MODULATION OF BEHAVIORAL OUTCOMES BY CONDITIONING COMPETING STATES, VALENCES, OR RESPONSES

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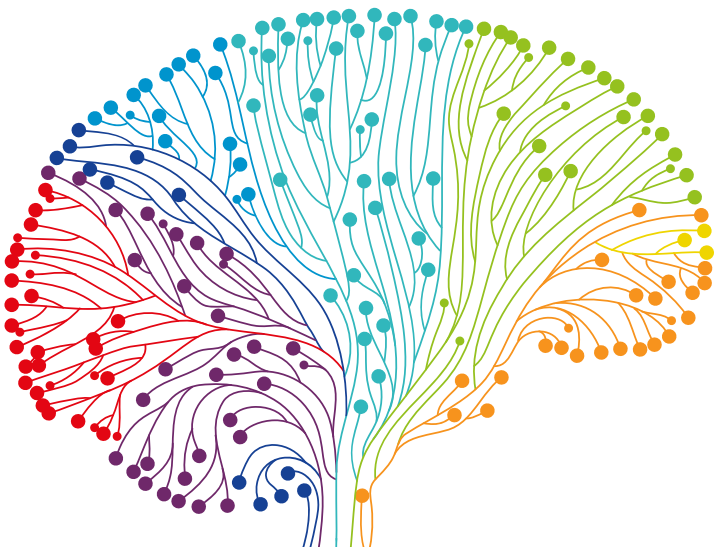

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MODULATION OF BEHAVIORAL OUTCOMES BY CONDITIONING COMPETING STATES, VALENCES, OR RESPONSES

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Editorial: Modulation of behavioral outcomes by conditioning competing states, valences, or responses

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Editorial on the Research Topic
Modulation of behavioral outcomes by conditioning competing states, valences, or responses

Introduction

At any moment, an organism encounters a wide array of stimuli that have the potential to drive learning and behavior. The majority of experience-dependent plasticity (a.k.a., learning) research focuses on investigating the parameters and mechanisms activated by pairing a novel stimulus with an established stimulus-response circuit, the crux of associative conditioning. However, different stimuli can often activate competing and/or opposing neural circuits. There are fewer recent studies that investigate how established circuits that produce opposing responses, states, or valences are integrated in learning. How the activation of opposing circuits are integrated and ultimately influence behavior will significantly inform our understanding of behavioral flexibility, where a response is modulated because of changing internal and external conditions. Furthermore, the associations of competing circuits have the potential for clinical significance as human behavioral states may be subject to the same conditioning and counter-conditioning principles. In this special issue, we feature an assortment of research and reviews that explore the "Modulation of Behavioral Outcomes by Conditioning Competing States, Valences or Responses" at many levels of investigation. This collection expands our understanding of how learning occurs between previously established and opposing circuits by highlighting recent findings and advances.

Circuit mechanisms

Gil-Lievana et al. and Laurent et al. investigated how learning is influenced by the salience and affective valence of incoming stimuli, respectively. Gil-Lievana et al. implicate VTA dopaminergic input to the insular cortex in consolidating taste recognition memory and identify a specific role for D1-like receptors in consolidating aversive, but not appetitive, taste memory. Laurent et al. report that "transreinforcer blocking" occurs with a positive valence, a phenomenon previously only demonstrated with negative valence, by using a stimulus that indicates the omission of an aversive US when presented in conjunction with a new stimulus and food presentation. Together, these studies show how opposing stimuli can rely on different signaling mechanisms, as is the case of stimulus salience, but produce a similar behavioral learning result, as with affective valence.

Pribic et al. and Orr et al. report that locomotor response pathways are also differentially influenced by either opposing response co-activation or by opposing internal states determined by social status, respectively. Pribic et al. begin to uncover how association of stimuli that drive opposing motor responses modifies locomotor behavior by taking advantage of the well-described neural circuitry of *C. elegans*. Pribic et al. show that pairing stimuli that produce opposite directional movement, results in a novel "pause" (cessation of locomotion) response to a stimulus that previously activated a backward locomotion pathway. Orr et al. found that the opposing states imposed by social hierarchy result in behavioral differences in locomotion and startle sensitivity when comparing dominant and subordinate Zebrafish (*Danio rerio*). Endocannabinoid signaling mediated a switch in locomotion strategy that was pharmacologically reversible, demonstrating that opposing social status can modify locomotion strategy. These studies provide examples of how multiple, competing signals drive response plasticity.

Behavioral flexibility

Animals have evolved to show adaptive behavioral responses that promote survival and reproduction. Behavioral flexibility allows animals to adjust responses to a changing environment. Hones and Mizumori describe a role for the lateral habenula (LHb) in facilitating response flexibility. LHb is postulated to drive alternative choices, behaviors, and/or internal state conditions by regulating hippocampal theta, a neural state associated with flexible associations, exploration, and greater attention and arousal. Accordingly, behavioral flexibility reflects not only the selection or competition between actions but also changes in neural state that enable consideration of different response options.

Devineni and Scaplen provide an overall conceptual framework for studies of behavioral flexibility in *Drosophila*, including the delineation of general principles of neural circuit organization and modulation that underlie behavioral flexibility across different systems, principles that are likely to extend to other species. Ortu and Bugg describe a model by which different response options or systems might arise and then ultimately, how the response is expressed and the decision for that expression comes about. The latter is proposed to depend on a striatal-thalamo-cortical circuit. Nemchek et al. report that responses are also impacted by predictive stimuli that occur prior to a learning assay by describing the influence that novel vs. familiar pre-test transportation cues can have on novel object exploration.

Overall, these contributions on the neural and behavioral mechanisms of behavioral flexibility inform future therapeutic interventions for disorders of behavioral control that are characterized by response inflexibility, such as, addiction and depression.

Clinical significance

Understanding the integration of opposing circuits can provide possible avenues for novel therapeutic approaches. Desrochers et al. propose a dual-system approach, which comprises the competing processes of reward processing and inhibitory control, to understand the mechanisms of impulsivity control. They review the role of serotonin signaling in modulating systems that drive and inhibit motivated behaviors respectively and suggest that pathological impulsivity likely arises because of the imbalance between these systems. Steinman et al. report that interventions that draw on the integration of opposing states to maximize the benefits of a learning-based therapy (novelty-facilitated extinction) do not uniformly translate to a sample from a clinical population.

Conclusions

These reports provide insights into how activation of opposing stimulus-response circuits can modulate future behavioral responses as well as how different qualities of a stimulus, an organism's state, or the environment influences the modulation of response circuitry. What plasticity mechanisms are involved when two or more established response circuits are co-activated remains to be clarified. Furthermore, how a conglomerate of circuits reconciles these competing signals to adapt to changing conditions requires further investigation. As we develop our understanding of these signal integration and potential decision mechanisms, future

clinical applications will benefit from a more nuanced understanding of experience-dependent plasticity that incorporates how previous experience is coded within a circuit.

Author contributions

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

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Conflict of interest

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

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Social Experience Regulates Endocannabinoids Modulation of Zebrafish Motor Behaviors

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Social status-dependent modulation of neural circuits has been investigated extensively in vertebrate and invertebrate systems. However, the effects of social status on neuromodulatory systems that drive motor activity are poorly understood. Zebrafish form a stable social relationship that consists of socially dominant and subordinate animals. The locomotor behavior patterns differ according to their social ranks. The sensitivity of the Mauthner startle escape response in subordinates increases compared to dominants while dominants increase their swimming frequency compared to subordinates. Here, we investigated the role of the endocannabinoid system (ECS) in mediating these differences in motor activities. We show that brain gene expression of key ECS protein pathways are socially regulated. Diacylglycerol lipase (DAGL) expression significantly increased in dominants and significantly decreased in subordinates relative to controls. Moreover, brain gene expression of the cannabinoid 1 receptor (CB_1R) was significantly increased in subordinates relative to controls. Secondly, increasing ECS activity with JZL184 reversed swimming activity patterns in dominant and subordinate animals. JZL184 did not affect the sensitivity of the startle escape response in dominants while it was significantly reduced in subordinates. Thirdly, blockage of CB_1R function with AM-251 had no effect on dominants startle escape response sensitivity, but startle sensitivity was significantly reduced in subordinates. Additionally, AM-251 did not affect swimming activities in either social phenotypes. Fourthly, we demonstrate that the effects of ECS modulation of the startle escape circuit is mediated via the dopaminergic system specifically via the dopamine D1 receptor. Finally, our empirical results complemented with neurocomputational modeling suggest that social status influences the ECS to regulate the balance in synaptic strength between excitatory and inhibitory inputs to control the excitability of motor behaviors. Collectively, this study provides new insights of how social factors impact nervous system function to reconfigure the synergistic interactions of neuromodulatory pathways to optimize motor output.

Keywords: social experience, aggression, zebrafish, Mauthner cell, endocannabinoid, 2-AG, motor circuits

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INTRODUCTION

Social status can be defined by a set of behaviors that accompanies an animal's position in a social hierarchy. Aggressive behavior typically displayed by dominant animals consists of either physical attacks or pursuit of conspecifics. When two adult male zebrafish are paired in a tank, they quickly establish a stable social relationship in which one fish is dominant and the other is subordinate. These social relationships can be used as the basis to study the effects of social status on behavior and brain function (Miller et al., 2017; Clements et al., 2018; Park et al., 2018).

Two fundamental behaviors in zebrafish, startle escape and swimming, are notable for the relative simplicity of the neural circuits that control them and the ease with which they can be studied behaviorally and physiologically. The neural circuits underlying these basic motor behaviors have been well-characterized in terms of their neuronal organization (Eaton et al., 2001) and the neurochemicals that modulate their activation (McLean and Fetcho, 2004). The startle escape response in zebrafish and other teleost fish is controlled by a group of reticulospinal neurons, namely the Mauthner cell (M-cell) and two serial homologs, MiD2cm and MiD3cm (Eaton et al., 2001). The firing of a single M-cell is necessary and sufficient for the initiation of a fast startle escape response. The M-cells act as integration centers for auditory, tactile, and visual inputs, and, as such, they are responsible for the initiation of startle escape behavior in response to auditory stimuli (Eaton et al., 2001). Auditory stimuli activate hair cells in the ear, which signals the M-cell via the VIIIth cranial nerve. A stimulus sufficient to activate the M-cell subsequently activates fast motor neurons (MNs) responsible for startle escape and inactivates slow MNs responsible for rhythmic swimming. This activation pattern generates a contralateral contraction of the trunk musculature producing a fast escape away from the stimulus (Eaton et al., 2001; **Figure 1A**).

Swimming is a well-conserved behavior whose neural circuit has been described in zebrafish (Fetcho and McLean, 2010; Kiehn, 2011). This behavior is controlled by a distributed network of neurons arranged hierarchically from the midbrain to the spinal cord. Initiation of locomotion begins in the mesencephalic locomotor region. This brain region sends descending inputs to reticulospinal neurons in the hindbrain, which project to the central pattern generators (CPGs). The CPG consists of two half-centers, one on either side of the midline (**Figure 1A**). Each half-center is composed of motor neurons, descending excitatory interneurons (e-INs), and commissural inhibitory interneurons (i-INs). The coordinated action of these neurons is responsible for the locomotor pattern generation (Roberts et al., 2008). The behavioral switch between startle escape and swimming is controlled by a hardwired neural circuit spanning from the hindbrain to the spinal cord. The threshold for this switch from swimming to startle has been shown to be modulated neurochemically by the endocannabinoid 2-arachidonoylglycerol (2-AG) (Song et al., 2015). It has also been demonstrated in goldfish that the reticulospinal M-cells release 2-AG in order to regulate their own excitability (Cachope et al., 2007).

The ECS is broadly involved in the central nervous system and functions via a retrograde signaling mechanism. This neurochemical system is composed of cannabinoid receptors and their endogenous lipid-based ligands, i.e., endocannabinoids. Two cannabinoid receptors have been identified in vertebrates, the cannabinoid 1 receptor (CB1) and the cannabinoid 2 receptor (CB2). While CB1 is the primary cannabinoid receptor found in the brain, CB2 is also present, although at much lower receptor number per cell and is found primarily on immune cells (Lam et al., 2006). The endogenous ligands anandamide and 2-AG are retrograde signaling molecules, which are synthesized "on demand" in response to post-synaptic depolarization (Kano et al., 2009). The synthesis of 2-AG in the post-synaptic neuron is triggered by intracellular increase in Ca^{2+} concentration resulting from cell depolarization. Binding and activation of presynaptic CB1 leads to the pre-synaptic closing of Ca^{2+} channels and/or opening of K^{+} channels. These cellular changes result in reduced neurotransmitter release (Hernandez and Cheer, 2015). After being transported into the presynaptic neuron by an unknown uptake mechanism (Fu et al., 2011), 2-AG is degraded by monoacylglycerol lipase (MAGL) as a mechanism to regulate 2-AG activity (Dinh et al., 2002).

It has been demonstrated that the endocannabinoid 2-AG acts as a molecular "clutch" in the zebrafish spinal cord circuit, setting the threshold for the switch from swimming to startle escape behavior (Song et al., 2015). Furthermore, evidence strongly suggests that the M-cell releases 2-AG (Cachope et al., 2007). It was found that activation of the group 1 metabotropic glutamate receptor (mGluR1) led to a lasting potentiation from the VIIIth nerve onto the M-cell. 2-AG is known to be synthesized and released from a post-synaptic cell in response to mGluR1 activation. Moreover, blockage of CB1 eliminated this potentiation (Cachope et al., 2007). These results suggest that the M-cell increases its own excitability by releasing 2-AG. The findings from these two studies set the stage to study the role of the ECS in balancing activation of the startle escape and swimming circuits based on social status.

Socially dominant fish show reduced startle escape sensitivity and higher swimming frequency, whereas socially subordinate fish show a shift in circuit activation toward higher sensitivity of the M-cell startle escape and lower activation of the swimming circuit resulting in lower swimming frequency (Miller et al., 2017). While the effects of social status on behavior are well-documented, the effects of social status on the molecular machinery responsible for shifting activation between the competing neural circuits of escape and swim is poorly understood. The known role of the ECS in switching activation between motor circuits suggests potential involvement in the facilitation of social status-dependent shifts in motor behavior (**Figure 1B**). However, this social role of the ECS remains undetermined. Here, we investigated the effects of ECS modulation on the social status-dependent activation of two competing motor circuits controlling the M-cell startle escape reflex and swimming behaviors.

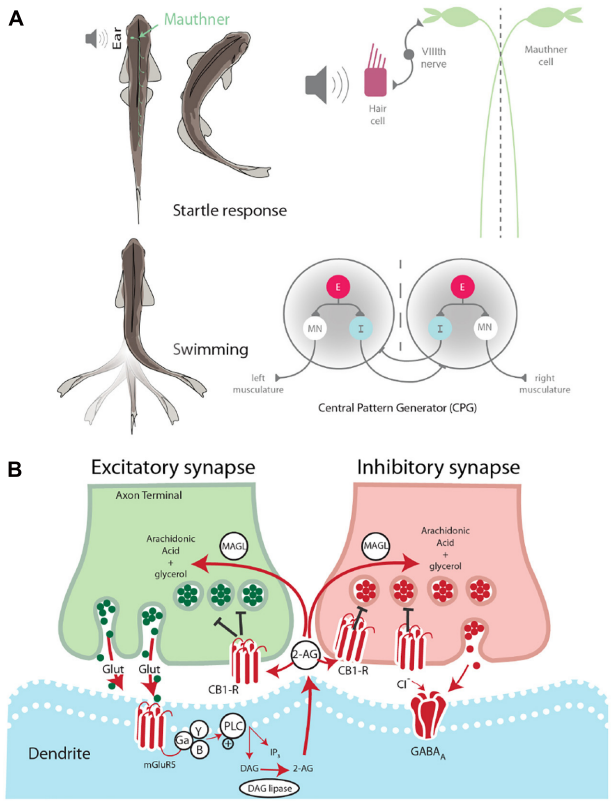

FIGURE 1 | Startle escape and swim motor behaviors are socially regulated. **(A)** Startle behavior in zebrafish is controlled by the M-cell startle escape circuit. The auditory startle escape response is activated when a sound activates hair cells within the ear. Next, the signal is sent from the VIIIth nerve to the M-cell, which activates contralateral fast motor neurons responsible for contraction of flexor muscles that leads to the startle escape response. The swimming motor pattern is controlled by the central pattern generators (CPGs) which repeat along the length of the spinal cord. Each half-center of the CPG is composed of an excitatory interneuron (E), an inhibitory interneuron (I), and a motor neuron (MN). The motor neurons project ipsilaterally to the trunk musculature and induce contraction. **(B)** Schematic model of endocannabinoid retrograde signaling. The endocannabinoid 2-AG is synthesized post-synaptically in response to neurotransmitter binding. Traditional model suggests that retrograde transmission of 2-AG inhibits further release from both excitatory and inhibitory neurons. DAG lipase synthesizes 2-AG post-synaptically. CB1 receptor binds 2-AG. MAGL degrades 2-AG in presynaptic terminal.

MATERIALS AND METHODS

Animal Maintenance

Zebrafish (*Danio rerio*) were housed at the Zebrafish Core Facility at East Carolina University. The facility was kept at a temperature of at 28°C under a 14 h/10 h light/dark cycle. Fish were fed daily with a high protein commercial food (Otohime B2, Reed Mariculture, Campbell, CA, United States) and with newly hatched artemia (Brine Shrimp Direct, Ogden, UT, United States). Wildtype (AB) zebrafish were group-housed in 10 gallon mixed-sex tanks prior to isolation and pairing. All experiments were performed in accordance with the Institutional Animal Care and Use Committee at East Carolina University (AUP #D320a). The dopamine type 1 receptor knockout line [drd1b$^{(-/-)}$] was generously provided by the Nicolson's lab (Oregon Health Sciences University). The line was originally constructed at the Sanger Institute with an AB genetic background (Busch-Nentwich et al., 2013) and later deposited at the Zebrafish International Resource Center (ZIRC).

Social Isolation and Pairing

Adult male fish (∼6–12 months old) were taken from their communal tanks and isolated in a tank for 1 week, separated spatially and visually from other fish to minimize pre-existing social experience (Miller et al., 2017). Subsequently, two animals of equal size were paired in a new tank for a 2-week period and their aggressive behavior was monitored daily for 5 min to assess dominance as described previously (Miller et al., 2017).

Experimental Setup

After the pairing phase was completed, fish were temporarily separated, and behavioral testing was performed on a single fish following the protocol described elsewhere (Issa et al., 2011). Each fish was placed in a testing chamber (dimensions: 11 × 4 × 3 cm). A pair of conductive electrodes placed on either side of the chamber recorded the electric field potentials. Bare electrodes were 1 mm in thickness with 3–5 mm metal exposure. Electrodes were connected to an AC differential amplifier (AM-Systems model 1700, Carlsborg, WA, United States), and signals were amplified 1,000-fold. Electrical signals were low-pass filtered at 300 Hz and high-pass filtered at 1 KHz. Electrical field potentials are generated by muscle contractions when the fish moves (Issa et al., 2011). These signals were digitized using a Digidata-1322A digitizer then stored using Axoscope software (Molecular Devices, Inc., Sunnyvale, CA, United States). The experimental animals were acclimatized for 30 min before behavioral testing was initiated. Swimming behavior was recorded immediately following acclimation. Immediately after, startle escape responses were recorded.

Determination of Startle Escape Sensitivity

Auditory pulses consisting of phasic 4 ms sine waves were generated using Audacity open-source audio editor and recorder software[1]. Sound intensity was measured and calibrated external to the tank using a decibel meter (Sinometer, MS6700). Sensitivity of the animal's auditory startle escape response was determined by tracking startle escape probability as a function of sound intensity. Activation of the M-cell mediated escape has a short latency of 5–15 ms. Non-Mauthner mediated responses with a time onset ranging from 15 to 40 ms were not counted, as these are controlled by an independent set of neural circuit that is not the target of our investigation (Eaton et al., 2001). Pulse intensity ranged from 70 to 100 dB with 5 dB increments. Pulse intensities were randomized and presented with a minimum of 2-min intervals to prevent habituation of the startle reflex.

[1] audacityteam.org

Response probability for each intensity was tabulated, and these probabilities were averaged across animals.

Measurement of Swimming Activity

Following the 30-min acclimation period, and before conducting startle escape experiments, the animal's swimming behavior was recorded for 1 min. The same methods of data acquisition, amplification, digitization, and storage were used as previously stated. Swimming activity was measured by counting swim bursts with Clampfit software. The "Threshold" function was used for this purpose. A potential was marked as a swim burst if it was at least 8 mV in total amplitude and 30–200 ms in duration. This range was chosen based on the typical characteristics of rhythmic swimming potentials that we observed. The timing of each swim burst was saved into a Microsoft Excel spreadsheet in reference to the recording start time.

Data Analysis

Startle escape and swimming behavioral data was analyzed using Prism (GraphPad software Inc., San Diego, United States) and IBM-SPSS (RRID:SCR_002865). Unless specified otherwise, all comparisons were first subjected to one-way ANOVA or mixed design (a mixture of between-group and repeated-measures variables) ANOVA (between factor as group; within-factors as treatment and decibel) followed by the least significant difference (LSD) or paired two-sided *t*-test *post hoc* test for all multiple comparisons. Before using mixed-design ANOVA, sphericity was tested by using Mauchly's test. When the assumption of sphericity was violated, the degree of freedom in Greenhouse-Geisser correction was used. For startle escape data, nonlinear regressions were performed using the Boltzmann sigmoidal equation:

$$Y = Bottom + (Top - Bottom)/(1 + exp((V50 - X)/Slope)).$$

Pharmacology

A day after initial behavioral testing, fish were treated with either AM-251 or JZL184 and re-tested according to the previously stated protocol. Paired fish were separated with a divider during the injection and post-testing phase. The acclimation period was initiated 2 h post-injection. Fish were treated with a drug injected intraperitoneally following the protocol of Song et al. (2015). Intraperitoneal injections are preferred over direct brain injections because there is less risk of altering behavior with the physical injection and because both drugs can effectively cross the blood-brain barrier (Song et al., 2015). The drugs AM-251 and JZL184 were dissolved in DMSO to produce a 40 mM stock solution. For injection, capillary tubing was used, having the dimensions 1.0 mm OD × 0.5 mm ID × 100 mm in length. These were pulled using Flaming/Brown Micropipette Puller – Model P-87 from Sutter Instrument Co. The 40 mM stock solution was diluted in saline to 400 μM AM-251 and 400 μM JZL184. The tip of the micropipette was broken off with a razor blade, before loading with the drug solution. Loaded micropipettes were placed in Pneumatic PicoPump PV 820 for drug administration. A 0.3 % tricaine solution was used to anesthetize the animal prior to injection. Zebrafish were determined to have an average weight of 100 mg, therefore 2 μL of drug was injected to achieve a concentration of 4 mg/kg AM-251 and 4 mg/kg JZL184. To control for injury from injection and possible effects from solvents, separate dominant-subordinate pairs were injected with 10% DMSO in saline. To control for social status, communal fish were injected with either AM-251 or JZL184.

Molecular Methods

ECS Signaling Molecules RNA Extraction and Reverse Transcription

Fish were euthanized by hypothermic shock for 10 min. Dissections were performed in ice cold reverse osmosis water and completed within 5 min of sacrifice. Whole-brain and Hindbrain tissues were collected and stored at −80°C until use. Samples were homogenized by sonication in TRIzol® (Life Technologies) and incubated at room temperature for 5 min. Chloroform was added (200 μL per 1 mL TRIzol®), mixed by inversion, and incubated at 4°C for 20 min with intermittent mixing. Samples were then centrifuged at 4°C for 15 min at 14,000 rpm. An equal volume of cold 100% ethanol was added, and sample was passed through a RNeasy® Mini Spin Column (Qiagen) according to RNeasy® protocols. RNA extracts were quantified by Nanodrop 2000 (Thermo Fisher Scientific) and stored at −80°C until use. cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen).

Qualitative Real-Time PCR

Primers used for the ECS genes are summarized in **Table 1**. qPCR was performed used the Quantstudio 12k Flex System (Applied Biosystems) to determine expression of dagl, mgl, and cb1r in whole-brain tissue. Beta-actin 2 (actb2) was used an internal reference gene. All samples were run in duplicate. Expression was normalized to actb2 and analyzed using the comparative ΔΔCt method with isolate animals as control.

Western Blot

For western blot analysis four separate western blot trials were conducted with 10 brains per trial for each social phenotype. Zebrafish were anesthetized with 0.02% MS-222 (1 min) then placed in iced water (10 min). Brains were dissected out, placed in a 1.5 mL microcentrifuge tube, and stored at -20°C until use. Then they were prepared using the total membrane isolation protocol. Brains were homogenized in 1 ml of resuspension buffer: 2.5 ml 2M Sucrose, 2 ml 10 × 10 mM Tris–HCl, 400 μL 0.25M EDTA, 40 μl 20x protease cocktail inhibitor. The homogenized brains were centrifuged (2,000 rpm, 4°C, 10 min) followed by ultra-centrifugation (37,000 rpm, 4°C, 60 min). Protein sample concentrations were determined with a Lowery protein assay. For Western blots, 10 μg of each protein sample was denatured using 4X buffer containing 10X reducing reagent at 70°C for 10 min and loaded onto a Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA, United States) and run ∼120 min at 60V. The proteins were transferred to a 0.2 μm Nitrocellulose membrane using *Trans*-Blot® Turbo™ RTA Mini Transfer Kit (Bio-Rad, Hercules, CA, United States). The membrane was blocked with PBS containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20 for 1 h at room temperature and then incubated with

TABLE 1 | Primers used for qPCR analysis.

Gene	Forward primer 5′-3′	Forward primer 5′-3′	Amplicon GenBank#	Size (bp)
actb2	CCAAACCCAAGTTCAGCCATGG	TGGATGGGAAGACAGCACGG	NM_181601	118
dagl	CCTGGACACCTCAAATTCGCC	TCCGGTGAGCACAATAGGGA	XM_691781	145
mgl	GGAGACGCCGACAAACTGTG	AGTCGTGATGTAGGGCATGGT	NM_200297	118
cblr	CTCTGGAAGGCCCACCATCAT	CGGATGTCCATGCGTGCC	NM_212820	128

primary antibodies overnight at 4°C. After three washes with PBS containing 0.1% (v/v) Tween 20, the membrane was incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Cell Signaling) secondary antibody for 1 h. After washing, protein detection was performed using Horseradish peroxidase (HRP) Detection kit (SuperSignal^{TM} West Pico, Thermo Fisher Scientific) and visualized using the ChemiDoc Imaging System (Bio-Rad). The intensity of bands was quantified with ImageLab software. Band intensity was normalized by calculating protein/β-actin ratio. Data from dominants and subordinates were then normalized to protein/β-actin values from communal fish (Bio-Rad, Hercules, CA, United States). Primary antibodies used were as follows: mouse anti-CB_1R (1:500 Novus Biologicals), Rabbit anti-DAGL (1:500 Bioss Antibodies), β-actin (1:1000 Cell Signaling).

Neurocomputational Model

We constructed a neurocomputational model network that is composed of one excitatory cell, one inhibitory cell, and one M-cell. In the model, auditory inputs are initially delivered to the excitatory cell, which then excites the M-cell and the inhibitory cell. The inhibitory cell inhibits the M-cell only. In other words, auditory inputs affect the M-cell via two paths: a direct path via the excitatory cell and an indirect path via the excitatory cell and then the inhibitory cell. All model neurons were modeled as a conductance-based modified Morris–Lecar model with additional calcium-dependent potassium current (Morris and Lecar, 1981; Izhikevich, 2007; Ermentrout and Terman, 2010; Miller et al., 2017; Park et al., 2018). The membrane potential of each cell obeys the following current balance equation:

$$C\frac{dv}{dt} = -I_{Ca} - I_K - I_L - I_{KCa} - I_{Tot} \quad (1)$$

where $I_K = g_K n\,(v - v_K)$, $I_{Ca} = g_{Ca} m_\infty\,(v)\,(v - v_{Ca})$, $I_{KCa} = g_{KCa}\left\{\frac{[Ca]}{[Ca]+k_1}\right\}(v - v_K)$, $I_L = g_L\,(v - v_L)$ represent the potassium, calcium, calcium-dependent potassium, and leak currents, respectively. $[Ca]$ represents intracellular calcium concentration. For all neurons, $g_K = 8$, $v_K = -84$, $g_L = 2$, $v_L = -60$, $g_{Ca} = 4$, $v_{Ca} = 120$, and $k_1 = 10$. In the M-cell, $g_{KCa} = 0.3$ and $C = 1$. In other neurons, $g_{KCa} = 0.25$ and $C = 20$.

m_∞ is an instantaneous voltage-dependent gating variable for the calcium current where,

$$m_\infty\,(v) = 0.5\left(1 + \tanh\left(\frac{v - v_1}{v_2}\right)\right) \quad (2)$$

with $v_1 = -1.2$ and $v_2 = 18$.

The concentration of intracellular Ca^{2+} is governed by the calcium balance equation:

$$\frac{d\,[Ca]}{dt} = \varepsilon\left(-\mu I_{Ca} - k_{Ca}\,[Ca]\right) \quad (3)$$

where $\varepsilon = 0.005$, $\mu = 0.19$ for all neurons. $k_{Ca} = 0.9$ in the M-cell and $k_{Ca} = 1$ in other neurons.

n is a gating variable for the potassium current obeying,

$$\frac{dn}{dt} = \frac{\phi\,(n_\infty\,(v) - n)}{\tau_n\,(v)} \quad (4)$$

$$n_\infty\,(v) = 0.5\left(1 + \tanh\left(\frac{v - v_3}{v_4}\right)\right) \quad (5)$$

$$\tau_n\,(v) = 1/\cosh\left(\frac{v - v_3}{2v_4}\right) \quad (6)$$

where $\phi = 0.23$, $v_3 = 12$, and $v_4 = 17$ for all neurons.

In an excitatory cell and an inhibitory cell, the synaptic variable, s, is modeled by an equation for the fraction of activated channels,

$$\frac{ds}{dt} = \alpha s_\infty\,(v)\,(1 - s) - \beta s \quad (7)$$

where $s_\infty\,(v) = 1/\left(1 + \exp\left(-\frac{v+\theta_s}{\sigma_s}\right)\right)$ with $\theta_s = 0$ and $\sigma_s = 4$. The parameters $\alpha = 15$ and $\beta = 0.3$ in an excitatory cell, and $\alpha = 8.5$ and $\beta = 0.046$ in an inhibitory cell.

I_{Tot} represents the total input that a cell receives and is composed of a fixed constant (I_0), the synaptic current (I_{syn}) which represents the sum of synaptic inputs from other cells, and an applied current $[I_{app}\,(t)]$. The synaptic current is given by:

$$I_{syn} = g_{syn}\left(v - v_{syn}\right)\sum_j s_j \quad (8)$$

where the summation is over s variables from all neurons projecting to a given neuron.

In the current neuronal network, an excitatory cell does not receive any synaptic input but receives an external stimulus to simulate the effect of an external stimulus from the sensory input. Thus, in an excitatory cell, $I_{Tot} = I_{E0} + I_{syn} + I_{app}\,(t)$ where $I_{E0} = 43.9$ is a fixed constant, $I_{syn} = 0$, and $I_{app}\,(t) = W_E I\,(\tau)$. Here, W_E is the stimulus strength, and $I(\tau)$ is the stimulus which resembles the square unit pulse with height 1 with duration of 2 ms starting at time τ.

In the M-cell, which receives synaptic inputs from an excitatory cell and an inhibitory cell in the network, the synaptic

input is,

$$I_{syn} = g_{\mathrm{E}\to\mathrm{M}}\,(v - v_{\mathrm{E}\to\mathrm{M}})\,s_E + g_{\mathrm{I}\to\mathrm{M}}\,(v - v_{\mathrm{I}\to\mathrm{M}})\,s_I + g_{\mathrm{M}\to\mathrm{M}}\,(v - v_{\mathrm{M}\to\mathrm{M}})\,s_M \quad (9)$$

where s_M is the synaptic variable from another M-cell, which is assumed to be a constant in the current study.

Now, calcium is known to modulate the presynaptic neurotransmitter release via retrograde signaling (Diana and Bregestovski, 2005). In the model M-cell we assumed that intracellular calcium level reciprocally modulates the presynaptic input to the M-cell and I_{syn} is updated as follows:

$$I_{syn} = g_{\mathrm{I}} * g_{\mathrm{E}\to\mathrm{M}} * (v - v_{\mathrm{E}\to\mathrm{M}})\,s_E + g_{\mathrm{I}} * g_{\mathrm{I}\to\mathrm{M}} * (v - v_{\mathrm{I}\to\mathrm{M}})\,s_I + g_{\mathrm{M}\to\mathrm{M}}\,(v - v_{\mathrm{M}\to\mathrm{M}})\,s_M \quad (10)$$

where g_{I} obeys the following equation:

$$\frac{dg_I}{dt} = \frac{\frac{g_{Imax}}{[Ca]+k_2} - g_{\mathrm{I}}}{\rho} \quad (11)$$

where g_{Imax} is the maximal g_{I} value, ρ is the time constant of g_{I}, and [Ca] is the intracellular calcium concentration of the M-cell. The total input to the M-cell is $I_{Tot} = I_{M0} + I_{syn}$ where $I_{M0} = 31$ is a fixed constant and the synaptic input I_{syn} is as in Eq. (10). Other parameter values are given as follows: $g_{\mathrm{E}\to\mathrm{M}} = 0.15$, $v_{\mathrm{E}\to\mathrm{M}} = 30$, $g_{\mathrm{I}\to\mathrm{M}} = 0.5$, $v_{\mathrm{I}\to\mathrm{M}} = -50$, $g_{\mathrm{M}\to\mathrm{M}} = 0.5$, $v_{\mathrm{M}\to\mathrm{M}} = -50$, $s_M = 0.029$, $g_{Imax} = 20$, $k_2 = 10$, and $\rho = 10000$.

An inhibitory cell does not receive any direct external stimulus [$I_{app}\,(t) = 0$] but receives a synaptic input from the excitatory cell. Thus, in the inhibitory cell, $I_{syn} = g_{\mathrm{I}} * g_{\mathrm{E}\to\mathrm{I}}\,(v - v_{\mathrm{E}\to\mathrm{I}})\,s_E$ where s_E is the synaptic variable from the excitatory cell. Thus, the total input to an inhibitory cell is $I_{Tot} = I_{I0} + I_{syn}$ where $I_{I0} = 36$, $v_{\mathrm{E}\to\mathrm{I}} = 30$, and $g_{\mathrm{E}\to\mathrm{I}} = 0.75$ for a dominant-like model and $g_{\mathrm{E}\to\mathrm{I}} = 0.7$ for a subordinate-like model.

Some parameters were modified to reflect different firing properties of each cell based on experiments (Eaton et al., 2001; Korn and Faber, 2005; Liao and Fetcho, 2008; Song et al., 2015). We note that all three cells are excitable cells so that they do not fire action potentials unless they receive enough excitatory inputs from other active cells or external stimulus. The main parameter that we controlled to implement these firing properties is the baseline level of a fixed constant I_0. Note that only the excitatory cell will receive the external stimulus which mimics the sensory input.

2-AG released from the M-cell modulates the release of the neurotransmitters in the pre-synaptic cells through CB_1R. To explore how 2-AG modulates the observed social status dependent escape responses to the external stimulus, we implemented the 2-AG modulation of synaptic inputs in the cells as follows.

In an inhibitory cell,

$$I_{Tot} = I_{I0} + I_{syn} = I_{I0} - g_{\mathrm{I}} * g_{\mathrm{E}\to\mathrm{I}}\,(1 + CB1R_{EI})\,(v - v_{\mathrm{E}\to\mathrm{I}})\,s_E \quad (12)$$

The main parameter $CB1R_{EI}$ depends on the social status and we let $CB1R_{EI} = 0.32$ for a dominant-like model and $CB1R_{EI} = 0.3$ for a subordinate-like model.

In the M-cell,

$$I_{Tot} = I_{M0} + I_{syn} = I_{M0} - g_{\mathrm{I}} * g_{\mathrm{E}\to\mathrm{M}}\,(1 + CB1R_{EM})\,(v - v_{\mathrm{E}\to\mathrm{M}})\,s_E - g_{\mathrm{I}} * g_{\mathrm{I}\to\mathrm{M}}\,(1 - CB1R_{IM})\,(v - v_{\mathrm{I}\to\mathrm{M}})\,s_I - g_{\mathrm{M}\to\mathrm{M}}\,(v - v_{\mathrm{M}\to\mathrm{M}})\,s_M \quad (13)$$

The main parameters $CB1R_{EM}$ and $CB1R_{IM}$ depend on the social status. We let $CB1R_{EM} = 0.27$ for a dominant-like model and 0.3 for a subordinate-like model. Similarly, $CB1R_{IM} = 0.2$ for a dominant-like model and 0.25 for a subordinate-like model.

Simulations were performed on a personal computer using the software XPP (Ermentrout, 2002). The numerical method used was an adaptive-step fourth order Runge-Kutta method with a step size 0.01 ms. The neurocomputational model is available online in ModelDB[2]. This website offers one of the largest opensource selections of neurocomputational models for various brain regions.

RESULTS

Social Status Regulation of Endocannabinoids' Signaling Pathways

Previously, we demonstrated that social status regulates the activation of the startle escape and swim behaviors. The sensitivity of the startle escape response significantly increases in subordinates relative to dominants and group-housed fish; while swimming frequency significantly increases in dominants and decreases in subordinates (Miller et al., 2017). Given that the escape and swim circuits receive descending and local neuromodulatory inputs, we hypothesized that the differences in excitability and behavioral selection are likely due to a rebalance in the strength of excitatory and inhibitory neuromodulatory inputs. One potential mechanism is the previously described retrograde release of 2-AG from the post-synaptic M-cell shown to regulate the M-cell's excitability by potentiating release from pre-synaptic dopaminergic inputs (Cachope et al., 2007). Moreover, the ECS is known to modulate other brain and spinal circuits involved in regulating motivated behavior (El Manira et al., 2008; Wenzel and Cheer, 2018). This lends credence to the notion that the ECS plays a key regulatory role in the molecular mechanism by which social status impacts circuit excitability. Therefore, we measured whole brain gene expression patterns of diacylglycerol lipase (DAGL), the primary enzyme that synthesizes 2-AG, monoacylglycerol lipase (MGL), the primary enzyme to degrade 2-AG, and Cannabinoid receptor type 1 (CB_1R) (Zou and Kumar, 2018). We found that the RNA gene expression of DAGL and CB_1R are socially regulated (**Figure 2A**). Dominant animals showed a significant increase in DAGL expression relative to subordinates, and subordinates showed a significant decrease in DAGL expression relative to controls [Kruskal–Wallis test, $p < 0.05$; **Figure 2A**]. We also observed that the expression of CB_1R was significantly increased

[2]https://senselab.med.yale.edu/modeldb/

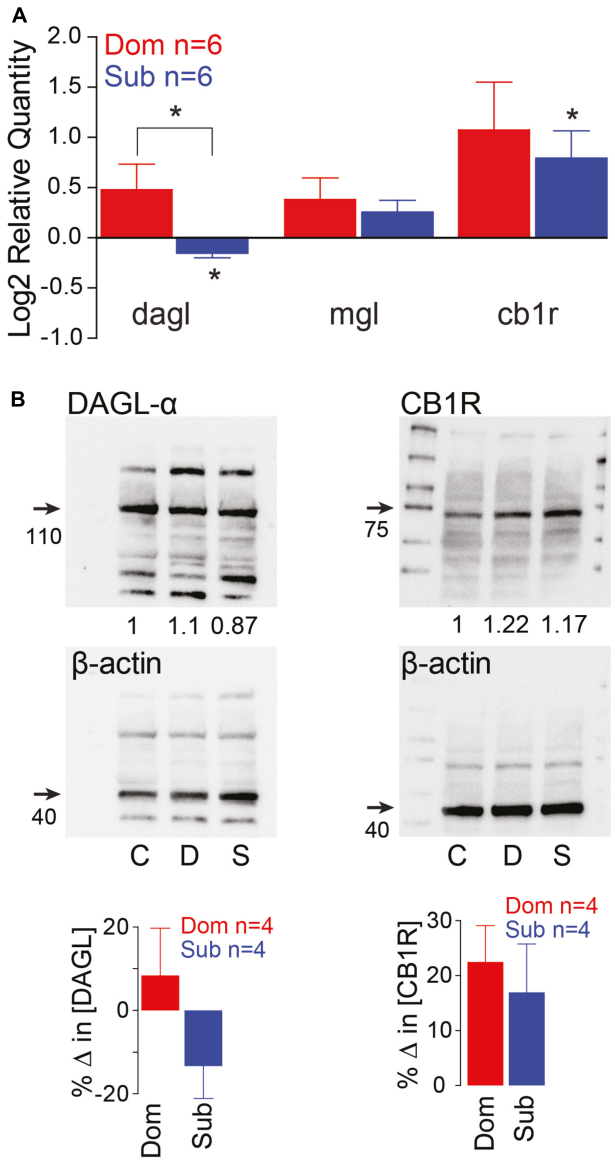

FIGURE 2 | Endocannabinoid signaling pathway is socially regulated and its modulation of M-cell excitability is status-dependent. **(A)** qPCR gene expression analysis of ECS signaling molecules. Data of dominants and subordinates normalized to control isolates ($n = 6$ pairs; control isolates $n = 6$; Kruskal–Wallis test, $^*p < 0.05$). **(B)** Western blot analysis of dagl-α and cb1 receptor. Each protein was tested concurrently with β-actin as a control. Protein expression of dominants and subordinates was normalized to communal controls as a ratio (illustrated values below each band); C, communals; D, dominants; S, subordinates. Bar graphs represent average % change in protein concentration of four replicates of samples each consisting of 10 brains normalized to WT communals.

in subordinates relative to controls [Kruskal–Wallis test, $p < 0.05$; **Figure 2A**], but CB_1R expression was not significantly different between dominants and subordinates. Western blot analysis of DAGL and CB_1R showed similar protein expression patterns (**Figure 2B**). DAGL western blots consistently showed multiple protein bands. Several causes for this are possible, including: denaturation of protein structure leading to increased antibody cross-reactivity with similar amino acid residues; differences in phosphorylation states of the DAGL proteins; and reduced antibody specificity for DAGL. Regardless, the largest bands were reliably ∼110 kDa, consistent with the size of DAGL. Such variations were not observed with CB_1R western blots that consistently showed single bands (**Figure 2B**).

2-AG Modulation of Motor Activities Is Social Regulated

These results led us to postulate that differences in the ECS may account for the social status-dependent differences in locomotor behavior. To test this hypothesis, we augmented 2-AG levels by injecting the animals with JZL184, an irreversible inhibitor of MGL (Long et al., 2009; **Figures 3A–C**). To compare differences in the startle escape response probabilities among the three animal groups and treatment (JZL184 injection), we performed a mixed-design ANOVA (between-subject factor as Group; within-subject factors as Treatment and Decibel). There was a significant main effect of Decibel [$F(3.35,107.06) = 338.337$, $p < 1.0e\text{-}16$; **Figures 3A–C**] and marginal main effect of Group [$F(2,32) = 3.023$, $p = 6.30e\text{-}2$; **Figures 3A–C**], but there was no effect of Treatment [$F(1,32) = 0.080$, $p > 0.05$; **Figures 3A–C**]. There were significant interactions of Group*Treatment [$F(2,32) = 15.69$, $p = 1.80e\text{-}5$; **Figures 3A–C**], Group*Decibel [$F(6.69,107.06) = 2.26$, $p = 3.75e\text{-}2$; **Figures 3A–C**], Treatment*Decibel [$F(3.75,120.06) = 2.18$, $p = 7.99e\text{-}2$; **Figures 3A–C**], and Group*Treatment*Decibel [$F(7.50,120.06) = 3.61$, $p = 1.12e\text{-}3$; **Figures 3A–C**]. We performed the *post hoc* test to determine which animal groups had higher escape response probability. We observed that the response probability for dominants was significantly lower compared to subordinates (LSD, $p = 3.95e\text{-}2$; **Figures 3A–C**), but there was no difference compared to communals (LSD, $p > 0.05$; **Figures 3A,B**). Moreover, the response probability for subordinates was also significantly higher compared to communals (LSD, $p = 4.41e\text{-}2$; **Figures 3A,C**).

We then performed further analysis to determine whether JZL184 injection affected the escape response in each animal group. In communals, there was significant main effect of Decibel [$F(3.13,31.27) = 107.87$, $p < 1.0e\text{-}16$; **Figure 3A**], but no effect of Treatment [$F(1,10) = 2.63$, $p > 0.05$; **Figure 3A**]. There was also no Treatment*Decibel interaction [$F(3.47,34.72) = 1.72$, $p > 0.05$; **Figure 3A**]. We also observed a significant difference of the startle escape responses due to JZL184 at 85 dB for communals [paired one-sample two-sided t-test, $t(10) = 3.08$, $p = 1.16e\text{-}2$; **Figure 3A**]. In dominants, there were significant main effects of Treatment [$F(1,11) = 7.57$, $p = 1.88e\text{-}2$; **Figure 3B**] and Decibel [$F(2.40,26.43) = 106.12$, $p < 1.0e\text{-}16$; **Figure 3B**]. There was no effect of Treatment*Decibel interaction [$F(2.90,31.89) = 1.43$, $p > 0.05$; **Figure 3B**]. We did not observe a significant difference after the treatment at a particular decibel although we observed that JZL184 injection increased the overall startle escape response over the wide range of decibel. In subordinates, there were significant main effects of Treatment [$F(1,11) = 30.08$, $p = 1.91e\text{-}4$; **Figure 3C**], Decibel [$F(2.15,23.65) = 134.28$, $p < 1.0e\text{-}16$; **Figure 3C**], and Treatment*Decibel [$F(3.14,34.59) = 5.67$,

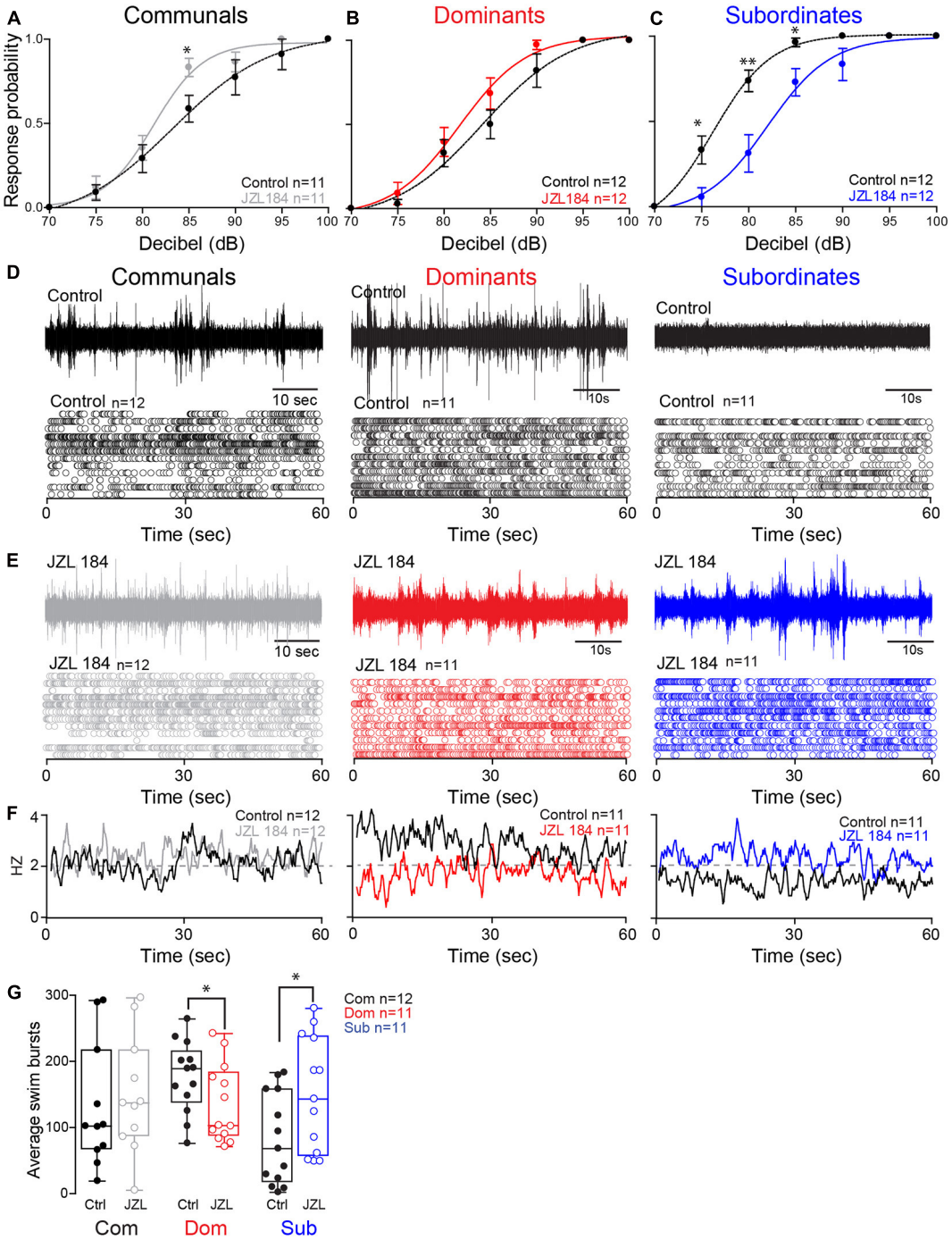

FIGURE 3 | 2-AG modulation of escape and swimming activities is social status-dependent. **(A–C)** Probability of startle escape response before (control) and after JZL184 injections for communals, dominants and subordinates, respectively. Asterisks (*$p < 0.05$, **$p < 0.005$) denote statistical difference between control and experimental condition at the specified dB level. **(D)** 1 min recoding of far field-potentials of spontaneous swimming activity before (control) and **(E)** after JZL184 injections for communal, dominants and subordinates, respectively, along with respective raster plots of each condition. **(F)** Average swimming frequency for all animals tested before and after JZL184 injection. **(G)** Box and whiskers plots of the average number of swim bursts per 1 min for each social phenotype. Dots represent individual animals. The box extends from the 25th to 75th percentiles, horizontal line is the median, and whiskers represent max/min values.

p = 8.50e-5; **Figure 3C**]. We observed that JZL184 injection significantly decreased the overall startle escape response over the wide range of decibels. In particular, we observed significant differences in the startle escape responses at 75 dB [paired one sample two-sided t-test; $t(11) = 3.45$, p = 5.46e-3; **Figure 3C**], at 80 dB [$t(11) = 3.82$, p = 2.86e-3; **Figure 3C**], and at 85 dB [$t(11) = 2.72$, p = 1.99e-2; **Figure 3C**]. In summary, the results show that blocking 2-AG

degradation increased the startle escape response sensitivity in communals and moderately increased it in dominants, but significantly decreased the startle escape sensitivity in subordinates.

Activation of the startle escape response suppresses swimming activity by inhibiting the slow motor neurons that drive swimming behavior (Svoboda and Fetcho, 1996; Satou et al., 2009). Given that the ECS is implicated in promoting motivated behavior, we hypothesized that increasing the availability of 2-AG is likely to promote a behavioral switch in the activation pattern that would favor motivated behavior (i.e., swimming) over submissive behavior (i.e., escape). The notion is that augmenting 2-AG would be sufficient to reverse the activation pattern of the swim circuit in a socially dependent manner as was observed with the escape response. Indeed, we found that injection of JZL184 had the opposite effects on dominants and subordinates in that subordinates significantly increased their swimming activity while dominants significantly decreased their swimming and communal animals showed only moderate change (**Figures 3D–G**).

We compared the difference of swim bursts among three animal groups and treatment (JZL184 injection) and performed a mixed-design ANOVA (between-subject factor as Group; within-subject factor as Treatment). We found no main effect of Group [$F(2,31) = 0.44$, $p > 0.05$; **Figures 3D–G**] and no effect of Treatment [$F(1,31) = 1.50$, $p > 0.05$; **Figures 3D–G**]. But there was a significant effect of Group*Treatment interaction [$F(2,31) = 10.89$, $p = 2.62\text{e-}4$; **Figures 3D–G**]. We performed further analysis to determine whether JZL184 injection affects the swim bursts in each animal group. In communals, we found a marginal main effect of Treatment [$F(1,11) = 3.65$, $p = 8.25\text{e-}2$; **Figures 3D–G**]. JZL184 injection slightly increased the swim bursts in communals. In dominants, there was a significant main effect of Treatment [$F(1,10) = 6.98$, $p = 2.46\text{e-}2$; **Figures 3D–G**]. JZL184 injection significantly decreased the swim bursts for dominants. In subordinates, there was a significant main effect of Treatment [$F(1,10) = 9.41$, $p = 1.19\text{e-}2$; **Figures 3D–G**]. JZL184 injection significantly increased the swim bursts for subordinates.

Effects of AM-251 on Startle Escape and Swim Activities in Dominant and Subordinate Animals

To determine whether these status-dependent differences are due to changes in CB_1R activity, we tested the startle escape response in the presence of AM-251, a specific CB_1R antagonist (Seely et al., 2012; **Figures 4A–C**). To compare the difference of the startle escape response probabilities to auditory pulses among three animal groups and treatment (AM-251 injection), we performed a mixed-design ANOVA (between-subject factor as Group; within-subject factors as Treatment and Decibel). There were significant main effects of Treatment [$F(1,27) = 10.32$, $p = 3.39\text{e-}3$; **Figures 4A–C**] and Decibel [$F(2.99,80.62) = 218.56$, $p < 1.0\text{e-}16$; **Figures 4A–C**]. But there was no main effect of Group [$F(2,27) = 2.22$, $p > 0.05$; **Figures 4A–C**]. We also observed significant interaction effects of Group*Decibel [$F(5.97,80.62) = 2.27$, $p = 4.55\text{e-}2$; **Figures 4A–C**] and Treatment*Decibel [$F(2.90,78.16) = 4.36$, $p = 7.41\text{e-}3$; **Figures 4A–C**]. But there were no effects of Group*Treatment interaction [$F(2,27) = 0.633$, $p > 0.05$; **Figures 4A–C**] and Group*Treatment*Decibel interaction [$F(5.79,78.16) = 0.76$, $p > 0.05$; **Figures 4A–C**]. We performed the *post hoc* test to determine which animal groups had higher probability. We observed that the response probability for subordinates was marginally higher compared to dominants (LSD, $p = 5.44\text{e-}2$; **Figures 4B,C**). But there were no differences between subordinates and communals (LSD, $p > 0.05$; **Figures 4A,C**) and between dominants and communals (LSD, $p > 0.05$; **Figures 4A,B**).

We then performed further analysis to determine whether AM-251 injection affects the escape response in each animal group. In communals, there was significant effect of Decibel [$F(2.70,23.30) = 46.88$, $p < 1.0\text{e-}16$; **Figure 4A**]. But there were no effects of Treatment [$F(1,9) = 3.32$, $p > 0.05$; **Figure 4A**] and Treatment*Decibel interaction [$F(3.04,27.31) = 0.41$, $p > 0.05$; **Figure 4A**]. In dominants, there was a significant main effect of Decibel [$F(2.16,19.41) = 77.29$, $p < 1.0\text{e-}16$; **Figure 4B**]. But there were no effects of Treatment [$F(1,9) = 1.74$, $p > 0.05$; **Figure 4B**] and Treatment*Decibel interaction [$F(1.74,15.66) = 1.26$, $p > 0.05$; **Figure 4B**]. In subordinates, there were significant main effects of Treatment [$F(1,9) = 13.50$, $p = 5.12\text{e-}3$; **Figure 4C**] and Decibel [$F(1.75, 15.72) = 139.13$, $p < 1.0\text{e-}16$; **Figure 4C**]. There was also a significant effect of Treatment*Decibel interaction [$F(2.84,25.60) = 5.65$, $p = 4.63\text{e-}3$; **Figure 4C**]. In particular, we observed significant differences in the startle escape responses at 80 dB [paired one sample two-sided t-test; $t(9) = 3.69$, $p = 5.02\text{e-}3$; **Figure 4C**], at 85 dB [$t(9) = 2.35$, $p = 4.34\text{e-}2$; **Figure 4C**], and at 90 dB [$t(9) = 2.51$, $p = 3.33\text{e-}2$; **Figure 4C**]. In summary, irrespective of social rank, AM-251 decreased startle escape sensitivity particularly so in subordinates. These findings supports Cachope et al. (2007) results in that 2-AG potentiates the sensitivity of the M-cell. We extend on their finding and show that the supply of 2-AG is socially regulated, and it modulates M-cell excitability in a status-dependent manner.

To determine whether CB_1R activity influences swimming patterns differently among the three social groups, we compared swim bursts among three animal groups and treatment (AM-251 injection) by performing a mixed-design ANOVA (between-subject factor as Group; within-subject factor as Treatment). There were main effects of Group [$F(2,27) = 4.25$, $p = 2.48\text{e-}2$; **Figures 4D–G**] and Treatment [$F(1,27) = 5.77$, $p = 2.34\text{e-}2$; **Figures 4D–G**]. But there was no effect of Group*Treatment interaction [$F(2,27) = 1.35$, $p > 0.05$; **Figures 4D–G**]. *Post hoc* tests showed that the swim bursts for dominants were significantly higher compared to communals (LSD, $p = 4.61\text{e-}2$; **Figures 4D–G**) and subordinates (LSD, $p = 9.19\text{e-}3$; **Figures 4D–G**). But there was no difference of swim bursts between communals and subordinates (LSD, $p > 0.05$; **Figures 4D–G**). We performed the further analysis to determine whether AM-251 injection affects the swim bursts in each animal group. In communals, there was a significant main effect of Treatment [$F(1,9) = 8.05$, $p = 1.95\text{e-}2$; **Figures 4D–G**]. AM-251 injection significantly decreased the swim bursts in communals. In dominants, there was no main effect

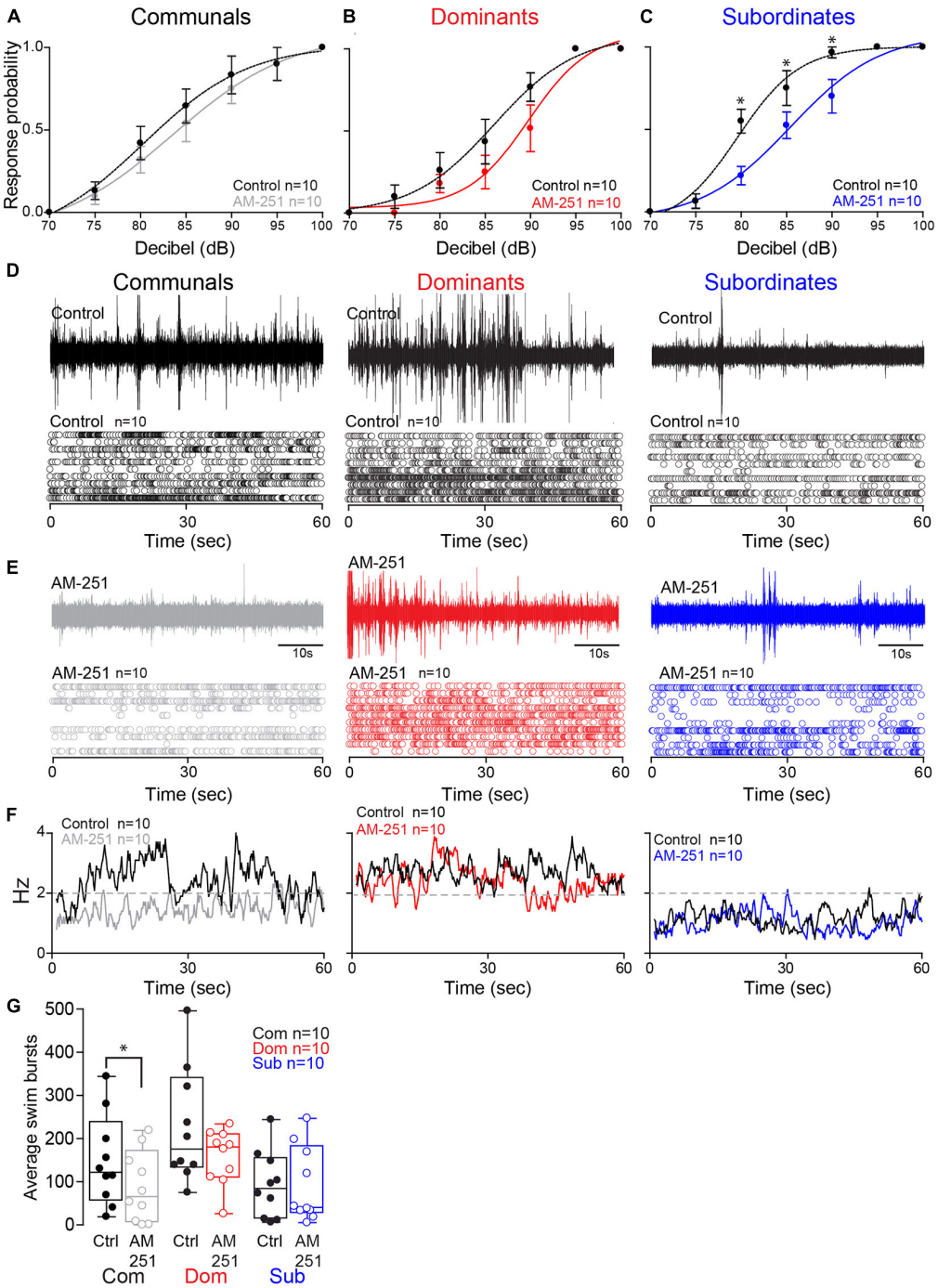

FIGURE 4 | Effect of AM-251 on status-dependent escape probability and swim frequency. **(A–C)** Probability of startle escape response before (control) and after AM-251 injections for communals, dominants and subordinates, respectively. Asterisks (*$p < 0.05$) denote statistical difference between control and experimental condition at the specified dB level. **(D,E)** 1 min recoding of far field-potentials of spontaneous swimming activity before (control) and after AM-251 injections for communal, dominants and subordinates, respectively, along with respective raster plots for all animals tested. **(F)** Average swimming frequency for all animals tested before and after AM-251 injection. **(G)** Box and whiskers plots of the average number of swim bursts per 1 min for each social phenotype. Box plot parameters are defined in **Figure 3G**.

of Treatment [$F(1,9) = 2.48$, $p > 0.05$; **Figures 4D–G**]. In subordinates, there was no main effect of Treatment [$F(1,9) = 0.004$, $p > 0.05$; **Figures 4D–G**]. Thus, blockage of CB_1R significantly decreased swimming in communals while no changes in swimming were observed in dominants or subordinates.

Collectively, the results of supplementing 2-AG and blockage of CB_1R indicate that 2-AG's regulation of the swim and escape behaviors is socially regulated. More importantly, 2-AG serves as a molecular switch in shifting the activation pattern between competing circuits by modulating the balance of excitatory and inhibitory inputs onto the two motor circuits in a social status-dependent manner.

2-AG Regulation of M-Cell Excitability Mediated via Dopaminergic Receptor Type 1b

Dopamine is known to be involved in social regulation (Watanabe and Yamamoto, 2015), motivation (Hamid et al., 2016), and aggression (Filby et al., 2010), and its function is highly interdependent with the ECS signaling pathway (Wenzel and Cheer, 2018). Specifically, ECS signaling can be mediated via dopamine receptor type 1 (DRD1) (Zenko et al., 2011). In zebrafish, the most compelling evidence is the capacity of 2-AG to potentiate mixed synaptic transmission to the M-cell that requires activation of DRD1 (Cachope et al., 2007). Moreover, evidence by Pereda et al. (1992) shows direct dopaminergic innervation of the M-cell, DA release directly potentiates M-cell excitability, and this potentiation can be blocked by antagonizing DRD1. We hypothesized that ECS signaling underlying status-dependent differences in escape sensitivity is mediated through DRD1. To test this hypothesis, we repeated our experiment of augmenting 2-AG levels but in DRD1 knockout zebrafish [drd1b$^{(-/-)}$] (Busch-Nentwich et al., 2013). We found that startle escape sensitivity was unaffected in drd1b$^{(-/-)}$ fish following JZL184 injection contrasting with the results of WT animals, in which an increase of 2-AG levels significantly increased startle escape sensitivity (**Figure 5A** compare with **Figure 3A**). We compared differences in the startle escape response probabilities to auditory pulses for DRD1 knockout zebrafish before and after the treatment of JZL 184 injection and performed repeated measures ANOVA (within-subject factors as Treatment and Decibel). We found a significant main effect of Decibel [$F(2.03,18.30) = 64.03$, $p <$ 1.0e-16; **Figure 5A**]. But there was no main effect of Treatment [$F(1,9) = 0.12$, $p > 0.05$; **Figure 5A**] and no Treatment*Decibel interaction [$F(2.77,24.92) = 0.75$, $p > 0.05$; **Figure 5A**]. Similarly, we found no change in swimming frequency in the drd1b$^{(-/-)}$ animals following JZL184 injection [$F(1,9) = 0.41$, $p > 0.05$; **Figures 5B,C**]. These results show that the potentiating effects of 2-AG on startle escape sensitivity are mediated through DRD1b.

Neurocomputational Analysis of 2-AG Regulation of the Escape Circuit

Our empirical results show that the ECS is affected by social experience to regulate the activation of the startle escape and swim behaviors. This complex interaction between social factors, neuromodulatory systems and motor circuits necessitated the development of a neurocomputational model to better understand how social status influences the ECS to modulate the excitability and pattern of motor activity. Toward this end, we developed a computational model whereby we simulated the M-cell along with two pre-synaptic cells, an excitatory cell (representing glutamatergic and/or dopaminergic neurons) and an inhibitory cell (representing GABAergic and/or glycinergic neurons) (see section "Materials and Methods," **Figure 6A**, inset).

Using this model, we tested the hypothesis that differences in the amount of 2-AG release due to differences in DAG lipase expression would account for how 2-AG sets the gain of the pre-synaptic excitatory and inhibitory inputs onto the M-cell. More specifically, we tested whether the behavioral differences in the escape circuit are modulated by changing network properties (synaptic strengths, $g_{E\to I}$) and three activity levels of CB_1Rs ($CB1R_{EM}$, $CB1R_{EI}$, and $CB1R_{IM}$) in the model to reflect different levels of 2-AG and social status. Here, $g_{A\to B}$ and $CB1R_{AB}$ represent the synaptic strength and the activity level of CB_1R from a neuron A to a neuron B where E, I, and M represent for the excitatory, inhibitory, and M-cell, respectively. We assumed that dominants and subordinates have different levels of a synaptic strength and activity levels of CB_1R. In particular, we assumed that low values of $CB1R_{EM}$ and $CB1R_{IM}$, high values of $CB1R_{EI}$ and $g_{E\to I}$ for a dominant-like model while high values of $CB1R_{EM}$ and $CB1R_{IM}$, low values of $CB1R_{EI}$ and $g_{E\to I}$ for a subordinate-like model. That is, for a dominant-like model, the inhibitory pathways were enhanced while a subordinate-like model had strong excitatory pathways.

Effect of Social Status on the Startle Escape Response in the Model

Depolarizing current pulses were applied to the model excitatory cell (50 stimuli for 2 ms duration with 1 sec inter stimulus interval) to determine the dynamic range of the model M-cell excitability for dominant-like and subordinate-like models. Here, the excitatory cell will excite both the inhibitory cell and the M-cell while the inhibitory cell inhibits the M-cell. In the simulation, the amplitude of the applied current, W_E in $I_{app}(t)$ on the excitatory cell, was gradually increased and the response probability of the M-cell was recorded. **Figure 6A** shows an example of the response of the M-cell when $W_E = 60$ for both model groups. Here, the response probabilities are 10/50 for a dominant-like model and 50/50 for a subordinate-like model. **Figure 6B** shows the response probabilities of the model M-cell for both model groups over the wide range of the applied currents (black curves). A subordinate-like model showed the significantly higher startle escape response probability compared to a dominant-like model. These results demonstrate that our model can reproduce the social status-dependent startle escape responses observed empirically. Different values in the synaptic strength and activation levels of CB_1Rs were sufficient to obtain the transition of activity patterns between a dominant-like model and a subordinate-like model while maintaining the same network architecture.

Social Status-Dependent Effects of 2-AG on the Startle Escape Response in the Model

To determine how the social status-dependent differences of 2-AG may account for the observed changes in the startle escape circuit, we tested the excitability of the M-cell by changing

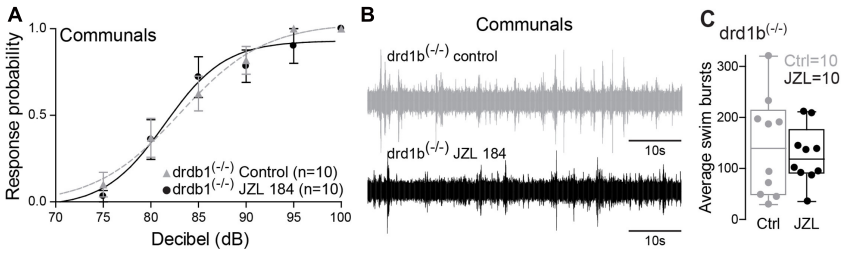

FIGURE 5 | drd1b expression is socially regulated and necessary for status-dependent ECS regulation of escape and swim circuits. **(A)** Probability of startle escape response of drd1b$^{(-/-)}$ communal zebrafish before (control) and after injection of JZL184. **(B)** 1 min recording of far field-potentials of spontaneous swimming activity before (control) and after JZL184 injections for drd1b$^{(-/-)}$ communal zebrafish. **(C)** Average number of swim bursts per 1 min for drd1b$^{(-/-)}$ communal fish ($n = 10$) before and after JZL184 injection.

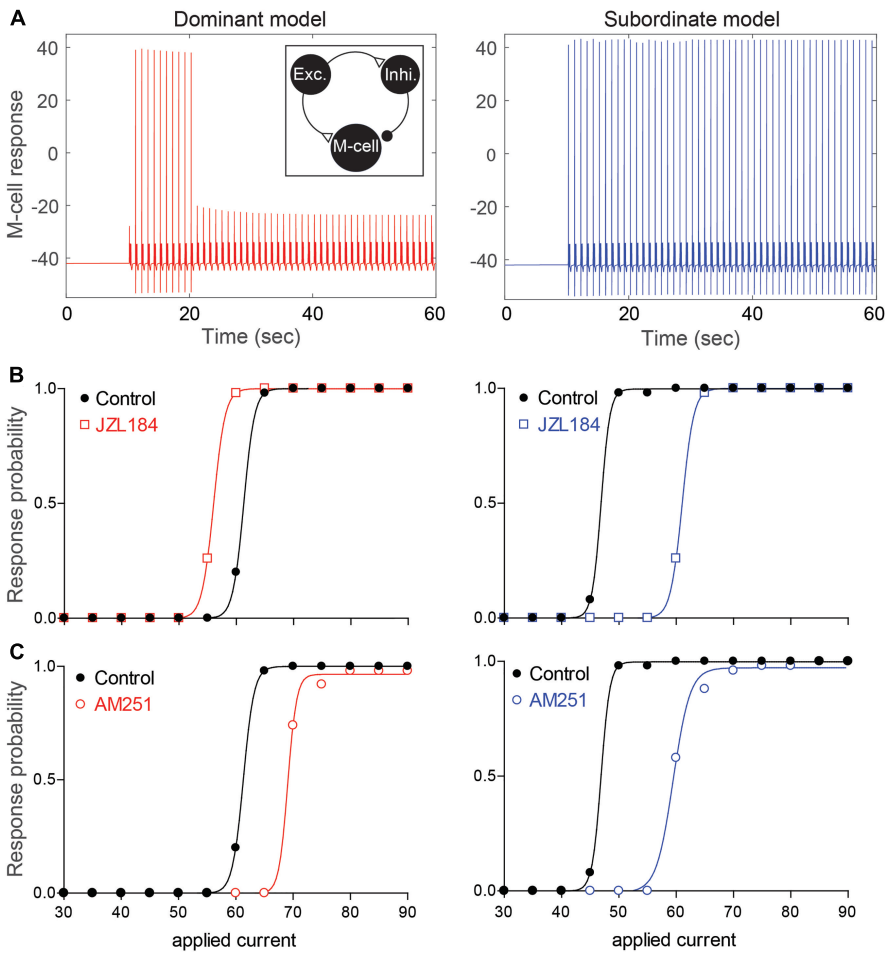

FIGURE 6 | Neurocomputational models simulating the effects of 2-AG on the escape response. **(A)** Examples of the response of the M-cell model cell within a simple model network (inset) to repeated suprathreshold applied current injection in dominant-like (left) and subordinate-like (right) models. **(B,C)** Results of dominant-like and subordinate-like models simulating the probability of startle escape response before (control, black solid line) and during [JZL184, **(B)**] and blockage of CB_1R [AM-251, **(C)**].

the activity levels of CB_1Rs on the presynaptic excitatory and inhibitory cells.

To mimic the observed effects of JZL184 on the startle response, we assumed that the activity levels of CB_1Rs on all cells are increased from baseline, but in different ratios depending on the social status. Since DAGL in dominants was already higher compared to that in subordinates (**Figure 2**), we assumed that 2-AG level in a dominant-like model is already near the maximum

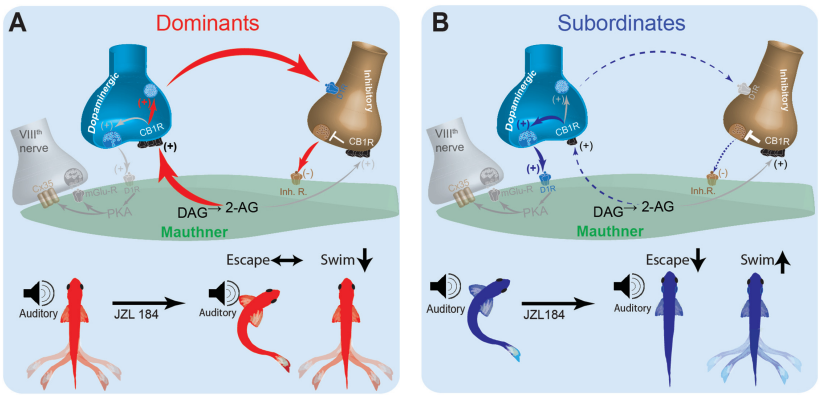

FIGURE 7 | Schematic model for social status-dependent regulation of neurochemical inputs to the M-cell: The M-cell (green) receives inputs from DA cells (blue), the excitatory VIIIth cranial nerve (gray), and inhibitory (brown). Our model predicts distinct neurochemical pathways in dominants **(A)** and subordinates **(B)** responsible for differences in startle escape sensitivity. These pathways are proposed based on differential effects of JZL184 treatment on startle escape behavior (bottom). Higher baseline 2-AG in dominants is responsible for activation of the "inhibitory pathway" via inhibitory neurotransmitter release. Lower baseline 2-AG in subordinates activates the lower threshold "excitatory pathway" via the VIIIth nerve, responsible for higher startle escape sensitivity.

capacity so that the effect of JZL184 would be negligible in a dominant-like model. On the other hand, we assumed that 2-AG level in a subordinate-like model is lower than the maximum capacity so that the effect of JZL184 would be significant in a subordinate-like model. Recall that we assumed that in dominant-like model, the activity levels of $CB1R_{EM}$ and $CB1R_{IM}$ are weak, but $CB1R_{EI}$ is strongly active. In subordinate-like model, on the other hand, we assumed that the activity levels of $CB1R_{EM}$ and $CB1R_{IM}$ are strong, but $CB1R_{EI}$ is weakly active. To model JZL184 injection, we used $CB1R_{IM} \rightarrow 1.6^{*}CB1R_{IM}$, $CB1R_{EM} \rightarrow 1.6^{*}CB1R_{EM}$, $CB1R_{EI} \rightarrow 1.4^{*}CB1R_{EI}$ for a dominant-like model and $CB1R_{IM} \rightarrow 1.7^{*}CB1R_{IM}$, $CB1R_{EM} \rightarrow 1.7^{*}CB1R_{EM}$, $CB1R_{EI} \rightarrow 2.7^{*}CB1R_{EI}$ for a subordinate-like model. We assumed that the activity level of $CB1R_{EI}$ for a subordinate-like model would be significantly increased in the presence of JZL184. In summary, JZL184 injection in the model will enhance the activity levels of these CB_1Rs in general. However, weakly activated CB_1Rs will be significantly increased while strongly activated CB_1Rs will be slightly increased although these increases of the activity levels still depend on the social status. The results show that simulated JZL184 injection slightly increased the escape response in a dominant-like model, but it significantly decreased the escape response in a subordinate-like model (compare **Figures 3B,C** with **Figure 6B**). Note that the assumption of the significant increase of the activity level of $CB1R_{EI}$ for a subordinate-like model was necessary for the significant decrease of the escape response while keeping all other biophysically driven parameters constants.

To mimic the effects of the AM-251 injection on dominant-like and subordinate-like models, we assumed that the activity levels of CB_1Rs on all cells are 0 after AM-251 injection. That is, $CB1R_{EM} = 0$, $CB1R_{EI} = 0$, and $CB1R_{IM} = 0$ for both model animal groups. These changes resulted in a slight decrease of the escape response in the dominant-like model, but a significant decrease in the subordinate-like model compared to their original response curves as we observed in the empirical results (compare **Figures 4B,C** with **Figure 6C**).

These results suggest that social status-dependent regulation of the pre-synaptic inputs of the M-cell may be mediated, in part, by intrinsic changes to M-cell excitability and retrograde activation of CB_1R (**Figure 7**). These changes are sufficient to replicate the escape response patterns of model animal groups as observed experimentally. Model simulations suggest that in a dominant-like model, the synaptic connections in the excitatory cell → inhibitory cell → M-cell were strengthened while the connection of the excitatory cell → M-cell was weakened as compared to a subordinate-like model (**Figure 7A**). On the other hand, in a subordinate-like model, the synaptic connection of the excitatory cell → M-cell was strengthened while the inhibitory pathway was weakened (**Figure 7B**). 2-AG also differently modulates the M-cell excitability in a social status-dependent manner so that a dominant-like model has a stronger activation of CB_1R from the excitatory cell to the inhibitory cell while a subordinate-like model has stronger activations of CB_1Rs in the excitatory cell → M-cell, and M-cell → inhibitory cell (**Figure 7**).

DISCUSSION

Despite extensive effort to understand the neural substrates underlying the selection of context-dependent behavioral output, our knowledge remains limited of how social status affects neuromodulatory systems that regulate motor behaviors. Our study was motivated by the fact that 2-AG plays a novel role in balancing the activation between competing motor circuits, and that the ECS of vertebrates is remarkably sensitive to social influences (Song et al., 2015; Morena et al., 2016). These studies pointed to the possibility that 2-AG plays a crucial role in shifting the balance in activation of motor circuits according to social status. Here, we demonstrated that social dominance regulates the activity of the ECS to modulate the startle escape and swim

motor behaviors in a behaviorally adaptive manner. We showed that JZL184 led to a partial reversal of social status-dependent motor behaviors in both dominant and subordinate zebrafish and this result provides evidence that the ECS plays a role in the neuronal modulation of social status-dependent control of the startle escape and swimming behaviors in zebrafish. Using drd1b$^{(-/-)}$ mutants, we also showed that ECS regulation of M-cell excitability is mediated, in part, via the dopaminergic system by the activation of DRD1b. However, it should be noted that detailed future examination of the effects of drd1b mutation on social interactions and aggressive activities will be invaluable. Our behavioral observations suggest drd1b$^{(-/-)}$ mutants pairs engaged in social agonistic interactions, formed stable dominance relationships and the time course of dominance formation mirrored that of WT animals.

One significant result of this study is the role of 2-AG in promoting synaptic transmission, a phenomenon that has been described in only a handful of studies (Cachope et al., 2007; Song et al., 2015) and is contrary to the prevalent notion that endocannabinoids suppress synaptic release (Alger, 2002; Gerdeman et al., 2002; Robbe et al., 2002; Brown et al., 2003; Chevaleyre et al., 2006; Kano et al., 2009). Our results show that prolonging 2-AG availability enhances M-cell excitability in communal animals and to a lesser extent in dominants but depresses it in subordinates. This suggests a social status-dependent dual role of the ECS within a given circuit. Studies of the ECS improved our understanding of its function in suppressing transmitter release to regulate anxiety (Moise et al., 2008), depression (Vinod and Hungund, 2006), aggression (Fontenot et al., 2018) and motor behavior (El Manira and Kyriakatos, 2010), all of which are behaviors that can be exhibited during social interactions. To our knowledge our results are novel because they demonstrate social status-dependent dual functionality of the ECS in promoting both excitation and inhibition of locomotor behavior within and across competing circuits as an adaptive strategy of social dominance.

Prolonging 2-AG availability had opposing effects on swimming activity in dominants versus subordinates. In subordinates, 2-AG increased swimming activity while in dominants swimming was decreased. How social experience influences the ECS to modulate swimming activity and the transition between escape and swimming remains unknown. A potential mechanism may lie in local control within the spinal cord motor network that modulates premotor interneurons. The fast motor neurons synthesize and release 2-AG (Song et al., 2015). It is thought that upon activation of the escape response, the fast motor neurons release 2-AG to momentarily disengage the swimming premotor elements (i.e., V2a, V1, and V0) and engage the escape circuit, thus providing a quick and local mechanism of behavioral selection (El Manira, 2014; Song et al., 2015). In this instance, the urgent execution of escape is prioritized over swimming. Although this scenario is possible in the case of subordinates, it does not explain how swimming can be selected over escape as the preferred behavior of dominants. The social regulation of swimming likely involves coordinated regulation from multiple levels of the nervous system. This is because swimming is a comparatively more flexible behavior so is likely susceptible to a greater degree of social modulation. Coordinated social regulation of swimming could take the form of chronic descending input from nuclei that control motivation coupled with spinal local control.

In addition to influencing swimming activity, prolonging 2-AG availability also induced opposite effects on the startle escape response in dominants versus subordinates. Startle escape sensitivity was significantly decreased in subordinates while it was moderately enhanced in dominants. In the startle escape circuit, it was found that 2-AG modulates the excitatory inputs to the M-cell and fast MNs (responsible for startle escape). The net effect of 2-AG is an activity-dependent potentiation of the escape circuit coinciding with a strong inhibition of the swimming circuit (Song et al., 2015). Our results for communals and dominants supported these findings although the increase of the startle escape response for dominants was moderate. This is the "clutch-like" mechanism that allows a smooth transition from swimming to startle escape, and then back to swimming (Song et al., 2015). On the other hand, our finding that 2-AG suppressed the escape circuit in subordinates is the opposite of expected effects based on the known excitatory actions of 2-AG (Song et al., 2015). Our findings suggest that, in the escape circuit, modulation of 2-AG is socially regulated, and 2-AG induces its effects by modulating presynaptic inputs onto the M-cell. Specifically, 2-AG potentiates the mixed synaptic input from the VIIIth auditory nerve onto the M-cell. The VIIIth nerve, in addition to exciting the M-cell, also excites commissural and collateral interneurons that inhibit the M-cell. M-cell firing only occurs when the direct excitatory input from the VIIIth nerve is sufficient to override the indirect inhibitory inputs (Korn and Faber, 2005).

Our results support previous findings that JZL184 treatment increases startle escape in communal zebrafish (Song et al., 2015). We found that JZL184 increased startle escape sensitivity in communals and dominants but decreased startle escape sensitivity in subordinates. Collectively, JZL184 treatment negated behavioral status-dependent differences in the startle escape response of zebrafish. This could be explained by the large increases in 2-AG concentration that ensues from inhibiting the degradative enzyme MAGL upon JZL184 administration. Previous research demonstrated that JZL184 led to a more than 5-fold increase in 2-AG levels in murine brains (Long et al., 2009). If 2-AG levels vary according to social status, then the large increase in 2-AG would eliminate any differences in 2-AG concentrations between dominants and subordinates that could be responsible for the original differences in startle escape sensitivity and explain why JZL184 administration abolished social status-dependent differences in startle escape sensitivity. Although we have no direct evidence, our results of differences in dagl expression suggest that synthesis of 2-AG is likely to differ between dominant and subordinates. Future studies quantifying 2-AG concentrations will be necessary to verify whether differences in dagl expression directly translate into differences in 2-AG availability.

Although our results of supplementing 2-AG showed clear status-dependent effects on motor activity, our results of blocking CB_1R using AM-251 were less definitive. On one hand, our results are consistent with previous work where application of

AM-251 induces a general decrease in both fictive swimming and startle escape response in communal zebrafish (Song et al., 2015). Our results along with Song et al. are in opposition to previous work in goldfish in which application of either AM-251 or SR141716 had no effect on the amplitude of the excitatory post-synaptic potential (EPSP) from the VIIIth nerve onto the M-cell (Cachope et al., 2007). These researchers reasoned that 2-AG is not released tonically from the M-cell, and so blocking the receptor would not affect the startle escape response. Our finding, that blocking CB_1R reduces startle escape sensitivity in subordinates, suggests either of several possibilities: (1) that the systemic application of AM-251 is blocking CB_1R upstream of the M-cell, or (2) that there is a tonic release of 2-AG in the M-cell of subordinate fish. Considering the first possibility, blocking CB_1R on hair cells could affect the startle escape response by influencing sensitivity to sound. However, there is currently no evidence that hearing is influenced by ECS activity. The second possibility is supported by the higher startle escape sensitivity in subordinates and the known potentiating effects of 2-AG on startle escape behavior. However, this is complicated by the result that JZL184, which is reported to increase 2-AG, also reduced startle escape sensitivity in subordinates. The findings by Song et al. (2015) are hard to reconcile with our results unless these researchers unintentionally selected subordinate or chronically stressed fish for their AM-251 experiments.

In an effort to reconcile these differences and probe possible cellular mechanisms of how social status affects ECS modulation of startle behavior, we built a neurocomputational model of the escape circuit based on a simplified representation of the properties of the relevant neurons. Although our simplified model did not include all the detailed neural elements that may act *in vivo*, it enabled the reproduction of several important network activity patterns in the escape circuit observed experimentally. Neurocomputational analysis suggested that social experience induces its regulatory effects on the ECS by shifting the balance between the excitatory and inhibitory neuromodulatory pathways that control M-cell excitability (**Figure 7**). In dominants, enhanced expression of DAGL increases 2-AG synthesis, which promotes dopaminergic release and activation of the inhibitory inputs that in turn inhibit the M-cell (**Figure 7A**). This would explain why supplementing 2-AG with JZL184 had minimal effect on M-cell excitability but significantly reduced swimming frequency in dominants. Conversely, reduction in DAGL expression in subordinates reduces dopaminergic release, which reduces the strength of the inhibitory input onto the M-cell (**Figure 7B**). In effect, in subordinates M-cell excitability is enhanced due to removal of inhibition. This model is supported by the fact that when subordinates were injected with JZL184, their startle escape sensitivity declined while swimming activity increased; thus, shifting their motor activity from subordinate-like to dominant-like behavior. This proposed model based on empirical data was faithfully recapitulated, in part, by our neurocomputational analysis.

Note that in the neurocomputational model, we changed four parameters: the synaptic strength $g_{E\rightarrow I}$ (the synaptic strength from the excitatory cell to the inhibitory cell) and three CB_1Rs. The differences of these four parameters between two model animal groups were about 10∼25% while network architecture was kept unchanged. Different values of CB_1Rs in two model animal groups were essential to mimic the different effects of JZL184 injection and AM-251 injection depending on the social status. Moreover, the different values of $g_{E\rightarrow I}$ between two model groups was also essential to mimic the effect of AM-251 injection. As shown in **Figure 4B**, after AM-251 injections the response curve of dominants was slightly lower compared to that of subordinates. This suggests that dominants have either strong inhibitory pathways or weaker excitatory pathways onto the M-cell compared to subordinates even without the effects of CB_1Rs. In the model, we varied values of $g_{E\rightarrow I}$ depending on the social status. One may vary other synaptic strengths ($g_{E\rightarrow M}$ or $g_{I\rightarrow M}$), but two model animal groups need to have at least one different value among three synaptic strengths to reflect different activity patterns depending on social rank.

To model the effects of JZL184, we increased the values of CB_1R at different ratios depending on the social status and locations of CB_1R. Some variations of these ratios qualitatively reproduced similar simulation results for both model animal groups. However, for a subordinate-like model, the incrementing ratio of $CB1R_{EI}$ due to JZL184 should be significantly higher compared to those in $CB1R_{EM}$ and $CB1R_{IM}$ to replicate the significant decrease of the startle escape. That is, we assumed JZL184 significantly increases the excitatory → inhibitory pathways which result in the significant inhibitory input to the M-cell. Further study is needed to test our hypothesis on this pathway.

It is noteworthy to mention some limitations of the computational model. First, our model does not include all the detailed neural components that may act *in vivo*. In particular, we built a neurocomputational model for the escape circuit consisting of one M-cell, one pre-synaptic excitatory cell of the M-cell, and one pre-synaptic inhibitory cell of the M-cell to explore how social factors regulate the neuromodulatory inputs onto the M-cell. Future computational approaches may extend on the model to incorporate other feed-forward and feed-backward neuromodulatory inputs (glutamatergic, dopaminergic, GABAergic, and glycinergic neurons) known to impinge on the escape and swim circuits. These various inputs are likely to work synergistically with the ECS to differentially regulate motor circuit excitability in dominants and subordinates. The second limitation is that the model does not incorporate the swim circuit. To further explore modulation of swimming, one could combine the current model of the pre-synaptic M-cell escape circuit with a model of the swim circuit (Miller et al., 2017). However, in the absence of definitive anatomical information of the swim circuit, in particular information regarding synthesis and release of 2-AG and expression patterns of CB_1R, results from neurocomputation models will be speculative and unlikely to provide representative insight into the ECS's specific role in the social modulation of swimming.

In our experiments, whole brain qPCR and protein expression analysis, while informative, were not sufficient in providing tissue-specific protein expression patterns. For instance, the whole brain approach could be masking differences in CB_1R

expression patterns between dominants and subordinates that would be otherwise detected with more targeted approach. Although we have no direct evidence, we suspect that detailed expression analysis (either via IHC or *in situ* hybridization) of CB_1R within the excitatory dopaminergic and inhibitory neurons are likely to show differences in expression patterns between the two social phenotypes. These future experiments will provide the necessary details to construct more accurate models and further our understanding of how social factors regulate network dynamics. Finally, although our study primarily focused on CB_1R, the contribution of CB_2R in regulating locomotor activity should not be overlooked. While CB_2R is mostly present on immune cells and is known to regulate immune responses and inflammatory pathways (Cabral and Griffin-Thomas, 2009; Atwood and MacKie, 2010), recent evidence suggests expression and functional effects of CB_2R in the brain in regulating anxiety-like behavior and swimming activity in zebrafish larvae (Chen et al., 2017; Acevedo-Canabal et al., 2019). Thus, future experiments examining the effects of social experience on brain expression of CB_2R and its regulation of the startle and swim circuits will provide added insights of how social factors impinge on nervous system function and will facilitate the development of computational models that more accurately represent the structural and functional connectivity of the motor networks.

CONCLUSION

We report that in zebrafish the ECS is regulated by social status to modulate the activation of startle escape and swimming in a behaviorally adaptive manner. We propose a model that this status-dependent regulation of motor activation is driven by a shift in the activation patterns of descending excitatory and inhibitory pathways that modulate the escape circuit. The report provides new insights into how social factors impact nervous system function to regulate the interactions of neuromodulatory pathways to optimize motor output.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the East Carolina University Institutional Animal Care and Use Committee. Animal Use Protocol #D320a.

AUTHOR CONTRIBUTIONS

SO and FI conceived and designed the experiments. SO, TM, and MK conducted the experiments. SO, TM, and FI analyzed the data. SO, SA, CP, and FI wrote and critically revised the manuscript. SA and CP developed the neurocomputational model. All authors have approved the final version of the manuscript and attested to the accuracy of the data presented.

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Altering Perceived Context: Transportation Cues Influence Novelty-Induced Context Exploration

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Context is the milieu in which everything occurs. Many research studies consider context, or even explicitly manipulate it; yet it remains challenging to characterize. We know that a context surrounds and influences tasks; however, the boundaries of its influence are difficult to define. In behavioral science, context is often operationalized by the physical environment in which the experiment takes place, and the boundaries of the context are assumed to begin at the entrance to that of the room or apparatus. Experiences during transportation to the testing space have been shown to impact rodent behavior and memory, but transportation's relationship with novelty and physical environment is not fully understood. The current study explored how familiar vs. novel cues, both within a physical environment and preceding it, impact the perception of a context. We manipulated context on three levels: physical testing environment, object cues within that environment, and transportation cues preceding entrance to the testing environment. We found that novel transportation cues can change rats' perception of both familiar and novel contexts. The effects of transportation on perceived context may be affected by the length of the retention interval, testing environment, and behavioral range. These data suggest that context is a broad concept that includes cues across time and is sensitive to small differences in experience.

Keywords: context, exploratory behavior, object play, novelty, retention interval, rat, transportation impacts context

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INTRODUCTION

Context is often mentioned, and even explicitly manipulated, in many research studies; yet it is rarely comprehensively defined. We know that a context surrounds and influences a task (Rosas et al., 2013); however, the boundaries of its influence are difficult to determine. Which stimuli make up a context and which are contained within it? How far does a context extend in time and space? In behavioral science, context is often operationalized as the physical environment in which the experiment takes place (e.g., the room or apparatus) and the boundaries of the context are assumed to begin at the entrance to that environment in both time and space. Manipulations of the physical testing environment (e.g., changes to flooring, lighting, or scent) are practical to carry out and are therefore common manipulations of context. While such manipulations have been used to demonstrate the importance of context in memory (Bouton and King, 1983; Wilson et al., 2013; Arias et al., 2015), the effects of shifting physical environments are far from uniform. When conditioned cues are presented in a different

context than training, the reinforcement is associated with the novel context but not the familiar cue until later in training when the combination of cue and environment is no longer novel. This suggests that memories may become more context-dependent when there is an added element of surprise or novelty (León et al., 2011). Novelty may also play a role in delineating cues from context. Novel stimuli are more salient and are more likely to be treated as a predictive cue (Mackintosh, 1973). Otherwise, familiar cues can fall into the "background" of the context (Nadel and Willner, 1980). The time between events is also well known to impact memory. In the novel object recognition literature, the delay between exposure to familiar objects and the test where a novel object is introduced (the retention period or test delay) impacts rodents' ability to distinguish between familiar and novel objects (Ennaceur and Delacour, 1988). Therefore, the length of a context in time is likely limited by memory and factors that influence memory.

While physical space—and the exteroceptive cues within that space—is the most commonly manipulated modality in experimental designs aimed at studying context effects, there are many other components that make up a context. Other well-studied contextual modalities are defined by interoceptive stimuli (Bouton, 2018) including hunger (Davidson, 1993; Schepers and Bouton, 2017), drug state (Lattal, 2007), and task demands (Smith and Mizumori, 2006). Experiences even during transportation to the testing space have also been shown to impact rodent behavior and memory (Bevins et al., 2000). Bevins and colleagues showed that when rats were transferred by being carried on an arm to an operant chamber where they had previously experienced a shock, they showed less fear behavior (i.e., freezing) than when transported using a cart as they were for fear conditioning previously. These results suggest that the rats had conditioned—at least in part—to the original transportation cues. Replacement of the original transportation cues with different transport cues was sufficient to reduce context freezing. It is not clear, however, if these rats learned to fear a context that was made up of both the physical environment and the set of transportation cues preceding that environment or if transportation cues were simply predictive of shock.

Current Study

The current study asks: how do familiar vs. novel cues, both within a physical environment and preceding it, impact contextual recognition? To examine rats' recognition of a context we utilized a modified novel object design and measured object exploration relating to changes in the environment, the object within that environment, and the transportation preceding entrance to the environment. We found that novel transportation disrupted context recognition and that this effect was modulated by testing delay, context familiarity, and behavioral range. The first study utilized a short retention interval of 1 h and was carried out in the familiar context, the second experiment showed that the effects of transportation are maintained with a long retention interval of 24 h and in a novel context, the last experiment (anchored object) showed that object play modulates the effects of novel transportation.

MATERIALS AND METHODS

Experimental Design

The Novel Transportation paradigm extends the logic of Novel Object Recognition tasks: less time spent exploring the object indicates that the subject retained object recognition memory (Ennaceur and Delacour, 1988). We used object exploration as an indirect measure of context familiarity; in a more familiar environment rats should explore the environment less and the object more. **Figure 1** demonstrates the Novel Transportation design. Rats undergo 2 days of familiarization where they are removed from their homecage with their cage mate, rest in a separate environment, and are allowed to explore the familiar environment and familiar object for 10 min before removal to the rest cage. Testing is conducted in a similar fashion; object, environment, and transportation could be either familiar or novel during the 3-min testing period.

Subjects

A total of 166 (short retention interval study: n = 24; long retention interval study: n = 96; anchored object study: n = 46) male Sprague-Dawley rats between 9 and 11 weeks old obtained from Envigo (Houston, TX). Rats were dual housed in clear plastic cages (27 × 48 × 20 cm) on a 12-h light-dark cycle. All experiments took place in the dark cycle. Food (standard rodent chow) and water were provided *ad libitum*. All procedures were conducted under the approval of the Institutional Animal Care and Use Committee at the University of Texas at Austin and in accordance with National Institutes of Health guidelines.

Transportation

Familiar Transportation

We chose variables that would influence a variety of sensory modalities that could change from day to day in a lab. During the familiarization period, and for those exposed to familiar transportation before testing, rats were transported between cages and testing environments rolled on a cart, covered in a dark shroud, in a cage with clean bedding by a single experimenter who wore their hair up and a lab coat.

Novel Transportation

Rats exposed to the novel transportation context were carried between testing environments, un-covered, in a cage with no bedding by a pair of experimenters (who conversed during transportation) wearing hair down, a face mask, and scrubs. These variables were counterbalanced across several cohorts of rats.

Objects

Light Objects

Object 1 was a pink plastic jax on a black plastic base (approximately 8 cm long × 4 cm wide), three objects were used interchangeably. The approximate weight was 8.0 g. Object 2 was a pink rubber ball affixed to plastic bases of the same dimensions. While the jax and the ball were approximately the same diameter (3 cm), the ball was heavier. Object 2 weighed about 29.5 g (see top panel **Figure 2**).

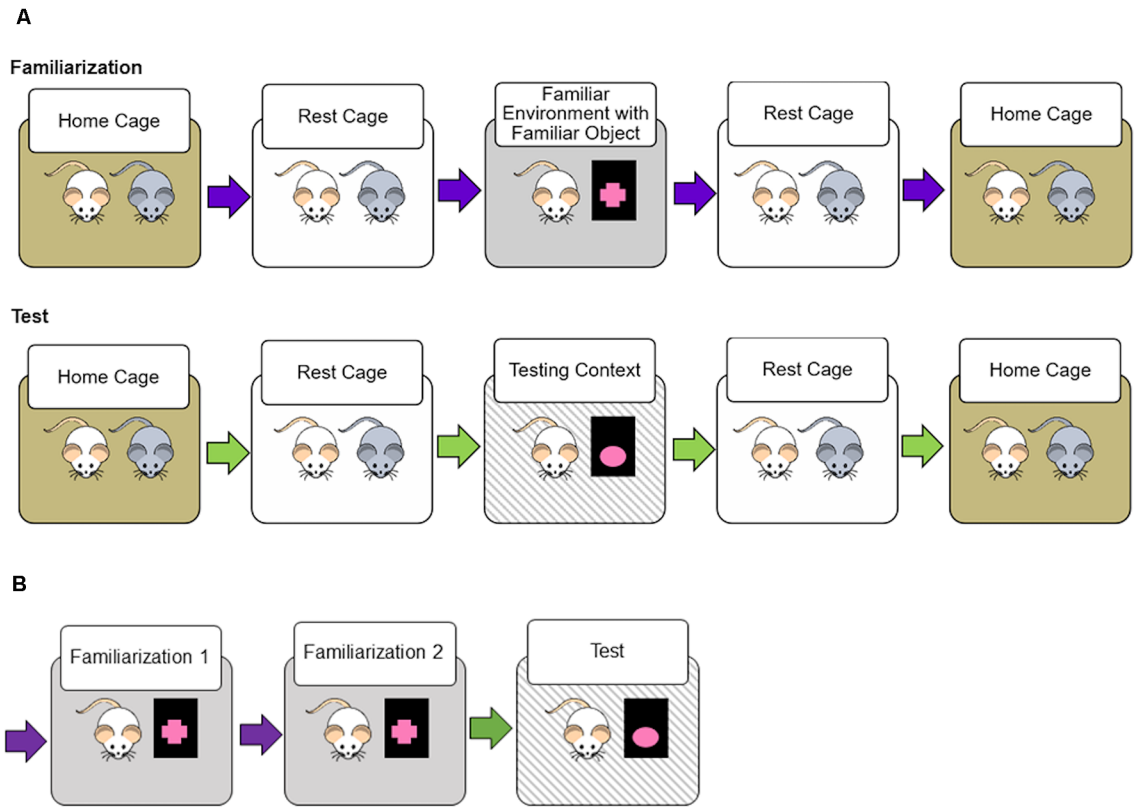

FIGURE 1 | Novel transportation procedure. **(A)** For two 10-min familiarization sessions rats were transported from their homecage to a rest environment with their cage mate. After a 5-min rest period, a single rat is transported to the familiarization context (familiar environment with a familiar object placed within). After the first rat in the pair has habituated for 5 min, the cage mate is then introduced to the familiarization context. Twenty-four hs after the second familiarization session the testing session occurs. Like before, both rats are removed from a homecage and placed in the same rest environment before entrance to the testing context. The testing context was either identical to the familiarization context, or a novel object, novel transportation cues, or both were introduced. **(B)** In each study, rats underwent two familiarization sessions spaced 24 h apart, followed by a 3-min testing session. In the short retention interval study, the delay between the second familiarization session was 1 h, for the long retention interval and anchored object studies the delay was 24 h. Different color arrows depict different transportation cues. All familiarization utilized familiar transportation cues.

Heavy Objects

Object 1 was a blue glass hexagonal doorknob on dark brown cast iron base 13 cm long × 9.5 cm wide × 5 cm high weighing approximately 380.8 g. Object 2 was a red cast iron hook on dark brown cast iron base 11.5 cm long × 7 cm wide × 6.5 cm high and weighing approximately 426.5 g (see bottom panel **Figure 2**).

Environments

Familiar: Homecage

The familiar environment was the homecage for each rat, with testing occurring in the colony room. This was chosen for two reasons: (1) to make the familiar environment as familiar as possible; and (2) to allow us to compare environments that were in completely different spaces so that the contexts were as distinct as possible. Since it was deemed best practice to pair-house the rats in these studies, both rats had to be removed from their homecage for it to be used for testing. Objects were added to the homecage after both rats were removed, and different objects were used for each cagemate. Additionally, in order to film the testing sessions, cages were placed on an empty shelf in the same colony room. Both the removal of the cagemate and relocation of the homecage were utilized during the familiarization phase, so these altered components were not novel during testing. Digital cameras were used to record behavior.

Novel: Operant Chamber

Novel environments were a standard conditioning chamber equipped with two clear plexiglass walls and two metal walls, stainless steel rod floors, and red lighting and were enclosed in acoustic isolation boxes (Coulbourn Instruments) were used as novel environments. The environment was lit with a red LED during testing and was cleaned with Windex between each testing session. Behavior was recorded with a digital camera mounted in the ceiling of each chamber.

Behavioral Scoring

The main behavioral measure used was the exploration of the object. Object exploration was defined using the traditional definition from the novel object literature: object exploration involves orienting the snout towards the object at a distance equal to or less than 2 cm, sniffing, licking, biting, or otherwise

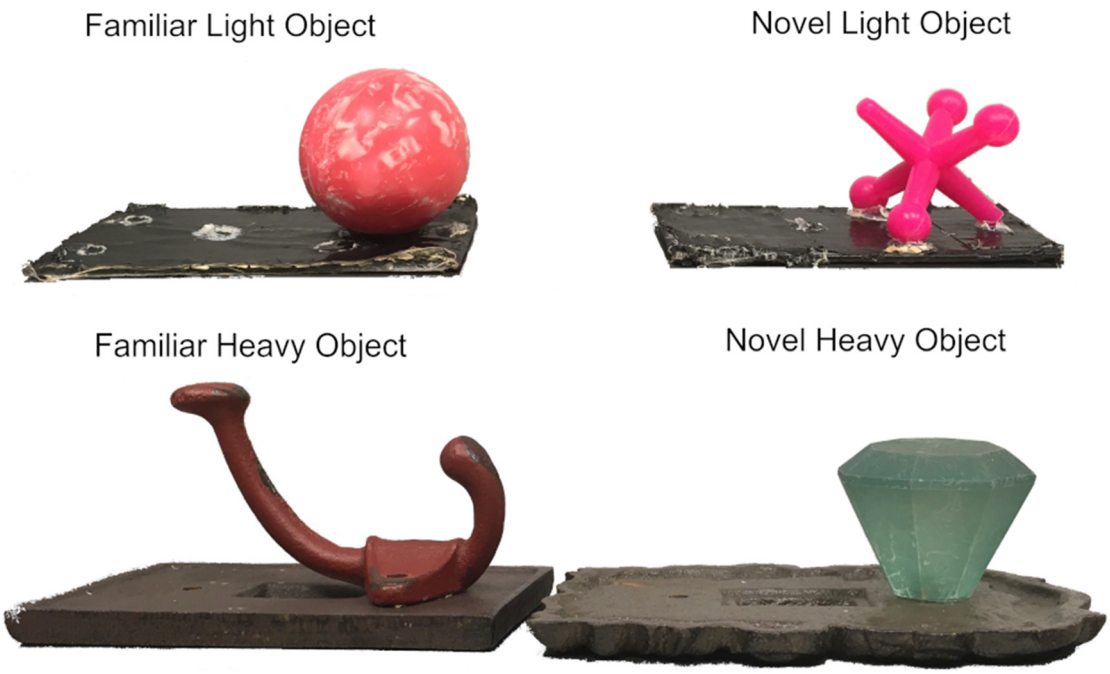

FIGURE 2 | Experiment 1 used light objects, experiment 2 used heavy objects. Light objects measured approximately 8 cm long × 4 cm wide × 3 cm high and weighed 7–30 g. Heavy objects measured approximately 13 cm long × 9.5 cm wide × 5 cm high and weighed 376–427 g. Familiar and novel objects were counterbalanced.

touching the object were included in object exploration; sitting, standing, or walking on the object were not considered object exploration (Ennaceur and Delacour, 1988). Based on novel object literature we know that exploration can be used to measure relative novelty, in this study we are ultimately interested in context novelty, but traditional definitions of object exploration could not be readily applied to environment exploration. Non-exploratory behaviors were defined as any behavior that is clearly neither exploration of either the object nor the surrounding environment, such behaviors included grooming and sleeping. While some stereotypical fear-induced freezing occurred, it was always accompanied by head scanning movements and was therefore categorized as context exploration. To assess context exploration, the total duration of object exploration and non-exploratory behaviors were subtracted from the total duration of the observation. Therefore, every second of the observation period was accounted for: if a rat was engaged in neither object exploration, nor non-exploratory behaviors the rat must be exploring the context. Using this definition, rats that were sniffing outside of the 2 cm perimeter of the object (including the walls and lid of the enclosure), digging through bedding, or moving throughout the enclosure were said to be exploring the environment.

During scoring an unanticipated behavior was noted. Rats were moving the object an unprecedented amount and in a way that was categorically different from how object exploration has been defined. Instead of sniffing, licking, or biting objects, rats were lifting them off the bottom of the enclosure, flipping them upside down, and running across the enclosure with it in their mouth. For simplicity's sake, we elected to call this heightened degree of object manipulation object play behavior. Object play was defined as a subset of object exploration in which rats move the object. For a behavior to count as object play a rat must be attending to the object (snout orienting less than 2 cm) and in some way move the object. For example, rolling the object upside down, running across the enclosure with the object in their mouth, and rotating the object using forepaws or mouth were all included as object play behavior. Sniffing or biting the object without movement or any object movement caused by back paws or tail were not considered object play behavior. Our definition of object play in rats aligns closely with how object play is defined for use in Avian studies (see O'Hara and Auersperg, 2017). Since object play behavior was defined as a subset of object exploration inclusion of object play does not affect the ratio of context to object exploration. While many measures of play behaviors involve scoring interactions between conspecifics (Pellis and Pellis, 1998; Whishaw and Kolb, 2020), in the current study object play was determined by interactions between the rat and the object.

All videos were scored using BORIS (Friard and Gamba, 2016). The time index that the subject began and ceased demonstrating a behavior was recorded. The total duration of each behavior was calculated by subtracting the start times from stop times and adding each instance of that type of behavior together. Scorers were blind to transportation and object conditions during scoring.

Procedure

Short Retention Interval

The short retention interval study utilized light objects in a modified novel object paradigm in a 2 × 2 between-subjects factorial design. Rats were habituated to the familiar object alone in the homecage for 10 min for 2 days. There were two levels of the object factor: familiar or novel. There were two levels of the transportation factor: familiar and novel. Rats were randomly assigned to one of these four groups.

The Novel Transportation procedure takes 2 days to run, after 3 days of handling to habituate rats to human contact. Familiarization to the familiar object takes place in two familiarization sessions. Both cage mates were removed from their homecage and placed into a rest cage and transported to a dark holding room for 5 min. One of two objects was chosen to become familiar and placed in the homecage (counterbalanced). The first rat was removed from the rest cage and placed in a transportation cage to be re-introduced to the homecage with the object. The first rat was allowed to explore the homecage and object for 10 min while their cage mate remained in the rest cage. The first rat was then removed and returned to the holding room. The object was switched out for the familiar object designated for the second rat. The second rat was transported back to the homecage for their 10-min exploration session. The second rat was removed from the homecage and reunited with its cage mate in the rest cage in the holding room before both rats are returned to their empty homecage. All transportation during familiarization was identical across all subjects. This procedure was repeated on day 2.

Testing occurred 1 h after the second familiarization session. Both rats were removed from their homecage and put into a rest cage and rest in the holding room for 5 min. It is at this point that some pairs of animals experienced novel transportation. Either the familiar or a novel object was placed in the testing environment. The first rat was then placed in a transportation cage and moved to the testing environment and recorded for 3 min. The first rat was returned to the holding room after testing and the second rat was removed and tested in the same way as the first rat. After the second rat had finished testing both rats were placed in the same rest cage and returned to the homecage

Long Retention Interval

The long retention interval study utilizes the same Novel Transportation paradigm with few alterations. This study employed a 2 × 3 between-subjects factorial design. Rats habituated to the light familiar object alone in the homecage for 10 min for 2 days. There were two levels of the object factor: familiar or novel. There were three levels of the context factor: familiar (homecage), novel (operant box), and novel transportation (novel transportation to operant box). Rats were randomly assigned to one of six groups.

The long retention interval procedure takes 3 days to run, after 3 days of handling to habituate rats to human contact. The first 2 days of the procedure are object familiarization. The third day is the testing day. One-third of the rats were transported using novel cues on this day. Either the familiar or a novel object was placed in the testing environment (the homecage is the familiar environment, a standard operant box is the novel environment, and the novel transportation context is the same operant box but with different transportation cues).

Anchored Object

In order to minimize potential confounds from object play, heavy objects were used to ensure that rats could not displace them in this study. Otherwise, the anchored object experiment was conducted identically to the long retention interval study.

RESULTS

R Studio was used to analyze the data. For the short retention interval study, a 2 × 2 object (familiar and novel) × transportation (familiar and novel) between-subject's analysis of variance (ANOVA) was conducted using the total duration of object exploration data. For the long retention interval study, a 2 × 3 object (familiar and novel) × context (familiar, novel, and novel transportation) between-subject's ANOVA was conducted using the total duration of object exploration data. This analysis was repeated using the total duration of object play behavior. The anchored object study also utilized a 2 × 3 object (familiar and novel) × context (familiar, novel, and novel transportation) between-subject's ANOVA to test the total duration of object exploration. Tukey's HSD tests were conducted *Post hoc* as necessary. All error values reported represent the standard error of the mean (SEM). See **Supplementary Materials** for additional analyses.

Short Retention Interval

This study demonstrates that transportation cues may be a part of a broader understanding of context. The total duration of object exploration differed as a function of object familiarity ($F_{(1, 21)}$ = 6.15, p = 0.022). Rats spend more time with novel objects (M = 74.5 ± 14.2 s) compared to familiar (M = 35.9 ± 8.2 s). There is a trend to suggest that object exploration also depends on transportation familiarity ($F_{(1, 21)}$ = 3.41, p = 0.071). Rats spend less time with the object after novel transportation (M = 40.8 ± 9.0 s) compared to familiar (M = 69.6 ± 14.8 s). There was no interaction between object and transportation familiarity ($F_{(1, 20)}$ = 1.308, p = 0.27). While the effect of transportation was only marginally significant, its effect size was large (partial η^2 = 0.14) and the relatively small group sizes (n = 6) contributed to the study's low power [(1− β) = 0.47]. Additionally, novelty-induced differences in exploration were demonstrated between subjects for the first time. The increased behavioral variability of a between-subjects design may have also obfuscated results.

Since environment exploration was calculated by subtracting object exploration and non-exploratory behaviors from the total duration of the observation, exploration of the environment and the object are mutually exclusive. As such, these data reflect increased interest in context as object exploration decreases. Therefore, context exploration increased with novel transportation (**Figure 3**), suggesting that transportation cues impact perceived context. We next asked if the effects of

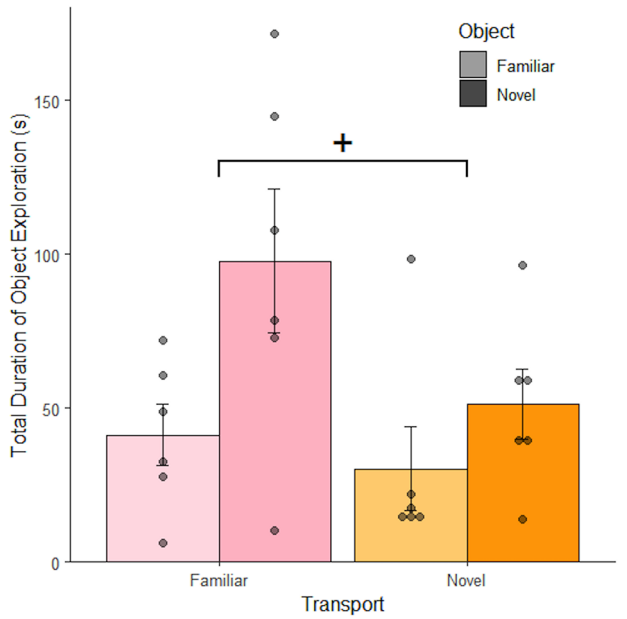

FIGURE 3 | Object exploration increased with novel objects and familiar transportation. Rats exposed to a novel object spent more time exploring it than rats exposed to a familiar object 1 h after the final object familiarization session. The introduction of novel transportation cues lead to a decrease in the mean total amount of time rats spent exploring the object; demonstrating that they were instead exploring the context (n = 24). Error bars report SEM for each group. Points indicate the total duration of object exploration for each subject grouped into bins of 5.5 s. $^{+}p < 0.1$.

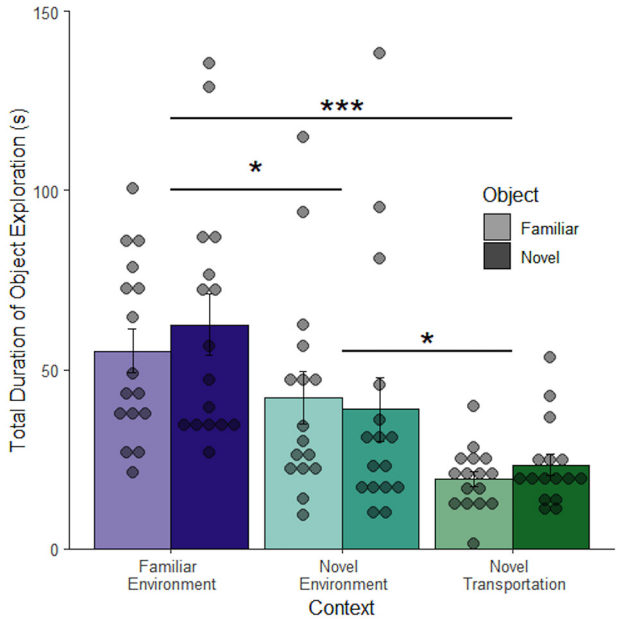

FIGURE 4 | Transportation but not object effects are retained in a novel environment. When the retention period was extended to 24 h there was no difference between mean exploration of a familiar compared to a novel object. Novel transportation to a novel environment leads to a decrease in mean object exploration, and therefore an increase in context exploration. There was a slight difference in exploration in familiar and novel environments proceeded by familiar transportation cues (n = 96). Error bars report SEM for each group. Points indicate the total duration of object exploration for each subject grouped into bins of 4.6 s. $^{***}p < 0.001$, $^{*}p < 0.05$.

transportation on context perception were robust enough to remain with a greater recall delay.

Long Retention Interval

Figure 4 shows that duration of object exploration differs depending on context ($F_{(2, 92)} = 16.70$, $p < 0.001$), but not object ($F_{(1, 92)} = 0.26$, $p = 0.61$) and no interaction between context and object ($F_{(2, 90)} = 0.35$, $p = 0.71$) 24 h after object familiarization (**Figure 4**). Rats spent significantly less time exploring the object after novel transportation ($M = 21.39 \pm 3.94$ s) than after familiar transportation to the familiar context ($M = 58.87 \pm 0.31$ s, adjusted $p < 0.001$) and novel context ($M = 40.52 \pm 0.86$ s, adjusted $p = 0.011$). There was also a difference between familiar and novel contexts (adjusted $p = 0.016$). This shows that novel transportation leads to increased context exploration. Since context exploration increased in the same novel environment with the addition of novel transport, novel transport may increase the perceived novelty of a context. Since the familiar and novel contexts were completely separate spaces in this study, these results show that relative exploration of either an object or the environment is an effective measure to compare rodent behavior across contexts. The lack of difference between the novel and familiar objects in the familiar environment contradicts what was expected based on the novel object literature (Dix and Aggleton, 1999; Antunes and Biala, 2012). However, the novel object novel context procedure used here differs considerably from traditional novel object paradigms. In this study, object exploration is compared to environment exploration rather than interaction with another object.

Intentional movement or manipulation of the object was deemed to be object play behavior, which was considered a subset of object exploration. Object play behavior changed depending on context ($F_{(2, 92)} = 12.43$, $p < 0.001$) with the most object play occurring in the familiar context ($M = 35.94 \pm 4.92$ s), less in the novel context ($M = 23.13 \pm 5.57$ s), and even less in the novel context after novel transportation ($M = 5.21 \pm 1.30$ s). In the novel transportation context, less than half of the rats exhibited any object play behavior, while every rat played with the object at least once in the familiar environment (**Figure 5**). Since object play differed across contexts it may have confounded the relative exploration of objects in different contexts. In order to eliminate this confound, the next study utilized immobile objects aimed at reducing object play behavior.

Anchored Object

The anchored object long retention interval study showed a similar pattern of results as the long retention interval study (**Figure 6**): there were clear effects of context but not object for every measure of exploration. The total duration of anchored object exploration was related to context familiarity ($F_{(2, 42)} = 65.25$, $p < 0.001$), but not object familiarity ($F_{(1, 42)} = 0.003$, $p = 0.96$) and no interaction between context

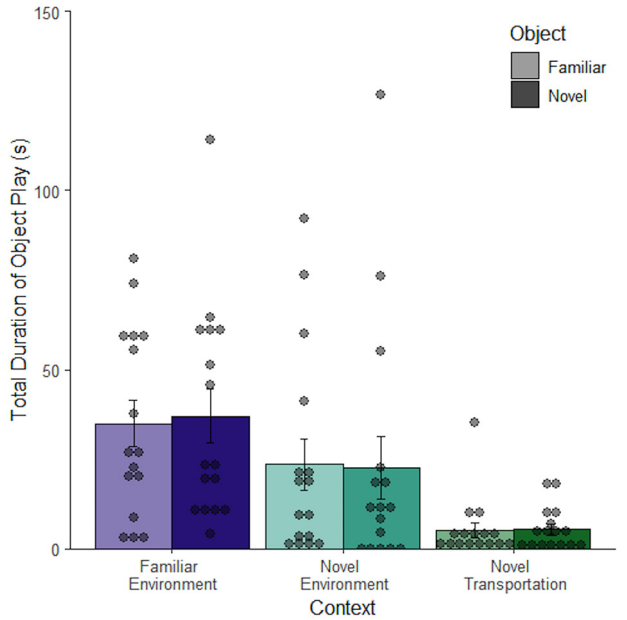

FIGURE 5 | Time spent playing with an object changes depending on context. Rats in a familiar context spend more time playing with an object than rats in a novel context. Novel transportation is also related to decreased mean object play (n = 96). Error bars report SEM for each group. Points indicate the total duration of object exploration for each subject grouped into bins of 4.2 s.

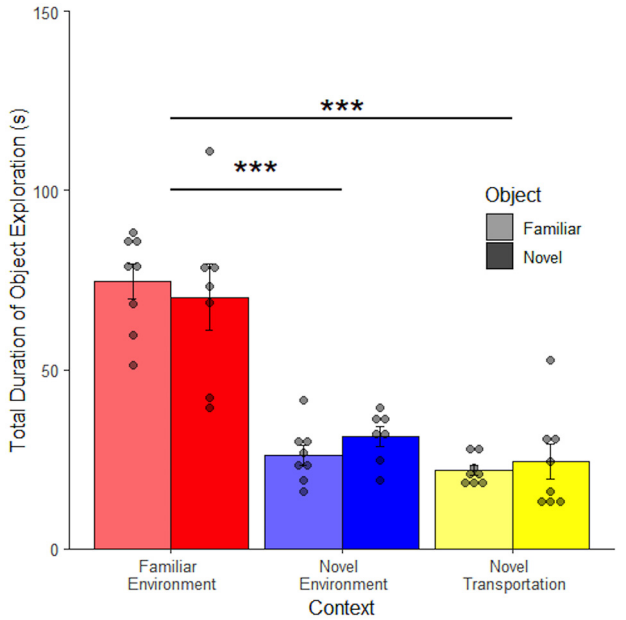

FIGURE 6 | Effects of novel transportation dissipate when an anchored object is introduced. There is no difference in the mean duration of object exploration between the novel and familiar objects or contexts with changes in transportation cues when object play is eliminated *via* anchoring of the object (n = 46). Error bars report SEM for each group. Points indicate the total duration of object exploration for each subject grouped into bins of 3.3 s. ***$p < 0.001$.

and object ($F_{(2, 40)} = 0.51$, $p = 0.60$). There was not a significant difference between familiar transportation ($M = 28.58 \pm 1.99$ s) and novel transportation ($M = 23.05 \pm 2.48$ s, adjusted $p = 0.47$) to a novel context. However, rats spent more time exploring an object in a familiar environment ($M = 72.57 \pm 4.84$ s) compared to novel independent of transportation (Familiar Transportation: adjusted $p < 0.001$, Novel Transportation: adjusted $p < 0.001$). The use of a heavy object to restrict object play behavior was effective since 0 s of object play were recorded during the second study. The total duration of non-exploratory behaviors also decreased; no non-exploratory behaviors occurred in the novel environment (**Supplementary Figure 3**).

DISCUSSION

Transportation Impacts Context

The three studies presented here were an investigation into multi-modal factors influencing novelty-induced exploration indicative of context-dependent memory in rats. In the novel transportation paradigm, a single object is placed in an environment as a way to indirectly measure context exploration: a rat is exploring a context if it is not exploring the object (or engaged in non-exploratory activity like grooming). Therefore, a decrease in object exploration indicates an increase in context exploration. The short retention interval study showed a trend indicating that object exploration changed after rats were transported *via* novel means: object exploration decreased after novel transportation and novelty-induced exploration of the novel object decreased relative to familiar transportation (**Figure 3**). These data suggest that transportation cues can lead to novelty-induced exploration of a context; indicating that transportation cues influence a rat's perception of context. Previous studies have shown that novel contexts can disrupt memory as demonstrated by decreased exploration of a novel object relative to a familiar one (Wilson et al., 2013; Arias et al., 2015). This suggests that transportation cues are part of a larger context that extends beyond the testing environment. Bevins et al. (2000) showed that rats conditioned, at least in part, to the transportation cues preceding entrance to an environment. The current studies extend upon these findings by showing that transportation cues are likely perceived as part of a context and are used in rats' broader contextual perception beyond predicting shock. The long retention interval study showed that transportation's effects of context remain when the delay between object familiarization and testing is increased to 24 h and in a novel environment. However, the anchored object study showed that when a rat's ability to play with the object is removed the effects of novel transportation disappear. It is unclear whether this is due to the object play itself, the decreased behavioral range, or the decreased saliency of the object within the context. A major limitation of these data is the lack of data on novel transportation preceding a familiar environment with a long retention interval. This makes some of our comparisons between studies indirect and it is, therefore, difficult to separate effects

caused by transportation-altered contexts and novelty-induced stress.

Modulations of Transportation Effects

Both studies that utilized a 24-h retention period (Long retention interval and Anchored Object) showed a similar pattern of results: object exploration changed depending on the environment, but not object, novelty. In the long retention interval study, which used light objects that the rats were able to play with, there were significant differences between the novel transportation and novel environment contexts indicating that the novel transportation cues presented in the novel transportation condition impacted how the rats interacted with the context. However, in the anchored object study objects were immobile and there was not a significant difference between the novel and novel transportation groups. Additionally, the tightened error in the anchored object study suggests that reducing object play also reduced the behavioral range available (**Figure 6**). Reduced behavioral range may also be related to reduced non-exploratory behaviors. A non-zero amount of non-exploratory behaviors was recorded in the second study; however, these behaviors were only recorded in the familiar environment (**Supplementary Figure 3**). The differences in behavior between the long retention interval study to the anchored object suggest that the removal of the object play variable influenced how the rats interacted with their environment. Object play is more typically measured as an interaction between rats (see Whishaw and Kolb, 2020). Future studies should address object play with both familiar and novel conspecifics to test the implications for play discussed in the present studies.

In the long retention interval study, object exploration showed a clear difference between novel and novel transportation contexts suggesting that transportation cues play an important role in how rats distinguish between contexts. However, this result was not reliably replicated in the anchored object study. This difference could be related to a smaller sample size in the anchored object study, but when collapsing by context the power is still sufficient to expect meaningful results. There is a possible ceiling effect when analyzing environment exploration; however, there is still no difference between novel and novel transportation contexts when looking at object exploration which does not suffer either ceiling or floor effects. Given the inhibited behavioral range and lower variability in the anchored object study, effects should be easier to detect, not harder. Therefore, the difference in context differentiation between the two studies is likely related to the ability to play in the long retention interval study but not in the anchored object study.

The heavier objects used in the anchored object study did not allow for rats to play with them as they tended to in the first two studies. This difference in object interaction may have led to differences in stimulus encoding. Less-salient cues can 'fade into the background' and become a feature of the environment rather than an independent cue (Nadel and Willner, 1980). Since rats could not interact with the anchored objects in the same way they may have been less salient and likely faded from the foreground in the first two studies and into the background and were encoded as a contextual cue rather than an independent cue in the anchored object study. The presence of an object distinct from the context is integral to the design of the current studies; without the addition of an object, there is no clear way to quantify context exploration. Do anchored objects have enough salience to be used in contrast to the surrounding context? While object play behavior was eliminated in the anchored object study, the mean total duration of exploration remained similar to the previous two studies. This suggests that rats distinguished between anchored objects and the surrounding context and that the logic of comparing relative exploration of the object and the context should still hold. Future work should clarify how cues and cue salience interact with the encoding of an environmental and contextual recall. However, differences in salience do not fully explain the differences in exploration between the familiar and novel transportation contexts seen in the first two studies.

Object play may affect both object and environment exploration behavior due to its relationship with stress. In the novel transportation paradigm rats are exposed to an operant chamber for the first time in the testing phase. This may cause greater stress compared to studies where rats have habituated to behavioral apparatuses as it is well known that novelty induces stress in rodents (Bassett and Cairncross, 1973; Baldwin et al., 1974). Additionally, introduction to the operant chamber during the test period in these studies may be more stressful than typical manipulations of context novelty in which rats are allowed to habituate to a behavioral apparatus as a familiar context. The rats in this study may have failed to successfully differentiate between familiar and novel objects after 24 h if they experienced increased stress. More work is needed to compare manipulations both within the same space and between distinct places across differing levels of novelty. Since stress has been known to impair both novel object recognition (Eagle et al., 2013) and contextual fear conditioning (Cordero et al., 2003), novelty-induced stress may have differentially impacted groups in the current studies. If rats that were able to reduce their stress by playing (Arelis, 2006), they may be better able to distinguish novel objects and contexts. Additionally, interoceptive cues related to stress have been shown to impact reinstatement of extinguished behavior (Schepers and Bouton, 2019). Stress may create a distinct set of interoceptive cues and therefore represent another contextual modality which influences behavior. A More direct measures of interoceptive cues related to play and stress is needed to understand the relationship between internal states and perceived context how interoceptive cues interact with exteroceptive cues. In the current study, the least amount of object play occurred in the most novel (novel transportation) context which presumably contains the greatest number of novel cues and therefore should elicit the most novelty-induced stress. The effects of play on behavioral variability, stress, and novelty-induced exploration of objects and environments need to be systematically tested in future experiments further refined.

The long retention interval study expanded upon the trend indicating novel transportation impacts perceived context from the short retention interval study, in a novel environment and with a longer retention period (**Figure 4**). However, rats did

not favor exploring novel objects with the longer retention period between object familiarization and testing. The lack of object differences was recapitulated in the anchored object study. Since the short retention interval study analyzed only familiar environments, it is therefore unclear whether increased stress from novel environments or the longer retention period accounts for the lack of object differentiation seen in the long retention interval and anchored object studies. While the novel transportation protocol used differs substantially from novel object recognition, differences between the novel and familiar objects were anticipated. The long retention interval and anchored object studies utilized a relatively long wait between object familiarization and recall (24 h) which, while common in the literature (see Antunes and Biala, 2012), may make the task prohibitively difficult and obscure results. Given this limitation of the current data it is unclear whether novel transportation to different environments has a differential impact on memory and behavior. Given the lack of object differentiation in familiar environments and what we know about object differentiation from other paradigms, it is likely that the retention period negatively impacted object differentiation (Ennaceur and Delacour, 1988; Antunes and Biala, 2012). More work is needed to determine the threshold retention period and whether additional factors such as sleep or consolidation impact object memory in this paradigm.

Conclusions

In this study, we sought to examine the effects of novel transportation cues on rats' experience of context. The Novel Transportation procedure, derived from Novel Object Recognition tasks, allowed us to quantify exploration of an environment relative to an object within the environment. Novel transportation led to increased context exploration and decreased differentiation between novel and familiar objects. These data suggest that novel transportation cues can lead to novelty-induced context exploration. Therefore, transportation cues influence a rat's perception of context and may also be a part of a larger context that extends beyond the testing environment. Transportation's effect on context may be moderated by retention period, the familiarity of testing environment, and behavioral range (i.e., ability to play with an object). These data highlight the importance of defining context broadly in behavioral science and suggest that future experimental manipulations of context should include transportation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.18738/T8/GVT0EE.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC at the University of Texas at Austin.

AUTHOR CONTRIBUTIONS

MM and VN designed the study. VN ran the study, analyzed the data, wrote the first draft of the manuscript, and approved the final version of the manuscript. MM helped with data analysis and interpretation, edited the manuscript, and approved the final version of the manuscript. SS, CM, LA, and MR helped with data interpretation, helped with running the experiments, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Neural Circuits Underlying Behavioral Flexibility: Insights From *Drosophila*

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Behavioral flexibility is critical to survival. Animals must adapt their behavioral responses based on changes in the environmental context, internal state, or experience. Studies in *Drosophila melanogaster* have provided insight into the neural circuit mechanisms underlying behavioral flexibility. Here we discuss how *Drosophila* behavior is modulated by internal and behavioral state, environmental context, and learning. We describe general principles of neural circuit organization and modulation that underlie behavioral flexibility, principles that are likely to extend to other species.

Keywords: *Drosophila*, environmental context, behavioral flexibility, neural circuits, internal state, behavioral state, learning, memory

INTRODUCTION

Across the animal kingdom, an animal's ability to modulate behavior in response to changing internal and external conditions is critical for survival. For example, animals are often faced with the challenge of finding food or water while simultaneously avoiding dangerous situations. If a predator's scent is detected, an animal might choose to hide. However, if they are hungry enough, they may prioritize finding food over staying hidden. Even a fruit fly is not always constrained by instinct but exhibits remarkable behavioral flexibility. For instance, carbon dioxide is released by fermenting fruit, a preferred food source for *Drosophila*, but may also represent a distress signal from other flies (Suh et al., 2004). Thus, when a fly smells carbon dioxide, it must decide: does it choose to seek a potential food source or avoid potential danger?

Behavioral flexibility can be defined as an animal's ability to adapt its behavioral responses to changing environmental contingencies or internal state (Kolb, 1990; Ragozzino et al., 1999; Floresco et al., 2009; Lea et al., 2020). *Drosophila melanogaster* is a powerful model organism for investigating the neural circuits underlying behavioral flexibility. Not only do these insects exhibit remarkable flexibility in their behavior, but the repertoire of advanced genetic tools available to study neural circuits is simply unprecedented. Genetic approaches enable us to target individual neurons or cell types in order to manipulate or record their neural activity. In addition, the recently published synaptic connectome of the fly brain (Scheffer et al., 2020) has greatly facilitated neural circuit identification and analysis. Using these tools, work in *Drosophila* has revealed the mechanisms underlying many different examples of behavioral flexibility, from learning and memory to state-dependent modulation.

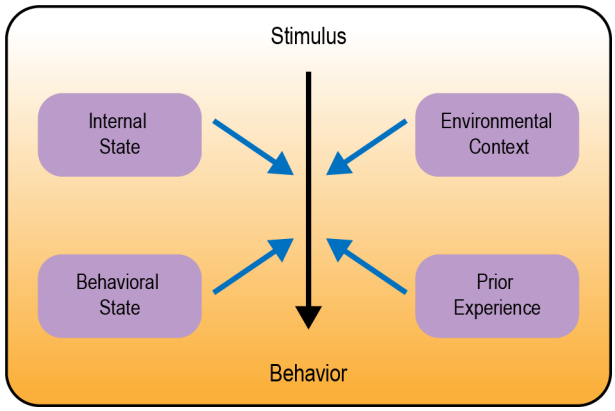

FIGURE 1 | Overview of different types of behavioral modulation. Neural circuits in the brain transform a sensory stimulus into a behavioral response (represented by the color gradient). Black arrow depicts the core circuit underlying behavior; blue arrows depict modulation of this circuit.

In this review we describe several examples of behavioral flexibility in *Drosophila*, focusing on four major categories: (1) modulation by internal state; (2) modulation by behavioral state; (3) modulation by environmental context; and (4) behavioral flexibility in learning and memory (**Figure 1**). We focus specifically on studies of adult *Drosophila*. There are a number of articles describing behavioral flexibility in *Drosophila* larvae that are not discussed here (e.g., Schroll et al., 2006; Schleyer et al., 2011, 2020; Allen et al., 2017; Mancini et al., 2019; Eschbach et al., 2020; Miroschnikow et al., 2020; Slankster et al., 2020; Gowda et al., 2021; Hernandez-Nunez et al., 2021; Vogt et al., 2021). Our goal is to highlight generalizable neural circuitry frameworks for how sensory cues, state, and experience are integrated to guide the flexible selection of appropriate behavior.

MODULATION BY INTERNAL STATE

Modulation by Hunger

The internal state of an animal can profoundly affect behavior. One such state is hunger, which is induced by energy deprivation. Hunger modulates a large repertoire of behaviors in order to promote food-seeking, food consumption, and, in some cases, to conserve energy. The impact of hunger state includes modulation of sensory processing as well as downstream pathways regulating locomotor and choice behaviors, as described below.

Several studies have revealed how hunger modulates sensory perception in flies. This often involves the modulation of parallel pathways that mediate opposing responses. Flies rely on smell to identify and navigate toward potential food sources. Olfactory detection is mediated by a large number of olfactory sensory neurons (OSNs) in the antenna and maxillary palp, which project to the antennal lobe of the brain (Montell, 2021). Different classes of OSNs express different olfactory receptors, which determine the set of odorants to which the neuron responds (Vosshall et al., 2000; Hallem et al., 2004; Fishilevich and Vosshall, 2005; Hallem and Carlson, 2006; Su et al., 2009). Olfactory attraction to vinegar, a natural food source, reflects a balance between two competing pathways: an attractive pathway mediated by Or42b-expressing OSNs and an aversive pathway mediated by Or85b-expressing OSNs, engaged at high vinegar concentrations (Semmelhack and Wang, 2009; Root et al., 2011; Ko et al., 2015). Hunger promotes attraction to vinegar by both enhancing the attractive olfactory pathway and suppressing the aversive olfactory pathway (**Figure 2A**). These parallel actions utilize distinct neuromodulatory and circuit mechanisms. The attractive OSNs release short neuropeptide F (sNPF), which acts in an autocrine manner to enhance their presynaptic activity (Root et al., 2011). In contrast, the activity of aversive OSNs is suppressed by tachykinin released from local interneurons (Ko et al., 2015). Both sNPF and tachykinin signaling in OSNs are regulated by insulin signaling, which represents a global satiety signal (Root et al., 2011; Ko et al., 2015).

Hunger-dependent modulation of food-seeking behaviors also relies on the integration of hunger and satiety signals in downstream processing centers. One such site is the mushroom body (MB), a high-level integration center essential for learning and memory (Heisenberg et al., 1985; Davis, 1993; de Belle and Heisenberg, 1994; Heisenberg, 1998, 2003; Zars, 2000; Pascual and Preat, 2001). Tsao et al. (2018) showed that a diverse group of hunger and satiety signals, including insulin, serotonin, and sNPF, regulate the activity of dopaminergic neurons (DANs) that innervate the MB. The activity of specific MB output neurons (MBONs) are modulated by hunger, likely *via* inputs from DANs, and these MBONs promote food-seeking when flies are hungry (Tsao et al., 2018). Once flies successfully find food, they should stop food-seeking in order to prioritize other behaviors. This behavioral switch is also mediated by the MB: in the presence of food, the persistence of food-seeking is inhibited by octopaminergic neurons innervating the MB (Sayin et al., 2019).

Similar to hunger-dependent modulation of olfaction, parallel modulation of opposing pathways also underlies hunger-dependent changes in taste sensitivity. Flies rely on taste in making the final decision about whether to consume food. Most studies of taste have focused on sweet and bitter tastes, which are detected by separate populations of neurons that promote or inhibit feeding, respectively (Montell, 2021). Hunger enhances taste sensitivity to sugar (Inagaki et al., 2012; Marella et al., 2012), which promotes energy consumption during this time of energy deficit. In parallel, hunger decreases bitter sensitivity (Inagaki et al., 2014), which increases a fly's willingness to consume food containing bitter-tasting contaminants. Like olfactory modulation, taste sensitivity is modulated at the level of sensory neurons and different channels are modulated by distinct mechanisms (**Figure 2B**). Dopamine release during hunger enhances the presynaptic activity of sugar-sensing neurons, which relies on upstream neuropeptide F (NPF) signaling (Inagaki et al., 2012, 2014). In contrast, hunger decreases octopamine signaling to suppress the activity of bitter-sensing neurons. This requires sNPF but not NPF signaling (Inagaki et al., 2014; LeDue et al., 2016).

In addition to modulating the strength of taste attraction or aversion, hunger can also elicit a behavioral switch in the taste response. Acetic acid is a natural food source that provides

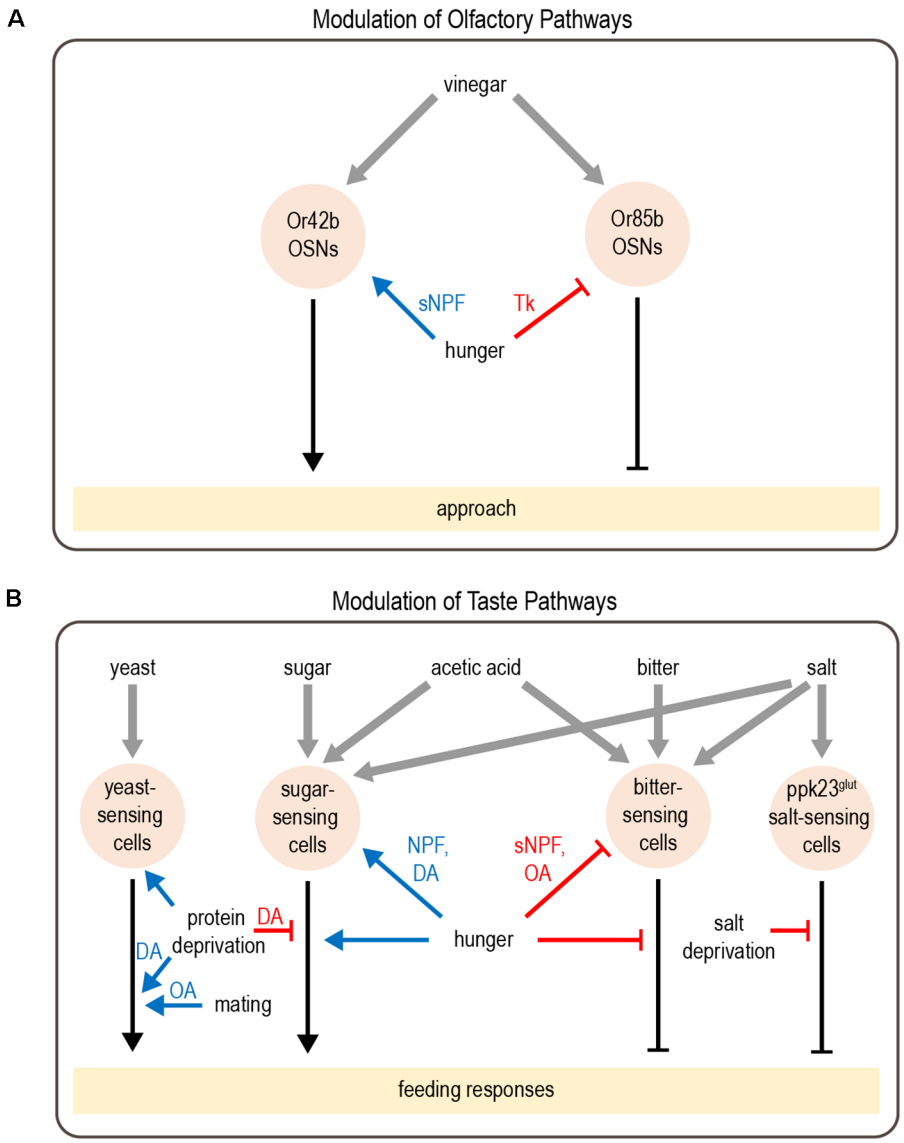

FIGURE 2 | Modulation of olfactory and taste pathways by internal states. **(A)** Parallel olfactory pathways are modulated by hunger at the level of sensory neuron output. **(B)** A variety of taste pathways are modulated by states such as hunger, protein or salt deprivation, or mating. This modulation can act at the level of sensory neurons or downstream. Most downstream neuronal targets of modulation have not yet been identified. Abbreviations not defined in text: tachykinin (Tk); octopamine (OA); dopamine (DA). Gray arrows depict sensory input; black arrows depict neural circuits that generate behavior; blue arrows depict excitatory modulation whereas red arrows depict inhibitory modulation.

calories, but it can also be toxic (Parsons, 1980; Hoffman and Parsons, 1984). Fed flies show taste aversion to acetic acid, whereas hungry flies show a strong appetitive response (Devineni et al., 2019). Acetic acid activates both the sugar- and bitter-sensing pathways, which respectively promote feeding attraction or aversion. The balance between these pathways determines the behavioral response. Hunger shifts this balance by enhancing the sugar-sensing pathway as well as suppressing the bitter pathway, resulting in a behavioral switch from aversion to attraction (**Figure 2B**). Although this modulation is consistent with the changes in sugar and bitter sensitivity described above, in this study the activity of taste sensory neurons showed very little modulation by hunger. Modulation of downstream taste pathways is therefore likely to be involved.

A theme that emerges is that, during hunger, distinct mechanisms function in parallel to modulate attractive and aversive food-sensing pathways. This affords greater control and flexibility in modulating behavior. For example, having distinct mechanisms for modulating sugar and bitter sensitivity allows these pathways to be modulated on different timescales (Inagaki et al., 2014). Mild starvation over short timescales (within 6 h) enhances sugar sensitivity, which represents a low-risk behavioral change. In contrast, prolonged starvation (at least 24 h) is required to decrease bitter sensitivity, which is a high-risk

change given that bitter compounds may be toxic. In olfaction, the aversive pathway for sensing vinegar is primarily engaged at high vinegar concentrations (Semmelhack and Wang, 2009; Ko et al., 2015), which are more likely to be toxic. Parallel modulation of the attractive and aversive pathways thus allows for differential modulation of vinegar attraction depending on its concentration.

In addition to modulating food-seeking behavior, another important survival strategy during starvation is to reduce one's metabolic rate in order to save energy. In ectothermic organisms such as *Drosophila*, metabolic rate depends on environmental temperature and can be reduced by moving to cooler temperatures. Indeed, starvation lowers a fly's preferred temperature by 2–3°C (Umezaki et al., 2018). Hunger elicits this behavioral change by modulating a specific set of thermosensory neurons, the anterior cells (AC). In starved flies, the ACs are activated by lower temperatures, which may lower the set point for the fly's preferred temperature (Umezaki et al., 2018). Unlike the changes in olfactory and taste sensitivity described above, the effect of hunger on ACs represents a tuning change rather than a gain change: the sensory neurons' preferred stimulus changes but their peak response amplitude remains constant. While gain changes can up- or downregulate specific behavioral responses, tuning changes provide a clearer mechanism to alter an animal's preference as it chooses between different stimuli of the same type.

Beyond modulation of sensory perception, hunger acts on central circuits to alter locomotor activity. Starved flies show increased locomotion (Lee and Park, 2004; Isabel et al., 2005), which may represent an enhanced exploratory drive and increase the likelihood of finding food. This change relies on bidirectional modulation by opposing metabolic signals, insulin (a satiety signal), and adipokinetic hormone (AKH, a hunger signal considered the analog of mammalian glucagon; Yu et al., 2016). AKH acts on a set of octopaminergic cells to promote hyperactivity during starvation. Conversely, insulin suppresses locomotor activity during satiety. AKH and insulin regulate locomotor activity by acting on the same set of cells, and this bidirectional regulation by hunger and satiety signals may ensure more robust control over behavior (Yu et al., 2016). Interestingly, the octopaminergic neurons that promote starvation-induced hyperactivity are not required for increased food consumption in starved flies, revealing that parallel mechanisms control distinct hunger-regulated behaviors (Yu et al., 2016).

Parallel regulation of different pathways thus emerges as a general principle for hunger modulation of behavior. As discussed above, the modulation of parallel pathways may allow for more flexibility. For example, behavior can be regulated on different timescales to promote different behavioral changes depending on whether an animal is facing mild or severe starvation. Another emerging principle is the role of slow-acting neurotransmitters and neuropeptides in hunger modulation. The release of neuromodulators during hunger or satiety states allows for state-dependent modulation of specific neural circuits that express the appropriate receptors. The strength of the modulation can therefore be tuned globally by altering the amount of neuromodulator that is released or locally by altering receptor expression levels.

Modulation by Other States Reflecting Changing Nutrient Demands

Although starvation has received the most attention, behavior is also modulated by other states reflecting changing nutrient demands. One example is the modulation of salt consumption. Similar to mammals, flies show taste attraction to low concentrations of salt, an essential nutrient, but avoid it at high concentrations. Depriving flies of salt reduces their aversion to high salt concentrations (Jaeger et al., 2018). Like acetic acid, salt activates multiple classes of taste sensory cells, including sugar- and bitter-sensing neurons as well as a population of Ppk23-expressing glutamatergic ($Ppk23^{glut}$) neurons that seem to be specific for salt-sensing. Salt deprivation specifically modulates the $Ppk23^{glut}$ taste pathway, ensuring that salt preference is modulated without affecting responses to sugar or bitter (**Figure 2B**). Activating $Ppk23^{glut}$ cells elicits lower salt aversion in salt-deprived flies than controls, demonstrating that state-dependent modulation occurs downstream of sensory cells (Jaeger et al., 2018).

Animals must also balance multiple nutritional needs, such as the need for carbohydrates vs. protein. Well-fed flies prefer to consume sugar over yeast, a good protein source, whereas protein-deprived flies shift their preference toward yeast (Ribeiro and Dickson, 2010; Vargas et al., 2010). Protein-deprived flies also show changes in foraging behavior: they reduce global exploration and focus on visiting yeast patches in a localized area (Corrales-Carvajal et al., 2016). Two DANs projecting to the "wedge" region of the *Drosophila* brain (DA-WED cells) promote yeast consumption and suppress sugar intake after protein deprivation (**Figure 2B**; Liu et al., 2017). The ability of DA-WED cells to regulate yeast and sugar intake in opposing ways is due to connections with distinct postsynaptic partners on different branches of the neuron. Postsynaptic neurons projecting to the fan-shaped body and lateral accessory lobe (FB-LAL cells) promote yeast consumption, whereas postsynaptic neurons projecting to the posterior lateral protocerebrum (PLP cells) regulate sugar consumption. Protein deprivation enhances the activity of DA-WED cells as well as inducing branch-specific plasticity that increases the number of synapses with FB-LAL neurons (Liu et al., 2017). Thus, internal state induces both functional and structural plasticity of neuromodulatory cells to exert opposing effects on distinct downstream circuits, resulting in a shift from sucrose to yeast consumption.

Mated females have higher protein and salt needs than males or virgin females due to the demands of egg production. When protein-deprived, mated females shift their preference toward yeast over sucrose much sooner than males or virgin females (Ribeiro and Dickson, 2010; Vargas et al., 2010). Similarly, mating causes females to increase salt consumption and shift their preference toward higher salt concentrations (Walker et al., 2015). The post-mating shift in both yeast and salt preference relies in part on sex peptide (Ribeiro and Dickson, 2010; Walker et al., 2015), a peptide present in male seminal fluid that is transferred to the female during mating (Chen et al., 1988). Sex

peptide acts on the sex peptide receptor (SPR), which is expressed in the female reproductive tract, and SPR activation represents a global post-mating signal that modulates a variety of behaviors (see below; Yapici et al., 2008; Hasemeyer et al., 2009). The post-mating shift in both yeast and salt preference is elicited even when egg production is blocked showing that it represents a feedforward modulation of nutrient intake that anticipates rather than reacts to changing nutrient needs (Ribeiro and Dickson, 2010; Walker et al., 2015, 2017). Downstream of SPR-expressing neurons, the mechanisms for modulating yeast and salt appetite diverge, since octopamine is required for enhanced preference toward yeast but not salt (Walker et al., 2015).

Interestingly, protein deprivation and mating enhance yeast preference by distinct mechanisms. This difference may arise because protein deprivation represents an urgent state of nutrient deficiency, whereas mating reflects the anticipation of increased nutrient demand in the future. Protein deprivation, but not mating, enhances the sensory responses of yeast-sensing taste neurons that drive feeding (Steck et al., 2018). A follow-up study performed functional imaging of yeast-evoked activity across the subesophageal zone (SEZ), the primary taste region of the fly brain (Münch et al., 2021). The SEZ contains the output projections of taste sensory neurons, the motor neurons that control feeding, and potentially the circuitry that connects them. Protein deprivation globally enhanced yeast-evoked activity across the SEZ and led to faster responses in motor regions. In contrast, mating had a more selective effect: it enhanced neural activity primarily in putative motor areas, an effect observed only when flies were also protein-deprived.

The modulatory effects of protein deprivation and mating can also be dissociated at the behavioral level by examining detailed metrics of foraging (Corrales-Carvajal et al., 2016). For instance, mating increases the probability that a fly will stop at a yeast patch it encounters. Amino acid deprivation does not affect this behavior in virgin flies but dramatically increases it in mated flies, revealing the synergistic effects of mating and protein deprivation. Future work will be needed to determine whether this synergy reflects convergence onto a common circuit or parallel activation of separate pathways.

Overall, we have just begun to scratch the surface in understanding how energy state and nutritional needs regulate feeding-related behaviors. Different states are typically sensed by different mechanisms, may be encoded by different neuromodulators, and may target distinct sensory or motor pathways. Even states that lead to similar behavioral changes—mating and yeast deprivation—engage distinct forms of modulation. The diversity of mechanisms capable of modulating the same neural circuits likely allows for more control and flexibility in adapting an animal's behavior to changing internal needs.

Emotion-Like States

Homeostatic internal states, such as those reflecting nutrient deprivation, are easily extended to model organisms such as *Drosophila*. Whether simple organisms also have emotion-like states is less clear. Anderson and Adolphs (2014) define emotion as an internal state encoded by specific patterns of neural activity that gives rise to observable behaviors. They argue that organisms as simple as flies have internal states sharing characteristics of emotion (Anderson and Adolphs, 2014). These emotion-like states are not necessarily homologous to human emotions, but they are similar in that they represent persistent internal states that modulate behavior. For example, repeated mechanical stimulation (strong air puffs) induces a state resembling arousal, in which flies show increased locomotor activity for ∼10 min and remain hypersensitive to startle-inducing stimuli even after the locomotor activity has normalized (Lebestky et al., 2009). This state relies on dopamine signaling in the ellipsoid body of the central complex. A similar arousal state can be induced by repeated presentation of a moving shadow, representing a visual threat (Gibson et al., 2015).

Sexual arousal is an internal state that has been well-studied in *Drosophila* males. Male sexual behavior toward females is controlled by a set of male-specific command neurons called P1 neurons, which integrate sensory cues from females and activate motor programs for courtship (Kohatsu et al., 2011; Clowney et al., 2015). P1 neurons also promote aggression toward other males, a male-typical behavior that is evoked by the presence of a female (Hoopfer et al., 2015). In addition to acutely promoting courtship or aggression, brief optogenetic activation of P1 neurons promotes a long-lasting increase in these behaviors (Hoopfer et al., 2015; Jung et al., 2020). This has been interpreted as a state of increased sexual or social arousal. For instance, low-intensity optogenetic stimulation of P1 neurons in solitary males may evoke no overt behavioral change, but these males show increased aggression when presented with another male after the stimulation has ended (Hoopfer et al., 2015). The internal state induced by P1 activation lasts for at least 10 min and decays over time. A similar state can be triggered more naturally by exposure to a female: males are more aggressive even after the female is removed (Jung et al., 2020).

How is a persistent state of sexual arousal encoded in the brain? Although this state is induced by stimulating P1 neurons, P1 neurons do not show persistent activity following stimulation (Hoopfer et al., 2015). Thus, the sexual arousal state must be encoded by downstream neurons. An imaging screen identified a set of neurons called pCd cells that show persistent activity in response to transient P1 activation (Jung et al., 2020). The activity of pCd neurons is required for the persistent courtship and aggression elicited by transient P1 activation or exposure to a female. Activating pCd neurons amplifies courtship and aggression behaviors but cannot elicit these behaviors in the absence of an appropriate target (a female or male fly, respectively) as P1 activation does. These results suggest that a persistent state of sexual arousal is encoded by persistent activity in pCd neurons and modulates the intensity of social behaviors (**Figure 3**).

A separate study found that mating drive over much longer timescales, such as days, is maintained by a recurrent circuit comprising pCd neurons and NPF-expressing neurons, which excite each other (Zhang et al., 2019). This is reminiscent of recurrent excitation in other systems, which generates persistent activity underlying functions such as working memory (Wang, 2008). Activity in this recurrent circuit parallels male mating

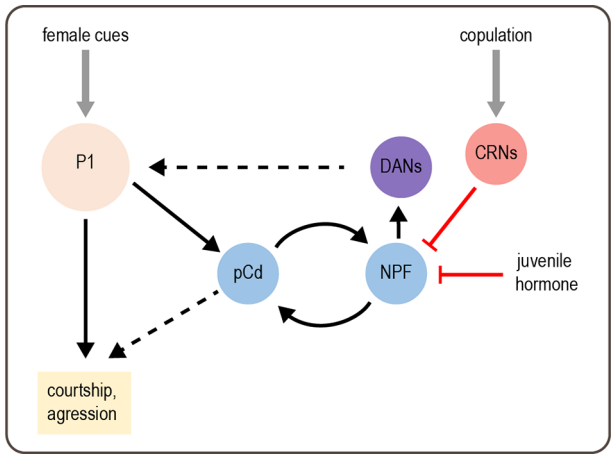

FIGURE 3 | Modulation of male behavior by sexual arousal state. Female cues activate P1 neurons, which acutely promote courtship and aggression. P1 activation also elicits a state of sexual arousal that lasts for several minutes and enhances sexual behaviors. This state is mediated by persistent activity in pCd neurons. On longer timescales, a recurrent circuit comprising pCd and NPF-expressing cells maintains a sexual arousal state. Activity in this circuit builds up over days if males do not copulate, and this activity promotes mating by enhancing P1 activity *via* DANs. Copulation suppresses activity in the recurrent circuit, as does juvenile hormone circulating in young males, thus suppressing mating behavior. Gray arrows depict sensory input; solid black arrows depict neural circuit connectivity; red arrows depict inhibitory modulation. Dashed black arrows represent more complex or unknown forms of modulation: DANs enhance P1 responses by desensitizing P1 to inhibition, and the targets of pCd are unknown.

drive, which is suppressed by repeated matings and gradually recovers over several days (Zhang et al., 2016, 2019). This suppression of courtship relies on copulation and is therefore distinct from courtship conditioning, a well-characterized effect in which males reduce courtship after being rejected by pre-mated females (Griffith and Ejima, 2009). The copulation-induced "sexual satiety" state likely represents an adaptive response that prevents males from expending energy on mating when their reproductive fluids have been depleted. A set of copulation reporting neurons (CRNs) in the abdominal ganglion are responsible for inducing the sexual satiety state. CRNs project dendrites to the genitalia and axons to the brain, where they inhibit the activity of NPF neurons in the recurrent circuit to decrease mating drive (**Figure 3**; Zhang et al., 2019).

Recovery from sexual satiety is correlated with a gradual increase in the intrinsic excitability of pCd and NPF neurons in the recurrent circuit (Zhang et al., 2019). Neuronal excitability is in turn controlled by CREB2, an activity-dependent transcription factor representing the homolog of mammalian CREB. CREB2 modulates neuronal excitability to prolong the satiety recovery time, which ensures that it parallels the replenishment of reproductive fluids after repeated mating. CREB2 is also involved in maintaining other long-lasting states, such as circadian rhythms and memory, demonstrating a conserved function across different systems (Lonze and Ginty, 2002). Interestingly, the same cellular and circuit mechanisms used to induce sexual satiety after mating are employed to suppress courtship in another context: in juvenile males, who do not court females. Juvenile hormone released in recently enclosed males suppresses the activity of recurrent pCd and NPF neurons to reduce mating drive (Zhang S. X. et al., 2021). Thus, different states can reuse the same molecular and circuit mechanisms to induce flexible behavior.

The work described above reveals how sexual arousal and satiety states are induced and maintained. How do they actually modulate behavior? The recurrent pCd/NPF circuit promotes mating drive by enhancing dopaminergic activity in the superior medial protocerebrum (**Figure 3**; Zhang et al., 2019). Dopamine modulates responses of P1 neurons, which integrate excitatory and inhibitory inputs to promote courtship (Clowney et al., 2015). Specifically, dopamine de-sensitizes the P1 response to inhibition from GABAergic neurons, suggesting that dopamine may help sustain P1 excitation during courtship (Zhang et al., 2018). These results suggest that P1 neurons not only induce a sexual arousal state but also represent a target of state-dependent modulation.

Sexual arousal state also modulates visual processing to enhance a male's ability to track a female during courtship. The underlying mechanisms have been studied using tethered males walking on a ball and tracking a moving visual stimulus representing a female (Ribeiro et al., 2018; Hindmarsh Sten et al., 2021). Increasing the male's sexual arousal state by brief P1 activation or presentation of female pheromones caused the male to track the stimulus more closely (Hindmarsh Sten et al., 2021). This arousal state gates the activity of LC10 visual neurons, which are required for tracking a female and elicit courtship behaviors in an arousal-dependent manner (Ribeiro et al., 2018). LC10 neurons respond strongly to the moving target only when males are aroused (Hindmarsh Sten et al., 2021), revealing dynamic modulation of sensorimotor processing by sexual arousal state.

Less work has been done on sexual arousal states in female flies, although it has long been known that recently mated females are less sexually receptive (Manning, 1962). As mentioned above, the female post-mating state is induced by the activation of SPR by sex peptide, contained in the male seminal fluid (Chapman et al., 2003; Liu and Kubli, 2003; Yapici et al., 2008). Sex peptide inhibits the activity of SPR-expressing neurons in the female reproductive tract, and this mating signal is then transmitted to postsynaptic sex peptide abdominal ganglion (SAG) neurons and downstream pC1 neurons in the brain (Hasemeyer et al., 2009; Feng et al., 2014; Wang et al., 2020).

Female pC1 neurons respond to stimuli that promote mating, including male courtship song and male pheromones, and PC1 activity promotes sexual receptivity (Zhou et al., 2014). The activity of pC1 neurons is decreased by sex peptide, representing a modulatory node to regulate post-mating behaviors. pC1 neurons provide input onto descending neurons [neurons projecting from the brain to the ventral nerve cord (VNC)] called vpoDNs that control vaginal plate opening, a key component of sexual receptivity (Wang et al., 2021). vpoDNs integrate excitatory signals from pC1 neurons as well as auditory neurons tuned to the male's courtship song. Because pC1 activity is lower after mating, this reduces the excitatory

drive onto vpoDNs and leads to lower receptivity. A parallel pathway independent of sex peptide also acts to suppress female receptivity on short timescales. Abdominal neurons detect copulation, likely based on mechanosensory cues, and activate a circuit comprising ascending neurons and central peptidergic neurons that reduce sexual receptivity (Shao et al., 2019).

pC1 neurons act *via* a similar but inverse circuit mechanism to regulate the post-mating switch in egg-laying behavior. pC1 cells activate inhibitory interneurons that suppress the activity of oviDN descending neurons, which promote egg-laying (Wang et al., 2020). Decreased pC1 activity after mating causes disinhibition of oviDN activity, which leads to increased egg-laying.

The activity of pC1 neurons in females has been proposed to encode a sexual arousal state analogous to that described above for males (Wang et al., 2021). Indeed, transient activation of pC1 neurons induces persistent changes in female behavior, similar to P1 activation in males. For several minutes following pC1 activation, females are more sexually receptive (Deutsch et al., 2020). They also show other behaviors such as shoving and chasing, which may represent aggression. These behaviors appear to be driven by a separate subset of pC1 neurons from those promoting receptivity. Imaging experiments reveal that transient pC1 activation elicits persistent activity in multiple brain areas and this is likely due to recurrent connectivity (Deutsch et al., 2020). Together, studies suggest that recurrent excitation likely maintains persistent neural activity and sexual arousal states in both males and females. These states modulate sexual and social behaviors to balance a fly's reproductive drive with other needs, such as conserving energy.

Competing Internal States

Most studies of internal state examine the effects of one state at a time. However, animals may also experience multiple internal states that reflect competing needs. For example, hunger and thirst are different states that lead an animal to prioritize different behaviors, namely food vs. water consumption. Food consumption alleviates hunger, but by increasing blood sugar levels it increases blood osmolality and exacerbates the need for water. Flies experiencing mild starvation consume less sugar if they are also water-deprived (Jourjine et al., 2016). Four interoceptive SEZ neurons (ISNs) integrate hunger and thirst cues and regulate sugar and water consumption in opposing ways (Jourjine et al., 2016). ISNs are directly activated by AKH, a hunger signal, and their activation promotes feeding. Conversely, ISN activity is inhibited by high extracellular osmolality, which signals thirst, and their activity inhibits water consumption. Thus, a single set of neurons integrates competing internal states in order to regulate consumption behaviors and maintain homeostasis.

Another study investigated how male flies choose between feeding and mating when they are both food- and sex-deprived (Cheriyamkunnel et al., 2021). The decision to mate or feed depends on the duration of food deprivation. After 15 h of starvation, male flies prioritize feeding over courtship. Their decision is also modulated by the quality of the food as flies show less preference for feeding over mating when presented with low-calorie food. Tyramine, a biogenic amine considered to be an analog of norepinephrine, modulates distinct pathways to regulate this choice. Tyramine receptor-expressing neurons in the posterior lateral protocerebrum ($TyrR^{PLP}$ neurons) promote feeding over courtship. Conversely, P1 neurons, previously identified as courtship command neurons, promote courtship over feeding. Tyramine acts as a satiety signal that modulates both of these pathways: tyramine inhibits the $TyrR^{PLP}$ neurons while activating P1 neurons, thus shifting the choice toward courtship over feeding.

A general principle emerging from these studies is that the same neuromodulator (tyramine) or set of neurons (ISN cells) may be used to modulate multiple pathways in opposing ways. By simultaneously activating and inhibiting different pathways that promote competing behaviors, the balance can be shifted toward one behavior or another. This principle is also reminiscent of behavioral switches that can occur during a single internal state, such as switching from acetic acid aversion to attraction during hunger or switching from sugar to yeast preference during protein deprivation (see above). In all of these examples, competing behavioral pathways are modulated in opposing ways by internal state.

MODULATION BY BEHAVIORAL STATE

Similar to internal states such as hunger, an animal's behavioral state can modulate how the animal responds to stimuli in the world. For instance, the same sensory cue may have different salience or even a different meaning depending on whether a fly is walking, flying, or stationary. Like modulation by internal state, flexibility due to behavioral state can reflect modulation of sensory processing, sensorimotor transformations, or motor responses. It is important to note that changes in behavioral state are likely to correspond with changes in internal state, whether it is the internal state that alters behavioral state (e.g., increased arousal promotes locomotor activity) or *vice versa* (e.g., being active increases arousal).

Modulation of Visual Motion Processing

Several examples of sensory modulation by behavioral state have been documented in the *Drosophila* visual system, particularly in motion-sensing pathways. These results parallel findings in the mammalian visual system (Niell and Stryker, 2010). Flies detect visual motion through horizontal (HS) and vertical (VS) neurons in the optic lobe. HS and VS cells show enhanced visual responses when flies are walking or flying, respectively (**Figure 4**; Chiappe et al., 2010; Maimon et al., 2010; Suver et al., 2012). This increased sensitivity could allow flies to more effectively use visual motion cues as they are actively navigating an environment (van Breugel et al., 2014). Walking also shifts the tuning of HS cells towards faster motion (Chiappe et al., 2010), a likely adaptation to the fact that the visual world moves more rapidly when flies are walking. Flying did not shift the peak of the tuning curve peak in VS cells, but it broadened tuning by enhancing responses to faster motion (Suver et al., 2012).

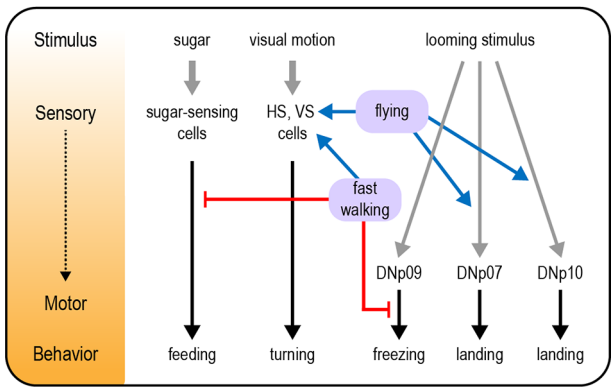

FIGURE 4 | Examples of modulation by behavioral state. Behavioral states such as walking or flying modulate neural pathways underlying a variety of behavioral responses, including feeding responses, visual motion processing, and responses to a looming stimulus. Modulation can occur at any level from sensory to motor processing (represented by the diagram and color gradient on the left). Gray arrows depict sensory input; black arrows depict neural circuits that generate behavior; blue arrows depict excitatory modulation whereas red arrows depict inhibitory modulation.

The HS cells receive multiple types of motor signals related to walking, which modulate the cells' membrane potential independently of visual input (Fujiwara et al., 2017). One component of the signal encodes the general walking state, whereas other components encode the speed and direction of walking. These motor signals modulate visual responses differently depending on whether the visual motion is in the same direction as expected from the fly's own movement. The integration of sensory and motor signals in HS cells may allow them to control walking behavior or create internal estimates of self-movement.

The enhancement of visual responses during flight or walking relies on modulation by octopamine (Suver et al., 2012; Strother et al., 2018), a mechanism also implicated in other insects (Longden and Krapp, 2010; Jung et al., 2011; Cheng and Frye, 2020). Octopamine has long been considered an analog of norepinephrine, a "fight-or-flight" signal that elicits a general state of arousal (Roeder, 2005). Flying enhances the activity of octopaminergic neurons innervating the optic lobe (Suver et al., 2012), but the circuits that elicit this activation are unknown. Although octopamine may represent a general arousal signal that enhances visual processing, the speed- and direction-selective motor signals transmitted to HS cells likely have a different source. These signals do not arise from visual or mechanosensory feedback generated during walking and may represent an internal copy of the motor command (Fujiwara et al., 2017). Similarly, motor command signals are conveyed to visual neurons during flight turns to suppress the visual response to self-motion (Kim et al., 2015, 2017). This ensures that flies do not inappropriately respond to perceived visual motion elicited by their own voluntary movement.

Overall, the modulation of visual motion processing by behavioral state likely plays several important roles in behavior. Gain changes may increase the salience of visual cues when the fly is walking or flying, whereas tuning changes ensure that moving flies detect motion at faster speeds. Speed- and direction-selective signals may allow flies to cancel out self-generated visual responses or use them to control their movement.

Modulation of Other Visually-Guided Responses

A fly's behavioral state also alters visual responses beyond motion-sensing. Positive phototaxis, or preference for light, is a well-studied innate behavior in *Drosophila* (Benzer, 1967). Interestingly, eliminating a fly's ability to fly by clipping or gluing the wings abolishes positive phototaxis and can switch its behavior to light aversion, even though this assay does not involve flight (McEwen, 1918; Gorostiza et al., 2016). This effect is not induced by damage to other organs (Gorostiza et al., 2016) and may have evolved because flightless flies are highly vulnerable to predators in the light. Dopaminergic and octopaminergic neurons were implicated in mediating photopreference (Gorostiza et al., 2016), but the mechanisms underlying the behavioral switch are still unknown. Presumably, flies must continuously monitor their ability to fly and modulate their visual responses accordingly.

Responses to looming visual stimuli are strongly modulated by behavioral state. Looming stimuli represent the approach of an object, often a predator. Flies respond to looming stimuli with defensive behaviors such as jumping, freezing, fleeing, or flight takeoff (Card and Dickinson, 2008; von Reyn et al., 2014; Zacarias et al., 2018). The response that a fly chooses depends on its behavioral state. Flies that are walking more slowly at the time of the threat are more likely to freeze than flee (Zacarias et al., 2018). This may be an adaptive response reflecting the fact that slowly moving flies cannot accelerate quickly enough to escape, so freezing is the better option. A pair of descending neurons, the DNp09 neurons, are required for freezing but not fleeing, demonstrating that these responses are mediated by distinct motor pathways (Zacarias et al., 2018). Optogenetic activation of DNp09 neurons causes flies to freeze, and the probability of freezing is higher if the flies are walking more slowly before stimulation. These results imply that information about walking speed is integrated downstream of DNp09 neurons (**Figure 4**), perhaps by gating motor neuron activation in the VNC.

Although a looming stimulus may represent the approach of a predator, this visual pattern also occurs when a moving fly intentionally approaches an object. In this case, a defensive response would be inappropriate. Indeed, the same looming stimulus that evokes escape behavior in a standing fly elicits a landing response in flying flies (Ache et al., 2019a). Two sets of descending neurons that promote landing responses have been identified, DNp07 and DNp10 (Ache et al., 2019a). Optogenetic activation of these neurons elicits landing-like motor responses even if the fly is not flying, revealing that the behavioral state acts upstream of these neurons to modulate their activity (**Figure 4**). Indeed, the visual responses of DNp07 and DNp10 are gated by state: they respond robustly to features of looming stimuli only during flight (Ache et al., 2019a). Thus, behavioral flexibility is achieved by modulating the sensorimotor transformation: visual

input is coupled to the landing motor pathways only during flight.

Modulation of Carbon Dioxide Responses

The behavioral state also modulates responses to other sensory stimuli, beyond visual cues. One prominent example is carbon dioxide. Carbon dioxide is released by animals during respiration and is emitted at three- to four-fold higher concentrations by flies when they are stressed (Suh et al., 2004). In a two-choice assay, flies robustly avoid the chamber containing carbon dioxide, suggesting that they may interpret carbon dioxide as a stress signal (Suh et al., 2004). However, flies switch their behavioral response from aversion to attraction when they are active, such as walking at high speeds or flying (Wasserman et al., 2013; van Breugel et al., 2018). Since carbon dioxide is emitted by fermenting fruit, attraction to carbon dioxide may help guide flies to their preferred food source. The aversive and attractive responses to carbon dioxide are mediated by separate olfactory receptors and neural pathways (Wasserman et al., 2013; van Breugel et al., 2018). Octopamine is required for flying flies to show carbon dioxide attraction (Wasserman et al., 2013), suggesting that neuromodulation may alter the gain of one or both of the olfactory pathways to shift the balance toward attraction—potentially similar to how hunger switches acetic acid aversion to attraction, as described above.

Other Examples of Modulation by Behavioral State

As described above, walking state modulates diverse responses such as visual motion processing, responses to looming stimuli, and the response to carbon dioxide. Indeed, locomotor activity seems to be one of the most critical behavioral states an organism needs to account for. Large-scale imaging studies have found that walking state increases global brain activity, suggesting that this state signal is transmitted to many different circuits with different functions (Aimon et al., 2019; Schaffer et al., 2021). One function of this state-dependent modulation may be to suppress behaviors that should not be expressed during walking. For example, flies must be stationary in order to feed on a substrate. Walking suppresses the initiation of feeding, and this is mediated by interneurons that are activated by mechanosensory inputs from the legs (**Figure 4**; Mann et al., 2013). Conversely, feeding seems to suppress locomotion: walking is reduced if the proboscis is maintained in an extended position, as occurs during feeding (Mann et al., 2013). The inhibition of one behavior during another is likely to be a general example of how behavioral state modulates neural processing.

Cande et al. (2018) took an unbiased approach to examine how a fly's current behavioral state modulates the behavioral responses induced by optogenetically activating subsets of descending neurons. They used an unsupervised method to characterize a variety of behaviors, such as locomotion, body movements, and grooming and found that behavioral changes elicited by optogenetic stimulation depended on what the fly was doing just before stimulation. In some cases, neuronal activation could induce different behaviors on different trials, and the fly's behavior before stimulation was highly predictive of which behavior was elicited by activation. These state-dependent effects reflect modulation downstream of the descending neurons, suggesting that significant modulation and processing occurs in the VNC.

Another study used a different unbiased approach to identify states that influence how males produce courtship song (Calhoun et al., 2019). Males modulate their song production depending on the female's distance, orientation, and movement as well as their own movement. However, the influence of these cues on song production varies depending on the male's state. Three states were identified using an unsupervised "GLM-HMM" approach and correspond roughly to periods when the male is chasing the female, close to the female without chasing, or residing far from the female and oriented away from her. The male is unlikely to sing in the latter state, whereas the former two states generate different types of song. Specific sensory cues, such as the male's velocity or female's distance, are weighted differently in different states in determining song production. This study reveals the power of unbiased behavioral analysis in identifying states that modulate the mapping of sensory input to behavior. Although the states identified in this study correspond to different behavioral states, such as the male chasing the female, this need not be the case.

Overall, behavioral states can modulate neural circuits at any level: from sensory processing to sensorimotor transformations to motor pathways. This is reminiscent of modulation by internal states such as hunger. Behavioral states can also be detected in multiple ways, such as by sensory feedback or an internal copy of the motor command. Ultimately this modulation may alter the likelihood of expressing a behavior or switch the behavioral response entirely.

MODULATION BY ENVIRONMENTAL CONTEXT

Thus far we have discussed how a fly's state, either an internal state or an ongoing behavioral state, modulates its behavioral responses and actions. In addition, a fly needs to continuously evaluate the environmental context in which stimuli are encountered. Context may be conveyed by the specific features of a stimulus or the presence of additional cues that modulate the response to a given stimulus.

Flexibility in the Response to a Single Stimulus

The response to a single stimulus can vary based on features of the stimulus that reflect its context. One example is the escape response to looming stimuli. Responses to looming stimuli are modulated by behavioral state, as described above, but also show additional flexibility. In a paradigm in which looming stimuli elicit flight takeoff, flies select between two takeoff modes: short and long (von Reyn et al., 2014). The short takeoff is faster but less controlled: flies trade off stability for speed. The same stimulus can evoke different takeoff modes on different trials, but flies choose the short takeoff more often if the stimulus is approaching faster.

The short and long takeoff modes are generated by different motor pathways. The giant fiber neurons, a pair of large descending neurons, mediate short but not long takeoffs (von Reyn et al., 2014). The giant fiber neuron is activated during both takeoff modes but spikes more quickly during short takeoff trials, suggesting a timing-based model: the motor pathway that is activated first determines the type of takeoff. The two motor pathways are differentially sensitive to specific features of the looming stimulus, including looming size and velocity, and these different features are processed in separate visual input pathways (von Reyn et al., 2017; Ache et al., 2019b). By integrating specific features of the stimulus and activating the appropriate motor pathway, flies can bias their behavior to maximize their chance of escape.

Context-Dependent Modulation of Carbon Dioxide Responses

The response to a stimulus can also vary based on the presence or absence of other cues within the environment. Previously we discussed how flies change their response to carbon dioxide depending on their behavioral state, and this may reflect a balance between the role of carbon dioxide as a potential distress signal as well as a product of fruit fermentation. In addition to behavioral state-dependent modulation, flies modulate their response to carbon dioxide depending on the presence of other olfactory cues. Flies display robust aversion to carbon dioxide in a T-maze (Suh et al., 2004), free-flying two-trap choice assay (Faucher et al., 2013), and four-field olfactometer assays (Faucher et al., 2006). Despite this innate aversion, when vinegar is present a starved fly significantly reduces its avoidance to carbon dioxide (Bracker et al., 2013; Lewis et al., 2015). Further, vinegar mixed with aversive concentrations of carbon dioxide is more appetitive to free-flying flies than vinegar alone (Faucher et al., 2013). In these instances, the fly appears to prioritize seeking food over avoiding carbon dioxide.

The MB, previously discussed as an integration center for hunger and satiety cues, appears to mediate the context-dependent modulation of carbon dioxide avoidance (Bracker et al., 2013). Initial studies revealed that MB inactivation during the presentation of carbon dioxide along with vinegar switches the response from aversion to attraction in both fed and starved flies. A follow-up study identified a specific set of glutamatergic MBONs that are activated by carbon dioxide and promote avoidance, and their response is diminished by the addition of vinegar (Lewis et al., 2015). This modulation is mediated by a set of DANs that innervate the same MB regions as the MBONs and likely act presynaptically. Optogenetic activation of these DANs suppresses carbon dioxide avoidance, and imaging data demonstrate that these DANs are strongly and specifically activated by vinegar. Previous work suggested that dopaminergic activity negatively regulates carbon dioxide avoidance (Siju et al., 2014), but this was the first study to identify specific DANs that play a critical role (Lewis et al., 2015). Together these data suggest a circuit mechanism in which glutamatergic MBONs promote carbon dioxide avoidance and this avoidance is suppressed in the presence of vinegar by activating DANs that inhibit MBON activity (**Figure 5A**). Interestingly, the suppression of carbon dioxide avoidance is not long-lasting. Thus, vinegar and the resulting DAN activation briefly inhibit carbon dioxide avoidance in an effort to prioritize food-seeking behaviors.

Cross-Modal Modulation

The behavioral response to a stimulus can also be modulated by sensory integration across multiple modalities. For instance, although feeding responses are primarily determined by the taste of a food source, they can be strongly modulated by its smell, texture, and temperature. Flies show a feeding response when their legs contact sugar and the presence of yeast odor enhances this response by acting through OR35a-expressing OSNs (Oh et al., 2021). Robust feeding responses also require mechanosensory input from the leg, which provides information related to the viscosity of the food. Both smell and touch work synergistically to enhance feeding responses elicited by sugar, enabling flexible feeding behavior based on multiple sensory properties of the food (Oh et al., 2021).

Other work demonstrates the importance of temperature in modulating taste perception and food preference. Like other animals, *Drosophila* show reduced taste attraction to sugar at lower temperatures (Li Q. et al., 2020). The lower preference for sucrose at cool temperatures persists despite starvation. The ethological relevance of this effect has not been demonstrated, but one possibility is that fresh fruit may be cooler than decaying fruit, which flies prefer. The reduction in sugar attraction could result from reduced activation of sugar-sensing neurons at cool temperatures. However, imaging and electrophysiological data suggest otherwise. Instead of suppressing sugar-sensing gustatory neurons, cool temperatures activate aversive bitter-sensing neurons and mechanosensory neurons to ultimately drive a decrease in feeding responses (Li Q. et al., 2020). Thus, feeding responses at cool temperatures are guided by the simultaneous activation of competing sensory pathways. This mechanism resembles the mechanism underlying the feeding response to acetic acid, described above, which is also determined by the balance between activation of the sugar- and bitter-sensing pathways (Devineni et al., 2019).

Cross-modal modulation of behavior also occurs while flies are in flight. Flies steer toward elongated vertical bars that visually resemble feeding sites, such as vegetation, but they turn away from aversive wind sources (Maimon et al., 2008). When aversive wind currents arise from the same location as the attractive visual cue, flies display a dynamic response in which they initially turn away and then slowly turn back toward the cue (Currier and Nagel, 2018). The aversive mechanosensory information arises, at least in part, from the antenna. Modeling studies suggest that visual and mechanosensory signals are independently processed to generate modality-specific turn commands. These signals converge and summate to ultimately drive turning behavior (Currier and Nagel, 2018).

Flies also use cross-modal integration to orient upwind in the presence of an attractive odor cue, which would enable them to navigate toward an odor source. Odor and wind cues are integrated in a region of the central complex called the fan-shaped body (FSB; Matheson et al., 2021). Output neurons from the MB and lateral horn, the two higher-order olfactory

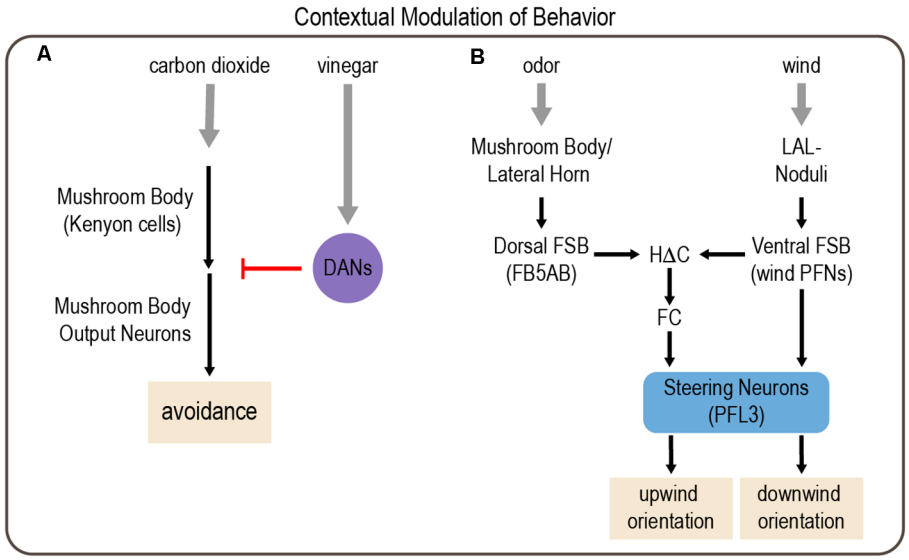

FIGURE 5 | Examples of modulation of behavior by environmental context involving integration of multiple sensory cues. **(A)** Context-dependent responses to carbon dioxide depend on the mushroom body (MB). Kenyon cells (KCs) in the MB are activated by carbon dioxide, which activates MB output neurons (MBONs) to drive avoidance responses. However, the presence of vinegar activates subsets of DANs, which inhibit MBON activation to reduce carbon dioxide avoidance. **(B)** Odor and wind cues are processed by parallel pathways that are integrated in the FSB to drive wind orientation. *Drosophila* typically orient downwind from a wind source. However, in the context of an appetitive odor cue, this behavior reverses, and flies orient upwind. Gray arrows depict sensory input; black arrows depict neural circuit connectivity; red arrow depicts inhibitory modulation.

processing centers (Marin et al., 2002; Su et al., 2009), converge on neurons that provide input to the dorsal FSB. A separate class of FSB neurons, hΔC neurons, integrate odor information from the dorsal FSB and wind direction cues from the ventral FSB. In the absence of odor, flies tend to orient downwind, suggesting that competing pathways operate in the presence and absence of odor. Circuit analysis and computational modeling suggest that a direct pathway from wind-activated FSB neurons to steering FSB output neurons (PFL3 cells) normally promotes downwind orientation, but the presence of an attractive odor activates an indirect pathway comprising hΔC neurons, which alters PFL3 activation to promote upwind orientation (**Figure 5B**; Matheson et al., 2021).

In contrast to elongated vertical bars, which flies steer toward, flies steer away from smaller objects, which are presumably interpreted as a threat (Maimon et al., 2008). This avoidance of small objects switches to attraction in the presence of an attractive odor, such as vinegar (Cheng et al., 2019). The presence of a food odor may signify that the small object is not a threat but in fact a potential food source. This odor-dependent switch from visual aversion to attraction can be mimicked by optogenetically activating modulatory octopaminergic neurons or motion-sensitive visual pathways in the optic lobe (Cheng et al., 2019). Previous work showed that vinegar activates octopaminergic neurons innervating the visual system (Wasserman et al., 2015). Cheng et al. (2019) propose a model whereby two different visual pathways compete to determine behavior: a motion vision pathway that drives approach and an object detection pathway that drives avoidance. An appetitive odor activates octopaminergic neurons, which enhances the gain of the motion vision pathway and tips the balance towards approach. As discussed above, flight itself recruits octopaminergic enhancement of visual motion responses (Suver et al., 2012). The additional role of octopamine in mediating odor-dependent modulation suggests that octopamine plays multiple roles in visual processing and its different effects can be superimposed.

Thus, the integration of different environmental cues is a common way in which behavioral responses exhibit flexibility. Classic studies have provided significant insight into sensory processing by presenting a single stimulus at a time, but paradigms with multiple stimuli will likely reveal far greater complexity in behavioral responses and the underlying neural circuitry. Moreover, how different cues are integrated and the behavioral responses they elicit are likely to differ across *Drosophila* species depending on the ecological niches that they occupy.

BEHAVIORAL FLEXIBILITY IN LEARNING AND MEMORY

Learning can be defined as an experience-dependent change in behavior. Storing past experiences as memories allows animals to apply learned information when faced with a similar situation. Thus, learning and memory is a prime example of flexible behavior. A wealth of studies investigating learning and memory in the fruit fly have provided significant insight into the underlying neural networks and how they are modified

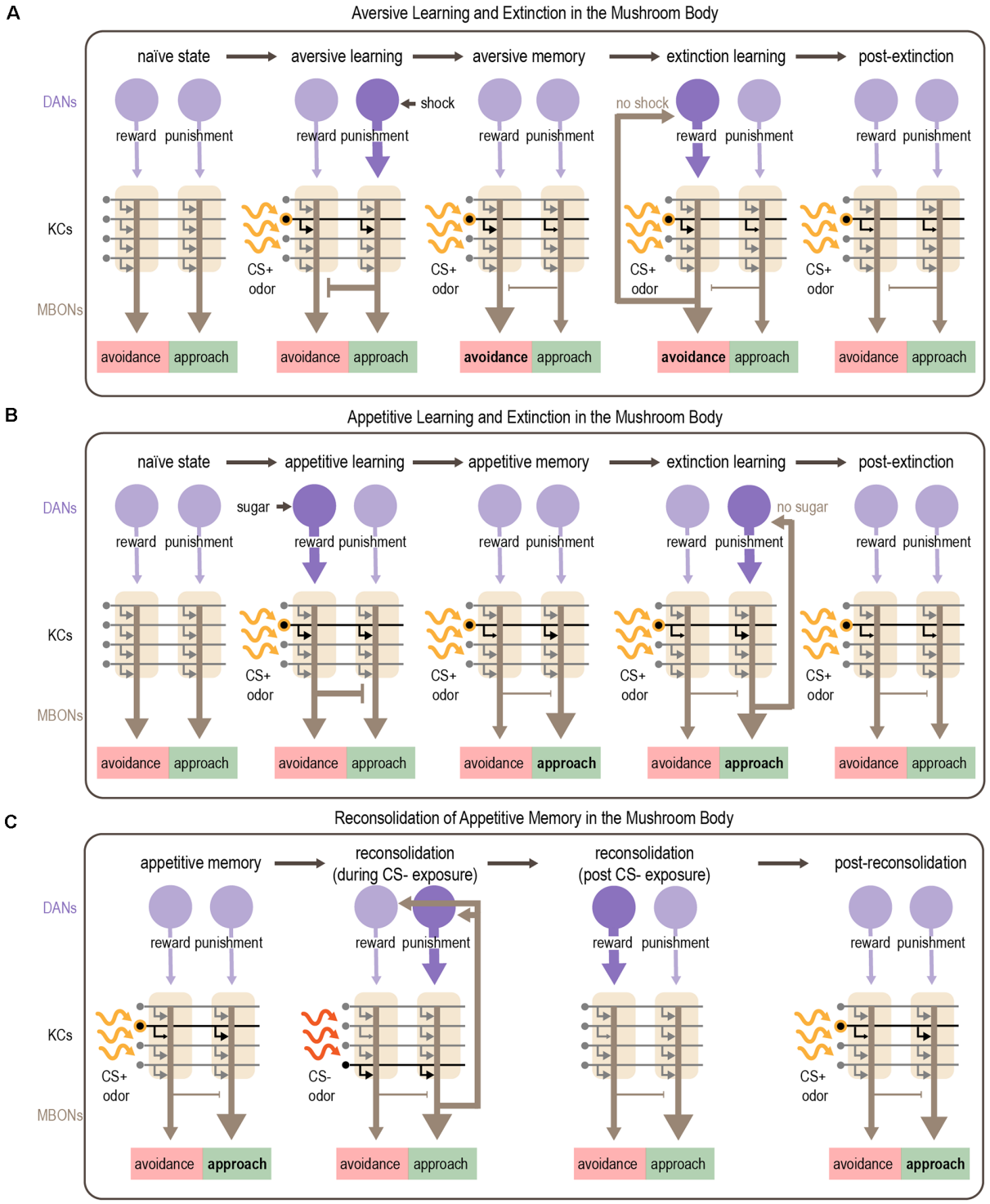

FIGURE 6 | Mechanism for associative learning, extinction learning, and reconsolidation in the MB. **(A,B)** Plasticity in MB circuits mediates aversive **(A)** and appetitive **(B)** learning. Odor sparsely activates KCs within the MB. Aversive cues activate punishment-encoding DANs, which modulate MBONs promoting approach, whereas appetitive cues activate reward-encoding DANs, which modulate MBONs promoting avoidance. Dopamine depresses active KC-MBON synapses. After aversive learning, this depression shifts the balance of MBON activity towards avoidance **(A)** whereas after appetitive learning the balance shifts towards approach **(B)**. Extinction of aversive or appetitive memories occurs by readjusting the balance of MBON activity. After aversive learning, presenting the CS+

(*Continued*)

FIGURE 6 | Continued
in the absence of anticipated electric shock causes avoidance-promoting MBONs to recurrently activate reward-encoding DANs, which encode a competing appetitive memory that reduces avoidance **(A)**. After appetitive learning, presenting the CS+ in the absence of anticipated sugar reward causes approach-promoting MBONs to recurrently activate punishment-encoding DANs, resulting in the formation of a competing aversive memory that reduces approach **(B)**. **(C)** Appetitive memories can be re-activated by exposure to the CS- which induces reconsolidation of the original memory. Reconsolidation requires recurrent DAN activation orchestrated by subsets of MBONs (MBON $\gamma 2\alpha' 1$). Activation of punishment-encoding DANs during CS- exposure and subsequent activation of rewarding-encoding DANs after CS- exposure results in reconsolidation of the original memory, although the exact mechanisms are unclear. Purple arrows depict DAN input to the MB; gray arrows depict KC axons innervating MBONs and are shown in black when activated by odor; brown arrows depict MBON output. Note that the middle panels of **(C)** show skewed MBON output as it would be elicited by the CS+, representing the CS+ memory; output in response to the CS- or in the absence of odor is not skewed.

over different timescales. In particular, the MB has received considerable attention (Cognigni et al., 2018; Modi et al., 2020). The MB consists of densely packed Kenyon cell (KC) fibers, which receive processed olfactory, visual, gustatory, and tactile sensory input (Marin et al., 2002, 2020; Liu et al., 2012, 2016; Caron et al., 2013; Gruntman and Turner, 2013; Vogt et al., 2014, 2016; Kirkhart and Scott, 2015; Yagi et al., 2016; Zheng et al., 2018; Li J. et al., 2020). The MB, therefore, serves as a multisensory integration hub for learning as well as other behaviors, as described above. Specific KCs synapse on distinct sets of GABAergic, cholinergic, and glutamatergic MBONs (Tanaka et al., 2008; Aso et al., 2014a). Several MBONs promote either odor-driven approach or avoidance, and the balance between these pathways seems to determine the fly's behavioral response to the odor (Aso et al., 2014b; Owald et al., 2015; Perisse et al., 2016). DANs innervating the MB encode valence-related signals and modulate the synapses between KCs and MBONs (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010; Sejourne et al., 2011; Burke et al., 2012; Liu et al., 2012; Hige et al., 2015). The MB is organized into distinct anatomical and functional modules, termed compartments (Aso et al., 2014a). Each compartment contains a defined subset of DAN inputs and MBON outputs, enabling specific valence signals to modulate specific output pathways and operate using distinct learning rules (Pai et al., 2013; Placais et al., 2013; Bouzaiane et al., 2015; Hige et al., 2015; Aso and Rubin, 2016; Perisse et al., 2016).

Although its function is complicated, the canonical view on associative learning in the MB is that different sets of DANs encode reward and punishment, and they mediate appetitive or aversive learning, respectively (**Figure 6**; Claridge-Chang et al., 2009; Aso et al., 2010, 2012; Burke et al., 2012; Liu et al., 2012; Waddell, 2013; Huetteroth et al., 2015; Yamagata et al., 2015). In general, reward-encoding DANs innervate compartments containing MBONs that promote avoidance, whereas punishment-encoding DANs innervate compartments containing MBONs that promote approach. Pairing an odor with a reward or punishment causes the odor-responsive KCs to be activated at the same time as the relevant DANs. The coincident activation of KCs and DANs in specific compartments typically induces synaptic depression of KC-MBON synapses within those compartments. Appetitive learning (associating odor with reward) reduces the activity of MBONs that promote avoidance, thereby skewing the network toward approach behavior (Owald and Waddell, 2015; Owald et al., 2015). Conversely, aversive learning (pairing odor with punishment, such as electric shock) reduces the activity of MBONs that promote approach, biasing the behavioral response towards avoidance (Sejourne et al., 2011; Aso et al., 2014b; Perisse et al., 2016; Felsenberg et al., 2018; McCurdy et al., 2021). Thus, memories are encoded by shifting the balance of the MB output network to bias the behavioral response and drive goal-directed behaviors (Owald and Waddell, 2015).

There are a number of studies and comprehensive reviews that discuss the synaptic and circuit mechanisms for encoding and retrieving memory (Zars, 2000; Heisenberg, 2003; Davis, 2011; Kahsai and Zars, 2011; Owald and Waddell, 2015; Cognigni et al., 2018; Boto et al., 2020; Modi et al., 2020). However, in a natural setting, associations between stimuli can quickly change, thus it is essential for animals to remain flexible and modify learned responses appropriately (Felsenberg, 2021). Here we will focus on how memories are modulated and updated, enabling behavioral flexibility beyond simple associative learning. In addition to the feedforward MB circuit organization described above, extensive feedback connections between MBONs and DANs also exist, both within and across MB compartments (Aso et al., 2014a; Li F. et al., 2020). Further anatomical work suggests that different MBONs are interconnected (Perisse et al., 2016; Felsenberg et al., 2018; Li F. et al., 2020; Scaplen et al., 2021). These lateral and recurrent connections provide a substrate to encode more complex relationships between stimuli, incorporate new information, update memories, and guide behavior.

Extinction

During associative learning, a stimulus acquires predictive value as it is associated with punishment or reward. When stimuli are no longer predictive, it is no longer adaptive to continue to avoid or approach the conditioned stimulus (CS+). In this case, the learned response is suppressed *via* extinction learning. Memory extinction refers to the decrease in the behavioral response to the CS+ when it is no longer paired with reward or punishment. A number of studies have described extinction learning in *Drosophila* in the context of aversive (Tempel et al., 1983; Tully and Quinn, 1985; Schwaerzel et al., 2002; Lagasse et al., 2009; Qin et al., 2012; Hirano et al., 2016; Felsenberg et al., 2018) and appetitive (Felsenberg et al., 2017; Wang et al., 2019) memories.

Early work suggested that extinction relies on distinct transcriptional and molecular signaling pathways from those that underlie initial associative learning (Qin and Dubnau, 2010; Hirano et al., 2016). Although it is widely accepted that extinguishing a behavioral response does not erase the original memory trace (Bouton, 2002; Eisenhardt and Menzel, 2007; Myers and Davis, 2007; Quirk and Mueller, 2008), until recently the circuitry mechanisms supporting this process remained unclear.

Felsenberg et al. (2017, 2018) describe a circuit basis for extinction learning in *Drosophila* (**Figures 6A,B**). They show that when a CS+ that was previously associated with punishment is no longer predictive, extinction learning forms a parallel, competing memory for the CS+ that engages the same circuitry used for appetitive learning. Inactivation of DANs involved in reward processing prevents the formation of this competing appetitive memory and extinction cannot occur (Felsenberg et al., 2018). However, a recent study showed that sugar reward learning and the extinction of aversive memory rely on different subsets of reward-encoding DANs that innervate the same compartment (Otto et al., 2020). An analogous circuit mechanism underlies the extinction of appetitive memories, in which an aversive CS+ memory competes with the original appetitive CS+ memory (Felsenberg et al., 2017, 2018). These studies also implicated feedback connections from MBONs to DANs in mediating extinction. A recent study tested a computational model of the *Drosophila* MB that includes separate reward and punishment pathways modulated by recurrence and mutual inhibition. This model reproduced the experimental results from Felsenberg et al. (2017, 2018) suggesting that these circuit motifs are important for mediating extinction (Springer and Nawrot, 2021).

A similar circuitry mechanism for extinction, also involving dopamine, has been described in rodents (Luo et al., 2018; Salinas-Hernandez et al., 2018). This suggests a general neural framework for extinction learning across species whereby the omission of a punishment is encoded as reward and the omission of reward is encoded as punishment. These newly formed CS+ memories compete with the original CS+ memory, resulting in the neutralization of a behavioral response.

The circadian system is a well-known regulator of learning and memory (Eckel-Mahan et al., 2008; Lyons and Roman, 2009; Gerstner and Yin, 2010; Le Glou et al., 2012; Smarr et al., 2014; Krzeptowski et al., 2018; Flyer-Adams et al., 2020; Inami et al., 2020) and also plays a role in the extinction of long-term memories (Pace-Schott et al., 2015; Sun et al., 2019; Zhang Y. et al., 2021). In *Drosophila*, a network of approximately 150 clock neurons orchestrates daily rhythms in physiology and behavior (Nitabach and Taghert, 2008). Recent evidence suggests that the activity of a subset of dorsal clock neurons is required for the extinction of long-term appetitive memories (Zhang Y. et al., 2021). Specifically, the inactivation of subsets of cryptochrome-positive dorsal neurons (DN1s) and downstream neurons expressing the peptide SIFamide disrupts extinction 24 h after initial learning. Further, artificial activation of DN1s potentiated extinction learning. Calcium imaging revealed that DN1 activity increased during extinction trials. DN1s promote sleep by acting on the circadian pacemaker cells (Guo et al., 2016), suggesting a connection between sleep, circadian rhythms, and extinction. It is not clear how clock neurons and their downstream targets interact with competing neural circuits within the MB, an important area for future study.

Reversal Learning

Reversal learning represents another example of behavioral flexibility in response to changes in expected contingencies (Jones and Mishkin, 1972). In a typical reversal learning paradigm, the animal first learns that stimulus A (CS+) predicts reward or punishment while stimulus B (CS-) does not. After learning, the stimulus-outcome contingencies are reversed: now stimulus B predicts the reward or punishment and stimulus A does not. Extinction may play a role in reversal learning as behavioral responses to the CS+ are diminished. However, reversal learning is more cognitively demanding because the reward or punishment is not merely absent but instead occurs with another stimulus that must now acquire predictive value (Izquierdo and Jentsch, 2012; Nilsson et al., 2015; Izquierdo et al., 2017). Thus, reversal learning is often considered the gold standard for assessing cognitive flexibility and, as such, is often disrupted in individuals suffering from neuropsychiatric disorders (Waltz and Gold, 2007; Murray et al., 2008; Leeson et al., 2009; Izquierdo and Jentsch, 2012; Gruner and Pittenger, 2017).

Early work in *Drosophila* highlighted the requirement of a GABAergic neuron that broadly innervates the MB for both olfactory and visual reversal learning, suggesting that it may inhibit the learned response in order to allow for new associations (Ren et al., 2012; Wu et al., 2012). The notion that reversal learning requires inhibition of the learned response has been discussed in the context of human behavior and mammalian models (Izquierdo and Jentsch, 2012). Other work in flies has highlighted the role and timing of dopamine activation in encoding memory and its reversal (Aso and Rubin, 2016; Berry et al., 2018; Handler et al., 2019).

How does reversal learning suppress the original memory? McCurdy et al. (2021) investigated this question using reversal learning in an aversive conditioning paradigm. During reversal trials when the original CS+ was no longer associated with electric shock, the activity of subsets of reward-encoding DANs increased, as also occurs during aversive extinction learning (Felsenberg et al., 2018; McCurdy et al., 2021). These DANs are activated through a recurrent circuit: the activity of punishment-encoding DANs is decreased when the expected shock is omitted, which increases the activity of a postsynaptic MBON that engages the reward-encoding DAN. Thus, a complex multi-compartment relay system is engaged to decrease CS+ avoidance and allow new associations to be learned.

Recent work has also highlighted the importance of nitric oxide, which is co-released with dopamine by DANs, in the rapid updating of memories (Aso et al., 2019). Nitric oxide acts antagonistically to dopamine to limit memory retention and enable the updating of memories in response to changing conditions. In optogenetic learning paradigms, impairing nitric oxide signaling led to longer memory retention and slower reversal learning. Nitric oxide acts in only a subset of MB compartments, enabling some MB pathways to form labile memories while other parallel MB pathways display more stable memory storage.

Reconsolidation

Considerable evidence suggests that the mere retrieval of a memory can cause the original memory trace to become labile and vulnerable to disruption or modification (Lee et al., 2017). As a consequence, a process called post-retrieval reconsolidation is

required in order to re-stabilize the memory trace. In a changing environment, reconsolidation likely provides an opportunity to incorporate new information and update a memory to maintain its relevance.

Similar to rodents (Nader et al., 2000), early work in flies demonstrated that aversive memory reconsolidation requires protein synthesis (Lagasse et al., 2009). After training, the ability to reactivate a memory was significantly reduced following treatment with protein synthesis inhibitors (Lagasse et al., 2009). More recent work identified the MB as a central brain region for reconsolidation (Felsenberg et al., 2017). In this study, appetitive learning was induced by pairing sugar with one odor (CS+) whereas another odor (CS-) was separately presented without reward, as a control. After learning, memory reactivation was triggered by presenting the CS-. Inactivation of specific DANs during CS- re-exposure disrupted reconsolidation, resulting in an impaired appetitive memory for the CS+. Interestingly, the MB compartments required for reconsolidation of appetitive memory are different from those involved in initial learning: in fact, reconsolidation engages circuits used for aversive learning. Imaging studies showed that the MBONs required for reconsolidation increased their response to the CS- after learning (Felsenberg et al., 2017). These data suggest a circuit framework in which a previously-stored appetitive memory is reactivated upon exposure to the CS-, which activates specific MBONs. These MBONs activate recurrently connected DANs, including reward-encoding DANs that reinforce the original appetitive association (**Figure 6C**). If this circuit is disrupted while the memory is still labile, the memory is lost.

Latent Learning

Thus far we have discussed how newly acquired information can modify memory-related circuits after learning to alter behavior. However, information acquired before associative conditioning can also modify subsequent learning. This can occur simply by exposure to an environment or stimulus in the absence of obvious reinforcement or motivation, known as latent learning. In one form of latent learning, called latent inhibition, pre-exposure to a stimulus diminishes its ability to acquire meaning at a later time (Lubow and Moore, 1959). Suppressing the ability to learn about irrelevant stimuli is likely advantageous as it allows an animal to focus its attention on relevant stimuli.

Latent inhibition has recently been reported for the first time in *Drosophila*. Jacob et al. (2021) showed that pre-exposure to the CS+ impairs the expression of subsequently learned appetitive memories. Interestingly pre-exposure to the CS+ did not impair the expression of subsequently learned aversive memories and in fact enhanced it. Pre-exposure to the CS+ creates an aversive memory that decays within several hours, which competes with newly formed appetitive memories but facilitates aversive memories. The aversive latent memory depends on punishment-encoding DANs and MB circuitry involved in long-term aversive learning (Aso and Rubin, 2016; Aso et al., 2019). Temporally controlled neuronal silencing experiments suggest that latent inhibition results from competing aversive and appetitive memory circuits interacting during memory retrieval, and not a disruption of memory acquisition. Thus, latent inhibition, like extinction, involves the formation of parallel memories for the same stimulus, which compete to drive behavioral choice. Competing memory traces are also thought to underlie these processes in mammals (Barad et al., 2004; Lingawi et al., 2017).

State-Dependent Flexibility in Learning and Memory

A fly's internal state, such as hunger or thirst, can have a profound impact on learning and memory (Krashes et al., 2009; Lin et al., 2014; Senapati et al., 2019). For example, flies need to be motivated by hunger in order to express a previously formed memory for sugar (Krashes and Waddell, 2008). This requires the hunger signal NPF and the expression of its receptor in subsets of DANs innervating the MB (Krashes et al., 2009).

More recent work revealed that the presence of food after learning an odor-sugar association determines whether or not sleep is required for the consolidation of long-term memory (Chouhan et al., 2021). When flies are starved after training, sleep is not required for memory consolidation. In contrast, when flies are fed after training, sleep is essential: sleep-deprived flies show significant reductions in the expression of sucrose memory 24 h after training. Whether sleep is required for consolidation was not dependent on caloric intake, but instead depends on the hunger signal NPF. Memory consolidation in starved flies that lacked either NPF or the NPF receptor was sleep-dependent. Strikingly, the consolidation of memories in these different contexts was mediated by distinct, non-overlapping MB neural circuits. Thus, experience and context can determine which neural circuits are required to consolidate the same memory.

DISCUSSION

In this review, we have highlighted examples of behavioral flexibility in *Drosophila* that are related to changes in internal or behavioral states, environmental context, or learning and memory. We note that there are many other examples not discussed here, such as behavioral flexibility in the context of social experience, circadian rhythms, and sleep (Allada and Chung, 2010; Kim et al., 2018; Beckwith and French, 2019; Bentzur et al., 2021; Li et al., 2021). We also have not discussed maladaptive forms of behavioral flexibility, such as addiction (Bainton et al., 2000; Devineni and Heberlein, 2009; Kaun et al., 2011; Landayan and Wolf, 2015; Lowenstein and Velazquez-Ulloa, 2018; May et al., 2019; Scaplen et al., 2020).

Nearly all forms of behavioral flexibility rely on the same principles: the state or context must be sensed and encoded, conveyed to relevant circuits, and must then modulate neural activity within those circuits to alter the behavioral output. Few examples exist where mechanisms for all of these processes have been identified. One of the most comprehensive examples identified is the circuit suppressing male sexual drive after copulation. Studies described above have identified the neurons sensing copulation and relaying this state to the brain (CRNs), the neurons that encode sexual arousal state (recurrently connected NPF/pCd neurons), how the activity of state-encoding neurons is regulated over long

TABLE 1 | Examples of behavioral flexibility described in this review.

Behavior	Modulator	Direction/type of change	Neuronal target of modulation	Modulators involved
Gain changes				
Vinegar attraction	Hunger	↑	Or42b- and Or85b-expressing OSNs	Insulin, sNPF, tachykinin
Food-seeking (yeast)	Hunger	↑	MB circuits	Dopamine, NPF, sNPF, serotonin, insulin, allatostatin A
Sugar attraction	Hunger	↑	Sugar-sensing neurons	Dopamine, NPF
Bitter aversion	Hunger	↓	Bitter-sensing neurons	AKH, sNPF, octopamine
Locomotor activity	Hunger	↑	Octopaminergic neurons	AKH, insulin
Aversion to high salt concentrations	Salt deprivation	↓	Downstream of ppk23^{glut} taste neurons	
Yeast consumption	Protein deprivation	↑	DA-WED cells; widespread changes in SEZ	Dopamine
Sugar consumption	Protein deprivation	↓	DA-WED cells	Dopamine
Yeast consumption (by females)	Mating	↑	Putative motor areas of SEZ	Sex peptide, octopamine
Salt consumption (by females)	Mating	↑		Sex peptide
Courtship of female (by males)	Long-term sexual arousal	↑	DANs, P1 neurons	NPF, dopamine
Visual pursuit of female (by males)	Short-term sexual arousal	↑	LC10a neurons	
Sexual receptivity (by females)	Mating	↓	pC1 neurons, vpoDNs	Sex peptide
Egg-laying (by females)	Mating	↑	pC1 neurons, oviDNs	Sex peptide
Sugar consumption (by starved flies)	Water deprivation	↓	ISNs	AKH
Motion sensing*	Walking, flying	↑	HS and VS cells	Octopamine
Feeding initiation	Walking	↓	Mechanosensory interneurons	
Walking	Extended proboscis state	↓		
Carbon dioxide avoidance	Vinegar	↓	DANs, glutamatergic MBONs	Dopamine
Sugar attraction	Yeast odor	↑	Downstream of Or35a-expressing OSNs	
Sugar attraction	Mechanosensation	↑	Downstream of hair plate mechanosensory neurons	
Sugar attraction	Cool temperatures	↓	Downstream of bitter-sensing and mechanosensing neurons	
Learned odor response	Reconsolidation (re-exposure to CS-)	↓ if not properly reconsolidated	Recurrent MB circuits	Dopamine
Learned odor response	Extinction (re-exposure to CS+ alone)	↓	Recurrent MB circuits	Dopamine
Expression of memory for odor-sugar association	Latent inhibition (pre-exposure to CS+)	↓	MB circuit	Dopamine
Expression of memory for odor-sugar association	Hunger	↑	DANs innervating MB	Dopamine, NPF
Learned odor association with sugar (in flies starved after training)	Sleep	↑	MB circuit	Dopamine, NPF

(Continued)

TABLE 1 | Continued

Behavior	Modulator	Direction/type of change	Neuronal target of modulation	Modulators involved
Tuning changes				
Temperature preference	Hunger	Shift to lower temperatures	AC cells	
Salt preference	Mating (in females)	Shift to higher concentrations		
Horizontal motion-sensing*	Walking	Shift to higher frequencies	HS cells	
Vertical motion-sensing*	Flying	Tuning broadened toward higher frequencies	VS cells	
Switch in behavior				
Acetic acid taste response	Hunger	Switch from aversion to attraction	Downstream of sugar- and bitter-sensing neurons	
Choice between feeding and mating (in males)	Hunger	Choice switches from courtship to feeding	$TyrR^{PLP}$ neurons, P1 neurons	Tyramine
Preference for light	Wing clipping or gluing	Switch from attraction to aversion		Dopamine, Octopamine
Response to looming stimulus	Fast walking	More likely to flee than freeze	Downstream of DNp09 neurons	
Response to looming stimulus	Flying	Switch from escape to landing	Upstream of DNp07 and DNp10	Octopamine
Carbon dioxide	Fast walking, flying	Switch from avoidance to approach		Octopamine
Takeoff response to looming stimulus	Context (looming speed)	Switch from long to short takeoff mode	LC4 and LPLC2 visual neurons, giant fiber neurons	
Steering response toward aversive wind and attractive visual cue	Context (presence of both cues together)	Switch from either aversion/attraction to turning sequence		
Small visual object	Attractive odor	Switch from avoidance to attraction	Motion-sensitive visual pathway	Octopamine
Odor approach or avoidance	Associative learning	Induce approach or avoidance	KC-MBON synapses in MB	Dopamine
Learned odor response	Reversal learning	Switch response to CS+ vs. CS-	Recurrent MB circuits	Dopamine

**These are not technically examples of behavioral modulation, but modulations of sensory processing that may relate to perception. OSNs, olfactory sensory neurons; sNPF, short neuropeptide F; NPF, neuropeptide F; AKH, adipokinetic hormone; MB, mushroom body; DANs, dopaminergic neurons; MBONs, MB output neurons; AC, anterior cells. Up or down arrows represent an increase or decrease in the behavior, respectively.*

timescales (CREB2 modulation of neuronal excitability), and how these neurons modulate circuits for behavior (dopaminergic modulation of P1 neurons). In the future, we expect to see more examples detailing comprehensive circuit mechanisms underlying behavioral flexibility.

Studies of behavioral flexibility in *Drosophila* reveal another general principle: circuits can be modulated at different levels, from sensory responses to motor pathways. Why might modulation at a specific level be advantageous? Modulating sensory responses early in the circuit, such as the sensory neurons themselves, would result in global modulation of all behaviors elicited by that cue. This could be advantageous if it is critical for an animal to perceive a cue as more or less salient, such as hunger-dependent enhancement of sugar sensitivity, but it does not allow for control over which behaviors are modulated. In contrast, modulating motor or pre-motor pathways would enable an internal state to gate the activation of specific behaviors or motor programs. Some forms of behavioral state-dependent modulation occur downstream of descending neurons and are likely to represent modulation of motor pathways. State-dependent modulation can also act in between sensory and motor pathways to modulate the sensorimotor transformation. Examples of this include the arousal-dependent gating of courtship pursuit and flight-dependent responses to looming stimuli.

The examples of behavioral flexibility that we have described primarily fall under three categories: (1) gain changes modulating the strength of a behavior, (2) tuning changes modulating the preferred range of stimuli that produce a response, and (3) a behavioral switch that changes the response entirely (**Table 1**). Shared principles may underlie these different categories of behavioral flexibility. For example, a behavioral switch can result from gain changes when there are two competing pathways that promote different behaviors (**Figure 7**). Enhancing or suppressing the output of either pathway beyond a certain threshold can shift their balance and thereby elicit a switch in the behavioral response. This mechanism underlies the hunger-dependent switch in the acetic acid response, the behavioral state-dependent responses to looming stimuli, and many of the learning-dependent changes that rely on MB output.

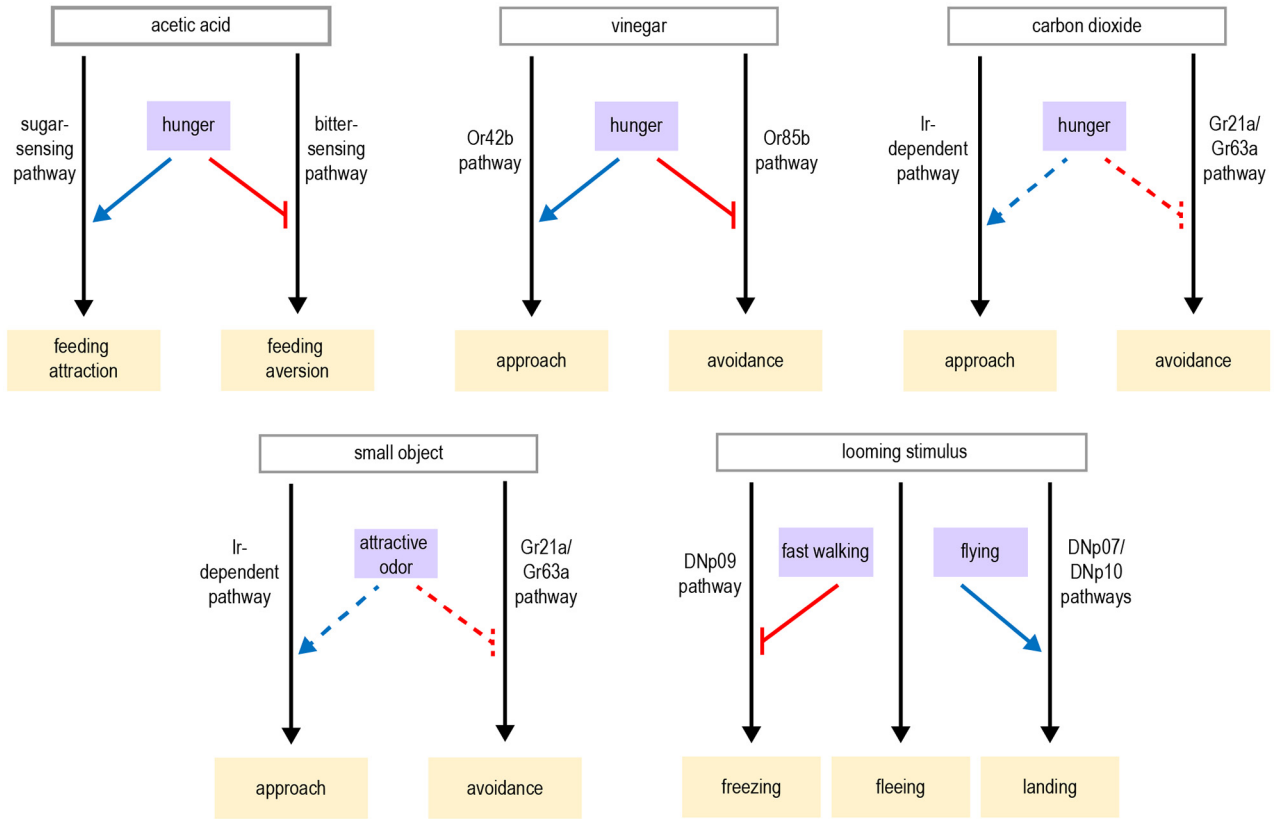

FIGURE 7 | Examples in which the same stimulus can elicit different behavioral responses through competing pathways that are gated by state or context. Black arrows depict neural circuits generating behavior; blue arrows depict excitatory modulation whereas red arrows depict inhibitory modulation. Dashed lines indicate putative types of modulation that have not been experimentally confirmed. For example, it is not known whether fast walking switches the behavioral response to carbon dioxide by enhancing the pathway promoting approach or suppressing the pathway for avoidance.

Moreover, examples of behavioral flexibility that we currently describe as gain changes may actually represent a behavioral switch if a broader set of behaviors were monitored. For instance, the suppression of PER during walking, or the suppression of courtship after males have recently copulated, likely allows for other behaviors to be expressed instead. It is important for an animal to adequately prioritize mutually exclusive goals. Recent studies investigating the behavioral competition, such as choosing between feeding and mating, highlight the interactions between different behaviors and their underlying circuits. As the field moves toward studying broader repertoires of behavior in more naturalistic paradigms, more of these interactions will likely emerge.

Overall, *Drosophila* has proven to be an excellent model system for dissecting the neural circuits underlying behavioral flexibility. Astounding progress has been made over the last decade in characterizing the synaptic wiring diagram of the fly nervous system and developing increasingly sophisticated genetic tools for circuit analysis. In parallel, methods for behavioral analysis and quantification have drastically improved (Pereira et al., 2020). These tools, combined with our ever-improving estimation of the fly's behavioral complexity, open the door to obtaining a comprehensive understanding of the molecular and neural circuit mechanisms underlying behavioral flexibility. This knowledge may in turn inform our understanding of the circuit mechanisms underlying behavioral inflexibility that occurs in psychiatric disorders (Barker et al., 2015; Gruner and Pittenger, 2017; Volkow et al., 2017).

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Response Systems, Antagonistic Responses, and the Behavioral Repertoire

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While response systems are often mentioned in the behavioral and physiological literature, an explicit discussion of what response systems are is lacking. Here we argue that response systems can be understood as an interaction between anatomically constrained behavioral topographies occasioned by currently present stimuli and a history of reinforcement. "New" response systems can develop during the lifetime as the organism gains instrumental control of new fine-grained topographies. Within this framework, antagonistic responses compete within each response system based on environmental stimulation, and competition is resolved at the striatum-thalamo-cortical loops level. While response systems can be by definition independent from one another, separate systems are often recruited at the same time to engage in complex responses, which themselves may be selected by reinforcement as functional units.

Keywords: response systems, behavioral repertoire, response competition, anatomical constraints, basal ganglia

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THE REPERTOIRE: SINGLE, INDEPENDENT, AND COORDINATED RESPONSE SYSTEMS

Instrumental conditioning provides a thoroughgoing account of how complex environment-behavior relations develop, and may be useful to neuroscientists as a framework for understanding the neurophysiological processes and mechanisms involved in the development of the behavioral repertoire (e.g., Donahoe et al., 1993). Comparing the behavior of an adult human to that of an infant or child makes clear the role of ontogenetic selection in the development of response systems. The human infant is capable at first of only gross motor responses. These "primordial" responses are differentiated over time into successively finer-grained responses, eventually permitting the full range of human conduct, or repertoire, from catching a ball, speaking in sentences, to driving a car—or any other behavioral topography available to the adult human.

But what is the behavioral repertoire in the first place? The repertoire is a construct referring to the behavior an organism is capable of as a result of both its phylogenetic and ontogenetic histories. Under certain conditions, one response is more probable than others due to a particular history of behaving under similar conditions (e.g., Catania, 2013). Over time, the experienced organism becomes capable of engaging in a wide-range of behavior under an equally wide-range of environmental conditions. As such, this large number of possible behavioral responses must

be coordinated to avoid the occurrence of chaotic behavior. To understand how this takes place, a neurobehavioral conceptualization of response systems may be of pragmatic value.[1] Our take on response systems is a preliminary effort focusing mostly on environmental cues in relation to responses and response systems, and not on the vital role of reinforcing stimuli in organizing response systems according to ethologically relevant categories (e.g., Graziano, 2006). Moreover, the current paper does not extensively address the complexities involved in rapid behavioral changes due to shifting contingencies; a relevant issue when an outcome following a specific action changes in value and the remote and recent history of learning interact.

A response system may be considered an anatomically constrained and defined subset of environment-behavior relations. Environment-behavior relations in turn are conceptualized here as cues increasing or decreasing the probability of certain behavior, based on previous instances of reinforcement and/or punishment when that behavior occurred in presence of those stimuli (for a review, see Dinsmoor, 1995a,b). A microwave oven emits a beeping sound, a person approaches the oven, opens its door, and retrieves a meal. But what about instances in which multiple activity patterns simultaneously occur? For instance, a skilled individual may be able to ride a bike, play guitar and sing at the same time. How does this take place?

Before tackling the complexities involved in multiple independent and coordinated response systems, let us then start from the assumption that within an individual response system, only one response occurs at any given time. Along those lines, Palmer (2009) mentions the following:

In any one response system, most responses are mutually incompatible. We can walk to the left of a tree or to the right of the tree, but we can't do both. We can say, "Hello," or "Hi there," but not both, at least not at the same time. Response competition is an important concept. If we tried to engage in every response that had some strength, our behavior would be chaotic. If two incompatible responses are roughly equipotent, only one will actually be emitted. The competing response appears to be inhibited (p. 56).

According to this conceptualization, within each response system, the strength of a response varies in a moment-to-moment fashion as a function of current environmental stimulation and an organism's learning history. Considering the multitude of behaviorally relevant stimuli encountered by an organism at any given moment, an important question is raised: What prevents multiple responses from occurring at the same time and potentially resulting in a maladaptive response topography? Let us consider Palmer's example of a person trying to go both left and right at the same time. Palmer suggests that at any given moment, the probability of emission for each response in the behavioral repertoire is influenced by current environmental stimulation: "shifts in stimulus control can favor the target response so that it becomes the dominant response in its response system" (p. 51). In other articles, behavioral scientists describe how current environmental stimulation potentiates a variety of responses within a given response system (e.g., Michael et al., 2011). For example, the production of the sounds making up vocal responses involves the coordination of a response system of facial and vocal tract muscles, as well as muscles belonging to the diaphragm and other components of the respiratory system. Given the anatomical organization of the human vocal apparatus as it is supported by thalamo-cortico-striatal circuitry, humans seem able to engage in a single vocal response at any given time (see **Figure 1**). Michael et al. (2011) elucidate this phenomenon:

When we see a dog, we cannot simultaneously say, dog, King, brown, Chihuahua, etc. [.]All behavior within a response system can be thought of as in competition with other behavior in that response system. Thus, many verbal responses may be relatively strong at a particular moment, but only one can be emitted at a time. Presumably one response, the prepotent response, is stronger because of its conditioning history, or perhaps because of the confluence of other evocative variables at the moment (p. 7).

Current stimulation, therefore, changes the probability of occurrence of many responses, as evidenced by a large number of priming experiments (e.g., Neely, 1991; for a behavior analytic interpretation see Palmer, 2009; Ortu, 2012). In a priming experiment, a priming stimulus is presented (for instance, the word "coffee") and is either followed by a "related" target stimulus (e.g., "milk") or an "unrelated" target stimulus (e.g., dog). In these experiments, carried out typically with a large number of trials, participants are faster in emitting a textual response after the "related" target compared to the unrelated target. The implication of these studies is that the presentation of the priming stimulus changes the response strength, or probability of emission, of a large number of potentially antagonistic responses.

The notion of response antagonism, or competition, might be expanded to account for multiple response systems (see **Figure 2**). For instance, if current textual stimulation (e.g., Starbucks, Latte, Cappuccino) increases the strength of the vocal response *coffee*, leading to the emission of a vocal response "coffee," emission of the vocal response should not prevent the organism from engaging concurrently in behavior involving another response system, for example, walking[2] (Palmer, 2009; for an alternative interpretation based on the spreading activation see also Klinger et al., 2000). Anatomically, the vocal apparatus and the locomotive apparatus appear relatively independent. Anatomy, then, imposes the

[1] The organization of behavior provides a framework to analyze neurophysiological events by defining the boundaries within which relevant physiological mechanisms operate (Donahoe and Palmer, 1994; Krakauer et al., 2017).

[2] In subvocal speech, a person is typically not able to engage in multiple concurrent vocal streams: It appears that the constraints typical of the vocal apparatus are maintained in subvocal speech. When the vocal response becomes subvocal through ontogenic development, what prevents individuals from learning to engage in multiple parallel vocal streams? The subvocal, or covert, version of the performance of interest also appears to be constrained by anatomical factors even when considering other behaviors. For instance, a guitar player who is covertly rehearsing a scale will not do so by using an arbitrary number of fingers and arms. Rather, the anatomical constraints that were present during learning still play a role in future covert practices. Similarly, when a person loses a limb or loses the ability to control some or all muscles due to paralysis or amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), they still appear to be able to retain covert speech and other covert topographies.

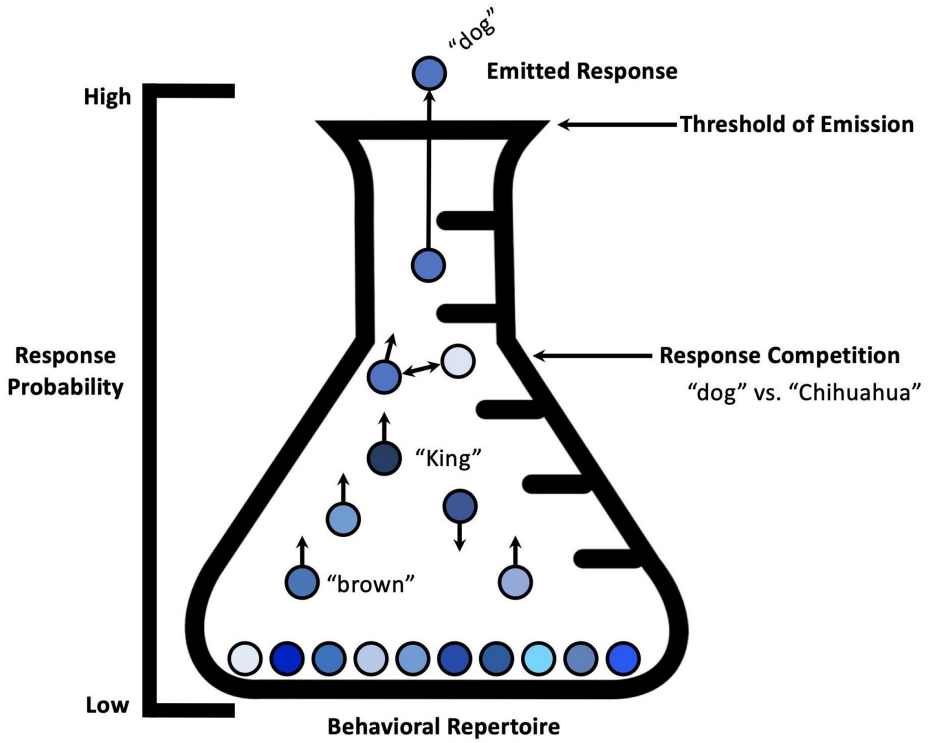

FIGURE 1 | Emission of a single response belonging to a single response system. A visual metaphor showing the emission of a single response, and the relative changes in response strength of responses within the same response system. Specific environmental conditions occasion a specific behavioral response, despite multiple members of the response class being suitable for a given situation. Response competition is characterized by bidirectional arrows between the particular responses within the response system. Adapted from "Response strength and the concept of the repertoire," by Palmer (2009).

boundaries within which ontogenetic development occurs; thus, response system organization develops from the interaction of anatomical constraints and learning variables (e.g., reinforcement learning/selection by consequences). As another example, given the anatomical organization of human arms and hands, multiple responses can be emitted concurrently; and within each hand, each finger can potentially respond independently from the others, provided the organism has learned to do so. Additionally, consider the hypothetical example of an individual born with six fingers on each hand learning how to play the piano. This person would have access to *topographic degrees of freedom* unavailable to a person born with five fingers on each hand. If we conceptualize each finger's movement as a new potentially independent response system, the addition of the sixth finger may add a new response system—a new "partition" within the overall repertoire.

Consider another example from the musical domain: Great drummers are known for acquiring complete limb independence. While reading a score or improvising, the responses of each limb are effectively independent of the others. This can be probably demonstrated best in an instance of sight-reading with a score the drummer has not seen before. Ultimately, however, anatomy never ceases to provide constraints. Even when full limb independence is gained, a drummer with especially long arms or legs may be able to execute parts unachievable to a drummer with shorter limbs, and vice-versa. This is also relatively evident in sports: A very tall basketball player may have access to topographies unavailable to a relatively short player, and vice-versa. Going back to the priming phenomenon, given that organisms are constantly "bombarded" by behaviorally relevant stimuli, and that the same stimulus may divergently affect *different* response systems, a further implication is suggested: At any given moment there may be as many instances of response competition as there are response systems.

As a corollary, if anatomical constraints are (at least in part) what is driving the separation across response systems, it is possible in principle that if those anatomical constraints were eliminated, then the organism—given the appropriate training—could learn to engage in a number of concurrent responses that would in principle be constrained only by the computational capabilities of the brain. Brain-Machine Interface (BMI) research has recently highlighted how monkeys with implanted electrodes in their brain cortex can learn to control additional robotic limbs (Carmena et al., 2003). Analogously, from a stimulus perspective, rats have been shown to be able to respond to changes in infrared light when an external sensor is connected to their brain cortex (Thomson et al., 2013), while concurrently discriminating other changes in the visible light spectrum. The same neural populations appear able to simultaneously "learn"

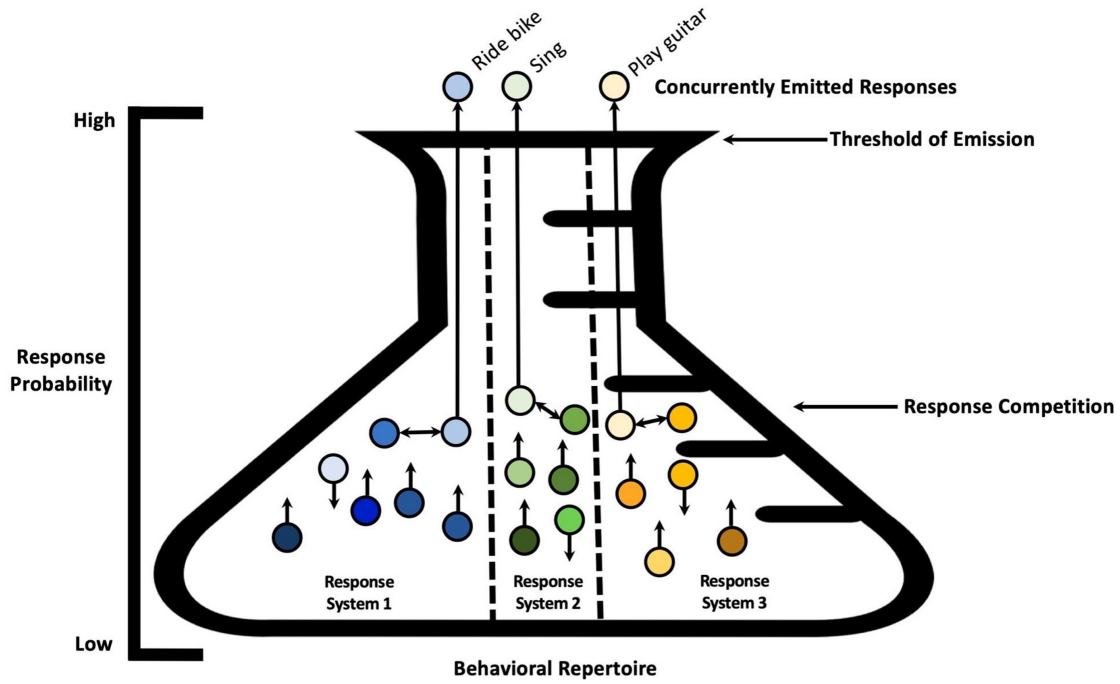

FIGURE 2 | Emission of three responses at a given time belonging to three separate response systems. A visual metaphor showing three response systems within the behavioral repertoire, and the simultaneous emission of three behavioral responses belonging to three separate response systems. Antagonistic responses within each response system are noted with bidirectional arrows between the responses. Since these responses belong to separate response systems, the individual can learn to emit them at the same time, as there are no anatomical limitations preventing them.

how to respond to multiple sources of stimulation, incoming from both biological and electronic receptors.

Given the possibility of n-number of additional non-biological interfaces to operate on the environment, and n-number of additional receptive mechanisms to detect incoming stimulation, the behaving capabilities of organisms appear potentially expandable beyond the current limitations dictated by the anatomical constraints due to natural selection. Organisms are at birth equipped with response systems that were congenial at points in time that may not reflect current environments. The possibility of adding response systems (e.g., robotic arms) and stimulus systems (e.g., robotic eyes) may greatly expand the behaving potential of organisms. Moreover, both response and stimulus systems need not be physically connected or located in the same environment as the biological organism. Response and stimulus systems can potentially be located in additional environments, with the biological organism constituting the physical locus, or hub, in which the actual, mechanistic selection of environment-behavior selection takes place at the neural level.

Whether or not response and stimulus systems are biological, we must consider both the potential gain of independence among response systems, as well as coordination among the partitions. When a person must jump suddenly to the right to avoid a ball thrown at him/her, an ensemble of potentially independent response systems is recruited at the same time, including leg muscles, postural muscles in the back, neck muscles, and others. How does this concurrent recruitment of potentially independent response systems occur, and happen so quickly? Is the coordinated response selected as a single functional unit, occasioned by specific stimulus conditions that differ from the ones that evoke responses from individual response systems? This leads us to another pressing concern: If these coordinated efforts involving multiple response systems are selected as units, i.e., a complex response unit,[3] is it the case that other, incompatible, complex response units are inhibited in the same way as they are within an individual response system when a dominant response has acquired critical strength? The behavioral account may be informed by the current understanding of the neural mechanisms related to these changes in probability and subsequent emission of

[3]Response complexity refers to both the diachronous emission of responses making up a particular motor program (see Jin et al., 2014 for a discussion of response "chunking"), as well as the synchronous emission of multiple responses (e.g., such as when a piano player learns to play one chord with the left hand and another with the right at the same time) unified into a single behavioral response pattern. Added to this consideration, responses of differing complexity may be evoked by stimuli that also vary in complexity. An individual relatively simple stimulus (e.g., a square) can be embedded within a simple context (e.g., a white background); a complex stimulus (e.g., a human face) can be embedded in a simple context or a complex context (e.g., a specific configuration of contextual stimuli). Stimuli—both objects and contexts—can also vary in complexity over time (e.g., a stimulus may morph from simple to complex within 5 s and at the same time the context can shift from complex to simple), and responses can potentially track these stimulus changes.

a particular response over others. Evidence at the thalamo-cortical-striatal level in particular may illuminate behavioral descriptions, especially "winner-takes-all" models of basal ganglia function.

NEURAL MECHANISMS SUPPORTING RESPONSE COMPETITION AND EMISSION ACROSS RESPONSE SYSTEMS

A review of the neuroanatomical literature, and especially of the topographically organized loops at the thalamo-cortical-striatal level, suggests a possible boundary-bridging convergence of the behavioral and neurological levels of analysis (e.g., Abrahamsen, 1987; Ortu and Vaidya, 2016). For instance, the continual restructuring of response systems at the behavioral level parallels selectionist accounts of the nervous system and the formation of cortical maps (Edelman, 1993). While the central nervous system facilitates the coordination of various effector systems, behavioral selection provides an ultimate cause for the organization of response systems making up the repertoire (Donahoe and Palmer, 1994).

From a response-outcome perspective, experiments have shown that the posterior dorsomedial striatum (pDMS) is involved in supporting rapid learning due to shifting environmental contingencies caused by changes in outcome value (Balleine and O'doherty, 2010; Shiflett et al., 2010). More specifically, the parafascicular thalamic nucleus (PF) is connected to the pDSM and contributes (via cholinergic interneurons) to selecting responses when a remote learning history "conflicts" with recent changes in outcome value (Bradfield et al., 2013).

From a stimulus-response perspective, the basal ganglia, the motor cortices, and the thalamus are involved in a neural loop critical in inhibiting competing responses once a dominant response has reached a critical point of "no return" (e.g., Middleton and Strick, 2000; Silkis, 2000, 2001; Haber and Mcfarland, 2001; McHaffie et al., 2005; Hayhow et al., 2013; Oldenburg and Sabatini, 2015). More specifically, the literature describes two co-active and interactive pathways—the direct and indirect—generally considered to be involved in response inhibition and selection (Hong and Hikosaka, 2011; Tecuapetla et al., 2016).

The thalamus plays a central role in these pathways due to its polymodal inputs from the sensory cortices and outputs to motor cortices. Further, it receives inhibitory GABAergic inputs from two striatal areas within the basal ganglia: the internal segment of the globus pallidus and the substantia nigra pars reticulata[4] (e.g., Hikosaka, 2007, see also McElvain et al., 2021). This interaction suppresses downstream initiation of movement by reducing overall thalamic activity (Plenz, 2003). Additionally, the striatum receives excitatory inputs from the cortex, resulting in the release of glutamate, and the subsequent activation of GABAergic neurons in the striatum. This release of GABA inhibits activity in the striatum, thus disinhibiting thalamic neurons and allowing the occurrence of a motor response (Chevalier and Deniau, 1990).

The indirect pathway has an equal role in inhibiting and disinhibiting motor responses. Within the basal ganglia, GABAergic neurons project from the globus pallidus and inhibit the neurons of the subthalamic nucleus, modulating striatal activity and broadly altering the interactions of the direct pathway. The indirect pathway is also at times activated by projections from the cortex inhibiting neurons in the globus pallidus. As a result, the activity of subthalamic neurons increases, and thus the inhibitory effect of the basal ganglia also increases resulting from the release of GABA. More recent research has proposed an additional "hyperdirect" pathway from cortical areas to the subthalamic nucleus (Nambu et al., 2000, 2002; Milardi et al., 2019; Chen et al., 2020). These mechanisms ultimately support the emission of responses via activation of neurons in motor areas[5].

Feedforward relations between premotor and supplementary motor areas eventually activate neurons in primary areas that are individually tuned in a very specific way to many motor features, similar to sensory-perceptual neurons in the IT cortex and their fine-tuned sensitivity to a large number of stimulus features (Desimone et al., 1984). Moreover, the classic idea that neurons in the primary motor cortex are organized in a one-to-one mapping with muscle effectors has been recently challenged by a more complex perspective involving the concept of many-to-many mapping. The many-to-many theory (e.g., Graziano, 2006; Huber et al., 2020) posits that individual neurons in primary motor areas can affect many different muscle effectors and that each effector can be controlled by many neurons in motor areas. From a response system perspective, this complexity allows for the dynamic and plastic recruitment of populations of neurons based on ethologically and behaviorally relevant categories.[6] These categories are not fixed but are repeatedly updated based on the feedforward and feedback interactions of dopaminergic signals from the ventral tegmental area and the substantia nigra to the dorsolateral striatum (e.g., Graybiel, 1990).

In conclusion, activation of effectors involved in a response system may then require the interaction between the basal ganglia and motor areas in concurrently inhibiting populations of neurons involved in competing responses and disinhibiting populations of neurons involved in the dominant response, based on current stimulation. Given the many-to-many mapping between neurons in motor areas and effectors, this population coding (Georgopoulos et al., 1986) may involve overlapping sets of neurons in motor areas in dominant and competing responses within a response system. When anatomical constraints are absent, multiple movements may occur concurrently due to

[4]The SNr and GPi also send GABAergic projections to a variety of other nuclei including the Superior Colliculus and the Pedunculupontine Nucleus.

[5]It is important to acknowledge that research on neural mechanisms involved in response competition is still partially speculative and based on physiologically informed computational models (e.g., Baston and Ursino, 2015).

[6]Ethologically and behaviorally relevant stimulus categories are coded by different subpopulations of the amygdaloid complex (e.g., Pignatelli and Beyeler, 2019).

the co-activation of multiple response systems. Under these conditions, the basal ganglia-motor cortex interaction may allow disinhibition—based on previous learning—of many populations of neurons coding for non-conflicting responses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DO wrote a first preliminary draft of the manuscript. RB contributed to all contents and sections in a deep and fundamental way. Both authors contributed to the article and approved the submitted version.

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REVIEW
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A Role for Serotonin in Modulating Opposing Drive and Brake Circuits of Impulsivity

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Impulsivity generally refers to a deficit in inhibition, with a focus on understanding the neural circuits which constitute the "brake" on actions and gratification. It is likely that increased impulsivity can arise not only from reduced inhibition, but also from a heightened or exaggerated excitatory "drive." For example, an action which has more vigor, or is fueled by either increased incentive salience or a stronger action-outcome association, may be harder to inhibit. From this perspective, this review focuses on impulse control as a competition over behavioral output between an initially learned response-reward outcome association, and a subsequently acquired opposing inhibitory association. Our goal is to present a synthesis of research from humans and animal models that supports this dual-systems approach to understanding the behavioral and neural substrates that contribute to impulsivity, with a focus on the neuromodulatory role of serotonin. We review evidence for the role of serotonin signaling in mediating the balance of the "drive" and "brake" circuits. Additionally, we consider parallels of these competing instrumental systems in impulsivity within classical conditioning processes (e.g., extinction) in order to point us to potential behavioral and neural mechanisms that may modulate the competing instrumental associations. Finally, we consider how the balance of these competing associations might contribute to, or be extracted from, our experimental assessments of impulsivity. A careful understanding of the underlying behavioral and circuit level contributions to impulsivity is important for understanding the pathogenesis of increased impulsivity present in a number of psychiatric disorders. Pathological levels of impulsivity in such disorders are likely subserved by deficits in the balance of motivational and inhibitory processes.

Keywords: serotonin, impulsivity, reward, inhibition, learning

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INTRODUCTION

Impulsivity is generally conceived of as a deficit in inhibitory control, resulting in unwanted actions. However, impulsive behavior has many diverse presentations and complex neurobiological underpinnings (Dalley and Robbins, 2017; Strickland and Johnson, 2021). Many lines of work have fractionated impulsivity into a number of different subtypes and components, with dissociable

biological bases (Winstanley et al., 2004; Robbins et al., 2012; Bari and Robbins, 2013; MacKillop et al., 2016; Dalley and Robbins, 2017; Nautiyal et al., 2017; Bailey et al., 2021). Impulsive choice is described as risky decision making, including discounting of delayed rewards. Alternatively, impulsive action is characterized by acting prematurely and/or the decreased ability to stop or withhold responding. In this review, we focus on the action component of impulsivity, exploring the idea that impulse control can be broadly described as competing circuits. One "drive" circuit encodes an initially learned response-reward outcome association, and the second "brake" circuit subserves an opposing and subsequently learned inhibitory association. The sum of the outputs of these circuits shapes the action plan determining whether to go or inhibit going. In particular, we highlight the role of serotonin signaling in modulating these oppositional circuits in the control of impulsive action.

Dysfunction in different nodes of these "drive" and "brake" neural circuits could result in the heterogeneity of phenotypic presentations of impulsivity. Therefore, careful dissection of the underlying behavioral and circuit level contributions to impulsivity is important for understanding the pathogenesis of increased impulsivity. This idea is highlighted in the dual systems and imbalance models of adolescent impulsivity which consider disproportionate development and changes in communication for brain areas involved in reward/motivation and inhibitory control (Somerville et al., 2010; Steinberg, 2010; Casey et al., 2011; Ellingson et al., 2013). Considering imbalance models in the context of preclinical studies aimed at understanding adult impulsivity could help elucidate different entry points to dysfunctional circuits responsible for pathological impulsivity.

This dual-systems perspective is also relevant to clinical populations with disorders in which impulsivity is dysregulated. For example, attention deficit hyperactivity disorder (ADHD) is characterized by inhibitory deficits, including increased impulsive action (Schachar and Logan, 1990; Nigg, 2001; Wright et al., 2014; Grandjean et al., 2021). Impulsivity is also a key phenotype found in substance use disorder, in which both reward system and inhibitory dysfunctions are present (Jentsch et al., 2014; Weafer et al., 2014). From the perspective of reward sensitivity, genetic risk for alcoholism is associated with increased sensitivity to sweet substances (Kampov-Polevoy et al., 2001, 2003). Poor inhibitory control is associated with sensitivity to amphetamines (Weafer and De Wit, 2013; Weafer et al., 2017) and chronic cocaine use (Fillmore and Rush, 2002). Increased impulsive action likely reduces the ability to withhold actions to obtain or consume drugs, though it is difficult to parse out the cause versus effect, as is common generally when studying psychiatric disorders. However, it is clear that impulsivity is both a predisposing factor and a result of drug use. Several studies which supports a role for impulsivity as a causal factor shows that subjects with familial history of drug dependence have higher impulsivity across many domains, including impulsive action (Acheson et al., 2011; Ersche et al., 2012; Kumar et al., 2018). Additionally, increased impulsivity and altered reward sensitivity are also found in gambling disorder (Sztainert et al., 2013; Hodgins and Holub, 2015; Wardell et al., 2015; Jiménez-Murcia et al., 2017; Ioannidis et al., 2019; Mestre-Bach et al., 2020), which, as a behavioral addiction, is free from the confound of pharmacological effects on these phenotypes. Indeed, Brevers et al. (2012) found that the severity of problem gambling was predicted by performance on a stop-signal test of impulsive action.

Assuming the presence of competing drive and brake processes in impulsivity, we can examine the behavioral/cognitive components and the underlying neural mechanisms of each of these components. This sets up the possibility to arrive at the endpoint of increased impulsive behavior via a number of different paths and combinations of intermediate phenotypes (**Figure 1**). For example, in a behavioral assay of impulsive action, increased maladaptive actions could arise from a stronger action-outcome association, an increased motivation or valuation of reward, a failure to learn the opposing behavioral response (inhibition), or even a failure to express the inhibition, despite it having been learned. Understanding which components contribute to impulsive phenotypes, can lead toward developing novel, specific treatments targeting dysfunction of neural circuitry more precisely.

Serotonin (5-HT) has been strongly implicated in encoding reward *and* mediating behavioral inhibition, and is poised to modulate the balance of reward-based approach and adaptive inhibition of action. Manipulation of serotonin neuron activity in preclinical models clearly show that serotonin is involved in waiting and inhibiting behavioral responses (Wogar et al., 1992; Fletcher, 1995; Jolly et al., 1999; Winstanley et al., 2004; Fonseca et al., 2015; Miyazaki et al., 2018, 2020). Studies using *in vivo* monitoring, through single-unit electrophysiology and photometric calcium monitoring, in the dorsal raphe also implicate serotonin neurons in encoding both rewards and associated cues (Cohen et al., 2015; Li et al., 2016; Zhong et al., 2017; Ren et al., 2019). A large number of studies have also investigated the role of serotonin signaling—through many of its 14 receptors—in both reward-related behaviors and behavioral inhibition. Though many have used pharmacological approaches with systemically administered drugs, some studies have targeted brain region specificity with local drug administration and cell- and circuit-specificity using genetic models (**Table 1**). Given that serotonin, as a neuromodulator, tunes synaptic signaling and guides plasticity to alter learning and motivated behaviors, it is relevant to explore the idea that serotonin acts at the convergence of the neural circuits governing "drive" and "brake" processes in impulsivity.

The first goal of this review is to synthesize studies, especially in preclinical animal models, that parse excitatory and inhibitory behavioral substrates that contribute to impulsive action. Next, we review potential circuit level mechanisms that underlie the interaction of these opposing learned associations in the generation of impulsivity. We focus on serotoninergic modulation of the underlying neural circuitry of both reward processing and inhibitory control, and also potentially in determining the balance of these competing systems to generate the output impulsive behavior. Finally, we discuss future research questions which examine the relative contributions of initial response-reward associations and subsequently learned

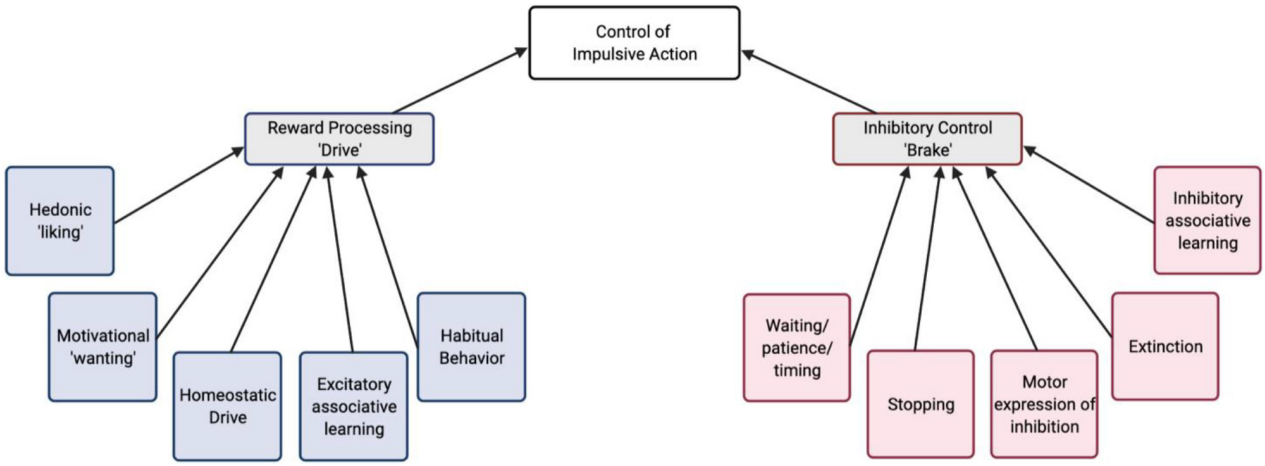

FIGURE 1 | A conceptual schematic of behavioral/cognitive processes that contribute to the control of impulsive action. These are organized into reward "drive" and inhibitory "brake" processes.

TABLE 1 | Effects of serotonin receptors on reward-related and impulsive action behavior from preclinical pharmacology and genetic mouse models.

Receptor	Behavioral effects	Reward	Behavioral inhibition: impulsive action
5-HT_{1A}	Modulates anxiety, depression, and the antidepressant response to SSRIs	Activation enhances sensitivity to reward[1–3]	Agonists increase impulsive action likely through inhibition of raphe/serotonin signaling[4–6]
5-HT_{1B}	Influences impulsive aggression and modulates social and drug reward	Activation reduces incentive motivation and knockout increases reward motivation[6–10]	Knockout increases impulsive action[8,11]
5-HT_{2A}	Pro-hallucinogenic, necessary for psychedelic effects	Activation decreases incentive motivation[12,13]	Activation increases impulsive action, antagonists reduce impulsivity[14–17]
5-HT_{2B}	Impulsivity, cognition, and anxiety	Knockout/blockade reduces reward sensitivity, and activation is required for some rewarding effects[18]	Knockout increases impulsivity[19]
5-HT_{2C}	Influences feeding, stress, and sex behavior	Activation reduces incentive motivation[13]	Activation decreases impulsive action[15,17]
5-HT_3	Nausea	Limited direct effects, but is necessary for the rewarding effects of MDMA, cocaine, morphine, and ethanol[12, 20–25]	No established effects
5-HT_4	Anxiety, depression, and feeding	No effect[26]	No established effects
5-HT_5	Memory and depression	No established effects	No established effects
5-HT_6	Memory, activity, and anxiety	Limited direct effects[27]; striatal expression facilitates cocaine reinforcement[28–31]	No effect[16,32]
5-HT_7	Depression and anxiety	No established effects	No established effects

Blue and orange shading represent directionality (decreased or increased, respectively) of receptor activation effects on reward-related behaviors and impulsive action. [1]*Balleine et al. (1996),* [2]*Fletcher et al. (1993),* [3]*Fletcher et al. (1995),* [4]*Groft et al. (2019),* [5]*Miyazaki et al. (2012),* [6]*Korte et al. (2017),* [7]*Harrison et al. (1999b),* [8]*Desrochers et al. (2021),* [9]*Acosta et al. (2005),* [10]*Fletcher et al. (2002),* [11]*Pattij et al. (2003),* [12]*Frick et al. (2015),* [13]*Fletcher et al. (2017),* [14]*Koskinen et al. (2000a),* [15]*Silveira et al. (2020),* [16]*Talpos et al. (2006),* [17]*Fletcher et al. (2007),* [18]*Doly et al. (2009),* [19]*Goldman et al. (2010),* [20]*Fletcher and Higgins (1997),* [21]*Roger-Sánchez et al. (2013),* [22]*Kelley and Hodge (2003),* [23]*Rodd-Henricks et al. (2003),* [24]*Rompré et al. (1995),* [25]*Higgins et al. (1992),* [26]*Reavill et al. (1998),* [27]*Mitchell et al. (2007),* [28]*Brodsky et al. (2016),* [29]*Ferguson et al. (2008),* [30]*Valentini et al. (2013),* [31]*da Silva et al. (2018), and* [32]*de Bruin et al. (2013).*

inhibitory associations to increased impulsivity in terms of both behavioral substrates and the underlying neural circuits.

THE DRIVE: CONTRIBUTIONS OF REWARD PROCESSING TO IMPULSIVITY

Impulsivity is innately tied to reward processing, with excitatory drive being a key aspect of motivated behavior. Importantly, before being able to consider an inhibitory process, a motivated behavior needs to exist. This commonly includes a learned cue-reward or action-outcome association. In other words, we first learn to respond to obtain rewarding outcomes, prior to learning to avoid responding in certain circumstances (an innate or learned propensity to "go"). Alterations in appetitive associations may change the strength of the drive for reward. This could include differences in the intrinsic value/pleasurability of a reward (liking), and/or changes in the motivational value of the reward/reward paired cues (wanting). Though these are experimentally separable (Peciña, 2008; Berridge and Robinson, 2016; Morales and Berridge, 2020), they are linked together

such that changes to either "liking" or "wanting" would likely increase actions in pursuit of reward, a characteristic of impulsive action. Therefore, superficially similar clinical presentations could actually be the result of dysfunction in different underlying neural mechanisms. Careful behavioral analysis using a variety of tests in different reward domains may allow us to identify the mechanisms contributing to pathological levels of impulsivity.

Reward Processing in Classical Conditioning

Though impulsivity is defined in terms of operant behavior, in which impulsive behavior is characterized by actions that have unwanted consequences, the processes that underlie impulsive behavior may also be measurable at the level of Pavlovian tasks if they include changes to reward processing. In other words, if an instance of increased impulsivity was due to a change in the drive process, it may be able to be seen in altered appetitive classical conditioning, when outcomes are independent of action. For example, changes to the magnitude/value of an unconditioned stimulus influences the associative strength of conditioned cues, resulting in enhanced conditioned responding (Rescorla and Wagner, 1972; Pearce and Hall, 1980; Van Den Bos et al., 2004; Morris and Bouton, 2006). For appetitive conditioning, increased reward value due to altered hedonic pleasure or homeostatic processes could therefore increase the salience or associative strength of a cue, such that vigor of responding correlates with the perceived magnitude. If the value of a reward was subjectively increased, either due to pathological neural changes or simply everyday variations in reward preference in non-pathological cases, we would expect that subjects would form a stronger association between the cue and the reward and therefore have generally increased responding. For example, the phenomenon of signtracking, where animals may interact with a manipulable cue as if it were the reward which it has come to be associated with, shows that a classically conditioned cue can acquire increased incentive salience (Flagel et al., 2011). In fact, rats bred for a high novelty responding phenotype had increased signtracking behaviors along with a decreased ability to withhold responding in the differential reinforcement of low-rate responding test of impulsive action (Flagel et al., 2010). This interestingly correlates incentive salience with impulsivity – either subserved by a single underlying endophenotype or possibly due to a causal link of increased incentive salience leading to increased impulsive action. Interestingly high novelty responding rats also increased preference for the large reward in a delay discounting test of impulsive choice. Overall this study supports the idea that increased reward sensitivity may underlie both the operant impulsive and Pavlovian signtracking phenotypes. Additionally, in a study of excitatory Pavlovian responding during the adolescent developmental period, which is often characterized by heightened reward reactivity and impulsivity, adolescents showed increased responding under partial reinforcement conditions compared to adults (Meyer and Bucci, 2016a). This suggests that developmentally mediated impulsivity and altered classical conditioning may be modulated by similar reward-based changes. Taken together, the consideration of the processes which contribute to responding in appetitive classical conditioning may shed light on the mechanisms through which reward processing contributes to impulsive behavior.

Multiple neural substrates have been implicated in assigning value to an outcome or cue and incentive motivation. Dysregulation of any number of highly interconnected implicated brain regions could therefore result in altered reward related behavior. Several regions appear to represent or integrate reward value, including the nucleus accumbens (NAc), ventral pallidum (VP), basolateral amygdala (BLA), and regions of the prefrontal cortex including the orbitofrontal cortex (OFC) (Amiez et al., 2006; Chen et al., 2015; Howard et al., 2015; Wassum and Izquierdo, 2015; Ottenheimer et al., 2018). In particular, distinct areas of both the NAc (Peciña and Berridge, 2005; Peciña, 2008; Castro and Berridge, 2014) and the VP (Tindell et al., 2006; Ahrens et al., 2016; Richard et al., 2016; Smith et al., 2009) have been implicated in hedonic "liking" of reward assessed through taste reactivity, as well as incentive motivation "wanting." The NAc is poised to integrate cortical and limbic information about reward and output to the VP, the subthalamic nucleus (STN), the substantia nigra, the ventral tegmental area (VTA), and the lateral hypothalamus, providing a mechanism for translating value assessment and motivation into behavior (Mogenson et al., 1983; Robbins and Everitt, 1996). Indeed, as reviewed by Day and Carelli (2007), the NAc and its connections are critical to appetitive Pavlovian cue-outcome learning, both in association acquisition and motoric expression. In sum, changes in brain regions involved in both "liking" and "wanting" aspects of reward processing could contribute to increased responding to conditioned stimuli during appetitive classical conditioning by subjectively increasing the outcome value.

Contributions of Serotonin to Classical Conditioning Through Modulation of Reward Processing

Many brain regions involved in reward encoding and classical conditioning are innervated by the serotonin system, rendering serotonin as a well-positioned modulator of reward processes (Huang et al., 2019; Ren et al., 2019; **Figure 2A**). Serotonergic neurons within the dorsal raphe nucleus (DRN) respond by increasing firing to both expected and unexpected rewards (Cohen et al., 2015; Li et al., 2016; Zhong et al., 2017), indicating that serotonin generally does not encode "surprise" or prediction-error. Rather, during classical conditioning, some DRN neurons develop a ramping response to reward-predictive cues, with response magnitude being commensurate to expected reward value (Nakamura et al., 2008; Bromberg-Martin et al., 2010). This response specifically requires that stimuli have acquired value through conditioning (Zhong et al., 2017), and differs from the response of other DRN neurons during aversive experiences (Hayashi et al., 2015). Thus, serotonergic signaling reflects the value (either learned, in the case of cues, or innate, in the case of rewards or punishments) of stimuli, with different populations (and projections; Ren et al., 2018) responding selectively to appetitive or aversive events.

Serotonin's involvement in reward processes ultimately depends on not only the activity of serotonergic neurons, but also the projection targets as well as the receptors expressed in

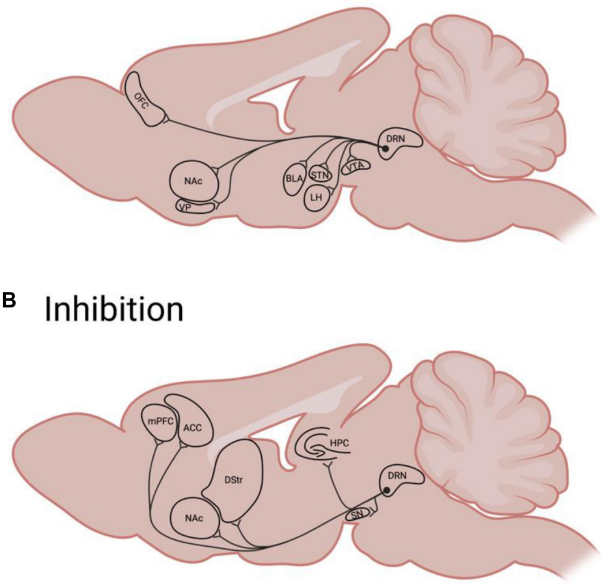

FIGURE 2 | A simplified schematic of rodent Dorsal Raphé serotonergic efferents to brain regions implicated in reward **(A)** or behavioral inhibition **(B)**. ACC, anterior cingulate cortex; BLA, basolateral amygdala; DRN, dorsal raphé nucleus; DS, dorsal striatum; HPC, hippocampus; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; OFC, orbitofrontal cortex; STN, subthalamic nucleus; SN, substantia Nigra; VP, ventral pallidum; VTA, ventral tegmental area.

those areas. Brain regions within canonical reward circuitry—and containing "hedonic hotspots" (Peciña et al., 2006)—receive dense innervation from the DRN and express various serotonin receptors. Serotonin signaling in some of these reward processing regions, specifically the NAc and VP, appears to mediate the euphoric effects of recreational drugs (Yoshimoto et al., 1992, 2012; Napier and Istre, 2008; Matsui and Alvarez, 2018). However, the hedonic effects of serotonin signaling are not consistent across all receptors, and specifically targeting distinct receptors manifests varied, and sometimes opposite, effects (**Table 1**). Manipulating general serotonin tone via systemic agonism of 5-HT_{1A} receptors (thought to increase synaptic serotonin by antagonizing autoreceptors) or genetic knockout of serotonin transporters (SERT; also thought to generally increase synaptic serotonin, Homberg et al., 2007) fails to alter hedonic liking of palatable tastes (Treit and Berridge, 1990; Caras et al., 2008) or incentive wanting for reward paired cues (Nonkes et al., 2014). However, subjects with depleted serotonin levels display reduced neural responsivity to rewards in fMRI (Seymour et al., 2012), and we recently demonstrated that mice lacking the 5-HT_{1B} receptor seem to have increased subjective reward valuation, including enhanced hedonic taste reactivity to sucrose in a lickometer test (Desrochers et al., 2021). Taken in totality, this evidence suggests that serotonin likely does not have a unified brain-wide role in reward processing. Rather, to accurately characterize serotonin's functions, region, cell-type, and receptor specificity must be considered.

Communication between subregions of the OFC and BLA is crucial in learning, representing, and using the value of cues to guide behavior (Malvaez et al., 2019; Lichtenberg et al., 2021; Sias et al., 2021), and serotonergic signaling regulates this communication. Serotonin neurons projecting to the BLA respond to both reward and punishment (Ren et al., 2018) and may have a general role in tuning the response of this region to stimuli; BLA serotonin activates GABAergic interneurons, which inhibit excitatory projection neurons (Rainnie, 1999; Bocchio et al., 2015), and should be expected to mute the area's response to salient stimuli. Interestingly, the dampening effect on BLA activity appears to depend on balanced serotonin signaling; while acute administration of serotonin excites inhibitory interneurons, prolonged exposure to serotonin (such as would occur in the absence of proper serotonin re-uptake) *reduces* the inhibitory output of interneurons on BLA principal neurons (Rainnie, 1999). In agreement with this, reduced 5-HT levels following excitotoxic lesion or 5-HT desensitization, leads to amygdalar over-activity and over-responding to reward paired cues (Nonkes et al., 2010; Man et al., 2012). Serotonin in the OFC regulates anticipatory encoding of reward in response to predictive cues (Zhou et al., 2015) and coordinates emotional and behavioral responses to those cues (Man et al., 2012). Overall changes to serotonin signaling in these areas results in deficits in the ability to represent and use expected outcome values and increases the likelihood of an animal's adopting inefficient behavioral strategies, such as seen in impulsivity.

Region-specific and receptor targeted parsing of the serotonin system is historically difficult, due to the system's complexity and the limitations of some tools in distinguishing varied components. For example, serotonergic neurons can corelease glutamate throughout the brain (Sengupta et al., 2017; Ren et al., 2018; Belmer et al., 2019; Wang H. L. et al., 2019). Monitoring DRN activity doesn't distinguish between the effect of serotonin and glutamate release, even when neurons are targeted in projection-specific approaches. Global manipulations of the serotonergic system, such as SERT KO or inhibition, produce manifold compensatory changes beyond simply altering the level of serotonin in the synapse (Homberg et al., 2007). Historically, the primary technique available for monitoring serotonin *in vivo* was microdialysis, which is sensitive to only one of the multiple timescales at which serotonergic neurons appear to operate (Cohen et al., 2015). Fortunately, recent advances in fluorescent-based in-vivo monitoring techniques now allow for direct monitoring of serotonin release at time scales compatible with understanding its role in reward processing, motivation, and impulsivity (Unger et al., 2020; Dong et al., 2021; Wan et al., 2021).

Reward and Impulsive Action

In addition to classical conditioning, reward processing is central to instrumental behavior, and increased impulsivity could result from the overvaluation or increased motivation for reward, which override the negative consequences associated with taking action. Difficulty in withholding or stopping ongoing responding

for reward in tests of instrumental behavior is a defining characteristic of impulsive action. Examples of paradigms used to assess this component of impulsivity include the Go/No-go (measuring the decreased ability to withhold responding when presented with a no-go cue), 5-choice serial reaction time test (5CSRTT; assessing premature responding), stop signal reaction time test (SSRT; testing the decreased ability to halt ongoing responding), and differential reinforcement of low rate responding (DRL; measuring the decreased ability to withhold responding for a wait period).

Importantly, an increased impulsive action phenotype may influence behavioral readouts in other operant paradigms testing motivation, such as random ratio and progressive ratio. Changes in excitatory responding (actions normally taken in pursuit of reward), for example the vigor of responding, which are subserved by changes in reward circuitry (Bailey et al., 2016, 2018) may make inhibiting the response more difficult. This could drive increased/disordered responding seen in these operant tasks, as well as in clinical cases of increased impulsivity. For example, dysfunctional reward processing is frequently comorbid in psychiatric disorders characterized by levels of increased impulsivity, including substance use, gambling disorders, and schizophrenia (Bjork et al., 2004; Monterosso et al., 2005; Rubio et al., 2008; Billieux et al., 2012). Preclinically, rats that show high levels of premature responding in the 5CSRTT are also more sensitive to cue-induced reinstatement of sucrose-seeking (Diergaarde et al., 2009). The question remains if the dysregulated impulsivity is causally linked to the reward system dysfunction. We recently developed a paradigm to show that increasing reward value on a trial-by-trial basis can lead directly to increased impulsive action in a Go/No-go paradigm (Desrochers et al., 2021). Future studies can expand on this to attempt to ameliorate disordered impulsivity by normalizing the aberrant reward processing.

Approaches to dissect the underlying neural circuits of co-occurring reward process and inhibitory dysfunction can determine if the neural circuit dysregulation is subserved by convergent mechanisms. Many of the same brain areas noted to be involved in reward and motivation have also been implicated in impulsive action. In particular, the NAc and its core and shell subregions have been extensively studied for their individual roles in impulsive action through reaction time tests, with pharmacological manipulations and deep brain stimulation of the shell subregion causing elevated premature responding (Sesia et al., 2010; Feja et al., 2014). Optogenetic stimulation of projections from the VTA to the NAc shell also increased premature responding during a long inter- trial interval in the 5CSRTT (Flores-Dourojeanni et al., 2021). Additionally, prefrontal cortical regions modulate impulsive action, though they are more often associated with assigning value to different decisions and choosing between actions (OFC, mPFC). Specifically, in an imaging study in humans, Mechelmans et al. (2017) found that impulsivity for high value reward cues in a 4CSRTT was accompanied by increased activity in the mOFC and in a monetary incentive delay task was associated with increased functional connectivity between the STN and left mOFC. In a rodent study of the 5CSRTT, rats that tended to respond prematurely had alterations in oscillatory patterns in the mPFC and NAc, which may cause abnormal reward encoding resulting in increased impulsive action (Donnelly et al., 2014).

The alterations in reward-related behavior in impulsivity could also be the result of impaired action selection supported by the dorsal striatum, which is important when there is an instrumental contingency between response and reward, as in many tests of impulsive action (Balleine et al., 2007; Corbit and Janak, 2007, 2010). Pharmacological manipulations of serotonin and glutamate receptors in the dorsal striatum modulate premature responding in the 5CSRTT (Agnoli and Carli, 2012). The varied regions associated with the control of impulsive action highlight the importance of considering reward processing in the study of impulsivity, as well as suggest that there may be many ways to cause an impulsive action "phenotype" through modulation of different behavioral endophenotypes. Behavioral analysis which considers the learning, hedonic, and motivational contributions to pathological cases of impulsivity may help clarify and point toward more specific neural targets for treatment.

Contributions of Serotonin to Impulsive Action Through Reward

Given that serotonin signaling is involved in many aspects of reward processing, it is relevant to consider how the influence of serotonin on reward contributes to its effect on impulsivity. As discussed in prior sections, most, if not all, rodent assays of action impulsivity involve approach toward appetitive cues and outcomes, and are therefore confounded by reward processing. As such, the effects of manipulations to serotonin signaling on tests of impulsive action may in some cases arise from effects on reward responsivity. Directly assessing the effects of experimental manipulations of serotonin signaling on reward related alterations is helpful to accurately interpret the effects in traditional tests of impulsivity.

Serotonin enables appropriate waiting for reward (Eagle et al., 2009; Miyazaki et al., 2014), and activation of serotonergic neurons in the DRN is correlated with (Miyazaki et al., 2011) and causally linked (Miyazaki et al., 2014) to waiting. The OFC, in addition to its previously described involvement in reward processing, mediates at least some of serotonin's action in impulse control, as stimulating serotonin release in the OFC almost fully recapitulates the effect of DRN stimulation on waiting (Miyazaki et al., 2020). These two sets of functions are likely intertwined. Serotonergic signaling in the OFC is often associated with impulsive choice (Wischhof et al., 2011; Darna et al., 2015), in part because of its well established contributions to tracking and representing the value of reward predictive cues (Clarke et al., 2007; Nonkes et al., 2010). However, there is evidence that it is also involved in the capacity to withhold premature responses, to stop ongoing behavior, and to perform other forms of response inhibition (Chudasama et al., 2003; Eagle et al., 2008b; Mechelmans et al., 2017). Yet, the OFC is not necessary for pure motor inhibition (Swick et al., 2008) nor does it encode the value of actions themselves but rather that of affective stimuli (Rolls, 2004; Rudebeck et al., 2008). Thus, the understanding of OFC serotonergic function in impulsivity should not be restricted to a choice vs. action binary nor to a pure response

inhibition framework. Rather, it appears related to the ability to withhold behavior generally and likely does so through its role in outcome value encoding.

Serotoninergic modulation of prospective reward encoding within the OFC is heterogeneous (Zhou et al., 2015), as are its effects on OFC neuronal activity (Wright et al., 2017, 2021). During reward prediction, OFC activity is distinct from baseline yet characterized by neither a gross increase nor decrease in activity (Shobe et al., 2017). This "activity-silent" state (Stokes, 2015) is similar to the heterogeneous responses observed in response to serotonin tone. The OFC communicates information regarding the prospective value of expected rewards to other limbic structures, its connectivity with the NAc being particularly important (Meyer and Bucci, 2016b; Wang Z. et al., 2019), thereby permitting the usage of that value in the computation of whether or not to initiate a behavioral response.

The mesolimbic pathway is another circuit in which serotonin regulates reward encoding and the generation of motivated behavior. In humans, genetic variation in a serotonin production enzyme, tryptophan hydroxylase-2, is associated with increased impulsivity and increased responsivity of NAc to reward (Neufang et al., 2016). Gross manipulations to serotonin tone within the limbic system in rodents alter motivational drive. For example, the impulsivity produced by serotonin depletion in the NAc does not appear to reflect an alteration in response inhibition (Fletcher et al., 2009). Specifically, while it increases the rate of responding in the DRL, it does not alter burst responding nor does it increase premature responding in the 5CSRTT. More specific manipulations to serotonergic signaling through either 5-HT_{1B} (by gene knockout) or 5-HT_{2C} receptors (by receptor antagonist) impairs response inhibition while also increasing mesolimbic DA release (Pennanen et al., 2013; Nautiyal et al., 2015), suggesting that these receptors may mediate the general effect of serotonin on NAc activity and behavior. The 5-HT_{1B} receptor is a likely substrate through which serotonin may influence reward and impulsive action: 5-HT_{1B} receptor knockout increases reward valuation in a lickometer test and false alarm rate for no-go trials in the Go/No-go test (Desrochers et al., 2021), and restoring 5-HT_{1B} receptor expression in adulthood reverses both action impulsivity and altered dopamine signaling in the NAc (Nautiyal et al., 2015). Interestingly, overexpression of 5-HT_{1B} receptors in NAc shell projections to the VTA also increases the rewarding effects of drugs of abuse (Neumaier et al., 2002; Furay et al., 2011). Additionally, reduced binding of these receptors in the NAc and VP is associated with major depressive disorder, of which insensitivity to reward (anhedonia) is a principal symptom (Murrough et al., 2011). More generally, 5-HT_{1B} signaling is linked to depression-like behavior in animal models (Svenningsson et al., 2006) and to cocaine or social reward through is localization in the NAc, (Dölen et al., 2013; Fontaine et al., 2021). Thus, 5-HT_{1B} under- *or* over-expression can contribute to altered reward processing, suggesting that normal function requires maintenance of balanced signaling.

In sum, there is evidence that serotonergic regulation of impulsivity occurs, in part, at the level of reward processing. Described above are proposed roles for serotonin in linking these processes in the OFC and mesolimbic pathway. However, the extent of serotonin's involvement in such a link in many other regions remains to be characterized. **Figure 2A** summarizes areas targeted by serotonergic neurons that are involved in reward and impulsivity which may be promising targets for such characterization. Research that seeks to bridge the gap between reward and impulsivity—such as through the use of batteries of behavioral assays that provide information across multiple dimensions—would greatly enhance our understanding of not only impulse control, but also the processes by which motivated behaviors and impulses are generated.

THE BRAKE: CONTRIBUTIONS OF INHIBITORY CONTROL TO IMPULSIVITY

Alternatively to increased reward drive, disordered impulsivity can be considered as a failure of inhibitory processes, even colloquially described as a lack of "self-control." In the impulsive action subtype of impulsive behavior, this presents as deficits in preventing responding or stopping ongoing responding. Withholding an action, or learning that the absence of response results in reward, is an action-outcome association that is necessary for successful performance in standard tests of impulsive action. This action-outcome association opposes the initially learned excitatory association in which the action led to reward. The ability to withhold responding, or inhibitory control, is often ascribed to higher executive functions and decision-making processes controlled by cortical areas, which act to modulate subcortical regions involved in "drive" (Dalley et al., 2011; Bari and Robbins, 2013). However, deficits in response inhibition also arise locally within lower neural areas involved in the volitional process (such as the NAc, which is usually associated with the "drive" component but may also have an inhibitory role). There also may be separable component processes underlying the acquisition/learning and the expression of inhibitory control, which would require carefully designed behavioral studies to separate.

Inhibition and Classical Conditioning

Learning about inhibitory associations is an important component to consider in understanding response inhibition in impulsive action. This is distinct from the behavioral/motor expression. Deficits in inhibitory learning could be a cause of deficits in response inhibition, or alternatively, could be intact with the impulsivity arising at other levels of processing. While impulsivity itself is not defined in the context of classical conditioning, a behavioral output may appear impulsive if there are underlying deficits in inhibitory learning. For example, an impulsive behavior could result from the lack of learning of the response omission – reward association, or from a decreased ability to withhold a response. The acquisition of inhibitory learning has been studied extensively in the context of classical conditioning.

A primary area of inhibitory learning is extinction, where a new inhibitory memory is acquired to compete against a previously established excitatory memory. Importantly, extinction is not an erasure of a memory, but rather a competition

of parallel associations, where old memories/behaviors can spontaneously renew (Bouton, 1993; Todd et al., 2014; Bouton et al., 2021). A deficit in the formation of the new inhibitory association or a failure of this association to successfully compete with the excitatory association, could result in altered impulsivity in classic tests of impulsive action. However, though there are many parallels between Pavlovian and operant extinction, there are also clear dissociations; for example, Pavlovian extinction does not usually transfer between conditioned stimuli, but operant extinction does (Trask et al., 2017; Bouton et al., 2021). Neurally, both the BLA and the infralimbic cortex, among others, are all involved in both Pavlovian and operant extinction, but the NAc shell is especially implicated in operant extinction (Millan and McNally, 2011; reviewed in Bouton et al., 2021). The hippocampus also seems to be involved in behavioral inhibition during extinction, as lesions to this region prevent extinction of a previously classically conditioned appetitive stimulus (Chan et al., 2003). All of these regions have also been implicated in the modulation of impulsive action, suggesting that deficits in extinction behavior may be involved in some presentations of impulsivity, or rely on dysfunction in similar neural mechanisms.

Another Pavlovian behavioral paradigm which could be useful in understanding the role of inhibitory learning in impulsive action is conditioned inhibition (reviewed by Sosa and dos Santos, 2019). Conditioned inhibition is a form of classical inhibitory learning where an inhibitory cue indicates the absence of an outcome when it is paired as a compound with a normally excitatory cue (A+, AX−; Pavlov, 1927). This inhibitor cue can then "transfer" and reduce responding when paired with other excitatory cues (BX−; Holland, 1989). Impulsive subjects which have diminished inhibitory control in operant paradigms may also fail to inhibit responding for the inhibitor-excitor compound cue in a test of Pavlovian conditioned inhibition, potentially suggesting common underlying mechanisms. Accordingly, He et al. (2011) found decreased expression of conditioned inhibition in a clinical population with personality disorders, often characterized by disinhibition and impulsivity. However, to dissociate the acquisition of this inhibitory learning from behavioral expression, acute time-limited experiments using optogenetic or chemogenetic inactivation of relevant neural targets during training vs. recall testing may be necessary.

Another version of Pavlovian inhibitory learning is negative occasion setting in which an inhibitory cue indicates that an outcome will not occur when presented in sequence with a normally excitatory cue (A+, X → A−). In this case, the conditioning is specific to the trained set of cues, and the inhibitor does not usually transfer to a different excitatory cue (Holland, 1989). Adolescent rats take longer to discriminate between reinforced and non-reinforced trials in a negative occasion setting paradigm when compared to preadolescents and adults, possibly due the functional immaturity of the PFC during this developmental period (Meyer and Bucci, 2017). Indeed, the prelimbic region of the PFC is necessary for learning this discrimination negative occasion setting, but not expressing it following training (MacLeod and Bucci, 2010). Additionally, these findings were replicated in a conditioned inhibition paradigm, where Meyer and Bucci (2014) found that lesions of the prelimbic region of the PFC decreased acquisition of conditioned inhibition learning, whereas lesions of the infralimbic cortex decreased behavioral expression following successful discrimination. Further testing inhibitory learning processes in established models for impulsive action or clinical populations are important next steps. These classical conditioning experiments could help elucidate the underlying behavioral/cognitive deficits present in specific cases of impulsivity, as well as suggesting potential shared neural substrates.

Contributions of Serotonin to Inhibitory Classical Conditioning

Though serotonin is strongly implicated in behavioral inhibition in instrumental conditioning, it is also involved in the acquisition and expression of inhibitory learning in these classical conditioning experiments. Lister et al. (1996) found that ablation of serotonergic pathways in rats reduced the acquisition of inhibitory associations, but left excitatory associations intact in a conditioned inhibition task. Knockout of the serotonin-transporter in rats also results in reduced latent inhibition, which is when a previously unpaired stimulus acquires inhibitory properties (Nonkes et al., 2012). Additionally, serotonin depletion impairs both Pavlovian and instrumental reversal learning, resulting in perseverative responding for a previously rewarded or safe stimulus (Cools et al., 2008; Kanen et al., 2021). This could be interpreted as a failure of behavioral flexibility, or as a failure to learn/express a new inhibitory association. One concept that unites these findings together is that serotonin signaling may play a role in the processing of aversive outcomes (including low reward, reward absence, or punishment; Crockett et al., 2012; Geurts et al., 2013; Unger et al., 2020). Specifically, serotonergic dysfunction (induced by tryptophan depletion) may cause a more positive estimation of the value of aversive outcomes, resulting in disinhibition of responding in both classical and operant conditioning (Dayan and Huys, 2008).

Serotonin is also involved in the extinction of classical conditioning. However, most research in this area has been conducted in fear extinction, rather than extinction of appetitive cues which correspond better to the inhibitory associations necessary for typical reward-based tests of impulsive action (though Pereyra et al., 2021b do describe serotonin effects on extinction of *operant* responding for reward). In fear conditioning, knockout of the serotonin transporter impairs extinction recall, though here the effect may be through the retention or expression, rather than the acquisition, of the new inhibitory association (Wellman et al., 2007; Narayanan et al., 2011; furthered reviewed in Bauer, 2015). Consideration of extending the role of serotonin signaling from operant behavioral inhibition to appetitive classical inhibitory conditioning (e.g., in tests of conditioned inhibition, negative occasion setting, and extinction) may help parse the behavioral and circuit mechanisms through which serotonin impacts inhibition. Importantly, inhibitory learning could be an important avenue through which serotonin modulates impulse control.

Impulsive Action and Response Inhibition

Though disordered impulsivity could occur because of differences in inhibitory Pavlovian associations, it is defined in the context of operant conditioning requiring inhibition of an action to obtain reward. Nevertheless, similarly to classically conditioned response inhibition, the inhibitory "brake" seems to rely heavily on prefrontal regions upstream of subcortical reward areas (see Bari and Robbins, 2013 for an extensive review of their search). In humans, several fMRI studies have identified neural correlates of inhibitory control during tests of impulsive action. Activity in the vlPFC was associated with successful response inhibition in no-go trials for larger monetary rewards in an incentivized inhibition task (Leong et al., 2018). Additionally, using a stop signal task, Weafer et al. (2019) found that decreased activity in the right PFC during response inhibition was associated with higher left ventral striatum activity during reward receipt, suggesting negative functional association between inhibitory control and reward drive modulated through cortico-striatal connections. The anterior cingulate cortex (ACC) has also been implicated in impulse control in subjects with ADHD (Baytunca et al., 2021), and its activity is related to error processing in a Go/No-go task (Hester et al., 2004).

There is also a large literature investigating the neural circuitry underlying cortical control of response inhibition in preclinical models. Pharmacological inactivation of various regions of the mPFC, especially the prelimbic and infralimbic regions, resulted in a loss of inhibitory control on no-go trials in a response inhibition task which included shock punishments (Verharen et al., 2019). Chemogenetic activation of the vmPFC to NAc shell pathway decreases motor impulsivity in a 1CSRTT and binge-eating in rats, suggesting that these higher order areas have inhibitory control over reward processing (Anastasio et al., 2019). Indeed, using optogenetics, Li et al. (2020) found that another cortical-subcortical connection from the dmPFC to the STN in mice was important for response inhibition in a Go/No-go task. In the ACC, inhibitory G proteins are involved in the control of premature responding in the 5CSRTT (van der Veen et al., 2021). Interestingly, these studies manipulate their pathways/regions only after subjects acquired baseline training performance, suggesting that these pathways play a role in the behavioral expression of inhibition, not necessarily the learning itself. There is also convergent human and animal evidence for a role of the OFC in response inhibition (reviewed in Winstanley et al., 2010), however, single-unit recordings by Bryden and Roesch (2015) revealed that OFC neuron activity seems to support the separation of similar actions rather than inhibition independently.

Beyond the cortex, there is also evidence for the contribution of subcortical areas to response inhibition during tests of impulsive action. Deep brain stimulation of the NAc core in rats decreased impulsivity as measured by premature responding in a reaction time test, while stimulation of the NAc shell increased impulsivity, suggesting that the different subregions of the NAc may functionally support both excitation and inhibition in pursuit of reward (Sesia et al., 2008). Also, in the NAc, local inhibitory control may occur through the activity of fast-spiking interneurons, which seem to constrain impulsive action in the 5CSRTT, likely by inhibiting signaling of medium spiny neurons (Pisansky et al., 2019). Finally, dopamine signaling in the dorsal striatum is also important for response inhibition in a stop-signal task (Robertson et al., 2015). Together, all these studies suggest that the inhibitory control of impulsive action relies both on cortical and local sub-cortical control of reward processing areas.

Contributions of Serotonin to Inhibitory Control of Impulsivity

Overall, the serotonin system is well-positioned to impact impulsive action through its ability to modulate components of this inhibitory control system. Serotonergic neurons from the DRN innervate many cortical (and some subcortical) regions implicated in behavioral inhibition (**Figure 2B**), and signal through a number of different serotonin receptors (**Table 1**). Classic research which implicates serotonin in the regulation of anxiety behavior, is sometimes extended to the behavioral inhibition concept. For example freezing behavior in response to aversive stimuli (e.g., Wise et al., 1973) or a lack of approach in a conflict test (Graeff et al., 1997) may be viewed as inhibited behavioral responses. However, while serotonergic neurons do respond to such anxiogenic and aversive stimuli (Wise et al., 1973; Ren et al., 2018), clinical observations failed to support the theory that serotonergic signaling generated aversion. Soubrié (1986) proposed a simple resolution to this apparent conflict: serotonin encoded not anxiety but the "stop" signal that such an emotional experience occasions. Subsequently, a great deal of work has sought to characterize the precise nature of serotonin's role in behavioral inhibition in both punishment (e.g., Crockett et al., 2009) and reward (Clark et al., 2005; Eagle et al., 2009; Nonkes and Homberg, 2013; Miyazaki et al., 2014, 2020; Nonkes et al., 2014; Odland et al., 2021).

Whereas serotonin's contributions to reward can be assessed directly outside of tests of impulsivity (as discussed), its role in behavioral inhibition is harder to extricate since tests of impulsive action are most commonly tests of behavioral restraint. Nevertheless, both behavioral evidence and a review of areas in which serotonin acts on behavioral restraint support a role for serotonin in "brake" processes. Global 5-HT depletion increases premature responding in the 5CSRT task (Harrison et al., 1997; Winstanley et al., 2004) and impairs behavioral restraint on "no-go" trials in the Go/No-go task (Harrison et al., 1999a; Masaki et al., 2006). Critically, the deficits induced by such depletion are specific to impulse control during the action preparation phase of behavior: both the ability to cease ongoing behavior, tested with the stop-signal reaction time task (Eagle et al., 2008a), and preference for smaller, immediate rewards in delay discounting (Winstanley et al., 2003; Worbe et al., 2014) are *insensitive* to serotonergic manipulation. In the other direction, stimulating serotonergic neurons enhances the ability to wait for reward delivery in "patience" based tasks, but is not, itself, rewarding (Miyazaki et al., 2014, 2018; Fonseca et al., 2015). Further, serotonergic release differentially mediates

waiting through actions in different cortical areas (Miyazaki et al., 2020). Specifically, stimulation of 5-HT release in the mPFC improves "patience" only during periods of waiting uncertainty (Miyazaki et al., 2020). Because the mPFC is generally thought to regulate the timing of behavior and encodes event and delay durations (Narayanan and Laubach, 2006; Kim et al., 2009; Xu et al., 2014; Tiganj et al., 2017), the selectivity of the effect of stimulated release suggests that prefrontal serotonin may contribute to action inhibition through a role in response timing. The many functions of the mPFC are mediated by dissociable, heterogeneous populations of projection neurons, which display a wide range of responses to affective stimuli (Grant et al., 2021) and regulate discrete aspects of motivated behavior (Otis et al., 2017, 2019). Serotonin is well positioned to modulate general cortical synchrony and the balanced activity of output pathways through tuning the activity of both inhibitory microcircuits and projection neurons (Puig and Gulledge, 2011; Dembrow and Johnston, 2014), as well as regulating the general cortical response to affective stimuli (Pereyra et al., 2021a).

The mPFC receives strong serotonin innervation from the DRN and is critical for behavioral control through both action selection and timing. Paradoxically, though elevated tonic extracellular levels of 5-HT in mPFC correlate with higher impulsive action in the 5CSRTT (Dalley et al., 2002), directly stimulating serotonin release in mPFC terminals increases wait times (i.e., decreases impulsivity) in a delayed reward task (Miyazaki et al., 2020). Resolution of this conflict will likely require characterization of both phasic serotonin release in the mPFC and neural responses to said release during such tasks. Furthermore, serotonergic neurons co-release glutamate in numerous other areas, including the amygdala (Sengupta et al., 2017) and VTA (Wang H. L. et al., 2019) while the presence of glutamate-serotonin co-transmission has not been characterized within the mPFC, DRN terminals in this area show robust coexpression of SERT and VGLUT3 (Belmer et al., 2019), indicating that the contribution of glutamatergic signaling to DRN stimulation must be considered.

The ACC is another locus for behavioral control that is modulated by serotonin. Altered serotoergic signaling in humans is associated with altered ACC activity that correlates with impoverished action monitoring and behavioral restraint (Holmes et al., 2010; Hong et al., 2018). 5-HT_{1B} receptor binding within the ACC is strongly associated with inhibiting responses to stimuli in an emotional Go/No-go task (da Cunha-Bang et al., 2017). Serotonin also acts at 5-HT_{1A} (Tian et al., 2017) and complexed 5-$HT_{2A/C}$ receptors (Price et al., 2019) within the ACC. However, the precise role that these latter receptors play in behavior remains unclear. The behavioral consequences of 5-HT_{2C} activation or inactivation are mixed, likely due to the lack of receptor specificity in some pharmacological manipulations, with some drugs impacting both the 2C and 2A receptors. Across multiple studies, systemic 5-HT_{2C} agonism has been observed to increase (Koskinen et al., 2000b; Blokland et al., 2005) or decrease (Fletcher et al., 2007, 2013) premature responding in the 5CSRTT. More specific systemic antagonism of the 5-HT_{2A} receptors reduces premature responding in the 5CSRTT (Fletcher et al., 2007). Interestingly, when infused directly into the ACC, a 5-$HT_{2A/2C}$ agonist (which had increased impulsive action in the 5CSRTT when administered systemically in the same study) had no effect on impulsivity (Koskinen et al., 2000b). Though the precise functional roles of ACC serotonin receptors clearly remain to be determined, they are likely related to the ACC's established roles in both maintaining representations of desired outcomes and inhibiting behaviors that interfere with outcome acquisition (Berkman et al., 2012). These functions of the ACC are accomplished, in part, by cortical inhibition of stimulus-response associations within the striatum to permit the control of behavior by action-outcome contingencies (Cools et al., 2008), and serotonin is a known modulator of these projections.

Serotonin also acts directly in both ventral and dorsal striatum, important targets of both the mPFC and ACC in their regulation of behavior, to regulate motivation and impulsivity. While serotonin in the ventral striatum *counters* anticipatory encoding of proximal rewards, serotonin in the dorsal striatum *enables* prospective encoding of distal rewards (Tanaka et al., 2007). In the latter system, serotonin facilitates information processing through the interplay between cortical input and 5-HT_{2A}/5-HT_{2C} signaling (Agnoli and Carli, 2012). In the ventral striatum both 5-HT_{2A} and 5-HT_{2C} antagonism reduce impulsivity, and 5-HT_{2A} antagonism appears to do this by generally suppressing motivated responding (Robinson et al., 2008). Serotonin depletion in the NAc increases the rate of responding in the DRL without appearing to influence motor impulsivity and is theorized to reflect a decreased tolerance for delayed reward (Fletcher et al., 2009). However, directly stimulating serotonergic terminals in the NAc does not increase waiting in a patience-based task (Miyazaki et al., 2020). Thus, serotonergic signaling within the ventral striatum appears to mediate proximal reward response and approach drive, while within the dorsal striatum it facilitates control of behavior in the face of delayed rewards.

In summary, the literature supports the following conclusions: (1) Serotonin receptors are positioned to regulate cortical microcircuits and projection neurons. (2) Serotonergic manipulations within prefrontal cortices and striatal outputs alter impulsive behavior. (3) The described functions of these cortical regions align best with "brake" processes in behavioral restraint rather than "drive" processes. To build a model of serotonergic impulsivity-regulation within cortical circuits, these ideas would ideally be integrated in a cohesive framework. For example, future work could characterize the role and impact of serotonergic signaling on the activity of functionally discrete cortical subpopulations (e.g., Hart et al., 2018; Grant et al., 2021). Causally linking serotonergic signaling within corticostriatal circuits to behavioral inhibition would be the next step. Behavioral assays targeted at cortical control processes including those targeting timing or action monitoring may help identify specific "brake" functions altered in impulsive animals. Meanwhile, tests sensitive to altered reward and motivation may serve to *exclude* changes in "drive" processes.

DISCUSSION: IMPULSIVITY AS AN IMBALANCE OF SYSTEMS AND FUTURE DIRECTIONS

Through this review, we have provided an overview of the behavioral and neural systems underlying impulsive action. Dysregulations of either reward or inhibition can create an imbalance of the neural systems responsible for impulse control. Neurally, we suggest that widespread DRN serotonergic projections (**Figure 2**) place serotonin, signaling through its various receptor types (**Table 1**), in a prime position to modulate both the excitatory and inhibitory components of these systems. Indeed, there may be multiple serotonin subsystems which separably mediate responses to rewarding or aversive outcomes (Ren et al., 2018). Either excess excitation or decreased inhibitory control could result in increased impulsive action as observed by a decreased ability to stop or withhold responding. In this case, the initially learned "go" association overrides the "no-go" or stop association. Increased impulsivity could also be the result of altered activity in both drive and brake processes. Ultimately, both processes compete over controlling the same endpoint: motor output. For animals to achieve efficient, flexible behavior, the drive and brake circuitry must each be responsive to task demands in guiding action selection.

Adolescence is an interesting case which allows us to probe the role of these two processes and how serotonin influences the balance. Specifically, adolescence is a developmental period characterized by increased impulsivity, risky decision making, and hyper reward-sensitivity. In the dimension of impulsive action, compared to children and adults, teenagers have more false alarms for no-go cues in the Go/No-go test (Somerville et al., 2011; Dreyfuss et al., 2014). This heightened impulsive action is thought to be the result of the linear development of the PFC and the nonlinear development of the ventral striatum and other components of the reward system, which peak in sensitivity during adolescence (Blakemore and Robbins, 2012). This results in an imbalance between the subcortical systems which motivate behavior and the cortical systems providing inhibitory control compared to childhood and adulthood (Casey et al., 2011). Substance use disorders have also been considered through a similar lens, with both increased appetitive drive and disordered executive control potentially resulting in impulsive behavior, though the extent to which impulsivity is causal or resultant to addiction is unclear (Bechara, 2005; Camchong et al., 2014; Jentsch et al., 2014; Kozak et al., 2019).

Importantly, the imbalance of reward and inhibitory processing could be the result of dysfunction of many different regions, cell types, and/or receptor types, which may each result in an impulsive action phenotype, albeit through different neural and behavioral processes. Therefore, careful dissections of the processes which contribute to impulsive action allows for the fractionation of different paths to an overall impulsive phenotype. Testing may use non-traditional tests for the study of impulsivity, including the consideration of Pavlovian and instrumental learning processes, the expression of behavioral inhibition, and reward processes. For example, in **Figure 3**, we show a chart with reward and behavioral inhibition as hypothetical dimensions characterizing different behavioral measures. Notably, tests like taste reactivity primarily measure a reward-related behavior; on the other hand tests like the Go/No-go are considered measures of impulsive action, but are also influenced by reward value (Desrochers et al., 2021). Desrochers et al. found that increasing reward quantity increases false alarm rates for no-go trials in control mice, while decreasing reward quantity reduced this measure in normally impulsive mice lacking the 5-HT_{1B} receptor. This suggests that some traditional measures of impulsive action are intrinsically tied to reward related behaviors. However, we highlight the idea that additional approaches to measuring impulsivity in the absence of learned appetitive motivators may allow the dissociation of reward processes from behavioral inhibition (see **Figure 3**). It is unclear to us whether any existing tests could specifically be used to measure impulsive action in the absence of reward. A possibility may be active avoidance, where animals learn to avoid the side of a shuttle box associated with an aversive outcome. Subjects with decreased behavioral inhibition may have enhanced active avoidance behaviors (more rapid acquisition of the behavior) in this task; indeed, selectively bred Roman-high avoidance rats also have increased premature responding in the 5CSRTT compared to low-avoidance rats (Moreno et al., 2010). Additionally, in these rat strains, 5-HT_{2A} binding levels are higher in the high-avoidance rats and correlate with impulsivity

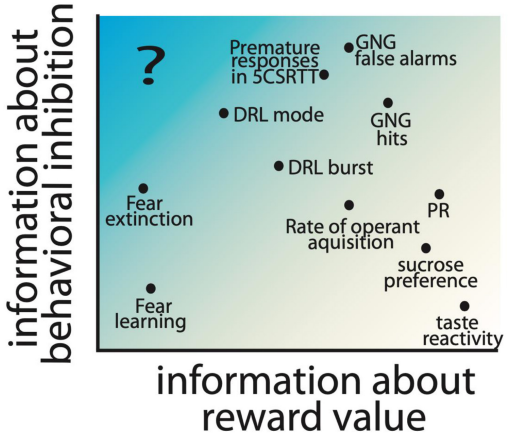

FIGURE 3 | Hypothesized contribution of reward value and behavioral inhibition factors to commonly used preclinical behavioral assays is shown based on the location of measures on the reward and inhibition axes. Parameters extracted from commonly used assays of impulsive action, including Go/No-go (GNG), 5 choice serial reaction time task (5CSRTT), and differential reinforcement of low-rate responding (DRL) are included, as well as other operant tasks which measure motivation, e.g., progressive ratio (PR) and hedonic responses (sucrose preference and taste reactivity). Fear learning and rate of extinction of fear learning are also included as measures with low information about reward value. Highlighted with a "?" is an information space that would provide measures of impulsivity independent of reward, and has limited preclinical behavioral assays.

in the 5CSRTT (Klein et al., 2014). This suggests a role for serotonin signaling in behavioral inhibition, independent of reward, which could be further studied using an active avoidance test. More studies testing traditional models for pathological impulsivity in active avoidance paradigms would be helpful to understand if this would be a useful approach to measuring impulsive action without an appetitive conditioning paradigm.

In conclusion, we support consideration of components of impulsivity beyond the overarching subtypes (i.e., action vs. choice), to include specific cognitive and behavioral substrates. Studies of disordered impulse control, both in human and animal models, would ideally use multiple and varied behavioral tests to determine the underlying component processes and tease apart influences of reward from inhibitory control. The understanding of the contributions of these processes can provide tractable targets for pursuing neural circuit mechanisms and potentially more individualized treatment approaches for pathological impulsivity. In particular, we propose that serotonin signaling is an important mechanism to explore in this context to understand the behavioral and neural bases of the control of impulsive action.

AUTHOR CONTRIBUTIONS

SD, MS, and KN contributed to the ideas, wrote the manuscript, and approved the final version.

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Photostimulation of Ventral Tegmental Area-Insular Cortex Dopaminergic Inputs Enhances the Salience to Consolidate Aversive Taste Recognition Memory *via* D1-Like Receptors

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Taste memory involves storing information through plasticity changes in the neural network of taste, including the insular cortex (IC) and ventral tegmental area (VTA), a critical provider of dopamine. Although a VTA-IC dopaminergic pathway has been demonstrated, its role to consolidate taste recognition memory remains poorly understood. We found that photostimulation of dopaminergic neurons in the VTA or VTA-IC dopaminergic terminals of TH-Cre mice improves the salience to consolidate a subthreshold novel taste stimulus regardless of its hedonic value, without altering their taste palatability. Importantly, the inhibition of the D1-like receptor into the IC impairs the salience to facilitate consolidation of an aversive taste recognition memory. Finally, our results showed that VTA photostimulation improves the salience to consolidate a conditioned taste aversion memory through the D1-like receptor into the IC. It is concluded that the dopamine activity from the VTA into IC is required to increase the salience enabling the consolidation of a taste recognition memory. Notably, the D1-like receptor activity into the IC is required to consolidate both innate and learned aversive taste memories but not appetitive taste memory.

Keywords: insular cortex, ventral tegmental area, salience, consolidation, aversive taste, D1-like receptor

INTRODUCTION

Taste memory evolves as a critical system for animal survival through the detection of taste attributes related to the hedonic value, degree of familiarity, and to remember their nutritive or toxic consequences of food to form a memory for future acceptance or avoidance responses (Bermúdez-Rattoni, 2004; Scott, 2005). Taste memory involves encoding, storing, and retrieving taste information due to neural plastic changes taking place in a complex network comprising many different brain areas, encompassing the insular cortex (IC), medial prefrontal cortex, basolateral

amygdala, nucleus accumbens, and ventral tegmental area (VTA), among others (Yamamoto, 2006, 2008; Ramírez-Lugo et al., 2007; Fontanini et al., 2009; Jezzini et al., 2013).

Several studies have demonstrated that taste learning requires dopaminergic neurotransmission to consolidate the memory representation of tastants (Guzmán-Ramos and Bermúdez-Rattoni, 2011; Yiannakas and Rosenblum, 2017). In this regard, the VTA is a vital dopamine supplier that serves a central role in motivating behavior and reward processing. Some evidence suggests that VTA dopaminergic neurons increase their firing rate to signal reward (Berridge and Robinson, 1998; Nomoto et al., 2010; Fiorillo, 2013). However, evidence shows VTA dopaminergic neurons also increase their activity after the presentation of an aversive stimuli (Brischoux et al., 2009; Bromberg-Martin et al., 2010; Núñez-Jaramillo et al., 2010). In this sense, it has been reported that the modulation of the dopaminergic neurons by rewarding or aversive stimuli depends on the brain area(s) to which these dopaminergic neurons project (Lammel et al., 2011; de Jong et al., 2019).

We recently described the functional characterization of the dopaminergic VTA-IC pathway. However, its role in consolidating taste recognition memory remains poorly understood. The photoactivation of ChR2 + neurons in TH-Cre mice (20 Hz, 15 mW laser power, 473 nm and 5 ms pulse width) for 20 min induces electrophysiological responses in VTA neurons, dopamine release (measured by *in vivo* microdialysis), and neuronal modulation in the IC (Gil-Lievana et al., 2020). Importantly, the IC contains the primary gustatory cortex, which serves as a critical structure to consolidate taste recognition memory (Bermudez-Rattoni and McGaugh, 1991; Rosenblum et al., 1993; Bermúdez-Rattoni, 2004; Chen et al., 2011; Bermudez-Rattoni, 2014). Accordingly, *in vivo* microdialysis studies show dopamine release triggered in the IC when a novel appetitive or aversive taste is presented (Guzmán-Ramos et al., 2010; Osorio-Gómez et al., 2017). Interestingly, dopamine released during the presentation of novelty enables memory consolidation through the D1-like receptor since post-trial cortical microinjection of D1-like receptor antagonist impedes consolidation of taste recognition memory (Osorio-Gómez et al., 2021). It has been suggested that phasic dopamine activity plays a major role in salience (Bromberg-Martin et al., 2010; Cho et al., 2017) mainly *via* D1-like receptor (Brenhouse et al., 2008). Salience can be signaled by multiple factors, including intrinsic physical and chemical properties of the stimuli, the association with valenced stimuli, and the physiological state of the organisms, among others (Hyman, 2005; Cowan et al., 2021). Salient stimuli prioritize the consolidation of the relevant over neutral information to drive goal-relevant behaviors (Payne et al., 2008; Moessnang et al., 2012; Alger et al., 2019). We define stimulus salience as allowing subthreshold taste stimuli to be consolidated in long-term memory.

In this work, we aimed to increase the salience of subthreshold aversive and appetitive taste stimuli through the photostimulation of the VTA-IC dopaminergic pathway to facilitate the consolidation of taste recognition memories. Here we found that the photostimulation of VTA increases the salience to facilitate consolidation of taste stimuli regardless of the natural hedonic value and without altering its taste palatability. Consequently, photostimulation of the VTA dopaminergic terminals into IC also increases the salience to facilitate the consolidation of appetitive and aversive taste stimuli. However, the D1-like receptor activity into the IC is only required to consolidate both innate and learned aversive taste recognition memory.

MATERIALS AND METHODS

Animals

TH-Cre mice (Tyrosine Hydroxylase, FI12 line) express Cre-recombinase protein under the control of the endogenous tyrosine hydroxylase (TH) promoter. Breeder mice were kindly donated by Rui M. Costa from the Champalimaud (Center for the Unknown) and crossed onto C57BL/6J mice for at least six generations. Two-month-old (25–30 g bodyweight) female and male TH-Cre mice were used for all experiments. No differences were found between male and female mice in all experiments. Mice were housed individually at 20 ± 2°C, 50 ± 5% humidity, under a 12:12 h light/dark cycle with free access to food and water all time except during behavioral testing. All experiments were conducted during the light phase of the room illumination cycle. All experiments were approved by Instituto de Fisiología Celular (FBR125-18) and complied per the Official Mexican Standard (NOM-062-ZOO-1999).

Genotyping

The genotyping procedure was previously reported (Gil-Lievana et al., 2020). Once the mice were 1 month old, a tail snipping procedure was performed, and 1 mm of the tail was removed with sanitized sharp scissors. We used the HotSHOT method for DNA extraction. Briefly, the tail snip was lysed in an alkaline reagent (25 mM NaOH, 0.2 mM disodium EDTA) under heat conditions (95°C, 1 h) and further neutralization with a suitable buffer (1 M Tris-HCl, pH 7.4). After centrifugation (2,500 rpm, 2 min, Hermle Z 233 MK-2), the DNA's supernatant was recovered. The DNA was used for PCR amplification (201443, QIAGEN). Primers sequences were as follows: Cre forward primer 5′-AGC CTG TTT TGC ACG TTC ACC-3″; Cre reverse primer 5′-GGT TTC CCG CAG AAC CTG AA-3′ (both primers were purchased from Sigma-Aldrich).

Viral Vector

The Cre-inducible adeno-associated virus (AAV) was obtained from the University of North Carolina (UNC) Gene Therapy Center Vector Core. The viral concentrations were as follows: 5.2 × 1012 viral units/ml for rAAV5/EfIα-DIO-hChR2(H134R)-eYFP (ChR2); 6.0 × 1012 viral units/ml for rAAV5/EfIα-DIO-eYFP (eYFP). All viruses were aliquoted and stored at −80°C until use.

Stereotaxic Surgery

Mice were induced to anesthesia with 3% Isoflurane and maintained with 1–1.5% isoflurane (VETone FlurisoTM; Matrix VIP 3000, Midmark) until the end of surgery. Once anesthetized,

mice were placed in a stereotaxic apparatus (51603, Stoelting) with an incisive adapter (923-B, KOPF instruments). A small incision in the scalp was made, and the head was adjusted to the horizontal plane. The microinjection needles (29-G) were connected to a 10 μl Hamilton syringe and filled with AAV. For all experiments, the mice were bilaterally injected with AAV (0.5 μl) at a rate of 0.1 μl/min with an additional 5 min for diffusion. Mice were implanted with core optic fibers (200 μm) through zirconia ferrules (1.25-mm-wide) in each hemisphere. The AAV was injected into the VTA [from Bregma (mm) AP: −3.08; ML: ± 0.60 ML; DV −4.80]. The optic fibers were implanted above the VTA [from Bregma (mm) AP: −3.08; ML: ± 1.20; DV: −4.30 at 10° angle] or above the IC [from Bregma (mm) AP: + 1.40; ML: ± 3.30, DV: −3.5]. For pharmacological experiments, mice were implanted with bilateral 23-gauge stainless steel cannulas (8 mm long, Small Parts, Logansport, Indiana, United States) into IC [from Bregma (mm) AP: + 1.40; ML: ± 3.30; DV: −3.0]. Coordinates were taken from Allens reference atlas of the mouse brain. The cannulas and ferrules were anchored with dental adhesive and dental acrylic cement. Stylets were inserted into guide cannulas to prevent clogging. Mice were allowed to recover for 3 weeks before behavioral procedures.

Optogenetic Stimulation

The conditions for photostimulation were previously reported (Gil-Lievana et al., 2020), briefly: optogenetic stimulation consisted of a diode-pumped-solid-state blue laser (473 nm, 15 mW, 20 Hz, 5 ms width; OEM Laser Systems) coupled to 62.5 μm core, 0.22 NA standard multimode hard-cladding optical fiber (ThorLabs, New Jersey, United States) that passed through a single-channel optical rotary joint (Doric Lenses) before being split 50:50 with a fused optical coupler. The light intensity output was 12–15 mW per split fiber for all experiments. These photostimulation parameters have previously been shown to increase dopamine release by *in vivo* microdialysis experiments, producing electrophysiological responses in VTA neurons and modulation of IC neurons (Gil-Lievana et al., 2020).

Behavioral Procedures

Mice were water-deprived only during experimental days. Every afternoon, mice were supplied with water for 10 min to avoid dehydration. All experiments were conducted during the light phase of the illumination cycle in an acrylic bowl (height 36 cm, diameter 40 cm, CMA 120 bowl, Harvard apparatus). During five consecutive days, two randomized bottles of water were presented for 20 min (baseline). The inclusion criteria were that mice must consume from both bottles. The mice with a bottle preference (consumption index >0.6) during the acquisition session were discarded. A new cohort of mice was used for each behavioral experiment.

Taste Intensity Detection Test

The next day after the last baseline session, two bottles of quinine (low: 126 μM or high: 504 μM) or saccharin (low: 5 mM or high: 15 mM) were presented for 20 min. Twenty-four hours later, during the memory test, two bottles with water/quinine (low or high) or water/saccharin (low or high) were presented to mice for 20 min. The consumption index during the novel tastant exposure session was calculated dividing the volume of the tastant consumed (ml) by the sum of the volume of tastant (ml) and the mean of the baseline consumed (ml): tastant/(tastant + mean baseline).

Concomitant Optogenetic Stimulation and Tastant Exposure

Next day after baseline, two bottles of 126 μM quinine or 5 Mm saccharin were presented for 20 min (novel tastant exposure); at the same time, photoactivation of VTA, or VTA projections in the IC was performed during the 20 min session.

Brief Access Taste Task

Mice were water-deprived for 23 h and placed in an operant chamber equipped with a central sipper (Med Associates Inc., Fairfax, VT, United States), where one of three tastants could be delivered (water, quinine 126 μM, or saccharin 5 mM) controlled by a solenoid valve (Parker, Mayfield Heights, OH, United States). In each trial, mice randomly received one tastant for 5 s (2 μl drop in each lick), mice decided whether they lick during the entire reward period (Garcia et al., 2021). To start a new trial, mice needed to refrain from licking for a 1–3 s inter-trial interval (ITI) and lick once again after the ITI was finished. Mice were trained during five sessions, and three additional sessions were performed with laser stimulation, in which mice were opto-stimulated during the entire task at 20 Hz.

Conditioned Taste Aversion and Pharmacological Manipulations

The next day after the last baseline session, mice were injected into IC with vehicle (0.9% saline solution), or dopamine D1-like receptor antagonist SCH23390 (2 μg/μl, dissolved in 0.9% saline solution, D054, Sigma-Aldrich). Ten minutes after the drug injection, two bottles of 5 mM saccharin were presented for 20 min; simultaneously, photoactivation of VTA was performed during the 20 min session (the photostimulation conditions were like those previously described). Ten minutes after the tastant exposure, mice received an intraperitoneal injection of 0.15 M LiCl at 48 mg/kg body weight or 0.15 M NaCl at a dose of 66 mg/kg body weight and returned to their households. The consumption index during the training session were calculated by dividing the volume of the tastant (ml) by the sum of the volume of the tastant (ml) and mean of the baseline (ml): tastant/(tastant + mean baseline).

Twenty-four hours after the training session, a memory test was performed in all cases; one bottle that contained the taste (used during the training session) and one bottle of water were presented to mice for 20 min. Consumption indexes for the test were calculated by dividing the volume of the tastant by the total volume consumed: tastant/(tastant + water).

Immunofluorescence

After the test, mice were sacrificed with an overdose of intraperitoneal pentobarbital monosodium (200 mg/kg). Intracardiac perfusion was performed with 0.9% saline

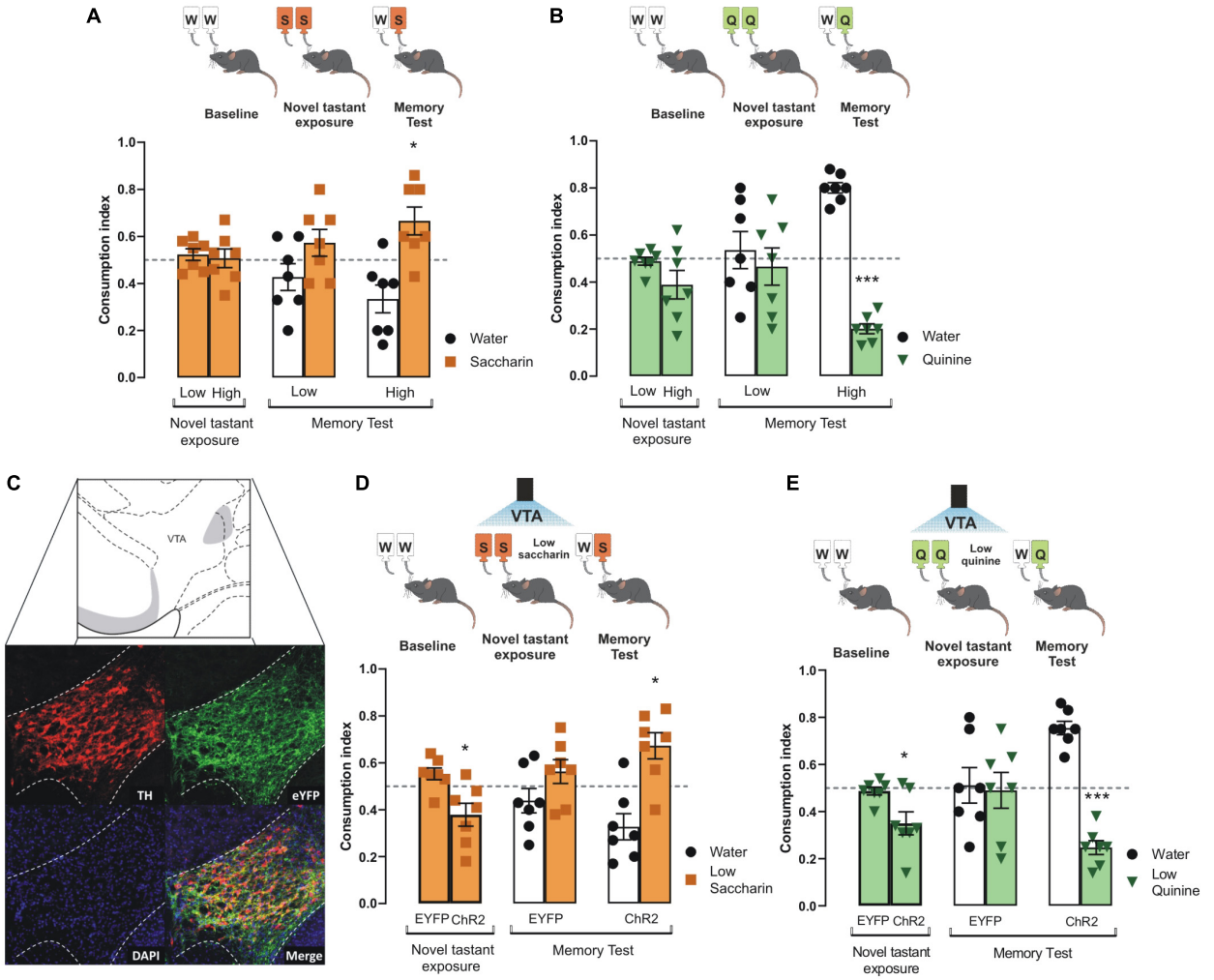

FIGURE 1 | Photoactivation of VTA dopaminergic neurons increases the salience of tastant stimuli regardless of the hedonic value. **(A)** During the novel tastant exposure session, no significant differences were reported between low and high saccharin (n = 7) in intact mice vs. randomness 0.5 [Low: one sample t-test, $t_{(6)}$ = 0.9305, p = 0.3880; High: one sample t-test, $t_{(6)}$ = 0.1805, p = 0.8627]. During the memory test, mice were not capable of discriminating low saccharin (5 mM)/water (n = 7) vs. randomness 0.5 [one sample t-test, $t_{(6)}$ = 1.283, p = 0.2470]. In comparison, high saccharin (15 mM)/water (n = 7) was discriminated vs. randomness 0.5 [one sample t-test, $t_{(6)}$ = 2.799, p = 0.0312]. **(B)** Mice do not show statistical significance in consumption index vs. randomness 0.5 for neither low or high quinine during the novel tastant exposure session vs. randomness 0.5 [Low: one sample t-test, $t_{(6)}$ = 0.6737, p = 0.5256; High: one sample t-test, $t_{(6)}$ = 1.834, p = 0.1164]. Accordingly, quinine memory test shows that low quinine (126 μM)/water (n = 7) was not discriminated vs. randomness 0.5 [one sample t-test, $t_{(6)}$ = 0.4336, p = 0.6797], while higher quinine (504 μM)/water (n = 7) was distinguished vs. randomness 0.5 [one sample t-test, $t_{(6)}$ = 14.01, $p < 0.0001$]. **(C)** Micrographs of the adenoviral infection in ventral tegmental area (VTA). Green shows the reporter protein enhanced yellow fluorescent protein (eYFP), red shows the expression of tyrosine hydroxylase (TH) protein, and the last micrograph shows the colocalization between eYFP and TH expression. VTA diagram adapted from Dong (2008). **(D)** During novel taste presentation, the optogenetic stimulation of VTA decreases the consumption index of low saccharin vs. randomness 0.5 [EYFP: one sample t-test, $t_{(6)}$ = 2.103, p = 0.0802; ChR2: one sample t-test, $t_{(6)}$ = 2.485, p = 0.0475]. The ability to discriminate saccharin solution was improved vs. randomness 0.5 during the memory test in ChR2 mice [ChR2 group n = 7, one sample t-test, $t_{(6)}$ = 3.101, p = 0.0211; EYFP group n = 7, one sample t-test, $t_{(6)}$ = 1.240, p = 0.2613]. **(E)** During novel taste presentation, the optogenetic of VTA decreases the consumption index of low quinine vs. randomness 0.5 [EYFP: one sample t-test, $t_{(6)}$ = 0.7756, p = 0.4675; ChR2: one sample t-test, $t_{(6)}$ = 3.086, p = 0.0215]. Accordingly, the performance of ChR2 mice to discriminate quinine vs. randomness 0.5 was improved during the memory test [EYFP group n = 7, one sample t-test, $t_{(6)}$ = 0.1320, p = 0.8993; ChR2 group n = 7, one sample t-test, $t_{(6)}$ = 8.526, p = 0.0001]. All data are shown as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

solution and pre-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution. Brains were removed and fixed in 4% paraformaldehyde and stored for 1 week. Brains were treated with 30% sucrose at least 2 days before slicing. Brains were sliced in 40 μm sections using a cryostat (Leica, CM1520). Free-floating sections were incubated with anti-TH (1:1,000, rabbit, P40101, Pel-Freez, Rogers, AR) overnight, at 4°C. Sections were washed with trizma buffer solution added with a triton (TBST; 150 mM NaCl, 100 mM trizma base, 0.1% triton X-100; all purchased from Sigma-Aldrich) incubated with CY3-conjugated goat anti-rabbit (1:250, AP132C, Millipore, Darm-Stadt, Germany) for 2 h. Antibodies were incubated in 5% bovine serum albumin in TBST.

Sections were washed with TBST and incubated with 300 nM 4',6-diamidino-2-fenilindol (DAPI, D9542, Sigma-Aldrich) for 1 min. After a final wash with TBST, sections were mounted in Dako fluorescence mounting medium. Immunofluorescence was observed using a ZEISS LSM 800 confocal microscope.

Statistical Analysis

For experiments, we used the minimum sample necessary to obtain a mean and standard deviation required for a Cohen's d parameter equals or greater than 0.8 (Lakens, 2013). Data were analyzed using GraphPad Prism software version 8.0 and Matlab R2021a. The Kolmogorov-Smirnoff test was performed for normal distribution. Data were plotted as mean ± SEM. One sample Student's *t*-test against 0.5 was performed for experiments showing consumption index during the memory tests and two-way ANOVA were used for raw data during the novel tastant exposure and the brief access taste task (BATT). For all statistical analyses $p < 0.05$ threshold was considered statistically significant.

RESULTS

The Activity of the Ventral Tegmental Area Dopaminergic Neurons Increases the Intensity of Appetitive and Aversive Taste Stimuli

To determine the role of VTA dopaminergic neurons to increase the intensity of innate appetitive and aversive taste stimuli, we tested the performance of mice to consolidate taste recognition memory (TRM) when two different concentrations of saccharin or quinine were presented. We did not find differences in consumption index for low and high saccharin (**Figure 1A**) nor quinine in intact mice during the novel tastant exposure session (**Figure 1B**). Interestingly, we found that only high, but not low, concentration of saccharin (15 mM, **Figure 1A**) or quinine (504 μM, **Figure 1B**) solution produced a reliable preference for saccharin and avoidance for quinine in mice during the memory test. However, mice did not recognize a very low concentration of either saccharin (5 mM, **Figure 1A**) or quinine solution (126 μM, **Figure 1B**) in comparison to water when presented during the memory test. Therefore, the low concentration of saccharin (5 mM) or quinine solution (126 μM) was insufficient to produce a TRM, and they were used as less-salient taste concentrations for our further experiments.

To study whether the VTA dopaminergic neurons increase the salience of appetitive and aversive taste stimuli, we injected TH-Cre mice with an adeno-associated virus encoding Cre-dependent channelrhodopsin-2 protein (ChR2). Enhanced yellow fluorescent protein (eYFP) was used as a reporter protein. Expression of eYFP reporter and TH immunoreactive neurons in coronal slices of VTA neurons is shown in **Figure 1C**. Colocalization analysis showed high expression of eYFP in VTA dopaminergic neurons with endogenous TH immunoreactivity (**Figure 1C**, merge). We found that the photostimulation of the VTA coupled to the presentation of low concentration saccharin (**Figure 1D**) or quinine (**Figure 1E**) solutions during the novel tastant exposure session decreased the consumption index in ChR2 mice, but not in eYFP mice. We found that the ChR2 mice showed a reliable TRM, measured by the strong preference for low saccharin solution (**Figure 1D**) or strong avoidance for low quinine solution (**Figure 1E**). However, during the memory test, eYFP mice did not consolidate a TRM to low concentrated saccharin or quinine solutions. These results suggest that VTA dopaminergic neurons increase the salience of low concentration appetitive and aversive taste stimuli to consolidate into TRM.

Photostimulation of Ventral Tegmental Area Dopaminergic Neurons Does Not Alter the Palatability of Appetitive or Aversive Tastes

To determine whether the behavioral effects induced by VTA dopaminergic neurons were due to a change in taste palatability, mice were placed in a Brief Access Taste Task (BATT). A BATT measures the oromotor responses (palatability) using the lick rate evoked by tastants during the 5 s reward period (Villavicencio et al., 2018). If dopaminergic neurons are related to taste palatability, we hypothesize that the stimulation would change the lick rate of familiar tastants (Garcia et al., 2021). During this task, mice received either low concentrated saccharin 5 mM, quinine 126 μM, or water for 5 s per trial (**Figure 2A**). As expected, eYFP mice exhibited a higher lick rate elicited by saccharin and a lower licking rate after quinine delivery (**Figure 2B**). Similar results were also observed when mice were tested at high tastants concentrations (**Figures 2E,F**). Importantly, taste palatability was not altered by the photostimulation of VTA dopaminergic neurons while mice licked for familiar tastes (**Figure 2C**). Specifically, the lick bout duration, a measure of palatability, in the ChR2 mice was not significantly different from eYFP mice (**Figure 2D**; two-way ANOVA, Factor mice, $F_{(1,21)} = 1.188$, $p = 0.28$). Collectively, our data demonstrate that the behavioral effects induced by photostimulation of VTA dopaminergic neurons were not related to taste palatability. Thus, it is more likely that stimulation of DA neurons affected saliency rather than taste palatability.

Specific Photostimulation of the Ventral Tegmental Area-Insular Cortex Dopaminergic Terminals Increases the Salience to Consolidate Taste Stimuli

To determine whether the VTA dopaminergic projections in the IC would solely consolidate taste information (Bermudez-Rattoni, 2014), we photostimulated the VTA dopaminergic terminals in the IC during the novel tastant exposure session. Congruently with prior studies, we found a greater level of off-target EYFP expression in the IC due to the tyrosine hydroxylase promoter used to control the expression of the transgenes in the TH-Cre mice (Lammel et al., 2015). The photostimulation of VTA dopaminergic projections into the IC (**Figure 3A**) did not

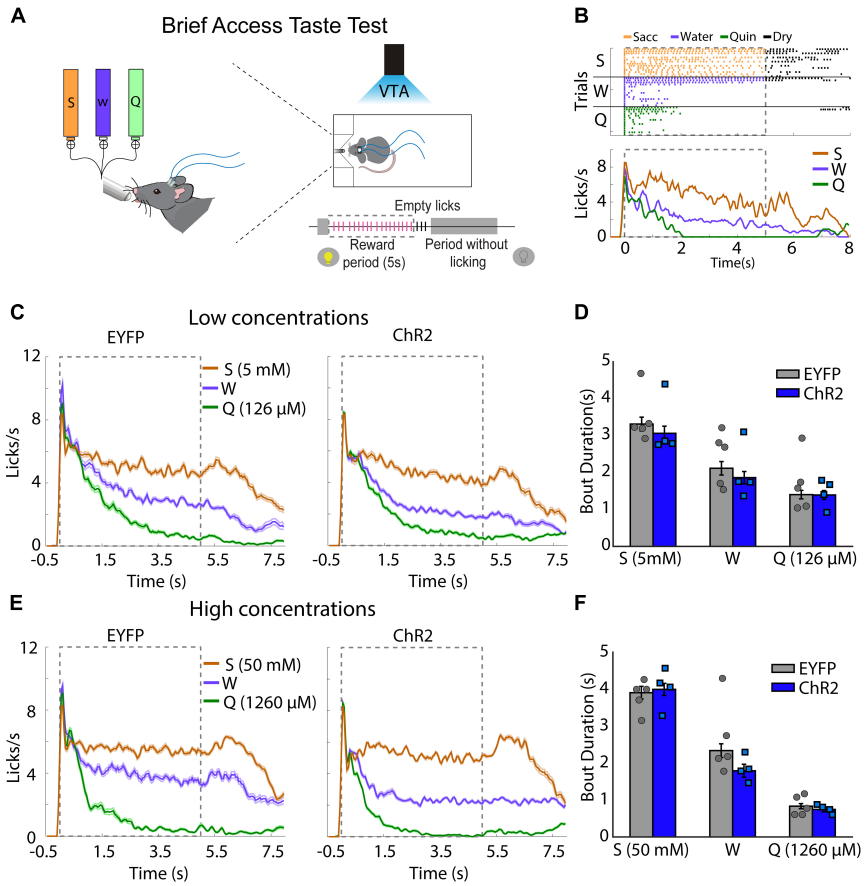

FIGURE 2 | Photoactivation of ventral tegmental area (VTA) dopaminergic neurons does not impact taste palatability. **(A)** Schematic representation of each trial, in which mice had access to different tastants for a brief time (5 s reward period) to either saccharin, water, or quinine, randomly. Mice received photostimulation during the complete session. **(B)** A representative raster plot for licking of one mouse and session. Each tick indicates a single lick and the color the tastant delivered (orange saccharin, blue water, green quinine); black ticks indicate empty (dry) licks. Below is the Peristimulus time histogram (PSTH) from a representative mouse. The dash and gray square indicate the reward period. **(C)** Population PSTH for all subjects and sessions for low concentrations. **(D)** Mean of lick bout duration (a measure of palatability, i.e., the longer, the more palatable) for each taste during the rewarded period. **(E,F)** like **(C,D)**, but for high concentrations. S, saccharin, W, water, or Q, quinine hydrochloride. Enhanced yellow fluorescent protein (eYFP) group (n = 5) and ChR2 group (n = 4). Differences were not observed in the bout duration between subjects for low concentrations [two-way ANOVA, Factor mice, $F_{(1,21)}$ = 1.188, p = 0.28] or high concentrations [two-way ANOVA, Factor mice, $F_{(1,21)}$ = 1.386, p = 0.25].

alter the consumption indexes in ChR2 mice of novel saccharin (**Figure 3B**) or quinine (**Figure 3C**) at low concentrations during the novel tastant exposure session. In contrast, ChR2 mice exhibited a reliable TRM during the memory test, as seen by the strong preference for saccharin relative to water (**Figure 3B**) and a strong avoidance for the quinine solution vs. water (**Figure 3C**). Our results showed that the photostimulation of the VTA-IC inputs is sufficient to consolidate a TRM in ChR2 mice but not in eYFP control mice.

The Salience to Consolidate Aversive, but Not Appetitive Taste Recognition Memory, Requires D1-Like Receptor Activity Into Insular Cortex

Having demonstrated the role of dopamine from VTA into the IC to process appetitive and aversive taste stimuli, we administered an antagonist of D1-like receptors (SCH23390) into the IC before the photostimulation of the VTA dopaminergic neurons in ChR2 and eYFP mice during the presentation of saccharin and quinine solutions. We found that the blockage of the D1-like receptors into the IC did not alter the consumption index during the novel tastant exposure session nor impair the salience to consolidate an appetitive TRM (**Figure 4A**), as measured by a preference for saccharin vs. water during the memory test. Nevertheless, the blockage of the D1-like receptors did not affect the consumption index during the novel tastant exposure session but impaired the salience to consolidate an aversive TRM (**Figure 4B**). These results suggest that the VTA-IC dopaminergic pathway increases the salience of taste stimuli regardless of the hedonic value to consolidate a TRM. Notably, the aversive but not appetitive taste stimuli require D1-like receptors into the IC to process the salience of TRM.

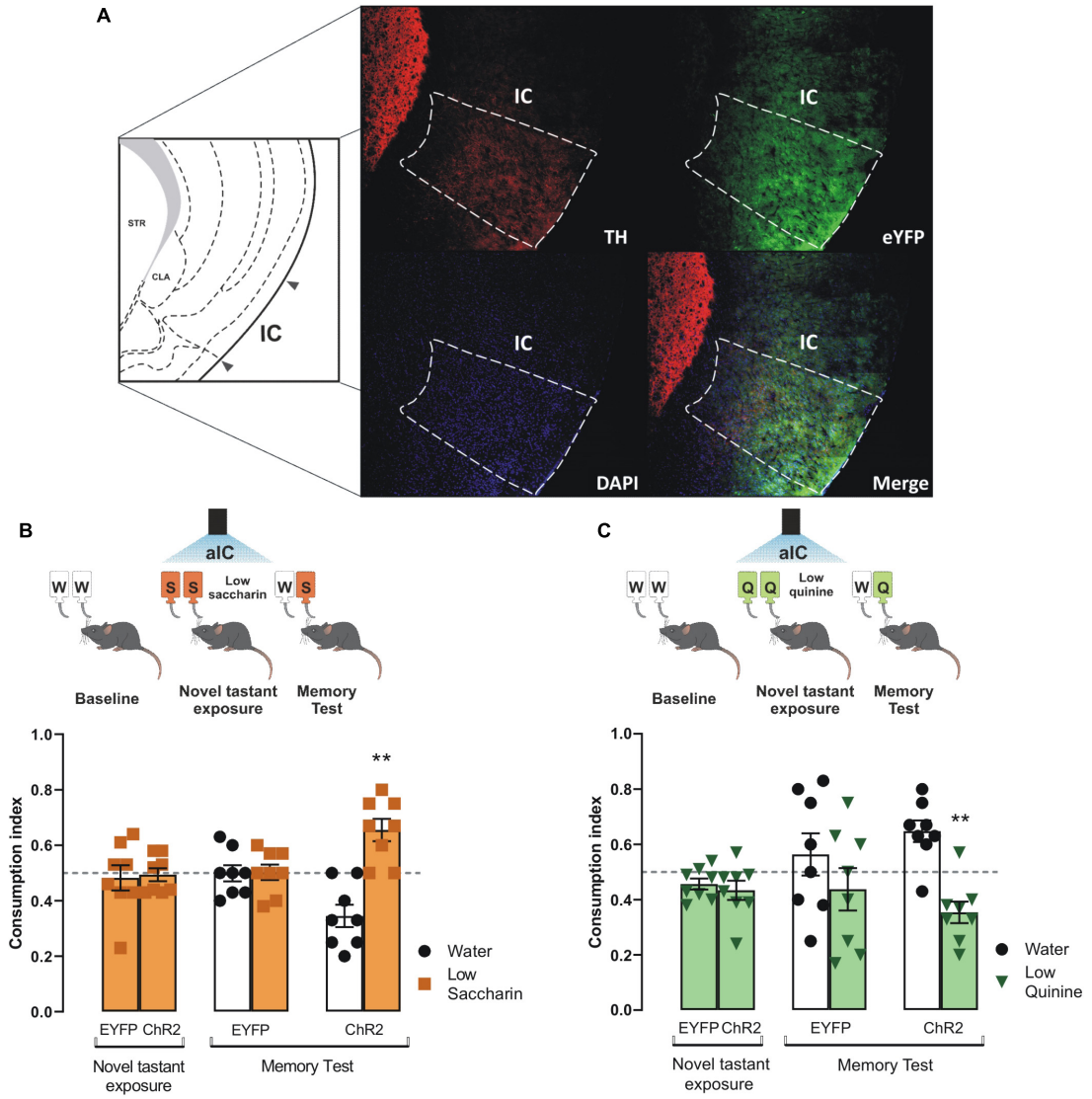

FIGURE 3 | Photostimulation of the ventral tegmental area (VTA)-insular cortex (IC) dopaminergic pathway increases the salience of taste stimuli. **(A)** Representative micrographs of triple immunofluorescence for tyrosine hydroxylase (TH) immunoreactive fibers in the IC (top, left), enhanced yellow fluorescent protein (eYFP) immunoreactive projections from the VTA into the IC (top, right), DAPI (bottom, left), and merge (bottom, right). IC diagram adapted from Dong (2008). **(B)** Optogenetic stimulation of VTA-IC dopaminergic projections during the novel tastant exposure session does not alter consumption index of low saccharin in ChR2 mice vs. randomness 0.5 [ChR2: one sample *t*-test, $t_{(7)} = 0.2666$, $p = 0.7975$; EYFP: one sample *t*-test, $t_{(7)} = 0.3856$, $p = 0.7113$]. However, in the memory test, the optogenetic stimulation of the VTA-IC dopaminergic projections in ChR2 mice ($n = 8$) enabled the preference for low saccharin solution vs. randomness 0.5 [one sample *t*-test, $t_{(7)} = 3.854$, $p = 0.0063$], but not in EYFP mice ($n = 8$) [one sample *t*-test, $t_{(7)} = 0.08904$, $p = 0.9315$]. **(C)** Optogenetic stimulation of VTA-IC dopaminergic projections in ChR2 mice during the novel tastant exposure session does not modify the consumption index of low quinine vs. randomness 0.5 [EYFP: one sample *t*-test, $t_{(7)} = 2.178$, $p = 0.0658$; ChR2: one sample *t*-test, $t_{(7)} = 1.910$, $p = 0.0978$]. Accordingly, during the memory test, the optogenetic stimulation of the VTA dopaminergic projections into the IC led ChR2 mice ($n = 8$) to avoid low quinine solution [one sample *t*-test, $t_{(7)} = 3.745$, $p = 0.0072$]. However, EYFP mice ($n = 8$) did not show a significant difference in consumption between low quinine vs. randomness 0.5 [one sample *t*-test, $t_{(7)} = 0.8147$, $p = 0.4421$]. All data are shown as mean ± SEM. $^{**}p < 0.01$.

Salience to Consolidate Conditioned Taste Aversion Requires D1-Like Receptor Activity Into Insular Cortex

Since the results showed that the VTA-IC dopaminergic pathways potentiated the salience of an innate aversive taste stimulus to consolidate a TRM through the D1-like receptors; we extended our research to study whether the same pathway is required to process the salience of associative aversive taste memories, such as a conditioned taste aversion memory. First, ChR2 and eYFP mice received VTA dopaminergic neurons photostimulation concomitantly with the presentation of the low saccharin solution (training session). ChR2 mice showed reduced consumption of low saccharin solution compared with eYFP mice (**Figure 5A**).

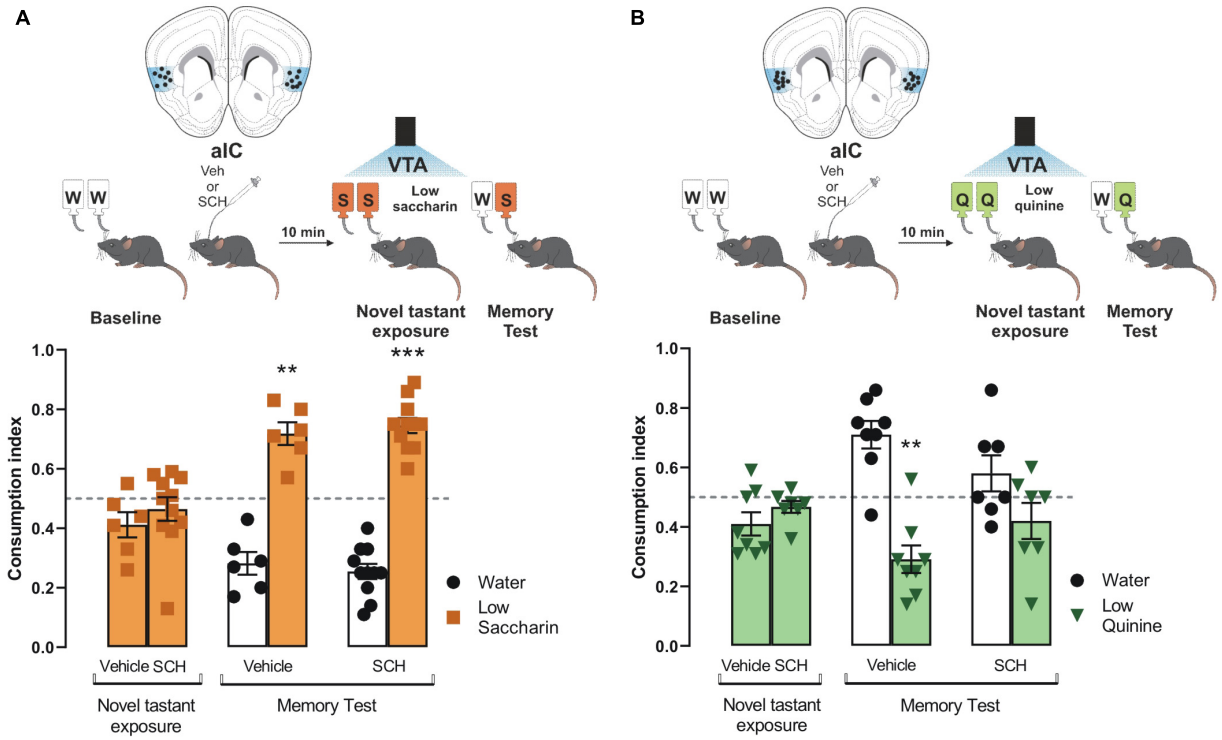

FIGURE 4 | Processing of aversive but not appetitive taste requires D1-like receptor activity into insular cortex (IC). Dopaminergic activity blockade in the IC impedes consolidation of aversive but not appetitive taste recognition memory (TRM). **(A)** ChR2 mice were administered with SCH23390 (n = 11) or vehicle (n = 6) before the photostimulation of the ventral tegmental area (VTA) dopaminergic neurons. No statistical significance was shown for consumption index of low saccharin in ChR2 mice/vehicle nor ChR2/SCH23390 mice vs. randomness 0.5 [ChR2/Vehicle mice: one sample t-test, $t_{(5)} = 2.077$, $p = 0.0925$; ChR2/SCH23390 mice: one sample t-test, $t_{(10)} = 0.8933$, $p = 0.3927$]. During the memory test, both mice groups exhibited a strong preference for the low saccharin solution vs. randomness [ChR2/Vehicle: one sample t-test, $t_{(5)} = 5.722$, $p = 0.0023$; ChR2/SCH23390: one sample t-test, $t_{(10)} = 9.670$, $p < 0.0001$]. IC coronal plane adapted from Dong (2008). **(B)** ChR2 mice were administered with SCH23390 (n = 7) or vehicle (n = 8) before the photostimulation of the VTA dopaminergic neurons. No statistical significance was shown for consumption index of low quinine in ChR2/Vehicle nor ChR2/SCH23390 vs. randomness 0.5 [ChR2/Vehicle: one sample t-test, $t_{(7)} = 2.313$, $p = 0.0540$; ChR2/SCH23390: one sample t-test, $t_{(6)} = 1.636$, $p = 0.1530$]. During the memory test, the group administered with SCH23390 before the optogenetic stimulation of the VTA-IC dopaminergic pathway impaired the avoidance behavior for the low quinine solution in the ChR2 mice vs. randomness 0.5 (n = 7) [one sample t-test, $t_{(7)} = 4.489$, $p = 0.0028$] but not in the ChR2 mice administered with vehicle vs. randomness 0.5 (n = 8) [one sample t-test, $t_{(6)} = 1.320$, $p = 0.2349$]. IC coronal plane adapted from Dong (2008). All data are shown as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

Ten minutes later, mice were randomly injected with a neutral stimulus (NaCl, 66 mg/kg) or a subthreshold of an unconditioned aversive stimulus (LiCl, 48 mg/kg) intraperitoneally. We found that neutral stimulation (NaCl) did not induce conditioned taste aversion. Moreover, eYFP mice could not discriminate between the low saccharin solution against water, but ChR2 mice preferred the low saccharin solution from water (**Figure 5A**). Interestingly, eYFP mice injected with the subthreshold LiCl (48 mg/kg) did not discriminate the low saccharin solution against water. Instead, ChR2 mice showed a robust conditioned taste aversion response due to the aversive association between the gastric malaise produced by the LiCl and the low saccharin solution (**Figure 5A**). Finally, using the protocol described above, ChR2 mice were injected with SCH23390 or vehicle into the IC 10 min before the conditioned taste aversion. Our results showed that the ChR2 mice injected with the vehicle had a strong conditioned aversive response, with a reliable avoidance behavior for the low saccharin solution associated with the gastric malaise produced by the LiCl. On the other hand, the ChR2 mice administered with SCH23390 were incapable of associating the unconditioned aversive stimulus with the low saccharin solution, showing a preference for the low saccharin solution rather than the water (**Figure 5B**). In sum, these results suggest that dopamine terminals activity from VTA to the IC also increases the salience of subthreshold stimuli, enhancing the association of an unconditioned aversive stimulus with an appetitive taste *via* D1-like receptors.

DISCUSSION

Our results demonstrate that the VTA photostimulation increases the salience of subthreshold taste stimuli, which are not likely to form long-term memory. The VTA photostimulation facilitates the consolidation of a taste recognition memory generated through naturally appetitive or aversive stimuli without an evident alteration on its taste palatability. In this sense, midbrain dopaminergic neurons modulate brain networks through phasic

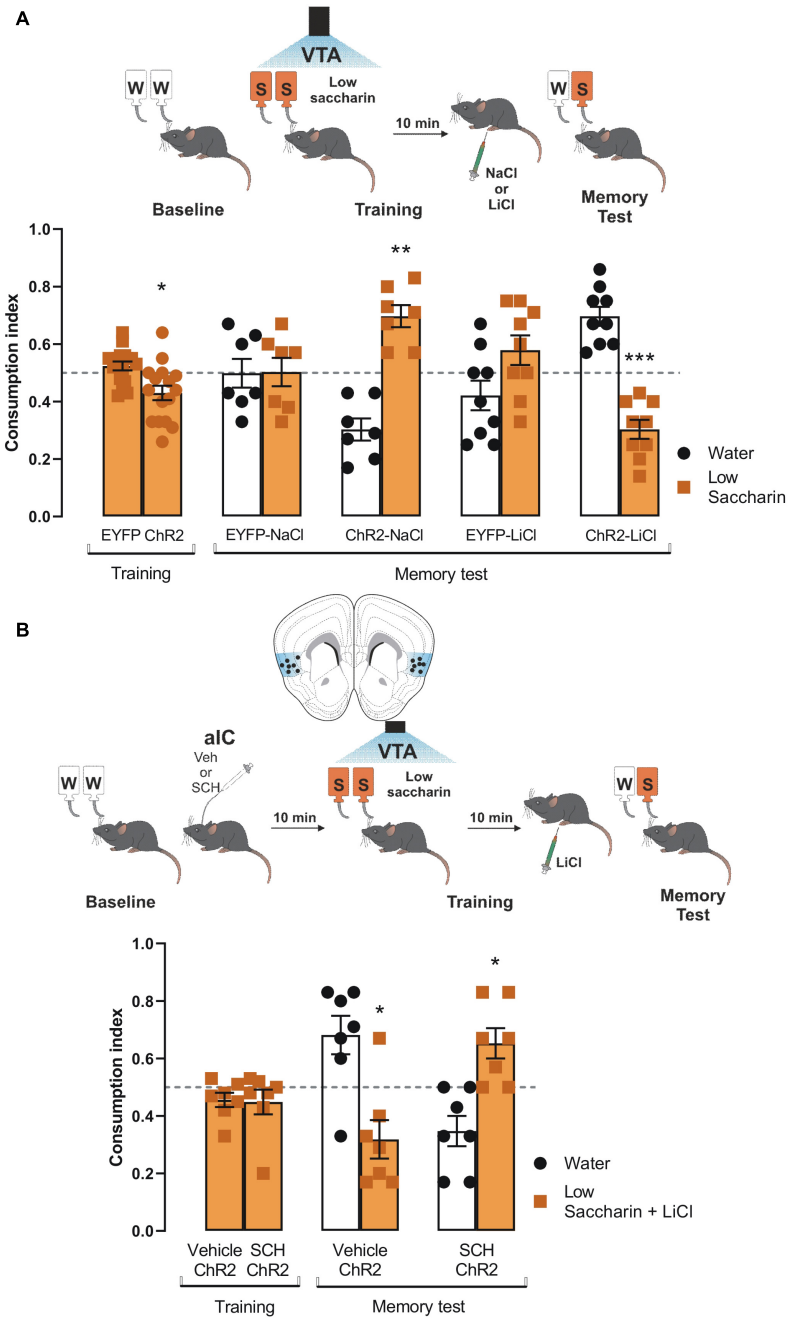

FIGURE 5 | Ventral tegmental area (VTA) photostimulation increases the salience of conditioned taste aversion for an appetitive taste. **(A)** ChR2 (n = 16) or EYFP (n = 16) mice were photostimulated into VTA when the low saccharin was presented during the training session. No statistical significance was shown for consumption index of low saccharin in EYFP nor ChR2 mice vs. randomness 0.5 [EYFP: one sample t-test, $t_{(15)}$ = 1.550, p = 0.1421; ChR2: one sample t-test, $t_{(15)}$ = 2.793, p = 0.0137]. After the training session, mice were intraperitoneally injected with an unconditioned stimulus NaCl (66 mg/kg) or LiCl (48 mg/kg). The administration of NaCl in ChR2 mice (n = 7) did not affect the preference for the low saccharin vs. randomness 0.5 due to the photostimulation of the VTA dopaminergic neurons [ChR2-NaCl: one sample t-test, $t_{(6)}$ = 5.109, p = 0.0022] but the consumption in EYFP was not affected [EYFP-NaCl: one sample t-test, $t_{(6)}$ = 0.05799, p = 0.9556]. The administration of LiCl in ChR2 mice decreased the consumption of the low saccharin vs. randomness 0.5 [ChR2-LiCl: one sample t-test, $t_{(8)}$ = 5.930, p = 0.0003], but the consumption of EYFP mice was not affected [EYFP-LiCl: one sample t-test, $t_{(8)}$ = 1.532, p = 0.1640]. **(B)** Diagram of coronal slice for insular cortex (IC) cannulation in ChR2 mice. SCH23390 (n = 7) or vehicle (n = 7) in ChR2 mice was administered before the photostimulation of the VTA dopaminergic neurons. No statistical significance was shown for consumption index of low saccharin in Vehicle/ChR2 mice nor SHC23390/ChR2 mice vs. randomness 0.5 [Vehicle/ChR2: one sample t-test, $t_{(6)}$ = 1.767, p = 0.1276; SCH23390/ChR2: one sample t-test, $t_{(6)}$ = 1.190, p = 0.2791]. Ten minutes later mice were intraperitoneally injected with a low dose of LiCl. The blockage of the D1-like receptors into the IC impairs the consolidation of the conditioned taste aversion in the SCH23390/ChR2 mice [one sample t-test, $t_{(6)}$ = 2.897, p = 0.0274]. In contrast, the vehicle/ChR2 mice showed a reliable conditioned taste aversion [one sample t-test, $t_{(6)}$ = 2.703, p = 0.0355]. IC coronal plane adapted from Dong (2008). All data are shown as mean ± SEM. *p < 0.05; **p < 0.01.

responses to encode not only the novelty, prominence, or surprise of rewarding stimuli but also aversive experiences (Schultz, 1998; Redgrave et al., 1999; Horvitz, 2000; Di Chiara, 2002; Joseph et al., 2003; Pezze and Feldon, 2004; Ungless et al., 2004; Lisman and Grace, 2005; Redgrave and Gurney, 2006; Schultz, 2007a; Moriya et al., 2018). The salience of stimuli, including taste, is adaptive because salient information tends to be preferentially consolidated into long-term memory (Shohamy and Adcock, 2010; Cowan et al., 2021). Importantly, we further extended our previous proposal by demonstrating that the increased VTA dopaminergic neuronal activity did not alter taste palatability. Our results agree with previous results, showing that dopaminergic neurotransmission affects novel taste intake without affecting taste evoked orofacial responses, another parameter of taste palatability (Treit and Berridge, 1990). Therefore, our findings suggest that the dopaminergic activity of the VTA pathway is involved in salience to consolidate the taste memory trace without affecting palatability. We observed a reduced consumption of the novel low concentration of tastants with the optogenetic stimulation of the VTA dopaminergic neurons, but not during the photostimulation of the VTA-IC dopaminergic pathway. However, the photostimulation of either VTA or VTA-IC terminals with low taste concentrations induced taste memory consolidation in both cases. These results suggest that in the first case, the photostimulation of VTA might trigger dopamine release into different brain structures potentially implicated in motivated behavior (Bromberg-Martin et al., 2010), motor control (Bourdy and Barrot, 2012), or even reward processing (Lammel et al., 2011). However, this unspecific effect does not interfere with taste memory consolidation.

After novel food consumption, c-fos activity is increased in the VTA and its projection areas within the mesolimbic and mesocortical dopaminergic systems, necessary to form memory (Kest et al., 2012; Dela Cruz et al., 2016). Additionally, we recently reported that photoactivation of the VTA dopaminergic neurons at 20 Hz for 20 min induces dopamine release in the IC, which modulates the neuronal activity in this cortical structure (Gil-Lievana et al., 2020). Here we found that VTA-IC dopaminergic activity increases the salience needed to preferentially facilitate consolidation of a taste recognition memory regardless of the hedonic value of taste stimuli. Similar outcomes were obtained by Kutlu et al. (2021), who show that dopamine contributes to fit valence-independent perceived salience across different learning paradigms. We hypothesize that the increased salience to facilitate consolidation into IC results from the synaptic potentiation induced by photostimulation of the VTA dopaminergic neurons. It is widely known that tonic and phasic dopaminergic release differentially modifies and modulates the synaptic strength and neuronal activity in many different structures, including the hippocampus, prefrontal, and insular cortices, to serve as a cellular mechanism to establish memory (Matsuda et al., 2006; Sheynikhovich et al., 2013; Rodríguez-Durán and Escobar, 2014; Otani et al., 2015; Navakkode et al., 2017; Papaleonidopoulos et al., 2018). In fact, findings support that brief exposure to a novel environment reduces the threshold to induce long-term potentiation. This facilitatory effect occurs for a short period of time following novelty exposure and depends on the activation of D1-like receptors but is absent in animals that explore a familiar environment (Li et al., 2003). Moreover, it has been demonstrated that aversive stimuli selectively modify synapses on dopaminergic neurons that project to cortical areas (Lammel et al., 2011). Furthermore, the dysfunctional dopaminergic activity alters the synaptic plasticity in the BLA-IC pathway to transform the long-term potentiation into long-term depression associated with memory impairment (Moreno-Castilla et al., 2016).

Although our results show that the VTA-IC dopaminergic activity is sufficient to increase the salience of aversive and appetitive stimuli, we found that D1-like receptor activity is necessary to integrate aversive taste information to facilitate the consolidation of an aversive taste recognition memory. Interestingly, the consumption index during the novel tastant exposure (acquisition) was not altered, regardless of the inhibition of D1-like receptors, but impairs the aversive long-term memory. Accordingly, the presentation of a novel taste stimulus, regardless of its valence, induces an increment of basal concentration of dopamine in the IC (Osorio-Gómez et al., 2021). However, previous reports have shown that the D1-like receptor is critical for aversive recognition memories formation, increasing neural activity within cortical networks (Heath et al., 2015; Saito et al., 2020). It is parsimonious to hypothesize that the VTA-IC dopaminergic pathway is one of many circuits encoding salience of taste stimuli regardless of the hedonic value due to the processing of salience to consolidate taste information involves multiple brain areas (Bromberg-Martin et al., 2010; Shiner et al., 2015; Cai et al., 2020; Kutlu et al., 2021). However, a D1-like receptor within IC is required for the consolidation and storage of mainly aversive taste recognition memory. Moreover, we found that D1-like receptor activity is also required to establish a conditioned taste aversion. Importantly, the blockage of the D1-like receptor into the IC before a conditioned taste aversion impairs the consolidation, but not the short-term memory, suggesting that the D1-like receptor is involved in the cognitive processing to consolidate the conditioned taste aversion (Osorio-Gómez et al., 2021). This study's results agree with reports suggesting that D1-like, but not D2/D3 dopaminergic receptor activity is associated with conditioned taste aversion (Fenu et al., 2001).

Dopamine plays a critical role in mediating reward and aversive signals of various stimuli, including visual, auditory, and mechanosensory stimuli (Wise, 2004; Schultz, 2007b; Zweifel et al., 2011; McCutcheon et al., 2012; Stelly et al., 2019). We show that although dopamine signaling in the IC is required to consolidate aversive and appetitive taste memories, the downstream molecular events may differ. Indeed, the PKC activity, a kinase involved in neural plasticity processes, is needed in the IC to establish aversive taste memory, but not for appetitive taste memory (Núñez-Jaramillo et al., 2007). We propose that dopamine from VTA encodes the salience of appetitive and aversive taste, but the interaction of different brain structures (e.g., basolateral amygdala and nucleus accumbens) and other neurotransmitters (i.e., glutamate) within the IC leads to the activation of different signaling pathways to facilitate consolidation of the aversive appetitive nature of the taste memory.

All in all, we found that the activity of the VTA-IC dopaminergic pathway increases the salience to facilitate the consolidation of aversive and appetitive taste stimuli. However, the IC only consolidates the aversive taste information through the D1-like dopaminergic receptors. Given the complexity of VTA connectivity, it is reasonable to hypothesize that the encoding of tastant's salience requires the processing of multiple parallel brain regions. Here we unveiled the VTA-IC dopaminergic pathway as an essential component of this complex circuitry involved in salience to facilitate the consolidation of taste recognition memories. Importantly, it is very likely our photostimulation parameters do not replicate any endogenous process in the VTA-IC pathway. Therefore, future studies should identify a more defined temporal window in which the VTA-IC dopaminergic pathway is required to increase the salience of novel taste stimuli to consolidate taste memory.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Instituto de Fisiología Celular (FBR125-18).

AUTHOR CONTRIBUTIONS

EG-L, GR-M, OU-M, RG, and FB-R designed the research and wrote the manuscript. EG-L, GR-M, and OU-M performed behavioral and optogenetic experiments. JL-I performed experiments for brief access taste test. EG-L and GR-M performed imaging analysis. All authors contributed to the article and approved the submitted version.

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Affective Valence Regulates Associative Competition in Pavlovian Conditioning

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Evidence suggests that, in Pavlovian conditioning, associations form between conditioned stimuli and multiple components of the unconditioned stimulus (US). It is common, for example, to regard USs as composed of sensory and affective components, the latter being either appetitive (e.g., food or water) or aversive (e.g., shock or illness) and, therefore, to suppose different USs of the same affective class activate a common affective system. Furthermore, evidence is growing for the suggestion that, in competitive learning situations, competition between predictive stimuli is primarily for association with the affective system activated by the US. Thus, a conditioned stimulus (CS) previously paired with one US will block conditioning to another CS when both are presented together and paired with a different US of the same affective class, a phenomenon called transreinforcer blocking. Importantly, similar effects have been reported when steps are taken to turn the pretrained CS into a conditioned inhibitor, which activates the opposing affective state to the excitor that it inhibits. Thus, an appetitive inhibitor can block conditioning to a second CS when they are presented together and paired with foot shock. Here we show that the same is true of an aversive inhibitor. In two experiments conducted in rats, we found evidence that an aversive inhibitor blocked conditioning to a second CS when presented in a compound and paired with food. Such findings demonstrate that affective processes and their opponency organize appetitive-aversive interactions and establish the valences on which they are based, consistent with incentive theories of Pavlovian conditioning.

Keywords: Pavlovian conditioning, incentive learning, appetitive-aversive interactions, prediction error, affect, valence, motivation

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INTRODUCTION

One consequence of pairing a neutral cue with an unconditioned stimulus is that the former can take on some of the motivational and affective properties of the latter through a process of Pavlovian incentive learning. Perhaps the most sophisticated account of Pavlovian incentive learning is that developed by Konorski (1967)—see **Figure 1**. In this view Pavlovian conditioning comes in two forms: *consummatory* and *preparatory*; the former driven by associations between a conditioned stimulus (CS) and the sensory properties of the unconditioned stimulus(US), producing discrete conditioned responses (CRs) such as chewing or blinking (Debold et al., 1965; Schmajuk and Christiansen, 1990), and the latter by associations with the affective properties of the US, producing

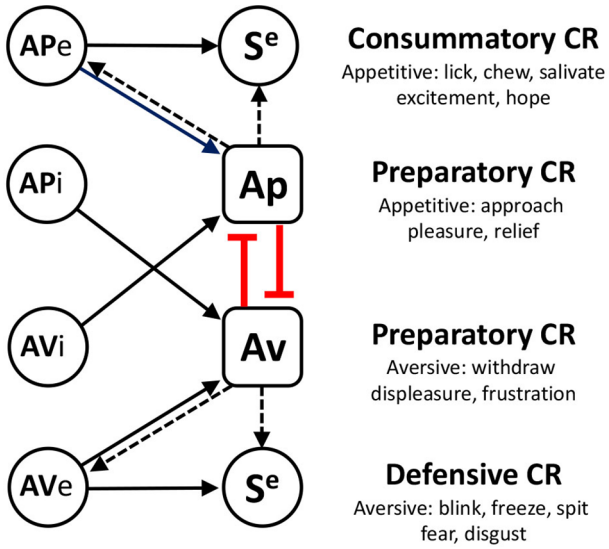

FIGURE 1 | A summary of Konorski's theory of Pavlovian incentive learning. Appetitive (APe) and aversive (AVe) excitors activate sensory specific (S^e) and affectively specific (Ap or Av) components of the US representation to generate consummatory (defensive) or preparatory conditioned responses (CR), respectively. At the heart of this position is an opponency between appetitive and aversive affective systems which are assumed mutually to inhibit one another. One consequence of this view is that it provides a principled account of conditioned inhibition. Thus, appetitive inhibitors (APi) are aversive and so activate the aversive system whereas aversive inhibitors (AVi) are appetitive and activate the appetitive system. As a consequence, inhibitors can influence their concomitant excitors *via* this mutual inhibitory process (shown in red), reducing preparatory CR's directly, and consummatory (and defensive) CR's indirectly by reducing affective activation (dashed arrows).

responses characteristic of the affective class to which that US belongs; approach when the US is appetitive and withdrawal when aversive (Dickinson and Dearing, 1979; Dickinson and Balleine, 2002). Although there is no doubting the importance of associations with the sensory properties of the US, the current research was focused on evaluating the nature of associations with the affective properties of the US.

In this context, the general claim, following Konorski, is that different USs from the same affective class activate a common affective system, perhaps the strongest evidence for which comes from studies of transreinforcer blocking. Blocking refers to the observation that a CS previously associated with a US can reduce, or block, conditioning to another CS when they are subsequently presented in compound and paired with the US (Kamin, 1968). However, whereas blocking typically employs the same US during initial and compound training, Bakal et al. (1974) observed that a CS pretrained with a foot shock US could block conditioning to a second CS when the compound was paired with a startle-eliciting auditory US, even though the sensory properties of these USs differ substantially. What they have in common, of course, is that they are aversive, and so transreinforcer blocking is usually taken as evidence that blocking is driven by competition for association with the affective system activated by the US. Transreinforcer blocking has also been observed using distinct appetitive USs; e.g., food pellets and sucrose solution for hungry rats (Rescorla, 1999) and food pellets and water for hungry and thirsty rats (Ganesan and Pearce, 1988).

Konorski also claimed that appetitive and aversive systems mutually inhibit one another, a claim supported by studies of counterconditioning in which pairing a CS with a mild paraorbital shock in rabbits was found to inhibit a previously established appetitive CRs to that CS (Lovibond and Dickinson, 1982) and *vice versa* (Scavio and Gormezano, 1980). Although such findings were important, perhaps even more important was the recognition that Konorski's account of this interaction between contrasting motivational systems also provides a principled explanation for both the associative and affective properties of conditioned inhibitors. A conditioned inhibitor is a stimulus that signals the omission of an otherwise predicted US. From an affective perspective, appetitive inhibitors—that signal the omission of USs like food—are aversive whereas aversive inhibitors—that signal the omission of USs like a shock—are clearly appetitive. Therefore, if competition in Pavlovian conditioning is for association with the affective properties of the US, an appetitive inhibitor should block conditioning to an aversive excitor and an aversive inhibitor should block conditioning to an appetitive excitor—see **Figure 1**.

The original findings supporting this claim were reported in a chapter some years ago (Dickinson and Dearing, 1979). More recently, we have been able to replicate one part of this report, finding evidence that a CS that predicts the omission of a food US blocks aversive conditioning of its associate across parings of the compound and foot shock (Laurent et al., 2018). The current experiments investigated the opposite arrangement, evaluating the capacity of an aversive inhibitor to block appetitive conditioning to its associate across pairings of the compound and a food US. The aversive inhibitory CS was generated using backward conditioning during which the stimulus was presented a few seconds after the administration of a foot shock US. To confirm the efficacy of this arrangement, an aversive excitory CS was also established through standard forward conditioning during which the stimulus immediately preceded the administration of the foot shock US. Experiment 1 investigated the effect of the backward CS-shock pairings (i.e., shock-S2) against forward pairings (i.e., S1-shock). Experiment 2 compared the influence of these forward and backwardly paired CSs against neutral control CSs.

METHODS

Subjects

The subjects were 24 experimentally naive male Sprague-Dawley rats (Rattus Norvegicus; 8–12 weeks old; 300–500g) obtained from the Animal Resources Center (Perth, WA). They were housed in plastic boxes (67 cm × 40 cm × 22 cm; eight rats per box) located in a climate-controlled colony room. The room was maintained on a 12 h light/dark cycle (lights on between 7 a.m and 7 p.m) and all procedures took place during the light cycle. Three days before the behavioral procedures, the rats were handled daily and put on a food deprivation schedule to maintain

them at around 90% of their free feeding weight. The Animal Care and Ethics Committee of the University of New South Wales approved all procedures.

Behavioral Apparatus

Training and testing took place in 12 Med Associates (St. Albans, VT, USA) operant chambers enclosed in sound- and light-resistant shells. The floors consisted of stainless-steel rods that were 3.8 mm in diameter, spaced 1.6 cm apart (center to center), and wired to a constant current generator that could deliver a shock. Each operant chamber was equipped with a pellet dispenser that could deliver a single grain pellet (45 mg; BioServe Biotechnologies) into a recessed magazine, an auditory stimulus generator, that could deliver a 1 kHz tone stimulus, and a 28 V DC mechanical relay that generated a 2 Hz clicker stimulus. There were three stimulus lights, two positioned on the same wall to either side of the magazine, providing a 2 Hz flashing visual stimulus when activated, and a 3 W, 24 V house light positioned on the opposite wall. An infrared photobeam allowed for the detection of magazine entries and a camera mounted on the back wall of each shell and connected to a monitor and a DVD recorder located in another room, recorded the behavior of each rat. An infrared light source illuminating each chamber was used to visualize behavior conducted in the dark (i.e., the house light was used as a discrete stimulus). A set of two microcomputers running proprietary software (Med-PC; MED Associates) controlled all experimental events and recorded magazine entries.

Behavioral Procedures

General Procedures

Four distinct stimuli were used (S1, S2, S3, and S4): clicker, tone, the constant house light, and flashing stimulus lights. The duration of the individual stimuli or compounds of these stimuli (always one visual and the other auditory) was 20 s. In any single session, presentations of the stimuli or compounds were separated by an inter-trial interval that ranged from 4 min to 6 min with an average of 5 min. In all experiments, the intensity of the foot shock was 0.5 mA with a duration of 0.5 s.

All experiments started with two sessions of pre-exposure across two consecutive days during which each stimulus was presented twice. Two sessions of magazine training were then given across two consecutive days for 30 min during which food pellets were delivered on random-time 60 s schedule. The aim of these sessions was to familiarize the rats with the pellets and overcome neophobia.

Experiment 1

Aversive training—Following pre-exposure and magazine training, two sessions of aversive training were given each day for six consecutive days. One session involved forward conditioning in which four presentations of S1 terminated in the delivery of foot shock. The other session involved backward conditioning in which each of the four deliveries of the foot shock were followed 5 s later by presentation of S2. The order of the sessions was counterbalanced across days. For half of the rats, S1 and S2 were auditory (tone and clicker, counterbalanced) whereas for the remainder they were visual (constant house light or flashing stimulus lights, counter balanced). Aversive responses (freezing) were recorded across conditioning in the presence of S1 and S2 and the 20 s period preceding (pre) either S1 or the shock in the case of the S2 sessions.

Appetitive training—The day after aversive training, rats received an additional magazine training session in the manner described. Next, appetitive training was given across two sessions on two consecutive days. In each session, S1 was presented in compound with S3 and S2 in compound with S4. Presentations of the S1S3 and the S2S4 compounds terminated in the delivery of a food pellet. All compounds were pseudo-randomly presented four times in each session. The stimuli were counterbalanced such that S3 and S4 were auditory in rats for which S1 and S2 were visual, whereas S3 and S4 were visual in rats for which S1 and S2 were auditory. Only two sessions of appetitive training were administered to avoid extinction of the aversive properties of the aversive CSs. Throughout these sessions, magazine entries were recorded and separated into stimulus and compound periods and pre-stimulus and pre-compound periods of equal length (20 s).

Appetitive testing—A single test session was administered 24 h after the end of appetitive training. The two previously trained compounds (S1S3 and S2S4) were presented once followed by the delivery of the food pellet outcome. Then, stimuli S3 and S4 were presented alone twice in the following order: S3-S4-S4-S3. Magazine entries were recorded and separated into stimulus/compound period and a pre-stimulus/compound period with equal length (20 s).

Experiment 2

Aversive training—Following pre-exposure and magazine training, eight consecutive days of aversive training were administered. There was a single daily session across the first 4 days. For half of the rats (Group Forward), the session involved forward conditioning to stimulus S1 in the manner described previously. For the other half of the rats (Group Backward), the session consisted of backward conditioning to stimulus S1 in the manner described previously. From day 5 of aversive training, the rats received an additional training session each day for the next 4 days during which a second stimulus S2 was presented without any consequence. The two daily sessions (the one with S1 and the one with S2) were given at least 2 h apart and the order in which they occurred was counterbalanced. For half of the rats in each group, S1 and S2 were visual stimuli (constant house light or flashing stimulus lights, counterbalanced), whereas they were auditory stimuli (clicker or tone, counterbalanced) for the other half. Aversive responses were recorded during the 20 s stimulus period and the immediately preceding 20 s period (pre) in the group that received forward conditioning and during the stimulus period and the 20 s period immediately prior to the delivery of the foot shock (pre) in the group that received backward conditioning.

Aversive training was followed by one session of magazine training, two daily sessions of appetitive training and one session of appetitive testing. These sessions were identical to those described in Experiment 1.

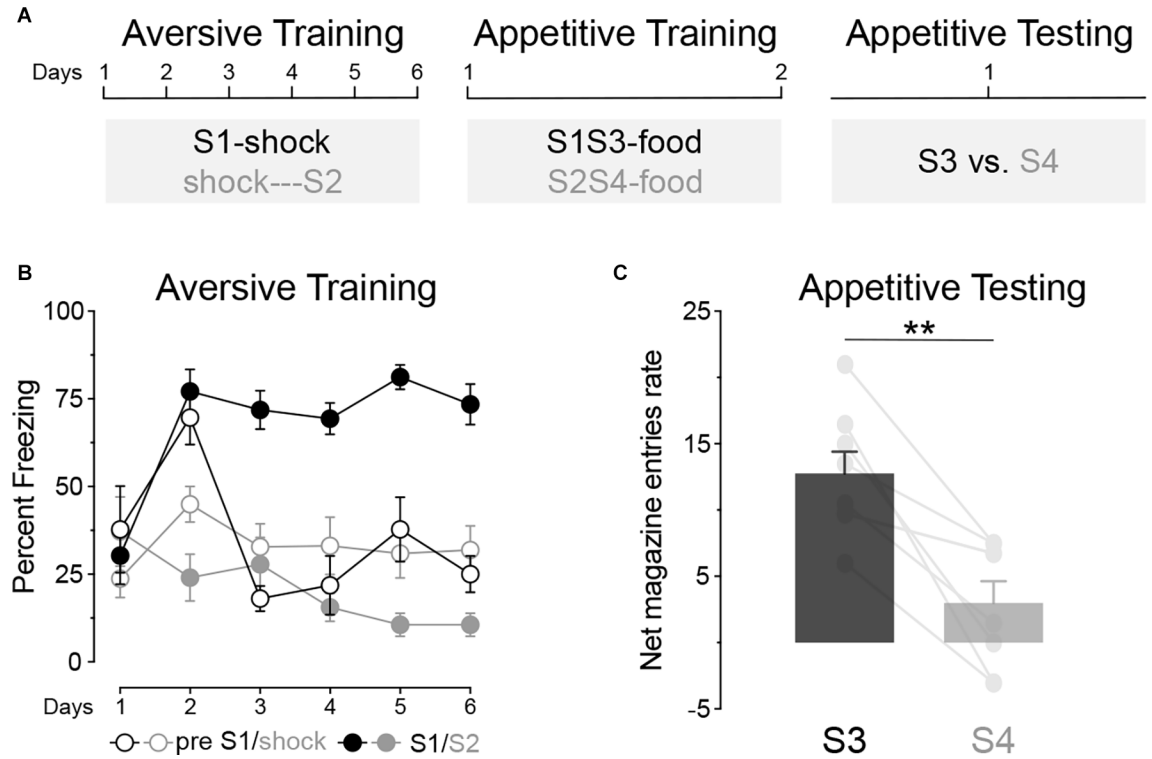

FIGURE 2 | The opposing effect of an aversive excitor and an aversive inhibitor on appetitive conditioning. **(A)** Design of Experiment 1; S1/S2/S3/S4: clicker, tone, flashing light or constant light (counterbalanced). **(B)** Aversive conditioning: S1 became an aversive excitor whereas S2 became into an aversive inhibitor. **(C)** Appetitive conditioning test: stimulus S3 elicited significantly more appetitive responding than stimulus S4 at test. Error bars denote ± 1 SEM. Asterisks denote significant effect (**$p < 0.01$).

Statistical Analyses

Appetitive responding was recorded by the Med Associates software. Aversive responding was rated in a time-sampling manner and judged as either freezing or not freezing every 2 s by a trained observer blind to the subjects' group assignment. A proportion of test data was cross-scored by a second naïve observer; there was a high level of agreement between observers (Pearson product moment correlation >0.9). Freezing was defined as the absence of all movement, except those related to breathing. The differences between groups or stimuli were analyzed by means of planned orthogonal contrasts. Within-session changes in responding were assessed by a planned linear trend analysis. All these procedures and analyses have been described by Hays (1973); see also Harris (1994) and were conducted in the PSY software (School of Psychology, The University of New South Wales, Australia). The Type I error rate was controlled at $\alpha = 0.05$ for each contrast tested. If interactions were detected, follow-up simple effects analyses were calculated to determine the source of the interaction.

RESULTS

Experiment 1

Experiment 1 used a within-subjects design to examine whether an aversive excitor and an aversive inhibitor exert distinct effects on appetitive conditioning. The design, shown in **Figure 2A**, had three stages: aversive conditioning, in which rats ($n = 8$) received S1 paired forwardly and S2 paired backwardly with foot shock, preceding S2 by a few seconds (Moscovitch and LoLordo, 1968); appetitive conditioning, in which S1 and S2 were presented in compound with two novel stimuli, S3 and S4, to form S1S3 and S2S4 compounds paired with a food pellet outcome; and a test phase in appetitive conditioning to S3 and S4 was assessed.

Training

During aversive training (**Figure 2B**), the forwardly paired S1 elicited more freezing than the backwardly paired S2 (Period; S1 vs. S2; $F_{(1,7)} = 107.24$, $p < 0.001$) and this difference grew larger as training progressed (Period × Days; $F_{(1,7)} = 17.19$, $p < 0.01$). Freezing in the absence of the stimuli (the "pre" period) was similar in the forward and backward sessions (pre S1 vs. pre shock; $F < 0.5$). Pre-responding was lower than that to S1 (pre vs. S1; $F_{(1,7)} = 51.63$, $p < 0.001$), confirming that fear was increased by S1. By contrast, pre-responding was greater than that elicited by S2 (pre vs. S2; $F_{(1,7)} = 7.894$, $p < 0.05$), confirming that fear was reduced by S2. These results suggest, therefore, that S1 became an aversive excitor and S2 an aversive inhibitor. During appetitive training (**Table 1**) no significant difference was found between the S1S3 and S2S4 compounds or in responding in the presence or absence of these compounds (Fs < 1.3).

TABLE 1 | Appetitive training data for Experiment 1—Magazine entries per minute (mean ± s.e.m) in the absence (pre) or presence of the two compounds S1S3 and S2S4.

Days	Magazine entries per minute ± (mean s.e.m)		
	pre	S1S3	S2S4
1	5.86 ± 1.05	5.53 ± 1.66	5.25 ± 1.23
2	6.61 ± 0.65	7.87 ± 1.86	7.13 ± 1.13

Test

The data from the final test are shown in **Figure 2C**. It is clear that stimulus S3 elicited more appetitive responding than stimulus S4 (S3 vs. S4; $F_{(1,7)} = 21.90$, $p < 0.01$) suggesting that the aversive inhibitor, S2, attenuated conditioning to the neutral S4 stimulus.

Experiment 2

Although consistent with the claim that the aversive inhibitor, S2 in Experiment 1, blocked appetitive conditioning to the added stimulus, S4, it is possible that the aversive excitor, S1, increased conditioning to the neutral stimulus S3 and, as it stands, it is not possible to establish whether conditioning was elevated to S3 or was depressed to S4. Experiment 2 sought to resolve this issue using the between-subject design shown in **Figure 3A**. In the first aversive conditioning stage, two groups were used, Group Forward ($n = 8$) received forward pairings of S1 and foot shock, whereas Group Backward ($n = 8$) received backward pairings of S1 and shock. For both groups a second stimulus, S2, was presented alone unpaired with shock. In appetitive conditioning the two pre-trained stimuli in compound with two distinct novel stimuli, S1S3 and S2S4 and both were paired with the delivery of a food pellet. Finally, appetitive conditioning to S3 and S4 was assessed in a final test stage. As a consequence, in both groups S4 was compounded with a neutral stimulus, S2, which acted as a control to evaluate the influence of an aversive excitor (S1 in Group Forward) or an aversive inhibitor (S1 in Group Backward) on appetitive conditioning to S3.

Training

For aversive training (**Figure 3B**) we first considered performance to S1. This training revealed a significant difference in freezing between the two groups of rats, Group Forward and Backward (Group: Forward vs. Backward; $F_{(1,14)} = 119.85$, $p < 0.001$), freezing in the presence vs. absence of S1 (Period: pre vs. S1; $F_{(1,14)} = 147.17$, $p < 0.001$) and in freezing across the course of training (Group × Days; $F_{(1,14)} = 15.95$, $p < 0.01$; and Period × Days; $F_{(1,14)} = 19.87$, $p < 0.001$). Group Forward displayed more freezing in the presence of S1 than in its absence (Period: S1 vs. pre; $F_{(1,7)} = 220.07$, $p < 0.001$) and this difference grew larger across training (Period × Days; $F_{(1,7)} = 16.22$, $p < 0.01$). By contrast, Group Backward quickly reduced responding to S1 (Days; $F_{(1,7)} = 5.82$, $p < 0.05$) such that freezing was similar in the presence and absence of S1 (Period: pre vs. S1; $F < 0.3$). These patterns of performance remained unaffected by the introduction of the S2 sessions on days 5–8. Overall, S2 elicited less aversive responding than S1 (Period 1: S2 vs. S1; $F_{(1,14)} = 164.79$, $p < 0.001$) but more than when the stimuli were absent (Period 2: S2 vs. pre; $F_{(1,14)} = 14.56$, $p < 0.01$). Yet, these differences depended on the group of rats considered (Period 1 × Days; $F_{(1,14)} = 120.59$, $p < 0.001$; Period 2 × Days; $F_{(1,14)} = 103.06$, $p < 0.001$). In group Forward, S2 elicited less aversive responding than S1 (S2 vs. S1; $F_{(1,4)} = 154.74$, $p < 0.001$) but more than when the stimuli were absent (S2 vs. pre; $F_{(1,7)} = 277.99$, $p < 0.001$). By contrast, in Group Backward, S2 elicited more aversive responding than S1 (S2 vs. S1; $F_{(1,7)} = 10.32$, $p < 0.05$) and more in the presence than in the absence of the stimuli (S2 vs. pre; $F_{(1,7)} = 14.99$, $p < 0.01$). Taken together, these results are consistent with the view that S1 became an aversive excitor in Group Forward and an aversive inhibitor in Group Backward, whereas S2 was neutral in both groups. In appetitive conditioning (**Table 2**) no significant differences were found between groups, compounds or responding in the presence or absence of these compounds (all Fs < 3.9).

Test

The data from the final test are presented in **Figure 3C**. Appetitive responding to S3 relative to S4 depended on group (Groups × Period; $F_{(1,14)} = 26.80$, $p < 0.001$). Thus, S3 elicited more responding than S4 in Group Forward (Period: S3 vs, S4; $F_{(1,7)} = 12.24$, $p < 0.05$) whereas it elicited less responding than stimulus S4 in Group Backward (Period: S3 vs, S4; $F_{(1,7)} = 15.64$, $p < 0.01$). These test data reveal, therefore, that, whereas an aversive inhibitor can block appetitive conditioning to a neutral CS, an aversive excitor can enhance appetitive conditioning when both are presented together and paired with food. This experiment suggests, therefore, that the effect in Experiment 1 was induced by both a facilitation of conditioning to S3 and by blocking conditioning to S4.

DISCUSSION

The results of the current experiments have four important implications for theories of Pavlovian conditioning generally and appetitive-aversive interactions in particular. The first is the most obvious; the current findings replicate those reported in the Dickinson and Dearing (1979) chapter, that aversive inhibitors and appetitive excitors have similar properties, and so support Konorski's view of Pavlovian incentive learning. Here we established that backwardly pairing a shock with a discrete CS is sufficient to allow that CS to compete with another CS for association with the appetitive affective system when both were presented in a compound and paired with food. In Experiment 1 this reduction was relative to a stimulus compounded with an aversive excitor and, therefore, we could not definitively establish whether the effect reflected a reduction in the former or an elevation in the latter. This was clarified in Experiment 2 using a between-subjects design, with the effect of the aversive inhibitor vs. the aversive excitor compared against a control CS. There it was established less ambiguously that the aversive inhibitor blocked conditioning to the added CS when presented in a compound and paired with food. Along with other examples of transreinforcer blocking, therefore, these results confirm that competitive learning processes in Pavlovian conditioning are

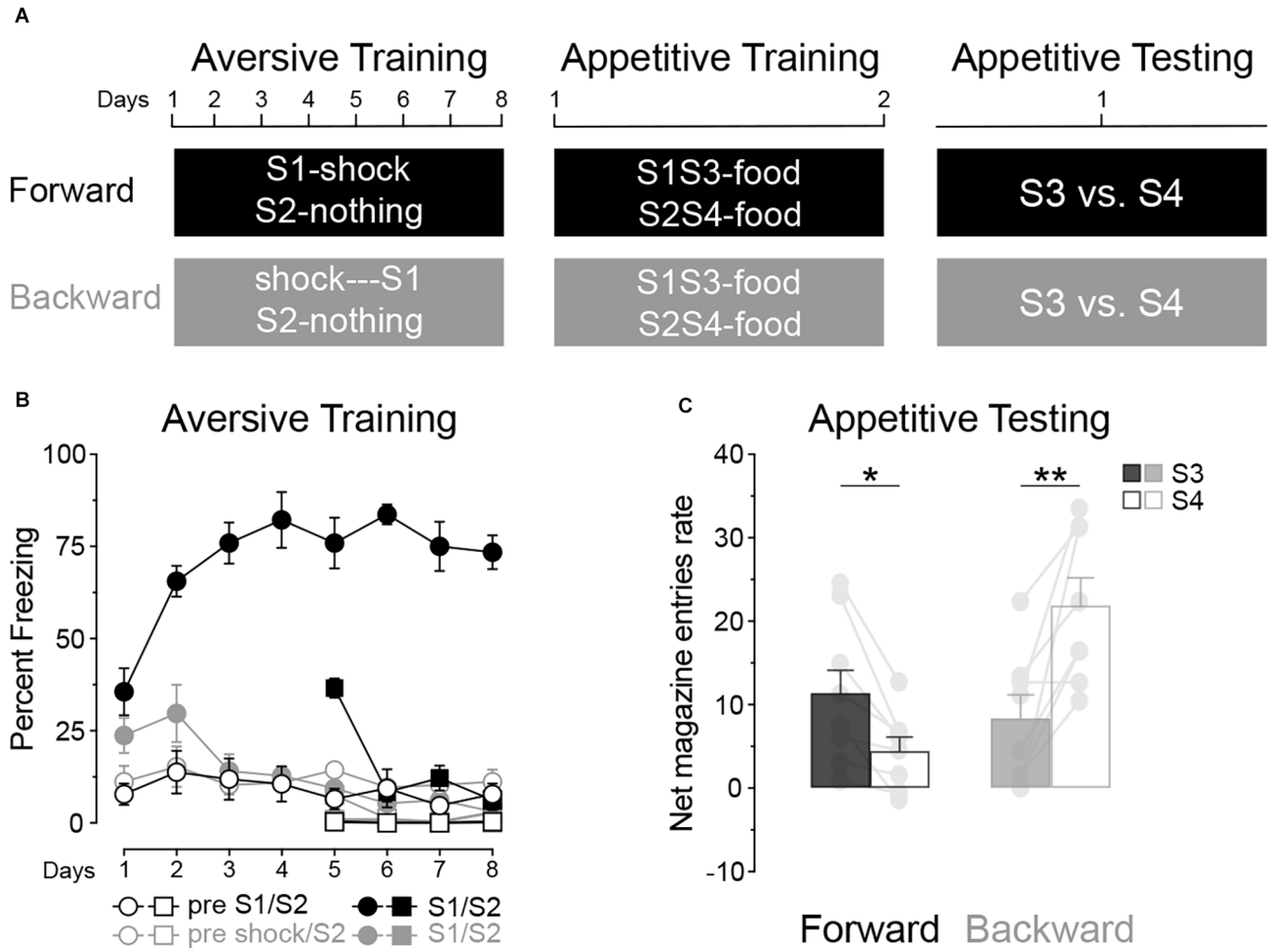

FIGURE 3 | An aversive excitor enhances, whereas an aversive inhibitor blocks, appetitive conditioning. **(A)** Design of Experiment 2; S1/S2/S3/S4: clicker, tone, flashing light or constant light (counterbalanced). **(B)** Aversive conditioning: S1 became an aversive excitor in group Forward and an aversive inhibitor in group Backward whereas S2 was neutral in both groups. **(C)** In group Forward, stimulus S3 elicited more appetitive responding than stimulus S4. The opposite was found in group Backward. Error bars denote ± 1 SEM. Asterisks denote significant effect (*$p < 0.05$; **$p < 0.01$).

best viewed as competing for association with the affective processes activated by the US, whether that competition is generated directly within affective systems or indirectly *via* their interaction.

Context Predictions

Evidence indicates that, as backward conditioning imbues a CS with inhibitory properties, it can also confer excitatory properties to the background context (Chang et al., 2003). This raises the possibility that the expectation of shock in the context influenced conditioning to the novel CSs across appetitive training. To minimize this possibility, our experiments included magazine training sessions before and immediately after aversive training to reduce the aversive properties of the context. The lack of differences in responding in the absence of the stimuli during appetitive training (**Tables 1** and **2**) indicates that these sessions successfully reduced context fear. More importantly, the within-subjects design employed in Experiment 1 ensured that the expectation of shock in the context was similar during presentations of the backwardly- and forwardly-trained stimuli with their associates during appetitive training, and the two stimuli influenced appetitive conditioning to their associates in an opposite manner. We are therefore confident that the effects reported here were due to the predictive properties of the aversive stimuli rather than those of the context.

Appetitive-Aversive Predictions and Prediction Errors

Experiment 2 also provided evidence for another phenomenon dependent on appetitive-aversive interaction: appetitive superconditioning. Dickinson (1977) reported that, when a novel CS was presented with a CS previously paired with food and the compound paired with shock, conditioning to the novel CS was enhanced relative to a control stimulus shocked in a compound with a CS unpaired with food. Here we found evidence that a

TABLE 2 | Appetitive training data for Experiment 2—Magazine entries per minute (mean ± s.e.m) in the absence (pre) or presence of the two compounds S1S3 and S2S4.

Days	Groups	Magazine entries per minute ± (mean s.e.m)		
		pre	S1S3	S2S4
1	Forward	8.30 ± 1.83	7.66 ± 0.87	6.75 ± 2.39
	Backward	11.20 ± 1.68	10.78 ± 2.22	7.97 ± 2.04
2	Forward	6.38 ± 1.77	10.84 ± 1.89	11.62 ± 3.08
	Backward	9.23 ± 1.89	14.5 ± 3.66	12.72 ± 3.04

novel CS presented in a compound with an aversive inhibitor and paired with food showed enhanced appetitive conditioning, again compared to a stimulus paired with food in a compound with CS unpaired with shock. Superconditioning is in many ways the opposite of blocking and is perhaps best interpreted as driven by the increased discrepancy between the predicted outcome, shock in the case of the S1S3 compound in Group Forward of Experiment 2, and the food that was actually delivered. Nevertheless, both the superconditioning induced by this enhanced prediction error in Group Forward and the transreinforcer blocking induced by the reduced prediction error in Group Backward present a problem. These explanations not only depend on the interaction between appetitive-aversive affective systems, they also depend on a common prediction error signal derived from that interaction. The question is how does that common prediction error arise? Although this issue has not been directly addressed in the current literature, these phenomena could be argued to suggest the existence of a substrate that establishes an appetitive-to-aversive continuum on which, for example, appetitive USs and CSs generate positively signed predictions and aversive USs and CSs negatively signed predictions, with predictions from appetitive and aversive inhibitors signed oppositely to their excitors (**Figure 4A**). Such a substrate would provide a straightforward basis for generating the net affective prediction necessary to calculate a common prediction error.

The alternative is that there exists a range of more specific motivational control processes that are independent but can interact in a manner able to emulate the activity of distinct affective systems (**Figure 4B**); e.g., excitatory interactions between pain and illness systems, productive of fear and disgust, could emulate general aversive activity whereas interactions between nutrient and fluidic predictions, driven by hunger and thirst systems, could emulate general appetitive activity, a suggestion for which there is some evidence (Balleine and Dickinson, 2006). In contrast, inhibitory interactions between such systems, or perhaps a subset of them, could subserve conditioned inhibition, with their net effects resulting in general excitatory and inhibitory predictions and prediction errors. There is, at present, little evidence on which decide between these accounts although, in a recent study, we found some evidence for the latter in showing that shifts in primary motivational state, for example a shift from hunger to satiety, reduced the impact of the prediction error on aversive superconditioning to a neutral CS when a compound composed of that CS and an appetitive excitor was paired with foot shock (Laurent et al., 2018; see also Balleine and Dickinson, 2006).

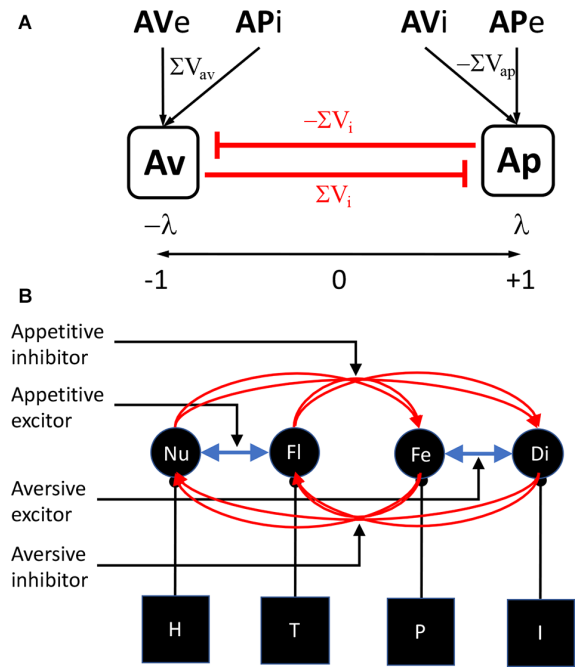

FIGURE 4 | Models of appetitive-aversive interaction. **(A)** An appetitive-aversive continuum account according to which appetitive (λ) and aversive ($-\lambda$) events are combined on a substrate to generate the prediction error term for appetitive-aversive interactions. The associative strength of aversive excitors and appetitive inhibitors (AVe+APi) sum (ΣV_{av}) to drive aversive predictions, aversive prediction errors ($-\lambda+\Sigma V_{av}$) and inhibition of the appetitive system (ΣVi) whereas the associative strength of appetitive excitors and aversive inhibitors (APe+AVi) sum (ΣV_{ap}) to drive appetitive predictions, aversive prediction errors ($\lambda-\Sigma V_{ap}$) and inhibition of the aversive system ($-\Sigma Vi$). **(B)** A motivational account on which specific motivational systems combine to emulate appetitive and aversive systems and drive inhibitory connections with systems to which they are not linked. In this example, a hunger (H)—nutrient (Nu) system joins a thirst (T)—fluid (Fl) system to generate appetitive activity and a pain (P)—fear (Fe) system joins an illness (I)—disgust (Di) system to generate aversive activity (blue arrows) and these pairs of systems maintain individual inhibitory links (red arrows) to generate appetitive-aversive interactions.

The Importance of Appetitive-Aversive Interactions

Generally, therefore, the current data, when combined with previous findings, suggest that experiments investigating the factors controlling appetitive-aversive interactions may provide an interesting test bed for theories of Pavlovian conditioning. Such interactions are, of course, very common—few situations are entirely appetitive or entirely aversive—and although most associative theories can be applied equally to appetitive or aversive conditioning, these theories have yet to develop a systematic approach to the opponency between affective processes at the heart of their interaction. There have certainly been very prominent opponent process theories, mostly concerned with specifying when and under what conditions appetitive and aversive states arise and that were successfully

applied to some behavioral phenomena—particularly those generated by addiction (Solomon and Corbit, 1974). Another approach was developed more formally into Wagner's (1981) Sometimes Opponent Processes (SOP) model of conditioning and, indeed some aspects of Konorski's theory of Pavlovian incentive learning permeated a later version of that model as an affective-emotional extension to SOP (i.e., AESOP; Wagner and Brandon, 1989). This later version explored the effects of partitioning the US into sensory and affective components but, unfortunately, did not extend the account to formally investigate how appetitive-aversive interactions might be considered or how excitatory and inhibitory processes of opposing valence interact.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Ethics Committee of the University of New South Wales.

AUTHOR CONTRIBUTIONS

VL and RW designed the experiments. VL conducted the experiments and analyzed the data. VL and BB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Response Flexibility: The Role of the Lateral Habenula

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The ability to make appropriate decisions that result in an optimal outcome is critical for survival. This process involves assessing the environment as well as integrating prior knowledge about the environment with information about one's current internal state. There are many neural structures that play critical roles in mediating these processes, but it is not yet known how such information coalesces to influence behavioral output. The lateral habenula (LHb) has often been cited as a structure critical for adaptive and flexible responding when environmental contexts and internal state changes. A challenge, however, has been understanding how LHb promotes response flexibility. In this review, we hypothesize that the LHb enables flexible responding following the integration of context memory and internal state information by signaling downstream brainstem structures known to drive hippocampal theta. In this way, animals respond more flexibly in a task situation not because the LHb selects a particular action, but rather because LHb enhances a hippocampal neural state that is often associated with greater attention, arousal, and exploration. In freely navigating animals, these are essential conditions that are needed to discover and implement appropriate alternative choices and behaviors. As a corollary to our hypothesis, we describe short- and intermediate-term functions of the LHb. Finally, we discuss the effects on the behavior of LHb dysfunction in short- and intermediate-timescales, and then suggest that new therapies may act on the LHb to alleviate the behavioral impairments following long-term LHb disruption.

Keywords: behavioral adaptation, lateral habenula, motivation, context memory, hippocampus, medial prefrontal cortex

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INTRODUCTION

One's ability to behave intentionally, especially when presented with options, involves a number of complex processes such as selectively attending to relevant sensory input, determining whether environmental context conditions have changed from what is expected based on past experience, selecting an appropriate action, assessing the outcome of the selected action relative to internal state information, and then updating one's knowledge about the context and response outcomes to be prepared for the next encounter. A common driver of all of these processes is not only one's memory but also the ability of information about one's internal state to modulate the efficiency of memory processing and thus memory's impact on subsequent behaviors.

Therefore, in order to fully understand real world goal-directed and flexible behavioral adaptation, it is necessary to understand not only how the brain processes new information to form memories, but it is essential to clarify how internal state (i.e., motivation) information comes to regulate the behavioral implementation of context memories.

There are a number of excellent and detailed reviews on brain mechanisms of context memory (e.g., Burgess et al., 2002; Eichenbaum, 2017; Lisman et al., 2017; Maurer and Nadel, 2021). Since the hippocampus (HPC) is known to be critical for context memory, and since hippocampal neurons are sensitive to a broad range of external and internal sensory information (including rewards and aversive stimuli), our definition of "context" extends beyond the external sensory environment. While we have made exciting and significant advances in our understanding of the molecular, as well as neural circuit and systems changes during context learning and memory, precisely how context memory intersects with information about one's current motivational state to promote adaptive behavioral outcomes is not clear. This is an important problem to solve for it applies to many of our everyday behaviors and decisions. As an example, while you may have learned about and understand the health benefits of exercise, your motivational state may conflict with this knowledge, resulting in you deciding not to go to the gym.

In this review, we focus specifically on the issue of how brain systems that integrate context memory and motivation information come to enable freely-navigating animals to quickly switch behaviors by flexibly responding to changes in environmental conditions. One approach to resolving this issue is to consider the advantage that cortical evolution may have conferred onto a pre-existing, evolutionarily conserved experience-dependent response flexibility brain system that involves the epithalamic structure, the habenula. In fish, amphibians, reptiles, and birds, the habenula receives sensory and internal state information while sending strong signals to midbrain structures that regulate overt actions (Stephenson-Jones et al., 2012). A critical role of the habenula in response flexibility is supported by findings that habenula disruption leads to impaired approach or avoidance responses to dynamic shifts in internal conditions or information, such as hormone levels (Ogawa et al., 2021), rules for task performance (Palumbo et al., 2020), or external environmental context cues. As the neocortex evolved in mammals, so did the prominence of the lateral division of the habenula (the lateral habenula, or LHb) as well as LHb connectivity with frontal cortical areas. Thus, it has been hypothesized that while the mammalian LHb continues to support flexible responding, it does so based not only on sensory and internal state input but also the frontal cortical (presumably context memory) input (Baker and Mizumori, 2017; Mizumori and Baker, 2017). Given the growing number of excellent and relevant published review articles, in the following sections, we only briefly highlight key findings that support the claim that the LHb plays important roles in context memory, processing motivation information, integrating context memory and motivational state information, and in response flexibility. Then we suggest a novel hypothesis to address the unanswered question of how the LHb enables adaptive context-dependent response flexibility.

LHb IS A HUB FOR MEMORY AND INTERNAL STATE INFORMATION

At any given point in time, the current constellation of neural activity and functional connectivity across the brain (considered here as the *internal state*) defines the neural foundation within which new information is processed. One often refers to such foundational neural states relative to a particular functional attribute such as a cognitive state, motivational state, and/or behavioral/response state. As an example, the patterns of cortical neural activity that exist prior to stimulus exposure (reflecting the cognitive state) effectively determine how new sensory information is processed and perceived, which in turn defines our interpretation and interactions with the world. In everyday life, different neural states do not function independently, but rather they are interdependent. For example, it is well known that altered motivational states, such as that which occurs when stressed, can bias the efficiency of information processing to improve (or impair) memory. The LHb presumably at least identifies, if not also retains (Andalman et al., 2019), current cognitive and motivational state information in order to adjust behavioral responding as task conditions change.

Cognitive State: Context Memory

Significant evidence supports the generally-accepted view that the HPC is critically important for context memories (e.g., Burgess et al., 2002; Howard and Eichenbaum, 2013; Smith et al., 2013; Eichenbaum, 2014; Place et al., 2016; Maurer and Nadel, 2021). Many studies have shown that place cells in the HPC fire strongly when an animal occupies a particular location (a place field; O'Keefe and Nadel, 1978; Gothard et al., 1996). When almost any feature of the context changes, HPC place fields remap or change their spatial and temporal patterns of firing. Additionally, place fields link to represent recent past, present, and future context information (e.g., Muller and Kubie, 1987; Wilson and McNaughton, 1993; Mizumori et al., 1999; Ferbinteanu and Shapiro, 2003; Leutgeb J. K. et al., 2005; Leutgeb S. et al., 2005; Smith and Mizumori, 2006; Nakashiba et al., 2009). In the natural world, it would be unfavorable for an animal to explore an environment similar to one that they have explored in the past that led to danger. Thus, when faced with a new but similar context, the HPC is thought to retrieve information about similar previous experiences (Spiers et al., 2015), then evaluate the extent to which the current context varies from the retrieved (expected) context (Mizumori et al., 1999; Vinogradova, 2001). The degree of similarity between expected and current context information seems reflected in HPC output signals (Mizumori, 2013). Without proper functioning of the HPC, context memory-guided behavior becomes significantly impaired (e.g., Morris et al., 1982; Bradfield et al., 2020; Gridchyn et al., 2020).

LHb Plays a Role in Context Memory

Early studies that probed LHb functions discovered that its inactivation results in analgesic-like effects at the time of tonic pain presentation (Fuchs and Cox, 1993). Soon after, it was shown that electrical stimulation of the LHb resulted in aversive behaviors perhaps by generating an aversive signal (Matsumoto and Hikosaka, 2009a; Friedman et al., 2011). Indeed, LHb terminals in the ventral tegmental area (VTA) and rostromedial tegmentum (RMTg) mediate behavioral avoidance (Lammel et al., 2012; Stamatakis and Stuber, 2012; Brown and Shepard, 2013). Therefore, LHb is often considered the "aversion center" of the brain (Baker et al., 2016). As a result of more recent studies, however, a number of functions are now attributed to the LHb in addition to the initial idea that it serves to provide an aversive signal. For instance, Congiu et al. (2019) found that aversive foot shocks not only excite the majority of LHb neurons, but also inhibit the activity of a small population of excitatory LHb neurons, indicating a more complex function of the structure. Also, neurons in the LHb have been found to exhibit changes in activity patterns to rewards, suggesting the LHb contributes to signaling information to *both* aversive and rewarding stimuli (Matsumoto and Hikosaka, 2009a). An important role of the LHb in HPC-related context memory has been suggested in numerous studies. For example, LHb inactivation results in disruption of memory retrieval as well as an inability to update the spatial configuration of the environment (Mathis et al., 2015). LHb neurons have also been shown to keep track of choice outcomes, implicating it in memory for decisions made during goal-directed tasks (Baker et al., 2015; Kawai et al., 2015). LHb, then, appears to participate in the signaling of memories in the recent past, as well as memories collected over longer periods, suggesting a perhaps more general modulatory role in memory (Bromberg-Martin et al., 2010b).

Numerous studies more explicitly show that the LHb is necessary for accurate context memory processing since its disruption impairs HPC-dependent context memory tasks such as the spatial delayed alternation and probabilistic reversal maze tasks (Baker et al., 2015; Barker et al., 2017; Baker et al., 2019). LHb is also involved in tasks such as novel object recognition (Goutagny et al., 2013) and the water maze task (Thornton and Davies, 1991; Lecourtier et al., 2004). It has also been shown that the spiking of LHb neurons aligns with the HPC theta phase, suggesting a crucial interaction between the two regions (Aizawa et al., 2013). In the case of context-dependent fear memory, which necessitates the association between a context and an aversive cue presentation, inactivation of the LHb impaired the ability to appropriately respond to an aversive context (Durieux et al., 2020). In sum, a role for the LHb in context memory seems clear.

What Is the Source of Context Information for the LHb?

An important outstanding question is the source of context information for the LHb. It is possible that the HPC relays contextual information to the LHb to guide proper behavioral responses. There are, however, no known direct connections between the HPC and the LHb, suggesting the involvement of an intermediary brain region. Many studies have demonstrated the importance of the communication between the cortex and HPC in context memory and response flexibility (Spellman et al., 2015; Tamura et al., 2017; Avigan et al., 2020). Notably, one cortical brain region that plays a crucial role in action selection when responding to changing contexts, as well as receiving strong synaptic innervation from the HPC, is the medial prefrontal cortex (mPFC; Gilmartin and Helmstetter, 2010; Brockett et al., 2020). When the infralimbic and prelimbic areas of the mPFC are inactivated, rats exhibit a significantly higher escape latency if the escape platform in a water maze is shifted to a different area of the maze compared to controls (de Bruin et al., 1994; Haddon and Killcross, 2011). Some mPFC neurons are preferentially active during specific behavioral states, giving rise to possible state information that the LHb can use to influence behavior (Halladay and Blair, 2015). Dysfunction of either the HPC or the mPFC often results in similar context memory and decision-making impairments, but several studies have attempted to disentangle their possible unique properties (Corcoran and Quirk, 2007). The HPC plays a greater role in the formation and retrieval of memories about spatial contexts (O'Keefe and Nadel, 1978; Maurer and Nadel, 2021), while the mPFC plays a more crucial role in the retrieval of distant memories that are generalizable to similar contexts (DeNardo et al., 2019; Samborska et al., 2021).

mPFC neurons display neuronal activity comparable to that of the LHb in that specific subpopulations display different activities in response to appetitive and aversive stimuli (Warden et al., 2012; Rubio et al., 2019; Capuzzo and Floresco, 2020). This suggests that the mPFC and LHb may have common functions and/or they both participate in behavioral flexibility. Such findings imply a potentially important interaction between the mPFC and LHb. Indeed, studies using both anterograde and retrograde tracers have identified mPFC fibers terminating at the LHb, suggesting a functional connection (Kim and Lee, 2012; Mathis et al., 2021). What might be the functions of mPFC-LHb connections? Along with the evolution of the cortex came the ability to have greater intentional control over the execution of behaviors. As the evolution of neocortical memory systems continued, so did the establishment of connectivity between cortical memory areas and subcortical structures that influence action, such as the LHb. mPFC signals in particular, then, become a strong candidate intermediary structure to communicate HPC-derived context and other valence-related information necessary for appropriate and adaptive behavior. Recent studies have attempted to uncover the nature of the signal from the mPFC to the LHb as well as its impact on behavioral output. Mathis et al. (2021) conducted a sequence of experiments characterizing the activity pattern of mPFC neurons that project to the LHb and their role in stress. They found that mPFC cells send signals to the LHb in the presence of a stressful event such as a foot shock. Moreover, the LHb cells that received signals from the mPFC had differential behavioral output based on the different network projection profiles. For instance, the mPFC-LHb-locus coeruleus projection played a major role in cocaine-seeking, implicating this projection in reward-seeking behavior.

The mPFC-LHb-raphe projection was implicated in freezing behavior in response to a stressor. Interestingly, these findings are consistent with the theory that the mPFC relays context-specific information to the LHb, which can serve as a brake signal (Sleezer et al., 2021) to cease behaviors when appropriate or to engage behaviors in other aversive contexts.

Another potential source of context information for the LHb is the septal complex. The septum is a subcortical midline structure that is divided into medial and lateral septal portions (MS and LS, respectively) each of which have strong connections with the HPC and LHb (Swanson and Cowan, 1979). Inactivation of the MS results in spatial working memory deficits, impairments in HPC place cell activity, as well as impairments in processing contextual information, implicating the septum in processing contextual memory (Mizumori et al., 1989b; Leutgeb and Mizumori, 1999; McGlinchey and Aston-Jones, 2018). Separating the functional contributions of the LS and MS to LHb has been challenging. The LS receives input primarily from the HPC, and projects to the MS, which projects back to the HPC *via* the fornix. A strong output of both the MS and LS is to the LHb (for an in-depth review of septal inputs and outputs, see Swanson and Cowan, 1979). Silencing either the LS or the MS results in the overall decrease in avoidance behavior, while stimulation of excitatory MS inputs to the LHb induced conditioned place aversion in a two-chamber avoidance task (Veening et al., 2009; Zhang et al., 2018). The aversion-induced behavioral effect was positively correlated with stimulation frequency, which suggests that MS inputs play a significant role in driving aversive behavior in response to an aversive context. In terms of septal influences on context memory, the MS and LS are thought to play a role in maintaining HPC theta that is necessary for proper spatial and contextual memory integration (Tsanov, 2018). While the MS has reciprocal connections with the HPC, the LS only receives unidirectional inputs from the HPC (Tsanov, 2018). Thus, the MS may be driving both LHb and HPC activity necessary for contextual information processing, while the LS may be filtering contextual HPC information and relaying this signal to the LHb for proper adaptive behavioral output (Yetnikoff et al., 2015; Tsanov, 2018; Wirtshafter and Wilson, 2019).

Motivational State

The primary motivating factor of a living organism is the need for survival. Thus, an animal's experiences and actions in the world are highly influenced by recent previous experiences and motivations. For instance, an animal's willingness to work for food is influenced by whether or not they have eaten recently. There are many outstanding reviews on the neural circuitry underlying motivational systems (e.g., Berridge, 2004; Bromberg-Martin et al., 2010a; Morales and Margolis, 2017; Petrovich, 2018; Burdakov and Peleg-Raibstein, 2020). The distinct and overlapping motivational systems contribute to a specific profile of a motivational state. Motivational systems research has typically focused on relating one's internal state to specific biologically-motivated behaviors such as hunger and feeding behaviors, as well as reproductive hormones and mate-seeking behaviors. Recent theories postulate that motivation structures such as the lateral hypothalamus serve as an interface between motivation and cognition systems (e.g., Petrovich, 2018; Burdakov and Peleg-Raibstein, 2020), but it is not yet known how motivational state influences the type of response flexibility needed for accurate goal-directed and context-dependent navigation.

Motivational Information Is Processed by the LHb

The LHb is known to receive a wide range of inputs relating to one's internal and external motivational state, such as value-based signals (Bianco and Wilson, 2009; Trusel et al., 2019), gustatory signals (Stamatakis et al., 2016), and circadian rhythm signals (Baño-Otálora and Piggins, 2017). For illustrative purposes, below we focus on only a few direct sources of motivation information that are known to modulate LHb activity, and consequently response flexibility.

The entopeduncular nucleus (EPN) provides significant motivational input to the LHb. Formerly thought to be primarily involved in the motor movement (Hauber, 1998), LHb lesions often resulted in cognitive and not motor-related deficits (Miller et al., 2006). Additional research unearthed another potential role for the EPN, implicating it in reward valuation (Hikosaka et al., 2006). Hong et al. (2011) found antidromic LHb signals in the globus pallidus (GP), a primate EPN analog, that differentially responds to reward. Bilateral inactivation of the GP led to the inability to learn new associations and task contingencies, implicating the GP in adaptive behavior (Piron et al., 2016). Interestingly, the EPN exhibits graded levels of firing activity corresponding to the expectation of an outcome, suggesting that the EPN encodes the value of an action as well as the outcome (Stephenson-Jones et al., 2016). These reward-related signals are sufficient to drive motivation. For example, Cerniauskas et al. (2019) found that most EPN neurons synapse onto VTA-projecting LHb neurons, driving LHb hyperexcitability and inducing motivational impairments. Thus, the EPN appears to communicate reward-related signals to the LHb, as well as contributes to driving motivation.

The lateral hypothalamus (LH) also provides behaviorally-relevant motivational information to the LHb.regulating anxiety and depressive-like behaviors. The LH is functionally heterogeneous, containing both glutamatergic and GABAergic neurons, activation of which results in different behavioral responses (Jennings et al., 2013; Trusel et al., 2019). The LH has prominent projections that terminate in the LHb, and stimulating this projection results in both excitatory and inhibitory responses in the LHb, suggesting a potential bidirectional influence of LH on LHb activity (Stamatakis et al., 2016). In another study, orexinergic LH signals that terminate in the LHb result in LHb inhibitory responses, as well as increases in aggressive behavior (Flanigan et al., 2020). Excitatory responses in the LHb as a result of glutamatergic LH stimulation result in aversive behavior and it plays a role in generating a prediction signal for future negative events (Lecca et al., 2017; Lazaridis et al., 2019). As such, the LH exhibits a two-factor influence on LHb activity and subsequent motivated behavior.

The lateral preoptic area (LPO) is a critical structure for motivational drive and it provides one of the largest inputs to the LHb (Yetnikoff et al., 2015). Comparable to other structures that bidirectionally influence LHb activity, the LPO also exerts bivalent control over the LHb, which influences the motivational state. Interestingly, glutamatergic and GABAergic LPO neurons simultaneously synapse on individual LHb neurons and both are activated by aversive stimuli (Barker et al., 2017). However, when stimulated individually, glutamatergic and GABAergic LPO inputs to the LHb produce divergent behavioral responses. This suggests that LPO activity is able to influence individual LHb neurons and drive opposing motivational states.

All mammals have a biological clock that regulates the activity of physiological functions (i.e., immune system coordination) and prepares them for specific motivated behaviors. The circadian rhythm is influenced by both intrinsic (i.e., physiological states, autonomic arousal) as well as extrinsic activity (i.e., daylight, food). For instance, animals that exhibit diurnal rhythms, like most primates, have increased motivation during the day to socialize and hunt for food. Without regular circadian rhythmicity, motivation lowers and animals tend to make less-optimal decisions (Acosta et al., 2020). Although many brain regions have been shown to play a role in this internal rhythmicity, the suprachiasmatic nucleus (SCN) is the most prominent. The SCN organizes the activity in the brain that inevitably influences the body through its inherent ability to oscillate and synchronize the activity of multiple brain regions. The LHb appears to play an important role in circadian rhythmicity (Sakhi et al., 2014; Baño-Otálora and Piggins, 2017; Mendoza, 2017), likely as a result of receiving significant input from the SCN. Paul et al. (2011) found that LHb lesions resulted in motor and circadian rhythm impairment, implicating the LHb in the relay of SCN circadian rhythmicity important for behavior. It is possible that the LHb is integrating information about circadian rhythms to appropriately time-motivated behaviors for optimal decisions (Mendoza, 2017). Lastly, reward-signaling structures (i.e., the VTA) have shown to exhibit circadian rhythm firing, highlighting the LHb's circadian rhythm regulation of reward systems more generally (Bussi et al., 2014).

While the EPN, LH, LPO, and the SCN each strongly and directly relays motivational information to the LHb, it is worth noting that motivation information may bias the nature of information arriving in LHb from other structures not traditionally considered to be related to motivation, such as the mPFC. For example, in times of deliberation, an animal must evaluate options and select an action that would lead to the most optimal outcome. These actions most often have to do with approaching reward or avoidance of punishment. Selecting the action with the most optimal outcome involves evaluating similar previous actions and their respective outcomes. This manifests itself in the form of reward and reward prediction error signals (Bromberg-Martin and Hikosaka, 2011). Such value-based (motivation-related) signals are encoded at the time of action selection and outcome evaluation and are used to inform future behavior. The VTA and nucleus accumbens (NAcc) are likely involved early in this decision process since they seem to track outcomes and generate reward prediction signals as shown by neural activity that correlates with behavior and motivational effort for both the VTA (Bromberg-Martin et al., 2010a) and NAcc (Hamid et al., 2016). NAcc activity, however, is not dependent on VTA input (Floresco et al., 1998). Instead, the basolateral amygdala (BLA), involved in reward-related associative learning, appears to drive NAcc reward firing. Importantly, these NAcc signals come to influence LHb activity which then drives downstream structures, such as the VTA, toward the facilitation of reward approach or punishment avoidance behaviors (Bianco and Wilson, 2009). It is unclear whether the direct projection from the NAcc to the LHb influences motivated behavior.

The Ventral Pallidum (VP) receives significant reward-related signals from the NAcc and it in turn relays this information to the LHb *via* both glutamatergic and GABAergic projections (Soares-Cunha et al., 2020; Stephenson-Jones et al., 2020). Inhibition of excitatory VP inputs to the LHb abolished reward-seeking behavior, while inhibition of inhibitory VP inputs to the LHb abolished behavioral avoidance (Knowland et al., 2017; Stephenson-Jones et al., 2020). Therefore, subpopulations of VP neurons (perhaps influenced by the NAcc) bi-directionally drive behavior *via* their inputs to the LHb in different motivational contexts.

The VTA-PFC projection has been implicated in many cognitive and behavioral processes such as mood regulation (Walsh and Han, 2014). Importantly, stimulation of VTA neurons induces neuroplastic strengthening of cortical inhibitory circuits, thereby inhibiting overall PFC activity (Zhong et al., 2020). Concurrently, dopamine injection increases theta coherence between the HPC and mPFC (Benchenane et al., 2010). It is possible that reward-related dopaminergic signals reach the PFC to update cortical information such that it more precisely represents the present situation relative to the HPC. In this way, information sent from the mPFC to the LHb is behaviorally relevant, thereby promoting timely and appropriate behaviors.

Motivational Influence on Contextual Information

Behaviorally, many experiments have exemplified the positive influence of motivation, induced by factors such as reward magnitude, on performance in context-related goal-oriented tasks (Sänger and Wascher, 2011). Additionally, the presence of reward in specific predictable locations results in animals returning to these rewarded locations at a higher rate in the future (see Anselme, 2021 for review). It is possible that context information within PFC signals and motivational information from a number of subcortical structures arrive at LHb in a temporally precise manner that biases LHb outputs appropriate for a particular context. For example, Chang et al. (1998) showed that a subset (∼5%) of PFC and NAcc neurons respond to a rewarding stimulus simultaneously. At the population level, theta oscillatory synchrony between the PFC and VTA increases during actions that were likely to result in a reward (Park and Moghaddam, 2017). The synchronized theta activity may be

linking the PFC contextual information with the VTA reward signal, allowing the LHb to receive in a timely manner input to associate motivational and contextual PFC information.

The mPFC is thought to serve as an inhibitory control for motivated behaviors by interacting with the HPC and retrieving context-appropriate memories to inform future action selection (McDonald et al., 2008; Chen et al., 2013; Zelikowsky et al., 2013; Porter et al., 2019). Interestingly, HPC neurons that project to the mPFC (Hsu et al., 2018), and mPFC neurons that project to the LHb (Mathis et al., 2021), appear to inhibit motivated behavior to seek reward, implicating this circuit in the motivational regulation of reward seeking. The HPC itself has been shown to monitor and respond to motivational states when associated with a particular context (Kennedy and Shapiro, 2009). Specifically, Kennedy and Shapiro (2009) show that HPC single units respond preferentially to a context paired with reward only when the rats were hungry or thirsty. This may be a result of motivational inputs directly influencing the HPC, as many of the aforementioned motivational circuits, such as the VTA (Gasbarri et al., 1994; Martig et al., 2009; Ghanbarian and Motamedi, 2013), SCN (Phan et al., 2011; McCauley et al., 2020), LH (Samerphob et al., 2015; Noble et al., 2019; Rezaee et al., 2020), EPN (Sabatino et al., 1986; Chen Y. et al., 2020), and amygdala (Sheth et al., 2008; Tsoory et al., 2008; Ghosh et al., 2013), synapse onto and influence HPC activity. In sum, these findings demonstrated that the LHb is not alone in integrating motivational and memory information since cortical memory information likely already incorporates some aspects of motivation. What distinguishes the LHb from cortical systems that may be influenced by motivational and memory states is that LHb output may more directly determine optimal and adaptive behavior.

Motivation and Memory-Guided Decisions

All previous experiences serve as roadmaps for future decisions, actions, and their respective outcomes. The selection of an action that leads to a particular outcome will occur only when an animal is motivated. As mentioned above, the experience of hunger/satiation, the state of the biological clock, and internal valuation of possible outcomes define one's motivational states which guide and direct goals. As such, there is a necessary link between motivations and decisions, where highly motivated animals exhibit more effortful behavior in order to obtain a reward. There are numerous studies examining the effect of motivational states on decision making, where animals must choose between small or large rewards that necessitate large or small amounts of effort, respectively (Floresco and Ghods-Sharifi, 2007; Mai et al., 2012). Animals will exert effort to seek reward up to a certain point until the effort required is too great and no longer worth the payoff. This threshold is influenced by internal state and motivation, often changing depending on context (Knauss et al., 2020). Past experiences, too, shape internal state and motivation (Dysvik and Kuvaas, 2013). As animals evoke memories of similar previous experiences to evaluate the current context and most optimal choice, the animal's associations with a previous choice will influence their motivation and, subsequently, their decisions and actions. As a result of the functional interactions between the LHb and mPFC, the LHb serves as an integrative node for motivational and contextual information for the purpose of ensuring adaptive and flexible responses.

LHb AND RESPONSE OR CHOICE FLEXIBILITY

Regardless of the species under study, it is often suggested that the habenula regulates an animal's ability to switch learned behavioral and cognitive strategies when a goal or context changes. This switch is likely, not due to successive learning by different memory systems since strategy switching occurs much more quickly than new learning, and since multiple memory systems are thought to essentially operate in parallel (e.g., Mizumori et al., 2004; White et al., 2013; Hasson et al., 2015). The ability to rapidly change behavioral strategies is often attributed to the mPFC (e.g., Dalley et al., 2004; Ragozzino, 2007), but species without a defined prefrontal cortex (e.g., fish; Agetsuma et al., 2010; Okamoto et al., 2012; Stephenson-Jones et al., 2016) show remarkable abilities to flexibly respond in adaptive ways when a change in either the external sensory environment (including social cues, Chou et al., 2016) or internal state (such as motivation or use of learned task rules; Parker et al., 2012; Randlett et al., 2015; Cherng et al., 2020; Palumbo et al., 2020) occurs. Fish habenula, as an example, is often suggested to enable response or behavioral flexibility by integrating the different types of information (including the evaluation of response outcomes, motivation state, and sensory cues) needed to strategically switch behavioral responses/strategies in simple and more cognitively demanding tasks.

The mammalian LHb (relative to the MHb) seems to have co-evolved with the cortex to process more complex sensory and memory-related information, and in this way enable more refined and flexible behavioral control when performing cognitive tasks that depend on limbic cortical processing (e.g., by HPC and mPFC; Ichijo and Toyama, 2015; Ichijo et al., 2017; Mizumori and Baker, 2017). Early reports of the effects of lesions on the mammalian LHb showed that rats became unable to switch or maintain learned behaviors when contingencies changed in appetitive, HPC-dependent tasks (Thornton and Evans, 1984; Thornton and Davies, 1991). Importantly, LHb inactivation or lesion do not affect working memory, nonspecific sensory processing, identification of spatial locations, new learning, memory retrieval, motivation, behavioral activation, or reward discrimination *per se* (e.g., Thornton and Evans, 1984; Thornton and Davies, 1991; Lecourtier et al., 2004; Stopper and Floresco, 2014; Baker et al., 2015, 2019; Mathis and Lecourtier, 2017; Mathis et al., 2017). The hypothesis that the LHb importantly contributes to cortically-mediated response flexibility received additional support when rats were tested in HPC and mPFC-dependent tasks for which there is no right or wrong response, but rather choice preferences reveal an animal's responsiveness to changing task conditions. Such tasks require continuous and subjective value assessments to direct choice responses. LHb inactivation was found to be sufficient to

disrupt such choice preferences when the probability of obtaining rewards shifted, or when the delay before reward access varied, during both operant testing (Shohamy et al., 2009; Dickerson et al., 2011; Delgado and Dickerson, 2012; Stopper and Floresco, 2014) and testing on open, elevated mazes (Baker et al., 2015, 2019). Importantly, spatial processing was not required for task performance, suggesting that the HPC involvement was related to other more integrative features of the task structure since changed preferences were observed only after the task structure shifted.

Understanding the specific processes and neural circuitry underlying the transformation between memory/motivational integration and the execution of behaviors is challenging since the LHb is not considered to be part of the motor output pathway that supports specific actions. Rather it is often explained that LHb's impact on response flexibility relies on its control over structures known to be important for the execution of voluntary actions (raphe nucleus and the VTA; e.g., Baker et al., 2019). Such an explanation is not satisfactory for it is still unclear how LHb-to-raphe or LHb-to-VTA signals can account for the type of response flexibility often attributed to the LHb. It is suggested here that one approach to resolving this apparent dilemma is to take a reverse engineering approach to this question. That is, the following starts by discussing the nature of the information represented by LHb neurons when rats are engaged in an HPC and mPFC-dependent natural foraging task. From there, we consider which of many brain structure(s) may be strategically informed by patterned LHb output to generate or enable flexible responses.

LHb Neural Representation During Goal-Oriented Free Navigation

If the LHb enables flexible behavioral responses to changing task conditions, one might expect LHb neural activity to somehow reflect this function. Indeed, the LHb has been shown to be a critical part of the neural circuit that generates prediction error signals when task conditions change. The well-known dopamine neural response to prediction errors is driven at least in part by the LHb (Christoph et al., 1986; Matsumoto and Hikosaka, 2007; Ji and Shepard, 2007; Bromberg-Martin and Hikosaka, 2011; Proulx et al., 2014; Baker et al., 2015; Tian and Uchida, 2015; Lalive et al., 2021), even though this occurs indirectly through the rostromedial tegmentum, or RMTg; Li et al., 2019). While these findings illustrate that flexible behavior is likely mediated by more than brain mechanism (e.g., Floresco, 2013), it clearly shows that LHb neural activity is driven by memory-based outcome expectations. Further evidence for the impact of experience on LHb neural responsiveness is the well-documented change in LHb cell firing that occurs during aversive task performance as well as duringstress (e.g., Stamatakis and Stuber, 2012). Is the coding and integration of mnemonic and motivational information sufficient to ensure flexible responding? Recordings of LHb neural activity during a navigation-based foraging task shed new light on this question.

Using a pellet-chasing task that did not require HPC-based context memory, Sharp et al. (2006) described striking velocity-correlated neural activity in rat LHb. A subsequent study by Baker et al. (2015) confirmed the existence of prominent and strong (often $r > \pm 0.85$) velocity-correlated LHb neural activity but this time as rats performed an HPC-dependent spatial working memory task. This result was surprising given the generally accepted view that the LHb contributes to learning and memory by signaling aversive/negative events/consequences/information (see review by Baker et al., 2016). However, Baker et al. (2015) also described another group of LHb neurons that responded to reward encounters, the expectation of rewards, and reward prediction errors in manners similar to the responses of primate LHb reward-responsive neurons described by Matsumoto and Hikosaka (2007). Further, about a third of the recorded LHb neurons showed conjunctive coding of reward and velocity information. During subsequent probe trials in which the reward condition or context was unexpectedly altered, the velocity correlate was retained albeit the overall firing rate was lowered. This pattern of reward and context coding by LHb neurons suggests that the LHb tracks the ongoing behavior of animals, but that the strength of movement state signals may be regulated by reward-related information. Perhaps this behavioral tracking feature is related to the recent report that LHb neurons encode a history of experiences (Andalman et al., 2019). A combination of reward and movement state neural signaling has also been reported for an important efferent structure of the LHb, the VTA (Puryear et al., 2010; Jo et al., 2013), and strong movement state information has been described as a major VTA afferent structure, the lateral dorsal tegmentum (LDTg; Redila et al., 2015). LDTg neurons were postulated to regulate reward responses of DA neurons according to the learned behaviors needed to obtain rewards. What might be the function of LHb movement state signals?

To aid in our understanding of the significance of LHb movement-related neural signals for response flexibility, it is helpful to first consider the finding by Aizawa et al. (2013) that the spiking of LHb neurons is preferentially related in time to the peaks of simultaneously recorded HPC theta rhythms. While a strong argument was made that the LHb spike-HPC theta phase coherence resulted from a common input from the vertical limb of the diagonal band of Broca, this explanation does not address the issue of interest here, and that is how might LHb enable task-specific response flexibility. To shed new light on this issue, we offer the following hypothesis:

Hypothesis*: During active navigation, the LHb may track and then relay information about one's ongoing behaviors to signal at appropriate times when downstream brainstem structures should drive hippocampal theta. In this way, LHb enables flexible responding not because e it selects a particular action, but rather because it enhances a hippocampal neural state that is often associated with greater attention, arousal, and exploration. In freely navigating animals, these are essential conditions that are needed to discover and implement appropriate alternative choices and behaviors.*

The HPC theta rhythm is known to encourage exploratory behaviors, increase arousal and attention, and improve learning and memory (e.g., Winson, 1978; Mizumori et al., 1989b; Leutgeb

and Mizumori, 1999; Lega et al., 2012; Buzsáki and Moser, 2013). Although the LHb does not have direct anatomical connections with the HPC, functional coupling between LHb and HPC has been demonstrated since LHb spikes exhibit cortical synchrony with the HPC theta phase (Aizawa et al., 2013). Such spike-phase coherence is thought to reflect periodic influences of one structure on another (Singer, 1993; Engel et al., 2001; Buzsáki, 2002). Recent evidence (described below) suggests that a number of prominent LHb efferent targets in the hindbrain area may serve as important nodes for communication from the LHb and HPC. These structures receive substantial input from the LHb, and they are considered to be critical pacemakers for HPC theta. Thus, spiking activity in the LHb may enable response and cognitive flexibility by regulating the HPC theta state to optimize exploration-related neural and memory plasticity. Such regulation could strengthen and/or maintain ongoing memory operations by the HPC during navigation of familiar contexts, as well as enable increased cognitive and response flexibility when task conditions change.

Transforming LHb Neural Signals Into Hippocampal Neural States That Support Cognitive and Response Flexibility

The diverse array of LHb efferent targets is well documented (as reviewed in Kim, 2009; Baker et al., 2016; Mizumori and Baker, 2017). Of particular interest here are those that are considered theta-pacemaker structures for the HPC, such as the nucleus incertus, supramammillary nucleus, the median raphe, and the locus coeruleus (**Figure 1**). In contrast to the traditional view that hindbrain structures regulate slow processes such as the general state of arousal, recent findings demonstrate temporally and spatially-specific regulation of HPC physiology and behavior by these brain regions.

The Nucleus Incertus (NI) lies in the midline periventricular central gray region of the pontine hindbrain, and it is generally considered to be an essential part of the ascending reticular activating system (Steriade and Glenn, 1982) since it innervates structures known to regulate HPC theta such as the MS (Vanderwolf, 1969; Vertes and Kocsis, 1997; Goto et al., 2001; Olucha-Bordonau et al., 2003; Ma et al., 2013). Also, stimulation or inhibition of the NI up- or down-regulates active locomotion (respectively), as well as modulates physiological indices of arousal and HPC theta (Nuñez et al., 2006; Lu et al., 2020). For example, NI neurons preferentially fire at the initial ascending phase of the HPC theta rhythm (Ma et al., 2013), possibly to reset theta (Martínez-Bellver et al., 2017). Evidence that NI impacts on HPC physiology have consequences for HPC-based memory has been demonstrated using a variety of behavioral tasks (Gil-Miravet et al., 2021), including context fear conditioning (Szönyi et al., 2019), various maze-based tasks (Nategh et al., 2015; Albert-Gascó et al., 2017), as well as spatial working memory operant tasks (Albert-Gascó et al., 2017; Garcia-Diaz et al., 2019).

GABA and glutamate incertus neurons (Lein et al., 2007; Cervera-Ferri et al., 2012) express receptors for stress-related hormones (corticotropin-releasing factor, or CRF) and contain multiple neuropeptide markers for stress and arousal such as neuromedin, relaxin, D2 dopamine receptors, and orexin/hypocretin (e.g., Jennes et al., 1982; Kubota et al., 1983; Ma et al., 2013; Lu et al., 2020). Therefore, the NI had been studied primarily for its role in different physiological states. However, as noted above, more recent emerging evidence clearly shows that especially the relaxin-3 NI neurons likely play a significant role in HPC-based memory (Gil-Miravet et al., 2021). For example, these relaxin-3 neurons are the ones that preferentially fire at the initial ascending phase of the HPCtheta rhythm (Ma et al., 2013). Furthermore, Szönyi et al. (2019) identified an incertus-HPCcircuit that may determine which CA1 pyramidal neurons take part in context memory processing: NI long-range GABAergic neurons project directly and indirectly (*via* the medial septum) to HPCsomatostatin neurons to regulate the excitatory/inhibitory balance in stratum oriens of CA1, thereby helping to select which cells participate in memory networks and which ones do not. Since the NI receives strong projections from the prefrontal cortex and the LHb (Goto et al., 2001; Lu et al., 2020), one or both of these incertus afferent systems may drive the NI to regulate HPC neural activity in context-specific ways.

The supramammillary nucleus (SUM) is another deep brain structure of interest when considering how LHb neural activity may translate to HPC-mediated response flexibility. The SUM is a hypothalamic structure that provides strong direct and indirect (*via* the medial septum) theta-rhythmic inputs to the HPC (Haglund et al., 1984; Vertes, 1992, 2015; Kirk and McNaughton, 1993; McNaughton et al., 1995; Kocsis and Vertes, 1997; Vertes and McKenna, 2000; Ito et al., 2018). The overall functional impact of the SUM input is to not only generate HPC theta (Pan and McNaughton, 2004), but SUM afferents amplify neocortical input to the HPC by increasing the responsiveness of dentate gyrus granule cells to input from the entorhinal cortex through disinhibition of inhibitory interneurons (Mizumori et al., 1989a). Such regulation of the excitatory state of granule cells may selectively promote information arriving from the entorhinal cortex. More recently, behaviorally-relevant functional coupling between the SUM and the HPC was shown when SUM spiking became HPC theta phase-modulated particularly before choice points in a continuous alternation task (Ito et al., 2018). Specifically, SUM cells became aligned to the later phases of the CA1 theta cycle, and SUM spiking began close to the time of firing by CA1 interneurons. In the same study, the SUM was demonstrated to be a critical coordinator of communication within the HPC-mPFC-nu. reuniens memory circuit. Thus, it was suggested that the SUM (and by extension, the LHb) dynamically coordinates HPC-related memory circuits by varying spiking relative to HPC theta (Ito et al., 2018).

Not only does the SUM regulate intra- and extra-HPC information processing, but recently it was elegantly demonstrated that the SUM may provide and/or facilitate specific types of information processing in the HPC *via* its

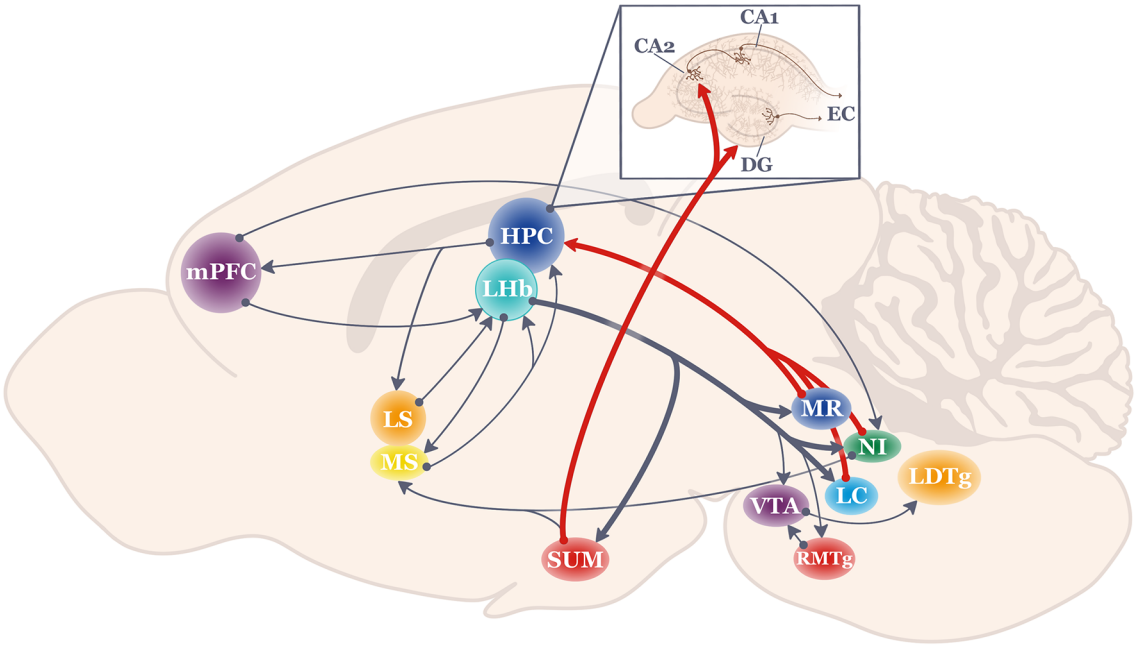

FIGURE 1 | Schematic of the anatomical projections to and from the lateral habenula (LHb; shown in teal). Thick lines highlight LHb projections most strongly related to hippocampal function. HPC, hippocampus; LC, locus coeruleus; LDTg, lateral dorsal tegmentum; LHb, lateral habenula; LS, lateral septum; mPFC, medial prefrontal cortex; MR, medial raphe; MS, medial septum; NI, nucleus incertus; RMTg, rostromedial tegmentum; SUM, supramammillary nucleus; VTA, ventral tegmental area.

distinct direct inputs to the dentate gyrus and region CA2 (Vertes and McKenna, 2000). The SUM to dentate gyrus input may signal contextual novelty while the SUM to CA2 input may signal social novelty (Chen S. et al., 2020). Thus, the SUM appears to be not only involved in the generation of the HPC theta, but it is also important for gating contextual and social information processing within the HPC. Further, SUM enables HPC to communicate with partnered mnemonic structures such as the mPFC and the nu. reuniens. Given the multiple ways that the SUM impacts HPC processing, it is not surprising that lesion or reversible inactivation of the SUM has been shown to impair different types of HPC-dependent behaviors as shown in spatial working memory and certain avoidance tasks (Shahidi et al., 2004a,b; Aranda et al., 2006, 2008), and to be activated during times of stress (Wirtshafter et al., 1998; Ito et al., 2009; Choi et al., 2012). Given the strong input from the LHb to the SUM (Kiss et al., 2002), the SUM is a strong candidate for linking functions of the LHb and HPC (as noted by Goutagny et al., 2013). Here we hypothesize more specifically that the theta-rhythmic firing of SUM neurons may be regulated by LHb behavioral/movement state signals, and in this way, LHb output can generate the conditions needed for animals to flexibly respond to changes in task conditions.

The median raphe (MR) has long been studied for its role in emotion and stress regulation (e.g., Graeff et al., 1996; Andrade et al., 2013). Recently (Baker et al., 2015), it was suggested that the MR may also play a critical role in the coordination of communication between the LHb and HPC since the MR receives strong input from the LHb (Quina et al., 2015; Metzger et al., 2017), the MR has strong projections to a broad extent of the HPC (Azmitia and Segal, 1978; Vertes et al., 1999) and the MR has been shown to significantly impact HPC-dependent behaviors and theta/ripple oscillations (Vertes and Kocsis, 1997; Wang et al., 2015) Thus, the MR is strategically situated to at least assist in the transformation of LHb signals to regulate HPC theta in a manner that facilitates response flexibility. Indeed, a link between serotonergic function and response flexibility is often discussed given the numerous studies that report alterations of serotonin receptors or neurotransmitter release results in difficulties performing classic response flexibility tasks such as set-shifting and strategy shifting (excellent reviews include Nilsson et al., 2015; Alvarez et al., 2021).

The locus coeruleus (LC) is another hindbrain structure that (in anesthetized and awake rats) is known to project to HPC to impact theta and gamma oscillations (Gray et al., 1975; Walling et al., 2011; Broncel et al., 2021), and to receive at least modest input from the LHb (Mathis et al., 2021). As reviewed by Sara (2009) and Poe et al. (2020), LC neural firing has long been observed to occur in response to novelty, to signal-mismatches events, and to attentional shifts (Aston-Jones and Bloom, 1981a,b; Aston-Jones et al., 1991; Sara and Segal, 1991; Hervè-Minivielle and Sara, 1995; Bouret and Sara, 2005). Our cumulative understanding of the broad impact of the LC across many brain regions as well as the diverse cell types and patterns of LC neural activity have led to theories that especially phasic LC activity enhances the execution of actions in response to unexpected stimuli (Aston-Jones and Cohen, 2005). LC activation, then, effectively resets neural networks to

enable rapid switches of cognitive representations or response strategies when needed (Sara and Bouret, 2012). These types of LC responses appear necessary for animals to exhibit adaptive behaviors (Yu and Dayan, 2005). Thus, in addition to the NI, SUM, and MR, the LC is a strong region of interest for its putative role in transmitting LHb signals to the HPC by establishing an HPC theta state that supports response and cognitive flexibility (**Figure 2**).

LHb CONTRIBUTES TO RESPONSE FLEXIBILITY ALONG MULTIPLE TIMESCALES: IMPLICATIONS FOR UNDERSTANDING LHb-RELATED PSYCHOPATHOLOGIES

Multiple Time Scales of LHb Neural Responses and the Impact on Response Flexibility

Based on our extant knowledge and theories about how the LHb contributes to response flexibility, an important and fundamental question arises: How does one reconcile findings that the LHb signals short duration aversive events (Matsumoto and Hikosaka, 2007), while also signaling longer-duration behavioral/movement states (Baker et al., 2015). One explanation is that while LHb broadly tracks the current sensory/behavioral situation, LHb neurons exhibit different patterns of firing depending on the types of information being tracked. LHb neurons may fire phasically to single, short-duration events such as sensory stimuli or a particular goal outcome, or they may fire tonically during longer-duration behavioral state conditions such as movement through space. This sort of dual-coding is not uncommon in the brain. One example is that the same VTA dopamine neurons fire phasically to rewards, and tonically when moving through space (e.g., Puryear et al., 2010; Jo et al., 2013). Either short- or long-duration firing could impact response flexibility that is enabled by the LHb efferent structures described earlier. For example, encountering an unexpected reward should result in phasic LHb cell firing, which would signal efferent structures to increase HPC theta to resolve a potential conflict between expected and actual context features (Mizumori et al., 1999). When tracking sensory/behavioral information across this relatively short period, the LHb may be serving as a brake signal to halt the initiation of potential inappropriate behaviors thereby allowing other adaptive behaviors to commence (Sleezer et al., 2021). When faced with other aversive situations, LHb output may instead result in the engagement of avoidance behaviors. In either case, LHb activity is considered critical during flexible action selection.

By comparison, tonic LHb neural activity for example during spatially-extended navigation, should signal hindbrain regions to elevate HPC theta for the duration of the translocation in space. Such signaling is postulated (see above) to enable the timely enhancement of neuroplasticity mechanisms needed to evaluate the current context so that memories can be updated in preparation for a future decision. That is, LHb-induced HPC theta may result in greater attention and arousal, along with a stronger neuroplasticity state that provides the basis for more efficient and timely context evaluation. Such context analysis is considered essential in order to then execute adaptive and flexible responses in the future. During real world navigation, the LHb engages in both short (sensory) and intermediate-term (during navigation) behavioral monitoring in the same context as evidenced by LHb neurons that show both reward and velocity-related neural codes (Baker et al., 2015). Such dual time frame coding may not be needed in Pavlovian or operant tasks that do not require animals to move about in a spatially-extended environment, but rather they only require information to be associated over relatively short time scales. Thus, assessing LHb in navigating animals may reveal an extended complement of potential contributions to adaptive responding.

In sum, we propose a) that the LHb contributes to response flexibility by tracking behavior across short (in the tens of ms to seconds range) and intermediate (in the range of seconds to hours or more) time frames. Prolonged disturbances of function in either of these time domains can result in different behavioral disorders and psychopathologies.

LHb Dysfunction in the Short and Intermediate-Term

LHb responses to short-term aversive or appetitive stimuli are necessary for proper adaptive behavior and do not typically result in behavioral maladaptations (Baker et al., 2015). As shown in many of the experiments described previously, inaccurate stimulus coding results in suboptimal decisions. If LHb encoding of behavioral and movement states are deficient, one would expect impaired and inappropriate activation of HPC theta, which could lead to memory deficits. We also know, based on extensive research on the role of LHb in processing aversive information, the LHb plays a substantial role in fear memories. For instance, in the case of fear conditioning, it is important to learn the association between a specific context and the potential threat. In aversive situations, not only does the LHb respond to the aversive stimulus, but also to the cue predicting the onset of the aversive stimulus (Lecca et al., 2017; Lazaridis et al., 2019; Trusel et al., 2019). It takes merely five trials in order for short-scale synaptic changes to occur in the LHb, likely between the LHb and the RMTg (Wang et al., 2017). Also, inactivation of the LHb following fear conditioning of a context resulted in context memory impairments (Durieux et al., 2020). Furthermore, in the same experiment, the c-fos expression following fear conditioning increased in the HPC, mPFC, as well as the LHb, suggesting an involvement of this circuit in contextual fear memories. Thus, in situations when the LHb does not appropriately function over periods of seconds to minutes to hours, one sees impaired decision making and poor context memory.

Prolonged LHb Dysfunction

Extended and hyperactive LHb aversive signaling can lead to long-lasting plasticity-related changes, resulting in psychiatric disorders such as depression, schizophrenia, and addiction (Metzger et al., 2021). In support of this theory, studies have

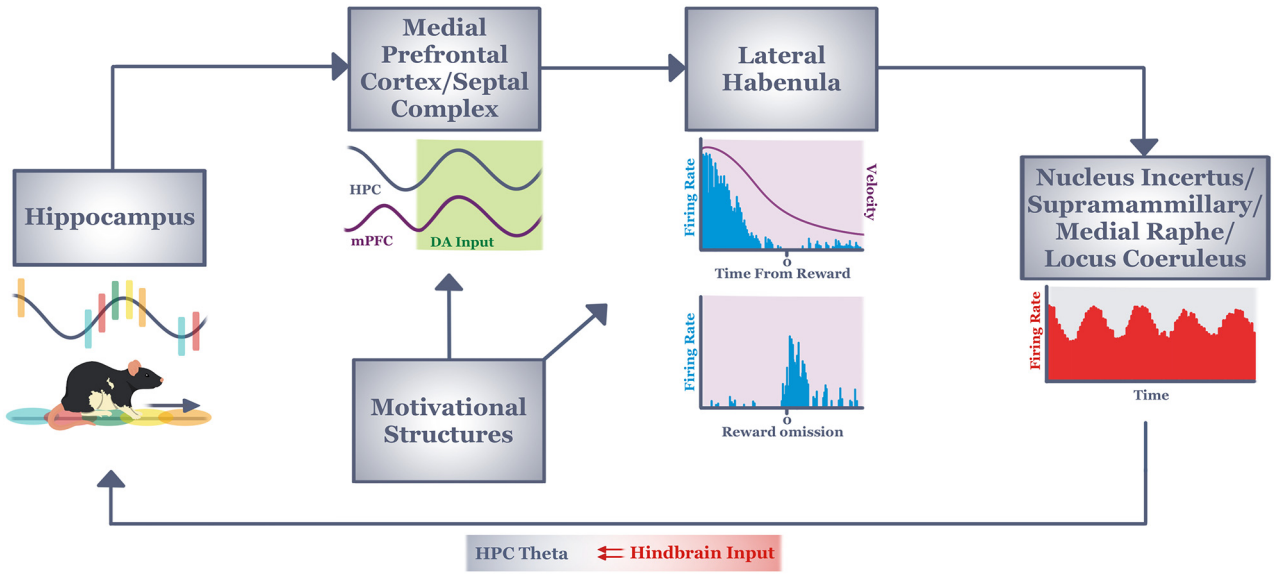

FIGURE 2 | Information flow through our hypothesized circuit (see text) that mediates context-dependent response flexibility during active navigation. Context information from the hippocampus (in the form of place field sequences) informs decision processes of the medial prefrontal cortex and septal complex *via* reward-modulated theta coherence. The output of this memory/decision system integrates with motivational information in the lateral habenula. Lateral habenula neurons integrate this information over many seconds to discharge tonically (to encode current behavioral state, or velocity) or phasically (to encode short duration stimuli/events such as reward outcome). Both types of signals are driven by motivational, memory and decision processing (see text). The output of lateral habenula is hypothesized to provide a signal to hindbrain regions known to regulate hippocampal theta. In this way, the lateral habenula may establish/maintain a neural state in hippocampus that encourages the increased attention and exploratory tendencies needed for animals to respond flexibly to changing task conditions. DA, dopamine; HPC, hippocampus; mPFC, medial prefrontal cortex.

shown that cocaine exposure induces increased AMPA receptor expression in excitatory LHb neurons, causing long-term potentiation and hyperexcitability (Maroteaux and Mameli, 2012). Similarly, prolonged exposure to an aversive stimulus, such as a foot shock, results in decreased GABAb receptor expression in excitatory LHb neurons, leading to disinhibition and hyperexcitability of LHb neurons (Lecca et al., 2016). Along with synaptic changes, stress and other environmental factors can induce changes in LHb gene expression (Levinstein et al., 2020). Specifically, expression of genes implicated in the RMTg, and not VTA or DRN, pathway is increased in the LHb following stress. These genetic changes alter the strength of the connections between the LHb and downstream structures, resulting in long-lasting changes in plasticity.

Excessive LHb activity can also lead to impaired motivated behavior and result in the pathophysiology of depression. In fact, the LHb is the only brain region that exhibits consistent hyperactivity in depression (Caldecott-Hazard et al., 1988; Andalman et al., 2019). One of the pathways implicated in the expression of depression-like symptoms is the LHb-RMTg pathway that inhibits dopaminergic activity in the VTA and other structures (Proulx et al., 2018). Another pathway underlying this disorder is the LHb's increased excitation of raphe inhibitory interneuron-based inhibition of serotonergic neurons, which causes a passive coping transition, a marker of depression (Amat et al., 2001; Andalman et al., 2019; Coffey et al., 2020). Ketamine, a common antidepressant medication, has been shown to elevate raphe activity in addition to decreasing the hyperactivity of the LHb, consistent with the role of this pathway in depression (Cui et al., 2018; Yang et al., 2018; López-Gil et al., 2019).

Maladaptive activity in the PFC is frequently tied to the phenotype of schizophrenia (Weinberger et al., 2001). Specifically, the established dopamine hypothesis of schizophrenia posits that schizophrenia arises as a result of dopaminergic hyperactivity in subcortical areas due to cortical dysfunction (Winterer and Weinberger, 2004). In a rat model of schizophrenia, Li et al. (2019) found that there was significant hypofunctionality in the LHb, potentially disinhibiting subcortical dopamine activity. In the same experiment, lesioning the LHb of schizophrenic rats resulted in a significant decrease in cortical activity. Interestingly, functional connectivity between the habenula and cortex increases in schizophrenic patients, suggesting that the habenula may be contributing to the cortical dysfunction seen in schizophrenia (Zhang et al., 2017). Moreover, serotonergic activity in the DRN increases in the pathophysiological profile of schizophrenia. Typically, in healthy brains, the LHb functions to inhibit serotonergic activity in the DRN. However, in schizophrenia, LHb activity is hypoactive, disinhibiting serotonergic activity in the DRN. When the LHb is lesioned in schizophrenic rats, serotonin levels in the DRN, as well as in the mPFC, increased (Li et al., 2019). Owing to these findings, hypofunction in the LHb may contribute to the pathophysiology of schizophrenia.

Likely as a consequence of its critical role in reward valuation and processing, the LHb plays a large role in addiction. Repeated drug exposure results in addiction, or excessive

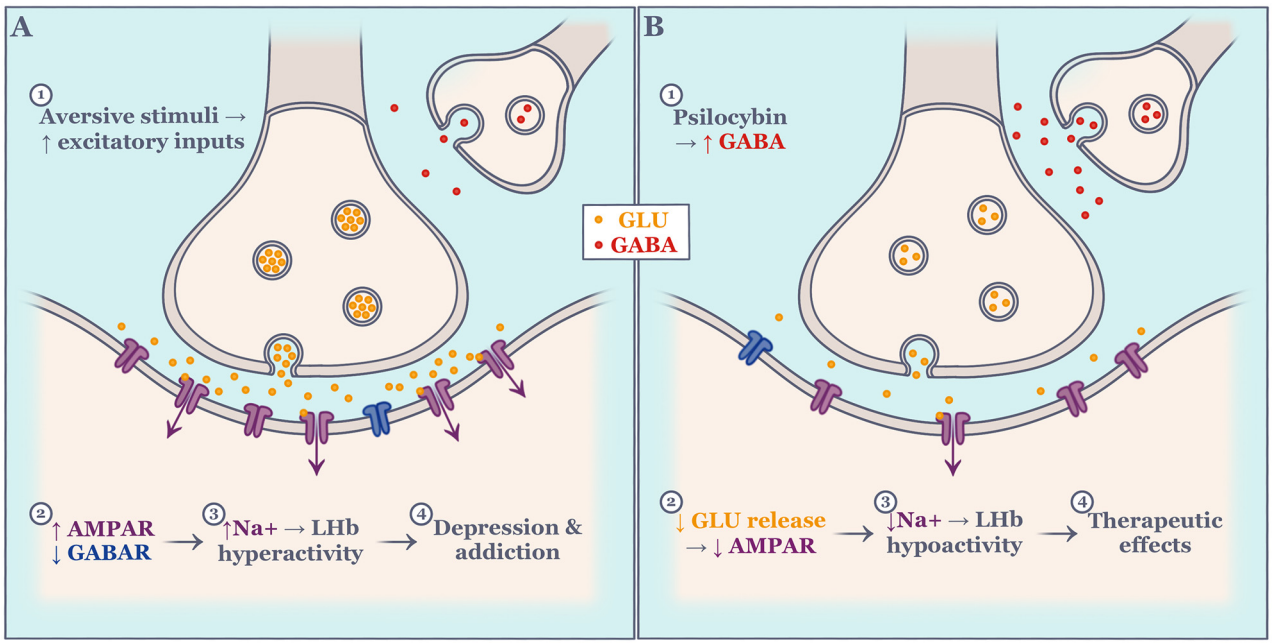

FIGURE 3 | Schematic of hypothesized molecular effect on excitatory lateral habenula neurons during long-term dysfunction **(A)** and psilocybin administration **(B)**. Events are numbered in succession. AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; GABA, gamma-aminobutyric acid; GABAR, gamma-aminobutyric acid receptor; GLU, glutamate; LHb, lateral habenula; Na, sodium.

drug-seeking behavior, and is tied to increases in LHb activity (Zhang et al., 2005). Although the LHb processes both aversive and rewarding properties of stimuli (Matsumoto and Hikosaka, 2009b), it appears that the LHb primarily responds to the aversive properties of drug exposure (Zhang et al., 2013). In particular, LHb activity is thought to underlie the negative effects of drug-seeking behavior through its projection to the RMTg (Meye et al., 2015). In support of this finding, Maroteaux and Mameli (2012) showed that cocaine exposure results in increased AMPA receptor expression in LHb neurons selectively projecting to the RMTg. Lastly, chronic drug exposure causes neurodegeneration in the main output fiber bundle of the LHb, the fasciculus retroflexus, disrupting the LHb's communication with and modulation of downstream monoaminergic structures (Lax et al., 2013).

Therapeutic Treatment for LHb Dysfunction

Reasoning that long-term LHb function underlies maladaptive behavior, clinicians recently target the LHb in an attempt to correct LHb dysfunction as a therapeutic approach. Deep brain stimulation (DBS) of the LHb has been one such therapeutic procedure that is producing encouraging results (Sartorius et al., 2010). Interestingly, DBS frequency parameters exhibit differential therapeutic outcomes in distinct disorders such as depression and addiction (Ferraro et al., 1996). For an in-depth discussion of DBS in the LHb, see the excellent review by Germann et al. (2021). Relatedly, ketamine has proven to be a successful therapeutic treatment that inhibits the LHb and disinhibits reward centers such as dopamine and serotonin (Yang et al., 2018).

Another emerging therapeutic approach for the development of treatments for LHb-related psychopathology focuses on the serotonergic system. Evidence suggests that serotonergic dysfunction results in the inability to adapt to changing environmental conditions, as seen in depression and addiction. Interestingly, the effects of serotonin depletion or overexpression do not always resemble one another across studies. For instance, Lapiz-Bluhm et al. (2009) showed that chronically stressed rats that were serotonin-depleted using *para*-chlorophenylalanine showed significant deficits in reversal learning which was rescued with a serotonin reuptake inhibitor. In a separate study, humans were exposed to a rapid tryptophan depletion paradigm on a reversal-learning task and exhibited slightly improved decision-making (Talbot et al., 2006). Such discrepancy in the literature may be attributed to inter-species differences, as well as the reliability of the drug cocktail protocol. Recent evidence has exposed distinct functions of serotonin subtypes as the main culprit (Alvarez et al., 2021). Importantly, Morris et al. (1999) showed that selective serotonin depletion causes significant increases in LHb activity, mimicking the neural correlate of depression, compared to other structures. Furthermore, tryptophan depletion causes a significant impairment in context memory in mice, implicating the serotonergic system in both motivation and context memory (Uchida et al., 2007). Serotonin 2A receptors subtype has been extensively studied in relation

to its influence on behavioral flexibility. A selective serotonin 2A receptor blockade significantly impaired performance on a spatial reversal learning task (Boulougouris et al., 2008). In the same study, a selective serotonin 2C receptor blockade improved performance on a spatial reversal learning task. Interestingly, serotonin 2C and serotonin 2A receptors are found on GABAergic dorsal raphe neurons (Serrats et al., 2005). Psilocybin, being studied as an antidepressant, is a strong serotonin 2A receptor agonist and serotonin 2A receptors are highly expressed in the LHb. This raises the question as to whether psilocybin has modulatory effects on LHb activity. Indeed, activation of serotonin 2A receptors inhibits excitatory LHb neurons, likely through the facilitation of GABAergic transmission, suggesting that psilocybin mimics a neuromodulator and works to rescue LHb dysfunction (**Figure 3**; Shabel et al., 2012; Metzger et al., 2017). In support of this, psilocybin administration has proven effective in drug-resistant depression (Carhart-Harris et al., 2018), and addiction (Johnson et al., 2014). Whether the therapeutic effects of psilocybin result from the direct action on LHb activity, however, is not yet clear and is in need of further study.

The diversity of LHb inputs from cortical and subcortical areas makes the LHb a prime region for integrating information related to context memory and motivation. Likewise, the LHb's downstream control of monoaminergic centers, as well as the HPC, implicates it in a number of psychiatric disorders, such as those described above. The reviewed data provide a compelling argument that the LHb should be one of the primary targets of therapeutic intervention, such as with psilocybin, for psychiatric disorders that manifest in context memory, motivational impairments, and certain disorders of behavioral control.

CONCLUSION

An outstanding and challenging question is how the LHb enables response flexibility. Here, it is hypothesized that LHb may enable response flexibility by integrating context memory and internal state information to provide critical feedback to memory systems (e.g., the hippocampus) about the outcome of choices and the status of behaviors (e.g., movement velocity). Importantly, this feedback may upregulate neural states in HPC when a context change requires flexible responding to maintain accurate decisions. The upregulation of at least HPC theta could enable the greater attention, arousal, and behavioral activation needed for response flexibility. This feedback system to the HPC, then, may represent a critical step in the loop of information processing between context memory and decision systems, intrinsic motivational systems, response implementation, and memory updating and retrieval that is needed to flexibly redirect responses. Supporting our hypothesis, improper functioning of the LHb results in impairments in behavior related to response flexibility such as those seen in psychiatric disorders.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.mizumorilab.com/.

AUTHOR CONTRIBUTIONS

VH and SM contributed to the conceptualization and writing of this review. All authors contributed to the article and approved the submitted version.

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A Preliminary Test of Novelty-Facilitated Extinction in Individuals With Pathological Anxiety

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Studies with rodents and healthy humans suggest that replacing the expected threat with a novel outcome improves extinction and reduces the return of conditioned fear more effectively than threat omission alone. Because of the potential clinical implications of this finding for exposure-based anxiety treatments, this study tested whether the same was true in individuals with pathological anxiety (i.e., met DSM-5 diagnostic criteria for an anxiety disorder and/or obsessive-compulsive disorder (OCD). In this preliminary test of novelty-facilitated extinction, 51 unmedicated individuals with pathological anxiety were randomized to standard extinction (n = 27) or novelty-facilitated extinction (n = 24). Participants returned 24 h later to test extinction recall and fear reinstatement. Skin conductance responses (SCR) were the dependent measure of conditioned fear. Participants in both groups learned the fear association but variably extinguished it. Novelty did not facilitate extinction in this preliminary trial. Findings underscore the importance of translating paradigms from healthy humans to clinical samples, to ensure that new treatment ideas based on advances in basic neuroscience are relevant to patients.

Keywords: extinction, anxiety, obsessive-compulsive disorder, cognition, skin conductance

INTRODUCTION

Exposure therapy, in which individuals confront feared stimuli in a gradual manner to reduce fear, is a proven treatment for individuals with anxiety disorders and obsessive-compulsive disorder (OCD; Deacon and Abramowitz, 2004). However, following exposure therapy, while some individuals maintain their gains, many others (e.g., up to 62%) experience a return of fear (Craske and Mystkowski, 2006; Craske et al., 2008). There is a pressing need to determine ways to reduce relapse. Pavlovian fear conditioning and extinction is a valuable model to develop and test innovative treatments for psychopathology. Under standard extinction protocols, some studies (albeit not all) find that anxiety

and OCD samples show deficits in learning and retaining extinction memories (Michael et al., 2007; Milad et al., 2013; Duits et al., 2016; Rabinak et al., 2017). The current study is a preliminary test of whether an augmented behavioral extinction strategy enhances fear extinction in individuals with pathological anxiety during a laboratory paradigm.

In typical lab-based extinction paradigms, participants learn that a conditioned stimulus (CS; e.g., a light) predicts an aversive unconditioned stimulus (US; e.g., a shock). The participants are then exposed to the CS multiple times without the US, leading to a reduction in defensive responses (often operationalized as "fear") to the CS (e.g., reduced freezing in rodents, reduced skin conductance in humans). However, fear responses to the CS often return following a delay (Vervliet et al., 2013).

One method to enhance extinction is by replacing, rather than merely omitting, the expected aversive outcome. Dunsmoor et al. (2015) developed an extinction paradigm in which the US was replaced with a novel neutral stimulus (i.e., a tone). This procedure, referred to as novelty-facilitated extinction (NFE), was effective at decreasing return of fear responses 24-h after extinction in rats and healthy humans. This work has been replicated and extended in healthy humans: Lucas et al. (2018) demonstrated that NFE was effective at diminishing reinstatement (response to CS following return of aversive stimulus) in healthy humans, and Dunsmoor et al. (2019) found that NFE may lead to more durable extinction *via* activating the ventromedial prefrontal cortex during extinction trials. If NFE were to have similar effects in individuals with pathological anxiety it would provide specific suggestions on how to modify exposures to reduce relapse.

Associative learning literature provides potential explanations for why pairing a CS with a novel outcome might have advantages over standard extinction procedures. For example, instead of promoting a sense of safety, the omission of an expected threat in standard extinction may render the meaning of a CS ambiguous (Bouton, 2002). Pairing the CS with a novel stimulus might reduce ambiguity generated by threat omission alone. Further, because extinction is new associative learning (Pearce and Hall, 1980; Larrauri and Schmajuk, 2008), a novel but neutral outcome might generate a more durable association than a CS-no US association.

Reducing ambiguity may be particularly important for individuals with anxiety, given that these individuals respond to ambiguity differently than healthy individuals. Specifically, individuals with anxiety tend to interpret ambiguous information as threatening, rather than benign (Mathews and MacLeod, 2005). Additionally, these individuals are less able to tolerate uncertainty (Gentes and Ruscio, 2011). In healthy humans, intolerance of uncertainty is related to return of fear following a standard extinction paradigm, but not following an NFE paradigm (Dunsmoor et al., 2015; Lucas et al., 2018). Further, intolerance of uncertainty has been implicated in reduced extinction learning in healthy humans (Morriss et al., 2015, 2016). Together, observations suggest that reducing ambiguity *via* NFE may reduce post-extinction recall of fear.

The current study is a preliminary test of the effects of NFE in a sample of unmedicated individuals who met the criteria for an anxiety disorder or OCD. We predicted that participants in the NFE group would have less return and reinstatement of fear 24 h after extinction than participants in the standard extinction group.

METHODS AND MATERIALS

Design

This study was conducted at the Anxiety Disorders Clinic, an outpatient research clinic, at the New York State Psychiatric Institute (NYSPI) and Columbia University Medical Center, and was approved by the NYSPI Institutional Review Board. Following informed consent, 67 unmedicated participants diagnosed with an anxiety disorder or OCD completed questionnaires and a differential fear conditioning paradigm followed by extinction. Participants were randomized to either standard extinction or NFE. Participants returned to the lab 24 h later to test post-extinction recall and reinstatement [skin conductance in response to conditioned stimulus (CS)].

Participants

Participants were recruited *via* advertisements and referrals from physicians. Participants included adults aged 18–50 with a DSM-5 diagnosis of OCD, social anxiety disorder (SAD), generalized anxiety disorder (GAD), and/or specific phobia (SP). Diagnoses were made by trained clinicians (e.g., doctoral student with master's degree, clinical psychologist, or psychiatrist) using a structured clinical interview (SCID; First and Spitzer, 1996). Participants were excluded if they used psychotropic medication in the last 4 weeks (8 weeks for selective serotonin reuptake inhibitors), had current diagnosis of major depressive disorder, or lifetime diagnosis of any psychotic disorder, bipolar disorder, or alcohol, or substance use disorder. Other exclusions included: acute suicidal risk; major medical or neurological problems that might interfere with study procedures or data interpretation or increased risk of participation (assessed *via* brief meeting with MD or Nurse Practitioner, e.g., cardiovascular disease, seizure disorder, head trauma); inability to refrain from caffeine (for 4 h) or nicotine (for 24 h) without withdrawal symptoms. Medication and drug use was established *via* self-report in the brief meeting with MD or Nurse Practitioner.

Assessments

Following informed consent, participants completed a series of questionnaires, including the 21-item Beck Depression Inventory II (BDI-II; Beck et al., 1996) to assess depressive symptomatology, the 40-item State-Trait Anxiety Inventory (STAI; Spielberger et al., 1983) to assess both state (current, in the moment) and trait (how one typically feels) anxiety, and the Intolerance of Uncertainty Scale (IUS; scores range from 27 to 135; Buhr and Dugas, 2002) to assess response to uncertainty.

Fear Acquisition, Extinction, and Recall

Participants completed the acquisition, extinction, and extinction recall, and reinstatement task over the course of 2 days. PsyLab (Contact Precision Instruments, Cambridge, MA, USA) was used to collect skin conductance data and run the acquisition, extinction, and recall tasks. On Day 1, two Ag/AgCl electrodes were affixed to the hypothenar eminence of the participants' left hand, to assess skin conductance, and two Ag/AgCl electrodes were affixed to the participants' right wrist, to deliver shocks. SignaGel Electrode Gel (Parker Laboratories, Inc., Fairfield, NJ, USA), a highly conductive saline gel, was used[1]. The shock was generated by the SHK1 Pain Stimulation Shocker (Contact Precision Instruments, Cambridge, MA, USA) and lasted 200 ms. A shock work-up was conducted to determine the level of shock that each participant found to be highly annoying, but not painful (Boucsein et al., 2012).

Following the work-up, participants were instructed to sit comfortably and pay attention to the images displayed on the screen. They were told they may or may not receive shocks, and that there would be an association between pictures and shocks, but they would need to learn it themselves. Participants wore headphones (Sennheiser PRO, Sennheiser electronic GmbH & Co. KG, Wennebostel, Wedemark, Germany) to block out noise and to deliver the novel tone to participants randomized to the NFE group.

Conditioned stimuli (CS) included two angry male faces (following Dunsmoor et al., 2015)[2]. Each trial included a CS displayed for 6 s, followed by a 12 s intertrial interval. The trial order was pseudorandomized so that no more than three trials of the same type occurred in a row. The conditioning session began with 10 habituation trials (five each of CS+ and CS-) to diminish initial orienting responses. This was followed by the first run of fear conditioning that included four CS+ trials that co-terminated with a shock to the wrist, seven CS+ trials unpaired with shock, and seven CS- trials. The second run of fear-conditioning included four CS+ trials paired with shock, eight CS+ trials unpaired with shock, and eight CS- trials. Conditioning was identical between groups. One rationale for using partial CS-US pairing is that continuous (100%) CS-US pairing rates can lead to a rapid decrease in conditioned responses (Grady et al., 2016) that would potentially obscure the effect of the extinction manipulation.

Following conditioning, the standard extinction group (EXT) underwent two runs of extinction that each included 10 CS+ trials unpaired with shock and 10 CS- trials. Following Dunsmoor et al. (2015), there was a very short pause (less than 1 min) between the first and second run of fear conditioning and extinction. For participants randomized to the NFE group, all CS+ extinction trials co-terminated with a low-volume 440-Hz tone for 1.5 s, delivered binaurally through headphones. Both the 200 ms shock (during acquisition for both groups) and the 1.5 s tone (during extinction for the NFE group) co-terminated with CS+. Thus, the onset of the tone during extinction preceded the onset of the shock during acquisition. The dB level of the tone was not recorded. The tone was meant to be perceptible but not loud or aversive. All of the subjects in the NFE condition were asked at the end of the experiment if they heard the tone, and all reported that they did.

Participants returned to the lab the following day. No new instructions were given for Day 2 that would indicate any departure from the procedures from the previous day. Electrodes were reattached and shock intensity was set at the level determined on Day 1. The recall included 10 CS+ trials unpaired with shocks (or tones) and 10 CS- trials. After these 20 CS trials, participants received three unsignaled shocks to the wrist to reinstate conditioned responses. The reinstatement test included 10 CS+ trials unpaired with shock and 10 CS- trials. See **Figure 1** for an illustration of the phases of the experiment.

Data Processing

Skin conductance responses (SCR) were collected using PsyLab (Contact Precision Instruments, Cambridge, MA, USA) at 500 Hz. Responses were calculated according to previous criteria (Dunsmoor et al., 2015) using a validated automated MATLAB (Mathworks, Inc., Natick, MA, USA) script (Green et al., 2014). CS+ trials paired with the shock during fear conditioning were not included in the analysis to avoid potential confounds introduced by the electrical shock. An SCR was considered related to the CS if the trough-to-peak deflection occurred within a 0.5–6.0 s time window starting at CS onset, if the responses lasted between 0.5 and 5.0 s, and if the response was greater than 0.2 microsiemens. If a response on a trial did not meet these criteria, it was scored as zero. Raw SCR values were square-root transformed to normalize the distribution. To account for different patterns of SCR over the course of learning, we divided the data into early and late phases. Early and late fear acquisition were defined as the first and second run of conditioning, respectively. Early extinction was defined as the first run of extinction. Late extinction was defined as the last 3 CS+ and 3 CS- trials, in order to capture responses toward the end of training (Dunsmoor et al., 2015). Early recall and reinstatement were likewise defined as the first three CS+ and three CS- trials, as prior research shows that extinction manipulations tend to affect early presentations of CS+ at extinction-retention tests (Milad et al., 2009; Schiller et al., 2010; Dunsmoor et al., 2015).

[1]Guidelines for assessing electrodermal activity (Boucsein et al., 2012) recommend against using non-isotonic gels, such as SignaGel, as it may artificially inflate skin conductance. However, the differential nature (CS+ vs. CS-) of our outcome variables likely protects the validity of our results.

[2]The task design was similar to the design from Dunsmoor et al. (2015). However, we added additional trials to the extinction session. This was done a priori with the assumption that anxiety patients would need more trials to fully extinguish. Indeed, despite this effort, many anxiety patients still failed to show evidence of extinction of SCRs by the end of the session. Angry faces were selected (vs. neutral sensory stimuli, such as colored squares) as CS because they are "fear relevant," and can be used to more reliably assess the effects of an enhanced extinction technique, as using a purely neutral CS can lead to rapid extinction. A tone was selected as the novel outcome because subjects would clearly process it as a concrete outcome that occurs at the moment in time that the shock is expected.

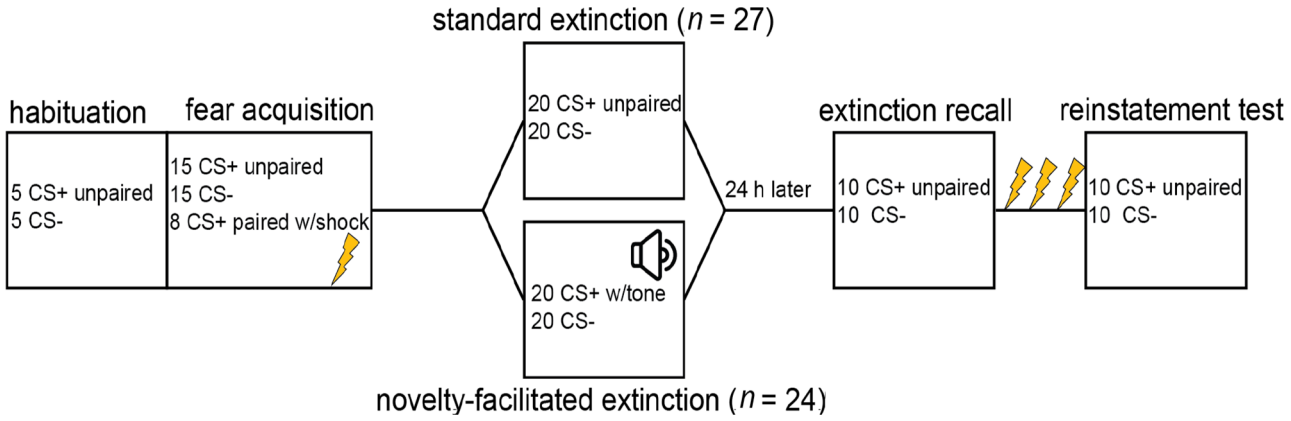

FIGURE 1 | Graphic illustrating phases of the experiment. *Note*. Early and late fear acquisition were defined as the first and second run of conditioning, respectively. Early extinction was defined as the first run of extinction. Late extinction was defined as the last three CS+ and three CS- trials. Early recall and reinstatement were defined as the first three CS+ and three CS- trials.

Statistical Analysis

Following prior fear conditioning studies (Duits et al., 2016), repeated measures Analyses of Variance (ANOVAs) were run separately for each phase of the experiment (early acquisition, late acquisition, late extinction, early recall, early reinstatement). In each ANOVA, Group (EXT vs. NFE) was included as a between-subjects factor and stimulus (CS+, CS-) was included as a within-subjects factor. We followed-up significant two-way interactions with *t*-tests. Statistical significance was defined as $p < 0.05$.

RESULTS

Demographics and Clinical Characteristics

Sixty-seven participants provided consent. Three were excluded from the analysis [did not return for the second day of study ($n = 1$), no longer met inclusion criteria ($n = 1$), technical errors ($n = 1$)]. An additional 13 participants were not included in the analysis due to a failure to show evidence of conditioned learning, as defined by no positive difference between mean SCRs to the CS+ vs. CS- during late conditioning (i.e., a difference of 0 or a greater mean SCRs to the CS- than CS+).

This resulted in 51 participants (60.80% female, *M* age = 25.23, *SD* age = 4.82, age-range = 18–41) included in the analysis: 27 in the EXT group and 24 in the NFE group. Participants in the two groups did not differ significantly in demographic or clinical characteristics (see **Table 1** for *M*, *SD*, and tests for differences between groups).

As expected, STAI scores suggest elevated state ($M = 44.14$, $SD = 9.39$) and trait anxiety ($M = 52.20$, $SD = 9.53$) in both groups. These scores are approximately one and two standard deviations, respectively, above scores of healthy individuals (Spielberger et al., 1983). IUS scores reveal elevated levels of intolerance of uncertainty ($M = 77.98$, $SD = 17.75$); this mean score is approximately three standard deviations above a college sample without anxiety disorders (Freeston et al., 1994). BDI-II scores suggest minimal depression ($M = 14.24$, $SD = 8.81$).

The sample was free from psychotropic medications at the time of the study. Most participants (37 participants) had never used psychotropic medications. Of the 14 participants that had, weeks since the last dose ranged from 5 to 150.86 ($M = 84.43$, $SD = 52.71$), except for one participant who had a 2 mg dose of clonazepam 3 weeks prior to consent.

Fear Acquisition, Extinction, Recall, and Reinstatement

See **Figure 2** for trial by trial SCR to CS+ and CS-, separated by Group. See **Figure 3** for means and standard errors of the means for each phase of the experiment, separated by Group. ANOVAs for early and late fear acquisition revealed significant effects of Stimulus (CS+, CS-), demonstrating that participants acquired conditioned SCRs to the CS+ compared to the CS- (early fear conditioning: $F_{(1,49)} = 23.31$, $p < 0.001$, $\eta_p^2 = 0.32$; late fear conditioning: $F_{(1,49)} = 19.56$, $p < 0.001$, $\eta_p^2 = 0.29$). There was no effect of Group for early fear acquisition ($F_{(1,49)} = 0.49$, $p = 0.487$, $\eta_p^2 = 0.01$), but results revealed a Stimulus by Group (NFE, EXT) interaction for early fear acquisition ($F_{(1,49)} = 4.72$, $p = 0.035$, $\eta_p^2 = 0.09$). Follow-up paired sample *t*-tests and visual inspection of means demonstrated that both groups successfully acquired conditioned fear during early fear conditioning (EXT: $t_{(26)} = 2.40$, $p = 0.024$, $d = 0.25$; NFE: $t_{(23)} = 4.07$, $p < 0.001$, $d = 0.52$), although the difference in response to CS+ to CS- was larger for the NFE group than the EXT group. For late fear acquisition, there was no effect of Group ($F_{(1,49)} = 1.55$, $p = 0.219$, $\eta_p^2 = 0.03$) or Stimulus by Group interaction ($F_{(1,49)} = 2.10$, $p = 0.154$, $\eta_p^2 = 0.04$), suggesting that during the second run of conditioning, participants successfully acquired similar levels of conditioned fear, regardless of group.

Contrary to expectations, the ANOVA for late fear extinction revealed a significant effect of Stimulus ($F_{(1,49)} = 4.17$, $p = 0.047$, $\eta_p^2 = 0.08$), indicating that SCRs remained elevated to the

TABLE 1 | Demographics and clinical characteristics.

	Standard extinction (EXT) *n* = 27 Mean (SD)	Novelty-facilitated extinction (NFE) *n* = 24 Mean (SD)	Statistical test for differences between groups
Age	24.33 (4.77)	26.24 (4.77)	$t_{(49)} = 1.43$, $p = 0.159$
Sex (*n*, %female)	15 (55.6%)	16 (66.7%)	$X^2_{(1)} = 0.66$, $p = 0.417$
Naïve to Psychotropic Medication (*n*, %)	19 (70.4%)	18 (75.0%)	$X^2_{(1)} = 0.14$, $p = 0.712$
Race (*n*, %)			$X^2_{(4)} = 1.40$, $p = 0.844$
White	17 (63%)	16 (66.7%)	
Black	3 (11.1%)	2 (8.3%)	
Asian	3 (11.1%)	2 (8.3%)	
American Indian/	1 (3.7%)	0 (0%)	
Other	3 (11.1%)	4 (16.7%)	
Ethnicity (*n*, %)			$X^2_{(1)} = 0.04$, $p = 0.835$
Hispanic or Latino	5 (18.5%)	5 (20.8%)	
Diagnosis (*n*, %)			$X^2_{(4)} = 1.94$, $p = 0.747$
GAD	1 (3.7%)	2 (8.3%)	
OCD	4 (14.8%)	5 (20.8%)	
PD	1 (3.7%)	0 (0%)	
SAD	7 (25.9%)	7 (29.2%)	
More than one anxiety or OCD diagnosis	14 (51.9%)	10 (41.7%)	
Years of Education	14.81 (2.71)	15.92 (2.69)	$t_{(48)} = 1.45$, $p = 0.153$
STAI Trait	51.37 (9.32)	53.13 (9.88)	$t_{(49)} = 0.65$, $p = 0.517$
STAI State	41.81 (7.49)	46.75 (10.71)	$t_{(49)} = 1.92$, $p = 0.060$
BDI-II	14.11 (8.03)	14.38 (9.79)	$t_{(49)} = 0.11$, $p = 0.916$
IUS	75.22 (19.82)	81.08 (14.91)	$t_{(49)} = 1.18$, $p = 0.243$

Note. GAD, Generalized Anxiety Disorder; OCD, Obsessive Compulsive Disorder; PD, Panic Disorder; SAD, Social Anxiety Disorder; STAI, State Trait Anxiety Inventory; BDI-II, Beck Depression Inventory II; IUS, Intolerance of Uncertainty Scale.

CS+ compared to the CS-. That is, participants still evinced heightened SCRs to the CS+ vs. the CS- in the last few trials of extinction, despite having been presented with 17 preceding CS+ trials during extinction without shock. Notably, there was no main effect of Group ($F_{(1,49)} = 0.12$, $p = 0.724$, $\eta_p^2 = 0.002$) or Stimulus by Group interaction ($F_{(1,49)} = 0.46$, $p = 0.502$, $\eta_p^2 = 0.01$). Overall, the extinction results suggest that conditioned fear was not extinguished in this anxiety sample, regardless of group.

Interpretation of the 24-h recall and reinstatement tests is complicated by the fact that neither group fully extinguished on Day 1. The ANOVA for 24-h recall revealed a significant effect of Stimulus ($F_{(1,49)} = 38.63$, $p < 0.001$, $\eta_p^2 = 0.44$), with no effect of Group ($F_{(1,49)} = 0.03$, $p = 0.863$, $\eta_p^2 = 0.001$) or stimulus by Group interaction ($F_{(1,49)} = 3.88$, $p = 0.055$, $\eta_p^2 = 0.07$). For the reinstatement test, there was no effect of stimulus ($F_{(1,49)} = 4.02$, $p = 0.050$, $\eta_p^2 = 0.08$), Group ($F_{(1,49)} = 0.23$, $p = 0.637$, $\eta_p^2 = 0.01$), or stimulus by Group interaction ($F_{(1,49)} = 0.01$, $p = 0.910$, $\eta_p^2 < 0.001$).

We also conducted an additional test to determine if there was an increase in differential responding from the end of extinction to the beginning of the 24-h recall test, despite incomplete extinction across participants. Specifically, we conducted a repeated measures ANOVA with Group (EXT vs. NFE) as a between subjects factor, and stimulus (CS+, CS-) and Phase (Late Extinction, Early Recall) as within subjects factors. Results revealed no effect of Group ($F_{(1,49)} = 0.10$, $p = 0.752$, $\eta_p^2 = 0.002$), Stimulus by Group interaction ($F_{(1,49)} = 0.31$, $p = 0.580$, $\eta_p^2 = 0.01$), Phase by Group interaction ($F_{(1,49)} = 0.03$, $p = 0.870$, $\eta_p^2 = 0.001$), or Phase by Stimulus by Group interaction ($F_{(1,49)} = 2.67$, $p = 0.109$, $\eta_p^2 = 0.05$). Additionally, there was no correlation between IUS and spontaneous recovery (as measured by SCR to CS+ during early trials of 24 h recall) in the entire sample ($r_{(49)} = 0.10$, $p = 0.508$), or in either group (EXT group: $r_{(25)} = 0.23$, $p = 0.242$; NFE group: $r_{(22)} = -0.13$, $p = 0.545$).

Contingency Awareness

At the end of the experiment, we asked participants how often the shock followed the CS+ and CS- face. 86.3% of subjects correctly responded that the CS+ face was sometimes paired with shock, and 72.5% correctly responded that the CS- face was never paired with shock. We did not exclude subjects for incorrect contingency awareness, given that the question was asked retrospectively at the end of the experiment on Day 2.

DISCUSSION

To our knowledge, this preliminary study is the first to test the effects of novelty-facilitated extinction(NFE) compared to standard extinction (EXT) in 51 unmedicated participants diagnosed with an anxiety disorder or OCD. Neither group demonstrated within-session fear extinction. Further, novelty did not facilitate extinction. Although difficult to interpret (given the lack of extinction), novelty did not lead to reduced recall or reinstatement 24-h after extinction trials.

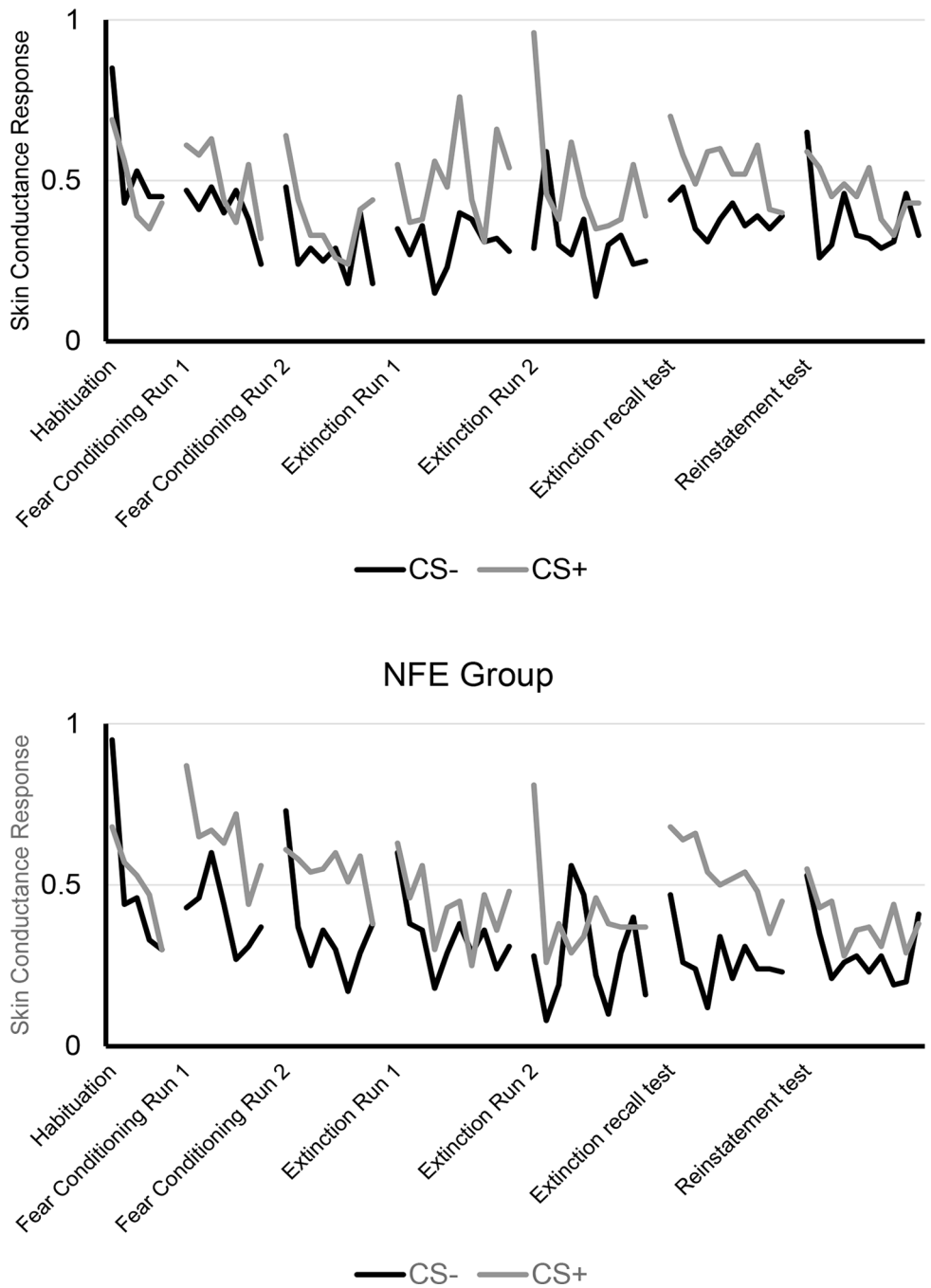

FIGURE 2 | Trial by trial skin conductance response to CS+ and CS-, separated by group. *Note*. EXT, Standard Extinction; NFE, Novelty-Facilitated Extinction; CS, Conditioned Stimulus.

That participants in our study did not extinguish on Day 1 was unexpected given prior studies using the same paradigm in healthy individuals (Dunsmoor et al., 2015; Lucas et al., 2018). One explanation is that the CS used in our study (angry faces) may have been particularly anxiety-provoking for our sample, given that 70.6% of our sample met the criteria for SAD and individuals with social anxiety tend to interpret facial expressions in a more threatening way than non-anxious individuals (Mohlman et al., 2007; Yoon and Zinbarg, 2008). Another explanation could be a general abnormality in fear extinction in those with pathological anxiety. Indeed, several studies (albeit not all) suggest that individuals who meet the

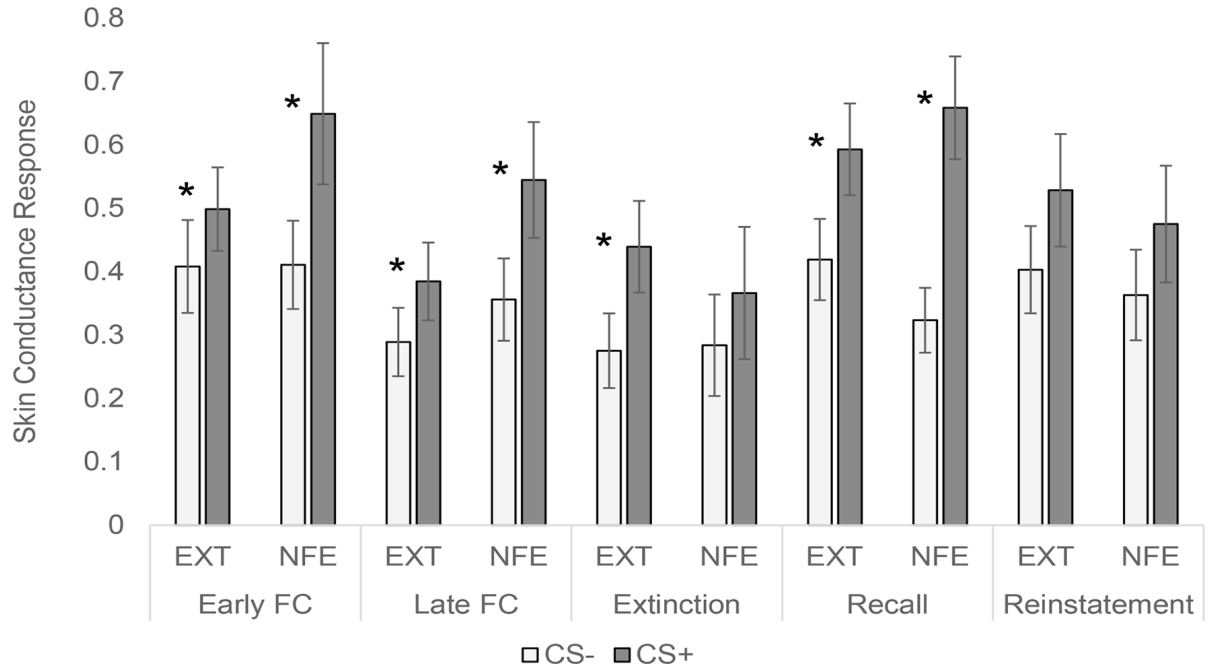

FIGURE 3 | Skin conductance response to conditioned stimuli across experimental phases, separated by group. *Note*. EXT, Standard Extinction; NFE, Novelty-Facilitated Extinction; CS, Conditioned Stimulus. Error bars represent the standard error of mean. *$p < 0.05$ for paired sample *t*-tests comparing CS- to CS+ in each group.

criteria for an anxiety disorder or OCD have extinction deficits compared to healthy control samples (Michael et al., 2007; Milad et al., 2013; Duits et al., 2016; Rabinak et al., 2017). Moreover, a meta-analysis by Duits et al. (2015) found a trend for increased response to CS+ vs. CS- during extinction in anxious individuals (defined as individuals that met criteria for DSM-IV anxiety disorders) compared to healthy controls. Another possibility is that tonic arousal is elevated, maintained, and carries over from acquisition into extinction in a clinically anxious sample. The result of impaired extinction learning could therefore be akin to the immediate extinction deficit described by Maren (2014), whereby elevated anxiety interferes with extinction learning processes. To address these different possibilities, future research testing novelty-facilitated extinction in a clinical sample should include a healthy control group, increase the number of extinction trials, use stimuli other than angry faces, and test the effect of immediate vs. delayed extinction.

The inability of the NFE paradigm to produce similar effects in clinically anxiety participants as shown previously in healthy adults (Dunsmoor et al., 2015, 2019; Lucas et al., 2018) raises important questions on how to leverage insights into the mechanisms of fear extinction to improve exposure-based therapy. There is an excitement that laboratory-based approaches centered on inhibitory learning and memory updating can translate to the clinic (Fullana et al., 2020). This includes techniques such as counter conditioning (Keller et al., 2020), memory reconsolidation updating, and pharmaceutical adjuncts to enhance learning during psychotherapy (Phelps and Hofmann, 2019). However, many issues remain in translating basic research in healthy adults to clinical populations, and much more work is needed to discover how to optimize behavioral protocols to yield similar effects in people with diagnosed anxiety disorders and OCD. Future designs should consider more extensive extinction-based training (e.g., multiple sessions) and be attentive to individual participants' ability to successfully extinguish conditioned fear as a precondition to test the return of fear.

This study has several strengths: an unmedicated, transdiagnostic sample with pathological anxiety, and the use of an established laboratory paradigm (Dunsmoor et al., 2015). Results should be considered in light of the limitations described above as well as the fact that our sample was predominantly white and non-Hispanic. Despite limitations, our preliminary findings provide insight into methodological considerations for future tests of novelty-facilitated extinction, and data can be included in future meta-analyses.

In summary, our preliminary data is in line with prior findings that demonstrate extinction deficits in those with anxiety disorders and OCD, and extend these findings to suggest that such individuals may process novel information differently than healthy individuals (though replication with a diagnosed vs. healthy sample is needed). In addition, our data highlight the importance of testing whether basic and clinical neuroscience findings gleaned in healthy populations translate to clinical samples. This will ensure that new treatment ideas based on basic neuroscience advances are indeed relevant for patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NYSPI Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SS: funding acquisition, conceptualization, formal analysis, investigation, writing—original draft, writing—review and editing. JD: conceptualization, methodology, resources, data curation, writing—review and editing. ZG: software, investigation, and resources. YS: investigation and project administration. OP and JP: project administration. EP: resources and supervision. AF: resources, supervision, writing—review and editing. HBS: resources, supervision, writing—review and editing. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Association of Two Opposing Responses Results in the Emergence of a Novel Conditioned Response

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Recent studies examining association of opposing responses, contrasting emotional valences, or counter motivational states have begun to elucidate how learning and memory processes can translate to clinical therapies for trauma or addiction. In the current study, association of opposing responses is tested in *C. elegans*. Due to its relatively simple and well-described nervous system, it was hypothesized that association of two oppositional stimuli presented in a delayed conditioning protocol would strengthen the behavioral response to the first stimulus (alpha conditioning). To test this, *C. elegans* were exposed to a tone vibration stimulus (to activate a mechanosensory-driven locomotor reversal response) paired with a blue light (to activate a forward locomotor response) at a 2-s delay. After five pairings, behavior was measured following a tone-alone stimulus. Worms that received stimulus pairing did not show an enhanced response to the first presented stimulus (tone vibration) but rather showed a marked increase in time spent in pause (cessation of movement), a new behavioral response (beta conditioning). This increase in pause behavior was accompanied by changes in measures of both backward and forward locomotion. Understanding the dynamics of conditioned behavior resulting from pairing of oppositional responses could provide further insight into how learning processes occur and may be applied.

Keywords: opposing responses, *C. elegans*, delayed conditioning, photosensory, mechanosensory, classical conditioning

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INTRODUCTION

Modifying behavior by conditioning a new response has numerous clinical applications. A "strong" memory like fear may be considered more resistant to disruption or alteration due to learning (Roozendaal and McGaugh, 2011). Recent work has attempted to counter-condition strong fear responses (anxiety disorder, post-traumatic stress disorder; PTSD) or drug cravings for the treatment of addiction. For instance, Hurd et al. (2019) reported a reduction in cue-induced craving and behavioral indicators of anxiety (increased heart rate, temperature and salivary cortisol levels) following repeated presentation of cannabidiol to counter feelings of stress brought on by heroin drug cues in opioid addicts (Fatseas et al., 2011; Preston et al., 2018). Using a similar rationale, better treatment outcomes for PTSD patients are reported when 3,4-methylenedioxymethamphetamine (MDMA) is delivered during psychotherapy sessions to counter the anxiety and/or fear-responses

activated during recall of traumatic memories (Mithoefer et al., 2019; Mitchell et al., 2021). At the simplest level, the approach of driving an opposing behavioral response (even if pharmacologically induced) to alter future behavior can be seen as a function of signal integration; thus, it is of interest to investigate how signal integration occurs in a nervous system.

In rodent models, conditioning of opposing responses has been tested by pairing two unconditioned stimuli (US-US conditioning); for instance, rats salivating in response to a footshock following food-shock pairing (Gormezano and Tait, 1976). However, there are few reports of this form of conditioning. More often, investigators counter-condition a previously conditioned response. For example, rabbits conditioned to perform an appetitive jaw-movement following tone-water conditioning were later trained with water-pre-orbital shock pairings and showed both jaw-movement and nictitating membrane responses to tone suggesting parallel opposing response pathways were activated to the conditioned tone stimulus (Tait et al., 1986). More recently it has been purported that individual neurons can be involved in driving opposing responses as mice conditioned to escape following photostimulation of neurons expressing channelrhodopsin (ChR2) in piriform cortex, will show a subsequent appetitive licking response when ChR2 activation of the same neurons is later paired with water presentation (Choi et al., 2011). A circuit-specific investigation of conditioning of opposing responses could elucidate the learning processes involved.

Part of the challenge to uncovering plasticity processes involved with conditioning opposing response behaviors is due to the complexity of the nervous systems employed. *Caenorhabditis elegans* (*C. elegans*) is a microscopic nematode with a neural connectome made up of 302 neurons (White et al., 1986). These neurons and their connections have been identified (Cook et al., 2019). A popular example of conditioning opposing outcomes in *C. elegans* is to generate an avoidance response to the attractant sodium chloride (NaCl) by pairing NaCl with the absence of food (Saeki et al., 2001). The same avoidance response can be conditioned to other water-soluble attractants as well [cyclic adenosine monophosphate (cAMP), biotin, lysine; Saeki et al., 2001]. Other examples of conditioning avoidance behavior to attractant signals in *C. elegans* include pairing cultivation temperature with starvation, or 1-propanolol with an aversive acidic pH environment produced by hydrochloric acid (Mohri et al., 2005; Amano and Maruyama, 2011). Some have also shown that an aversive stimulus (1-nonanol or ultraviolet light) can be conditioned to drive an appetitive response when paired with an attractant (potassium chloride or food; Nishijima and Maruyama, 2017; Ozawa et al., 2022). Thus, *C. elegans* are capable of updating response circuits when stimuli are presented with opposing conditions.

In each of these examples of *C. elegans* opposing state conditioning, the conditioned response activates the same forward locomotion neural circuitry in order to produce differential migration patterns (i.e., forward movement toward or away from the conditioned stimulus). The aim of the current study was to investigate response conditioning by associating two stimuli that drive opposing locomotor responses: generalized mechanosensory stimulus (vibration produced by a tone; Chen and Chalfie, 2014) and a blue light stimulus. Worms typically respond to a non-localized mechanosensory stimulus (e.g., tap to the side of the petri dish) with backward locomotion (Wicks and Rankin, 1995). Conversely, worms respond with forward locomotion in response to whole-body blue light illumination (Edwards et al., 2008). The neural circuitry for responses driven by each of these forms of stimuli has been largely identified (see **Figure 1**) and includes the potential for signal integration at several interneurons (see **Table 1**). Using a delayed conditioning protocol, it was expected that paired presentation of a tone (vibration) stimulus with a blue light would result in a strengthening of the mechanosensory reversal response as the onset of the blue light presentation was delayed. However, responses to both stimuli appeared to be modulated following conditioning.

METHODS

Strains and Strain Maintenance

N2 (Wild Type, acquired originally from the *Caenorhabditis* Genetics Center, U Minnesota) colonies were maintained on NGM agar plates at 20°C and fed the OP50 *E. coli* strain (Stiernagle, 2006). Worm colonies were age-synchronized, cultivated at 20°C and tested 4 days later (day L4 + 1). This adult stage was chosen for testing to ensure complete maturation of mechanosensory neurons, as the touch-sensitive AVM neuron matures in a late larval stage (Chalfie et al., 1985). Age synchronization entailed picking 10–12 adult worms and submersing them in a ∼5 ul drop of 2:1 bleach to 1 M NaOH solution on a seeded plate and testing surviving progeny (see Stiernagle, 2006; Meneely et al., 2019).

Behavioral Assay and Apparatus

Behavioral training and testing was conducted on seeded NGM plates with ∼20 worms per plate. During testing, on average ∼10 worms were video captured per plate. Training and testing were conducted in the dark with blue light filters on all computer monitors and the microscope stage, to control for blue light from other light sources. Worms were acclimated to the training/testing conditions for 2 min prior to any stimulus delivery. Worms in the Naïve group were positioned in the training/testing apparatus for an equal period of time as the stimulus conditions but Naïve received no blue light or tone stimuli. The conditioning assay consisted of five pairings of a 5-s 300 Hz tone vibration, produced by placing a speaker (XM12001 X-Vibe 3. 0 Vibration Speaker by XDream) next to and in contact with the worm-containing agar plate on the microscope stage (Olympus SZ7), with 2-s delayed onset of a 3-s 470 nm blue light stimulus at 1,000 mA (Mightex LED) placed above the plate. Training stimuli were presented at a 1-min interstimulus interval (ISI). For the Tone Alone condition, worms were exposed to five presentations of the 5-s tone stimulus at a 1-min (ISI) and for the Light Alone condition worms were exposed to the 3-s blue-light stimulus at a 1-min ISI (see **Figure 2A**). Testing consisted of a 5-s tone vibration stimulus alone delivered at 5 and 10 min

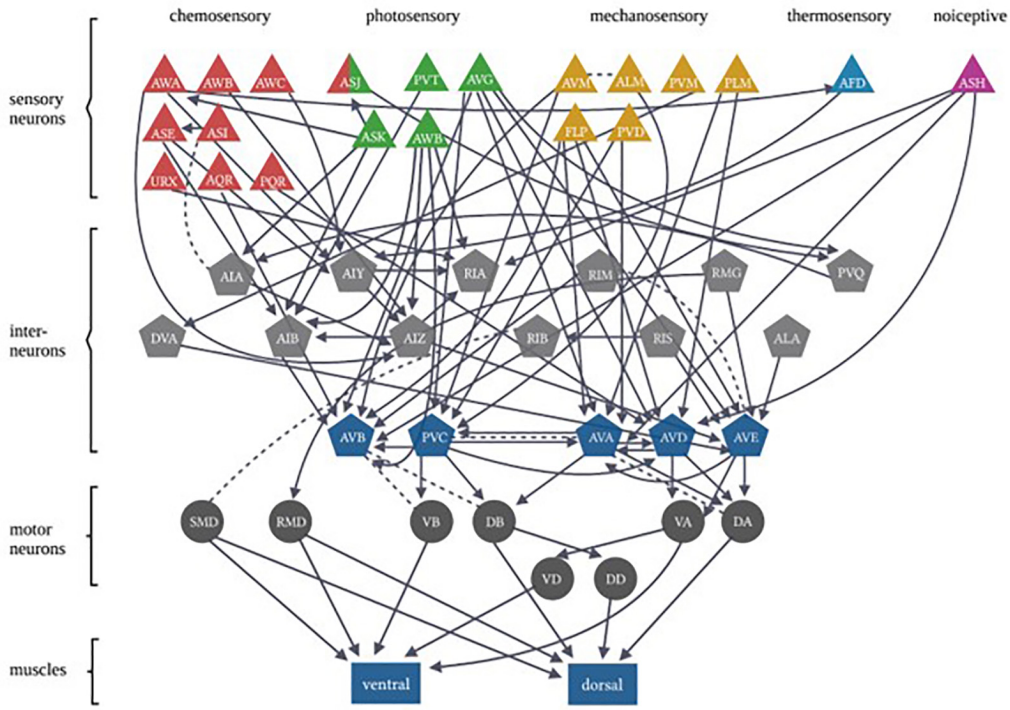

FIGURE 1 | *Caenorhabditis elegans* photo- and mechanosensory neural circuits are embedded within a broader set of multiple sensory-response circuits. Sensory neurons are represented by triangles, with colors differentiating between different sensory inputs. Gray pentagons represent interneurons and blue pentagons represent command interneurons. Motor neurons are indicated by circles that extend connections to body-wall muscles represented by rectangles (Figure adapted from Fang-Yen et al. (2015), with permission from the Royal Society, United Kingdom).

after the last pairing or following a comparable time period on the microscope for the Naïve group.

Video of the locomotor response to tone vibration was captured *via* CCD Camera (AmScope MUB2003). Videos of behavior were captured for the duration of stimulus presentation plus 10 or 60 s. Behavior was only analyzed for the periods after stimulus presentation because of minor video distortion due to vibration during stimulus presentation, so stimulus offset was used to consistently mark the beginning of analyses. Each trial had a minimum of three replicates and each group had trials that were tested over a minimum of three different days with trained and untrained matched controls for every test day to randomize any effects due to environmental fluctuations. For the motion mode analysis (**Figure 2**) data was captured from a total of 130 worms with 5 worms not responding for the recording period. For locomotor metrics (**Figure 3**), data was derived from 1,327 different worm trajectories. Data recorded at 5 and 10 min post-training were captured from the same plates of worms for both time points. No worms were excluded from analysis.

Behavior Analysis

All behavior videos were analyzed using TierPsy Multi-Worm tracker (V1.4.0; Javer et al., 2018a,b). This program is a free download, found here: https://github.com/ver228/tierpsy-tracker. Descriptions of output files for TierPsy are found here: https://github.com/ver228/tierpsy-tracker/blob/master/docs/OUTPUTS.md.

TABLE 1 | Neurons involved in photosensory processing.

Photosensory neurons	**PVT**	**AVG**	**ASJ**	**ASK**	**AWB**
Interneurons	**RIG**	**PVC**	AIM	**AIA**	**AIB**
	RIH	**PVP**	PVQ	**AIB**	**AIZ**
	AVK	PVQ		**RIF**	**AVB**
	DVC	PVT		AIM	**RIA**
	AIB	**AVA**			RIR
		AVB			
		AVD			
		AVE			
		AVJ			
Interneurons/motor neurons	**SMB**	PVN	AVF		**SMB**
	AVL	AVF			
	RMF	AVL			
Motor neurons	RME	VA	**HSN**		
		DA			
Sensory neurons		PHA	**ASK**	**CEP**	**ADF**
				AWA	**ASG**
				ASJ	**ASH**

Bold indicates neurons also have a reported role in detecting and responding to mechanosensory stimuli (compiled from Chalfie et al., 1985; Von Stetina et al., 2006; Ward et al., 2008; Haspel et al., 2010; Bhatla and Horvitz, 2015).

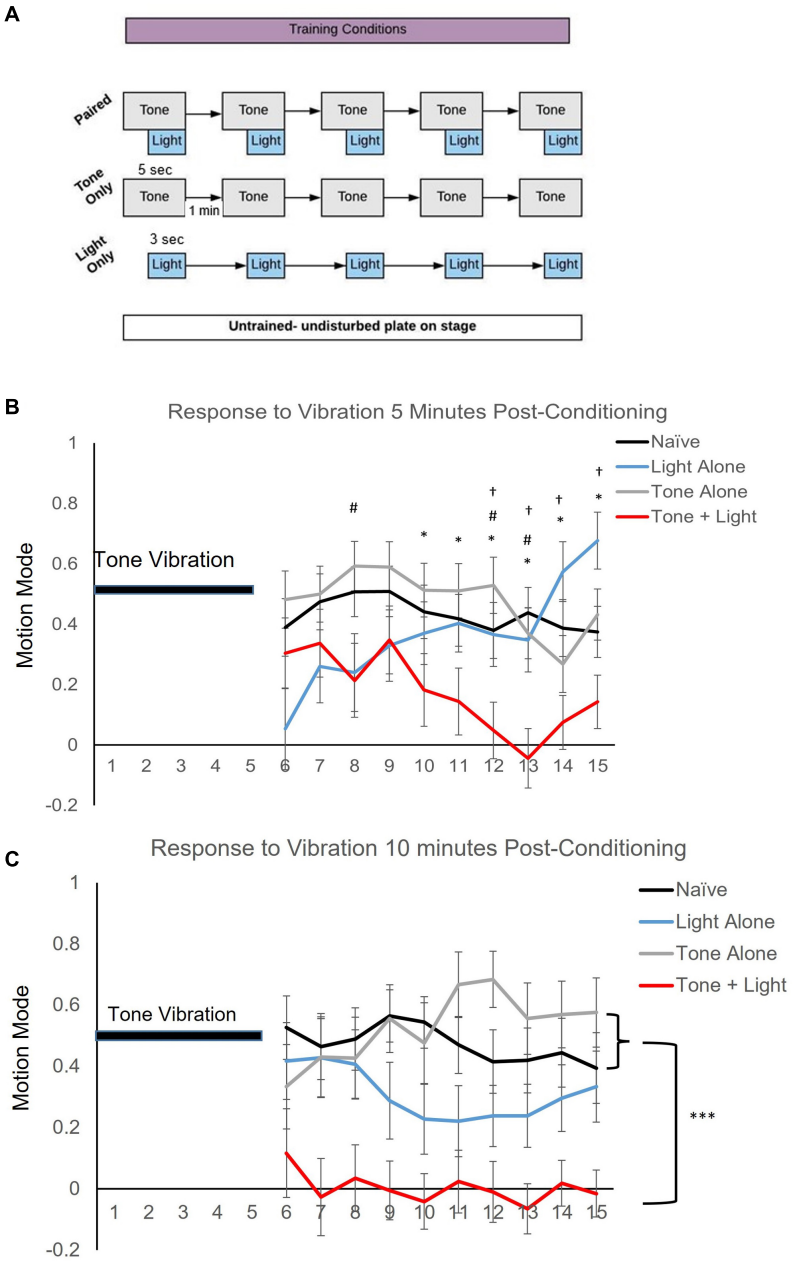

FIGURE 2 | Training condition affects locomotor direction of worms following a single tone stimulus. **(A)** Training protocol showing Naïve (untrained), Tone Alone, Light Alone and Paired conditioning. Stimulus pairing consisted of five presentations of a 5-s tone vibration stimulus delivered with a 3-s blue light stimulus at a 2-s delayed onset. Testing consisted of a single 5-s tone stimulus. **(B)** Mean motion mode (± SEM) (indicates average vector of movement direction whereby + values signify forward and − values signify backward locomotion direction) captured for the 10-s period following the 5-s test tone at 5 min after conditioning. *Post-hoc* analyses indicate: $^{*}p < 0.05$ between Naïve ($n = 46$) and Paired ($n = 29$); $^{\#}p < 0.05$ between Tone Alone and Paired ($n = 25$); $^{\dagger}p < 0.05$ between Light Alone ($n = 30$) and Paired training conditions. **(C)** Mean motion mode (± SEM) captured for a 10-s period following a 5-s test tone at 10 min after conditioning. *Post-hoc* analyses indicate: $^{***}p < 0.001$ of main effects.

Tierpsy Criteria for Locomotor Responses

Forward: The animal is moving in the "head direction" for at least 0.5 s and at least 5% of its length.

Backward: The animal is moving in the "tail direction" for at least 0.5 s and at least 5% of its length.

Pause: The animal is moving neither in the head nor tail direction for at least 0.5 s.

Omega Turns: The animal moves forward; the head side then turns back at a sharp angle to become even with the tail and swims off in the direction at which the animal was coming from in a forward motion.

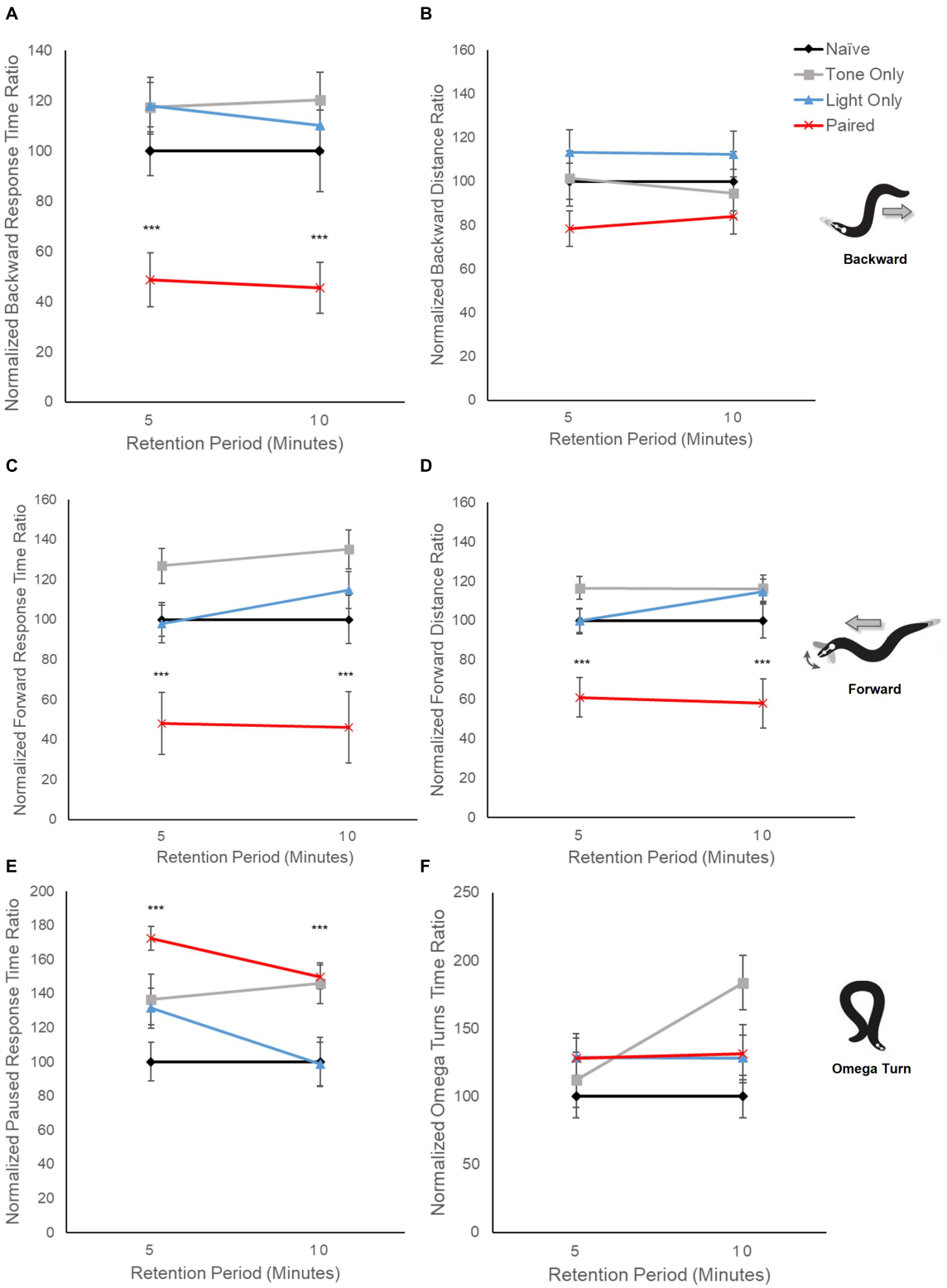

FIGURE 3 | Locomotion metrics indicate differences in locomotor response circuits following conditioning. Graphs display averaged response metrics for a period of 60 s following a 5-s tone stimulus delivered at 5 min and 10 min after conditioning (retention period). Average response metrics of all training groups are expressed relative to the average of Naïve worm responses. Mean (± SEM) for: **(A)** backward response time ratio, **(B)** backward distance ratio, **(C)** forward time ratio, **(D)** forward distance ratio, **(E)** paused time ratio, and **(F)** omega turn time ratio. ***$p < 0.001$ between Naïve and Paired groups. Worm movement illustrations adapted with permission from Broekmans et al. (2016).

Behavior Metrics

Descriptions of the calculated locomotor measures provided in Tierpsy software:

Time Ratio: (no units) ratio between the time spent at the event over the total trajectory time. This is calculated for forward, reversal, pause, and omega turn behaviors.

Distance Ratio: (no units) ratio between the total distance traveled during an event and the total distance traveled during the whole trajectory. This is calculated for only forward and reversal behaviors.

Motion Mode: vector indicating if the worm is moving forward (1), backward (−1), or is paused (0). This measure is unique for every animal, as it assigns a number for the above movements for every frame that animal is in.

Statistical Analysis

Analysis of behavioral data was performed using Mixed repeated measures ANOVAs for motion mode with least significant difference for *post-hoc* analyses ($p < 0.05$ significance). Locomotor metrices were analyzed with two-way ANOVAs, with Dunnett's *post-hoc* comparisons to determine the effect of training, by comparing the results of vibration-light training, light-only and tone-only to the appropriate untrained group, of the same retention period ($p < 0.05$ significance). Analyses were completed in R, using package "car" for analysis and "ggplot2" for data visualizations or IBM SPSS Statistics (Ver. 26, Microsoft). For response metrics, visualization of behavioral data for trained, light-only, or tone-only animals is normalized to data from untrained animals (% Naïve) and presented as normalized mean. Lucidchart, Adobe Illustrator and Excel were used to create diagrams and schematics.

RESULTS

Repeated pairings of a mechanosensory tone stimulus with a blue light stimulus result in a difference in response to the tone stimulus alone. Motion mode (vector indicator where + values indicate forward motion and−values indicate backward motion) was captured for a 10-s period after a vibration test stimulus presentation. Results show a statistically significant interaction between training and time on locomotion direction at 5 min post-training ($F_{3.817,11.452} = 3.478$, $p < 0.001$ with Greenhouse-Geisser correction; **Figure 2B**). Despite the variability of behavior across trials from animals in all training conditions in the initial seconds following delivery of the tone stimulus, only the Paired group shows locomotion direction at ∼0 (meaning worms were not moving forward or backward, but were likely paused) for the latter time-period measured (5–10 s after tone stimulus delivery). At 10 min after conditioning, there was no interaction of training across time ($F_{10.101,420.880} = 1.013$, $p > 0.05$) as there was no statistical difference in response direction over time ($p > 0.05$); however, there was a significant effect of training condition ($p < 0.001$). Multiple comparisons across training conditions (corrected for multiple tests) indicate significant differences between the Paired group and both the Naïve ($p < 0.001$) and the Tone Alone ($p < 0.001$) groups. Multiple comparisons did not reveal a significant difference between the Light Alone group and any of the other training conditions ($p > 0.05$) though there was a trend toward significance between the Paired and Light Alone group ($p = 0.076$; **Figure 2C**). These data indicate that pairing of a tone vibration with a blue light stimulus to drive opposing locomotor responses affects subsequent responding to the tone stimulus.

Examination of distinct locomotor response elements provides some indication as to how behavior has changed following conditioning. When the relative time spent performing backward locomotion is captured and averaged over a longer period following the mechanosensory tone stimulus presentation (60 s), trained worms show a notable decrease compared to control groups ($F_{3,633} = 26.30$, $p < 0.001$; **Figure 3A**). This overall difference in response behavior cannot be accounted for by pseudoconditioning to either the tone stimulus or the light stimulus alone as worms repeatedly exposed to a single tone stimulus or light stimulus did not show the same decline in backward relative response time ($p > 0.10$). Interestingly, the decrease in backward locomotion response time did not correspond to a significant decrease in relative backward distance traveled ($F_{3,483} = 2.230$, $p = 0.08$; **Figure 3B**). For forward locomotion, the relative amount of time trained worms spent moving in a forward direction was also significantly decreased across retention periods compared to the control groups ($F_{3,633} = 45.11$, $p < 0.001$; **Figure 3C**); however, a significant decrease in the relative distance traveled while moving forward was also noted for the trained group ($F_{3,633} = 43.72$, $p < 0.001$; **Figure 3D**). Together, these data suggest that responding in the mechanosensory-driven reversal circuit and the light-driven forward circuit appear altered following conditioning.

As earlier data indicated consistent average motion mode direction around zero in trained worms (see **Figures 2B,C**), we also examined relative time spent performing pause behavior (neither forward nor backward locomotion) and found a significant increase in paused behavior response time ratio for trained worms compared to controls ($F_{3,557} = 6.46$, $p < 0.001$; **Figure 3E**). This is interesting as it could suggest that neither locomotor circuit is activated following conditioning, or conversely that both are now activated, and the effects cancel out. The tone-only experimental group also showed a significant increase in relative pause time but only at the 10-min retention interval ($p < 0.001$).

To probe if the increase in paused behavior and decrease in both backward and forward locomotion could reflect an overall change in locomotor responding a separate avoidance behavior metric was analyzed across groups. Omega turns are avoidance responses whereby worms reverse into a circular body position in order to change direction. Interestingly, there appeared to be no significant difference in the time spent performing Omega turns across groups ($F_{3,347} = 1.99$, $p = 0.11$) suggesting the effects on locomotor behavior are not due to a generalized locomotor deficit.

DISCUSSION

Associative conditioning of opposing response circuits results in a change in locomotor behavior following delivery of a single tone test stimulus (**Figures 2B,C**). As well, the shift to a pause response to the tone vibration appears to become more consistent at 10 min after conditioning compared to 5 min. As the same worm plates are tested at both time points, it is possible that learning increases by 10 min post-conditioning if the tone-CS presentation at 5 min serves as reminder (Spear, 1973). Other work in *C. elegans* has shown enhanced learning following subsequent presentation of learning stimuli (Rose et al., 2002; Amano and Maruyama, 2011). It is also possible that some gradual plasticity process or memory consolidation mechanism is activated following the repeated pairings. Examples of this include mitogen-activated protein kinase (MAPK)-dependent sensitization of the siphon withdrawal reflex in *Aplysia* following repeated shock delivery (Sutton et al., 2002: Sharma et al., 2003), increased calcium levels in associated neurons after odor-shock pairing in *Drosophila* (Wang et al., 2008), and time-dependent enhancement of contextual fear memory when multiple shocks are given during conditioning (Poulos et al., 2016).

It was anticipated that initial onset of the tone vibration stimulus would prime any response modulation to be entrained only to the tone as response modification with US-US delayed conditioning should result in the first stimulus being influenced by the second stimulus in the pairing (Schreurs and Alkon, 1990). Preliminary data from trials where the blue light was presented first with delayed tone onset suggest that tone presentation could modulate response to blue light in a similar way (see **Supplementary Figures 1A–C**) though technical challenges limit interpretation of these data (see **Supplementary Material**). As there are a number of neurons included in both the mechanosensory and the photosensory neural circuits (see **Table 1**), it is possible that repeated pairing stimulates some plasticity mechanism that alters the response probabilities for both stimuli.

When backward and forward locomotion were analyzed separately, trained worms showed significant decreases in the relative time spent performing either forward or backward locomotion following a tone vibration test stimulus compared to naïve and to single-stimulus training groups (**Figures 3A,C**). Interestingly, trained worms also showed a significant increase in relative time spent paused (no locomotion; **Figure 3E**). In rodents, individual neurons in olfactory piriform cortex can be involved in both appetitive and avoidance responses such that when a neuron activated with an aversive outcome is re-activated in an appetitive environment neither the appetitive nor avoidance behavior occurs but instead a freezing response is initially seen (Choi et al., 2011). Thus, it is possible that the immediate effect of co-activation of opposing response circuits is a similar "freezing" response in the worm. Future research will need to examine further how this cessation in locomotion occurs following conditioning as well as determine the duration this increase in pause behavior persists.

As mentioned, there are both independent and overlapping neurons included in both the mechanosensory and photosensory neural circuitry (**Figure 1**). The majority of neurons involved in both circuits are interneurons (**Table 1**), in particular, all of the locomotor command interneurons (AVA, AVD, AVE, AVB, PVC) thus again identifying these neurons as possible sites for neural signal integration leading to a decision of locomotor response behavior (Chalfie et al., 1985; Piggott et al., 2011; Kaplan et al., 2018). In mammals, the ventral pallidum has been reported to contain both positive- and negative-valence specific neurons that differentially influence what behavior is expressed in response to environmental conditions (Stephenson-Jones et al., 2020). In *C. elegans*, behavioral flexibility derived from signal integration (described as a "hub and spoke"' model) has been reported for multiple sensory systems (Macosko et al., 2009; Rabinowitch et al., 2013; Summers et al., 2015). This form of neural signaling architecture has been previously reported to mediate responses driven by opposing outcomes (threat vs. reward) in worms (Ghosh et al., 2016). Plasticity within a hub interneuron could explain the ability for rapid response modulation following co-activation of the opposing locomotor response circuitry.

There are many questions that remain unanswered with regards to how organisms process and reconcile information from competing and oppositional stimuli. From the current study, future research will need to uncover the extinction parameters for each stimulus, the duration for which this learning persists, and address if additional pairings prolong retention. As well, determining if this form of learning is vulnerable to a stimulus pre-exposure effect that could reduce the efficacy of the association conditioning protocol (Randich and LoLordo, 1979). Finally, it is of great interest to delve in to the neural signaling mechanisms that underlie learning resulting from the co-occurrence of competing inputs. It is likely that similar to other models as well as previous reports of *C. elegans* learning that glutamate signaling and perhaps calcium/calmodulin-dependent kinase II (CaMKII) activation is involved (Amano and Maruyama, 2011; Stein and Murphy, 2014; Ozawa et al., 2022). Given the simplicity of the approach and the relatively rapid conditioning time scale, it may be possible to employ neuron activation tools to capture signaling changes in real-time allowing us to answer many questions about how neurons integrate competing signals to guide behavior. The implications of these results could offer insight into the mechanisms and the efficacy of this conceptual approach in applied settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MP performed the experiment and analyzed the data except for the testing in response to light stimulus and composed sections of the initial draft. AHB performed the testing in response to the light experiment and analyzed the data. ADB composed multiple

sections of the initial draft and compiled **Figure 1** and **Table 1**. JG and LC composed sections of the initial draft. JR conceptualized the study, consulted and supervised on data collection and analysis, and composed and refined all of the manuscript drafts. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.852266/full#supplementary-material

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