Shih et al., 2019). While many cell types exist in the fly brain, in this study, we analyzed only a small fraction of them, focusing mostly on NPFR neurons. Nevertheless, the transcriptome profiles of other neuronal populations in this dataset can serve as a resource for labs investigating other neurons.

We took two complementary pairwise based approaches to investigate the relative signature of NPFR neurons: the first approach comparing profiles of each of the populations to those of all neurons and subsequently comparing the DEGs across populations; and the second approach performing pairwise comparisons between NPFR and each of the nine neuronal populations. Although the two approaches highlighted different number of differentially expressed genes, they resulted in similar hierarchical clustering patterns, and complemented the picture describing the distinct molecular landscape of NPFR neurons.

By comparing expression profiles of different neuronal populations to those of all neurons, we found that NPF expressing neurons represent a much more unique population than NPFR neurons. This may result from differences in cell number (40 NPF vs. ∼100 NPFR cells) or may be associated with the heterogenous expression profile of NPFR as receptor neurons. The second explanation is supported by the enriched levels of receptors for neuropeptides and neuromodulators we detected, a finding that is in agreement with those of previous studies showing anatomical overlap between NPFR cells and some NPF and TH neurons (Shao et al., 2017; Zhang et al., 2019). Still, it is not known if NPFR neurons receive multiple inputs from many neuromodulatory systems, or whether they are composed of diverse groups of neurons with distinct combinations of receptors. This question could be addressed in future studies by dissecting NPFR neuronal population into smaller subsets of cells using genetic intersection approaches or by single cell RNA-seq analysis of NPFR positive neurons.

The second part of this study investigated the functional relevance of the unique transcriptome identified with our genomic approach. We discovered that global perturbation of RNA editing and protein ubiquitination programs in NPFR neurons resulted in dramatic behavioral phenotypes. Tissue-specific knockout of Ubc7 in male flies resulted in a strong motivation to court female flies that could possibly stem from increased level of arousal as reflected by enhanced velocity. The enhanced courtship display found upon the tissue specific knockout of Ubc7 is in contrast with the complete loss of courtship behavior documented in male flies that lack Ubc7 in all cells (Orgad et al., 2000). This apparent discrepancy can be easily explained by distinct roles of Ubc7 in different tissues, the lack of which in NPFR possibly perturb the proper function of NPFR in restraining courtship as shown by Liu et al. (2019). Interestingly, a subset of NPFR-dopamine neurons has been shown to promote mating drive (Zhang et al., 2019), strengthening the notion that different sub-populations of NPFR neurons have distinct roles in regulating the motivation to court, and stressing the need for dissecting NPFR cells into smaller groups of neurons to analyze their transcriptomes and functions.

In addition to the increased motivation to court, we also documented increased frequencies of male-male social interactions, including increased levels of song and chase behaviors, which are normally absent in socially experienced male flies that are housed in groups (Bentzur et al., 2021). This manifestation, together with the increased walking velocity and lack of social clustering behavior observed in groups of Ubc7 KO flies, resemble the behavioral properties of male flies exposed to social isolation, a condition that is known to promote aggression (Wang et al., 2008; Liu et al., 2011). Given that the physical features of the FlyBowl set-up prevent the expression of aggression displays such as lunging, the increased chase behavior documented in the FlyBowl setup may be indicative of the male-male aggressive behavior that is normally suppressed by NPF action on NPFR neurons (Dierick and Greenspan, 2007) and that was promoted by perturbing the proteome balance in NPFR neurons.

We have previously shown that different neuronal populations possess unique RNA editing profiles (Sapiro et al., 2019), suggesting that RNA editing may account for some functional differences between neuronal populations in the brain. The pronounced behavioral outcome of perturbing RNA editing in NPFR neurons supports this hypothesis and shows that RNA editing is necessary for the proper function of these neurons. The phenotypic resemblance to Ubc7 K.O in NPFR neurons suggests that both manipulations perturb the function of NPFR in regulating social interaction, but closer inspection reveals the existence of interesting differences. While perturbing protein ubiquitination affects activity/arousal that may stimulate chase behavior, lack of RNA editing leads to pronounced increase in approach behavior, interaction, chase and chaining behaviors without changing activity levels. These differences suggest that

perturbing RNA-editing and protein ubiquitination do not lead to global malfunction of NPFR but rather affect distinct targets that regulate different features of NPFR physiology and function.

The behavioral phenotypes of reducing ADAR levels were more pronounced in NPFR than in CRZ neurons, suggesting that RNA editing does not have a uniform role in all neurons but rather shapes the diversity of expressed proteins in different neurons to allow their distinct function. Our findings join a previous study that demonstrated the spatial requirements of ADAR expression in regulating locomotor behavior (Jepson and Reenan, 2009), emphasizing the need to extend this to other behavioral paradigms, neuronal populations and even to studying the tissue specific role of specific editing events.

To conclude, in this study we demonstrated that the function of NPFR neurons in suppressing certain aspects of social behavior depends strongly on the integrity of its transcriptome and proteome. This finding highlights the importance of cell specific posttranscriptional mechanisms in regulating the abundance and function of certain RNA molecules and proteins, the action of which determines the output function of the neuron. Based on the function of the NPF/R system in regulating other types of motivational behavior we expect the unique transcriptome of NPFR to regulate also feeding behaviors, appetitive memory, ethanol related behaviors and sleep.

Considering the technology driven revolution in deciphering the connectome of the fly brain (Zheng et al., 2018; Scheffer et al., 2020), the next challenge in understanding the neurobiology of complex behavior will be to combine these static 3D maps with the molecular programs that function within defined circuits. This will be especially important for understanding a long-standing question of how a given circuit is shaped by context and internal states to produce different outcomes from a seemingly similar input? A good starting point toward solving this question will be to focus on circuits that are regulated by neuromodulators, such as NPFR neurons, and use cell specific transcriptomics to identify dynamic changes in expression pattern under various conditions corresponding to different internal states. Newly emerging technologies that allow the profiling of smaller amounts of cells (Croset et al., 2018) and spatially resolved transcriptomics (Alon et al., 2021) can open the way for identifying key cellular pathways that encode changes in motivation. Studying the functional relevance of such regulatory events requires manipulating their expression in the most accurate spatial-temporal context as best demonstrated by the different outcomes of eliminating Ubc7 expression in the whole animal vs. specifically in NPFR neurons.

Lastly, the similar patterns of social responses observed across the animal kingdom suggest, that certain social behaviors originated early in evolution, and that similar ancient biological principles and genes are involved in these processes. An example for this is the functional conservation of the NPF/R system from worms to humans in regulating social behavior, feeding, sleep-wake, ethanol related behaviors and the response to stress (De Bono and Bargmann, 1998; Tokuno et al., 2002; Rogers et al., 2003; Sokolowski, 2003; Davies et al., 2004; Heilig, 2004; Kalra and Kalra, 2004; Karl and Herzog, 2007; Sparta et al., 2007; Briggs et al., 2010; Xu et al., 2010; Wiater et al., 2011; Shohat-Ophir et al., 2012; Chung et al., 2017; Robinson and Thiele, 2017; Li et al., 2018). While the transcriptome of NPF and NPFR neurons support this notion and illuminate some of the cellular pathways participating in these behaviors (Ubc7 for courtship and the insulin pathway for the regulation of feeding), further work is needed to comprehensively decipher their function and extend these findings beyond flies.

MATERIALS AND METHODS

Fly Lines and Culture

Drosophila melanogaster CS flies were kept in 25°C, ∼50% humidity, light/dark of 12:12 h, and maintained on cornmeal, yeast, Molasses, and agar medium. NPFR-GAL4 was a gift from the Truman lab (HHMI Janelia Campus), CRZ-GAL4 was a gift from the Heberlein lab (HHMI Janelia Campus), UAS-dicer, UAS-dADAR RNAi was a gift from the Lee lab (Stanford University), UAS-CAS9.c was a gift from the Schuldiner lab (Weizmann Institute). Vasa-CAS9 was a gift from Gershon lab (Bar-Ilan University), y^1w^{67c23};P{CaryP}attP1 (BestGene BL#8621).

Determining Gene Expression Levels From RNA-Seq

Previously published RNA-seq data was used (Sapiro et al., 2019). Reads were trimmed using cutadapt (Martin, 2011) and mapped to *Drosophila melanogaster* (BDGP6) genome using STAR (Dobin et al., 2013) v2.4.2a (with EndToEnd option and out FilterMismatchNoverLmax was set to 0.04). Counting proceeded over genes annotated in Ensembl release 31, using htseq-count (Anders et al., 2015) (intersection-strict mode). DESeq2 (Love et al., 2014) was used to measure differential expression analysis with the betaPrior, cooks Cutoff and independent Filtering parameters set to False. Genes were filtered in a pairwise manner according to the following parameters: $\log_2$-fold change of at least $|1|$, adjusted *P*-value lowers than 0.05 (Benjamini and Hochberg procedure) and a minimum of least of 30 normalized counts in one of the repeats.

Accession Numbers

Raw data is available at GEO with accession GSE113663.

FlyBowl

FlyBowl experiments were conducted as described in Bentzur et al. (2021). In brief: groups of 10 male flies, which were socially raised in groups of 10 for 3–4 days, were placed in FlyBowl arenas, and their behavior was recorded at 30 fps for 15 min and were tracked using Ctrax (Branson et al., 2009). Automatic behavior classifiers and Per-frame features were computed by JABBA (Kabra et al., 2013) tracking system. Data of all behavioral features was normalized to % difference from the average of each experiment for visualization. Details about the different features are found in **Supplementary Figure 1**.

Courtship Assay (ubc7)

Four to Five days old naive males were placed with 4–5 days old virgin females in round courtship arenas (0.04 cm^3 in volume), one male and one female in each arena. Courtship arenas were placed in behavior chambers, under controlled temperature and humidity (25°C, 70% humidity). Behavior was recorded for 10 min from the introduction of male and female pairs using Point-Grey Flea3 cameras (1,080 × 720 pixels at 60 fps). Latency to copulation attempt and latency to copulation were quantified for each pair relative to the first wing vibration the male exhibited. Statistics: Mann-Whitney test.

Generation of gRNA, Transgenic Constructs and Transgenic Flies

gRNA sequences were selected using the Fly- CRISPR algorithm[1], contain 20 nucleotides each (PAM excluded), and are predicted to have zero off-targets. Two different gRNA sequences were selected for Ubc7, both within the coding region of the gene, but not overlapping each other. Both gRNA sequences were cloned into the pCFD4 plasmid (**Figure 6A**). Cloning into pCFD4 was done using Q5® High-Fidelity DNA Polymerase (BioLabs). gRNA-harboring constructs were injected to Drosophila embryos and integrated into attP landing sites using the φC31 system into attP1 (BL#8621) on the second chromosome. Injections were performed as services by BestGene[2].

gRNA sequences:
GTTAACACTTGACCCGCCCG
GCCCCATCAGCGAGGACAAC.

Generation of the Germline ubc7 Indel Mutant

Transgenic flies expressing gRNA pCFD4 were crossed to flies expressing Vas-Cas9. Flies containing both the gRNAs and nos-Cas9 were crossed to a Fm7a balancer line, offspring were then collected and checked for the presence of an indel using DNA seq. The resulting indel is a deletion of 18–23 bp (**Figure 6B** and **Supplementary Figure 4**).

Primers for DNA sequencing:
Forward: AGAAAGCCACTCGATTCATTCGATA
Reverse: GTCCAGAGCGTGGAGAAGAT.

Generation of Tissue Specific CRISPR

Transgenic flies expressing gRNA pCFD4 were crossed to flies expressing NPFRG4/+; UAS-Cas9.c/+.

Statistical Analysis

Data of each behavioral feature per experiment was tested for normality and consequently tested by either One-way ANOVA or Kruskal-Wallis tests followed by Turkey's or Dunn's *post hoc* tests using Prism. FDR correction for multiple comparisons was performed for all features. Statistical overrepresentation was generated using PANTHER (Thomas et al., 2006; Mi et al., 2019)[3].

Hierarchical clustering dendrogram with *p*-values was done using the R package pvclust[4], with multiscale bootstrap resampling of 10,000 iterations to assess statistical significance, represented by a 1–100 score. Hierarchical clustering was performed using average linkage method with Euclidian distance as the distance measure.

Graphics

Figures 6A,B were Created in BioRender.com.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AB, JR, AS, and GS-O: conceptualization. AB and JR: methodology and statistical analysis. JR, AB, OS, SD, MT, and ML: investigation. JR, AB, and GS-O: writing. GS-O: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Israel Science Foundation Grant 384/14 and Israel Science Foundation Grant 174/19.

ACKNOWLEDGMENTS

We thank all members of the Shohat-Ophir lab for fruitful discussions and technical support. We express special thanks to C. Andrew Frank (University of Iowa) and Dion Dickman (University of Southern California) for their productive suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.628662/full#supplementary-material

Supplementary Figure 1 | List of behavioral features presented in **Figures 5**, **6**.

Supplementary Figure 2 | NPFR expressing neurons exhibit intricate expression patterns of receptors for neuropeptides and neuromodulators.

Supplementary Figure 3 | NPFR expressing neurons exhibit intricate expression of ion channels.

Supplementary Figure 4 | Sequenced 750 bp of Ubc7 DNA displaying 18–23 bp deletion in 4 male flies harboring Vas-Cas9; Ubc7 gRNA. Yellow and purple colors represent gRNA1 and 2 complementary sequences. Underlined sequences represent the deleted sequences.

[1] http://flycrispr.molbio.wisc.edu/

[2] https://www.thebestgene.com/

[3] http://pantherdb.org/citePanther.jsp

[4] https://academic.oup.com/bioinformatics/article/22/12/1540/207339

REFERENCES

Abruzzi, K. C., Zadina, A., Luo, W., Wiyanto, E., Rahman, R., Guo, F., et al. (2017). RNA-seq analysis of *Drosophila* clock and non-clock neurons reveals neuron-specific cycling and novel candidate neuropeptides. *PLoS Genet.* 13:e1006613. doi: 10.1371/journal.pgen.1006613

Agrawal, P., Chung, P., Heberlein, U., and Kent, C. (2019). Enabling cell-type-specific behavioral epigenetics in *Drosophila*: a modified high-yield INTACT method reveals the impact of social environment on the epigenetic landscape in dopaminergic neurons. *BMC Biol.* 17:30. doi: 10.1186/s12915-019-0646-4

Alon, S., Goodwin, D. R., Sinha, A., Wassie, A. T., Chen, F., Daugharthy, E. R., et al. (2021). Expansion sequencing: spatially precise in situ transcriptomics in intact biological systems. *Science* 371:eaax2656. doi: 10.1126/science.aax2656

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq-a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Anderson, D. J. (2016). Circuit modules linking internal states and social behaviour in flies and mice. *Nat. Rev. Neurosci.* 17, 692–704. doi: 10.1038/nrn.2016.125

Andrews, J. C., Fernández, M. P., Yu, Q., Leary, G. P., Leung, A. K. W., Kavanaugh, M. P., et al. (2014). Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in *Drosophila* males. *PLoS Genet.* 10:e1004356. doi: 10.1371/journal.pgen.1004356

Anpilov, S., Shemesh, Y., Eren, N., Harony-Nicolas, H., Benjamin, A., Dine, J., et al. (2020). Wireless optogenetic stimulation of oxytocin neurons in a semi-natural setup dynamically elevates both pro-social and agonistic behaviors. *Neuron* 107, 644–655.e7. doi: 10.1016/j.neuron.2020.05.028

Arias-Carrión, O., Caraza-Santiago, X., Salgado-Licona, S., Salama, M., Machado, S., Nardi, A. E., et al. (2014). Orquestic regulation of neurotransmitters on reward-seeking behavior. *Int. Arch. Med.* 7:29. doi: 10.1186/1755-7682-7-29

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., et al. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *Elife* 3:e04580. doi: 10.7554/eLife.04580.039

Bargmann, C. I., and Marder, E. (2013). From the connectome to brain function. *Nat. Methods* 10, 483–490. doi: 10.1038/nmeth.2451

Bentzur, A., Ben-Shaanan, S., Benichou, J. I. C. Costi, E., Levi, M., Ilany, A., et al. (2021). Early life experience shapes male behavior and social networks in *Drosophila*. *Curr. Biol.* 31, 486-501.e3. doi: 10.1016/j.cub.2020.10.060

Bentzur, A., Shmueli, A., Omesi, L., Ryvkin, J., Knapp, J. M., Parnas, M., et al. (2018). Odorant binding protein 69a connects social interaction to modulation of social responsiveness in *Drosophila*. *PLoS Genet.* 14:e1007328. doi: 10.1371/journal.pgen.1007328

Beshel, J., and Zhong, Y. (2013). Graded encoding of food odor value in the *Drosophila* brain. *J. Neurosci.* 33, 15693–15704. doi: 10.1523/JNEUROSCI.2605-13.2013

Branson, K., Robie, A. A., Bender, J., Perona, P., and Dickinson, M. H. (2009). High-throughput ethomics in large groups of *Drosophila*. *Nat. Methods* 6, 451–457. doi: 10.1038/nmeth.1328

Briggs, D. I., Enriori, P. J., Lemus, M. B., Cowley, M. A., and Andrews, Z. B. (2010). Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. *Endocrinology* 151, 4745–4755. doi: 10.1210/en.2010-0556

Cabrera, C. V. (1992). The generation of cell diversity during early neurogenesis in *Drosophila*. *Development* 115, 893–901.

Callis, J. (2014). The ubiquitination machinery of the ubiquitin system. *Arabidopsis Book* 12:e0174. doi: 10.1199/tab.0174

Certel, S. J., Leung, A., Lin, C. Y., Perez, P., Chiang, A. S., and Kravitz, E. A. (2010). Octopamine neuromodulatory effects on a social behavior decision-making network in *Drosophila* males. *PLoS One* 5:e13248. doi: 10.1371/journal.pone.0013248

Certel, S. J., Savella, M. G., Schlegel, D. C. F., and Kravitz, E. A. (2007). Modulation of *Drosophila* male behavioral choice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4706–4711. doi: 10.1073/pnas.0700328104

Chen, P., and Hong, W. (2018). Neural circuit mechanisms of social behavior. *Neuron* 98, 16–30. doi: 10.1016/j.neuron.2018.02.026

Chen, R., Wu, X., Jiang, L., and Zhang, Y. (2017). Single-cell RNA-seq reveals hypothalamic cell diversity. *Cell Rep.* 18, 3227–3241. doi: 10.1016/j.celrep.2017.03.004

Chung, B. Y., Ro, J., Hutter, S. A., Miller, K. M., Guduguntla, L. S., Kondo, S., et al. (2017). *Drosophila* neuropeptide F signaling independently regulates feeding and sleep-wake Behavior. *Cell Rep.* 19, 2441–2450. doi: 10.1016/j.celrep.2017.05.085

Croset, V., Treiber, C. D., and Waddell, S. (2018). Cellular diversity in the *Drosophila* midbrain revealed by single-cell transcriptomics. *Elife* 7:e34550. doi: 10.7554/eLife.34550.026

Datta, S. R., Anderson, D. J., Branson, K., Perona, P., and Leifer, A. (2019). Computational neuroethology: a call to action. *Neuron* 104, 11–24. doi: 10.1016/j.neuron.2019.09.038

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, Ł, et al. (2018). A single-cell transcriptome atlas of the aging *Drosophila* brain. *Cell* 174, 982–998.e20. doi: 10.1016/j.cell.2018.05.057

Davies, A. G., Bettinger, J. C., Thiele, T. R., Judy, M. E., and McIntire, S. L. (2004). Natural variation in the npr-1 gene modifies ethanol responses of wild strains of C. elegans. *Neuron* 42, 731–743. doi: 10.1016/j.neuron.2004.05.004

Davis, F. P., Nern, A., Picard, S., Reiser, M. B., Rubin, G. M., Eddy, S. R., et al. (2020). A genetic, genomic, and computational resource for exploring neural circuit function. *Elife* 9:e50901. doi: 10.7554/eLife.50901

De Bono, M., and Bargmann, C. I. (1998). Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. *Cell* 94, 679–689. doi: 10.1016/S0092-8674(00)81609-8

Desai, S. J., Upadhya, M. A., Subhedar, N. K., and Kokare, D. M. (2013). NPY mediates reward activity of morphine, via NPY Y1 receptors, in the nucleus accumbens shell. *Behav. Brain Res.* 247, 79–91. doi: 10.1016/j.bbr.2013.03.018

Dierick, H. A., and Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat. Genet.* 39, 678–682. doi: 10.1038/ng2029

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

Erion, R., King, A. N., Wu, G., Hogenesch, J. B., and Sehgal, A. (2016). Neural clocks and neuropeptide F/Y regulate circadian gene expression in a peripheral metabolic tissue. *Elife* 5:e13552. doi: 10.7554/eLife.13552.023

Franco, S. J., and Müller, U. (2013). Shaping our minds: stem and progenitor cell diversity in the mammalian neocortex. *Neuron* 77, 19–34. doi: 10.1016/j.neuron.2012.12.022

Goodson, J. L., and Bass, A. H. (2001). Social behavior functions and related anatomical characteristics of vasotocin / vasopressin systems in vertebrates. *Brain Res. Rev.* 35, 246–265. doi: 10.1016/S0165-0173(01)00043-1

Gray, J. M., and Spiegel, I. (2019). Cell-type-specific programs for activity-regulated gene expression. *Curr. Opin. Neurobiol.* 56, 33–39. doi: 10.1016/j.conb.2018.11.001

He, C., Cong, X., Zhang, R., Wu, D., An, C., and Zhao, Z. (2013a). Regulation of circadian locomotor rhythm by neuropeptide Y-like system in *Drosophila melanogaster*. *Insect Mol. Biol.* 22, 376–388. doi: 10.1111/imb.12027

He, C., Yang, Y., Zhang, M., Price, J. L., and Zhao, Z. (2013b). Regulation of sleep by neuropeptide Y-like system in *Drosophila melanogaster*. *PLoS One* 8:e74237. doi: 10.1371/journal.pone.0074237

Heilig, M. (2004). The NPY system in stress, anxiety and depression. *Neuropeptides* 38, 213–224. doi: 10.1016/j.npep.2004.05.002

Henry, G. L., Davis, F. P., Picard, S., and Eddy, S. R. (2012). Cell type-specific genomics of *Drosophila* neurons. *Nucleic Acids Res.* 40, 9691–9704. doi: 10.1093/nar/gks671

Herranz, H., Morata, G., and Milán, M. (2006). Calderón encodes an organic cation transporter of the major facilitator superfamily required for cell growth and proliferation of *Drosophila* tissues. *Development* 133, 2617–2625. doi: 10.1242/dev.02436

Ida, T., Takahashi, T., Tominaga, H., Sato, T., Kume, K., Ozaki, M., et al. (2011). Identification of the novel bioactive peptides dRYamide-1 and dRYamide-2, ligands for a neuropeptide Y-like receptor in *Drosophila*. *Biochem. Biophys. Res. Commun.* 410, 872–877. doi: 10.1016/j.bbrc.2011.06.081

Jepson, J. E. C., and Reenan, R. A. (2009). Adenosine-to-inosine genetic recoding is required in the adult stage nervous system for coordinated behavior in *Drosophila*. *J. Biol. Chem.* 284, 31391–31400. doi: 10.1074/jbc.M109.035048

Kabra, M., Robie, A. A., Rivera-Alba, M., Branson, S., and Branson, K. (2013). JAABA: interactive machine learning for automatic annotation of animal behavior. *Nat. Methods* 10, 64–67. doi: 10.1038/nmeth.2281

Kacsoh, B. Z., Lynch, Z. R., Mortimer, N. T., and Schlenke, T. A. (2013). Fruit flies medicate offspring after seeing parasites. *Science* 339, 947–950. doi: 10.1126/science.1229625

Kalra, S. P., and Kalra, P. S. (2004). NPY and cohorts in regulating appetite, obesity and metabolic syndrome: beneficial effects of gene therapy. *Neuropeptides* 38, 201–211. doi: 10.1016/j.npep.2004.06.003

Karl, T., and Herzog, H. (2007). Behavioral profiling of NPY in aggression and neuropsychiatric diseases. *Peptides* 28, 326–333. doi: 10.1016/j.peptides.2006.07.027

Keegan, L. P., Brindle, J., Gallo, A., Leroy, A., Reenan, R. A., and O'Connell, M. A. (2005). Tuning of RNA editing by ADAR is required in *Drosophila*. *EMBO J.* 24, 2183–2193. doi: 10.1038/sj.emboj.7600691

Kim, D. H., Shin, M., Jung, S. H., Kim, Y. J., and Jones, W. D. (2017). A fat-derived metabolite regulates a peptidergic feeding circuit in *Drosophila*. *PLoS Biol.* 15:e2000532. doi: 10.1371/journal.pbio.2000532

Krashes, M. J., DasGupta, S., Vreede, A., White, B., Douglas Armstrong, J., Waddell, S., et al. (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

LeGates, T. A., Kvarta, M. D., Tooley, J. R., Francis, T. C., Lobo, M. K., Creed, M. C., et al. (2018). Reward behaviour is regulated by the strength of hippocampus–nucleus accumbens synapses. *Nature* 564, 258–262. doi: 10.1038/s41586-018-0740-8

Li, X., Overton, I. M., Baines, R. A., Keegan, L. P., and O'Connell, M. A. (2014). The ADAR RNA editing enzyme controls neuronal excitability in *Drosophila melanogaster*. *Nucleic Acids Res.* 42, 1139–1151. doi: 10.1093/nar/gkt909

Li, X., Qu, M.-J., Zhang, Y., Li, J.-W., and Liu, T.-X. (2018). Expression of neuropeptide F gene and its regulation of feeding behavior in the pea aphid, *Acyrthosiphon pisum*. *Front. Physiol.* 9:87. doi: 10.3389/fphys.2018.00087

Lingo, P. R., Zhao, Z., and Shen, P. (2007). Co-regulation of cold-resistant food acquisition by insulin- and neuropeptide Y-like systems in *Drosophila melanogaster*. *Neuroscience* 148, 371–374. doi: 10.1016/j.neuroscience.2007.06.010

Liu, W., Ganguly, A., Huang, J., Wang, Y., Ni, J. D., Gurav, A. S., et al. (2019). Neuropeptide f regulates courtship in drosophila through a male-specific neuronal circuit. *Elife* 8:e49574. doi: 10.7554/eLife.49574

Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y. H., Zhang, J. X., et al. (2011). Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in *Drosophila*. *Nat. Neurosci.* 14, 896–902. doi: 10.1038/nn.2836

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Maldonado, C., Alicea, D., Gonzalez, M., Bykhovskaia, M., and Marie, B. (2013). Adar is essential for optimal presynaptic function. *Mol. Cell. Neurosci.* 52, 173–180. doi: 10.1016/j.mcn.2012.10.009

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17:10. doi: 10.14806/ej.17.1.200

Mcrobert, S. P. (1991). The effect of the homoeotic mutation, spineless-aristapedia, on female receptivity to Male courtship in drosophila melanogaster. *J. Neurogenet.* 7, 253–256. doi: 10.3109/01677069109167437

Meltzer, H., Marom, E., Alyagor, I., Mayseless, O., Berkun, V., Segal-Gilboa, N., et al. (2019). Tissue-specific (ts)CRISPR as an efficient strategy for in vivo screening in *Drosophila*. *Nat. Commun.* 10:2113. doi: 10.1038/s41467-019-10140-0

Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P. D. (2019). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* 47, D419–D426. doi: 10.1093/nar/gky1038

Mickelsen, L. E., Bolisetty, M., Chimileski, B. R., Fujita, A., Beltrami, E. J., Costanzo, J. T., et al. (2019). Single-cell transcriptomic analysis of the lateral hypothalamic area reveals molecularly distinct populations of inhibitory and excitatory neurons. *Nat. Neurosci.* 22, 642–656. doi: 10.1038/s41593-019-0349-8

Mo, A., Mukamel, E. A., Davis, F. P., Luo, C., Henry, G. L., Picard, S., et al. (2015). Epigenomic signatures of neuronal diversity in the mammalian brain. *Neuron* 86, 1369–1384. doi: 10.1016/j.neuron.2015.05.018

Orgad, S., Rosenfeld, G., Greenspan, R. J., and Segal, D. (2000). courtless, the *Drosophila* UBC7 homolog, is involved in male courtship behavior and spermatogenesis. *Genetics* 155, 1267–1280.

Palladino, M. J., Keegan, L. P., O'Connell, M. A., and Reenan, R. A. (2000). A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. *Cell* 102, 437–449. doi: 10.1016/S0092-8674(00)00049-0

Parker, K. E., Pedersen, C. E., Gomez, A. M., Spangler, S. M., Walicki, M. C., Feng, S. Y., et al. (2019). A paranigral VTA nociceptin circuit that constrains motivation for reward. *Cell* 178, 653–671.e19. doi: 10.1016/j.cell.2019.06.034

Perisse, E., Owald, D., Barnstedt, O., Talbot, C. B. 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

Poe, A. R., Wang, B., Sapar, M. L., Ji, H., Li, K., Onabajo, T., et al. (2019). Robust CRISPR/CAS9-mediated tissue-specific mutagenesis reveals gene redundancy and perdurance in drosophila. *Genetics* 211, 459–472. doi: 10.1534/genetics.118.301736

Pu, Y., Zhang, Y., Zhang, Y., and Shen, P. (2018). Two *Drosophila* neuropeptide Y-like neurons define a reward module for transforming appetitive odor representations to motivation. *Sci. Rep.* 8:11658. doi: 10.1038/s41598-018-30113-5

Robie, A., Kabra, M., Hirokawa, J., Korff, W., and Branson, K. (2012). High-throughput behavioral screen from data collection to analysis. *Front. Behav. Neurosci.* 6, 451–457. doi: 10.3389/conf.fnbeh.2012.27.00315

Robinson, S. L., and Thiele, T. E. (2017). "The role of neuropeptide Y (NPY) in alcohol and drug abuse disorders," in *International Review of Neurobiology*, Vol. 136, ed. T. E. Thiele (Cambridge, MA: Academic Press Inc), 177–197. doi: 10.1016/bs.irn.2017.06.005

Rogers, C., Reale, V., Kim, K., Chatwin, H., Li, C., Evans, P., et al. (2003). Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat. Neurosci.* 6, 1178–1185. doi: 10.1038/nn1140

Rosenthal, J. J. C., and Seeburg, P. H. (2012). A-to-I RNA editing: effects on proteins key to neural excitability. *Neuron* 74, 432–439. doi: 10.1016/j.neuron.2012.04.010

Sapiro, A. L., Shmueli, A., Lee Henry, G., Li, Q., Shalit, T., Yaron, O., et al. (2019). Illuminating spatial A-to-I RNA editing signatures within the *Drosophila* brain. *Proc. Natl. Acad. Sci. U.S.A.* 116, 2318–2327. doi: 10.1073/pnas.1811768116

Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S. Y., Hayworth, K. J., et al. (2020). A connectome and analysis of the adult *Drosophila* central brain. *bioRxiv* [Preprint], 1–83. doi: 10.1101/2020.04.07.030213

Schnell, J. D., and Hicke, L. (2003). Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J. Biol. Chem.* 278, 35857–35860. doi: 10.1074/jbc.R300018200

Schützler, N., Girwert, C., Hügli, I., Mohana, G., Roignant, J. Y., Ryglewski, S., et al. (2019). Tyramine action on motoneuron excitability and adaptable tyramine/octopamine ratios adjust *Drosophila* locomotion to nutritional state. *Proc. Natl. Acad. Sci. U.S.A.* 116, 3805–3810. doi: 10.1073/pnas.1813554116

Senapati, B., Tsao, C. H., Juan, Y. A., Chiu, T. H., Wu, C. L., Waddell, S., et al. (2019). A neural mechanism for deprivation state-specific expression of relevant memories in *Drosophila*. *Nat. Neurosci.* 22, 2029–2039. doi: 10.1038/s41593-019-0515-z

Shao, L., Saver, M., Chung, P., Ren, Q., Lee, T., Kent, C. F., et al. (2017). Dissection of the *Drosophila* neuropeptide F circuit using a high-throughput two-choice assay. *Proc. Natl. Acad. Sci. U.S.A.* 114, E8091–E8099. doi: 10.1073/pnas.1710552114

Shih, M. F. M., Davis, F. P., Henry, G. L., and Dubnau, J. (2019). Nuclear transcriptomes of the seven neuronal cell types that constitute the *Drosophila* mushroom bodies. *G3* 9, 81–94. doi: 10.1534/g3.118.200726

Shohat-Ophir, G., Kaun, K. R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual deprivation increases ethanol intake in *Drosophila*. *Science* 335, 1351–1355. doi: 10.1126/science.1215932

Sokolowski, M. B. (2003). NPY and the regulation of behavioral development. *Neuron* 39, 6–8. doi: 10.1016/S0896-6273(03)00398-2

Sparta, D. R., Fee, J. R., Knapp, D. J., Breese, G. R., and Thiele, T. E. (2007). Elevated anxiety-like behavior following ethanol exposure in mutant mice lacking neuropeptide Y (NPY). *Drug Alcohol Depend.* 90, 297–300. doi: 10.1016/j.drugalcdep.2007.04.001

Stapleton, M., Carlson, J. W., and Celniker, S. E. (2006). RNA editing in *Drosophila melanogaster*: new targets and functional consequences. *RNA* 12, 1922–1932. doi: 10.1261/rna.254306

Sternson, S. M. (2020). Exploring internal state-coding across the rodent brain. *Curr. Opin. Neurobiol.* 65, 20–26. doi: 10.1016/j.conb.2020.08.009

Thomas, P. D., Kejariwal, A., Guo, N., Mi, H., Campbell, M. J., Muruganujan, A., et al. (2006). Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucleic Acids Res.* 34, W645–W650. doi: 10.1093/nar/gkl229

Thornquist, S. C., Langer, K., Zhang, S. X., Rogulja, D., and Crickmore, M. A. (2020). CaMKII measures the passage of time to coordinate behavior and motivational state. *Neuron* 105, 334–345.e9. doi: 10.1016/j.neuron.2019.10.018

Tokuno, H., Chiken, S., Kametani, K., and Moriizumi, T. (2002). Blockade of central neuropeptide Y (NPY) Y2 receptors reduces ethanol self-administration in rats. *Neurosci. Lett.* 332, 1–4. doi: 10.1016/S0304-3940(02)00904-7

Tsao, C. H., Chen, C. C., Lin, C. H., Yang, H. Y., and Lin, S. (2018). *Drosophila* mushroom bodies integrate hunger and satiety signals to control innate food-seeking behavior. *Elife* 7:e35264. doi: 10.7554/eLife.35264.042

Venken, K. J. T., Simpson, J. H., and Bellen, H. J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron* 72, 202–230. doi: 10.1016/j.neuron.2011.09.021

Waddell, S., Owald, D., Lin, S., and Waddell, S. (2015). 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

Wang, L., Dankert, H., Perona, P., and Anderson, D. J. (2008). A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5657–5663. doi: 10.1073/pnas.0801327105

Wen, T., Parrish, C. A., Xu, D., Wu, Q., and Shen, P. (2005). *Drosophila* neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2141–2146. doi: 10.1073/pnas.0406814102

Wiater, M. F., Mukherjee, S., Li, A.-J., Dinh, T. T., Rooney, E. M., Simasko, S. M., et al. (2011). Circadian integration of sleep-wake and feeding requires NPY receptor-expressing neurons in the mediobasal hypothalamus. *Am. J. Physiol. Integr. Comp. Physiol.* 301, R1569–R1583. doi: 10.1152/ajpregu.00168.2011

Wilinski, D., Winzeler, J., Duren, W., Persons, J. L., Holme, K. J., Mosquera, J., et al. (2019). Rapid metabolic shifts occur during the transition between hunger and satiety in *Drosophila melanogaster*. *Nat. Commun.* 10:4052. doi: 10.1038/s41467-019-11933-z

Winnubst, J., Bas, E., Ferreira, T. A., Wu, Z., Economo, M. N., Edson, P., et al. (2019). Reconstruction of 1,000 projection neurons reveals new cell types and organization of long-range connectivity in the mouse brain. *Cell* 179, 268–281.e13. doi: 10.1016/j.cell.2019.07.042

Wu, Q., Wen, T., Lee, G., Park, J. H., Cai, H. N., and Shen, P. (2003). Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron* 39, 147–161. doi: 10.1016/S0896-6273(03)00396-9

Wu, Q., Zhang, Y., Xu, J., and Shen, P. (2005). Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13289–13294. doi: 10.1073/pnas.0501914102

Xu, J., Li, M., and Shen, P. (2010). A G-protein-coupled neuropeptide Y-like receptor suppresses behavioral and sensory response to multiple stressful stimuli in *Drosophila*. *J. Neurosci.* 30, 2504–2512. doi: 10.1523/JNEUROSCI.3262-09.2010

Xu, S., Yang, H., Menon, V., Lemire, A. L., Wang, L., Henry, F. E., et al. (2020). Behavioral state coding by molecularly defined paraventricular hypothalamic cell type ensembles. *Science* 370:eabb2494. doi: 10.1126/science.abb2494

Xue, Z., Wu, M., Wen, K., Ren, M., Long, L., Zhang, X., et al. (2014). /Cas9 mediates efficient conditional mutagenesis in *Drosophila*. *G3* 4, 2167–2173. doi: 10.1534/g3.114.014159

Yizhar, O. (2012). Optogenetic insights into social behavior function. *Biol. Psychiatry* 71, 1075–1080. doi: 10.1016/j.biopsych.2011.12.029

Zer-Krispil, S., Zak, H., Shao, L., Ben-Shaanan, S., Tordjman, L., Bentzur, A., et al. (2018). Ejaculation induced by the activation of Crz neurons is rewarding to *Drosophila* males. *Curr. Biol.* 28, 1445–1452.e3. doi: 10.1016/j.cub.2018.03.039

Zhang, S. X., Miner, L. E., Boutros, C. L., Rogulja, D., and Crickmore, M. A. (2018). Motivation, perception, and chance converge to make a binary decision. *Neuron* 99, 376–388.e6. doi: 10.1016/j.neuron.2018.06.014

Zhang, S. X., Rogulja, D., and Crickmore, M. A. (2019). Recurrent circuitry sustains *Drosophila* courtship drive while priming itself for satiety. *Curr. Biol.* 29, 3216–3228.e9. doi: 10.1016/j.cub.2019.08.015

Zheng, Z., Lauritzen, J. S., Perlman, E., Robinson, C. G., Nichols, M., Milkie, D., et al. (2018). A complete electron microscopy volume of the brain of adult *Drosophila melanogaster*. *Cell* 174, 730–743.e22. doi: 10.1016/j.cell.2018.06.019

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 21 April 2021
doi: 10.3389/fnbeh.2021.660464

Insects Provide Unique Systems to Investigate How Early-Life Experience Alters the Brain and Behavior

*Rebecca R. Westwick and Clare C. Rittschof**

Department of Entomology, University of Kentucky, Lexington, KY, United States

Early-life experiences have strong and long-lasting consequences for behavior in a surprising diversity of animals. Determining which environmental inputs cause behavioral change, how this information becomes neurobiologically encoded, and the functional consequences of these changes remain fundamental puzzles relevant to diverse fields from evolutionary biology to the health sciences. Here we explore how insects provide unique opportunities for comparative study of developmental behavioral plasticity. Insects have sophisticated behavior and cognitive abilities, and they are frequently studied in their natural environments, which provides an ecological and adaptive perspective that is often more limited in lab-based vertebrate models. A range of cues, from relatively simple cues like temperature to complex social information, influence insect behavior. This variety provides experimentally tractable opportunities to study diverse neural plasticity mechanisms. Insects also have a wide range of neurodevelopmental trajectories while sharing many developmental plasticity mechanisms with vertebrates. In addition, some insects retain only subsets of their juvenile neuronal population in adulthood, narrowing the targets for detailed study of cellular plasticity mechanisms. Insects and vertebrates share many of the same knowledge gaps pertaining to developmental behavioral plasticity. Combined with the extensive study of insect behavior under natural conditions and their experimental tractability, insect systems may be uniquely qualified to address some of the biggest unanswered questions in this field.

Keywords: critical period, phenotypic plasticity, genetic toolkit, trauma, DNA methylation

OPEN ACCESS

Edited by:
Tom V. Smulders,
Newcastle University, United Kingdom

Reviewed by:
Bryan Kolb,
University of Lethbridge, Canada
Lars Chittka,
Queen Mary University of London, United Kingdom

***Correspondence:**
Clare C. Rittschof
clare.rittschof@uky.edu

Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *29 January 2021*
Accepted: *16 March 2021*
Published: *21 April 2021*

Citation:
Westwick RR and Rittschof CC (2021) Insects Provide Unique Systems to Investigate How Early-Life Experience Alters the Brain and Behavior. Front. Behav. Neurosci. 15:660464. doi: 10.3389/fnbeh.2021.660464

INTRODUCTION

Early-life experiences can have profound consequences for adult phenotypes, particularly behaviors (Beach and Jaynes, 1954), a phenomenon called developmental behavioral plasticity (sensu West-Eberhard, 2003, 2005). Although this phenomenon is well-established, its mechanistic basis remains a persistent research puzzle that touches many behavioral neuroscience disciplines and applications (Beldade et al., 2011; Snell-Rood, 2013; Reh et al., 2020). Brain development is fundamentally complex—it is a dynamic interaction between endogenous, gene-guided programs and environmental inputs (Boyce et al., 2020; Reh et al., 2020). Thus, determining how experiences are "embedded" requires knowledge at multiple levels of organization,

from molecules to neural structure (Champagne, 2012; Cardoso et al., 2015; Curley and Champagne, 2016; Sinha et al., 2020). Moreover, individual differences can extend to peripheral tissues, which are also shaped by developmental experience and interact with the brain to influence adult behavioral expression (**Figure 1**). Finally, in addition to triggering behavioral change, environmental conditions dictate the adaptive consequences of behavioral expression. Understanding these consequences may allow researchers to predict the types of experiences that cause lasting or transient behavioral impacts. However, adaptive consequences of behavioral expression are difficult to ascertain in traditional lab-based model systems alone (Yartsev, 2017).

Fortunately, developmental behavioral plasticity occurs in animals as complex as humans and as simple as nematodes (Jobson et al., 2015; Kundakovic and Champagne, 2015). In this mini review, we explore how the insects are surprisingly well-suited to provide unique contributions to the study of this phenomenon. First, we highlight the strong ecological basis of insect behavior research (Schowalter, 2016), reviewing the exceptionally diverse systems available to explore the neurobiological basis of developmental behavioral plasticity in natural contexts with adaptive significance. Second, we provide an overview of the extensive examples of homology of function between insect and vertebrate nervous systems, despite their phylogenetic distance. We highlight the fact that a variety of mechanisms that embed developmental experience are broadly shared across groups. We conclude that insects offer a fertile and exciting area of future comparative research that explores the complex relationships between early-life experiences and adult behavioral expression.

INSECTS AS MODELS FOR DEVELOPMENTAL BEHAVIORAL PLASTICITY IN NATURAL CONTEXTS

Extensive previous studies show that the developmental environment has diverse adaptive consequences for insect behavior. Such a perspective is valuable to behavioral neuroscience because environmental context defines the cues, sensory systems, and central processing dynamics that underpin behavioral change. Knowledge of environmental context may also be useful in establishing a general understanding of the types of conditions that give rise to transient versus lasting behavioral effects, a long-term goal in behavioral neuroscience. We highlight some of the established relationships between developmental experience and adult behavioral variation in insects, focusing on three major types of common environmental inputs: season, feeding experience, and interactions with other organisms.

Season

Many insects integrate seasonal cues during development and adaptively tune their adult behavioral expression to match environmental conditions (De Wilde, 1962; Benoit, 2010; Buckley et al., 2012). For example, in the butterfly *Bicyclus anynana*, males produce a costly nutritional gift they provide to females in order to improve their mating chances. The costs and benefits of this gift change from the wet to the dry season, and accordingly, males adjust their gift production and courtship efforts depending on developmental moisture conditions (Prudic et al., 2011). In ground crickets (*Allonemobius fasciatus*), developmental temperature constrains male singing ability (Olvido and Mousseau, 1995), and as a result, females adjust their species-specific song preferences in response to their experience of temperature and day length during development (Grace and Shaw, 2004). Subtle differences in developmental temperature (e.g., developing in shaded versus sun-exposed shallow underground nests) can have profound behavioral impacts in female *Lasioglossum baleicum* bees; they shift from a cooperative reproductive tactic to a solitary one when developing in shadier locations (Hirata and Higashi, 2008). This selection of examples shows that the insects provide opportunities to investigate how simple developmental cues like temperature impact sophisticated phenotypes involving high level sensory integration and complex behaviors.

Feeding Experience

Developmental feeding conditions can convey a variety of information. For example, because many insects are short-lived, developmental diet often predicts the state of nutritional resources available to the adult insect and even its offspring. Females of many insects, particularly moths, prefer to lay eggs on the same species of plant they fed on during development (Petit et al., 2015), a phenomenon often referred to as Hopkins' Host Selection Principle (Hopkins, 1917). This pattern may minimize search time for suitable host plants for offspring. Though the mechanistic basis of this phenomenon remains controversial, experience-based developmental preferences for or against certain host plants or olfactory cues have been shown in multiple insect clades (Barron, 2001; Rietdorf and Steidle, 2002; Akhtar and Isman, 2003; Blackiston et al., 2008; Akhtar et al., 2009; Videla et al., 2010; Anderson et al., 2013; Anderson and Anton, 2014; König et al., 2015; Lhomme et al., 2017). Developmental feeding conditions can also indirectly signal the degree of intraspecific competition in the immediate environment, triggering mechanisms that alter myriad traits including adult body size, dispersal strategy, activity level, and exploratory behavior (**Figure 1**; Moczek and Emlen, 2000; Tripet et al., 2002; Tremmel and Müller, 2012).

Diverse neurobiological mechanisms are implicated in the response to developmental feeding experience. For example, plant volatile cues and the olfactory system play a strong role in butterfly and moth larval host plant identification (Petit et al., 2015). In other cases, including in some beetles, bees, aphids, and planthoppers, food intake itself is a cue leading to altered insulin and hormone signaling, which coordinate both peripheral and cognitive processes during development and throughout adulthood (Ament et al., 2008; Snell-Rood and Moczek, 2012; Zhang et al., 2019). More work is needed to understand how physiological processes like insulin signaling affect sensory perception and integration throughout adulthood, a topic that is currently of general interest in vertebrate cognitive neuroscience (Arvanitakis et al., 2020).

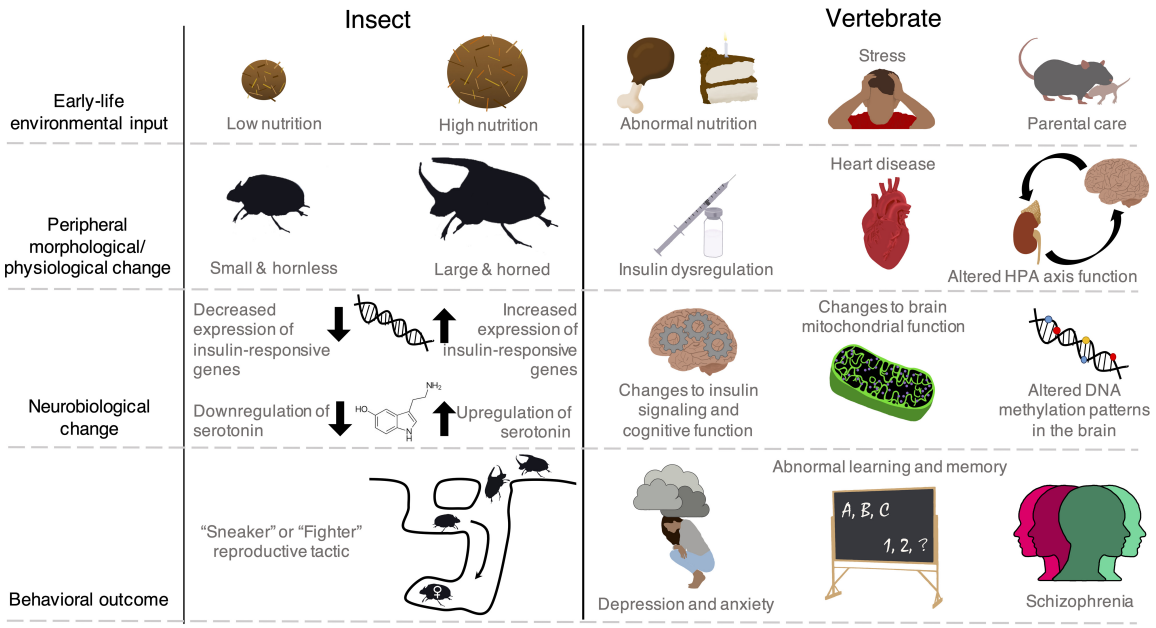

FIGURE 1 | The impacts of early-life experiences extend beyond the brain to peripheral physiological systems and even body morphology in insect and vertebrate species. The brain and peripheral systems interact to shape adult behavioral expression in ways that remain poorly understood. Though these brain-peripheral connections are common across animals including vertebrates, and specifically humans, some insects show particularly conspicuous and discrete changes in morphology, presenting interesting systems to investigate behavioral regulation. Moreover, despite the more noticeable phenotypic differences in some insects, there are examples of common regulatory mechanisms (e.g., insulin signaling) that underpin behavioral dynamics across the insect and vertebrate phylogenetic space. Left: In some beetles (*Onthophagus spp.*), males that are provided high amounts of nutrition during development emerge as large adults with horns (Emlen, 1997). Horns give males a benefit in competition over female mates, which nest in sub-terranean tunnels under dung piles (Moczek and Emlen, 2000). These morphological changes are associated with changes in brain insulin and serotonin signaling (Snell-Rood and Moczek, 2012; Newsom et al., 2019) and result in two distinct male reproductive tactics. Large, horned males will guard female tunnels and compete with other rivals, while small, hornless males dig side tunnels and sneak around large males to reach the female (Emlen, 1997; Moczek and Emlen, 2000). Right: In vertebrates, early-life nutrition, stress, and social interactions cause coordinated changes in peripheral physiological function (Barker, 1995; Champagne and Curley, 2005; Avitsur et al., 2015) as well as brain hormone signaling, bioenergetics, and gene regulation (Hochberg et al., 2011; Korosi et al., 2012; Hoffmann and Spengler, 2018). These changes can give rise to cognitive and mental health disorders (Avishai-Eliner et al., 2002; van Os et al., 2010; Chen and Baram, 2016; Sripetchwandee et al., 2018).

Interactions With Other Organisms

Other animals (but see also Schretter et al., 2018; Schwab et al., 2018 for the role of microbiota) commonly shape the insect developmental environment. For example, in a variety of insects, conspecific density and predation pressure induce developmental behavioral plasticity (Walzer and Schausberger, 2011; Müller et al., 2016). One famous case involves the transition from the solitary to gregarious phase in migratory locusts. Increased frequency of physical contact during early life (a result of high conspecific density) gives rise to diverse morphological and behavioral changes, culminating in massive swarming events that disperse individuals to new locations with greater resources (Gillett, 1973; Simpson et al., 2001).

A variety of insect species (e.g., many ants, bees, wasps, and termites) live in complex eusocial societies where certain members forego reproduction to help raise the offspring of their relatives (Oster and Wilson, 1978). Individuals of these species interact socially with conspecifics throughout life, including during development. Female caste differentiation, where females can develop into either a reproductive queen or a non-reproductive worker, is a well-studied example of developmental behavioral plasticity in these eusocial insects (Schwander et al., 2010). Queen/worker caste determination is typically a function of larval nutrition (at least in part) and mediated by adult "nurses" who provide food to larvae (Brian, 1956; Gadagkar et al., 1991; Page and Peng, 2001; Liu et al., 2005; Smith et al., 2008). In some eusocial insects, particularly ants, developmental dietary differences also give rise to behaviorally and morphologically distinct "soldiers" (female workers specialized for defense; Rajakumar et al., 2018).

There are other more subtle effects of the developmental social environment in eusocial insects (Miura, 2004; Traynor et al., 2014; Wang et al., 2014). For example, worker honey bees express different levels of defensiveness during adulthood depending on the defensiveness of the nestmates who rear them; this effect may be mediated by diet, but it is subtle enough that it does not alter body morphology (Rittschof et al., 2015). Adult wasps use vibratory signals directed at larvae, in combination with dietary interventions, to influence adult behavior, again without conspicuous changes in morphology (Jandt et al., 2017). More primitive social insects also show effects of developmental social interactions. For example, in the twig-nesting small carpenter bee (*Ceratina calcarata*), a mother's removal from the nest during the larval stage eliminates maternal grooming activity and

increases defensive and avoidant behaviors once offspring reach adulthood (Arsenault et al., 2018). Behavioral differentiation in developing insects involves a variety of cue types (e.g., nutrition, pheromone, vibratory, or tactile signals), often acting in combination, suggesting that diverse sensory and physiological systems are integrated to give rise to behavioral effects.

HOMOLOGY IN INSECT AND VERTEBRATE NERVOUS SYSTEM FUNCTION AND PLASTICITY

Insects have a popular reputation of having simplistic, decentralized nervous systems (Schaefer and Ritzmann, 2001). While it is true that some processes are locally guided by "ganglia," semi-autonomous central nervous system components along the ventral nerve cord (Klowden, 2013), the brain is still required for sensory integration, decision-making, navigation, and learning (Pringle, 1940; Reingold and Camhi, 1977; Zill, 1986; Wessnitzer and Webb, 2006). Indeed, insects are capable of an impressive array of cognitive abilities, such as numeracy and social learning, because of their integrative brains (Chittka and Geiger, 1995; Giurfa et al., 1996, 2001; Dyer, 1998; Crist, 2004; Coolen et al., 2005; Avarguès-Weber, 2012; Pahl et al., 2013; Alem et al., 2016).

Insect brain structure and function is well studied (Ito et al., 2014), giving a strong basis to evaluate mechanisms of developmental plasticity from a comparative perspective. Extensive previous studies illuminate examples of homology of function with vertebrate systems (Simons and Tibbetts, 2019). Below we briefly review these general similarities, and then we focus on the specific neural mechanisms that encode developmental experience, many of which are also shared.

Homology of Function Between Insect and Vertebrate Brains

Insect and vertebrate central nervous systems have similar functions (Kinoshita and Homberg, 2017), and many general features are shared, although notably, the evolutionary origin of these similarities remains controversial (Farris, 2008; Holland et al., 2013). For example, many of the same chemicals act as neurohormones and neurotransmitters, and even in conserved behavioral and cognitive contexts (Bicker et al., 1988; Osborne, 1996; Wu and Brown, 2006; Byrne and Fieber, 2017). In both vertebrates and insects including honey bees, bumble bees, fruit flies, and crickets, dopamine is involved in learning, novelty, reward prediction, and locomotion (Barron et al., 2008; Cohn et al., 2015; Gadagkar et al., 2016; Perry et al., 2016; Hattori et al., 2017; Terao and Mizunami, 2017; Felsenberg et al., 2018; Sovik et al., 2018). Likewise, serotonin modulates appetite, sleep, learning, social behavior, and aggression across a similar range of insect examples (Vleugels et al., 2015; Rillich and Stevenson, 2018; Bubak et al., 2020). Even insect-specific hormones have clear functional analogs in vertebrates. Insect juvenile hormone and vertebrate thyroid hormone both act through type II nuclear receptors, and they show similar growth and developmental functions (Flatt et al., 2006; Charles et al., 2011). Octopamine is an insect-specific neurohormone that is analogous to norepinephrine, and both compounds control stress response, motivation, and aggression (Roeder, 2005; Prieto Peres and Valença, 2010; Alfonso et al., 2019).

Beyond neurochemicals, recent studies suggest extensive homology between insect and vertebrate brain genome dynamics and protein function. Genes responsible for brain developmental patterning are surprisingly conserved (Lichtneckert and Reichert, 2005; Tessmar-Raible et al., 2007; Reichert, 2009; Loesel, 2011; O'Connell, 2013), and there is even evidence for functional conservation of genes associated with complex behaviors like territorial aggression, foraging, and brood care (Toth and Robinson, 2007; Rittschof et al., 2014; Toth et al., 2014; Saul et al., 2019; Shpigler et al., 2019). Cell types in the brain show similarities in structure and function. Like vertebrate brains, insect brains contain neurons and various types of glia (Losada-Perez, 2018), and the metabolic relationships between these cell types are similar across groups (Rittschof and Schirmeier, 2017). Neural activity is well-known for its energetic demands (Peters et al., 2004; Niven and Laughlin, 2008), and insects and vertebrates share some neural adaptations to high energy need (Robertson et al., 2020) and increased cognitive demands; the latter even shows a similar developmental basis (Farris, 2008).

Despite extensive similarities, insects do show some profound differences in nervous system structure and function compared to vertebrates. For example, insect neurons are unmyelinated, they have different classes of olfactory and photoreceptors compared to vertebrates, and neuronal polarity is often different (Chittka and Niven, 2009; Kaupp, 2010; Gutierrez et al., 2011; Rolls and Jegla, 2015; Albert and Kozlov, 2016). Another conspicuous difference between insects and most vertebrates is the structure of early-life development (**Figure 2**), including the somewhat extreme behavioral and morphological changes that occur during insect metamorphosis. Metamorphosing amphibians and fish are notable exceptions within vertebrates and provide an exciting avenue for comparative work (Gilbert et al., 1996; Heyland and Moroz, 2006; Shi, 2013; Lowe et al., 2021). As with outward appearance, the structure and function of the nervous system can change dramatically during metamorphic developmental transitions in insects (Wolbert and Kubbies, 1983; Weeks and Truman, 1986; Gilbert et al., 1996). For instance, butterflies transition from relatively sessile plant-eating caterpillars to flighted adults with distinct diets, behavioral traits, sensory structures, and motor and cognitive capabilities (André, 1991; Ebenman, 1992). About 80% of all insect species (including ants, bees, wasps, butterflies, beetles, and flies, among others) experience this extreme form of metamorphosis ("complete metamorphosis," Rolff et al., 2019). Most other insects experience incomplete metamorphosis, where the pupal stage is absent and the body plan in early life is more similar to that of the adult form (except for the absence of wings). Notably, some of these species still show radical differences in life history between juvenile and adult stages (Corbet, 1957; Gabbutt, 1959). The variation in development patterns in insects make them exciting but perhaps challenging subjects for comparative study of developmental behavioral plasticity.

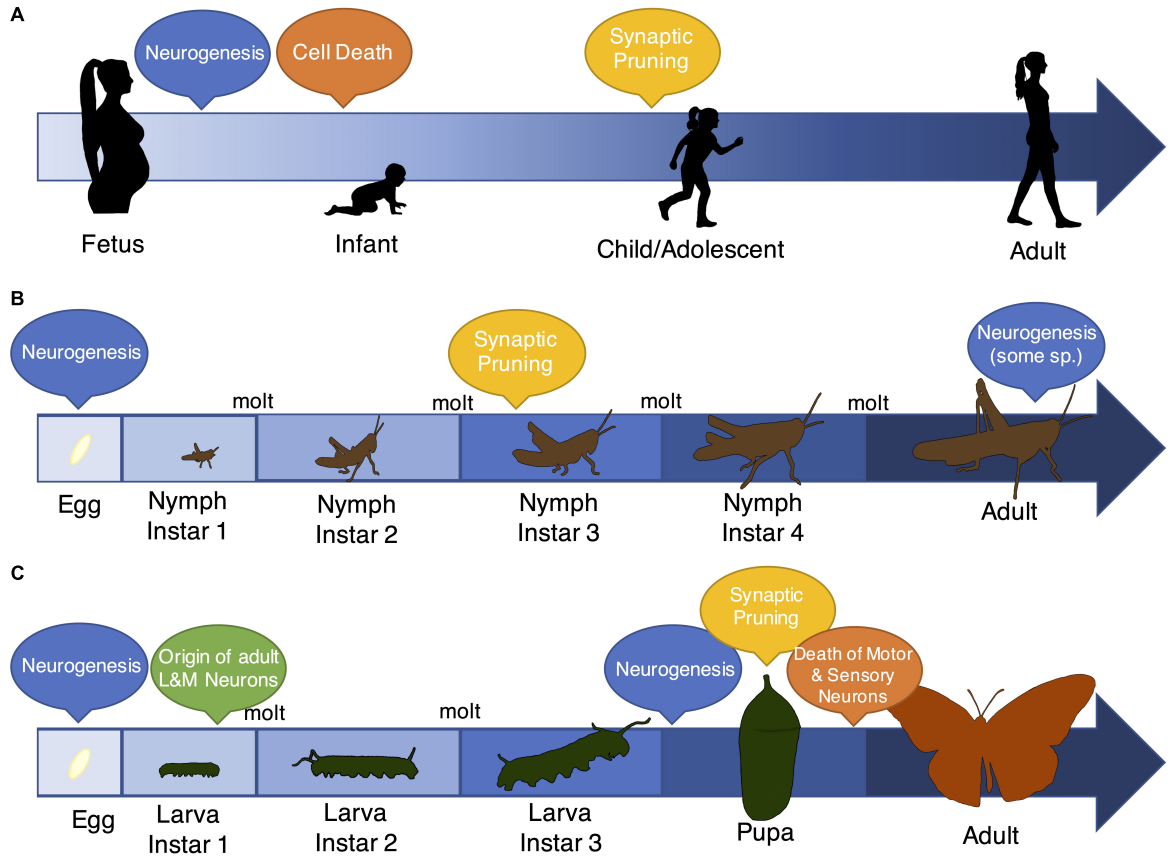

FIGURE 2 | Patterns of development, specifically the timing of neurobiological events, vary across vertebrates and insects. Although insects and vertebrates show remarkable overlap in the types of mechanisms that characterize brain development and entrain early-life experience (Watson, 1992; Pearson, 1993; Reh and Cagan, 1994; Salzberg and Bellen, 1996; Luo and O'Leary, 2005; Bello et al., 2008), the progression of early-life, and specifically the timing of events like neurogenesis, programmed cell death ("Cell Death"), and synaptic pruning, differs markedly across these groups. **(A)** Most vertebrates show gradual changes in body size and tissue morphology. In the brain, they experience massive neurogenesis early in life followed by cell death and pruning through adolescence and early adulthood (Watson et al., 2006). Notably, more limited neurogenesis also occurs during adulthood (Zhao et al., 2008). **(B)** Some insects also show a pattern of gradual development (called "incomplete metamorphosis"), where juvenile stages resemble the basic body plan of adults. However, these insects still shed their exoskeletons in order to grow, and as a result, they transition through distinct developmental stages. Relatively little is known about neurobiological events in these species, although there is evidence of extensive neurogenesis both prior to egg hatch and during adulthood (Cayre et al., 1994). There is also evidence for synaptic pruning dynamics that resemble vertebrate mechanisms (Lnenicka and Murphey, 1989). **(C)** The majority of insects (~80% of species) show a pattern of complete metamorphosis, where life stages have distinct morphologies, and adult behaviors and body plans vastly differ from juveniles. Data from several representatives of this group again suggest multiple periods of neurogenesis, both early in life and during the pupal stage (Booker and Truman, 1987; Truman and Bate, 1988). Interestingly, the timing of neurogenesis and programmed cell death and the retention of neurons through the life stages is brain region (and thus, functionally) specific (Wegerhoff, 1999; Tissot and Stocker, 2000). For example, a small number of neurons responsible for learning and memory originate early in the larval period and persist through adulthood, but most motor and sensory neurons are completely remodeled during the pupal phase (Cantera et al., 1994).

Despite their developmental complexities, one unique benefit to insect study is that in some species, particularly those that undergo complete metamorphosis, only a subset of neurons is retained between the juvenile and adult stages (**Figure 2**; Cantera et al., 1994; Wegerhoff, 1999; Tissot and Stocker, 2000). This feature narrows the target populations for studies of early-life environmental effects. For example, in the sensory integration and learning and memory centers of the brain (primarily the "mushroom bodies"), adult neurons typically originate during early larval life, suggesting adequate opportunity to retain environmental information into adulthood; this is in contrast to sensory neurons, which are completely distinct between the larval and adult stages (Cayre et al., 1994; Tissot and Stocker, 2000). Moreover, even though the degree of neuronal remodeling may be relatively extreme in insects compared to vertebrates, the components of the remodeling process closely resemble the types of developmental changes that also occur in vertebrates (Luo and O'Leary, 2005; Bello et al., 2008). For example, analogous to developing vertebrates, different neuron populations in circuits associated with learning and memory display a coordinated process of pruning and regrowth during metamorphosis in *Drosophila melanogaster* (Spear, 2013; Mayseless et al., 2018). These features of insect neurodevelopment provide unique opportunities to study the complex neural mechanisms of developmental behavioral plasticity in careful detail.

Homology of Function in Neural Mechanisms that Encode Developmental Experience

Early-life cues change adult behavior by persistently altering the structure and/or function of the nervous system (Odeon et al., 2013). Though the precise dynamics of these changes remain poorly understood in any system, in general terms, known mechanisms are similar when comparing vertebrates to insects (Watson, 1992; Pearson, 1993; Reh and Cagan, 1994; Salzberg and Bellen, 1996). Major categories of mechanisms include epigenetic modifications, changes in the quantity of neurochemicals and/or their receptors, and brain structural changes (Elekonich and Robinson, 2000; Kretzschmar and Pflugfelder, 2002; Fahrbach, 2006; Schoofs et al., 2017; Glastad et al., 2019). These mechanisms are not mutually exclusive, and one long-term challenge in behavioral neuroscience for insects and vertebrates alike is to understand how these mechanisms are integrated to alter dynamic behaviors (Wolf and Linden, 2011). However, here we highlight some known insect examples of epigenetic, neurochemical, and structural mechanisms that encode developmental experience.

Chemical modifications to brain DNA are proposed to be critical mediators of early-life effects on adult behavior in vertebrates (Aristizabal et al., 2019). DNA methylation and histone post-translational modifications are the most well-studied among these mechanisms (Smallwood and Kelsey, 2012; Paredes et al., 2016). Not all insects possess appreciable levels of DNA methylation (Deobagkar, 2018; Deshmukh et al., 2018), but some, including many social insects, do (Li-Byarlay, 2016; Yagound et al., 2020). Some studies show that developmental experience-induced changes in DNA methylation impact adult behavioral phenotypes (Linksvayer et al., 2012; Patalano et al., 2012; Weiner and Toth, 2012; Yan et al., 2014; Alvarado et al., 2015). For example, the variation in larval diet that gives rise to queen versus worker female honey bees acts at least in part through DNA methylation changes in both the head and peripheral tissues (Kucharski et al., 2008; Shi et al., 2011; Wang et al., 2020). Similarly, studies in termites and locusts demonstrate a relationship between differential DNA methylation and developmentally induced adult behavioral variation (e.g., in the solitary versus gregarious phases of migratory locusts, Lo et al., 2018). Other known epigenetic mechanisms also play a role in developmental behavioral plasticity in insects, including histone modifications and long non-coding RNAs (Simola et al., 2016; Glastad et al., 2019).

The relationship between brain epigenetic modifications and gene expression patterns varies across species and is not well-understood. For example, whereas DNA methylation in gene regulatory regions tends to suppress gene expression in vertebrates, in insects, gene body methylation, which is thought to regulate alternative splicing, is more common (Feng et al., 2010; Zemach et al., 2010; Glastad et al., 2014; Schmitz et al., 2019). Furthermore, some studies have shown surprisingly weak relationships between DNA methylation dynamics and behavioral expression (Herb et al., 2012; Libbrecht et al., 2016). More data is necessary to understand how DNA methylation dynamics correspond to both gene expression dynamics and behavior (Flores et al., 2012; Li-Byarlay, 2016; Jeong et al., 2018), including whether the presence and degree of DNA methylation and other epigenetic modifications predict capacity for behavioral plasticity (Kapheim et al., 2015; Lo et al., 2018). These are general challenges facing vertebrate research as well (Di Sante et al., 2018), which could benefit from a comparative approach.

The developmental environment can cause lasting behavioral effects by altering neurochemical processes, e.g., circulating levels of hormones and neurotransmitters in the central nervous system. For example, changes in brain insulin, juvenile hormone, prothoracicotropic hormone, octopamine, and serotonin signaling are prominent correlates of insect developmental behavioral plasticity (De Wilde and Beetsma, 1982; Rachinsky, 1994; Paulino Simões et al., 1997; Moczek and Emlen, 2000; Snell-Rood and Moczek, 2012; Erion and Sehgal, 2013; Newsom et al., 2019). These chemicals impact behaviors like aggression, gregariousness, feeding, locomotion, and non-aggressive social interactions (Iba et al., 1995; Anstey et al., 2009; Erion and Sehgal, 2013) in a number of species, including the cricket and locust examples above. The degree to which neurochemical systems comparably regulate behaviors across vertebrates and invertebrates is a matter of debate (Bubak et al., 2020), and thus an important area of on-going study, especially in the context of developmental behavioral plasticity.

A final common way the developmental environment affects the nervous system is through brain structural changes (Teicher et al., 2016; Saleh et al., 2017; Hall and Tropepe, 2020). For example, in flies, high conspecific density during development results in larger mushroom bodies and enhanced olfactory processing abilities (Heisenberg et al., 1995). Similar conditions in wasps lead to increased overall adult brain size, and larger-volume mushroom bodies and regions required for visual processing (Groothuis and Smid, 2017). Gregarious locusts have larger integrative mushroom bodies, while solitary individuals show neural adaptations associated with enhanced sensory sensitivity (Ott and Rogers, 2010). Female social insects often show variation in relative brain region size as a function of behavioral specialization (Lucht-Bertram, 1961; Wheeler and Nijhout, 1984; Vitt and Hartfelder, 1998; Page and Peng, 2001; Muscedere and Traniello, 2012). Insect and vertebrate nervous systems not only exhibit many of the same developmental plasticity mechanisms, but they also face many of the same conceptual challenges associated with connecting developmental experience to behavioral expression. These extensive similarities suggest many potential benefits to comparative study.

DISCUSSION

Predicting, and in some cases changing, adult behavioral effects of early-life experience are challenges relevant to diverse fields of behavioral neuroscience (West-Eberhard, 2003; Beldade et al., 2011; Bryck and Fisher, 2012; Snell-Rood, 2013; Stamps and Biro, 2016; Danese, 2020; Reh et al., 2020). Behavioral effects of early-life experience are commonplace among animal species, presenting the opportunity to use comparative approaches to

identify the general principles of developmental behavioral plasticity. Many fundamental questions that are common to both insects and vertebrates remain to be resolved, for example, how the brain integrates early-life experience across multiple levels of organization, and whether specific mechanisms like DNA methylation universally predict long term behavioral impacts. Moreover, it remains unclear how developmental experiences are integrated with other sources of information (e.g., genetic variation, parental transgenerational effects) that also influence behavior (Dall et al., 2015; Stamps and Frankenhuis, 2016; Stein et al., 2018; Rösvik et al., 2020), and whether these outcomes can be modified by additional information later in life. Though these sources of complexity apply to both insect and vertebrate species, certain characteristics of insects, like their relatively short lifespans, may alter the ecological selection pressures that shape information integration. With respect to the evolution and expression of behavioral plasticity, diverse comparative approaches may illuminate both broad, general features and taxon-specific patterns.

In insects, studies of behavioral plasticity largely focus on processes during the adult stage, and although many patterns of nervous system development are known (Prillinger, 1981; Rospars, 1988; Hähnlein and Bicker, 1997; Cayre et al., 2000; Awasaki et al., 2008), precisely how these patterns respond to early-life environmental stimuli remains poorly understood. However, the deep research history of insects in natural ecological contexts provides diverse, tractable systems for future work that fills this research gap. The developmental environment, including simple abiotic factors like temperature and moisture, impacts a variety of sophisticated behaviors from dispersal patterns (Zera and Denno, 1997; Alyokhin and Ferro, 1999; Benard and McCauley, 2008) to social and reproductive tactics (Radwan, 1995; Emlen, 1997; Taborsky and Brockmann, 2010; Łukasik, 2010; Kasumovic and Brooks, 2011). Thus, in controlled but environmentally relevant experiments, it is possible to assess how specific types of developmental inputs shape both sensory and integrative processes (Anton and Rossler, 2020; Fernandez et al., 2020; Gonzalez-Tokman et al., 2020) relevant to many different behavioral phenotypes. In addition, the short generation time of insects is ideal for life-long studies of behavior.

On the neurobiological level, developmental behavioral plasticity in insects is mediated through familiar neural plasticity mechanisms like epigenetic modifications, neurochemical changes, and changes to neural structure (LeBoeuf et al., 2013). Some of these mechanisms can be, and have been, explored in the context of traditional learning and memory frameworks, which also are well established in insects (Tully et al., 1994; Blackiston et al., 2008; Yang et al., 2012; Alloway, 2015; Tan et al., 2015). Though most learning and memory research has focused on dynamics during the adult stage (Fahrbach et al., 1998; Ravary et al., 2007; Li et al., 2017; Jernigan et al., 2020), many insights from this work are likely applicable to the pre-adult life stages as well. Moreover, in what may be the majority of insect species, only a subset of the brain survives the transition from the juvenile life-stage to adulthood, presenting a narrow range of target areas in which to carefully investigate how neural plasticity mechanisms give rise to complex behaviors. However, some challenges to comparative work remain. For instance, it is unclear which insect life stages are comparable to the early-life timeframe in vertebrates, or whether retention of early-life effects in insects is fundamentally constrained by their extensive morphological and neurobiological remodeling (Vea and Minakuchi, 2020).

Despite these challenges, insects have a history of contributing surprisingly general insights into complex behavioral phenotypes relevant to vertebrate species. For example, eusocial insects present detailed systems to address general neurobiological principles of developmental behavioral plasticity in the context of complex social living. Because insect societies show patterns of organization that can be generalized to other social species (Seeley, 1995; Bonner, 2004; Ireland and Garnier, 2018), they have tremendous promise for investigating both the causes and consequences of developmental plasticity in vertebrates. This comparison may even extend to humans, where many persistent effects of the early-life environment on behavior and mental health are social in nature (Miller et al., 2009; Nothling et al., 2019). It is possible that behavioral plasticity in social contexts has unique neurobiological features (Taborsky and Oliveira, 2012), and social insects will continue to serve as excellent models to examine this idea.

Although this review is specifically focused on insect contributions to behavioral neuroscience in a comparative framework, the uniqueness of this animal group, as well as its ecological and economic importance, cannot be overstated. These aspects provide further motivation for study of developmental behavioral plasticity in this group. Many bee species are important agricultural pollinators (Winfree et al., 2011; Reilly et al., 2020). The ongoing locust outbreak in East Africa is anticipated to cause enormous economic loss and endanger food security (Peng et al., 2020). Many agricultural pests are metamorphosing insects with destructive larval feeding stages (e.g., beetles and moths). Understanding the natural history of these organisms, as well as the range of neural and behavioral responses to developmental experience (Haynes, 1988; Desneux et al., 2007; De França et al., 2017; Müller, 2018; Sehonova et al., 2018) will improve environmental management in addition to deepening our understanding of the general principles of developmental behavioral plasticity.

AUTHOR CONTRIBUTIONS

RW and CR conceptualized and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture Hatch Program (accession number 1012993, CR) and the Foundation for Food and Agriculture Research Pollinator Health Fund (549049, CR).

REFERENCES

Akhtar, Y., and Isman, M. B. (2003). Larval exposure to oviposition deterrents alters subsequent oviposition behavior in generalist, trichoplusia ni and specialist, *Plutella xylostella* Moths. *J. Chem. Ecol.* 29, 1853–1870. doi: 10.1023/A:1024802328458

Akhtar, Y., Shikano, I., and Isman, M. B. (2009). Topical application of a plant extract to different life stages of Trichoplusia ni fails to influence feeding or oviposition behaviour. *Entomol. Exp. Appl.* 132, 275–282. doi: 10.1111/j.1570-7458.2009.00895.x

Albert, J.T., and Kozlov, A.S. (2016). Comparative aspects of hearing in vertebrates and insects with antennal ears. *Curr. Biol.* 26, R1050–R1061. doi: 10.1016/j.cub.2016.09.017

Alem, S., Perry, C. J., Zhu, X., Loukola, O. J., Ingraham, T., Søvik, E., et al. (2016). Associative mechanisms allow for social learning and cultural transmission of string pulling in an insect. *PLoS Biol.* 14:e1002564. doi: 10.1371/journal.pbio.1002564

Alfonso, S., Sadoul, B., Gesto, M., Joassard, L., Chatain, B., Geffroy, B., et al. (2019). Coping styles in European sea bass: the link between boldness, stress response and neurogenesis. *Physiol. Behav.* 207, 76–85. doi: 10.1016/j.physbeh.2019.04.020

Alloway, T. M. (2015). Retention of learning through metamorphosis in the grain Beetle Tenebrio molitor. *Am. Zool.* 12, 471–477. doi: 10.1093/icb/12.3.471

Alvarado, S., Rajakumar, R., Abouheif, E., and Szyf, M. (2015). Epigenetic variation in the Egfr gene generates quantitative variation in a complex trait in ants. *Nat. Commun.* 6:6513. doi: 10.1038/ncomms7513

Alyokhin, A. V., and Ferro, D. N. (1999). Modifications in dispersal and oviposition of Bt-resistant and Bt-susceptible Colorado potato beetles as a result of exposure to *Bacillus thuringiensis* subsp. tenebrionis Cry3A toxin. *Entomol. Exp. Appl.* 90, 93–101. doi: 10.1046/j.1570-7458.1999.00426.x

Ament, S. A., Corona, M., Pollock, H. S., and Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4226–4231. doi: 10.1073/pnas.0800630105

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

Anderson, P., Sadek, M. M., Larsson, M., Hansson, B. S., and Thöming, G. (2013). Larval host plant experience modulates both mate finding and oviposition choice in a moth. *Anim. Behav.* 85, 1169–1175. doi: 10.1016/j.anbehav.2013.03.002

André, H. M. (1991). Stase, metamorphosis and competition in insects and other arthropods. *Belg. J. Zool.* 121, 3–25.

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

Anton, S., and Rossler, W. (2020). Plasticity and modulation of olfactory circuits in insects. *Cell Tissue Res.* 383, 149–164. doi: 10.1007/s00441-020-03329-z

Aristizabal, M. J., Anreiter, I., Halldorsdottir, T., Odgers, C. L., McDade, T. W., Goldenberg, A., et al. (2019). Biological embedding of experience: a primer on epigenetics. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23261–23269. doi: 10.1073/pnas.1820838116

Arsenault, S. V., Hunt, B. G., and Rehan, S. M. (2018). The effect of maternal care on gene expression and DNA methylation in a subsocial bee. *Nat. Commun.* 9:3468. doi: 10.1038/s41467-018-05903-0

Arvanitakis, Z., Wang, H. Y., Capuano, A. W., Khan, A., Taib, B., Anokye-Danso, F., et al. (2020). Brain insulin signaling, alzheimer disease pathology, and cognitive function. *Ann. Neurol.* 88, 513–525. doi: 10.1002/ana.25826

Avarguès-Weber, A. (2012). Face recognition: lessons from a Wasp. *Curr. Biol.* 22, R91–R93. doi: 10.1016/j.cub.2011.12.040

Avishai-Eliner, S., Brunson, K. L., Sandman, C. A., and Baram, T. Z. (2002). Stressed-out, or in (*utero*)? *Trends Neurosci.* 25, 518–524. doi: 10.1016/s0166-2236(02)02241-5

Avitsur, R., Levy, S., Goren, N., and Grinshpahet, R. (2015). Early adversity, immunity and infectious disease. *Stress* 18, 289–296. doi: 10.3109/10253890.2015.1017464

Awasaki, T., Lai, S.-L., Ito, K., and Lee, T. (2008). Organization and postembryonic development of glial cells in the adult central brain of *Drosophila*. *J. Neurosci.* 28, 13742–13753. doi: 10.1523/jneurosci.4844-08.2008

Barker, D. J. P. (1995). Fetal origins of coronary heart disease. *BMJ* 311, 171–174. doi: 10.1136/bmj.311.6998.171

Barron, A. (2001). The life and death of hopkins' host-selection principle. *J. Insect Behav.* 14, 725–737. doi: 10.1023/A:1013033332535

Barron, A. B., Maleszka, R., Helliwell, P. G., and Robinson, G. E. (2008). Effects of cocaine on honey bee dance behaviour. *J. Exp. Biol.* 212, 163–168. doi: 10.1242/jeb.025361

Beach, F. A., and Jaynes, J. (1954). Effects of early experience upon the behavior of animals. *Psychol. Bull.* 51, 239–263. doi: 10.1037/h0061176

Beldade, P., Mateus, A. R. A., and Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. *Mol. Ecol.* 20, 1347–1363. doi: 10.1111/j.1365-294X.2011.05016.x

Bello, B. C., Izergina, N., Caussinus, E., and Reichert, H. (2008). Amplification of neural stem cell proliferation by intermediate progenitor cells in *Drosophila* brain development. *Neural Dev.* 3:5. doi: 10.1186/1749-8104-3-5

Benard, M. F., and McCauley, S. J. (2008). Integrating across life−history stages: consequences of natal habitat effects on dispersal. *Am. Nat.* 171, 553–567. doi: 10.1086/587072

Benoit, J. B. (2010). "Water management by dormant insects: comparisons between dehydration resistance during summer aestivation and winter diapause," in *Aestivation: Molecular and Physiological Aspects*, eds C. Arturo Navas and J. E. Carvalho (Berlin: Springer), 209–229. doi: 10.1007/978-3-642-02421-4_10

Bicker, G., Schafer, S., Ottersen, O. P., and Storm-Mathisen, J. (1988). Glutamate-like immunoreactivity in identified neuronal populations of insect nervous systems. *J. Neurosci.* 8, 2108–2122. doi: 10.1523/jneurosci.08-06-02108.1988

Blackiston, D. J., Silva Casey, E., and Weiss, M. R. (2008). Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? *PLoS One* 3:e1736. doi: 10.1371/journal.pone.0001736

Bonner, J. T. (2004). Perspective: the size-complexity rule. *Evolution* 58, 1883–1890. doi: 10.1111/j.0014-3820.2004.tb00476.x

Booker, R., and Truman, J. W. (1987). Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* 255, 548–559. doi: 10.1002/cne.902550407

Boyce, W. T., Sokolowski, M. B., and Robinson, G. E. (2020). Genes and environments, development and time. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23235–23241. doi: 10.1073/pnas.2016710117

Brian, M. V. (1956). Studies of caste differentiation inMyrmica rubra L. 4. Controlled larval nutrition. *Insectes Soc.* 3, 369–394. doi: 10.1007/BF02225758

Bryck, R. L., and Fisher, P. A. (2012). Training the brain: practical applications of neural plasticity from the intersection of cognitive neuroscience, developmental psychology, and prevention science. *Am. Psychol.* 67:87. doi: 10.1037/a0024657

Bubak, A. N., Watt, M. J., Yaeger, J. D. W., Renner, K. J., and Swallow, J. G. (2020). The stalk-eyed fly as a model for aggression - is there a conserved role for 5-HT between vertebrates and invertebrates? *J. Exp. Biol.* 223(Pt 1), jeb132159. doi: 10.1242/jeb.132159

Buckley, L. B., Hurlbert, A. H., and Jetz, W. (2012). Broad-scale ecological implications of ectothermy and endothermy in changing environments. *Glob. Ecol. Biogeogr.* 21, 873–885. doi: 10.1111/j.1466-8238.2011.00737.x

Byrne, J. H., and Fieber, L. A. (2017). *Neurotransmitters and Neuropeptides of Invertebrates*. Oxford: Oxford University Press.

Cantera, R., Nässel, D. R., and Veenstra, J. A. (1994). Postembryonic development of corazonin-containing neurons and neurosecretory cells in the blowfly, Phormia terraenovae. *J. Comp. Neurol.* 350, 559–572. doi: 10.1002/cne.903500405

Cardoso, S. D., Teles, M. C., and Oliveira, R. F. (2015). Neurogenomic mechanisms of social plasticity. *J. Exp. Biol.* 218(Pt 1), 140–149. doi: 10.1242/jeb.106997

Cayre, M., Malaterre, J., Charpin, P., Strambi, C., and Strambi, A. (2000). Fate of neuroblast progeny during postembryonic development of mushroom bodies in the house cricket, *Acheta domesticus*. *J. Insect Physiol.* 46, 313–319. doi: 10.1016/S0022-1910(99)00184-5

Cayre, M., Strambi, C., and Strambi, A. (1994). Neurogenesis in an adult insect brain and its hormonal control. *Nature* 368, 57–59. doi: 10.1038/368057a0

Champagne, F. A. (2012). Interplay between social experiences and the genome: epigenetic consequences for behavior. *Adv. Genet.* 77, 33–57. doi: 10.1016/B978-0-12-387687-4.00002-7

Champagne, F. A., and Curley, J. P. (2005). How social experiences influence the brain. *Curr. Opin. Neurobiol.* 15, 704–709. doi: 10.1016/j.conb.2005.10.001

Charles, J.-P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J., and Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. U.S.A.* 108, 21128–21133. doi: 10.1073/pnas.1116123109

Chen, Y., and Baram, T. Z. (2016). Toward understanding how early-life stress reprograms cognitive and emotional brain networks. *Neuropsychopharmacology* 41, 197–206. doi: 10.1038/npp.2015.181

Chittka, L., and Geiger, K. (1995). Can honey bees count landmarks? *Anim. Behav.* 49, 159–164. doi: 10.1016/0003-3472(95)80163-4

Chittka, L., and Niven, J. (2009). Are bigger brains better? *Curr. Biol.* 19, R995–R1008. doi: 10.1016/j.cub.2009.08.023

Cohn, R., Morantte, 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

Coolen, I., Dangles, O., and Casas, J. (2005). Social learning in noncolonial insects? *Curr. Biol.* 15, 1931–1935. doi: 10.1016/j.cub.2005.09.015

Corbet, P. S. (1957). The life-history of the emperor dragonfly Anax imperator Leach (Odonata: Aeshnidae). *J. Anim. Ecol.* 26, 1–69. doi: 10.2307/1781

Crist, E. (2004). Can an insect speak? The case of the honeybee dance language. *Soc. Stud. Sci.* 34, 7–43. doi: 10.1177/0306312704040611

Curley, J. P., and Champagne, F. A. (2016). Influence of maternal care on the developing brain: mechanisms, temporal dynamics and sensitive periods. *Front. Neuroendocrinol.* 40:52–66. doi: 10.1016/j.yfrne.2015.11.001

Dall, S. R., McNamara, J. M., and Leimar, O. (2015). Genes as cues: phenotypic integration of genetic and epigenetic information from a Darwinian perspective. *Trends Ecol. Evol.* 30, 327–333. doi: 10.1016/j.tree.2015.04.002

Danese, A. (2020). Annual research review: rethinking childhood trauma-new research directions for measurement, study design and analytical strategies. *J. Child Psychol. Psychiatry* 61, 236–250. doi: 10.1111/jcpp.13160

De França, S. M., Breda, M. O., Barbosa, D. R., Araujo, A. M., and Guedes, C. A. (2017). "The sublethal effects of insecticides in insects," in *Biological Control of Pest and Vector Insects*, ed. V. D. C. Shields (London: IntechOpen), 23–39.

De Wilde, J. (1962). Photoperiodism in insects and mites. *Annu. Rev. Entomol.* 7, 1–26. doi: 10.1146/annurev.en.07.010162.000245

De Wilde, J., and Beetsma, J. (1982). "The physiology of caste development in social insects," in *Advances in Insect Physiology*, eds M. J. Berridge, J. E. Treherne, and V. B. Wigglesworth (Cambridge, MA: Academic Press), 167–246. doi: 10.1016/s0065-2806(08)60154-x

Deobagkar, D. (2018). Epigenetics with special reference to the human X chromosome inactivation and the enigma of *Drosophila* DNA methylation. *J. Genet.* 97, 371–378. doi: 10.1007/s12041-018-0937-5

Deshmukh, S., Ponnaluri, V. C., Dai, N., Pradhan, S., and Deobagkar, D. (2018). Levels of DNA cytosine methylation in the *Drosophila* genome. *PeerJ* 6:e5119. doi: 10.7717/peerj.5119

Desneux, N., Decourtye, A., and Delpuech, J.-M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* 52, 81–106. doi: 10.1146/annurev.ento.52.110405.091440

Di Sante, J., Ismaylova, E., Nemoda, Z., Gouin, J. P., Yu, W. J., Caldwell, W., et al. (2018). Peripheral DNA methylation of HPA axis-related genes in humans: Cross-tissue convergence, two-year stability and behavioural and neural correlates. *Psychoneuroendocrinology* 97, 196–205. doi: 10.1016/j.psyneuen.2018.07.019

Dyer, F. C. (1998). "5 - Spatial cognition: lessons from central-place foraging insects," in *Animal Cognition in Nature*, eds R. P. Balda I, M. Pepperberg, and A. C. Kamil (London: Academic Press), 119–154.

Ebenman, B. (1992). Evolution in organisms that change their niches during the life cycle. *Am. Nat.* 139, 990–1021. doi: 10.1086/285370

Elekonich, M. M., and Robinson, G. E. (2000). Organizational and activational effects of hormones on insect behavior. *J. Insect Physiol.* 46, 1509–1515. doi: 10.1016/S0022-1910(00)00101-3

Emlen, D. J. (1997). Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Behav. Ecol. Sociobiol.* 41, 335–341. doi: 10.1007/s002650050393

Erion, R., and Sehgal, A. (2013). Regulation of insect behavior via the insulin-signaling pathway. *Front. Physiol.* 4:353. doi: 10.3389/fphys.2013.00353

Fahrbach, S. E. (2006). Structure of the mushroom bodies of the insect brain. *Annu. Rev. Entomol.* 51, 209–232. doi: 10.1146/annurev.ento.51.110104.150954

Fahrbach, S. E., Moore, D., Capaldi, E. A., Farris, S. M., and Robinson, G. E. (1998). Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn. Mem.* 5, 115–123.

Farris, S. M. (2008). Structural, functional and developmental convergence of the insect mushroom bodies with higher brain centers of vertebrates. *Brain Behav. Evol.* 72, 1–15. doi: 10.1159/000139457

Felsenberg, J., Jacob, P. F., Walker, T., Barnstedt, O., Edmondson-Stait, A. J., Pleijzier, M. W., et al. (2018). Integration of parallel opposing memories underlies memory extinction. *Cell* 175, 709.e15–722.e15. doi: 10.1016/j.cell.2018.08.021

Feng, S., Cokus, S. J., Zhang, X., Chen, P.-Y., Bostick, M., Goll, M. G., et al. (2010). Conservation and divergence of methylation patterning in plants and animals. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8689–8694. doi: 10.1073/pnas.1002720107

Fernandez, M. P., Pettibone, H. L., Bogart, J. T., Roell, C. J., Davey, C. E., Pranevicius, A., et al. (2020). Sites of circadian clock neuron plasticity mediate sensory integration and entrainment. *Curr. Biol.* 30, 2225.e5–2237.e5. doi: 10.1016/j.cub.2020.04.025

Flatt, T., Moroz, L. L., Tatar, M., and Heyland, A. (2006). Comparing thyroid and insect hormone signaling. *Integr. Comp. Biol.* 46, 777–794. doi: 10.1093/icb/icl034

Flores, K., Wolschin, F., Corneveaux, J. J., Allen, A. N., Huentelman, M. J., and Amdam, G. V. (2012). Genome-wide association between DNA methylation and alternative splicing in an invertebrate. *BMC Genomics* 13:480. doi: 10.1186/1471-2164-13-480

Gabbutt, P. D. (1959). The bionomics of the wood cricket, *Nemobius sylvestris* (Orthoptera: Gryllidae). *J. Anim. Ecol.* 28, 15–42. doi: 10.2307/2011

Gadagkar, R., Bhagavan, S., Chandrashekara, K., and Vinutha, C. (1991). The role of larval nutrition in pre-imaginal biasing of caste in the primitively eusocial wasp *Ropalidia marginata* (Hymenoptera: Vespidae). *Ecol. Entomol.* 16, 435–440. doi: 10.1111/j.1365-2311.1991.tb00236.x

Gadagkar, V., Puzerey, P. A., Chen, R., Baird-Daniel, E., Farhang, A. R., and Goldberg, J. H. (2016). Dopamine neurons encode performance error in singing birds. *Science* 354, 1278–1282. doi: 10.1126/science.aah6837

Gilbert, L. I., Tata, J. R., and Atkinson, B. G. (1996). *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. London: Academic Press.

Gillett, S. D. (1973). Social determinants of aggregation behaviour in adults of the desert locust. *Anim. Behav.* 21, 599–606. doi: 10.1016/S0003-3472(73)80022-3

Giurfa, M., Eichmann, B., and Menzel, R. (1996). Symmetry perception in an insect. *Nature* 382, 458–461. doi: 10.1038/382458a0

Giurfa, M., Zhang, S., Jenett, A., Menzel, R., and Srinivasan, M. V. (2001). The concepts of 'sameness' and 'difference'in an insect. *Nature* 410, 930–933. doi: 10.1038/35073582

Glastad, K. M., Hunt, B. G., and Goodisman, M. A. D. (2014). Evolutionary insights into DNA methylation in insects. *Curr. Opin. Insect Sci.* 1, 25–30. doi: 10.1016/j.cois.2014.04.001

Glastad, K. M., Hunt, B. G., and Goodisman, M. A. D. (2019). Epigenetics in insects: genome regulation and the generation of phenotypic diversity. *Annu. Rev. Entomol.* 64, 185–203. doi: 10.1146/annurev-ento-011118-111914

Gonzalez-Tokman, D., Cordoba-Aguilar, A., Dattilo, W., Lira-Noriega, A., Sanchez-Guillen, R. A., and Villalobos, F. (2020). Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biol. Rev. Camb. Philos. Soc.* 95, 802–821. doi: 10.1111/brv.12588

Grace, J. L., and Shaw, K. L. (2004). Effects of developmental environment on signal-preference coupling in a Hawaiian cricket. *Evolution* 58, 1627–1633. doi: 10.1111/j.0014-3820.2004.tb01744.x

Groothuis, J., and Smid, H. M. (2017). Nasonia parasitic wasps escape from Haller's rule by diphasic, partially isometric brain-body size scaling and selective neuropil adaptations. *Brain Behav. Evol.* 90, 243–254. doi: 10.1159/000480421

Gutierrez, D. V., Oh, E., and Herlitze, S. (2011). "Vertebrate and invertebrate rhodopsins: light control of G-Protein signaling," in *Photosensitive Molecules for Controlling Biological Function*, eds J. J. Chambers, and R. H. Kramer (Berlin: Springer), 133–146. doi: 10.1007/978-1-61779-031-7_7

Hähnlein, I., and Bicker, G. (1997). Glial patterning during postembryonic development of central neuropiles in the brain of the honeybee. *Dev. Genes Evol.* 207, 29–41. doi: 10.1007/s004270050089

Hall, Z. J., and Tropepe, V. (2020). Using teleost fish to discern developmental signatures of evolutionary adaptation from phenotypic plasticity in brain structure. *Front. Neuroanat.* 14:10. doi: 10.3389/fnana.2020.00010

Hattori, D., Aso, Y., Swartz, K. J., Rubin, G. M., Abbott, L. F., and Axel, R. (2017). Representations of novelty and familiarity in a mushroom body compartment. *Cell* 169, 956.e17–969.e17. doi: 10.1016/j.cell.2017.04.028

Haynes, K. F. (1988). Sublethal effects of neurotoxic insecticides on insect behavior. *Annu. Rev. Entomol.* 33, 149–168. doi: 10.1146/annurev.en.33.010188.001053

Heisenberg, M., Heusipp, M., and Wanke, C. (1995). Structural plasticity in the *Drosophila* brain. *J. Neurosci.* 15, 1951–1960. doi: 10.1523/jneurosci.15-03-01951.1995

Herb, B. R., Wolschin, F., Hansen, K. D., Aryee, M. J., Langmead, B., Irizarry, R., et al. (2012). Reversible switching between epigenetic states in honeybee behavioral subcastes. *Nat. Neurosci.* 15, 1371–1373. doi: 10.1038/nn.3218

Heyland, A., and Moroz, L. L. (2006). Signaling mechanisms underlying metamorphic transitions in animals. *Integr. Comp. Biol.* 46, 743–759. doi: 10.1093/icb/icl023

Hirata, M., and Higashi, S. (2008). Degree-day accumulation controlling allopatric and sympatric variations in the sociality of sweat bees, Lasioglossum (Evylaeus) baleicum (Hymenoptera: Halictidae). *Behav. Ecol. Sociobiol.* 62:1239. doi: 10.1007/s00265-008-0552-1

Hochberg, Z., Feil, R., Constancia, M., Fraga, M., Junien, C., Carel, J. C., et al. (2011). Child health, developmental plasticity, and epigenetic programming. *Endocr. Rev.* 32, 159–224. doi: 10.1210/er.2009-0039

Hoffmann, A., and Spengler, D. (2018). The mitochondrion as potential interface in early-life stress brain programming. *Front. Behav. Neurosci.* 12:306. doi: 10.3389/fnbeh.2018.00306

Holland, L. Z., Carvalho, J. E., Escriva, H., Laudet, V., Schubert, M., Shimeld, S. M., et al. (2013). Evolution of bilaterian central nervous systems: a single origin. *EvoDevo* 4:27. doi: 10.1186/2041-9139-4-27

Hopkins, A. (1917). A discussion of CG Hewitt's paper on "Insect Behaviour". *J. Econ. Entomol.* 10, 92–93.

Iba, M., Nagao, T., and Urano, A. (1995). Effects of population density on growth, behavior and levels of biogenic amines in the cricket, *Gryllus bimaculatus*. *Zool. Sci.* 12, 695–702. doi: 10.2108/zsj.12.695

Ireland, T., and Garnier, S. (2018). Architecture, space and information in constructions built by humans and social insects: a conceptual review. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 373:20170244. doi: 10.1098/rstb.2017.0244

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

Jandt, J. M., Suryanarayanan, S., Hermanson, J. C., Jeanne, R. L., and Toth, A. L. (2017). Maternal and nourishment factors interact to influence offspring developmental trajectories in social wasps. *Proc. Biol. Sci.* 284:20170651. doi: 10.1098/rspb.2017.0651

Jeong, H., Wu, X., Smith, B., and Yi, S. V. (2018). Genomic landscape of methylation islands in hymenopteran insects. *Genome Biol. Evol.* 10, 2766–2776. doi: 10.1093/gbe/evy203

Jernigan, C. M., Halby, R., Gerkin, R. C., Sinakevitch, I., Locatelli, F., and Smith, B. H. (2020). Experience-dependent tuning of early olfactory processing in the adult honey bee, *Apis mellifera*. *J. Exp. Biol.* 223(Pt 1):jeb206748. doi: 10.1242/jeb.206748

Jobson, M. A., Jordan, J. M., Sandrof, M. A., Hibshman, J. D., Lennox, A. L., and Baugh, L. R. (2015). Transgenerational effects of early life starvation on growth, reproduction, and stress resistance in *Caenorhabditis elegans*. *Genetics* 201, 201–212. doi: 10.1534/genetics.115.178699

Kapheim, K. M., Pan, H., Li, C., Salzberg, S. L., Puiu, D., Magoc, T., et al. (2015). Genomic signatures of evolutionary transitions from solitary to group living. *Science* 348, 1139–1143.

Kasumovic, M. M., and Brooks, R. C. (2011). It's all who you know: the evolution of socially cued anticipatory plasticity as a mating strategy. *Q. Rev. Biol.* 86, 181–197. doi: 10.1086/661119

Kaupp, U. B. (2010). Olfactory signalling in vertebrates and insects: differences and commonalities. *Nat. Rev. Neurosci.* 11, 188–200. doi: 10.1038/nrn2789

Kinoshita, M., and Homberg, U. (2017). "Insect brains: minute structures controlling complex behaviors," in *Brain Evolution by Design: From Neural Origin to Cognitive Architecture*, eds S. Shigeno, Y. Murakami, and T. Nomura (Tokyo: Springer), 123–151. doi: 10.1007/978-4-431-56469-0_6

Klowden, M. J. (2013). *Physiological Systems in Insects*. London: Academic press.

König, K., Krimmer, E., Brose, S., Gantert, C., Buschlüter, I., König, C., et al. (2015). Does early learning drive ecological divergence during speciation processes in parasitoid wasps? *Proc. R. Soc. B Biol. Sci.* 282:20141850. doi: 10.1098/rspb.2014.1850

Korosi, A., Naninck, E. F., Oomen, C. A., Schouten, M., Krugers, H., Fitzsimons, C., et al. (2012). Early-life stress mediated modulation of adult neurogenesis and behavior. *Behav. Brain Res.* 227, 400–409. doi: 10.1016/j.bbr.2011.07.037

Kretzschmar, D., and Pflugfelder, G. O. (2002). Glia in development, function, and neurodegeneration of the adult insect brain. *Brain Res. Bull.* 57, 121–131. doi: 10.1016/S0361-9230(01)00643-8

Kucharski, R., Maleszka, J., Foret, S., and Maleszka, R. (2008). Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319, 1827–1830. doi: 10.1126/science.1153069

Kundakovic, M., and Champagne, F. A. (2015). Early-life experience, epigenetics, and the developing brain. *Neuropsychopharmacology* 40, 141–153. doi: 10.1038/npp.2014.140

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

Lhomme, P., Carrasco, D., Larsson, M., Hansson, B., and Anderson, P. (2017). A context-dependent induction of natal habitat preference in a generalist herbivorous insect. *Behav. Ecol.* 29, 360–367. doi: 10.1093/beheco/arx173

Li, L., MaBouDi, H., Egertova, M., Elphick, M. R., Chittka, L., and Perry, C. J. (2017). A possible structural correlate of learning performance on a colour discrimination task in the brain of the bumblebee. *Proc. Biol. Sci.* 284:20171323. doi: 10.1098/rspb.2017.1323

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

Li-Byarlay, H. (2016). The function of DNA methylation marks in social insects. *Front. Ecol. Evol.* 4:57. doi: 10.3389/fevo.2016.00057

Lichtneckert, R., and Reichert, H. (2005). Insights into the urbilaterian brain: conserved genetic patterning mechanisms in insect and vertebrate brain development. *Heredity* 94, 465–477. doi: 10.1038/sj.hdy.6800664

Linksvayer, T. A., Fewell, J. H., Gadau, J., and Laubichler, M. D. (2012). Developmental evolution in social insects: regulatory networks from genes to societies. *J. Exp. Zool. Part B Mol. Dev. Evol.* 318, 159–169. doi: 10.1002/jez.b.22001

Liu, Y., Henderson, G., Mao, L., and Laine, R. A. (2005). Effects of temperature and nutrition on juvenile hormone titers of *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Ann. Entomol. Soc. Am.* 98, 732–737. doi: 10.1603/0013-87462005098[0732:Eotano]2.0.Co;2

Lnenicka, G. A., and Murphey, R. K. (1989). The refinement of invertebrate synapses during development. *J. Neurobiol.* 20, 339–355. doi: 10.1002/neu.480200507

Lo, N., Simpson, S. J., and Sword, G. A. (2018). Epigenetics and developmental plasticity in orthopteroid insects. *Curr. Opin. Insect Sci.* 25, 25–34. doi: 10.1016/j.cois.2017.11.003

Loesel, R. (2011). "Neurophylogeny: retracing early metazoan brain evolution," in *Evolutionary Biology – Concepts, Biodiversity, Macroevolution and Genome Evolution*, ed. P. Pontarotti (Berlin: Springer), 169–191. doi: 10.1007/978-3-642-20763-1_11

Losada-Perez, M. (2018). Glia: from 'just glue' to essential players in complex nervous systems: a comparative view from flies to mammals. *J. Neurogenet.* 32, 78–91. doi: 10.1080/01677063.2018.1464568

Lowe, W. H., Martin, T. E., Skelly, D. K., and Woods, H. A. (2021). Metamorphosis in an era of increasing climate variability. *Trends Ecol. Evol.* 36, 360–375. doi: 10.1016/j.tree.2020.11.012

Lucht-Bertram, E. (1961). Das postembryonale wachstum von hirnteilen bei *Apis mellifica* L. und *Myrmeleon europaeus* L. *Zeitschrift für Morphol. Ökol. Tiere* 50, 543–575. doi: 10.1007/bf00389832

Łukasik, P. (2010). Trophic dimorphism in alternative male reproductive morphs of the acarid mite *Sancassania berlesei*. *Behav. Ecol.* 21, 270–274. doi: 10.1093/beheco/arp186

Luo, L., and O'Leary, D. D. (2005). Axon retraction and degeneration in development and disease. *Annu. Rev. Neurosci.* 28, 127–156. doi: 10.1146/annurev.neuro.28.061604.135632

Mayseless, O., Berns, D. S., Yu, X. M., Riemensperger, T., Fiala, A., and Schuldiner, O. (2018). Developmental coordination during olfactory circuit remodeling in *Drosophila*. *Neuron* 99, 1204.e5–1215.e5. doi: 10.1016/j.neuron.2018.07.050

Miller, G. E., Chen, E., Fok, A. K., Walker, H., Lim, A., Nicholls, E. F., et al. (2009). Low early-life social class leaves a biological residue manifested by decreased glucocorticoid and increased proinflammatory signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14716–14721. doi: 10.1073/pnas.0902971106

Miura, T. (2004). Proximate mechanisms and evolution of caste polyphenism in social insects: from sociality to genes. *Ecol. Res.* 19, 141–148. doi: 10.1111/j.1440-1703.2003.00618.x

Moczek, A. P., and Emlen, D. J. (2000). Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? *Anim. Behav.* 59, 459–466. doi: 10.1006/anbe.1999.1342

Müller, C. (2018). Impacts of sublethal insecticide exposure on insects — Facts and knowledge gaps. *Basic Appl. Ecol.* 30, 1–10. doi: 10.1016/j.baae.2018.05.001

Müller, T., Küll, C. L., and Müller, C. (2016). Effects of larval versus adult density conditions on reproduction and behavior of a leaf beetle. *Behav. Ecol. Sociobiol.* 70, 2081–2091. doi: 10.1007/s00265-016-2212-1

Muscedere, M. L., and Traniello, J. F. (2012). Division of labor in the hyperdiverse ant genus Pheidole is associated with distinct subcaste-and age-related patterns of worker brain organization. *PLoS One* 7:e31618. doi: 10.1371/journal.pone.0031618

Newsom, K. D., Moczek, A. P., and Schwab, D. B. (2019). Serotonin differentially affects morph-specific behavior in divergent populations of a horned beetle. *Behav. Ecol.* 31, 352–360. doi: 10.1093/beheco/arz192

Niven, J. E., and Laughlin, S. B. (2008). Energy limitation as a selective pressure on the evolution of sensory systems. *J. Exp. Biol.* 211, 1792–1804. doi: 10.1242/jeb.017574

Nothling, J., Malan-Mueller, S., Abrahams, N., Hemmings, S. M. J., and Seedat, S. (2019). Epigenetic alterations associated with childhood trauma and adult mental health outcomes: a systematic review. *World J. Biol. Psychiatry* 21, 493–512. doi: 10.1080/15622975.2019.1583369

O'Connell, L. A. (2013). Evolutionary development of neural systems in vertebrates and beyond. *J. Neurogenet.* 27, 69–85. doi: 10.3109/01677063.2013.789511

Odeon, M. M., Salatino, A. E., and Acosta, G. B. (2013). Consequences of postnatal stress: maternal separation in rats induces long-lasting changes on glutamate transporters. *Clin. Exp. Pharmacol.* 3:121.

Olvido, A. E., and Mousseau, T. A. (1995). Effect of rearing environment on calling-song plasticity in the striped ground cricket. *Evolution* 49, 1271–1277. doi: 10.2307/2410452

Osborne, R. H. (1996). Insect neurotransmission: neurotransmitters and their receptors. *Pharmacol. Therapeut.* 69, 117–142. doi: 10.1016/0163-7258(95)02054-3

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 solitarious phase. *Proc. R. Soc. B Biol. Sci.* 277, 3087–3096. doi: 10.1098/rspb.2010.0694

Page, R. E., and Peng, C. Y. S. (2001). Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Exp. Gerontol.* 36, 695–711. doi: 10.1016/S0531-5565(00)00236-9

Pahl, M., Si, A., and Zhang, S. (2013). Numerical cognition in bees and other insects. *Front. Psychol.* 4:162. doi: 10.3389/fpsyg.2013.00162

Paredes, U., Radersma, R., Cannell, N., While, G. M., and Uller, T. (2016). Low incubation temperature induces DNA hypomethylation in lizard brains. *J. Exp. Zool. A Ecol. Genet. Physiol.* 325, 390–395. doi: 10.1002/jez.2024

Patalano, S., Hore, T. A., Reik, W., and Sumner, S. (2012). Shifting behaviour: epigenetic reprogramming in eusocial insects. *Curr. Opin. Cell Biol.* 24, 367–373. doi: 10.1016/j.ceb.2012.02.005

Paulino Simões, Z. L., Boleli, I. C., and Hartfelder, K. (1997). Occurrence of a prothoracicotropic hormone-like peptide in the developing nervous system of the honey bee (*Apis mellifera* L). *Apidologie* 28, 399–409. doi: 10.1051/apido:19970607

Pearson, K. (1993). Common principles of motor control in vertebrates and invertebrates. *Annu. Rev. Neurosci.* 16, 265–297. doi: 10.1146/annurev.ne.16.030193.001405

Peng, W., Ma, N. L., Zhang, D., Zhou, Q., Yue, X., Khoo, S. C., et al. (2020). A review of historical and recent locust outbreaks: links to global warming, food security and mitigation strategies. *Environ. Res.* 191:110046. doi: 10.1016/j.envres.2020.110046

Perry, C. J., Baciadonna, L., and Chittka, L. (2016). Unexpected rewards induce dopamine-dependent positive emotion-like state changes in bumble bees. *Science* 353, 1529–1531. doi: 10.1126/science.aaf4454

Peters, A., Schweiger, U., Pellerin, L., Hubold, C., Oltmanns, K. M., Conrad, M., et al. (2004). The selfish brain: competition for energy resources. *Neurosci. Biobehav. Rev.* 28, 143–180. doi: 10.1016/j.neubiorev.2004.03.002

Petit, C., Le, Ru, B., Dupas, S., Frérot, B., Ahuya, P., et al. (2015). Influence of dietary experience on the induction of preference of adult moths and larvae for a new olfactory cue. *PLoS One* 10:e0136169. doi: 10.1371/journal.pone.0136169

Prieto Peres, M. F., and Valença, M. M. (2010). "Chapter 60 - Headache: endocrinological aspects," in *Handbook of Clinical Neurology*, eds M. J. Aminoff, F. Boller, and D. F. Swaab (Amsterdam: Elsevier), 717–737.

Prillinger, L. (1981). Postembryonic development of the antennal lobes in *Periplaneta americana* L. *Cell Tissue Res.* 215, 563–575. doi: 10.1007/BF00233532

Pringle, J. (1940). The reflex mechanism of the insect leg. *J. Exp. Biol.* 17, 8–17.

Prudic, K. L., Jeon, C., Cao, H., and Monteiro, A. (2011). Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. *Science* 331, 73–75. doi: 10.1126/science.1197114

Rachinsky, A. (1994). Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. *J. Insect Physiol.* 40, 549–554. doi: 10.1016/0022-1910(94)90141-4

Radwan, J. (1995). Male morph determination in two species of acarid mites. *Heredity* 74, 669–673. doi: 10.1038/hdy.1995.91

Rajakumar, R., Koch, S., Couture, M., Fave, M. J., Lillico-Ouachour, A., Chen, T., et al. (2018). Social regulation of a rudimentary organ generates complex worker-caste systems in ants. *Nature* 562, 574–577. doi: 10.1038/s41586-018-0613-1

Ravary, F., Lecoutey, E., Kaminski, G., Chaline, N., and Jaisson, P. (2007). Individual experience alone can generate lasting division of labor in ants. *Curr. Biol.* 17, 1308–1312. doi: 10.1016/j.cub.2007.06.047

Reh, R. K., Dias, B. G., Nelson, C. A. III, Kaufer, D., Werker, J. F., Kolb, B., et al. (2020). Critical period regulation across multiple timescales. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23242–23251. doi: 10.1073/pnas.1820836117

Reh, T., and Cagan, R. (1994). Intrinsic and extrinsic signals in the developing vertebrate and fly eyes: viewing vertebrate and invertebrate eyes in the same light. *Perspect. Dev. Neurobiol.* 2, 183–190.

Reichert, H. (2009). Evolutionary conservation of mechanisms for neural regionalization, proliferation and interconnection in brain development. *Biol. Lett.* 5, 112–116. doi: 10.1098/rsbl.2008.0337

Reilly, J., Artz, D., Biddinger, D., Bobiwash, K., Boyle, N., Brittain, C., et al. (2020). Crop production in the USA is frequently limited by a lack of pollinators. *Proc. R. Soc. B* 287:20200922. doi: 10.1098/rspb.2020.0922

Reingold, S. C., and Camhi, J. M. (1977). A quantitative analysis of rhythmic leg movements during three different behaviors in the cockroach, *Periplaneta americana*. *J. Insect Physiol.* 23, 1407–1420. doi: 10.1016/0022-1910(77)90166-4

Rietdorf, K., and Steidle, J. L. (2002). Was Hopkins right? Influence of larval and early adult experience on the olfactory response in the granary weevil *Sitophilus granarius* (Coleoptera, Curculionidae). *Physiol. Entomol.* 27, 223–227. doi: 10.1046/j.1365-3032.2002.00289.x

Rillich, J., and Stevenson, P. A. (2018). Serotonin mediates depression of aggression after acute and chronic social defeat stress in a model insect. *Front. Behav. Neurosci.* 12:233. doi: 10.3389/fnbeh.2018.00233

Rittschof, C. C., and Schirmeier, S. (2017). Insect models of central nervous system energy metabolism and its links to behavior. *Glia* 66, 1160–1175. doi: 10.1002/glia.23235

Rittschof, C. C., Bukhari, S. A., Sloofman, L. G., Troy, J. M., Caetano-Anolles, D., Cash-Ahmed, A., et al. (2014). Neuromolecular responses to social challenge:

common mechanisms across mouse, stickleback fish, and honey bee. *Proc. Natl. Acad. Sci. U.S.A.* 111, 17929–17934. doi: 10.1073/pnas.1420369111

Rittschof, C. C., Coombs, C. B., Frazier, M., Grozinger, C. M., and Robinson, G. E. (2015). Early-life experience affects honey bee aggression and resilience to immune challenge. *Sci. Rep.* 5:15572. doi: 10.1038/srep15572

Robertson, R. M., Dawson-Scully, K. D., and Andrew, R. D. (2020). Neural shutdown under stress: an evolutionary perspective on spreading depolarization. *J. Neurophysiol.* 123, 885–895. doi: 10.1152/jn.00724.2019

Roeder, T. (2005). Tyramine and Octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50, 447–477. doi: 10.1146/annurev.ento.50.071803.130404

Rolff, J., Johnston, P. R., and Reynolds, S. (2019). Complete metamorphosis of insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374:20190063. doi: 10.1098/rstb.2019.0063

Rolls, M. M., and Jegla, T. J. (2015). Neuronal polarity: an evolutionary perspective. *J. Exp. Biol.* 218, 572–580. doi: 10.1242/jeb.112359

Rospars, J. P. (1988). Structure and development of the insect antennodeutocerebral system. *Int. J. Insect Morphol. Embryol.* 17, 243–294. doi: 10.1016/0020-7322(88)90041-4

Rösvik, A., Lhomme, P., Khallaf, M. A., and Anderson, P. (2020). Plant-induced transgenerational plasticity affecting performance but not preference in a polyphagous moth. *Front. Ecol. Evol.* 8:254. doi: 10.3389/fevo.2020.00254

Saleh, A., Potter, G. G., McQuoid, D. R., Boyd, B., Turner, R., MacFall, J. R., et al. (2017). Effects of early life stress on depression, cognitive performance and brain morphology. *Psychol. Med.* 47, 171–181. doi: 10.1017/S0033291716002403

Salzberg, A., and Bellen, H. J. (1996). Invertebrate versus vertebrate neurogenesis: variations on the same theme? *Dev. Genet.* 18, 1–10. doi: 10.1002/(sici)1520-6408(1996)18:1<1::aid-dvg1>3.0.co;2-d

Saul, M. C., Blatti, C., Yang, W., Bukhari, S. A., Shpigler, H. Y., Troy, J. M., et al. (2019). Cross-species systems analysis of evolutionary toolkits of neurogenomic response to social challenge. *Genes Brain Behav.* 18:e12502. doi: 10.1111/gbb.12502

Schaefer, P. L., and Ritzmann, R. E. (2001). Descending influences on escape behavior and motor pattern in the cockroach. *J. Neurobiol.* 49, 9–28. doi: 10.1002/neu.1062

Schmitz, R. J., Lewis, Z. A., and Goll, M. G. (2019). DNA methylation: shared and divergent features across eukaryotes. *Trends Genet.* 35, 818–827. doi: 10.1016/j.tig.2019.07.007

Schoofs, L., De Loof, A., and Van Hiel, M. B. (2017). Neuropeptides as regulators of behavior in insects. *Annu. Rev. Entomol.* 62, 35–52. doi: 10.1146/annurev-ento-031616-035500

Schowalter, T. D. (2016). *Insect Ecology: An Ecosystem Approach*. London: Academic press.

Schretter, C. E., Vielmetter, J., Bartos, I., Marka, Z., Marka, S., Argade, S., et al. (2018). A gut microbial factor modulates locomotor behaviour in *Drosophila*. *Nature* 563, 402–406. doi: 10.1038/s41586-018-0634-9

Schwab, D. B., Casasa, S., and Moczek, A. P. (2018). On the reciprocally causal and constructive nature of developmental plasticity and robustness. *Front. Genet.* 9:735. doi: 10.3389/fgene.2018.00735

Schwander, T., Lo, N., Beekman, M., Oldroyd, B. P., and Keller, L. (2010). Nature versus nurture in social insect caste differentiation. *Trends Ecol. Evol.* 25, 275–282. doi: 10.1016/j.tree.2009.12.001

Seeley, T. D. (1995). *The Wisdom of the Hive: The Social Physiology of Honey Bee Colonies*. Cambridge, MA: Harvard University Press.

Sehonova, P., Svobodova, Z., Dolezelova, P., Vosmerova, P., and Faggio, C. (2018). Effects of waterborne antidepressants on non-target animals living in the aquatic environment: a review. *Sci. Total Environ.* 63, 789–794. doi: 10.1016/j.scitotenv.2018.03.076

Shi, Y. Y., Huang, Z. Y., Zeng, Z. J., Wang, Z. L., Wu, X. B., and Yan, W. Y. (2011). Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, Apidae). *PLoS One* 6:e18808. doi: 10.1371/journal.pone.0018808

Shi, Y.-B. (2013). *Animal Metamorphosis*. London: Academic Press.

Shpigler, H. Y., Saul, M. C., Murdoch, E. E., Corona, F., Cash-Ahmed, A. C., Seward, C. H., et al. (2019). Honey bee neurogenomic responses to affiliative and agonistic social interactions. *Genes Brain Behav.* 18:e12509. doi: 10.1111/gbb.12509

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

Simons, M., and Tibbetts, E. A. (2019). Insects as models for studying the evolution of animal cognition. *Curr. Opin. Insect Sci.* 34, 117–122. doi: 10.1016/j.cois.2019.05.009

Simpson, S. J., Despland, E., Hägele, B. F., and Dodgson, T. (2001). Gregarious behavior in desert locusts is evoked by touching their back legs. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3895–3897. doi: 10.1073/pnas.071527998

Sinha, S., Jones, B. M., Traniello, I. M., Bukhari, S. A., Halfon, M. S., Hofmann, H. A., et al. (2020). Behavior-related gene regulatory networks: a new level of organization in the brain. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23270–23279. doi: 10.1073/pnas.1921625117

Smallwood, S. A., and Kelsey, G. (2012). De novo DNA methylation: a germ cell perspective. *Trends Genet.* 28, 33–42. doi: 10.1016/j.tig.2011.09.004

Smith, C. R., Anderson, K. E., Tillberg, C. V., Gadau, J., and Suarez, A. V. (2008). Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. *Am. Nat.* 172, 497–507. doi: 10.1086/590961

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. *Anim. Behav.* 85, 1004–1011. doi: 10.1016/j.anbehav.2012.12.031

Snell-Rood, E. C., and Moczek, A. P. (2012). Insulin signaling as a mechanism underlying developmental plasticity: the role of FOXO in a nutritional polyphenism. *PLoS One* 7:e34857. doi: 10.1371/journal.pone.0034857

Sovik, E., Berthier, P., Klare, W. P., Helliwell, P., Buckle, E. L. S., Plath, J. A., et al. (2018). Cocaine directly impairs memory extinction and alters brain DNA methylation dynamics in honey bees. *Front. Physiol.* 9:79. doi: 10.3389/fphys.2018.00079

Spear, L. P. (2013). Adolescent neurodevelopment. *J. Adolesc. Health* 52, S7–S13.

Sripetchwandee, J., Chattipakorn, N., and Chattipakorn, S. C. (2018). Links between obesity-induced brain insulin resistance, brain mitochondrial dysfunction, and dementia. *Front. Endocrinol.* 9:496. doi: 10.3389/fendo.2018.00496

Stamps, J. A., and Biro, P. A. (2016). Personality and individual differences in plasticity. *Curr. Opin. Behav. Sci.* 12, 18–23.

Stamps, J. A., and Frankenhuis, W. E. (2016). Bayesian models of development. *Trends Ecol. Evol.* 31, 260–268. doi: 10.1016/j.tree.2016.01.012

Stein, L. R., Bukhari, S. A., and Bell, A. M. (2018). Personal and transgenerational cues are nonadditive at the phenotypic and molecular level. *Nat. Ecol. Evol.* 2, 1306–1311. doi: 10.1038/s41559-018-0605-4

Taborsky, B., and Oliveira, R. F. (2012). Social competence: an evolutionary approach. *Trends Ecol. Evol.* 27, 679–688. doi: 10.1016/j.tree.2012.09.003

Taborsky, M., and Brockmann, H. J. (2010). "Alternative reproductive tactics and life history phenotypes," in *Animal Behaviour: Evolution and Mechanisms*, ed. P. Kappeler (Berlin: Springer), 537–586. doi: 10.1007/978-3-642-02624-9_18

Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y., and Nieh, J. C. (2015). A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci. Rep.* 5:10989. doi: 10.1038/srep10989

Teicher, M. H., Samson, J. A., Anderson, C. M., and Ohashi, K. (2016). The effects of childhood maltreatment on brain structure, function and connectivity. *Nat. Rev. Neurosci.* 17, 652–666. doi: 10.1038/nrn.2016.111

Terao, K., and Mizunami, M. (2017). Roles of dopamine neurons in mediating the prediction error in aversive learning in insects. *Sci. Rep.* 7:14694. doi: 10.1038/s41598-017-14473-y

Tessmar-Raible, K., Raible, F., Christodoulou, F., Guy, K., Rembold, M., Hausen, H., et al. (2007). Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. *Cell* 129, 1389–1400. doi: 10.1016/j.cell.2007.04.041

Tissot, M., and Stocker, R. F. (2000). Metamorphosis in *Drosophila* and other insects: the fate of neurons throughout the stages. *Prog. Neurobiol.* 62, 89–111. doi: 10.1016/s0301-0082(99)00069-6

Toth, A. L., and Robinson, G. E. (2007). Evo-devo and the evolution of social behavior. *Trends Genet.* 23, 334–341. doi: 10.1016/j.tig.2007.05.001

Toth, A. L., Tooker, J. F., Radhakrishnan, S., Minard, R., Henshaw, M. T., and Grozinger, C. M. (2014). Shared genes related to aggression, rather than chemical communication, are associated with reproductive dominance in paper wasps (*Polistes metricus*). *BMC Genomics* 15:75. doi: 10.1186/1471-2164-15-75

Traynor, K. S., Le Conte, Y., and Page, R. E. (2014). Queen and young larval pheromones impact nursing and reproductive physiology of honey bee (*Apis mellifera*) workers. *Behav. Ecol. Sociobiol.* 68, 2059–2073. doi: 10.1007/s00265-014-1811-y

Tremmel, M., and Müller, C. (2012). Insect personality depends on environmental conditions. *Behav. Ecol.* 24, 386–392. doi: 10.1093/beheco/ars175

Tripet, F., Jacot, A., and Richner, H. (2002). Larval competition affects the life histories and dispersal behavior of an avian ectoparasite. *Ecology* 83, 935–945. doi: 10.1890/0012-9658(2002)083[0935:lcatlh]2.0.co;2

Truman, J. W., and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* 125, 145–157. doi: 10.1016/0012-1606(88)90067-x

Tully, T., Cambiazo, V., and Kruse, L. (1994). Memory through metamorphosis in normal and mutant *Drosophila*. *J. Neurosci.* 14, 68–74. doi: 10.1523/jneurosci.14-01-00068.1994

van Os, J., Kenis, G., and Rutten, B. P. (2010). The environment and schizophrenia. *Nature* 468, 203–212. doi: 10.1038/nature09563

Vea, I. M., and Minakuchi, C. (2020). Atypical insects: molecular mechanisms of unusual life history strategies. *Curr. Opin. Insect Sci.* 43, 46–53. doi: 10.1016/j.cois.2020.09.016

Videla, M., Valladares, G., and Salvo, A. (2010). Differential effects of experience on feeding and ovipositing preferences of a polyphagous leafminer. *Entomol. Exp. Appl.* 137, 184–192. doi: 10.1111/j.1570-7458.2010.01053.x

Vitt, H. H., and Hartfelder, K. (1998). Scientific Note neurogenesis detected by BrdU incorporation in brains of larval honey bees, *Apis mellifera* L. (HYMENOPTERA : APIDAE). *Int. J. Insect Morphol. Embryol.* 27, 351–354. doi: 10.1016/S0020-7322(98)00028-2

Vleugels, R., Verlinden, H., and Vanden Broeck, J. (2015). Serotonin, serotonin receptors and their actions in insects. *Neurotransmitter* 2, 1–14.

Walzer, A., and Schausberger, P. (2011). Threat-sensitive anti-intraguild predation behaviour: maternal strategies to reduce offspring predation risk in mites. *Anim. Behav.* 81, 177–184. doi: 10.1016/j.anbehav.2010.09.031

Wang, H., Liu, Z., Wang, Y., Ma, L., Zhang, W., and Xu, B. (2020). Genome-wide differential DNA methylation in reproductive, morphological, and visual system differences between queen bee and worker bee (*Apis mellifera*). *Front. Genet.* 11:770. doi: 10.3389/fgene.2020.00770

Wang, Y., Kaftanoglu, O., Fondrk, M. K., and Page, R. E. (2014). Nurse bee behaviour manipulates worker honeybee (*Apis mellifera* L.) reproductive development. *Anim. Behav.* 92, 253–261. doi: 10.1016/j.anbehav.2014.02.012

Watson, A. H. D. (1992). Presynaptic modulation of sensory afferents in the invertebrate and vertebrate nervous system. *Comp. Biochem. Physiol. Part A Physiol.* 103, 227–239. doi: 10.1016/0300-9629(92)90573-9

Watson, R. E., Desesso, J. M., Hurtt, M. E., and Cappon, G. D. (2006). Postnatal growth and morphological development of the brain: a species comparison. *Birth Defects Res. B Dev. Reprod. Toxicol.* 77, 471–484. doi: 10.1002/bdrb.20090

Weeks, J., and Truman, J. (1986). Hormonally mediated reprogramming of muscles and motoneurones during the larval-pupal transformation of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 125, 1–13.

Wegerhoff, R. (1999). GABA and serotonin immunoreactivity during postembryonic brain development in the beetle Tenebrio molitor. *Microsc. Res. Techn.* 45, 154–164. doi: 10.1002/(sici)1097-0029(19990501)45:3<154::Aid-jemt3<3.0.Co;2-5

Weiner, S. A., and Toth, A. L. (2012). Epigenetics in social insects: a new direction for understanding the evolution of castes. *Genet. Res. Int.* 2012:609810.

Wessnitzer, J., and Webb, B. (2006). Multimodal sensory integration in insects—towards insect brain control architectures. *Bioinspir. Biomimet.* 1:63. doi: 10.1088/1748-3182/1/3/001

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. Oxford: Oxford University Press.

West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proc. Natl. Acad. Sci. U.S.A.* 102(Suppl. 1), 6543–6549. doi: 10.1073/pnas.0501844102

Wheeler, D. E., and Nijhout, H. F. (1984). Soldier determination in *Pheidole bicarinata*: inhibition by adult soldiers. *J. Insect Physiol.* 30, 127–135. doi: 10.1016/0022-1910(84)90116-1

Winfree, R., Gross, B. J., and Kremen, C. (2011). Valuing pollination services to agriculture. *Ecol. Econ.* 71, 80–88. doi: 10.1016/j.ecolecon.2011.08.001

Wolbert, P., and Kubbies, M. (1983). The cell cycle of epidermal cells during reprogramming in the pupa of Galleria. *J. Insect Physiol.* 29, 787–794. doi: 10.1016/0022-1910(83)90008-2

Wolf, C., and Linden, D. E. J. (2011). Biological pathways to adaptability – interactions between genome, epigenome, nervous system and environment for adaptive behavior. *Genes Brain Behav.* 11, 3–28. doi: 10.1111/j.1601-183X.2011.00752.x

Wu, Q., and Brown, M. R. (2006). Signaling and function of insulin-like peptides in insects. *Annu. Rev. Entomol.* 51, 1–24. doi: 10.1146/annurev.ento.51.110104.151011

Yagound, B., Remnant, E. J., Buchmann, G., and Oldroyd, B. P. (2020). Intergenerational transfer of DNA methylation marks in the honey bee. *Proc. Natl. Acad. Sci. U.S.A.* 117, 32519–32527. doi: 10.1073/pnas.2017094117

Yan, H., Simola, D. F., Bonasio, R., Liebig, J., Berger, S. L., and Reinberg, D. (2014). Eusocial insects as emerging models for behavioural epigenetics. *Nat. Rev. Genet.* 15, 677–688. doi: 10.1038/nrg3787

Yang, E.-C., Chang, H.-C., Wu, W.-Y., and Chen, Y.-W. (2012). Impaired olfactory associative behavior of honeybee workers due to contamination of imidacloprid in the larval stage. *PLoS One* 7:e49472. doi: 10.1371/journal.pone.0049472

Yartsev, M. M. (2017). Rebalancing diversity of animal models in neuroscience research. *Science* 358, 466–469. doi: 10.1126/science.aan8865

Zemach, A., McDaniel, I. E., Silva, P., and Zilberman, D. (2010). Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science* 328, 916–919. doi: 10.1126/science.1186366

Zera, A. J., and Denno, R. F. (1997). Physiology and ecology of dispersal polymorphism in insects. *Annu. Rev. Entomol.* 42, 207–230. doi: 10.1146/annurev.ento.42.1.207

Zhang, C.-X., Brisson, J. A., and Xu, H.-J. (2019). Molecular mechanisms of wing polymorphism in insects. *Annu. Rev. Entomol.* 64, 297–314. doi: 10.1146/annurev-ento-011118-112448

Zhao, C., Deng, W., and Gage, F. H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell* 132, 645–660. doi: 10.1016/j.cell.2008.01.033

Zill, S. N. (1986). A model of pattern generation of cockroach walking reconsidered. *J. Neurobiol.* 17, 317–328. doi: 10.1002/neu.480170406

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 28 June 2021
doi: 10.3389/fnbeh.2021.659615

Sexual Dimorphism in Aggression: Sex-Specific Fighting Strategies Across Species

Matias Pandolfi[1†], Maria Florencia Scaia[1] and Maria Paz Fernandez[2]*

[1] Department of Biodiversity and Experimental Biology, University of Buenos Aires, Buenos Aires, Argentina, [2] Department of Neuroscience and Behavior, Barnard College of Columbia University, New York, NY, United States

Aggressive behavior is thought to have evolved as a strategy for gaining access to resources such as territory, food, and potential mates. Across species, secondary sexual characteristics such as competitive aggression and territoriality are considered male-specific behaviors. However, although female–female aggression is often a behavior that is displayed almost exclusively to protect the offspring, multiple examples of female–female competitive aggression have been reported in both invertebrate and vertebrate species. Moreover, cases of intersexual aggression have been observed in a variety of species. Genetically tractable model systems such as mice, zebrafish, and fruit flies have proven extremely valuable for studying the underlying neuronal circuitry and the genetic architecture of aggressive behavior under laboratory conditions. However, most studies lack ethological or ecological perspectives and the behavioral patterns available are limited. The goal of this review is to discuss each of these forms of aggression, male intrasexual aggression, intersexual aggression and female intrasexual aggression in the context of the most common genetic animal models and discuss examples of these behaviors in other species.

Keywords: aggression, invertebrates, model system, sexual dimorphism, territoriality

OPEN ACCESS

Edited by:
Rui F. Oliveira, University Institute of Psychological, Social and Life Sciences (ISPA), Portugal

Reviewed by:
Hans A. Hofmann, University of Texas at Austin, United States
Sergio Marcello Pellis, University of Lethbridge, Canada

***Correspondence:**
Maria Paz Fernandez
mfernand@barnard.edu

[†] *Deceased*

Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *27 January 2021*
Accepted: *02 June 2021*
Published: *28 June 2021*

Citation:
Pandolfi M, Scaia MF and Fernandez MP (2021) Sexual Dimorphism in Aggression: Sex-Specific Fighting Strategies Across Species. Front. Behav. Neurosci. 15:659615. doi: 10.3389/fnbeh.2021.659615

INTRODUCTION

Aggression is a complex, plastic behavior whose manifestation depends on an animal's internal physiological state, sensory stimuli, and previous social experiences. Agonistic behavior is a more broadly defined concept, an adaptive act that arises from a conflict between two members of the same species. These behaviors play roles in conflict resolution when animals compete for specific resources such as territory, mates, or food sources and may involve intimidation of conspecifics by threat displays and can result in submissive responses like freezing, passive coping, or escape. Dominance in animals is established through repeated agonistic interactions that result in one animal controlling a contested resource. In animals living in groups, individuals who win agonistic encounters will become dominant, and losers often become subordinated, ultimately generating a hierarchical social organization. Some species establish a social hierarchy during the reproductive season that grants increased access to resources and reproduction to the highest-ranked individuals (Dewsbury, 1982). In social animals, at least three different types of social conflicts can be observed: between dominant and subordinates (Clement et al., 2005), among subordinates (Alonso et al., 2012), and between territorial neighbors (Muller and Manser, 2007).

Across species, competitive aggression is more common among males than females, and these differences are typically attributed to the action of steroid hormones during development or in adulthood (Gatewood et al., 2006). This is also the case in the best-studied rodent models in laboratory settings, including mice, rats, prairie voles, and hamsters. However, in all of these species, both males and females exhibit patterns of agonistic behavior, although often in different contexts (Been et al., 2019). Although female–female aggression is usually considered a behavior displayed almost exclusively to protect offspring, there are multiple instances of competitive aggression among females, both over high-value mates and food sources, in invertebrate and vertebrate species. Moreover, cases of intersexual aggression have been observed in a variety of species, particularly in fish.

Aggressive behavior studies in the laboratory have shed light on the genetic and neural basis of this behavior in animals that range from crustaceans to primates (Nelson, 2006; Anderson, 2012; Asahina, 2017). In particular, genetically tractable models have proven extremely valuable for our understanding of the underlying neuronal circuitry and the genetic architecture of aggressive behavior. Of all genetically tractable model organisms, the mouse nervous system is the most similar to ours. Studies in mice have shed light on mechanisms that appear to also regulate aggression in humans and may provide insight into psychiatric disorders associated with pathological aggression. Research in invertebrate models, *Drosophila* in particular, has also contributed to our understanding of neuronal mechanisms underlying this highly conserved behavior (Kravitz and Fernandez, 2015; Asahina, 2017). However, studies in genetic model systems also have limitations, particularly the lack of an ethological perspective. In this review, we compare the most commonly used genetic model systems, mice, zebrafish, and *Drosophila*, and discuss their advantages and limitations for studying three different forms of aggressive behavior: male intrasexual aggression, intersexual aggression, and female intrasexual aggression. In each case, we also discuss behavioral repertoires from other rodent, fish, and invertebrate species, that make them interesting and valuable models for the study of aggressive behavior.

MALE AGGRESSION: MODELS FOR COMPETITION AND TERRITORIALITY

Mice are currently the most common laboratory animal model for the study of aggressive behavior. Aggression in mice is almost exclusively observed among males, and the most common test is the resident-intruder test, in which a resident animal confronts an intruder. This test allows the manifestation of both offensive and defensive behaviors. Early in the study of the neurobiological basis of aggression, rats, and hamsters were used for lesion experiments because they were the organisms most commonly used in behavioral psychology (Huhman, 2006). Those studies proved particularly insightful for our understanding of the brain regions that control male aggressive behavior, particularly the hypothalamic area, which has been linked to aggression for almost a century (Kruk, 1991). Studies in rats revealed that electrical stimulation of the so-called hypothalamic attack area (HAA) induces escalated aggression, which can be directed toward both males or females, or even mice (Kruk, 1991; Hrabovszky et al., 2005). However, in these approaches the spatial resolution for localizing specific neuronal populations involved in aggression is quite limited (Anderson, 2012). In contrast, the genetic tractability of mice has allowed an increasingly detailed mapping of neural circuits underlying aggression (Takahashi and Miczek, 2014).

Genetic tools available in mice, such as optogenetics, have also made it possible to explore the connection between areas related to aggression and other brain centers, and the tools for manipulation of neuronal activity and addressing neuronal connectivity in this species have enabled the identification of a specific population within the hypothalamus as crucial for male aggressive behavior. Studies using channelrodopsin2 showed that optogenetic stimulation of neurons in the ventrolateral subdivision (VMHvl) of the ventromedial hypothalamus (VMH) elicits aggression in male mice toward other males but also females and inanimate objects (Lin et al., 2011). Interestingly, this structure also contains neurons that appear to be active during mating (Lin et al., 2011). Silencing of the same neuronal population via an ivermectin (IV)-gated chloride channel led to a significant reduction in aggression, and in some cases its complete suppression, without affecting male mating behavior. Genetic tools have also allowed to study the connections between neuronal circuits that control aggression and brain areas that regulate other behaviors. For example, a recent study showed that circadian regulation of male aggression is mediated by a polysynaptic pathway from the suprachiasmatic nuclei to VMHvl neurons (Todd et al., 2018).

Although the mouse has many advantages as a model system, particularly the comparative ease of genetic manipulations and the possibility of circuit mapping, it also has limitations, notably the lack of extensive information about behavior in natural environments. However, experimental methods using environments more similar to the natural burrow system of the ancestral species have been developed to address some of the shortcomings of the standard behavioral tests and allow more ethologically relevant studies of dominance relationships in large groups (Williamson et al., 2017, 2019). Work from the Curley laboratory showed that groups of 12 outbred males mice living in large and complex environments establish linear and stable social dominance hierarchies (So et al., 2015; Williamson et al., 2016). Authors were able to subdivide the animals into three broad social status categories. Once a hierarchy is established each animal displays agonistic or subordinate behaviors to other males depending on the other animal's relative social rank (Lee et al., 2018). Subordinate individuals are less likely to initiate fights than alpha or subdominant mice and lose far more contests than they win. Alpha males, which have the highest social rank, rarely lose fights and initiate a large fraction of agonistic interactions. Besides the behavioral consequences of establishing dominance these males also show higher levels of major urinary proteins and increased their feeding and drinking levels (Lee et al., 2017, 2018; Williamson et al., 2017). In addition, studies of large groups in complex environments revealed that socially dominant males

had significantly higher oxytocin receptor (OTR) binding in the nucleus accumbens core than subordinate animals. Alpha males also showed higher OTR binding in several brain regions, while alpha males had lower vasopressin 1a receptor (V1aR) compared to subordinates (Lee et al., 2019).

Finally, the sensitivity of neural circuits mediating aggression and the behavioral responses to the effects of steroid hormones can vary greatly across species (Romeo et al., 2003). In mice, the direct connection between elevated testosterone and increased aggression is clear, whereas in other species such as Syrian hamsters (*Phodopus sungorus*), there relationship is reversed: testosterone decreases aggression in adult males housed under short-day conditions (Jasnow et al., 2000). Conversely, males from other rodent species, such as Mongolian gerbils (*Meriones unguiculatus*) and prairie voles (*Microtus ochrogaster*), do not exhibit diminished aggression in response to castration (Christenson et al., 1973; Demas et al., 1999).

Aggression in Teleosts

In vertebrates, most social behaviors are regulated by the Social Decision-Making Network (SDMN), an evolutionarily conserved brain network in which consensus homologies for most relevant brain areas have been already identified in mammals, birds/reptiles, amphibians, and fish (O'Connell and Hofmann, 2011, 2012). Considering that the neural substrate for social behaviors is phylogenetically conserved and that fish species present a vast repertoire of reproductive and parental care behaviors, teleost fish constitute a group of growing interest in the study of aggressive behavior. In particular, the zebrafish (*Danio rerio*), native to freshwater habitats in South Asia, has become a widely used vertebrate model organism. Some of the advantages of zebrafish for aggression studies are their relatively small size, short generation time, and early onset of displays of social behaviors (Dreosti et al., 2015). Due to its fully sequenced genome, a wide offer of genetic tools including mutant lines, CRISPR/Cas9 genome engineering (Prykhozhij et al., 2017), and optogenetics (Del Bene and Wyart, 2012) has been established, as well as robust behavioral tests (Norton and Bally-Cuif, 2010). In recent years, zebrafish has been used to investigate several aspects of aggressive behavior, including the search for novel drugs that modulate aggressive behavior (Gutierrez et al., 2020).

Zebrafish male–male aggression is assessed during dyadic fights, after which a winner and a loser emerge as a consequence of a clear asymmetry of expressed behaviors, such as displays, circles, bites, chases, strikes, flees, and freezing (Oliveira et al., 2011; Teles and Oliveira, 2016). The temporal organization of these behavioral patterns allowed identifying highly structured patterns of aggressive behaviors. Not only contributing genes but also underlying neuronal pathways have been identified in this species, such as the hypothalamo-neurohypophysial or hypothalamo-pituitary-gonadal systems, and the histamine pathway, novel for non-mammalian systems (Filby et al., 2010). Interestingly, two subregions of the dorsal habenula antagonistically regulate the outcome of social conflict in zebrafish. While silencing the lateral subregion of dorsal habenula causes a stronger predisposition to lose a fight, silencing the medial subregion of the dorsal habenula is linked to winning the encounter, suggesting that both subregions of the habenula and their projections to the interpeduncular nucleus constitute a dual control system for conflict resolution (Chou et al., 2016). Remarkably, 69% of zebrafish genes have human orthologs. One of the limitations of zebrafish is that very little information is available about its behavior in natural environments. Therefore, while it is a useful model for studying the neural circuits that modulate aggressive behavior, currently studies in this model do not include an ecological or evolutionary perspective.

An attractive fish model to study aggression is the Siamese fighting fish *Betta splendens*. Popular in the aquarium trade, in the wild this species is found in standing waters of canals, rice paddies, and floodplains (Mendez-Sanchez and Burggren, 2014). Most of the animals used in research came from laboratory-reared animals, which are larger, more colorful, and substantially more aggressive than their wild counterparts, occasionally exhibiting lethal aggression between males in laboratory conditions. The differences between wild and laboratory-reared fish are quantitative and also involve divergent behavioral patterns (Ramos and Goncalves, 2019). The neural circuits and brain areas involved in aggressive behaviors in *B. splendens* have been studied primarily in strains that were artificially selected. *B. splendens* exhibit robust and highly stereotyped displays of aggressive behavior. Although it is not a genetically tractable model, CRISPR/Cas9 gene editing can be successfully implemented in this species (Andres Bendesky, personal communication). Gene editing techniques are also been implemented in other fish species (Gratacap et al., 2019; Warren et al., 2021).

From an ethological perspective, Cichlid fish are a particularly interesting model. The African cichlids, *Oreochromis mossambicus*, which is found on the Limpopo and Zambezi Rivers, and *Astatotilapia burtoni*, from Lake Tanganyika [reviewed by Fernald and Maruska (2012)], are the most extensively studied species to date. Cichlids form hierarchical social systems in which dominant individuals defend their status by aggressive displays toward other submissive, lower-ranked animals (Maruska, 2014). In particular, *A. burtoni* is a maternal mouth-brooding species living in a lek like social system, in which males can adopt two distinct reversible phenotypes: while dominant males are brightly colored and represent only 10–30% of the population, subordinate males present faded coloration and make up the majority of male population (Maruska and Fernald, 2013). Dominant males defend territories providing food, shelter, and substrate for spawning. While subordinates do not hold territories, they typically do not reproduce but school with females and other subordinates.

Since suitable territories are often limited, and females are less prone to mate outside shelters, *A. burtoni* males often engage in high-intensity aggressive encounters. They can reversibly switch between dominant and subordinate states, which has profound effects on behavioral and physiological mechanisms regulating reproduction. This fish model offers several important advantages for the study of the physiological basis of aggression: as in *B. splendens*, social change in males is signaled by obvious color differences which occurs within a few minutes, this species offers relatively easy access to the brain, facilitating

sampling, and also has a fully sequenced genome (Fernald, 2012; Maruska and Fernald, 2013). Moreover, their social system can be replicated and manipulated under laboratory conditions, for example, mimicking natural changes to identify key physiological mechanisms regulating aggressive behavior and their impact on reproduction.

As in many other species, aggression in *A. burtoni* appears to be inhibited by serotonin (5-HT). Serotonin receptors in the telencephalon play a role in social status, and dominant males have more 5-HT cells in the raphe than their subordinate counterparts (Loveland et al., 2014). In addition, arginine vasotocin (AVT) regulates different aspects of male social behavior. While neuronal subpopulations in the parvocellular pathway are involved in the activation and modulation of submissive neural circuits or inhibition of aggressive/dominance networks, gigantocellular pathways have been associated with an upregulation of both courtship and aggression (Greenwood et al., 2008).

Steroid hormones have also been shown to play a role in aggressive behavior in *A. burtoni*. Growing evidence suggests that 17b-estradiol (E2) and brain aromatase, the enzyme that converts testosterone (T) to E2, have a central role in regulating male aggression. Dominant and territorial males also present higher circulating plasma levels of T, 11-ketotestosterone (11-KT, one of the most relevant androgens in fish), progesterone (P), and E2 when compared to subordinate males (Maruska and Fernald, 2013). When males are given a chance to raise their rank, T, 11-KT, E2, and P plasma levels increase due to changes in social status, suggesting that interactions occurring during the establishment of dominance modulate sex-steroid levels (Maruska et al., 2013). Both dominant and submissive males show differences in androgen and estrogen receptor mRNA levels in several brain regions within the SDMN (Maruska et al., 2013). Furthermore, since aromatase promotes aggression through actions in the preoptic area and estradiol promotes male aggression (Huffman et al., 2013), elevated T levels in dominant males can regulate aggression through their aromatization to E2 and a concomitant activation via estrogen receptors in the brain (Renn et al., 2008).

Although most of the research has focused on African cichlids, male aggressive behavior has also been studied in several Neotropical cichlid species. *Cichlasoma dimerus* (Chanchita) is an appealing model for studying the relations between hormones, social context, and behavior (Scaia et al., 2020). Unlike African cichlids, Chanchita is a monogamous species with biparental care, in which both males and females aggressively defend their territory (Pandolfi et al., 2009). This allows the study of aggressive parental behavior and underlying physiological mechanisms in both males and females. In this species, 5-HT also plays a key role in regulating male aggressive behavior. Evidence suggests that incorporating the rate-limiting substrate for 5-HT synthesis, the amino acid *L*-tryptophan, into the diet reduces the motivation to attack and modulates both aggressive and submissive behaviors (Morandini et al., 2019). After hierarchy establishment, subordinate males showed increased soma area of the parvocellular AVT subpopulation compared to territorial males, suggesting that changes in the synthesis or accumulation of AVT are necessary for the modulation of social behaviors (Ramallo et al., 2012). Steroid hormones also play a role in *C. dimerus*; while territorial, dominant males with high levels of aggression show higher T and 11-KT plasma levels than non-territorial males, the opposite is true for E2 (Ramallo et al., 2015).

Invertebrate Models of Aggression

Invertebrates have proven to be excellent models for studying the neurobiological bases of aggression. Long before the introduction of *Drosophila*, the most widely used invertebrate genetic model, work on several invertebrate species, particularly crustaceans, revealed key aspects of the neural architecture underlying aggressive behavior, the formation and maintenance of dominance relationships, and the neurochemical mechanisms involved in the manifestation of aggression. These species typically have highly structured, accessible nervous systems, and aggressive behavior is highly stereotyped (Huber et al., 1997b; Kravitz and Huber, 2003). In crustaceans such as lobsters and crayfish, winners raise their legs and direct their antennae forward to display a dominant posture while losers adopt submissive postures (Huber et al., 1997b; Kravitz and Huber, 2003). Lobsters (*Homarus americanus*) became a model for the study of aggression largely due to their modular neural system, with few aminergic neurons (Kravitz and Huber, 2003). Amine neurons, in particular serotonin and octopamine, regulate their agonistic behavior, escalation of fights, and establishment of dominance (Kravitz, 2000). Laboratory studies on lobster aggression have focused on male–male encounters, in which opponents can cause serious injuries to one another. Agonistic encounters involve highly stereotyped behavioral patterns, progressing through visual displays to physical attacks of increasing intensity. Males initiate agonistic encounters even in the absence of females or resources, and unlike social animals, they form strong dominance relationships purely based on physical superiority (Huber et al., 1997a).

One of the main advantages of *Drosophila melanogaster* as a model for the study of aggression is its unparalleled genetic tools, which allow for high spatial and temporal resolution in manipulations of gene expression and neuronal activity (Venken and Bellen, 2007; Bellen et al., 2010; Kravitz and Fernandez, 2015). In addition, its highly stereotyped patterns of aggressive behaviors are robust across laboratory settings and make quantification straightforward and suitable for automatic tracking methods (Dankert et al., 2009; Kravitz and Fernandez, 2015; Asahina, 2017; Chowdhury et al., 2021). As in most species, males exclusively attack other males. *D. melanogaster* males are territorial, and that they fight over resources such as females and food. After several encounters, dominance relationships are established, and animals that have lost fights are less likely to engage in aggressive interactions against naïve individuals or familiar winners (Penn et al., 2010; Trannoy et al., 2015, 2016). Selecting for highly aggressive lines over several generations allows the generation of hyper-aggressive lines, and the study of the genetic contributions to this behavioral phenotype (Dierick and Greenspan, 2006; Penn et al., 2010). In male–male encounters, hyper-aggressive animals show shorter latencies to fight and increased retaliation frequency, and win the

vast majority of fights against males of the original parental line. In *D. melanogaster* as well as in other invertebrates, serotonin seems to increase, rather than decrease, aggression (Huber et al., 1997b; Dierick and Greenspan, 2007). Interestingly, studies in other species suggest that this inverse relationship between serotonin and aggression does not hold across all invertebrates (Stevenson et al., 2000; Bubak et al., 2020). The brain connectome is close to completion in *D. melanogaster* (Li et al., 2020; Scheffer et al., 2020). When combined with existing genetic, physiological, and behavioral methods, this new knowledge will undoubtedly improve our understanding of how neural circuits control complex and plastic behaviors like aggression.

Both male and female *Drosophila* show aggressive behavior toward individuals of their same sex, but the behavioral patterns employed are highly dimorphic (Dow and von Schilcher, 1975; Jacobs, 1978; Nilsen et al., 2004). Moreover, only males establish dominance (Nilsen et al., 2004). The latency to start a fight is usually defined as the latency to the first *lunge*. Lunging is the most distinctive male pattern of aggression, a direct attack in which a male fly rises on its hind legs and snaps down on the opponent. Eventually, the dominant male gains control of the contested resources, after which the defeated animal retreats (Yurkovic et al., 2006; Miczek et al., 2007). In recent years, putative pheromones, as well as some olfactory and gustatory receptors, have been shown to play key roles in *Drosophila* aggression (Yew et al., 2009; Fernandez et al., 2010; Wang and Anderson, 2010; Wang et al., 2011), and the vast and versatile genetic toolkit available this species has made it possible to map neuronal circuits underlying this behavior (Asahina et al., 2014). However, as is the case for mice and zebrafish, studies in this species lack an ethological perspective.

The first descriptions of *Drosophila* aggression were those by Alfred Sturtevant in 1915, working mainly with *D. ampilophila* (Sturtevant, 1915). In an article about sex recognition and sexual selection, he was the first to mention male intrasexual aggression which appeared to be in the context of competition for mating partners. One of the patterns that he described for males appears to be similar to the "head-butt" pattern seen in *D. melanogaster* female fights (Nilsen et al., 2004). A few decades later A. Hoffmann used *D. melanogaster* and *D. simulans* and created a complete ethogram of agonistic interactions between males of the two species. Escalation of fights in *D. simulans* was more frequent and depended on body weight differences, and encounters lasted longer. More *D. simulans* males exhibited territorial behaviors (Hoffmann, 1987a,b).

A particularly interesting aggression phenotype that highlights the role of ethologically relevant environments has been described in males of the Mediterranean field cricket *Gryllus bimaculatus*. Similar to male lobsters, fights between male crickets follow a stereotyped sequence of escalating intensity. Initial encounters involve antennae, then proceed to spread mandibles displays, then interlocking mandibles and eventually engaging in "wrestling." Losers tend to avoid further aggressive encounters. Remarkably, being allowed to fly after losing a fight restores their willingness to engage in subsequent fights, since losers regain their aggressiveness after being repeatedly thrown into the air (Hofmann and Stevenson, 2000). The majority of the losers re-engage in aggressive interactions with their previous opponent, and can escalate to the same level as naive animals. This is a rare example of activation of a motor pattern immediately after an aggressive interaction affecting the dynamics of the fight. Amine neurons have been mapped in the *G. bimaculatus* nervous system, and depletion of biogenic amines affects male aggression: the aggressiveness of crickets is reduced after depleting octopamine and dopamine from the CNS but is unaffected by serotonin depletion (Stevenson et al., 2000), suggesting that amines used to control aggression play different roles in insects and crustaceans (Stevenson et al., 2000; Murakami and Itoh, 2001). Moreover, the frequency and intensity of fighting can vary markedly within cricket species (Sakaluk, 1987; Jang et al., 2008; Kim et al., 2011).

In contrast to *Drosophila*, male–male fights in lobsters, crayfish, and crickets involve high intensity patterns of aggression and may result in physical harm. Recently, CRISPR/Cas9 gene editing has begun to be used to manipulate gene expression in crustaceans (Martin et al., 2016; Xu et al., 2020). This opens the possibility of expanding the applications of some of the classical invertebrate models to study of the neurobiology of aggression by adding genetic tools that could enable, for example, optogenetic control of neuronal activity during fights in natural or semi-natural environments.

INTERSEXUAL DISPLAYS OF AGGRESSION

In mice, female aggression toward males is rare and has been described mostly in the context of maternal aggression. Female aggressive behavior is frequent before gestation, increases shortly postpartum, and then declines (Noirot et al., 1975; Erskine et al., 1978). Maternal aggression includes both defensive and offensive behavioral patterns. Lactating females engage in defensive attacks toward males and offensive attacks toward female intruders (Lucion and de Almeida, 1996). Unlike male–male aggression, which has been widely studied in species ranging from invertebrates to primates, little is known about mechanisms underlying male attacks toward conspecific females. Males from the most widely used mammalian genetic model, mice, do not normally attack females under laboratory conditions. However, optogenetic activation of the VMH elicits male aggression toward females and toward inanimate objects (Lin et al., 2011).

In contrast, pair-bonded prairie voles exhibit one of the most robust aggressive responses from a male rodent toward a female. Specifically, once a pair bond has been formed, males exhibit aggression toward conspecific females but not toward their partners. This response appears to be mediated by dopamine receptor expression in the nucleus accumbens (Young and Wang, 2004). Vasopressin also plays a role, and the anterior hypothalamus (AH)-AVP system appears to mediate aggression toward females in hamsters as well as in other rodents (Ferris et al., 1989; Motta et al., 2009). Interestingly, cohabitation with females in the absence of mating does not induce male attacks toward novel females (Insel et al., 1995;

Wang et al., 1997). Males that mated for 24 h and formed bonds attacked both male and female intruders and expressed higher levels of Fos-ir expression in the medial amygdala (meA) (Wang et al., 1997). Selective aggression appears to serve the role of maintaining the monogamous pair bond (Resendez and Aragona, 2013), and is a rare example of a case in which rodent males display aggression toward a sexually receptive female that does not represent a threat. Thus, the prairie vole is considered an outstanding model for studying the neuronal mechanisms underlying monogamy in rodents.

Intersexual Aggression in Teleosts

Intersexual aggression in zebrafish has been assessed mainly in the context of how size-selective harvesting (e.g., fisheries) can directionally change sexually selected traits. To study the role of the size-selective harvesting on the evolution of mating behavior, size-matched spawning trials were performed among different size-harvested lines of zebrafish (Sbragaglia et al., 2019). Evidence suggests that while male aggression is lower when random-harvested males were crossed with females from the small and random harvested lines, male aggression is higher when large and small harvested males were crossed with females from the random harvested line. Moreover, females from the large harvested line experience lower levels of male aggression than females from the random and small harvested lines. Interestingly, since evidence on intersexual aggression in zebrafish focuses on male aggression because of its key importance in mating behavior of this species (Spence et al., 2008), female aggression toward males is still understudied.

Betta splendens males and females intensely and frequently attack each other regardless of the reproductive context and males often attack females for long periods of time. Females also attack males, but less frequently. In contrast, intersexual aggression in cichlids is often associated with pair-bonding and reproductive behavior. The convict cichlid (*Amatitlania siquia*) is a serially monogamous fish in which both intrasexual competition and intersexual selection influence the mating pattern. In this species, both sexes are highly aggressive, and the winner of aggressive encounters is usually the larger individual regardless of sex (Leese, 2012). Several monogamous species demonstrate size-assortative mating patterns, showing a positive correlation between male and female sizes of mate pairs within a population. In the case of the convict cichlids, oftentimes pairs are formed in which males are larger than females both under laboratory (Beeching and Hopp, 1999) and field conditions (Wisenden, 1994). Intersexual selection in this species influences size-assortative mating, and most studies have focused on female preference for larger males (Gagliardi-Seeley et al., 2009). However, when males are forced to pair with a smaller or a larger female, pair formation only occurs when the female is smaller than the male, while larger female shows high aggression to the male (Bloch et al., 2016). This suggests that intersexual aggression from females toward males limits size-assortative mating. Moreover, there is also evidence of intersexual aggression from males toward novel females (Leese, 2012). Besides cichlids, another interesting organism for the study of intersexual aggression is the electric fish *Gymnotus omarorum*, which shows non-breeding intrasexual and intersexual territorial aggression, does not exhibit sexual dimorphism in body size and in which body size and not sex is the best predictor of dominance in intersexual aggressive encounters (Batista et al., 2012).

Intersexual Aggression in *Drosophila*

Intersexual aggression in *D. melanogaster* has not been observed in the absence of manipulations of neuronal activity or gene expression, at least under laboratory conditions. High levels of female aggression toward males can be elicited by masculinization of the female nervous system either via expression of the male form of *fruitless* (Vrontou et al., 2006) or through RNA interference (RNAi)-mediated silencing of the sex determination gene *transformer* (Chan and Kravitz, 2007). Such "masculinized" females attack females of the same genotypes, mutant, or transgenic males that exhibit female aggression patterns, and wild-type males. However, these manipulations do not trigger female aggression toward wild-type females.

Drosophila males attack only other males and do not attack females (Kravitz and Fernandez, 2015). However, when the pheromonal profile of females is genetically masculinized, these females can elicit aggression from males (Fernandez et al., 2010). The composition of female cuticular hydrocarbons (CHs), which serve as contact pheromones, can be changed to that normally found on surfaces of the males by expression of a *transformer* RNAi transgene (Fernandez et al., 2010). These females exhibit pheromonal profiles similar to those of wild-type males, with high levels of monoenes and low levels of dienes. Males also attack females with a masculinized central nervous system, indicating that behavioral cues displayed by the females can override their chemical cues (Fernandez et al., 2010). In addition, males in which the nervous system is feminized by expression the female form of *fruitless* (Vrontou et al., 2006) or in which *transformer* is ectopically expressed (Chan and Kravitz, 2007) exhibit aggression toward females. Interestingly, activation of tachykinin-expressing neurons in males can elicit male aggression toward females (Asahina et al., 2014).

FEMALE–FEMALE AGGRESSION

Although not as extensively studied as male intrasexual aggression, female intrasexual aggression occurs in vertebrate and invertebrate species (Clutton-Brock, 2009). Exploring the dynamics underlying this behavior in taxa with different evolutionary histories would help better understand the selective pressures driving the evolution of female aggression. Female–female aggression has been postulated to be the by-product of genetic correlations with males (Lande, 1980). According to this hypothesis, traits that are advantageous for males, like aggression toward other males, are often expressed in females as well (Forstmeier et al., 2011). An alternative explanation is that female–female aggression has evolved from direct selection on females themselves and likely functions in competition over reproductive and social benefits (Tobias et al., 2012; Stockley and Campbell, 2013).

One reason why studies of aggression have focused on males is the potentially confounding behavioral effects of steroid hormone level oscillations during the estrus cycle. Estradiol, the main steroid hormone in females, has been implicated in female aggressive behavior (Rosvall et al., 2012), and several rodent species such as rats and hamsters are less likely to exhibit aggression during the estrus cycle (Wise, 1974; Davis and Marler, 2004). However, in these species, as well as in mice, the effect of the estrus cycle on aggression remains unclear. As in males, brain regions in the social behavior network [meA, bed nucleus of the stria terminalis (BNST), lateral septum (LS), medial preoptic area (mPOA), AH, VMH, and periaqueductal gray (PAG)] as well as the mesocorticolimbic dopamine pathway have been found to form the basis of the neural circuit regulating aggression, though there are some important sex differences (Duque-Wilckens and Trainor, 2017).

Intrasexual competition and hierarchy formation in female mice appears to be rare when population density is low and increase as population size increases, but little is known about the formation of female social hierarchies in mice (Yasukawa et al., 1985; Weidt et al., 2018). As it is the case for males, studies in more naturalistic environments revealed novel aspects of female social behaviors that were not observed under standard laboratory conditions (Williamson et al., 2019). Female mice living in large, complex environments are able to form linear hierarchies that emerge quickly, are stable for around 2 weeks and do not appear to be affected by the estrous cycle. Interestingly, these females housed under these conditions showed an extended estrous cycle (Williamson et al., 2019). Dominant females spent significantly longer in estrus than subordinate females, which subordinate showed higher levels of plasma corticosterone than dominant females, suggesting that they may be more susceptible to social stress.

Unlike most female laboratory rodents, which rarely display spontaneous aggression, female Syrian hamsters exhibit a range of competitive strategies. Females are able to form robust and stable hierarchal relationships and even inhibit the reproductive capacity of other females (Albers et al., 2002). Interestingly, clear sex differences in the neural regulation of dominance and aggression have been reported in this species. While hypothalamic injection of a 5-HT1a agonist stimulated aggression in females and inhibited aggression in males, injection of AVP had the opposite effects on both males and females. In addition, formation of female dominance was associated with activation of 5-HT neurons within the dorsal raphe while formation of male dominance was associated with activation of AVP neurons in the hypothalamus. Interestingly, fluoxetine increased female aggression while it substantially reduced aggression in males, an observation with obvious implications for psychiatry (Terranova et al., 2016).

Teleosts and Female Dominance

Analyses of aggression in fish have focused on male intrasexual competition. However, female dominance behaviors can also be observed in common laboratory models and in domestic fish. In zebrafish, although female intrasexual encounters are less aggressive (i.e., fewer attacks in the same time interval), evidence suggests that dominant females display significantly more aggressive displays than subordinate females (Filby et al., 2010). During the spawning period, dominant females are less aggressive toward their subordinate same-sex counterparts than dominant males toward theirs (Paull et al., 2010). Given that zebrafish is a popular vertebrate model for studying the neuronal basis of behavior, female aggression is surprisingly understudied. For example, a systematic quantification of aggressive behavior patterns is not yet available, and the role of all brain activation across the SDMN in aggressive displays remains unknown.

Betta splendens females exhibit a fighting pattern similar to that of males when in small aquariums (Braddock and Braddock, 1955). Fights between females end with submissive behaviors displayed by one of the individuals, while the dominant female continues to exhibit aggression for a short period of time. When housed in mixed large groups, female–female fights are less frequent than male–male fights (Elcoro et al., 2008). Aggression in female wild-types has also been described and compared to a strain that was selected for more aggression ("fighters") by replicating a mating scheme commonly used by local breeders in Thailand, in which sibling males of a winner are mated with sibling females from another breeder (Ramos and Goncalves, 2019). 'Fighter' females are more aggressive than wild-type females, but the differences are quantitative rather than qualitative. Even though both strains show similar behavioral patterns (frontal displays, lateral displays, charge, caudal swing, and approach), behavioral correlation networks of the two strains are similar when females are paired with conspecifics but different in the mirror trials. Higher aggression in fighter females may be an adaptation to captivity, with more aggressive females having higher survival rates.

There are several examples of species in which females display high levels of aggressive behavior. Fish present a wide variety of reproductive and parental strategies, and cichlid fish are particularly interesting models to study both male and female aggression. For example, both intrasexual male and female aggression has been reported in dyadic agonistic encounters in the cooperatively breeding cichlid *Neolamprologus pulcher* (Taves et al., 2009), and aggression levels are similar between the sexes. Newly dominant females have higher plasma testosterone (T) but similar 11-KT levels in comparison with newly subordinate females (Taves et al., 2009). By contrast, newly dominant males have higher 11-KT but similar T levels relative to subordinate males. Female aggressive behavior of an intensity comparable to that in males has also been reported in the cichlid *A. siquia* (Bloch et al., 2016).

In contrast, females of the cichlid *A. burtoni* are usually not aggressive and do not form social hierarchies. However, when they are placed in all-female communities, they develop social hierarchies, display aggression, and exhibit male-like patterns of behavior (Renn et al., 2012; O'Connell et al., 2013). Interestingly, in a recently collected stock of fish, females of this species show aggressive behaviors toward male intruders if they are taking care of their brood (Renn et al., 2009). When comparing the neuroendocrine regulation of aggression in male and female dominants and subordinates, there are sex-specific and status-specific patterns of hormonal regulation of dominance

(Renn et al., 2012; O'Connell et al., 2013). Moreover, evidence on neural gene expression suggests that there are specific modules and functional gene ontology categories that can explain either dominance or reproductive state when comparing brooding females with dominant and subordinate males (Renn et al., 2008). In females, gene expression patterns reveal a core module of genes associated with social dominance and up-regulation of genes previously identified as male-biased (Renn et al., 2016). However, even if aggressive behavior in *A. burtoni* females is observed in recently collected fish or in tanks in only in all-female groups, these behaviors have been studied in the context of maternal aggression and not in neutral aquaria. Monogamous cichlids, as well as species without lek-like system, are interesting models for the study of female territoriality and the underlying neuroendocrine mechanisms (Reddon et al., 2013).

Females of the neotropical cichlid *C. dimerus* can be as aggressive as males, as dominant, reproductive females aggressively defend their territory from subordinate, lower-ranked animals (Ramallo et al., 2014). The highest aggression levels in pre-spawning females are associated with larger GnRH-3 nuclear and somatic area and peaks in androgen and E2 plasma levels (Tubert et al., 2012). Comparisons of male–male and female–female encounters in neutral arenas do not reveal significant differences between sexes in terms of latency to attack, time of resolution, or frequency of aggressive displays, suggesting that females are as aggressive as males (Scaia et al., 2018b). Moreover, female winners show higher E2 levels before the agonistic encounter than female losers, while there are no differences on T and 11-KT levels (Scaia et al., 2018a). These results suggest that in *C. dimerus* female aggression is associated with initial levels of E2, and that estrogen levels could predict female aggression.

Female Aggression in Invertebrate Models

Aggression in *D. melanogaster* females was first described by Ueda and Kidokoro (2002). The authors described the female behavior as being similar to those of males identified and identified several behavioral patterns, including "lunge." The "lunge" described in this study was different from the pattern used currently to quantify male aggression (Nilsen et al., 2004), since the female lunge did not involve rising. Ueda and Kidokoro reported that female aggression levels were dependent on rearing conditions, since isolated females were more aggressive than their group-housed counterparts, and on the quality of the food source, which suggested defense of potential future egg-laying sites.

Recent studies showed that female aggression in *D. melanogaster* is influenced by mating via an associated seminal fluid protein called sex peptide (Bath et al., 2017). Although the majority of the work on *D. melanogaster* aggression has been done in males, a growing number of studies have focused on female intrasexual aggression. Neuronal populations that mediate female-to-female aggression have been identified (Palavicino-Maggio et al., 2019), such as the *doublesex*-expressing pC1 cluster (Deutsch et al., 2020). Optogenetic activation of a subset of the neurons derived from the aIP-g neuroblast (Cachero et al., 2010) increases female aggression in the absence of aggression-promoting cues (Schretter et al., 2020). As is the case with males, connectomics studies in combination with genetic tools will likely help our understanding of how neuronal circuits control aggression with a level of resolution that is not possible in other invertebrate species. However, aggression in female *D. melanogaster* is less frequent and substantially less intense than in males, and females do not establish dominance (Nilsen et al., 2004). Studies focused on female competitive aggression in other non-social insects would allow for an examination of other aspects of this form of aggression. Unfortunately, there are relatively few such studies, as most focus on female aggression in social insects.

Female aggression in the context of nestmate recognition has been explored in multiple insect species such as ants, bees, wasps, and termites. One of the best described cases of female aggression in invertebrates is that of honey bees (Nouvian et al., 2016). Unlike other eusocial species, like ants and termites, guard, and soldier bees do not exhibit obvious morphological differences. Nest guards remain at the hive entrance to determine whether incoming individuals belong to the nest or are unfamiliar. Aggression is context-dependent and can be influenced by food availability: guards are less likely to attack non-nestmates when the colony has and guarding is decreased under high predation pressure [reviewed in Nouvian et al. (2016)]. The presence of a queen also affects honeybee defensive behavior: without a queen, all individuals participate in nest defense (Naeger et al., 2013). As in other invertebrates, central biogenic amines play a role in mediating aggressive behaviors: for example, octopamine decreases the activity of the stinger (Burrell and Smith, 1995). In addition, the genetic architecture of honeybee aggression has been well described (Hunt, 2007), and aggression-related changes in gene expression have been reported (Alaux et al., 2009).

CONCLUDING REMARKS

Over the past two decades, genetic models such as *Drosophila* and mice have been extremely useful for understanding not only the role of genes but the neuroarchitecture underlying aggressive behavior. A sophisticated and increasingly versatile repertoire of genetic tools has enabled the identification of specific neuronal populations involved in aggression, and manipulation of gene expression and neuronal activity specifically in those neurons to elucidate their roles in aggressive interactions. Despite a recent increase in attention to females, the vast majority of the literature has focused exclusively in male–male interactions. Although these models, especially mice, have made unparalleled contributions to our understanding of the neurobiological mechanisms underlying aggressive behavior, each model has distinct limitations that are sometimes ignored. The ethological perspective is arguably the most important aspect missing from studies in genetic models. The frequency and patterns of behaviors manifested by animals under laboratory conditions often differ greatly from those displayed in the wild or even semi-natural environments. Technological advances will undoubtedly soon allow us to overcome some of the limitations, e.g., by adapting optogenetics manipulations to freely moving animals in large spaces.

More broadly, technology will also soon allow genetic manipulations in species that had been beyond experimental reach. CRISPR is already being used in a wide range of species, many of which are potentially valuable models for the study of aggression because they exhibit patterns of behaviors absent in mice or flies, such as lethal aggression or high-intensity patterns of female intrasexual aggression. These tools will expand the repertoire of organisms amenable to experimental manipulation and could help to bring back classical non-genetic models such as crustaceans, in which fights are highly stereotyped and have long-lasting consequences. In this new era, rather than being limited to the inquiries that can be made in a few laboratory models, researchers seeking a neuroethological perspective will be empowered to select their model organisms based on the biological question of interest.

AUTHOR CONTRIBUTIONS

MP and MPF conceived and wrote the manuscript. MFS wrote the manuscript. MFS and MPF gave their approval for final submission. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants PICT-2016/0086 (Agencia de Promoción Científica y Técnica, Argentina) and UBACyT 2016/0038 (Universidad de Buenos Aires, Argentina) awarded to MP and Barnard College start-up funds supported the work of MPF.

ACKNOWLEDGMENTS

We thank Edward Kravitz, Orie Shafer, Sarah Certel, Russell Romeo, Clare Rittschof, Olga Alekseyenko, and Andres Bendesky for helpful discussions and Sarah Certel for critically reading a previous version of the manuscript. This article is dedicated to the memory of MP.

REFERENCES

Alaux, C., Sinha, S., Hasadsri, L., Hunt, G. J., Guzman-Novoa, E., DeGrandi-Hoffman, G., et al. (2009). Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15400–15405. doi: 10.1073/pnas.0907043106

Albers, H. E., Huhman, K. L., and Meisel, R. L. (2002). "6–Hormonal basis of social conflict and communication," in *Hormones, Brain and Behavior*, eds D. W. Pfaff, A. P. Arnold, S. E. Fahrbach, A. M. Etgen, and R. T. Rubin (San Diego, CA: Academic Press), 393–433. doi: 10.1016/b978-012532104-4/50008-1

Alonso, F., Honji, R. M., Guimaraes Moreira, R., and Pandolfi, M. (2012). Dominance hierarchies and social status ascent opportunity: anticipatory behavioral and physiological adjustments in a Neotropical cichlid fish. *Physiol. Behav.* 106, 612–618. doi: 10.1016/j.physbeh.2012.04.003

Anderson, D. J. (2012). Optogenetics, sex, and violence in the brain: implications for psychiatry. *Biol. Psychiatry* 71, 1081–1089. doi: 10.1016/j.biopsych.2011.11.012

Asahina, K. (2017). Neuromodulation and strategic action choice in *Drosophila* aggression. *Annu. Rev. Neurosci.* 40, 51–75. doi: 10.1146/annurev-neuro-072116-031240

Asahina, K., Watanabe, K., Duistermars, B. J., Hoopfer, E., Gonzalez, C. R., Eyjolfsdottir, E. A., et al. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. *Cell* 156, 221–235. doi: 10.1016/j.cell.2013.11.045

Bath, E., Bowden, S., Peters, C., Reddy, A., Tobias, J. A., Easton-Calabria, E., et al. (2017). Sperm and sex peptide stimulate aggression in female *Drosophila*. *Nat. Ecol. Evol.* 1:0154.

Batista, G., Zubizarreta, L., Perrone, R., and Silva, A. (2012). Non-sex-biased dominance in a sexually monomorphic electric fish: fight structure and submissive electric signalling. *Ethology* 118, 398–410. doi: 10.1111/j.1439-0310.2012.02022.x

Beeching, S. C., and Hopp, A. B. (1999). Male mate preference and size-assortative pairing in the convict cichlid. *J. Fish Biol.* 55, 1001–1008. doi: 10.1111/j.1095-8649.1999.tb00735.x

Been, L. E., Gibbons, A. B., and Meisel, R. L. (2019). Towards a neurobiology of female aggression. *Neuropharmacology* 156:107451. doi: 10.1016/j.neuropharm.2018.11.039

Bellen, H. J., Tong, C., and Tsuda, H. (2010). 100 years of *Drosophila* research and its impact on vertebrate neuroscience: a history lesson for the future. *Nat. Rev. Neurosci.* 11, 514–522. doi: 10.1038/nrn2839

Bloch, A. N., Estela, V. J., Leese, J. M., and Itzkowitz, M. (2016). Male mate preference and size-assortative mating in convict cichlids: a role for female aggression? *Behav. Processes* 130, 81–85. doi: 10.1016/j.beproc.2016.07.010

Braddock, J. C., and Braddock, Z. I. (1955). Aggressive behavior among females of the Siamese fighting fish, *Betta splendens*. *Physiol. Zool.* 28, 152–172. doi: 10.1086/physzool.28.2.30163682

Bubak, A. N., Watt, M. J., Yaeger, J. D. W., Renner, K. J., and Swallow, J. G. (2020). The stalk-eyed fly as a model for aggression–is there a conserved role for 5-HT between vertebrates and invertebrates? *J. Exp. Biol.* 223:jeb132159.

Burrell, B. D., and Smith, B. H. (1995). Modulation of the honey bee (*Apis mellifera*) sting response by octopamine. *J. Insect Physiol.* 41, 671–680. doi: 10.1016/0022-1910(95)00022-m

Cachero, S., Ostrovsky, A. D., Yu, J. Y., Dickson, B. J., and Jefferis, G. S. (2010). Sexual dimorphism in the fly brain. *Curr. Biol.* 20, 1589–1601. doi: 10.1016/j.cub.2010.07.045

Chan, Y. B., and Kravitz, E. A. (2007). Specific subgroups of FruM neurons control sexually dimorphic patterns of aggression in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19577–19582. doi: 10.1073/pnas.0709803104

Chou, M. Y., Amo, R., Kinoshita, M., Cherng, B. W., Shimazaki, H., Agetsuma, M., et al. (2016). Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. *Science* 352, 87–90. doi: 10.1126/science.aac9508

Chowdhury, B., Wang, M., Gnerer, J. P., and Dierick, H. A. (2021). The divider assay is a high-throughput pipeline for aggression analysis in *Drosophila*. *Commun. Biol.* 4:85.

Christenson, T., Wallen, K., Brown, B. A., and Glickman, S. E. (1973). Effects of castration, blindness and anosmia on social reactivity in the male Mongolian gerbil (*Meriones unguiculatus*). *Physiol. Behav.* 10, 989–994. doi: 10.1016/0031-9384(73)90177-7

Clement, T. S., Parikh, V., Schrumpf, M., and Fernald, R. D. (2005). Behavioral coping strategies in a cichlid fish: the role of social status and acute stress response in direct and displaced aggression. *Horm. Behav.* 47, 336–342. doi: 10.1016/j.yhbeh.2004.11.014

Clutton-Brock, T. (2009). Sexual selection in females. *Anim. Behav.* 77, 3–11. doi: 10.1016/j.anbehav.2008.08.026

Dankert, H., Wang, L., Hoopfer, E. D., Anderson, D. J., and Perona, P. (2009). Automated monitoring and analysis of social behavior in *Drosophila*. *Nat. Methods* 6, 297–303. doi: 10.1038/nmeth.1310

Davis, E. S., and Marler, C. A. (2004). c-fos Changes following an aggressive encounter in female California mice: a synthesis of behavior, hormone changes

and neural activity. *Neuroscience* 127, 611–624. doi: 10.1016/j.neuroscience.2004.05.034

Del Bene, F., and Wyart, C. (2012). Optogenetics: a new enlightenment age for zebrafish neurobiology. *Dev. Neurobiol.* 72, 404–414. doi: 10.1002/dneu.20914

Demas, G. E., Moffatt, C. A., Drazen, D. L., and Nelson, R. J. (1999). Castration does not inhibit aggressive behavior in adult male prairie voles (*Microtus ochrogaster*). *Physiol. Behav.* 66, 59–62. doi: 10.1016/s0031-9384(98)00268-6

Deutsch, D., Pacheco, D., Encarnacion-Rivera, L., Pereira, T., Fathy, R., Clemens, J., et al. (2020). neural basis for a persistent internal state in *Drosophila* females. *Elife* 9:e59502.

Dewsbury, D. A. (1982). Dominance rank, copulatory behavior, and differential reproduction. *Q. Rev. Biol.* 57, 135–159. doi: 10.1086/412672

Dierick, H. A., and Greenspan, R. J. (2006). Molecular analysis of flies selected for aggressive behavior. *Nat. Genet.* 38, 1023–1031. doi: 10.1038/ng1864

Dierick, H. A., and Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat. Genet.* 39, 678–682. doi: 10.1038/ng2029

Dow, M. A., and von Schilcher, F. (1975). Aggression and mating success in *Drosophila melanogaster*. *Nature* 254, 511–512. doi: 10.1038/254511a0

Dreosti, E., Lopes, G., Kampff, A. R., and Wilson, S. W. (2015). Development of social behavior in young zebrafish. *Front. Neural Circuits* 9:39. doi: 10.3389/fncir.2015.00039

Duque-Wilckens, N., and Trainor, B. C. (2017). *Behavioral Neuroendocrinology of Female Aggression*. Oxford: Oxford University Press.

Elcoro, M., Silva, S. P., and Lattal, K. A. (2008). Visual reinforcement in the female Siamese fighting fish, Betta splendens. *J. Exp. Anal. Behav.* 90, 53–60.

Erskine, M. S., Barfield, R. J., and Goldman, B. D. (1978). Intraspecific fighting during late pregnancy and lactation in rats and effects of litter removal. *Behav. Biol.* 23, 206–218. doi: 10.1016/s0091-6773(78)91814-x

Fernald, R. D. (2012). Social control of the brain. *Annu. Rev. Neurosci.* 35, 133–151.

Fernald, R. D., and Maruska, K. P. (2012). Social information changes the brain. *Proc. Natl. Acad. Sci. U.S.A.* 109(Suppl. 2), 17194–17199. doi: 10.1073/pnas.1202552109

Fernandez, M. P., Chan, Y. B., Yew, J. Y., Billeter, J. C., Dreisewerd, K., Levine, J. D., et al. (2010). Pheromonal and behavioral cues trigger male-to-female aggression in *Drosophila*. *PLoS Biol.* 8:e1000541. doi: 10.1371/journal.pbio.1000541

Ferris, C. F., Axelson, J. F., Martin, A. M., and Roberge, L. F. (1989). Vasopressin immunoreactivity in the anterior hypothalamus is altered during the establishment of dominant/subordinate relationships between hamsters. *Neuroscience* 29, 675–683. doi: 10.1016/0306-4522(89)90140-1

Filby, A. L., Paull, G. C., Hickmore, T. F., and Tyler, C. R. (2010). Unravelling the neurophysiological basis of aggression in a fish model. *BMC Genomics* 11:498. doi: 10.1186/1471-2164-11-498

Forstmeier, W., Martin, K., Bolund, E., Schielzeth, H., and Kempenaers, B. (2011). Female extrapair mating behavior can evolve via indirect selection on males. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10608–10613. doi: 10.1073/pnas.1103195108

Gagliardi-Seeley, J., Leese, J., Santangelo, N., and Itzkowitz, M. (2009). Mate choice in female convict cichlids (*Amatitlania nigrofasciata*) and the relationship between male size and dominance. *J. Ethol.* 27, 249–254. doi: 10.1007/s10164-008-0111-2

Gatewood, J. D., Wills, A., Shetty, S., Xu, J., Arnold, A. P., Burgoyne, P. S., et al. (2006). Sex chromosome complement and gonadal sex influence aggressive and parental behaviors in mice. *J. Neurosci.* 26, 2335–2342. doi: 10.1523/jneurosci.3743-05.2006

Gratacap, R. L., Wargelius, A., Edvardsen, R. B., and Houston, R. D. (2019). Potential of genome editing to improve aquaculture breeding and production. *Trends Genet.* 35, 672–684. doi: 10.1016/j.tig.2019.06.006

Greenwood, A. K., Wark, A. R., Fernald, R. D., and Hofmann, H. A. (2008). Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. *Proc. Biol. Sci.* 275, 2393–2402. doi: 10.1098/rspb.2008.0622

Gutierrez, H. C., Vacca, I., Schoenmacker, G., Cleal, M., Tochwin, A., O'Connor, B., et al. (2020). Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil. *Eur. Neuropsychopharmacol.* 30, 17–29. doi: 10.1016/j.euroneuro.2019.10.005

Hoffmann, A. A. (1987a). A laboratory study of male territoriality in the sibling species *Drosophila melanogaster* and *D.simulans*. *Anim. Behav.* 35, 807–818. doi: 10.1016/s0003-3472(87)80117-3

Hoffmann, A. A. (1987b). Territorial encounters bewteen *Drosophila* males of different sizes. *Anim. Behav.* 35, 1899–1901. doi: 10.1016/s0003-3472(87)80085-4

Hofmann, H. A., and Stevenson, P. A. (2000). Flight restores fight in crickets. *Nature* 403:613. doi: 10.1038/35001137

Hrabovszky, E., Halasz, J., Meelis, W., Kruk, M. R., Liposits, Z., and Haller, J. (2005). Neurochemical characterization of hypothalamic neurons involved in attack behavior: glutamatergic dominance and co-expression of thyrotropin-releasing hormone in a subset of glutamatergic neurons. *Neuroscience* 133, 657–666. doi: 10.1016/j.neuroscience.2005.03.042

Huber, R., Orzeszyna, M., Pokorny, N., and Kravitz, E. A. (1997a). Biogenic amines and aggression: experimental approaches in crustaceans. *Brain Behav. Evol.* 50(Suppl. 1), 60–68. doi: 10.1159/000113355

Huber, R., Smith, K., Delago, A., Isaksson, K., and Kravitz, E. A. (1997b). Serotonin and aggressive motivation in crustaceans: altering the decision to retreat. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5939–5942. doi: 10.1073/pnas.94.11.5939

Huffman, L. S., O'Connell, L. A., and Hofmann, H. A. (2013). Aromatase regulates aggression in the African cichlid fish *Astatotilapia burtoni*. *Physiol. Behav.* 112–113, 77–83. doi: 10.1016/j.physbeh.2013.02.004

Huhman, K. L. (2006). Social conflict models: can they inform us about human psychopathology? *Horm. Behav.* 50, 640–646. doi: 10.1016/j.yhbeh.2006.06.022

Hunt, G. J. (2007). Flight and fight: a comparative view of the neurophysiology and genetics of honey bee defensive behavior. *J. Insect Physiol.* 53, 399–410. doi: 10.1016/j.jinsphys.2007.01.010

Insel, T. R., Preston, S., and Winslow, J. T. (1995). Mating in the monogamous male: behavioral consequences. *Physiol. Behav.* 57, 615–627. doi: 10.1016/0031-9384(94)00362-9

Jacobs, M. E. (1978). Influence of beta-alanine on mating and territorialism in Drosophila melanogaster. *Behav. Genet.* 8, 487–502. doi: 10.1007/bf01067478

Jang, Y., Gerhardt, H. C., and Choe, J. C. (2008). A comparative study of aggressiveness in eastern North American field cricket species (genus *Gryllus*). *Behav. Ecol. Sociobiol.* 62, 1397–1407. doi: 10.1007/s00265-008-0568-6

Jasnow, A. M., Huhman, K. L., Bartness, T. J., and Demas, G. E. (2000). Short-day increases in aggression are inversely related to circulating testosterone concentrations in male Siberian hamsters (*Phodopus sungorus*). *Horm. Behav.* 38, 102–110. doi: 10.1006/hbeh.2000.1604

Kim, H., Jang, Y., and Choe, J. C. (2011). Sexually dimorphic male horns and their use in agonistic behaviors in the horn-headed cricket *Loxoblemmus doenitzi* (Orthoptera: Gryllidae). *J. Ethol.* 29, 435–441. doi: 10.1007/s10164-011-0277-x

Kravitz, E. A. (2000). Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. *J. Comp. Physiol. A* 186, 221–238. doi: 10.1007/s003590050423

Kravitz, E. A., and Fernandez, M. P. (2015). Aggression in *Drosophila*. *Behav. Neurosci.* 129, 549–563.

Kravitz, E. A., and Huber, R. (2003). Aggression in invertebrates. *Curr. Opin. Neurobiol.* 13, 736–743. doi: 10.1016/j.conb.2003.10.003

Kruk, M. R. (1991). Ethology and pharmacology of hypothalamic aggression in the rat. *Neurosci Biobehav Rev* 15, 527–538. doi: 10.1016/s0149-7634(05)80144-7

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution* 34, 292–305. doi: 10.2307/2407393

Lee, W., Hiura, L. C., Yang, E., Broekman, K. A., Ophir, A. G., and Curley, J. P. (2019). Social status in mouse social hierarchies is associated with variation in oxytocin and vasopressin 1a receptor densities. *Horm. Behav.* 114:104551. doi: 10.1016/j.yhbeh.2019.06.015

Lee, W., Khan, A., and Curley, J. P. (2017). Major urinary protein levels are associated with social status and context in mouse social hierarchies. *Proc. Biol. Sci.* 284:20171570. doi: 10.1098/rspb.2017.1570

Lee, W., Yang, E., and Curley, J. P. (2018). Foraging dynamics are associated with social status and context in mouse social hierarchies. *PeerJ* 6:e5617. doi: 10.7717/peerj.5617

Leese, J. M. (2012). Sex differences in the function of pair bonding in the monogamous convict cichlid. *Anim. Behav.* 83, 1187–1193. doi: 10.1016/j.anbehav.2012.02.009

Li, F., Lindsey, J. W., Marin, E. C., Otto, N., Dreher, M., Dempsey, G., et al. (2020). The connectome of the adult *Drosophila* mushroom body provides insights into function. *Elife* 9:e62576.

Lin, D., Boyle, M. P., Dollar, P., Lee, H., Lein, E. S., Perona, P., et al. (2011). Functional identification of an aggression locus in the mouse hypothalamus. *Nature* 470, 221–226. doi: 10.1038/nature09736

Loveland, J. L., Uy, N., Maruska, K. P., Carpenter, R. E., and Fernald, R. D. (2014). Social status differences regulate the serotonergic system of a cichlid fish, *Astatotilapia burtoni*. *J. Exp. Biol.* 217, 2680–2690.

Lucion, A. B., and de Almeida, R. M. M. (1996). On the dual nature of maternal aggression in rats. *Aggress. Behav.* 22, 365–373. doi: 10.1002/(sici)1098-2337(1996)22:5<365::aid-ab5>3.0.co;2-i

Martin, A., Serano, J. M., Jarvis, E., Bruce, H. S., Wang, J., Ray, S., et al. (2016). CRISPR/Cas9 Mutagenesis reveals versatile roles of hox genes in crustacean limb specification and evolution. *Curr. Biol.* 26, 14–26. doi: 10.1016/j.cub.2015.11.021

Maruska, K. P. (2014). Social regulation of reproduction in male cichlid fishes. *Gen. Comp. Endocrinol.* 207, 2–12. doi: 10.1016/j.ygcen.2014.04.038

Maruska, K. P., and Fernald, R. D. (2013). Social regulation of male reproductive plasticity in an African cichlid fish. *Integr. Comp. Biol.* 53, 938–950. doi: 10.1093/icb/ict017

Maruska, K. P., Zhang, A., Neboori, A., and Fernald, R. D. (2013). Social opportunity causes rapid transcriptional changes in the social behaviour network of the brain in an African cichlid fish. *J. Neuroendocrinol.* 25, 145–157. doi: 10.1111/j.1365-2826.2012.02382.x

Mendez-Sanchez, J. F., and Burggren, W. W. (2014). Environmental modulation of the onset of air breathing and survival of *Betta splendens* and *Trichopodus trichopterus*. *J. Fish Biol.* 84, 794–807. doi: 10.1111/jfb.12322

Miczek, K. A., de Almeida, R. M., Kravitz, E. A., Rissman, E. F., de Boer, S. F., and Raine, A. (2007). Neurobiology of escalated aggression and violence. *J. Neurosci.* 27, 11803–11806. doi: 10.1523/jneurosci.3500-07.2007

Morandini, L., Ramallo, M. R., Scaia, M. F., Hocht, C., Somoza, G. M., and Pandolfi, M. (2019). Dietary L-tryptophan modulates agonistic behavior and brain serotonin in male dyadic contests of a cichlid fish. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 205, 867–880. doi: 10.1007/s00359-019-01373-x

Motta, S. C., Goto, M., Gouveia, F. V., Baldo, M. V., Canteras, N. S., and Swanson, L. W. (2009). Dissecting the brain's fear system reveals the hypothalamus is critical for responding in subordinate conspecific intruders. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4870–4875. doi: 10.1073/pnas.0900939106

Muller, C. A., and Manser, M. B. (2007). 'Nasty neighbours' rather than 'dear enemies' in a social carnivore. *Proc. Biol. Sci.* 274, 959–965. doi: 10.1098/rspb.2006.0222

Murakami, S., and Itoh, M. T. (2001). Effects of aggression and wing removal on brain serotonin levels in male crickets, *Gryllus bimaculatus*. *J. Insect Physiol.* 47, 1309–1312. doi: 10.1016/s0022-1910(01)00120-2

Naeger, N. L., Peso, M., Even, N., Barron, A. B., and Robinson, G. E. (2013). Altruistic behavior by egg-laying worker honeybees. *Curr. Biol.* 23, 1574–1578. doi: 10.1016/j.cub.2013.06.045

Nelson, R. J. (2006). *Biology of Aggression*. Oxford, NY: Oxford University Press.

Nilsen, S. P., Chan, Y. B., Huber, R., and Kravitz, E. A. (2004). Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12342–12347. doi: 10.1073/pnas.0404693101

Noirot, E., Goyens, J., and Buhot, M.-C. (1975). Aggressive behavior of pregnant mice toward males. *Horm. Behav.* 6, 9–17. doi: 10.1016/0018-506x(75)90018-5

Norton, W., and Bally-Cuif, L. (2010). Adult zebrafish as a model organism for behavioural genetics. *BMC Neurosci.* 11:90.

Nouvian, M., Reinhard, J., and Giurfa, M. (2016). The defensive response of the honeybee *Apis mellifera*. *J. Exp. Biol.* 219, 3505–3517. doi: 10.1242/jeb.143016

O'Connell, L. A., Ding, J. H., and Hofmann, H. A. (2013). Sex differences and similarities in the neuroendocrine regulation of social behavior in an African cichlid fish. *Horm. Behav.* 64, 468–476. doi: 10.1016/j.yhbeh.2013.07.003

O'Connell, L. A., and Hofmann, H. A. (2011). 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

Oliveira, R. F., Silva, J. F., and Simoes, J. M. (2011). Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. *Zebrafish* 8, 73–81. doi: 10.1089/zeb.2011.0690

Palavicino-Maggio, C. B., Chan, Y.-B., McKellar, C., and Kravitz, E. A. (2019). A small number of cholinergic neurons mediate hyperaggression in female *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 116, 17029–17038. doi: 10.1073/pnas.1907042116

Pandolfi, M., Canepa, M. M., Meijide, F. J., Alonso, F., Vazquez, G. R., Maggese, M. C., et al. (2009). Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Percifomes, Cichlidae). *Biocell* 33, 1–18. doi: 10.32604/biocell.2009.33.001

Paull, G. C., Filby, A. L., Giddins, H. G., Coe, T. S., Hamilton, P. B., and Tyler, C. R. (2010). Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success. *Zebrafish* 7, 109–117. doi: 10.1089/zeb.2009.0618

Penn, J. K., Zito, M. F., and Kravitz, E. A. (2010). A single social defeat reduces aggression in a highly aggressive strain of *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12682–12686. doi: 10.1073/pnas.1007016107

Prykhozhij, S. V., Caceres, L., and Berman, J. N. (2017). New developments in crispr/cas-based functional genomics and their implications for research using zebrafish. *Curr. Gene Ther.* 17, 286–300.

Ramallo, M. R., Birba, A., Honji, R. M., Morandini, L., Moreira, R. G., Somoza, G. M., et al. (2015). A multidisciplinary study on social status and the relationship between inter-individual variation in hormone levels and agonistic behavior in a Neotropical cichlid fish. *Horm. Behav.* 69, 139–151. doi: 10.1016/j.yhbeh.2015.01.008

Ramallo, M. R., Grober, M., Canepa, M. M., Morandini, L., and Pandolfi, M. (2012). Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*. *Gen. Comp. Endocrinol.* 179, 221–231. doi: 10.1016/j.ygcen.2012.08.015

Ramallo, M. R., Morandini, L., Alonso, F., Birba, A., Tubert, C., Fiszbein, A., et al. (2014). The endocrine regulation of cichlids social and reproductive behavior through the eyes of the chanchita, *Cichlasoma dimerus* (Percomorpha; Cichlidae). *J. Physiol. Paris* 108, 194–202. doi: 10.1016/j.jphysparis.2014.08.004

Ramos, A., and Goncalves, D. (2019). Artificial selection for male winners in the Siamese fighting fish *Betta splendens* correlates with high female aggression. *Front. Zool.* 16:34.

Reddon, A. R., Balk, D., and Balshine, S. (2013). Probing aggressive motivation during territorial contests in a group-living cichlid fish. *Behav. Processes* 92, 47–51. doi: 10.1016/j.beproc.2012.10.005

Renn, S. C., Aubin-Horth, N., and Hofmann, H. A. (2008). Fish and chips: functional genomics of social plasticity in an African cichlid fish. *J. Exp. Biol.* 211, 3041–3056. doi: 10.1242/jeb.018242

Renn, S. C., Carleton, J. B., Magee, H., Nguyen, M. L., and Tanner, A. C. (2009). Maternal care and altered social phenotype in a recently collected stock of *Astatotilapia burtoni* cichlid fish. *Integr. Comp. Biol.* 49, 660–673. doi: 10.1093/icb/icp085

Renn, S. C., Fraser, E. J., Aubin-Horth, N., Trainor, B. C., and Hofmann, H. A. (2012). Females of an African cichlid fish display male-typical social dominance behavior and elevated androgens in the absence of males. *Horm. Behav.* 61, 496–503. doi: 10.1016/j.yhbeh.2012.01.006

Renn, S. C., O'Rourke, C. F., Aubin-Horth, N., Fraser, E. J., and Hofmann, H. A. (2016). Dissecting the transcriptional patterns of social dominance across teleosts. *Integr. Comp. Biol.* 56, 1250–1265. doi: 10.1093/icb/icw118

Resendez, S. L., and Aragona, B. J. (2013). Aversive motivation and the maintenance of monogamous pair bonding. *Rev. Neurosci.* 24, 51–60.

Romeo, R. D., Schulz, K. M., Nelson, A. L., Menard, T. A., and Sisk, C. L. (2003). Testosterone, puberty, and the pattern of male aggression in Syrian hamsters. *Dev. Psychobiol.* 43, 102–108. doi: 10.1002/dev.10125

Rosvall, K. A., Burns, C. M. B., Barske, J., Goodson, J. L., Schlinger, B. A., Sengelaub, D. R., et al. (2012). Neural sensitivity to sex steroids predicts individual differences in aggression: implications for behavioural evolution. *Proc. R. Soc. B Biol. Sci.* 279, 3547–3555. doi: 10.1098/rspb.2012.0442

Sakaluk, S. K. (1987). Reproductive behaviour of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae): calling schedules, spatial distribution, and mating. *Behaviour* 100, 202–225. doi: 10.1163/156853987x00134

Sbragaglia, V., Gliese, C., Bierbach, D., Honsey, A. E., Uusi-Heikkila, S., and Arlinghaus, R. (2019). Size-selective harvesting fosters adaptations in mating

behaviour and reproductive allocation, affecting sexual selection in fish. *J. Anim. Ecol.* 88, 1343–1354. doi: 10.1111/1365-2656.13032

Scaia, M. F., Cavallino, L., and Pandolfi, M. (2020). Social control of spermatogenesis and steroidogenesis in cichlid fish: a comparative approach. *Reproduction* 159, R31–R43.

Scaia, M. F., Morandini, L., Noguera, C., Trudeau, V. L., Somoza, G. M., and Pandolfi, M. (2018a). Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. *Physiol. Behav.* 194, 481–490. doi: 10.1016/j.physbeh.2018.06.028

Scaia, M. F., Morandini, L., Noguera, C. A., Ramallo, M. R., Somoza, G. M., and Pandolfi, M. (2018b). Fighting cichlids: dynamic of intrasexual aggression in dyadic agonistic encounters. *Behav. Processes* 147, 61–69. doi: 10.1016/j.beproc.2017.12.015

Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S. Y., Hayworth, K. J., et al. (2020). A connectome and analysis of the adult *Drosophila* central brain. *Elife* 9:e57443.

Schretter, C. E., Aso, Y., Robie, A. A., Dreher, M., Dolan, M. J., Chen, N., et al. (2020). Cell types and neuronal circuitry underlying female aggression in *Drosophila*. *Elife* 9:e58942.

So, N., Franks, B., Lim, S., and Curley, J. P. (2015). A social network approach reveals associations between mouse social dominance and brain gene expression. *PLoS One* 10:e0134509. doi: 10.1371/journal.pone.0134509

Spence, R., Gerlach, G., Lawrence, C., and Smith, C. (2008). The behaviour and ecology of the zebrafish, *Danio rerio*. *Biol. Rev. Camb. Philos. Soc.* 83, 13–34. doi: 10.1111/j.1469-185x.2007.00030.x

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

Stockley, P., and Campbell, A. (2013). Female competition and aggression: interdisciplinary perspectives. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368:20130073. doi: 10.1098/rstb.2013.0073

Sturtevant, A. H. (1915). Experiments on sex recognition and the problem of sexual selection in *Drosophila*. *J. Anim. Behav.* 5, 351–366. doi: 10.1037/h0074109

Takahashi, A., and Miczek, K. A. (2014). Neurogenetics of aggressive behavior: studies in rodents. *Curr. Top. Behav. Neurosci.* 17, 3–44. doi: 10.1007/7854_2013_263

Taves, M. D., Desjardins, J. K., Mishra, S., and Balshine, S. (2009). Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). *Gen. Comp. Endocrinol.* 161, 202–207. doi: 10.1016/j.ygcen.2008.12.018

Teles, M. C., and Oliveira, R. F. (2016). Quantifying aggressive behavior in zebrafish. *Methods Mol. Biol.* 1451, 293–305. doi: 10.1007/978-1-4939-3771-4_20

Terranova, J. I., Song, Z., Larkin, T. E. II, Hardcastle, N., Norvelle, A., Riaz, A., et al. (2016). Serotonin and arginine-vasopressin mediate sex differences in the regulation of dominance and aggression by the social brain. *Proc. Natl. Acad. Sci. U.S.A.* 113, 13233–13238. doi: 10.1073/pnas.1610446113

Tobias, J. A., Montgomerie, R., and Lyon, B. E. (2012). The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 2274–2293. doi: 10.1098/rstb.2011.0280

Todd, W. D., Fenselau, H., Wang, J. L., Zhang, R., Machado, N. L., Venner, A., et al. (2018). A hypothalamic circuit for the circadian control of aggression. *Nat. Neurosci.* 21, 717–724. doi: 10.1038/s41593-018-0126-0

Trannoy, S., Chowdhury, B., and Kravitz, E. A. (2015). Handling alters aggression and "loser" effect formation in *Drosophila melanogaster*. *Learn. Mem.* 22, 64–68. doi: 10.1101/lm.036418.114

Trannoy, S., Penn, J., Lucey, K., Popovic, D., and Kravitz, E. A. (2016). Short and long-lasting behavioral consequences of agonistic encounters between male *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4818–4823. doi: 10.1073/pnas.1520953113

Tubert, C., Lo Nostro, F., Villafane, V., and Pandolfi, M. (2012). Aggressive behavior and reproductive physiology in females of the social cichlid fish *Cichlasoma dimerus*. *Physiol. Behav.* 106, 193–200. doi: 10.1016/j.physbeh.2012.02.002

Ueda, A., and Kidokoro, Y. (2002). Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. *Physiol. Entomol.* 27, 21–28. doi: 10.1046/j.1365-3032.2002.00262.x

Venken, K. J., and Bellen, H. J. (2007). Transgenesis upgrades for *Drosophila melanogaster*. *Development* 134, 3571–3584. doi: 10.1242/dev.005686

Vrontou, E., Nilsen, S. P., Demir, E., Kravitz, E. A., and Dickson, B. J. (2006). fruitless regulates aggression and dominance in *Drosophila*. *Nat. Neurosci.* 9, 1469–1471. doi: 10.1038/nn1809

Wang, L., and Anderson, D. J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in *Drosophila*. *Nature* 463, 227–231. doi: 10.1038/nature08678

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J. C., Miyamoto, T., et al. (2011). Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*. *Nat. Neurosci.* 14, 757–762. doi: 10.1038/nn.2800

Wang, Z., Hulihan, T. J., and Insel, T. R. (1997). Sexual and social experience is associated with different patterns of behavior and neural activation in male prairie voles. *Brain Res.* 767, 321–332. doi: 10.1016/s0006-8993(97)00617-3

Warren, W. C., Boggs, T. E., Borowsky, R., Carlson, B. M., Ferrufino, E., Gross, J. B., et al. (2021). A chromosome-level genome of Astyanax mexicanus surface fish for comparing population-specific genetic differences contributing to trait evolution. *Nat. Commun.* 12:1447.

Weidt, A., Gygax, L., Palme, R., Touma, C., and Konig, B. (2018). Impact of male presence on female sociality and stress endocrinology in wild house mice (*Mus musculus* domesticus). *Physiol. Behav.* 189, 1–9. doi: 10.1016/j.physbeh.2018.02.039

Williamson, C. M., Lee, W., and Curley, J. P. (2016). Temporal dynamics of social hierarchy formation and maintenance in male mice. *Anim. Behav.* 115, 259–272. doi: 10.1016/j.anbehav.2016.03.004

Williamson, C. M., Lee, W., DeCasien, A. R., Lanham, A., Romeo, R. D., and Curley, J. P. (2019). Social hierarchy position in female mice is associated with plasma corticosterone levels and hypothalamic gene expression. *Sci. Rep.* 9:7324.

Williamson, C. M., Romeo, R. D., and Curley, J. P. (2017). Dynamic changes in social dominance and mPOA GnRH expression in male mice following social opportunity. *Horm. Behav.* 87, 80–88. doi: 10.1016/j.yhbeh.2016.11.001

Wise, D. A. (1974). Aggression in the female golden hamster: effects of reproductive state and social isolation. *Horm. Behav.* 5, 235–250. doi: 10.1016/0018-506x(74)90032-4

Wisenden, B. D. (1994). Factors affecting mate desertion by males in free-ranging convict cichlids (*Cichlasoma nigrofasciatum*). *Behav. Ecol.* 5, 439–447. doi: 10.1093/beheco/5.4.439

Xu, S., Pham, T. P., and Neupane, S. (2020). Delivery methods for CRISPR/Cas9 gene editing in crustaceans. *Mar. Life Sci. Technol.* 2, 1–5. doi: 10.1007/s42995-019-00011-4

Yasukawa, N. J., Monder, H., Leff, F. R., and Christian, J. J. (1985). Role of female behavior in controlling population growth in mice. *Aggress. Behav.* 11, 49–64. doi: 10.1002/1098-2337(1985)11:1<49::aid-ab2480110107>3.0.co;2-a

Yew, J. Y., Dreisewerd, K., Luftmann, H., Muthing, J., Pohlentz, G., and Kravitz, E. A. (2009). A new male sex pheromone and novel cuticular cues for chemical communication in *Drosophila*. *Curr. Biol.* 19, 1245–1254. doi: 10.1016/j.cub.2009.06.037

Young, L. J., and Wang, Z. (2004). The neurobiology of pair bonding. *Nat. Neurosci.* 7, 1048–1054. doi: 10.1038/nn1327

Yurkovic, A., Wang, O., Basu, A. C., and Kravitz, E. A. (2006). Learning and memory associated with aggression in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17519–17524. doi: 10.1073/pnas.0608211103

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership