INVERTEBRATE MODELS OF NATURAL AND DRUG-SENSITIVE REWARD

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INVERTEBRATE MODELS OF NATURAL AND DRUG-SENSITIVE REWARD

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Drosophila melanogaster self-administering drug.
Image: Craig Bell and Brad Phalin.

The rewarding properties of drugs depend on their capacity to activate appetitive motivational states. Because the mechanisms underlying natural reward are an important life-sustaining process and strongly conserved throughout metazoan evolution, invertebrate models provide a powerful complement to the mammalian systems traditionally used in addiction research. A wide range of organizational complexity, combined with genetically manipulable, and relatively simple, accessible nervous systems, make invertebrates excellent models in which to explore general addiction principles. These include the role of natural reward systems in learning, the basic biological mechanisms of drug addiction, and the long-term effects of early drug exposure. The contributions to this e-book illustrate the current state of invertebrate addiction research. The chapters show that the reward circuits of invertebrate taxa

are surprisingly sensitive to human drugs of abuse. Employing learning paradigms typically used in vertebrate studies (viz., conditioned place preference and operant, self-administration paradigms), invertebrates are shown to exhibit aspects of the addiction cycle from activational effects of common psychostimulants, sensitization with repeated application, to extinction, withdrawal, and reinstatement. This highlights the value of the comparative approach for both exploring conserved mechanisms underlying drug addiction and the utility of invertebrate models in seeking potential solutions.

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SECTION 4

ALCOHOL USE DISORDER

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EDITORIAL
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Editorial: Invertebrate Models of Natural and Drug-Sensitive Reward

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Editorial on the Research Topic

Invertebrate Models of Natural and Drug-Sensitive Reward

The relationship between insects and humans is a complex one, characterized biotically as commensalism, mutualism, or parasitism. This collection of papers reveals yet another dimension, in which shared history invests invertebrate models with the power to interrogate critical challenges to the human reward system. As humans we take pride in approaching a given scenario rationally, in considering the possible options, assigning them values, and then choosing that which maximizes one's individual outcomes. So, why do drug addicts make choices that inevitably lead to ruinous consequences? Fundamentally, addiction appears to impair the very ability to form considered judgments, as it strips the afflicted of this most essential of human faculties. What are *Homo sapiens* bereft of their "sapient" power? Despite widespread recognition of the devastating and lasting effects of addiction, there is little consensus regarding its mechanistic and perceptual causes, or on effective therapeutic interventions. Policy makers, healthcare specialists, and the general public, frequently view drug dependence as an incompetent life choice, or moral failure resulting from a fundamental lack of willpower. However, the moralistic perspective falls short in generating effective solutions. It provides little help to the addicted, offers no support to those in the addict's immediate social circle, and gives no guidance in addressing the significant and growing societal burden posed by substance abuse (Florence et al., 2016). In contrast, the US National Institute of Drug Abuse has strongly advocated for a brain disease model of addiction (BDMA), and empirical findings in behavioral neuroscience have advanced promising avenues for reframing this phenomenon from a more structural perspective (Volkow and Koob, 2015). Viewed as a chronic, relapsing brain disorder, addiction is characterized as a dysregulation of reward, motivation, judgment and memory, with associated changes in neuronal structure and function, and where medical technologies offer the clearest path to treatment. A third, more holistic perspective, dissents rather forcefully from the medical cure inherent in the BDMA approach (Lewis, 2015). In this view, addiction results from the development of all-consuming patterns of conduct in which initially formed mental and behavioral habits become self-perpetuating. Liberation from the yoke of addiction thus will come from engraving new beneficial patterns over the existing harmful ones, and will depend crucially on a conducive social environment. Regardless of specific distinctions, all of these explanatory models share the fundamental assumption that addiction resides squarely within the cognitive domain.

A unifying view emerging from this compilation of drug-sensitive reward in invertebrates suggests that we may do well to critically evaluate fundamental notions about addiction vulnerabilities and the causes of compulsive drug taking. First, we ought to recognize addiction as a phenomenon with exceedingly deep evolutionary roots. Though commonly referred to as "human drugs of abuse," addictive plant alkaloids emerged as potent chemical defenses against insect herbivory (Wink). The Achilles heel of animals then, resides in the tradeoffs required for efficient learning–balancing specificity with generalization, and computational speed with

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behavioral flexibility. Shaped by an evolutionary arms race, compounds such as nicotine, cathinone, and morphine, evolved to interfere with essential functions of learning in their insect pests. Acting as weaponized disruptors of conserved learning machinery, they commandeer neural drivers for motivated search and dysregulate the circuits for reward perception. The very nature of this interference limits the evolution of effective countermeasures. For instance, mutations lessening an individual's sensitivity to these defenses inevitably incur significant side effects of reduced initiative, lowered reward perceptions, and critically impaired decision-making. Within this broader context, addicted humans represent collateral damage arising from the homology of ancestral learning mechanisms tracing back to the early divergence of bilateral metazoa. Aside from fetal drug exposure or postoperative analgesics, initial drug consumption is generally a choice and not an evolutionary imperative. However, once addictive alkaloids have compromised the basic learning circuitry, higher-order executive functions (including cognition and willpower) have limited authority against them.

Second, we should acknowledge that these deep evolutionary roots imbue invertebrate models with unique power to reveal the origin and development of reward system function. The papers gathered in this volume demonstrate the rich spectrum of behavioral and neural consequences exhibited by invertebrate preparations in response to drug exposure. These addiction-like phenomena parallel the full range of effects identified in mammalian models ranging from activational responses associated with common psychostimulants, sensitization on repeated application, extinction, withdrawal, and reinstatement. Drug self-administration, considered the most valid experimental model for drug-seeking and -taking behaviors, and the final step in preclinical testing of potential treatments, is observed in roundworms self-exposure to cocaine, nicotine and methamphetamine (Engleman et al.), and in crayfish self-administering amphetamine (Datta et al.). Invertebrate models also exhibit strong tendencies for relapse after extended periods of abstinence. Søvik et al. employ a proboscis extension reflex learning paradigm to explore whether cocaine affects memory processing independently of its effect on incentive salience in honey bees. Their finding that cocaine strongly impairs consolidation of extinction memory is key to understanding how cocaine exerts enduring impacts on behavior.

We underscore the benefits of broad taxonomic inclusion for illuminating aspects of addiction that are of clinical importance, but for which few suitable animal models exist. For instance, Lee et al. describe spontaneous amphetamine-induced escape swims in a sea slug, *Tritonia diomedea*, triggered by false perceptions of predator contact (i.e., hallucinations). Unconditioned exposure to mammalian drugs of abuse generate a variety of stereotyped behaviors in crayfish, stimulating exploration and appetitive motor patterns along with molecular processes for drug conditioned reward in novel contexts (Shipley et al.). Step-wise alteration of the phenotype in taxa with incomplete metamorphosis permits longitudinal analysis of behavioral states with a degree of precision that is otherwise difficult to achieve.

Third, since natural reward is a life-sustaining process central to learning, comparison with drug-sensitive reward can identify the genetic and neural mechanisms underlying appetitive reward, and most urgently, the factors contributing to addiction vulnerabilities. What variation promotes the progression from initial drug use to addiction? Important insights come from contributions in *Drosophila*, a model system with powerful genetic tools and a vibrant research community. With an emphasis on how motivational states shape the value of the rewarding experience, Ryvkin et al. modeled different aspects of natural and drug rewards. They conclude that it is social isolation, pain, deprivation and stress that shape the repertoire/function of proteins and mediate reward processing. Lowenstein and Velazquez-Ulloa establish the functional modularity of reward circuits in *Drosophila* resembles that of mammals.

Aminergic systems in associative learning transmit prediction error signals and convey stimulus prediction signals for the execution of conditioned responses. Mizunami and Matsumoto review work in crickets, honey bees, and fruit-flies, demonstrating the conserved nature of reward systems in insects and mammals, along with diversity in the neurotransmitters mediating appetitive signaling. Elucidating the computational rules underlying such activity at the molecular level requires a combination of functional and behavioral analyses. Lanzo et al. used cell-specific knockdown *in vivo*, and cultured neurons *in vitro*, to demonstrate that the dopamine transporter in the cell membrane of *C. elegans* functions primarily to reuptake dopamine from the synaptic cleft back into the dopaminergic neurons, an activity altered by drugs of abuse such as amphetamine.

A major challenge to the discovery of effective addiction therapies is our limited understanding of underlying mechanisms and of potential therapeutic targets. One promising approach aims to assess comparative reward strength of individual drugs using operant, self-administration or conditioned place preference paradigms, with the high-throughputs made possible by invertebrates. Engleman et al. propose just such a system, presenting data for *C. elegans* with predictive validity as a behavioral medication screen.

Alcohol Use Disorder is a major health, social and economic problem with few effective treatments. Four studies illustrate invertebrate contributions at levels from molecular to complex memory phenotypes. *Drosophila* develop a preference for ethanol, an effect that is reversed by the opiate antagonist naltrexone (Koyyada et al.). Guevara et al. examined developmental alcohol exposure on feeding behavior in *Drosophila*, where NPF signaling plays a critical role in alcohol-reduced food intake for both larvae and adults. Neural and molecular mechanisms underlying persistent memories of intoxication induce cravings and trigger relapse in recovering individuals. The work of Nunez et al. characterizes the dose-dependent nature of ethanol on the memory expression of intoxication experience, highlighting the advantage of sophisticated behavioral analysis. Fruit flies possess both acute and persistent memories for ethanol-conditioned odor cues, and the state of intoxication influences the retention and expression of associated

memories. Finally, Swierzbinski and Heberholz combine electrophysiological and neuropharmacological techniques to exploit the occurrence of two escape mechanisms in crayfish. Their findings suggest intriguing effects of alcohol on the GABAergic system.

The invertebrate taxa reported on in this volume trace the origins of addiction-like phenomena to at least 950 MYA (Dohrmann and Wörheide, 2017). Placozoa occupy a basal position in the metazoan phylogeny and lack a nervous system. The recent demonstration that they coordinate sophisticated behavioral sequences, such as feeding, using an intercellular peptidergic signaling system (Varoqueaux et al., 2018) is the clearest indication yet that the revelatory power of invertebrate models for understanding the intricacies of natural- and drug-sensitive reward has only just begun.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Plant Secondary Metabolites Modulate Insect Behavior-Steps Toward Addiction?

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Plants produce a diversity of secondary metabolites (PSMs) that serve as defense compounds against herbivores and microorganisms. In addition, some PSMs attract animals for pollination and seed dispersal. In case of pollinating insects, PSMs with colors or terpenoids with fragrant odors attract pollinators in the first place, but when they arrive at a flower, they are rewarded with nectar, so that the pollinators do not feed on flowers. In order to be effective as defense chemicals, PSMs evolved as bioactive substances, that can interfere with a large number of molecular targets in cells, tissues and organs of animals or of microbes. The known functions of PSMs are summarized in this review. A number of PSMs evolved as agonists or antagonists of neuronal signal transduction. Many of these PSMs are alkaloids. Several of them share structural similarities to neurotransmitters. Evidence for neuroactive and psychoactive PSMs in animals will be reviewed. Some of the neuroactive PSMs can cause addiction in humans and other vertrebrates. Why should a defense compound be addictive and thus attract more herbivores? Some insects are food specialists that can feed on plants that are normally toxic to other herbivores. These specialists can tolerate the toxins and many are stored in the insect body as acquired defense chemicals against predators. A special case are pyrrolizidine alkaloids (PAs) that are neurotoxic and mutagenic in vertebrates. PAs are actively sequestered by moths of the family Arctiidae and a few other groups of arthropods. In arctiids, PAs are not only used for defense, but also serve as morphogens for the induction of male coremata and as precursors for male pheromones. Caterpillars even feed on filter paper impregnated with pure PAs (that modulate serotonin receptors in vertebrates and maybe even in insects) and thus show of behavior with has similarities to addiction in vertebrates. Not only PA specialists, but also many monophagous herbivores select their host plants according to chemical cues i.e., PSMs) and crave for plants with a particular PSMs, again a similarity to addiction in vertebrates.

Keywords: plant secondary metabolites, pharmacology, toxicology, plant-insect interactions, neurotoxicity, psychoactive natural products

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EVOLUTION AND FUNCTION OF PLANT SECONDARY METABOLITES[1]

Since the early days of plant evolution in the Devonian period, plants had to cope with herbivores, but also with bacteria, fungi and viruses around them. Plants cannot run away when attacked by an herbivore, nor do they possess an adaptive immune system as present in vertebrates against microbial infections (Wink, 1988, 2003).

Similar to the situation of other immobile or slow-moving organisms (amphibians, slugs, cnidarians, and sponges) plants invested into the production of a wide diversity of organic compounds, the so-called secondary metabolites (PSMs). The structures of PSMs underwent several rounds of selection; thus their structures were shaped in such a way that they could interfere with the metabolism, neuronal transmission or reproduction of an herbivore or microbe. In consequence, nearly all PSMs exhibit some sort of biological activity and PSMs support plants to ward off herbivores and microbial infections (Wink, 1988, 2003). Plants also employ other strategies in this context, such as an impenetrable bark and cuticles, thorns, spikes and stinging hairs; furthermore, plants possess the capacity of open growth. Thus, they can renew parts that had been damaged by an herbivore.

Plants produce a substantial structural diversity of PSMs, such as alkaloids, amines, cyanogenic glucosides, glucosinolates, non-protein amino acids, organic acids, terpenoids, phenolics, quinones, polyacetylenes, and peptides. Over 100,000 individual structures have been elucidated already (Wink, 1988, 2003). Plants do not produce a single compound for defense, but usually a complex mixture of PSMs from different structural classes that can attack multiple molecular targets at the same time and often in a synergistic fashion (Wink, 2008, 2015; Mason and Singer, 2015). The composition of these mixtures is not fixed, but varies in terms of both concentration and composition. Thus, mixtures differ between organs, developmental stages and within populations. We had previously suggested that this variation is an important strategy to avoid the adaptation and resistance of herbivores and pathogens against the chemical defense. It is widely known from medicine, that treatment of bacteria or viruses with a single drug will give raise to resistant strains in a rather short time (e.g., antibiotic resistance).

PSMs evolved as an important line of defense, but some of them are further used for other purposes. Flowering plants often employ insects as pollinators, and also a few other arthropods and vertebrates. These pollinators are attracted to flowers by their color or smell; color is usually due to the production of flavonoids, anthocyanins, or carotenoids, whereas terpenoids, amines and phenylpropanoids exhibit distinctive odors that are recognized by pollinators (not necessarily by all animals). However, pollinators should be attracted to flowers but should not eat them. Thus, the attractant PSMs and other compounds are toxic and deterrent for a pollinator that tries to feed on flowers. Instead, flowers produce sugar-rich nectar as a reward for pollinating animals that they normally prefer over other flowering material (Wink, 1988, 2003; Detzel and Wink, 1993). Plants try to disperse their seeds beyond the direct neighborhood of the producing mother plants. Also in this context, animals are being manipulated as fruit–and seed dispersers. Mature fruits are usually sweet and show attractive colouration and smell. Fruit-eating animals (frugivores) are adapted to eat ripe fruits; but they do not destroy the seeds, that pass the intestinal tract without harm. Furthermore, as frugivores will deposit their faces far away from the fruiting tree, the seeds become dispersed and furthermore they are dropped together with potential fertilizers. Some PSMs also serve the producing plants directly as antioxidants, nitrogen storage compounds or for UV protection. Thus, most PSMs have multiple functions for a plant producing them (Wink, 1988, 2003).

A special case is the production of PSMs that interfere with the nervous system in animals. In vertebrates, several small-molecule neurotransmitters are known that modulate the activity of neuroreceptors (Wink, 2000). Among the most important neurotransmitters are acetylcholine, GABA, serotonin, dopamine, adrenaline, noradrenaline, adenosine, histamine, glutamate, and endorphins. Some of the PSMs that mimic the structure of neurotransmitters are CNS stimulants, others psychedelic and hallucinogenic (especially those binding to serotonin and dopamine receptors). Because herbivores that feed on psychoactive PSMs, often become addicted to the drugs, such compounds appear to be counterproductive, as they will attract herbivores. However, in the wild, the survival of an intoxicated herbivore is probably quite short. It will either fall from trees and rocks or will be an easy prey for the predators which are abundant in most ecosystems.

PHARMACOLOGY AND TOXICOLOGY OF PLANT SECONDARY METABOLITES

Among alkaloids, several modulate neuronal signal transduction and are thus often toxic for herbivores. Ion channels, neurotransmitter receptors, neurotransmitter inactivating enzymes and transporters play an important role. Examples for alkaloids, known to interfere with these targets (mostly in vertebrates) are documented in **Table 1.**

When PSMs modulate elements of neuronal signal transduction, the concentrations of neurotransmitters, the activity of neurotransmitter receptors or their expression can be changed. This can lead to severe changes in physiology and often in the behavior of an animal. Addiction can be one of them.

Many PSMs can modulate the bioactivity and/or 3D structure of proteins. Among them are some specific inhibitors (such as colchicine, inhibiting microtubule assembly). The majority of the widely distributed phenolic compounds can modulate the 3D structure of proteins by forming multiple hydrogen and ionic bonds with them (**Table 2**; Wink, 2008, 2015). In addition, some

[1]In this review, I often cite results from the research of myself or my co-workers. I am aware that many other scientists have also worked in this field and published thousands of scholarly papers, which could have been cited instead (apology to all colleagues, whom I did not cite). The review does not cover the complete literature that exists on this topic. If complete, the review would have been very long and outside the scope of the journal. Therefore, this invited article presents my personal and certainly limited view.

TABLE 1 | Examples for alkaloids and other PSMs that modulate neuronal signal transduction (more details in Wink, 2000; Wink and Schimmer, 2010).

Target	Alkaloids	Activity
ION CHANNELS		
	Aconitine	Agonist
	Ajmaline	Antagonist
	Berbamine	Antagonist
	Capsaicin	antagonist
	Cocaine	Antagonist
	Dicentrine	Antagonist
	Ervatamine	Antagonist
	Glaucine	Antagonist
	Hirsutine	Antagonist
	Liriodenine	Antagonist
	Paspaline	Antagonist
	Phalloidin	antagonist
	Quinidine	Antagonist
	Ryanodine	Agonist
	Sparteine	Antagonist
	Strychnine	Antagonist
	Veratrine	Agonist
	Vincamine	Antagonist
	Zygademine	Agonist
NEUROTRANSMITTER RECEPTORS		
Nicotinic acetylcholine receptor (nAChR)		
	Anabasine	Agonist
	Boldine	Antagonist
	Coniine	Agonist
	Cytisine, lupanine	Agonist
	Erythrina alkaloids	Antagonist
	Methyllycaconitine	Antagonist
	Nicotine	Agonist
	Tubocurarine	Antagonist
Muscarinic acetylcholine receptor (mAChR) (GPCR)		
	Arecoline	Agonist
	Cryptolepine	Agonist
	Ebeinone	Antagonist
	Himbacine	Antagonist
	Hyoscyamine, scopolamine	Antagonist
	Muscarine	Agonist
	Pilocarpine	Agonist
	Sparteine	agonist
Dopamine receptor (GPCR)		
	Agroclavine	Agonist
	Bulbocapnine	Antagonist
	Epinine	Agonist
	Ergot alkaloids	Agonist
	Loline	Agonist
	Salsolinol	Agonist
	Tyramine	Antagonist
Serotonin receptor (mostly GPCR)		
	Akuammine	Antagonist
	Bufotenine	Agonist
	Confusameline	antagonist
	Ergot alkaloids	Antagonist
	Harman and other harmine alkaloids	Agonist
	Liridinine	Antagonist
	Mescaline	Agonist
	Mitragynine	Agonist
	N-Methyltryptamine	Agonist
	Psilocine	Agonist
Adenosine receptor (GPCR)		
	Caffeine, theobromine, theophylline	Antagonist
GABA receptor (mostly ICR)		
	Bicuculline	Antagonist
	Corlumine	Antagonist
	Hydrastine	Antagonist
	Muscimol	Agonist
	Securinine	Antagonist
Glutamate receptor (mostly ICR)		
	Acromelic acid	Agonist
	Domoic acid	Agonist
	Ibogaine	Antagonist
	Ibotenic acid	Agonist
	Kainic acid	Agonist
	Nuciferine	Antagonist
	Quisqualic acid	Agonist
	Willardiine	Agonist
Noradrenaline receptor (GPCR)		
	Ajmalicine	Antagonist
	Berbamine	Antagonist
	Berberine	Antagonist
	Boldine	Antagonist
	Bulbocapnine	Antagonist
	Corynanthine	Antagonist
	Crebanine	Antagonist
	Dispegatrine	Antagonist
	Ergot alkaloids	Agonist / antagonists
	Glaucine	Antagonist
	Octopamine	Agonist
	Predicentrine	Antagonist

(Continued)

TABLE 1 | Continued

Target	Alkaloids	Activity
	Yohimbine	Antagonist
Endorphine receptor (GPCR)		
	Akuammine	Agonist
	Ibogaine	Agonist
	Mitragynine	Agonist
	Morphine	Agonist
INACTIVATION OF NEUROTRANSMITTERS		
Acetylcholine esterase		
	Berberine	Antagonist
	Galantamine	Antagonist
	Harmaline	Antagonist
	Huperzine	Antagonist
	Physostigmine	Antagonist
	Sanguinarine	Antagonist
	Solanum alkaloids	Antagonist
	Vasicinol	Antagonist
Monoamine oxidase (MAO)		
	Alstovenine	Antagonist
	Carnegine	Antagonist
	N,N-dimethyltryptamine	Antagonist
	Harmaline and other harman alkaloids	Antagonist
	Saracodine	Antagonist
	Salsolidine	Antagonist
TRANSPORTER FOR NEUROTRANSMITTERS		
	Annonaine	Inhibition of DA reuptake
	Arecaidine	Inhibition of GABA reuptake
	Cocaine	Inhibition of DA uptake
	Ephedrine	Release of NA from synaptic vesicles; inhibition of NA reuptake
	Ibogaine	Modulates DA, NA and 5-HT transporters in synaptic vesicles
	Norharman	Inhibitor of DA and tryptamine uptake
	Reserpine	Depletes stores of NA and 5-HT in synaptic vesicles
	Salsolinol	Inhibitor for uptake of biogenic amine neurotransmitters
	Veratramine	Releaser and uptake inhibitor for 5-HT
ION PUMPS		
Na^+/K^+-ATPase		
	Ouabain and other cardiac glycosides	Inhibitor

In case of neurotransmitter receptors, some are ion channel coupled receptors (nAChR, 5-HT3, NMDA, AMPA, kainate, GABAA) (=ICR), the other metabotropic receptors coupled with G-protein (GPCR)

TABLE 2 | PSMs interfering with proteins (more details in Wink, 2008, 2015; Wink and Schimmer, 2010).

Activity	PSMs	Examples	Comments
COVALENT BONDS WITH PROTEINS			
With SH-groups	With SH groups	Allicin and similar PSMs	
With amino groups	With epoxy groups		
	With exocyclic methylene groups	Sesquiterpene lactones	
Non-covalent bonding with proteins	With amino groups	Phenolics, polyphenols	Hydrogen and ionic bonds
	With hydroxyl groups	Phenolics, polyphenols	Hydrogen and ionic bonds
	With lipophilic sites	Lipophilic terpenes	Hydrophobic attraction

PSMs possess chemically reactive functional groups by which they can form covalent bonds with amino, sulfhydryl or hydroxyl groups of amino acid residues of proteins (**Table 2**). Lipophilic terpenes can assemble in the inner hydrophobic core of globular proteins that thus can change their 3D structures.

A special case of protein inhibitors are those which can interfere with protein synthesis in ribosomes, such as lectins (e.g., ricine, abrine), emetine, and lycorine (Wink and Schimmer, 2010; Wink, 2015).

Biomembranes that surround all living cells and intracellular compartments, are the target for lipophilic PSMs (**Table 3**). They can be trapped inside the biomembrane and thus change its fluidity and permeability. Typical lipophilic PSMs include mono-, sesqui-, di-, and triterpenes, steroids, mustards oils, and phenylpropanoids. A special case are saponins that consist of a lipophilic steroid or triterpene moiety and a hydrophilic sugar chain. These compounds are amphiphilic and can lyse biomembrane by complexing membrane cholesterol (**Table 3**). Also antimicrobial peptides (AMPs) that are part of the innate immune system of most organisms, interfere with biomembranes of microbes but also of eukaryotic cells.

Several PSMs can interfere with nucleic acid and enzymes that metabolize them (Wink and Schimmer, 2010). We can distinguish between DNA intercalating and DNA alkylating compounds (**Table 4**). Lipophilic, aromatic and planar PSMs (such as furanocoumarins, berberine, sanguinarine) can intercalate between the stacks of DNA-base pairs. Intercalators stabilize DNA and can prevent the activity of helicases and RNA polymerases; they can be mutagenic (because of frame shift), genotoxic, and cytotoxic (**Table 4**). Alkylating agents directly bind to nucleotide bases and form covalent bonds. They also lead to mutations and genotoxicity (**Table 4**).

PLANT–INSECT INTERACTIONS

Among all multicellular living organisms, plants and insects exhibit the largest diversity with more than 1 million described

arthropod taxa (mostly insects) and more than 350,000 plant taxa. Amongst eukaryotes, the diversity of plants and metazoans pales in comparison to the diversity amongst fungal taxa (albeit non-described so far; Yahr et al., 2016). Although flowering plants (angiosperms) evolved already during the Cretaceous, an extensive radiation took place at the start of the Tertiary, 66 million years ago. Evidence suggests that parallel to the angiosperm radiation, a radiation of insects set in as well. If both radiations were true co-evolutionary processes is an open debate. Many insects are pollinators, others are herbivorous. Among the herbivores, we can distinguish polyphagous species that feed on many plant species, oligophagous species that love a selection of plants and monophagous species that are adapted to individual species or species groups which produce similar PSMs (Ali and Agrawal, 2012; Mason and Singer, 2015).

The herbivorous insects had and still have to cope with the PSMs in their food plants (Detzel and Wink, 1993; Linde and Wittstock, 2011). They have evolved several mechanisms to tolerate or detoxify PSMs. Mostly, the generalists have very active enzymes that either inactivate (via CYP) or quickly eliminate (via ABC transporter) toxic PSMs. Another strategy is to feed not only on one plant, but to sample from several species (with low PSMs content) thus diluting any toxic effect. Often herbivores have a fast digestion, by which they absorb nutrients quicker than any toxins that are quickly eliminated in the feces. For detoxification, some herbivores obtain help from symbiotic intestinal microorganisms that often can degrade or inactivate toxic material (Pennisi, 2017).

From a plant side of view, the specialists have won the evolutionary arms race. They can harm their host plants severely if their numbers are large. This can be seen in areas where *Senecio jacobaea* plants (producing PAs) are abundant. If the PA specialist moth *Tyria jacobaeae* occurs in the same area, a *Senecio* population may suffer seriously. But even under these conditions *Tyria* will not completely wipe out its host plants (Wink and Legal, 2001). A predator–prey equilibrium will emerge in the long run.

TABLE 3 | PSMs interfering with biomembranes (more details in Wink, 2008, 2015; Wink and Schimmer, 2010).

Activity	PSMs	Examples	Comments
Increasing membrane fluidity	Lipophilic terpenoids	Monoterpene, sesquiterpenes	Membranes become leaky or disintegrate
Lysis of membranes	Triterpene and steroidal saponins		Saponins bind to membrane cholesterol and induce cell lysis
	AMPs	Widely distributed	Part of the innate immune system

UTILIZATION OF PLANT SECONDARY METABOLITES BY INSECTS

Among monophagous insects, several specialists have been described that apparently love their toxic host plants. These specialists often not only tolerate the toxic PSMs of the host plant, but actively sequester them in their body (Wink, 1992, 1993; Brown and Trigo, 1995; Hartmann and Witte, 1995; Hartmann, 1999, 2004; Petschenka and Agrawal, 2016). Thus, these specialists can store substantial amounts of toxic PSMs and use them for their own defense against predators (Mason and Singer, 2015). Such specialist have been described for toxic cardiac glycosides, aristolochic acids, cyanogenic glucosides, iridoid glucosides and several toxic alkaloids (aconitine, pyrrolizidines, quinolizidines) (Wink, 1992, 1993; Sime et al., 2000; Dobler, 2001; Zagrobelny and Møller, 2011; Kelly and Bowers, 2016;

TABLE 4 | Examples for PSMs interfering with nucleic acids (DNA, RNA) (more details in Wink, 2008, 2015; Wink and Schimmer, 2010).

Target	PSMs	Activity	Comments
DNA INTERCALATION			
	Berbamine	Strong intercalator	Also inhibition of replication and ribosomal protein biosynthesis
	Berberine	Strong intercalator	Also inhibition of replication and ribosomal protein biosynthesis
	Dictamnine and other furaquinoline alkaloids	Strong intercalator	After UV activation also DNA alkylation
	Ellipticine	Strong intercalator	
	Harmine and other Harman alkaloids	Strong intercalator	Also inhibition of replication and ribosomal protein biosynthesis
	Psoralen and other furanocoumarins	Strong intercalator	After UV activation also DNA alkylation
	Rutacridone and other acridone alkaloids	Strong intercalator	After UV activation also DNA alkylation
	Sanguinarine	Strong intercalator	Also inhibition of replication and ribosomal protein biosynthesis
DNA ALKYLATION			
	Aristolochic acid	Mutagenic after metabolic activation	
	Cycasin	Methylazooxymethanol is the active mutagen	
	Furanoquinoline alkaloids	Mutagenic after metabolic activation	
	Ptaquiloside	Active after removal of glucose from glycoside	
	Safrole and other phenylpropanoids	Mutagenic after metabolic activation	
	Senecionine and other PAs	Mutagenic after metabolic activation	

Petschenka and Agrawal, 2016). These specialist often exhibit warning colors, i.e., they are aposematic; and thus advertise their potential toxicity to any predator.

In most instances, we do not know how these specialists circumvent the inherent toxicity of PSMs. For some insects that sequester cardiac glycosides, it could be shown, that the binding site of their molecular target, the Na^+, K^+-ATPase, has been changed through point mutations in such a way, that cardiac glycosides no longer bind to it. Thus, Monarch butterflies can tolerate high concentrations of cardiac glycosides that would kill any poly- or oligophagous species (Holzinger et al., 1992; Holzinger and Wink, 1996; Dobler et al., 2012; Aardema and Andolfatto, 2016). In most other cases, we do not have a clear evidence, how an insensitivity has been accomplished.

ADDICTION OF INSECTS TO PLANT CHEMISTRY?

As mentioned above, monophagous species [mostly butterflies and moths, aphids and other hemipterans) only feed on a single particular plant species that produces a certain kind of toxin, such as cardiac glycosides, iridoid glycosides, glucosinolates, cyanogenic glucosides, or alkaloids [pyrrolizidine (PA), quinolizidine alkaloids (QA)] (Boppré, 1984; Wink, 1992, 1993; Brown and Trigo, 1995; Hartmann and Witte, 1995; Hartmann, 1999, 2004; Klitzke and Trigo, 2000; Laurent et al., 2005; Hilker and Meiners, 2011; Macel, 2011; Trigo, 2011; Cogni et al., 2012). If related plants produce similar toxins, such as in *Brassica* species that all produce glucosinolates, then even a monophagous species may feed on more than a single host plant because they love these particular PSMs. But they will not live on plants with different kinds of PSMs.

Who decides on the choice of a food plant? In most instances, it will be the female with fertile eggs that will search for its specific food plants that it can identify because of their typical PSMs profile. In case of plants from the family Brassicaceae that all produce glucosinolates (which release the often odorant mustard oils), it has been shown that the mustard oils guide a butterfly to its appropriate host plant (Renwick and Lopez, 1999). Apparently, specific odorant receptors have evolved in such butterflies (like Garden Whites, Pieridae) that are activated if the odorant from cabbage plants pass along their antennae. In this instance, the plant odorant appears to work like the pheromones that are used by insects to attract potential mates. Food consumption by larvae of *Pieris rapae* that love food plant with glucosinolates, has been compared with addiction in vertebrates (Renwick and Lopez, 1999).

A similar situation has been described from arctiid moths with sequester PAs, such as *Utetheisa* and *Creatonotus*. We have studied PAs in *Creatonotus* for several years in collaboration with Dietrich Schneider, who had discovered the strange relationship between moths and PAs (Boppré, 1986; Wink and Schneider, 1988, 1990; von Nickisch-Rosenegk et al., 1990; von Nickisch-Rosenegk and Wink, 1993). The caterpillars can be reared on artificial diets without PAs. But the hairy caterpillars of *Creatonotus gangis* and *C. transiens* clearly prefer any plant that produces PAs. Plants with other toxic alkaloids are usually avoided. The larvae appear to be addicted to PAs, because they will even chew filter paper that was impregnated with pure PAs. Normally, they would never touch filter paper, even when hungry. This indicates that PAs induce a very strong feeding stimulus, similar to the situation of the behavior of humans toward addictive drugs.

Addiction in humans implies a craving for a certain chemical whose consumption would confer happiness, good feeling or hallucinations. Addiction will change the personality of the consumer as a strong urge appears once the level of the addictive chemical has dropped in the body.

PAs are actively absorbed by the larvae: PAs mostly occur as polar PA N-oxides which cannot pass biomembranes by simple diffusion. There is evidence that transporter proteins exist at the gut epithelium that can transport the polar alkaloids into the haemolymph (Wink and Schneider, 1988). An alternative mechanism was also found, in that PA become reduced to the more lipophilic free base in the gut which can pass the membranes by simple diffusion. Once the alkaloids have reached the haemolymph, they will be re-oxidized to PA N-oxides (Wang et al., 2012). The PAs do not stay in the haemolymph, but are sequestered into the integument of the larvae (Egelhaaf et al., 1990; von Nickisch-Rosenegk et al., 1990; Wink et al., 1990; von Nickisch-Rosenegk and Wink, 1993), where they serve as defense compounds against predators (Martins et al., 2015).

However, the situation becomes more complex if we look closer into male and female larvae after metamorphosis into adult insects: In female larvae, PAs will be sequestered to some degree in the integument, but a large part is transferred to the orange colored eggs that thus gain chemical protection (von Nickisch-Rosenegk et al., 1990). PAs as a nuptial gift for the defense of the eggs has also been described for other arctiids *Utetheisa ornatrix* and *Cosmosoma myrodora* (González et al., 1999; Conner et al., 2000; Bezzerides and Eisner, 2002; Cogni et al., 2012).

Males produce impressive coremata (these are inflatable sacks at the abdomen which are covered with many hairs) that are inflated during courtship and which will dissipate pheromones to attract female partners (**Figure 1**). Dietary PAs serve as a morphogen that induces the formation of coremata. If a caterpillar did not obtain PAs, then only very small coremata will develop in the imagines (**Figure 1**; Schneider et al., 1982; Boppré, 1986). Thus, the more PAs were ingested, the bigger the corema (von Nickisch-Rosenegk et al., 1990). It seems that co-evolution proceeded even a step further in this system (Schneider, 1992). The pheromones that are dissipated via the coremata, consist of hydroxydanadial (and others) that is derived from dietary PAs (Boppré, 1986; Wink et al., 1988; Schulz et al., 1993; Schulz, 1998). And evidence shows that female moths like males with an abundant PA perfume. And for good reason: we detected that the male spermatophore was also filled with dietary PAs that were transferred as a nuptial gift during copulation to the female increasing the PA contents of the eggs. Thus, males can contribute to the fitness of their offspring. Hydroxydanaidal that is produced by many PA plants is also a signal for other PA insects (Bogner and Boppré, 1989). However, arctiid caterpillars have

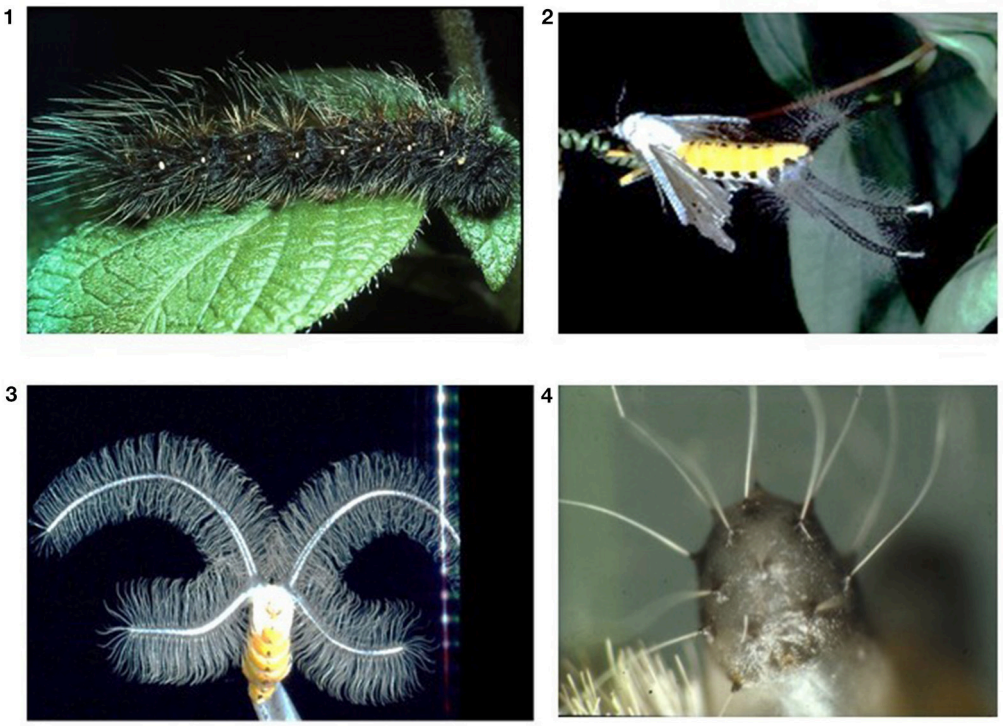

FIGURE 1 | Influence of PAs on the development of male corema in *Creatonotos transiens*. (1) Caterpillar, (2) Adult male with inflated corema, (3) Large corema of a male whose larva had PA-rich food, (4) Minute corema of a male whose larva had no PAs in its food.

taste receptor neurons which are dedicated to the perception of PAs and PA-N oxides (Bernays et al., 2002, 2003).

As shown in **Table 1**, many PSMs modulate the activity of neuroreceptors in vertebrates; what about insects? Insects have similar neurotransmitters, such as acetylcholine, GABA, glutamate, histamine, tyramine, dopamine, octopamine, and serotonin (Vleugels et al., 2015) indicating that neuroreception and corresponding mechanisms are evolutionary old features. Octopamine in insects is similar to noradrenaline in mammals (Vleugels et al., 2015). It is likely, that at least some of the PSMs listed in **Table 1** as modulators of neuroreceptor activity for vertebrates, will also work on insect neuroreceptors. For example, there is evidence that insects lose their coordination when exposed to cocaine that binds to octopamine receptors. Serotonin receptors are expressed in the brain but also in the intestinal tract of animals. Serotonin is involved in the regulation of appetite, mood and emotion, sleep, sexual activity, pain, learning and memory (Vleugels et al., 2015). As serotonin agonist often induce euphoria and hallucinations in vertebrates, we can only speculate that maybe also insects react to serotonin receptor agonists (Vleugels et al., 2015). In vertebrates, PA bind to serotonin receptors (Schmeller et al., 1997). We do not know if this is also the case of serotonin receptors of insects that are also involved in the regulation of feeding, food choice and sleep (Vleugels et al., 2015). The addictive behavior of arctiid moths toward PAs, described above, would be plausible if this would be the case. This is an open question that needs to be addressed experimentally.

CONCLUSIONS

Many PSMs interfere with neuroreceptors and neurotransmitters in vertebrates (Wink, 2000; Wink and Schimmer, 2010). Since neuroreception is on old evolutionary invention, insects share many neuroreceptors with vertebrates, but have tyramine and octopamine receptors in addition (Schneider, 1992; Vleugels et al., 2015). Many insects feed on a single or a few often phylogenetically related food plants. It has been demonstrated, that PSMs serve as olfactory cues for insects to identify their appropriate food plants (Brown and Trigo, 1995). The behavior of insects toward such chemical cues reminds of drug addiction in humans and other vertebrates. It is a challenge for physiologist to discover how PSMs modulate neuroreception, and thus food choice. Since many psychoactive PSMs affect the serotoninergic and dopaminergic system in vertebrates (**Table 1**), it would be worth studying their effects on insects and find out if they also trigger addiction and behavioral changes in invertebrates. There is good evidence for cocaine and nicotine that these alkaloids are active in this context (Barron et al., 2009; Baracchi et al., 2017).

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Cocaine Directly Impairs Memory Extinction and Alters Brain DNA Methylation Dynamics in Honey Bees

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Drug addiction is a chronic relapsing behavioral disorder. The high relapse rate has often been attributed to the perseverance of drug-associated memories due to high incentive salience of stimuli learnt under the influence of drugs. Drug addiction has also been interpreted as a memory disorder since drug associated memories are unusually enduring and some drugs, such as cocaine, interfere with neuroepigenetic machinery known to be involved in memory processing. Here we used the honey bee (an established invertebrate model for epigenomics and behavioral studies) to examine whether or not cocaine affects memory processing independently of its effect on incentive salience. Using the proboscis extension reflex training paradigm we found that cocaine strongly impairs consolidation of extinction memory. Based on correlation between the observed effect of cocaine on learning and expression of epigenetic processes, we propose that cocaine interferes with memory processing independently of incentive salience by directly altering DNA methylation dynamics. Our findings emphasize the impact of cocaine on memory systems, with relevance for understanding how cocaine can have such an enduring impact on behavior.

Keywords: addiction, *Apis mellifera*, DNMT3, demethylation, epigenomics, TET

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INTRODUCTION

Commonly abused drugs cause debilitating drug addiction in a small fraction of users (McLellan et al., 2000). Addiction is a chronic, relapsing condition marked by compulsive drug seeking and craving (Robinson and Berridge, 2003). Recovering addicts suffer high relapse rates due to persistent drug associated memories (Hser et al., 2001). This has led some authors to conclude that drug addiction is a disease of learning and memory (Hyman, 2005; Hyman et al., 2006). Most drugs of abuse are strongly reinforcing and have high incentive salience (Siegel, 2005; Robinson and Berridge, 2008): that is, motivation to seek out drugs or drug associated cues is strong (Robinson and Berridge, 1993). Consequently, stimuli associated with drug administration are readily learned, and memories of them are persistent (Uslaner et al., 2006). It has been argued that increased incentive salience is why drug associated memories are particularly difficult to extinguish (Stewart, 2000), resulting in frequent relapses (Weiss et al., 2001). In mammalian brains, many drugs of abuse alter neurotransmission in the dopaminergic midbrain pathway either by increasing release or inhibiting clearance of dopamine (Kuhar et al., 1991; Han and Gu, 2006), thereby increasing the incentive salience of a given stimuli (Berridge, 2007).

Altered incentive salience is not the only way by which memory processing can be altered, however. Many drugs also interfere with memory processing directly (Mittenberg and Motta, 1993). Cocaine induces widespread changes in DNA methylation patterns (Anier et al., 2010). This is of particular interest, since both DNA methyltransferase enzymes (DNMT) and Ten-eleven translocation (TET) proteins, responsible for methylation and demethylation of DNA, respectively, are both vital for memory formation (Day et al., 2013; Alaghband et al., 2016; Kennedy and Sweatt, 2016). Previous studies have shown cocaine-associated memories to be correlated with changes in DNA methylation (Tian et al., 2012) and to be highly resistant to extinction (Di Ciano and Everitt, 2004). It is not yet known, however, if the enduring nature of cocaine-associated memory is due to cocaine directly affecting the DNA methylation machinery, or if it is an indirect consequence of altered incentive salience.

Honey bees have long been used to study mechanisms of associative learning (Menzel et al., 1974; Bitterman et al., 1983; Hammer and Menzel, 1995), and more recently as a valuable model system for elucidating the effects of pharmacological manipulations on learning and memory (Felsenberg et al., 2011; Maleszka, 2014). Bees have functional DNA methylation and demethylation systems (Wang et al., 2006; Lyko and Maleszka, 2011; Wojciechowski et al., 2014; Maleszka, 2016), that are involved in memory processing. Following olfactory conditioning altered methylation patterns can be seen across the entire honey bee genome (Li et al., 2017).

DNMT function is required for forming stimulus-specific olfactory memories (Biergans et al., 2012, 2016), potentially due to its activity in the antennal lobes (Biergans et al., 2017). Pharmacological inhibition of DNMTs has also been shown to interfere with consolidation extinction of appetitive memories (Lockett et al., 2010; Gong et al., 2016). *DNMT1b* and *DNMT3* and *Tet* are all upregulated following olfactory conditioning (Biergans et al., 2015), but no direct function of TET proteins during learning has been demonstrated in bees so far.

Because cocaine results in similar behavioral and neurochemical responses in bees and mammals (Barron et al., 2009; Søvik, 2013; Søvik et al., 2013, 2014), it presents itself as a valuable system to explore the basic interactions between drugs of abuse, epigenomic modifications and behavior (Søvik and Barron, 2013; Maleszka, 2014, 2016). Here we investigated the effects of cocaine on acquisition, consolidation, and retrieval of memories in honey bees when drug delivery was dissociated from conditioning, and explored whether cocaine affected brain DNA methylation systems.

MATERIALS AND METHODS

Animals

European honey bees, *Apis mellifera*, of the standard commercial strain available in New South Wales, Australia were used for all experiments. Adult bees were collected on emergence from brood cells, placed in mesh cages (20 × 16 × 3 cm) with *ad libitum* access to honey (80 bees per cage) and housed in an incubator at 34°C for 6 days prior to learning experiments. Cage rearing offers greater control of bees' age and experience it differs fundamentally from life in the hive. This can be problematic for some experiments, but as it does not significantly affect brain development (Maleszka et al., 2009) or ability to retain olfactory memories (Arenas and Farina, 2008), we decided it was the best approach for our experiments. Behavioral experiments 1–4 were conducted at The Australian National University, Canberra, while remaining experiments were conducted at Macquarie University, Sydney.

Drug Treatments

The treatments used for all experiments consisted of either 3 μg of freebase cocaine (cocaine) dissolved in 1 μL dimethylformamide (DMF) or 1 μL DMF on its own (control). All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA). The treatments were given topically by placing 1 μL of the solution onto the dorsal thorax of bees with a microcapillary pipette. Care was taken to prevent treatments from spreading to wing joints or across the wings. DMF rapidly penetrates bee cuticle and can conduct compounds into the haemolymph of the bees' open circulatory system, from where small compounds can access the brain and nervous system (Barron et al., 2007; Okada et al., 2015). This administration method has previously been shown to be effective for delivering cocaine to honey bees (Barron et al., 2009; Søvik et al., 2013, 2016; Scheiner et al., 2014).

Training Protocols

At 6 days of age, bees were harnessed for proboscis extension response (PER) conditioning (Bitterman et al., 1983). The thorax and abdomen of bees were lightly restrained in 8 mm diameter metal tubes by a thin piece of tape placed behind the neck so the head was kept in place, but antennae and proboscis were free to move (Maleszka et al., 2000; Si, 2004; Lockett et al., 2014). Each bee was fed 2 drops (approx. 30 μL) of 1.5 M sucrose, and left overnight. On the following morning, bees were trained in either a differential (experiment 1–5), or absolute (experiment 6) conditioning paradigm. For differential conditioning bees were trained to distinguish two odors (limonene and natural vanilla), one paired to reward and the other to punishment. For absolute conditioning only a single odor associated with reward was used.

Reward training involved touching a droplet of 2 M sucrose solution to the antennae followed by offering sucrose to the proboscis. Punishment consisted of touching saturated NaCl solution to the antennae, which is strongly aversive to bees (Maleszka et al., 2000; de Brito Sanchez et al., 2005; Lockett et al., 2010, 2014). Presentation of sucrose to bees results in proboscis extension, and following paired presentation of odor and sucrose bees learn to extend their proboscis to an odor that is predictive of sucrose delivery. Following training with the aversive salt solution the proboscis is actively withheld (Smith et al., 1991). For acquisition training odors were presented for 3 s on their own, and for 2 s simultaneously with the reward/punishment. For extinction training odors were presented on their own for 5 s.

For both absolute and differential conditioning bees were given 3 learning trials, each separated by 6 min (acquisition training). In the evening bees were fed with 2 drops of 1.5 M

sucrose and left overnight. On the following morning, 24 h after conditioning, bees were tested by presenting training odors alone, and whether or not bees extended their proboscis to the odors was recorded (acquisition test). For the differential conditioning, we immediately discarded all bees that were deemed non-learners after the acquisition test (i.e., responded to the odor associated with the punishment, failed to respond to the odor associated with the reward, or both). Because of this, only bees that had had the correct response (proboscis extension to the rewarding odor only) were included in the extinction training when using the differential training protocol, whereas all bees were included in extinction training for the absolute conditioning. Therefore, the training curves for extinction training in experiments with differential conditioning start with all bees responding, while those using absolute conditioning start with the same proportion of responses as seen in the acquisition test.

Testing was immediately followed by an additional four presentations of odors alone, each separated by 2 min (extinction training). Five hours later bees were tested again (odor presentation alone) and proboscis extension recorded (extinction test). This method follows the conditioning paradigm used by Maleszka et al. (2000) and training schedule of Lockett et al. (2010). For molecular experiments, only bees that responded correctly during the acquisition test was used for experiments that used extinction training. Analyzed bees were drawn randomly from a cohort of bees that had gone through the exact same training or drug treatment protocol.

DNA Methylation Enzyme Activity Quantification

To assess the activity of DNA methylation enzymes in individual bee brains, honey bees were chilled to −20°C for 3 min before the central brain (excluding optic lobes and gnathal ganglia) was removed in freshly prepared PBS solution. This procedure was performed 1 h after cocaine delivery. Nuclear proteins were extracted using the EpiQuik™ Nuclear extraction kit (Epigentek Group Inc., Farmingdale, NY) and DNA methylation enzyme activity was then measured using an EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Epigentek Group Inc., Farmingdale, NY), according to the manufacturer instructions. Protein concentration was determined with a Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA), and DNA methylation enzyme activity was estimated as follows:

$$\text{DNA methylation enzyme activity} = 1,000 \times \frac{\text{Sample OD} - \text{Blank OD}}{\text{Protein amount} \times \text{Incubation time}}$$

Gene Expression Analysis

To examine transcription levels of *DNA methyltransferase 3* (*AmDNMT3*, GenBank gene ID: 410798) and *TET dioxygenase* (*AmTET,* GenBank gene ID: 412879 412878) following cocaine treatment and extinction training, whole bees were frozen in liquid nitrogen 1 h after treatment (2 h after extinction training). Heads were partially lyophilized prior to the removal of central brains. Dissected brains were stored at −80°C until RNA extraction with the PureLink® RNA Mini Kit (Ambion®, Life Technologies, Grand Island, NY) following supplied guidelines. Each biological replicate consisted of RNA extracted from a single brain. For each experiment, six replicates were performed for each treatment group. Total RNA was reverse transcribed using SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen™, Life Technologies, Grand Island, NY). Transcription levels were assessed by quantitative real time polymerase chain reactions (qRT-PCR). We performed 10 μL reactions using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA). Primers used for *AmDNMT3* were: forward: 5′-GAA CTCGTTGAAGCAAGGCA-3′; reverse: 5′-AACGTTTGCACG CTCCAAGA-3′, and for *AmTET*: forward 5′-GTCAGTGAG ATCAGAGGAGC-3′; reverse 5′-TGGTGCAAGGCTGAGGTA CA-3′. The housekeeping genes *AmUGT* (*uridine 5′-diphospho-glucuronosyltransferase*) and *AmeIF.S8* (*eukaryotic initiation factor S8)* were used as controls (*AmUGT*, GenBank gene ID: 412198, forwards primer: 5′-CGTTGATGCTGATCAGGT TG-3′; reverse primer: 5′-CGTCGAAATCGCTTCAAGTC-3′; *AmeIF.S8*, GenBank gene ID: 551184, forwards primer: 5′-TGAGTGTCTGCTATGGATTG CAA-3′; reverse primer: 5′-TCGCGGCTCGTGGTAAA-3′). All primers crossed an intron junction in order to avoid potential problems with contamination from genomic DNA, and have previously been used successfully (Foret et al., 2012; Wojciechowski et al., 2014).

Specimen Preparation for Quantification of Cocaine in Honey Bee Brains by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

Bees were treated with cocaine in the same manner as for behavioral experiments and placed in an incubator for 15, 30 min, 1, 4, or 24 h, after which central brains were extracted and stored at −80°C until analysis. For analysis brains were resuspended in 60 μL pH 6.0 phosphate buffer and lysed by sonication. Cellular debris was collected by centrifugation at 12,000 g for 10 min at 4°C and the supernatant was removed for analysis. A separate set of untreated brains were processed as above ($n = 3$–6 for each concentration point) and spiked with concentrations of cocaine ranging from 1 to 0.1 ng/mL. These served as a standard for quantification.

LC-MS/MS

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) experiments were performed with an AB SCIEX QTRAP 5500 (SCIEX, Framingham, MA, USA) triple quadrupole linear ion trap mass spectrometer. The LC system used was an Agilent 1100 (Agilent, Santa Clara, CA, USA). Analyst® TF software (version 1.6.2) was used for acquisition and quantitation. Chromatographic separation was achieved using a Zorbex SB-C18 column (150 × 0.5 mm) (Agilent). Elution was performed isocratically with 35% methanol/65% (0.1% formic acid) in H_2O at a flow rate of 30 μL/min for 8 min total run time. Ten microliter injection volume was used.

MS data was collected in positive ion mode. For quantification and validation of cocaine, the following transitions were monitored, as previously described by Shakleya and Huestis (2009) m/z 304 to 182 (quantification) and m/z 304 to 82 (validation). Peak areas were measured for quantification of each sample.

Statistical Tests

To determine if the cocaine treatment had any effect on learning a χ^2-test was conducted for each of the two tests (acquisition and extinction). For acquisition and extinction curves a χ^2-test was used for each point in the curve, adjusted using Bonferroni's correction to account for multiple testing. Effect sizes were estimated using Pearson's φ. Mann-Whitney U-test was used to compare DNA methylation enzyme activity between cocaine and control treated bees. The effect size was estimated using rank biserial correlations (r). Transcript levels were compared by t-tests, with effect sizes given as Cohen's d. All statistical analysis were conducted in R 3.4.1 (R Development Core Team, 2017).

Experimental Order

In order to examine the effects of cocaine on learning, independently of its effects on reward perception, we treated bees with cocaine at various time points before and after acquisition and extinction training. Since we found that cocaine most strongly inhibited consolidation of extinction memory (see below), we tried testing for acquisition memory at the same time point to ensure the effect of cocaine was specific to consolidation of extinction memory. In all of the experiments so far, we used a differential conditioning paradigm. In this paradigm bees are exposed to both aversive and appetitive conditioning. To ensure that the effect of cocaine on consolidation on extinction memory was not the result of an interaction between the processing of these two distinct kinds of memories we repeated the whole conditioning procedure, treating bees 1 h after extinction training, with an absolute conditioning paradigm.

RESULTS

Cocaine Weakly Inhibits Acquisition and Recall of Memory

Cocaine treatment applied 1 h before training (**Figure 1**) does not alter the rate of acquisition or extinction of learning of an appetitive memory as evidenced by comparison of acquisition and extinction learning curves for cocaine treated and control bees for any of the experiments (**Figures 2**, **3**). There were, however, differences in the recall of acquisition memory for bees treated with cocaine 1 h before (Experiment 1: $\chi^2 = 8.8245$, $p = 0.0030$, $n = 236$, $\phi = 0.1933$, **Figure 2A**) and after acquisition training (Experiment 2: $\chi^2 = 3.9503$, $p = 0.0469$, $n = 234$, $\phi = 0.1299$, **Figure 2B**), and 2 h before the recall of acquisition memory (Experiment 3: $\chi^2 = 12.043$, $p = 0.0005$, $n = 139$, $\phi = 0.2943$, **Figure 2C**). In other words, bees that had been treated with cocaine prior to the acquisition test (Experiments 1–3), regardless of time point (before conditioning, after conditioning, or before recall) performed worse in the recall of acquisition test than control bees (**Figure 2**). There was no difference in response rate to the aversive memory for any of the treatment timepoints (Figure S1).

Cocaine Strongly Inhibits Consolidation of Extinction

When we tested the recall of extinction conditioning, however, it was only when cocaine was administered 1 h post-extinction training that there was a difference between cocaine and control groups (Experiment 4: $\chi^2 = 16.7965$, $p < 0.00001$, $n = 66$, $\phi = 0.5044$, **Figure 3D**). Here, cocaine treatment impaired consolidation of extinction conditioning resulting in a stronger response to the training odor during the extinction test (**Figure 3**). Because the extinction test was 5 h after conditioning (as opposed to the 24 h gap between acquisition training and testing) we could not be sure if the effect seen was specific to the extinction paradigm or a general performance change 4 h after cocaine treatment. We therefore treated bees with cocaine 1 h after acquisitioning training and tested them 4 h later. We did not detect any difference between treatment and control treated bees in response to odor paired with sucrose (Experiment 5: $\chi^2 = 0.5489$, $p = 0.4588$, $n = 101$, **Figure 4**) or NaCl (Figure S2). This suggests the effect seen in **Figure 3** is specific to consolidation of extinction memory.

We further examined the robustness of this phenomenon by training bees in an absolute conditioning paradigm with a rewarded odor only. When cocaine treatment was given 1 h after training the same effect was seen (Experiment 6: $\chi^2 = 21.2706$, $p < 0.000001$, $n = 84$, $\phi = 0.5032$, **Figure 5**). Thus, we conclude that cocaine has a strong inhibitory effect on consolidation of extinction memory.

Cocaine Affects DNA Methylation Dynamics

DNA methylation enzyme activity was increased in honey bee brains 1 h following cocaine administration (Experiment 7: Mann-Whitney $U = 29$, $p = 0.01276$, $r = 0.4994$, **Figure 6A**). Cocaine treatment did not affect transcription levels of *AmDNMT3*, which is believed to be responsible for *de novo* methylation in honey bees (Wang et al., 2006), in bees 1 h after cocaine treatment (Experiment 7: *AmUGT*: $t_{10} = 1.3439$, $p = 0.1940$; *AmeIF.S8*: $t_{10} = 0.0001$, $p = 0.9999$; **Figure 6B**). We next examined the effects of cocaine treatment and associative learning on levels of the honey bee homolog of TET dioxygenase, which has been shown to be responsible for removal of methylation marks in honey bees (Wojciechowski et al., 2014). Cocaine treatment caused a significant decrease in transcript levels of *AmTET* (Experiment 7: *AmUGT*: $t_{10} = -5.0172$, $p < 0.0001$, $d = -1.5866$; *Ame.IF.S8*: $t_{10} = -5.3780$, $p < 0.0001$, $d = -1.7007$; **Figure 6C**).

The Effects of Cocaine on TET Is Context Dependent

The effects of cocaine on *AmTET*, but not *AmDNMT3*, expression levels varied with the learning experience of the bees. Levels of *AmDNMT3* mRNA were not affected by cocaine in bees that had gone through extinction conditioning

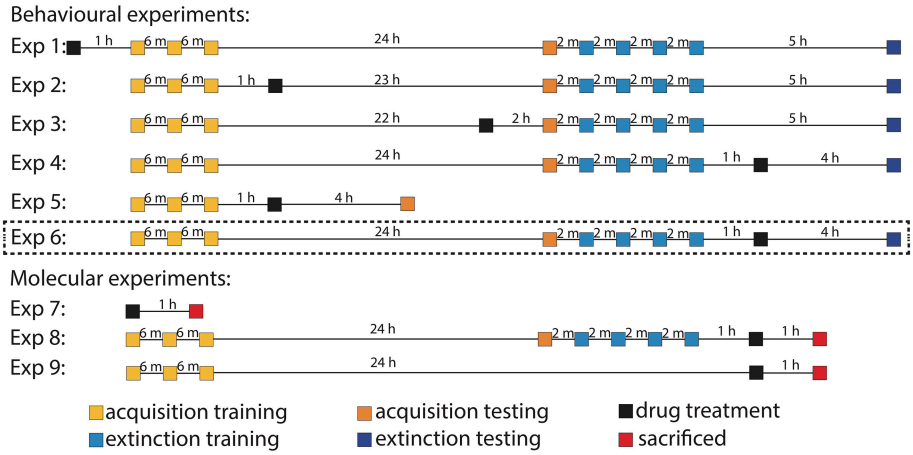

FIGURE 1 | Schematic of training schedules and treatment regimens. Acquisition and extinction trials are presented in yellow and blue, respectively. The darker shades represent test of the same type of memory. The black blocks signify when drug treatment was given, while red blocks show when animals were sacrificed. Note that time is not presented as a linear scale in diagram (h, hours; m, minutes). Dashed lines denote absolute conditioning was used instead of differential.

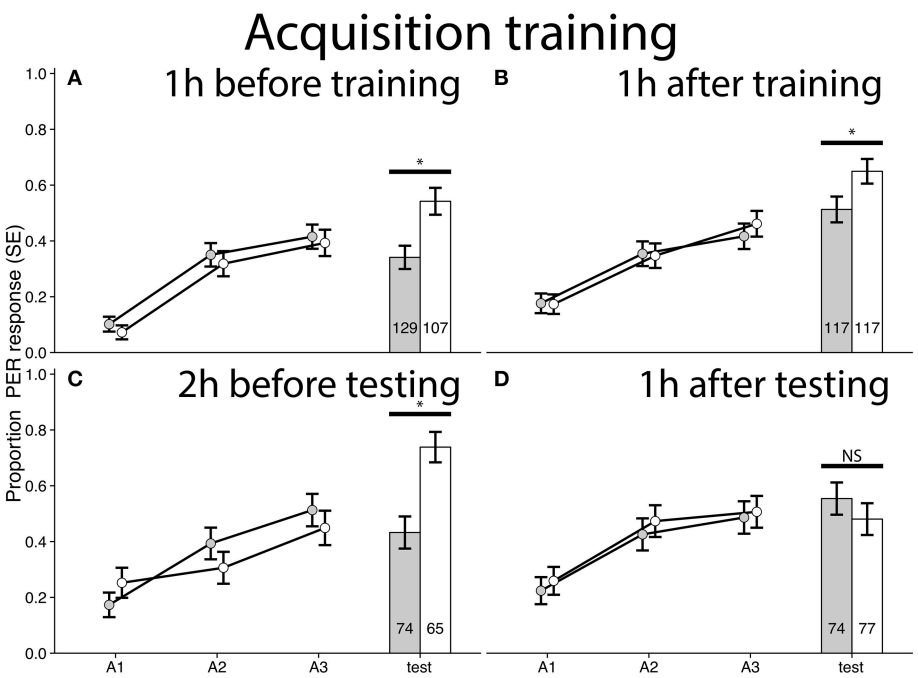

FIGURE 2 | Acquisition curves and test results for bees trained in a differential conditioning PER training paradigm. A1–A3 refers to each odor exposure during conditioning. Gray bars/dots represent bees treated with cocaine and controls are in white. Responses shown for the odor paired with sucrose reward, see Figure S1 for responses of the odor paired with NaCl. **(A)** Experiment 1: Bees treated with cocaine 1 h before training ($\chi^2 = 8.8245$, $p = 0.002972$, $n = 236$, $\phi = 0.1933$). **(B)** Experiment 2: Bees treated 1 h after training ($\chi^2 = 3.9503$, $p = 0.04686$, $n = 234$, $\phi = 0.1299$). **(C)** Experiment 3: Bees treated 2 h before testing ($\chi^2 = 12.043$, $p = 0.0005$, $n = 139$, $\phi = 0.2943$). **(D)** Experiment 4: Bees treated 1 h after testing ($\chi^2 = 0.5491$, $p = 0.4587$, $n = 151$). *Denotes statistically significant differences.

(Experiment 8: *AmUGT*: $t_{10} = 0.8532$, $p = 0.4135$; *AmeIF.S8*: $t_{10} = -0.6977$, $p = 0.4927$; **Figure 7A**). Cocaine treatment did not alter *AmDNMT3* levels in bees that had not gone through extinction training either (Experiment 9: *AmUGT*: $t_{10} = 0.9113$, $p = 0.3836$; *AmeIF.S8*: $t_{10} = 1.8837$, $p = 0.0729$; **Figure 7B**). In contrast, levels of *AmTET* were significantly reduced following cocaine treatment after extinction conditioning (Experiment 8: *AmUGT*: $t_{10} = -3.6832$, $p = 0.0013$, $d = -1.1105$; *AmeIF.S8*: $t_{10} = -7.8450$, $p < 0.0001$, $d = -2.3653$; **Figure 7C**). Intriguingly, when bees that were treated with cocaine after acquisition training *AmTET* levels increased significantly (Experiment 9: *AmUGT*: $t_{10} = 14.1652$, $p < 0.0001$,

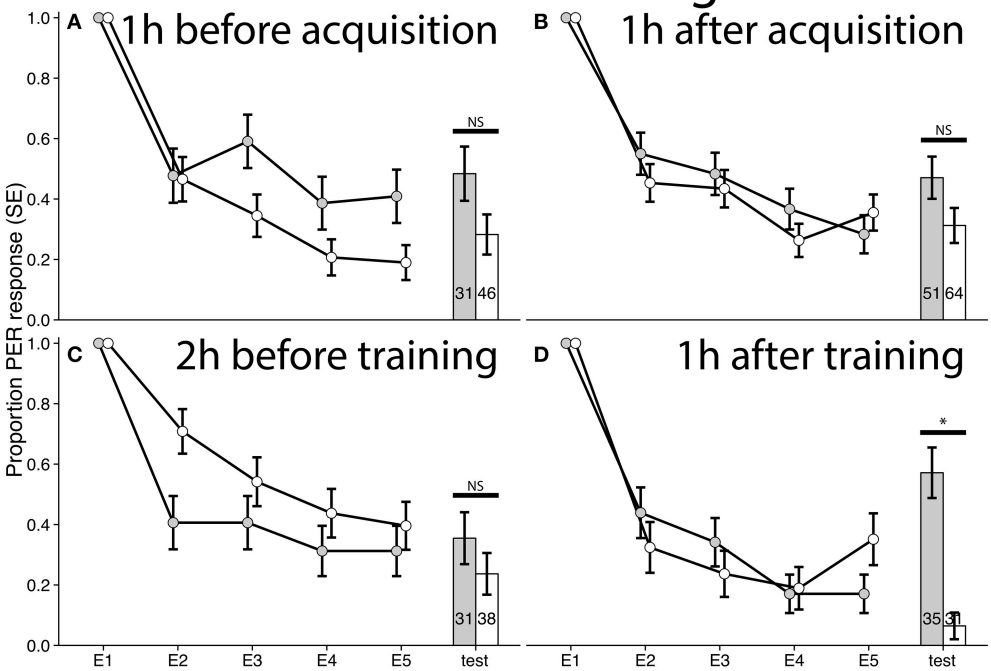

FIGURE 3 | Extinction curves and test results for bees trained in a differential conditioning PER training paradigm. E1–E5 refers to each odor exposure during extinction. Gray bars/dots represent bees treated with cocaine and controls are in white. **(A)** Experiment 1: Bees treated with cocaine 1 h before acquisition training ($\chi^2 = 2.4304$, $p = 0.119$, $n = 77$). **(B)** Experiment 2: Bees treated 1 h after acquisition training ($\chi^2 = 2.3709$, $p = 0.1236$, $n = 115$). **(C)** Experiment 3: Bees treated 2 h before extinction training ($\chi^2 = 0.6527$, $p = 0.4192$, $n = 69$). **(D)** Experiment 4: Bees treated 1 h after extinction training ($\chi^2 = 16.7965$, $p < 0.00001$, $n = 66$, $\phi = 0.5044$). Note that for extinction memory, lack of PER expression signifies successful memory formation. *Denotes statistically significant differences.

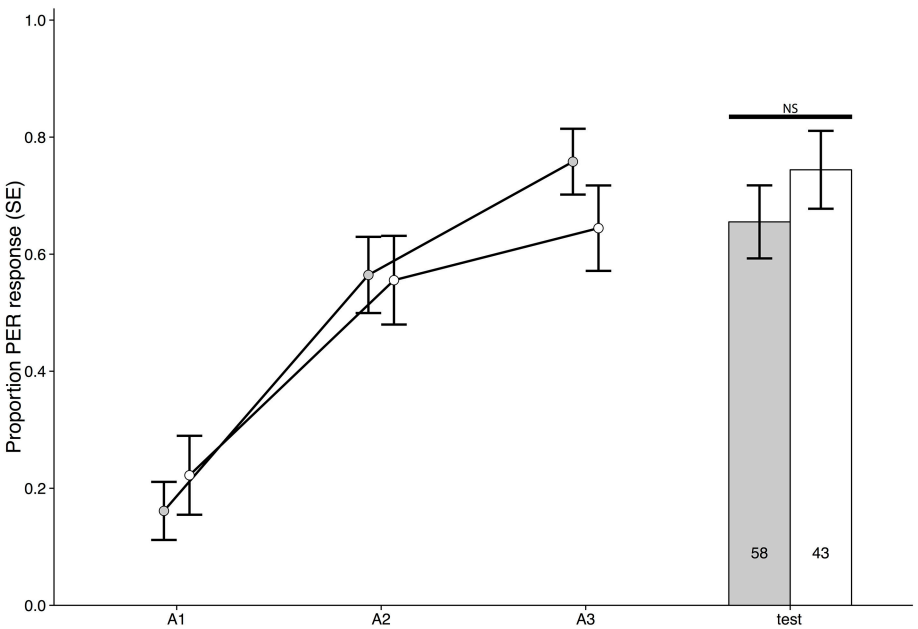

FIGURE 4 | Experiment 5: acquisition curve and test results for bees trained in a differential conditioning PER paradigm and treated with cocaine 1 h after acquisition training, but tested 5 h after training ($\chi^2 = 0.5489$, $p = 0.4588$, $n = 101$). Responses shown for the odor paired with sucrose reward, see Figure S2 for responses of the odor paired with NaCl.

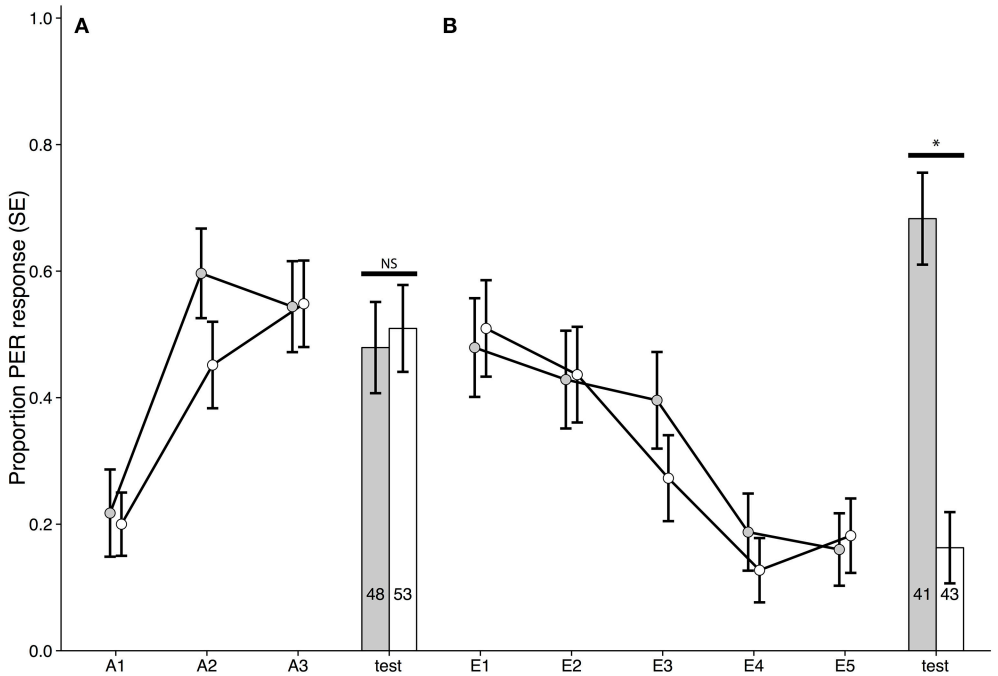

FIGURE 5 | Experiment 6: results for bees trained in a single odor conditioning PER paradigm. Cocaine treatment was given 1 h after extinction training. **(A)** Acquisition test ($\chi^2 = 0.0109$, $p = 0.9167$, $n = 101$). **(B)** Extinction test ($\chi^2 = 21.2706$, $p < 0.000001$, $n = 84$, $\phi = 0.5032$). Note that for extinction memory, lack of PER expression signifies successful memory formation. *Denotes statistically significant differences.

$d = 4.2710$; *AmeIF.S8*: $t_{10} = 6.9097$, $p < 0.0001$, $d = 2.0834$; **Figure 7D**). Taken together, these results suggest that the effects of cocaine on *AmTET* levels were dependent on the learning experience of the bees.

Retention of Cocaine Post-treatment

The total amounts of cocaine present in brains peaked 30 min after treatment and gradually declined to almost zero over a 4 h period (**Figure 8**).

DISCUSSION

Our experiments show a far stronger effect of cocaine on consolidation of extinction memory (**Figures 3D**, **5**) than consolidation of acquisition memory (**Figures 2B**, **4**), as evidenced by the difference in effect size between these experiments. Cocaine treatment affects performance in the recall of acquisition memory when it was applied before testing (**Figure 2C**). Treatment prior to acquisition training does not affect the rate of acquisition, but still impair memory formation (**Figure 2A**). Cocaine did not affect naïve odor responses as no difference was seen between treatment groups on the first exposure to odors in any of our experiments where cocaine was administered prior to acquisition training.

The lack of any effect of cocaine on rate of learning (demonstrated by the overlapping acquisition and extinction curves of the two experimental groups, **Figures 2**–**4**) was expected, as we intentionally chose time points for cocaine administration that would not cause cocaine to interfere with perception of stimuli during acquisition and extinction conditioning. This design allowed us to examine any direct effects of cocaine on learning and memory, rather than the effects of changed incentive salience for the conditioned stimuli. The strongest effect of cocaine, seen in this context, was an impairment of the consolidation of extinction memory (**Figures 3D**, **5**). This suggests that part of the reason why cocaine-associated memories are so hard to extinguish, could be that in addition to increasing the incentive salience of stimuli (Uslaner et al., 2006), cocaine also actively inhibits consolidation of extinction. A likely mechanistic explanation for this phenomenon could be that cocaine interferes with the epigenetic mechanisms of memory consolidation (Day and Sweatt, 2010; Robison and Nestler, 2011).

There is ample evidence from the mammalian literature that chronic cocaine administration interferes with DNA methylation dynamics (Robison and Nestler, 2011). In mice levels of *DNMT3a*, but not *DNMT1* and *DNMT3b*, increase following chronic cocaine exposure (LaPlant et al., 2010). This increase persist for at least 28 days after the end of drug treatments (LaPlant et al., 2010). The altered levels of *DNMT3a* have been shown to affect the DNA methylation patterns in the brains of mice following cocaine exposure (Anier et al., 2010). Further, Feng et al. (2015) has shown that levels of *TET* are also decrease after chronic cocaine exposure. The effects on both *DNMT3a* and *TET* have been shown to be localized to particular brain regions and affect the methylation and demethylation of specific

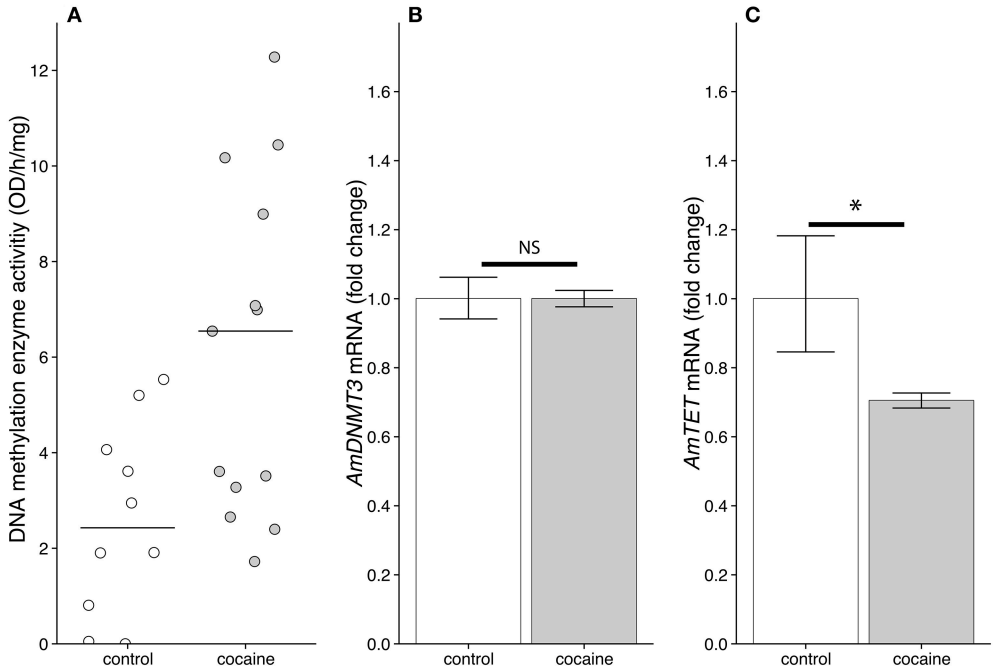

FIGURE 6 | The effects of cocaine on DNA methylation enzyme activity and *AmDNMT3* and *AmTET* and transcription levels, 1 h after cocaine treatment. **(A)** DNA methylation enzyme activity following cocaine administration is displayed as a function of relative optic density. Black lines show median values, dots represent values for samples consisting of four pooled brains. DNMT activity was significantly increased following exposure to 3 μg of cocaine (Mann-Whitney $U = 29$, $p = 0.01276$, $r = 0.4994$). **(B)** There was no significant difference in *AmDNMT3* mRNA levels following cocaine or control treatments (*AmUGT*: $t_{10} = 1.3439$, $p = 0.1940$; *AmelF.S8*: $t_{10} = 0.0001$, $p = 0.9999$). **(C)** There was a significant reduction in levels of *AmTET* mRNA in bees treated with cocaine (*AmUGT*: $t_{10} = -5.0172$, $p < 0.0001$, $d = -1.5866$; *Ame.IF.S8*: $t_{10} = -5.3780$, $p < 0.0001$, $d = -1.7007$). *Denotes statistically significant differences.

genomic regions (LaPlant et al., 2010; Feng et al., 2015). Thus, it is becoming increasingly clear that changes to brain DNA methylation dynamics play a role in the behavioral outcomes associated with cocaine abuse.

In this study we show that in bees a single cocaine treatment can alter DNA methylation enzyme activity levels (**Figure 6A**) and transcription of *AmTET* (**Figures 6C**, **7C,D**). So far the results for *AmTET* largely mimics what is seen in mammalian systems, however, unlike studies in mice, we failed to detect any change in *AmDNMT3* levels in both naïve and trained bees. The most likely explanation for this difference is that mice were treated chronically over the course of 28 days, while the bees in our study received a single cocaine treatment. It is of course possible that *AmDNMT3* levels are affected in bees following chronic exposure.

Another possibility is that *AmDNMT3* is not a primary *de novo* methyltransferase in honey bees (Wedd and Maleszka, 2016). Recent studies suggest that the classical roles of DNMT1 and DNMT3s in establishing methylation patterns need to be redefined to include the evident *de novo* activity of DNMT1 and DNMT3s' involvement in maintenance (Jeltsch and Jurkowska, 2014). The variation of DNMTs across invertebrates is also suggestive of diverse roles for these enzymes. In the honey bee, the DNA methylation toolkit consists of two copies of DNMT1 and one copy of DNMT3, but many insects lack DNMT3 and still methylate their genome implying that DNMT1 provides *de novo* activity in these organisms (Wedd and Maleszka, 2016).

Perhaps the most surprising result is the context dependency of the effects of cocaine on *AmTET* levels. We can only speculate why levels were shown to increase in animals following acquisition conditioning, while they were decreased in naïve animals and animals that had gone through extinction training, but it is a clear demonstration that when considering how drugs of abuse might interact with the DNA methylation/demethylation machinery it is important to remember that what the animal is actively doing or exposed to can play an important role. A further issue that must be taken seriously is where in the brain the enzymes responsible, for methylation and demethylation, act during memory formation and where cocaine is exerting its effects. In this study we used a systemic cocaine treatment and all analysis of gene expression or enzyme activity occur at the whole-brain level, we are thus not able to conclude anything clearly about this question. Biergans et al. (2017) beautifully demonstrated how DNMT activity in the antennal lobes mediates odor specificity during learning. Another interesting to note is that many of the methylation related genes shown to have

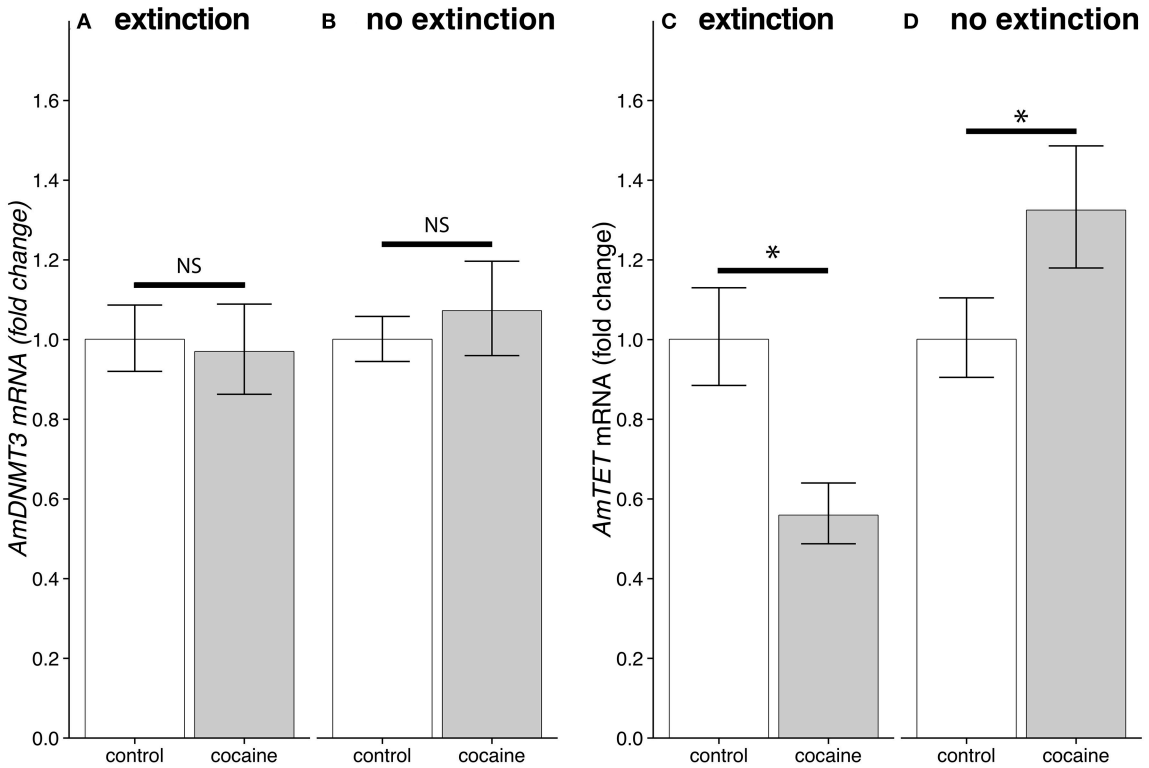

FIGURE 7 | The effects of cocaine and extinction training on levels of DNA methylation enzymes 1 h after treatment. **(A)** There was no significant difference in *AmDNMT3* mRNA levels following cocaine or control treatments given after extinction training (*AmUGT*: $t_{10} = 0.8532$, $p = 0.4135$; *AmelF.S8*: $t_{10} = -0.6977$, $p = 0.4927$). **(B)** Similarly, levels were not affected by cocaine in bees that did not go through extinction training (*AmUGT*: $t_{10} = 0.9113$, $p = 0.3836$; *AmelF.S8*: $t_{10} = 1.8837$, $p = 0.0729$). **(C)** There was a significant reduction in levels of *AmTET* mRNA in bees treated with cocaine following extinction training (*AmUGT*: $t_{10} = -3.6832$, $p = 0.0013$, $d = -1.1105$; *AmelF.S8*: $t_{10} = -7.8450$, $p < 0.0001$, $d = -2.3653$). **(D)** Intriguingly, bees that only went through acquisition training, prior to being treated with cocaine showed the opposite effect (*AmUGT*: $t_{10} = 14.1652$, $p < 0.0001$, $d = 4.2710$; *AmelF.S8*: $t_{10} = 6.9097$, $p < 0.0001$, $d = 2.0834$). *Denotes statistically significant differences.

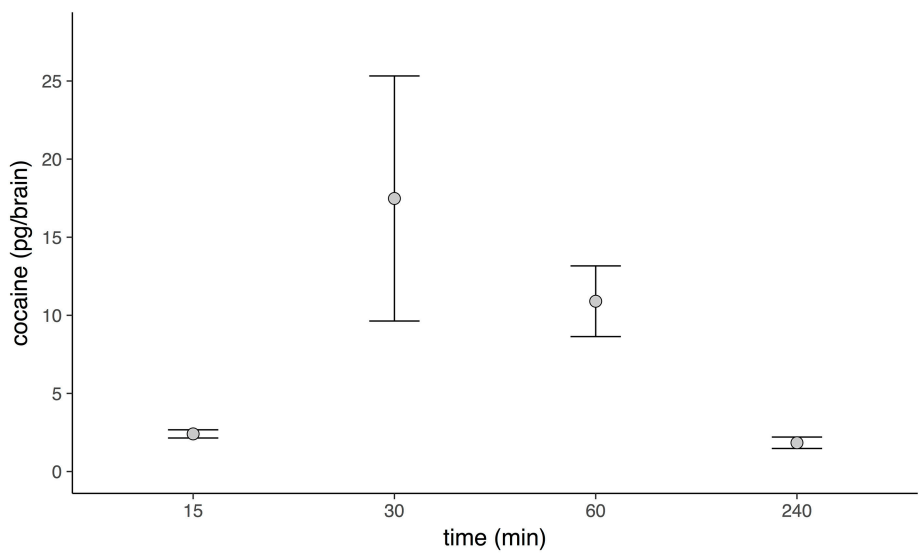

FIGURE 8 | Amounts of cocaine in the honey bee brain after topical treatment. Levels of cocaine increased sharply 30 min after treatment, thereafter levels gradually decreased, until they were almost absent at 4 h.

altered expression patterns following olfactory conditioning are highly expressed in the honey bee mushroom bodies (Biergans et al., 2015). We therefore think that the antennal lobe and mushroom bodies are key structures for future investigations.

The finding that cocaine interferes with consolidation of extinction learning, potentially by changing levels of *AmTET*, has implications for the application of extinction-based therapies. While this method has worked well when attempting to extinguish fearful memories (Schiller et al., 2010), it has been less successful for treating addiction (Conklin and Tiffany, 2002), as marked by very high rates of relapse in recovering addicts (McLellan et al., 2000; Hser et al., 2001). If cocaine uniquely interferes with mechanisms involved in consolidation of extinction memory, it could potentially mean that extinction therapies are severely compromised in recovering addicts who still occasionally use cocaine.

AUTHOR CONTRIBUTIONS

ES, AB, and RM conceived the study. ES, PD, WK, PH, EB, and JP performed the experiments. ES analyzed the data. ES, AB, RM, PD, WK, PH, EB, and JP wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Sensitivity of the Crayfish Reward System to Mammalian Drugs of Abuse

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The idea that addiction occurs when the brain is not able to differentiate whether specific reward circuits were triggered by adaptive natural rewards or falsely activated by addictive drugs exist in several models of drug addiction. The suitability of crayfish (*Orconectes rusticus*) for drug addiction research arises from developmental variation of growth, life span, reproduction, behavior and some quantitative traits, especially among isogenic mates reared in the same environment. This broad spectrum of traits makes it easier to analyze the effect of mammalian drugs of abuse in shaping behavioral phenotype. Moreover, the broad behavioral repertoire allows the investigation of self-reinforcing circuitries involving appetitive and exploratory motor behavior, while the step-wise alteration of the phenotype by metamorphosis allows accurate longitudinal analysis of different behavioral states. This paper reviews a series of recent experimental findings that evidence the suitability of crayfish as an invertebrate model system for the study of drug addiction. Results from these studies reveal that unconditioned exposure to mammalian drugs of abuse produces a variety of stereotyped behaviors. Moreover, if presented in the context of novelty, drugs directly stimulate exploration and appetitive motor patterns along with molecular processes for drug conditioned reward. Findings from these studies indicate the existence of drug sensitive circuitry in crayfish that facilitates exploratory behavior and appetitive motor patterns via increased incentive salience of environmental stimuli or by increasing exploratory motor patterns. This work demonstrates the potential of crayfish as a model system for research into the neural mechanisms of addiction, by contributing an evolutionary, comparative context to our understanding of natural reward as an important life-sustaining process.

Keywords: amphetamine, appetitive motor patterns, crayfish, drugs of abuse, exploratory behavior

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INTRODUCTION

As individuals experience repeated exposure to opiates and other psychoactive drugs, vulnerable individuals enter an addictive cycle that is triggered by several mechanisms. These drugs initially function as reinforcers that strengthen behaviors associated with drug intake. After a short period of time, the resulting tolerance and dependence lead to progressively higher doses to maintain a desired effect (Wise and Koob, 2014). At this stage, compulsive drug-seeking behaviors become evident, even when paired with negative consequences (Wise, 1998). To identify useful targets for the development of future therapeutic interventions for drug-seeking behaviors, several studies explored the central components of drug-sensitive reward processes in both vertebrate and invertebrate species. Much of these efforts have been focused on an evolutionary basis of

drug reward as an entrenched process within natural reward systems (Higgins and Fletcher, 2003; Panksepp and Huber, 2004; Nathaniel et al., 2009; Huber et al., 2011). Findings from most of these studies reveal that mammalian drugs of abuse typically exploit the natural reward systems, which align with the species' adaptive needs. These drugs function by supplanting the individual's inherent pursuit of its basic needs, such as nourishment, shelter, and reproduction, with a search for the drug instead (Koob and Le Moal, 2001). Findings from these studies provided the opportunity to investigate common neural substrates underlying reward in a model system that has previously shown remarkable success under similar conditions and, to date, has provided major insights into wide-ranging behavioral occurrences. The first part of this review discusses the core neural pathways associated with drug addiction. The importance of invertebrate model systems in drug addiction research is then highlighted. Finally, a series of experiments that support crayfish as a powerful invertebrate model system for the study of drug addiction are discussed.

NEURAL PATHWAYS IN DRUG ADDICTION

Dopamine is considered the primary neural pathway underlying the neural causations of excitement, curiosity, and exploration (Alcaro et al., 2007). Several studies in the past have challenged a unitary role of the pathway in "pleasure." The common neural pathways surrounding mesolimbic dopaminergic neurons are commonly thought to mediate subjective reward and maintain reinforcement processes via hedonic affect (Schultz, 1997). Dopamine alters behavior via incentive salience in which motivational components are applied to stimuli that have shown to be rewarding in the past (Johanson et al., 1976; Robinson and Berridge, 1993; Spanagel and Weiss, 1999). Mesolimbic and neostriatal dopamine systems exhibit residual reward capacity even after depletion of dopamine, which demonstrates a value in learning that is independent of hedonia and strict reward-based learning (Berridge and Robinson, 1998). The concept of "wanting" has been defined from the idea of reward-related stimuli conferring a motivational value to an organism, which is distinct from hedonia (Panskepp, 1998; Panksepp, 2005). The "wanting" mechanism may be modulated by dopamine systems via perceived attractiveness, rather than the traditional view of receiving pleasure, or "liking" a stimulus.

The distinction between "wanting" and "liking" is important as it appears that drug-mediated dopamine responses progress by "wanting" something more but "liking" it less (Robinson and Berridge, 2001; Berridge and Robinson, 2003). Drugs can be associated with certain contextual cues, such as a novel environment. For example, when an organism is conditioned to receive a psychoactive drug paired with a sensory cue, associated neural functions are activated in response to the environmental cue. In the absence of the drug itself, the effect goes so far to re-activate and sustain drug seeking behavior (Davis and Smith, 1976; Cervo et al., 2003; Burbassi and Cervo, 2008).

The dopaminergic pathways are responsible for feelings of desire and reward in humans through their influence on the ventral tegmental region, medial forebrain bundle and the nucleus accumbens (Alcaro et al., 2007), and can modulate compulsive behavior characteristic of drug addiction in several mammalian models. Dopamine is also implicated in a more direct learning process, in which mesolimbic dopamine neurons fire unconditionally in affiliation with natural rewards often associated with survival. Over time, however, this dopaminergic activity will shift from firing in response to the reward itself to firing in response to the cue that is predictive of the novel reward (Schultz, 1997; Vanderschuren and Kalivas, 2000). Although reward can be grouped into a few separate processes; an object's incentive value, the connective learning process of predictive cues and the object of attraction including the object's ability to produce hedonism are distinct in their own way and they each relate to a dopaminergic response that reinforces reward (Wise, 1998; Ikemoto and Panksepp, 1999; Kelley, 1999; Everitt et al., 2001; Panksepp and Huber, 2004). It is seemingly paradoxical, that humans and animals are susceptible to addictive effects of cocaine, a neurotoxic chemical that has been shown to be evolutionarily adapted to protect the coca plant from insect herbivory by interfering with motor control in the organisms that consume coca plant (Nathanson et al., 1993). The dopaminergic system should be affected by cues that provide reward, not a plant neurotoxin that is designed to thwart predation. Several theories have been proposed that attempt to provide an evolutionary explanation for this phenomenon, ranging from co-evolution of herbivores and plants, to simple fundamental differences in response to the chemical by mammals compared to arthropods (Nathanson et al., 1993).

INVERTEBRATE MODEL SYSTEMS IN DRUG ADDICTION RESEARCH

The introduction of invertebrate model systems in evolutionarily relevant studies of drug-induced reinforcement, compulsion, withdrawal, reinstatement, and addiction has greatly broadened this field of research. These systems have shown to be powerful tools in the understanding of the neuroanatomical and behavioral processes underlying the addictive process. Benefits of invertebrates, aside from being more cost effective, offering reduced moral concerns, and behaviors patterned by experimentally accessible neural structures, are shared homologies with mammals in the key neurochemical aspects of reward, including receptor elements (Hen, 1992a, 1993), neuropharmacology (Tierney, 2001), mechanisms of action (Vernier et al., 1995, 1997), deactivation (Pörzgen et al., 2001), and association with similar behavioral contexts (Kravitz et al., 1980; Kravitz, 2000). Monoamine systems developed during the transition to metazoan life, where they were used to adapt functions of individual cells to disturbances within their environment (Gillette, 2006). Importantly, dopamine and serotonin receptors predate the chordate lineage (Hen, 1992b; Peroutka and Howell, 1994; Vernier et al., 1995; Walker et al., 1996), and divergence has given rise to considerable diversity in specific subtypes within different lineages, along with some unique differences in receptor subunits and pharmacological properties in both vertebrates and invertebrates. As a result of the divergence during evolution, mammals utilize oxidation and methylation while flies use N-acetylation and β-alanylation

for dopamine (DA) metabolism (Yamamoto and Seto, 2014). Indeed, flies lack the genes required to synthesize norepinephrine and epinephrine, and these are two major catecholamines derived from DA that function in neuromodulation signaling in mammals (Yamamoto and Seto, 2014). A cloned dopamine receptor from *D. melanogaster* has similar structural and functional properties with vertebrate D1-type receptors, but the pharmacological properties are very different (Gotzes and Baumann, 1996; Schetz et al., 2011). The characterization of the sensitivity of *D. melanogaster* to cocaine in an *in situ* hybridization study demonstrates that dopamine transporter (dDAT) lacks all the structural components that are found in the mammalian catecholamine transporters (Pörzgen et al., 2001). Moreover, cocaine displayed a lower affinity for dDAT when compared with serotonin transporter (Pörzgen et al., 2001). This study provides evidence that the structural and pharmacological profiles of dDAT is different from the DAT of vertebrate species. In addition, it indicates that injected cocaine, methamphetamine or morphine agonists or antagonists may function differently in vertebrate and invertebrate models of addiction. Despite the differences that exist between vertebrates and invertebrates, crayfish, *D. melanonogaster* and other invertebrate model systems will continue to provide new insights into the regulatory mechanisms of DA signaling drug addiction research.

With the expansion of drug-addiction research into invertebrate models, identification of behavioral stereotypes and profiles have become evident (Palladini et al., 1996; McClung and Hirsh, 1998; Torres and Horowitz, 1998). Fruit flies are a popular model system and have been shown to behaviorally sensitize in a fashion similar to that of the mammalian neurochemical and behavioral response to psychostimulants (Pierce and Kalivas, 1997; Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999). Behavioral sensitization in fruit flies is regarded to have an opposite effect of tolerance and is characterized by an increased intensity of drug cravings and associated behaviors (Robinson and Berridge, 1993). Strengthening the argument for invertebrate models, an important commonality between the two models suggests that catecholamine circuits in flies bear a strong resemblance to the mammalian sensitization process (Wolf, 1999; Wolf and Heberlein, 2003). For behavioral sensitization to occur in both flies (Li et al., 2000) and rats (Kalivas, 1995), stimulation of the pre-synaptic monoamine sites must occur. The post-synaptic sites also play an important role in the cocaine response as flies that under-express these receptors exhibit a reduced response to an initial exposure to the drug (Li et al., 2000). The opposite is true for mutants that over-express the receptor. In each of these mutant cases, the flies will not sensitize as the wild-type flies do. Vertebrate dopamine receptor antagonists can block cocaine-induced behaviors in fruit flies (Torres and Horowitz, 1998) and planarians (Palladini et al., 1996), strongly suggesting that dopamine is implicated in the resulting altered motor behaviors. Tyramine has been revealed as a vital part of the sensitization process in a number of animal models, including drosophila. Mutant individuals exhibiting lowered amounts of this amine are affected normally by the initial effects of cocaine but are less likely to sensitize. An increase in the individual's tyramine will result in a stereotypical sensitization akin to the wildtype counterparts (McClung and Hirsh, 1999). The *per* gene has an interactive role with tyramine, in that those lacking the gene will not undergo a normal sensitization process when stimulated with a vertebrate D2 agonist (Andretic et al., 1999; Andretic and Hirsh, 2000). The recent work revealing the activity of tyramine and the *per* gene in invertebrates has suggested that these processes could be conserved across a wide range of taxa. Tyramine has been likened to amphetamine's pharmacological profile as it inhibits membrane transporter uptake and alters synaptic catecholamines (Sitte et al., 1998). This work on the transcription of the *per*

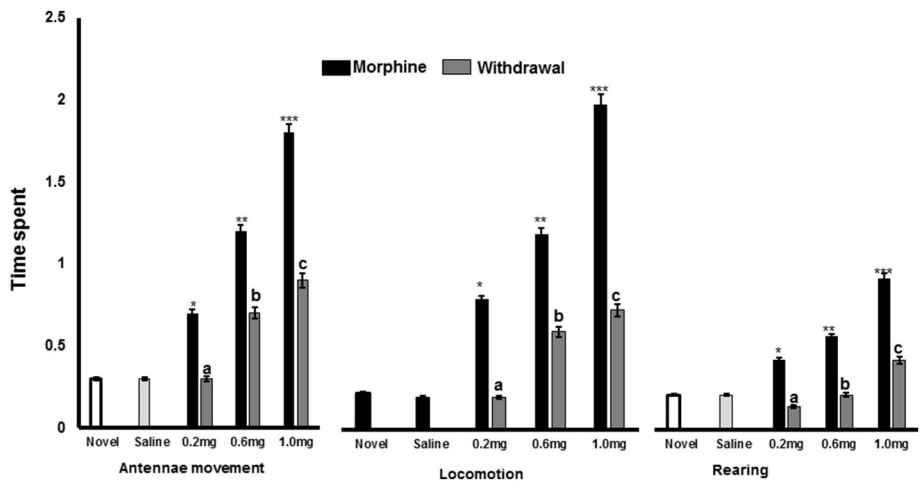

FIGURE 1 | Effects of repeated morphine injections for 5 days on crayfish exploratory behaviors. Results are presented as mean percentage of time. *Post hoc* analysis revealed differences for 0.2 mg/kg ($^{a}P < 0.01$), 0.6 mg/kg ($^{b}P < 0.001$), and 1.0 mg/kg ($^{c}P < 0.0001$) for antennae movements, locomotion, and rearing behaviors. Morphine injections increased antennae movements, locomotion and rearing behaviors for 0.2 mg/kg ($*P < 0.01$), 0.6 mg/kg ($**P < 0.001$), and 1.0 mg/kg ($***P < 0.0001$) doses.

gene has led to its demonstration in mammalian dorsal striatal regions receiving input from midbrain dopaminergic neurons (Nikaido et al., 2001). A recent study (Northcutt et al., 2016) identified genes for 34 distinct ion channel types, 17 biogenic amine and 5 GABA receptors, 28 major transmitter receptor subtypes including glutamate and acetylcholine receptors and 6 gap junction proteins—the innexins in the nervous system of Jonah crab (*Cancer borealis*) and the American lobster (*Homarus americanus*). These genes are associated with neural function in the crustacean systems and could provide important new insights to understand the organization of circuits in the control of behaviors. Other recent studies (Søvik et al., 2014; Zhu et al., 2014; Davies et al., 2015; Grotewiel and Bettinger, 2015; Hawkins et al., 2015; Engleman et al., 2016) indicate that an invertebrate system is a powerful tool that can be used to investigate the neuroanatomical, molecular and behavioral processes underlying the addictive process. Highlighting these accomplishments is vital in showing how simpler model systems can lead to exploration and discovery in mammalian systems as well.

The desire to more firmly establish invertebrate models in the study of drug addiction is driven by the lower cost and easy genetic manipulability of invertebrate models. To prove their effectiveness as a model, the biological and behavioral overlap between the two separate model systems needs to be demonstrated. The invertebrate model has been well established in the rewarding properties for psychostimulants (Wolf, 1999; Kusayama and Watanabe, 2000; Panksepp and Huber, 2004; Müller et al., 2007), opioids (Vanderschuren et al., 1997; Srivastava and Singh, 2006; Nathaniel et al., 2009, 2010), alcohol (Parsons, 1980; Bellen, 1998; Cadieu et al., 1999; Abramson et al., 2000, 2004), nicotine (Singaravelan et al., 2005), and caffeine (Singaravelan et al., 2005). Analogous to mammalian models, invertebrates also exhibit behavioral and motor stereotypes after the administration of cocaine. These studies show that fruit flies (McClung and Hirsh, 1998; Torres and Horowitz, 1998) and planarians (Palladini et al., 1996) exhibit increased locomotion and appetitive activities (Bellen, 1998; Torres and Horowitz, 1998; Wolf, 1999; Kusayama and Watanabe, 2000) which strongly resemble corresponding behaviors in mammals. Fruit flies have also been shown to demonstrate functional tolerance via a central nervous system adaptation with the administration of ethanol, mimicking mammalian tolerance and behavioral adaptation (Scholz et al., 2000). Land snails learn to self-administer electric current pulses into areas of the brain associated with sexual behavior (Balaban and Chase, 1991) and not administer treatments for areas controlling escape. This suggests that land snails feature distinct pathways involved with reward and punishment (Balaban, 1993; Balaban and Maksimova, 1993). Planarians exhibit susceptibility to place conditioning, as individuals will switch to non-preferred environments if it is paired with a psychostimulant. This effect could be subsequently blocked by administering selective vertebrate D1 and D2 antagonists (Kusayama and Watanabe, 2000).

CRAYFISH AS AN INVERTEBRATE MODEL OF DRUG ADDICTION RESEARCH

Some crayfish-specific benefits in drug addiction studies includes a body size that allows for easy handling and a relatively simple neuroanatomical composition. Moreover, the crayfish

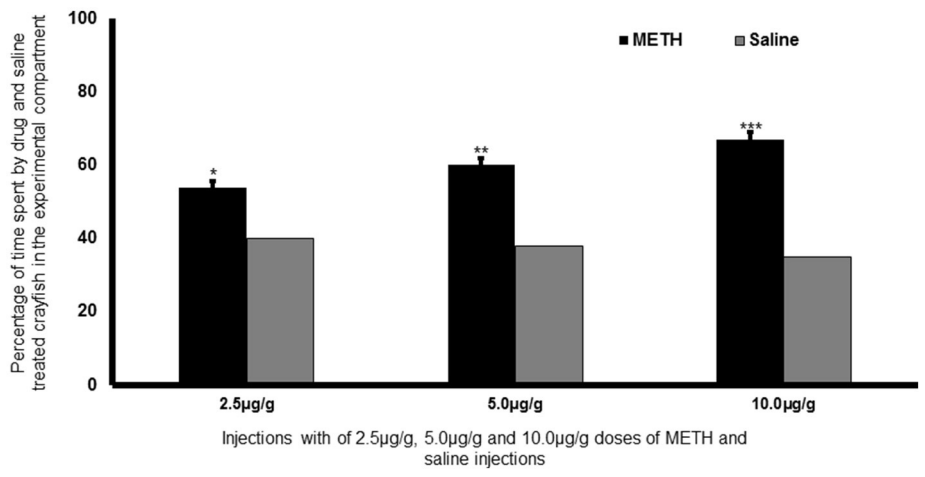

FIGURE 2 | Repeated infusions of METH induced CPP in crayfish in the hard-textured experimental arena. There was a significant preference for the hard-textured compartment vs. soft textured-compartment following 5 days of injections with 2.5, 5.0, and 10.0 μg/g doses of METH, such that a conditioned place preference was established. *Post hoc* test comparison indicates that crayfish treated with 5.0 and 10.0 μg/g (***$P < 0.05$ and **$P < 0.01$) were higher and different from the crayfish treated with 2.5 μg/g of METH (*$P < 0.001$) when compared with the saline-paired crayfish. The METH-conditioning effects were shown when crayfish that were treated with METH were paired with the naturally non-preferred hard environment. ANOVA [$F_{(4, 30)} = 21.13$; $P < 0.001$] reveals a significant effect, indicating a larger amount of time being spent in the METH-paired, hard-textured compartment when compared to saline conditioning, such that a conditioned place preference was established. The ANOVA factor revealed that the METH conditioning effect on crayfish was high (statistical power; $1 - \beta = 1.00$) indicating that METH-induced CPP can be consistently replicated with a high degree of reliability.

amine system consists of fewer than 1,000 neurons, including 30–35 dopamine neurons in the brain and nerve cord (Furshpan and Potter, 1959; Tierney, 2001), axons with far reaching projections and large somata (Tierney et al., 1999), and a complex and easily identifiable behavioral set that offers convenient experimentation. Crayfish have already proven their effectiveness in exploring the proximate neural mechanism of behavioral decisions (Mulloney, 2003) and neurochemical mechanisms in neuroethological studies (Panksepp and Huber, 2002), showing their diverse uses outside of modeling reward to psychostimulants. The extensive usage of crayfish and lobster in various neuroethological studies (Livingstone et al., 1981; Edwards et al., 2003) has led to their use in studies for drug reward. The neuroanatomical and physiological characteristics of the crayfish allow for easy accessibility in pharmaco-behavioral manipulative studies (Huber and Delago, 1998; Panksepp and Huber, 2002), and evidence for conserved monoamine re-uptake systems in invertebrates (Corey et al., 1994; Demchyshyn et al., 1994; Pörzgen et al., 2001) showcase their ability to demonstrate mechanisms of reward resulting from psychostimulant administration (Robinson and Becker, 1986).

An initial set of experiments (Panksepp and Huber, 2004) characterized behavioral and locomotor effects for intracardial infusions of cocaine and amphetamines. For example, introduction of cocaine produced rapid backwards walking, waving of the claws, and escape behavior, such as tail flips. "Static posturing" was exhibited where the crayfish flexes the abdomen and walking legs, with claws pointed anteriorly and downward. Amphetamines induced muscle tremors in the walking legs, as well as the crayfish moving to the corner of the aquarium and appearing to investigate the surrounding with its antennae. A subsequent study of morphine injections resulted in an overall increase of exploration of the environment with recognizable patterns of locomotion and antenna movements (Nathaniel et al., 2010). Stimulated by tactile and olfactory cues to the antennae and antennules, this information is processed by the olfactory lobes and modulated by serotonin and dopamine (McMahon et al., 2005; Sullivan and Beltz, 2005; Patullo and Macmillan, 2006). Moreover, this site is recognized for its role in the rewarding action of cocaine and other psychostimulant addictive drugs (Nathaniel et al., 2012b).

DRUGS OF ABUSE AUGMENT STEREOTYPIC BEHAVIORS (UNCONDITIONED STUDIES)

Exploration is a major component of the reward system that exists in the crayfish model of drug addiction. An expression of appetitive motivational states, exploration entails various

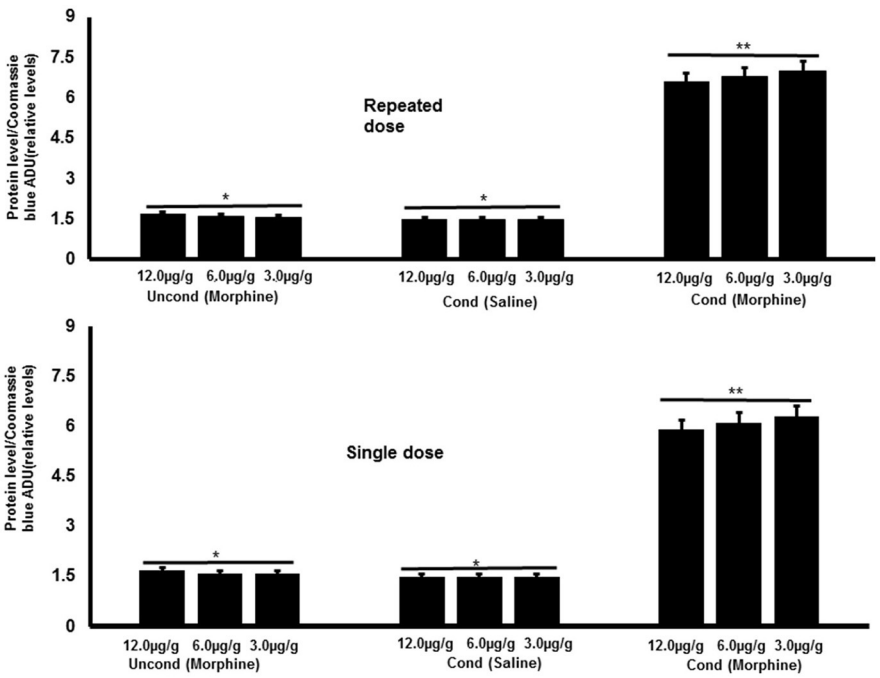

FIGURE 3 | The effect of morphine (3.0, 6.0, and 12.0 μg/g doses) and different environmental treatments on the alteration of c-Fos protein expression for the single and repeated drug treatments regime. $N = 7$ for all treatment doses (3.0, 6.0, and 12.0 μg/g) for each environmental treatment. Normalization was determined with signal intensities of c-Fos proteins to the gels stained with Coomassie blue as a ratio to produce relative abundance units (Dosimetry; ADU). Levels of c-Fos proteins were expressed as a ratio of ADU value to the whole protein in Coomassie blue-stained gels. For the single morphine treatment, there was a significant effect of the environment [$F_{(1, 54)} = 90.23$, $P < 0.001$] such that morphine conditioned environment significantly increased c-Fos (**$P < 0.05$) when compared with the effect of conditioned saline (*$P < 0.05$) or morphine unconditioned environment (*$P < 0.05$). The effect of the environment was also significant for the repeated treatment [$F_{(1, 54)} = 50.25$, $P < 0.001$], such that there was a significant effect of morphine conditioned-environment on the alteration of c-Fos expression (**$P < 0.05$) when compared with saline conditioned (*$P < 0.05$) or morphine unconditioned environment (*$P < 0.05$).

approaches in seeking a reward or positive outcome. Mammalian drugs of abuse promote unconditioned behavioral responses along with increased exploratory activity through approach behaviors (Panksepp and Huber, 2004). Approach behaviors such as the use of tactile and visual information are displayed in everyday life when searching for natural rewards such as food and shelter. In other words, mammalian drugs of abuse are particularly powerful in their ability to gain control of exploration behaviors, as the brain cannot distinguish whether reward circuits are being activated by genuine natural reward stimulus (such as food and shelter) or are being falsely triggered by psychostimulants, particularly amphetamine, cocaine, and morphine (Nathaniel et al., 2012b). When injected with drugs, the neural processes involved in appetitive motivation are stimulated and exploratory behavior is enhanced. The specific and differential effects of psychostimulant drugs (cocaine, amphetamine), and opioids (morphine) on the unconditioned behavioral response of crayfish at different doses over a period of 3 days was investigated (Nathaniel et al., 2012b). There was a significant effect of drugs on mobility when compared to the control group irrespective of drug. In a conditioning testing, morphine significantly increased locomotion at different doses (0.2, 0.6, and 1.0 mg), while locomotion was reduced in crayfish following repeated saline injections or withdrawal for 5 days in the previously morphine paired gravel background arena (**Figure 1**). This result indicates that paring with saline in the absence of morphine provided measures of the incentive properties of morphine in crayfish. For this reason, the reduction in exploratory behavior in the absence of response contingent drug availability probably led to the observed decline in the significance of the drug-paired stimuli in crayfish. Exploration of the environment as shown by patterns for locomotion, rearing and antenna movements increased in crayfish that were tested in the gravel environment, compared to crayfish that were tested in the plain background environment. The results indicate that novel stimuli can directly promote exploratory behaviors that are typically used to search for natural rewards.

In a qualitative analysis of drug-induced stereotypic behavior in crayfish, all three drugs produced distinct stereotypic behaviors. Following the cocaine injections at both low and high doses, crayfish adopted a static posture with legs flexed below the thorax and claws held downward. They remained static only

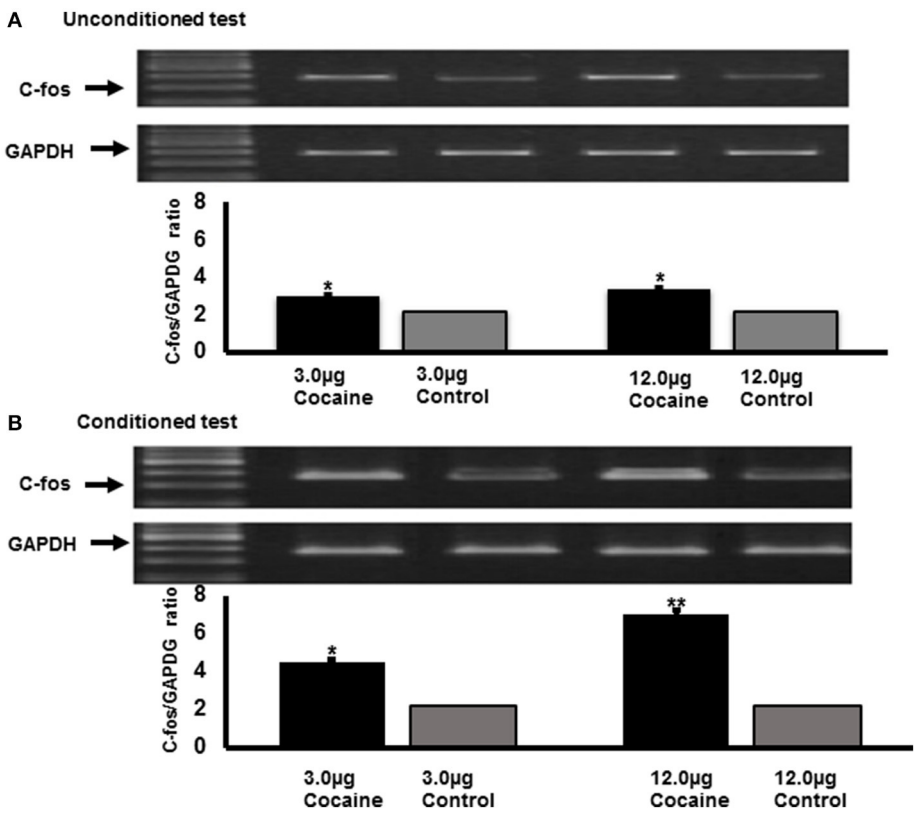

FIGURE 4 | C-Fos mRNA alterations in cocaine treated animals (3 and 12 µg/g) in unconditioned **(A)** and conditioned environment **(B)**. The effect of cocaine on c-Fos mRNA expression was measured by quantitative RT-PCR (Top panel) and normalized with GAPDH (panel below). Data represent mean ± S.E.M. at 35 min following CPP and non-conditioning after 5 days of cocaine injections ($n = 9$). Different doses of cocaine (3 and 12 µg/g) induced a significant [$F_{(3,23)} = 62.05$, $P < 0.001$] change in c-Fos mRNA expression in unconditioned treatment with cocaine when compared with the control animals without cocaine injection. The effects of 3 and 12 µg/g doses of cocaine were not significantly different (3 µg/g, $^{*}P < 0.05$; 12 µg/g, $^{*}P < 0.05$). The conditioning effect of cocaine was significant [$F_{(3,27)} = 92.12$, $P < 0.001$] when compared with the control group. The expression of C-Fos mRNA was significantly higher at a higher dose of cocaine (12 µg/g, $^{**}P < 0.05$) when compared with a lower dose (3 µg/g, $^{*}P < 0.05$).

for a few minutes before becoming mobile again. Following the low dose amphetamine injection, crayfish slowly approached the corners of the aquarium and consistently contacted the walls with their antennae. There was no static posture following the high dose amphetamine injection, but walking leg tremors, grooming, and perimeter exploration were observed. Low dose of cocaine injections produced enhanced rearing, antennae movement, and exploration of the corner of the experimental aquarium (Imeh-Nathaniel et al., 2017). A prior study investigated the effect of cocaine on specific locomotive traits (Nathaniel et al., 2012a), where intrapericardial injections of repeated doses of cocaine over the course of 3 days, decreased dose dependent lingering, increased speed of locomotion, distance traveled, and mobility, as well as increased immobility. This result suggests that each sub-component of locomotion is targeted by the effects of cocaine. The increased immobility is attributed to a potential desensitization of the involved receptors. These results revealed cocaine can produce distinct effects on movement and non-movement activities, indicating that cocaine impacts crayfish behavior in a way that is more specific to sub-locomotion components facilitated by the injected drugs.

Since repeated injections of cocaine are known to alter patterns of locomotion in crayfish, other studies in crayfish determined the relationship of single and repeated morphine injections on immediate and long-term effects of unconditioned behavior in crayfish. Significant effects of dose and time for single and repeated morphine treatments compared to saline controls, produced comparable long-term effects on locomotion. Even 5 days post treatment, these effects were maintained. These findings suggest that single and repeated doses of morphine can induce long-term behavioral sensitization including grooming, tail-flipping, movement of mouthparts, continuous exploration of aquarium corners, and mild tremors in the walking legs (Nathaniel et al., 2009).

NOVEL STIMULI DIRECTLY AUGMENT EXPLORATION AND APPETITIVE MOTOR PATTERNS IN CRAYFISH (CONDITIONED STUDIES)

Drug addiction studies in humans, mammals, and more recently, crustaceans, utilize conditioned place preference (CPP) paradigms to examine the rewarding effects of mammalian drugs of abuse. CPP illustrates that a psychostimulant paired with an environmental cue increases preference for the latter, with dopamine neuronal activity shifting from direct association with the stimulant to the presentation of the environmental cue (Waelti et al., 2001). In such instances, even in the absence of the drug, the conditioned cue is sufficient to re-establish drug seeking behaviors in an individual (Davis and Smith, 1976). In crayfish a CPP protocol was used to examine unconditioned preferences for environments, followed by a drug-paired, conditioning phase and CPP test.

In three different doses (2.5, 5.0, and 10.0 μg/g), methamphetamine induced a significant CPP for the hard-textured environment (**Figure 2**), with the higher doses (5.0 and 10.0 μg/g) of both drugs having a more pronounced effect of CPP. CPP was not established in the initially preferred soft textured environment when compared with the control group (Imeh-Nathaniel et al., 2016). Similarly, in a study investigating the effects of different visual cues on CPP when paired with morphine, crayfish initially showed an unconditioned preference for a white walled environment (Dziopa et al., 2011). After conditioning, crayfish showed preference for striped environment when paired with single and multiple morphine injections, at all doses.

These results were consistent with a previous study when crayfish was paired with environment showing textural differences (Nathaniel et al., 2009). The similarity in findings from these studies indicate that irrespective of the drug or its dosage, mammalian drugs of abuse prove to be rewarding to the crayfish when paired with a textural or visual environment. The significance of this observation is that the textural and visual stimuli are novel to the crayfish. An important question relevant to this review is, "how do crayfish find the hard texture novel?" As part of adaptation, the crayfish's brain is able to integrate appetitive motor responses such as seeking out for food and shelter. Their preference for a hard environment may be related to the intrinsic capability to use tactile cues, such as in the test environment, for survival. It is possible that crayfish might have explored and perceived the hard texture to be relatively attractive or novel when compared with the soft environments, suggesting that stimulus salience when paired with drugs indicates the significance or noticeability of the hard texture or striped visual environment as novel by crayfish. Similar findings were shown when varying doses of amphetamine were injected into the crayfish head ganglion during exposure to a novel environment (Alcaro et al., 2011). The administration of psychostimulants directly into the head ganglion enhanced active exploration of the novel environment. This indicates that the dopamine-mediated appetitive motivational states stimulated by drugs of addiction, conditions animals to pursue objects and environments for survival. It is possible that such an effect may enhance an adaptive behavior including exploration, and the acquired affective incentive value for cues associated with natural and drug rewards (Imeh-Nathaniel et al., 2016).

Exploratory behaviors such as locomotion, rearing, and antennae movements enhanced the ability of crayfish to seek rewards. A previous study characterized morphine-induced conditioned exploratory patterns and quantified atypical behaviors associated with termination of drug administration (Imeh-Nathaniel et al., 2014). In this study, when morphine was paired with a novel environment, locomotion, antennae movements, and rearing were enhanced in crayfish. Changes in exploratory behavior were diminished when morphine treatments were terminated and saline injections were given instead for five days. Locomotion was still elevated in withdrawal animals when compared to the saline control suggesting that morphine priming can reinstate an already established increase in locomotion irrespective of dose. This observation reveals the effects of morphine induced locomotion as well as the ability to restore exploratory behavior after extinction (Imeh-Nathaniel et al., 2014).

MOLECULAR ALTERATIONS ASSOCIATED WITH DRUG CONDITIONED REWARDS IN CRAYFISH

The conditioned association between environmental cues and drug-activated reward circuitry are known to be a key point in drug relapse in humans (Childress et al., 1988; Zahm et al., 2010). The neuronal alterations that occur in this process are linked to certain transcription factors, such as ΔFosB and the cAMP-response component binding protein (CREB), whose activity is altered through changes in gene expression. The c-Fos proteins (catecholamine reuptake transporters) are linked to the morphine response by regulating *Fos* gene expression levels in dopamine neurons (Curran et al., 1996). The c-Fos protein has been studied in mammals in regard to activation of brain regions by drugs of abuse and, when activated, plays a role in signal transduction and genetic modifications. This protein has not been studied extensively in invertebrate models, but an investigation of c-fos gave insights into the molecular alterations associated with drug reward in invertebrates (Dziopa et al., 2011). The single and repeated injections of morphine at 3.0, 6.0, and 12.0 μg/g (**Figure 3**) in an unconditioned experiment did not reveal a significant increase in c-Fos expression. However, in the conditioned experiment, 5 days of repeated morphine treatments paired with a novel environment produced a significant increase in c-Fos expression. The intensities in c-Fos bands were increased in both single and repeated morphine treatment groups, but were higher in the repeated morphine treatment group. The levels of c-Fos expression remained constant in the control group. This result suggests that novel environment when paired with drugs impacts gene regulatory processes (Dziopa et al., 2011).

In a similar study with cocaine (Nathaniel et al., 2012b), there was a significant increase in the expression of c-Fos following the injections of 3.0 and 12.0 μg/g doses of cocaine in a conditioned test when compared with the unconditioned test (**Figure 4**). Maximal intensities in c-Fos bands were observed with a high dose of cocaine (12.0 μg/g) when compared with a low dose (3.0 μg/g). Collectively, these results show that cocaine-induced reward paired with a hard environment is associated with the enhancement of c-Fos mRNA expression in the accessory lobe of a crayfish (Nathaniel et al., 2012b). This indicates that cocaine produced a context specific reward in the novel hard-texture environment, and that the repeated injections of the drug are also associated with the increase of c-Fos mRNA expression in the accessory lobe of the crayfish. In mammals, c-Fos mRNA markers have been reported as an indication of activated brain regions associated with drug usage, and at specific targets (Zawilska, 2003; Perrotti et al., 2004; Yamada et al., 2007; Zavala et al., 2007; Xu, 2008; Velázquez-Sánchez et al., 2009; Watanabe et al., 2009). The increase of c-Fos mRNA expression in the accessory lobe of the crayfish brain suggests that the accessory lobe of the crayfish may play a role analogous to the higher brain structures in the frontal regions of the cerebral cortex of mammals. Such areas include the medial prefrontal cortex, anterior cingulate cortex, or orbitofrontal cortex, responsible for high-order choices made within its environment in regard to the search for food, shelter or conspecifics (Sandeman et al., 1992).

CONCLUSION

These studies offer insight into potential mechanisms that remain unexplored within the crayfish model in drug addiction research. Crayfish as a model organism features a highly modular, experimentally accessible nervous system, yet capable of substantial behavioral complexity. With strongly conserved evolutionary mechanisms for behavioral sensitization, drug dependence, and drug-induced reward seeking, crayfish demonstrate significant vulnerability to human drugs of addiction. Research in crustaceans thus offers a valuable perspective for studying the neural implementation of conserved behavioral phenomena, including motivation, escape, aggression, and drug-sensitive reward.

AUTHOR CONTRIBUTIONS

AS, VO, and LW, reviewed articles related to this manuscript and wrote the initial draft. AI-N, RH, and TN reviewed the contents, data and the final draft of the manuscript.

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Crayfish Self-Administer Amphetamine in a Spatially Contingent Task

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Natural reward is an essential element of any organism's ability to adapt to environmental variation. Its underlying circuits and mechanisms guide the learning process as they help associate an event, or cue, with the perception of an outcome's value. More generally, natural reward serves as the fundamental generator of all motivated behavior. Addictive plant alkaloids are able to activate this circuitry in taxa ranging from planaria to humans. With modularly organized nervous systems and confirmed vulnerabilities to human drugs of abuse, crayfish have recently emerged as a compelling model for the study of the addiction cycle, including psychostimulant effects, sensitization, withdrawal, reinstatement, and drug reward in conditioned place preference paradigms. Here we extend this work with the demonstration of a spatially contingent, operant drug self-administration paradigm for amphetamine. When the animal enters a quadrant of the arena with a particular textured substrate, a computer-based control system delivers amphetamine through an indwelling fine-bore cannula. Resulting reward strength, dose-response, and the time course of operant conditioning were assessed. Individuals experiencing the drug contingent on their behavior, displayed enhanced rates of operant responses compared to that of their yoked (non-contingent) counterparts. Application of amphetamine near the supra-esophageal ganglion elicited stronger and more robust increases in operant responding than did systemic infusions. This work demonstrates automated implementation of a spatially contingent self-administration paradigm in crayfish, which provides a powerful tool to explore comparative perspectives in drug-sensitive reward, the mechanisms of learning underlying the addictive cycle, and phylogenetically conserved vulnerabilities to psychostimulant compounds.

Keywords: addiction, amphetamine, invertebrate reward, crayfish, operant learning

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INTRODUCTION

The activation of natural reward pathways signifies the perception of a positive outcome in adaptive situations, such as when the individual manages to satisfy its demands for food, sex, or contact comfort (Kelley and Berridge, 2002). Prior studies have demonstrated that these circuits are sensitive to stimulation by a number of plant secondary compounds (Wink, 2015), even in the absence of any inherent beneficial outcomes (Koob, 2015). Cues experienced during such exposure, whether novel or previously encountered, acquire special salience and become labeled as rewarding. As vulnerable individuals enter an addictive cycle, they increasingly pursue

conditions that enhance access to both the drugs themselves and the cues with which they are paired (Robinson and Berridge, 2000; Hyman and Malenka, 2001). The commonly accepted view that addiction is an exclusively human and cognitive phenomenon, is erroneous, and has for far too long hindered the emergence of a comprehensive understanding of addiction processes. The ability to duplicate drug-associated neural properties and behavioral consequences in other mammals, both primate and non-primate, resulted in the use of an expanded taxonomic range in preclinical addiction studies (Deneau et al., 1969; Collins et al., 1983; Bergman et al., 1989; Spealman et al., 1989; Sanchis-Segura and Spanagel, 2006). More recently, interest has focused on the potential utility of invertebrate model systems as we have come to appreciate that addiction represents a much more fundamental biological phenomenon of associative learning than had previously been thought. This perspective becomes somewhat less radical when one considers that the majority of addictive substances are defensive plant alkaloids to deter insect herbivory (Wink, 2015). Invertebrate models including *Drosophila*, honeybees, nematodes, and recent work on crayfish, have significantly enriched perspectives on addiction research. This 'simpler systems' approach (Wolf and Heberlein, 2003; Burne et al., 2011; Søvik and Barron, 2013; Yartsev, 2017) capitalizes on the structural efficiency, and unique accessibility to experimental manipulation that is inherent in invertebrate nervous systems. Most importantly, invertebrate and vertebrate models (humans included) are united by the conserved nature of reward mechanisms, sharing the same neurotransmitter systems with homo- and paralogous receptors, and featuring matched signaling pathways underlying behavioral addiction (Hen, 1992, 1993; Vernier et al., 1995, 1997; Porzgen et al., 2001; Tierney, 2001; Tierney et al., 2003).

A host of advantages make decapod crustaceans (i.e., crayfish, lobsters) a very suitable, and historically successful, model organism for exploring the neural machinery of behavior. Molecular, neurophysiological, and neurobehavioral experimentation (Clarac and Pearlstein, 2007) on the mechanisms of natural and drug-sensitive reward profits from a highly modular neural structure, conserved monoaminergic, neuromodulatory systems, a relatively small number of large and individually identifiable neurons, and high sensitivity toward human drugs of abuse. Amphetamine (Alcaro et al., 2011), cocaine (Nathaniel et al., 2012a,b), morphine (Nathaniel et al., 2010), and cathinones (Gore et al., unpublished data) exhibit potent psychostimulant properties, which sensitize with repeated exposure (Nathaniel et al., 2010, 2012b; Dziopa et al., 2011). Moreover, in a conditioned place preference paradigm (CPP), these substances trigger the formation of strong associations between drugs and the cues with which they are paired (Panksepp and Huber, 2004; Nathaniel et al., 2009). Discontinuing drug access produces withdrawal (Nathaniel et al., 2009; Huber et al., 2011), and a single, small priming dose is sufficient to fully reinstate a drug-induced CPP following a period of abstinence (Nathaniel et al., 2009). The present work expands on recent findings in which crayfish quickly learned to avoid areas paired with mild electric shock punishment (Bhimani and Huber, 2016). Here we advance a novel system for automated drug self-administration in crayfish, and explore whether, and to what extent, amphetamine reward alters crayfish behavior in an operant conditioning paradigm.

Conditioned place preference paradigm provides a measure for the reinforcing nature of a drug. However, because it relies on behavioral responses to conditioned stimuli, it is only an indirect assessment of a drugs affective properties. A more direct metric for an individual's motivation to acquire drugs, and hence a drug's inherent reward strength, derives from changes in operant behavior during a self-administration paradigm. In such a scenario the subject is able to control drug delivery by performing a learned, operant task (Gardner, 2000; Deroche-Gamonet et al., 2004; Belin et al., 2009), where successful task completion delivers a bolus of the substance. The ability to associate performance of the operant behavior and its earned drug infusion, is facilitated by both precise timing of drug delivery as well as by a rapid physiological response. Although a significant challenge for many smaller invertebrate study systems (Søvik and Barron, 2013), a rapid and precise drug delivery via an indwelling cannula is quite achievable in crayfish.

Using a fully-automated approach to crayfish behavior in a learned spatial task, we first assess baseline, unconditioned space use of an arena featuring distinct substrate textures. In a second step, we then reward each entrance into a particular substrate region with a bolus of drug. The study aims to determine whether individual crayfish can learn to perform tasks that gain them infusions of amphetamine by using their movement patterns to specifically revisit areas of the arena paired with drug. Effective demonstration of such an operant, self-administration paradigm would permit direct measurement and comparisons of relative reward strength of human drugs of abuse in crayfish, a quintessential model for behavioral neuroscience research.

MATERIALS AND METHODS

Animals

Crayfish (*Orconectes rusticus*) were captured from the Portage River near Bowling Green, OH, United States (41.377965–83.475812). They were maintained in the laboratory under controlled environmental conditions in an aerated community tank (at 20°C, pH 7, 12 h L:12 h D) and fed twice a week with rabbit chow. Three days prior to the experiment, intermolt males (7–14 g) with all appendages intact were selected, individually housed in perforated plastic containers (Ø: 140 mm, ht: 70 mm), and placed in holding trays supplied with continuously circulating, filtered, aerated water from a large supply tank.

Experimental Procedure

Training trials were performed in a circular polyethylene arena (Ø: 0.5 m, ht: 0.25 m) with the floor divided into four quadrants of two different substrates arranged diagonally. Tiles of white Plexiglas presented a smooth, hard surface, while tiles coated with a white, polyester mesh (Nonadhesive Easy Shelf Liner, Duck

Brand, OH, United States) provided a soft, textured contrast. The arena was rotated between each trial. A custom, open-source video tracking solution (available for free download[1]) was used to record the movements of the test animal and to deliver a bolus of drug in real-time when the operant behavior was performed. Each experiment employed a new set of individuals, which were treated as described below.

Experiment 1 explored the unconditioned substrate preference, locomotion and arena use by drug naïve crayfish. For this experiment, spatial responses in two distinct groups of individuals that received saline injection either in the vicinity of supraesophageal ganglion ($n = 6$) or into the pericardium ($n = 9$) were recorded. Movements of saline treated individuals were recorded across a 5-h experimental time line. These provide the relevant baseline data for subsequent comparison with amphetamine-associated behavioral changes observed in Experiments 2 and 3.

The efficacy of amphetamine as a reinforcer under an operant conditioning paradigm was assessed in Experiments 2 and 3. In Experiment 2, the infusion cannula was implanted into the pericardial sinus for systemic application of drug at one of several dosages. Each experimental session lasted 3 h, during which movement of the individual into a quadrant with a particular texture earned a bolus of drug. Reinforcement was delivered for every instance of operant response. Following an operant response, a 5 s time timeout period was instated during which additional responses initiated did not result in drug infusions. Subjects (n = 12 per group) were randomly assigned to one of five drug dose categories. Under each dose category, animals were further classified either as: (1) Drug-Master individuals that received drug contingent to their entry into a particular substrate or (2) Drug-yoked animals that received an equal amount of amphetamine at the same time as the drug-master individual to which they were yoked. While the treatment animals had the opportunity to associate their action to the delivery of reward, individuals in the yoke group received drug infusions independent of their actions. Each drug dose level was thus evaluated in combinations of six master-yoke pairs. A saline group (n = 9) that received behaviorally contingent injections of saline served as the vehicle control.

The reward contingency for the two substrates (hard vs. soft) was counterbalanced among the individuals in each dose category. Learning of reward contingency was consequently evaluated for the hard substrate in three master- yoke pairs and for the soft substrate in another set of three master-yoke pairs. Experiment 3 was conducted in the same manner as that described above, except that the cannula for drug delivery was implanted directly over the supraesophageal ganglion (i.e., SEG, brain) of the crayfish. In this iteration the bolus was therefore delivered in close proximity to the neural tissues of the head ganglion, rather than reaching it indirectly via the general circulation. Previous work focusing on psychostimulant effects had demonstrated that injection in the head region resulted in stronger behavioral effects and a more rapid response for a given drug infusion (Alcaro et al., 2011).

[1]http://iEthology.com/

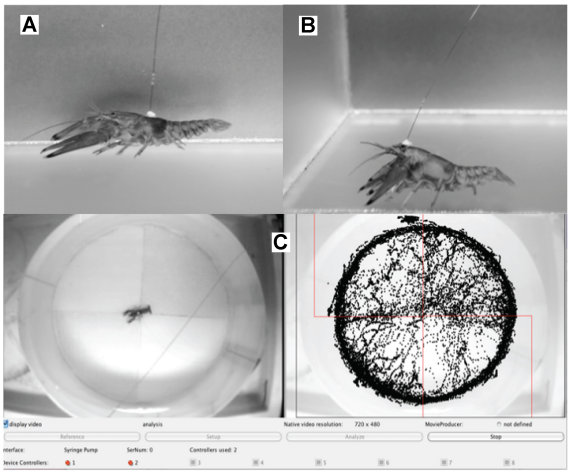

FIGURE 1 | Positions of the cannula for the two different anatomical locations. Cannula implanted in the pericardial cavity **(A)** vs. in the vicinity of the supraesophageal ganglion **(B)**. Movements of the animal were monitored in real time and visualized using a computerized tracking framework. Drug reward was automatically delivered following the occurrence of an operant response. The quadrants with reward paired entries on the representative tracking window **(C)** are outlined in red. Location of the animal (depicted as black dots on the tracking window) captured at a sampling rate of 2 Hz. Time stamps, x and y Cartesian coordinates, and instances of operant responses were obtained and saved into a text file for subsequent analysis.

Surgery

Prior to surgery, animals were cold anesthetized for 20 min in ice. Cannulae were implanted through the carapace to deliver drug either into the general circulation via the pericardial sinus (Experiment 2), or directly over the SEG (Experiment 3). Precise positioning of the cannula (**Figures 1A,B**) was informed through a series of preliminary dissections. For systemic infusion (Experiment 2) a 26.5 gauge needle was used to drill through the exoskeleton into the anterior end of the sinus, and slightly lateral of the midline, to avoid damaging the underlying heart. A 50 mm section of deactivated, fused silica material (Agilent 160-2655, i.d. = 50 μm, o.d. = 250 μm) was inserted through the opening such that 3 mm entered the pericardial sinus, and attached to the carapace with cyanoacrylate and bonding material. For Experiment 3 the cannula was placed over the SEG at the same insertion depth. Following surgery, the animals were allowed to recover overnight in their holding containers.

Drug and Injection Protocol

Tygon microbore tubing (Fisher Scientific ND 100-80, i.d. = 250 μm) was used to connect a 0.5 m section of deactivated, fine-bore, fused silica needle material (Agilent 160-1010, i.d. = 100 μm, o.d. = 190 μm, 0.5 m long) to the implanted animal stub on one end and the blunt-tipped needle on a 1 ml glass syringe (SGE Analytical Sciences, Model# 008100)

on the other. A syringe pump (Razel R-99E with R-ACC-6 Multi Micro Syringe Adapter) was positioned above the experimental arena, allowing concurrent drug application to multiple animals.

Doses of D-amphetamine sulfate (Sigma-Aldrich A 5880, St. Louis, MO, United States) were prepared in 125 mM saline (NaCl) and tested for their ability to support self-administration at two anatomical locations: pericardium (Experiment 2: five doses of amphetamine: 0.1, 0.3, 1, 3, and 10 μg/bolus), and supraesophageal ganglion (Experiment 3: three doses of amphetamine: 0.1, 0.3, and 1 μg/bolus).

Behavioral Analysis

Movements of the animal within the experimental arena were captured using the JavaGrinders tracking framework. The analog signal from an overhead camera (Sony HDR-HC5 HDV 1080i) was digitized via an A/D converter (Canopus ADVC-110, 720x480 pixel resolution) on an Apple Macintosh computer (iMac, OSX 10.7.4). A collection of freeware programming functions for the analysis of behavior (available for free download[1]) were employed to capture time-stamped coordinates in a 2D Cartesian plane at a sampling rate of 2 Hz (**Figure 1C**). A minimum distance of 3.5 pixels between captures was required for inclusion as a movement event, to distinguish these from actions associated with grooming bouts. Operant tasks were defined as all instances in which the test individual crossed from an unpaired substrate into a reward paired one. The syringe was controlled by the tracking framework via a serial interface (USB/serial adapter DB-9RS-232). Each instance of operant response triggered the infusion of a 5 μl bolus containing a particular treatment delivered over a period of 1 s. This automated system offered reliable and rapid response-reward pairing over the course of extended trials. Movement descriptors, operant behaviors, and drug delivery were extracted post-trial from the time-stamped data logged to a file. Enhanced locomotion necessarily emerges from unconditioned psychostimulant effects and thus inevitably results in increased rates of operant responses. To distinguish between unconditioned and conditioned psychostimulant effects we calculated the number of valid responses per distance traveled as a measure of how effective movements were used to activate the pump [i.e., operant index (OI)].

Statistical Analyses

Statistical analyses were conducted using R (Version 3.4.3, R Development Core Team, 2008). Levels of significance were set at $p \leq 0.05$ for all tests. Substrate and quadrant preferences were assessed using a two-tailed, within subject design. Since OI values were neither normal (Shapiro–Wilk W-test, $p < 0.001$) nor homoscedastic (Brown–Forsythe test, $p < 0.001$), a conservative approach was adopted and original values of the variable were replaced by their rank equivalents. For Experiments 2 and 3, each 3 h experiment was binned into 20 min intervals and a mean OI was calculated for each time segment and effect of reward contingency tested with a repeated measures design.

RESULTS

Experiment 1: Unconditioned Substrate Preference, Locomotion, and Arena Use

This study analyzed spatial responses in 15 saline treated individuals (six receiving brain infusions, nine receiving pericardial infusions) prior to, or in the absence of drug conditioning, across the 5-h experimental time line. These provide the relevant baseline data for subsequent analysis of amphetamine-associated behavioral changes, substrate preferences, locomotor activity, and space utilization summarized in **Figure 2**. When placed into the arena, drug-free crayfish spend much of their time following the circular outer wall, only occasionally leaving the periphery to cross the central, open portion of the arena. Initial walking speeds are consistent and high, occasionally interrupted by brief moments of hesitation when they approach the transition between substrate textures. Initial locomotion is paired with intense tactile and olfactory sampling indicative of exploration, but over the first hour mean speeds slow considerably as crayfish increasingly settle into stationary periods along the perimeter wall. Preferred places to settle appear to be the soft-textured side adjacent to a hard quadrant border. This is reflected in a significant preference for soft quadrants (mean p[soft] ± SE, $p = 0.581 \pm 0.015$), which begins to emerge as a significant effect (one-sample t-test versus a hypothetical population mean $p = 0.5$, $t_{[14]} = 5.2865$, $p < 0.001$)

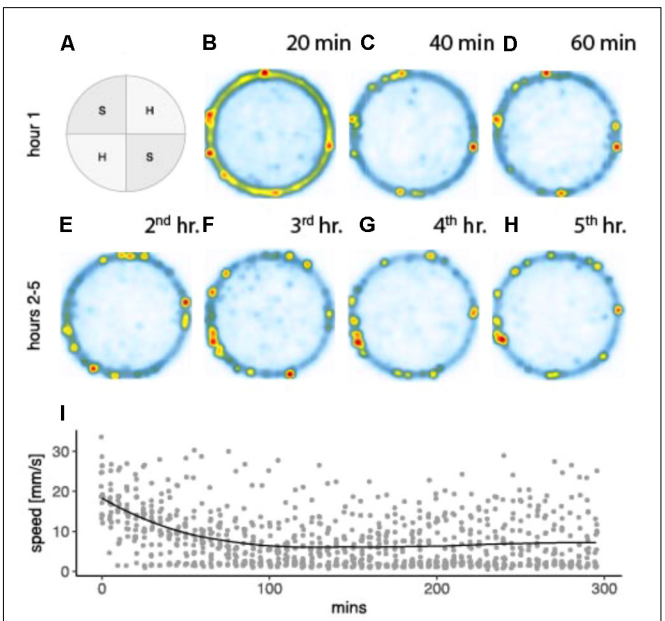

FIGURE 2 | Spatial preferences combined for 15 drug naive crayfish in an arena with two soft and two hard textured quadrants arranged diagonally **(A)**. Heat maps depict utilization distributions obtained from two dimensional kernel density estimation (*kde2d*, package MASS, R Version 3.4.2) for 20 min segments of the first hour **(B–D)**, and 60 min segments for hours 2–5 **(E–H)**. Pixel densities range from low (white and blue) to high (yellow and red). Mean walking speeds for 5 min time segments are plotted for the 5 h time line with geometric loess smoothing and estimated standard error region **(I)**.

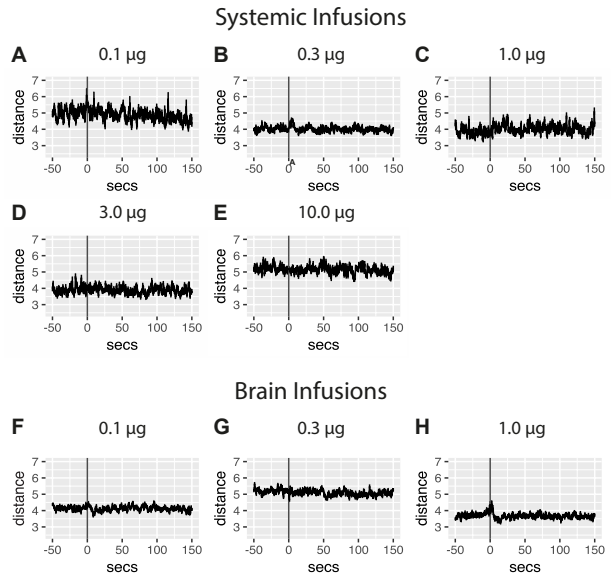

FIGURE 3 | Unconditioned psychostimulant effects on locomotion were examined with stimulus averaging of repeated amphetamine infusions administered to yoked controls. Traces are aligned to the time of infusion (gray line) with average measures of locomotion plotted 50 s prior to and 150 s past the administration of a 5 µl bolus containing one of several drug amounts into the pericardial sinus **(A–E)** or brain **(F–H)**. No effects of pericardial infusions were detected although more subtle changes in traces may exist in **(A)** 0.1 µg (451 infusions in five individuals), **(B)** 0.3 µg (1321 infusions in six individuals), **(C)** 1 µg (521 infusions in five individuals), **(D)** 3 µg (566 infusions in five individuals), and **(E)** 10 µg doses of amphetamine (647 infusions in six individuals). Averaged traces for distance traveled following infusions above the crayfish brain of **(F)** 0.1 µg (1290 infusions in six individuals), **(G)** 0.3 µg (1042 infusions in six individuals), and **(H)** 1 µg amphetamine (927 infusions in six individuals). Traces indicate a brief, dose-dependent psychostimulant effect, followed by a short period of psychodepression.

40 min into the trial. With their locomotor responses, control individuals earned saline infusions at a mean rate (± SE) of 30.43 infusions per hour. A repeated measures analysis confirmed that the rate of infusions was a direct linear function of locomotion (1.28 infusions per meter traveled $F_{[1,55]} = 1840.834$, $p < <0001$, adjacent $r^2 = 0.959$) and that this relationship remained constant over the 5 h time period ($F_{[4,55]} = 1.642$, $p = 0.177$).

Experiments 2 and 3: Unconditioned Psychostimulant Effects of Amphetamine

Individuals in the yoked groups received amphetamine infusions contingent on their master's operant responses and independent of their own behavior. In this group then, observed responses to the drug can thus inform amphetamine's unconditioned behavioral effects. Individuals from the brain master group earned an hourly average of 43.1 (0.1 µg), 34.8 (0.3 µg), and 30.9 (1 µg/bolus) infusions respectively. Infusions at the highest dose are accompanied by a brief, dose-dependent psychostimulant effect, followed by a short period of psychodepression (**Figure 3**). Data also demonstrate that pericardial infusions of amphetamine were unassociated with distinct changes in levels of locomotion.

Experiment 2: Pericardial Infusions – Effect of Reward Conditioning on Operant Responding

Treatment and yoke pairs for each dose category ($n = 6$ pairs/dose) were compared based on OI (Experiment 2; **Figure 4**) using a within-subject design. No clear distinction in the levels of operant responding between treatment and their yokes was observed for any of the doses assayed. While higher OI scores of treatment relative to the yoked group were observed, most prominently at the drug dose of 0.3 and 1.0 µg/infusion, they failed to reach statistical significance. For comparable dose categories, systemic amphetamine injection produced less distinct differences in OI scores between self-administering and yoke groups relative to brain infusions.

Experiment 3: SEG Infusions – Effect of Reward Conditioning on Operant Responding

The effect of injection site on reward strength was examined by comparing the previously described systemic injections (Experiment 2) with those infused near the brain (Experiment 3; **Figure 5**). Effects of reward contingency over the duration of the conditioning session was significant when examined using a repeated measures design (Treatment × Time interaction: $F_{[8,3]} = 68.29$, $p < 0.05$). OI scores of the treatment animals in the 1.0 µg dose group showed an increase after 1.5 h, whereas OI scores of the yoke remained unchanged across the trial. The evaluation of 3.0 and 10 µg/infusion doses were restricted to pericardial administration. When injected near the brain, these higher doses produced strong motor responses (including tail flips and excessive grooming), which precluded normal locomotion. The increase in OI scores of treatment- relative to the yoke groups was also observed for both the intermediate- (0.3 µg/infusion) and the lowest doses (0.1 µg/infusion) but was not statistically significant. OI scores of treatment and yoke groups appeared to be more similar when operant tasks were rewarded with lower doses of amphetamine, indicative of a dose-dependent increase in reward strength. The difference in OI scores between treatment and their yoked counterparts was maximum for the highest dose assayed.

DISCUSSION

Crayfish placed in a novel arena show enhanced levels of locomotion and antennal movements while actively exploring their surroundings. In the natural context, this active seeking drive is essential for encountering critical resources. As crayfish become familiar with their environment, a reduction in locomotion is observed, and animals tend to settle along the perimeter walls of the test arena. The ability of amphetamine to increase motor activity and stereotypy in mammals has been widely documented (Fog, 1969; Schiorring, 1971;

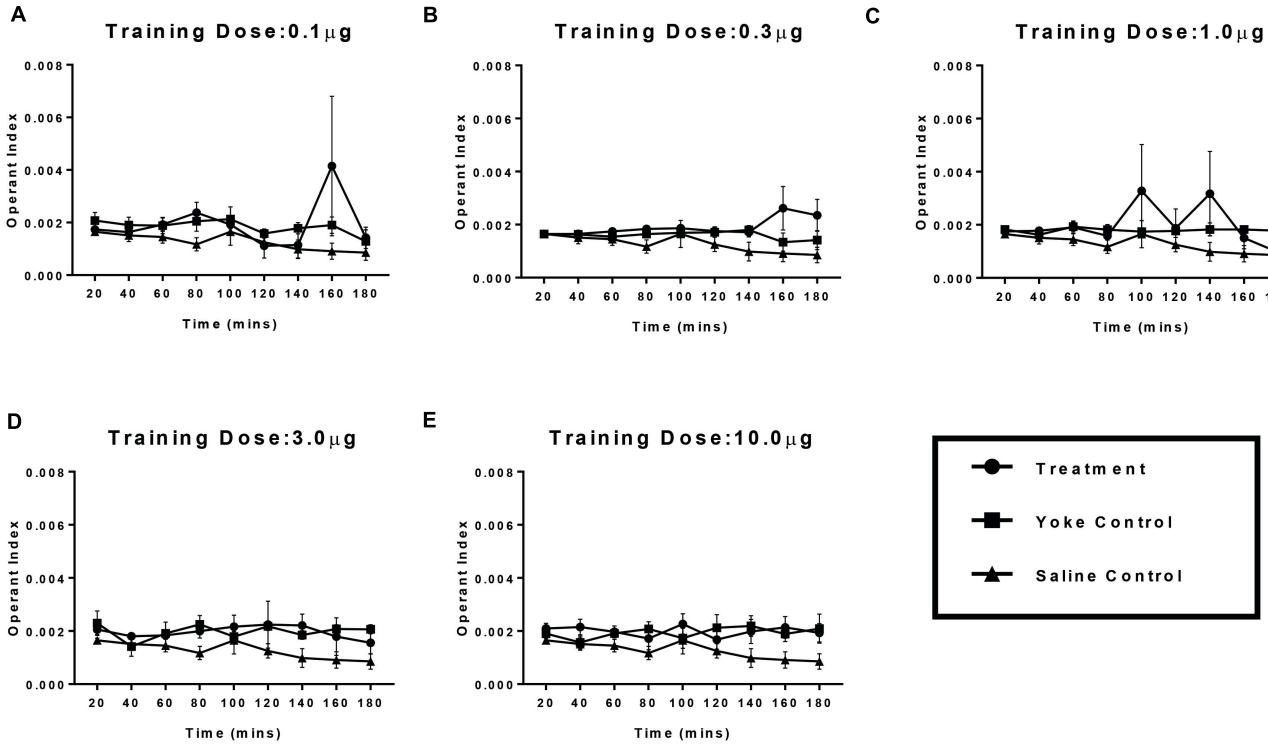

FIGURE 4 | Operant conditioning of a spatial task paired with amphetamine reward, delivered into the pericardial sinus. Treatment animals received amphetamine infusions upon entering a chosen substrate. Yoked controls received the drug infusions at time points identical to their treatment partners. Vehicle controls received contingent saline infusions. Learning of reward contingency was compared using operant index (OI) between treatment and yoke groups for each dose category **(A–E)**. Operant conditioning in crayfish receiving behavior contingent injections of amphetamine at one of several doses **(A)** 0.1 μg, **(B)** 0.3 μg, **(C)** 1 μg, **(D)** 3 μg, and **(E)** 10 μg doses of amphetamine into the pericardium is less robust than animals receiving the drug over the supraesophageal ganglion. Mean OI for 20 min time segments are plotted for the 3 h session with standard error of mean.

Segal and Mandell, 1974; Hoebel et al., 1983). Using the distance traveled by yoke individuals that experienced the drug in a non-contingent fashion, we found that the unconditioned effects of the drug did not vary in a dose-dependent manner. Levels of locomotion were identical for all dose categories. A lack of amphetamine-induced increase in measures of locomotion for crayfish has previously been observed (Panksepp and Huber, 2004). One possible explanation for unchanged locomotory response level includes increased time spent in tactile exploration of the arena. In crayfish, exploration of surroundings is strongly dependent on mechanoreception using active movements of the antenna (Basil and Sandeman, 2000; Koch et al., 2006). Therefore, it is possible that stimulation of the appetitive motivational states by amphetamine results in increased tactile investigation of the surroundings via sensory appendages rather than increases in locomotion *per se*.

The present paper demonstrates the ability of crayfish to self-administer amphetamine in an operant conditioning paradigm. Free moving, behaving crayfish learn to self-inject amphetamine under continuous reinforcement schedules. We found the rewarding potential of amphetamine to be dose dependent, and the reward potency to vary with the site of injection. Injections near the supraesophageal ganglion exhibited stronger reinforcing qualities than did systemic infusions of the drug. With the establishment of a self-administration paradigm utilizing an automated and targeted drug delivery technique through implanted cannulae, we introduce an invertebrate system whose properties closely resemble those of mammalian self-administration models.

The ability of amphetamine to act as a reinforcer in the crayfish nervous system has previously been demonstrated using a CPP (Panksepp and Huber, 2004). In that study, amphetamine-evoked CPP appeared after just a single exposure, was persistent, and displayed prompt reinstatement. Here we have demonstrated that under a spatially contingent, operant conditioning paradigm, crayfish can learn to execute tasks paired with amphetamine infusions. Crayfish that experienced amphetamine reward contingent on their behavior displayed significantly higher OI scores. In contrast, yoked individuals that received amphetamine injections on the same temporal

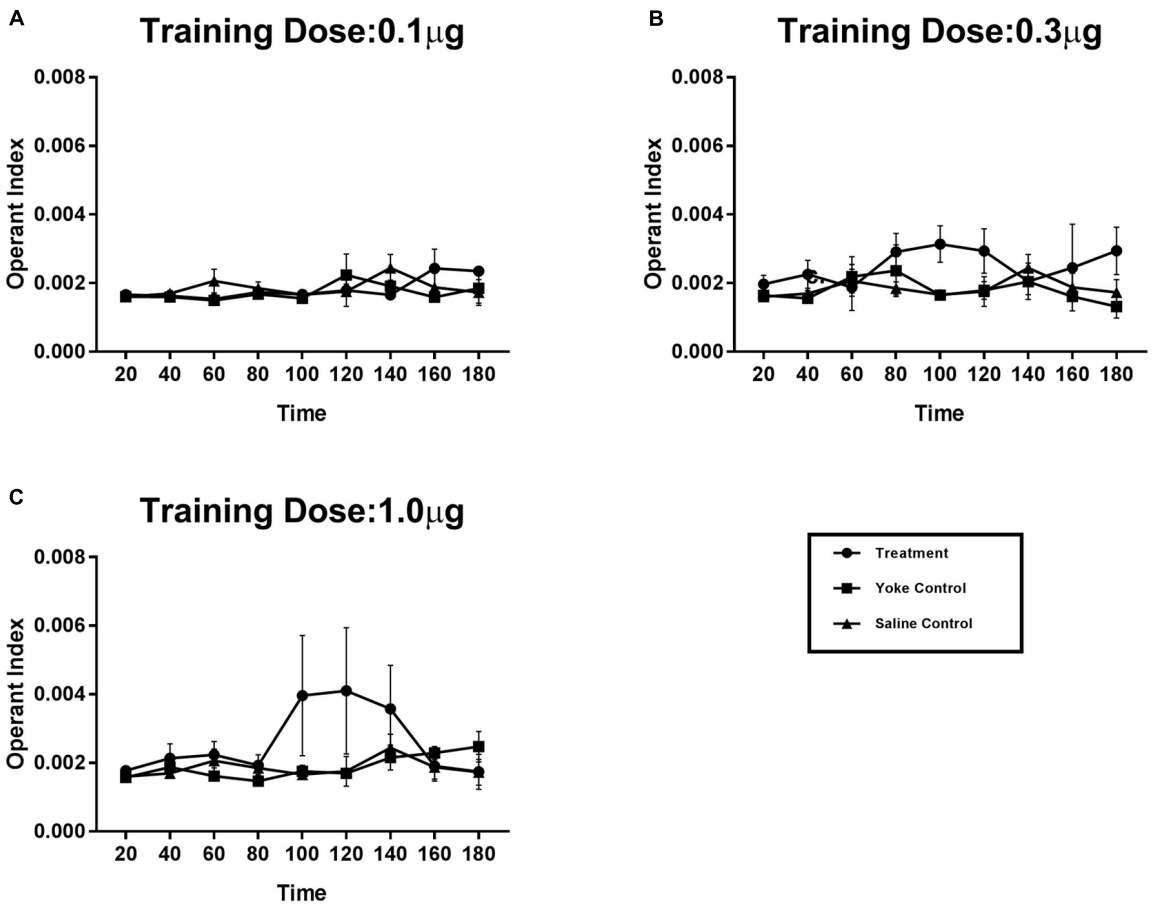

FIGURE 5 | Operant conditioning of a spatial task using amphetamine reward delivered at the supraesophageal ganglion. Treatment animals received amphetamine infusions upon entering a chosen substrate. Yoked controls received the drug infusions at time points identical to their treatment partners. Vehicle controls received contingent saline infusions. Learning of reward contingency was compared using operant index (OI) between treatment and yoke groups for each dose category **(A–C)**. Increases in OI appeared in dose dependent manner. Lower doses of amphetamine **(A)** 0.1 μg, and **(B)** 0.3 μg per infusion display more subtle effects on operant index. Learning of reward contingency appeared prominently at the higher dose of **(C)** 1.0 μg per infusion, with self-administering animals selectively engaging in the drug paired behavior more than their yoked counterparts. Operant index scores of treatment group show an increase after 80 min of exposure to reward conditioning compared to the yoke group (Treatment X Time interaction: $F[8,3] = 68.29$, $p < 0.05$) indicating the time frame necessary for the learning of reward contingency. Mean Operant learning index for 20 min time segments are plotted for the 3 h session with standard error of mean.

pattern but in a manner unrelated to their own behavior, did not display a similar increase. The vehicle control group that received contingent injections of saline also displayed no change in their OI scores across the session. Under the current paradigm, OI measures the individuals' efficiency of movement to regulate self-administration through the activation of the infusion pump. The higher OI scores solely in individuals that were controlling their exposure to the drug (self-administering individuals) indicates that when crayfish are offered the opportunity to control delivery of drug reward, they will increasingly engage in behaviors that allows them to obtain the drug.

Operant conditioning using amphetamine reward in crayfish appeared in a dose dependent manner. Studies in rodent models have demonstrated that the rate and probability of acquisition of self-administration are positively correlated with the unit dose (van Ree et al., 1978; Carroll and Lac, 1997). Low unit doses of amphetamine (0.1 and 0.3 μg/infusion) were unable to act as a reinforcer of sufficient strength in our operant conditioning paradigm. Identical scores for OI were observed in self-administering individuals and their yoke at low unit doses, indicating that crayfish made no particular effort to self-administer the drug at these doses. Differences in OI scores were observed at the 0.3 μg unit dose but failed to achieve statistical significance. For 1.0 μg, the highest dose included in our study for supraesophageal ganglion drug administration, a significant increase in OI was observed in animals experiencing the reward contingently compared to their yokes. OI scorers in the self-administering group rose rapidly midway through the conditioning session, indicating learning of the reward contingency and the onset of active drug seeking.

Toward the end of the session, OI scores tended to decrease, suggesting that there is a ceiling for amphetamine intake which is likely a function of both the total amount of drug injected and the unit dose per injection. Plateauing amphetamine intake after a period of self-administration is indicative of a decrease in reinforcement efficacy, either because the amount of amphetamine injected established internal levels of the drug that reached satiation, or because they generated aversive states beyond a given level. Previous studies conducted in our lab have indicated that amphetamine at higher doses (5 mg/kg) increases the occurrence of tail flips and convulsions (Alcaro et al., 2011). Since tail flips are innate escape responses of crayfish employed under perceptions of serious threat, it is likely that at higher doses amphetamine generates aversive states that constrain further drug intake.

Self-administering individuals displayed higher variance in their OI scores compared to the yoke and vehicle control groups, as indicated by large error bars in the dose response curve. Large inter-individual differences in response to drugs have also been observed in humans and other animal models (de Wit et al., 1986; Piazza et al., 1998; Marinelli, 2005). Although self-administration may be acquired with relative ease by some individuals, others tend to be more resistant. Another factor potentially contributing to this large variance is the source of the sample. Since our *O. rusticus* sample is derived from a wild population, the error bars reflecting between-subject variability in the acquisition of operant responding are likely to be large.

Brain injections of amphetamine were self-administered more readily compared to systemic injections of amphetamine. Although systemic injections with a broad range of doses were tested, we observed few apparent changes in OI scores of the treatment groups relative to the yoke group. It was previously demonstrated that administration of D-amphetamine directly into the crayfish brain is more efficient than pericardial injections at enhancing exploratory behaviors (Alcaro et al., 2011). Cumulatively, these findings indicate that the potential target of amphetamine reward indeed resides in the crayfish brain. Application of the drug directly over the supraesophageal ganglion minimizes the time delay between operant response and the experience of reward thus increasing the effectiveness of the conditioning paradigm. Findings from both invertebrate (Kusayama and Watanabe, 2000; Panksepp and Huber, 2004; Carvelli et al., 2010; Alcaro et al., 2011) and vertebrate models highlight the role of the monoaminergic pathway in amphetamine reward (Sora et al., 2010; Howell and Negus, 2014; Wiers et al., 2016). In crayfish, monoamines have been demonstrated to modulate motor control, exploration, and more complex behaviors such as aggression and anxiety-like responses (Fossat et al., 2014). Considering the highly conserved functions of biogenic amines, they are also likely to play a role in reward processes in both natural and abnormal contexts (e.g., behaviors displayed under the influence of addictive drugs such as drug seeking, self-administration, and relapse). Both dopamine (Tierney et al., 2003) and serotonin (Sandeman and Sandeman, 1987; Sandeman et al., 1988) innervations occur prominently in the accessory lobe of crayfish. The accessory lobe, a structure capable of processing higher-order multimodal inputs, may thus be a critical brain region involved in the implementation of reward in crayfish.

Although the reward seeking circuit (Ferenczi et al., 2016; Otis et al., 2017) in crayfish brain is yet to be mapped out in its entirety. Nonetheless, with an amine system consisting of fewer than 1,000 neurons (30–35 dopamine neurons in the brain and nerve cord) and a well-characterized set of behaviors associated with drug reward, crayfish is a model amenable to the exploration of reward mechanisms (Shipley et al., 2017). The establishment of an automated, operantly conditioned self-administration paradigm in crayfish sets the stage for more nuanced studies of the processes underlying invertebrate reward. Such studies should aim to understand the implementation of an appetitive/seeking disposition in what is a relatively simple neural system, and by what particular mechanism/s this disposition is targeted by the rewarding action of drugs of abuse.

ETHICS STATEMENT

Invertebrates are animals; however, no animal use protocols are required.

AUTHOR CONTRIBUTIONS

UD designed and carried out experiments, acquisition of data, analysis and interpretation of data, drafting and revising the article. RH helped with instrumentation and design, analysis, and interpretation of data. MvS assisted in conception and design, analysis and interpretation of data, drafting and revising of article.

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Caenorhabditis elegans Show Preference for Stimulants and Potential as a Model Organism for Medications Screening

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The nematode *Caenorhabditis elegans* (*C. elegans*) is a popular invertebrate model organism to study neurobiological disease states. This is due in part to the intricate mapping of all neurons and synapses of the entire animal, the wide availability of mutant strains, and the genetic and molecular tools that can be used to manipulate the genome and gene expression. We have shown that, *C. elegans* develops a conditioned preference for cues that had previously been paired with either cocaine or methamphetamine exposure that is dependent on dopamine neurotransmission, similar to findings using place conditioning with rats and mice. In the current study, we show *C. elegans* also display a preference for, and self-exposure to, cocaine and nicotine. This substance of abuse (SOA) preference response can be selectively blocked by pretreatment with naltrexone and is consistent with the recent discovery of an opioid receptor system in *C. elegans*. In addition, pre-exposure to the smoking cessation treatment varenicline also inhibits self-exposure to nicotine. Exposure to concentrations of treatments that inhibit SOA preference/self-exposure did not induce any significant inhibition of locomotor activity or affect food or benzaldehyde chemotaxis. These data provide predictive validity for the development of high-throughput *C. elegans* behavioral medication screens. These screens could enable fast and accurate generation of data to identify compounds that may be effective in treating human addiction. The successful development and validation of such models would introduce powerful and novel tools in the search for new pharmacological treatments for substance use disorders, and provide a platform to study the mechanisms that underlie addictions.

Keywords: addiction research, cocaine, nicotine, invertebrate models, self-administration models, high-throughput screening assays

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INTRODUCTION

The impact of addiction on our society is profound and by all accounts is increasing. It has been estimated that in the United States alone, addiction costs approach 200 billion dollars (U.S. Department of Justice, 2010). Clearly, there is an acute need for a better understanding of the neurobiological basis of addiction, as well as better and more effective treatments to confront this growing epidemic. Animal models have provided much of our

current understanding about the neuroscience of addiction (Edwards and Koob, 2012). In particular, behavioral measures used to model and study human addiction in animals (cf., Bell and Rahman, 2016) in conjunction with functional neurobiological studies have provided us with an understanding of basic reward circuitry (Koob and Volkow, 2010). Development of pharmacotherapeutic medications is a promising avenue to reduce the impact of substance use disorders; however, few such treatments are currently available. Thus, additional efforts are needed to identify molecular targets and novel compounds for medications development. Work from our group and others shows that the effects of substances of abuse (SOAs) on neurobiology and behavior is phylogenetically ancient, suggesting that invertebrates possess some of the mechanisms that underlie addiction. Techniques historically used to study behavioral aspects of SOAs in vertebrates such as conditioned place preference (CPP) and SOA self-administration (Tzschentke, 2007) have also been developed for invertebrates. Elegant studies have shown SOA reward, withdrawal, and seeking to opiates, cocaine, and amphetamines in crayfish (Huber et al., 2011). In *Drosophila melanogaster*, ethanol (EtOH) conditioning and self-administration paradigms have demonstrated that flies develop conditioned preference responses to cues previously paired with EtOH (Kaun et al., 2011). These data show that even simple invertebrate animals can model what are widely considered to be highly complex behaviors. However, this should not be surprising as behavioral models using invertebrates have played a key role in discovering the underlying molecular mechanisms that provide the basis for learning and memory (Kandel, 2001).

The nematode *Caenorhabditis elegans* has some major advantages as a model organism to study neurobiology and disease states (Hulme and Whitesides, 2011). *C. elegans* have conserved neurobiological systems with established mapping of all neurons and synapses in the entire animal. We have shown that, *C. elegans* develops a conditioned preference for cues that had previously been paired with either cocaine or methamphetamine exposure that is similar to findings using place conditioning with rats and mice (Musselman et al., 2012; Katner et al., 2016). Moreover, conditioning required functional dopamine neurotransmission (Musselman et al., 2012). Additionally, with SOA pre-exposure, *C. elegans* demonstrate tolerance (Grotewiel and Bettinger, 2015) and sensitization (Lee et al., 2009) which are hallmarks of addiction in humans. Together, these data indicate that *C. elegans*, show behavioral responses to SOAs that are consistent with those of higher level organisms. These data also indicate that invertebrates, specifically *C. elegans* in this case, show behavioral responses to addictive SOAs that are consistent with those seen in more complex animals. Recent research has established that *C. elegans* display depressed locomotion and functional tolerance after contact with EtOH which is mediated, in part, through the BK potassium channel which may mediate behavioral sensitivity to EtOH in many species including humans (Bettinger and Davies, 2014; Davis et al., 2014). Importantly, the internal tissue concentration leading to the effects of EtOH on locomotor activity in *C. elegans* is strikingly similar to blood alcohol levels that produce intoxication in humans (Alaimo et al., 2012). These data suggest that *C. elegans* show a concentration-dependent attraction to EtOH that results in EtOH self-exposure and significant tissue concentrations of EtOH. We have discovered that this EtOH preference response can be selectively blocked by pretreatment with the pan-opioid receptor antagonist, a treatment for alcohol and opiate use disorders, naltrexone, which is consistent with the recent discovery of an opioid receptor system in *C. elegans* that mediates responses to both appetitive stimuli (Cheong et al., 2015) as well as nociception (Mills et al., 2016). In the current work, we have expanded the use of such a treatment approach on cocaine and nicotine preference and have examined the effects of the nicotinic cholinergic partial agonist, and smoking cessation treatment, varenicline, on nicotine preference (Gomez-Coronado et al., 2018). The results suggest an opportunity to establish and validate a high-throughput *C. elegans* behavioral medications screening model for stimulant addiction. The successful development and implementation of such models would provide the field with powerful and novel tools in the search for new pharmacological treatments for addictions, and provide a platform to study the underlying mechanisms of these agents.

Objective: To determine if *C. elegans* may be used to model the behavioral aspects of stimulant self-administration and to screen for putative addiction treatments.

MATERIALS AND METHODS

Drugs

Cocaine hydrochloride was received from the NIDA Drug Supply Program, nicotine bitartrate was purchased from Sigma Aldrich (St. Louis, MO, United States), and varenicline tartrate was purchased from Biotang (Lexington, MA, United States). Vehicle (0.97 mM HCl; salt equivalent of naltrexone HCl) and naltrexone HCl (N-3136; FW 377.9; Sigma-Aldrich) were used to pretreat animals prior to SOA preference testing. Benzaldehyde (#418099; 99.5%; Sigma-Aldrich; FW 106.12) was used to test for non-selective effects of naltrexone HCl. 2-nonanone (99%; CAS 821-55-6; FW 142.24; Arcos Organics) was used to show that animals could move away from the SOA target zone.

Culture and Maintenance of Strains

The N2 Bristol wild-type (WT) strain was used in all assays. All animals were maintained at 22°C, and all general culturing techniques have been described previously by Nass and Hamza (2007). Worms were grown with *E. coli* strain NA22 as a food source on maintenance plates, produced by filling 60-mm petri dishes with 10-ml regular NGM agar [25 g bacto agar, 20 g bacto peptone, 3 g NaCl, 1 L H20, 1 ml cholesterol (5 mg/ml 95% ethanol), 1 ml 1 M CaCl2, 1 ml 1 M MgSO4, and 25 ml of potassium phosphate buffer]. The potassium phosphate buffer contained 5 g of K2HPO4 dibasic/anhydrous, 30 g of KH2PO4 monobasic, and 500 ml of H20, pH adjusted to 6.0 (Bianchi and Driscoll, 2006).

Adult worms were used for all experiments to control for any effects of different sensitivities and responses to SOAs at varying developmental stages. Worms were age synchronized by lysing gravid adults with bleach and sodium hydroxide, allowing eggs to be released into solution and hatched in M9 buffer (Bianchi and Driscoll, 2006). After 18 h, hatched L1 larvae were washed three times with water, plated, and maintained on NGM plates with NA22 *E. coli* bacterial lawns until reaching adulthood. Testing began approximately 72 h post-plating the L1 larvae, when worms were adults.

6-well Costar^{TM} cell culture plates were used to determine SOA preference (Fisher cat. no. 07-200-80). Clear templates were taped to the bottom each 6-well plate to create two 1.2 cm diameter circular target zones within the 3.5 cm diameter of each well. Test plates were produced by filling each well of the plates with 3.8 ml of NaCl free agar (17 g bacto agar, 2.5 g bacto peptone, 1 L H20, 1 ml 1 M CaCl2, 1 ml 1 M MgSO4, and 25 ml of potassium phosphate buffer). Cholesterol was not included in the salt-free agar in order to obtain clearer images of worms during testing. Although the lack of salts and cholesterol in the agar may have long-term effects on worms, our previous work indicating intact cue-conditioned learning (Musselman et al., 2012; Katner et al., 2016) and the differential responses with the SOAs vs. controls (food or benzaldehyde) show that the agar preparation as used in this paradigm does not prevent normal chemotaxic responses.

Treatment Agent Pretreatment Prior to SOA Preference Testing

Worms were washed off maintenance plates with 15 ml of water and transferred to 15 ml centrifuge tubes. Adults were allowed to settle on the bottom of each tube for 5 min and then the supernatant was removed. This was repeated two more times with 10 ml of water to remove the majority of bacteria from the worms. After the final removal of the supernatant, approximately 0.3–0.5 ml of worms were transferred to a 5 ml Eppendorf tube and 3 ml of vehicle (0.97 mM HCl) or treatment agent naltrexone HCl (10 mM; dose selected from Cheong et al. (2015), or varenicline (1.0 or 9.0 mM) was added to each tube. The tubes were placed on a nutator for 30 min prior to SOA preference testing. Following vehicle or treatment agent, tubes were taken off nutator and worms were allowed to settle to the bottom of each tube for approximately 3 min and the supernatant was removed to a point where worms were diluted to a ratio of approximately one part worms to two parts vehicle or treatment agent solution. Then, 4 ul aliquots, containing approximately 40–80 worms, were pipetted into the center of each well of a 6-well testing plate and excess liquid was removed from the worms using a Kimwipe. Images of each well were taken 10 and/or 30 min after placing worms on test plates. It should be noted that although 0.97 mM HCl controlled for the HCl ions in the 10 mM naltrexone, there was an osmotic difference between the vehicle control and naltrexone exposure group and washing with water may induce osmotic stress. Thus, control experiments were conducted examining locomotor behavior (body bends) and movement to control attractants (benzaldehyde and food) to determine if such treatments affect either locomotor activity or normal chemotaxis to other attractants. Moreover, we (and others) have conducted washings with diH2O without effects on locomotion or the ability to develop and display learned responses to cues or preference responses to benzaldehyde (Law et al., 2004; Musselman et al., 2012; Katner et al., 2016). As with our previous work (Musselman et al., 2012; Katner et al., 2016) we are interested in only counting worms in the target zones because it provides comparable measures of elective responses of animals moving into zones that contain either the SOA or the vehicle. The vehicle zone controls for the application of a substance of the same volume as the SOA target zone and effects of that application to that space on the agar.

SOA Preference Testing Procedure

In general, 4 μl of vehicle and a SOA solution were applied to the center of the 1.2 cm target zones of each well. These spotting solutions were allowed to absorb into agar for 30 min prior to testing. Cocaine preference was tested with 0, 50, 250, and 500 μM cocaine HCl concentrations. Nicotine preference was tested with 0, 5, 50, and 100 mM nicotine concentrations. Vehicle (water) and inhibitor agent solutions were prepared fresh, prior to each day of testing. All concentrations of SOAs include the salt and each experiment was conducted over 2 to 4 days.

Food and Benzaldehyde Preference

Food: 1 μl of water or food (NA22 bacterial solution) was spotted to the two target zones of each well. Images were taken at 30 min. Benzaldehyde: 2 μl of a 1%(v/v) benzaldehyde solution dissolved in 25%(v/v) EtOH was spotted in one target zone, while 25% EtOH was spotted in the opposite target zone, 30 min before testing. Images were taken at 30 min. The amounts/concentrations of food and benzaldehyde were selected to produce preference indices similar to those observed with cocaine and nicotine in this paradigm.

Nonanone Aversion

Nonanone (an aversive compound) was spotted [2 μl of 10%(v/v)] to the outer edge of the SOA target zone of each well (i.e., between the edge of the SOA target zone and the outer edge of the well; see **Figure 1**) immediately after taking 30 min images for SOA preference experiments in order to determine if animals were capable of moving away from the SOA target zones and were not rendered ataxic by the SOA test compounds themselves. Therefore, images were taken immediately before and 10 min after placing nonanone into each well. Pre- and post-nonanone preference indexes (PIs) (as described below under SOA preference testing) were calculated for each well in order to compute the change in preference from the SOA target zone in response to nonanone. In this way the effects of nonanone are tested at the time and under the conditions in which the animals are displaying the preference response.

Body Bend Assay

The body bend assay used here was adapted from Hart (2006). After 30 min pretreatment with vehicle (0.97 mM HCl) or

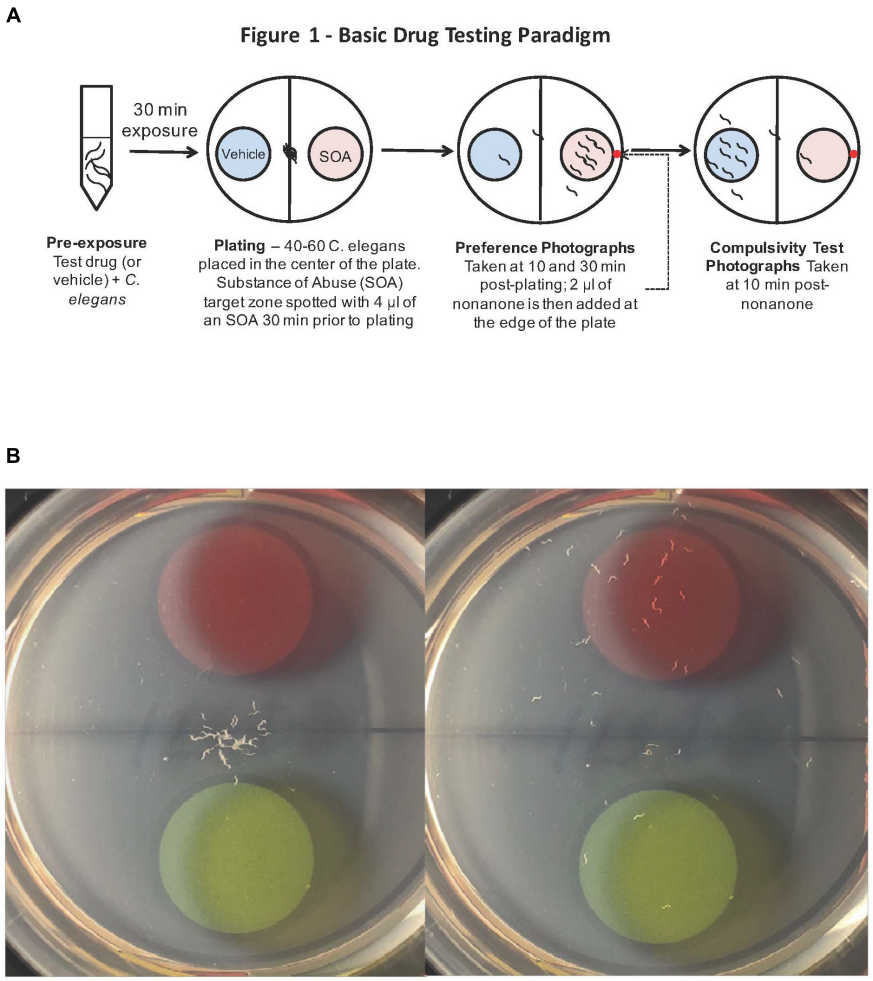

FIGURE 1 | (A) *Caenorhabditis elegans* screen for medications development. A popular method to assess how *C. elegans* respond behaviorally to a chemical is the simple chemotaxis assay, which is a type of voluntary self-exposure paradigm. Worms were exposed to either vehicle (0.97 mM HCl) or test treatment agent (naltrexone or varenicline) in a centrifuge tube for 30 min prior to the SOA preference testing to either cocaine or nicotine. Animals were then pipetted into the center of each well of a 6-well agar test plate containing two target zones; one spotted with SOA and the other with vehicle. Images were taken 30 min after plating worms to determine a SOA preference index for each well. The chemotaxic (preference/avoidance) index (CI) is calculated by dividing the number of worms in the target zone containing the test substance by the total sum of worms counted in both zones. To indicate that the animals are not simply anesthetized by the SOA, after the preference test has been established, a 2 µl drop of the aversive stimulus nonanone (red dot) is placed between the edge of the plate and the target zone and another photograph is taken 5 min later to assess ability to determine if the presence in the target zone is the result of anesthesia. **(B)** A single well of a 6-well plate spotted with 1% benzaldehyde (red target zone) and vehicle (green target zone): Images were taken immediately (left) and 30 min after (right) placing worms in the center of the well. The benzaldehyde preference index for the well on the right at the 30 min time point was 83.3%.

naltrexone HCl (10 mM), 2 µl of worms diluted in a ratio of one part worms to two parts vehicle or inhibitor agent (as described above in the pretreatment section) were placed on a microscope slide on the stage of a microscope (Bausch & Lomb ASZ45L3 45X). After selecting a single worm to track, the number of times the worm's tip crossed this midline and extended to about a 45–90 degree arc over a 20 s period was recorded. Only instances where the midline was completely crossed were counted.

Imaging and Worm Counting

Worms were imaged by taking pictures or video with a smartphone positioned on top of a light box, which emitted light indirectly and underneath each 6-well plate. Video time-course files were compressed to time-lapse.mov files to illustrate worm activity during preference tests (± pretreatment with naltrexone) and the response to nonanone. Individual images were analyzed using ImageJ software to count the number of worms in the target zones of the test plates. Using ImageJ, the target zone was cropped from each photo and the color threshold of the image was adjusted. Specifically, threshold color was set to red, color space was set to RGB, and color threshold was adjusted so worms were highlighted in red. Particles were analyzed with a pixel size of 80 to infinity. The number of worms counted in each target zone was recorded and analyzed in Microsoft Excel.

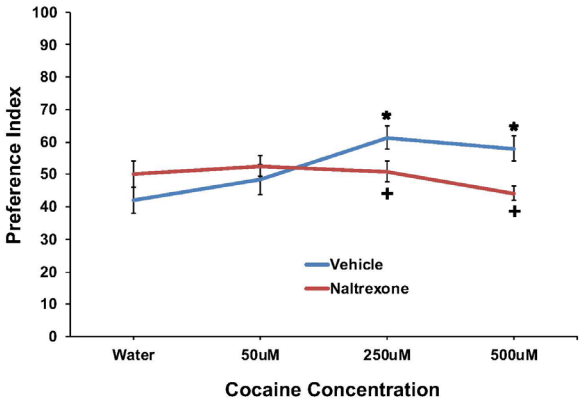

FIGURE 2 | Pretreatment with 10 mM naltrexone decreased cocaine preference in N2 *C. elegans*. A two-way ANOVA found a main effect of cocaine concentration and a significant interaction between pretreatment and cocaine concentration on cocaine preference. Significant factors were decomposed using one-way ANOVAs followed by LSD *post hoc* tests. *Significant ($p < 0.05$) increase in cocaine preference in vehicle treated worms compared to respective 0 mM cocaine (water). +Significant ($p < 0.05$) decrease in preference for cocaine following 10 mM naltrexone pretreatment compared to respective vehicle condition. The number of wells analyzed for the vehicle treated groups were 24 (water), 18 (50 μM), 18 (250 μM), and 18 (500 μM), and for the naltrexone treated groups were 24 (water), 24 (50 μM), 24 (250 μM), and 24 (500 μM).

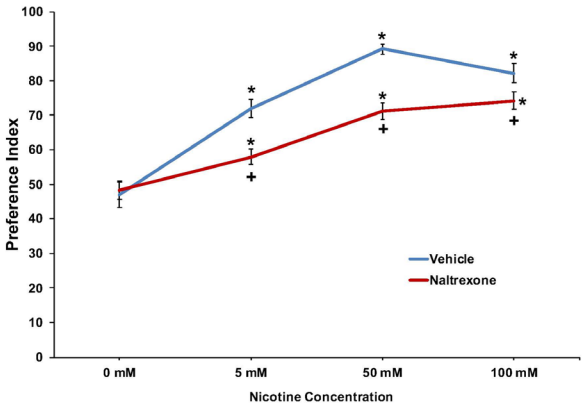

FIGURE 3 | Pretreatment with 10 mM naltrexone decreased nicotine preference in N2 (wild-type) *C. elegans*. For naltrexone, a two-way ANOVA decomposed and followed by LSD *post hoc* tests where appropriate found main effects of concentration and treatment, and a significant interaction between concentration and treatment on nicotine preference at 30 min. *Significant ($p < 0.05$) increase in nicotine preference in vehicle and naltrexone treated worms compared to respective 0 mM nicotine. +Significant ($p < 0.05$) decrease in preference for nicotine following 10 mM naltrexone pretreatment compared to respective vehicle condition. The number of wells analyzed for the vehicle treated groups were 36 (0 mM), 36 (5 mM), 36 (50 mM), and 24 (100 mM), and for the naltrexone treated groups were 36 (0 mM), 36 (5 mM), 36 (50 mM), and 24 (100 mM).

A chemotaxic PI for each SOA concentration was then calculated by dividing the number of worms in the SOA target zone by the total sum of worms counted in both the SOA and vehicle zones converted to a percentage.

Statistical Analyses

All analyses were analyzed using one-, two-, or three-way ANOVAs, followed by decomposition of factors and *post hoc* tests as appropriate and previously conducted (Musselman et al., 2012; Katner et al., 2016). Independent or paired *t*-tests were used to compare two samples between or within groups, respectively. Values in figures and tables are presented as mean ± SEM.

RESULTS

Cocaine/Naltrexone

The effect of cocaine (0, 50, 250, or 500 μM) to induce preference and the effect of pretreatment with 10 mM naltrexone to inhibit the response are shown in **Figure 2**. Preference was found for cocaine at the 250 and 500 μM concentrations, which was eliminated by naltrexone pretreatment. A two-way ANOVA found a main effect of cocaine concentration [$F(3,174) = 2.7$; $p < 0.05$] and a significant interaction between pretreatment and cocaine concentration [$F(3,174) = 4.4$, $p < 0.006$] on cocaine preference. For vehicle pretreated worms, a one-way ANOVA found a main effect of cocaine concentration on preference [$F(3,77) = 5.0$; $p < 0.004$], and *post hoc* tests revealed significant ($p < 0.05$) preference indices for 250 and 500 μM cocaine compared to water. *Post hoc* tests also found significant ($p < 0.05$) differences in cocaine preference between the 50 μM and 250 μM cocaine concentrations, for vehicle treated worms. For naltrexone pretreated worms, a one-way ANOVA did not find a main effect of cocaine concentration on preference [$F(3,95) = 1.3$; ns]. One-way ANOVAs examining differences between vehicle and naltrexone pretreatment for each cocaine concentration found no main effect of pretreatment on water [$F(1,47) = 2.0$; ns] or 50 μM cocaine [$F(1,41) = 0.6$; ns] preference. However, naltrexone pretreatment decreased 250 μM [$F(1,41) = 4.8$, $p < 0.04$] and 500 μM [$F(1,41) = 10.8$, $p < 0.003$] cocaine preference compared to vehicle pretreatment.

Nicotine/Naltrexone

The effect of nicotine (0, 5, 50, or 100 mM) to induce a preference response and the effect of pretreatment with 10 mM naltrexone to inhibit the response is shown in **Figure 3** and **Supplementary Figure 1**. For naltrexone, a two-way ANOVA found main effects of concentration [$F(3, 263) = 65.0$; $p < 0.001$] and treatment [$F(1,263) = 25.3$; $p < 0.001$], and a significant interaction between concentration and treatment [$F(3,263) = 5.4$; $p < 0.002$] on nicotine preference at 30 min. Overall, the findings indicate a significant preference response at each nicotine concentration that is significantly reduced by pretreatment with naltrexone (**Figure 3** and **Supplementary Figure 1**).

Nicotine/1 mM Varenicline

Varenicline pretreatment revealed robust effects at the 10 min time point in some cases, thus preference data were analyzed and presented for both the 10 and 30 min time points. The effect of pretreatment with 1 mM varenicline to inhibit the

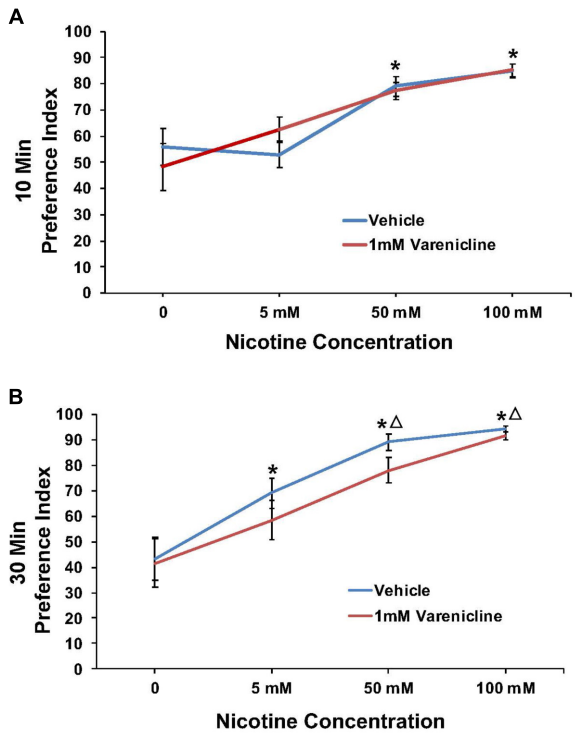

FIGURE 4 | Pretreatment with 1 mM varenicline failed to modify nicotine preference in N2 (wild-type) *C. elegans*. **(A,B)** Represent 10 and 30 min nicotine preference, respectively. A two-way ANOVA decomposed and followed by LSD *post hoc* where appropriate, found a main effect of nicotine concentration on nicotine preference at 10 and 30 min. There was however, no effect of 1 mM varenicline pretreatment on nicotine preference at either time-point or nicotine concentration ($p > 0.05$). *Significant ($p < 0.05$) increase in nicotine preference in vehicle and varenicline treated worms compared to respective 0 mM nicotine. $^{\Delta}$Significant ($p < 0.05$) increase in preference for nicotine compared to respective 5 mM nicotine condition. The number of wells analyzed for the vehicle treated groups were 12 (0 mM), 12 (5 mM), 12 (50 mM), and 9 (100 mM), and for the varenicline treated groups were 12 (0 mM), 12 (5 mM), 12 (50 mM), and 11 (100 mM).

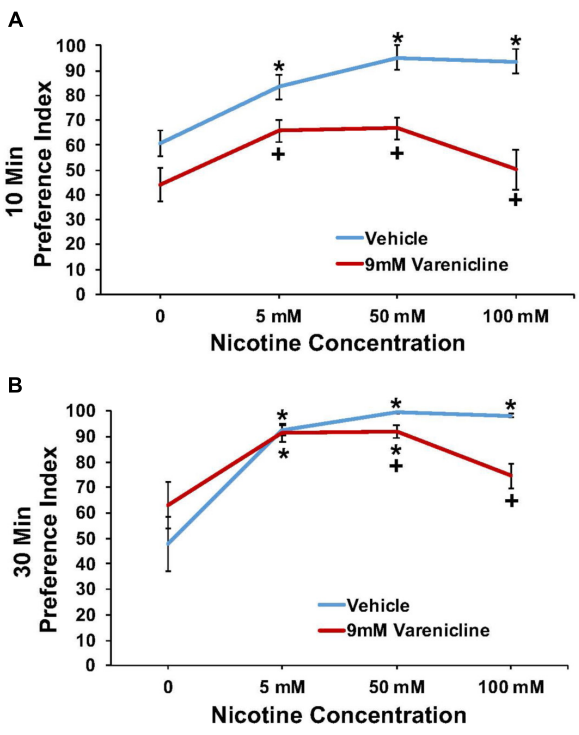

FIGURE 5 | Pretreatment with 9 mM varenicline decreased nicotine preference in N2 (wild-type) *C. elegans*. **(A,B)** represent 10 and 30 min nicotine preference, respectively. A three-way ANOVA decomposed and followed by LSD *post hoc* tests where appropriate, found main effects of time (10 and 30 min), concentration, treatment, a significant interaction between concentration and treatment, and a significant interaction between time and treatment on nicotine preference. *Significant ($p < 0.05$) increase in nicotine preference in vehicle and varenicline treated worms compared to respective 0 mM nicotine. $^{+}$Significant ($p < 0.05$) decrease in preference for nicotine following varenicline pretreatment compared to respective vehicle condition. The number of wells analyzed for the vehicle treated groups were 12 (0 mM), 12 (5 mM), 10 (50 mM), and 11 (100 mM), and for the varenicline treated groups were 11 (0 mM), 12 (5 mM), 12 (50 mM), and 12 (100 mM).

nicotine preference response is shown in **Figure 4** at both the 10 min (A) and 30 min (B) time points. For 1 mM varenicline, a two-way ANOVA found a main effect of nicotine concentration [$F(3,91) = 23.9$; $p < 0.001$] on nicotine preference at 10 and 30 min. There was however no effect of 1 mM varenicline pretreatment on nicotine preference at either time-point or nicotine concentration ($p > 0.05$).

Nicotine/9 mM Varenicline

The effect of pretreatment with 9 mM varenicline to inhibit the nicotine preference response is shown in **Figure 5** at both the 10 min (A) and 30 min (B) time points. A three-way ANOVA found main effects of time (10 and 30 min) [$F(1,84) = 20.4$; $p < 0.001$], concentration [$F(3,84) = 24.5$; $p < 0.001$], treatment [$F(1,84) = 65.0$; $p < 0.001$], a significant interaction between concentration and treatment [$F(3,84) = 5.2$; $p < 0.003$], and a significant interaction between time and treatment [$F(1,84) = 16.3$; $p < 0.001$] on nicotine preference. Overall, pretreatment with 9.0 mM varenicline significantly reduced the preference response to nicotine at both time points.

Benzaldehyde Preference

The effect of pretreatment with 10 mM naltrexone to modify benzaldehyde preference was conducted in order to examine the effect naltrexone on the preference response to a known volatile attractant. An independent *t*-test found that naltrexone (10 mM) pretreatment had no significant effect [$t = 0.97$; ns] on 1% (v/v) benzaldehyde preference (PI = 84.4 ± 4.0%; $n = 12$) compared to vehicle (0.97 mM HCl) pretreatment (PI = 84.9 ± 8.6%; $n = 12$).

Nonanone Aversion

In order to determine if animals were anesthetized after moving into target zones containing either cocaine or nicotine, the aversive compound nonanone was applied between the target zone and the edge of the plate after the animals had established a preference response. Independent *t*-tests found significant effects

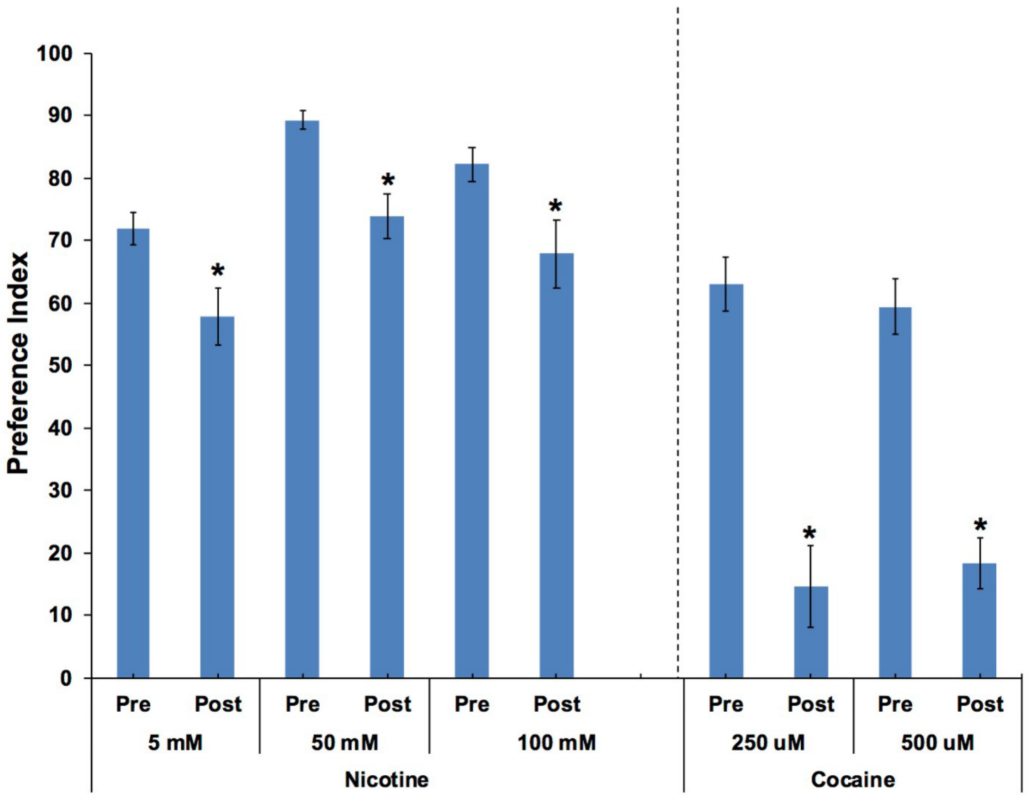

FIGURE 6 | *Caenorhabditis elegans* retained the ability to move away from the aversive compound, nonanone. Nonanone application significantly reduced the preference value for nicotine (5, 50, and 100 mM) or cocaine (250 and 500 μM). Independent *t*-tests found significant effects of nonanone on 250 and 500 μM cocaine preference ($p < 0.001$). Paired *t*-tests found significant effects of nonanone on 5, 50 and 100 mM nicotine preference ($p < 0.05$). *Significant ($p < 0.05$) decrease in SOA preference post nonanone compared to respective pre-nonanone preference for a given SOA concentration. For nicotine, the number of wells analyzed for the pre-nonanone condition were 36 (5 mM), 36 (50 mM), and 24 (100 mM), and for the post-nonanone condition were 36 (5 mM), 36 (50 mM), and 24 (100 mM). For cocaine, the number of wells analyzed for the pre-nonanone condition were 12 (250 μM) and 12 (500 μM), and for the post-nonanone condition were 6 (250 μM) and 6 (500 μM).

of nonanone on 250 and 500 μM cocaine preference ($p < 0.001$; **Figure 6**). Paired *t*-tests found significant effects of nonanone on 5, 50, and 100 mM nicotine preference ($p < 0.05$; **Figure 6**).

Video of SOA Preference, the Response to Nonanone, and the Effect of Naltrexone Pretreatment

To better illustrate the development of drug preference and the aversive response to nonanone in this paradigm, videos of a preference test with 100 mM nicotine and the subsequent response to nonanone were recorded and compressed into time-lapsed format and are presented in **Supplementary Video 1**. A similar time-lapsed video illustrating the effects of pretreatment with 10 mM naltrexone on the preference test with 100 mM nicotine is presented in **Supplementary Video 2**.

Food Preference

For vehicle and naltrexone pretreatment, both conditions showed a normal chemotaxis to food and food preference was 86.1 ± 2.8 and 80.3 ± 5.7%, respectively (**Table 1**). An independent *t*-test found no significant effect of pretreatment (vehicle vs. naltrexone) on food preference [$t = 2.1$; ns]. In addition, 9 mM varenicline pretreatment did not significantly affect food preference (**Table 1**). An independent *t*-test found no effect of varenicline on food preference [$t = 2.2$; ns].

Locomotor Activity

Naltrexone: We found that exposure to 10 mM naltrexone for 30 min prior to testing had no significant effect on locomotor activity compared to vehicle exposure [$F(1,11) = 0.02$; ns]. Specifically, the number of body bends in 20 s for vehicle and

TABLE 1 | Food preference in N2 *C. elegans* following vehicle and inhibitor agent treatment.

Agent	Vehicle	Inhibitor Treated
Naltrexone (10 mM)	86.1 ± 2.8%	80.3 ± 5.7%
Varenicline (9 mM)	84.0 ± 3.6%	80.7 ± 5.0%

t-tests found that inhibitor agent treatments had no effect on food preference. The number of wells analyzed for naltrexone were 12 for vehicle and 12 for naltrexone. The number of wells analyzed for varenicline were 12 for vehicle and 12 for varenicline.

naltrexone treatments were 54 ± 7 (n = 6) and 53 ± 3 (n = 6), respectively.

Varenicline: Mean (±SEM) body bends/20 s for vehicle treated worms were 46.3 ± 3.6 (n = 9), while body bends were 47.0 ± 3.6 (n = 8) for varenicline (9 mM) treated worms. Independent t-tests found no significant difference in body bends after varenicline treatment.

DISCUSSION

The present studies found a concentration-dependent attraction by *C. elegans* to the SOAs cocaine (**Figure 2**) and nicotine (**Figures 3–5** and **Supplementary Video 1**). Naltrexone pretreatment selectively reduced preference for both cocaine (**Figure 2**) and nicotine (**Figure 3**, **Supplementary Figure 1** and **Supplementary Video 2**) in this paradigm, but had no effect on preference for food or benzaldehyde preference. Moreover, the SOA preference response was not likely due to an anesthetic or paralytic effect, since worms were able to move away from the SOA target zones following the application the chemorepellent, nonanone (**Figure 6** and **Supplementary Video 1**), and continue to move in the SOA target zone after entering (**Supplementary Video 1**). Pretreatment with varenicline, a treatment agent approved for smoking cessation in humans, also was found to reduce the nicotine preference response at concentrations that did not affect locomotor activity or food preference. These data are consistent with observations in vertebrate animal models showing efficacy and selectivity of the SOAs and begins to provide face and predictive validity for the model in medications screening applications. Importantly, the 6-well plates enable a high-throughput system for behavioral screening, and are able to reduce the number of *C. elegans* needed to conduct preference testing. This also limits the time required for imaging, and ultimately enhances throughput. Combined, these findings suggest that procedures using *C. elegans* may be developed to screen medications for the treatment of substance use disorders. In addition, the development of this technology will allow for the future investigation of the molecular mechanisms that underlie the efficacious effects of novel agents using the fully tractable *C. elegans* model.

A popular method to assess how *C. elegans* respond behaviorally to a chemical or substance is the simple chemotaxis assay (Bargmann and Horvitz, 1991; Bargmann, 2006) which, in fact, is a type of voluntary self-exposure paradigm. In the current studies, we employed a modified version of this assay, in which a 6-well agar test plate was prepared with a SOA placed in a defined target region on one side of each well and the vehicle, usually water, placed in a target zone on the other side of each well. The current experiments build on our previous work showing that *C. elegans* show conditioned attraction to cues (either a salt or food cue) previously paired with cocaine or methamphetamine which utilized a procedure analogous to Pavlovian conditioning models of reward in rodents (Musselman et al., 2012; Katner et al., 2016). The current work examines preference responses to two of the most widely abused stimulants, cocaine and nicotine (Lee et al., 2009; Ward et al., 2009; Musselman et al., 2012; Sellings et al., 2013). Few studies have examined the reinforcing properties of stimulants in *C. elegans*; however, Sellings et al. (2013), demonstrated that *C. elegans* show a concentration dependent attraction to nicotine applied to agar test plates which were confirmed in the current findings with nicotine. It should be noted that the concentrations of treatment agents needed to produce effects in *C. elegans* in these studies and in the current study are often high due to the waxy cuticle that encases the animal and functions as a barrier to entry (Epstein, 1995; Davies et al., 2003; Cheong et al., 2015). In the current work, animals counted in the target zone containing the stimulant (either cocaine or nicotine) are in contact with the SOA and thus demonstrating self-exposure to the SOA. This is also a true choice behavior, since the current study found that the addition of the aversive compound nonanone near the SOA target zone, after the preference response has been established, caused the animals to immediately move away from the SOA target zone, inducing a measurable aversive response. These findings confirm that the SOAs tested here are not simply functioning as a simple locomotor anesthetic or paralytic agent in this procedure.

Consistencies in responses to SOAs across phyla led to the hypothesis that *C. elegans* may be a viable model system to screen potential candidate treatment for substance use disorders. Recently, *C. elegans* were found to have functional opioid-like receptors (Cheong et al., 2015). Thus, to determine the predictive validity of the model, we tested the effectiveness of naltrexone to decrease preference responses, as it is one of the very few compounds shown consistently to reduce alcohol and other SOAs intake and seeking behavior in animal models as well as humans (Heilig and Egli, 2006). Using vertebrate models, naltrexone has been demonstrated to reduce cocaine intake (Mello et al., 1990; Corrigall and Coen, 1991; Ramsey and van Ree, 1991) and seeking (Giuliano et al., 2013); opioid intake in animal models and humans (Negus and Banks, 2013), and has recently been shown to decrease cannabis self-administration and subjective effects in chronic cannabis users (Haney et al., 2015). Naltrexone has shown mixed effects on nicotine use in humans (Aboujaoude and Salame, 2016; Barboza et al., 2016; Kirshenbaum et al., 2016). Rodent studies indicate that naltrexone can reduce nicotine-induced locomotor sensitization (Goutier et al., 2016) self-administration at 2.0 mg/kg (Guy et al., 2014) but not 1.0 mg/kg or below (Le et al., 2014). Also, treatment with naloxanazine (a selective mu1 opioid receptor antagonist) significantly reduced nicotine self-administration in rats (Liu and Jernigan, 2011). However, some work suggests that naltrexone may have more consistent effects to reduce conditioned responses to nicotine (Liu et al., 2009). Other opioid receptors may also be efficacious targets, with the kappa-opioid receptor antagonist nor-binaltorphimine reducing nicotine seeking behavior (Grella et al., 2014). Together, these studies support a role for opioid systems in stimulant reinforcement and use and are consistent with findings in the current screen with *C. elegans*. However, much additional investigation is needed to identify how the opioid system may be involved in nicotine self-administration and how agents that target these systems may reduce tobacco or cocaine use in humans.

Based on the data presented here, we anticipate that potential compounds that have efficacy in reducing SOA intake and/or seeking in vertebrate models and humans will also inhibit the SOA preference response in *C. elegans*. Naltrexone pre-exposure clearly reduced SOA preference (**Figures 2–3**) at concentrations that do not inhibit food consumption (**Table 1**), benzaldehyde chemotaxis, or locomotor activity (body-bend data). These data are consistent with rodent data showing that naltrexone can inhibit intake of SOAs at doses that do not affect sucrose intake or body weight (Henderson-Redmond and Czachowski, 2014). In most instances, little or no prior work has been published to determine if treatment agents used to treat stimulant addictions have effects on models of addictive responses to stimulants in *C. elegans*. However, varenicline pre-exposure has been shown to reduce chemotaxis to nicotine in *C. elegans* (Sellings et al., 2013). Our data are consistent with these data and show selectivity and predictive validity of varenicline in this screening model. Varenicline is a partial agonist at the α4β2 receptor in vertebrates and is an approved treatment for nicotine addiction (Crooks et al., 2014). Although it is still unclear how varenicline reduces nicotine preference in *C. elegans*, attraction to nicotine may be mediated through the acr-5 and acr-15 nicotinic acetylcholine receptors (Sellings et al., 2013). Other possible mechanisms such as changes in drug metabolism or subtle changes in sensory systems have yet to be investigated. Interestingly, the effect of varenicline to inhibit nicotine self-exposure in this paradigm is evident at the 10-min time point and, although still evident at 30 min, appears to degrade over time (**Figure 5**). This could be a reflection of the apparent strength of the nicotine preference response, or possibly rapid clearance of varenicline. In support of this idea is the apparent greater strength of the preference response, and resistance to nonanone for nicotine compared with cocaine at the concentrations used in this study (**Figure 6**). Although it is somewhat difficult to make direct comparisons between the cocaine and nicotine data due to the differences in systems and mechanisms, and also in concentrations used to produce the respective preference responses, there are clear differences in the response to nonanone. One possible explanation is that animals are being paralyzed by the SOAs at these concentrations. This cannot be completely ruled out as previous work has indicated that nicotine uniformly mixed in agar to concentrations from 1 to 10 mM can induce paralysis (Sobkowiak et al., 2011). However, other evidence argues against the idea that the worms are paralyzed. First, although the concentrations of cocaine and nicotine contacting the worms in the target zones are not known, only 4 μl of SOA was absorbed into a target zone in a well containing 3.8 mls of agar, indicating the concentrations contacting the worms was likely much lower than the concentrations added to the target zones. Secondly, both groups of animals show a significant effect of nonanone to move the animals from the SOA target zone (although the magnitude of the effect was less for nicotine), indicating that they are not paralyzed. This hypothesis is further supported by examination of time-lapse videos (**Supplementary Video 1**) which clearly show animals continuing to move in the nicotine target zone after entering during a preference test on a plate spotted with 100 mM nicotine, and a clear movement out of the zone after the addition of nonanone. One possible explanation for the greater effect of nonanone to displace cocaine exposing animals compared to nicotine is the somewhat stronger preference response observed with nicotine vs. cocaine in these assays (**Figure 6**). The increased preference response for nicotine over cocaine suggests a greater reinforcing property of the SOA as tested and as such would confer greater aversion resistance. Future experiments will provide additional evaluation and characterization of varenicline and other compounds to inhibit the SOA preference response. Such work is needed to further demonstrate predictive validity and provide a strong case for the model's utility as a screening tool to help identify compounds that have potential as treatments for SOA and alcohol use disorders.

Behavioral studies of addictive SOAs in *C. elegans* to date have mostly focused on EtOH (Grotewiel and Bettinger, 2015; Engleman et al., 2016). Additional studies with other SOAs are needed to better characterize the mechanisms that underlie addictive properties of SOAs across the many classes of SOAs and how they may be consistent or divergent across species. In the few studies conducted thus far, several molecular targets have been identified in various behavioral paradigms across SOA classes using *C. elegans* (Engleman et al., 2016). Thus far, it appears that genes involved in monoamine neurotransmission mediate at least some behaviors induced by each SOA (Bettinger and McIntire, 2004; Lee et al., 2009; Ward et al., 2009; Musselman et al., 2012; Sellings et al., 2013; Topper et al., 2014; Matsuura and Urushihata, 2015). In particular, mutation of the gene coding for tyrosine hydroxylase (*cat-2*) reduced or inhibited SOA-induced behaviors for each SOA of abuse (Bettinger and McIntire, 2004; Musselman et al., 2012; Matsuura and Urushihata, 2015). It is widely thought that the dopamine neurotransmitter system plays an important role in drug abuse (Koob, 1992; Koob and Volkow, 2010) and all of the SOAs discussed here have effects on dopamine neurotransmission. Similarly, in agreement with the current data, previous work has shown that manipulations that inhibit cholinergic neurotransmission in *C. elegans* will also affect the behavioral response to nicotine (Feng et al., 2006; Sellings et al., 2013) and the effects of varenicline may be due in part to its effect in modulating dopamine neurotransmission (Crooks et al., 2014). Overall, the known mechanisms of action of SOAs in vertebrate animals thus far show parallel findings in *C. elegans* and other invertebrates. The effects of SOAs in *C. elegans* appear likely to be mediated by neurobiological systems associated with many genes and proteins that are known to mediate and/or support neuronal function in higher level organisms (for review see Engleman et al., 2016). However, since most of the work in *C. elegans* thus far has focused heavily on EtOH, additional studies are needed to determine if these mechanisms are also involved in other SOAs using the *C. elegans* as a model system. Since the contributions of the olfactory/chemosensory systems and specific mechanisms of attraction and self-exposure will likely changes across various classes of drugs of abuse, such differences may provide further insight into the addictive properties of individual drugs and will be a key focus of future studies.

Although *C. elegans* phenotypes are surprisingly highly conserved functionally, with few clear differences in

neurobiology, pharmacology, and molecular systems between vertebrates and *C. elegans*. Moreover, *C. elegans* simple nervous system lacks the complex neurocircuitry of mammals that have been found to be involved in addiction (Koob and Volkow, 2010). However, the similarities in responses to SOAs between *C. elegans* and mammals suggests that the behavioral responses to SOAs may rely more on functional similarities in terms of how SOAs affect systems that mediate survival (food) of the species rather than complexities in the neuroanatomy. Interestingly, differences in receptor systems and molecular pharmacology in *C. elegans* could also provide an advantage of this model providing a unique perspective concerning how some classes of putative treatments affect SOAs. As an example, topiramate is under investigation as a possible treatment for EtOH use disorders (Johnson, 2004). Topiramate has a rich pharmacology and there are several possible molecular mechanisms for reducing EtOH drinking behavior. One suggested mechanism is activity at voltage-sensitive sodium channels (Johnson, 2004), which are not present in *C. elegans* (Bargmann, 1998). If topiramate were to be found ineffective in *C. elegans* assays of EtOH self-exposure, the data would support the contention that sodium channels may have a role in reducing SOA intake/seeking in vertebrates. Thus, cross-species findings could be assessed with respect to molecular homology of the mechanisms thought to be mediating SOA taking and/or preference. This could be conducted across SOA classes to identify the effects of divergent molecular structure or function on the results. Overall, such investigations may help to characterize the molecular and pharmacological foundations of the effects of these compounds, whether or not the findings are consistent with the anticipated results.

Further development of the model employed here is anticipated to provide the field with a new and powerful tool to discover novel targets and treatments for addiction. This work will combine the advances in our knowledge of human addiction and insight gained through the use of vertebrate behavioral models, and apply them to invertebrate models with tremendous advantages and potential for discovery on a number of levels: (a) the current work contributes to the establishment of a new behavioral model in *C. elegans* for screening candidate compounds to treat stimulant addictions; (b) in addition, the ability to manipulate genes and gene expression quickly, and the availability of many mutant strains in this well studied and simple organism, greatly enhances the capability to discover specific genes and proteins involved in SOA preference behavior in this model; (c) the low cost and potential to fully automate the assays allows for a dramatic increase in the number of experiments that can be conducted for a fraction of the cost and time needed with other animal models. Thus, this model might be used in conjunction with gene editing techniques like CRISPR where *C. elegans* receptors can be replaced with their human orthologs to create transgenic *C. elegans* that might show human-like pharmacology. Such an application could improve the translational utility of the model and possibly enhance predictive validity.

Once a high-throughput system is fully established, one could conceivably screen entire potential treatment agent libraries using tiny amounts of expensive compounds relative to other animal models. Future collaborative projects will employ transgenic approaches to express human genes in this model to enhance the predictive validity of the model. Finally, the data will be bi-directionally informative with other animal models of medications development for substance use disorders and compounds in clinical trials, such that the diversity in pharmacology and molecular systems between different species will help to better identify the mechanisms of action of putative and/or validated treatment compounds.

AUTHOR CONTRIBUTIONS

EE and SK wrote the initial drafts and designed and directed the experiments. KS, KB, MB, and HK performed the experiments and provided critical feedback for method development and manuscript preparation. RB and BN-B reviewed content, data, and final draft of the manuscript.

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An Argument for Amphetamine-Induced Hallucinations in an Invertebrate

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Hallucinations – compelling perceptions of stimuli that aren't really there – occur in many psychiatric and neurological disorders, and are triggered by certain drugs of abuse. Despite their clinical importance, the neuronal mechanisms giving rise to hallucinations are poorly understood, in large part due to the absence of animal models in which they can be induced, confirmed to be endogenously generated, and objectively analyzed. In humans, amphetamine (AMPH) and related psychostimulants taken in large or repeated doses can induce hallucinations. Here we present evidence for such phenomena in the marine mollusk *Tritonia diomedea*. Animals injected with AMPH were found to sporadically launch spontaneous escape swims in the absence of eliciting stimuli. Deafferented isolated brains exposed to AMPH, where real stimuli could play no role, generated sporadic, spontaneous swim motor programs. A neurophysiological search of the swim network traced the origin of these drug-induced spontaneous motor programs to spontaneous bursts of firing in the S-cells, the CNS afferent neurons that normally inform the animal of skin contact with its predators and trigger the animal's escape swim. Further investigation identified AMPH-induced enhanced excitability and plateau potential properties in the S-cells. Taken together, these observations support an argument that *Tritonia*'s spontaneous AMPH-induced swims are triggered by false perceptions of predator contact – i.e., hallucinations—and illuminate potential cellular mechanisms for such phenomena.

Keywords: hallucinations, invertebrate, *Tritonia,* amphetamine, mollusk

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INTRODUCTION

Invertebrate models have become increasingly valuable for investigating how addictive drugs exert their effects on the nervous system and behavior (Kusayama and Watanabe, 2000; Wolf and Heberlein, 2003; Carvelli et al., 2010; Kennedy et al., 2010; van Swinderen and Brembs, 2010; Alcaro et al., 2011; Huber et al., 2011; Kaun et al., 2012; Musselman et al., 2012; Ramoz et al., 2012; Walters et al., 2012)[1]. The replication of many human drug-related behaviors in invertebrates suggests that underlying mechanisms may have been preserved across diverse nervous systems. For example, the *Drosophila* mutant *Radish* displays reduced attention-like behavior that is partly reversed by the ADHD drug methylphenidate (van Swinderen and Brembs, 2010). In addition, methamphetamine-induced anorexia, and d-amphetamine-, cocaine-, and opioid-associated drug seeking and addiction

[1]https://www.frontiersin.org/research-topics/5495/invertebrate-models-of-natural-and-drug-sensitive-reward

behaviors have been described in crayfish (Alcaro et al., 2011; Huber et al., 2011), *Drosophila* (Kaun et al., 2012; Walters et al., 2012), *Caenorhabditis elegans* (Carvelli et al., 2010; Musselman et al., 2012), planaria (Kusayama and Watanabe, 2000), and *Lymnaea stagnalis* (Kennedy et al., 2010). Although the behavioral effects of psychostimulants and classical hallucinogens have been studied in invertebrates (Witt, 1971; Nichols et al., 2002; Wolf and Heberlein, 2003), to our knowledge hallucinations themselves have yet to be demonstrated, or even suggested to occur.

Hallucinations are defined as perceptions of stimuli (visual, auditory, tactile) that don't actually exist (Esquirol, 1965; DSM-IV, 2000). They occur in several psychiatric and neurological diseases, as well as in response to certain drugs of abuse (Asaad and Shapiro, 1986; Brasic, 1998). One of these is the psychostimulant amphetamine (AMPH) and its derivatives. Chronic, or in some cases even single high doses of AMPH can induce a paranoid psychotic state closely resembling that of schizophrenia, complete with vivid hallucinations (Connell, 1958; Angrist and Gershon, 1970; Bell, 1973; Snyder et al., 1974; Groves and Rebec, 1976; Seiden et al., 1993). One well-known type of hallucination induced by AMPH and its derivatives is formication—the sensation of "bugs" biting or crawling on the skin (Ellinwood, 1967; Smith and Crim, 1969; Stanciu et al., 2015). Amphetamine also induces what have been speculated to be hallucinations in non-human animals, including monkeys (Nielsen et al., 1983), rats (Nielsen et al., 1980), and mice (Tadano et al., 1986). Understanding the cellular mechanisms that cause neural networks to generate false perceptions is of great importance to both clinical neuroscience and behavioral biology. Unfortunately, since animals cannot report their subjective experiences, little progress has been made on this topic.

Tritonia diomedea is a marine nudibranch mollusk attractive for neurophysiological studies because of its large pigmented neurons, many of which are individually identifiable from animal to animal. Upon skin contact with its seastar predators, *Tritonia* launches a rhythmic escape swim consisting of a series of alternating ventral and dorsal whole-body flexions (**Figure 1A**). The animal rarely displays this behavior spontaneously. Here we demonstrate that *Tritonia* injected with large or repeated doses of amphetamine (AMPH) launch sporadic escape swims in the absence of any apparent stimulus. The neural circuit mediating this behavior is well understood (**Figure 3A**; Getting, 1983; Frost et al., 2001) and can be studied in deafferented brain preparations where real stimuli can play no role. This allowed us to investigate the neural basis of these unusual drug-induced escape behaviors.

MATERIALS AND METHODS

Behavioral Experiments

Tritonia diomedea were obtained from two sources. Those used in the initial pilot behavioral experiment were collected near Dash Point, Puget Sound, WA and maintained in running seawater tanks (11–13°C) at Friday Harbor Laboratories, Friday Harbor, WA, United States. Those used in all remaining experiments were obtained from Living Elements, Vancouver, BC, Canada, and maintained at 11°C in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH, United States) at Rosalind Franklin University of Medicine and Science. Animals were injected into the body cavity near the buccal mass with either artificial seawater or D-amphetamine sulfate (Sigma) mixed in artificial seawater. Fresh stock solutions of AMPH were prepared each day. Animals were injected to produce the desired concentration of AMPH (3–40 mg/kg), assuming full diffusion into the animal's volume, calculated as 1 ml per gm of body weight. Controls were injected with a weight-equivalent amount of artificial seawater. A test animal injected with fast green (Sigma) stained completely, indicating that injected substances do spread from the hemocoel throughout the body tissues. For comparison purposes, common doses of D-amphetamine used in behavioral studies in vertebrate animals range from 1 – 20 mg/kg i.p. (Randrup and Munkvad, 1967), and human amphetamine abusers have been estimated to experience a dosage range of 5–25 mg/kg/day (Trulson and Jacobs, 1979). In mammals, D-amphetamine equilibrates in brain tissue at a higher concentration than the injected i.p. concentration. For example, an 8 mg/kg i.p. injection in rats reaches 25 mg/kg in the brain (Maickel et al., 1969), a value slightly higher than the Day 3 dose of our progressive behavioral experiment.

Electrophysiological Experiments

The cellular experiments utilized both semi-intact animal and isolated brain preparations. Semi-intact preparation. This consisted of the brain and body, but with the internal organs removed. This preparation was used to obtain intracellular recordings from swim circuit neurons during swim motor programs (SMPs) elicited by natural skin stimulation. The details of this dissection procedure were as previously described (Lee et al., 2012). Isolated brain preparation. The brain, consisting of the fused cerebral–pleural ganglia and the pedal ganglia (with the pedal–pedal commissures cut), was dissected from the animal and pinned dorsal side-up in a Sylgard-lined recording chamber perfused with artificial seawater at 4–6°C. After dissecting away the connective tissue sheath covering the dorsal side of the cerebral–pleural ganglia, a polyethylene suction electrode was attached to left pedal nerve 3 (for nomenclature, see Willows et al., 1973a). The perfusion temperature was then raised to 11°C for the recording session. Intracellular recordings were made with 15–40 MΩ electrodes filled with 3 M KCl or 3 M *K*-acetate. Neurons were identified on the basis of their location, size, color, synaptic connections with other identified neurons, and activity during the SMP, as described previously (Getting, 1983; Frost and Katz, 1996; Frost et al., 2001). Swim motor programs were elicited by applying a 10 Hz, 2 s train of 5 ms 10 V pulses to the suction electrode attached to pedal nerve 3. The AMPH was prepared in artificial seawater at the concentration to be used, and applied via a gravity-driven perfusion system by switching a stopcock between instant ocean and AMPH sources. Data were digitized at 1000 Hz with a Biopac MP150 data acquisition system. Normal saline consisted of (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 HEPES, pH 7.6, and 11 D-glucose. In 0 calcium experiments, the calcium was replaced with the same concentration of either $CoCl_2$ or $BaCl_2$. Experiments

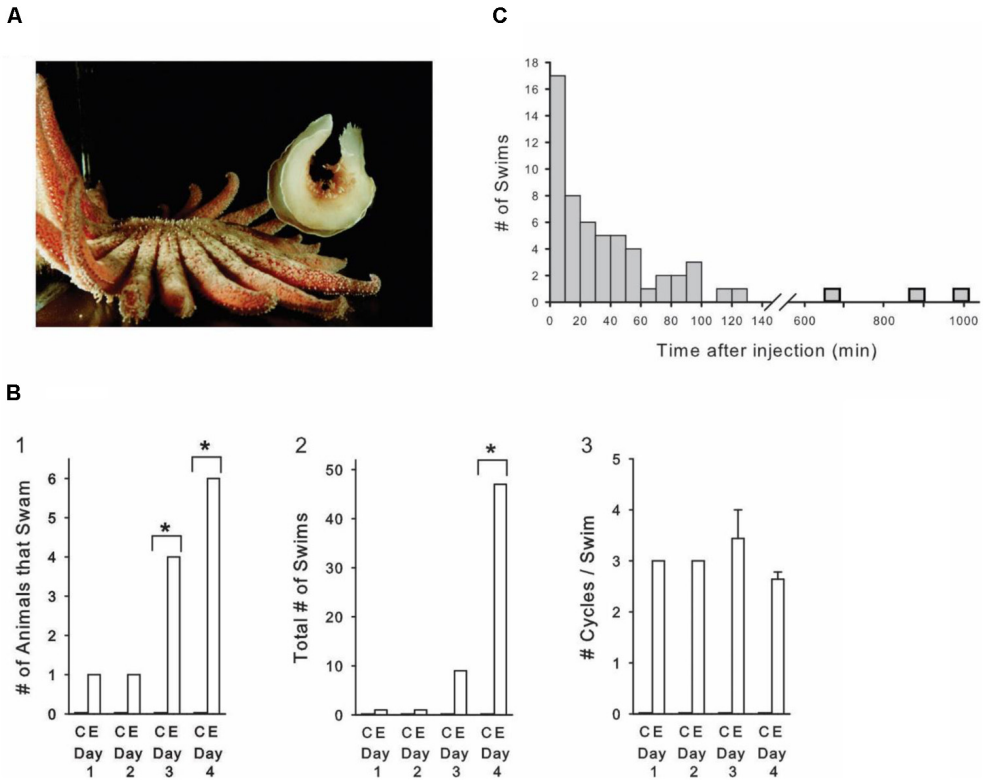

FIGURE 1 | Amphetamine induces sporadic, spontaneous escape swims in freely behaving animals. **(A)** *Tritonia* at maximal dorsal flexion during an escape swim triggered by skin contact with a predator, the seastar *Pycnopodia helianthoides*. **(B)** Progressive AMPH dose experiment. **(B1)** The number of AMPH-injected animals displaying spontaneous swims significantly increased over the course of the 4-day experiment, while controls never swam. **(B2)** The total number of swims in the AMPH-injected group significantly increased over the course of the 4-day experiment. **(B3)** The number of cycles per swim was not affected by the progressive AMPH regimen. AMPH dose on day 1: 5 mg/kg, day 2: 10 mg/kg, day 3: 20 mg/kg, day 4: 40 mg/kg. C, Controls; E, Experimentals. **(C)** Time after AMPH injection of all 58 spontaneous swims that occurred in the progressive dose experiment. AMPH-induced swims occurred sporadically, with the majority occurring more than 20 min after the injection, and nearly one-fourth occurring an hour to many hours after. Asterisks indicate significance at $p < 0.05$.

applied AMPH at concentrations ranging from 10 to 1000 μm, similar to the range of 1 to 300 μM used in some vertebrate electrophysiological studies (Mercuri et al., 1989). Throughout results, means are reported ± standard error.

RESULTS

AMPH Induces Sporadic, Spontaneous Escape Swims in Freely Behaving *Tritonia*

Skin contact with the tube feet of its seastar predator triggers *Tritonia's* escape swim, consisting of an alternating series of ventral and dorsal whole-body flexions that propel it away to safety (**Figure 1A**). The escape swim has a high threshold, and in laboratory tanks does not normally occur in the absence of suitably aversive skin stimuli, which include predator contact, bites from conspecifics, or strong salt applied to the skin. We were therefore intrigued to find that *Tritonia* occasionally exhibited spontaneous escape swims in the minutes to hours after being injected with AMPH. In an initial pilot experiment, 25 drug-naïve experimental animals received AMPH injections (3 −15 mg/kg in a saline vehicle), after which they were filmed for 2 h. Some of the animals received additional injections at later times and were again filmed. In response to 48 total injections, 9 of the animals spontaneously swam at least once, with 19 spontaneous swims recorded overall. The swims ranged from 2 – 11 flexion cycles in duration, typical of stimulus-elicited escape swims in this animal. None of the 10 control animals receiving weight-matched injections of the saline vehicle swam.

In a second experiment (**Figures 1B,C**), 10 drug-naïve experimental animals were injected with progressively increasing AMPH doses (see **Figure 1** legend for details), once-per-day for 4 days (mean weight = 95.0 g, range 45 – 200 g). A group of 10 control animals were injected on the same schedule with the saline vehicle (mean weight = 95.0 g, range 15 – 130 g). During the experiment, animals were individually housed in 2 rows of 5 compartments that were pressed against the clear front wall of their home aquarium, where they could be filmed 10 at a time. All animals were filmed continuously with time-lapse video for 4 days (white light 12 h, red light, 12 h), allowing every swim in every animal to be observed over this period. Control and experimental animals were randomly distributed among the different compartments, and the individual viewing

the videotapes was blind to which animals received AMPH vs. artificial seawater. All animals were drug-naïve at the start of the experiment.

As in the pilot experiment, several AMPH-injected animals displayed spontaneous swims of 2 or more cycles in the absence of any apparent stimulus, while saline-injected animals never swam. On the first day, 1 of the 10 experimental animals swam after AMPH injection, whereas by day 4, 6 animals swam (**Figure 1B1**). Over the course of the experiment, there was a significant overall difference in the number of animals that swam in the experimental group ($p < 0.01$, Cochran Q Test for dichotomous nominal scale data). Day-by-day between-group comparisons indicated that AMPH injections produced a significant increase in the number of animals that swam on day 3 and day 4 ($p = 0.043$ and $p = 0.005$, respectively, Fisher-exact Test, One-tailed). In addition, the total number of swims markedly increased over the course of the experiment (**Figure 1B2**). A two-way repeated measures ANOVA indicated a significant interaction between the AMPH vs. saline injected groups and treatment day [$F(3,54) = 4.708$, $p = 0.005$]. *Post hoc* Student–Newman–Keuls comparisons between the AMPH and saline groups for each day indicated that the number of SMPs was significantly different on Day 4, the highest dose of AMPH. Thus, while one animal swam once on day 1, by day 4, six animals swam a total of 47 times ($p < 0.001$). In vertebrates, AMPH is well known to produce behavioral sensitization – increased responsiveness over time when the drug is administered in repeated fashion (Robinson and Becker, 1986). We did not attempt to determine whether sensitization contributed to the increased responsiveness observed with our progressive-dose drug regimen. A one-way repeated measures ANOVA indicated that, in spite of the above effect of this progressive AMPH administration regimen on swim occurrence, it had no effect on the number of cycles per swim [$F(3,8) = 1.349$, $p = 0.325$], which averaged 3.0 ± 0.2 across the 4 days of the experiment (**Figure 1B3**).

A notable feature of the AMPH-induced swims was their unpredictability. Rather than occurring immediately after injection, as swims do when *Tritonia* are injected with the neurotransmitter serotonin (McClellan et al., 1994), the AMPH-induced swims occurred sporadically, anywhere from several minutes to several hours following injection of the drug. The time after injection for all 58 AMPH-induced spontaneous swims in the progressive dose experiment is shown in **Figure 1C**. While 17 swims occurred in the first 10 min after injection, the majority occurred later, with 27 occurring more than 30 min following injection, including 3 that occurred at 11.2, 14.7, and 16.5 h post-injection.

Other AMPH-Induced Behaviors

Amphetamine is well known to produce unusual and repeated stereotyped behaviors in vertebrates, including twitching, rearing, and biting (Groves and Rebec, 1976; Rebec and Bashore, 1984; Seiden et al., 1993). A final behavioral experiment focused on whether AMPH elicits any repetitive stereotyped behaviors in *Tritonia*. Seven drug-naïve experimental animals were injected with a single dose of 20 mg/kg AMPH (mean weight = 86.0 g, range 20–232 g), while 7 controls were injected with artificial seawater (mean weight = 82.3 g, range 22–252 g). After injection, each animal was placed in a Plexiglas box and filmed for 3 h using a tripod-mounted camera and a time lapse VCR to record general activity. In addition, during the first hour an observer visually monitored the animal's mouth region, using a mirror as needed through the transparent bottom of the tank to record instances of spontaneous mouth opening and/or biting. After all animals were filmed separately, videos of saline- and AMPH-injected pairs were mixed into side-by-side videos to allow simultaneous viewing at 24x speed in order to determine whether there were characteristic effects of AMPH on ongoing behavior.

Amphetamine-injected animals displayed several stereotypic behaviors that were either unique to the drug, or occurred with much greater frequency than in saline-injected controls. *Biting*. During the hour of direct visual observation of their mouth region, AMPH-injected animals exhibited significantly increased spontaneous mouth opening and/or biting vs. controls (**Figure 2A**; mean = 11.7 ± 3.5 vs. 1.6 ± 0.7 events, t-test, $t = 2.81$, $p = 0.016$), involving mouth opening events which often included the full odontophore grasping and radular scraping components of a normal bite, but with nothing in contact with the mouth region. During the full 3 h of post-injection videotaped behavior AMPH-injected animals exhibited several additional behaviors not normally seen. *Ventral flexions*. Drug-injected animals displayed several spontaneous single ventral twitches or flexions. (**Figure 2B**; mean = 7.7 ± 2.0 vs. 0.0 ± 0.0 flexions, $t = 3.86$, $p = 0.002$). *Head rearing*. AMPH-injected animals often crawled with their front foot margin and oral veil raised above the substrate, which we referred to as head rearing behavior. To document this, we counted the number of minutes when any instance of head rearing behavior occurred during the 3-h post-injection observation period. AMPH injected animals showed significantly more head rearing than controls (**Figure 2C**; mean = 27.6 ± 9.4 vs. 0.0 ± 0.0 min in which rearing events occurred, $t = 2.92$, $p = 0.013$). *Raised tail*. AMPH-injected animals also often crawled with their tail raised off the substrate, a behavior not seen in the saline-injected controls (**Figure 2D**; mean = 37.9 ± 12.5 vs. 0.0 ± 0.0 min in which such events occurred, $t = 3.03$, $p = 0.010$). These results appear consistent with amphetamine's ability to induce repeated stereotyped behaviors in vertebrates, in spite of *Tritonia*'s markedly different invertebrate CNS organization.

The AMPH-Induced Swim Initiates From Within the Brain, Rather Than in Response to a Real Skin Stimulus

While the AMPH-induced swims in the behavioral experiments appeared to be spontaneous in origin, it was possible that the drug enhanced the animals' awareness of, or responsiveness to, real skin irritants that are normally below threshold for eliciting the swim. This issue has plagued interpretation of the origin of "spontaneous" AMPH-induced behaviors in vertebrate studies (see Discussion). To address this issue we next tested whether AMPH would induce SMPs in deafferented, isolated brain preparations, where sensations elicited by skin stimuli

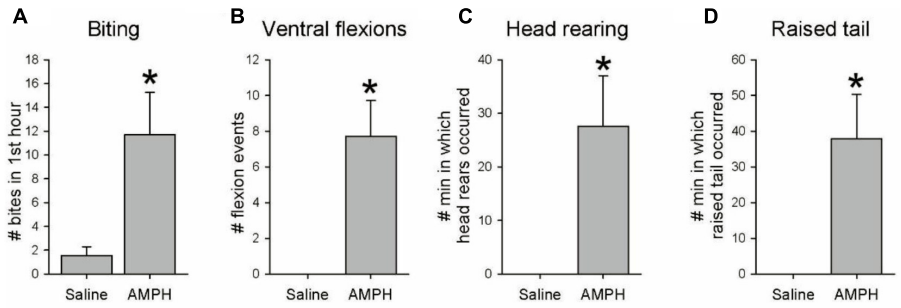

FIGURE 2 | Other behavioral effects of amphetamine. Seven drug-naïve animals were injected with 20 mg/kg AMPH, and seven others with a weight-matched amount of saline vehicle. Animals were videotaped for 3 h after injection, with the mouth area watched live for the first hour. **(A–D)** AMPH-injected animals exhibited significantly more spontaneous biting ($p = 0.016$), non-swimming single ventral flexions/twitches ($p = 0.002$), head rearing ($p = 0.013$) and crawling with raised tail ($p = 0.010$). Asterisks indicate significance at $p < 0.05$.

cannot occur. SMPs elicited by nerve stimulation in the isolated brain preparation (**Figure 3B1**) were similar in appearance to those elicited by aversive skin stimuli in semi-intact animal preparations (**Figure 3D**), consistent with the well-documented negligible role of sensory feedback in this centrally generated motor program (Dorsett et al., 1973; Frost et al., 2001).

In decades of work with *Tritonia* isolated brain preparations, we had never observed a spontaneous SMP in normal saline. It was therefore striking that in all 30 drug-naive isolated brains in which it was attempted, perfusion with 50 μM to 1 mM AMPH led to several (range = 1–19 per preparation) spontaneous, AMPH-induced swim motor programs (AMPH-SMPs) (**Figure 3B2**; 50 μM: 7.0 ± 1.6 AMPH-SMPs per preparation, 2.9 ± 0.2 cycles per motor program, range = 2–4 cycles, 5 preparations; 100 μM: 10.4 ± 1.8 AMPH-SMPs per preparation, 3.0 ± 0.5 cycles per motor program, range = 2–7 cycles, 7 preparations; 1 mM: 3.3 ± 0.7 AMPH-SMPs per preparation, 3.1 ± 0.2 cycles per motor program, range 2–7 cycles, 18 preparations). In these experiments, each brain was exposed once to a single concentration of AMPH. AMPH perfused at 10 or 20 μM did not induce AMPH-SMPs in single experiments tried at each of these lower concentrations, but more work is needed to reliably determine the threshold dose. AMPH-SMPs were similar in appearance to SMPs elicited by real sensory input – they began abruptly from a normal baseline of neuronal activity and then proceeded through several cycles of rhythmic firing (**Figure 3B**). From these isolated brain results we conclude that the drug-induced swims observed in AMPH-injected intact behaving animals appear to be triggered, not by actual skin stimuli, but instead by spontaneous activity originating within the nervous system.

The AMPH-Induced Swim Motor Program Originates With Spontaneous Bursts of Activity in the Swim Afferent Neurons

We next sought the site of origin of the AMPH-SMPs in the swim circuit. Because direct intracellular stimulation of several individual command and CPG interneurons can effectively bypass the S-cells and elicit the SMP in normal saline (Getting, 1977; Frost et al., 2001; Katz et al., 2004), there were multiple potential sites of origin of the AMPH-SMP in the swim circuit. We therefore obtained intracellular recordings from neurons at all hierarchical levels of the swim circuit during spontaneous AMPH-SMPs to determine where the circuit activity originated. For example, if the AMPH-SMP originates in the CPG, then the upstream neurons that normally fire to trigger the skin-elicited SMP would be expected to remain largely silent. Over the course of 38 preparations, which included those described above, we obtained multiple intracellular recordings from most of the known members of the swim circuit during spontaneous AMPH-SMPs, including the DRI swim command neurons ($N = 2$), the C2 ($N = 9$) and DSI ($N = 44$) CPG neurons, and the DFN ($N = 11$) and VFN ($N = 14$) flexion neurons. We found that all sampled interneurons and flexion neurons participated during the spontaneous AMPH-SMP (**Figures 3B,C**) as they normally do in response to real sensory input (**Figures 3B,D**; Getting, 1983; Frost et al., 2001).

Having traced the origin of the spontaneous AMPH-SMP as far as the swim command neurons, we next turned to their input, the well-characterized swim afferent neurons (S-cells). The S-cells have their cell bodies located in a cluster on the dorsal side of each pleural ganglion (Getting, 1976; Megalou et al., 2009). Each pseudounipolar S-cell sends one or more axons out peripheral nerves to innervate specific regions of the skin (Getting, 1976; Frost et al., 2003). Within the brain, each S-cell makes monosynaptic excitatory connections onto the Tr1 and DRI command neurons to initiate the SMP (**Figure 3A**) (Frost and Katz, 1996; Frost et al., 2001). **Figure 3D** shows how S-cells, together with the swim command neuron DRI and CPG neuron DSI fire in response to an SMP-initiating aversive salt stimulus applied to the skin in a semi-intact animal preparation. Such stimuli elicit a vigorous burst of firing in those S-cells having receptive fields in the stimulated skin region. The S-cell burst then terminates soon after the SMP gets underway.

In 18 of the 38 isolated brain preparations comprising the above swim network survey, one to three S-cells were simultaneously recorded together with a DSI neuron, which was included to indicate the onset time of each AMPH-SMP, as well as

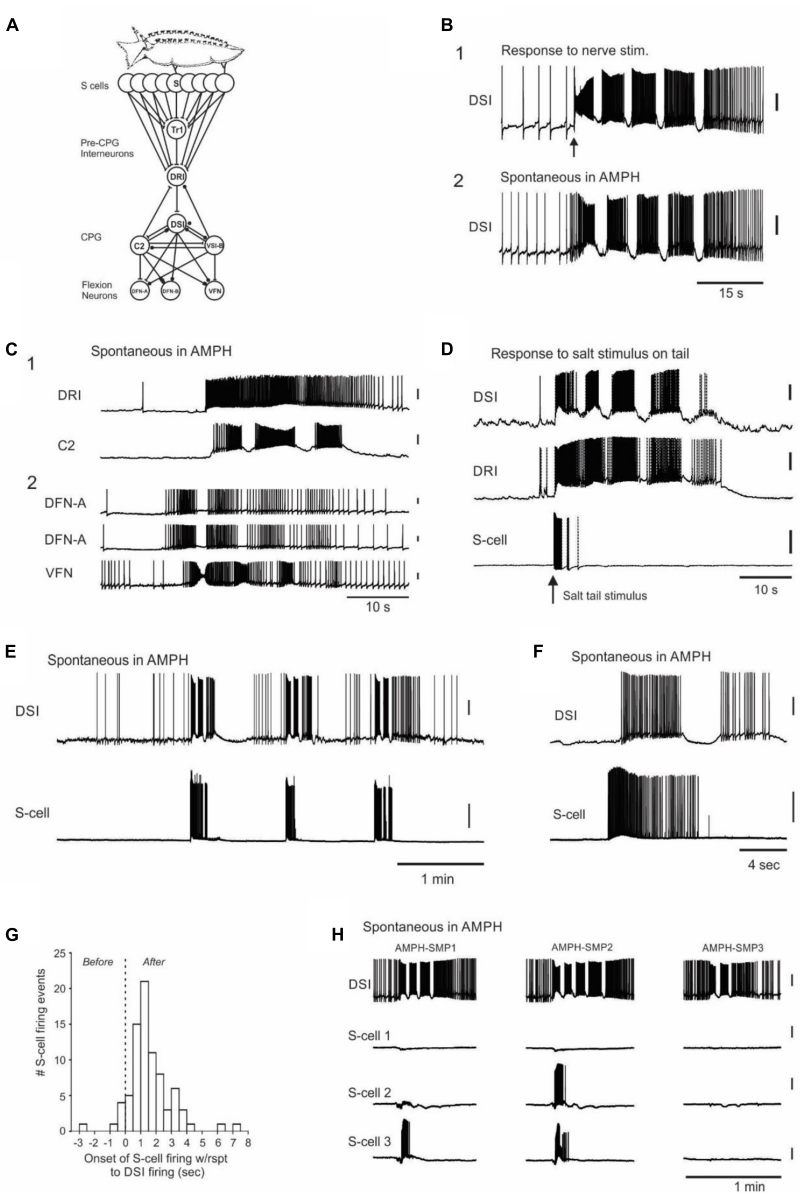

FIGURE 3 | Neurophysiological evidence that the AMPH-induced swims originate within the CNS, with spontaneous bursts in the normally silent afferent neuron population that detects the animal's seastar predators. **(A)** *Tritonia* escape swim circuit. Skin stimuli elicit the motor program by exciting the S-cells (afferent neurons) in the brain, which in turn activate pre-CPG command interneurons, CPG interneurons and efferent flexion neurons. S, S-cells; Tr1, Trigger-type 1 command neuron; DRI, dorsal ramp command neuron; DSI, dorsal swim interneuron; C2, Cerebral neuron 2; VSI-B, Ventral swim interneuron type B; DFN-A, Dorsal flexion neuron type A; DFN-B, Dorsal flexion neuron type B; VFN, Ventral flexion neuron. **(B)** Similarity of sensory-elicited vs. AMPH-induced swim motor programs (AMPH-SMPs). **(B1)** Stimulus-elicited SMP in normal saline, elicited via brief suction electrode stimulation (10 Hz, 1 s, 10 V) of Pedal Nerve 3, a peripheral nerve containing S-cell axons. **(B2)** Spontaneous AMPH-SMP that occurred 50 min after switching perfusion from normal saline to 50 μM AMPH saline. The two recordings are from different preparations. As can be seen here and in other panels, spontaneous AMPH-SMPs are very similar in appearance to normal, stimulus-elicited SMPs. **(C)** A survey of circuit neurons traced the origin of the AMPH-SMP to the CPG or more afferent network loci. **(C1)** Simultaneous recording from the pre-CPG command neuron DRI and the CPG interneuron DSI during a spontaneous AMPH-SMP. **(C2)** Simultaneous recording in a different preparation from three flexion neurons during a spontaneous AMPH-SMP. Both experiments in 1 mM AMPH. **(D)** Swim motor program elicited by salt applied to the skin in a semi-intact animal preparation. Stimulus-elicited SMPs begin with a burst of action potentials in the S-cells, which converge onto the single command neuron DRI that in turn directly drives the DSI neurons of the CPG. **(E)** Three consecutive spontaneous motor programs in an isolated brain preparation recorded in 50 μM AMPH. Each AMPH-SMP began with a burst of spikes in the recorded S-cell. **(F)** Recording of a spontaneous S-cell burst that began shortly before the onset of the AMPH-SMP. **(G)** The time of onset of all recorded S-cell bursts with respect to the first action potential of the speed-up of DSI firing rate that signaled AMPH-SMP onset. **(H)** Simultaneous recording from DSI and three S-cells during three spontaneous AMPH-SMPs in an isolated brain perfused with 100 μM AMPH. Different combinations of S-cells initiate each AMPH-SMP, consistent with a shifting body location for the perceived but non-existent predator contact. The motor programs occurred at 7.0, 10.9, and 30.1 min after the onset of AMPH perfusion. All but **(D)** are from isolated brain preparations. All vertical scale bars = 20 mV.

its number of cycles. Across these preparations, AMPH perfusion induced a total of 114 spontaneous SMPs of 2 or more cycles (mean = 8.8 SMPs ± 1.9, range = 1–19 per preparation), yielding a dataset of 238 recordings of how 40 S-cells did or did not fire during AMPH-SMPs. In total we recorded 81 S-cell firing events during 59 AMPH-SMPs in 18 preparations. These firing events were typically vigorous (mean = 69.9 ± 5.2 spikes; range = 3–223 spikes), with firing rates reaching 33 Hz.

S-cells in the isolated brain are silent at rest, and their only known synaptic input is inhibitory, from the normally silent Pl9 neuron that gets its input from the S-cells (Frost et al., 2003). We therefore did not expect them to fire in association with the AMPH-SMP, when there is no possibility of skin stimulation. However, of the above 40 S-cells, 12 (30%) fired a burst of spikes before or during the initial part of the first spontaneous AMPH-SMP that occurred during their recording (**Figures 3E–H**). Assuming our sampling was random from the 80 S-cells estimated to be in the recorded pleural ganglion (Getting, 1976), this suggests that approximately 24 S-cells erupt into activity at the time of onset of the AMPH-SMP. Since a prior study found that directly driving a minimum of 5 S-cells is needed to initiate the SMP in normal saline (Getting, 1976), this large-scale firing event in the S-cell population appears more than sufficient to trigger the AMPH-SMP.

In isolated brain studies of the swim network in normal saline, the motor program is typically triggered by trains of short electrical pulses applied to PdN3, such as in **Figure 3B1**. In that highly artificial case, 100% of the directly driven S-cells will start firing before the CPG's DSI neurons, since the latter are two synapses downstream from the S-cells (**Figure 3A**). **Figure 3G** shows the time of onset of all 81 recorded S-cell bursts with respect to the first action potential of the speed-up of DSI firing rate that signals the first hint of motor program onset. This S-cell firing, because it was not forced by direct stimulation of S-cell axons in a peripheral nerve, was not synchronous in onset. The very first S-cells to fire, such as those first responders shown in **Figure 3G**, would act to start increasing the DSI firing rate. Then, as increased numbers of S-cells rapidly join the population burst, they drive the accelerating DSI activity that becomes the first motor program cycle.

The S-cells somatotopically innervate the body surface (Getting, 1976; Slawsky, 1979; Frost et al., 2003), thus each cell normally informs the animal of a stimulus at a specific region of the body. Of the 11 S-cells that fired in preparations with multiple AMPH-SMPs, 9 did so in some SMPs but not others (**Figure 3H**). From this we conclude that the body location of the perceived skin stimulus apparently shifts from episode to episode (see Discussion).

In a further test of the hypothesis that spontaneous bursts in the S-cells are the origin of the AMPH-SMP, we also examined whether AMPH could induce the motor program when applied only to a small well beside the isolated brain containing the cut end of PdN3, which remained attached to the brain via a Vaseline-sealed slit. Many S-cells send their peripheral axons in PdN3 to innervate the skin. In the 2 preparations in which this was tried, exposing the nerve alone to 1 mM AMPH led to 14 total spontaneous SMPs, during which 3 of 6 recorded S-cells fired a burst at swim onset. As a peripheral nerve, PdN3 primarily contains the axons of afferent and efferent neurons, so this observation supports our conclusion that the AMPH-SMP originates with spontaneous bursts in the S-cells, rather than with network interneurons, whose processes are not known to travel in peripheral nerves.

Possible Mechanisms Contributing to the AMPH-Induced Afferent Neuron Population Burst

Prior studies estimated the size of the S-cell population to be approximately 160 neurons (∼80 per pleural ganglion) (Getting, 1976, 1983). The above finding that 30% of recorded S-cells fired a burst of spikes before or during the initial part of at least one AMPH-SMP suggests that a sizable portion of the S-cell population spontaneously erupts into activity from a silent baseline to trigger each AMPH-SMP. AMPH thus transforms the normally silent and non-interactive S-cell population into one that is sporadically eruptive. Further experiments exposed possible contributing processes.

In 15 experiments from the above dataset, S-cells were impaled and driven at regular intervals with 3 or 5 s depolarizing constant current pulses (36 total S-cells, 2 – 3 per preparation) while recording a DSI CPG neuron to monitor SMP occurrence during AMPH perfusion. Depending on the preparation, current pulses were administered at either 1, 2, or 5 min intervals, beginning several minutes before, and continuing for several minutes after the start of perfusion of either 0.05 mM (N = 5 preparations) or 1 mM (N = 10 preparations) AMPH.

Enhanced S-Cell Efficacy and Excitability

A striking finding was that in AMPH, 14 of the 36 S-cells (38.9%) across 10 preparations triggered an SMP in response to the firing of that single neuron driven by a current pulse (**Figure 4A**). Five of these 14 S-cells, all in different preparations, triggered SMPs on multiple trials (range 2–4 trials). Because SMPs also occurred spontaneously in the presence of AMPH (mean = 4.73 ± 0.76 SMPs, range = 1–10 per preparation), an SMP was considered to have been triggered by the S-cell current pulse if the speed-up of DSI tonic firing signaling motor program onset began within 2 s after the end of the S-cell current injection. Such triggering of SMPs by single S-cells has never been observed by us in normal saline, either during this study, or across several years of work with S-cells (Frost et al., 2001, 2003; Megalou et al., 2009; Lee et al., 2012). Ten of the 36 recorded S-cells, in 7 preparations, also exhibited firing that continued beyond the end of the current pulse in AMPH (mean = 24.08 ± 8.55 extra spikes, range = 1–101), a phenomenon also never observed in normal saline in these experiments or in prior work (**Figures 4A,B**).

Occasionally the S-cell firing elicited by the constant current pulses appeared to spread to other recorded S-cells in AMPH (**Figure 4B**). This occurred with 5 (13.9%) of the 36 stimulated S-cells on 1 or more current pulse trials, in 3 of the 15 preparations, involving both 0.05 and 1.0 mM AMPH. The mechanism of this rapid spread of firing in the S-cell population remains unknown. Prior studies have reported no direct

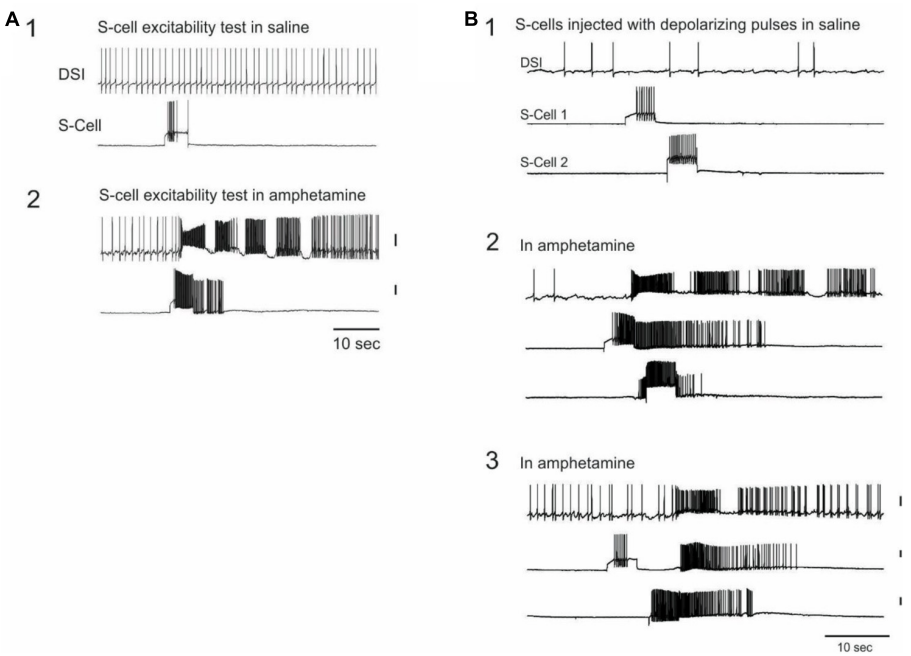

FIGURE 4 | Single S-cells can trigger the AMPH-SMP. **(A)** In AMPH, S-cells respond to constant current injections with increased firing, which can continue after the current injection and can trigger the swim motor program. The same two neurons are simultaneously recorded across both panels. **(B)** In AMPH, S-cells can excite strong firing in other S-cells. The same three neurons were simultaneously recorded across the panels. **(B1)** in saline, both S-cells responded to the current injection with modest firing. **(B2)** in 1 mM AMPH, the firing of the first neuron to the constant current test pulse triggered an SMP. The firing continued after the test pulse and also spread to the other S-cell. **(B3)** Injecting the same constant current pulses triggered a one-cycle SMP that originated this time from the other S-cell. The amount of injected current was constant across panels for each neuron. All vertical scale bars = 20 mV.

excitatory synaptic connections among the S-cells (Getting, 1976), consistent with our own observations before this study.

AMPH-Dependent Plateau Potentials in S-Cells

As a further test of whether AMPH acts directly on the S-cells, we repeated the prior constant current test pulse protocol in calcium-free AMPH saline, in which the normal 11 mM calcium chloride was replaced by either 11 mM cobalt chloride or 11 mM barium chloride. Twenty five S-cells were recorded in 0 calcium saline in 8 new preparations (range = 1–4 S-cells per preparation). Before the addition of 1 mM AMPH, 3–5 s depolarizing constant current pulses delivered at 1–2 min intervals elicited S-cell firing that always ceased with the end of the pulse (**Figure 5A1**). After 1 mM AMPH was added, 12 of the 25 S-cells sporadically exhibited firing that continued beyond the end of the current pulse (mean of the largest such event for each cell = 30.92 ± 8.29 extra spikes, range 1–84; **Figure 5A2**).

The sporadic nature of this post-current injection firing in AMPH may be associated with AMPH-induced plateau potential properties in the S-cells that are variably triggered by the current pulses. Plateau potentials are induced in many invertebrate and vertebrate neurons by monoamine and other modulatory transmitters (Kiehn, 1991), and cause cells to exhibit sustained firing in response to brief inputs. Once triggered, plateau potentials either spontaneously terminate, or can be terminated by brief hyperpolarizing inputs. In calcium free, 1 mM AMPH saline the prolonged S-cell firing that continued after injections of depolarizing current could be abruptly terminated by injecting brief hyperpolarizing current pulses (**Figures 5A2–A4**), suggesting they are plateau potential based. In 9 of the above 25 S-cells examined in 0 calcium AMPH saline, injecting sufficient depolarizing current to fully accommodate the S-cell action potential sporadically evoked large-amplitude spike-free plateau potentials that outlasted the current pulse by several seconds to over 1 min (**Figure 5B1**). In 5 out of 6 S-cells in which it was attempted, these large depolarization-induced plateau potentials were abruptly terminated by brief hyperpolarizing current pulses (**Figure 5B2**, 5 preparations). While many plateau potentials are calcium-dependent (Kiehn, 1991), calcium-independent examples, such as shown here, have also been described in both vertebrates (Llinas and Sugimori, 1980; Hoehn et al., 1993) and invertebrates (Angstadt and Choo, 1996).

Consistent with AMPH inducing instability in the S-cell population through direct action on these cells, when isolated from spike-mediated chemical synaptic inputs by 0 calcium saline S-cells were still observed to sporadically burst spontaneously. Eight of the 25 S-cells examined in 0 calcium AMPH gave forth spontaneous bursts (mean of the largest such burst for all such cells = 36.00 ± 9.91 spikes in a burst duration of 4.37 ± 1.06 s; range = 9–84 spikes; mean max frequency = 16.05 Hz, **Figure 5A5**). Spontaneous bursts were never observed during the pre-AMPH testing period in 0 calcium saline.

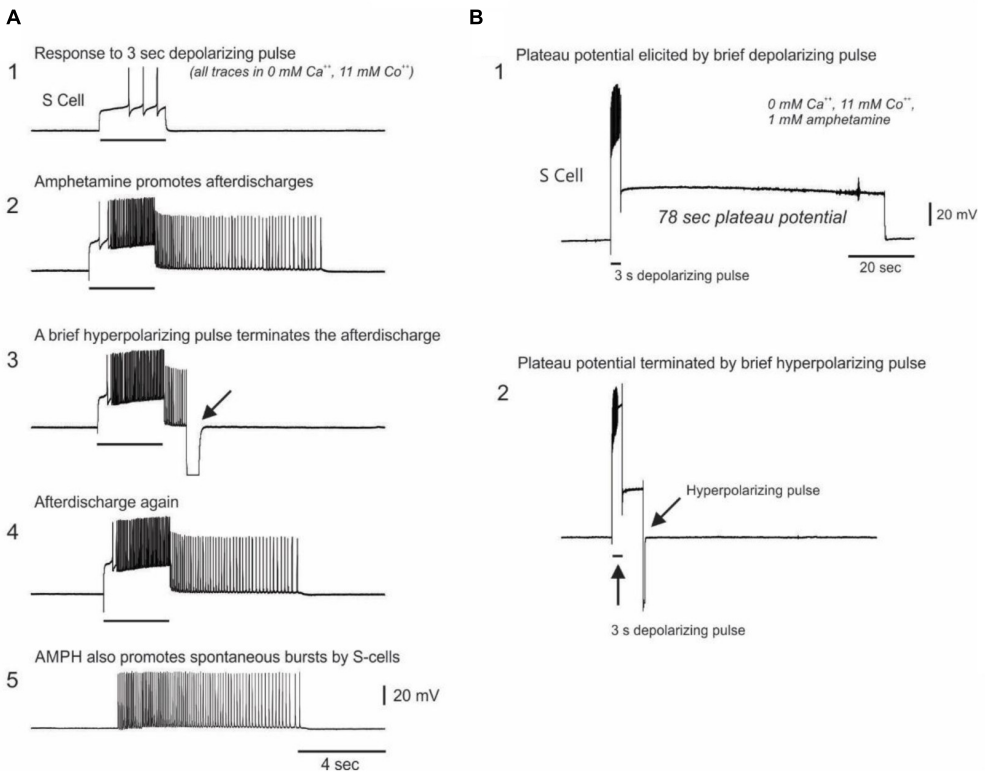

FIGURE 5 | Evidence for AMPH-induced plateau potential properties in S-cells. **(A)** Top trace: In saline in which calcium was replaced with the calcium channel blocker cobalt, constant current test pulses elicited modest firing. Second trace: In 0 Ca^{2+}, 11 mM Co^{2+}, 1 mM AMPH, the same test pulse elicited greater firing, followed by post-burst firing that long outlasted the test pulse. Third trace: the prolonged post-burst firing could be abruptly terminated by a brief hyperpolarizing pulse, a characteristic feature of plateau potentials. Fourth trace: Another test pulse again elicited post-burst firing. Fifth trace: 32% of the S-cells recorded in 0 calcium AMPH emitted sporadic spontaneous bursts. All traces are from the same neuron in one preparation. **(B)** In 0 calcium AMPH, injecting sufficient depolarizing current to fully accommodate the action potential often triggered a pronounced plateau potential which could last over a minute. This plateau potential could be terminated early with a brief injection of hyperpolarizing current. Both traces are from the same neuron.

Taken together, the above 0 Ca^{2+} results are consistent with the hypothesis that AMPH exerts its effects either directly on the S-cells, or perhaps on as-yet unidentified monoaminergic terminals synapsing directly onto the S-cells. In both invertebrates and vertebrates, amphetamine acts as an indirect monoaminergic agonist, promoting calcium-independent transmitter release from the presynaptic terminal, in part by reversing presynaptic monoaminergic reuptake transporters (Seiden et al., 1993; Sulzer et al., 1995). Both serotonin and dopamine are present in the *Tritonia* CNS (McCaman et al., 1973; Sudlow et al., 1998; Fickbohm et al., 2001). Dopamine has been implicated in the animal's cilia-mediated crawling behavior (Woodward and Willows, 2006), but was found to inhibit the nerve-elicited SMP in isolated brain preparations (McClellan et al., 1994). Serotonin's role in *Tritonia's* escape swim has been well-studied. Serotonin elicits the animal's escape swim (McClellan et al., 1994), and the serotonergic DSI neurons of the swim CPG can drive the escape SMP and produce intrinsic neuromodulation of other neurons in the swim circuit (Katz et al., 1994; Katz, 1998). Possible direct modulation of the S-cells by either of these monoamine transmitters has not yet been examined.

DISCUSSION

Evidence for an Invertebrate Model of Drug-Induced Hallucinations

The present study originated from curiosity about how AMPH, a commonly abused drug in humans, would act in a well-studied invertebrate with a highly tractable nervous system. We found that AMPH induced spontaneous escape swims in freely behaving animals, in the absence of any apparent stimulus. More surprisingly, we found that drug-induced escape SMPs sporadically occurred in deafferented isolated brain preparations, and traced their origin to spontaneous bursts in the afferent neuron population that normally informs the animal of skin contact with its seastar predators. Here we present an argument that *Tritonia's* AMPH-induced spontaneous swims are initiated in response to drug-induced perceptions of non-existent aversive skin stimuli, i.e., hallucinations.

Hallucinations were first formally described as perceptions of stimuli that do not actually exist (Esquirol, 1965). The DSM-IV definition is "a sensory perception that has the compelling sense of reality of a true perception, but that occurs without external stimulation of the relevant sensory

organ" (DSM-IV, 2000). We suggest that *Tritonia's* response to AMPH conforms to both components of this definition. Since the origin of the AMPH-SMPs was traced to spontaneous S-cell bursts in isolated brain preparations, they involve sensory neuron activity in the absence of an actual stimulus. Moreover, because the animal responds by launching its high-threshold escape swim, this sensory activity is clearly both perceived by and compelling to the animal. Since S-cells respond most strongly to skin contact with a chemical substance in the tube feet of the animal's seastar predators (**Figure 3B**; Getting, 1976), *Tritonia's* AMPH-induced hallucinations appear to be of predator contact. To our knowledge, the present study represents the first evidence for hallucinations in an invertebrate.

Many stimulus-elicited behavioral responses can be classed as simple, graded reflexes, in which response magnitude is proportional to stimulus strength. In contrast, *Tritonia's* escape swim is a complex, high threshold, all-or-none command neuron driven behavior (Frost and Katz, 1996; Frost et al., 2001) that only occurs to suitably aversive stimuli. For example, *Tritonia* do not swim in response to either tactile (Willows et al., 1973b; Mongeluzi et al., 1998) or even many tissue damaging skin stimuli (unpublished observations), presumably because of the behavior's high cost for the animal. Its thrashing, dorsal-ventral body flexions typically lift the animal into water currents that can carry it far away from food and potential mates (Willows, 2001; Wyeth and Willows, 2006). Below threshold stimuli produce graded, reflex withdrawal of the affected body region. Slightly stronger stimuli may elicit bilateral withdrawal of the gills and rhinophores, and even whole-body stiffening, all normal preparatory components of the swim itself, and yet the swim will not be launched unless the stimulus is sufficiently strong or prolonged (Willows et al., 1973b). Taken together, these findings are consistent with the AMPH-elicited swims being launched in response to compelling perceptions of skin contact with non-existent predators.

Behavioral studies of chronic AMPH exposure in vertebrate animals have led investigators to suggest the possible occurrence of AMPH-induced hallucinations in monkeys (Nielsen et al., 1983; Castner and Goldman-Rakic, 1999), cats (Trulson and Jacobs, 1979), rats (Nielsen et al., 1980), and mice (Tadano et al., 1986) (several studies are reviewed in Ellison, 1991). However, such studies have so far been unable to determine whether drug-induced behaviors such as repeated digging at the skin, or turning to stare or vocalize at objects unseen by human observers, represent true perceptual hallucinations, altered perceptions of real stimuli, or motor automatisms. If, in our *Tritonia* studies, AMPH had induced the motor program by activating the swim CPG directly, without prior activation of the sensory neurons, these behaviors would have been classified as automatisms rather than hallucinations. We similarly would not have posited hallucinations had we recorded just one or two spontaneous S-cell action potentials, with no observable effect on downstream circuitry or behavior. Such modest afferent neuron activity is well below threshold for triggering the escape swim behavior (Getting, 1976), and thus could not reasonably be classified as being compelling to the animal.

The majority of our cellular studies of AMPH-induced SMPs involved drug-naïve preparations. AMPH-induced hallucinations in humans, as well as those posited to occur in vertebrate animal studies, characteristically occur after repeated or continuous drug administration (Ellison, 1991). However, several publications, involving both emergency room admissions (Connell, 1958; Gold and Bowers, 1978) as well as controlled drug administration studies in hospital settings (Angrist and Gershon, 1970; Bell, 1973; Snyder et al., 1974; Seiden et al., 1993), have documented the occurrence of hallucinations in response to initial acute exposure to AMPH, at times in individuals believed to have no prior experience with the drug.

Chronic amphetamine abuse can produce delusional parasitosis in humans, involving formication: vivid tactile hallucinations of invertebrates biting or crawling on the skin (Stanciu et al., 2015). Given the encoding function of *Tritonia's* S-cells, the present study suggests that amphetamines can apparently induce surprisingly similar aversive perceptions, albeit operating at an unconscious level, in invertebrates themselves.

False Perceptions Need Not Be Conscious to Be Considered Hallucinations

Being an invertebrate, *Tritonia's* hallucinations are presumably non-conscious. [However, it seems worth noting that the impressive cognitive abilities of certain invertebrates have led many authors to suggest that such organisms may be capable of some degree of conscious awareness (Walters et al., 1981; Griffin and Speck, 2004; Edelman et al., 2005; van Swinderen, 2005; Smith, 2009)]. While the notion of unconscious hallucinations may be unfamiliar, it is well known that sensory information in humans is routed to, and perceived in detail, in both conscious and unconscious brain regions (Sahraie et al., 1997; Anders et al., 2004; Augusto, 2010; Fahrenfort et al., 2017). A well-studied example is that of "blindsight," in which individuals unable to see due to damage to their primary visual cortex are nonetheless able to use unconscious perception to navigate around obstacles, point to the locations of objects (Cowey, 2010), and even identify the emotional tone of pictures of human faces (Danckert and Rossetti, 2005). Additional studies have demonstrated unconscious visual and tactual perceptual abilities in healthy individuals (Imanaka et al., 2002). Many studies have concluded that human unconscious perception is as richly detailed as conscious perception, able to support perceptual, evaluative and motivational guidance to behavioral choice (Marcel, 1983; Bargh and Morsella, 2008; Hassin, 2013) and even a degree of rational deliberation (Garrison and Handley, 2017) and the setting and pursuit of goals (Custers and Aarts, 2010). In addition to such parallel pathways for processing perception, it is well established that learning and memory have distinct conscious (explicit) and unconscious (implicit) components that are processed, stored and accessed separately (Schacter, 1992; Squire, 2009). In fact, it is often suggested that

the unconscious, rather than the conscious mind is actually better suited for reaching decisions involving complex issues – the familiar example of "sleeping on it" to achieve clarity with regard to selecting a best course of action (Dijksterhuis and Nordgren, 2006).

Given that a portion of perception in humans appears to be mediated by unconscious networks, drug-induced spontaneous activity in such networks may reasonably be hypothesized to trigger hallucinations that, while not perceived consciously, might nonetheless affect an individual's affect and behavior. This notion is consistent with the large psychoanalytic literature on the significant role that "phantasies," defined as "the primary content of unconscious mental processes," play in human experience (Spillius, 2001; Ogden, 2011). From this perspective, it seems reasonable to posit that the nervous systems of animals living entirely unconscious lives may also, under the influence of psychostimulants such as AMPH, generate and respond to hallucinations of non-existent stimuli.

Generalizability of Invertebrate Mechanisms to Higher Animals

Invertebrates have long been successfully used to pursue general principles of nervous system function (Clarac and Pearlstein, 2007). For example, results from marine mollusks have been found to generalize to vertebrates across several levels of complexity, including mechanisms of action potential generation (Catterall et al., 2012), learning and memory (Pittenger and Kandel, 2003; Glanzman, 2010), and even sleep (Michel and Lyons, 2014). *Tritonia* research has identified the first cellular mechanisms mediating prepulse inhibition, an important sensory gating process common to both vertebrates and invertebrates (Mongeluzi et al., 1998; Frost et al., 2003; Lee et al., 2012). Prepulse inhibition deficits are a core feature of schizophrenia, and have been linked to several cognitive disorders of the disease, including psychosis (Braff et al., 2001; Quednow et al., 2008; Ziermans et al., 2011, 2012).

How likely is it that hallucinations will share at least some features in common between invertebrates and humans? One relevant perspective has been raised by several authors—that some brain mechanisms operating in the non-conscious, more ancestral regions of the human brain appear to have changed little through evolutionary time (Reber, 1992; Augusto, 2010). Another is that while the subjective content of human hallucinations varies with their location of origin in the brain, the triggering mechanisms may be more parsimonious. This hypothesis is supported by several decades of electrical microstimulation of cortex during brain surgery in awake humans, which has shown that very different subjective experiences and memories can be elicited by this uniform type of stimulation, simply by varying the locus of stimulation (Curot et al., 2017). Thus, while our example involves AMPH-induced instability in a sensory neuron population, locating the same mechanism in interneuronal networks in different regions of the mammalian brain would be expected to trigger, once elaborated by local cortical processing, conscious hallucinations of diverse and complex character.

Several features of the results seem consistent with the potential for using *Tritonia* to investigate the poorly understood network instability mechanisms that trigger hallucinations. First, the AMPH-induced swims in intact animals, and the corresponding AMPH-induced motor programs in isolated brains occur sporadically and without warning in the minutes to hours after AMPH administration, much as hallucinations of varied causes do in humans. Second, while AMPH can produce elevated excitability in vertebrate neurons (Jahromi et al., 1991; Ma et al., 2013), its effect on *Tritonia's* S-cells is of particular interest due to its sporadic nature. Even when tested in 0 Ca^{2+} saline, where spike-mediated synaptic inputs can play no role, *Tritonia's* AMPH-induced S-cell plateau potentials occurred on some test depolarizations and not on others. This trial-to-trial variability of AMPH's effect on S-cell excitability resembles the sporadic nature of hallucinations themselves. Finally, AMPH is well known in vertebrates to elevate monoamine release, including serotonin (Hernandez et al., 1987; Jones and Kauer, 1999), by reversing transmitter reuptake transporters (Fleckenstein et al., 2007). While we have not determined whether AMPH promotes serotonin release via this mechanism in *Tritonia*, it has been shown to do so with respect to dopamine in the gastropod *Planorbis*, thus this basic mode of action is common to invertebrates (Sulzer et al., 1995). The facts that serotonin injections trigger *Tritonia's* escape swim, and that serotonin receptors mediate the actions of many classical hallucinogens in vertebrates (Halberstadt, 2015), are consistent with a possible role for this transmitter in mediating AMPH-induced hallucinations in this invertebrate model system.

AUTHOR CONTRIBUTIONS

AL conducted the experiments, analyzed the data, and participated in writing the paper. CB conceived of the project, conducted the experiments, and analyzed the data. JW conducted the experiments. WF conducted the experiments, analyzed the data, participated in writing the paper, and made the figures.

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REVIEW
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A Fly's Eye View of Natural and Drug Reward

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Animals encounter multiple stimuli each day. Some of these stimuli are innately appetitive or aversive, while others are assigned valence based on experience. Drugs like ethanol can elicit aversion in the short term and attraction in the long term. The reward system encodes the predictive value for different stimuli, mediating anticipation for attractive or punishing stimuli and driving animal behavior to approach or avoid conditioned stimuli. The neurochemistry and neurocircuitry of the reward system is partly evolutionarily conserved. In both vertebrates and invertebrates, including *Drosophila melanogaster*, dopamine is at the center of a network of neurotransmitters and neuromodulators acting in concert to encode rewards. Behavioral assays in *D. melanogaster* have become increasingly sophisticated, allowing more direct comparison with mammalian research. Moreover, recent evidence has established the functional modularity of the reward neural circuits in *Drosophila*. This functional modularity resembles the organization of reward circuits in mammals. The powerful genetic and molecular tools for *D. melanogaster* allow characterization and manipulation at the single-cell level. These tools are being used to construct a detailed map of the neural circuits mediating specific rewarding stimuli and have allowed for the identification of multiple genes and molecular pathways that mediate the effects of reinforcing stimuli, including their rewarding effects. This report provides an overview of the research on natural and drug reward in *D. melanogaster*, including natural rewards such as sugar and other food nutrients, and drug rewards including ethanol, cocaine, amphetamine, methamphetamine, and nicotine. We focused mainly on the known genetic and neural mechanisms underlying appetitive reward for sugar and reward for ethanol. We also include genes, molecular pathways, and neural circuits that have been identified using assays that test the palatability of the rewarding stimulus, the preference for the rewarding stimulus, or other effects of the stimulus that indicate how it can modify behavior. Commonalities between mechanisms of natural and drug reward are highlighted and future directions are presented, putting forward questions best suited for research using *D. melanogaster* as a model organism.

Keywords: *Drosophila*, natural reward, drug reward, ethanol, nicotine, cocaine, amphetamine, methamphetamine

Abbreviations: AC, adelylyl cyclase; CS, conditioned stimulus; DA, dopamine; MB, mushroom bodies; MNC, median neurosecretory cells; nAChR, nicotinic acetylcholine receptor; NPF, neuropeptide F; OA, octopamine; PAM, protocerebral anterior medial; PI, pars intercerebralis; PPL1, protocerebral posterior lateral; PPM3, protocerebral posterior medial; SOG, subesophageal ganglion; TβH, tyramine beta hydroxylase; US, unconditioned stimulus.

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INTRODUCTION

Animals need to distinguish beneficial stimuli in order to survive. There is partial conservation among reward systems across species (Scaplen and Kaun, 2016). Mammalian models of reward have allowed for the dissection of the several components of reward as well as the mapping of these components for different neural circuits and neurotransmitters. Further dissection of reward circuits using large-scale genetic screens could help to elucidate the genetic and molecular mechanisms of reward. For this complementary approach, the *Drosophila melanogaster* model system is ideally suited and allows for targeted genetic manipulations, which are necessary to determine causality for the identified genetic factors (Venken and Bellen, 2014). *Drosophila* also allows for targeted genetic manipulations, which are necessary to determine causality.

Drosophila has been a primary model organism for elucidating the role of genes and identifying molecular mechanisms and neural circuits underlying biological processes (Rubin and Lewis, 2000; Bellen et al., 2010; Venken et al., 2011). The whole genome of the fly has been sequenced and annotated. It is believed that between 65 and 75% of human disease-causing genes have a functional homolog in *Drosophila* (Reiter et al., 2001; Chien et al., 2002; Yamamoto et al., 2014). The fast life cycle, high fecundity, smaller space needed to maintain colonies, lower cost of fly husbandry, and the wide array of commercially available transgenic fruit fly strains make the fruit fly a great model organism for forward and reverse genetic screens as well as genomic approaches for identification and rapid validation of genes involved in reward. Knowledge gathered about *Drosophila* has been compiled in databases that specialize in different content (modENCODE, Celniker et al., 2009; FlyAtlas, Chintapalli et al., 2007; FlyBase, Gramates et al., 2017; Larval Olympiad data set and FlyEM, HHMI Janelia Research Campus; Virtual Fly Brain, Milyaev et al., 2012; Flybrain Neuron Database, The University of Tokyo). *Drosophila* labs and public institutions across the world develop and maintain collections of mutants and transgenic tools that allow for probing the function of nearly every gene in the fly with exquisite spatial and temporal control, including single cell resolution and restriction of the manipulation to specific developmental stages or segments of a behavioral task (Brand and Perrimon, 1993; Jenett et al., 2012; Venken and Bellen, 2014).

The possibility of altering gene expression or controlling neuronal activity at the single-cell level makes flies an ideal model to dissect reward circuits and allows for mapping how specific genetic networks act within specific cells in a neuronal circuit. These tools have allowed for the mapping of genes and neuronal circuits that control natural and drug reward, revealing similarities in the organization of the reward system in mammals and *Drosophila*, including the role of DA and the general rules for reward processing (de Araujo, 2016; Scaplen and Kaun, 2016).

Behavioral assays have been developed to study natural and drug reward in *D. melanogaster* (Kaun et al., 2012). The behavioral assays for studying reward vary between mammals and insects. Mammalian assays of natural reward regularly involve operant conditioning, such as pressing a lever, and are dependent on a specific action by the animal to obtain an US (Perry and Barron, 2013). In contrast, the paradigms used to study insect reward involve presentation of the CS and US by the researcher, and are thus independent of the action of the animals (Perry and Barron, 2013). The study of reward in *D. melanogaster* has largely focused on classical conditioning learning paradigms using either innately rewarding or punishing US paired with a CS. Learning is said to take place based on the response to the CS after training. Assays to test other aspects of the reinforcing stimuli involve voluntary consumption and two-choice preference assays. These assays provide a broad picture of reinforcing stimuli, ultimately determining whether these stimuli have reinforcing properties for short and/or long-term memories. Studies have probed multiple aspects of natural reward across developmental stages, including appetitive and aversive stimuli in larval and adult stage *Drosophila* (Diegelmann et al., 2013; Perry and Barron, 2013; Davis, 2015; Landayan and Wolf, 2015; Das et al., 2016; Scaplen and Kaun, 2016; Kaun and Rothenfluh, 2017; Cognigni et al., 2018).

Only recently has the study of drug reward in *Drosophila* used similar assays to those used to dissect natural reward (Kaun et al., 2012). Palatability, voluntary consumption, and conditioned odor preference behavior assays have identified genes, molecular pathways, and neural circuits underlying drug reward, and have demonstrated that certain drugs, such as ethanol, can be rewarding for flies. Much more is known about the acute effects of drugs in *Drosophila*. However, these acute drug assays do not focus on reward, but on the locomotor effects of the drugs. Nonetheless, some of the genes and neural circuits that have been identified with acute drug exposure match those underlying feeding (Landayan and Wolf, 2015). This suggests common factors in the mechanisms underlying drug and natural reward.

Recent reviews on this topic have focused on reward processing and the similarities between the *Drosophila* and mammalian reward systems (de Araujo, 2016; Scaplen and Kaun, 2016; Kaun and Rothenfluh, 2017; Cognigni et al., 2018). Research studying reward in *Drosophila* larva has been reviewed by Diegelmann et al. (2013). A comprehensive review by Das et al. (2016) focuses on food reward in *Drosophila*. Another recent review highlights the neurotransmitters and neural circuits that mediate both feeding and drug effects (Landayan and Wolf, 2015).

In this review, we have compiled information about the mechanisms underlying natural and drug reward in *Drosophila* organizing information according to the behavior elicited by the natural or drug stimulus. We focus on appetitive behaviors that indicate that a given stimulus is palatable, preferred when given a choice, and serves a reinforcement in a learning and/or memory assay, indicating its rewarding value. These behaviors in combination provide a view of the underlying mechanisms of reward from perception to reinforcement. We limit this review to three assays for natural rewards: palatability, assessed by the proboscis extension reflex response; preference, assessed in a choice assay; and reward, assessed in a conditioned odor preference assay. The natural stimuli included in this review are

sugars, proteins, fatty acids, and water. The drugs included are ethanol, cocaine, amphetamine/methamphetamine, and nicotine. Most research on drug reward has focused on ethanol, for which studies about its palatability, preference, and reward have been conducted. Ethanol and other drugs have also been examined by looking at their locomotor effects. We included these, as neural circuits and genes that mediate locomotor drug effects show partial overlap with those of natural and ethanol reward (Landayan and Wolf, 2015; this review). The figures in this review highlight common factors and are meant to help identify gaps in knowledge.

NATURAL REWARD

Rewarding stimuli are attractive, eliciting a subjective degree of pleasure, a hedonic value, or 'liking' (Berridge and Kringelbach, 2008). In mammals, 'liking' is identified by studying facial expressions (Steiner et al., 2001; Berridge and Kringelbach, 2008; Berridge and Robinson, 2016). 'Liking' in mammals is mediated by opioid, endocannabinoid, and GABA-benzodiazepine signaling, and is localized to hedonic hot spots distributed throughout the limbic system (Berridge et al., 2009). Rewards also have incentive salience, evoking a strong desire or craving for the reward or 'wanting' in mammals this is largely mediated by the mesocorticolimbic system, with DA as the main neurotransmitter (Berridge et al., 2009; Berridge and Robinson, 2016).

'Wanting' in insects can be assessed by focusing on approach and consummatory responses in instrumental learning paradigms (Perry and Barron, 2013). Natural rewards have intrinsic incentive salience and can be used as unconditioned stimuli (US). Incentive salience can also apply to Pavlovian conditioned stimuli (CS), which are learned stimuli that are originally neutral but become predictors of reward through stimulus-stimulus association (Berridge et al., 2009).

A different way to dissect reward is to focus on how rewarding stimuli affect the activity of the neural circuits underlying both approach and consummatory behavior, from perception to motor function. The first neural circuits to be activated by either rewarding or punishing stimuli are the sensory systems. Rewarding stimuli are salient, and hence activate the areas of the nervous system that encode attention in the brain. Learned rewards activate learning and memory circuits. Lastly, rewarding stimuli elicit behavior, which is directed by activation of motor circuits (Schultz, 2015).

Below, we present an overview of the mechanisms involved in palatability, preference and reinforcement elicited by the following natural food stimuli: sugar, protein, fatty acid, and water. We focus on sugar reward and how sweet taste versus nutritive value are encoded. We organize the information by assay, as each assay probes different aspects of the rewarding stimulus. Palatability focuses on the initial perception of the stimulus. Consumption and preference are tested using a two-choice assay to study consummatory behavior. Lastly, conditioned odor preference reflects the reinforcing properties of the stimulus.

Sugar Palatability and Preference

Palatability: Proboscis Extension Reflex

The proboscis extension reflex (PER) can be used to measure the palatability of a stimulus. Sugars are detected by gustatory receptor neurons located in the tarsae and mouthparts of *Drosophila* (Wang et al., 2004; Amrein and Thorne, 2005). PER response to sugar is partly mediated by Gr5a, a gustatory receptor expressed in specific sensory neurons (Wang et al., 2004). It has been shown that both sweet and nutritious and also sweet but non-nutritious sugars elicit PER responses in flies (Dus et al., 2011; Fujita and Tanimura, 2011). A single dopaminergic ventral unpaired medial neuron (TH-VUM), which has projections in the SOG, is sufficient to elicit a PER response to sucrose (**Figure 1**) (Marella et al., 2012). The DA receptor 2 (*Dop2R*) was required for DA-induced PER (Marella et al., 2012). Another neurotransmitter involved in the PER is serotonin (Albin et al., 2015). Activating a subset of serotonergic neurons, R50H05, increased PER responses in sated flies, which normally would be low (Albin et al., 2015).

The number of trials to the first PER response is an indication of sucrose responsiveness and has been shown to correlate with the PER habituation, which is the reduction of PER response upon repetition of the stimulus (Çevik and Erden, 2012). Flies with lower sucrose responsiveness habituate faster, and flies with high sucrose responsiveness habituate slower (Scheiner et al., 2014). OA was implicated in PER habituation indirectly, via modulation of sucrose responsiveness (Scheiner et al., 2014). Flies

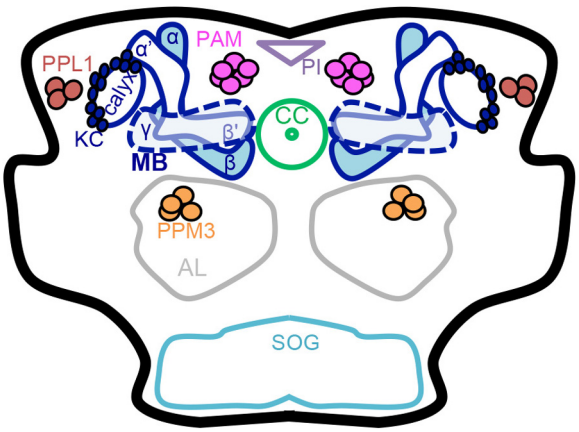

FIGURE 1 | Schematic of the adult *Drosophila* brain. Dopaminergic neurons (PAM, PPL1 and PPM3 clusters) and the mushroom body have a role in learning, memory, natural reward, and drug effects. Dopaminergic neurons have been thoroughly characterized anatomically into defined clusters that project to specific regions in the mushroom body lobes. Mushroom body output neuron connections with intrinsic mushroom body Kenyon cells and dopaminergic neurons form 15 compartments that appear to be functionally independent (Mao and Davis, 2009; Chiang et al., 2011; Aso et al., 2014a; Das et al., 2016; Kaun and Rothenfluh, 2017). MB mushroom body (α/β, α′/β′, and γ lobes) (blue), KC Kenyon cells (blue), CC central complex (light green), AL antennal lobe (gray), SOG subesophageal ganglion (teal), PAM protocerebral anterior medial neurons (pink), PPM3 protocerebral posterior medial 3 neurons (orange), PPL1 protocerebral posterior lateral 1 neurons (brown), PI pars intercerebralis (light purple).

with mutations in the gene for the rate limiting enzyme for OA synthesis, *Tyramine β hydroxylase* (*Tβh*), have unaffected PER habituation rates but decreased response to sucrose, which can be rescued by supplementing OA by feeding or by expressing OA specifically in octopaminergic neurons of the ventro medial cluster of the SOG (Scheiner et al., 2014). This suggests that OA promotes sucrose responsiveness (Scheiner et al., 2014).

Voluntary Consumption and Two-Choice Preference

Flies display preference for sugars, but this preference is not solely based on taste. In a two-choice preference assay, flies first chose the sweetest sugar but after 5 min, flies started favoring the nutritious sugar (Dus et al., 2015). The continued ingestion of nutritious sugar induced PER response and activated food processing in the gut (Dus et al., 2015). Flies develop preference for nutritious sugar even in the absence of taste input, but only after a long period of starvation (Dus et al., 2011). After 5 h of starvation, which correlates with decreased hemolymph levels of glucose and trehalose, taste receptor mutant flies preferentially ate agar with sucrose in a two-choice agar plate (Dus et al., 2011). Another study using the two-choice CApillary FEeder (CAFE) assay confirmed that flies choose sugars according to sweetness but that this initial preference shifts toward sugars with higher nutritional value after 12 h, which suggests that this phenomenon is experience dependent (Ja et al., 2007; Stafford et al., 2012).

Preference for a nutritious sugar is mediated by Dh44 neurons, which produce and release the Diuretic hormone 44 neuropeptide (the homolog of corticotropin-release hormone in mammals), which activates the Dh44 receptor R1 (Dus et al., 2015). Dh44 neurons mediating preference for nutritious sugars are located in the PI and their neurites project to the dorsal region of the subesophageal zone (the basal region of the supraesophageal ganglion fused to the SOG) and also innervate the gut (Dus et al., 2015; Hartenstein et al., 2018). In Dh44 neurons, a nutritious sugar stimulus causes changes in calcium oscillation frequency and duration, suggesting neuropeptide secretion (Thorner et al., 1988; Dus et al., 2015). Sugar transport into Dh44 cells and glucose metabolism are necessary to induce calcium oscillations and for nutritious sugar choice (Dus et al., 2015). These results show that nutritious sugars directly activate Dh44 neurons via a sugar-metabolism-dependent pathway resulting in Dh44 neuropeptide secretion, which conveys the signal of nutritious sugars to other regions of the brain.

Dh44 binds to and activates two receptors in *Drosophila*: R1, expressed in the brain and ventral nerve chord, and R2, expressed in the gut (Dus et al., 2015). The Dh44 receptors R1 and R2 are necessary for preference for nutritious sugars (Dus et al., 2015). Dh44 R1 neuron activation elicits PER responses, while the *Dh44 R2* gene is implicated in gut motility (Dus et al., 2015). Dh44 R1 expressing neurons have neurites in the PI and in the dorsal region of the subesophageal zone (Dus et al., 2015). Projections of Dh44 R1 neurons do not contact muscles in the labella and thus Dus et al. (2015) propose that these neurons synapse onto motoneurons in the subesophageal zone, which would in turn elicit PER (Dus et al., 2015).

The cAMP pathway has been implicated in learning, memory, and reward in *Drosophila,* and it was found that the cAMP pathway in neurons also mediates preference for nutritious sugars (Davis and Kiger, 1981; Tempel et al., 1983; Schwaerzel et al., 2003; Stafford et al., 2012). The shift in preference toward nutritious sugars occurred faster in hungry flies (Stafford et al., 2012). The insulin pathway is also involved in nutritious sugar preference (Stafford et al., 2012). Both the insulin receptor (*InR*) in the thoracic ganglion and the insulin-like peptides *dilp2* and *dilp3* in adult MNC mediate preference for nutritious sugars (Ikeya et al., 2002; Stafford et al., 2012). The serotonergic pathway has also been implicated in nutritious sugar preference (Albin et al., 2015). Activation of the R50H05 subset of serotonergic neurons in sated flies increased their preference for a nutritious sugar, mimicking the effects of starvation on nutritious sugar preference (Albin et al., 2015).

Figure 2 summarizes results from the studies about sugar preference above, which suggest that preference for a nutritious sugar is mediated by the cellular signaling cAMP pathway along with the insulin, Dh44 neuropeptide, and a subset of serotonergic neurons. This subset of serotonergic neurons also has a role in the PER for sugars, which are perceived by gustatory receptor neurons.

Hence, sugars are palatable for flies and flies use different neurotransmitter systems to convey hunger signals based on nutrient levels, which are correlated to calcium oscillations. Hunger modulation results in increased PER responses and preference for a nutritious sugar. Next, we examine the evidence supporting sugars as natural rewards for fruit flies and the mechanisms of sugar as reinforcement for short and long-term memories.

Sugar Reward: Appetitive Olfactory Conditioned Memory

Sugar has been used by multiple investigators as an US in olfactory conditioned learning and memory assays. Quinn et al. (1974) pioneered reward research in *D. melanogaster*, developing a classical conditioning assay in which a neutral odor and an aversive stimulus are paired (Tully and Quinn, 1985). The appetitive version of this assay uses sugar as attractive stimulus, pairing it to an odor (Tempel et al., 1983). This pioneering work identified mutant flies (*rutabaga* and *dunce*) with mutations in the cAMP pathway that had sugar memory defects (Tempel et al., 1983). Later it was shown that this defect could be rescued when wild-type *rutabaga* was expressed specifically in mushroom body Kenyon cells (Schwaerzel et al., 2003). Early studies tested involvement of the cAMP pathway shortly after training (Schwaerzel et al., 2003). It was subsequently shown that the cAMP pathway is also needed in *Drosophila* mushroom body for long-term appetitive memories (Krashes and Waddell, 2008).

The dopa decarboxylase (*Ddc*) gene, whose gene product catalyzes the synthesis of DA and serotonin, was also found to mediate sugar learning (Tempel et al., 1984). Involvement of OA has also been shown, as flies with mutations in *Tβh*, the enzyme that converts tyramine to OA, were impaired in sugar memory performance. This phenotype was rescued by expressing wild-type *Tβh* in mutant flies (Schwaerzel et al., 2003).

Some sugars are perceived as sweet and some are not; some sugars can be metabolized and some cannot (Burke and Waddell,

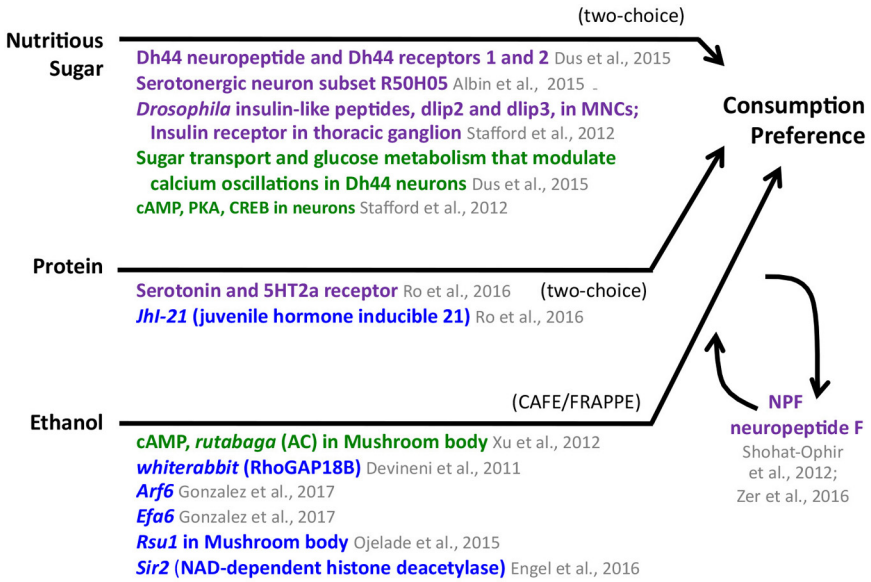

FIGURE 2 | Consumption preference for nutritious sugar, protein, and ethanol in adult *Drosophila*. The appetitive stimulus is shown on the left side, and the behavior, as measured in specific assays, is shown on the right. The compiled genes, signaling pathways, and neural structures mediating the behavior connect the stimulus and the behavior. Consumption preference for nutritious sugar and protein was tested using a two-choice preference assay; ethanol consumption preference was identified using the CAFE/FRAPPE assay. Neuronal pathways (dark purple), cellular pathways (dark green), genes/proteins (royal blue). MNCs median neurosecretory cells.

2011; Fujita and Tanimura, 2011). To further probe appetitive learning, researchers investigated whether the sweetness or the nutritional value could both function as reinforcements in appetitive learning and how the brain encodes these two aspects of sugar. Flies can learn the nutritional value of a non-sweet stimulus such as D-sorbitol in an olfactory conditioned memory assay; this learning is dependent on synapsin (*Syn*) (Fujita and Tanimura, 2011). Flies can form short-term memories with nutritious sugars sucrose or fructose and also with arabinose or xylose, which cannot be metabolized by flies (Burke and Waddell, 2011). However, long-term memory formation is much stronger for the nutritious sugars (Burke and Waddell, 2011).

Octopamine and Dopamine Mediate Short-Term Memories With Sweet Sugars as Reinforcement

Next, studies identified the neurotransmitter systems that convey sweetness versus those that convey nutritional information. OA signaling is needed for flies to form short-term appetitive memories with sweet taste as reinforcement (Burke et al., 2012). However, OA-dependent memories require DA signaling as well (Burke et al., 2012). Activation of a subcluster of dopaminergic neurons in the PAM cluster is sufficient to induce appetitive olfactory memory in starved flies, showing that DA signaling is downstream of OA-mediated short-term appetitive memory formation (Burke et al., 2012; Liu et al., 2012). Neurons in this PAM subcluster have dendrites in the anterior medial protocerebrum and presynaptic terminals in the tip of the mushroom body β′ and γ lobes. GFP reconstituted across synaptic partners (GRASP) analysis suggests that octopaminergic neurons make synapses with neurons in this PAM dopaminergic subcluster (Burke et al., 2012). The subgroup of PAM dopaminergic neurons that mediate OA-dependent olfactory memories express the Ca^{2+}-coupled α-adrenergic-like OA (OAMB) receptor, which is necessary for OA-dependent memories (Burke et al., 2012). A subset of OAMB OA receptor neurons within the PAM cluster project to the $\beta'_{2\ am}$ and γ_4 regions of the mushroom body and convey the short-term reinforcing effect of sweet taste (Huetteroth et al., 2015).

Octopamine also mediates olfactory memories via activation of the octopaminergic receptor, Octβ2R expressed in MB-MP1 dopaminergic neurons, which are part of the dopaminergic PPL1 cluster and innervate the mushroom body heel (γ_1, α/β peduncle) (Krashes et al., 2009; Aso et al., 2010; Burke et al., 2012). Burke et al. (2012) proposed a model in which OA mediates appetitive reinforcement via OAMB signaling by modulating the activity of positive PAM dopaminergic neurons and Octβ2R signaling by modulating the activity of negative PPL1 MB-MP1 dopaminergic neurons (Burke et al., 2012).

The *Drosophila* DA 1-like receptor 1 (DopR1) expressed in mushroom body intrinsic neurons (Kenyon cells) is required for OA-mediated appetitive short-term memory (Kim et al., 2007; Burke et al., 2012; Liu et al., 2012). It has not been tested whether DopR1 is needed in the specific mushroom body compartments that mediate sweet taste short-term memories.

Dopamine Mediates Long-Term Memories With Nutritional Sugars as Reinforcements

Octopamine signaling and PAM dopaminergic neurons expressing the OAMB receptor are not required for nutritional value reinforced memories (Burke et al., 2012). A different subset

of PAM neurons mediates nutritional value reinforced olfactory memory formation (Burke et al., 2012). PAM dopaminergic neurons that project to the γ_{5b} region of the mushroom body convey the long-term reinforcing effect of nutritional value (Huetteroth et al., 2015). Activation of the dopaminergic neurons innervating the β_1, β_2, and adjacent α_1 regions of the mushroom body is sufficient for long-term memory (Huetteroth et al., 2015; Yamagata et al., 2015). Among these sets of PAM neurons, blocking PAM-α_1 neurons impaired long-term memory formation with a non-nutritious sugar supplemented by a non-sweet nutritious sugar without affecting short-term memory formation, and selective activation of these neurons in hungry flies induced long-term appetitive memory (Yamagata et al., 2015). Hence, PAM-α_1 neurons are necessary and sufficient for long-term memory formation (Yamagata et al., 2015). PAM-α_1 neurons receive input from glutamatergic MBON-α_1 neurons, a specific type of mushroom body output neuron with dendrites in the α_1 mushroom body compartment (Ichinose et al., 2015). Moreover, PAM-α_1 neurons and MBON-α_1 neurons are required for acquisition and consolidation of long-term appetitive memories (Ichinose et al., 2015).

In addition to their role in short-term memory formation, PPL1 MB-MP1 dopaminergic neurons are also necessary and sufficient to convey the nutritional value to the mushroom body (Musso et al., 2015). PPL1 MB-MP1 neuron activity is needed for the establishment of long-term memory after training but not during training (Musso et al., 2015).

The *Drosophila* DA 1-like receptor 2 (DopR2) expressed in mushroom body neurons mediates appetitive long-term memories; this receptor seems to be activated by PPL1 MB-MP1 dopaminergic neurons signaling (Musso et al., 2015).

Hunger Modulation of Sugar Memories

Dopaminergic PPL1 MB-MP1 neurons express the NPF receptor 1 (NPFR1); NPF is the *Drosophila* homolog of mammalian NPY. Dopaminergic PPL1 MB-MP1 neurons are inhibited by NPF in hungry flies, allowing for the retrieval of appetitive memories (Krashes et al., 2009). PPL1 MB-MP1 neurons seem to function as a gate at the mushroom body, providing tonic inhibition when flies are fed and relieving this inhibition when they in turn become inhibited by NPF during food deprivation (Krashes et al., 2009). NPF stimulation increases appetitive memory performance in fed flies, mimicking performance of hungry flies (Krashes et al., 2009). Hence, starvation modulates appetitive olfactory memory formation centrally via NPF signaling at the PPL1 MB-MP1 dopaminergic neurons.

PPL1 MB-MP1 neurons have spontaneous calcium oscillations that change according to hunger state (Plaçais et al., 2012; Plaçais and Preat, 2013). These oscillations increase in frequency and quality factor 30 min after training with a nutritious sugar compared to training with a non-nutritious sugar (Musso et al., 2015). This delayed calcium trace in PPL1 MB-MP1 neurons correlates with the nutritional value of the sugar reward and with appetitive long-term memory formation (Musso et al., 2015). More recently, it has been shown that a subset of serotonergic neurons encodes the hunger signal. Activating these neurons results in fed flies eating as if they were starved (Albin et al., 2015).

Musso et al. (2015) proposed a two-step mechanism for appetitive memory formation: (1) integration of olfactory and gustatory sensory information and (2) post-ingestion energetic value (Musso et al., 2015). The nutritional value of food is the critical signal for generating long-term memory (Musso et al., 2015). Flies develop long-term memories when given a non-nutritious sugar only when fed a nutritious sugar immediately after training to mimic a post-ingestion signal (Musso et al., 2015). Long-term memory formation is impaired when intestinal glucose transport is blocked, which lowers glucose levels in the hemolymph (Musso et al., 2015). Sugar levels in hemolymph after sugar ingestion may represent their nutritional value (Yamagata et al., 2015). Fructose is sensed by the Gr43a receptor in the brain. Blocking Gr43a-expressing neurons during appetitive reward training impaired long-term memory formation while sparing short-term memory (Yamagata et al., 2015). Gr43a expressing neurons and their neuronal projections locate to the lateral protocerebrum in the same region where dendrites from PAM neurons that mediate long-term memories are located (Yamagata et al., 2015).

Figure 3 summarizes the findings showcased above, which demonstrate that sugars are natural rewards with the ability to serve as reinforcements for both short and long-term memories in *D. melanogaster*. The mechanisms underlying sugar reward show that parallel pathways for short versus long-term memory exist in the fly brain and each pathway involves different sets of neurotransmitters systems: OA and DA for sweet taste short-term memories, and DA for nutritious value long-term memories. These parallel circuits and the role of DA as a central neurotransmitter in reward memory formation reveals that *Drosophila* reward circuits are surprisingly more similar to mammals than previously thought. This further validates fruit flies as a valuable model organism to help elucidate the organizing principles of the reward circuits to complement research in mammalian systems.

Sugar reward in fruit flies has been studied the most. However, research to determine palatability and preference of other natural food stimuli in the context of reward has begun to reveal interesting similarities and differences to sugar reward mechanisms.

Protein and Fatty Acid Palatability and Preference

Medium-chain fatty acids elicit PER responses at a significantly higher rate than water (Masek and Keene, 2013). Medium-chain fatty acids are sensed by peripheral sugar-sensing sensory receptor neurons that express the Gr64f receptor. Silencing these neurons abolishes not only the PER response to sugar, but also the PER response to the medium-chain fatty acids (Masek and Keene, 2013). The Phospholipase C (PLC) homolog "no receptor potential A" (*norpA*) is required in Gr64f neurons for PER responses to fatty acids. *norpA* mutants have significantly lower PER responses to fatty acids, while the PER responses to sugar are unaffected (Masek and Keene, 2013). Neurons expressing the

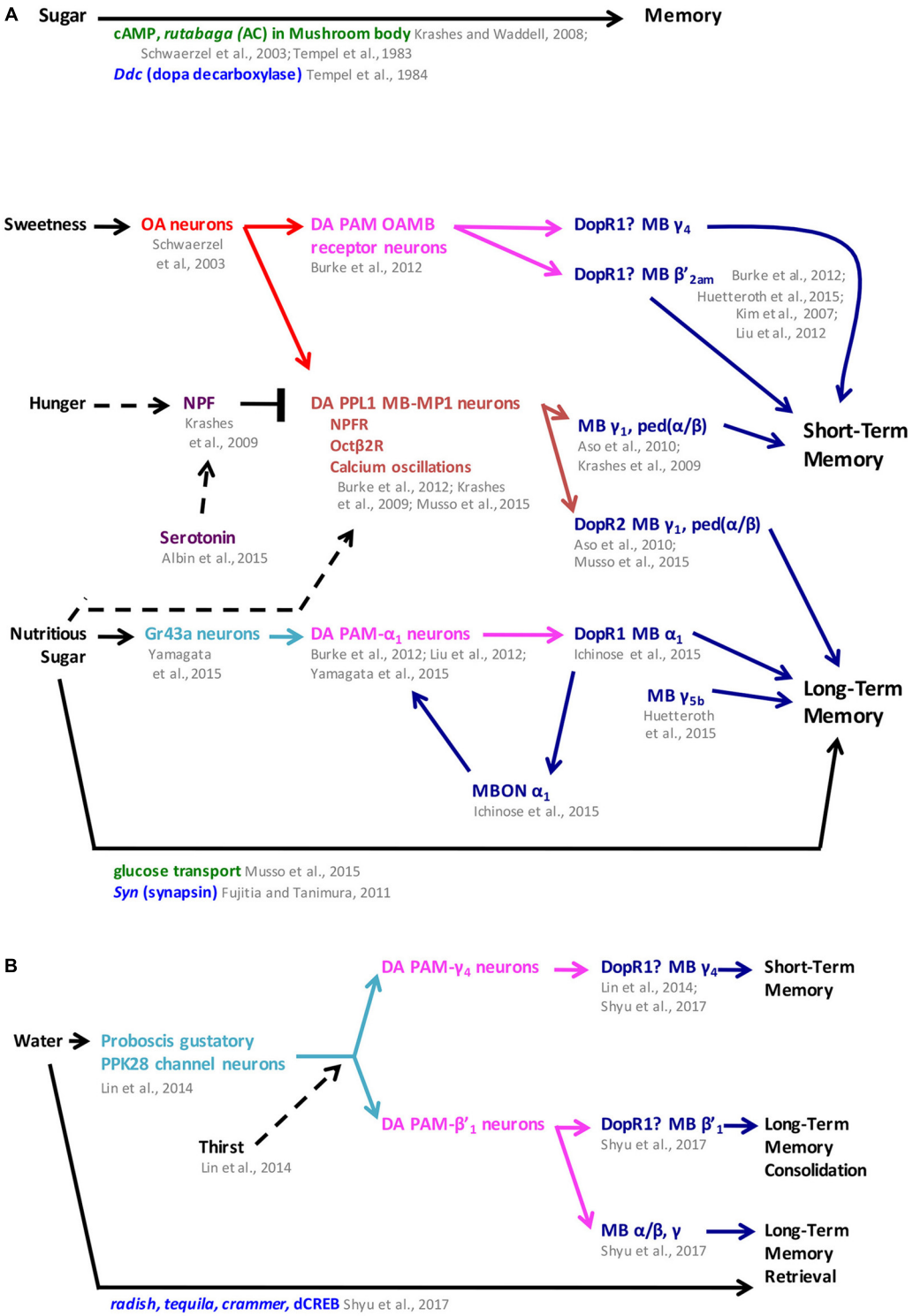

FIGURE 3 | Sugar and water memory follow parallel pathways in *Drosophila*. **(A)** Sweetness and nutritional value reinforce short- and long-term memory formation, respectively, through dopaminergic-mushroom body circuitry with hunger modulating both short- and long-term memory. **(B)** Water memory is mediated by dopaminergic PAM clusters through the mushroom body and is modulated by thirst. In some cases, mushroom body compartments were identified independently from the dopaminergic receptor function. In the figure, question marks next to dopaminergic receptors indicate when their function has been localized to the mushroom body but has not been narrowed down to a specific compartment. MB mushroom body (α/β, α′/β′, and γ lobes) (blue), MBON mushroom body output neurons (blue), OA octopaminergic (red), gustatory neurons (teal), PAM protocerebral anterior medial (pink), PPL1 protocerebral posterior lateral 1 (brown), neuronal pathways (dark purple), cellular pathways (dark green), genes/proteins (royal blue). NPF neuropeptide F; ped (α/β): peduncle of α/β.

ionotropic receptor 56d (IR56d) respond to short and medium-chain fatty acids; *norpA* is also required in these neurons for fatty acid PER responses (Tauber et al., 2017). A subpopulation of neurons that co-express Gr64F and IR56d mediates fatty acid taste and PER responses (Tauber et al., 2017). Even though these neurons respond to both sucrose and fatty acids, flies can distinguish between these two stimuli and form independent memories for sugar and fatty acids in an aversive memory test (Tauber et al., 2017).

In addition to eliciting PER, flies also prefer medium-chain fatty acids over water or low concentrations of sucrose (<1 mM) in the two-choice CAFE assay (Masek and Keene, 2013). Flies can also develop preference for protein. Starved flies developed preference for sugar food with added protein over sugar alone, while fed flies preferred sugar-only food (Ro et al., 2016). This protein preference is mediated by serotonin signaling acting through the 5HT2a receptor (Ro et al., 2016). Serotonergic signaling is needed during starvation to form protein preference but is not necessary during food-choice (Ro et al., 2016). Activation of serotonergic neurons results in protein preference in fed-flies, which suggests that serotonin increases the value of protein-food and that this value changes according to energy state (Ro et al., 2016). Protein preference is also mediated by the juvenile hormone inducible 21 (*JhI-21*) gene, a homolog of SLC7A5 (a mammalian leucine transporter), and seems to act upstream of serotonin signaling (Ro et al., 2016).

A summary of the results from the studies above is found in **Figure 2**. Flies find fatty acids palatable, based on their ability to elicit PER responses, and there seems to be some overlap with sugar palatability. It would be interesting to determine whether proteins also elicit PER responses. Flies show preference for both fatty acids and protein. Protein preference is mediated by *JhI-21* and serotonergic signaling. The mechanism mediating fatty acid preference is not currently know. It would be interesting to determine whether fatty acids or proteins can act as reinforcements in either short or long-term memory and whether these memories are encoded by additional, not yet identified parallel pathways to those of sugar memories. The study of another natural stimulus, water, suggests there are indeed additional parallel pathways for conveying different natural stimuli.

Water Reward

Water is rewarding for thirsty flies, as tested in a 3-min water-mediated learning assay or a 30-min water short-term memory assay (Lin et al., 2014). In flies, Pickpocket 28 (PPK28), an osmosensitive channel expressed in gustatory neurons in the proboscis, detects water (Cameron et al., 2010). Flies avoid water when not thirsty, but display approach behavior after water deprivation (Lin et al., 2014). Pairing water with a neutral odor is an effective reinforcement in an olfactory appetitive learning assay and is conveyed by a specific subpopulation of dopaminergic neurons separate from those involved in sugar reward (Lin et al., 2014). Pickpocket 28 mediates water reinforcement, as *ppk28* mutants are deficient in water-dependent learning but are able to detect water and other smells (Lin et al., 2014). A subset of PAM cluster dopaminergic neurons with projections to the γ_4 region of the mushroom body was required for water learning acquisition (Lin et al., 2014). The DopR1 receptor was required in γ lobe mushroom body neurons for water learning, while OA was not required (Lin et al., 2014). Naïve water-seeking behavior is mediated by a different pathway than water-learning behavior. PAM neurons innervating the β'_2 region of the mushroom body lobe mediated naïve water seeking, but the DopR1 receptor was not involved (Lin et al., 2014).

Another study distinguished short- versus long-term water memories, and identified additional dopaminergic clusters that mediate these memories (Shyu et al., 2017). PAM-γ_4 neurons mediate short-term water memory in thirsty flies (Lin et al., 2014; Shyu et al., 2017). Water reward also produces a protein synthesis-dependent long-term memory when tested 24 h after conditioning. Long-term water memory is disrupted by cycloheximide, and is also negatively affected in *radish*, *crammer*, *tequila,* and *dCREB* mutants. Long-term water memory is mediated by PAM dopaminergic neurons that innervate the β'_1 region of the mushroom body lobes (Shyu et al., 2017). The DopR1 DA receptor is required in α'/β' neurons for long-term water memory (Shyu et al., 2017). Different subsets of mushroom body neurons are required for consolidation and retrieval of long-term water memories. Output from α'/β' is needed for consolidation, while output from γ and α/β neurons is needed for memory retrieval (Shyu et al., 2017).

Figure 3 summarizes the findings about water reward and highlights the similarities and differences in the pathways that mediate sugar memories versus those that mediate water memories. Next, we switch focus to what is currently known about drug reward in *D. melanogaster*. Ethanol has been studied the most and has been shown to be rewarding for fruit flies. Comparison of genes, neurotransmitters and neural circuits that are involved in locomotor effects of ethanol against those for ethanol reward reveal overlap, suggesting shared mechanisms. With this insight, we include data from locomotor assays for additional drugs of interest. Genes, molecular pathways, and neural circuits underlying locomotor drug effects may provide hints for additional mechanisms for drug reward to be investigated in the future.

DRUG REWARD

Reward systems require the integration of sensory information and the formation of memory to assign beneficial or harmful associations to the stimuli and result in motivated behavior. There are three main theories of addiction. The incentive sensitization theory of addiction postulates that repetitive exposure to drugs of abuse persistently modifies the neurons and neural circuits that mediate incentive salience attributed to the drug stimulus and also drug-associated cues to the point of reaching a pathological level of 'wanting' for the drug (Robinson and Berridge, 2008). This theory of addiction focuses heavily on 'wanting' and its neural correlate of mesolimbic DA sensitization, which is most common after repeated, spaced apart, high dose exposure to drugs (Berridge and Robinson, 2016). A second theory of addiction has developed around the concept

of allostasis and opponent-process theory, including changes in neurotransmitter systems, neural circuits, and stress systems that result in an alternative homeostatic condition in response to drugs of abuse (Koob and Le Moal, 2008; Wise and Koob, 2014). This leads to a 'chronic elevation of reward set point' (Koob and Le Moal, 2008; Wise and Koob, 2014). Lastly, the third theory of addiction attributes the shift from voluntary drug taking to compulsive drug abuse to alterations in neurocircuitry involving habit systems and the development of 'habit-based learning' (Everitt et al., 2008; Everitt and Robbins, 2016). These theories continue to evolve as we gain insight into the mechanisms of both natural and drug reward.

Drug reward research in *D. melanogaster* has focused on identifying genes and neural circuits underlying the reinforcing properties of drugs. In the next section of this review, we discuss palatability, preference, and rewarding properties of ethanol. We delve into the genetic and neural mechanisms of ethanol's locomotor effects, which include changes in neurotransmitter systems and neural circuits. Lastly, we compiled data on mechanisms mediating the locomotor effects of cocaine, amphetamine, methamphetamine and nicotine.

Ethanol: Palatability and Preference

Palatability: Proboscis Extension Reflex

Studies have shown that ethanol is not an appetitive tastant for flies upon initial exposure. In one study, ethanol concentrations ranging from 0.1 to 40% failed to elicit PER responses (Devineni and Heberlein, 2009). When these concentrations were mixed with 100 mM sugar, which elicits reliable PER responses, there was an ethanol-concentration-dependent decrease in PER response frequency (Devineni and Heberlein, 2009). These results were replicated by Xu et al. (2012), who showed that ethanol preference did not significantly decrease PER responses for ethanol-laced sucrose food at low ethanol concentrations (Xu et al., 2012).

However, Devineni and Heberlein (2009) found that flies develop preference for ethanol-laced food over time, with flies exhibiting a mild preference for ethanol after a single day of consumption and increasing preference over the next 4 days (Devineni and Heberlein, 2009). Even though ethanol is not palatable to naïve flies, flies prefer olfactory traps with an ethanol smell, showing that ethanol smell is attractive to flies (Devineni and Heberlein, 2009; Schneider et al., 2012). It would be interesting to test PER in flies that have developed preference for ethanol.

Voluntary Consumption and Two-Choice Preference

Devineni and Heberlein (2009) modified the capillary feeder (CAFE) assay by Ja et al. (2007) to quantify voluntary ethanol consumption over time in chambers that included a choice between food laced with ethanol and food without the ethanol (Ja et al., 2007; Devineni and Heberlein, 2009). A comparison between the amount of ethanol food versus non-ethanol food consumed over time was then used to calculate a preference index. Results from this assay showed that flies develop a dose-dependent preference for food containing ethanol. Pohl et al. (2012) also showed that flies prefer ethanol-containing food (Pohl et al., 2012). Flies increase their ethanol consumption over time, are willing to overcome an aversive stimulus of quinine to consume ethanol food, and will go back to ingesting large amounts of ethanol following a deprivation period (Devineni and Heberlein, 2009). Ethanol preference in the CAFE assay is mediated by the cAMP pathway in the mushroom body. The adenylyl cyclase gene, *rutabaga*, is required in mushroom body neurons for flies to develop ethanol preference (Xu et al., 2012). Further investigation will be required to determine which specific mushroom body neurons mediate ethanol preference.

Ethanol preference in the CAFE assay was not based on nutritional value, as flies are not able to efficiently utilize ethanol calories for survival (Xu et al., 2012). The FRAPPE, a novel high-throughput ethanol consumption preference assay that measures the consumption of individual flies, further showed that ethanol preference in *Drosophila* is not driven by calories (Peru y Colón de Portugal et al., 2014). This study demonstrated that ethanol preference in fruit flies is an experience-dependent process in which ethanol is mildly aversive to naïve flies. However, flies develop long-lasting preference for ethanol food after 20 min of ethanol vapor pre-exposure (Peru y Colón de Portugal et al., 2014). Flies also developed ethanol preference when the pre-exposure was achieved by pre-feeding flies with ethanol-laced food both in a no-choice and in a two-choice configuration in the CAFE. This result shows that different routes of ethanol pre-exposure all lead to ethanol preference (Peru y Colón de Portugal et al., 2014).

A follow up study by Devineni et al. (2011) identified additional genes that regulate voluntary ethanol consumption, including *whiterabbit*, which codes for RhoGAP18B, a GTP-ase activating protein of the Rho family (Devineni et al., 2011). Flies with *whiterabbit* mutations had decreased voluntary ethanol consumption in the two-choice CAFE assay (Devineni et al., 2011). Other genes shown to act in the same pathway as RhoGAP18B also have ethanol consumption phenotypes. Unlike wild-type flies that require ethanol pre-exposure to develop preference for ethanol, naïve *Arf6* and *Efa6* mutant flies display a high and unchanging preference for ethanol food (Gonzalez et al., 2017). *Rsu1* mutants also have naïve preference for ethanol and acquire ethanol preference over time. A targeted decrease in *Rsu1* in the mushroom body resulted in flies with no naïve preference for ethanol or gradual ethanol preference, which showed that *Rsu1* in the mushroom body mediates gradual ethanol preference, while *Rsu1* acts in neurons outside the mushroom body to mediate naïve preference (Ojelade et al., 2015). Another gene, *Sir2*, also mediates ethanol preference and encodes for NAD-dependent histone deacetylase sirtuin-2 (Engel et al., 2016). *Sir2* mutant flies have high naïve preference for ethanol food but fail to develop ethanol preference after ethanol pre-exposure (Engel et al., 2016).

Ethanol preference can be modified by social experience, specifically sexual experience (Shohat-Ophir et al., 2012). Sexually rejected males have higher ethanol consumption and ethanol preference than mated males (Shohat-Ophir et al.,

2012). Mating status was correlated with levels of NPF (Shohat-Ophir et al., 2012). NPF transcript and protein levels were higher in mated males compared to rejected males (Shohat-Ophir et al., 2012). NPF pathway activity mediated ethanol preference, increasing ethanol preference when it was downregulated and decreasing ethanol preference when it was artificially activated (Shohat-Ophir et al., 2012). Notably, activation of the NPF pathway was found to be rewarding for flies in a conditioned odor preference assay (Shohat-Ophir et al., 2012). In addition, artificial activation of the NPF pathway abolished the preference for ethanol (Shohat-Ophir et al., 2012). It was also shown that the ethanol exposure regime that was rewarding for flies increased NPF levels (Shohat-Ophir et al., 2012). This study suggests a homeostatic model of reward in which the NPF pathway signals reward level status in *Drosophila*. This means that experiences that lower NPF signaling promote reward-seeking behaviors, while experiences that increase NPF signaling decrease reward-seeking behaviors (Shohat-Ophir et al., 2012; Devineni and Heberlein, 2013). These results have been replicated in a methods paper that details this novel experimental design to study reward in the fly (Zer et al., 2016). This experimental design has two components: the first consists of exposing the flies to either rewarding or non-rewarding experiences and the second consists of determining their voluntary ethanol consumption as a measure of motivation to seek a drug reward (Zer et al., 2016). This assay can be used to study how experience modulates drug reward and to identify novel genes and neural circuits that mediate reward (Zer et al., 2016).

Figure 2 summarizes the studies on palatability and preference for ethanol. The experience-dependent and delayed preference for ethanol described above is reminiscent of how preference for a nutritious sugar develops. One similarity is the role of the cAMP pathway as a mediator of both sugar and ethanol preference. It would be interesting to test if ethanol elicits calcium oscillations, as sugar does. There are also differences, for example the involvement of RhoGAP18 and the NAD-dependent histone deacetylase sirtuin-2 in ethanol preference.

Neuropeptide F is also a common factor between sugar and ethanol. NPF has been shown to be involved in hunger modulation of sugar memories by inhibiting specific dopaminergic neurons and allowing retrieval of sugar memories in hungry flies (**Figure 3**). Research on the role of NPF in ethanol preference has shown a negative correlation between levels of this neuropeptide and ethanol preference, either promoting ethanol consumption when NPF levels are low or decreasing ethanol consumption when NPF levels are high. A similar scenario for sugar would be that high levels of NPF correlate with hunger, which increases appetitive olfactory memory performance, a measure of increased sugar reward; low levels of NPF correlate with the sated state, which decreases appetitive memory performance and sugar reward. More is known about how NPF levels are modulated by hunger. It will be interesting to determine if similar mechanisms affect either sweet or nutritious sugar preference.

Oviposition Preference for an Ethanol Substrate

Flies also display preference for ethanol as a substrate for oviposition. It has been shown that flies prefer a substrate with 5% ethanol on a two-choice oviposition preference assay (Azanchi et al., 2013). Flies are attracted to acetic acid or the bitter compound lobeline for oviposition, while displaying positional aversion for these substrates, and the mushroom body was implicated in these behaviors (Joseph et al., 2009; Joseph and Heberlein, 2012). Flies did not show positional aversion or attraction to ethanol at the concentrations that elicited oviposition preference (Azanchi et al., 2013). Dopaminergic neurons of the PAM and the PPM3 clusters promote oviposition preference for ethanol, while PPL1 MB-MP1 neurons in the PPL1 cluster inhibit oviposition preference for ethanol (Azanchi et al., 2013). Both PAM and PPL1 dopaminergic neurons innervate the mushroom body, while PPM3 neurons innervate the ellipsoid body of the central complex (Mao and Davis, 2009; Kong et al., 2010b; Aso et al., 2012). The α′/β′ mushroom body neurons mediated oviposition preference, as did the ring R2 neurons of the ellipsoid body (Azanchi et al., 2013). The role of dopaminergic receptors in the mushroom and ellipsoid bodies was also tested. It was shown that decreasing DopR2 in the mushroom body increased oviposition preference, while decreasing either DopR1 or DopR2 in the ellipsoid body each had the effect of increasing oviposition (Azanchi et al., 2013). A model was proposed in which the PAM and PPM3 neurons signal an appetitive stimulus and promote oviposition preference, while the PPL1 MB-MP1 neurons signal an aversive stimulus and suppress oviposition preference (Azanchi et al., 2013).

Figure 4 summarizes the findings described above. Even though oviposition preference for ethanol at first glance may seem a very different assay to sugar preference or sugar reward, the apparent similarities in the neural circuits warrant further consideration about what this assay may be able to tell us about reward. Indeed, there are also similarities in the mechanisms underlying oviposition preference for ethanol and conditioned odor preference for ethanol, as shown in the next section.

Ethanol Reward: Conditioned Odor Preference

The conditioned odor preference assay for ethanol reward developed by Kaun et al. (2011) is the most sophisticated drug reward assay for *Drosophila*, and was designed with the specific goal of establishing a model of drug reward using *D. melanogaster* (Kaun et al., 2011). In this assay, neutral odors are paired with a moderately intoxicating dose of ethanol during training. During testing, each odor is streamed from opposite ends of a Y-maze. Flies are placed in the bottom of the Y-maze and given 2 min to climb up the maze, either toward the arm where the odor associated with the ethanol is being streamed or to the arm with an unpaired odor. The number of flies in each side of the Y-maze is counted and a preference index is calculated. Using this assay, Kaun et al. (2011) demonstrated that flies develop conditioned odor preference for moderate concentrations of ethanol that elicit locomotor hyperactivity. Flies showed aversion to ethanol when tested 30 min after

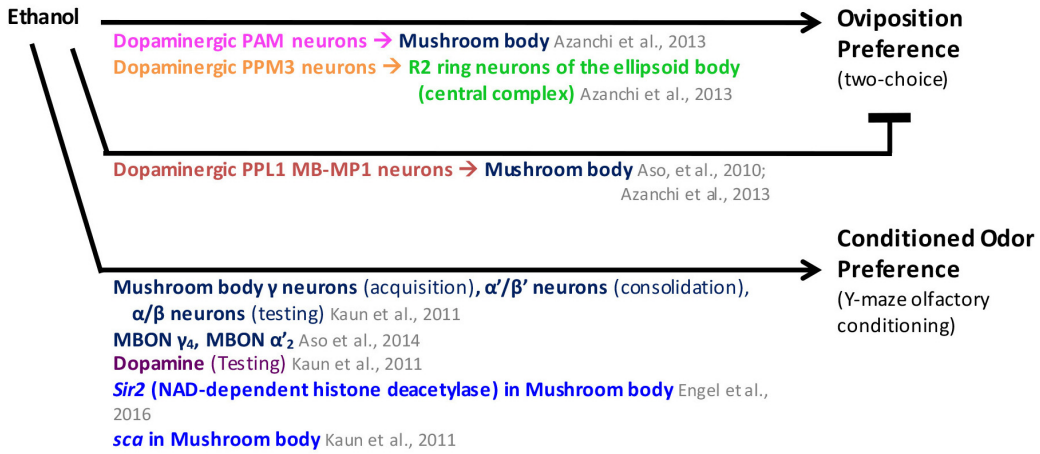

FIGURE 4 | Adult *Drosophila* choose an ethanol-associated stimulus in oviposition and conditioned odor preference assays. MB mushroom body (α/β, α′/β′, and γ lobes; blue), MBON mushroom body output neurons (blue), CC central complex (light green), PAM protocerebral anterior medial neurons (pink), PPL1 protocerebral posterior lateral 1 neurons (brown), neuronal pathways (dark purple), cellular pathways (dark green), genes/proteins (royal blue).

training but exhibited conditioned odor preference for ethanol 24 h after training, with preference first detected 12 h after training (Kaun et al., 2011). Conditioned preference for ethanol is dose-dependent and does not occur at lower doses that do not elicit behavioral effects in fruit flies, or at high doses that elicit sedation (Kaun et al., 2011). These results show that ethanol reward is long-lasting (Kaun et al., 2011). Interestingly, flies will overcome an electric shock to reach the Y-maze arm containing the odor associated with ethanol exposure (Kaun et al., 2011).

Dopamine was shown to be required for conditioned odor preference memory expression during preference testing but not during training or memory consolidation (Kaun et al., 2011). Activity in mushroom body neurons was needed for conditioned odor preference in sequence, such that γ neurons were needed during acquisition, α′/β′ neurons during consolidation, and α/β neurons during testing (Kaun et al., 2011). Given that dopaminergic neurons and α/β neurons in the MB were both needed during expression of the ethanol memory, it was proposed that ethanol reward memory is mediated by dopaminergic neurons that innervate the α/β neurons (Kaun et al., 2011). The mushroom body output neurons MBON-γ_4 and MBON-α'_2 were involved in conditioned odor preference for ethanol 24 h after training (Aso et al., 2014b). A genetic screen of a subset of mutant strains with GAL-4 reporter expression in the mushroom body identified a strain with persistent aversion that had a mutation in *scabrous* (*sca*), a fibrinogen-related peptide that functions via the Notch pathway and was found to be expressed in α/β and γ mushroom body neurons among other regions (Kaun et al., 2011). Another study showed that *Sir2* mutants had reduced conditioned odor preference for ethanol, suggesting that ethanol is not rewarding for *Sir2* mutants (Engel et al., 2016). *Sir2* appears to be required in mushroom body neurons for ethanol reward, as flies with reduced *Sir2* expression in the mushroom body did not display conditioned odor preference for ethanol (Engel et al., 2016).

A summary of the results from the studies above can be found in **Figure 4**. It is still unknown which dopaminergic clusters convey ethanol memories. However, the similarity between the neurons and neurotransmitter systems involved in oviposition preference for ethanol and conditioned odor preference for ethanol suggests that both are mediated through the same neural circuits (Kaun and Rothenfluh, 2017). In this proposed pathway, ethanol is a stimulus with dual properties: aversion and attraction. Appetitive reinforcement from ethanol exposure would be conveyed by activation of the dopaminergic neurons of the PAM cluster, while aversive reinforcement would be conveyed by activation of the dopaminergic neurons of the PPL1 cluster, (Kaun and Rothenfluh, 2017).

The experiments described above show that ethanol is rewarding to flies and also display preference for ethanol. Studies of ethanol preference demonstrated that this preference can be modulated, identifying NPF as a key modulator. NPF is also a modulator in sugar reward. Future research could determine whether NPF plays a role in modulation of ethanol reward.

In the next section, we move from the traditional assays used to study drug reward to measuring acute drug effects. These assays have identified additional mechanisms of drug action. Some of these mechanisms may provide new insights into genes and molecules that have not yet been implicated in ethanol and natural reward.

Ethanol Locomotor Effects

Ethanol exposure elicits different locomotor effects, including hyperactivity and loss of postural control. However, flies develop tolerance to these effects when re-exposed to ethanol (Kaun et al., 2012; Devineni and Heberlein, 2013). Using these behaviors as a marker for ethanol sensitivity, many genes, molecular pathways and neural structures have been identified as mediators for ethanol's effects (Kaun et al., 2012; Devineni and Heberlein, 2013).

Ethanol Hyperactivity

Ethanol exposure can increase locomotion in fruit flies (Wolf et al., 2002). Ethanol hyperactivity is modulated by hunger, with starvation increasing ethanol hyperactivity (Kliethermes, 2013). Interestingly, feeding flies just before exposure to ethanol with standard food or sucrose (but not yeast) blocked this effect of food deprivation (Kliethermes, 2013).

The dopaminergic pathway, specifically, a subset of dopaminergic neurons in the PPM3 cluster, mediates ethanol-induced hyperactivity (Bainton et al., 2000; Kong et al., 2010b). These neurons project to the ellipsoid body region of the central complex, known for its role in motor control. Moreover, specific neurons within the ellipsoid body, the ring neurons (R) R2/R4, have been implicated in ethanol-hyperactivity. These neurons express *DopR1*, which is required for ethanol-induced hyperactivity (Kong et al., 2010b). Alcohol dehydrogenase (*Adh*) and the cAMP pathway have also been shown to play a role in ethanol hyperactivity (Wolf et al., 2002). The *whiterabbit* gene, specifically the isoform RhoGAP18B-RA, promotes ethanol hyperactivity (Rothenfluh et al., 2006).

The *tao* gene, which encodes a serine-threonine kinase in the Mst/Ste20 family, has a role in adult nervous system development including mushroom body development (King et al., 2011). α/β mushroom body neurons and Tao through Par-1 mediate ethanol hyperactivity (King et al., 2011). *tao* mutants showed an increase in Tau phosphorylation, a microtubule stabilizing protein that is normally phosphorylated by Par-1. This suggests that *tao* exerts its effect on ethanol hyperactivity through a pathway that controls microtubule dynamics during development (King et al., 2011).

The epidermal growth factor receptor (EGFR) and the fibroblast growth factor receptor (FGFR) pathways have also been shown to modulate ethanol hyperactivity in opposing ways, suppressing and promoting ethanol hyperactivity, respectively (King et al., 2014). EGFR signaling, JNK signaling, and *tao* have been shown to act together in mushroom body development, which is a likely mechanism underlying ethanol hyperactivity (King et al., 2011, 2014).

Figure 5 summarizes the findings described above. Similarities between the ethanol hyperactivity and natural reward include the modulation by hunger, the involvement of the dopaminergic and the cAMP pathways.

Ethanol Sedation

If flies are exposed to ethanol continuously, the hyperactivity phase is followed by a loss of postural control, and the flies will eventually become sedated. Early work implicated the cAMP pathway in ethanol sensitivity (Moore et al., 1998). The *whiterabbit* gene product, isoform RhoGAP18B-RC, plays a role in ethanol sedation and was shown to function in adult flies through Rho1 and Rac1, which are small GTP-ases (Rothenfluh et al., 2006). A follow up study showed that RhoGAP18B-RC acts together with Rac1, the small GTPase Arf6, and *Drosophila* Arfaptin (Arfip) in adult neurons to regulate ethanol sedation (Peru y Colón de Portugal et al., 2012). Arfip interacts with GTP-bound Arf6 and GTP-bound Rac1, while Arf6 acts downstream of RhoGAP18B, Arfip, and Rac1 to mediate normal ethanol sedation (Peru y Colón de Portugal et al., 2012). Different RhoGAP18B isoforms act via specific Rho-family GTPases, which in turn regulate cofilin activity, an actin depolymerizing protein (Ojelade et al., 2015). Cofilin mutants had decreased sensitivity to ethanol sedation, and functioned downstream of RhoGAP18B-PC and –PD isoforms; these isoforms inhibited Rac1 and in turn regulated cofilin activity, leading to differences in actin dynamics (Ojelade et al., 2015).

The insulin pathway has been previously implicated in ethanol sedation and in mediating the effects of developmental ethanol exposure (Corl et al., 2005; McClure et al., 2011). The Insulin receptor (*InR)* is upstream of Arf6, which acts upstream of the p70 S6 kinase (S6k) to modulate ethanol sedation (Acevedo et al., 2015). Completing this ethanol sedation pathway, it was found that integrin signaling is upstream of Rac1 and that Ras suppressor 1 (Rsu1) inhibits Rac1 (Ojelade et al., 2015). A new study added Efa6 to the pathway, which is a guanine exchange factor for *Drosophila* Arf6 (Gonzalez et al., 2017). Like *Arf6* mutants, *Efa6* mutant flies have increased sensitivity to ethanol sedation and it was shown that *Efa6* acts upstream of *Arf6* and normally functions to activate Arf6. Together, *Efa6* and *Arf6* modulate ethanol sensitivity (Gonzalez et al., 2017).

Ethanol sensitivity is also regulated by *dLmo* genes, which are members of the LIM-homodomain transcription factor family that functions in fly circadian pacemaker neurons that express the pigment dispersing factor neuropeptide (Tsai et al., 2004; Lasek et al., 2011). The clock gene period (*per*) also modulates ethanol sedation (De Nobrega and Lyons, 2016; Liao et al., 2016). The NPF pathway, the EGFR/Erk and the PI3K/Act pathways have also been implicated in ethanol sedation (Wen et al., 2005; Corl et al., 2009; Eddison et al., 2011). More recently, it was found that the *Drosophila* dopamine/ecdysteroid receptor (DopEcR) mediates ethanol sedation by inhibiting EGFR/Erk signaling to promote ethanol sedation (Petruccelli et al., 2016). The GABA-B receptor, the *aru* gene, which encodes a homologous adaptor protein to mammalian Epidermal Growth Factor Receptor Substrate 8, the tumor suppressor homolog gene *tank,* and the *gfa* gene, a Dα7 nAChR subunit, have also been found to play a role in ethanol sedation (Dzitoyeva et al., 2003; Eddison et al., 2011; Devineni et al., 2013; Velazquez-Ulloa, 2017). *homer* function was needed in R2/R4 ellipsoid body neurons for ethanol sedation (Urizar et al., 2007). Corazonin neurons, which express the neuropeptide corazonin and the transcription factor *apontic (apt)* also modulate ethanol sedation (McClure and Heberlein, 2013). The autophagy gene *Atg16* acts in corazonin-expressing neurosecretory cells to regulate ethanol sedation, and seems to regulate corazonin transcript and protein levels (Varga et al., 2016).

Examination of gene expression on a microarray after 30 min of 60% ethanol vapor compared to flies exposed to water vapor identified several genes with altered expression in ethanol-exposed flies (Kong et al., 2010a). These genes had functions in serine biosynthesis, olfaction, transcriptional regulation, cytoskeletal organization, immunity and metabolism

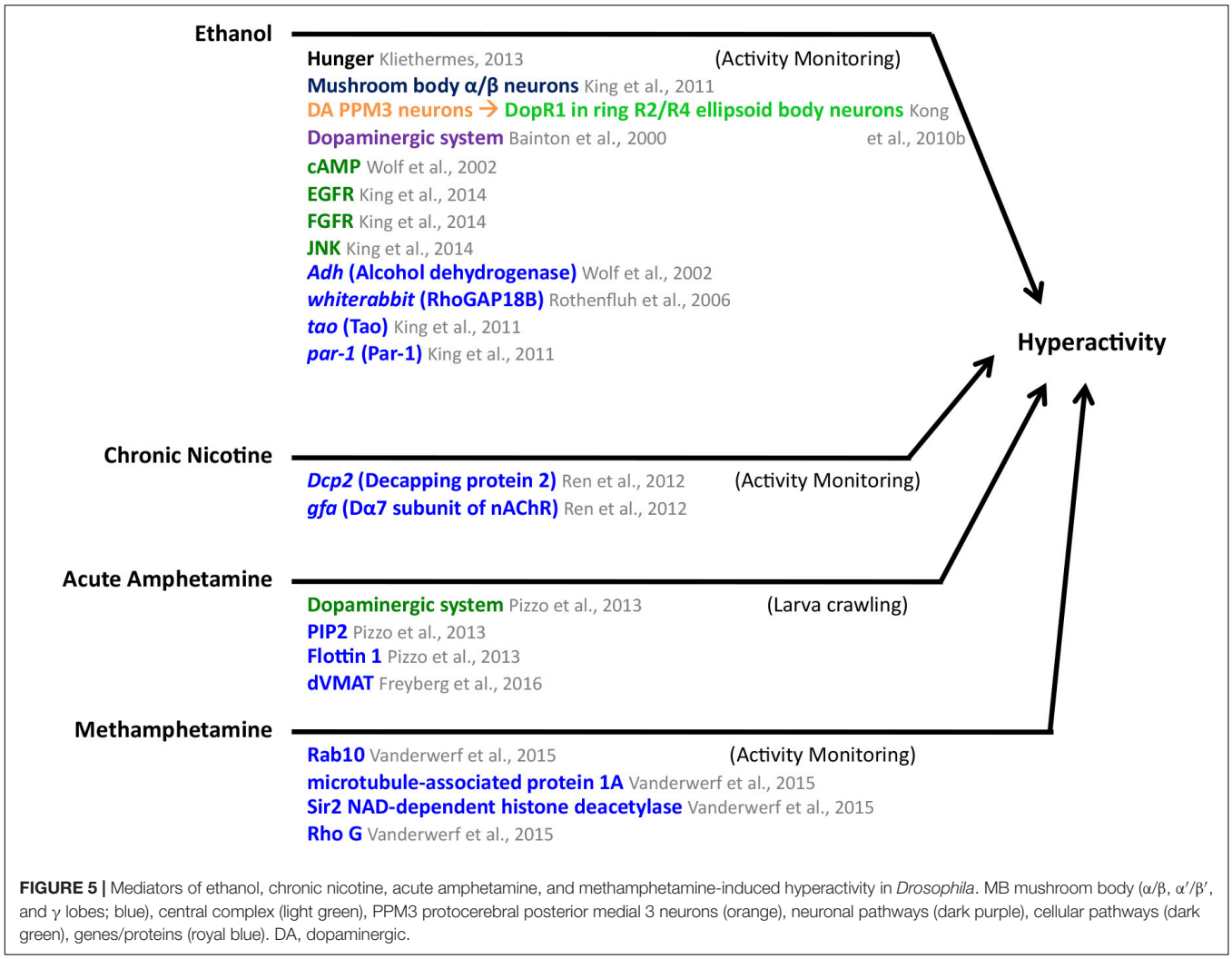

FIGURE 5 | Mediators of ethanol, chronic nicotine, acute amphetamine, and methamphetamine-induced hyperactivity in *Drosophila*. MB mushroom body (α/β, α′/β′, and γ lobes; blue), central complex (light green), PPM3 protocerebral posterior medial 3 neurons (orange), neuronal pathways (dark purple), cellular pathways (dark green), genes/proteins (royal blue). DA, dopaminergic.

(Kong et al., 2010a). *Sir2* transcript and protein expression was greatly reduced after ethanol exposure (Morozova et al., 2006; Kong et al., 2010a; Engel et al., 2016). Along with decreased expression, acetylation of Histone 3 at Lysine 9 (H3K9) was increased (Morozova et al., 2006; Engel et al., 2016). This is consistent with *Sir2*'s role as a deacetylase that targets H3K9 (Engel et al., 2016). *Sir2* mutants had decreased ethanol sedation sensitivity and ethanol sedation tolerance, and it was further showed that *Sir2* is required in adult mushroom body α/β lobe neurons for these effects (Engel et al., 2016). Synapsin (*Syn*) expression was greatly decreased in *Sir2* mutants and it was further shown that *Syn* expression decreased after ethanol exposure in wild-type but not in *Sir2* mutant flies (Engel et al., 2016). The protein levels of Syn were also decreased in ethanol treated brains (Engel et al., 2016). As expected, *Syn* mutants had decreased ethanol sensitivity and tolerance (Engel et al., 2016).

The results described above are summarized in **Figure 6**. Common factors in the mechanisms for ethanol hyperactivity and ethanol sedation include roles for RhoGAP18B, the EGFR pathway, and the gene *tao*. Common factors between ethanol sedation mechanisms and those of natural reward include roles for the cAMP and insulin pathways and the *Sir2* and *Syn* genes.

Ethanol Tolerance

Ethanol tolerance is the sedation response after a second ethanol exposure. The ethanol tolerance assay has identified several genes and molecular pathways in *Drosophila* that mediate this effect. The OA pathway was found to mediate ethanol tolerance (Scholz et al., 2000; Berger et al., 2004). The *hangover* gene (*hang*), a zinc finger protein, and *jwa,* which are genes involved in stress responses, mediate ethanol tolerance (Scholz et al., 2005; Li et al., 2008). The *slowpoke* gene (*slo*), which encodes a BK-type Ca-activated K channel, is also involved in ethanol tolerance (Cowmeadow et al., 2005, 2006). The GABA-B receptor and the gene *homer*, which interacts with metabotropic glutamate receptors on the post-synaptic site, have also been implicated in ethanol tolerance (Dzitoyeva et al., 2003; Urizar et al., 2007). Homer function was needed in R2/R4 ellipsoid body neurons for ethanol tolerance (Urizar et al., 2007). The pre-synaptic genes *synapsin*, *syntaxin 1A*, and *shibire* were also found to regulate ethanol tolerance (Godenschwege et al., 2004;

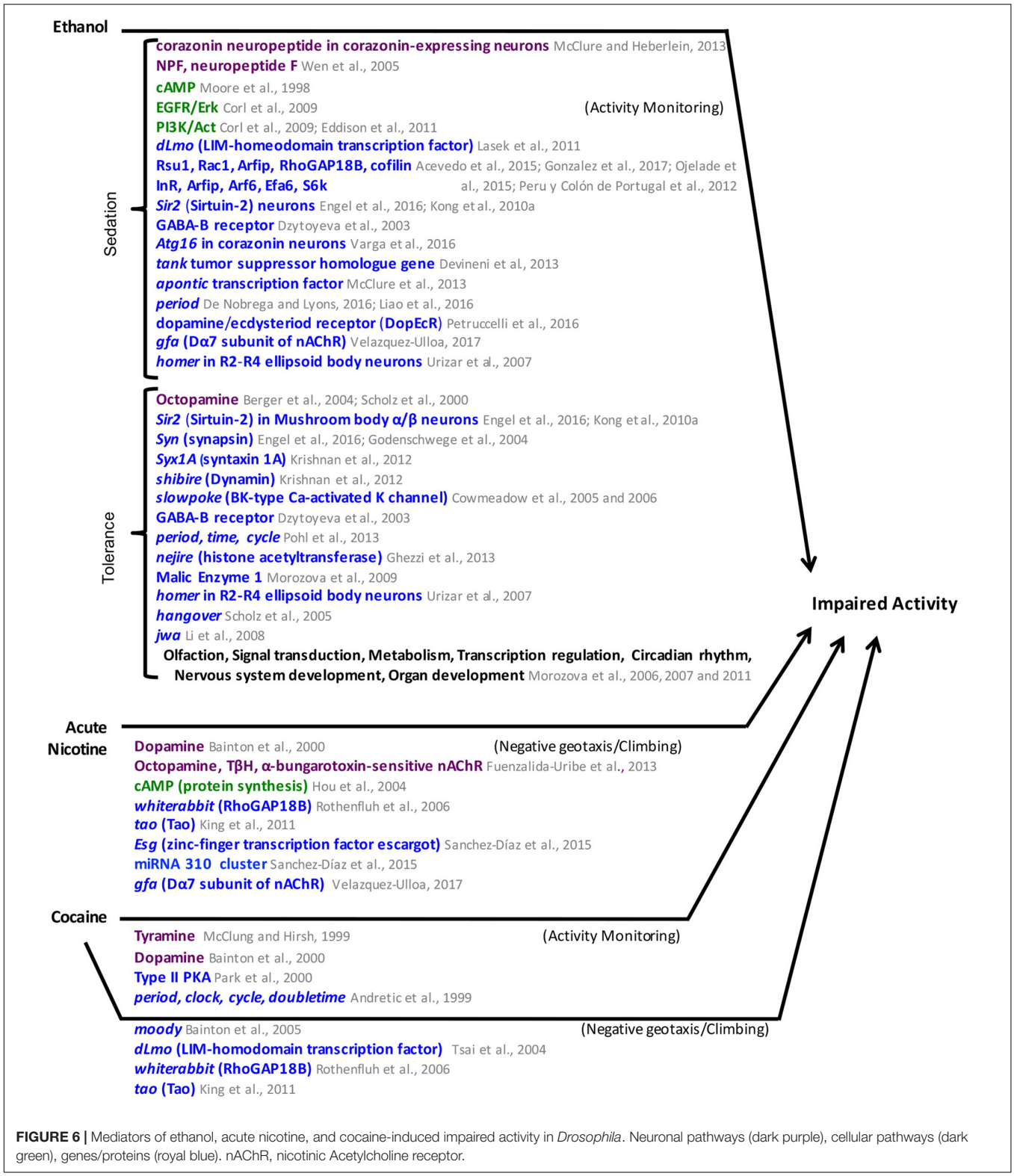

FIGURE 6 | Mediators of ethanol, acute nicotine, and cocaine-induced impaired activity in *Drosophila*. Neuronal pathways (dark purple), cellular pathways (dark green), genes/proteins (royal blue). nAChR, nicotinic Acetylcholine receptor.

Krishnan et al., 2012). *Sir2* mutants had reduced ethanol sedation tolerance (Kong et al., 2010a). The clock genes *per*, *tim*, and *cyc* also modulate ethanol tolerance (Pohl et al., 2013). More recently it has been shown that ethanol exposure results in the histone acetylation of genes that form a network for ethanol tolerance. The histone acetyltransferase that mediates these histone modifications is coded by the gene *nejire*, the *Drosophila* ortholog of mammalian CBP (Ghezzi et al., 2013).

A different approach taken to identify genes that regulate ethanol sensitivity and tolerance is to determine global changes in gene expression after ethanol exposure. In one study, transcript expression level was analyzed in flies exposed to ethanol during a postural control assay and again 2 h later (Morozova et al., 2006). This study identified downregulation of genes that function in olfaction and upregulation of signal transduction genes after a single ethanol exposure, and downregulation of metabolic enzymes, and upregulation of transcriptional regulators and circadian genes only after a second exposure to ethanol (Morozova et al., 2006). Another approach using artificial selection for ethanol sensitivity identified 32 mutants with significantly different responses to ethanol compared to their genetic control; 23 of these had human orthologs (Morozova et al., 2007). These genes were involved in carbohydrate metabolism, lipid metabolism, nervous system development, transcription regulation, and signal transduction (Morozova et al., 2007). Analysis of the variation in ethanol tolerance in 40 inbred lines with genome-wide variation in a gene expression study identified genetic networks that mediate this effect, including a network with Malic Enzyme 1 (Morozova et al., 2009). A new approach combined screening a co-isogenic P-element insertion mutant collection to identify lines with differential ethanol sensitivity, and then used computational approaches to build genetic networks based on transcription correlation from whole-genome expression data (Morozova et al., 2011). This approach identified focal genes in the networks that were validated as having a role in ethanol sensitivity in wild-type flies, and also validated that these genes worked in a single network (Morozova et al., 2011).

Figure 6 summarizes the results of the studies above. Common genes that mediate ethanol tolerance and ethanol sedation include the GABA-B receptor, *homer*, *synapsin*, and *Sir2*. Whole genome analysis of gene expression after ethanol exposure identified metabolism genes among the genes regulated by ethanol exposure. It would be interesting to try a similar approach to identify gene expression changes after exposure to natural rewards. Future research could also examine whether some of the pathways involved in ethanol's effects like Corazonin, EGFR/PI3K, or RhoGAP18 and cytoskeleton regulation also have roles in natural reward.

Ethanol has been studied more than other drugs but the current data shows similarities in the genes and pathways mediating the effects of ethanol and the drugs discussed below.

Other Drugs

Cocaine

There are parallels between the results from these studies with research in mammals that suggest *Drosophila* is a viable model to study cocaine reward. Flies exhibit specific locomotor effects when exposed to cocaine and develop sensitization after repeated exposure (McClung and Hirsh, 1998). Several molecular pathways have been implicated in cocaine's effects in the fly (Hirsh, 2001; Heberlein et al., 2009). Type II PKA activity mediates cocaine sensitization (Park et al., 2000). The dopaminergic pathway and tyramine also modulate cocaine sensitivity (McClung and Hirsh, 1999; Bainton et al., 2000). The *moody* gene, which encodes a G-protein-coupled receptor that regulates blood-brain-barrier permeability in flies, *whiterabbit* and *tao* also mediate cocaine sensitivity (Bainton et al., 2005; Rothenfluh et al., 2006; King et al., 2011). Cocaine sensitivity is also mediated by *dLmo* (Tsai et al., 2004). Mutant flies for several circadian genes fail to develop cocaine sensitization, including flies mutant for *period*, *clock*, *cycle*, and *doubletime* (Andretic et al., 1999). These circadian genes were first linked to cocaine sensitization in flies, and have now been linked to cocaine sensitization and reward in mammals (Abarca et al., 2002; McClung et al., 2005).

Amphetamines and Methamphetamine

There have not been any studies to determine whether amphetamines are rewarding in fruit flies. However, the acute locomotor effects of amphetamine have allowed for the identification of the conserved effects of amphetamines in flies. Amphetamine increases locomotion in *Drosophila* larvae (Pizzo et al., 2013). The effects of amphetamine are mediated by DA, DA transporter phosphorylation, and membrane raft protein Flotillin 1 (Pizzo et al., 2013). Another study found a contribution of PIP_2 in mediating the locomotor effects of amphetamine in *Drosophila* (Hamilton et al., 2014). It was shown that the DA transporter associates with PIP_2 in cell culture and that this interaction is needed for amphetamine-induced DA efflux and for amphetamine-induced locomotion in *Drosophila* (Hamilton et al., 2014). More recently it was shown that the *Drosophila* vesicular monoamine transporter (dVMAT) is also needed for the amphetamine-induced hyperlocomotion in fruit flies (Freyberg et al., 2016). Flies with a null mutation in dVMAT did not develop amphetamine-induced hyperlocomotion (Freyberg et al., 2016).

Methamphetamine has also been shown to increase locomotion in adult flies through Rab10, a GTP-binding protein present in membrane rafts that regulates intracellular membrane trafficking (Vanderwerf et al., 2015). Rab10's abundance within rafts is decreased after methamphetamine exposure (Vanderwerf et al., 2015). Flies with a mutant form of Rab10 had decreased sensitivity to methamphetamine-induced increased locomotion and needed a larger methamphetamine dose to display significantly increased locomotion compared to the controls (Vanderwerf et al., 2015). Other proteins whose abundance was affected by methamphetamine exposure included the microtubule-associated protein 1A, the NAD-dependent histone deacetylase Sirtuin-2, and the Rho-related GTP-binding protein Rho G (Vanderwerf et al., 2015).

Nicotine

Nicotine reward has not been established in flies. However, probing the acute effects of nicotine has revealed molecular mechanisms similar to cocaine (Bainton et al., 2000). The dopaminergic pathway, which modulates nicotine sensitivity, was tested in a climbing assay based on flies' natural behavior of negative geotaxis (Bainton et al., 2000). Flies acutely exposed to nicotine became unable to climb, but this effect was reduced when DA was depleted (Bainton et al., 2000). OA was also shown to mediate nicotine's effects on a similar assay, as flies with decreased

OA were not affected by nicotine (Fuenzalida-Uribe et al., 2013). OA release is mediated by the activation of α-bungarotoxin-sensitive nAChRs in the brain (Fuenzalida-Uribe et al., 2013).

The cAMP pathway mediates the effect of nicotine on negative geotaxis (Hou et al., 2004). Flies with increased levels of cAMP were more sensitive to nicotine's effects in the climbing assay, and flies with mutations in PKA were less sensitive to the effects of nicotine (Hou et al., 2004). Repeated exposure to nicotine in adult flies increased the effect of nicotine on the flies' ability to climb when tested at 4, 8, and 20 h after the first nicotine exposure (Hou et al., 2004). The sensitization of the response to nicotine is mediated by the cAMP pathway, including *dCREB*, and requires protein expression (Hou et al., 2004).

Additional genetic mechanisms mediating nicotine's effects in the climbing assay have been uncovered. Flies with mutations in the *whiterabbit* or in *tao* have decreased sensitivity to nicotine in a negative geotaxis climbing assay (Rothenfluh et al., 2006; King et al., 2011). A genetic screen identified two mutant lines with increased sensitivity to nicotine that had significantly longer recovery times after nicotine exposure (Sanchez-Díaz et al., 2015). The mutations mapped onto the transcription factor gene *escargot* (*esg*) and the miRNA 310 cluster (Sanchez-Díaz et al., 2015).

A different study characterized the effects of chronic nicotine exposure in adult flies and found that flies became hyperactive (Ren et al., 2012). This study identified *Dcp2*, the gene encoding the decapping protein 2, as a mediator of this chronic nicotine-induced locomotor hyperactivity (Ren et al., 2012). This study also identified the *gfa* gene, which encodes for the Dα7 nAChR subunit, as a mediator of chronic nicotine-induced locomotor hyperactivity, as flies with downregulated Dα7 did not develop hyperactivity (Ren et al., 2012).

The studies described above focus on the effects of nicotine exposure in adult flies. Developmental nicotine exposure in *Drosophila* also affects how exposed flies respond to nicotine when they are adults (Velazquez-Ulloa, 2017). After developmental nicotine exposure, flies display decreased sensitivity to acute nicotine exposure in the climbing assay. They also display decreased sensitivity to ethanol as adults in an ethanol sedation assay (Velazquez-Ulloa, 2017). In addition, developmental nicotine exposure resulted in decreased survival, increased developmental time and decreased weight (Velazquez-Ulloa, 2017). The nAChR subunit Dα7 mediated the effects of developmental nicotine on survival and developmental time, and may also mediate the effects on nicotine sensitivity (Velazquez-Ulloa, 2017). Different studies examining genetic variation associated with larval resistance to nicotine on a survival assay using the *Drosophila* Synthetic Population Resource identified *Ugt86Dd* as a locus that confers differential sensitivity to nicotine (Marriage et al., 2014; Highfill et al., 2017).

These studies identify DA as a common mediator of drug effects. Other neurotransmitters have been shown to play a role in the effects of specific drugs. More testing is needed to determine if these neurotransmitters mediate responses to most drugs. RhoGAP18B along with proteins both upstream and downstream, mediate effects of ethanol. These pathways regulate cytoskeleton dynamics. It will be interesting to determine the involvement of these pathways in mediating the effects of other drugs. Circadian genes and genes that encode proteins that modify histones are also common factor mediators of drug effects that warrant additional investigation (**Figures 5**, **6**).

PERSPECTIVE ON THE COMMON MECHANISMS OF NATURAL AND DRUG REWARD

Analysis of the scientific literature included here suggests that there are parallel circuits mediating perception and reward for each appetitive natural stimulus. Sensory receptors in the periphery are activated by different taste modalities. This sensory information is conveyed to different neuronal circuits in the *Drosophila* central brain, including the activation of specific subsets of dopaminergic neurons that connect to distinct mushroom body compartments that encode either short or long-term memories. Memory formation requires the cAMP pathway in mushroom body neurons to mediate the synaptic plasticity for encoding memories. These natural reward memories are homeostatically modulated by hunger and thirst. Serotonin and NPF convey nutrient signals via activation of PPL1 MB-MP1 neurons, which have calcium oscillations that are modulated by hunger state and represented by sugar levels in the hemolymph. The receptors, dopaminergic neurons, and mushroom body compartments have been determined for sugar and water reward, but have not yet been identified for protein or fatty acid reward. The neural circuits that mediate conditioned odor preference and oviposition preference for ethanol are remarkably similar to those for sugar and water reward, but seem to be a parallel circuit. More detailed mapping of ethanol reward circuits will determine if there is overlap between ethanol, sugar and other rewards.

Palatability and preference for different nutrients and ethanol also have common factors. Serotonin plays a role in both sugar and protein preference, while the cAMP pathway plays a role in sugar and ethanol consumption preference. Several neuropeptides mediate nutrient preference including insulin, juvenile hormone inducible 21, and Dh44. Dh44-expressing neurons mediate preference for a nutritious sugar, and similar to PPL1 MB-MP1 neurons, exhibit calcium oscillations that are modulated by glucose levels in the hemolymph. Hence, calcium oscillation modulation by nutrient levels in the hemolymph seems to be a common mechanism for encoding hunger.

The study of natural reward in *Drosophila* has developed around testing the reinforcing properties of stimuli that lead to either appetitive or aversive memories and mapping the neural circuits underlying these memories with continuously improving resolution. The study of drug reward began by focusing on acute effects of drugs and then identifying the genes that mediated the acute effects. More work needs to be done to map where the genes, proteins and signaling cascades function in the neural circuits that mediate drug reward. In addition, it would be interesting to test whether genes and signaling pathways that mediate drug effects also have roles on natural reward.

Of particular interest are the signaling cascades with RhoGAP18B at the center that involve cytoskeleton dynamics, genes involved in development of reward brain centers such as *tao*, circadian genes, and histone modification genes such as *Sir2* and *nejire*.

A model is emerging for parallel circuits for reward from sensory perception to behavior segregated by the type of stimulus. The reward system is centered around dopaminergic neurons as carriers of the reinforcement signal with the mushroom body as coincidence detector center, where integration of information occurs at specific compartments of the mushroom body, which in turn recruit different sets of mushroom body output neurons (de Araujo, 2016; Scaplen and Kaun, 2016; Kaun and Rothenfluh, 2017; Cognigni et al., 2018).

Future studies of drug reward with assays that focus on the reinforcing properties of the drugs instead of just the acute effects will make it possible to determine the similarities and differences in the encoding of natural and drug reward in *D. melanogaster*. The unparalleled genetic and molecular tools available for *Drosophila* research will continue to allow for the mapping of neuronal circuits at single-cell resolution. Combining this approach with the ability to manipulate genes in individual cells makes *Drosophila* an ideal model organism to dissect the mechanisms of both natural and drug reward.

AUTHOR CONTRIBUTIONS

NV-U wrote the manuscript draft and provided the feedback on the figures. EL wrote the sections of the manuscript, edited the full draft, and prepared the figures and all other sections of the paper.

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REVIEW
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Mechanisms Underlying the Risk to Develop Drug Addiction, Insights From Studies in *Drosophila melanogaster*

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The ability to adapt to environmental changes is an essential feature of biological systems, achieved in animals by a coordinated crosstalk between neuronal and hormonal programs that allow rapid and integrated organismal responses. Reward systems play a key role in mediating this adaptation by reinforcing behaviors that enhance immediate survival, such as eating or drinking, or those that ensure long-term survival, such as sexual behavior or caring for offspring. Drugs of abuse co-opt neuronal and molecular pathways that mediate natural rewards, which under certain circumstances can lead to addiction. Many factors can contribute to the transition from drug use to drug addiction, highlighting the need to discover mechanisms underlying the progression from initial drug use to drug addiction. Since similar responses to natural and drug rewards are present in very different animals, it is likely that the central systems that process reward stimuli originated early in evolution, and that common ancient biological principles and genes are involved in these processes. Thus, the neurobiology of natural and drug rewards can be studied using simpler model organisms that have their systems stripped of some of the immense complexity that exists in mammalian brains. In this paper we review studies in *Drosophila melanogaster* that model different aspects of natural and drug rewards, with an emphasis on how motivational states shape the value of the rewarding experience, as an entry point to understanding the mechanisms that contribute to the vulnerability of drug addiction.

Keywords: *Drosophila melanogaster*, reward, ethanol, addiction, learning and memory, natural reward, drug reward, animal models

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INTRODUCTION

From insects to humans, organisms living in complex environments need to respond quickly and appropriately to different stimuli by choosing one action over another to increase their chances of survival and reproduction. Reward systems play a key role in promoting this aim by motivating animals to repeat behaviors that increase their fitness, such as eating, drinking, sexual interaction, and parental behaviors. Drugs of abuse affect the same brain regions used for the processing of natural rewards, creating the pleasurable feeling indicative of a fitness benefit, and with repeated use can lead to compulsive drug abuse and addiction (Nesse and Berridge, 1997; Koob, 2009).

The American Psychiatric Association defines addiction as "maladaptive pattern of substance use manifested by recurrent and significant adverse consequences related to the repeated use of substances" (American Psychatric Association, 2013). This is characterized by a sequence of stages: (1) initial voluntary consumption of the drug, accompanied by an acute hedonic drug response, (2) repeated use, leading to compulsive and uncontrolled drug use, and finally, (3) physical and mental dependence (Koob and Bloom, 1988; Wolffgramm and Heyne, 1995; Koob, 1997, 2009; Nesse and Berridge, 1997).

Understanding the complex nature of human addiction is one of the greatest challenges in contemporary neuroscience, requiring parallel efforts of many scientific disciplines. One important approach is the use of animal systems to model certain features of the process, such as the reinforcing properties of drug rewards. Early studies by Karl von Frisch demonstrated the ability of sugar reward to reinforce preference for certain colors in honey bees (von Frisch, 1914). Subsequent studies by Olds and Milner demonstrated that rodents can learn to press a lever to receive intracranial self-stimulation (ICSS), facilitating the discovery of brain areas that encode reward (Olds and Milner, 1954). These seminal studies paved the path for the development of complex behavioral paradigms that measure the rewarding effects of drugs. Examples include self-administration paradigms, in which voluntary lever pressing results in delivery of a drug dose (Weeks, 1962; Thompson and Schuster, 1964), and conditioned place preference, where animals learn to associate a certain environment with receiving a drug, and the preference for this environment is tested afterwards in the absence of the drug (Rossi and Reid, 1976). Although the existing models do not entirely recapitulate the complexity of human addiction, they model important features of drug addiction (Koob, 2009; Lynch et al., 2010). For example, the positive reinforcing actions of binge intoxication is captured using self-administration paradigms in rodents and monkeys (Johanson and Balster, 1978; Collins et al., 1984), while the negative reinforcing properties of the withdrawal phase are measured by increased anxiety-like responses (Sanchis-Segura and Spanagel, 2006). The craving stage can be modeled by "cue-induced reinstatement," in which the reinstatement of drug seeking is tested after the induction of drug cues following drug self-administration training (Sanchis-Segura and Spanagel, 2006; Liu et al., 2008; Mantsch et al., 2016).

Although it is more common to use mammals to study addiction, insect behavior is no less organized and driven by reward. Many studies over the years have established the fruit fly *Drosophila melanogaster* as a non-conventional but very relevant model to explore molecular mechanisms underlying drug response. These have mostly focused on ethanol, modeling early stages of ethanol exposure, including its immediate locomotor effects (reviewed extensively in Rodan and Rothenfluh, 2010; Kaun et al., 2012; Devineni and Heberlein, 2013; Ghezzi et al., 2013a), its hedonic value, as reflected by voluntary consumption (Devineni and Heberlein, 2009), and the formation of long-lasting memories for the rewarding experience (Kaun et al., 2011; **Figure 1**). This review will present recent progress in which fruit flies were used to uncover genetic and environmental elements that influence the likelihood of progressing from initial exposure to repeated drug use. It will focus on drug-oriented studies and those that are not drug oriented but share mutual mechanisms and principles with addiction, such as learning and memory, and neuronal mechanisms that encode and process natural rewards. Together, the cellular pathways, neuronal circuits and newly discovered principles that govern reward processing can serve as a conceptual framework for understanding the mechanisms that underlie the risk to develop addiction.

METHODS OF STUDYING ETHANOL RELATED BEHAVIORS IN FRUIT FLIES

Flies encounter ethanol in their natural habitat, and as such, acquired many adaptations that enable them to survive and thrive in ethanol-rich environments (Gibson et al., 1981). Flies exhibit natural preference for ethanol: the smell of ethanol was shown to be an attractive cue using olfactory trap (Reed, 1938; Dudley, 2002; Devineni and Heberlein, 2009), and females show preference to lay eggs on ethanol containing substrates (Siegal and Hartl, 1999; Azanchi et al., 2013; Kacsoh et al., 2013). Flies develop preference to consume ethanol-containing food in a two-choice consumption paradigm. The kinetics of their preference and its extent depend on genetic background (Merçot et al., 1994; Devineni and Heberlein, 2009), prior exposure to ethanol (Peru y Colón de Portugal et al., 2014), sampling time (Devineni and Heberlein, 2009), and prior sexual experience (Shohat-Ophir et al., 2012). Importantly, flies display similar behavioral responses to acute exposure to ethanol as mammals: increased motor response when exposed to a low dose of ethanol, and sedation when reaching higher doses (Singh and Heberlein, 2000). Repeated exposure to ethanol results in functional tolerance and increases the time and dose needed to induce sedation. This reflects neuronal plasticity that corresponds to tolerance (**Figure 1**), but can also be caused by changes in ethanol metabolism (Scholz et al., 2000).

Over the years there have been several experimental systems to study the locomotor response to ethanol intoxication, the first of which was the inebriometer system (Cohan and Graf, 1985; Cohan and Hoffmann, 1986; Weber, 1988), in which flies lose their postural control when exposed to ethanol vapor. The system was later adapted for high throughput functional genetic screens by the Heberelin lab (Moore et al., 1998), and was subsequently replaced by video tracking systems that measure changes in fly velocity during acute intoxication, and assays that measure loss of righting response when reaching sedating levels (Wolf et al., 2002; Maples and Rothenfluh, 2011). Many genes and cellular pathways in neurons and glia cells have been shown to modulate the sensitivity of flies to both the positive and negative motor responses upon exposure to ethanol vapor, and the development of tolerance (Moore et al., 1998; Scholz et al., 2000, 2005; Berger et al., 2004; Ghezzi et al., 2004, 2013b; Corl et al., 2005; Cowmeadow et al., 2006; Kong et al., 2010; King et al., 2011, 2014; Kapfhamer et al., 2012; Krishnan et al., 2012, 2016; Devineni et al., 2013; Li et al., 2013; McClure and Heberlein, 2013; Pohl et al., 2013; Troutwine et al., 2016). Some of the identified fly genes, pathways and principles paved the way for parallel studies

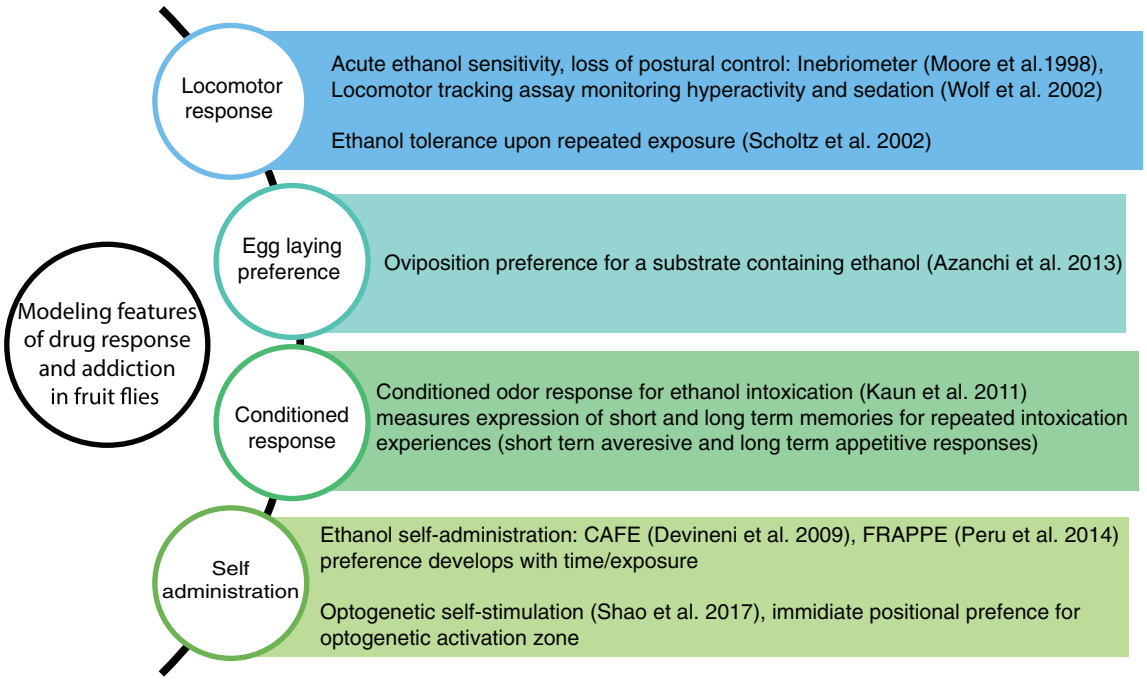

FIGURE 1 | Schematic illustration of the behavioral paradigms that are used to model different features of drug addiction in *Drosophila*.

in mammals (Corl et al., 2009; Lasek et al., 2011a,b,c; Maiya et al., 2012, 2015; Kapfhamer et al., 2013).

A breakthrough in modeling aspects of drug reward in flies was the introduction of two paradigms: a conditioned response to ethanol vapor (Kaun et al., 2011), and a two-choice assay to measure voluntary ethanol consumption (Ja et al., 2007; Devineni and Heberlein, 2009). In the first paradigm, flies learn to associate cues with ethanol intoxication and develop long-lasting attraction for an ethanol-paired cue (Kaun et al., 2011). A demonstration for the relevance of this model as a system to study aspects of drug reward was the finding that flies are willing to tolerate electric shock in order to approach an odor cue predicting ethanol reward (Kaun et al., 2011). The two-choice ethanol consumption paradigm measures motivation to obtain drug rewards, where flies can choose to feed from ethanol or non-ethanol containing food in a capillary feeder system (CAFE) (Devineni and Heberlein, 2009; Pohl et al., 2012; Shohat-Ophir et al., 2012; Xu et al., 2012; Ojelade et al., 2015; Zer et al., 2016). Another two-choice ethanol consumption paradigm is the FRAPPE (Fluorometric Reading Assay of Preference Primed by Ethanol), a novel assay based on the CAFE system, which allows precise and high throughput measurement of consumption in individual flies (Peru y Colón de Portugal et al., 2014; **Figure 1**). Lastly, a recent study by Shao, et al. established a new reward self-administration paradigm that is based on optogenetic stimulation of neurons that encode positive valence (Shao et al., 2017). In this assay, flies harboring the red shifted channel rhodopsin CsChrimson (Inagaki et al., 2014a) in NPF neurons prefer to be in a zone that triggers optogenetic stimulation of their NPF expressing neurons (Shao et al., 2017; **Figure 1**). Although this assay does not measure drug related responses, it facilitates the identification of neurons that induce immediate pleasure, and conceptually resembles the rodent intracranial self-stimulation (ICSS) paradigm (Olds and Milner, 1954).

DRUG-UNRELATED STUDIES AND THEIR CONTRIBUTION TO UNDERSTANDING THE MOLECULAR BASIS OF ADDICTION: THE CASE OF LEARNING AND MEMORY

Addiction is frequently referred to as pathological usurpation of learning and memory mechanisms that are normally used to predict the occurrence of natural rewards (Nestler, 2002; Hyman, 2005; Hyman et al., 2006; Kalivas and O'Brien, 2008; Duan et al., 2016; Patrono et al., 2016). This part of the review will explore the contribution of the field of learning and memory in flies to understanding drug related behaviors and possibly addiction, by covering two major directions in the field: traditional forward genetic screens, and more recent circuitry-oriented studies.

Genes and Cellular Pathways That Constitute the Basic Machinery Encoding Learning and Reward

Seymour Benzer and colleagues were the first to demonstrate that one can use a genetic scalpel to identify genes and pathways that are necessary for the formation of memory (Quinn et al., 1974). Learning and memory can be studied in flies using both reward or avoidance of punishment based assays, by pairing a neutral cue to the presence of sucrose (positive reinforcement)

or electric shock (punishment) (Quinn et al., 1974; Tempel et al., 1983). The memory for the experience is measured by testing the avoidance or attraction of the flies to the odor that was previously paired (conditioned stimulus) with the experience (unconditioned stimulus). Many studies over the years identified mutants in different stages of the process, some of which showed virtually no learning during shock training, like the mutant *turnip* (Quinn et al., 1979), *dunce* (Dudai et al., 1976), and *rutabaga* (Aceves-Piña and Quinn, 1979), while others learned normally but forgot the task of shock and sucrose training faster than wild type flies, like *amnesiac* (Quinn et al., 1979; Tempel et al., 1983). The elucidation of the molecular functions of the affected genes shed light on the biochemical mechanisms mediating learning and memory, and indicated a pivotal function for the cAMP pathway; *rutabaga (rut)* encodes for the Ca^{2+}/CaM-sensitive adenylyl cyclase (Livingstone et al., 1982), and *Dunce* has cAMP phosphodiesterase activity (Byers et al., 1981). In addition to the cAMP pathways, other studies identified additional players that regulate memory related plasticity events, such as Ca^{2+}/CaM Kinase II (Joiner and Griffith, 1997) and Orb2, a CPEB protein that functions in synaptic plasticity-required protein synthesis (Keleman et al., 2007).

Studying Neuronal Circuits That Encode Associative Learning; The Mushroom Bodies as an Association Center

Recent technological advances in neurogenetics led to the emergence of powerful genetic tools such as optogenetics, *in vivo* Ca^{2+} imaging, and the ability to manipulate single neurons in behaving animals. This resulted in an explosion of studies on mechanisms that encode associative learning and the processing of natural rewards (reviewed by Owald et al., 2015). A central player in integrating the conditioned and unconditioned stimuli of a given experience into an associative memory is the Mushroom Body (MB), a brain region extensively studied with classical conditioning assays and genetic manipulations (Heisenberg et al., 1985; Connolly et al., 1996; Wolf et al., 1998; Waddell et al., 2000; McGuire et al., 2001; Liu et al., 2007; Thum et al., 2007; Aso et al., 2009) (reviewed by Kaun and Rothenfluh, 2017; Cognigni et al., 2018). Below we introduce some basic principles that govern the function of the MB, as an introduction to the neuronal machinery that processes positive reinforcement, which is required for reward learning. As such, this is not intended to be a comprehensive review of the MB [for detailed up to date reviews on the wiring and function of the MB see (Scaplen and Kaun, 2016; Felsenberg et al., 2017; Kaun and Rothenfluh, 2017; Cognigni et al., 2018)].

The MB is a brain area where visual (Vogt et al., 2014), gustatory (Kirkhart and Scott, 2015), thermal (Yagi et al., 2016), and olfactory (Stocker et al., 1990; Wong et al., 2002; Tanaka et al., 2004; Liu et al., 2008, 2012; Caron et al., 2013) information (conditioned stimuli) reaches a set of intrinsic neurons called Kenyon Cells (KC). KC axons run in parallel through MB lobes and synapse with different subsets of Mushroom Body Output Neurons (MBON) (Takemura et al., 2017), forming functionally segregated compartments. MBONs integrate sensory information with the valence of the experience (Hige et al., 2015), generating an association between the conditioned and unconditioned stimuli, and leading to associative memory formation. For this to happen, specific sub-populations of Dopaminergic Neurons (DAN) that innervate each compartment deliver information about the valence of the experience (unconditioned stimulus) (Thum et al., 2007; Aso et al., 2010, 2014a,b; Liu et al., 2012; Caron et al., 2013; Clowney et al., 2015) (reviewed by Das et al., 2016).

Activation of different populations of DANs is sufficient for aversive or appetitive memory formation when paired with a CS (reviewed by Waddell, 2013). Further functional dissections revealed that different subpopulations of DANs and MBONs encode information regarding the sweet vs. caloric value of the ingested food (Das et al., 2014), water reward (Shyu et al., 2017), aversive taste (Masek et al., 2015), electric shock memory (Unoki et al., 2005; Aso et al., 2010), and even specific short and long-term memory formation (Aso et al., 2014b). Memory formation, consolidation, retrieval, reconsolidation and/or extinction have been shown to occur via neuronal activities in specific parts of the MBONs and specific subsets of reinforcing DANs (Berry et al., 2012, 2015; Shuai et al., 2015; Aso and Rubin, 2016; Ichinose and Tanimoto, 2016) reviewed by Cognigni et al. (2018). Intriguingly, re-evaluation of previously learned appetitive memory was shown to be conveyed by the activity of a subset of MBONs that is anatomically connected to both aversive and appetitive DANs, and that extinction or re-consolidation of appetitive memories requires activity of both during re-evaluation (Felsenberg et al., 2017). Finally, a recent comprehensive connectome map of the entire MB alpha lobe that was generated by electron microscopy imaging, demonstrated that the interconnectivity between KCs, DANs and MBONs is even more intricate than previously thought, paving the path for further delineation of the underlying neurobiological principles of this brain region (Takemura et al., 2017).

Shared Molecular Machinery of Memory, Reward, and Drug-Related Behaviors in Model Organisms

Drug rewards converge on molecular and neural pathways that encode memory for natural rewards, and induce similar neuroplastic changes as natural rewards (reviewed by Hyman et al., 2006; Kauer and Malenka, 2007; Kalivas and O'Brien, 2008; Koob and Volkow, 2010). The cAMP, CREB dependent and ΔFosB pathways play a prominent role in mediating these long-term adaptive changes in neuronal function (Nestler, 2002; Mameli and Lüscher, 2011). An example of the crosstalk between natural reward, drug reward and neuroplasticity is demonstrated in studies where periods of abstinence from sexual experience increase the sensitization of rats to amphetamine reward (Bradley and Meisel, 2001; Pitchers et al., 2010). This sex experience-induced plasticity, which in turn causes enhanced drug reward, was shown to be mediated by dopamine 1 receptor (D1R)-dependent induction of ΔFosB in the nucleus accumbens (NAc) (Pitchers et al., 2013). A similar phenomenon was also documented in *Drosophila*, in which sexual deprivation increased

the motivation to consume ethanol as a drug reward, by regulating the brain levels of neuropeptide F (NPF) (Shohat-Ophir et al., 2012).

As stated previously, the dopaminergic system plays a central role in processing natural rewards, and represents one way by which drugs of abuse induce changes in memory-related mechanisms (Di Chiara, 1999). In mammals, dopaminergic neurons show characteristic burst-firing activity during mating and food consumption (Dackis and O'Brien, 2001). Cocaine increases dopaminergic neurotransmission by blocking dopamine transport, preventing its removal from the synaptic cleft (Dackis and O'Brien, 2001). Reducing dopamine levels in fruit flies, using a competitive agonist to tyrosine hydroxylase (which converts tyrosine to L-Dopa), diminishes their sensitivity to cocaine and nicotine (Bainton et al., 2000). Dopamine release is also required for the expression of ethanol reward in fruit flies, as temporal block of neurotransmission in dopaminergic neurons prevented conditioned preference for ethanol-associated cues (Kaun et al., 2011). In addition, artificial activation of a certain dopamine neurons such as the protocerebral anterior medial (PAM neurons) is rewarding *per se*, as it induces robust appetitive odor memory in the absence of natural or drug reward (Liu et al., 2012).

Shared Mechanisms for Ethanol-Related Behaviors and Learning and Memory in Flies

Examining the connection between neuroplasticity and drug response, several studies tested whether established learning and memory mutants also depict aberrant behavioral phenotypes in acute ethanol response. The mutant *cheapdate,* which is an allele of the memory mutant *amnesiac,* caused increased sensitivity to the sedating effects of ethanol (Moore et al., 1998; Wolf and Heberlein, 2003). Another mutant, *rut,* exhibited increased ethanol hyperactivity and sensitivity (Wolf et al., 2002; Heberlein et al., 2004). In addition to acute responses to ethanol, learning and memory mutants revealed altered rapid and chronic tolerance responses to ethanol (for detailed list of genes see (Berger et al., 2008). For instance, the long-term memory mutant *john* displayed enhanced chronic tolerance in response to prolonged exposure (20–28 h) to low concentration of ethanol vapor (Berger et al., 2008).

Krasavietz (or *exba*), which encodes a translation initiation factor, is an example of a gene involved in learning and memory whose mutation affects both acute ethanol response and the motivation to consume ethanol. *Krasavietz* mutant flies exhibit decreased sensitivity to ethanol sedation (Berger et al., 2008), defects in the development of ethanol tolerance (Berger et al., 2008), and reduced voluntary consumption of ethanol (Devineni and Heberlein, 2009). Moreover, the expression of the memory gene *rut* in mushroom body (MB) neurons is necessary for robust ethanol consumption (Xu et al., 2012).

Recent studies identified new players that connect neuroplasticity and the formation of memories to the rewarding effects of ethanol intoxication. *scabrous,* which encodes a fibrinogen-related peptide that regulates Notch signaling, was shown to be necessary for the rewarding effects of ethanol intoxication (Kaun et al., 2011). Another study discovered that the sirtuin gene *Sir2 (Sirt1),* which deacetylates histones and transcription factors, is regulated by exposure to ethanol vapor, and is required for normal ethanol sensitivity, tolerance, and for ethanol preference and reward (Engel et al., 2016).

Lastly, although this review focuses on ethanol related behaviors, it is important to mention a study that tested the role of memory mutants in nicotine-induced motor sensitivity (Hou et al., 2004). Using a startle-induced climbing assay, measuring the effect of nicotine vapor on climbing ability, Hou et al. demonstrated that *dunce* mutant flies, which harbor higher basal levels of cAMP, exhibited increased sensitivity to the depressing effects of nicotine. In contrast, DCO^{H2} ($Pka\text{-}C1^{H2}$) and DCO^{B3} ($Pka\text{-}C1^{B3}$) mutants that are defective in PKA showed low sensitivity to nicotine (Hou et al., 2004).

MOTIVATIONAL STATES AS AN ORGINIZING PRINCIPLE THAT SHAPES REWARD PROCESSING

Animals continuously integrate their internal physiological state with environmental signals, and subsequently choose one action over another to increase their chances of survival and reproduction. As such, the state of the organism defines which stimuli are positively reinforced, negatively reinforced or considered negligible. A classic example of this is that fruit flies have to be hungry to express appetitive memory for sugar (Krashes and Waddell, 2008; Krashes et al., 2009; Gruber et al., 2013), highlighting the ability of internal signals such as hunger to modulate learned responses of cues associated with food.

An example of the interplay between states and reward processing can also be seen in aversive conditioning in fruit flies. Pairing a neutral odor with electric shock forms an association that predicts the arrival of pain. Conversely, presenting the odor following electric shock promotes appetitive behavior, and predicts the relief of pain, implying that the end of an aversive state can also be rewarding (Tanimoto et al., 2004). This indicates that even in flies, reward is not an absolute experience, but is relative to the state in which it is perceived. Repeated stressful experiences, such as repeated exposure to heat or electric shocks, where the fly cannot evade punishment by walking away, can induce a depression-like state, leading to decline in walking activity, similar to learned helplessness paradigms in rodents (Yang et al., 2013). Uncontrollable repeated mechanical stress in flies can induce long-lasting changes in motivational states, exhibited by reduced motivation to seek rewards and reduced 5HT (serotonin) levels (Ries et al., 2017). This depression-like state can be relieved by lithium treatment or artificial activation of serotonergic neurons that project to the MB (Ries et al., 2017).

Another aspect of the interplay between motivational states and reward is the concept that different motivational states are associated with particular drives (reward seeking behavior) and specific sensory sensitivity. For instance, food deprivation and satiety affect the extent of foraging behavior and food consumption, and modulate sensory perception of food related

sensory stimuli (Lee and Park, 2004; Yu et al., 2004; Wu et al., 2005; Root et al., 2011; Inagaki et al., 2012, 2014b; Marella et al., 2012; Beshel and Zhong, 2013; Wang et al., 2013; Ko et al., 2015; Jourjine et al., 2016). This is achieved by coordinated regulation of several different neuropeptide and hormonal systems that integrate nutrient signals and metabolic inputs into regulation of homeostatic drives and modulation of sensory systems (Lee and Park, 2004; Yu et al., 2004; Wu et al., 2005; Inagaki et al., 2012, 2014b; Marella et al., 2012; Gruber et al., 2013; Wang et al., 2013; Jourjine et al., 2016) (reviewed by Landayan and Wolf, 2016). This presumably occurs via the activation of specific DAN innervating the MB. For example, it was recently shown that insulin triggers the opposing functions of two neuropeptide systems: short neuropeptide F (sNPF) and tachykinin, and this in turn regulates the sensitivity toward appetitive and aversive odors (Ko et al., 2015). Serotonergic neurons were also shown to modulate motivational states that regulate feeding behavior and sugar associated reward (Burke et al., 2012; Sitaraman et al., 2012). Recently, a set of 15 serotonergic neurons was identified, that when activated, induces a fed fly to eat as if it was food deprived, and promotes the formation of appetitive memory (Albin et al., 2015). These findings imply that specific sub-populations of neurons act to shift motivational states, and thus control the way by which sensory stimuli that is associated with the experience is processed and affects behavior.

NPF System as a Molecular Signature for Reward States

The NPF/NPF-receptor system is emerging as a central player in modulating and encoding motivational states associated with sugar reward, sexual, and drug reward, and the homeostatic regulation of motivational responses. The activity of NPF-expressing neurons mimics a state of food deprivation, and promotes rewarding memories in satiated flies, via a subset of downstream NPF receptor expressing dopaminergic neurons that innervate the MB (Krashes et al., 2009). Additional studies revealed NPF's role in encoding other motivational aspects of feeding, such as promoting feeding (Wu et al., 2005), encoding the valence/attractiveness of food related odors (Beshel and Zhong, 2013; Beshel et al., 2017), and enhancing sugar sensitivity in sugar-sensing sensory neurons (Inagaki et al., 2014b). In addition, NPF serves as a homeostatic integration point of two interconnected systems: sleep and feeding. NPF regulates starvation, which induces sleep suppression, suggesting that the NPF system acts to encode a hunger signal that promotes an arousal state associated with high motivation to seek food (Keene et al., 2010; He et al., 2013; Chung et al., 2017).

Another example that demonstrates the interplay between motivational states and ways by which reward stimuli are perceived, is the role of NPF in integrating sexual deprivation and drug related rewards. Male flies perceive both mating interactions and ethanol intoxication as rewarding (Kaun et al., 2011; Shohat-Ophir et al., 2012). Mated male flies exhibited reduced motivation to consume ethanol containing food and have had high levels of NPF transcript, while sexually deprived male flies exhibited higher motivation to consume ethanol containing food and lower NPF transcript levels. Furthermore, activation of NPF neurons is rewarding in itself, reduces ethanol consumption, and prevents the formation of appetitive memory toward ethanol. This implies that experiences that modulate motivational states, can affect the reinforcing value of other rewarding stimuli.

The causal link between environmental stimuli, NPF levels and modulation of motivational behaviors has been documented in several studies. Reduction in NPF transcription and the activity of NPF-positive neurons was observed in response to negative environmental inputs, such as the presence of parasitic wasps and sexual deprivation, while NPF induction occurred in response to mating and ethanol intoxication (Shohat-Ophir et al., 2012; Kacsoh et al., 2013; Gao et al., 2015). Altogether, this suggests that NPF neuronal systems are central to the interplay between states and reward processing. Still, further studies are required to uncover the mechanism that connect NPF neuronal activity to activity of all specific DANs that project to the MB, and the neuronal and cellular mechanisms that allow this system to represent and affect a general reward state in the brain.

The different roles of NPF/R system in regulating motivational and homeostatic features of behavior are conserved between flies and mammals. A large number of studies demonstrate the central role of NPY (the mammalian homolog of NPF) in regulating feeding and the motivation to feed (Tatemoto et al., 1982; Clark et al., 1984; Flood and Morley, 1991; Kalra et al., 1997; Bannon et al., 2000; Day et al., 2005; Keen-Rhinehart and Bartness, 2007). A recent study uncovered a functional link between firing activities of NPY/AgRP neurons and energy homeostasis, wherein starvation induces an increase in NPY/AgRP firing rate, which in turn promotes re-feeding (He et al., 2016). The NPY system also functions in regulating sleep and wake homeostasis (Szentirmai and Krueger, 2006; Wiater et al., 2011; He et al., 2013). A study performed in zebrafish (Danio rerio) identified NPY signaling and NPY expressing neurons as regulators of zebrafish sleep, promoting sleep by inhibiting noradrenergic signaling, thus linking NPY signaling to an established arousal promoting system (Singh et al., 2017). In addition to its role in regulating natural physiological response, NPY has long been implicated in regulating drug addiction (for review on its role in ethanol addiction see (Thorsell and Mathé, 2017). NPY administration relieves the negative affective states of drug withdrawal and depression (Stogner and Holmes, 2000; Redrobe et al., 2002). Recently, a neuronal mechanism for the interplay between stress and reward systems on ethanol binge drinking was dissected in mice and monkeys, providing the first evidence for NPY and CRF functional interaction within neurons of the BNST (a limbic brain structure that is enriched with NPY and CRF neurons) (Pleil et al., 2015). Activation of the NPY Y1 receptor in the BNST led to enhanced inhibitory synaptic transmission in CRF neurons, which reduced binge alcohol drinking (Pleil et al., 2015). Their findings propose CRF neuronal function as a target for future therapies aimed to prevent and treat alcohol abuse.

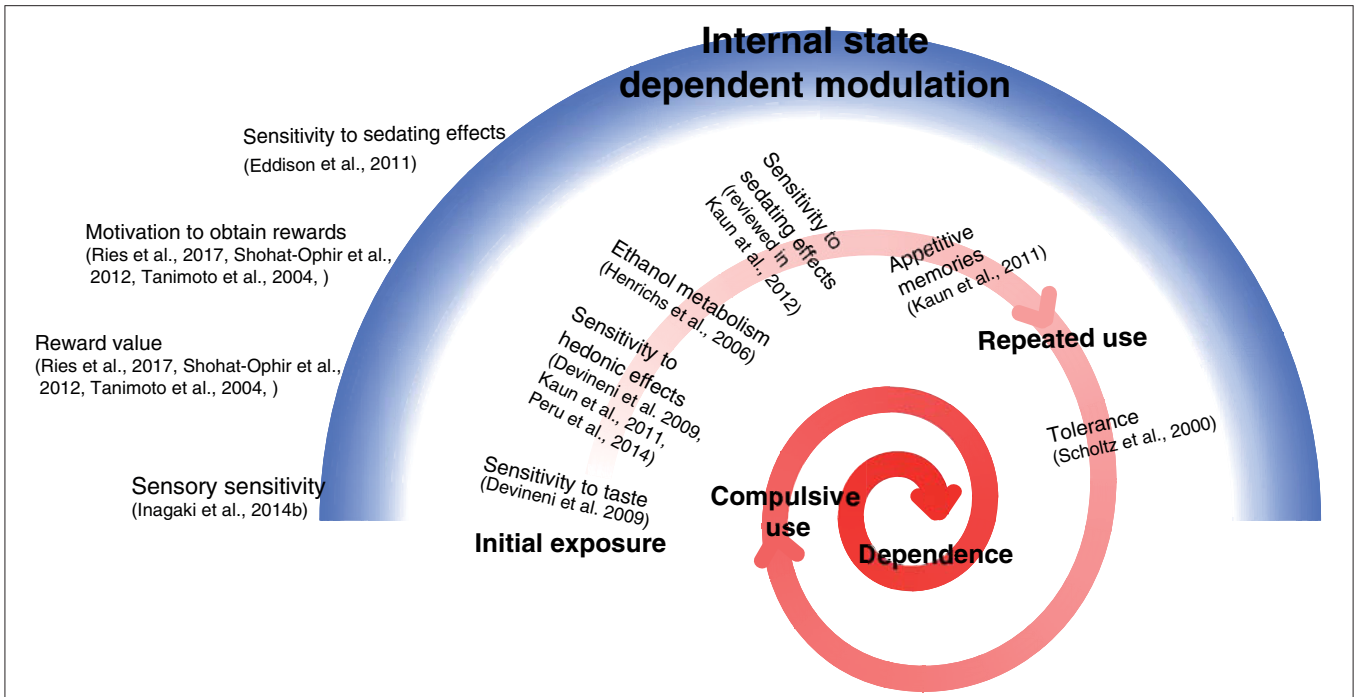

FIGURE 2 | Schematic model illustrating the genetic and motivational elements that influence the likelihood of progressing from initial exposure to repeated use. Red spiral depicts the multistage progression from initial drug exposure to drug dependence and addiction and the behavioral features that are shaped by molecular and neuronal mechanisms. Blue arc depicts the way by which internal state can modulate different features in reward processing via molecular and neuronal mechanisms affecting sensory sensitivity to reward related cues, the motivation to seek and obtain rewards, and the reinforcing value of the consumed reward.

CONCLUDING REMARKS

The risk of developing addiction is determined by molecular and neuronal mechanisms that influence the likelihood of progressing from initial drug exposure to repeated use. These mechanisms can shape the experience of initial consumption, the amount consumed, and the relative value of its reinforcing properties (**Figure 2**). For instance, genetic variations in bitter taste receptor and ethanol metabolism pathway influence the risk to develop addiction (Hinrichs et al., 2006; Yu and McClellan, 2016). Enhanced sensitivity to bitter taste is associated with reduced risk, and variations in ethanol metabolism lead to enhanced negative side effects and reduce the likelihood of repeated use, and therefore the risk to develop addiction (**Figure 2**). Studies in *Drosophila* demonstrated the functional link between ethanol metabolism and sensitivity to acute ethanol exposure (Ogueta et al., 2010). Other genetic components that control sensitivity to the hedonic and sedating effects of ethanol play a role in determining the extent of initial consumption and likelihood of repeated use. Upon repeated use, genetic factors that determine the extent of tolerance to ethanol-mediated responses can also shape the amount that is needed to reach the euphoric state (**Figure 2**).

An analogy for reward states can be proposed in which high reward state is illustrated by a full "reservoir" and low state by an empty "reservoir." One can speculate that vulnerability to addiction is related to the size of "reservoir" to be filled (Bar, 2012). According to this model, bigger reservoir will require greater amounts of rewarding experiences in order to be filled. In addition, individuals can possess different sensitivity to fluctuations in the levels of reward within the reservoir, where sensitive individuals have increased motivation to fill up the reservoir with any type of reward, while others will be less affected by fluctuations, corresponding to reduced reward-seeking behavior.

Lastly, prior experience/motivational states can also enter into this equation, modulating different aspects of drug response. For instance, social isolation affects sensitivity to ethanol sedation (Eddison et al., 2011), pain can modulate the perception of reward-related cues (Tanimoto et al., 2004), while sexual deprivation and stress modulate the motivation to seek and obtain rewards (Shohat-Ophir et al., 2012; Ries et al., 2017; **Figure 2**). It is postulated that these different conditions shape the repertoire and function of proteins within neurons that mediate reward processing. As a consequence, the reward baseline is shifted, which presumably modulates the motivation to obtain rewards, the value of the consumed reward, and the likelihood to continue consuming drug rewards (**Figure 2**). Still, the means by which different conditions and prior experiences are encoded in the reward system and lead to changes in motivational states are largely unknown.

Recent advances in the ability to purify RNA from genetically tagged neuronal populations (Henry et al., 2012;

Abruzzi et al., 2015), coupled with improvement in RNAseq technologies, make it now possible to bridge the gap between the specific transcriptomic repertoire and specific experiences/states. In this respect, it is now possible to profile the repertoire of coding mRNA, non-coding RNAs, and RNA modifications such as RNA editing, as well as the metabolome and proteome of specific neurons in every state. This can facilitate studies exploring the contributions of co-transcriptional mechanisms such as RNA editing, post-transcriptional mechanisms such as RNA methylation, and post-translational mechanisms in shaping the vulnerability to drug addiction. Further in-depth mechanistic studies will be required to connect specific regulation events to their functional relevance in shaping the transition from initial drug use to addiction.

AUTHOR CONTRIBUTIONS

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

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frontiers in Physiology

REVIEW
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Roles of Octopamine and Dopamine Neurons for Mediating Appetitive and Aversive Signals in Pavlovian Conditioning in Crickets

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Revealing neural systems that mediate appetite and aversive signals in associative learning is critical for understanding the brain mechanisms controlling adaptive behavior in animals. In mammals, it has been shown that some classes of dopamine neurons in the midbrain mediate prediction error signals that govern the learning process, whereas other classes of dopamine neurons control execution of learned actions. In this review, based on the results of our studies on Pavlovian conditioning in the cricket *Gryllus bimaculatus* and by referring to the findings in honey bees and fruit-flies, we argue that comparable aminergic systems exist in the insect brain. We found that administrations of octopamine (the invertebrate counterpart of noradrenaline) and dopamine receptor antagonists impair conditioning to associate an olfactory or visual conditioned stimulus (CS) with water or sodium chloride solution (appetitive or aversive unconditioned stimulus, US), respectively, suggesting that specific octopamine and dopamine neurons mediate appetitive and aversive signals, respectively, in conditioning in crickets. These findings differ from findings in fruit-flies. In fruit-flies, appetitive and aversive signals are mediated by different dopamine neuron subsets, suggesting diversity in neurotransmitters mediating appetitive signals in insects. We also found evidences of "blocking" and "auto-blocking" phenomena, which suggested that the prediction error, the discrepancy between actual US and predicted US, governs the conditioning in crickets and that octopamine neurons mediate prediction error signals for appetitive US. Our studies also showed that activations of octopamine and dopamine neurons are needed for the execution of an appetitive conditioned response (CR) and an aversive CR, respectively, and we, thus, proposed that these neurons mediate US prediction signals that drive appetitive and aversive CRs. Our findings suggest that the basic principles of functioning of aminergic systems in associative learning, i.e., to transmit prediction error signals for conditioning and to convey US prediction signals for execution of CR, are conserved among insects and mammals, on account of the fact that the organization of the insect brain is much simpler than that of the mammalian brain. Further investigation of aminergic systems that govern associative learning in insects should lead to a better understanding of commonalities and diversities of computational rules underlying associative learning in animals.

Keywords: octopamine, dopamine, appetitive learning, aversive learning, insects, classical conditioning

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INTRODUCTION

Elucidation of neural systems that mediate appetite and aversive signals in associative learning is an important subject in neuroscience. By associative learning, animals can acquire knowledge in their environments, which allow them, for example, to find suitable food, avoid toxic compounds, and escape from predators. Efforts have been made to elucidate neural systems mediating appetitive and aversive signals in associative learning in many animals, including mammals (Schultz, 2013, 2015), insects (Hammer and Menzel, 1998; Schwaerzel et al., 2003; Mizunami and Matsumoto, 2010; Waddell, 2013), and mollusks (Hawkins and Byrne, 2015). Prediction error, i.e., the discrepancy, or error, between the actual unconditioned stimulus (US) and the predicted US, represents a key determinant for whether a US-paired stimulus is learned (Rescorla and Wagner, 1972; Schultz, 2013, 2015). There is evidence that some classes of midbrain dopamine neurons mediate prediction error signals for appetitive events (Schultz, 2013, 2015), and some researchers have suggested that other classes of midbrain dopamine neurons mediate prediction error signals for aversive events (Matsumoto and Hikosaka, 2009; Matsumoto H. et al., 2016). Other classes of midbrain dopamine neurons control the execution of both appetitively and aversively learned actions (Berridge et al., 2009; Bromberg-Martin et al., 2010).

This review deals with results of our studies on the roles of biogenic amines in appetitive and aversive learning in crickets. Crickets are useful insects for the study of neurotransmitter mechanisms of learning and memory. First, they have excellent capabilities of olfactory and visual learning. For example, they exhibit lifetime olfactory memory (Matsumoto and Mizunami, 2002a), simultaneous memorization of seven pairs of odors (Matsumoto and Mizunami, 2006), context-dependent discriminatory learning (Matsumoto and Mizunami, 2004), and higher-order associative learning such as second-order conditioning (Mizunami et al., 2009) and sensory preconditioning (Matsumoto et al., 2013a). They also exhibit excellent capability to learn color and pattern of visual targets (Unoki et al., 2006; Nakatani et al., 2009; Matsumoto et al., 2013b). Second, applications of pharmacological studies (Unoki et al., 2005, 2006; Matsumoto et al., 2006, 2016; Matsumoto Y. et al., 2009; Mizunami et al., 2014; Sugimachi et al., 2016), gene knockdown by RNA interference (RNAi; Takahashi et al., 2009; Awata et al., 2016), and genome editing by the CRISPR/Cas9 system (Awata et al., 2015) are feasible, thereby greatly facilitating the analysis of molecular basis of learning and memory. Indeed, it can be stated that crickets are one of the best insect models for pharmacological analysis of learning and memory (Mizunami et al., 2013). Third, much information on the brain and behavior of crickets has been obtained as crickets have been used in diverse neuroethological studies (Stevenson and Schildberger, 2013; Hedwig, 2016). We first deal with the recent debate about whether appetite and aversive signals are conveyed by octopamine and dopamine neurons, respectively, as has been suggested in honey bees and crickets, or whether both appetitive and aversive signals are mediated by dopamine neurons, as has been suggested in fruit-flies. Next, we discuss the results of our studies suggesting (1) that activations of octopamine neurons and activation of dopamine neurons are needed for responding to an appetitive conditioned stimulus (CS) and an aversive CS, respectively, and (2) that conditioning is governed by US prediction error and that octopamine neurons mediate the prediction error signals for appetitive learning.

CONDITIONING PROCEDURES

We have established four different conditioning procedures for crickets (Matsumoto and Mizunami, 2000, 2002a,b; Matsumoto et al., 2015). Among them, we used a "classical conditioning and operant testing procedure" (Matsumoto and Mizunami, 2002b; Matsumoto et al., 2003), which is based on the transfer of memory formed during classical conditioning to an operant testing situation. Crickets were individually placed in a beaker and deprived of drinking water for 3 days to enhance motivation to uptake water. For conditioning of an odor (CS) with water US, a filter paper soaked with an odor was presented to the antennae of the cricket for 3 s, and then a drop of water was applied to the mouth. For conditioning of an odor with sodium chloride US, an odor was presented to the antennae and then a drop of 20% sodium chloride solution was applied to the mouth. Crickets were eager to drink water when it was applied to the mouth, whereas they immediately retracted from sodium chloride solution, indicating that the former serves as an appetitive stimulus and that the latter serves as an aversive stimulus. Odor preferences of individual crickets were tested before and after conditioning. In the test, crickets were individually placed in a test chamber and allowed to freely visit two odor sources, a conditioned odor and a control odor, for 4 min. The time that the cricket spent exploring each odor source with its mouth or palpi was recorded for evaluation of the relative odor preference of each cricket.

For conditioning of a visual pattern, presentation of water or sodium chloride solution to the mouth was paired with either a black-center and white-surround pattern or with its reverse pattern (Unoki et al., 2006). In the pattern preference test, the two patterns were simultaneously presented on the wall of the test chamber, and the time that the cricket spent touching each of the patterns was recorded for evaluating the relative preference between the two patterns. For color conditioning, crickets were presented with purple and green disks paired with water or sodium chloride US (Nakatani et al., 2009), and the two disks were presented simultaneously on the wall of the test chamber for the color preference test.

We also used conditioning of maxillary palpi extension response (MER) with odor CS and water or sodium chloride US, which allowed us to investigate the memory acquisition process (Matsumoto et al., 2015). Crickets often extend their maxillary palpi and then vigorously swing them when a drop of water is applied to their antenna or to the mouth, and we refer to this behavior as the MER. Crickets often exhibited MER to some odors such as vanilla and maple odors, whereas they rarely exhibited MER to other odors such as peppermint and apple odors. We showed that the MER to peppermint or apple odor is increased by pairing the odor with water US (Matsumoto et al., 2015). MER conditioning is analogous to the conditioning of

proboscis extension responses (PERs) with odor CS and sucrose US in honey bees (Menzel and Giurfa, 2006; Giurfa and Sandoz, 2012). Moreover, we also observed that the MER to vanilla or maple odor is decreased by pairing an odor with sodium chloride. Therefore, MER conditioning allows appetitive conditioning and aversive conditioning to be achieved in a similar experimental situation, as in the case of a classical conditioning and operant testing procedure.

ROLES OF OCTOPAMINE AND DOPAMINE IN APPETITIVE AND AVERSIVE LEARNING

Previous studies done in the honey bee *Apis mellifera* (Hammer and Menzel, 1998) and the fruit-fly *Drosophila melanogaster* (Schwaerzel et al., 2003) suggested that octopamine and dopamine neurons play critical roles in appetitive and aversive olfactory conditioning, respectively (for alternative view, see later section). We first investigated whether this was the case in crickets using a classical conditioning and operant testing procedure (Unoki et al., 2005). Crickets were injected with an octopamine receptor antagonist (epinastine or mianserin) into the hemolymph prior to the conditioning of an odor with water. In a post-training retention test, they did not exhibit an increase of preference for the odor conditioned with water. However, crickets showed normal scores of aversive conditioning with sodium chloride, and the scores being as high as those for control crickets that had been injected with cricket's saline solution. The latter observation indicates that octopamine receptor antagonists do not impair sensory function, motor function, or motivation necessary for learning. We also observed that crickets injected with a dopamine receptor antagonist (fluphenazine, chlorpromazine, spiperone, or flupentixol: Different dopamine receptor types are not discriminated by these drugs, see Mustard et al., 2005) exhibited no aversive learning with sodium chloride US, but appetitive learning with water US was unaffected. Sensory function, motor function, or motivation necessary for learning is not affected by dopamine receptor antagonists. Similar results were obtained in a recent study using olfactory conditioning of MER (Matsumoto et al., 2015). We, thus, suggest that octopamine codes for appetitive signals, and that dopamine neurons transmit aversive signals in two different forms of Pavlovian conditioning in crickets. Notably, crickets that were injected with octopamine or dopamine receptor antagonist exhibited a normal appetitive or aversive response, respectively, when water or sodium chloride solution was applied to the mouth. Hence, these neurotransmitters are not involved in the execution of a behavioral response to appetitive or aversive US.

We also investigated whether the blockade of octopaminergic and dopaminergic transmissions impairs appetitive and aversive conditioning, respectively, of a visual pattern (Unoki et al., 2006) and a color cue (Nakatani et al., 2009). For conditioning of a visual pattern, we observed that crickets injected with an octopamine receptor antagonist (epinastine or mianserin) exhibited no appetitive learning with water, but they exhibited normal aversive learning with sodium chloride solution. In contrast, crickets injected with a dopamine receptor antagonist (spiperone, chlorpromazine, or fluphenazine) exhibited no aversive learning, but appetitive learning was unaffected (Unoki et al., 2006). In color conditioning, crickets injected with an octopamine receptor antagonist (epinastine or mianserin) exhibited impaired appetitive color learning, but aversive color learning was unaffected. In contrast, crickets injected with a dopamine receptor antagonist (flupentixol, fluphenazine, or chlorpromazine) exhibited impaired aversive color learning, whereas appetitive color learning was unaffected (Nakatani et al., 2009). The results indicate that octopamine and dopamine neurons convey signals about an appetitive vs. an aversive US, regardless of the specific paradigm used, thereby suggesting the action of separate neurotransmitter systems to mediate appetitive and aversive signals, respectively, in associative learning in crickets.

ROLES OF OCTOPAMINE AND DOPAMINE IN APPETITE AND AVERSIVE LEARNING CONFIRMED BY RNAi AND TRANSGENIC CRICKETS

Recent studies on neurotransmitters mediating appetitive and aversive signals for Pavlovian conditioning in the fruit-fly, have yielded conclusions that differ from those obtained in crickets (Burke et al., 2012; Liu et al., 2012). In the fruit-fly, different sets of dopamine neurons mediate appetitive and aversive signals, such as sucrose and electric shock signals, respectively, to intrinsic neurons (Kenyon cells) of the mushroom body (MB), via the type 1 dopamine receptor Dop1, in the MB lobes (Kim et al., 2007; Burke et al., 2012; Liu et al., 2012; Perry and Barron, 2013; Waddell, 2013; Ichinose et al., 2015). Octopamine neurons in the subesophageal ganglion receive sweet taste signals from sugar receptor neurons and relay the signals to dopamine neurons in the protocerebrum that project to the MB lobes (Burke et al., 2012). Therefore, octopamine neurons have a peripheral role for relaying sweet taste signals (Huetteroth et al., 2015), whereas dopamine neurons transmit appetitive US signals to the MB to associate them with an olfactory CS (Burke et al., 2012). Considering that octopamine neurons play roles in mediating appetitive signals in flies, a critical difference between flies and crickets is that dopamine neurons mediate appetitive signals in flies but not in crickets. We considered three possible reasons for this difference, and we investigated them in crickets. The first possible reason is the use of different methods to inhibit dopaminergic signaling: while the use of transgenic techniques in flies allows a sophisticated way to silence dopamine or octopamine signaling, efficacies and specificities of antagonists used in the cricket may not be perfect. For example, a recent study in honey bees suggested that epinastine and mianserin antagonize not only OA1 octopamine receptors but also Dop2 dopamine receptors (Beggs et al., 2011), which raises the possibility that impairment of appetitive learning by epinastine and mianserin might be mediated via blockade of Dop2 receptors, instead of or in addition to OA1 receptors. The second possible reason is the use of different kinds of appetitive US for conditioning. We used water as US in our studies on crickets, whereas sucrose was

used in studies on flies except for two studies using water (Lin et al., 2014; Shyu et al., 2017). We, thus, considered the possibility that dopamine conveys sucrose US but not water US in crickets. The third possible reason is that neurotransmitters mediating appetitive signals are not the same in flies and crickets.

For clarifying the issues discussed above, we prepared transgenic crickets with *Dop1* gene knockout using the CRISPR/Cas9 system [clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system; Awata et al., 2015]. Dop1 is known to be highly enriched in the MB in fruit-flies (Kim et al., 2007) and honey bees (Mustard et al., 2005). *Dop1* knockout crickets exhibited no obvious abnormality in behavior and external morphology. Our conditioning experiments showed that *Dop1* knockout crickets exhibited no aversive learning with sodium chloride US but exhibited normal appetitive learning with water US or sucrose US (Awata et al., 2015). The latter finding indicates that the impairment of aversive learning was not due to the impairment of sensory or motor functions or motivation necessary for learning and for responding to the conditioned odor in the post-training test. The results suggest that Dop1 participates in aversive learning with sodium chloride but not in appetitive learning with water or sucrose in crickets. This differ from the findings in flies in which Dop1 is required for both appetitive learning with water or sugar US and aversive learning with electric shock (Kim et al., 2007; Burke et al., 2012; Liu et al., 2012).

It could be argued, however, that knockout of *Dop1* might have caused an abnormality in the development of neural circuits in the brain necessary for aversive learning, not that Dop1 has acute roles in learning in adults. For further clarification of this issue, we investigated the effects of silencing the expression of genes that code the OA1 octopamine receptor and the Dop1 and Dop2 dopamine receptors by RNAi in adult crickets (Awata et al., 2016). In those studies, we used olfactory conditioning of MER to investigate the effect of gene silencing on the acquisition process. Crickets were injected with dsRNA-targeting *OA1*, *Dop1*, or *Dop2* into the hemolymph and subjected 2 days later to conditioning trials to associate an odor with water or sodium chloride. Studies with quantitative real-time PCR (qPCR) confirmed a significant reduction in the mRNA level of each gene 2 days after dsRNA injection. *OA1*-silenced crickets exhibited no appetitive learning, but they exhibited normal scores in aversive learning. In contrast, *Dop1*-silenced crickets exhibited no aversive learning but exhibited normal scores in appetitive learning. *Dop2*-silenced crickets, as well as control crickets injected with *DsRed* dsRNA, showed normal scores in both appetitive learning and aversive learning. We, thus, conclude that octopamine mediates appetitive signals via OA1 receptors, whereas dopamine mediates aversive signals via Dop1 receptors in crickets. The perfect agreements of the results of pharmacological, transgenic, and RNAi studies provide decisive evidence that neurotransmitters and receptors that mediate appetitive signals indeed differ in crickets and flies. Our findings in crickets are in accordance with the findings in honey bees, where it has been suggested that appetitive learning is mediated by octopamine neurons via OA1 receptors (Hammer, 1993; Hammer and Menzel, 1998; Farooqui et al., 2003) and that aversive learning is mediated by dopamine neurons (Vergoz et al., 2007; the types of dopamine receptors involved are not known). Neurotransmitters involved in appetitive and aversive learning in other species of insects, however, remain elusive. More studies on various species of insects are needed to elucidate the diversity and evolutionary history of the neurotransmitters in mediating appetite and aversive signals in insects.

In associative learning in mammals, there is evidence that some classes of midbrain dopamine neurons convey signals about appetitive events (Schultz, 2013, 2015), whereas other classes may convey signals about aversive events (Matsumoto and Hikosaka, 2009; Matsumoto H. et al., 2016) (for more details, see Discussion in a later section). Hence, biogenic amines mediating appetitive signals are not the same between crickets and mammals, although the roles of dopamine in mediating aversive signals may be conserved between them. Dopamine has been reported to mediate appetitive signals in the mollusk *Aplysia* (Brembs et al., 2002). The origin of octopamine signaling for mediating appetite signals in crickets remains to be studied.

ROLES OF OCTOPAMINE AND DOPAMINE IN EXECUTION OF APPETITIVE AND AVERSIVE CONDITIONED RESPONSES

We next investigated whether administration of octopamine and dopamine receptor antagonists affects the performance of conditioned responses (CRs; or memory retrieval) after appetitive or aversive conditioning. Crickets were subjected to appetitive or aversive olfactory conditioning and then they received an injection of either octopamine or dopamine receptor antagonist before a retention test (Mizunami et al., 2009). Crickets injected with an octopamine receptor antagonist (epinastine) exhibited no CR to the odor associated with water, whereas they exhibited normal CR to the odor associated with sodium chloride. The latter indicates that epinastine had no effect on sensory and motor functions as well as the motivation necessary to perform a CR. This is in contrast to the finding that crickets injected with a dopamine receptor antagonist (flupentixol) exhibited no CR to the odor conditioned with aversive US but that they showed a normal CR to the odor conditioned with appetitive US. The latter finding indicates that flupentixol had no effect on sensory and motor functions as well as the motivation necessary to perform a CR. After recovery from the effect of the antagonists, crickets exhibited normal CRs. These observations are in accordance with the evidence from honey bees in which a disruption of antennal lobe (i.e., the primary olfactory center) octopaminergic transmission by either the octopamine receptor antagonist mianserin or RNAi of the *OA1* gene, disrupted the execution of an appetitive CR (or of appetitive memory retrieval; Farooqui et al., 2003). Moreover, visual pattern conditioning for appetitive or aversive CRs was impaired by injections of an octopamine or dopamine receptor antagonist, respectively (Mizunami et al., 2009). Therefore, we conclude that the execution of appetitive and aversive CRs for olfactory and visual cues requires intact octopaminergic or dopaminergic transmission, respectively.

Our findings were not in accordance with a neural model of classical conditioning proposed by Schwaerzel et al. (2003) (**Figure 1A**), which was designed to account for the roles of

intrinsic neurons (Kenyon cells) and extrinsic (output) neurons of the MB lobes in conditioning of an odor with sugar or electric shock US in the fruit-fly. The model assumed that (1) "CS" neurons (Kenyon cells) carry CS signals and make synaptic connections with dendrites of "CR" neurons (output neurons of the lobes), activation of which leads to a CR, (2) these synaptic connections are silent or very weak prior to conditioning, (3) octopamine and dopamine neurons projecting to the lobes ("OA/DA" neurons) convey signals for appetitive and aversive US, respectively, and make synaptic connections with axon terminals of "CS" neurons (in recent models of fruit-flies, "OA/DA" neurons have been replaced with different sets of DA neurons. See Burke et al., 2012; Liu et al., 2012.), and (4) coincident activation of "CS" neurons and "OA/DA" neurons in conditioning strengthens the efficacy of synaptic transmission from "CS" neurons to "CR" neurons.

We proposed a novel neural model of classical conditioning for the cricket (Mizunami et al., 2009) with minimal modifications of the model proposed for the fruit-fly by Schwaerzel et al. (2003). In our model (**Figure 1B**), it is assumed that (1) coincident activation of "CS" neurons and "OA/DA" neurons is required for activating "CR" neurons (AND gate) and producing a CR after conditioning and (2) simultaneous activation of "CS" and "OA/DA" neurons from CS/US pairing strengthens the synaptic connection between "CS" and "OA/DA" neurons. Following conventional learning theory, the model proposed for the fly is termed as S-R (or CS-CR) model, as it assumes the formation of stimulus-response (CS-CR) sensorimotor pathways by conditioning, whereas our model is termed as S-R and S-S (or CS-US) hybrid model, which assumes the formation of S-R connections and CS-US connections; the latter of which enables the CS to activate internal representation of US (for details, see Mizunami et al., 2009; Mizunami and Matsumoto, 2010). In our model, the extent by which the CS activates "OA/DA" neurons represents the extent by which the CS predicts the US, and the requirement of activated "OA/DA" neurons for execution of a CR indicates that US prediction guides the execution of the CR, as assumed in S-S learning theory (see Mizunami et al., 2009; Mizunami and Matsumoto, 2010). This is analogous to the findings that some classes of midbrain dopamine neurons govern the execution of learned actions in Pavlovian conditioning in mammals (Balleine et al., 2007; Bromberg-Martin et al., 2010). Following the terminology of human psychology, it has been stated that dopamine neurons confer a "wanting" attribute to the CS to drive actions to seek a US (Berridge et al., 2009). Our findings suggest that motivational mechanisms that govern the execution of a CR in insects are analogous to those in mammals.

ROLES OF OCTOPAMINE IN MEDIATING PREDICTION ERROR FOR APPETITE US

Finally, we address the question of what computational rules govern the learning process in crickets. In mammals, a discrepancy, or an error, between the actual US and the predicted US facilitates the classical conditioning for a stimulus paired with the US (Schultz, 2013, 2015). This theory emerged from the finding of "blocking" in rats (Kamin, 1969), in which pairing of stimulus X with US, and subsequent pairing of a compound of stimulus X and another stimulus Y with the US, blocked the learning of stimulus Y. Kamin (1969) argued that blocking requires surprise for learning, whereas learning does not occur when the animal fully predicts the occurrence of the US, and this argument was formulated into the prediction error theory of the Rescorla–Wagner model (Rescorla and Wagner, 1972). Activation of dopamine neurons in the mammalian ventral tegmental area is thought to mediate the prediction error signals for rewarding events in Pavlovian and instrumental conditioning (Waelti et al., 2001; Schultz, 2013, 2015). However, blocking can also be accounted for theories other than the prediction error

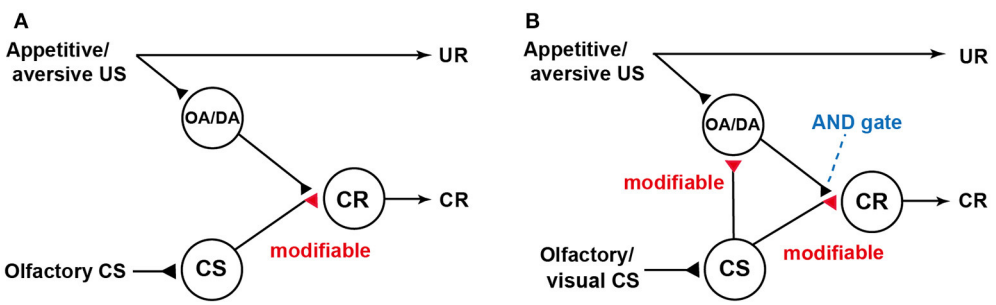

FIGURE 1 | Models of classical conditioning in flies and crickets. **(A)** A model proposed to account for the roles of intrinsic and extrinsic (output) neurons of the mushroom body (MB) in olfactory conditioning in fruit-flies (Schwaerzel et al., 2003). Octopamine neurons and dopamine neurons ("OA/DA" neurons) convey signals for appetitive and aversive US, respectively (In recent models in fruit-flies, "OA/DA" neurons have been replaced to different sets of DA neurons,). "CS" neurons, which convey signals for olfactory CS, make synaptic connections with "CR" neurons that induce a CR, which mimics an unconditioned response (UR). "OA/DA" neurons make synaptic connections with axon terminals of the "CS" neurons. The efficacy of the "CS-CR" synaptic connection is strengthened by coincident activation of "CS" neurons and "OA/DA" neurons by conditioning. For recent elaborations of the model in fruit-flies, see Hige (2017). **(B)** Our model of classical conditioning proposed for crickets (Mizunami et al., 2009). The model assumes that (1) efficacy of synaptic transmission from "CS" neurons to "OA/DA" neurons is strengthened by conditioning and that (2) coincident activation of "OA/DA" neurons and "CS" neurons is needed to activate "CR" neurons (AND gate) and to produce a CR. Synapses for which the efficacies are modifiable by CS-US pairings are shown as open triangles and marked "modifiable". Following the terminology of learning theories in mammals, the model in flies is characterized as an S-R model assuming formation of CS-CR connections, while our model is characterized as an S-R and S-S hybrid model assuming formation of CS-CR and CS-US connections. Modified from Mizunami et al. (2009).

theory, such as attentional theory (Mackintosh, 1975; Pearce and Hall, 1980) and retrieval theory (Miller and Matzel, 1988), and decisive evidence to discriminate prediction error theory from competitive theories has not been obtained in any learning systems of animals (Miller et al., 1995; Pearce, 2008; Mazur, 2013). Therefore, unambiguous demonstration of the validity of the prediction error theory remains to be achieved.

We performed experiments to investigate whether blocking occurs in classical conditioning in crickets (Terao et al., 2015). No convincing evidence of blocking has yet been obtained in any species of insects. In honey bees, for example, it has been concluded that blocking is not a robust phenomenon (Guerrieri et al., 2005; Blaser et al., 2006, 2008). We first investigated whether blocking of learning of an odor occurs. One group of crickets (blocking group) was subjected to pairing of a visual pattern (X) with a water US (reward) (X+ training) and then subjected to pairing of a pattern (X)-odor (Y) compound with water (XY+ training). An unpaired group received unpaired presentations of a visual pattern (X) and reward and then XY+ training. The blocking group exhibited no learning of the odor (Y), whereas the unpaired group exhibited normal learning of the odor (Y). We found that blocking of visual pattern learning also occurs (Terao et al., 2015). In a test of the prediction error theory, 1-trial XY+ conditioning should be successful, whereas in attentional theory, it should not be successful. We observed successful 1-trial XY+ conditioning, which matches with the prediction error theory but not with the attentional theory (Terao et al., 2015).

We revised our previous model (**Figure 1B**; Terao et al., 2015) for Pavlovian conditioning, thereby matching the prediction error theory (**Figure 2A**). How this model accounts for blocking is shown in **Figure 2B**. We noticed that the model predicted that the application of an octopamine receptor antagonist (epinastine) before Y+ training impairs the learning of Y but does not disrupt the formation of reward prediction by Y (see legend of **Figure 2**). Therefore, the model predicts that crickets that received Y+ training under the condition of application of epinastine and then Y+ training after recovery from the effect of epinastine exhibit no learning of Y. Indeed, crickets that received such training exhibited no learning of Y (Terao et al., 2015). The "auto-blocking" phenomenon can be easily accounted for by the prediction error theory. However, it cannot be accounted for by any of the competitive theories, as these theories assume cue competition to account for blocking, but it does not occur in an auto-blocking experiment (Terao et al., 2015). The occurrence of blocking and auto-blocking in the same learning system of the same species provides rigorous evidence for validity of the prediction error theory. Moreover, our observation that injection of an octopamine receptor antagonist leads to auto-blocking suggests that reward prediction error signals in crickets are mediated by octopamine neurons. Further neuroanatomical and electrophysiological studies of dopamine neurons are needed to elucidate neural circuit mechanisms for computation of the prediction error in crickets. Investigation is also needed to determine whether dopamine neurons mediate prediction error for aversive US.

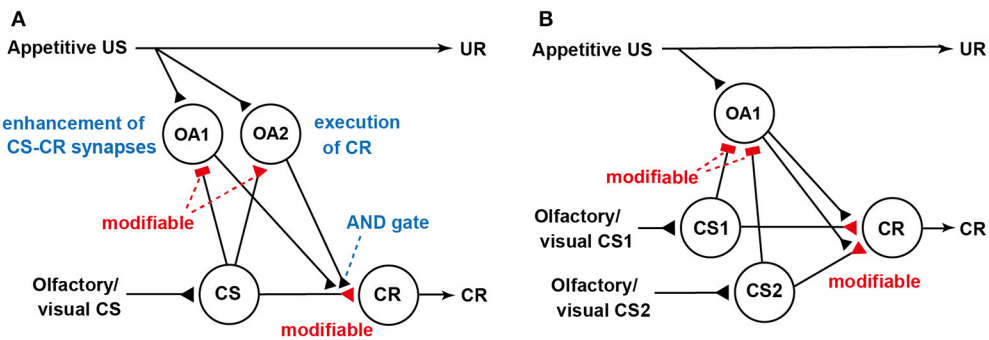

FIGURE 2 | Our updated model of the roles of octopamine neurons in appetitive conditioning. **(A)** Our updated model of classical conditioning established by modifying our previous model (**Figure 1B**) to match the prediction error theory (Terao et al., 2015). The model retains basic feature of the S-R and S-S hybrid model but assumes the presence of two classes of octopamine neurons, namely, "OA1" neurons that govern enhancement of "CS-CR" synapses (but not execution of a CR) and "OA2" neurons that govern execution of a CR or memory retrieval (but not a conditioning process). OA2 neurons, but not OA1 neurons, govern the "AND gate". In this figure, we focus on the roles of "OA1" neurons: "OA2" neurons are not illustrated for simplicity. We assume that "OA1" neurons receive no or very weak inhibitory synaptic input from "CS" neurons before training and that the efficacy of the inhibitory synapses is strengthened by CS-US pairing in training. During training, "OA1" neurons receive excitatory synaptic input (triangle) representing actual US and inhibitory input (rectangle) from "CS" neurons representing "predicted US" from the CS. Thus, activities of "OA1" neurons represent US prediction errors. Synapses for which the efficacies are modifiable by CS-US pairings are shown as open rectangles or open triangles and are marked "modifiable". **(B)** The model accounts for blocking and auto-blocking. In the figure, "OA2" neurons are omitted to focus on the roles of "OA1" neurons. The model assumes that pairing of a stimulus (CS1) with appetitive US leads to (1) enhancement of inhibitory pathways from "CS1" neurons to "OA1" neurons and (2) enhancement of excitatory synapses from "CS1" neurons to "CR" neurons. During pairing of a compound of CS1 and CS2 with US after sufficient repetition of CS1-US pairing trials, "OA1" neurons are inhibited by activation of "CS1" neurons and thus activation of "OA1" neurons in response to US presentation is inhibited. As a result, enhancement of "CS2-OA1" synapses and "CS2-CR"synapses, in which "CS2" neurons mediate CS2, does not occur. Therefore, no learning of CS2 occurs. The model also predicts that injection of an octopamine receptor antagonist before CS1-US conditioning trials impairs enhancement of "CS1-CR" synapses but not "CS-OA1" synapses. Therefore, no enhancement of "CS1-CR" synapses should occur in subsequent training even after recovery from the effect of the drug. We refer to this phenomenon as auto-blocking. Modified from Terao et al. (2015).

CONCLUSIONS

Most animals possess neural mechanisms that allow modification of their behavior for receiving appetitive stimuli and avoiding aversive stimuli. We showed that some octopamine and dopamine neurons play critical roles in appetitive and aversive learning, respectively, and more specifically, the octopamine neurons mediate reward prediction error in appetitive learning in crickets. Moreover, we suggested that some octopamine and dopamine neurons mediate signals about the extent by which the CS predicts the US and such signals drive appetitive and aversive CRs, respectively. Those roles of aminergic neurons in crickets match the S-S learning theory (see Mizunami et al., 2009) and are analogous to the roles of midbrain dopaminergic neurons in the execution of learned actions in mammals (Balleine et al., 2007; Berridge et al., 2009; Bromberg-Martin et al., 2010). We propose that the basic principles of information processing in associative learning are conserved among insects and mammals, on account of the fact that the organization of the insect brain is much simpler than that of the mammalian brain (Mizunami et al., 1999, 2004; Menzel and Giurfa, 2006, Menzel, 2012). Further studies on insect Pavlovian conditioning should pave the way for elucidating the diversity and evolution of associative learning mechanisms in animals.

In addition, our studies have demonstrated that crickets are one of most suitable animals for pharmacological analysis of learning and memory, and crickets may, thus, also be efficient model animals for screening drugs that affect motivational states of animals, and such screening may contribute to therapeutic applications in humans.

AUTHOR CONTRIBUTIONS

MM and YM wrote the manuscript and approved the final version.

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Silencing of *Syntaxin 1A* in the Dopaminergic Neurons Decreases the Activity of the Dopamine Transporter and Prevents Amphetamine-Induced Behaviors in *C. elegans*

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The dopamine transporter (DAT) is a cell membrane protein whose main function is to reuptake the dopamine (DA) released in the synaptic cleft back into the dopaminergic neurons. Previous studies suggested that the activity of DAT is regulated by allosteric proteins such as Syntaxin-1A and is altered by drugs of abuse such as amphetamine (Amph). Because *Caenorhabditis elegans* expresses both DAT (DAT-1) and Syntaxin-1A (UNC-64), we used this model system to investigate the functional and behavioral effects caused by lack of expression of *unc-64* in cultured dopaminergic neurons and in living animals. Using an inheritable RNA silencing technique, we were able to knockdown *unc-64* specifically in the dopaminergic neurons. This cell-specific knockdown approach avoids the pleiotropic phenotypes caused by knockout mutations of *unc-64* and ensures the transmission of dopaminergic specific *unc-64* silencing to the progeny. We found that, similarly to *dat-1* knockouts and *dat-1* silenced lines, animals with reduced *unc-64* expression in the dopaminergic neurons did not respond to Amph treatment when tested for locomotor behaviors. Our *in vitro* data demonstrated that in neuronal cultures derived from animals silenced for *unc-64*, the DA uptake was reduced by 30% when compared to controls, and this reduction was similar to that measured in neurons isolated from animals silenced for *dat-1* (40%). Moreover, reduced expression of *unc-64* in the dopaminergic neurons significantly reduced the DA release elicited by Amph. Because in *C. elegans* DAT-1 is the only protein capable to reuptake DA, these data show that reduced expression of *unc-64* in the dopaminergic neurons decreases the capability of DAT in re-accumulating synaptic DA. Moreover, these results demonstrate that decreased expression of *unc-64* in the dopaminergic neurons abrogates the locomotor behavior induced by Amph. Taken together these data suggest that Syntaxin-1A plays an important role in both functional and behavioral effects caused by Amph.

Keywords: Syntaxin-1A, dopamine, amphetamine, *C. elegans*, dopamine transporter

INTRODUCTION

The DAT is a plasma membrane protein which reuptakes the DA released in the synaptic cleft back into the neurons. By so doing, DAT plays a central role in controlling the extracellular content of DA and regulating the amplitude of the dopaminergic signaling. DAT has been involved in the etiology and treatment of various neurologic disorders including schizophrenia, attention deficit hyperactivity disorder (ADHD) and Parkinson's disease (Miller et al., 1999; Wang et al., 2013; Markota et al., 2014). Interestingly, DAT is also one of the major targets for psychostimulants such as cocaine and Amph; and while cocaine is a DAT blocker, Amph is also a DAT substrate, thus preventing DA uptake, and a releaser causing DA release by inducing reverse transport of DA from inside to outside the neurons through DAT (Khoshbouei et al., 2003). The resulting increase of extracellular DA is believed to be the first step that ultimately generates the behavioral outcomes produced by Amph.

Previous data showed that the regulatory effects of Amph on DAT require the participation of ancillary proteins such as Syntaxin-1A (Binda et al., 2008). Syntaxin-1A is a member of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins complex involved in the process of membrane vesicle fusion which leads to exocytosis and thus neurotransmitter release (Salaün et al., 2004). The neuronal specific isoform can bind to and regulate different plasma membrane proteins including ion channels (Naren et al., 1998; Arien et al., 2003; Condliffe et al., 2004; Tsuk et al., 2004) and neurotransmitter transporters (Deken et al., 2000; Geerlings et al., 2001; Haase et al., 2001; Horton and Quick, 2001; Quick, 2003, 2006; Sung et al., 2003; Wang et al., 2003; Fan et al., 2006). Studies focused on the DAT/ Syntaxin-1A interaction have showed that Syntaxin-1A directly binds at the N-terminal domain of DAT (Lee et al., 2004), reduces the capability of DAT to reuptake DA (Cervinski et al., 2010) and increases the ability of Amph to cause DA efflux (Binda et al., 2008). However, few studies have been performed to assess whether the interaction and/or lack of interaction between Syntaxin-1A and DAT may cause behavioral outcomes in living animals (Carvelli et al., 2008; Cartier et al., 2015).

Previously, we showed that, like in mammals, the *C. elegans* Syntaxin-1A homolog UNC-64 interacts with the *C. elegans* DAT (DAT-1) and regulates the electrogenic properties of DAT-1 (Carvelli et al., 2008). Here we investigated the effects caused by reduced expression of the *unc-64* gene in the dopaminergic neurons on DAT-1 function and DA-mediated behaviors. Because the *unc-64* knockout animals die shortly after embryogenesis (van Swinderen et al., 2001), we created transgenic animals expressing heritable and cell-specific knockdown of the *unc-64* gene in the dopaminergic neurons (*pdat-1::unc-64 sas*). We found that the *pdat-1::unc-64 sas* animals show a slight but statistically significant reduction in body bends when tested in plates without bacteria but they swim normally. However, when treated with Amph, the percentage of animals exhibiting SWIP was significantly reduced with respect to wild type animals. These behavioral results were supported by *in vitro* data showing that cultured neurons isolated from *pdat-1::unc-64 sas* animals exhibit reduced DA uptake and reduced DA release induced by Amph. Taken together these data suggest that Syntaxin-1A is required to mediate the physiological and behavioral effects caused by Amph in *C. elegans*.

Abbreviations: *C. elegans*, *Caenorhabditis elegans*; BSR, basal slowing response; DAT, dopamine transporter; DA, dopamine; Amph, amphetamine; GFP, green fluorescent protein; sas, sense and antisense; RNAi, RNA interference; SWIP, swimming-induced paralysis.

MATERIALS AND METHODS

Worms Husbandry and Strains

All *C. elegans* strains were cultivated under standard conditions, in non-crowded conditions, at 20°C on NGM plates seeded with the OP50 or NA22 *Escherichia coli* strains (Brenner, 1974), except for worms that were grown on *E. coli* strain HB101. The N2 wild type (Bristol variety), RM2702 *dat-1(ok157) III*, CB1112 *cat-2(e1112) II*, OH7547 *otIs199* [*pcat-2::GFP*; *prgef-1::dsRed*; *rol-6 (su1006)*], and CB246 *unc-64(e246) III* strains were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, United States). BY200 *vtIs1* [*pdat-1::GFP*; *rol-6(su1006)*] V strain was obtained from Dr. Randy Blakely at the Brain Institute, Florida Atlantic University. To knockdown GFP encoding gene (*gfp*) in the dopaminergic neurons under the promoter of *cat-2* (*pcat-2*), the following transgene was used: *gbEx525* [GBF312 *pcat-2::gfp RNAi sas*; *podr-1::RFP*]; whereas, under the promoter of *dat-1* (*pdat-1*) we used: *gbEx572* [GBF326 *pdat-1::gfp RNAi sas*; pJM371 *pelt-2::NLS::RFP*]. To knockdown the *dat-1* gene under *pdat-1*, the following transgenes were used: *gbEx584* [GBF334 *pdat-1::dat-1 RNAi sas*; *podr-1::RFP*; EM282 *pcat-2::GFP*] and *gbEx624* [GBF334 *pdat-1::dat-1 RNAi sas*; *podr-1::RFP*; GBF325 *pdat-1::GFP*]. To knockdown the *cat-2* gene: *gbEx574* [GBF327 *pdat-1::cat-2 RNAi sas*; *podr-1::RFP*]. To knockdown the *kal-1* gene: *gbEx599* [GBF339 *pdat-1::kal-1 RNAi sas*; *podr-1::RFP*; GBF325 *pdat-1::GFP*]. To knockdown the *unc-64* gene: *gbEx585* [GBF335 *pdat-1::unc-64 RNAi sas*; *podr-1::RFP*; EM282 *pcat-2::GFP*] and *gbEx613* [GBF335 *pdat-1::unc-64 RNAi sas*; *podr-1::RFP*; GBF325 *pdat-1::GFP*].

Construction of Transgenes for Neuron-Specific Knockdown

The construction of transgenes for neuron-specific knockdown was made by PCR fusion as previously described (Esposito et al., 2007). Genomic sequences, corresponding to the target gene and to promoters were amplified separately from *C. elegans* genomic DNA, unless otherwise noted. The promoter regions of *cat-2* or *dat-1* genes were chosen based on the cis-regulatory modules (CRM) necessary to drive expression specifically in dopaminergic neurons (Flames and Hobert, 2009; Illiano et al., 2017). The promoter specific expression in dopaminergic neurons was experimentally controlled by fusing them to *gfp* and by confirming that both were expressed in all dopaminergic neurons and only in them (data not shown). For the *cat-2* promoter a

600 bp fragment, upstream of the ATG, was amplified using the following primers: Pf *cat-2*: ataataaaactgcgtggcgtg; Pr *cat-2*: ctcttccaatttttcaagggg. The nested primer used for the second step of fusion was: Pf*2 *cat-2*: cgtgttgttaagaacgtgcttgatcg. For the *dat-1* promoter a 795 bp fragment, upstream of the ATG, was amplified using the following primers: Pf *dat-1*: aaagtctttctgcccacacaa; Pr *dat-1*: agtaaaccgtagcgggatcag. The nested primer used for the second step was: Pf* *dat-1*: cgacctcatacactttctctcg. To amplify the *C. elegans* target genes to be silenced, we amplified the same exon rich regions that have been used for RNAi by feeding experiments (Kamath et al., 2003). For *gfp* expression and knockdown the fragment to be fused were amplified from A. Fire (Stanford University, United States) plasmids pPD95.75 and L4417, respectively. Primers used to fuse the *gfp* fragment to *dat-1* promoter for expression were: Tf *pdat-1::gfp sas*: ctgatcccgctacggtttactTCACTATAGGGAGACCGGCA; Tr *pdat-1::gfp sas*: ctgatcccgctacggtttactTCACTATAGGGCGAATTGGG. Primers used to fuse the *gfp* fragment to *cat-2* promoter for expression were: Tf *pcat-2::gfp sas*: ccccttga aaaattggaagagTCACTATAGGGAGACCGGCA; Tr *pcat-2::gfp sas*: ccccttgaaaaattggaagagTCACTATAGGGCGAATTGGG. Primers used to amplify the *gfp* region (890 bp) for knockdown were: Tfa *gfp sas*: gttgtaaaacgacggccagt; Tfs *gfp sas*: GGCCGATTCATTAATGCAG; Tf* *gfp sas*: tcactataGGGAGACCGGCA; Tr* *gfp sas*: tcactatagggcgaa ttggg. Primers used to amplify the *cat-2* region (1092 bp) for knockdown were: Tfa *cat-2 sas*: caagctct tgtgatccgtga; Tfs *cat-2 sas*: acaatctgctgaacgccttt Tf* *cat-2 sas*: GA AATTCTCGATTTTCGCCA; Tr* *cat-1 sas*: CTTCTTTGCA CAACCCGAAT. Primers used to fuse the *cat-2* fragment to *dat-1* promoter for knockdown were: Tf *pdat-1::cat-2 sas*: ctgatcccgctacggtttactGAAATTCTCGATTTTCGCCA; Tr *pdat-1::cat-2 sas*: ctgatcccgctacggtttactCTTCTTTGCACAACCCGAAT. Primers used to amplify the *dat-1* region (1190 bp) for knockdown were: Tfs *dat-1 sas*: TTCGAACCTGAT CTCAACCC; Tfa *dat-1 sas*: TGCAGTTGGTGCCTACA GG; Tf* *dat-1 sas*: AAGCAAATGCACCGAACTCT; Tr**dat-1 sas*: AGCTCCAGCAAAACTTCCAA. Primers used to fuse the *dat-1* fragment to *dat-1* promoter for knockdown were: Tf *pdat-1::dat-1 sas*: ctgatcccgctacggttt actAAGCAAATGCACCGAACTCT; Tr *pdat-1::dat-1 sas*: ctga tcccgctacggtttactAGCTCCAGCAAAACTTCCAA. Primers used to amplify the *unc-64* region (1999 bp) for knockdown were: Tfa1 *unc-64 sas*: cttttcgtgtcgagacctgtc; Tfs *unc-64 sas*: AATGCCAGGAATATACTGAATGAG; Tr* *unc-64* (1) *sas*: CTC AATTCGATCAACCATCTCTC; Tf* *unc-64* (1) *sas*: AGAGA TTCGTGGAAGTGTGGATA. Primers used to fuse *unc-64* fragments to *dat-1* promoter for knockdown were: Tf *pdat-1::unc-64 sas*: ctgatcccgctacggtttactAGAGATTCGTGGAAGTGTG-GATA; Tr *pdat-1::unc-64 sas*: ctgatcccgctacggtttactCTCAATT CGATCAACCATCTCTC. All the Tf and Tr primers had at their 5′-end 20/21 additional nucleotides complementary to 3′ end of the promoter used to drive the knockdown. A mixture of sense and anti-sense PCR fusion product at the concentration of 50 ng/μL was microinjected together with co-transformation markers into the gonad of animals using standard microinjection technique (Mello et al., 1991). The following co-transformation markers were injected at the concentration of 30 ng/μL: p*odr-1::RFP*, expressed in the AWB and AWC neurons, a kind gift from C. Bargmann, Rockefeller University, United States; pJM371 p*elt-2::NLS::RFP*, expressed in intestinal cell nuclei, a kind gift from J. D. McGhee, University of Calgary, United States; EM#282 *pcat-2::GFP* expressed in dopaminergic neurons, a kind gift from S. Emmons, Albert Einstein College of Medicine, New York, United States. To test the silencing of *gfp* under the control of *dat-1* or *cat-2* promoters, the two PCR constructs were injected in two different integrated transgenic strains, in which the *gfp* is expressed in dopaminergic neurons with a "complementary" approach, i.e., *pdat-1::gfp (RNAi sas)* silencing construct was injected in *otIs199* [p*cat-2::GFP*] transgenic strain and vice versa, *pcat-2::gfp (RNAi sas)* silencing construct was injected in BY200 (*vtIs1* [p*dat-1::GFP*; *rol-6(su1006)*]) transgenic strain to avoid any disturbance of the same promoter on gene knockdown and on gene expression. To follow dopaminergic neurons in cultures some of the transgenic lines (i.e., *gbEx624*, *gbEx613*, and *gbEx599*) were obtained adding to the injection mix also GBF325 *pdat-1::GFP* fusion construct at 1 ng/μL. At least two lines for each genotype were analyzed in all cases and data pooled together.

Microscopy and Imaging

Animals were mounted and anesthetized with 0.01% tetramisole hydrochloride (Sigma-Aldrich, St. Louis, MO, United States) on 4% agar pads. The analysis of GFP expression and *gfp* knockdown in dopaminergic neurons was performed using Zeiss Axioskop microscopes (Carl Zeiss, Oberkochen, Germany).

Basal Slowing Response Assay

Basal slowing response assay was performed as previously described (Chase et al., 2004). Young adults, 18–22 h post-L4 stage at 20°C, were assayed. The locomotion rate of young adult animals was quantified by counting the number of body bends completed in five consecutive 20-s intervals in the presence or in absence of HB101 bacteria. Data were collected for six animals per condition, for a total of 30 measurements per condition. The percent of slowing was calculated by dividing the difference between locomotion rates on and off food by the locomotion rate off food.

Swimming-Induced Paralysis Assays

Animals were grown in agar plates seeded with NA22 bacteria and SWIP assays were performed as previously described (Carvelli et al., 2010). Briefly, in each SWIP trial, 8–16 age-synchronized larva-4 animals were placed in 40 μl of vehicle (200 mM sucrose) with or without 0.5 mM Amph (NIDA, Research Triangle Institute) in a single well of a Pyrex spot plate (Thermo Fisher Scientific, Waltham, MA, United States). Paralyzed animals were counted after 10 min using an inverted microscope (Carl Zeiss, Inc., Thornwood, NY, United States). The number of paralyzed animals was reported as a percentage of the total number of animals observed in each test ± standard error.

Caenorhabditis elegans Primary Cultures, [^{3}H]DA Uptake and Release Experiments

We prepared embryonal cultures from animals grown on NA22 bacteria, as previously described (Carvelli et al., 2004). Briefly, 2-day-old embryonic cells (10^6 cells/well) were pre-loaded with 5 nM [^{3}H]DA (NEN) for 30 min at room temperature. Cells were washed five times with bath solution containing 145 mM NaCl or $NMDG^+$, 5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, and 20 mM D-glucose (pH 7.2 and 350 osmolarity) and 100 μM Amph or bath solution were then applied for 1 min to induce DA release. Samples were collected and counted for radioactivity. For uptake experiments 2×10^6 cells were plated per well. Two days after seeded, cells were washed with bath solution and incubated with 50 nM [^{3}H]DA for 5 min at room temperature. Uptake was terminated by washing the cells three times with ice-cold bath solution. Cells were quickly lysed using 1% SDS and samples were collected in vials to count radioactivity.

Statistical Analysis

GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, United States) software was used for statistical analyses. The statistical significance was determined using one-way ANOVA with Bonferroni post-test, Kruskal–Wallis and Student's *t*-tests. The SWIP data passed the Shapiro–Wilk normality test ($\alpha = 0.05$). Data are reported as averages of multiple observations ± Standard error.

RESULTS

Cell-Specific RNAi Efficiently Silences Genes in Dopaminergic Neurons

unc-64 null mutants exhibit larval lethality, locomotion abnormalities, pharyngeal pumping and defecation defects (van Swinderen et al., 2001). Knockdown of *unc-64* using systemic RNAi causes growth and locomotion defects (Shephard et al., 2011). These defects do not allow using these deficient *unc-64* animals to perform behavioral assays and to explore the role of *unc-64* specifically in the dopaminergic neurons. To dissect the role played by *unc-64* specifically in dopaminergic neurons, in otherwise wild type animals, we created transgenic animals in which a dopaminergic specific promoter drives the expression of part of the gene, in the sense and antisense directions (RNAi sas) (Esposito et al., 2007; Gallotta et al., 2016). We initially tested the specificity and the efficiency of two dopaminergic-specific promoters, *pcat-2* and *pdat-1* (Flames and Hobert, 2009). The *cat-2* gene encodes the enzyme homolog to tyrosine hydroxylase, the rate-limiting enzyme required to produce DA. The *dat-1* gene encodes the DA transporter homolog to DAT, required to regulate synaptic DA signaling by controlling extracellular DA levels (Jayanthi et al., 1998). In *C. elegans*, these genes have been described to be specifically expressed only in the dopaminergic neurons (Flames and Hobert, 2009; Illiano et al., 2017). After confirming the dopaminergic-specific expression of *pcat-2* and *pdat-1* using a reporter gene (data not shown), we tested their efficiency in silencing the GFP in transgenic lines where GFP is constitutively expressed in all dopaminergic neurons (**Figure 1A**). The *pdat-1* promoter was largely more efficient in silencing the *gfp* (strain *pdat-1::gfp sas*), with only 15% of all dopaminergic neurons detectable by fluorescence microscopy ($p < 0.001$ vs control), while when using *pcat-2* (strain *pcat-2::gfp sas*), 90% GFP-positive neurons were still detectable, a percentage very similar to controls (98%). These results showed that the transgene driven neuron-specific silencing technique (RNAi sas) can be successfully applied to the dopaminergic neurons, that *pdat-1* is the appropriate promoter for this approach and we therefore used only this promoter in all the experiments. We then tested the efficiency of the *dat-1* promoter in silencing a gene known to play a function in the dopaminergic neurons. Thus, we evaluated the ability of *pdat-1* to knockdown *cat-2* gene by performing BSR experiments. This behavioral assay tests the ability of wild type animals to slow down their rate of locomotion when they encounter a bacterial lawn. This behavior is mediated by DA; in fact, *cat-2* null mutants do not exhibit BSR (Sawin et al., 2000). We used the BSR assay to evaluate the efficiency of the *cat-2* gene knockdown in dopaminergic neurons using the RNAi sas technique (**Figure 1B**). We observed that, similar to *cat-2(e1112)* null mutants, the transgenic animals silenced for *cat-2* (*pdat-1::cat-2 sas*) exhibited a defective BSR with respect to control animals (*pdat-1::gfp sas*). By representing BSR as the ratio between the locomotion rate off food and on food (% of Slowing in **Figure 1B**) we found that, similar to the *cat-2(e1112)* null mutants, the *pdat-1::cat-2 sas* animals exhibited a reduction in BSR, 6 and 15%, respectively. On the other hand, both the *pdat-1::gfp sas* animals (controls) and the wild type animals exhibited normal BSR values (45%). These results demonstrate that the RNAi sas technique is efficient, gene specific, and allows altering a DA-mediated behavior.

Dopamine Neuron-Specific Silencing of *unc-64* Causes DA-Dependent Behaviors

The results shown in **Figure 1B** encouraged us to apply the RNAi sas technique to specifically knockdown *unc-64* in the dopaminergic neurons. Therefore using the *dat-1* promoter, we created the *pdat-1::unc-64 sas* mutants. Contrary to what observed in *unc-64* null or hypomorphic mutants or after systemic RNAi (van Swinderen et al., 2001; Shephard et al., 2011), the *pdat-1::unc-64 sas* animals were viable and did not present obvious developmental defects. To assess whether *unc-64* was knocked down in the dopaminergic neurons, we tested these mutants for BSR. As mentioned above, the BSR phenotype depends on DA and specifically extracellular DA released by the dopaminergic neurons. Since, UNC-64 is an essential factor for vesicular fusion and neurotransmitter release, we hypothesized that the lack of function of UNC-64 would prevent DA release and consequently would cause defective BSR. Our results show that like the null mutants *cat-2(e1112)*, which cannot synthesize DA, the *pdat-1::unc-64 sas* lines, which cannot release DA via vesicle fusion, failed to show BSR, 6.4 and 3%, respectively (**Figure 2A**). For these experiments we created, as negative control, transgenic animals that were knocked down for the

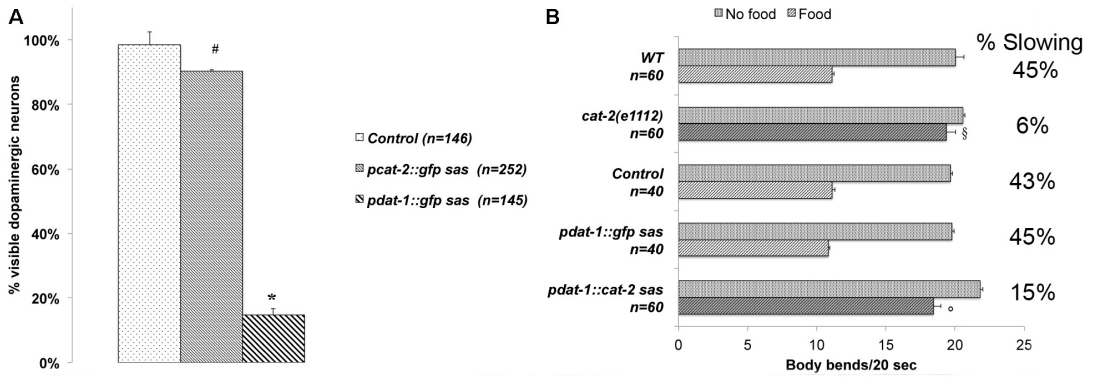

FIGURE 1 | (A) *dat-1* promoter is more efficient in silencing GFP expression in the dopaminergic neurons than the *cat-2* promoter. Using a cell-specific RNAi technique (Esposito et al., 2007), the two promoters *pcat-2* and *pdat-1* were employed to silence the *gfp* gene specifically expressed in the dopaminergic neurons. Animals carry an integrated transgene for the expression of GFP only in dopaminergic neurons. The percentage of visible dopaminergic neurons expressing GFP over the number of neurons expected (eight per animal) is reported. *pdat-1* promoter was significantly more efficient ($^{*}p < 0.001$, control vs *pdat-1::gfp sas*) in silencing the GFP expression than *pcat-2* ($^{\#}p > 0.01$, control vs *pcat-2::gfp sas*). *n* is the number of animals observed. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. **(B)** *cat-2* silencing through the *dat-1* promoter is sufficient to mimic a *cat-2* dependent behavior. Similar to *cat-2(e1112)* null mutants (Sawin et al., 2000), *cat-2* silenced animals (*pdat-1::cat-2 sas*) do not slowdown in presence of food. On the other hand, wild type, controls (non-transgenic siblings) and GFP silenced (*pdat-1::gfp sas*) animals reduce their locomotion rate when encounter bacteria. The locomotion rate for 20 s off and on food is reported as percent of slowing (% Slowing) calculated by dividing the difference between locomotion rates on and off food by the locomotion rate off food. *n* represents the number of animals tested. Kruskal–Wallis non-parametric with Dunn's multiple comparison post-test shows significant difference between wild type animals on food vs *cat-2(e1112)* on food ($^{\S}p < 0.001$) and *pdat-1::gfp sas* on food vs *pdat-1::cat-2 sas* on food ($^{\circ}p < 0.001$).

kal-1 gene using *pdat-1* (*pdat-1::kal-1 sas*). We reasoned that *kal-1* silencing represents the proper control because, while *gfp* is exogenously injected in worms to create transgenic animals, *kal-1* is natively expressed in most *C. elegans* neurons (Rugarli et al., 2002). As expected, both the wild type and *pdat-1::kal-1 sas* animals exhibited BSR when encountering food (58.3 and 48.4%, respectively; **Figure 2A**). In absence of food, we observed a slight but statistically significant 23% reduction ($^{\#}p < 0.001$) of body bends in the *pdat-1::unc-64 sas* animals with respect to controls (*pdat-1::kal-1 sas*). These data suggest that the *pdat-1::unc-64 sas* animals have a modest reduction in locomotion. To better understand the extent of this reduction, we tested the hypomorphic *unc-64(e246)* mutant. Although non-lethal, the *unc-64(e246)* mutation produces a point mutation in the *unc-64* gene (Ogawa et al., 1998) which causes severe locomotion defects. In fact, in our BSR experiments the *unc-64(e246)* animals besides lacking the BSR phenotype, also showed a strong reduction of body bends (4 ± 0.4) in absence of food with respect to wild type animals (13.6 ± 0.5; $^{\wedge}p < 0.0001$). Since the number of body bends in absence of food of the *pdat-1::unc-64 sas* animals was significantly higher (10 ± 0.4) then those observed in *unc-64(e246)* mutants, this result might suggest that in the *pdat-1::unc-64 sas* mutants the *unc-64* silencing occurs in a restricted number of cells, most likely the dopaminergic neurons, rather than malfunctioning in every cells, as in *unc-64(e246)* animals. On the other hand, the number of body bends of the *pdat-1::unc-64 sas* tested on food was significantly higher than those measured in the *pdat-1::kal-1 sas* animals ($^{\&}p < 0.001$, Kruskal–Wallis test). This result shows that *pdat-1::unc-64 sas* mutants are impaired in the BSR behavior compared to the control animals (*pdat-1::kal-1 sas* and wild type).

To further investigate the lack of function of UNC-64 in the dopaminergic neurons in the *pdat-1::unc-64 sas* animals, we utilized another DA-dependent behavior: SWIP. Previously, we showed that when immersed in liquid solutions, wild type worms swim vigorously for hours; however, *dat-1* null mutants exhibit SWIP, i.e., animals sink to the bottom of the well and do not move (McDonald et al., 2007). Since we showed that SWIP is in part caused by an increase of extracellular DA due to lack of function of DAT-1 (McDonald et al., 2007; Carvelli et al., 2008, 2010), we hypothesized that SWIP observed in *dat-1* null mutants can be recovered by knocking down *unc-64* in the dopaminergic neurons. In this case, even if DAT-1 cannot reuptake DA (*dat-1 ko*), the animals would not show SWIP because DA cannot be released (*pdat-1::unc-64 sas*). Thus, using the *pdat-1*, we knocked down *unc-64* in the *dat-1* null mutants and create the *dat-1 ko;pdat-1::unc-64 sas* double mutants. We found that the double mutants did not exhibited SWIP ($p < 0.001$ vs *dat-1 ko*, **Figure 2B**), confirming therefore that the RNAi sas technique we used, causes genetic ablation of *unc-64* in the dopaminergic neurons and, as a consequence, it prevents DA release. No SWIP was observed in control animals (wild type and *pdat-1::kal-1 sas*) or *pdat-1::unc-64 sas* mutants (**Figure 2B**). Taken together, these results suggest that the *pdat-1::unc-64 sas* mutants have reduced ability to release DA most likely because of the reduced expression of *unc-64* in the dopaminergic neurons.

Dopamine Neuron-Specific Silencing of *unc-64* Reduces Amph-Induced Behaviors

Previously, we demonstrated that Amph, a drug that increases extracellular DA (Di Chiara and Imperato, 1988), causes SWIP

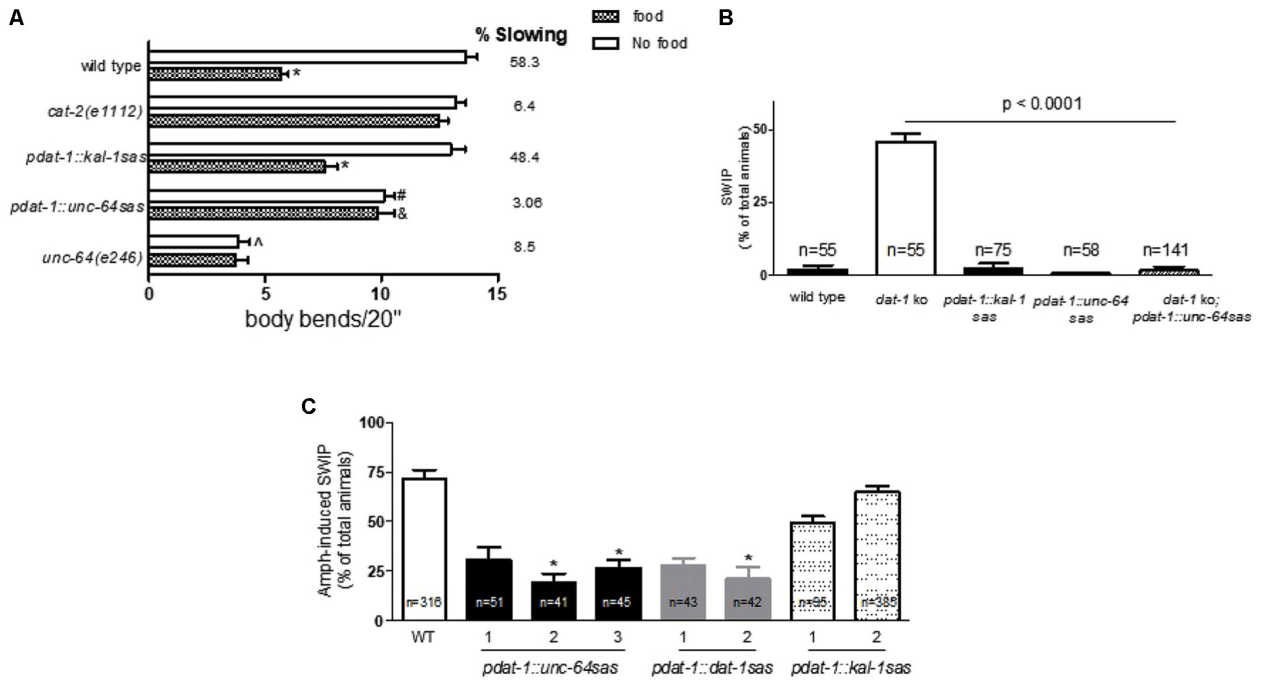

FIGURE 2 | Dopamine neuron-selective *unc-64* knockdown alters DA-dependent behaviors. **(A)** Similarly to *cat-2(e1112)* knockouts (6.4%), the *pdat-1::unc-64 sas* lines exhibit highly reduced BSR (3%) with respect to wild type and *pdat-1::kal-1 sas* animals, 58 and 48%, respectively (*$p < 0.0001$). In presence of food, the *pdat-1::unc-64 sas* mutants show a significant 33% increase ($^{\&}p < 0.001$) in body bends with respect to the control (*pdat-1::kal-1 sas*). The *pdat-1::unc-64 sas* mutants exhibit a slight but significant 21% reduction in body bends in absence of food with respect to *pdat-1::kal-1 sas* animals ($^{\#}p < 0.001$). Under the same conditions, the *unc-64(e246)* mutants exhibit a 71% reduction in body bends with respect to wild type animals ($^{\wedge}p < 0.0001$). Animals were assayed as in **Figure 1B**. At least 18 animals were tested per each sample. **(B)** *unc-64* silencing in the dopaminergic neurons recovers the *dat-1* knockout phenotype. *dat-1 ko* animals exhibit SWIP when forced to swim for 10 min (McDonald et al., 2007). This phenotype was recovered in the *dat-1 ko;pdat-1::unc-64 sas* double mutants (Kruskal–Wallis non-parametric test). Wild type, *pdat-1;kal-1 sas* (controls) and *pdat-1::unc-64* animals do not exhibit SWIP. **(C)** Similarly to *dat-1* null mutants (Carvelli et al., 2010) and the two *dat-1* silenced strains (*pdat-1::dat-1 sas*), animals that are silenced for *unc-64* specifically in the dopaminergic neurons using the *dat-1* promoter (*pdat-1::unc-64 sas*) exhibit reduced Amph-induced swimming-induced paralysis (SWIP). Two lines of *kal-1* silenced animals (*pdat-1::kal-1 sas*), used as negative control, exhibit AMPH-induced SWIP values that are comparable to those observed in wild type animals. The three black bars for *pdat-1::unc-64 sas*, two gray bars for *pdat-1::dat-1 sas* and two dotted bars for *pdat-1::kal-1 sas* represent independent lines. In **(A–C)**, statistical analysis was performed using one-way ANOVA followed by both Bonferroni and Kruskal–Wallis non-parametric post-tests. *n* represents the number of animals tested per each strain.

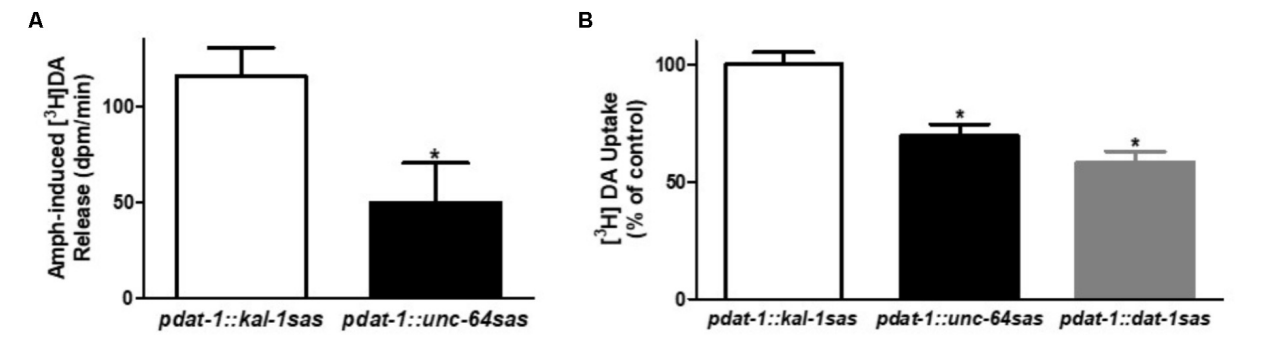

FIGURE 3 | Dopamine neuron-selective *unc-64* knockdown decreases DA release and DA uptake. Cultured dopaminergic neurons isolated from transgenic animals were used to measure the Amph-induced DA release **(A)** and DA uptake **(B)**. **(A)** *unc-64* silenced cells (*pdat-1::unc-64 sas*) show a statistically significant reduction of [^{3}H]DA release (*$p \leq 0.0001$) with respect to control cells (*pdat-1::kal-1 sas*). *t*-Test was used to performed statistical analysis. Data are presented as the difference of dpm (disintegration per minute) between samples treated with Amph and samples treated with control solution. **(B)** *pdat-1::unc-64 sas* cells show a statistically significant 30% reduction of [^{3}H]DA uptake (*$p \leq 0.001$) with respect to control neurons (*pdat-1::kal-1 sas*). A similar reduction (40%) was observed in cells silenced for *dat-1* (*pdat-1::dat-1 sas*). Statistical analysis was performed using one-way ANOVA and Bonferroni post-test. Both data are average of values obtained from three independent experiments.

in *C. elegans* (Carvelli et al., 2010). Here we investigated whether reduced expression of *unc-64* in the dopaminergic neurons (*pdat-1::unc-64 sas*) would alter Amph-induced SWIP. We found that three independent transgenic lines of *pdat-1::unc-64 sas* animals treated with Amph exhibited significantly reduced SWIP with respect to wild type animals (black bars in **Figure 2C**; $^{*}p < 0.0001$). We also measured SWIP in transgenic animals that were silenced for the *dat-1* (*pdat-1::dat-1 sas*) or *kal-1* (*pdat-1::kal-1 sas*) genes, used as positive and negative controls, respectively. Interestingly, the reduction observed in the *pdat-1::unc-64 sas* animals was comparable to that observed in the two independent transgenic lines of *pdat-1::dat-1 sas* animals (gray bars in **Figure 2C**; $^{*}p < 0.0001$). And, as expected, the two independent transgenic lines of *pdat-1::kal-1 sas* animals did not show significant change in Amph-induced SWIP with respect to wild type animals (dotted bars in **Figure 2C**). These results suggest that *unc-64* might play a role in the mechanism of action of Amph that ultimately generates SWIP.

Dopaminergic-Specific Silencing of *unc-64* Reduces Amph-Induced DA Release and DA Uptake

The strong reduction of Amph-induced SWIP measured in the *pdat-1::unc-64 sas* animals suggested that UNC-64, like its mammalian homolog Syntaxin-1A, is a protein required by Amph to induce DA efflux (Binda et al., 2008; Daberkow et al., 2013). To test this hypothesis we performed *in vitro* experiments using *C. elegans* primary cultures (Christensen et al., 2002; Carvelli et al., 2004). After preloading the embryonic cells with [^{3}H]DA, cells were treated with Amph to induce release of [^{3}H]DA. We found that cells derived from *pdat-1::unc-64 sas* animals had significant reduced [^{3}H]DA release with respect to the control *pdat-1::kal-1 sas* cells (**Figure 3A**; $^{*}p < 0.05$, *t*-test).

Previous studies showed that Syntaxin-1A interacts and regulates the activity of DAT (Binda et al., 2008; Carvelli et al., 2008; Cervinski et al., 2010). For example, Cervinski et al. (2010) showed that heterologous co-expression of Syntaxin-1A with rat DAT led to a reduction in DAT surface expression, which resulted in a reduction of DA uptake. We tested if this was also true in our native cultured cells by performing [^{3}H]DA uptake experiments (**Figure 3B**). We found a significant reduction (31 $\pm$ 5%) in the uptake of the *pdat-1::unc-64 sas* cells with respect to the controls (*pdat-1::kal-1 sas* cells). Interestingly, this reduction was comparable to that obtained in *pdat-1::dat-1 sas* cells derived from animals in which *dat-1* gene was silenced using the RNAi sas technique (42 $\pm$ 5%). Because DA is accumulated inside the neurons by DAT, these results suggest that reduced expression of UNC-64 in the dopaminergic neurons causes a reduction of DAT activity or, as Cervinski et al. (2010) previously showed, a reduction of DAT expression on the cell membrane. Moreover, these results suggest that the reduced [^{3}H]DA release observed in the *pdat-1::unc-64 sas* cells (**Figure 3A**) may result from less [^{3}H]DA moving inside the neurons as seen in our uptake results (**Figure 3B**).

DISCUSSION

In the present study, we explored the role played by the Syntaxin-1A *C. elegans* homolog, *unc-64*, in the dopaminergic neurons. We used *C. elegans* because this model is amenable to genetic manipulations and conserves a high homology with the human dopaminergic-signaling pathway. To overcome the limitations presented by genetic null/hypomorphic mutants or systemic RNAi technique, we applied an alternative RNAi strategy, named RNAi sense and antisense (RNAi sas), to generate transgenic animals in which the function of *unc-64* gene was knocked down only in the dopaminergic neurons. This strategy, originally developed in our laboratory (Esposito et al., 2007), has been adopted by several groups to successfully silence various genes in almost all *C. elegans* tissues including neurons. In this study, we determined that the dopaminergic-specific promoter *pdat-1* is able to specifically and efficiently drive the knockdown of a reporter-gene, such as *gfp*, in the dopaminergic neurons only. The fact that *pdat-1* was more efficient than *pcat-2* in silencing the reporter gene may be due to a higher transcriptional activity of the promoter sequence we have chosen; hence a higher concentration of RNA silencing molecules was produced. Indeed, a clear difference in *gfp* intensity was visible when the same promoter was used to express the GFP as reporter gene (data not shown). The *dat-1* promoter sequence we chose contains three CRM or DA motifs, which are required and sufficient to drive expression in all dopaminergic neurons; on the other hand, the *cat-2* promoter we used had only one CRM motif (Flames and Hobert, 2009). Importantly, the *dat-1* promoter was very efficient in silencing genes that are endogenously expressed in the dopaminergic neurons, i.e., *cat-2* and *dat-1*, and therefore, *pdat-1* became the candidate promoter for silencing *unc-64* in these neurons.

Several studies have recognized the SNARE protein Syntaxin-1A as a key player of neurotransmitter release (Salaün et al., 2004) and one of the regulatory proteins of DAT. By binding to the DAT N-terminal domain (Lee et al., 2004), Syntaxin-1A modulates the release, transport, and ion channel activities of DAT (Binda et al., 2008; Carvelli et al., 2008; Cervinski et al., 2010). Thus, Syntaxin-1A may represent an important key element in the dopaminergic circuit that controls the amount of DA in the synaptic cleft. Using the RNAi sas technique, we created transgenic lines silenced for *unc-64* specifically in the dopaminergic neurons that are viable, able to grow and do not exhibit severe locomotor dysfunctions. This allowed us to overcome the limitations observed using classical *unc-64* RNAi, such as defects in growth and in locomotion (Shephard et al., 2011). Moreover, since the RNAi sas is a transgenic-based approach, the silencing constructs are heritable. This was a crucial requirement for the feasibility of both our *in vivo* and *in vitro* experiments (**Figures 2**, **3**). Two distinct behavioral assays, BSR and SWIP, were used to test whether we effectively silenced the expression of *unc-64* in the dopaminergic neurons of *pdat-1::unc-64 sas* mutants. Both assays depend on the ability of the dopaminergic neurons to release DA, which ultimately makes the animals to slow down when they encounter food, BSR (Sawin et al., 2000), or to stop swimming if the DAT-1

function is ablated, SWIP (McDonald et al., 2007). The *pdat-1::unc-64 sas* mutants did not exhibit BSR, suggesting therefore that the lack of DA release is caused by the silencing of *unc-64* in the dopaminergic neurons (**Figure 2A**). Moreover, the SWIP behavior observed in the *dat-1 ko* mutants was recovered in the *dat-1 ko;pdat-1::unc-64 sas* double mutants, i.e., in animals that cannot release DA. Taken together, these results suggest that the RNAi sas strategy we used effectively silences *unc-64* in the dopaminergic neurons. We cannot exclude that the *pdat-1::unc-64 sas* mutants are deficient of *unc-64* in additional cells other than the dopaminergic neurons; however, we can speculate that the lack of obvious developmental defects seen in these animals makes this possibility quite unlikely. This hypothesis is also supported by our behavioral data (**Figure 2A**). In fact, while the hypomorphic *unc-64* mutants (*unc-64(e246)*) move very slowly in absence of food (four body bends/20 s), the *pdat-1::unc-64 sas* mutants move almost three times faster (10 body bends/20 s) than the *unc-64* hypomorphic mutants, and only slightly slower than the wild type animals (13 body bends/20 s).

Three *pdat-1::unc-64 sas* transgenic lines obtained with the RNAi sas technique (**Figure 2C**), exhibited a strong reduction in Amph-induced SWIP. Because the increase of extracellular DA is one of the causes generating Amph-induced SWIP (Carvelli et al., 2010; Safratowich et al., 2013, 2014), the reduction in SWIP seen in *pdat-1::unc-64 sas* animals treated with Amph could be due to a reduced amount of DA released in the synaptic cleft. We tested this hypothesis by performing *in vitro* assays, and we found that indeed the release of DA triggered by Amph was diminished in dopaminergic neurons of *pdat-1::unc-64 sas* mutants. Previous studies have shown that Amph evokes DA release through two separate mechanisms (Siciliano et al., 2014), one vesicle-independent and DAT-mediated (Sulzer et al., 1993; Jones et al., 1998) and one DAT independent and vesicle-mediated (Daberkow et al., 2013). Because UNC-64 is required to dock and fuse the vesicles at the cell membrane such that the neurotransmitter can be released, the reduced Amph-induced DA release measured in neurons silenced for *unc-64* could indicate that fewer vesicles empty their DA into the synaptic cleft. We also found that the reduced expression of *unc-64* in the dopaminergic neurons (*pdat-1::unc-64 sas*) diminished the DA uptake (**Figure 3B**). These results are in accordance with previous published data showing that Syntaxin-1A decreases DA uptake by reducing the amount of DAT on the cell membrane (Cervinski et al., 2010). If we assume that a reduction in the number of DAT proteins is responsible of the diminished DA uptake observed in the *pdat-1::unc-64 sas* neurons (**Figure 3B**), then we can speculate that less Amph is taken up inside these neurons since Amph, like DA, is a DAT substrate. This, in turn, would be the cause of the reduced DA release we measured in the *pdat-1::unc-64 sas* neurons (**Figure 3A**). In fact, with less Amph moved inside, we get less DA released out. Regardless of these speculations and interpretations, we may conclude here that in *C. elegans*, Syntaxin-1A besides controlling the basal release of DA also moderates the behavioral effects generated by Amph by reducing the amount of DA in the synaptic cleft.

AUTHOR CONTRIBUTIONS

AL carried out the molecular genetic manipulations, microscope analysis, and BSR assays. BDS performed SWIP assays, the [^{3}H]DA release and uptake experiments. IG did the initial molecular genetic manipulations and initial characterization. GZ participated in the genetic manipulations. SRK performed the BSR assays. LC and EDS conceived, designed, coordinated the study and wrote the manuscript. All authors read and approved the final manuscript.

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Cue-Induced Ethanol Seeking in *Drosophila melanogaster* Is Dose-Dependent

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Alcohol use disorder generates devastating social, medical and economic burdens, making it a major global health issue. The persistent nature of memories associated with intoxication experiences often induces cravings and triggers relapse in recovering individuals. Despite recent advances, the neural and molecular mechanisms underlying these memories are complex and not well understood. This makes finding effective pharmacological targets challenging. The investigation of persistent alcohol-associated memories in the fruit fly, *Drosophila melanogaster*, presents a unique opportunity to gain a comprehensive understanding of the memories for ethanol reward at the level of genes, molecules, neurons and circuits. Here we characterize the dose-dependent nature of ethanol on the expression of memory for an intoxication experience. We report that the concentration of ethanol, number of ethanol exposures, length of ethanol exposures, and timing between ethanol exposures are critical in determining whether ethanol is perceived as aversive or appetitive, and in how long the memory for the intoxicating properties of ethanol last. Our study highlights that fruit flies display both acute and persistent memories for ethanol-conditioned odor cues, and that a combination of parameters that determine the intoxication state of the fly influence the seemingly complex retention and expression of memories associated with intoxication. Our thorough behavioral characterization provides the opportunity to interrogate the biological underpinnings of these observed preference differences in future studies.

Keywords: ***Drosophila*, ethanol, alcohol-use disorder, memory, addiction, reward**

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INTRODUCTION

A critical component of the recurring nature of alcohol use disorder (AUD) involves the cravings elicited by ethanol exposures (priming doses), cues, and stress (Ludwig and Wikler, 1974; Hodgson et al., 1979; Le et al., 1998, 1999; Gass and Olive, 2007). Cue reactivity to ethanol-conditioned cues is an indicator of urges, predictor of relapse, and used to monitor putative treatments (Niaura et al., 1988; Monti et al., 1993; Rohsenow et al., 1994; Sayette et al., 1994; McGeary et al., 2006; Witteman et al., 2015). Although these studies recognize the importance of cue reactivity, the biological underpinnings of cue reactivity are not fully delineated. Moreover, in a natural environment cravings may be elicited in a more complex manner. Comprehensively understanding how ethanol-associated cue memories are formed and expressed may provide valuable insight to understanding the recurring nature of AUD.

Model systems provide the opportunity to characterize the biology underlying cue-induced cravings. Memory for ethanol associated cues are demonstrated in a wide range of species, from nematodes to primates (Smith et al., 1984; Reid et al., 1985; Bozarth, 1990; Colombo et al., 1990; Suzuki et al., 1992; Lee et al., 2009; Mathur et al., 2011). Although rodent models are the predominant model organism used to study cue-induced ethanol seeking, *Drosophila melanogaster* offer distinct advantages in defining the biology of cue-induced ethanol seeking. Not only do the genetic tools available in *Drosophila* permit precise spatial and temporal control of gene expression (Venken and Bellen, 2005; del Valle Rodriguez et al., 2011), but *Drosophila* show persistent preference for an odor cue previously associated with ethanol intoxication (Kaun et al., 2011). This provides the ability to define precise circuit motifs, and the accompanying molecular mechanisms required for behavior. However, before leveraging these tools in *Drosophila,* extensive characterization of factors impacting cue-induced ethanol preference is required. This is a critical step in avoiding mis- or overinterpretation of the results derived from future mechanistic studies.

In humans, dose-response relationships for addictive substances such as ethanol follow an inverted 'U' shaped curve where the ascending slope builds towards a peak appetitive response associated with reward and euphoria, and the descending slope depicts aversive states of dysphoria, anxiety and withdrawal (Van Etten et al., 1995; Tomie et al., 1998; Uhl et al., 2014). Similarly, in rodent models the dose and duration of ethanol intoxication affects the valence and strength of memories for a cue-associated experience (Bozarth, 1990; Risinger and Oakes, 1996; Shimizu et al., 2015). Although sensitivity and tolerance to ethanol have been well characterized in *Drosophila*, less is understood about the behavioral intricacies underlying the appetitive and aversive properties of the intoxication experience. We hypothesized that the extent and timing of intoxication would impact an animal's preference for cues associated with alcohol. Using a conditioned preference assay to test preference for an olfactory cue previously associated with ethanol intoxication, we characterized how intoxication affects valence and magnitude of cue memory for intoxication in *Drosophila*. We also characterized the administered dose concentration, duration, number of exposure sessions, latency between exposures, and time until testing to understand how these variables shape preference. This extensive characterization provides a framework within which future investigations will inform behavioral and pharmacological interventions to inhibit cue-induced cravings and relapse.

MATERIALS AND METHODS

Stocks and Conditions

Canton-S (CS) wild-type flies were used for all experiments. Flies were reared at 25°C and 70% humidity on a 12:12 Light:Dark (L:D) cycle with lights on at 8:00am. Flies were raised in 9.5 cm (height) × 2.5 cm (diameter) polypropylene vials on standard Bloomington cornmeal, molasses, and yeast media. Groups of 50 male flies were collected 0–1 days after eclosion under CO_2-induced anesthesia. Flies were given 2 days to recover from the CO_2 anesthesia, stored in groups of 50 in food vials at 25°C and 70%, on the same 12:12 L:D cycle. Behavior experiments were initiated when flies were 3–5 days old (adult flies). Importantly, because flies were sacrificed following each test, different groups of flies were used for each experiment reported here.

Environmental Conditions for Behavior Experiments

All behavioral experiments were based on the original cue-induced ethanol seeking or 'ethanol reward memory' behavior paradigm outlined in Kaun et al. (2011), and described in more detail below (also see **Figure 1**): For all behavioral experiments, flies were not food- or water-deprived prior to training. Throughout training and testing periods, flies were kept in a dark-room under red-light at 22–23°C and 70% humidity. The temperature was controlled with an oil-filled radiator (DeLonghi TRD0715T, Dubuque, IA, United States) and humidity controlled with a warm-mist humidifier (Vicks V745A, Procter & Gamble, San Ramon, CA, United States). Temperature and humidity were constantly monitored throughout training and testing to ensure consistent conditions across experiments.

Ethanol-Odor Training

Flies were trained in perforated vials (2.5 cm diameter and 9.5 cm height) containing 1 mL of 1% agar. Vials contained 64 evenly-spaced perforations (∼1 cm spacing throughout 74 cm^2 surface area of vial without top and bottom circular surfaces) and a mesh lid to facilitate uniform distribution of ethanol within the vials. Vials were placed into a test-tube holder in a 30 cm length × 15 cm height × 15 cm width training chamber (Aladin Enterprises, Inc., San Francisco, CA, United States). The training chamber contained three nozzles to allow for air/odorants/ethanol to stream in and one exhaust nozzle for waste. Flies were given a 10–15 min acclimation period within the training chamber prior to the start of experiments.

Humidified air was bubbled through 95% ethanol to vaporize ethanol, which was then combined with humidified air in various proportions. Humidified air was streamed over odors placed in a 2.5 cm diameter and 13 cm height cylinder at a flow rate of 130 U for training and 100 U for tests (where 100 U is equal to 1.7 L/min at room temperature). Odor flow rates were decreased during the test to ensure that the odors do not intermix in the Y-ends, allowing the flies to sufficiently discriminate between the two different odors during the choice test. The odors we used were either 3 mL iso-amyl alcohol (1:36 in mineral oil) or a mixture of 2 mL ethyl acetate (1:36 in mineral oil) and 1 mL acetic acid (1:400 in mineral oil). Odors were replaced daily to reduce any effects of odor evaporation. Humidified air (130 U) was flowed through training boxes during acclimation and rest periods.

Reciprocal odor training was performed to account for any inherent odor preference. Unless stated otherwise, a training

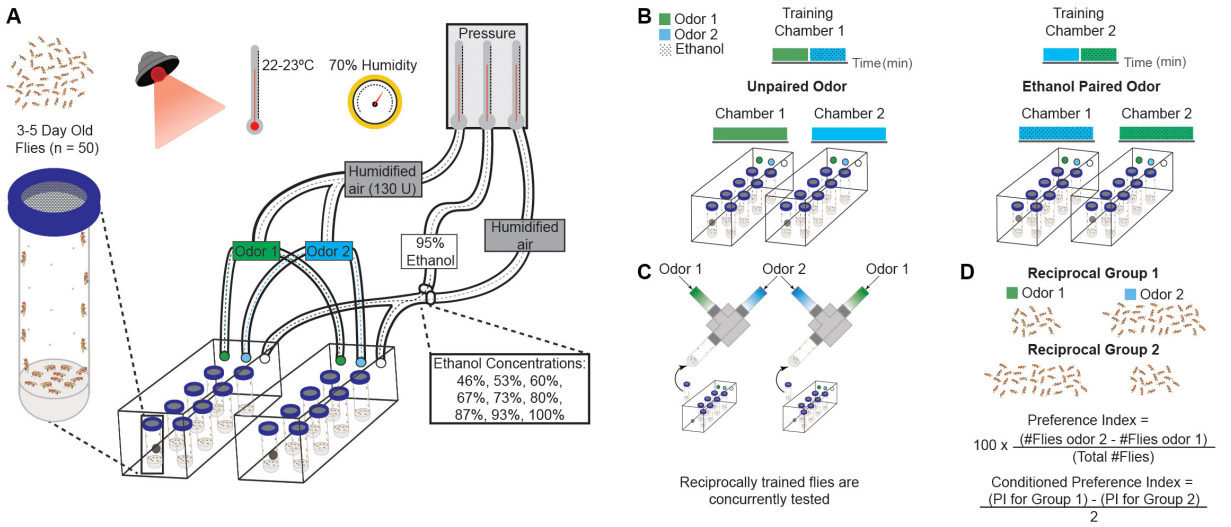

FIGURE 1 | Ethanol conditioning assay. **(A)** Schematic of behavioral training apparatus and fly training conditions. Behavioral training is done under red light, with temperature and humidity controlled throughout. Male flies (n = 50) are transferred into perforated vials with 1 mL of 1% agar and acclimated in the behavioral boxes for 15 min with 130 U of humidified air. Air pressure for ethanol and humidified air is controlled to adjust the administered dose. A passive vacuum is present within the behavioral boxes for air/odorant removal. **(B)** Olfactory-cues are paired with vaporized ethanol during training. Varying ethanol concentrations are achieved by adjusting the flow rates of 95% vaporized ethanol and humidified air. Flies are trained with two neutral or appetitive odors: one unpaired with the ethanol and the other paired with ethanol. A reciprocal paradigm is used to control for innate odor preference. **(C)** Flies are tested with the two odor cues in a Y-maze (displayed above). Reciprocally trained flies are tested concurrently. **(D)** A preference index (PI) is calculated by subtracting the flies that move towards odor one from the flies that move towards odor two, dividing the resultant number by the total number of flies, and multiplying that number by 100. A conditioned preference index (CPI) is calculated by subtracting the preference index for odor two from the preference index for odor one and dividing the resulting number by two. A positive CPI value indicates preference, while a negative value indicates aversion. Each individual sample (N) is composed of 100 flies.

period generally consisted of exposure to odor cue 1, followed by exposure to odor 2 with vaporized ethanol. A separate group of flies was simultaneously trained with exposure to odor 2, then odor 1 with vaporized ethanol (**Figure 1**). These training periods varied in exposure duration, number of training sessions and rest periods throughout this study. Vials of flies from Group 1 and Group 2 were paired according to placement in the training chamber and tested simultaneously. Vials tested simultaneously were averaged together to get a conditioned preference index.

Conditioned Odor Preference Test

The testing chamber was a 6 cm cube with a mesh Y-maze in the middle (Aladin Enterprises, Inc., San Francisco, CA, United States). During testing periods odors were streamed in through opposite arms of the Y (each 6 cm). Vials of flies were placed at the base of the Y and flies climbed up the mesh cylinder, where they chose between opposing arms of the Y that were capped with collection vials (2.5 cm diameter, 9.5 cm height). After 2 min, vials were removed, plugged, and covered with tape to trap flies within the collection tubes. The number of flies that moved into the odor 1 and odor 2 vials were counted after vials were frozen at either −20°C for 1 h, or −80°C for 20 min. Preference index (PI for each group was calculated as [(# flies in odor 1 vial – # flies in odor 2 vial) / total # flies] * 100. A conditioned performance index (CPI) for conditioned odor preference or aversion was calculated by subtracting the PI for reciprocal group 2 from the PI of reciprocal group 1 and dividing by 2.

Memory was tested either 30 min or 24 h post-training. For all flies tested 24 h post-training, yeast pellets were carefully added to the training vials 1 h post-training to ensure flies did not become food deprived prior to testing. For characterization experiments that took place across several days, flies were trained on food containing 10 g yeast, 10 g sugar and 4 g agar boiled in 200 mL water.

Statistical Analysis

All conditioned preference indexes are plotted as bars representing means +/− standard error. Individual data points plotted represent N = 1 (∼100 flies) calculated by averaging preference indexes per reciprocally trained groups (∼50 flies), accounting for any innate odor preference. On all data plotted here, CPIs of zero depict no memory formation, CPIs greater than zero depict appetitive memory, and CPIs less than zero depict aversive memory (see 'Test for Conditioned Odor Preference' above and **Figure 1** for a more detailed explanation).

Statistical analysis was performed using JMP® Pro 13.2.0 licensed to Brown University. Comparisons were made between Preference Indexes for each reciprocal group within a condition. This tested whether preference for the paired odor was significantly different than for the unpaired odor, while controlling for innate preferences for either odor. All data conformed to equal sample sizes and the assumption of normality (Shapiro-Wilk test). The data between different doses did not consistently meet the assumption of equal

variances using a Brown-Forsythe test, thus, a test that permits comparisons between groups with unequal variances was deemed necessary. Data was considered statistically significant when $p < 0.05$ using a Welch's unequal variances two-tailed *t*-test. No data were removed as outliers in order to provide an accurate depiction of variability within the data.

RESULTS

Ethanol Dose Influences Valence of Cue Memories

In *Drosophila*, ethanol dose affects ethanol-induced increases in locomotion, sedation, tolerance, and consumption (Moore et al., 1998; Scholz et al., 2000; Singh and Heberlein, 2000; Wolf et al., 2002; Berger et al., 2004; Devineni and Heberlein, 2009; Kaun et al., 2012). We previously showed that three doses of 53% vaporized ethanol (approximately 6mM or 0.025 g/dl body alcohol content per dose) induces an aversive memory shortly after exposure, and an appetitive memory 18 h to 7 days after exposure (Kaun et al., 2011). We sought to understand how changing the parameters of odor-ethanol pairings affected expression of memory for the odor cue.

We first characterized single exposure trainings across ethanol concentrations and exposure durations (10, 15, and 20 min), followed by testing for preference at 30 min and 24 h post-training. Of note, most of the single-dose characterizations did not display statistical significance, so we focus on observed trends to guide the following experiments and interpretation in our study. When testing preference 30 min after a single 10 min exposure there is a significant appetitive memory when training with an ethanol concentration of 87% (**Figure 2A**), however, this memory did not last 24 h (**Figure 2B**). Interestingly, we observed that a dose that induced a trend toward aversive memory 30 min after training (67% ethanol, **Figure 2A**), resulted in a lasting appetitive memory 24 h later (**Figure 2B**). This lasting appetitive dose for ethanol corresponding to a single low-dose results in approximating 9 mM body ethanol concentration or 0.04 g/dl (corrected for baseline) (Kaun et al., 2011). Thus, we used 67% and the slightly lower 60% ethanol doses as a reference to try increasing the duration of ethanol exposure to 15 or 20 min. 46% and 100% were included as lower and upper limits accordingly.

Fifteen minute exposures showed a similar trend to 10 min exposures where seemingly aversive 30 min memories corresponded to 24 h appetitive memories, however, none of these results were statistically significant (**Figures 2C,D**). Training with 20 min exposures results in a significant appetitive preference at 46% ethanol 30 min but not 24 h after training, whereas a 67% concentration results in an appetitive preference 24 h but not 30 min after training (**Figures 2E,F**). Thus, although single ethanol exposures don't produce a large conditioned preference score, the most notable observation from the data is that the subtle shift from aversive or neutral valence towards an appetitive preference 24 h later is consistent across many doses (**Figures 2G,H**).

Binge-Like Intoxication Induced Short-Lived Appetitive Cue Memories

Repeated exposure to the same cue during intoxication strengthens the memory for that cue, making it a more salient predictor of ethanol reward (Tomie et al., 2002; Krank, 2003). Drinking norms observed in social environments often involve binge-consumption of ethanol, in which consecutive drinks are consumed before the effects of the first drink tapers. How this affects initial memory for cues associated with intoxication is, for the most part, unknown. We found 53% ethanol vapor did not significantly affect memory 30 min or 24 h after training (**Figures 3A,B**). Two, or three doses of 60% ethanol trended towards an appetitive memory 30 min after training (**Figure 3C**), which persisted 24 h after training following two but not three doses of 60% ethanol (**Figure 3D**). Interestingly, two, three or four consecutive doses of 67% ethanol induced a small but significant memory 30 min after training (**Figure 3E**). This memory did not persist 24 h after training (**Figure 3F**). Together, the trends in our data suggest that initially a single exposure of alcohol may result in an aversive memory whereas, two or more binge-like low-dose ethanol exposures trend towards a short-lived appetitive memory with few lasting effects (**Figures 3G,H**).

Number of Spaced Ethanol Doses Determines Valence of Cue Memories

Long-lasting memory is induced by associations spaced by rest periods (Spreng et al., 2002; Commins et al., 2003). In the context of memories associated with alcohol intoxication, one might consume two or more glasses of wine over several hours. The wine may be consumed at a slow pace and consistently spaced over time, thus maintaining a mild euphoria throughout consumption. This consistent spacing doesn't allow for inebriation to occur. Alternatively, it may be consumed more quickly and promote inebriation, rather than constant mild euphoria.

To test how spacing ethanol exposures over time affects cue memory, we exposed flies to two, three or four ethanol-odor pairings with a 50 min rest period in between pairings (**Figure 4**). This rest period was sufficient to decrease body ethanol concentration to ethanol-naive levels (Kaun et al., 2011). Three spaced pairings between an odor and 53% vaporized ethanol resulted in a significant aversive memory for an odor cue 30 min after training (**Figure 4A**), and appetitive memory 24 h after training (**Figure 4B**). Spaced-training with 60% ethanol vapor resulted in no 30 min memory (**Figure 4C**), but an appetitive memory trend after two pairings, and a significant aversive memory after three pairings (**Figure 4D**). Spaced training with 67% ethanol induced no 30 min memory (**Figure 4E**), but resulted in a significant appetitive memory after two training sessions (**Figure 4F**). Together, this data suggests that the strongest lasting appetitive response occurs after low dose exposures that include two spaced pairings with each dose approximating 8–9 mM (0.03–0.04 g/dl), or three spaced pairings of 6 mM (0.025 g/dl). Further, the trends suggest that too many ethanol exposures result in either the absence

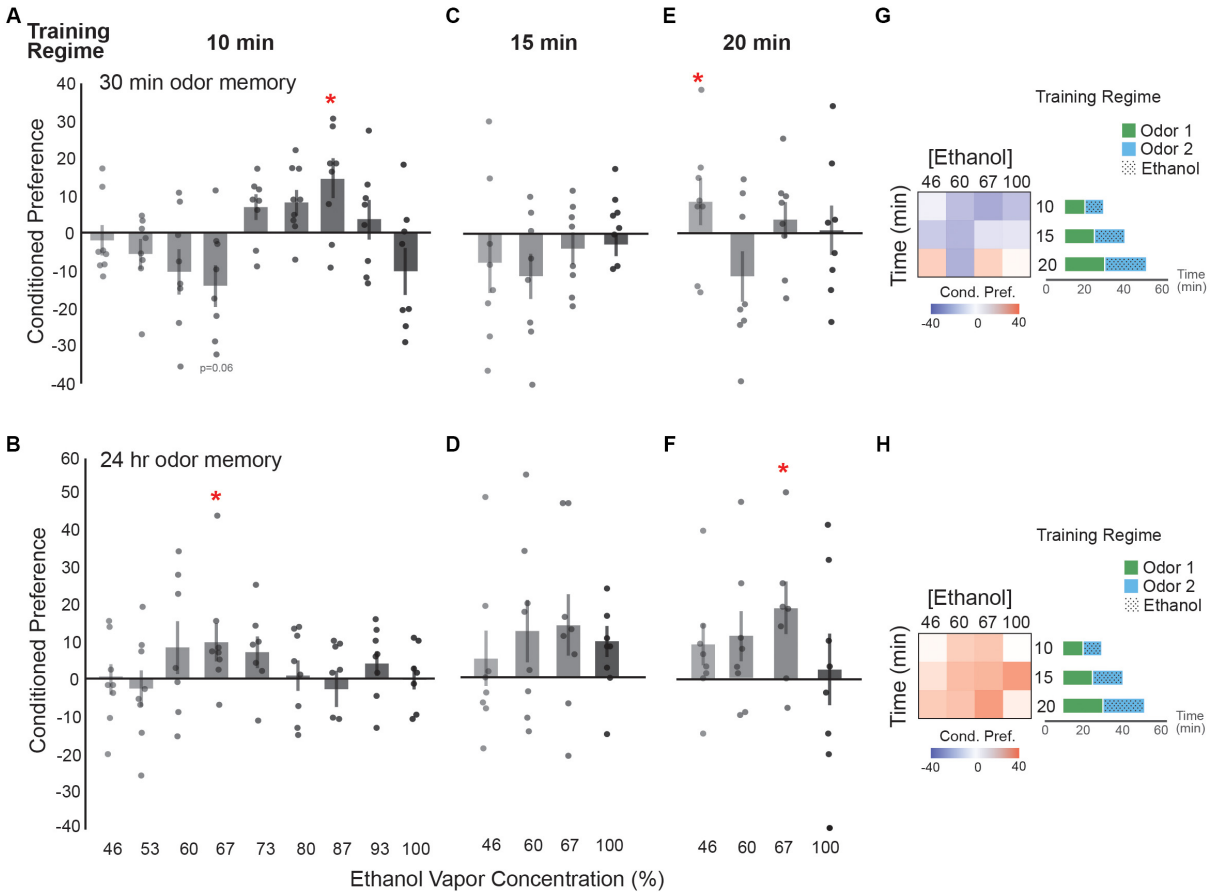

FIGURE 2 | Ethanol preference is dose-dependent. **(A)** Distinct groups of flies were exposed to ethanol at increasing concentrations and sacrificed prior to calculating a preference score. Flies receiving a 67% dose of ethanol vapor showed a trending aversive memory [$t(13.75) = 2.08$, $p = 0.06$] whereas flies receiving a 87% ethanol vapor dose show a significant appetitive memory [$t(13.81) = -2.34$, $p = 0.04$]. **(B)** Flies receiving a 67% ethanol vapor dose show a significant appetitive memory [$t(14.00) = -2.56$, $p = 0.02$]. **(C)** A non-significant trend toward aversive preferences occurs at 30 min post-training when exposure duration is increased to 15 min. **(D)** A non-significant trend toward appetitive ethanol preference is displayed at 24 h with a 15 min exposure duration. **(E)** Increasing the exposure duration to 20 min results in significant appetitive memory at 30 min post-training with 46% ethanol [$t(14.00) = 2.74$, $p = 0.02$]. **(F)** Exposure duration of 20 min results in a long-term appetitive ethanol preference with 67% ethanol [$t(11.96) = -3.51$, $p = 0.004$]. **(G)** Heat map summary of 30 min ethanol preference across different exposure durations suggesting that single 10 and 15 min exposure trainings trend toward an aversive (blue) response to ethanol at 30 min post-training. Schematics of single exposure training paradigm are depicted. **(H)** Heat map summary suggests a trend towards appetitive (red) ethanol preference at 24 h. Bars represent mean +/− standard error. N∼8 per group where individual data points represent $N = 1$ (∼100 flies) CPI. $^*p < 0.05$.

of 24 h memory or an aversive 24 h memory. This suggests that perhaps mild intoxication with sufficient rest to account for metabolism, rather than inebriation, is initially most appetitive. Additionally, reminiscent of memory after a single ethanol-odor pairing (**Figure 2**), conditions that trended toward short-term aversion also trended toward appetitive memory 24 h later (**Figures 4F,G**).

Daily Ethanol Induces Long-Lasting Appetitive Cue Memories

Although a single early experience with ethanol can induce a lasting appetitive response (Warner and White, 2003), repeated daily ethanol consumption is more characteristic of consumption in modern society (Grant et al., 2017). Thus, we tested whether spacing single odor-intoxication pairings by 1 day induced a dose-dependent, lasting appetitive memory. We found that conditioned preference for an odor cue associated with 53 or 60% ethanol vapor generally increased as the number of training days increased (**Figures 5A,B**). A significant appetitive memory was observed at 4 and 5 days of training with 53% ethanol (**Figure 5A**), and after 2 days with 60% ethanol (**Figure 5B**). Increasing the number of days of training to 4 days with 60% ethanol appeared to increase the appetitive memory (**Figure 5B**). Training with 67% ethanol vapor resulted in a significant appetitive memory after 1 day of training, with a trend towards a decrease in preference as the number of days of training increased (**Figure 5C**). This suggests that daily doses of approximately 8mM (0.03 g/dl) produce the strongest cue-induced ethanol seeking (**Figure 5D**). This data is also most reminiscent of the U-shaped curve, where very low dose exposures for few days does not produce a lasting memory, moderately low dose exposures for

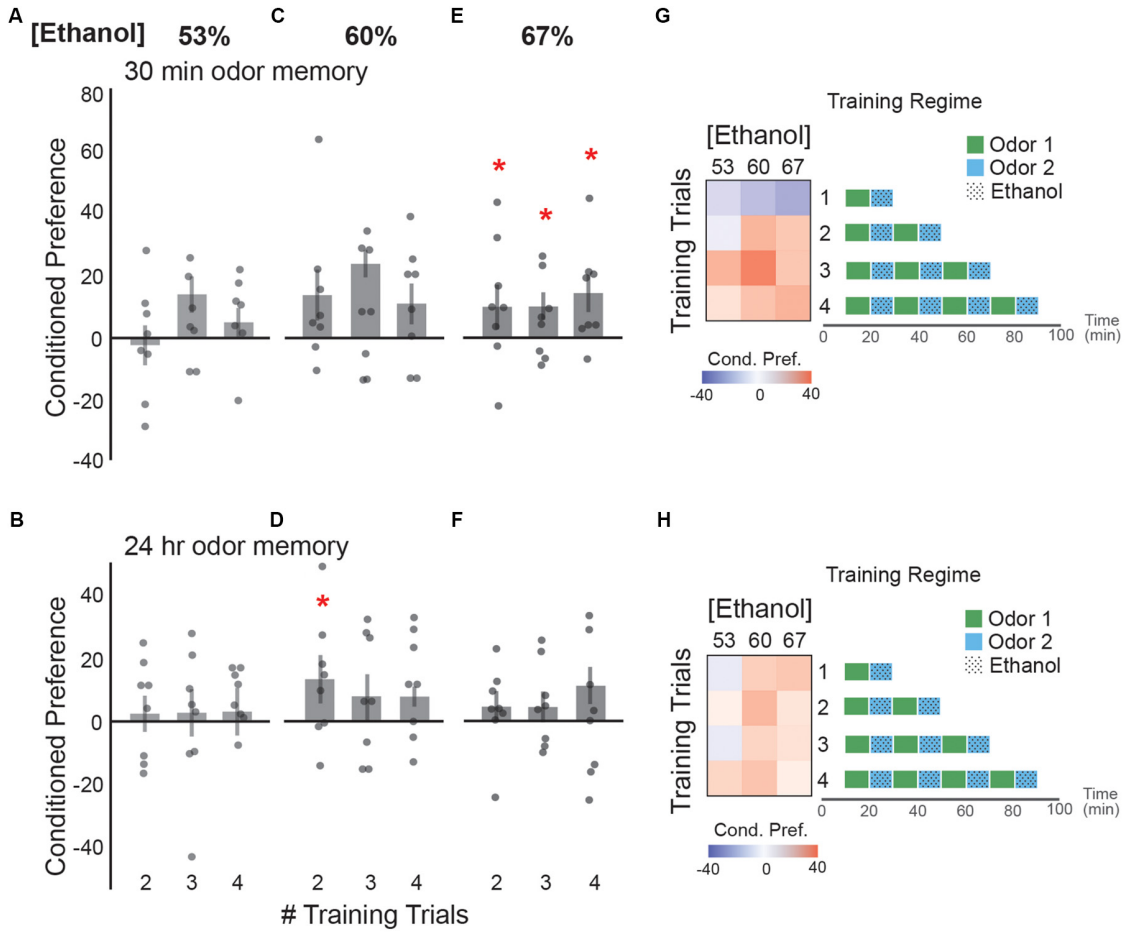

FIGURE 3 | Binge-like alcohol-odor pairings results in an appetitive ethanol preference. No statistically significant ethanol preference is observed **(A)** 30 min post-training or **(B)** 24 h post-training with two, three, or four consecutive training trials of 53% ethanol. **(C)** Two [$t(13.37) = -2.14$, $p = 0.051$] or three [$t(12.88) = -2.11$, $p = 0.054$] consecutive doses of 60% ethanol trend towards an appetitive ethanol preference 30 min. **(D)** There is an observed 24 h ethanol preference following two, but not three or four consecutive doses of 60% ethanol [$t(13.86) = -2.33$, $p = 0.04$]. **(E)** Appetitive ethanol preference is observed at 30 min following two [$t(13.89) = -2.26$, $p = 0.04$], three [$t(13.89) = -2.26$, $p = 0.04$] or four [$t(12.98) = -2.35$, $p = 0.03$] consecutive doses of 67% ethanol. **(F)** No significant ethanol preferences are displayed 24 h following two, three, or four consecutive doses of 67% ethanol. **(G)** A heat map summary of the data from both the single exposure training and binge-like training demonstrates that binge-like training results in trend towards an appetitive ethanol preference at 30 min with multiple consecutive pairings. Schematic of the training paradigms are depicted. **(H)** A heat map summary of conditioned preference at 24 h post-training suggests very little lasting appetitive preference regardless of concentration and number of training trials. Bars represent mean +/− standard error. N~8 per group where individual data points represent $N = 1$ (~100 flies) CPI. *$p < 0.05$.

a moderate number of days produces a strong appetitive memory, and moderately high dose exposures for many days does not produce a lasting memory.

Ethanol Intoxication, Not Odor, Induces Cue Memories

In our paradigm, flies are exposed to ethanol odor simultaneously with a neutral or appetitive odor cue. Although it is unclear whether flies can form an associative memory between two odors, 2 min training sessions with sucrose are sufficient to produce a memory for the associated cue (Schwaerzel et al., 2003; Burke et al., 2012). Importantly, 2-min exposures of 53% ethanol vapor are not sufficient to produce the locomotor stimulatory effects of ethanol (Kaun et al., 2011). Three 2-min pairings between an odor cue and 53% ethanol, spaced by 1 h rest periods produced no preference 30 min or 24 h after training (**Figure 6A**). Similarly, single 2 min pairings between 53% ethanol and an odor cue across 4 training days resulted in no odor preference (**Figure 6B**). Since similar training paradigms produced persistent memory when the ethanol exposure was long-enough to produce locomotor stimulatory effects (Kaun et al., 2011), this suggests that flies are forming memories between the pharmacological or intoxicating properties of ethanol rather than the odor of the ethanol vapor.

DISCUSSION

In order to further our understanding of how ethanol can co-opt the natural reward systems within the brain, it is important to

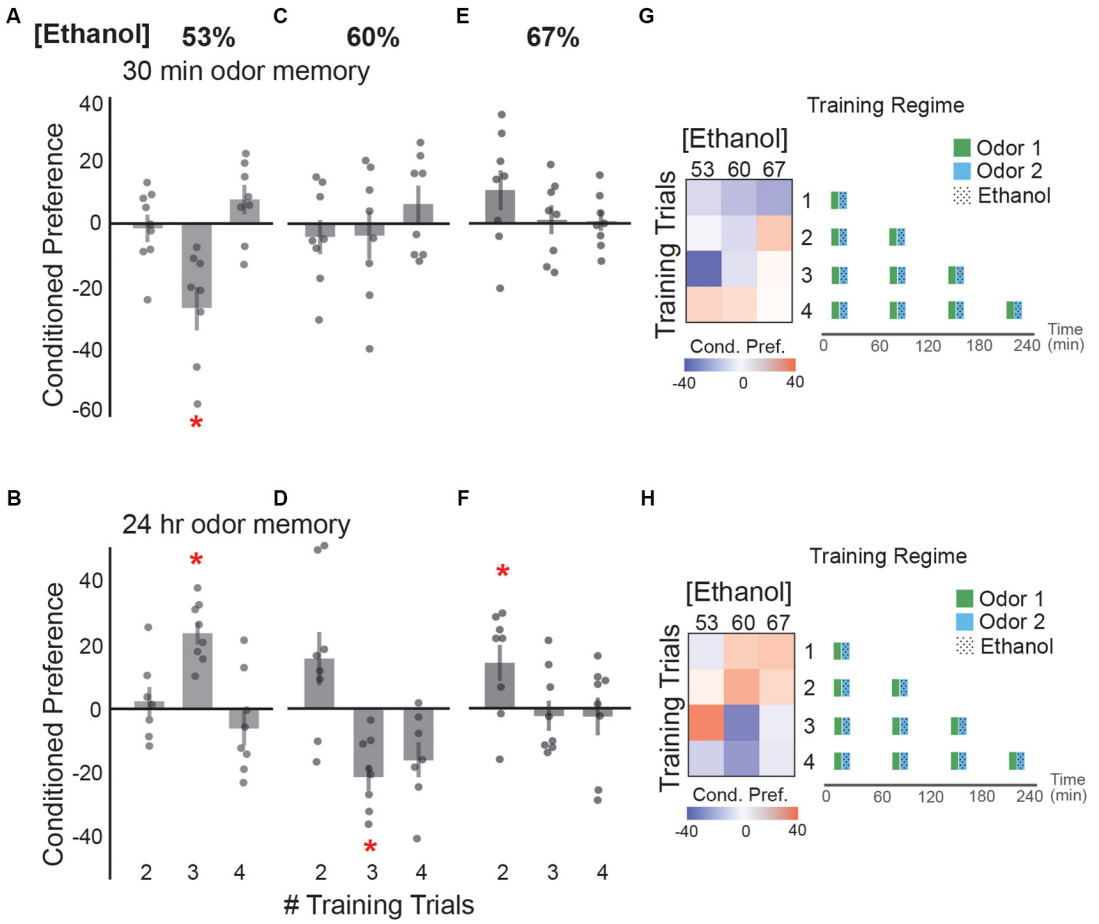

FIGURE 4 | Preferences following spaced training depend on ethanol dose. **(A)** Three spaced training sessions of 53% ethanol concentration resulted in a significant aversive preference at 30 min [$t(13.45) = 3.57$, $p = 0.003$]. **(B)** The same training conditions induced a significant appetitive preference when tested at 24 h [$t(12.58) = -6.57$, $p = 0.0001$]. **(C)** Spaced training sessions with 60% ethanol concentration do not induce a preference at 30 min, but **(D)** result in a trend toward an appetitive memory after two trials [$t(11.79) = -0.83$, $p = 0.09$] and an aversive memory after three trials [$t(11.86) = 3.92$, $p = 0.002$] at 24 h post- training. **(E)** Spaced sessions with 67% ethanol concentration do not induce significant preference at 30 min, but **(F)** result in an appetitive ethanol preference after two trials [$t(13.05) = -2.49$, $p = 0.03$] at 24 h post-training. **(G)** Heat maps summarizing the 30 min preference relationships with training trial number and ethanol concentration suggest a complex dose relationship where an increase in the number of training trials and dose of ethanol trends towards a switch from appetitive (red) to aversive (blue) memory. Schematic of the training paradigms are depicted. **(H)** Inversely, heat maps displaying the preference trends at 24 h suggest that an increase in the number of training trials and dose of ethanol trends towards a switch from appetitive (red) to aversive (blue) memory. Bars represent mean +/− standard error. N∼8 per group where individual data points represent $N = 1$ (∼100 flies) CPI. $^*p < 0.05$.

understand how exposure parameters affect the reward system. Ethanol displays a dose-dependent relationship in humans that can drastically alter the displayed physiological response, and, importantly, the consumption of ethanol (Van Etten et al., 1995). Understanding how different concentrations can alter memory of the intoxication experience, and ultimately cravings, can inform our understanding of the neurobiology underlying AUD.

Drosophila have proven to be an effective model to study mechanisms of ethanol-induced hyper-locomotion, tolerance, reward, and sedation (Moore et al., 1998; Scholz et al., 2000; Singh and Heberlein, 2000; Wolf et al., 2002; Berger et al., 2004; Devineni and Heberlein, 2009; Kaun et al., 2011, 2012; Robinson et al., 2012; van der Linde et al., 2014; Zer et al., 2016). The genetic tractability of *Drosophila* has allowed researchers to identify genetic components underlying these ethanol-related behaviors (Heberlein et al., 2004; Kong et al., 2010; Rodan and Rothenfluh, 2010; Devineni et al., 2011). Advancements in the field have also implicated the complicated nature of ethanol-related behaviors, where an animal's internal-state (hunger, circadian rhythm, sexual deprivation, stress, etc.) is an important factor to consider (Corl et al., 2005; van der Linde and Lyons, 2011; Shohat-Ophir et al., 2012). However, the behavioral parameters mediating the aversive or rewarding properties of ethanol memory in *Drosophila* is less understood.

In this study we provide a comprehensive characterization of a behavioral paradigm for memory of cues associated with intoxication in *Drosophila*, where we analyze the relationships between: dose concentration, number of exposure pairings, exposure duration, training paradigm, testing period, and observed preference. We found that all these factors affect the

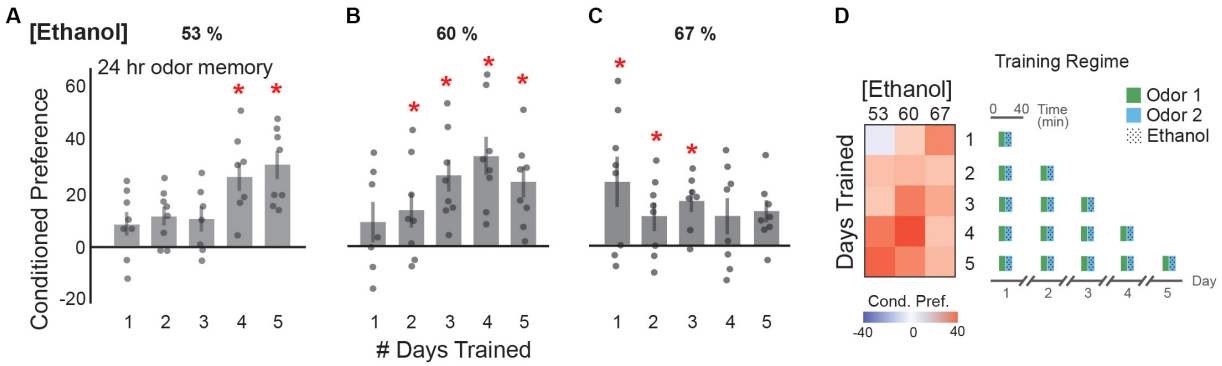

FIGURE 5 | Single daily exposures result in an appetitive ethanol preference. **(A)** Appetitive ethanol preference at 24 h increases with the number of single daily training exposures with a 53% ethanol concentration [4 trials $t(11.66) = -5.76$, $p = 0.0001$; 5 trials $t(13.01) = -4.70$, $p = 0.0004$]. **(B)** Similarly, the number of daily exposures correlates with the magnitude of appetitive ethanol preference observed at 24 h with a 60% ethanol concentration [2 trials $t(13.83) = -2.27$, $p = 0.04$; 3 trials $t(13.09) = -4.36$, $p = 0.0007$, 4 trials $t(8.42) = -5.36$, $p = 0.0006$; 5 trials $t(11.01) = -4.07$, $p = 0.002$]. **(C)** Daily sessions of 67% ethanol concentration suggest saturated appetitive ethanol preference with greater than three training days, [1 trial $t(9.82) = -2.95$, $p = 0.01$; 2 trials $t(10.33) = -2.43$, $p = 0.03$, 3 trials $t(11.43) = -4.27$, $p = 0.001$]. **(D)** Heat map summaries suggest that the number of daily exposure trainings correlate with the observed appetitive preference at 24 h, with the strongest responses in the middle, characteristic of a U-shaped response. Schematic of the training regimes are depicted. Bars represent mean +/− standard error. N∼8 per group where individual data points represent $N = 1$ (∼100 flies) CPI. $^*p < 0.05$.

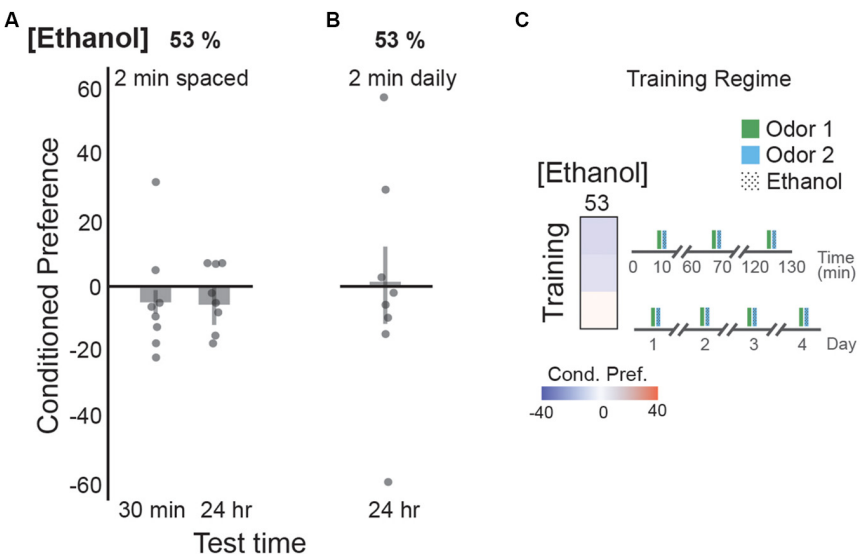

FIGURE 6 | Memory is formed for ethanol intoxication, not odors. **(A)** Two minute exposure durations resulted in no preference valence with the spaced-training paradigm at 30 min, or 24 h post-training with a 53% ethanol concentration. **(B)** Single 2 min duration exposures across 4 days is not sufficient to form a cue-memory with a 53% ethanol concentration, when tested on the fifth day. Two minute exposures are not sufficient to yield increased locomotor responses that correlates with ethanol intoxication (Kaun et al., 2011). **(C)** Heat map summaries suggest no strong preference valence for either training paradigm with a 2 min exposure duration. Schematic of the training regimes are depicted. Bars represent mean +/− standard error. N∼8 per group where individual data points represent $N = 1$ (∼100 flies) CPI.

observed ethanol preference, with the most important factor being the administered dose.

Ethanol-Dose Is a Major Determinant of Displayed Preference

In humans, there is a dose-dependent relationship between ethanol consumption and the dose concentration (Van Etten et al., 1995). Ethanol consumption increases as a function of increasing ethanol concentration. This trend continues up to a peak concentration at which point further increases in concentration result in less ethanol consumption. This inverted U-shaped relationship is conserved in rodents, where the conditioned lever-press responses increase as the dose of injected ethanol increases (Tomie et al., 1998). Once the maximum conditioned response is reached, the average lever presses decline with increasing ethanol dose (Tomie et al., 1998).

We observed that single exposure training sessions across moderate ethanol vapor concentrations display a mild trend towards an inverted U-shaped dose-preference relationship in *Drosophila* when tested 30 min post-training (**Figure 2A**). However, these memories were not long-lasting (**Figure 2B**). Similarly, we observe a mild U-shaped trend 24 h after training with a single exposure of low ethanol vapor concentrations (**Figure 2B**). The data that most resembles a U-shaped curve, however, is when flies are exposed to a single dose of ethanol vapor once a day for up to 5 days (**Figure 5**). The trend in this data suggests that doses too infrequent and too low, or conversely too frequent and too high do not produce a strong appetitive response. The 'goldilocks' training paradigm to produce the strongest appetitive response appears to be a dose of ethanol approximating 8mM (0.03 g/dl) body alcohol content, once a day for 4 days. Remarkably, this dose of alcohol approximates one that induces a mild euphoria in humans due to about one drink.

This dose-dependent response profile is notably similar to that seen in humans and rodents, suggesting that similar biological mechanisms may be underlying these preferences. However, it is of note that initial studies looking at these dose response relationships in humans and rodents are vastly different. The timescale across studies is not consistent, where these observations are made across weeks and months in rodent and human studies. Additionally, the methodology is vastly different. Although we are similarly looking at cue-induced responses, we are using different measures to characterize this. Whether it is *via* volitional intake of alcohol with lever-press studies in rodents, or through looking at physiological responses and cravings in clinical studies.

Understanding which ethanol concentration distinguishes between an aversive or appetitive response to an ethanol-associated cue can shape the way we understand how levels of intoxication are perceived and stored as memories. We speculate that both the immediate and long-term preferences are dose-dependent, but the level of intoxication dictates whether the flies find the associated cue appetitive or aversive. Lower concentrations are initially aversive, whereas slightly higher concentrations are sufficiently intoxicating to overcome ethanol's aversive properties. This reflects the first exposure to ethanol being initially aversive in humans, until an association is formed between the drug and subsequent euphoria.

Our data also demonstrate that low doses of alcohol (0.025 – 0.04 g/dl body alcohol content) result in the highest appetitive memory 24 h after exposure. Despite low-dose ethanol being most behaviorally relevant in inducing alcohol preference, limited attention has been given to understanding the molecular and cellular targets of *in vivo* low-dose ethanol responses (Cui and Koob, 2017). Our results highlight the relevance of using *Drosophila* to investigate how low doses of alcohol influence the neural and molecular mechanisms underlying memory formation and behavioral decisions.

Differences in Training Sessions Drastically Alter Choice Outcome

Training paradigms in which ethanol exposures are given in consecutive short intervals reflect the preferences that are observed in single exposure training. Flies have heightened levels of intoxication following multiple consecutive binge-like exposures (no 'rest' period between exposure pairings), which is rewarding shortly after training (**Figure 3**). This acute reward, however, does not induce a strong lasting memory. Intriguingly, when exposures were spaced by 1 h to allow sufficient ethanol metabolism, lower doses that were aversive shortly after training were remembered as more appetitive the following day (**Figure 4**). This is consistent with the observation that many abused substances are initially aversive until the rewarding properties are learned to be associated with the drug (Wise et al., 1976; White et al., 1977; Riley, 2011).

This stark switch in valence hints at the complex nature of how drugs of abuse may unnaturally act on the reward system. In rodents pre-exposure to ethanol conditions an appetitive memory for ethanol (Bienkowski et al., 1995; Cunningham et al., 1997; Cunningham and Henderson, 2000; Carrara-Nascimento et al., 2014). Similarly, in humans, a priming dose directly affects subsequent craving responses (Ludwig and Wikler, 1974; Hodgson et al., 1979). We speculate that this priming dose functions to initially activate circuits mediating aversion. This is later followed by the simultaneous inhibition of aversion circuits and stimulation of reward circuits inducing an enhanced appetitive response. This is affirmed by observation that single exposures of ethanol across multiple days results in stronger appetitive memories. In this case, the first day of training is a priming dose, and further activation of this reward circuitry by subsequent training increases preference.

Relevance for Understanding Cue-Induced Cravings

Behavioral characterization in this present study highlights the similarities and differences shared across animal models in cue-induced ethanol memories. Initially, our study looks at cue-induced ethanol memory immediately following a single exposure pairing (30 min post-training) and the following day (24 h post-training) across different ethanol concentrations. As stated previously, we observe that cue-induced memory valence and strength depends on the ethanol concentration. This relationship is similar to those observations in rodent and human literature, where conditioned responses are shaped by the concentration used (Bozarth, 1990; Monti et al., 1993; Van Etten et al., 1995; Risinger and Oakes, 1996; Tomie et al., 1998; Uhl et al., 2014; Shimizu et al., 2015).

However, it is important to highlight that rodent and human studies observe these effects on a different timescale than fruit flies. Training paradigms for rodent studies typically require weeks of training, while most human studies look at patients with a history of alcohol-dependence that developed after years of alcohol abuse. Similarly, when flies are trained on a longer time

scale, such as once a day for 4 days, they maintain an appetitive memory of the experience as long as the dose of alcohol is enough to be intoxicating (unlike in **Figure 6**) but not too high (as in **Figures 5A,B** but not **Figure 5C**).

Additionally, we show that concentrations that are initially found to be aversive tend to result in long-term appetitive memories. This is reminiscent of studies where ethanol is initially found to be aversive, and priming doses are used in training to elicit conditioned responses (Ludwig et al., 1974; Bienkowski et al., 1995; Cunningham et al., 1997; Cunningham and Henderson, 2000; Carrara-Nascimento et al., 2014). Interestingly, this valence switch is not observed across all concentrations and training paradigms in our study. The conditions resulting in this valence switch may be more comparable to current rodent and human studies, but training conditions that do not result in this switch may provide valuable information missing from the field. The flexibility of our behavioral system allows us to change training conditions with ease and test how different parameters result in different conditioned preferences. Thus, the behavioral flexibility provided by *Drosophila* allows us to ask questions that may be more costly in other model systems, while preserving the behavioral responses.

Our careful characterization of how ethanol concentration, timing, and number of exposures influence expression of memory for a cue associated with intoxication provides a framework for investigating the circuit, cellular and molecular mechanisms affected by low-dose ethanol exposure. This affirms the viability of *Drosophila* as a model to study mechanisms underlying cue-induced cravings at multiple levels: from molecules to single cells to network activity within a relatively complex circuit.

AUTHOR CONTRIBUTIONS

KK designed, performed, and analyzed the experiments. RA designed and developed the experimental apparatus. KN and KK wrote and revised the manuscript.

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Naltrexone Reverses Ethanol Preference and Protein Kinase C Activation in *Drosophila melanogaster*

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Alcohol use disorder (AUD) is a major health, social and economic problem for which there are few effective treatments. The opiate antagonist naltrexone is currently prescribed clinically with mixed success. We have used naltrexone in an established behavioral assay (CAFE) in *Drosophila melanogaster* that measures the flies' preference for ethanol-containing food. We have confirmed that *Drosophila* exposed to ethanol develop a preference toward this drug and we demonstrate that naltrexone, in a dose dependant manner, reverses the ethanol-induced ethanol preference. This effect is not permanent, as preference for alcohol returns after discontinuing naltrexone. Additionally, naltrexone reduced the alcohol-induced increase in protein kinase C activity. These findings are of interest because they confirm that *Drosophila* is a useful model for studying human responses to addictive drugs. Additionally because of the lack of a closely conserved opiate system in insects, our results could either indicate that a functionally related system does exist in insects or that in insects, and potentially also in mammals, naltrexone binds to alternative sites. Identifying such sites could lead to improved treatment strategies for AUD.

Keywords: ethanol, CAFE assay, PKC, opiate antagonist, *Drosophila melanogaster*, naltrexone

INTRODUCTION

Alcohol abuse and alcohol use disorder (AUD, commonly referred to as alcohol addiction) are global health problems with major social, mental health, and economic consequences (Gilmore et al., 2016). AUD is a complex disease affected by both genetic and environmental factors (Flatscher-Bader and Wilce, 2009). The molecular mechanisms resulting from alcohol consumption and leading to alcohol use disorder are still not completely understood. Clinically, AUD is currently treated with mixed success using both psychological and drug therapies. With respect to the latter, acamprosate (Kufahl et al., 2014), naltrexone (Hendershot et al., 2016) and more recently nalmefene (Soyka, 2016) have been the most widely used drugs

for treating AUD. However, clinical studies have yet to conclusively demonstrate the general effectiveness of these drugs (Arias and Sewell, 2012). Naltrexone is an opiate antagonist believed to exert its action on alcohol craving and relapse by blocking the μ opiate receptors which are involved in the molecular mechanisms of addiction (Gilpin and Koob, 2008). Although the mechanisms of ethanol induced behavioral changes are not well understood, it is known that ethanol alters the function of a number of neurotransmitters receptors (Liang and Olsen, 2014) and affects signal transduction including an increase in Protein Kinase C activity (Wilkie et al., 2007), which in turn also affects neurotransmitter receptors (Kumar et al., 2006).

Opiate peptides and receptors have been implicated in addiction mechanisms in response to many psychoactive substances including alcohol (Koob and Volkow, 2016). However, the potential of using opiate receptors as a therapeutic target for AUD remains controversial and indeed the use of naltrexone and nalmefene in the clinic has arisen from empirical observations rather than an understanding of their mechanism of action.

A variety of rodent models have been developed to try dissecting the molecular components of addictive behaviors (Crabbe, 2014). The fruit fly *Drosophila melanogaster* has proven to offer several advantages which include displaying simple alcohol-induced behaviors such as motor impairment and sedation, and the availability of a wide range of mutants for both reverse and forward genetics (Devineni and Heberlein, 2013; Park et al., 2017). *Drosophila* have an intrinsic capacity of sensing alcohol and indeed, identifying alcohol sources in rotting fruit, is part of the female's egg-laying strategy when deciding where to position the eggs for the maximal benefit to the larvae (Yang et al., 2008). Several studies have shown that when flies are repeatedly exposed to ethanol levels of up to 10–15% they develop a behavior that suggests that the flies have had a rewarding experience and that they seek more ethanol (Devineni and Heberlein, 2013; Peru Y Colón de Portugal et al., 2014). The capillary feeder assay (CAFE) is a convenient method for assessing the flies preference for alcohol (Ja et al., 2007) and was used here to determine whether naltrexone could alter the observed development of preference toward alcohol-containing food.

The choice of *Drosophila* for this study may seem controversial due to the lack of evidence for mammalian-like opiate systems in *Drosophila* or indeed in insects and other invertebrates. Recently however, behavioral effects of morphine have been reported in ants (Entler et al., 2016), crayfish (Huber et al., 2011), and *C. elegans* (Cheong et al., 2015). Additionally, two G-protein coupled receptors with structural homology to mammalian opioid/somatostatin receptors, but activated by allatostatin-like peptides, have been described in *Drosophila* (Lenz et al., 2000; Kreienkamp et al., 2002). The existence of these opiate-like systems which may have different activators or effectors, but result in similar behaviors, is in itself an important area of investigation because it may elucidate novel mechanisms in mammalian systems.

We show here that naltrexone reduces the preference for consumption of alcohol-containing food in flies previously exposed to alcohol and in the same flies it reduces the alcohol-induced increase of Protein Kinase C (PKC) activity. This study thus reinforces the need to further investigate novel targets or mechanism of action for opiate antagonists in treating AUD.

MATERIALS AND METHODS

Fly Maintenance

Wild type Drosophila Canton S were obtained from Bloomington Centre (Stock 64349) and maintained at 24°C, 70% humidity 12 h light/dark cycle on ready made mixed dried food (Batch no: B8A03876 obtained from Phillip Harris). For all experiments 1–3 day old male flies were used.

CAFE Assay

The previously described CAFE method was adopted (Ja et al., 2007). The CAFE apparatus consisted of 9 × 1.5 cm (height × diameter) tubes where the fly chamber was limited by inserting a cotton plug (flugs, Dutscher cat 789036) to create two chambers within the tube. To provide humidity, water (2 ml) was added to the lower chamber through a small hole created with a hot needle and plugged with plasticine. The top chamber was 5 cm high and hosted the flies. All incubations were carried out in the incubator at 24°C, 70% humidity. Four 5 μl capillary tubes (cat: CAP-TF-5 Jaytec Glass Ltd UK) were inserted in the top flug via cut-off pipette tips. Liquid food (5% Sucrose w/v, 5% w/v yeast extract) with or without 15% ethanol or naltrexone was loaded into the capillary tubes. Eight 1–3 day old male flies were anesthetized with CO_2 and placed in the chamber. Occasionally during the whole treatment some flies died, tubes with less than six flies were discarded. Flies were fed via capillaries for 2 days with liquid food with or without ethanol (pre-treatment). The duration of pre-treatment (48 h) and the concentration of ethanol (15%) were chosen after initial optimization for maximum preference response and are consistent with other reports (Ja et al., 2007; Devineni and Heberlein, 2013). Capillaries were reloaded with food or food plus naltrexone for 24 h. Capillaries were removed for 24 h. During this starvation period humidity was maintained by the presence of water in the lower chamber. Starvation increases consumption during the assay and reduces variability between groups. Four capillaries reintroduced where two capillaries contained food and the other two contained food plus 15% ethanol. The amount of food consumed was measured in the same batch of flies after 2 and 24 h by placing each capillary tube under a dissecting microscope aligned to a ruler with millimeter divisions. A tube containing no flies was used as control for liquid evaporation and the values were subtracted from the experimental tubes (corrected values). The preference index was calculated as the ((corrected ethanol consumption) − (corrected food consumption))/(corrected total consumption). Variations of the above protocol are described in the text.

Protein Kinase C Assay

Protein Kinase C (PKC) activity was measured using the kit from Abcam UK (cat 789036). This is an ELISA-based system where a peptide with the specific substrate sequence for the PKC protein family is immobilized on the walls of the

microtiter plate wells. Samples putatively containing PKC are incubated in the wells. Antibodies specifically recognizing the phosphorylated form of the immobilized peptides are added and detected by enzyme-linked secondary antibodies. Flies were fed via capillary tubes with either just food (prepared as above), or food with 15% ethanol for 48 h. Flies were then either exposed to food or food and 0.1% naltrexone for 24 h and then sacrificed by snap freezing in liquid nitrogen. Fly heads were separated by vortexing and homogenized in lysis buffer [20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF)] and either stored at −20°C or used immediately according to the manufacturer's instructions. Absorbance of each well was measured in a microtiter plate scanner. The protein content of the samples were estimated by a Bradford assay using bovine serum albumin as a standard. The specific activity of protein kinase C was calculated as absorbance value of the ELISA assay divided by absorbance value of the protein assay.

Statistical Calculations

Data was analyzed with the statistical package Graph Pad. Data were first analyzed for normal distribution by Shapiro-Wilk normality test. If it passed the normality test (alpha = 0.05) parametric tests were used (**Figures 1**–**3**) alternatively non-parametric tests were used (**Figures 4**, **5**) A preference index calculated from one tube containing 6–8 flies was considered as $n = 1$. Results were considered statistically significant if $p < 0.05$.

RESULTS

Alcohol Preference Is Induced by Ethanol Pre-exposure and Inhibited by Naltrexone

Drosophila were housed in the CAFE apparatus for 2 days and either fed liquid food or, in separate tubes, liquid food with 15% ethanol. After a 24 h starvation period flies were offered a choice of food with and without ethanol and the consumption from the capillary tubes was measured at 2 and 24 h (**Figure 1**). Flies with previous exposure to ethanol showed preference for ethanol-containing food, unlike the naive flies. Similar levels of preference were observed whether the first 2 or 24 h of food consumption were measured suggesting that the effect is due to the pre-exposure to ethanol rather than familiarity with the apparatus during the assay. The assay is thus measuring an established rather than a developing behavior.

To test the effect of naltrexone on alcohol preference, ethanol pre-exposed flies were fed food containing 0.05–0.5% naltrexone for 24 h, then starved for 24 h before testing for alcohol preference in the CAFE assay (**Figure 2**). The naltrexone dose range was chosen to include approximate equivalent values of the mg/kg bodyweight amounts used in mammalian systems (Critcher et al., 1983). The results in **Figure 2** indicate that naltrexone had an overall significant ($p < 0.0001$) effect in reversing ethanol preference however there was no significant difference between adjacent doses tested. This result suggests that naltrexone acts on a specific target to induce its effect. High doses of naltrexone appeared to cause an avoidance of ethanol (negative preference values shown in **Figure 2**) however, naive flies exposed to 0.1% naltrexone did not show negative preference (data not shown), thus the effect of naltrexone appears to be related to the response to ethanol.

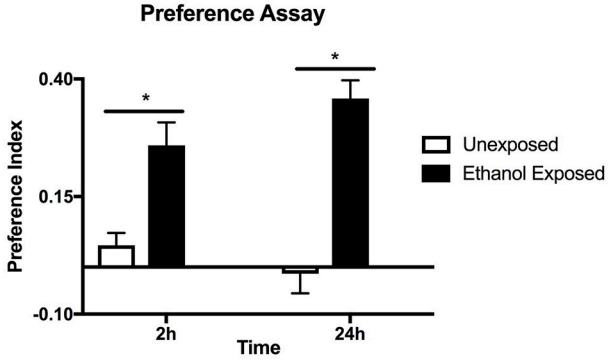

FIGURE 1 | Preference assay for naive or ethanol exposed flies. Preference indices were measured at 2 and 24 h in the same batch of flies. Columns represent three independent experiments, each consisting of three assay tubes containing 6–8 flies each. $n = 9$. Error bars are SEM. Statistical significance was measured by two-way ANOVA with Bonferroni multiple comparisons test. The effect of alcohol treatment was highly significant $p < 0.0001$ (horizontal line with*). The effect of time was not significant $p = 0.635$.

The Effect of Naltrexone on Alcohol Preference Is Not Permanent

To test whether naltrexone permanently reverses alcohol preference in *Drosophila* we introduced an additional step in the treatment of the flies whereby after the naltrexone treatment (0.05%), flies were fed normal food for 24 h before being starved and tested in the CAFE assay. This was carried out to allow naltrexone to be fully metabolized and thus presumably being absent during the CAFE assay. Flies treated in this manner showed preference for alcohol equal to those never exposed to naltrexone, while as previously shown in **Figure 2**, in the flies tested in the CAFE assay within 24 h of the end of the naltrexone treatment, the preference for ethanol was no longer detectable (**Figure 3**).

Total Food Consumption Is Not Affected by Ethanol or Naltrexone

To emphasize the concept that ethanol alters a decision making process (preference) rather than an instinctive physiological behavior (food consumption) we present the data for total food consumption, i.e., the sum of "food only" and "food + ethanol" consumed by the flies in each vial. The data shown in **Figure 4** is derived from sets of triplicate assays carried out on the same batch of flies for each experiment. No significant difference ($p > 0.9$) can be observed between the total food consumptions when comparing flies exposed or not exposed to ethanol(15%) whether the preference assay is carried out for 2 or 24 h or when comparing flies exposed to ethanol (15%) alone with flies exposed

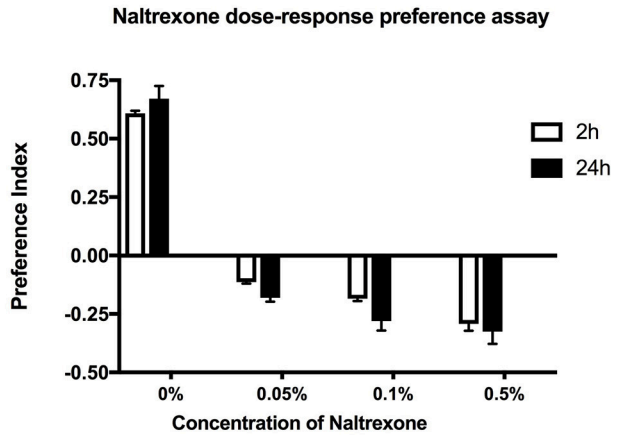

FIGURE 2 | Concentration dependent effect. Preference assay for ethanol exposed flies fed with naltrexone (0–0.5% w/v) for 24 h before being starved for 24 h and tested for ethanol preference in the CAFE assay. Preference indices measured at 2 and 24 h. Columns represent three independent experiments, each consisting of three assay tubes containing 6–8 flies each. $n = 9$. Error bars are SEM. Statistical significance was measured by two-way ANOVA with Bonferroni multiple comparisons test. The effect of naltrexone treatment was overall highly significant $p < 0.0001$ but there was no statistical difference between consecutive naltrexone concentrations tested. The effect of time was not significant $p = 0.175$.

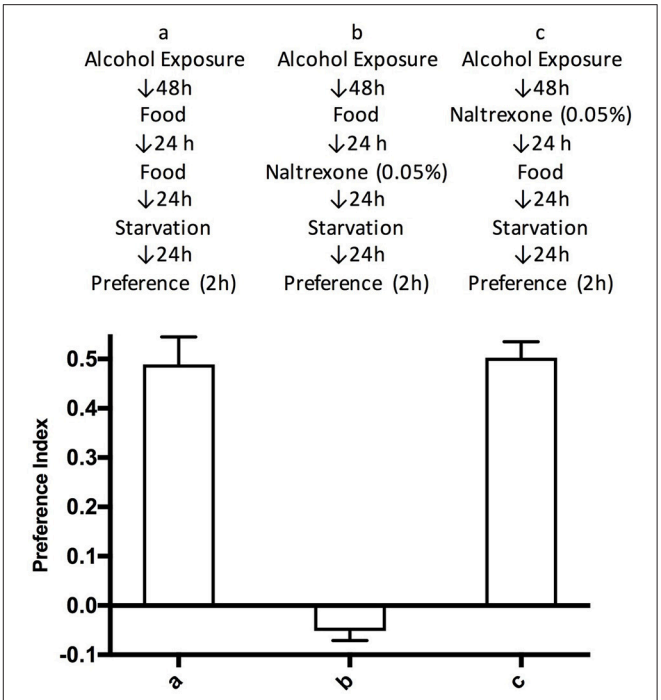

FIGURE 3 | Time duration of naltrexone effect. Flies were treated according to schemes a–c described in the top part of the figure. Preference indices were measured after 2 h. Each bar represents three experiments with triplicate assays containing 9 flies each, $n = 9$. Error bars are SEM. One-way ANOVA with Bonferroni multiple comparison analysis showed a significant difference between group a and b, and b and c, $p < 0.001$, but not between a and c with $p = 0.506$.

to ethanol(15%) and naltrexone (**Figure 4**). We did observe some variation between batches of flies: for example the total food consumption of the flies labeled as "exposed to ethanol and 0% naltrexone" is slightly higher (but not significantly, $p = 0.99$) than flies labeled as "ethanol exposed" which is the effectively the same treatment. These small variations in total food consumption occur between different batches of flies and may be due to factors such as age distribution (all flies are between 0 and 5 days old at the start of the experiment) room temperature, small differences in the time of the day the experiment is carried out. However, despite these small insignificant variations in total food consumption (see Supplementary Table 1), we consistently observe significant changes in the preference index induced by ethanol and suppressed by naltrexone as shown in **Figures 1**–**3**.

Naltrexone Affects Ethanol-Induced PKC Activity

In order to broaden the investigation of the behavioral effect of naltrexonon alcohol induced events, we chose to biochemically investigate the known phenomenon of the increase of level of PKC following ethanol stimulation. Using an ELISA assay to measure PKC activity in fly head extracts, we have confirmed that like in mammals, ethanol consumption (food with 15% ethanol for 48 h followed by food only for 24 h) induced a statistically significant increase ($p= 0.037$) in PKC activity (**Figure 4**). However, flies exposed to naltrexone (food with 15% ethanol for 48 h followed by food with 0.1% naltrexone for 24 h) showed no statistically significant increase in PKC, indicating that naltrexone affected the ethanol-induced increase in PKC activity. Flies exposed to naltrexone alone, in the absence of any alcohol treatment, showed no change in basal PKC activity (**Figure 5**).

DISCUSSION

In this work we have used the CAFE assay (Ja et al., 2007) to study long term *Drosophila* response to alcohol consumption. We have confirmed that preference for ethanol-containing food is induced by previous exposure to ethanol as opposed to being induced by preference in taste or immediate reward, because naive flies that were exposed to the ethanol-containing food for 24 h did not show significant preference (**Figure 1**). The novel aspect of our work is that we have provided evidence that the opioid antagonist naltrexone can neutralize ethanol preference in flies previously exposed to alcohol. The overall effect of naltrexone was dose dependant and at higher doses naltrexone caused a negative preference (repulsion) for ethanol-containing food (**Figure 2**). It is not possible to conclude from these experiments whether the reduction of preference and the induction of aversion are part of the same phenomena or are two separate processes requiring different concentrations of naltrexone.

The effect of naltrexone appeared to be short lived: ethanol pre-exposed flies that were allowed to recover a total of 48 h (24 h food + 24 h starvation) after naltrexone treatment before being tested in the CAFE assay, showed the same level of ethanol

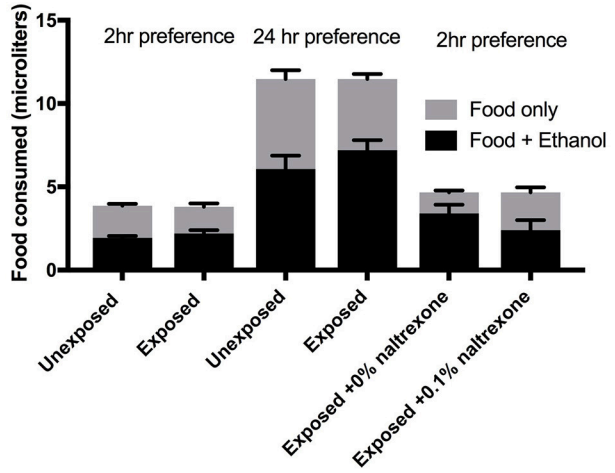

FIGURE 4 | Food consumption. Flies were either exposed or unexposed to 15% ethanol for 48 h or exposed to ethanol for 48 h followed by treatment with 0 or 0.1% naltrexone. The consumption was measured over a period of 2 or 24 h as indicated. Each column represents triplicate assays containing 6 flies each, $n = 3$. Error bars are SEM. One-way ANOVA with Kruskall-Wallis multiple comparison analysis showed no significant difference ($p > 0.9$) between any of the groups where consumption was measured for the same length of time. The Preference indices for the data shown for this figure for the 6 columns left to right are 0.0; 0.16; 0.02; 0.26; 0.48; 0.03.

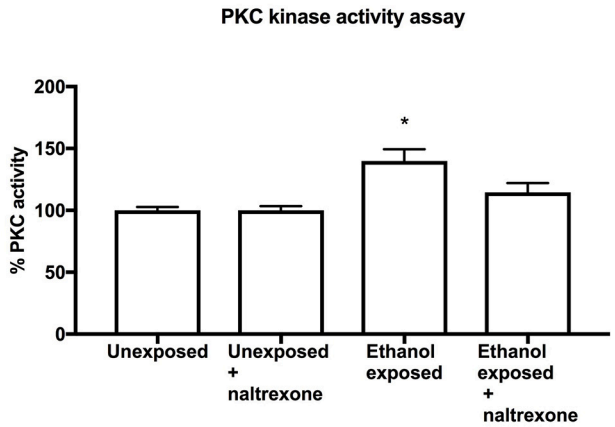

FIGURE 5 | PKC Kinase activity assay. PKC assay of homogenates from heads of flies exposed to food for 72 h (Unexposed), exposed to food for 48 h and treated with naltrexone for 24 h (Unexposed + naltrexone) exposed to ethanol for 48 h and food for 24 h (Ethanol exposed) or exposed to ethanol 48 h followed by naltrexone for 24 h (Ethanol exposed + naltrexone). Each bar represent two independent experiments each consisting of triplicate assays containing 15 fly heads each. $n = 6$. Error bars are SEM. Data was analyzed by non-parametric one way ANOVA Kruskal-Wallis with *post-hoc* comparison to Unexposed flies. The only statistical difference was between Unexposed and Ethanol exposed $^*p = 0.037$.

preference as flies that had not been exposed to naltrexone (**Figure 3**). The fact that the ethanol-induced alcohol preference is longer lived than the effect of naltrexone would suggest that while alcohol has a chronic effect that persists beyond the time in which alcohol is still present in the system, naltrexone has a more acute effect. This would suggest that either naltrexone activates a system that counteracts the alcohol-induced effect or that naltrexone antagonizes an opiate-like system that is an integral part of the development of the alcohol-induced alcohol preference. From the behavioral experiments presented here it is not yet possible to determine the exact mechanism of action of naltrexone in *Drosophila*. To exclude possible confounding factors we have observed that ethanol treatment with or without naltrexone did not affect the total amount of food consumed when the flies were given the choice of food with or without ethanol; indeed the total consumption of any food at any stage of the experiment showed no significant variations (**Figure 4**). Additionally, administration of naltrexone prior to the initial 48 h ethanol exposure did not affect the induction of ethanol preference (data not shown). It thus appears that naltrexone affects preferentially the behavioral seeking of ethanol in ethanol-exposed flies. The dopaminergic system is known to be implicated in addictive mechanisms in *Drosophila* (Azanchi et al., 2013; Aranda et al., 2017) and in the mammalian nervous system this is influenced by the opiate system (Koob and Volkow, 2016), further work is required to investigate this relationship in *Drosophila.*

The underlying theory of addiction behavior is that psychoactive substances cause long term changes at the cellular and molecular level which then result in behavioral changes (Nestler, 2014). To investigate whether naltrexone altered any of the known ethanol-induced biochemical changes we chose to investigate its impact on PKC activation. In mammals, chronic ethanol exposure causes an increase in PKC activity (Wilkie et al., 2007) while in *Drosophila* inactivation of PKC genes cause a desensitization to ethanol (Chen et al., 2010). Our data indicates that PKC phosphorylation is elevated in flies exposed to ethanol as compared to naive flies. This result, which to our knowledge is the first direct measurement of ethanol-induced PKC increase in *Drosophila*, further justifies the use of *Drosophila* as a model for the study of mammalian addiction mechanisms. Moreover, we demonstrate here that naltrexone affected the ethanol-induced increase of PKC to the extent that in naltrexone-treated ethanol-exposed flies PKC activity was no longer significantly different from unexposed flies (**Figure 5**). It should be noted that the specificity for PKC in this assay is based on the sequence of the peptide immobilized on the ELISA plates, it is possible that other kinases may have contributed to the phosphorylation process. The results shown in this study do not provide details of the mechanism of action for naltrexone with respect to PKC activity, but confirm the ability of naltrexone to alter alcohol-induced phenomena. Previous work in mammalian systems on the effect of naltrexone on PKC have reported an increase in PKC expression (Yu et al., 2011) and an antagonistic effect on ethanol induced increase of PKC activity (Oh et al., 2006). While further elucidating the role of PKC in addiction processes would be of interest, our aim for this study was to demonstrate that naltrexone reduces both an alcohol-induced behavior (ethanol-induced alcohol preference) and an alcohol-induced biochemical process (ethanol-induced increase in PKC activity). Taken together these findings justify further

work to investigate the mechanism of action of naltrexone in *Drosophila* and in mammalian systems. Indeed, it would also be of interest to understand how the putative naltrexone response system interacts with the dopaminergic system which is known to be involved in addiction behaviors and other related functions such as memory (Kaun and Rothenfluh, 2017) and circadian rhythms (De Nobrega and Lyons, 2016).

Understanding the mechanism of action of naltrexone in *Drosophila* is complicated by the fact that unlike other mammalian neurotransmitter receptors, the opioid receptors are not highly conserved in *Drosophila*. Two opioid/somatostatin-like receptors Drostar-1 and -2 and their endogenous allatostatin-like peptides have been identified in *Drosophila* (Lenz et al., 2000; Kreienkamp et al., 2002), however further work would be required to investigate whether naltrexone interacts with drostar receptors which do not respond to mammalian opiate peptides (Kreienkamp et al., 2002). The implication of this work is that either naltrexone binds in *Drosophila* to an as yet unidentified receptor which is functionally but not structurally related to mammalian opiate receptors or that naltrexone operates through another target and mechanism in *Drosophila*. In the latter case it would be of interest to identify such a *Drosophila* target as there may be an homologous mammalian target that could help elucidate the mechanism of action of naltrexone and possibly be a target for improved treatment of AUD.

AUTHOR CONTRIBUTIONS

RK obtained most of the data presented, NL contributed to the initial idea of using naltrexone, JJ and NL carried out initial experiments to develop the techniques, SA and OC contributed ideas to the project, analyzed the results and edited the manuscript, SC lead the project and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00175/full#supplementary-material

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Developmental Ethanol Exposure Causes Reduced Feeding and Reveals a Critical Role for Neuropeptide F in Survival

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Food intake is necessary for survival, and natural reward circuitry has evolved to help ensure that animals ingest sufficient food to maintain development, growth, and survival. Drugs of abuse, including alcohol, co-opt the natural reward circuitry in the brain, and this is a major factor in the reinforcement of drug behaviors leading to addiction. At the junction of these two aspects of reward are alterations in feeding behavior due to alcohol consumption. In particular, developmental alcohol exposure (DAE) results in a collection of physical and neurobehavioral disorders collectively referred to as Fetal Alcohol Spectrum Disorder (FASD). The deleterious effects of DAE include intellectual disabilities and other neurobehavioral changes, including altered feeding behaviors. Here we use *Drosophila melanogaster* as a genetic model organism to study the effects of DAE on feeding behavior and the expression and function of Neuropeptide F. We show that addition of a defined concentration of ethanol to food leads to reduced feeding at all stages of development. Further, genetic conditions that reduce or eliminate NPF signaling combine with ethanol exposure to further reduce feeding, and the distribution of NPF is altered in the brains of ethanol-supplemented larvae. Most strikingly, we find that the vast majority of flies with a null mutation in the NPF receptor die early in larval development when reared in ethanol, and provide evidence that this lethality is due to voluntary starvation. Collectively, we find a critical role for NPF signaling in protecting against altered feeding behavior induced by developmental ethanol exposure.

Keywords: Neuropeptide Y, feeding behavior, *Drosophila melanogaster*, developmental ethanol exposure, developmental lethality

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INTRODUCTION

Pediatricians often tell parents that their child won't starve themselves to death, and will eat when hungry. This is largely true: feeding behavior in both invertebrates and vertebrates is driven by two factors: hunger (induced by reduced energy availability) and food reward, and these two factors combine to ensure that animals consume sufficient food to allow further growth and survival. Nevertheless, there are developmental conditions that reduce the ability or willingness of children to eat. One such condition is Fetal Alcohol Spectrum Disorder (FASD), a collection of neurobehavioral and physical abnormalities that are a result of developmental alcohol exposure (DAE) (Jones and Smith, 1973; Kvigne et al., 2004; Dörrie et al., 2014). Feeding abnormalities, including anorexia and dysphagia, are commonly associated with FASD (Clarren and Smith, 1978),

and feeding anomalies associated with chronic ethanol exposure have previously been characterized in adult mammals (Štrbák et al., 1998). However, despite the growing body of research on chronic ethanol exposure and feeding, investigations into changes in feeding behavior after DAE are nearly non-existent.

Both hunger and food reward appear to be regulated by the appetite-stimulating molecule Neuropeptide Y (NPY), a 36-amino acid neuropeptide that signals through a variety of G-protein-coupled receptors (GPCRs) (Clark et al., 1984; Segal-Lieberman et al., 2002). Injections of NPY into the hypothalamus of rats induce feeding (Clark et al., 1984), while NPY ablation in mice results in an impaired refeeding response after fasting (Segal-Lieberman et al., 2002). Hypothalamic NPY expression is increased by fasting, an effect that is reversed by refeeding (reviewed in Heilig et al., 1994). Neuropeptide F (NPF), the sole *Drosophila* ortholog of NPY (Brown et al., 1999), signals through NPFR1, a GPCR related to mammalian NPY receptors. Like NPY, NPF regulates feeding behavior. In flies, NPF is expressed in six neurons in the third instar larval central nervous system: two pairs in the medial and lateral protocerebrum, and one pair in the subesophageal ganglion (SEG). Expression in the adult brain is more widespread (Brown et al., 1999; Lee et al., 2006). Reduced NPF signaling causes decreased feeding in larvae, and changes in NPF expression regulate developmental changes in foraging behavior, with high levels of NPF driving foraging in younger larvae (Wu et al., 2003).

NPY/NPF is implicated in the regulation of both natural rewards, such as food and sex, as well as drug rewards. Food containing a high concentration of sugar (20%) increases both NPF mRNA expression and NPF release in larvae (Shen and Cai, 2001), and overexpression of the fly NPF receptor (NPFR1) is sufficient to induce well-fed larvae to consume noxious food, while silencing of NPFR1 neurons reduces consumption of noxious food in food-deprived larvae (Wu et al., 2005).

Altered NPY/NPF signaling also results in changes in ethanol-induced behaviors. For example, in mice, knocking out NPY or its receptor NPY-Y1 leads to decreased ethanol sensitivity as measured by time to return to normal posture after an intraperitoneal inebriating dose of ethanol (loss of righting reflex). NPY knockout mice were able to right themselves significantly faster than control mice. In addition, mice deficient in NPY signaling show increased ethanol consumption compared to wildtype animals (Thiele et al., 1998, 2002). Similarly, flies with a loss of function in *npf* or *npfr1* display decreased ethanol sensitivity, as measured by the time it takes animals to become immobile when exposed to a sedating concentration of ethanol vapor (Wen et al., 2005). Finally, in sexually deprived male flies there is a decrease in NPF expression and a concomitant increase in ethanol consumption, while activation of NPF neurons reduces ethanol reward, as measured by the preference of animals for ethanol-containing food over food without ethanol (Shohat-Ophir et al., 2012).

In the wild, female *Drosophila* preferentially deposit their eggs in rotting fruit, resulting in larval exposure to concentrations of ethanol ranging from 6 to 11%, much higher than those usually tolerated by insects, and this is due in part to high levels of expression of the alcohol-detoxifying enzyme alcohol dehydrogenase (Gibson et al., 1981; McKechnie and Morgan, 1982). Ethanol at these concentrations is nonetheless toxic to developing *Drosophila* larvae, leading to decreased cell division, slow growth, and, sometimes, to the deaths of at least 50% of the flies (McClure et al., 2011).

Several hypotheses have been proposed to explain the preference of flies for egg deposition sites with high ethanol concentrations. At low concentrations, ethanol is beneficial to fly development (Parsons et al., 1979); thus the consumption of toxic levels may be merely a consequence of selection for preference of lower, healthful ethanol concentrations. Alternatively, as ethanol is also toxic to many of the organisms that prey on developing fly larvae and well as other insects with which the larvae compete for resources, ethanol preference may have evolved as a way to utilize an environment that competitors and parasites find intolerable (Milan et al., 2012).

Here we use our previously-established *Drosophila* model for DAE (McClure et al., 2011; Logan-Garbisch et al., 2014) to examine the effects of DAE on feeding behavior and investigate the hypothesis that DAE leads to reduced hunger or food reward. We show that ethanol-supplemented flies consistently eat less than control animals, at every stage of development. Additionally, we find that NPF expression is increased in the brains of ethanol-supplemented larvae, and loss of NPF signaling enhances ethanol-induced anorexia. Finally, we show that while loss of NPF signaling normally has no effect on survival, loss of function of the NPF receptor (NPFR1) combined with rearing in ethanol-supplemented food results in early larval lethality, and provide evidence that this lethality is due to decreased food intake. Our data raise the possibility that NPF signaling during larval development is an adaptation that helps to allow *Drosophila* larvae to exploit environments with a high concentration of ethanol.

MATERIALS AND METHODS

Fly Stocks, Genetics, and Husbandry

Fly stocks were maintained at 25°C on standard corn meal and molasses medium. Fly strains were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana) and the strains used were: w^{1118}; *PBac{PB}npfr*^{c01896} (Bloomington Stock #10747), w^{1118}; *da-GAL4* (Bloomington Stock #12429), and *UAS-npf*^{RNAi} (Bloomington Stock #27237). For the *npf* RNAi experiments, *da-GAL4/da-GAL4* virgin females were crossed with *UAS-npf*^{RNAi}/*UAS-npf*^{RNAi} males. Background controls for RNAi experiments were generated by crossing *da-GAL4/da-GAL4* virgin females to males from our standard laboratory stock strain (w^{1118}, *Wild-Type Berlin (w:WTB)*), or *UAS- UAS-npf*^{RNAi}/*UAS-npf*^{RNAi} males to *w; WTB* virgin females. The *npfr1*^{c01896} mutation behaves as a genetic null allele; Lee and colleagues found that the electrophysiological phenotypes of flies homozygous for the *npfr1*^{c01896} mutation were indistinguishable from flies transheterozygous for *npfr1*^{c01896} and a deletion uncovering *npfr1* (Lee et al., 2017).

Throughout the manuscript, "food" refers to fly food prepared according to the Bloomington Stock Center's Cornmeal, Molasses

and Yeast Recipe (https://bdsc.indiana.edu/information/recipes/molassesfood.html), with additions as described in the text.

Ethanol Rearing

Eggs were collected on 35 mm Petri dishes containing standard fly food. One hundred eggs (per vial) were transferred to vials containing fly food with 7% ethanol or no ethanol (control). For ethanol-containing food, food is allowed to cool to 70–75°C, at which point ethanol is added to the appropriate concentration (and the same volume of water is added to control food). Vials are transferred to a closed 40-cup food storage container (Rubbermaid Home Products, Fairlawn, OH) filled with 1 L of 7% ethanol (experimental conditions) or deionized water (control conditions). The ethanol bath ensures that animals are exposed to ethanol throughout development, which continues for another 10–16 days. Newly eclosed adult flies were counted and collected daily and kept at 25°C (∼12 h light, ∼12 h dark), and these data were used to calculate the percentage of flies that survived to adulthood.

To determine critical periods for the deaths of NPF signaling mutants on ethanol-containing food, larvae were collected from control food plates as they reached the desired developmental stage (first, second, or third larval instar), transferred to 7%-ethanol-containing food (or control food) and grown as described above. The total number of pupae was counted for each vial, and used to calculate: (1) The percentage of larvae that survived to pupation, and (2) the percentage of pupae that survived to adulthood.

Feeding Assays

Adult feeding assays were conducted on mated females. Flies analyzed for behavior were aged 2–5 days after eclosion and anesthetized briefly with CO_2 (<5 min) no less than 24 h before feeding assays. To measure feeding motivation, mated females were collected and kept food-deprived in vials with a 25 mm-diameter circle of water-saturated Whatman Grade 1 filter paper for 6 h prior to feeding. Twenty-five flies were allowed to feed on food mixed with 0.5% v/v of FD&C Blue Dye #1, and confirmation of food consumption was performed by visual assay for the presence of blue dye in the gut. Motivation was calculated as the proportion of flies that had eaten within 3–4 min of the start of the assay. For **Figure 1A**, we tested 12 control and 14 ethanol-reared groups of 25 flies each. For **Figure 2A**, we tested 3 groups of 25 flies for each combination of condition and genotype.

For larval feeding assays, first instar or young third instar larvae were collected at 16 or approximately 72 h after egg-laying (AEL), respectively. Third instar larvae were kept food-deprived for 2 h prior to feeding, while first instar larvae were not starved. 30 larvae were placed onto 3% agarose plates and allowed to feed on yeast paste containing 0.5% v/v FD&C Blue Dye #1 for 20 min. A larva was considered to have eaten by the presence of blue dye in 3/4 its length. This measurement is a slight modification of the protocol published by Wu et al. (2005), with an increased length that helps ensure accuracy in scoring. For **Figure 1B**, we tested 7 control and 7 ethanol-reared groups of 30 larvae each. For **Figure 2B**, we tested 6–11 groups of 30 larvae for each combination of condition and genotype. For **Figures 3A,B**, we tested 10–12 groups of 30 larvae for each combination of condition and genotype.

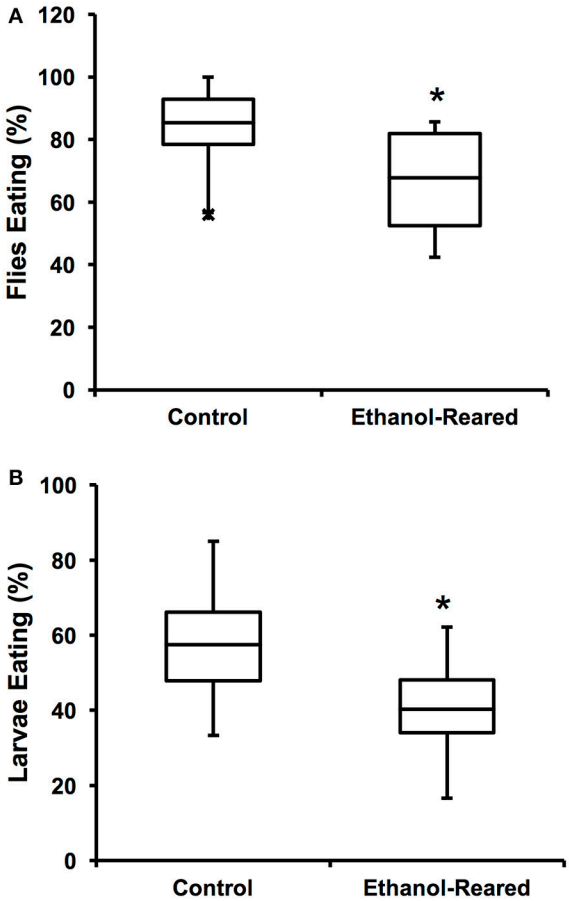

FIGURE 1 | Ethanol-rearing results in reduced feeding. **(A)** Percentage of adult female flies that ate within a 3-min interval after 6 h of food deprivation. ($N = 12$ for control, 14 for ethanol-reared. $P = 0.0056$, Student's *t*-Test). **(B)** Percentage of early third instar larvae that ate within a 20-min interval after 2 h of food deprivation ($N = 7$, $P = 0.035$, Student's *t*-Test). Center lines show the sample mean; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by "x." *$P < 0.05$.

Locomotion Assay

To measure locomotion, first instar larvae were transferred to a pre-marked spot on a 3% agarose plate. Larvae were allowed to move for 3 min, then their final position was marked on the plate using the point of a needle. Total distance traveled was measured as the distance of the direction connection from the starting point to the end point, and reported in mm.

Immunostaining and Imaging

Larvae were dissected in PBS + 0.3% Triton X-100 and tissues were fixed in 4% paraformaldehyde for 30 min. Tissues were then washed with 1x PBST and incubated for 2 days in a 1:750 dilution

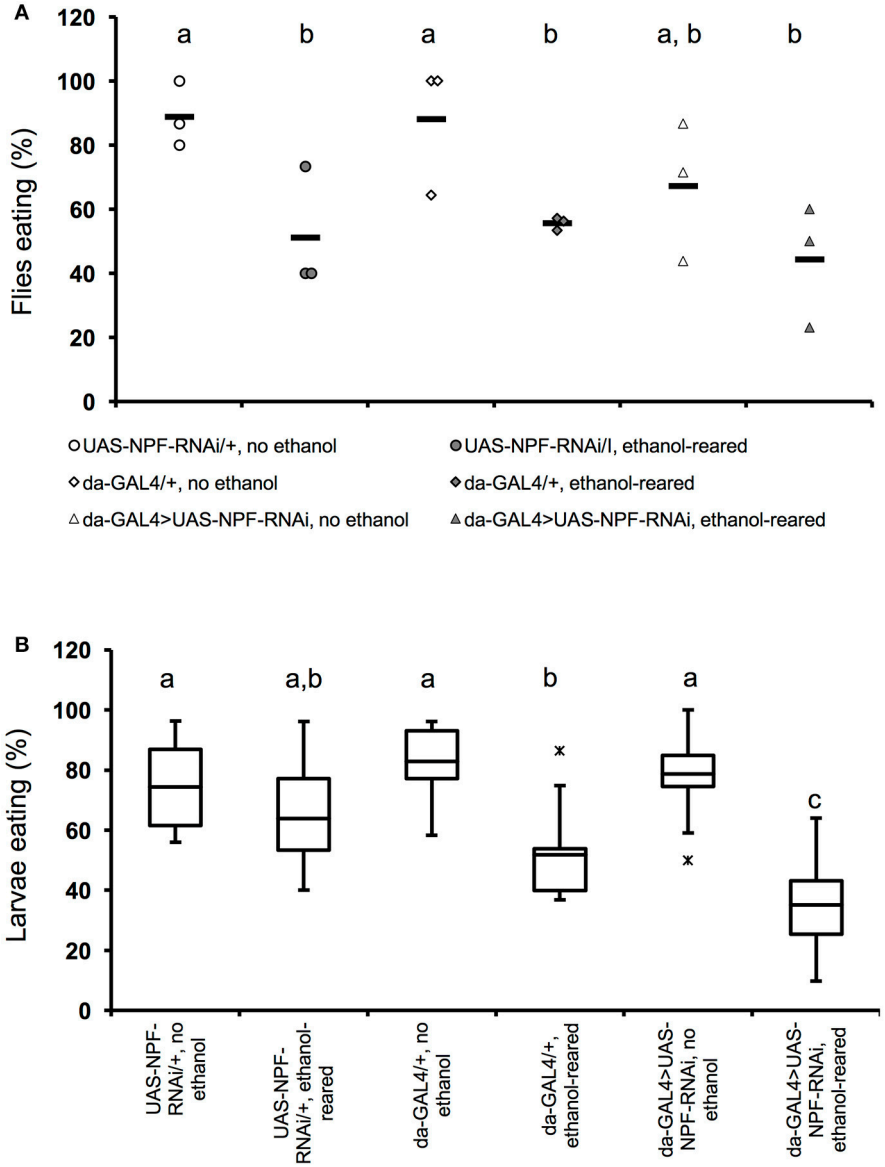

FIGURE 2 | Flies with partial loss of *npf* signaling show reduced feeding when ethanol-supplemented. **(A)** Percentage of adult female flies that ate within a 4-min interval after 6 h of food deprivation. ($N = 3$ for all conditions and genotypes, $P = 0.0022$ for the effect of ethanol-rearing, NS for the effect of loss of *npf* ($P = 0.24$, two-way ANOVA with Tukey's *post-hoc* analysis). Solid bars indicate means; individual data points are represented as open or closed circles. **(B)** Percentage of early third instar larvae that ate within a 20-min interval after 2 h of food deprivation ($N = 11, 11, 8\ 6, 10, 8$. $P < 0.0001$ for the effect of ethanol-rearing, $P = 0.11$ for the effect of loss of *npf*, $P = 0.0053$ for the interaction between genotype and condition, two-way ANOVA with Tukey's *post-hoc* analysis). Center lines show the sample mean; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by "x." Boxes sharing the same letter do not differ significantly, while boxes with different letters are significantly different ($P < 0.05$).

of rabbit anti-NPF (Ray Biotech, Norcross, GA) in 1X PBST. Secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Jackson ImmunoResearch, Burlington, Ontario) were diluted 1:750 in 1X PBST plus 5% normal goat serum. Stained samples were mounted in Vectashield anti-fade mounting medium for imaging and all images were collected on a Zeiss LSM 700 confocal microscope using a 20X objective.

Confocal images were quantitated using NIH Image J. For pixel area, thresholded pixels were counted for each image (threshold set to 90). For total fluorescence, the integrated density of pixels for entire images was measured. Images were then calibrated for background by calculating the average mean fluorescence of four circular regions of each image, multiplying that average by the total pixel area of the image, and subtracting that number from the integrated pixel density of the image.

Statistical Analyses

All samples were tested for normality using the Shapiro-Wilk normality test (Shapiro and Wilk, 1965). Data that were non-normal were log-transformed, and statistical analyses conducted

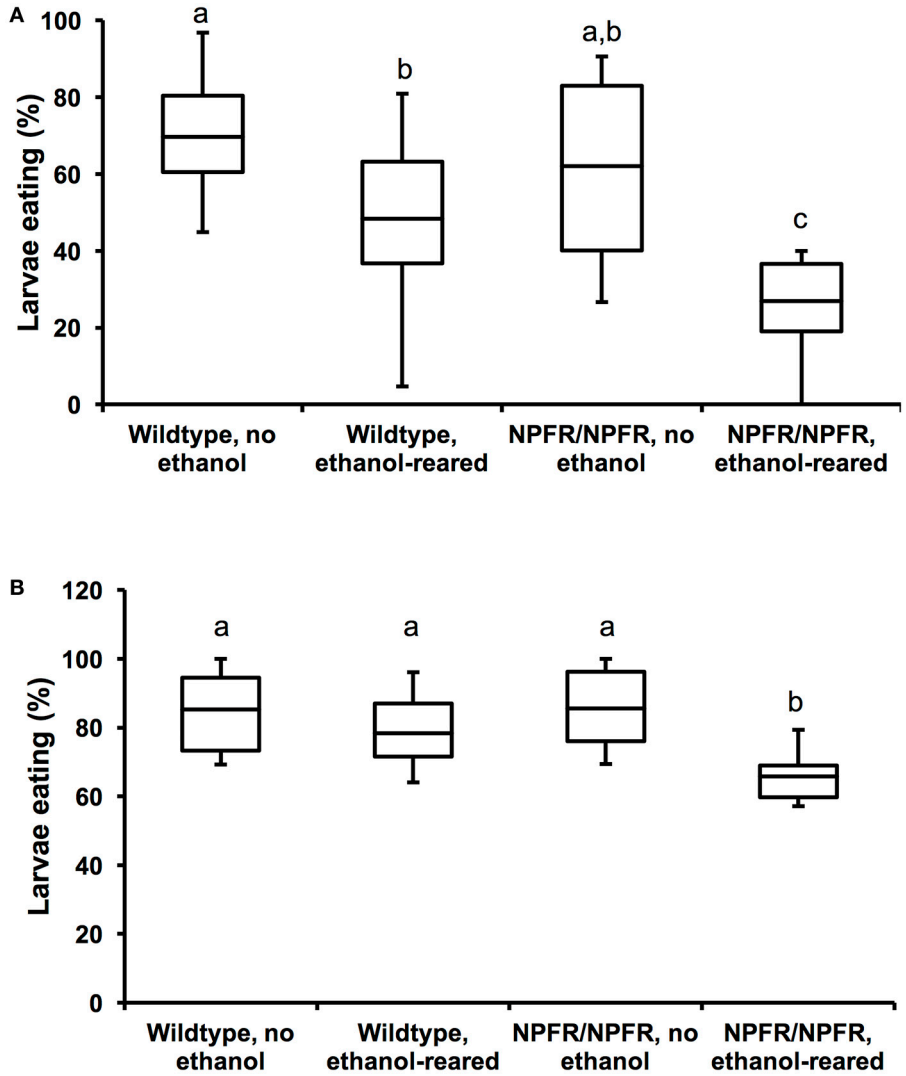

FIGURE 3 | Loss of NPF signaling enhances feeding deficits in ethanol-supplemented flies**. (A)** Percentage of unstarved first instar larvae that ate during a 20-min interval. Both ethanol-rearing and loss of *npfr1* result in reduced feeding ($N = 12, 11, 11, 11$, $P < 0.001$ for the effect of ethanol-rearing, $P = 0.009$ for the effect of mutation of *npfr1,* two-way ANOVA with Tukey's *post-hoc* analysis). **(B)** Percentage of unstarved first instar larvae that ate during a 45-min interval. ($N = 10, 10, 12, 10$, $P = 0.003$ for the effect of ethanol-rearing, $P = 0.13$ for the effect of *npfr1* mutation, $P = 0.046$ for the interaction between ethanol and genotype, two-way ANOVA with Tukey's *post-hoc* analysis). Center lines show the sample mean; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Boxes sharing the same letter do not differ significantly, while boxes with different letters are significantly different ($P < 0.05$).

on log-transformed data (**Supplemental Figure 1**). All statistical analyses were conducted using two-way ANOVA with a Tukey HSD *post-hoc* or Student's *T*-test unless otherwise indicated.

RESULTS

Ethanol-Rearing Results in Reduced Feeding

In order to assess the effects of DAE on feeding, we measured the flie's motivation to feed, as defined as the probability that a fly will choose to consume food within a specified time frame. To measure motivation, 25 adult female flies were starved for 6 h, then introduced into vials containing standard fly food mixed with blue dye and allowed to feed for 3 min. Feeding was assessed by the presence of blue color in the gut. Within 3 min of being transferred to blue food, 85 ± 3.6% of control animals contained food in 3/4 the length of the gut, compared with 68 ± 4.4% of ethanol-supplemented flies (**Figure 1A**, $N = 12–14$, $P = 0.0056$, Student's *t*-Test). These results demonstrate that DAE leads to a reduction of food present in the gut, a result similar to the reported effects of fetal alcohol exposure in humans and rodent models.

Next, we asked whether DAE also reduces larval feeding. We tested the feeding motivation of early third instar larvae

(at this stage of development, larvae are still actively eating). Our results were similar to those seen with adult flies: over the course of 20 min, 57.5 ± 6.5% of control larvae fed, compared with 40.3 ± 5.8% of ethanol-supplemented larvae (**Figure 1B**, $N = 7$, $P = 0.035$, Student's *t*-Test). As with the adult flies, these results demonstrate that the guts of ethanol-supplemented animals contain less food than those of control animals.

Ethanol-Induced Changes in Feeding Are Mediated by Neuropeptide F

In addition to being a known "hunger" signal, Neuropeptide F (NPF) has been implicated as a regulator of response to acute ethanol exposure (Wen et al., 2005). Specifically, flies with a partial loss of function in *npf* or *npfr1,* the gene encoding the NPF receptor, displayed resistance to ethanol-induced sedation after being exposed to ethanol vapor, whereas overexpression of *npf* resulted in increased ethanol sensitivity. Since both food and alcohol activate reward pathways (Devineni and Heberlein, 2013), and ethanol-supplemented wild-type flies eat less following starvation, we hypothesized that starved flies with decreased NPF signaling would eat less compared to genotypic controls after being reared in ethanol-supplemented food.

To test this hypothesis we used the ubiquitously-expressed GAL4 line *da-GAL4* to drive expression of a double-stranded RNA interference construct targeting *npf* (*UAS-npf^{RNAi}*). As expected, in adult animals, ethanol-rearing lead to a reduction in feeding. While 88–89% of unexposed genetic background controls ate during the 4-min observation window (88.9 ± 5.9% for *UAS-npf^{RNAi}/+;* 88.1 ± 11.9% for *da-Gal4/+*), only 51–56% of ethanol-supplemented controls ate (51.1 ± 11.1% for *UAS-npf^{RNAi}/+;* 55.6 ±1.2% for *da-Gal4/+*). (**Figure 2A**, $N = 3$ for all conditions, $p = 0.0022$ for the effect of ethanol, two-way ANOVA with Tukey HSD *post-hoc* analysis). The longer feeding window (4 vs. 3 min) in this experiment reflects the fact that, at earlier time points, differences in food intake were not significantly different, unlike in wildtype animals.

Consistent with its role in feeding, reducing *npf* expression also resulted in reduced feeding. Only 67.3 ± 12.6% of unexposed *da-Gal4/+; UAS-npf^{RNAi}/+* fed. Finally, rearing *da-Gal4/+; UAS-npf^{RNAi}/+* animals in ethanol reduced feeding still further: only 44.4 ± 11% of ethanol-supplemented *da-Gal4/+; UAS-npf^{RNAi}/+* animals ate. (**Figure 2A**). The effect of genotype on feeding was not statistically significant, likely due to small sample size ($N =$ 3 for all combinations, $P = 0.24$, two-way ANOVA with Tukey *post-hoc* analysis). Thus, animals with reduced NPF signaling ate less than animals with intact NPF signal transduction, and this effect may combine with the strong effect of ethanol to reduce feeding still further.

In third instar larvae, we saw similar results—ethanol rearing alone significantly reduces feeding motivation: 74–83% of unexposed genetic background controls ate during the observation window (74.3 ± 4.4% for *UAS-npf^{RNAi}/+;* 82.8 ± 4.5% for *da-Gal4/+*), only 52–64% of ethanol-supplemented controls ate (63.8 ± 5.8% for *UAS-npf^{RNAi}/+;* 51.9 ± 7.5% for *da-Gal4/+*). (**Figure 2B**, $N = 6$–11, $p < 0.0001$ for the effect of ethanol, two-way ANOVA with Tukey HSD *post-hoc* analysis). In larvae with reduced NPF, we saw no effect on feeding in the absence of ethanol (78.8 ± 4.1% of unexposed *da-Gal4/+; UAS-npf^{RNAi}/+* ate during the observation window), but when *da-Gal4/+; UAS-npf^{RNAi}/+* larvae were reared in ethanol, we saw a significant effect on feeding: only 35 ± 6.1% of animals ate during the observation period (**Figure 2B**). The effect of genotype on feeding alone was not statistically significant ($N =$ 8–10, $p = 0.11$ for the effect of genotype, two-way ANOVA with Tukey HSD *post-hoc* analysis). However, we detected a significant interaction between genotype and ethanol, and *post-hoc* analyses determined that this interaction was due to the effect of ethanol on feeding in *da-Gal4/+; UAS-npf^{RNAi}/+* larvae ($P = 0.0053$ for the interaction between ethanol and genotype, two-way ANOVA with Tukey HSD *post-hoc* analysis). Thus, as with the adult experiments described above, reducing NPF signaling alone did not have a significant effect on feeding, but in this case there was a strong effect of ethanol on feeding in *da-Gal4/+; UAS-npf^{RNAi}/+* animals.

In order to further test for an effect of NPF signaling on feeding in ethanol-supplemented animals, we attempted to test the feeding behavior in animals homozygous for a genetically null (Lee et al., 2017) mutation in the fly NPF receptor *npfr1* (*npfr1^{c01896}).* Surprisingly, we found that we could recover very few *npfr1^{c01896}/npfr1^{c01896}* adults or third instar larvae when the flies were reared in ethanol. These results are described in detail below, and in **Figure 3**. As a result of this lethality, we decided to test first instar larval feeding behavior.

When unstarved, ethanol-supplemented first instar larvae (approximately 16 h post hatching) are allowed to feed on blue food for 20 min, 48.4 ± 6.5% of wildtype and 26.9 ± 3.7% of *npfr1^{c01896}/npfr1^{c01896}* larvae eat, compared with 69.7 ± 4.2% of unexposed wildtype and 62.1% of unexposed *npfr1^{c01896}/npfr1^{c01896}* animals (**Figure 3A**, $N =$ 11–22, $p <$ 0.0001 for the effect of ethanol, $p = 0.009$ for the effect of genotype, two-way ANOVA with Tukey HSD *post-hoc* analysis). Most strikingly, as with *da-Gal4/+; UAS-npf^{RNAi}/+* larvae, there is little effect of the *npfr1^{c01896}* mutation on feeding under control conditions, but when *npfr1^{c01896}/npfr1^{c01896}* mutant animals are reared in ethanol, there is a dramatic reduction in feeding (**Figure 3A**). Thus, while individually, loss of NPF signaling and ethanol reduce feeding by 25 and 42%, respectively, the combination of the two conditions reduces feeding by 61%.

We repeated this assay for a longer feeding time (45 min), and the results were similar: 78.3 ± 3.6% of wildtype ethanol-supplemented larvae and 65.8 ± 2.5% of *npfr1^{c01896}/npfr1^{c01896}* larvae ate, compared with 85.3 ± 3.9 and 85.5 ± 3.2% of unexposed larvae. In this experiment, we again see no effect of the *npfr1^{c01896}* mutation on feeding under control conditions, and ethanol-supplemented flies appeared to "catch up" over the longer observation time, such that there is no significant effect of ethanol on feeding (**Figure 3B**, $N =$ 20–22, $p = 0.126$ for the effect of ethanol, two-way ANOVA with Tukey HSD *post-hoc* analysis).

However, there was a significant effect of genotype, as well as a significant interaction between ethanol and genotype, and this interaction is again due to the reduction in feeding by ethanol-supplemented *npfr1^{c01896}/npfr1^{c01896}* larvae (**Figure 3B**,

$p = 0.003$ for the effect of genotype, $p = 0.046$ for the interaction between genotype and ethanol, two-way ANOVA with Tukey HSD *post-hoc* analysis). Thus, the combination of ethanol exposure during development and loss of NPF signaling results in a greater reduction in feeding than either condition alone.

In order to test whether reduced feeding in first-instar larvae could be a result of increased ethanol-induced sedation, as animals in this assay were taken directly from ethanol-containing (or control) food for use in the assay, we measured the distance traveled in 3 min by first-instar larvae under each set of conditions (**Supplemental Figure 1**). This experiment showed that ethanol does not decrease movement of the animals; in fact, the only effect of ethanol was to increase the average distance traveled in wildtype ethanol-supplemented animals ($N = 10$, $p = 0.025$, two-way ANOVA with Tukey HSD *post-hoc* analysis), while there was no difference between mutant and wildtype animals, nor any effect of ethanol-rearing on the movement of mutant animals ($N = 10$ for all conditions, $p = 0.82$, two-way ANOVA with Tukey HSD *post-hoc* analysis). It should be noted, however, that animal-to-animal variability in distance traveled is large under all conditions.

Loss of NPF Signaling Enhances Ethanol-Induced Developmental Lethality

Ethanol exposure during larval development leads to a reduction in survival and induces a developmental delay (McClure et al., 2011). In addition, downregulation of *npf* using *npf*-GAL4 drivers in younger larvae results in cessation of feeding and onsets of social behavior (cooperative burrowing) indicative of older third instar larvae, suggesting that NPF signaling induces changes during development (Wu et al., 2003). Finally, NPF signaling is required for adult ethanol sensitivity (Wen et al., 2005). However, to our knowledge, there is no known effect of loss of NPF signaling on survival. We were therefore surprised to discover that homozygosity for $npfr1^{c0189}$ drastically reduces survival of ethanol-supplemented flies. 59 ± 3.3% of control flies survived to eclosion when reared in food containing 7% ethanol ($N = 12$), whereas only 21 ± 3.2% of *npfr1* mutant flies survived ($N = 12$). We found a significant interaction between genotype and condition ($N = 48$, $P < 0.0001$ for the interaction between genotype and condition, two-way ANOVA with Tukey HSD *post-hoc* analysis). Survival of *npfr1* mutant flies was no different from wildtype when reared in control food (81 ± 1.6% for wildtype; 73 ± 1.9% for *npfr1*, $N = 12$ for each condition, insignificant according to Tukey's HSD *post-hoc* analysis), confirming that *npfr1* is not required for survival under normal conditions (**Figure 4A**). These results indicate that NPF signaling is protective against ethanol-induced developmental lethality.

Ethanol-Induced Lethality in *npfr1* Mutant Flies Occurs During Early Larval Development

Because NPF signaling is involved in food reward, and ethanol rearing causes reduced food intake, we hypothesized that animals lacking NPF signaling might not eat enough to survive. Flies eat the most during early larval development, stopping in the late third instar prior to pupation. To assess for ethanol-induced toxicity in *npfr1* mutant flies, we reared flies on food supplemented with 7% ethanol for discrete developmental periods and measured survival to pupation. The results of this analysis are presented in **Table 1**.

In this experiment, only 15.8 ± 1.7% of *npfr1* mutant flies exposed to ethanol for the entirety of larval development pupated, compared with 60.3 ± 2.7% of wildtype flies. When the exposure period was limited to the second and third larval instars, 36 ± 14.6% of *npfr1* mutant animals pupated, while 71.3 ± 6.1% of wildtype animals began metamorphosis. However, when animals were exposed only during the third larval instar, *npfr1* mutant survival was comparable to that of controls: 76 ± 1.5% of *npfr1* mutant flies pupated, and, of those, 85.6 ± 7.4% survived to adulthood. Similarly, 72.5 ± 3.4% of wildtype animals exposed to ethanol during the third instar pupated, and, of those, 77.8 ± 1.9% survived to adulthood. Taken together, these data demonstrate that the critical period for ethanol-induced toxicity in *npfr1* mutant flies is primarily during the first and second instar larval stages, while *npfr1* mutant animals are relatively insensitive to ethanol exposure during the third instar and metamorphosis.

Developmental Ethanol Exposure Alters NPF Expression in Larval Brains

Adult flies ablated of NPF/NPFR1 neurons show decreased sensitivity to ethanol-induced sedation; in addition, sexual deprivation in male flies results in both increased drinking and downregulation of NPF (Wen et al., 2005; Shohat-Ophir et al., 2012). Further, activation of NPF-expressing neurons reduces ethanol reward (Shohat-Ophir et al., 2012). Thus, NPF signaling is a likely molecular target of ethanol exposure. However, little is known about the expression of NPF in response to ethanol exposure, and nothing is known about the effects of chronic developmental ethanol exposure on NPF expression.

To examine NPF expression in ethanol-supplemented larvae, we labeled third instar larval brains with anti-NPF antibodies (**Figures 4B,C**). NPF is expressed in four cells in the larval protocerebrum (Brown et al., 1999), which send projections to the central brain as well as the subesophageal ganglion (SEG). The SEG contains nerves that control larval foraging and feeding (Altman and Kien, 1987) (**Figures 4B,C**). Staining of the cell bodies is intense in both conditions, and we see no change in this staining in the brains of ethanol-reared animals. However, we observed differences in the distribution of fluorescence in the brains of ethanol-supplemented larvae, in both the central brain and the SEG (**Figures 4C,D**). In particular, there is an increase of NPF-expressing neuronal projections to the SEG, very similar to the results obtained when larvae are fed on highly palatable diets (Shen and Cai, 2001). We confirmed these observations through quantitation of both fluorescence and pixel density. We find that total pixel area is significantly increased in the brains of ethanol-supplemented larvae (**Figure 4D**, $N = 7$ brains for each condition, $P = 0.0473$, Student's *t*-Test), while overall fluorescence is no different (**Figure 4E**, $N = 7$ brains

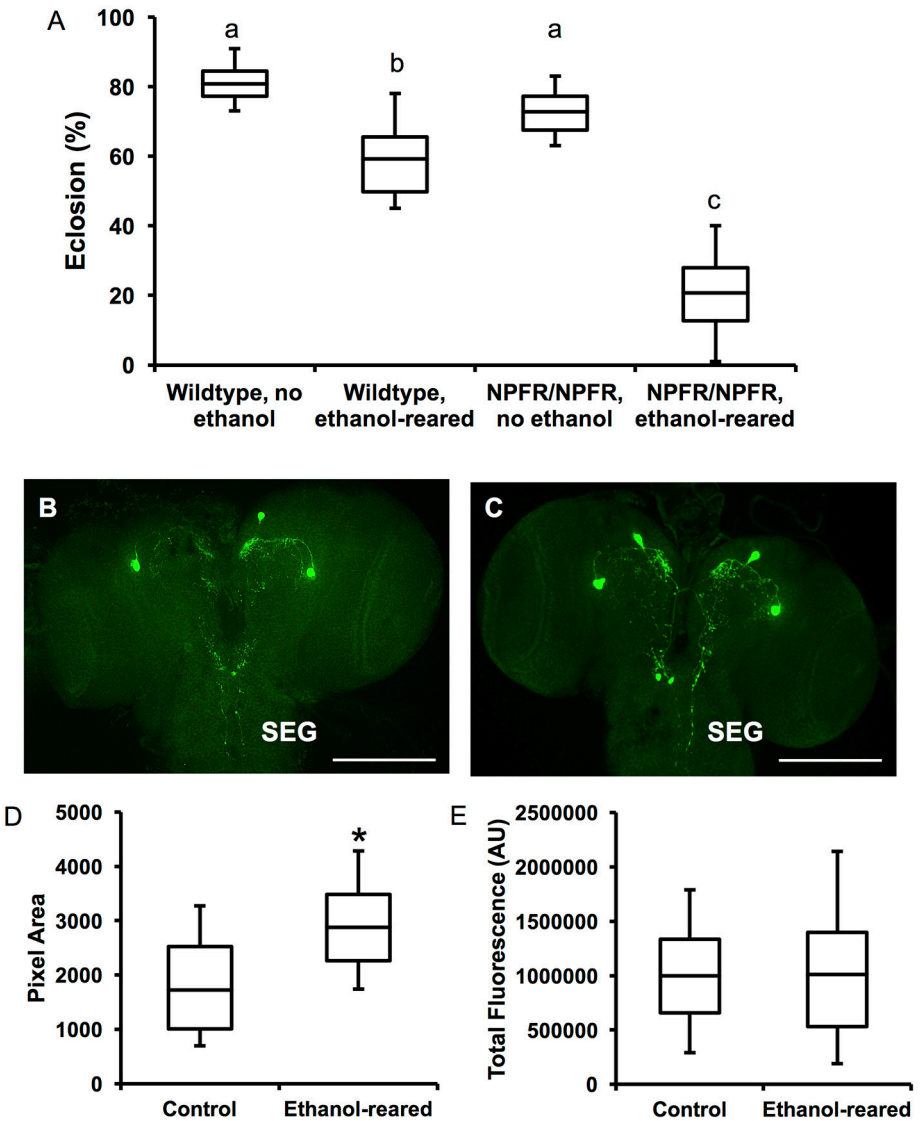

FIGURE 4 | *npfr1* is necessary for survival of ethanol-supplemented flies, and developmental ethanol exposure induces a change in NPF distribution in larval brains. **(A)** Survival to adulthood of *npfr1* mutant flies reared in ethanol. ($N = 12$ for all conditions and genotypes, $P < 0.0001$ for the effect of ethanol-rearing, $P < 0.0001$ for the effect of mutation of *npfr1*, and $P < 0.0001$ for the interaction between treatment and genotype, two-way ANOVA with Tukey's *post-hoc* analysis.) Boxes sharing the same letter do not differ significantly, while boxes with different letters are significantly different ($P < 0.05$). **(B)** Confocal reconstruction of NPF-expressing neurons in the brain of a 3rd-instar larva reared in ethanol-free control food. Scale bar = 0.5 μM. **(C)** Confocal reconstruction of NPF-expressing neurons in the brain of a 3rd-instar ethanol-reared larva. Scale bar = 0.5 μM. **(D)** Quantitation of pixel area for anti-NPF fluorescence in control and ethanol-reared third-instar larval brains. ($N = 7$ brains for each condition, $P = 0.0473$, Student's *t*-Test). **(E)** Quantitation of total anti-NPF fluorescence in control and ethanol-reared third-instar larval brains. ($N = 7$ brains for each condition, $P = 0.97$, Student's *t*-Test.) Center lines show the sample mean; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. *$P < 0.05$.

for each condition, $P = 0.97$, Student's *t*-Test). These results, which indicate more overall foci of NPF fluorescence without an increase in the total amount of fluorescence, suggest that DAE alters the distribution of NPF in the larval brain, perhaps enhancing the activity of the NPF circuitry. We hypothesize that this co-opting of the natural reward circuitry by ethanol may also serve as a compensatory mechanism that normally prevents starvation of ethanol-exposed larvae by increasing feeding and foraging behavior.

DISCUSSION

Drugs of abuse, including alcohol, engage the natural reward systems that animals have evolved to ensure pursuit of food and sex, which are essential to the continued existence of both the individual and the species (Kelley and Berridge, 2002; Koob, 2009; Kaun et al., 2011). The neuropeptide NPY/NPF modulates both food and mating reward, and, in adult animals, NPF signaling appears to reduce both the rewarding effects and the

TABLE 1 | Critical periods for ethanol-induced toxicity in *npfr1* mutant flies.

Ethanol exposure	% Survival to pupation (± s.e.m.)	
	Wildtype	*npfr1/npfr1*
No exposure	87.5 (±1.9)	85.3 (±2.6)
L1–L3	60.3 (±2.7)	15.8 (±1.7)***
L2–L3	71.3 (±6.1)	36.0 (±14.6)*
L3	72.5 (±3.4)	76 (±1.5)
M to adult	77.8 (±1.9)	85.6 (±7.4)

*L1, first larval instar; L2, second larval instar; L3, third larval instar, M, metamorphosis. Each rearing condition represents four vials of 100 animals/vial (n = 4). Data are presented as mean ± s.e.m. *P < 0.05, ***P < 0.0001, two-way ANOVA with Tukey HSD post-hoc analysis.*

sedative effects of ethanol (Thiele et al., 1998; Shohat-Ophir et al., 2012).

Ethanol exposure during development results in a variety of phenotypes in flies and mammals, including decreased survival, developmental delays, increased oxidative stress and changes in fat metabolism, and a variety of behavioral changes (Jones and Smith, 1973; Clarren and Smith, 1978; Kvigne et al., 2004; McClure et al., 2011; Dörrie et al., 2014; Logan-Garbisch et al., 2014). Most relevant to the current work are altered feeding behavior and responses to drugs of abuse. Children with FASD can have a variety of feeding problems (Clarren and Smith, 1978), and DAE causes reduced ethanol sedation in both flies and mammals (Middaugh and Ayers, 1988; McClure et al., 2011).

We have a well-established fly model for DAE, and here we have used it to investigate the possible effects of DAE on feeding and reward. Here, we show that ethanol-supplemented flies are less likely to eat than control animals, and that this ethanol-induced behavior change is exacerbated by loss of NPF signaling. Additionally, we show that DAE results in altered NPF signaling in larval brains. Finally, we demonstrate that ethanol-supplemented *npfr1* mutant animals die in early larval development and provide evidence that the cause of death may be starvation.

DAE Changes Feeding Behavior Through an Unknown Mechanism

DAE results in reduced feeding in flies at all stages of development tested. Flies may display reduced motivation to feed for at least three reasons: they may feel less hungry, they may be behaviorally less able to find or consume food, or they may find food less rewarding and thus be less likely to eat (and eat less when they do feed). Our data do not distinguish directly between these possibilities. We are currently investigating the effects of ethanol-rearing on additional mutations that alter feeding and foraging behavior, in order to begin to address this question.

In addition, it is possible that, because ethanol is an energy-providing nutrient, animals reared in ethanol-supplemented food may find the "test" food, which lacks ethanol, to be a lower-quality food source, and stop or slow down feeding temporarily. We think this explanation is unlikely, because previous results have shown ethanol-containing food to be unpalatable (Kaun et al., 2011). However, we are unable to formally rule out such an explanation.

DAE Leads to Altered Distribution of NPF in Larval Brains

Third instar larvae reared in ethanol show increased anti-NPF fluorescence in the axons of NPFergic neurons projecting to the central brain as well as the SEG. This is consistent with the known effects of sugar on NPF cell projections (Shen and Cai, 2001), and would be expected to increase foraging behavior for at least two reasons: first, the SEG contains both afferent and efferent nerves that regulate larval foraging behavior (Altman and Kien, 1987). Second, increased NPF signaling prolongs foraging and feeding behavior in third instar larvae (Wu et al., 2003). In addition, because ethanol-containing food is unpalatable (Kaun et al., 2011), and NPF signaling increases the willingness of larvae to ingest unpalatable food (Wu et al., 2005), this increase would be expected to increase the overall amount of food consumed.

It is also possible that NPF release is being inhibited in ethanol-reared animals, and the increased anti-NPF foci in the SEG reflects a loss of NPF neuron function rather than increased signaling in these animals. We think this explanation is less likely due to previous data demonstrating the NPF signaling is enhanced by rewarding substances, and that the activation of NPF neurons in flies is sufficient to mimic the effects of ethanol reward, suggesting that alcohol does not prevent the release of NPF in flies, but, rather, tends to enhance it (Shohat-Ophir et al., 2012).

NPF is expressed in both the brain and in enteroendocrine cells in the midgut of larvae as well as adult flies (Brown et al., 1999). Here, we focus on the expression of NPF in the larval brain, but it should be noted that our experiments would alter midgut NPF expression, and we have not investigated possible involvement of midgut cells in the regulation of hunger in ethanol-supplemented animals.

NPF Signaling Is Essential in the Presence of Ethanol

Loss of NPF signaling through a genetically null mutation in *npfr1* leads to death during early larval stages for most ethanol-reared animals. This was a surprising result, as, to our knowledge, there has been no lethality previously associated with loss of NPF signaling. In our experiments, *npfr1* is not required for survival under normal environmental conditions, as homozygosity for a null mutation in *npfr1* results in no significant change in survival compared to wildtype. Thus, we have identified an environmental condition under which *npfr1* is an essential gene.

Signaling by the Drosophila insulin-like peptides (DILPs) also affects food intake (Wu et al., 2005). Specifically, it is thought that DILPs signal to and inhibit the activity of NPFR1-expressing cells, such that, when animals are well-fed, DILP signaling leads to reduced feeding (and reduced acceptance of noxious foods). We have previously shown that DAE leads to a 75% reduction in expression of the Drosophila insulin receptor (InR) (McClure et al., 2011). Thus, it is interesting to speculate that the combination of reduced insulin signal and increased NPF signal

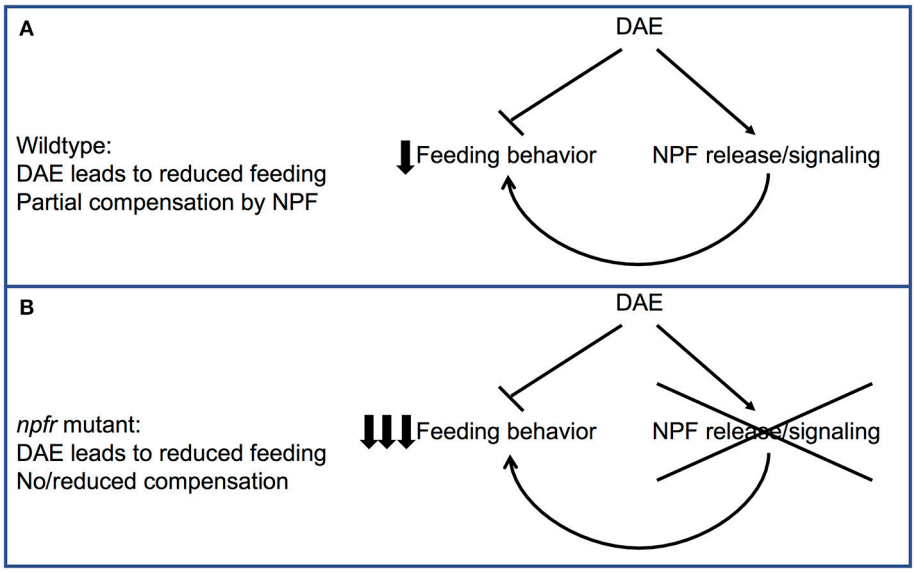

FIGURE 5 | Model for the effect of loss of NPF signaling on feeding in ethanol-supplemented larvae. **(A)** In wildtype animals, eating food containing ethanol leads to a decrease in feeding behavior as well as an increase in NPF release/signal transduction. This increase in NPF release would be expected to partially compensate for the effect of ethanol on feeding. **(B)** In *npfr* mutant animals, this compensatory upregulation in feeding behavior cannot occur, leading to a much larger reduction in feeding.

upon DAE would lead to increased acceptance of ethanol-tainted food. This hypothesis predicts that mutations leading to reduced insulin signaling should exert a protective effect in the presence of an NPFR mutation, and, conversely, that overexpression of DILPs should exacerbate the effects of an NPFR mutation. We are currently performing experiments to test these predictions.

Taken together, these data suggest a model in which DAE causes increased NPF signaling, as well as abnormal feeding through as-yet unidentified molecular targets (**Figure 5A**). In this model, increased NPF expression in ethanol-supplemented animals has evolved in part as a compensatory or protective mechanism, in which NPF expression offsets to some degree the reduced food intake caused by DAE. When flies lack the ability to increase NPF signaling due to a mutation in *npfr1*, the combination of reduced feeding due to ethanol exposure and loss of a reward pathway that would serve to drive increased foraging and food intake may result in larvae that eat too little to sustain growth and development (**Figure 5B**).

It should be noted that, though feeding was significantly reduced in *npfr1* mutant animals reared in ethanol, more than half of these animals have nevertheless consumed food by the end of a 45-min observation period (**Figure 3B**). It is possible that, despite this, the volume of food consumed by these animals is insufficient to sustain growth, leading to the lethality associated with the combination of ethanol and reduced NPF signaling. It is also possible that NPF signaling is affecting survival through an as-yet-unidentified mechanism that impacts survival. One possibility is that ethanol metabolism is altered in *npfr1* mutant animals, such that ethanol is toxic to these mutants at the concentrations described here. We think that possibility is unlikely, given that we see no sedative effects on locomotion in *npfr1* mutant animals when reared in food containing 7% ethanol (**Supplemental Figure 1**).

In conclusion, we have shown that flies reared in ethanol display feeding changes consistent with the effects of FASD in mammals, and that DAE induces NPF signaling to regions of the central nervous system that drive foraging and food intake, and, finally, that loss of this compensatory mechanism results in additional reductions in food intake and a very high rate of developmental lethality, suggesting that the cause of death for *npfr1/npfr1* mutant larvae may be "voluntary" starvation. Our data also suggest that NPY receptor agonists may have potential for treating feeding difficulties associated with FASD.

AUTHOR CONTRIBUTIONS

AG and RF: Conceived and designed the experimental plan; AG, HG, and BU: Performed the experiments; AG, HG, BU, and RF: Analyzed the data; AG and RF: Drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00237/full#supplementary-material

Supplemental Figure 1 | Ethanol-rearing does not cause sedation in first instar larvae. Distance traveled in in ethanol-free medium in 3 min by first instar larvae. Distance traveled was similar for wildtype larvae reared in control food, *NPFR1/NPFR1* larvae reared in control food, and *NPFR1/NPFR1* larvae reared in ethanol-containing food. Wildtype larvae reared in ethanol-containing food moved more, on average, than all other conditions. Locomotion data were not normally distributed. Statistics were performed on log-transformed data. ($N = 10$ for all conditions, $P = 0.82$ for the effect of genotype, $P = 0.025$ for the effect of ethanol, $P = 0.079$ for the interactions between ethanol and genotype.) Center lines show the back-transformed sample mean; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

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Effects of Ethanol on Sensory Inputs to the Medial Giant Interneurons of Crayfish

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Crayfish are capable of two rapid, escape reflexes that are mediated by two pairs of giant interneurons, the lateral giants (LG) and the medial giants (MG), which respond to threats presented to the abdomen or head and thorax, respectively. The LG has been the focus of study for many decades and the role of GABAergic inhibition on the escape circuit is well-described. More recently, we demonstrated that the LG circuit is sensitive to the acute effects of ethanol and this sensitivity is likely mediated by interactions between ethanol and the GABAergic system. The MG neurons, however, which receive multi-modal sensory inputs and are located in the brain, have been less studied despite their established importance during many naturally occurring behaviors. Using a combination of electrophysiological and neuropharmacological techniques, we report here that the MG neurons are sensitive to ethanol and experience an increase in amplitudes of post-synaptic potentials following ethanol exposure. Moreover, they are affected by GABAergic mechanisms: the facilitatory effect of acute EtOH can be suppressed by pretreatment with a GABA receptor agonist whereas the inhibitory effects resulting from a GABA agonist can be occluded by ethanol exposure. Together, our findings suggest intriguing neurocellular interactions between alcohol and the crayfish GABAergic system. These results enable further exploration of potentially conserved neurochemical mechanisms underlying the interactions between alcohol and neural circuitry that controls complex behaviors.

Keywords: alcohol, crayfish, neurons, muscimol, inhibition

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INTRODUCTION

Alcohol is one of the most abused drugs worldwide with devastating impacts on health and economy. Despite its well-documented negative effects, research aimed at understanding the underlying neurobehavioral mechanisms has progressed slowly. Unlike other drugs of abuse, alcohol exposure produces biphasic behavioral responses, which are expressed by initial hyperexcitability followed by motor incoordination and sedation. In addition, alcohol exerts its cellular effects by interacting with multiple neurotransmitter system, namely serotonin (Barr et al., 2003; Wolf and Heberlein, 2003; Ferraz and Boerngen-Lacerda, 2008) and y-amino-butyric acid (GABA) (Mehta and Ticku, 1988; Mihic et al., 1997; Lobo and Harris, 2008; Kumar et al., 2009).

Given the complexity of alcohol's interplay with neural function, more recent research efforts have focused on animal models that display easily quantifiable behaviors and nervous systems that

contain fewer and more accessible neurons. Across invertebrate studies, the symptoms of ethanol (EtOH) intoxication are highly conserved. This includes research performed in nematodes (Topper et al., 2014), fruit flies (Lee et al., 2008), and crayfish (Friedman et al., 1988; Macmillan et al., 1991; Blundon and Bittner, 1992; Swierzbinski et al., 2017). Similar to vertebrates, lower doses produce disinhibition while higher doses produce increased incoordination and/or decreased activity. The nematode, *Caenorhabditis elegans*, has proven a powerful tool for investigating the cellular and molecular targets of addictive substances, including alcohol (Schafer, 2004; Mitchell et al., 2007; Topper et al., 2014). The fruit fly, *Drosophila melanogaster*, has been used to study the effects of alcohol on behavior after genetic manipulations, which provided new insights into the interactions between EtOH and neurobehavioral mechanisms (Kong et al., 2010; van der Linde and Lyons, 2011; Devineni and Heberlein, 2013; Robinson and Atkinson, 2013). Crayfish have been used to study the effects of EtOH on behavior and synaptic transmission at the neuromuscular junction. Friedman et al. (1988) demonstrated an increase in righting time (when crayfish were placed on their back) in newly intoxicated animals, but not in those that had been chronically exposed. This study also reported a dose-dependent effect, with lower concentrations of EtOH increasing, and higher concentrations reducing, transmitter release and synaptic potentials at the neuromuscular junction. In addition, crayfish have been used to study drugs of abuse other than alcohol. For example, Huber et al. (2011) demonstrated that crayfish display conditioned place preference following injections of cocaine and amphetamines, and morphine injections into the crayfish brain have been shown to facilitate locomotion and exploratory behaviors (Imeh-Nathaniel et al., 2014).

Previous results from our lab demonstrated that juvenile crayfish are behaviorally and physiologically sensitive to EtOH and that this sensitivity is affected by recent social experience (Swierzbinski et al., 2017). When placed in a water-filled tank that contained various concentrations of EtOH, crayfish became more intoxicated over time in a dose-dependent manner, and they progressed through distinct stages of behavioral change. This included increased locomotor activity displayed by spontaneous tail-flips in the absence of any threat, which was followed by decreased activity and incoordination when they fell on their backs, eventually unable to right themselves. Surprisingly, animals housed with conspecifics prior to EtOH exposure progressed through these stages of increasing intoxication more rapidly than animals that were socially isolated. Importantly, we were further able to demonstrate parallel effects of EtOH on single neurons using intracellular electrophysiology. We found that the excitability of the crayfish's lateral giant (LG) escape neurons was facilitated by EtOH exposure and, similar to our observations in freely behaving animals, the amount of EtOH-induced facilitation was dependent on recent social experience. Lastly, we also found that removing brain-derived tonic GABAergic inhibition to the local LG circuit reduced the sensitivity of the LG neurons to EtOH exposure, which led us to hypothesize that EtOH interacts with GABA receptors in the LG circuit.

Our current work expands on this notion utilizing a different set of crayfish giant neurons, which are key components of the medial giant (MG) escape circuit. We decided to target the MG neurons for three main reasons: (1) To see if the effects of EtOH would generalize across different escape circuits in crayfish, and (2) To contribute to our understanding of the neurochemical mechanisms in a circuit that is currently understudied, and (3) To provide a first glimpse into the interplay between alcohol and GABAergic inhibition in a circuit that receives multi-modal sensory activation and is of behavioral relevance.

While the LG circuit is one of the best described neural circuits in the animal kingdom, the MG circuit is much less well understood (Edwards et al., 1999). Both the LG and the MG circuits are considered to be hardwired and to produce reflexive, stereotyped escape behaviors. If a predatory attack is directed to the rear, activation of the LG neurons is sufficient to initiate an escape response that pitches the animal upward and forward away from the stimulus. Conversely, a frontal attack will activate the MG neurons and elicit a behavioral sequence that thrusts the animal backward (Herberholz et al., 2004). The LGs and MGs have many features in common: They can both be activated by strong and phasic sensory inputs, and a single impulse is necessary and sufficient to coordinate the entire escape response. Both travel the length of the nervous system and share most of their motor and inhibitory elements, which are often connected by electrical synapses (Wine and Krasne, 1982). However, the circuits also exhibit some important differences, which led to an imbalance in research efforts. Most experiments have been devoted to the LG circuit, which receives mechanosensory inputs from innervated hairs and proprioceptors on the abdomen and tail as well as subthreshold modulatory inputs from rostral body areas (Liu and Herberholz, 2010). The physiological characteristics of the LG circuit can be studied in each of the six abdominal body segments, which are readily accessible, and can be isolated from the rest of the animal. Analysis of the MG neurons has progressed much slower. The MG neurons, a pair of cells that are electrically coupled to each other, have their cell bodies located in the supraesophageal ganglion (brain). Activation of one MG in the brain typically activates the other MG, resulting in a pair of action potentials that descend toward the abdomen. The MG axons project along the entire nerve cord where the descending action potentials activate motor neurons connected to flexor muscles in all abdominal segments (Wiersma, 1947; Wine and Krasne, 1972; Wine, 1984).

The role of inhibition has been studied intensively in the LG circuit, but is mostly unknown for the MG circuit. The LG neurons undergo both phasic and tonic GABAergic inhibition (Roberts, 1968; Vu and Krasne, 1993; Vu et al., 1997). Ambient GABA released "globally" by interneurons descending from the brain has prolonged, modulatory function in regulating LG neuron excitability. The synapses for tonic inhibition are located on the dendrites of the LG neurons, and the inhibitory effects are mediated by ligand-gated chloride channels (Vu and Krasne, 1993; Vu et al., 1993). Up- and down-regulation of tonic inhibition and corresponding neuronal threshold has been observed in a number of situations, e.g., during feeding or restraint (Krasne and Wine, 1975; Krasne and Lee, 1988).

In addition, the LG neurons also rapidly inhibit themselves after they discharge. This phasic "recurrent" inhibition is thought to happen more proximally, near the spike initiation zone, which is located at the initial axon segment. The behavioral purpose of recurrent inhibition is to prevent subsequent activation of the neurons after producing a single tail-flip. Recurrent inhibition can prevent firing of the LG neurons regardless of the magnitude of excitatory inputs, whereas strong excitation can override tonic inhibition. Chloride-mediated inhibition in crayfish often causes neurons to depolarize due to an outflow of chloride ions. In the LG circuit, the opening of such chloride channels shunts the current that flows toward the LG spike initiation zone (Roberts, 1968; Edwards et al., 1999).

Unlike the LG neurons, which receive primarily mechanosensory excitatory inputs, sensory activation of the MG neurons has not been mapped out in detail. However, Glantz and Viancour (1983) showed that they receive excitatory inputs from the antenna I, the main olfactory organ, and the antenna II, the primary mechanosensory organ. The antenna II also contains a smaller number of bimodal chemotactile receptors in a number of crustaceans, but it is unknown whether these non-olfactory receptors provide any inputs to the MG neurons (Sandeman et al., 1992; Liu and Herberholz, 2010; Derby and Weissburg, 2014). Visual activation of the MG neurons has been confirmed behaviorally (Liden and Herberholz, 2008; Liden et al., 2010). For example, when juvenile crayfish were presented with a threatening visual stimulus while they were approaching a food odor release point, they either displayed a freezing response or an escape tail-flip. All tail-flips were mediated by activity in the MG neurons, which was confirmed by using a pair of bath electrodes located in the water to record the large field potentials generated by the MG neurons during the tail-flip (Liden and Herberholz, 2008). Moreover, when the food odor concentration was increased, MG-mediated tail-flips were suppressed as animals decided to freeze (and stay close to the food) rather than escape. This suggests that the response of the MG neurons to visual stimulation was modulated by olfactory signals (Liden et al., 2010). Internal state, such as hunger, also affected MG threshold in these experiments (Schadegg and Herberholz, 2017). Together, these behavioral experiments illustrated the sensitivity of the MG neurons to multi-modal sensory cues as well as intrinsic signals.

The MG circuit also plays a significant role during crayfish aggression (Edwards and Herberholz, 2005). The formation of a social dominance relationship between two crayfish includes escalating levels of aggression, and activation of the MG neurons is often observed during the decision point when the future dominants and subordinates are determined. A sharp transition in behavior typically identifies the loser of a fight, and MG-mediated tail-flips are used by the emerging subordinate to break off an escalated encounter (Herberholz et al., 2001). In response to attacks from a natural predator, the MG circuit is engaged more than other escape circuits (Herberholz et al., 2004), and much like the LG circuit, it is also susceptible to other strong and phasic mechanosensory stimuli (Herberholz, 2009).

Given the known influences of alcohol on invertebrate behavior and the extensive background on escape circuitry in crayfish, the MG circuit presents a well-suited experimental model for testing the effects of EtOH on the function of identified neurons that are involved in a number of important behavioral outputs.

MATERIALS AND METHODS

Juvenile crayfish (*Procambarus clarkii*) were used for all electrophysiological experiments. Animals were purchased from Atchafalaya Biological and housed in large communal tanks (76 cm × 30 cm × 30 cm, L:W:H) with 50–100 crayfish until social isolation. Since social experience significantly affects alcohol sensitivity in crayfish, both behaviorally and physiologically (Swierzbinski et al., 2017), we decided to use only pre-isolated animals for the current study. All animals were socially isolated for 7–10 days in small individual tanks (15 cm × 8 cm × 10 cm, L:W:H) prior to the experiments. Approximately 2 cm of gravel covered the bottom of the isolation tanks, and each tank was oxygenated using air stones (BubbleMac Industries). Before social isolation, animals were checked for intactness (no signs of any major bodily injury), and only animals that had not recently molted (within 48 h) were used. On the day of social isolation, crayfish were given one single shrimp pellet (Aqua Pets Americas). Before surgical procedure, each animal was measured from their rostrum to their telson (tail-fan). The average body length of all crayfish used in the experiments was 3.5 ± 0.21 cm (N = 35). Each animal was only used once.

Surgery and Electrophysiology

Animals were chilled on ice for 15 min and pinned down ventral side up in a Sylgard-lined dish filled with 40 ml of crayfish saline. Pins were inserted into the telson (tail-fan) and thorax to secure the animal in place. Ventral cuticle was removed from the abdomen in order to expose the ventral nerve cord (VNC) of the abdomen and cut all motor roots of the abdominal ganglia in order to reduce movements induced by activation of the MG neurons. Cuticle rostral to the mandibles was removed and the green glands were extracted to expose the brain connectives (BC) where the impalement of the MG neuron was performed (**Figure 1A**). The MG was impaled using sharp micropipette electrodes pulled (Sutter Micropipette Puller; Sutter Instruments) from glass capillary tubes (World Precision Instruments; outer diameter: 1 mm, inner diameter: 0.58 mm). Intracellular electrodes were backfilled with 2 M potassium acetate and had resistances between 20 and 35 MΩ. The antennal II nerve was exposed by removing a rectangular piece of cuticle from the basal segment of the antenna. An extracellular silver wire hook electrode (Teflon coated wire; AM-Systems; uncoated diameter 0.127 mm) was placed on the nerve. Contact with the antenna II nerve was verified through observation of spontaneous and tactile-evoked action potentials. Post-synaptic potentials (PSPs) in the MG neuron were elicited through electrical stimulation of the ipsilateral antenna II nerve using a Grass stimulator (Model S88). Stimulation of one antenna II nerve almost

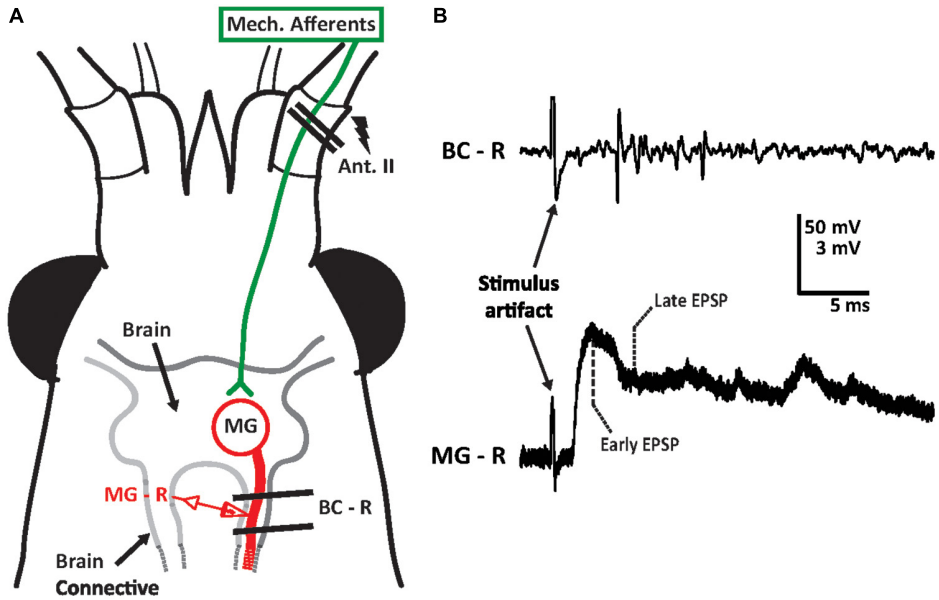

FIGURE 1 | Medial giant (MG) circuit diagram for antenna II inputs and sample recording trace. **(A)** Crayfish head depicted with location of the antenna II. Sensory inputs from other sensory systems are not shown. Supraesophageal ganglion (brain) of the crayfish was surgically exposed from the ventral surface. Extracellular hook electrodes (two parallel black lines) were used to electrically stimulate the sensory afferents of the antenna II nerve (lightning bolt) and to record evoked activity in the ipsilateral brain connective (BC-R). Sharp intracellular glass electrodes were inserted into the MG neuron in the brain connective ipsilateral to the stimulated antenna II nerve to record MG neuron activity (MG–R). The size of the crayfish nervous system is not to scale. **(B)** A sample recording trace of the brain connective (BC–R) and MG neuron (MG–R) in response to electrical stimulation of the antenna II nerve (at 3 V). Extracellular activity (top trace) as well as MG's early (3 ms after stimulus artifact) and late (6 ms after stimulus artifact) post-synaptic potentials (bottom traces) are shown.

never led to an action potential in MG, even at voltages just below direct (non-synaptic) stimulation of MG. However, post-synaptic potentials of several millivolts in amplitude could be reliably evoked. Intracellular signals were amplified using a microelectrode amplifier (Axoclamp 900A, Molecular Devices). Extracellular recordings were amplified using an A-M Systems differential amplifier (Model 1700) and digitized using a Digidata 1440A (Molecular Devices). The stimulating voltage was increased from 0 V until a sizable PSP could be observed, then increased until additional voltage produced no further change in the PSP. The voltage was then decreased to a voltage roughly at the midpoint between these two values. An inter-stimulus interval (ISI) of 90 s was used for all experiments. All experiments were conducted in a grounded Faraday cage.

Medial giant PSP amplitudes were analyzed at several time intervals after the start of the experiments. For each time point, two measurements were made (**Figure 1B**): the PSP amplitude at 3 ms following the stimulus artifact (termed "early") and the PSP amplitude at 6 ms after the stimulus artifact (termed "late"). This analysis was based on previous experiments in the LG circuit; here, the early peak of the PSP reflects a mostly excitatory component, while the later time point is associated with postexcitatory inhibition, or a combination of excitatory and inhibitory inputs. Sweeps recorded during the saline baseline (∼10 min) were averaged for each animal and early and late component amplitudes were recorded. For each experiment, averaged PSP values recorded during baseline, experimental, and washout phases were normalized to the baseline average from all animals. Therefore, values above 100% of baseline demonstrate an increase over the averaged starting amplitudes, while values below 100% of baseline demonstrate a decrease. Electrophysiological data was recorded and stored using Molecular Devices pClamp 10 software. Data analysis was performed using Clampfit.

Pharmacology

Solutions were introduced to crayfish preparations through a gravity-flow superfusion system consisting of glass reservoirs of solutions placed on top of the Faraday cage. The flow rate of the superfusion was held constant at 5 ml/min using a Baxter flow control device (Baxter International Inc.). This flow rate was checked before each experiment. Excess solution was removed from the dish through the use of a peristaltic pump (Thermo-Scientific FH100). All crayfish preparations were immersed in a modified van Harreveld's solution (van Harreveld, 1936), a standard crayfish saline consisting of the following salts (in concentrations in mM): 202 NaCl, 5.37 KCl, 13.53 $CaCl_2$, 2.6 $MgCl_2$, and 2.4 HEPES (Antonsen et al., 2005; Liu and Herberholz, 2010). Saline was also used as a vehicle to deliver pharmacological agents to the preparations.

Experiment Procedures

Experiment 1 (MG PSP Changes Over Time)

Previously, repeated stimulation of the LG while superfused with normal crayfish saline produced weak sensitization in some

preparations (Swierzbinski et al., 2017). To measure how MG PSPs are affected during long-term repeated stimulations while superfused with normal crayfish, preparations were exposed to a 10 min saline baseline followed by 90 min of normal crayfish saline.

Experiment 2 (Alcohol Effects on MG PSP)

We have previously demonstrated the sensitivity of the LG circuit to 100 mM EtOH. To see if EtOH's effects generalize across tail-flip circuits, preparations were exposed to baseline, then 60 min of 100 mM EtOH (4.6 g of ethyl alcohol solution mixed in 1 L of saline solution), and finally 60 min of washout with normal crayfish saline.

Experiment 3 (GABAergic Pharmacology of MG)

To further explore the presence of GABAergic inhibition in the MG circuit, preparations were exposed to the $GABA_A$ antagonist, picrotoxin or the GABA agonist, muscimol. These preparations received 10 min of saline superfusion (baseline), followed by 30 min of drug exposure (25 μM PTX or 25 μM muscimol), and finally 60 min of saline (washout). In a subgroup of muscimol-exposed preparations (N = 3), the MG neuron was impaled with two intracellular electrodes to measure the change in input resistance during muscimol exposure. The input resistance of the MG was measured through injection of positive and negative currents (−40 to 40 nA) for 30 ms using the second intracellular electrode placed in the MG neuron in close proximity to the recording electrode. Voltage changes in MG membrane potential caused by current injections were recorded. In between injections, the ipsilateral antenna was electrically stimulated to produce MG PSPs to obtain recordings of both input resistances and post-synaptic potentials before (baseline), during muscimol application, and after (washout).

Experiment 4 (Interactions Between Muscimol and EtOH)

To test the interactions of muscimol and EtOH, preparations were exposed to 25 μM muscimol before being exposed to 100 mM EtOH. Preparations were given 10 min saline (baseline), 30 min of muscimol exposure, followed by 30 min of EtOH exposure, and finally 60 min of normal saline (washout).

Statistical Analysis

All data is presented as means ± SEM except for animal sizes where standard deviation is shown. Statistical tests were performed using IBM SPSS (Version 23). Since some of our data failed normality as determined by Shapiro–Wilk Test, we used non-parametric tests throughout. We used Friedman as our omnibus test followed by pairwise comparison with Wilcoxon Signed Rank Test. We did not apply Bonferroni adjustment because our comparison across multiple levels (e.g., time points) would have likely resulted in a type II error. Statistical results are reported in the text and indicated in the figures.

RESULTS

MG Action Potentials and PSPs Change Over Time

We found that antenna II nerve stimulation reliably produces MG PSPs, but it generally fails to evoke an action potential in MG. Increasing the stimulus voltage will increase MG PSP amplitudes to a maximum level that is below spiking threshold. Increasing voltage further can fire MG directly (i.e., non-synaptically). Although we used voltage levels below the maximum in our experiments, in a few cases MG fired in response to antenna II stimulation when the appropriate stimulus voltage was determined. **Figure 2A** shows one such example. The MG spike rises from the early part of the PSP (3.5 ms after the stimulus artifact) suggesting that this part of the PSP consists of mostly excitatory synaptic inputs following antenna II stimulation.

Since our previous work had shown that the crayfish LG neurons become modestly sensitized after repeated sensory stimulation in normal crayfish saline (Swierzbinski et al., 2017), we first tested the effect of repeated stimulation on MG neuron excitability. After 10 min of baseline recordings, preparations (N = 5; 3.6 ± 0.16 cm) were perfused for 90 min with fresh crayfish saline and the antenna II nerve was stimulated every 90 s. We observed minor fluctuations in PSP amplitudes in both early and late PSP components throughout this time period (**Figure 2B**). Although PSPs changed slightly compared to baseline level at 15 min (Early = 106.2 ± 15.3%, Late = 96.0 ± 17.7%), 30 min (Early = 113.9 ± 16.2%, Late = 99.8 ± 15.5%), 60 min (Early = 112.5 ± 20.1%, Late = 96.9 ± 14.4%), and 90 min (Early = 110.0 ± 20.3%, Late = 80.7 ± 16.2%), none of these changes were significantly different from average baseline values (Friedman Tests; Early: Chi-Square = 1.914, df = 5, p = 0.861; Late: Chi-Square = 2.829, df = 5, p = 0.726). This is similar to what we observed in our earlier study of the LG circuit (Swierzbinski et al., 2017), and confirms that no major changes occur in these preparations over the course of 90 min of continuous stimulation and recordings.

Alcohol Effects on MG PSP

To investigate the effect of EtOH on the MG neuron, preparations (N = 8; 3.48 ± 0.23 cm) were exposed to 100 mM EtOH, a concentration found to be effective in socially isolated crayfish LG preparations (Swierzbinski et al., 2017). Similar to the LG, we found that EtOH exposure increased both early and late MG PSP amplitudes (**Figure 3**). The increases from baseline were significant (Friedman Test: Chi-Square = 12.929, df = 5, p = 0.024) for the early component after 15 min (150.6 ± 18.8%, Wilcoxon Signed Rank Test; z = −2.521, p = 0.012), 30 min (162.1 ± 24.3%, Wilcoxon Signed Rank Test; z = −2.380, p = 0.017), and 60 min (156.9 ± 21.9%, Wilcoxon Signed Rank Test; z = −2.240; p = 0.025). For the late PSPs, the responses to EtOH (15 min: 140.5 ± 19.3%; 30 min: 151.6 ± 30.3%; 60 min: 133.5 ± 27.3%) were not significant (Friedman Test; Chi-Square = 8.768, df = 5, p = 0.118).

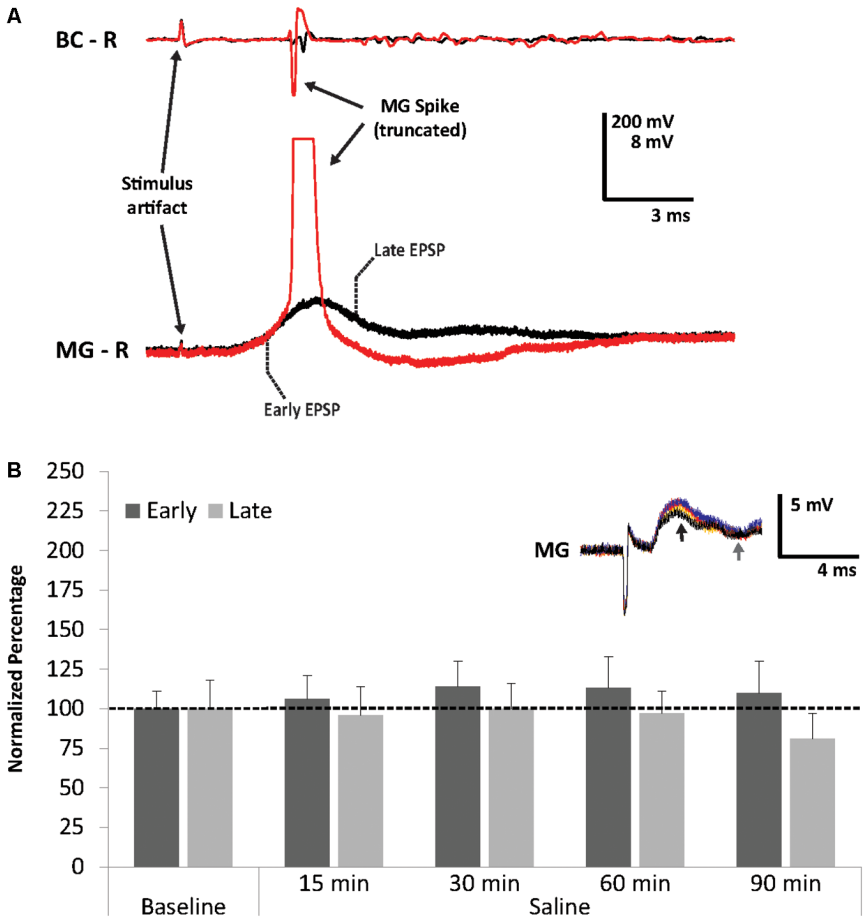

FIGURE 2 | Superthreshold nerve shock and effects of repeated sensory stimulation on early and late MG PSPs. **(A)** MG action potential in response to antenna II nerve stimulation. **(B)** Average percent of PSP baseline values during 90 min of saline superfusion of the MG neuron (N = 5) and repeated stimulation (ISI = 90 s) of the antenna II nerve. Means ± SEM are presented. *Inset*: Example of a single MG PSP recording; black trace = baseline, orange trace = 30 min, red trace = 60 min, blue trace = 90 min. Arrows indicate early (black) and late (gray) PSPs.

The early MG PSP was resistant to saline washout. Average amplitudes of the early PSP recorded after 30 min (156.0 ± 25.0%; Wilcoxon Signed Rank Test: $z = -1.960$, $p = 0.05$) and 60 min (156.1 ± 22.5%; Wilcoxon Signed Rank Test; $z = -2.380$, $p = 0.017$) of washout remained significantly higher than average baseline level. Late PSP, however, decreased during washout from levels recorded during EtOH exposure and measured 128.8 ± 36.6% (30 min) and 116.9.3 ± 30.9% (60 min); these amplitudes were not significantly different from baseline level. The result suggests that EtOH has a facilitatory effect on the early MG PSP and a weaker non-significant effect on the late PSP. This is similar to what was observed in the LG circuit where EtOH made LG more excitable and increased its firing rate (Swierzbinski et al., 2017).

GABAergic Pharmacology of MG

The LG is known to be inhibited through GABAergic mechanisms (Edwards, 1990; Vu and Krasne, 1992; Vu et al., 1997), but little is known regarding the MG. We next explored the role of GABAergic effects by measuring the effects of a GABA receptor antagonist (picrotoxin; PTX) and agonist (muscimol) on MG PSPs evoked through electrical stimulation of antenna II afferents.

Higher concentrations of PTX (>25 μM) often produced convulsions in the pinned-down preparation and MG spikes rising from the later PSP components. An example is shown in **Figure 4A**. Although we used 25 μM PTX to avoid evoking these spikes in subsequent experiments, the observation suggests that the later MG PSP component consists of PTX-sensitive inhibition. Since excitatory inputs likely contribute to the late PSP as well, this excitation is released from inhibition following PTX application (Vu et al., 1997). Because we rarely observed spikes less than 8 ms after the stimulus artifact during PTX treatment, this supports our notion that MG PSPs are similar to LG PSPs where an early, mostly excitatory PSP is followed by a later component, which is dominated primarily by inhibition (Roberts, 1968).

However, both early and late MG PSP components experienced significant changes when preparations (N = 5; 3.38 ± 0.31 cm) were exposed to 25 μM PTX and during washout

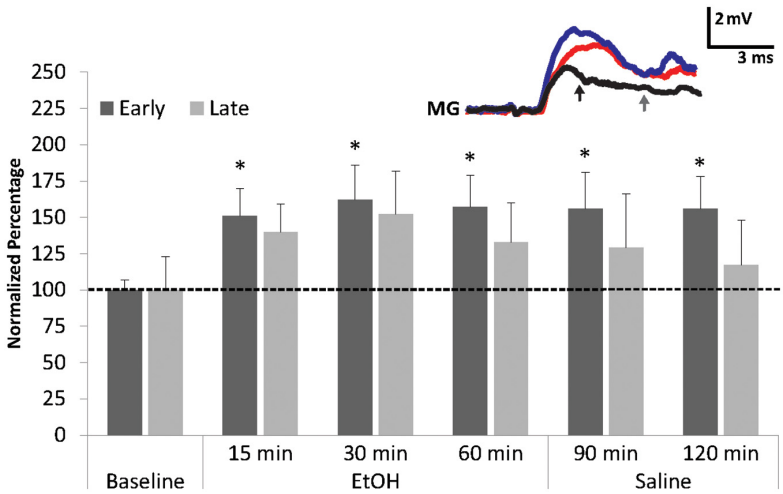

FIGURE 3 | Effects of EtOH application on early and late MG PSPs. MG preparations (N = 8) were exposed to 10 min of saline baseline (Baseline), 60 min of 100 mM EtOH (EtOH), and finally 60 min of washout with normal saline (Saline). * indicates values that were significantly different ($p < 0.05$) from baseline. Means ± SEM are presented. *Inset*: Example of a single MG PSP recording; black trace = baseline, red trace = 60 min, blue trace = 120 min. Arrows indicate early (black) and late (gray) PSPs. A Gaussian low-pass filter was applied to reduce electrical noise in the recording.

(Friedman Tests; Early: Chi-Square = 16.800, df = 4; p = 0.002; Late: Chi-Square = 14.720, df = 4; p = 0.005). The early PSP was significantly facilitated at 15 min (117.5 ± 17.6; Wilcoxon Signed Rank Test: z = −2.023, p = 0.043) and 30 min (128.5 ± 19.6; Wilcoxon Signed Rank Test: z = −2.023, p = 0.043) of PTX treatment compared to average baseline (**Figure 4B**). Early PSP amplitudes did not change significantly during washout at 30 min (76.5 ± 16.9%; Wilcoxon Signed Rank Test: z = −1.753, p = 0.08) and 60 min (47.3 ± 14%; Wilcoxon Signed Rank Test: z = −1.753, p = 0.08). Late PSPs were not significantly affected by PTX treatment at 15 min (106.9 ± 22.8; Wilcoxon Signed Rank Test: z = −0.405, p = 0.686) and 30 min (139.7 ± 37.6; Wilcoxon Signed Rank Test: z = −1.483, p = 0.138). After 30 min of saline washout, late PSP amplitudes changed compared to baseline (61.9 ± 27.6%; z = −1.483, p = 0.138) and were significantly lower than baseline after 60 min of saline washout (22.9 ± 10.5%; Wilcoxon Signed Rank Test: z = −2.023, p = 0.043).

Surprisingly, muscimol, a known ionotropic GABA receptor agonist (Johnston, 2014), produced only small PSP amplitude changes in all preparations (N = 8; 3.45 ± 0.14 cm) during application, but significant suppression of both early and late PSPs during washout (Friedman Tests; Early: Chi-Square = 14.700, df = 4; p = 0.005; Late: Chi-Square = 10.100, df = 4; p = 0.039) similar to those observed in PTX (**Figure 4C**). Amplitudes measured after 15 min of exposure changed to 113.4 ± 22.8% (Wilcoxon Signed Rank Test: z = −0.420, p = 0.674) and 97.9 ± 19.7% (Wilcoxon Signed Rank Test: z = −0.840, p = 0.401) for the early and late MG PSP, respectively. After 30 min of muscimol exposure, little additional change was observed for the early PSP (110.8 ± 21.4%; Wilcoxon Signed Rank Test: z = −0.420, p = 0.674) and late PSP (90.4 ± 12.5%; Wilcoxon Signed Rank Test: z = −0.700, p = 0.484). During washout, the early PSP was reduced to 74. 5 ± 16.0% at 30 min (Wilcoxon Signed Rank Test: z = −1.820, p = 0.069) and 55.3 ± 13.3% at 60 min, the latter being a significant decrease from baseline (Wilcoxon Signed Rank Test: z = −2.240, p = 0.025). The late PSP was reduced significantly at both the 30 min (47.7 ± 7.4%; Wilcoxon Signed Rank Test: z = −2.240, p = 0.025) and the 60 min (31.7 ± 9.8%) time points (Wilcoxon Signed Rank Test: z = −2.100, p = 0.036).

To determine whether a relationship exists between muscimol application and MG input resistance, possibly indicating an effect of muscimol on MG itself, we measured MG input resistance in a subset of these preparations (N = 3; 3.5 ± 0.17 cm). The average input resistance was 75 ± 21.4 KΩ, which is comparable to an existing report of MG input resistance measured in adult crayfish (Glantz and Viancour, 1983). In parallel to small changes in PSP amplitudes over the course of 30 min of muscimol exposure in both the early (93.7 ± 49.3%) and the late (97.2 ± 21.9%) PSPs, the input resistance changed only marginally (107.1 ± 28.0%) compared to the input resistance measured during baseline. PSP amplitudes decreased after 60 min of saline compared to baseline (Early = 49.5 ± 27.6%; Late = 51.3 ± 25.5%), and input resistance of the MG neuron showed a parallel decline (81.8 ± 13.6%).

Interactions Between Muscimol and EtOH

To explore the interactions between the GABA receptor agonist muscimol and EtOH on MG PSPs, preparations (N = 6; 3.6 ± 0.16 cm) were treated with 25 μM of muscimol for 30 min, then exposed to 100 mM EtOH for another 30 min, and finally to saline for 60 min (**Figure 5**). Muscimol exposure produced only small changes in early and late MG PSPs compared to baseline. Early PSP amplitude values measured 114.4 ± 20.9% (15 min) and 124.9 ± 29.6% (30 min), and late PSPs measured 117.5 ± 24.5% (15 min) and 129.8 ± 41.7% (30 min). After EtOH was added to the preparations, both the early PSP

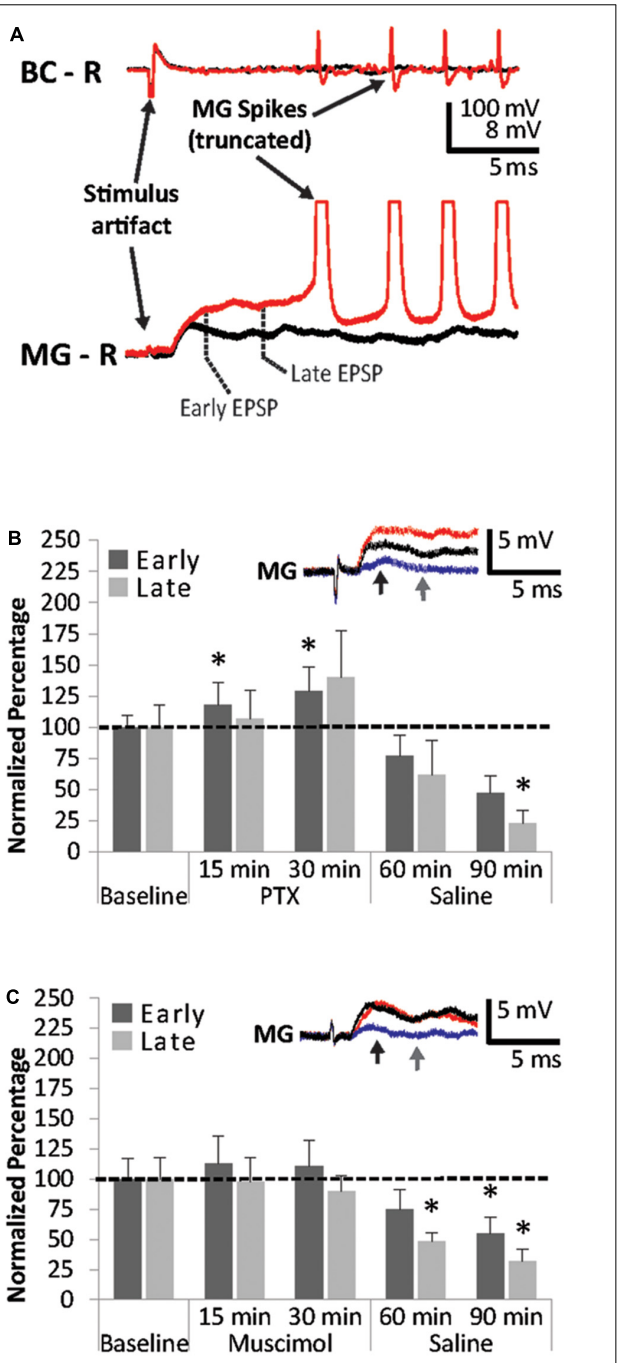

FIGURE 4 | Effects of PTX and muscimol applications on early and late MG PSPs. **(A)** Superfusion of high concentrations of PTX cause repeated MG spikes rising from the late PSP. **(B)** MG preparations (N = 5) were exposed to 10 min of saline baseline (Baseline), 30 min of 25 μM picrotoxin (PTX), and 60 min of washout with normal saline (Saline). Early and late PSPs are compared to their average baseline values. **(C)** MG preparations (N = 8) were exposed to 10 min of saline baseline (Baseline), 30 min of 25 μM muscimol (Muscimol), and 60 min of washout with normal saline (Saline). Early and late PSPs are compared to their baseline values. * indicates values that were significantly different ($p < 0.05$) from baseline. Means ± SEM are presented. *Inset*: Example of a single MG PSP recording; black trace = baseline, red trace = 30 min, blue trace = 90 min. Arrows indicate early (black) and late (gray) PSPs.

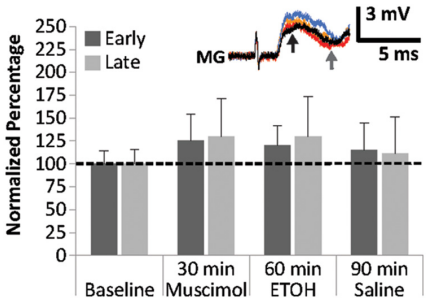

FIGURE 5 | Effects of muscimol application prior to EtOH exposure on MG PSPs. Preparations (N = 6) were exposed to 10 min of saline baseline, 30 min of 25 μM muscimol, 30 min of 100 mM EtOH, and 60 min of saline washout. Means ± SEM are presented. *Inset*: Example of a single MG PSP recording; black trace = baseline, orange trace = 30 min, red trace = 60 min, blue trace = 90 min. Arrows indicate early (black) and late (gray) PSPs.

amplitude (15 min: 124.5 ± 25.1%; 30 min: 119.5 ± 21.8%) and the late PSP amplitude (15 min: 140.1 ± 39.9%, 30 min: 129.7 ± 43.8%) exhibited only minor changes. In addition, saline washout produced only minor effects in early PSPs (30 min: 114.8 ± 29.7%, 60 min: 125.1 ± 37.9%) and late PSPs (30 min: 110.5 ± 40.5%, 60 min: 130.0 ± 52.2%). None of the changes in early or late MG PSP were statistically significant from average baseline (Friedman Tests; Early: Chi-Square = 3.000, df = 6; p = 0.809; Late: Chi-Square = 4.214, df = 6; p = 0.648). Two important conclusions can be drawn from this experiment: Pre-exposure to muscimol prior to EtOH application suppresses the facilitating effect of EtOH compared to when EtOH is applied alone, and EtOH exposure after muscimol eliminated the reduction of PSP amplitudes typically seen during muscimol washout.

DISCUSSION

The cellular workings underlying the complex interplay between alcohol and nervous system function are still poorly understood. The crayfish present a highly suitable model to probe into the neurocellular and neurochemical mechanisms, and it allows linking drug-induced changes in neural activity to whole animal behavior. We have previously reported that crayfish are behaviorally sensitive to EtOH exposure and progress through quantifiable, discrete stages of intoxication (Swierzbinski et al., 2017). Behavioral sensitivity to EtOH is dependent on social history of the individual, which we demonstrated by showing that communally housed crayfish respond to EtOH more quickly than socially isolated conspecifics. Lastly, we were able to determine that behavioral effects evoked by EtOH in crayfish are paralleled on the level of single neurons, the LG interneurons.

In our current study, we expanded on these prior findings. We focused our work on a different escape circuit, the MG, because we wanted to know if the effects of EtOH would generalize across neural circuits in crayfish. We selected the MG circuit due to its higher complexity compared to LG, which is illustrated by integration of multimodal sensory signals as well as superior

relevance for behaviors such as predator escape, aggression, decision-making and risk-taking (Herberholz et al., 2001, 2004; Liden et al., 2010). Although our current study focused on antenna II stimulation of MG, this work can be expanded by including other sensory modalities (e.g., visual) in the future (Liu and Herberholz, 2010).

We found that MG neurons respond to sensory afferent stimulation in similar fashion compared to LG neurons (Liu and Herberholz, 2010). The evoked compound post-synaptic potential (PSP) is of mostly biphasic shape (**Figure 1**), separated by a downward deflection, which has been attributed to post-excitatory inhibition in LG (Vu et al., 1997). Unlike LG, however, the MG is nearly impossible to activate in a restrained preparation with sensory nerve stimulation alone. Previous work has shown that coincident inputs from the contralateral MG and subthreshold antenna II inputs can bring MG to threshold (Herberholz and Edwards, 2005). In addition, when subthreshold antenna II inputs are combined with superfusion of 500 μM serotonin, MG action potentials can be evoked (Hu et al., 2014). In our current experiments, we observed MG spikes only on rare occasions when the baseline stimulus voltages were determined, and we found that the MG action potential rises from the early PSP (**Figure 2A**) similar to previous observations made on the LG neuron. Application of high concentrations of the GABA-receptor antagonist PTX also evoked MG spikes, which always originated from the later parts of the PSP (**Figure 4A**). This is likely due to MG spike activation via uninhibited excitation and has been shown to occur in the LG (Vu et al., 1997). In LG, excitatory mechanosensory interneurons (e.g., Interneuron C) produce bursts of action potentials that contribute to the late PSP (e.g., Zucker et al., 1971). The occurrence of multiple MG spikes indicates that proximal recurrent inhibition has been eliminated by PTX, similar to what has been described for LG (Roberts, 1968). While these observations may suggest that the early MG PSP component is primarily of excitatory nature and the later part comprised of (mostly) inhibitory and excitatory inputs (similar to LG PSPs), additional work is necessary to fully characterize the contributions of antenna II synaptic inputs to the MG PSPs.

When we exposed preparations to 100 mM EtOH, the alcohol concentration was higher than those that produce effects in humans (e.g., the legal blood alcohol driving limit in the United States is ∼17 mM), but in line with other alcohol literature. In fact, one controversy of alcohol research (including rodent work) is the perplexing result that EtOH concentrations within the "normal biological range" are often insufficient to evoke neurophysiological responses (Aguayo et al., 2002). Recently, progress has been made to understand the mechanisms underlying this reduced sensitivity, which seem to be related, in part, to the GABA receptor subunits (Cui and Koob, 2017). We have shown previously that the LG neurons of socially experienced crayfish respond to lower EtOH concentrations (10–20 mM) compared to socially isolated animals (20–100 mM), which were used in the current study.

Exposure of the preparations to EtOH produced significant increases in the early MG PSPs (**Figure 3**). Although late PSPs increased as well, the changes were not statistically significant. Thus, EtOH facilitated early sensory inputs to the MG neuron, which is similar to LG where excitability in response to synaptic inputs from tail afferents was significantly enhanced by EtOH (Swierzbinski et al., 2017). We found that EtOH-induced facilitation was resilient to wash out (with saline) for the early MG PSPs, suggesting strong binding affinity of EtOH to post-synaptic receptors or sustained facilitation of synaptic inputs that produce the early PSP component.

It is unclear at this point how EtOH causes the increase in early MG PSP. Both pre- and post-synaptic effects are possible as well as interactions with multiple neurotransmitter systems. Our initial attempt focused on the role of the GABAergic system since the interplay between alcohol and GABA has been described in numerous publications and investigated in a large number of animal systems (e.g., Koob et al., 1998; Davies, 2003). In addition, GABAergic inhibition, including tonic inhibition, of the LG neurons is well established (Vu and Krasne, 1993; Vu et al., 1993; Edwards et al., 1999), and its role in regulating behavior has been documented (Krasne and Wine, 1975; Krasne and Lee, 1988). However, until now inhibitory mechanisms of the MG circuit other than those related to motor outputs have not been studied.

Application of a non-competitive GABA receptor antagonist (picrotoxin; PTX) resulted in larger PSPs (**Figure 4B**). The result was only significant for the early component suggesting that the early PSP is more affected by PTX than the late PSP. The effect is likely due to reduced GABAergic inhibition; however, since PTX is generally assumed to block the channel pore and prevent flow of chloride ions, it could also partially be attributed to an overall increase in MG input resistance. In addition, PTX interactions with invertebrate GABA receptors are more complex than in vertebrate systems where it reliably blocks ionotropic GABA receptors although more effectively for $GABA_A$ receptors than $GABA_C$ (also known as $GABA_{Arho}$) receptors (Bormann and Feigenspan, 1995; Wang et al., 1995). PTX has been shown to block crustacean non-GABA mediated chloride channels (Albert et al., 1986), and crayfish interneurons vary in their response to PTX, some being highly sensitive and others being unaffected (Sherff and Mulloney, 1996; Miyata et al., 1997). This prior work suggested that at least some GABA-gated channels in crayfish have low affinity for PTX or are entirely non-sensitive to the antagonist.

A similar result to our finding (i.e., increase of early PSP) has been described by Vu and Krasne (1993) for the LG neuron. In intact preparations that contained the entire nervous system, PTX caused an increase in the early excitatory component (i.e., β-component) of the LG PSP evoked with tail afferent inputs. However, when the brain was separated from the tail (the location of the LG neurons) by sucrose gap or transection of the ventral nerve cord, PTX had no effect on the β-component. These differences were explained by changes in tonic inhibition of the LG, which originates in the crayfish brain and projects to the abdomen via descending interneurons (Vu and Krasne, 1993; Vu et al., 1993). Our work on the MG was done in restrained intact preparations where tonic inhibition was likely to occur; it is therefore possible that the PTX-induced increase in early PSP is related to suppression of tonic inhibition.

Tonic inhibition is widely distributed across the mammalian brain (e.g., Farrant and Nusser, 2005; Kullmann et al., 2005), produced by low levels of extracellular GABA, and mediated by slow-desensitizing ionotropic GABA receptors of distinct subunit composition, which can be blocked by PTX (Yeung et al., 2003; Wei et al., 2004). Interestingly, those receptors are also highly sensitive to alcohol (Sundstrom-Poromaa et al., 2002; Paul, 2006; Smith and Gong, 2007) and are considered a major cellular target for the drug in the mammalian nervous system (Valenzuela and Jotty, 2015).

In our own earlier work (Swierzbinski et al., 2017), we found that LG neurons of crayfish tail preparations (i.e., without tonic inhibition) were less excited by EtOH than semi-intact preparations (i.e., with tonic inhibition). This indicated that EtOH might interact with brain-derived tonic inhibition. The fact that lower concentrations of EtOH were required to activate the tonically inhibited LG than the disinhibited LG suggested that EtOH blocks tonic inhibition. Together with our current findings, one – of several – possibilities is that EtOH binds competitively to the receptors mediating tonic inhibition. EtOH has previously been shown to promote *or* prevent GABA receptor activation by the natural ligand in different mammalian neurons. EtOH-mediated *inhibition* of GABA receptor activity is widely known for the $GABA_C$ receptor (Mihic and Harris, 1996; Yamakura and Harris, 2000; Borghese et al., 2016). Interestingly, it has been suggested that (some) invertebrate GABA receptors are similar to this receptor type, in part because they both are insensitive to the antagonist bicuculline (Swensen et al., 2000). Moreover, $GABA_C$-like receptors have been identified in cultured thoracic neurons of lobster (Jackel et al., 1994), and cloning and expression of a GABA receptor subunit from the X-organ of crayfish revealed a homomeric, bicuculline-insensitive receptor similar to the vertebrate $GABA_C$ receptor (Jiménez-Vázquez et al., 2016). However, no invertebrate GABA receptor has yet been determined to be fully homologous to the vertebrate $GABA_C$ receptor (Martínez-Delgado et al., 2010). Importantly, $GABA_C$ receptors are known to mediate tonic inhibition in the retina of mammals and possibly other areas of the brain as well (Jones and Palmer, 2009).

Taken together, one mechanism by which EtOH could facilitate early MG PSPs is by interfering with GABA activation of $GABA_C$-like receptors located on the MG, and likewise, on the LG. These receptors could be located intra- or extra-synaptically and respond to phasic or tonic inhibition. However, this is only one possible scenario and several others must be considered. For example, EtOH could exert its effects presynaptically by facilitating transmitter release from sensory pathways that stimulate MG, or it could interact with post-synaptic receptors other than GABA. The purpose of our current paper was not to solve these questions. They provide, however, a useful concept and exciting avenues for additional experimentation.

Washout of PTX caused reduction of MG PSP amplitudes compared to baseline in our experiments, which was significant for the late PSP amplitude, indicating onset of strong inhibition after the GABA receptor blocker was removed. At this point, the mechanisms underlying this "rebounding" inhibition are unclear. It seems possible that GABA accumulates over time due to continuous tonic release and after repeated stimulation, and once the channel blocker is removed, strong GABA-mediated inhibition follows. Importantly, the aforementioned $GABA_C$ receptor fails to desensitize even with maintained GABA (or other agonist) application, and PTX has lower efficacy for blocking this receptor compared to the $GABA_A$ receptor when expressed in *Xenopus* oocytes (Wang et al., 1995; Chang and Weiss, 1999). Since GABA is cleared rapidly (i.e., within a few hundred microseconds) from the synaptic cleft in mammalian neurons (Nusser et al., 2001), build-up of tonically released GABA (and/or diffusion/spillover from phasic release) and subsequent activation of inhibitory receptors might be a more plausible explanation.

While we expected muscimol (an agonist for ionotropic GABA receptors) to reduce MG PSP amplitudes, it actually produced no statistically significant effect during exposure (**Figure 4C**). This is not in agreement with some reports in the literature showing that muscimol is capable of agonizing certain GABA receptors in crayfish (Hori et al., 1978; Krause et al., 1981; El Manira and Clarac, 1991). However, no prior experiments exist that measured the effects of muscimol on the MG circuit, and the types of GABA receptors present in this circuit are unknown. In mammals, muscimol competitively agonizes the $GABA_A$ receptor, but only acts as a partial agonist on the $GABA_C$ receptor. Thus, it can occupy the binding site and reduce the receptor's response to the natural ligand, basically acting as an antagonist (Johnston, 2014). As mentioned earlier, it has been recognized that (at least some) crustacean GABA receptors, including those in crayfish, are indeed structurally and functionally similar to the vertebrate $GABA_C$ receptor.

Along those lines, a GABA receptor subunit sequenced from crayfish (*P. clarkii*) showed high sequence similarity to a GABA receptor subunit previously cloned from lobster (*Homarus americanus*) and expressed in human embryonic kidney cells (Hollins and McClintock, 2000). Interestingly, for both the crayfish and lobster GABA receptor, pharmacology revealed that PTX blocked receptor currents, but bicuculline did not, and muscimol was much less effective as an agonist than GABA itself. Using American Lobster and Jonah crab, Northcutt et al. (2016) recently applied deep sequencing of transcriptomes and identified orthologs of two $GABA_B$ type subunits as well as three $GABA_A$ type subunits with similarity to *Drosophila* RDL, LCCH3, and GRD receptors. The sequence of the RDL-like receptor identified in their study was most similar to the crayfish and lobster GABA receptors described earlier. Importantly, the *Drosophila* RDL receptor also experiences lower binding affinity to muscimol than GABA (Buckingham et al., 1994), is insensitive to bicuculline, and shares other similarities with the vertebrate $GABA_C$ receptor (Hosie et al., 1997).

During saline washout of muscimol, we found that PSP amplitudes decreased significantly for both the early and late PSPs compared to baseline levels. Since muscimol is expected to compete with GABA at the receptor binding site, this result may suggest that removal of muscimol during washout clears the binding sites for ambient GABA that has accumulated due to either ongoing tonic release, or is present during stimulus-evoked synaptic release. This effect could be potentiated since muscimol

also acts as a weak GABA uptake inhibitor (Johnston, 2014). Although the described hypothesis is mostly speculative at this point, it is supported by prior work showing that GABA receptors exposed to long-term bath application of muscimol have high affinity for the natural ligand (i.e., GABA) after washout (Chang et al., 2002).

When we tested MG's input resistance during and after muscimol exposure, we found no major changes. A minor increase with muscimol exposure was followed by a minor decrease during washout. Our sample size was small (N = 3), and the result should be interpreted with caution. Nonetheless, it hints at the possibility that the effects of muscimol are post-synaptic rather than presynaptic, and they are related to muscimol acting as a competitive partial agonist during exposure (as described for $GABA_C$ receptors), which is then displaced by GABA during washout. Alternatively, tonic presynaptic effects could lead to prolonged transmitter release and increase in MG input resistance. A clearer picture would emerge if GABA currents could be measured in MG and more localized drug application was used, which might be possible in the future. Those additional experiments are needed to unambiguously identify post- and/or pre-synaptic mechanisms.

The suppression of PSP amplitudes for both PTX- and muscimol-treated animals and the decrease in MG input resistance during washout may also suggest "run-down" of the preparations at these late experimental stages. However, in our other experiments (e.g., saline; **Figure 2B**), the PSPs showed no sign of reduction for long-lasting experiments, indicating healthy preparations. Moreover, resting membrane potentials did not change (aside from small fluctuations) over the course of the experiments. Thus, we believe these effects to be based on GABA receptor interactions during washout of the drugs.

When we exposed our preparations to muscimol *before* EtOH was applied, we made two interesting and possibly interconnected observations (**Figure 5**): First, the facilitating effect of EtOH on the early MG PSP was eliminated after muscimol pre-treatment. Second, the significant decrease in MG PSP during washout of muscimol was muted when EtOH (instead of saline) was added to the preparations.

This may indicate that the addition of EtOH after muscimol produced an effect similar to saline washout of muscimol, and thus the normally observed increase of PSP amplitudes after EtOH application was counterbalanced by parallel suppression of MG PSPs via GABAergic inhibition. Although EtOH would possibly compete with GABA for receptor occupancy once the binding sites become available, EtOH can either activate or inhibit GABA receptors in mammalian neurons (e.g., Lobo and Harris, 2008). Thus, the stimulating effects of EtOH on the sensory-evoked MG PSPs could be in part mediated by its interaction with GABA (i.e., inhibition of GABA receptors), or produced independently. Conversely, it seems possible that GABAergic inhibition that normally follows muscimol washout and produces a reduction in MG PSP amplitudes has been counterbalanced by the stimulating effects of EtOH, either by EtOH-GABA interactions, via independent routes, or a combination thereof.

It is well known that EtOH affects other neurotransmitter systems, including serotonin (Barr et al., 2003; Ferraz and Boerngen-Lacerda, 2008). In crayfish, serotonin modulates the excitability of the LG circuit (Glanzman and Krasne, 1983; Teshiba et al., 2001), and this modulation is dependent on the social status of the animal (Yeh et al., 1996). Although our recordings were made in the MG neurons, the results have revealed some similarities between the two neural circuits, and thus serotonergic modulation of MG excitability may be expected. EtOH has also been shown to interact with the dopaminergic system in both invertebrates and vertebrates (e.g., Brodie et al., 1990; Yoshimoto et al., 1992; Bainton et al., 2000). More work is needed to further identify the neurocellular and pharmacological interactions between alcohol and crayfish neurotransmitters systems. Having identified two neural circuits that are accessible for single cell electrophysiology, and are both modulated by EtOH in a similar fashion, opens up new opportunities.

In summary, we found that EtOH increases post-synaptic potentials evoked by sensory inputs from the antenna II to the MG neuron. Although we only have indirect evidence that these early inputs are excitatory, it suggests that the stimulating effects of EtOH generalize across crayfish giant circuits. We also found that muscimol, a GABA receptor agonist, blocks the EtOH-induced facilitation of early post-synaptic potentials, which suggests interactions between EtOH and the GABAergic system, although they may not be direct. This is further supported by the lack of inhibition normally observed during muscimol washout with saline when saline is being replaced with EtOH. A possible role for tonic inhibition and $GABA_C$ -like receptors is discussed in this context and provides an exciting framework that warrants further investigation.

ETHICS STATEMENT

The study was exempt from ethical approval procedures because invertebrate animals were used.

AUTHOR CONTRIBUTIONS

JH and MS designed the study, contributed to data analysis, and wrote the manuscript. MS performed the experiments.

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