HIGHER-ORDER CONDITIONING: BEYOND CLASSICAL CONDITIONING

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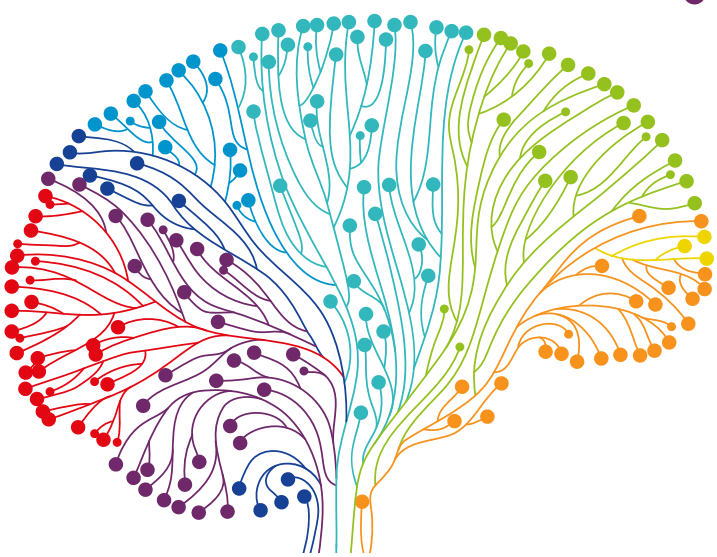
PUBLISHED IN: Frontiers in Behavioral Neuroscience



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ISSN 1664-8714
ISBN 978-2-88976-418-1
DOI 10.3389/978-2-88976-418-1

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HIGHER-ORDER CONDITIONING: BEYOND CLASSICAL CONDITIONING

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Citation: Busquets-Garcia, A., Holmes, N., eds. (2022). Higher-Order Conditioning: Beyond Classical Conditioning. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-418-1

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EDITORIAL
published: 25 May 2022
doi: 10.3389/fnbeh.2022.928769

Editorial: Higher-Order Conditioning: Beyond Classical Conditioning

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Keywords: higher-order conditioning, sensory preconditioning, second-order conditioning, rodent model, humans

Editorial on the Research Topic

Higher-Order Conditioning: Beyond Classical Conditioning

INTRODUCTION

Pavlovian conditioning is the means by which animals learn about cues that signal biologically significant events, such as the presence of food or danger. In the laboratory, it is studied in a range of species, including fish, crabs, snails, birds, rodents, primates, and human subjects. Studies of so-called "higher-order" Pavlovian conditioning have provided specific insights to how animals learn about cues that signal innocuous events, how different types of associations are linked in a memory network, and how memories are retrieved from a network to guide behavior. Such studies are rapidly gaining popularity in the field of behavioral neuroscience. They have the potential to accelerate our understanding of how learning and memory is organized in the brain, and thereby, disturbances of learning and memory that underlie various brain pathologies.

This Research Topic consists in a series of empirical and theoretical papers that analyze the two types of higher-order conditioning: sensory preconditioning and second-order conditioning. These papers specifically address what is learned in sensory preconditioning and second-order conditioning, and how this learning is expressed in behavior; the pharmacological and neural processes that regulate the two types of conditioning; and points of contact between studies of higher-order conditioning in animal and human subjects.

The first set of papers addresses what is learned during sensory preconditioning and second-order conditioning; and how this learning is retrieved/expressed in behavior. Prével and Krebs review findings that the level of responding to a sensory preconditioned or second-order conditioned stimulus can be independent of the level of conditioning to its first-order conditioned stimulus-associate; and consider implications of this independence for classic and contemporary theories of learning and memory. Gostolupce et al. review factors that influence sensory preconditioning and second-order conditioning; what is learned in different types of sensory preconditioning and second-order conditioning protocols, and how an appreciation of these differences might help to identify the functions of specific brain regions. Honey and Dwyer provide a formal analysis of higher-order conditioning according to their model, HeiDI (How excitation and inhibition determine ideo-motion), which attributes sensory preconditioned and second-order conditioned responding to complex (but principled) chains of associations that form in training; that is, they explicitly address how the two forms of higher-order conditioning are expressed in behavior. Finally, Muñiz-Diez et al. show that, when second-order conditioning is established using a feature negative discrimination across multiple training sessions, the second-order stimulus initially elicits responding, gradually ceases to elicit responding and eventually passes a retardation test for the presence of inhibition.

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

Received: *26 April 2022*
Accepted: *09 May 2022*
Published: *25 May 2022*

Citation:
Busquets-Garcia A and Holmes NM (2022) Editorial: Higher-Order Conditioning: Beyond Classical Conditioning. Front. Behav. Neurosci. 16:928769. doi: 10.3389/fnbeh.2022.928769

The second set of papers examines/reviews the pharmacological underpinnings of sensory preconditioning and second-order conditioning. It shows that associative formation between affectively neutral stimuli in sensory preconditioning is enhanced by a systemic injection of an opioid receptor antagonist (Michalscheck et al.); impaired by a systemic injection of a dopamine receptor antagonist (D1 or D2; Roughley et al.); and also impaired by a systemic or intra-hippocampal injection of a cannabinoid receptor antagonist (CB1; Ioannidou et al.). It also shows that midbrain dopamine regulates appetitive second-order conditioning in the same way that it regulates associative formation in sensory preconditioning (Seitz et al.); and opioid receptor-dependent signaling regulates aversive second-order conditioning in the same way that it regulates associative formation in sensory preconditioning (Michalscheck et al.). Taken together, these studies imply that: (1) despite their co-evolution, endocannabinoid and opioid receptors influence sensory preconditioning in different ways; (2) despite their differences, sensory preconditioning and second-order conditioning share the same pharmacological underpinnings in the brain; (3) given the links between error-correction and midbrain dopamine in appetitive protocols, error-correction drives the learning that occurs in appetitive second-order conditioning; and (4) given the links between error-correction and opioid receptor-signaling in aversive protocols, error correction drives the learning that occurs in aversive second-order conditioning.

Fournier et al. extend this analysis by reviewing the brain regions that are engaged during sensory preconditioning and/or second-order conditioning. These regions include the hippocampus (Busquets-Garcia et al., 2018), amygdala (Parkes and Westbrook, 2011), orbitofrontal, perirhinal, and retrosplenial cortices (Robinson et al., 2014; Holmes et al., 2018; Sadacca et al., 2018). Fournier et al. focus on the retrosplenial cortex which is shown to encode sensory preconditioned associations in the absence of reinforcement. On the basis of these demonstrations, they argue that future work should examine how the retrosplenial cortex interacts with other regions during sensory preconditioning as this will shed light on how this brain region encodes and stores very basic types of information. More generally, it will be important to assess how the aforementioned brain regions interact with each other during both forms of higher-order conditioning as this will lay a foundation for discovering how the brain encodes and stores different types of information.

The remaining papers in our Research Topic include studies of higher-order conditioning in people. While higher-order conditioning has been demonstrated many times in animal subjects (Gewirtz and Davis, 2000), there are relatively few demonstrations in humans; so much so that it has been described as experimentally elusive in these subjects. In this respect, Lee recognizes difficulties in performing second-order conditioning experiments in humans and identifies critical parameters for establishing reliable effects in these subjects. Dhamija et al. provide a novel demonstration of second-order conditioning in humans using electrophysiological responses as a measure of performance; and some evidence that first- and higher-order conditioning might be supported by different neural substrates. Bouchekioua et al. review evidence that has been taken to indicate the use of reasoning-like processes when navigating in a new environment; and show that goal-directed navigation can be explained as the result of higher-order associative learning rather than by appeal to reason or inference. Finally, Wang et al. examine the episodic-like basis of sensory preconditioning in human subjects; and suggest that distinct memories might be manipulated to achieve better outcomes in the treatment of various pathologies [e.g., post-traumatic stress disorders (PTSD)].

CONCLUDING REMARKS

Second-order conditioning and sensory preconditioning were first-described many decades ago: the former by Pavlov (1927) and the latter by Brogden (1939). It is now recognized that the study of higher-order conditioning has the potential to answer fundamental questions about how the brain processes and integrates different types of information; and that the answers to these questions will advance our understanding of how the brain works under normal and pathological conditions. However, two major gaps must be addressed before such understanding can be attained. First, it will be important for higher-order conditioning to be reliably established in laboratory studies with human subjects, as this will expand analysis of the ways in which environmental stimuli influence decision making and contribute to psychopathology. Second, we must improve the dialogue between researchers that study higher-order conditioning in animals and clinicians that treat psychopathology (including PTSD, addictions and anxiety disorders) to ensure that gains in knowledge from basic science research are useful and applied in the development of therapeutic strategies.

All of this is to say that, while our understanding of the behavioral, pharmacological and neural substrates of higher-order conditioning has advanced over the past few decades, much work remains to be done. Our Research Topic identifies lines of inquiry that could and should be pursued; and, ultimately, how theories of higher-order conditioning might be grounded in the brain and used to inform the management/treatment of psychopathology.

AUTHOR CONTRIBUTIONS

AB-G and NH have contributed equally to this work and approved it for publication.

FUNDING

This work was supported by the IBRO Return Home Fellowships 2019 and the ERC Starting Grant (HighMemory,

#948217) received by AB-G. AB-G is a Ramon y Cajal researcher (RYC-2017-21776, funded by MCIN/AEI/10.13039/501100011033 and FSE). Finally, NH was supported by an Australian Research Council (ARC) Future Fellowship (FT190100697).

ACKNOWLEDGMENTS

We would like to thank all the authors participating in this Research Topic and also all the reviewers that have been involved in the revision processes.

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 21 April 2021
doi: 10.3389/fnbeh.2021.632548

Second-Order Conditioning and Conditioned Inhibition in Different Moments of the Same Training: The Effect of A+ and AX− Trial Number

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The feature negative discrimination (A+/AX−) can result in X gaining excitatory properties (second-order conditioning, SOC) or in X gaining inhibitory properties (conditioned inhibition, CI), a challenging finding for most current associative learning theories. Research on the variables that modulate which of these phenomena would occur is scarce but has clearly identified the trial number as an important variable. In the set of experiments presented here, the effect of trial number was assessed in a magazine training task with rats as a function of both the conditioning sessions and the number of A+ and AX− trials per session, holding constant the total number of trials per session. The results indicated that SOC is most likely to be found at the beginning of training when there are many A+ and few AX− trials, and CI (as assessed by a retardation test) is most likely to be found at the end of training when there are few A+ and many AX− trials. Both phenomena were also found at different moments of training when the number of A+ trials was equal to the number of AX− trials. These results cannot be predicted by acquisition-focused associative models but can be predicted by theories that distinguish between learning and performance.

Keywords: feature negative discrimination, second-order conditioning, conditioned inhibition, cue interaction, associative learning models

OPEN ACCESS

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

Received: *23 November 2020*
Accepted: *24 February 2021*
Published: *21 April 2021*

Citation:
Muñiz-Diez C, Muñiz-Moreno J and Loy I (2021) Second-Order Conditioning and Conditioned Inhibition in Different Moments of the Same Training: The Effect of A+ and AX− Trial Number. Front. Behav. Neurosci. 15:632548. doi: 10.3389/fnbeh.2021.632548

INTRODUCTION

The feature negative discrimination task consists of pairing an initially neutral stimulus (A) both with an unconditioned stimulus (US) and with another initially neutral stimulus (X) in the absence of the US (Pavlov, 1927/1960). This training, represented as A+/AX−, can result either in X gaining excitatory properties, a phenomenon known as second-order conditioning (SOC), or in X acquiring inhibitory properties, a phenomenon known as conditioned inhibition (CI). The fact that opposite results can be obtained constitutes a challenge for current associative learning theories, as most of them were developed in the light of cue competition phenomena. For instance, the highly influential Rescorla and Wagner (1972) model was aimed to account for learning phenomena such as blocking (Kamin, 1968) or degraded contingency (Rescorla, 1968). It is for this reason that these theories readily explain the phenomenon of CI, which can be considered a form of cue competition. However, cue facilitation effects, such as SOC, are not predicted by most of these models. An exception to this would be the models that are able to differentiate between acquisition and performance, such as the one proposed by Stout and Miller (2007) or Pineño (2007). Thus,

research on the circumstances under which cue competition or cue facilitation emerge is of great theoretical importance (for a review, see Urcelay, 2017).

The circumstances that lead to SOC or CI are still unclear, but previous research has focused in three main variables: the temporal relationship of the stimuli in the compound (AX−), the number of trials employed, and the order of presentation of the AX− trials in relation to the A+ trials. Regarding the temporal relationship of the stimuli in the compound, Pavlov (1927/1960) pointed out some methodological details that need to be taken into account to produce one phenomenon or the other. For example, if the stimuli in the compound (AX) overlap to any extent, CI is observed, but if the new stimulus (X; hereafter referred to as second-order stimulus) is presented just before the onset of the conditioned stimulus (CS; A; hereafter referred to as first order stimulus), then CI is observed less frequently. On the contrary, if the interval between them is increased, SOC is found. Pavlov also mentioned that the duration of the interval should be increased according to the increasing intensity of the second-order stimulus in order to achieve SOC. However, this statement was proved wrong in subsequent studies with different procedures in which SOC was found in spite of overlapping presentations of the first- and second-order stimuli (e.g., Maisiak and Frey, 1977; Rescorla, 1982). Kehoe et al. (1981), in a study of the rabbits' nictitating membrane response, found an inverse relationship between responding to the second-order stimulus and the interval between the first- and second-order stimuli. This result was replicated by Gibbs et al. (1991) with the same procedure. Taken together, these conflicting results suggest that the temporal relationship between the stimuli in the compound is not a critical variable to find either SOC or CI and that its effect, if any, is modulated by other experimental parameters.

As already mentioned, another variable that has been examined is the number of trials. Kehoe et al. (1981) also found that the excitatory response to X followed an inverted U shape as the sessions progressed, although the response was significantly higher than in the control group throughout the experiment, which indicated that SOC was maintained. Gibbs et al. (1991), again using the rabbits' nictitating membrane response, assessed this issue varying the number of AX− trials per session (5, 15, 25, or 50 for each group, respectively), while the number of A+ trials was kept fixed (30). The results indicated that SOC occurred in all groups, as confirmed by the significant differences with the respective unpaired control groups, and that responding followed an inverted U shape as a function of the number of trials (i.e., responding was greater in groups that received 15 and 25 AX− trials per session). Despite this, none of these studies clearly demonstrated CI, as tests for the inhibitory properties were not performed. Rescorla (1972, 1973) and Holland and Rescorla (1975), employing, respectively, a conditioned suppression and a magazine training procedure in rats, showed excitatory conditioning to X at the beginning of training that decreased as the sessions progressed. After training, the inhibitory properties of X were confirmed by a summation test, i.e., the stimulus was able to reduce responding to an excitatory stimulus (transfer excitor) that had been trained independently. All in all, the studies that assess the effect of number of trials indicate that SOC is found early in training and tends to fade out after a certain number of sessions, eventually turning into CI.

Finally, the third variable that interacts with the effect of the number of trials is whether training is performed in two phases or not (Yin et al., 1994). SOC is usually found when A is reinforced in a phase previous to AX− training (e.g., Rizley and Rescorla, 1972), whereas CI is usually found when A+ and AX− trials are interspersed in one single phase (e.g., Pavlov, 1927/1960). Yin et al. (1994) carried out three experiments to determine if the number of trials and the use of phases were of significant importance for the finding of SOC or CI. The results of these experiments indicated that SOC was found only with few AX− trials (a total of four trials across training), no matter if they were presented after or interspersed with 96 A+ trials, and that CI is found when there are many AX− trials (48 across training) interspersed with 96 A+ trials. Stout et al. (2004), using the same procedure, examined the effect of the temporal relationship of A and X in those trials. They presented, across training, either few (four), intermediate (20), or many (100) AX− trials interspersed with 48 A+ trials, and in the AX− trials, the stimuli were presented either serially (the offset of X coincided with the onset of A) or simultaneously (X and A overlapped). The results indicated that the two variables interacted significantly, so with a few trials both temporal arrangements led to SOC, with many trials both temporal arrangements led to CI, and with an intermediate number of trials, if the AX− compound was presented serially, it led to SOC, and if it was presented simultaneously, it led to CI.

The number of trials is a variable that has been studied in two different ways in the reviewed studies. Some of the studies took into account the number of sessions and found that the development of SOC is attenuated with extended training and that, at the end of training, CI is developed. On the other hand, some other studies manipulated the number of AX− trials per session. Importantly, in this case, the total number of trials and the intertrial interval (ITI) differed between groups, thus being potentially confounding variables. The present set of experiments aimed to further examine the transition from SOC to CI throughout the sessions by holding constant the total number of trials per session and ITI and by manipulating the number of A+ and AX− trials per session. Whereas the literature clearly shows that the number of trials is a key variable in finding SOC or CI, the effect of the temporal relationship of the stimuli in the compound and the order of presentation of AX− trials in relation to A+ trials is not so clear. These variables were out of the scope of the present experiments, so A+ trials were presented interspersed with AX− trials as in the study by Stout et al. (2004), and the AX− compound was presented in a simultaneous way as in the study by Yin et al. (1994).

EXPERIMENT 1

The design of Experiments 1–3 is depicted in **Table 1**. In Experiment 1, two groups of rats were trained in a magazine procedure, where the US was a food pellet, and the conditioned

TABLE 1 | Experimental designs.

Experiment		Group	Conditioning	Retardation test
Exp1	14-2	Experimental	14A+ / 12F− / 2AX− / 2X−	10X+
		Control	14A+ / 12F− / 2BX− / 2X−	10X+
Exp2	8-8	Experimental	8A+ / 6F+ / 6F− / 8AX− / 2X−	10X+
		Control	8A+ / 6F+ / 6F− / 8BX− / 2X−	10X+
Exp3	5-11	Experimental	5A+ / 9F+ / 3F− / 11AX− / 2X−	10X+
		Control	5A+ / 9F+ / 3F− / 11BX− / 2X−	10X+

A represents tone presentation, B represents light presentation, AX represents tone-click compound presentation, F represents lever presentation and X represents click presentation. The numbers before the letters indicate the number of trials that the stimulus was presented in each session. The + symbol represents that the stimuli were followed by a food pellet and the − symbol represents that the stimuli were not followed by a food pellet.

response (CR) was the number of entries into the food delivery site in the presence of the CS. During training, both groups received 14 A+ trials and two non-reinforced compound trials per session across 20 sessions. In each session, X− alone trials were included to test the CR controlled by this stimulus. The difference between groups was that, in one group, the compound was formed by A and X, whereas in the other group, the compound was formed by B and X, thus acting as a control for SOC and CI. After conditioning, both groups were tested for inhibitory properties using a retardation test, i.e., presenting X followed by the US. It was expected that the subjects in the experimental group would develop a higher responding to X in the first sessions of the experiment, which would indicate SOC, and that, with extended training, responding would equate with the control group. Regarding CI, according to the results reported by Rescorla (1972, 1973) and by Holland and Rescorla (1975), it would be expected to occur, but based on the results by Yin et al. (1994) and Stout et al. (2004), with few AX− trials only SOC would be expected.

Method

Subjects

The sample size needed was first calculated using G*Power (Faul et al., 2007). The total sample size needed to achieve an effect size f of 0.25, with the level of significance $\alpha = 0.05$ and power $1\text{-}\beta = 0.95$, was 14. Two subjects were added in case there was some sample loss (which was not the case for this experiment), so the subjects were 16 experimentally naive male Wistar rats that were 100 days old and had an *ad libitum* weight of 408 g (range, 343–474 g). All procedures related to the maintenance and use of animals were in accordance with the European Law of Animal Welfare and were approved by the Animal Welfare Committee of the University of Oviedo. They were housed in cages, each of which contained four rats that received the same training during the experiment. The weight of the animals was gradually reduced by controlled feeding to 85% of their individual free-feeding weights and was kept at that level throughout the experiment. Each day, in the housing room, there was 12 h of light, beginning at 8 a.m. The experiment was run during this light phase.

Apparatus

Eight identical conditioning chambers (24 × 29 × 38 cm: height × width × depth; Med Associates) were placed in a sound- and light-attenuating shell that incorporated a ventilation fan, which maintained the background noise at 62 dB(A). Background light was turned off for the experiment. The front and back walls were constructed from aluminum, the side walls and the ceiling were of clear methacrylate, and the floor was formed from 0.4 cm stainless steel rods, spaced 1 cm apart. A recessed food well (6 × 3.5 × 6 cm) was placed at the center of the front wall, 0.5 cm above the floor. Foods pellets (45 mg, Test Diet-MLab Rodent Tablet) were delivered to the food well and played the role of the US. The food well was equipped with photocells that allowed the presence of the rat in the well to be automatically recorded, playing the role of the response. A speaker that produced a 600 Hz and 76 dB(A) tone was mounted on the front wall, 8 cm over the food magazine. Above this speaker, there was another speaker that generated a second auditory stimulus: a 3,000 Hz and 82-dB(A) intermittent click. A 2 W and 24 V light was situated just above the food magazine. A stainless steel retractable lever (4.8 × 0.55 × 1.9 cm) was located 3 cm to the left of the food well. The depression of the lever was not recorded as a response nor had any scheduled consequence. The presence of the lever in the chamber was used as a stimulus, and when not active, it was retracted into the chamber wall. The tone, click, light, and presence of the lever all lasted 10 s and were used as stimuli as described in the procedure section below.

Procedure

Rats were randomly assigned to two groups of eight subjects each and then received 4 days of magazine training followed by 20 sessions of conditioning followed by four sessions of retardation test. The groups were labeled 14-2Exp and 14-2Ctrl.

Magazine training

On days 1–4, the subjects received a 20 min session of magazine training. In each session, food pellets were delivered according to a variable time 120 s schedule. Four pellets were placed in the magazine before the beginning of these sessions.

Conditioning

Conditioning began on day 5 and continued throughout day 24 (a total of 20 sessions). Each session lasted 52 min. The subjects in group 14-2Exp received 14 tones followed by a food pellet (A+), two non-reinforced tone-click compounds (AX−), 12 non-reinforced presentations of the lever (F−), and two non-reinforced clicks (X−) per session. Stimuli were presented in random order within the session. The ITI had a mean duration of 80 s (range, 50–110 s). The first and last 100 s had no event scheduled. Training for 14-2Ctrl group was identical to 14-2Exp, except that two light-click compounds (BX−) were presented instead of two tone-click compounds (AX−). The function of the lever presentations was twofold: they were included to control the total amount of reinforcement received per session across the experiments presented here, in such a way that all subjects received 14 food pellets per session in all experiments, and they also allowed to slow down the development of excitatory responding to A. This, as shown in preliminary unpublished

studies from our laboratory, was necessary to observe excitatory responding to X. Click-alone presentations were included to test the CR to this stimulus.

Retardation Test

On days 25–28, all subjects received a 20-min retardation test. In each session, 10 clicks followed by a food pellet (X+) were presented, with a mean ITI of 80 s (range, 50–110 s).

Data Analysis

Food well entries were registered during the 10 s that preceded the presentation of the CS and during the presentation of the CS itself. The CR controlled by the CS was computed as the difference in responding during the CS and the pre-CS periods, which was averaged for each session. The rationale for choosing this measure was that it allows to control for the general activity differences that can be seen between subjects. All the analyses reported here were performed on the mean differences per session. SPSS 24 (IBM Corp., 2016) was used to analyze the data. The analyses were mixed-model ANOVAs. The level of significance used was $\alpha = 0.05$. The effect sizes for ANOVAs are reported as partial Eta-square (η_p^2).

Results

As can be seen in **Figure 1**, during the first sessions of the conditioning phase, the subjects in group 14-2Exp, the one in which A+ was presented 14 times and AX− was presented twice, showed higher responding to X than the control group, for which BX− instead of AX was used as a compound. Responding in group 14-2Exp matched the responding in group 14-2Ctrl at around session 7. A mixed-model ANOVA with a between-subjects factor Group (experimental or control) and a within-subjects factor Session found a significant main effect of Session, $F(19,266) = 7.001$, $p < 0.001$, $\eta_p^2 = 0.333$, and of Session × Group interaction, $F(19,266) = 1.876$, $p = 0.016$, $\eta_p^2 = 0.118$, but not a main effect of Group, $F(1,14) = 3.175$, $p = 0.096$, $\eta_p^2 = 0.185$. Bonferroni-corrected pairwise comparisons for the interaction showed that there were significant differences between the experimental and control groups in session 1, $MD = 2.063$, $SE = 0.912$, $p = 0.04$, and session 6, $MD = 4.125$, $SE = 1.663$, $p = 0.026$. These analyses indicate that the subjects in the group that received 14 A+ and two AX− presentations per session developed a significantly higher response to the click (X) than the subjects that received 14 A+ and two BX− presentations per session in sessions 1 and 6, a result that is congruent with the development of SOC.

In the retardation test, responses to X in the group that received the 14A+/2AX− treatment (group 14-2Exp) showed no differences with the control group in the first two sessions. In contrast, responding to X by group 14-2Exp was higher than in the control group in sessions 3 and 4, as can be seen in **Figure 2**. A mixed-model ANOVA with the between-subjects factor Group (experimental or control) and the within-subjects factor Session found a significant main effect of Session, $F(3,42) = 7.344$, $p < 0.001$, $\eta_p^2 = 0.344$, but not of Group, $F(1,14) = 1.2$, $p = 0.292$, $\eta_p^2 = 0.079$, or Session × Group interaction, $F(3,42) = 2.084$, $p = 0.117$, $\eta_p^2 = 0.13$. This analysis indicated that both groups increased their responding to X over sessions in a similar way. The absence of a significant group effect in the analysis indicated that, in group 14-2Exp, X did not gain inhibitory properties.

Taken together, the results of this experiment indicated that the group that was trained with 14 A+ and two AX− presentations per session showed an increase in responding to X in sessions 1 and 6, which might indicate the development of SOC in those sessions. However, the absence of a difference between the two groups in the retardation test indicates that it did not develop CI. These results are consistent with the previous literature (Rescorla, 1972, 1973; Holland and Rescorla, 1975), as excitatory responding to X is developed in two sessions at the beginning of training and disappears with extended training. The absence of CI is not consistent with the results obtained by Rescorla (1972, 1973) and Holland and Rescorla (1975) but is consistent with the results found by Yin et al. (1994) and Stout et al. (2004) when they used few AX− trials.

EXPERIMENT 2

Experiment 2 aimed to find excitatory properties at the beginning of training and inhibitory properties at the end. In order to achieve this, the number of A+ trials was lowered from 14 to eight, and the number of AX− trials was increased from two to eight, thus maintaining the total number of trials presented per session equal to the total number of trials per session presented in the previous experiment. In short, the experimental group received eight A+ and eight AX− trials, whereas the control group received eight A+ and eight BX− trials. This experiment included a retardation test identical to the ones employed in the experiments above.

Method

Subjects and Apparatus

The sample size was calculated as in the previous experiment, but in this case, one of the rats died. Hence, the subjects were 15 experimentally naive male Wistar rats that were 105 days old and that had an *ad libitum* weight of 459 g (range, 420–515 g). Housing, deprivation schedule, and apparatus were identical to those of Experiment 1.

Procedure

The rats were randomly assigned to two groups and then received four days of magazine training followed by 20 sessions of conditioning and four sessions of retardation test. The groups were labeled 8-8Exp and 8-8Ctrl. Group 8-8Exp had eight subjects and group 8-8Ctrl had seven subjects.

The subjects in group 8-8Exp received eight tones followed by a food pellet (A+), eight non-reinforced tone-click compounds (AX−), six non-reinforced presentations of the lever (F−), six presentations of the lever followed by a food pellet (F+), and two non-reinforced clicks (X−) per session. Six of the 12 lever presentations were reinforced in order to equate the number of reinforcers received per session with that of the previous experiment. The training for 8-8Ctrl group was identical to the one for 8-8Exp, except that eight light-click compounds (BX−)

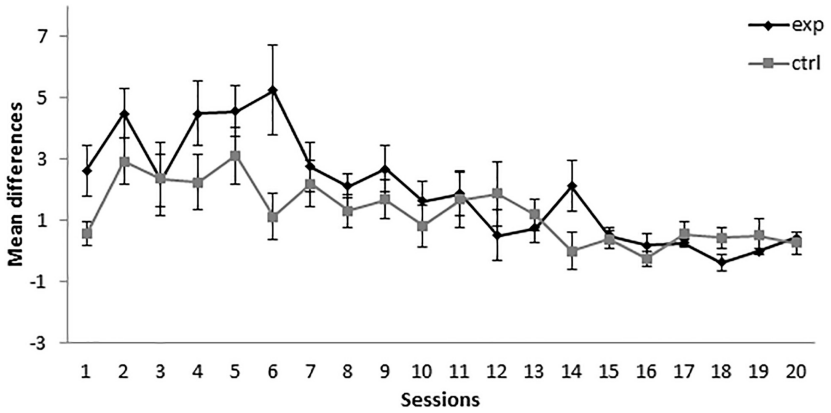

FIGURE 1 | Conditioning phase in Experiment 1. PreX-X differences (±SEM), averaged for the two X– presentations per session in conditioning, are displayed. The black line represents the group that was trained with 14 A+, two AX–, 12 F–, and two X– presentations per session in conditioning. The gray line represents the group that was trained with 14 A+, two BX, 12 F–, and two X– presentations per session.

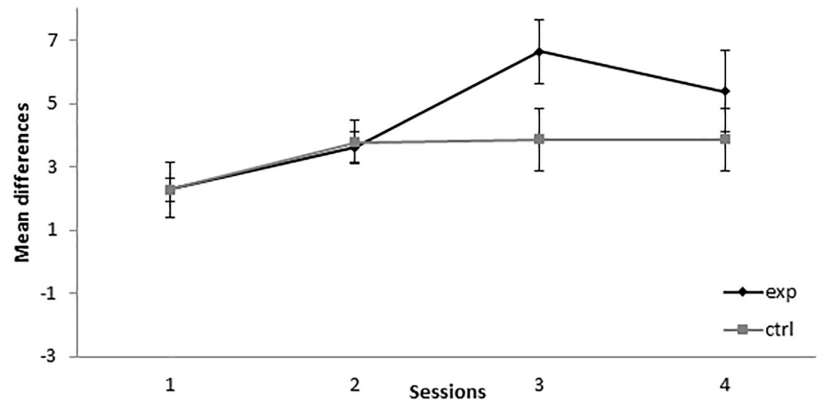

FIGURE 2 | Retardation test in Experiment 1. PreX-X differences (±SEM), averaged for the 10 X+ presentations per session in the retardation test, are displayed. The black line represents the group that was trained with 14 A+, two AX–, 12 F–, and two X– presentations per session in conditioning. The gray line represents the group that was trained with 14 A+, two BX, 12 F–, and two X– presentations per session. In the retardation test, both groups received 10 X+ presentations per session.

were presented instead of eight tone-click compounds (AX−). All other details were identical to those of Experiment 1.

Results

During the first 11 sessions of conditioning, the subjects in group 8-8Exp (i.e., trained with 8A+ and 8AX−) showed a higher responding to X than the subjects in the control group (8-8Ctrl), for which BX− instead of AX− was used as a compound (see **Figure 3**). A mixed-model ANOVA found statistically significant differences for the main effects Session, $F(19,247) = 3.777$, $p < 0.001$, $\eta_p^2 = 0.225$, Group, $F(1,13) = 18.485$, $p = 0.001$, $\eta_p^2 = 0.587$, and Session × Group interaction, $F(19,247) = 3.081$, $p < 0.001$, $\eta_p^2 = 0.192$. Bonferroni-corrected pairwise comparisons for the interaction showed that there were significant differences between the experimental and the control groups in session 1, $MD = 2.857$, $SE = 1.258$, $p = 0.041$, session 2, $MD = 2.438$, $SE = 0.992$, $p = 0.029$, session 3, $MD = 1.83$, $SE = 0.79$, $p = 0.038$, session 4, $MD = 2.723$, $SE = 0.652$, $p = 0.001$, session 5, $MD = 1.589$, $SE = 0.606$, $p = 0.021$, session 7, $MD = 1.705$, $SE = 0.712$, $p = 0.032$, session 11, $MD = 1.696$, $SE = 0.682$, $p = 0.027$, and session 13, $MD = -1.75$, $SE = 0.6$, $p = 0.012$. The experimental group showed an increase in responding to X in sessions 1, 2, 3, 4, 5, 7, and 11, which is congruent with subjects acquiring SOC. There was also a significant higher responding in control group in session 13.

As can be seen in **Figure 4**, during the retardation test, the experimental group showed a lower responding than the control group across all sessions. A mixed-model ANOVA found statistically significant effects for the main effects Session, $F(3,39) = 10.688$, $p < 0.001$, $\eta_p^2 = 0.451$, and Group, $F(1,13) = 7.745$, $p = 0.016$, $\eta_p^2 = 0.373$, but not for the Session × Group interaction, $F(3,39) = 0.931$, $p = 0.435$, $\eta_p^2 = 0.067$. This analysis showed that both groups increased their responding to X across sessions, but there was retardation in the acquisition of conditioning in the experimental group compared with the control group, thus indicating that X gained inhibitory properties.

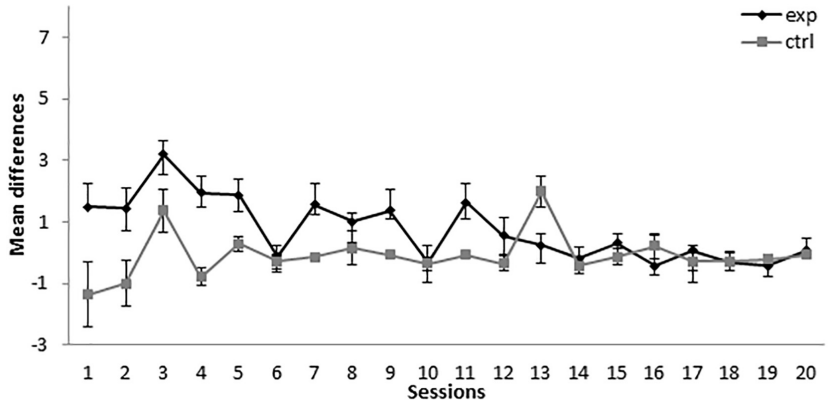

FIGURE 3 | Conditioning phase in Experiment 2. PreX-X differences (±SEM), averaged for the two X– presentations per session in conditioning, are displayed. The black line represents the group that was trained with eight A+, eight AX–, six F–, six F+, and two X– presentations per session in conditioning. The gray line represents the group that was trained with eight A+, eight BX–, six F–, six F+, and two X–presentations per session.

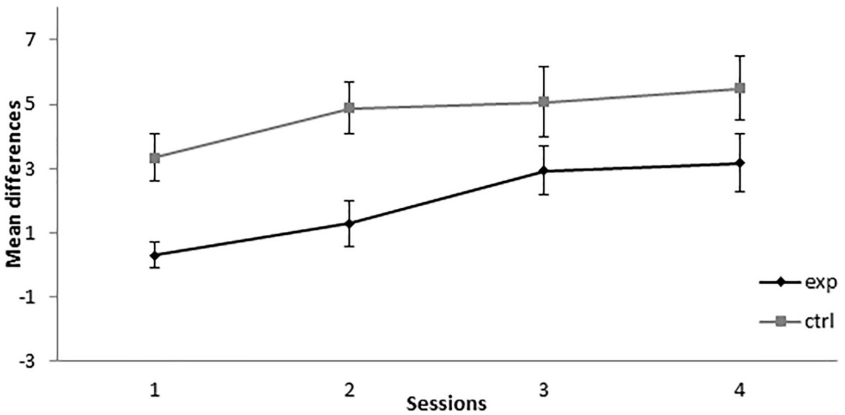

FIGURE 4 | Retardation test in Experiment 2. PreX-X differences (±SEM), averaged for the 10 X+ presentations per session in retardation, are displayed. The black line represents the group that was trained with eight A+, eight AX–, six F–, six F+, and two X– presentations per session in conditioning. The gray line represents the group that was trained with eight A+, eight BX–, six F–, six F+, and two X– presentations per session. In the retardation test, both groups received 10 X+ presentations per session.

The results of this experiment altogether indicated that, when 8A+ and 8AX− trials are presented per sessions, responding to X increases in the first sessions, a result consistent with SOC, and that, at the end of training, X showed a significant retardation in conditioning when paired with the US, thus indicating inhibitory properties consistent with CI. These results are consistent with the results found by Rescorla (1972, 1973) and Holland and Rescorla (1975), as SOC was found at the beginning of training, fading as sessions progressed, and CI was found at the end of training. The aforementioned authors demonstrated CI based on a summation test, whereas in this experiment CI was demonstrated based on a retardation test.

EXPERIMENT 3

Taking into account that Yin et al. (1994) and Stout et al. (2004) found that, with many trials, only CI was developed, it would be interesting to assess if a greater number of AX− trials would prevent that development of SOC while not affecting the development of CI. Experiment 3 was designed to assess this question by increasing the number of AX− trials and decreasing, accordingly, the number of A+ trials. In order to achieve this, the experimental group of this experiment received five A+ and 11 AX− presentations per session. It was compared with a control group that received five A+ and 11 BX− presentations per session.

Method

Subjects and Apparatus

The sample size was calculated as in the previous experiments. However, two rats died, so the subjects were 14 experimentally naive male Wistar rats that were 71 days old and had an *ad libitum* weight of 247 g (range, 224–279 g). Housing, deprivation schedule, and apparatus were identical to those of experiments 1 and 2.

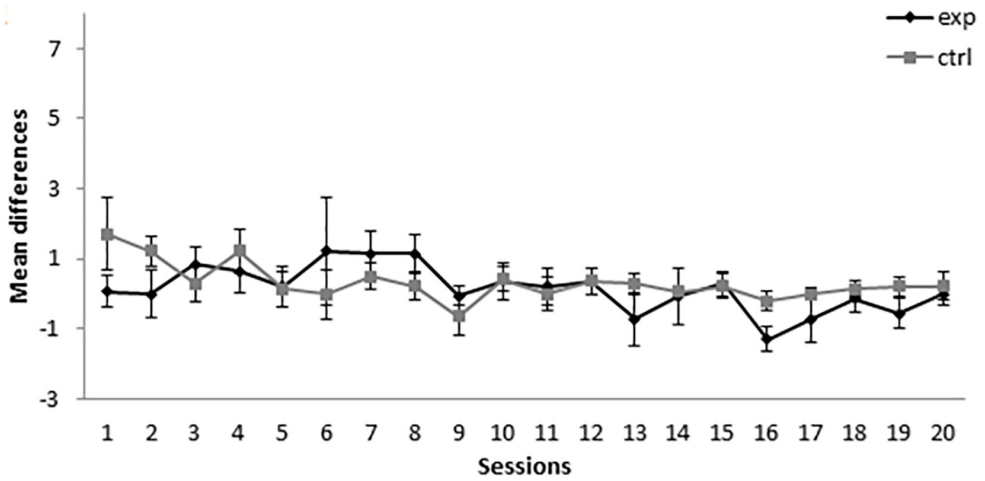

FIGURE 5 | Conditioning phase in Experiment 3. PreX-X differences (±SEM), averaged for the two X– presentations per session in conditioning, are displayed. The black line represents the group that was trained with five A+, 11 AX–, three F–, nine F+, and two X– presentations per session in conditioning. The gray line represents the group that was trained with five A+, 11 BX–, three F–, nine F+, and two X–presentations per session.

Procedure

The rats were randomly assigned to two groups of seven subjects each and then received four days of magazine training followed by 20 sessions of conditioning and four sessions of retardation test. The groups were labeled 5-11Exp and 5-11Ctrl.

The subjects in group 5-11Exp received five tones followed by a food pellet (A+), 11 non-reinforced tone-click compounds (AX−), three non-reinforced presentations of the levers (F−), nine presentations of the lever followed by a food pellet (F+), and two non-reinforced clicks (X−) per session. The training for the 5-11Ctrl group was identical to the one of 5-11Exp, except that 11 light-click compounds (BX−) were presented instead of 11 tone-click compounds (AX−). All other details were identical to experiments 1 and 2.

Results

As can be seen in **Figure 5**, the subjects in groups 5-11Exp (for which A+ was presented five times and AX− was presented 11 times) and 5-11Ctrl (for which BX− was used as a compound instead of AX−) showed a similar level of responding throughout the conditioning phase of the experiment. A mixed-model ANOVA with the between-subjects factor Group (experimental or control) and the within-subjects factor Session found no statistically significant differences nor a significant interaction [Session: $F(19,228) = 1.420$, $p = 0.119$, $\eta_p^2 = 0.106$, Group: $F(1,12) = 0.681$, $p = 0.425$, $\eta_p^2 = 0.054$, Session × Group: $F(19,228) = 0.941$, $p = 0.533$, $\eta_p^2 = 0.073$]. The absence of significant differences indicates that X did not acquire excitatory properties at any point of the experiment.

In the retardation test, 5-11Exp showed a lower level of responding than the control group in all sessions except for session 2, as can be seen in **Figure 6**. A mixed-model ANOVA with the between-subjects factor Group (experimental or control) and the within-subjects factor Session found a significant main effect of Session, $F(3,36) = 7.18$, $p = 0.001$, $\eta_p^2 = 0.374$, and of Group, $F(1,12) = 5.692$, $p = 0.034$, $\eta_p^2 = 0.322$, but not of the Session × Group interaction, $F(3,36) = 2.091$, $p = 0.119$, $\eta_p^2 = 0.148$. This analysis indicates that, even when both groups increased their responding to X across sessions, there is a consistently lower responding in the group that was trained with five A+ and 11 AX− trials, thus indicating that CI was developed in the 5-11Exp group.

All in all, the results of Experiment 3 showed that the group that was trained with five A+ and 11 AX− presentations per session did not developed SOC at any point of the experiment. However, in the retardation test, the pattern of the results was congruent with the development of CI in group 5-11Exp. These results are consistent with the results reported by Yin et al. (1994) and by Stout et al. (2004) in the experiments where many AX− trials were used as they did find CI but not SOC.

GENERAL DISCUSSION

These experiments show that, when 14 A+ trials and two AX− trials were presented in each of the training sessions (Experiment 1), the subjects showed an increase in responding to X congruent with SOC in sessions 1 and 6 that faded out in the last sessions. Moreover, these subjects did not show retardation of conditioning to X at the end of training, thus indicating that CI was not developed. Contrastingly, the subjects that were trained with eight A+ and eight AX− trials in each session (Experiment 2) showed an increase in responding to X in the first half of the training sessions, consistent with a SOC effect, and a retardation of conditioning to X in the retardation test, which shows that CI was developed. Finally, those subjects that received five A+ and 11 AX− trials per session (Experiment 3) did not show an increase in responding to X at any moment of the experiment, proving that SOC was not developed, but they did show a

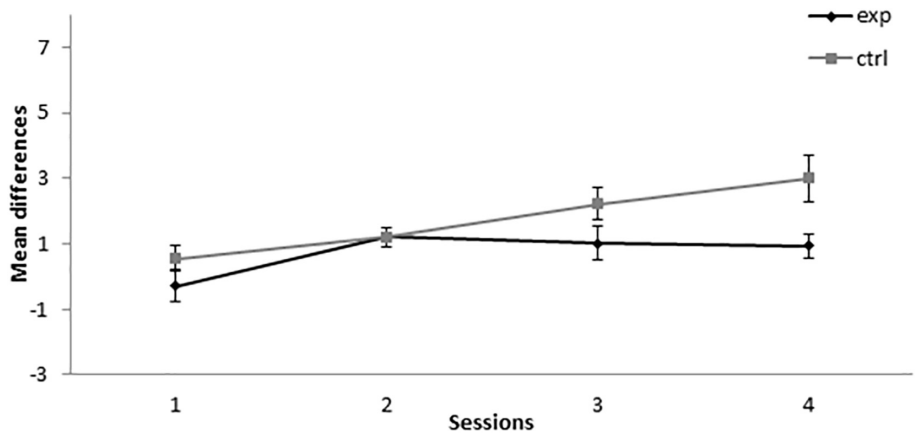

FIGURE 6 | Retardation test in Experiment 3. PreX-X differences (±SEM), averaged for the 10 X+ presentations per session in retardation, are displayed. The black line represents the group that was trained with five A+, 11 AX–, three F–, nine F+, and two X– presentations per session in conditioning. The gray line represents the group that was trained with eight A+, five A+, 11 BX–, three F–, nine F+, and two X– presentations per session. In the retardation test, both groups received 10 X+ presentations per session.

retarded acquisition of conditioning to X in the retardation test, congruent with the development of CI.

Taken together, these results indicate that both the number of A+ and AX− trials and the progression of the sessions are important variables that determine if SOC or CI would occur. These variables also seem to interact with each other. SOC appeared with many A+ and few AX− trials as well as with an equal number of A+ and AX− trials (14–2 and 8–8, respectively), but as the sessions progressed, SOC was no longer evident. CI is demonstrated at the end of training with an equal number of A+ and AX− trials, with few A+ and many AX− trials (8–8 and 5–11, respectively). It is worth noting that the results of the present study are consistent with the previous ones in which the number of AX− trials per session was manipulated (Yin et al., 1994; Stout et al., 2004), with the novelty that, in this study, the total number of trials per session and the ITI was held constant. However, as both A+ and AX− trials were varied, it is not clear if the results found were due to the number of A+ trials, the number of AX− trials, or the conjoint effect of both trial numbers. Further investigation is needed to address this question.

Furthermore, group 8-8Exp in Experiment 2 replicates the findings reported by Rescorla (1972, 1973) and by Holland and Rescorla (1975), as SOC is shown at the beginning of training and CI is observed at the end of training. CI was assessed with different tests. Whereas previous studies employed a summation test to assess the inhibitory properties of the additional cue X, in the present study a retardation test was used. It has been argued that using both summation and retardation tests, the so-called two-test strategy, is the best way to test the inhibitory properties of a stimulus as it allows one to rule out alternative explanations based on attentional shifts (Rescorla, 1969). Reduced attention to a stimulus can account for the retardation effect but will not affect responding to a transfer excitor, i.e., there will be no reduction in responding in the summation test. Conversely, increased attention to a stimulus would decrease responding to a transfer excitor in a summation test but would not produce a retardation effect, so if a stimulus passes both summation and retardation tests, it cannot be due to an attentional shift. However, some authors have claimed that both tests might not be sufficient nor necessary to assess the inhibitory properties of a stimulus (Williams et al., 1992). In fact, according to Papini and Bitterman (1993), in the A+/AX− design, a retardation test would be sufficient as long as the experiment includes a control group in which the putative inhibitory stimulus receives a treatment that is assumed to be less inhibitory or not inhibitory at all compared to the treatment received by the experimental group, as is the case of the present experiments. According to Papini and Bitterman (1993), as in the present study a control group trained with a BX compound was included, a retardation test could be sufficient, given that attention cannot readily be assumed to be less in the experimental than in the control group.

As noted earlier, these results are challenging for most theories of associative learning, as most of these models simply cannot predict the existence of SOC (e.g., Rescorla and Wagner, 1972; Mackintosh, 1975; Pearce and Hall, 1980; Pearce, 1987). However, Stout et al. (2004) noticed that their results might be explained by the models proposed by Wagner (1981), by Sutton and Barto (1981), and by McLaren and Mackintosh (2000). These models explain SOC as an associative chain involving an association between X and A and an association between A and the US, so X indirectly activates the representation of the reinforcer. They are also able to explain CI, given that, with extended training, X is associated with the absence of the reinforcer that was expected due to the presence of A. However, this explanation of SOC requires the treatment to be performed in two phases, that is, A+ should be first conditioned to the asymptotic level in a phase prior to the presentation of the AX− compound. It is only under these circumstances that A can function as a reinforcer for X. To illustrate this, simulations of Wagner's Sometimes Opponent Processes (SOP) model for the present experiments were performed using the SOP model simulator (Byers et al., 2017). As can be seen in the left panel of **Figure 7**, in Experiment

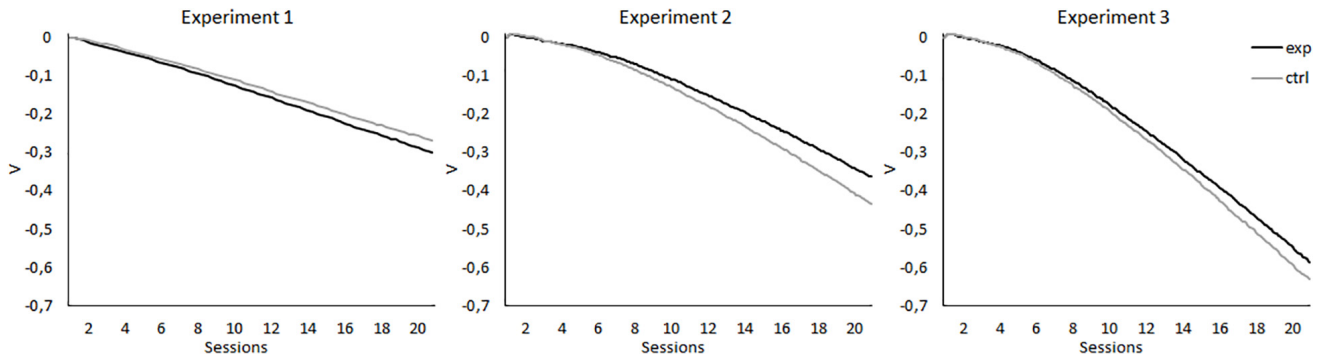

FIGURE 7 | Simulations of associative properties acquired by X according to Wagner's Sometimes Opponent Processes (SOP) model, performed using the SOP model simulator (Byers et al., 2017), for the 20 sessions of the experimental designs reported. The left panel displays the simulation for Experiment 1, in which the experimental group (black line) was trained with 14 A+, two AX–, 12 F–, and two X– trials per session, and the control group (gray line) was trained with 14 A+, two BX–, 12 F–, and two X– trials per session. The central panel displays the simulations for Experiment 2, in which the experimental group (black line) was trained with eight A+, eight AX–, six F–, six F+, and two X– trials per session, and the control group (gray line) was trained with eight A+, eight BX–, six F–, six F+, and two X– trials per session. The right panel displays the simulations for Experiment 3, in which the experimental group (black line) was trained with five A+, 11 AX–, three F–, nine F+, and two X– trials per session, and the control group (gray line) was trained with five A+, 11 BX–, three F–, nine F+, and two X– trials per session.

1, for both the group that was trained with 14 A+ and two AX− trials per session and the group that was trained with 14 A+ and two BX− trials per session, the model predicts the development of an inhibitory link between X and the US, not predicting that X would gain excitatory properties at any point of the experiment. It is worth noting that, although it does not predict SOC, the predictions are consistent with the results on the retardation test, where both groups showed similar levels of responding. The simulations for Experiment 2 are displayed in the central panel of **Figure 7**. For both the group that was trained with eight A+ and eight AX− per session and the group that was trained with eight A+ and eight BX− per session, the model predicts that X would develop inhibitory properties, with the strength of this inhibition being stronger in the control group. Thus, the results of the simulations are not consistent with the results obtained in Experiment 2, as they predict neither the development of SOC during the first sessions nor the retardation that X shows in the experimental group in the retardation test. For Experiment 3, the model predicts an inhibitory relationship between X and the US for both groups, with the inhibition being stronger in the control group, especially in the last sessions. The results of the simulations are consistent with the absence of excitatory properties of X in the first sessions of Experiment 3, but not with the results of the retardation test, given that in the experiment it was found that X had acquired inhibitory properties in the 5-11Exp group compared with the 5-11Ctrl group. In conclusion, for the present experiments, this model cannot predict SOC through an associative chain that involves the association between X and A and the association of A with the US. It does predict the acquired inhibitory properties for X. However, it does not predict the results of the retardation tests, as according to the model, X would acquire similar or stronger inhibitory properties for the control groups than for the experimental ones.

Another significant exception are the models that distinguish between acquisition and performance (see Miller, 2006, for a review). The models described previously share the assumption that the response to a stimulus depends only on the associative status of that stimulus and that cue competition occurs in acquisition. In performance-focused models, such as the comparator hypothesis proposed by Miller and Matzel (1988), associations are acquired in a non-competitive fashion, in such a way that all associations are excitatory, and inhibition is a result of the interaction between them, so inhibition is due to a process of comparison between stimuli at the moment of responding, in such a way that responding to a stimulus depends not only on its association with the reinforcer but also on the association with the reinforcer that has been acquired by other stimuli. In our experiments, CI would be the result of this comparison process, as the association between X and the reinforcer is 0, given that they are never presented together, and the comparison term value is high, as it depends on the association between X and A, and the association between A and the reinforcer. SOC would be predicted by the presence of a switching operator in the response rule that makes the result of the comparison excitatory in the first sessions and that, with the repeated presentation of the stimulus X, switches so that the net result of the comparison becomes inhibitory (Stout and Miller, 2007). It is worth noting that Pineño (2007) proposed a similar response rule but that can be applied in conjunction with acquisition rules from competitive acquisition models. According to this rule, competition occurs during acquisition, whereas facilitation occurs during performance, as a result of summing the associative strength of the stimulus X and the associative strength of the stimuli associated with it, weighted by the strength of the within-stimuli association and the novelty of the stimulus X. The transition from facilitation to competition is due to the decreased novelty of the stimulus X as training progresses. Although the acquisition mechanism is different in these two proposals and the comparison process in responding is slightly different, both can account for the present results.

CONCLUSION

To sum up, the present set of experiments provide a demonstration of the modulatory effect of the number of trials per session and the number of sessions on associative learning phenomena, adding evidence to the available literature that demonstrates that cue interactions can be facilitative and competitive (Urcelay, 2017). The results presented here are problematic for most learning theories, being more easily explained by theories that distinguish between what is learned and what is overtly displayed through behavior.

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 Welfare Committee of the University of Oviedo.

AUTHOR CONTRIBUTIONS

CM-D, JM-M, and IL contributed to the study design and final revision, and approved the final version of the manuscript. CM-D implemented the study, analyzed the results, and wrote the manuscript. JM-M contributed to the implementation of the study. JM-M and IL revised the manuscript. All the authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the project PSI2015-66974-P by the Ministerio de Economía y Competitividad of the Spanish Government (P.I.: IL), the grant Severo Ochoa BP16-049 awarded to CM-D by the Government of the Principality of Asturias, and by the Department of Psychology of the University of Oviedo.

ACKNOWLEDGMENTS

The authors would like to thank Beatriz Álvarez for the time and effort dedicated to the review of the manuscript.

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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.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 19 May 2021
doi: 10.3389/fnbeh.2021.682426

Cortical Contributions to Higher-Order Conditioning: A Review of Retrosplenial Cortex Function

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In higher-order conditioning paradigms, such as sensory preconditioning or second-order conditioning, discrete (e.g., phasic) or contextual (e.g., static) stimuli can gain the ability to elicit learned responses despite never being directly paired with reinforcement. The purpose of this mini-review is to examine the neuroanatomical basis of high-order conditioning, by selectively reviewing research that has examined the role of the retrosplenial cortex (RSC) in sensory preconditioning and second-order conditioning. For both forms of higher-order conditioning, we first discuss the types of associations that may occur and then review findings from RSC lesion/inactivation experiments. These experiments demonstrate a role for the RSC in sensory preconditioning, suggesting that this cortical region might contribute to higher-order conditioning via the encoding of neutral stimulus-stimulus associations. In addition, we address knowledge gaps, avenues for future research, and consider the contribution of the RSC to higher-order conditioning in relation to related brain structures.

Keywords: higher-order conditioning, sensory preconditioning, second-order conditioning, retrosplenial cortex, associative learning

OPEN ACCESS

Edited by:
Nathan Holmes, University of New South Wales, Australia

Reviewed by:
Mihaela D. Iordanova, Concordia University, Canada
Justine Fam, University of New South Wales, Australia

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

Received: *18 March 2021*
Accepted: *20 April 2021*
Published: *19 May 2021*

Citation:
Fournier DI, Cheng HY, Robinson S and Todd TP (2021) Cortical Contributions to Higher-Order Conditioning: A Review of Retrosplenial Cortex Function. Front. Behav. Neurosci. 15:682426. doi: 10.3389/fnbeh.2021.682426

INTRODUCTION

Associative learning is one process by which animal behavior can be modified based on experience. One example of this is Pavlovian conditioning, in which animals learn predictive relationships between stimuli (Pavlov, 1927). In *first-order* conditioning, an excitatory association is formed between a conditioned stimulus (CS) and an unconditioned stimulus (US) that are directly paired together, if the CS provides predictive information about the US (Rescorla, 1972). Through these direct pairings, the CS will acquire the ability to elicit a conditioned response (CR). Stimuli can also acquire the ability to elicit CRs through *higher-order* conditioning, in which the CS is never directly paired with the US. Higher-order learning is critical for survival and likely contributes to a wide range of adaptive behaviors (Gewirtz and Davis, 2000), but may also contribute to the development and maintenance of psychiatric disorders, such as post-traumatic stress disorder (PTSD; Wessa and Flor, 2007).

Higher-order conditioning can be studied through two paradigms: sensory preconditioning and second-order conditioning (see **Figure 1**). In sensory preconditioning, two initially neutral stimuli (e.g., S2 and S1) are repeatedly presented together. One stimulus (S1) is then paired with the US.

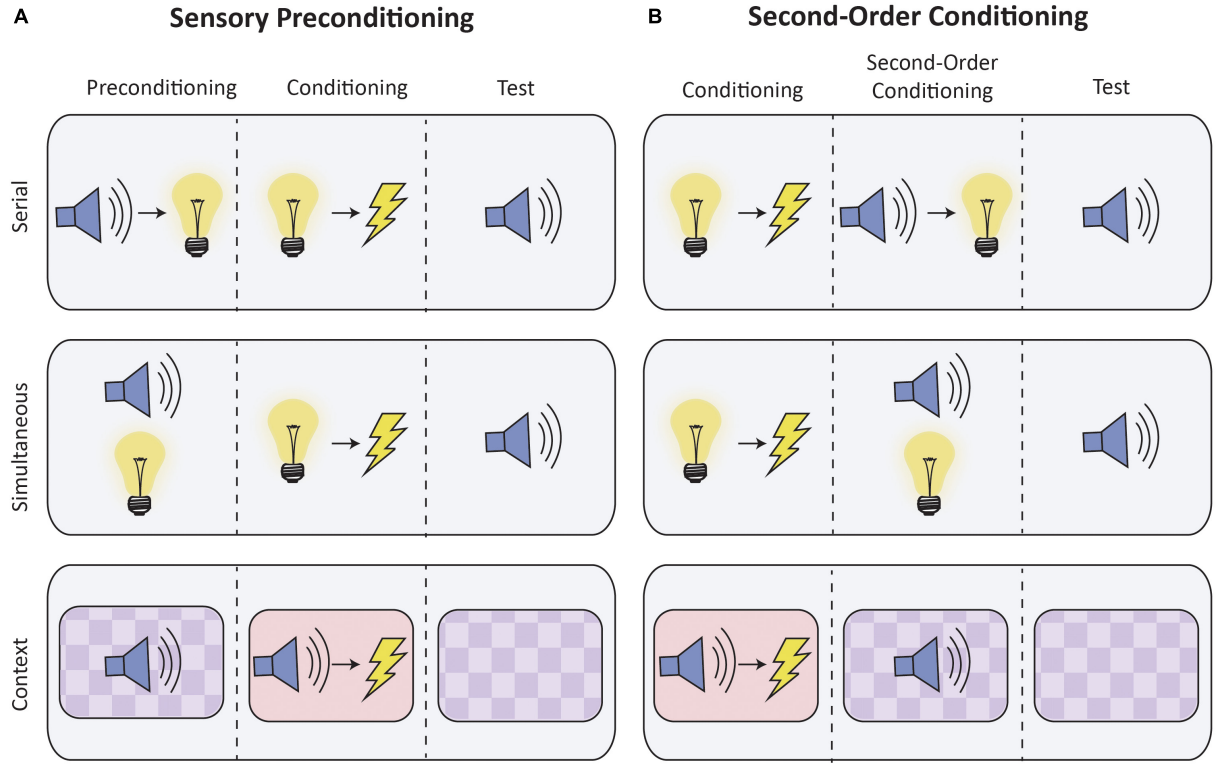

FIGURE 1 | Schematic of higher-order conditioning procedures. The figure depicts typical experimental conditioning for sensory preconditioning **(A)** and second-order conditioning **(B)**, which are contrasted with control conditions (not shown). Discrete stimuli can be presented either serially (top row) or simultaneously (middle row). Higher-order conditioning of contextual stimuli is presented in the bottom row. Contexts are operationally defined as the static background stimuli provided by conditioning apparatus, and typically differ with respect to visual, tactile and olfactory characteristics. In the figure, contexts are distinguished by color and background.

These phases are reversed during second-order conditioning: S1 is first directly paired with the US, after which it is then paired with S2. Importantly, in both sensory preconditioning and second-order conditioning, S2 acquires the ability to elicit a CR despite never being directly paired with the US. Through higher-order conditioning, both briefly presented discrete stimuli and static contextual stimuli can gain the ability to elicit responses (e.g., Rizely and Rescorla, 1972; Helmstetter and Fanselow, 1989; Iordanova et al., 2011; Robinson et al., 2018).

In the present article, we will consider the neuroanatomical basis of higher-order conditioning by selectively reviewing research that has examined the role of the retrosplenial cortex (RSC) in sensory preconditioning and second-order conditioning. For both forms of higher-order conditioning, we first briefly summarize the types of associations that may be formed and then we describe the putative role, if any, for the RSC. In addition, we identify gaps in the literature as well as avenues for future research. Finally, we consider the RSC's role in higher-order conditioning with respect to other related structures.

RSC Anatomy and Connectivity

The RSC (Brodmann area 29 and 30) was first described in humans but is evolutionarily conserved and is found in non-human primates and rodents (Vann et al., 2009). In rats, the RSC is located on the dorsomedial surface of the cerebrum and is cytoarchitecturally separated into dysgranular (Brodmann area 30) and granular RSC (Brodmann area 29). Connectomic studies using a combination of retrograde and anterograde tracers reveal extensive reciprocal connections of the RSC with multiple higher-order cortical structures including the hippocampal formation, parahippocampal region (e.g., perirhinal and postrhinal cortex) and the orbitofrontal cortex (see **Figure 2**; Van Groen and Wyss, 1990, 1992, 2003; Wyss and Van Groen, 1992; Miyashita and Rockland, 2007; Sugar et al., 2011). In addition, the RSC is well-connected with multiple sensory cortical areas; it receives inputs from auditory cortex and is reciprocally connected with the visual cortex (Vogt and Miller, 1983; Van Groen and Wyss, 1992, 2003; Todd et al., 2016b). The RSC also has reciprocal subcortical connections with several thalamic nuclei, the most prominent of which is the anterior thalamic nuclei (Sripanidkulchai and Wyss, 1986; Van Groen and Wyss, 1990, 1992, 2003). Functionally, the RSC contributes to several aspects of learning and memory, including spatial navigation, contextual and trace fear conditioning, and some aspects of Pavlovian and instrumental conditioning (see reviews by Vann et al., 2009; Bucci and Robinson, 2014; Miller et al., 2014; Mitchell et al., 2017; Corcoran et al., 2018; Todd et al., 2019). RSC pathology is also present in several disorders that include memory dysfunction,

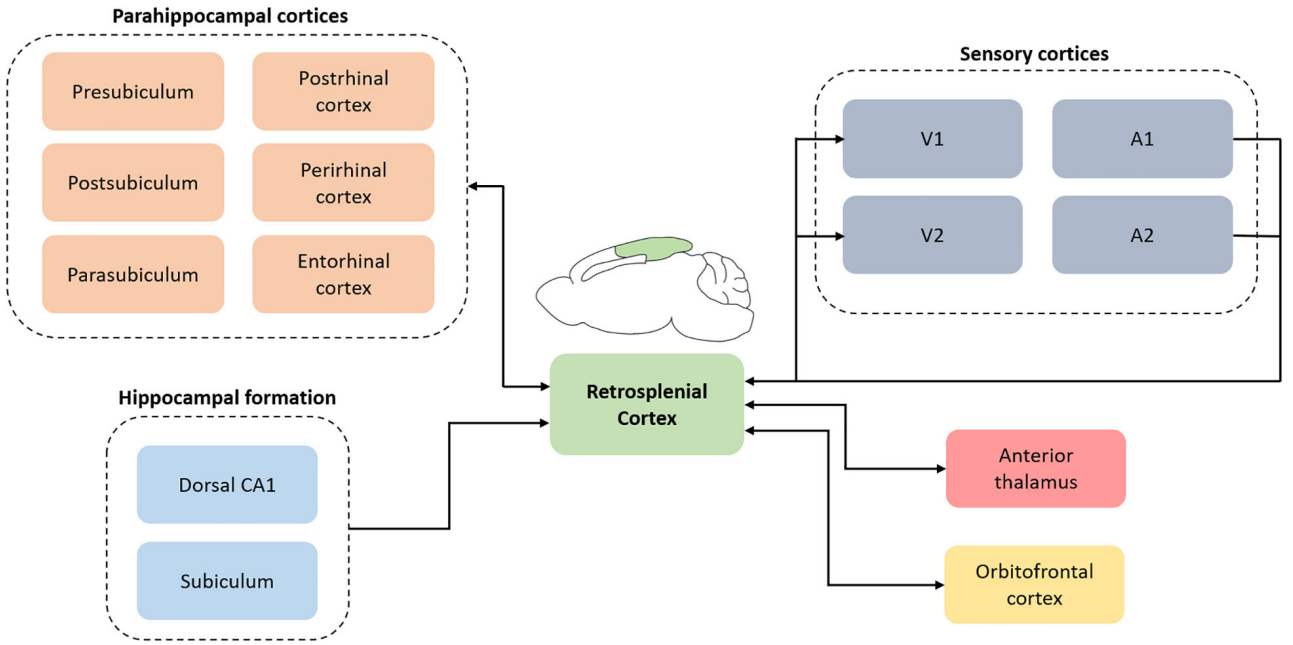

FIGURE 2 | A simplified schematic depicting retrosplenial connections with cortical and subcortical regions. The connectomic diagram is centered around retrosplenial cortex and does not include the complex interactions between all regions. V1, primary visual cortex; V2, secondary visual cortex; A1, primary auditory cortex; A2, secondary auditory cortex.

such as Alzheimer's disease (Buckner et al., 2005) and PTSD (Sartory et al., 2013).

RSC and Higher-Order Conditioning

Sensory Preconditioning

As previously noted, sensory preconditioning is an associative learning procedure in which a stimulus elicits a CR despite having never been directly paired with a US (**Figure 1**; Brogden, 1939). A key event in sensory preconditioning is thought to be the formation of stimulus-stimulus (S2–S1) associations that are acquired during the preconditioning phase when two neutral stimuli are presented together (Rizely and Rescorla, 1972; Rescorla and Cunningham, 1978). Importantly, this association is established prior to any presentation of a biologically significant US that will be later paired with S1 (e.g., Rizely and Rescorla, 1972; Rescorla, 1980). After a S2–S1 association is established, there are at least two ways by which S2 can gain the ability to elicit a CR (see Wong et al., 2019). One possibility is through an associative "chain," such that S2 → S1 → US (Rizely and Rescorla, 1972). A second possibility is that during first-order conditioning of S1, the initial S2–S1 association allows for the retrieval of S2, which is then associated with the US (Holland, 1981).

Several studies have demonstrated that disruption of the RSC impairs sensory preconditioning in rats. For example, in an experiment by Robinson et al. (2011), rats first received either pre-training electrolytic or sham lesions of the RSC. During preconditioning, all rats received pairings of a discrete auditory stimulus followed immediately by a discrete visual stimulus, whereas a second auditory stimulus was presented alone. During first-order appetitive conditioning, the visual stimulus was then directly paired with a US (food pellets). Finally, responding to the auditory stimulus that was initially paired with the visual stimulus ("Paired"), and the auditory stimulus presented alone ("Unpaired"), was assessed in a test session in which no food was delivered. In this and all subsequent appetitive conditioning experiments, the response measured was the amount of time rats spent in the food cup during each stimulus presentation. Although sham rats demonstrated sensory preconditioning by responding more during the Paired vs. Unpaired stimulus, lesions of the RSC eliminated this effect. The finding was recently replicated and extended by Fournier et al. (2020), who demonstrated that pre-training neurotoxic or electrolytic lesions of the RSC prevent appetitive sensory preconditioning when auditory stimuli were used for *both* the first- and higher-order stimuli. Thus, the RSC appears to have an important role in forming associations both within and across sensory modalities.

The aforementioned studies utilized pre-training permanent lesions and therefore do not isolate a specific role for the RSC in sensory preconditioning. It is possible, for instance, that the RSC contributes to sensory preconditioning via either encoding or retrieval of S2–S1 associations, or both. However, an additional appetitive conditioning study by Robinson et al. (2014) demonstrated impaired sensory preconditioning when the RSC was temporarily inactivated (via chemogenetic methods) only during the preconditioning phase. This experiment therefore separated encoding from retrieval, by specifically targeting the RSC during preconditioning, and thus suggests an important role for the RSC in the initial encoding of neutral S2–S1 associations.

A recent experiment demonstrated a role for the RSC in higher-order conditioning using a version of sensory preconditioning that involved both discrete as well as static contextual stimuli and an aversive footshock US (Robinson et al., 2018). During preconditioning, rats were exposed to two contexts (A and B) that had distinct olfactory and visual characteristics. A tone stimulus was repeatedly presented in Context A, and a white noise stimulus in Context B. Thus, during preconditioning rats had the opportunity to associate each context with a specific auditory stimulus. During conditioning in a third context (C), one auditory stimulus was paired with shock and one was not. Finally, higher-order conditioning was assessed by measuring freezing behavior when rats were re-exposed to Contexts A and B in the absence of shock or auditory stimuli. Note that with this design, Contexts A and B were never directly paired with the shock. Instead, one context had been associated with an auditory stimulus that now predicted shock ("Paired" context) and the other context had been associated with an auditory stimulus that now predicted no shock ("Unpaired" context). Robinson et al. (2018) observed that control rats froze more in Paired vs. Unpaired context, however, rats with pre-training electrolytic lesions of the RSC froze equally in both contexts. One interpretation of these findings is that lesions of the RSC prevented the formation of associations between stimuli and the contexts in which they occurred.

Second-Order Conditioning

As a procedure, second-order conditioning is very similar to sensory preconditioning with the exception that the order of the initial two phases are reversed (see **Figure 1**). Thus, in second-order conditioning, S1 is first paired with the US, after which S2 is then paired with S1. The ability of S2 to elicit a CR can theoretically be mediated by one of several associations. For instance, S2 might elicit a CR due to an association between S2 and the *response* elicited by S1 (S–R), or an association between S2 and S1 (S–S). It is also possible that during the second phase, S1 evokes a representation of the US which is then associated with S2 (mediated conditioning). Which association occurs depends on how the stimuli are initially presented, as well as the overall experience with S1 (Rescorla, 1982). For example, sequential presentation of S2 and S1 appears to produce an S–R association, whereas simultaneous presentation results in an S–S association. In addition, Rescorla (1982) noted that extensive exposure to S1, either reinforced or non-reinforced, reduces S–S learning and permits S-R learning even when S2 and S1 were presented simultaneously.

To our knowledge, only one study to date has examined the role of the RSC in second-order conditioning (Todd et al., 2016a). In this conditioned suppression experiment, rats received either pre-training electrolytic lesions or sham lesions of the RSC. Next, both Sham and RSC-lesioned rats received first-order conditioning in which one visual stimulus was paired with shock (V1+), and one visual stimulus was presented alone (V2−). During first-order conditioning, both groups of rats first showed high levels of conditioned suppression to both V1+ and V2−, with Sham lesioned rats gradually reducing fear to V2−. However, RSC-lesioned rats were much slower to reduce fear to V2−, demonstrating a clear impact of the lesions on behavior. At the end of first-order conditioning, when both groups were successfully discriminating V1+ from V2−, each visual stimulus was then paired in a serial fashion with an auditory stimulus; V1+ was followed by A1 and V2− was followed by A2. Overall, there was greater conditioned responding to A1 than A2, and this did not differ between sham and RSC-lesioned rats. Thus, lesions of the RSC did not impair second-order conditioning. Todd et al. (2016a) suggested that the discrepancy between the involvement of the RSC in second-order conditioning and sensory preconditioning may be related to the type of association that is acquired. Indeed, in that experiment, the first- and second-order stimuli were presented serially, and subjects received an extensive amount of prior training with the first-order stimulus. As noted, both of these factors tend to promote S–R over S–S learning.

Knowledge Gaps and Additional Considerations

Although the aforementioned experiments demonstrate a role of the RSC in sensory preconditioning with both discrete and contextual stimuli, several unanswered questions remain. For instance, no study to date has selectively inhibited RSC activity during either conditioning or testing of sensory preconditioning. Thus, although Robinson et al. (2014) demonstrated that the RSC is necessary for encoding of S–S associations, it is unknown if the RSC is also necessary for the retrieval, updating and/or reconsolidation of such associations. The role of the RSC in these phases might ultimately depend on the type of behavioral mechanism that is operating. One possibility is that RSC activity may be necessary during conditioning if, during S1–US pairings, S1 retrieves the representation of S2 such that S2 then undergoes mediated conditioning (Holland, 1981). An alternative possibility, which is not mutually exclusive from the first, is that RSC activity might be necessary during testing if the S2 → S1 → US chain is integrated during the final test phase. Interestingly, all prior discrete stimuli experiments have involved serial presentations of the higher- and first-order stimuli, which may involve chaining at the time of test (Sadacca et al., 2016; Sharpe et al., 2017; but see Wong et al., 2019).

In contrast to sensory preconditioning, there is currently no available data to support involvement of the RSC in second-order conditioning. However, before ruling out a role for the RSC completely, future experiments should examine if the RSC is involved in second-order conditioning with simultaneous presentation of S2 and S1, given that such presentation tends to promote S–S associations as in sensory preconditioning (Rescorla, 1982). These studies will be valuable in determining if the form of associations acquired (S–R or S–S) influence the recruitment of the RSC to second-order conditioning. In addition, such studies will provide valuable information about whether the RSC contributes to S–S associations when one stimulus is already associated with the US, or if the role of the RSC is specific to the encoding, storage, and/or retrieval of *neutral* S–S associations as in sensory preconditioning.

Apart from the types of associations that can be formed, other aspects of the procedure might impact whether or not the RSC is engaged during second-order conditioning. For instance, Holmes et al. (2018) demonstrated that a "dangerous" background context can impact where the brain stores S–S associations. When these associations are formed in a safe context, they involve the perirhinal cortex, but when they are formed in a dangerous context they rely on the amygdala. Critically, the presence of danger is typically a component of aversive second-order conditioning experiments, because the aversive US occurs during the first-order conditioning phase that by definition must precede the second-order phase. In contrast, this is often not the case in sensory preconditioning experiments, in which the US is typically not presented until the conditioning phase. Thus, the discrepancy in the contribution of the RSC to second-order conditioning and sensory preconditioning may be related to the valence of the context during the time that the higher-order associations are formed.

Finally, we note that the role of the RSC in sensory preconditioning is perhaps consistent with its role in other aspects of learning and memory, most notably contextual fear conditioning. Indeed, learning and memory for contexts is often thought to involve the integration of multiple sensory features in the environment (Fanselow, 2010), even in the absence of reinforcement, which is reminiscent of the task requirements inherent to sensory preconditioning. Further understanding of the role for the RSC in higher-order conditioning may thus inform the degree to which RSC function overlaps in these aspects of learning and memory.

Roles of Related Cortical Regions

The experiments reviewed here demonstrate a role for the RSC in sensory preconditioning, specifically for the encoding of neutral S–S associations. Drawing from prior studies, it is possible to speculate how RSC function intersects with other circuits during preconditioning. For instance, inhibiting neural activity or protein synthesis in the perirhinal cortex (PER) following preconditioning reduces responding at test (Holmes et al., 2013; Wong et al., 2019). Further, inactivation of the orbitofrontal cortex (OFC) during preconditioning also impairs responding to a preconditioned cue (Hart et al., 2020), and *in vivo* extracellular recordings indicate that OFC activity represents S–S associations acquired during preconditioning (Sadacca et al., 2018). Thus, the RSC, PER, and OFC may act in concert to facilitate the encoding of associations during preconditioning.

As described previously, S2–S1 associations encoded during preconditioning may allow S2 to be updated during conditioning of S1. This updating requires PER. For instance, blocking protein synthesis in PER immediately after conditioning impairs responding at test (Wong et al., 2019). It is possible that S–S associations encoded within the RSC are also updated during conditioning, although as noted, this has not been specifically tested. Nevertheless, it has been suggested that information encoded within the RSC might be updated through connections with the postrhinal cortex (POR; Bucci and Robinson, 2014); a suggestion that is supported by the putative role of POR in information processing that involves stimuli that undergo change (Ho and Burwell, 2014). Considering the direct anatomical projections between PER and POR (Furtak et al., 2007), it is possible that updating during conditioning might depend upon a distributed cortical network including PER, POR, and RSC.

A second form of integration we have described is one that occurs during the final test phase. In this case, initially encoded S–S associations are integrated with the conditioning memory as an associative chain to drive behavior (Sharpe et al., 2017). Inactivation of OFC during testing impairs responding to a preconditioned stimulus (Jones et al., 2012), suggesting that during testing the OFC is necessary for connecting associations acquired during the preconditioning and conditioning phases (Gardner and Schoenbaum, 2021). Although it is currently unknown if the RSC is also involved with integration at the time of test, such a role is perhaps consistent with the notion that the RSC is necessary when there is mismatch between previously acquired representations (Nelson et al., 2018). For example, although the S2–S1 association was initially encoded while both stimuli were neutral, during testing S2 now predicts S1 which has undergone a change in associative value. Future research is necessary to determine the role of the RSC during testing, and how it might contribute to a larger cortical network that supports higher-order conditioning.

CONCLUSION

Here we examined the neural underpinnings of higher-order conditioning by reviewing the role of the RSC in sensory preconditioning and second-order conditioning. While several studies have demonstrated involvement of the RSC in sensory preconditioning, there is currently no evidence to suggest a role of the RSC in second-order conditioning. This apparent discrepancy may be related to several factors, including the type of associations formed in the two procedures (Todd et al., 2016a), or the status of the background context during the formation of higher-order associations (Holmes et al., 2018). Although there is a need to further examine the contributions made by the RSC to higher-order conditioning, especially second-order conditioning, the results from sensory preconditioning experiments indicate a role for the RSC in forming neutral stimulus-stimulus associations in the absence of reinforcement.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by the National Institute of Mental Health of the National Institutes of Health under award numbers K01MH116158 and R01MH118734. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health.

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

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 08 July 2021
doi: 10.3389/fnbeh.2021.672628

Second-Order Conditioning in Humans

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In contrast to the large body of work demonstrating second-order conditioning (SOC) in non-human animals, the evidence for SOC in humans is scant. In this review, I examine the existing literature and suggest theoretical and procedural explanations for why SOC has been so elusive in humans. In particular, I discuss potential interactions with conditioned inhibition, whether SOC is rational, and propose critical parameters needed to obtain the effect. I conclude that SOC is a real but difficult phenomenon to obtain in humans, and suggest directions for future research.

Keywords: second-order conditioning, associative learning, predictive learning, feature negative, conditioned inhibition, causal learning

OPEN ACCESS

Edited by:
Arnau Busquets-Garcia, Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Spain

Reviewed by:
Mario A. Laborda, University of Chile, Chile
Shauna L. Parkes, Centre National de la Recherche Scientifique (CNRS), France

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

Received: *26 February 2021*
Accepted: *14 June 2021*
Published: *08 July 2021*

Citation:
Lee JC (2021) Second-Order Conditioning in Humans. Front. Behav. Neurosci. 15:672628. doi: 10.3389/fnbeh.2021.672628

INTRODUCTION

Second-order conditioning (SOC) describes a phenomenon whereby a conditioned stimulus (CS) acquires the ability to elicit a conditioned response (CR) without ever being directly paired with an unconditioned stimulus (US). SOC is an example of higher-order conditioning as it demonstrates how learned responses can transfer to stimuli outside of a conditioning episode. Pavlov (1927) first demonstrated SOC in a procedure with two training phases. First, a CS is conditioned by pairing it with a US (CS1-US), and in a subsequent phase, a second-order CS is paired with the first-order CS (CS2-CS1). Critically, the US is not presented on these latter trials to preclude the possibility of an association forming between the second-order CS and the US. SOC is demonstrated if, at test, CS2 elicits the CR, and the effect is associative in nature (i.e., is not elicited in explicitly unpaired control conditions). SOC has been documented in a number of conditioning preparations and animal species including rats (Rizley and Rescorla, 1972; Rescorla, 1982), pigeons (Rescorla, 1979), rabbits (Kehoe et al., 1981), snails (Loy et al., 2006), goldfish (Archer and Sjöden, 1982), and fruit flies (Tabone and de Belle, 2011). Although it is generally acknowledged that SOC is intrinsically weaker than first-order conditioning (Gewirtz and Davis, 2000), it is clear from the animal literature that SOC is a reliable phenomenon.

Historically, SOC has been theoretically important for a number of reasons. SOC explains how conditioned responses form to stimuli that signal seemingly innocuous events, and how they can spread from motivationally-relevant stimuli to distal ones. It therefore expands the explanatory scope of Pavlovian conditioning. SOC has been used as a tool to investigate the fundamental properties of associative learning, and the ability of a CS1 to serve as an effective reinforcer in SOC has proved to be a useful alternative measure of learning (Rescorla, 1980). A large amount of research has been devoted to investigating the associative structure of SOC, with the aim of uncovering which properties of reinforcers animals learn about. The second-order CS could become associated with the first-order CS (a *chained* associative structure, CS2-CS1-US; Hall, 1996), directly with the US evoked by the first-order CS (a *direct* CS2-US structure; Konorski, 1967), or with the response elicited by the US (stimulus-response or *S-R* structure; Rescorla, 1973a).

Evidence that SOC largely survives extinction of the first-order CS [e.g., Nairne and Rescorla (1981) and Rizley and Rescorla (1972), but see Rescorla (1982) and Rashotte et al. (1977)], as well as devaluation of the US (e.g., Rescorla, 1973b; Holland and Rescorla, 1975), suggests that SOC in animals is independent of the first-order association and primarily driven by S-R learning. This conclusion has clinical implications if S-R learning in SOC is accepted as a mechanism for the formation of specific phobias. If second-order stimuli are capable of eliciting fear or anxiety (i.e., the CR) by themselves, treatments that target the original (i.e., first-order) source of fear will not be effective (Rescorla, 1973a; Cook and Mineka, 1987).

An intriguing consequence of withholding presentation of the US on CS2-CS1 trials is that the training procedures that produce SOC can also generate *conditioned inhibition* (Pavlov, 1927; Rescorla, 1973a; Yin et al., 1994). The SOC procedure employs *feature negative* contingencies, which involve learning that a target predicts an outcome (A+), but not when combined in compound with the feature X (AX-). Here, the target (A) can be seen as the first-order CS, and the feature (X) can be seen as the second-order CS. Note that the feature X has a negative relationship with the outcome. According to traditional associative models, X should accrue negative associative strength and become a conditioned inhibitor (Rescorla and Wagner, 1972). Empirically, SOC is typically found early in training while conditioned inhibition emerges with additional training (Herendeen and Anderson, 1968; Yin et al., 1994; Stout et al., 2004; Muñiz-Diez et al., 2021). Rescorla (1973a, 1980) proposed that both effects could be captured using a single dimension of associative strength if it is assumed that SOC is a transient and earlier phase of conditioned inhibition, with second-order excitatory learning gradually being erased or overridden by the developing inhibitory learning.

Despite the theoretical utility of SOC, investigation of SOC in humans has been limited, with only a handful of studies demonstrating the effect in conditioning and causal learning tasks. The purpose of this review is to provide a brief overview of the studies investigating SOC in humans, propose reasons for why SOC has proven to be so elusive, and suggest some directions for future research.

Studies Demonstrating Second-Order Conditioning

The scope of this mini-review is limited to studies in humans using Pavlov's (1927) SOC procedure (CS1+/CS2-CS1) using forward conditioning [see Prével et al. (2019) for SOC demonstrated using backward conditioning]. Note that although this procedure typically presents the two trial types in separate training blocks, I will also count instances where the two trial types are intermixed as instances of SOC [as opposed to sensory preconditioning where the order of phases is reversed (CS2-CS1/CS1+)].

Davey and Arulampalam (1982) first demonstrated SOC in humans using a fear conditioning procedure. In phase 1, they paired a geometric shape (CS1) with an aversive loud noise (US). In phase 2, they paired a picture (CS2) with the geometric shape (CS1), while another control picture (CS0) was presented alone. In phase 3, participants received extinction of CS1, and then CS2 and CS0 were both tested under extinction. Participants showed SOC in skin conductance responses (CS2 > CS0) in the experimental group, but not in a control group who received unpaired presentations of CS1 and the US in Phase 1 [see also Davey and McKenna (1983)]. SOC in electrodermal responses has also been demonstrated with shock and noise USs (Siddle et al., 1987).

The first studies demonstrating SOC in a human causal learning task were reported by Jara et al. (2006). In phase 1, participants made predictions about the appearance of a blood substance (US) given a particular disease (CS1) in a patient. In phase 2, participants learned that a chemical (CS2) produced the disease (CS1). At test, participants were asked to rate to what degree they thought the chemical caused the blood substance, ranging from "never" to "always." In Experiments 1a and 1b, participants rated CS2 higher than control stimuli presented without their paired associates in either phase.

Karazinov and Boakes (2007) administered feature negative training (A+/AX−) in a predictive learning task where participants assumed the role of a doctor diagnosing the foods causing migraines in a fictitious patient. They found that participants who only had 3 s to make a prediction about the migraine outcome on each training trial (i.e., paced training) showed higher predictive ratings during an unpaced test phase to cue X than to a control cue (M) trained in compound and shown to produce no outcome (LM-). This result was interpreted as evidence of SOC, and was found when the feature negative contingencies were presented in separate training blocks (Experiment 1) or intermixed (Experiment 2). Similar results were found by Lee and Livesey (2012) under intermixed training and more strict time conditions (1.5 s to respond). Lee and Livesey (2012) found that when cue X was combined with a transfer excitor (B +) in a novel compound (BX), participants gave higher predictive ratings at test compared to when the same transfer excitor was combined with a non-causal control cue trained alone (C-) or in compound (DE-). Craddock et al. (2018) also demonstrated SOC in a predictive learning scenario with serial (as opposed to simultaneous) presentation of the compound trials (i.e., CS2 → CS1) where participants made predictions about the occurrence of an outcome (the text "WIN" presented on screen).

Finally, an effect analogous to SOC has been found using a contingency learning task with probabilistic relations. Baetu and Baker (2009) asked participants to learn about the causal relations between three lights (A, B, and C). On A-B trials, light C was covered and participants were asked to make predictions about whether light B was on or off, given trials with light A being on or off. The B-C trials were similar except that light A was covered and participants made predictions about light C given light B. The A-B (second-order) and B-C (first-order) trials were intermixed and participants received feedback. In the "Positive-Positive" conditions, the contingency (Δp) between lights A-B and between B-C was positive, meaning that the normative answer for the contingency between A-C was also positive since it could be derived from their product. At test, participants

were asked to judge the relationship between lights A and C, providing a causal rating ranging between perfect prevention and perfect causation. In two experiments, participants did indeed give positive causal ratings for the A-C relation, but they were much closer to 0 than anticipated by the normative answer.

What Do Humans Learn in Second-Order Conditioning?

Some of the studies reviewed above included various post-SOC manipulations to investigate the content of the second-order association. Unfortunately, the studies provide mixed results regarding the associative structure of SOC, offering evidence inconsistent with all three accounts (chain, direct, S-R). Davey and Arulampalam (1982) and Davey and McKenna (1983) found SOC in skin conductance responses despite successful extinction of the first-order association, suggesting that the second-order association does not depend on the first-order association. However, both studies lacked a control group who did not receive extinction of CS1. Thus, it is not known whether the SOC effect would have been larger in the absence of extinction trials. Jara et al. (2006) did include an appropriate (within-subjects) control, and were able to show that extinction of CS1 had no effect on causal ratings to CS2. Jara et al. (2006) concluded that SOC was best described by an independent (direct link between CS2-US) rather than a chained (CS2-CS1-US) causal structure, but noted that their results might also be consistent with the S-R view if it was assumed that the causal judgment itself was the conditioned response.

Craddock et al. (2018) found the opposite result–attenuation of SOC following extinction of CS1 when the CS2-CS1 compound was presented serially, supporting the associative chain-view. It should be noted the dependent variable in this study was slightly unusual, involving a single transformed score combining participants' binary predictions of the outcome and their normalized reaction times [see Craddock et al. (2012)]. Nevertheless, the study used a serial temporal arrangement between CSs that is known to promote SOC (Pavlov, 1927; Stout et al., 2004), and support for the associative chaining mechanism can be found in demonstrations of sensory preconditioning in humans with adequate controls [e.g., Brodgen (1947) and Chernikoff and Brogden (1949), see Seidel (1959) for a review]. In sensory preconditioning, the first- and second-order CSs are first presented in the absence of a US (CS2-CS1), and then the first-order CS is reinforced (CS1+). Thus, any transfer of conditioned responding to the non-reinforced stimulus (CS2) must be learned via a chained associative structure (CS2-CS1-US), since there is no US representation nor CR to become associated with CS2 in the initial phase. The story is complicated somewhat by studies showing that SOC and sensory preconditioning are differentially affected by post SOC-devaluations, suggesting that different associative structures underly these types of higher-order conditioning in animals (e.g., Rizley and Rescorla, 1972). The literature on sensory preconditioning in humans is also scarce, making it difficult to assess whether SOC and sensory preconditioning are learned in similar ways in humans.

Finally, Davey and McKenna (1983) found that SOC was attenuated in a subset of participants for whom habituation to the aversive tone US successfully revalued its valence. In contrast to the majority of animal studies, this finding suggests that SOC can be sensitive to the value of the US, providing evidence against the S-R view. Davey and McKenna explained their results by suggesting that in animals, the US elicits more salient and emotional CRs compared to humans. Thus, the CR is more likely to overshadow the more neutral CS1 in its association with CS2 and lead to S-R learning in animals. This idea is broadly consistent with claims that the associative structure of SOC might depend on the conditioning preparation (Rescorla, 1980), and the modality or salience of the stimuli (Nairne and Rescorla, 1981).

Due to the small number of studies investigating post-SOC manipulations, it is currently unclear what associative structure underlies SOC in humans, and whether differences in procedure, stimuli, or outcomes are responsible for the discrepant findings. Given the potential applicability of SOC to explaining the maintenance of specific phobias, studies investigating the associative structure of SOC will be an important avenue for future research in humans.

What Are the Necessary Conditions for Second-Order Conditioning in Humans?

The studies demonstrating SOC in humans share one important procedural detail–participants are either specifically instructed or encouraged to learn the association between CS2 and CS1. This detail is critical because, as discussed above, CS2 has a negative contingency with the US and can sometimes become a conditioned inhibitor. If SOC is an earlier transient phase of conditioned inhibition (and humans learn quickly), or if inhibition competes with SOC, then researchers might need to implement special measures in order to observe SOC.

The studies in this review certainly seem to incorporate such measures. Davey and Arulampalam (1982) and Davey and McKenna (1983) informed participants prior to each training phase what pairings would be presented, essentially directing them to learn the relevant associations needed to display SOC. In Jara et al. (2006), the cover story instructed participants that their task was to identify whether the diseases (CS1) were related to the blood substances (USs), and whether the chemical substances (CS2) were related to the diseases (CS1). Critically, they were not instructed to learn whether the chemical substances were related to the blood substances. Karazinov and Boakes (2007) and Lee and Livesey (2012) both implemented time pressure during training such that participants had limited time to make a prediction about the outcome. Lee and Livesey (2012) speculated that this manipulation served to disrupt the encoding of prediction error (and therefore conditioned inhibition), since prediction error can only be encoded if participants have the opportunity to encode the stimuli and make a prediction about the outcome. Indeed, in both experiments, Lee and Livesey (2012) found that separate groups of participants given unlimited time to respond during training showed predictions that were more consistent with conditioned inhibition. In Baetu and Baker's (2009) contingency

learning task, the light corresponding to the US was covered while participants observed the lights corresponding to CS2 and CS1. The authors reported that successful simulation of the empirical results depended on the covered light being encoded as "undefined" in the auto-associator, rather than "off" (which resulted in inhibition after a brief excitatory period). Finally, Craddock et al. (2018) specifically instructed participants to learn the associations between the first- and second-order stimuli, and participants were not asked to make predictions about the outcome during the CS2-CS1 pairings.

In summary, while SOC in humans is probably parameter-dependent, one detail that appears to be crucial is whether participants are encouraged to encode the association between CS2 and CS1 (i.e., the within-compound associations), and/or discouraged from encoding the association between CS2 and the absence of the outcome. Otherwise, some form of inhibitory learning may occur [see Lee and Lovibond (2021), Lovibond and Lee (2021) for different types of inhibitory learning]. Future studies could test whether parameters known to promote SOC over conditioned inhibition have similar effects in humans. For instance, SOC tends to be found early in training, using a small number of training trials (Herendeen and Anderson, 1968; Rashotte et al., 1981; Yin et al., 1994; Stout et al., 2004; Muñiz-Diez et al., 2021). While SOC has been demonstrated with simultaneous presentation of the XA compound (Rescorla, 1973a), serial presentation of the XA compound tends to be better than simultaneous presentation at promoting SOC (Stout et al., 2004), while intermixing or blocking the feature negative contingencies seems to have no effect when the number of trials is small in both animals (Yin et al., 1994) and humans (Karazinov and Boakes, 2007). Consistency between species in the effect of these parameters would provide support for the idea that the same associative mechanisms underlie the development of SOC in humans and non-human animals.

Is Second-Order Conditioning Rational?

A related reason that SOC may be difficult to observe is that in a scenario where participants are asked to predict the occurrence of the US, SOC as a phenomenon, is irrational (Karazinov and Boakes, 2007). As noted above, the second-order CS does not predict that the US will occur. In fact, it predicts its absence. There is thus a contradiction between what the second-order CS predicts (its informational or predictive properties), and what it brings to mind (its associative or referential properties). If SOC is a referential effect, it is questionable whether causal judgments or outcome predictions are appropriate ways to measure SOC, as these measures are designed to index the predictive properties of cues. Indeed, Gewirtz and Davis (2000) recommend choosing dependent measures for SOC that are not affected by conditioned inhibition.

A study by Mitchell et al. (2007) provides support for the idea that outcome predictions are not an ideal measure for SOC. Mitchell et al. (2007) administered feature negative training (A+/AX-) to participants, and found evidence of inhibitory learning of X in a forced-choice prediction test. However, the same participants were faster to associate X with its inhibited outcome compared to another familiar but unrelated outcome in a speeded categorization task. Mitchell et al. (2007) interpreted this result as participants learning an excitatory association between X and its respective outcome (i.e., X "went with" O), but learning and expressing an inhibitory causal relationship when asked to make predictions about the outcome (i.e., X prevents O). The authors interpreted their results as refuting the idea that associative strengths translate directly into causal judgments; claiming instead that an extra inferential step was needed (see Mitchell et al., 2009).

However, SOC has been shown in predictive ratings when time pressure is applied during training (Karazinov and Boakes, 2007; Lee and Livesey, 2012). One way to reconcile these findings with those of Mitchell et al. (2007) is to assume that learned associations can translate directly into predictive judgments, but only when conditioned inhibition has not developed. Indeed, Lee and Livesey (2012) showed that when the feature negative contingencies and transfer test were administered to participants in summary form (A + /AX-/B + / C-, test BX vs. BC), participants who had shown SOC after paced training reversed their pattern of judgments and subsequently showed conditioned inhibition once given ample time to think about the contingencies. An interesting direction for future research is to determine whether SOC is overridden by conditioned inhibition, or if a given cue can simultaneously possess both excitatory and inhibitory properties.

In the context of causal reasoning, SOC can be considered rational if the events are assumed to form a causal chain (CS2 causes CS1, CS1 causes the US, e.g., Jara et al., 2006). Baetu and Baker's (2009) results show that under these conditions, participants do infer a positive contingency, albeit with a slight underestimation. Baetu and Baker's (2009) suggested that the underestimation of causal strength may be due to low confidence in judging an unobserved relationship. An alternative possibility is that despite censoring the C light, participants nevertheless encoded the C light as "off" during the A-B trials, resulting in some degree of conditioned inhibition that counteracted SOC and lowered contingency ratings [see Lee et al. (2021) for a discussion of learning from censored information]. Somewhat paradoxically, in a causal chain (A → B → C) where B completely mediates the relationship between A and C, participants tend to *overestimate* the contribution of the irrelevant A event when estimating C from B, a violation of the Markov assumption [see Rottman and Hastie (2014) for a review]. Intriguingly, Rottman and Hastie (2014) suggest SOC as an explanation for why participants fail to disregard the irrelevant A event. Associative learning may therefore be useful in explaining departures from rationality in causal inferences. Further studies are needed to better understand how SOC in associative learning is applicable to causal reasoning phenomena, and whether a similar interaction between excitatory and inhibitory processes occurs in these types of tasks.

CONCLUSION

In conclusion, the evidence suggests that SOC in humans is a real phenomenon, but may be difficult to obtain. The

procedural similarities between those that generate SOC and those that generate conditioned inhibition may mean that SOC is always accompanied by some degree of inhibitory learning. Experimental manipulations that encourage learning of the association between the second-order stimulus and the first-order stimulus, instead of with the absence of the outcome, may be necessary to observe SOC. Suggested avenues for future research include systematic manipulation of experimental parameters to examine the interaction between conditioned inhibition and SOC, post-SOC manipulations to test what kinds of associations underpin SOC, and exploring SOC from the perspective of causal reasoning and rationality. SOC and other forms of higher-order conditioning have broad implications for explaining behaviors ranging from conditioned fear responses to causal inferences. They provide an opportunity to understand the content of learned associations as building blocks of complex memory networks. Given that second-order associations outnumber first-order associations, higher-order conditioning may be a better model for the majority of learning that occurs in the real world (Gewirtz and Davis, 2000). SOC has proven to be an important phenomenon in understanding associative learning in animals, and may prove to be just as useful in humans.

AUTHOR CONTRIBUTIONS

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

FUNDING

This research was supported by an Australian Research Council Discovery Early Career Discovery Research Award (DE210100292).

ACKNOWLEDGMENTS

The author would like to thank Peter Lovibond for helpful comments on an earlier draft.

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

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 06 August 2021
doi: 10.3389/fnbeh.2021.722796

Neural Substrates of Incidental Associations and Mediated Learning: The Role of Cannabinoid Receptors

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OPEN ACCESS

Edited by:
Liana Fattore,
National Research Council (CNR), Italy

Reviewed by:
George Panagis,
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Travis P. Todd,
Dartmouth College, United States

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

Received: 09 June 2021
Accepted: 14 July 2021
Published: 06 August 2021

Citation:
Ioannidou C, Busquets-Garcia A, Ferreira G and Marsicano G (2021) Neural Substrates of Incidental Associations and Mediated Learning: The Role of Cannabinoid Receptors. Front. Behav. Neurosci. 15:722796. doi: 10.3389/fnbeh.2021.722796

The ability to form associations between different stimuli in the environment to guide adaptive behavior is a central element of learning processes, from perceptual learning in humans to Pavlovian conditioning in animals. Like so, classical conditioning paradigms that test direct associations between low salience sensory stimuli and high salience motivational reinforcers are extremely informative. However, a large part of everyday learning cannot be solely explained by direct conditioning mechanisms – this includes to a great extent associations between individual sensory stimuli, carrying low or null immediate motivational value. This type of associative learning is often described as incidental learning and can be captured in animal models through sensory preconditioning procedures. Here we summarize the evolution of research on incidental and mediated learning, overview the brain systems involved and describe evidence for the role of cannabinoid receptors in such higher-order learning tasks. This evidence favors a number of contemporary hypotheses concerning the participation of the endocannabinoid system in psychosis and psychotic experiences and provides a conceptual framework for understanding how the use of cannabinoid drugs can lead to altered perceptive states.

Keywords: CB1, endocannabinoids, higher-order conditioning, sensory preconditioning, incidental learning, incidental associations, mediated learning

INTRODUCTION

In order to make decisions in daily life, we often rely on our previous experiences. We tend to repeat actions that were profitable in the past and, conversely, to avoid those that led to negative consequences. Therefore, the vast majority of learning and memory studies tends to focus on similar situations, where a neutral stimulus (i.e., carrying low salience levels *per se*) is directly associated with a biologically significant, highly salient stimulus (food, electric shock, etc.), producing a new learned response in the individual. However, more often than not, in our

environment we are exposed to novel and ambiguous settings, where direct experience is sparse and where a more flexible approach is required to predict – or guess – how a decision might turn out. In reality, while we engage in a particular activity, we are simultaneously surrounded by many incidental associations that might as crucially influence our future choices, as our direct experiences. Both humans and other animals have been shown to learn about the external world using such associations, often with the involvement of similar neural systems. Contrary to classical conditioning that generally produces solid and long-lasting responses, the memory of incidental associations is intrinsically labile. Rather than providing the individuals with direct information about the external world, it involves a large degree of "ambiguity." Such ambiguity provides a level of flexibility that may be highly adaptive in changing environments. However, forming a mental association between two stimuli could also prove less beneficial if their co-occurrence is simply by chance and doesn't represent truly an association. Incidental information is therefore constantly weighed against expectancies and environmental input to test its adherence to reality. Thus, whereas the ability to form incidental associations offers a way to better respond to unpredictable future challenges, a failure to precisely revise them and update them according to incoming information can account for "learning errors." This can be observed for example in individuals who experience psychotic symptoms like delusions, who can rapidly accept incidental stimuli and events as meaningful and link them in unusual ways.

Understanding the cognitive and neurobiological mechanisms underlying these processes can therefore provide valuable insight both into the complicated abstract ways we learn, as well as into a potential source of cognitive dysfunctions in many mental illnesses. On that account, non-human animal models of incidental learning are crucial in contemporary neuroscientific research.

HIGHER-ORDER CONDITIONING, INCIDENTAL ASSOCIATIONS AND REPRESENTATION-MEDIATED LEARNING

Classical Pavlovian conditioning described how the brain represents dependent relationships between environmental stimuli and still remains the best-characterized associative learning model (Pavlov, 2010). In first-order Pavlovian conditioning, a conditioned stimulus (CS, such as a tone or light) acquires motivational significance by being paired with an intrinsically rewarding or aversive unconditioned stimulus (US, such as food or foot shock). Learning is evaluated by the ability of the CS to elicit a conditioned response (CR) in anticipation of the occurrence of the US. Although traditional views for Pavlovian conditioning described it as the transfer of an unconditioned reflex from the US to the CS, most contemporary learning theories agree that it involves the establishment of associations between internal memory representations of the CS, US, and their relationship (Fanselow and Wassum, 2015).

Although extremely informative, Pavlovian first-order conditioning is not sufficient for representing more ambiguous situations, such as the majority of the ones occurring in every-day life. In fact, a large part of the learning processes to represent our external world involves higher-order conditioning based on associations between low salience sensory stimuli, whose simultaneous or contiguous occurrence is stored because of its potential value for future choices. In higher-order conditioning a CS (S2) acquires associative strength by being paired with another CS (S1), rather than with a US. Two higher-order conditioning paradigms have been mainly used to assess higher-order conditioning in humans and animals, second-order conditioning, and sensory preconditioning. In second-order conditioning, the S1–S2 pairing can occur after S1 has been paired with the US, whereas in sensory preconditioning S1–S2 pairing precedes the S1-US (Gewirtz and Davis, 2000). In both cases subjects eventually display a conditioned response to a stimulus that was never explicitly paired with the reinforcer and thus higher-order conditioning tasks have been largely used to evaluate forms of indirect learning.

Sensory preconditioning in particular represents the most common behavioral protocol for studying incidental associations among relatively neutral or low-salience stimuli. In a typical experiment, two low-salience stimuli are first presented jointly during a preconditioning phase (S1–S2), then followed by classical conditioning of one of these stimuli by pairing it with a biologically meaningful (high salience) unconditioned reinforcer, like food or a foot shock (S1-US). Finally, exposing the subjects to either of the original stimuli (the one directly associated with the reinforcer and the one never associated) reveals the retrieval of direct and indirect memories, respectively (Brogden, 1939). Across a range of species (Karn, 1947; Hall and Suboski, 1995; Kojima et al., 1998; Muller et al., 2000; Wimmer and Shohamy, 2012), subjects' response to the indirect preconditioned stimulus (S2) is found to be similar to that evoked by the directly conditioned cue (S1), assuming an association between the two has been formed.

Two prominent theoretical accounts are generally applied to explain the cognitive processes that underlie sensory preconditioning: the first one is the "associative chain" model, where the different associations are formed during the first and second phases of training allowing inference at test. In this account, the S1–S2 learning (phase 1) and the S1-US learning (phase 2) occur independently of each other and memories are integrated at the time of the testing, by recalling the two associations in order to infer on-the-fly the outcome that will likely follow (Rizley and Rescorla, 1972; Jones et al., 2012; Sharpe et al., 2017a; Sadacca et al., 2018; Wong et al., 2019; Wang et al., 2020). The second account, does not require memory integration at the time of testing, and refers to a process through which the preconditioned stimuli directly acquire positive or negative value during conditioning, due to a "unified representation" of S1 and S2. Through this process, often termed mediated or representation-mediated learning, presentation of S2 during the second phase of training activates a mental representation of S1, so that that this associatively retrieved memory might become further associated with the experience of the US. Eventually

presentation of the S1 during test, retrieves this mediated S1-US association, and thus, elicits the observed response (Holland, 1981b; Hall, 1996; Wheeler et al., 2008; Wimmer and Shohamy, 2012; Schlichting and Preston, 2015; Lin and Honey, 2016). Representation-mediated learning was originally described by Holland (1981a, 1990), whose work demonstrated that animals can learn not only about directly perceived stimuli, but also about indirect, associatively retrieved representations of that stimuli. Auditory or visual stimuli (Holland, 1981a) or contexts (Dwyer, 1999, 2001) were initially paired with a flavored solution. When the tone, light or context were later paired with a gastric malaise, they served as substitutes for their associated flavor stimuli. This paradigm differs from a classical sensory preconditioning task in that these stimuli (tone, light, or context) did not form any appreciable first-order association with the illness, however, the associatively activated taste representations did support taste-aversion learning.

BRAIN REGIONS INVOLVED IN INCIDENTAL LEARNING

Imaging studies in humans as well as experiments in rodents have provided insights into a network of brain regions that are involved in sensory preconditioning. The orbitofrontal cortex (OFC) has been shown to be necessary for forming value-neutral sensory associations, since both entire and selective inactivation of the OFC impairs inference about previously acquired stimulus-stimulus associations during the testing phase of sensory preconditioning (Jones et al., 2012). Moreover, single-unit recording experiments showed that neural activity in the lateral OFC reflects the acquisition of the associative information during the initial phase of training (Sadacca et al., 2018), and that optogenetic silencing of the OFC during this phase completely eliminates responding to the preconditioned cue during testing (Hart et al., 2020). Other structures, like the perirhinal and retrosplenial cortices have also been implicated. Lesions of the perirhinal cortex or its inactivation during preconditioning abolished sensory preconditioning (Nicholson and Freeman, 2000; Holmes et al., 2013; Wong et al., 2019), whereas chemogenetic silencing of the retrosplenial cortex during the preconditioning phase prevented inference at test without influencing direct conditioning (Robinson et al., 2014).

Interestingly, all aforementioned cortical regions are directly and indirectly interacting with the hippocampus (Agster and Burwell, 2013; Ritchey et al., 2015; Witter et al., 2017). Decades of research have characterized how the hippocampus critically contributes to representing and processing both real and abstract associative information (Port et al., 1987; Manns and Eichenbaum, 2009; Zeithamova et al., 2012; Voss et al., 2017) and many studies have highlighted its importance in sensory preconditioning both in humans (Bornstein and Daw, 2012, 2013; Wimmer and Shohamy, 2012; Shohamy and Turk-Browne, 2013) and in animals (Iordanova et al., 2009, 2011; Wheeler et al., 2013; Lin et al., 2016; Barron et al., 2020). Recent work additionally shows that a crosstalk between the hippocampus and the orbitofrontal cortex is important for inferring future outcomes during sensory preconditioning (Wang et al., 2020). Notably, in some studies, hippocampal activation has been demonstrated during the testing phase, suggesting its involvement primarily in the retrieval of the sensory-sensory associations (Talk et al., 2002; Barron et al., 2020). However, in other studies, hippocampal activation has been also shown during the conditioning phase of sensory preconditioning, as well as during the initial stimulus-stimulus associations, supporting a widespread hippocampal involvement and suggesting that this brain region may be particularly important not only for retrieval but also for the encoding of the incidental associations between neutral stimuli (Wang et al., 2020). This is consistent with evidence showing that the hippocampus is essentially involved in the acquisition of information, which can then be used by different brain regions to guide flexible behavior (Elliott Wimmer and Büchel, 2019; Schuck and Niv, 2019). In the following paragraphs we argue that one possible mechanism for the formation of low-salience stimulus-stimulus associations in the hippocampus during sensory preconditioning is involving the tight regulation of hippocampal GABAergic interneurons by cannabinoid receptors.

CANNABINERGIC CONTROL OF INCIDENTAL ASSOCIATIONS

Originally discovered as the endogenous targets of the cannabis plant psychotropic derivative Δ^9-tetrahydrocannabinol (THC), cannabinoid receptors and specifically type 1 cannabinoid receptors (CB1Rs) are key neuromodulatory elements of synapses. Physiologically, cannabinoid receptors are the main targets of endogenous signaling molecules called endocannabinoids, forming, together with the enzymatic machinery for their synthesis and degradation, the so-called endocannabinoid system (ECS) (Piomelli, 2003; Lu and Mackie, 2016). CB1 receptors are likely the most abundant G protein-coupled receptors in the brain, with amounts of protein comparable to NMDA and GABAA receptors (Herkenham et al., 1990; Howlett, 2002; Freund et al., 2003). The expression levels of CB1 receptors can drastically differ among different cell types and can diverge between different brain regions (Han et al., 2012; Busquets-Garcia et al., 2018a). In cortical areas such as the hippocampus and neocortex, both glutamatergic principal neurons and GABAergic interneurons contain CB1 receptors, with the latter expressing the highest levels (Marsicano and Lutz, 1999; Marsicano and Kuner, 2008). The ECS has been involved in many forms of direct learning such as fear conditioning through CB1R in the amygdala (Marsicano et al., 2002; Metna-Laurent et al., 2012), conditioned taste aversion through CB1R in insular cortex (Kobilo et al., 2007), conditioned odor aversion through CB1R in medial habenula (Soria-Gomez et al., 2015) or conditioned odor preference through CB1R in the anterior piriform cortex (Terral et al., 2019), among others. Interestingly, the involvement of the ECS in direct conditioning appears to be more prominent in the modulation of the behavioral expression

of the acquired memory, rather than its formation (Kobilo et al., 2007). However, despite the fact that CB1 receptor plays crucial roles in different phases of learning and memory processes (Rueda-Orozco et al., 2008; Marsicano and Lafenêtre, 2009; Akirav, 2011; Drumond et al., 2017), not many studies have addressed the physiological role of endocannabinoid signaling in higher-order learning.

In our previous work (Busquets-Garcia et al., 2018b) we evaluated the role of CB1R during the formation of incidental associations, using two different sensory preconditioning protocols in mice. Mice were first preconditioned by repeated exposure to pairs of low- salience sensory stimuli (pairing of an odor with a taste, or a light with a tone) forming an association between them. On subsequent days, mice were classically conditioned to associate one of these sensory stimuli (but not the other) with either an aversive or an appetitive stimulus. At the time of testing, both the directly conditioned stimulus but also the incidental preconditioned stimulus produced an aversion/preference, indicating the acquisition of both direct learning and mediated learning, respectively. Using this task, we showed that CB1R blockade upon preconditioning impaired the expression of mediated learning, however, CB1R blockade (or activation) at the stage of the testing did not affect the response to the preconditioned cue, strongly arguing for a specific role of endocannabinoid signaling in the initial processing of incidental stimulus-stimulus associations. Importantly, this effect did not appear to be limited to the specific sensory modality of the stimuli – whether those were olfactory and gustatory, or visual and auditory. The involvement of the ECS in different experimental conditions suggests broad common mechanisms underlying higher-order learning processes independently of the sensory modalities used and of the nature (aversive or appetitive) of the reinforcer.

With the hippocampus being a key brain region for sensory preconditioning, we addressed the role of hippocampal CB1R in these processes. In mice lacking CB1Rs selectively in the hippocampus or in forebrain GABAergic interneurons, mediated learning was compromised, yet direct learning was unaffected. Further experiments revealed that CB1Rs in hippocampal GABAergic neurons are indeed crucial for incidental learning, demonstrating a physiological link between hippocampal GABAergic signaling and associative memory between low-salience events. In fact, the paired presentations of the low-salience sensory cues during the initial, preconditioning phase induced a specific protein synthesis-dependent enhancement of hippocampal CB1R expression and facilitated long-term synaptic plasticity at hippocampal inhibitory synapses, suggesting that incidental learning might involve synthesis of new CB1Rs in hippocampal interneurons (Busquets-Garcia et al., 2018b). Interestingly, midbrain dopaminergic signaling has been shown to be both necessary and sufficient for the formation of incidental associations (Sharpe et al., 2017b). Dopamine function is also tightly regulated by and regulating the hippocampus (Lisman and Grace, 2005), and recently CB1 receptors have been identified in a subpopulation of hippocampal D1R-positive interneurons, where they control memory processes (Oliveira da Cruz et al., 2020). Therefore it is possible that endocannabinoids modulate incidental learning at hippocampal level through dopaminergic circuits, and further research should address this hypothesis.

FROM INCIDENTAL LEARNING TO REALITY TESTING: A ROLE FOR CB1 RECEPTOR SIGNALING

Contrary to classical conditioning between a conditioned stimulus and an unconditioned stimulus that generally produces solid and long-lasting responses, an elemental characteristic of incidental associations between stimuli is that they are intrinsically weak (McDannald and Schoenbaum, 2009). Several studies have shown that, when studied through sensory preconditioning paradigms, the establishment of incidental learning requires a certain amount of training/paired presentations between the preconditioned stimuli. Paradoxically though, extending this training or pairings during preconditioning abolishes its expression (Holland, 2005; Holland et al., 2008; Busquets-Garcia et al., 2017), suggesting that the sensitivity to incidental learning can change as training proceeds. One explanation for this phenomenon suggests that, with moderate preconditioning, animals form a unified mental representation of the different preconditioned stimuli (S1+S2). However, with prolonged exposure to the stimuli, the subjects acquire more information about these stimuli, allowing them to separate their specific sensory features and consequently their associated outcomes (McDannald and Schoenbaum, 2009). As the preconditioned cues are indeed separated entities in reality, researchers defined this process as "reality testing," following the basis of reality monitoring, the ability of individuals to distinguish real from illusory patterns and associations (Johnson and Raye, 1981; McDannald and Schoenbaum, 2009). An important aim down the road is therefore to unravel the complex biological processes that allow animals to switch from a unified representation of the different stimuli to their discrimination as independent entities ("reality testing").

Type 1 cannabinoid receptors appear to be a key element of this switch. Our studies using reality testing protocols revealed that cannabinoids could disrupt this fundamental adaptive process, since acute administration of the main psychoactive component of cannabis, THC, was shown to impair reality testing, through activation of hippocampal CB1Rs (Busquets-Garcia et al., 2017). Thus, there is a dual impact of hippocampal CB1R signaling: whereas a *minimal activation* of CB1Rs is required for incidental learning in order to form unified stimuli representations, their *excessive stimulation* impedes testing of the real nature of these representations (reality testing). The data collected so far indicate that there seems to be a descending gradient of CB1R signaling during the switch between incidental learning and reality testing. On one hand, ECS activity has to be sustained at the moment of forming incidental learning, during which individuals collect possible useful information from seemingly unrelated stimuli. On the other hand, CB1R signaling has to be reduced when this potential information is contrasted to reality. In other words, more ECS activity leads to the generation of "open possibilities," whereas the "closing" of

these possibilities when they do not adhere to reality requires a decrease of CB1R signaling.

CONCLUDING REMARKS: FROM IMAGINATION TO PSYCHOSIS?

The formation of incidental associations can underlie particular human abilities such as imagination and creativity, which are characterized by the ability to assume connections between unrelated phenomena in order to construct new ideas and imagine future scenarios (Schacter et al., 2012; Uddin, 2021). Cannabis use and creativity are also often portrayed as linked (LaFrance and Cuttler, 2017), with their connection culturally and commonly accepted. Cannabis intoxication has been shown to promote divergent thinking, the ability to see connections between distant concepts and reveal something new (Eisenman et al., 1980; Morgan et al., 2010), but at the same time to impair convergent thinking, the ability to reason based on logical inference (Oomen et al., 2018). This disparity could result in connections being made between seemingly unrelated concepts or ideas, which are then linked together and elaborated upon, a characteristic of creative thinking but also of the development of a delusional system, often present in psychiatric conditions such as schizophrenia and psychosis. Interestingly, the reconceptualization of schizophrenia symptoms as aberrant perceptions (hallucinations) (Corlett et al., 2019) and beliefs (delusions) (Feeney et al., 2017), has provided the framework to be studied through associative learning tasks in both humans and animals (Powers et al., 2017; Dwyer, 2018; Koh and Gallagher, 2020). Indeed, impaired "reality testing" was recently demonstrated in several animal models of schizophrenia in a way that mimics psychotic-like percepts (McDannald et al., 2011; Kim and Koh, 2016; Busquets-Garcia et al., 2017; Koh et al., 2018; Fry et al., 2019), with recent evidence suggesting that such phenomena involve dopamine signaling (Schmack et al., 2021).

Cannabis has been linked to the development of psychotic symptoms since a long time (Zuardi, 2006) and is well known to produce a range of immediate-onset psychotomimetic symptoms (Solymosi and Kofalvi, 2017), while alterations in the endocannabinoid system have also been implicated to the pathogenesis of schizophrenia and similar psychotic disorders (Muller-Vahl and Emrich, 2008). Given the general importance of the endocannabinoid system in the modulation of sensory perception (Soria-Gomez et al., 2014) and the fact that this function is centrally altered in psychotic states, it has been suggested that one important mechanism of cannabinoid-induced psychoses is linked to the alteration of perception of the external world. We therefore argue that the control of cannabinoid receptors over the formation and updating of incidental associations is contributing in orchestrating learning and associative thinking, in a continuum from normal perception to altered perceptual states.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by INSERM, European Research Council (Endofood, ERC-2010-StG-260515 and CannaPreg, ERC-2014-PoC-640923, MiCaBra, and ERC-2017-AdG-786467), Fondation pour la Recherche Medicale (DRM20101220445), and the Human Frontiers Science Program, Region Nouvelle Aquitaine, Agence Nationale de la Recherche (ANR, NeuroNutriSens ANR-13-BSV4-0006; ORUPS ANR-16-CE37-0010-01; CaCoVi ANR 18-CE16-0001-02; MitObesity ANR 18-CE14-0029-01; and BRAIN ANR-10-LABX-0043).

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 30 August 2021
doi: 10.3389/fnbeh.2021.740992

Dopamine D1 and D2 Receptors Are Important for Learning About Neutral-Valence Relationships in Sensory Preconditioning

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Dopamine neurotransmission has been ascribed multiple functions with respect to both motivational and associative processes in reward-based learning, though these have proven difficult to tease apart. In order to better describe the role of dopamine in associative learning, this series of experiments examined the potential of dopamine D1- and D2-receptor antagonism (or combined antagonism) to influence the ability of rats to learn neutral valence stimulus-stimulus associations. Using a sensory preconditioning task, rats were first exposed to pairings of two neutral stimuli (S2-S1). Subsequently, S1 was paired with a mild foot-shock and resulting fear to both S1 (directly conditioned) and S2 (preconditioned) was examined. Initial experiments demonstrated the validity of the procedure in that measures of sensory preconditioning were shown to be contingent on pairings of the two sensory stimuli. Subsequent experiments indicated that systemic administration of dopamine D1- or D2-receptor antagonists attenuated learning when administered prior to S2-S1 pairings. However, the administration of a more generic D1R/D2R antagonist was without effect. These effects remained constant regardless of the affective valence of the conditioning environment and did not differ between male and female rats. The results are discussed in the context of recent suggestions that dopaminergic systems encode more than a simple reward prediction error, and provide potential avenues for future investigation.

Keywords: dopamine, preconditioning, pavlovian, learning, D1, D2

OPEN ACCESS

Edited by:
Arnau Busquets-Garcia, Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Spain

Reviewed by:
Evan Hart, National Institute on Drug Abuse (NIDA), United States
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Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *14 July 2021*
Accepted: *04 August 2021*
Published: *30 August 2021*

Citation:
Roughley S, Marcus A and Killcross S (2021) Dopamine D1 and D2 Receptors Are Important for Learning About Neutral-Valence Relationships in Sensory Preconditioning. Front. Behav. Neurosci. 15:740992. doi: 10.3389/fnbeh.2021.740992

INTRODUCTION

Dopamine neurotransmission has been shown to be critical for aspects of reward-related learning in many different preparations, making it of key interest in the study of disorders involving dysregulation of the reward system, such as addiction, depression, ADHD, and schizophrenia. However, it has proven difficult to pinpoint the precise function(s) of dopamine with respect to associative learning (the formation of connections between cues or actions and their associated outcomes) and motivational processes. Whilst there is strong background literature implicating dopamine in aspects of effort (Salamone et al., 2007, 2016; Salamone and Correa, 2012), desire (Berridge, 2007; Flagel et al., 2011) and reward (Wise, 2006, 2008), there is also substantial evidence

highlighting its importance in the prediction error mechanisms important for associative formation in appetitive Pavlovian conditioning and instrumental reinforcement learning (Schultz et al., 1997; Schultz and Dickinson, 2000; Schultz, 2007; Balleine et al., 2009; Wassum et al., 2012; Steinberg et al., 2013; Chang et al., 2016).

Although it seems very likely that dopamine in fact plays a role in most (if not all) of these processes, it is not straightforward to isolate the different neurochemical mechanisms, and their neuroanatomical loci, through which dopamine is able to perform these different functions. One reason for this is that the majority of work investigating these functions naturally takes place in the context of reward-based conditioning procedures, making it difficult to tease apart the separate processes involved and study them in isolation. Accordingly, in order to get a clear picture of dopamine's role in learning, it may help to look beyond reward-learning procedures such as appetitive conditioning and reinforcement learning and explore dopamine's involvement in other associative preparations. For example, there is evidence to suggest that dopamine may be important in neutral-valence, stimulus-stimulus learning (Young et al., 1998; Sharpe et al., 2017).

One protocol used to examine this type of learning is sensory preconditioning (e.g., Holmes et al., 2013). In the first stage of a sensory preconditioning procedure, animals are exposed to pairings between two innocuous sensory cues (S2-S1; e.g., a tone and a light). In Stage 2, S1 is then paired with a mildly aversive unconditioned stimulus (US), such as a foot-shock (S1-US; e.g., light-shock). The degree to which animals learned about the S2-S1 relationship can then be assessed by measuring fear expressed to S2 (which was never directly paired with the fear-inducing US); Animals learn to fear S1 *via* S1-US conditioning, and as a consequence of having already learned the S2-S1 relationship, come to fear S2 by association. This procedure provides an opportunity to study the neural mechanisms involved in the formation of associations between stimuli that, at the time of learning, have no motivational significance—thereby separating the associative learning process from potentially confounding motivational functions. In addition, the nature of the stimuli and patterns of presentation are more closely matched to previous protocols demonstrating an impact of dopaminergic manipulation than other commonly employed sensory preconditioning procedures such as those involving flavour-flavour associations.

The basic notion that dopamine may be involved in learning about sensory stimuli is evidenced by early work demonstrating dopamine neurons fire in response to novel or high-intensity stimuli, or unexpected stimuli of a sort capable of eliciting a behavioural response (e.g., orienting) before these are ever paired with reward (Schultz and Romo, 1990; Ljungberg et al., 1992; Horvitz, 2000). In the context of sensory preconditioning, dopamine levels have been shown to increase in the nucleus accumbens during sensory S2-S1 learning (but not unpaired S2/S1 presentations) and subsequent tests of both S2 and S1 (Young et al., 1998). Similarly, dopamine neurons in the ventral tegmental area have been observed to fire in response to preconditioned sensory cues never directly paired with reward (Sadacca et al., 2016) and activation of these neurons has been shown to be necessary and sufficient to drive S2-S1 learning (Sharpe et al., 2017).

These studies demonstrate that dopamine is involved in learning processes that occur in the absence of any explicit reward factor, which has important implications for our broader understanding of dopamine function. However, much remains to be explored with regards to the nature of dopamine's involvement in this learning. For example, the evidence to date primarily stems from studies investigating dopamine release and/or activity in dopaminergic neurons. Little is known about downstream mechanisms involving activity at specific dopamine receptor subtypes or the neural populations and circuits in which these are expressed.

Evidence from the appetitive literature highlights that distinct dopamine receptor subtypes can serve complementary functions in some situations, and competing functions in others. For example, some have found evidence of D1R- and D2R-activation working in concert (Capper-Loup et al., 2002; Perreault et al., 2014; Kupchik et al., 2015; Hasbi et al., 2018) In contrast, it has been shown that Pavlovian cued food approach is impaired by D1R antagonism but enhanced by D2R antagonism (Eyny and Horvitz, 2003). Similarly, in an instrumental paradigm, administration of amphetamine promotes the development of habitual behaviour and this effect is prevented by blockade of D1R but enhanced by blockade of D2R (Nelson and Killcross, 2006, 2013).

Furthermore, we have shown that the acquisition of anticipatory approach behaviour towards the location of predicted reward delivery in an appetitive Pavlovian conditioning procedure requires activity at dopamine D1-like receptors (D1R) but not D2-like receptors (D2R; Roughley and Killcross, 2019). In contrast, acquisition of approach behaviour towards a *cue* that predicts reward delivery requires activity at both D1R and D2R (Roughley and Killcross, 2019). These findings are broadly consistent with other evidence that appears to suggest that D1R, and the phasic firing pattern of dopamine neurons for which these receptors have a preferential affinity (Wall et al., 2011), might be particularly important for learning predictive relationships between contingent events in general (Schultz, 2007; Zweifel et al., 2009), whereas D2R, more sensitive to tonic dopamine release resulting from basal level firing, may be more selectively involved in motivational aspects of learning and performance (Niv, 2007; Salamone and Correa, 2012; Gallo, 2019). In light of the substantial similarity between D1- and D5-receptors and D2- and D3/D4-receptors (including with respect to the specificity of dopaminergic agonists/antagonists), it should be noted that whilst we refer in this article to D1- and D2-receptors, these terms relate to D1-like and D2-like receptor families more broadly.

It would be of interest to explore the potentially differential or cooperative role of D1R and D2R in the context of sensory stimulus-stimulus learning. Accordingly, the aim of the present set of experiments is to determine whether the activity at D1R and/or D2R is important for the acquisition of S2-S1 associations in a sensory preconditioning procedure. In the interest of comparison with appetitive conditioning

procedures, a further aim is to investigate whether the nature of any D1R/D2R involvement is influenced by the motivational context in which learning takes place. In this way, we hope to demonstrate the utility of higher-order conditioning procedures like sensory preconditioning in enhancing our understanding of the neurochemical mechanisms at play in psychological disorders, such as addiction, that involve dysregulated associative learning processes.

In Experiment 1 we provide a demonstration of sensory preconditioning in a neutral vs. motivationally attractive context and confirm that, in both cases, the fear expressed to S2 at test is specifically a function of learned associations between S2-S1 and S1-US, and not due to generalisation effects or inherent conditioning properties of the stimuli (Holmes et al., 2013). Experiment 2 examines the importance of D1R and D2R for S2-S1 learning through the systemic administration of selective D1R- or D2R-antagonists prior to Stage 1 of the sensory preconditioning procedure. Experiment 3 also investigates the role of D1R and D2R in S2-S1 learning, but in this case it takes place in an environment already established as attractive. Finally, in Experiment 4 we examine whether the effects of D1R and D2R antagonism on S2-S1 learning are additive, and again, whether this differs according to the motivational relevance of the learning environment.

MATERIALS AND METHODS

Subjects

Subjects were experimentally naïve, male and female Long-Evans rats (UNSW Psychology breeding colony), between 12–16 weeks of age at the beginning of experimental procedures. Rats were housed in groups of four, in a temperature- and humidity-controlled environment (22°C) operating on a 12 h light/dark cycle (lights on at 07:00 h). Experimental procedures took place during the light cycle. For Experiments 1, 3, and 4 rats were placed on a restricted food schedule prior to behavioural training to induce appetitive motivation for food. Weights never reduced past 85% of free-feeding values and water was continuously available in home-cages. In Experiment 2 both food and water were continuously available. Animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80–23, revised 1996), and were approved by the UNSW Animal Care and Ethics Committee.

Apparatus

Behavioural training and testing took place in eight standard operant chambers (30 cm × 24 cm × 22 cm; MED Associates Inc., St. Albans, VT, USA), each housed in a light- and sound-attenuating compartment. The sidewalls of each chamber were constructed of aluminium, and the back wall, ceiling, and hinged front wall were made of clear Perspex. Floors consisted of 19 stainless-steel bars (4 mm diameter; 1.5 cm apart), aligned perpendicular to the back wall of the chamber. A constant current shock generator (MED Associates Inc., St. Albans, VT, USA) was used to deliver a brief duration electric current to the grid floor of the chamber (0.8 mA intensity; 0.5 s duration). Floors were cleaned after each experimental session.

The auditory stimulus used was a 70 dB 1 kHz square-wave tone produced through a speaker located at the top back left corner of the chamber. The visual stimulus was a 28 W light located on the ceiling of the compartment, flashing at a rate of approximately 3 Hz. The physical identity of the stimuli was fully counterbalanced in each experiment. Chambers were also equipped with a recessed food magazine located at the bottom Centre of the right-hand wall, into which reward pellets could be delivered from a pellet dispenser (Experiments 1, 3, and 4). Head entries to the magazine were detected by breaks of an infrared beam across the opening of the magazine.

Each chamber was illuminated *via* an infrared light source on the compartment ceiling. Cameras mounted on the back wall of each compartment recorded rats' behaviour during training and test sessions. Recordings were stored on an external hard drive. Experimental events were controlled and recorded *via* a PC running Med-PC software.

Drugs

Dopamine receptor antagonists were dissolved in 0.9% saline (w/v) and injected subcutaneously at a volume of 1 ml/kg 15 min prior to sensory preconditioning (Stage 1 training session). For Experiments 2 and 3, the antagonists used were the selective D1R antagonist SCH39166 (Tocris Bioscience; Bristol, UK), administered at a dose of 0.0125 mg/kg (Low), 0.025 mg/kg (Mid), or 0.05 mg/kg (High), and the selective D2R antagonist eticlopride hydrochloride (Sigma-Aldrich; Sydney, Australia), administered at a dose of 0.003 mg/kg (Low), 0.0125 mg/kg (Mid), or 0.03 mg/kg (High). For Experiment 4, the antagonist used was the non-selective dopamine receptor antagonist α-flupenthixol (flupenthixol dihydrochloride; Sapphire Bioscience; Redfern, Australia), administered at a dose of 0.5 mg/kg. SCH39166 and eticlopride doses were determined on the basis of the range observed within our laboratory to be behaviourally effective in an appetitive conditioning context (e.g., Roughley, 2017; Roughley and Killcross, 2019). The dose of α-flupenthixol was deliberately chosen to be at the high end of that range; at this dose, motor function remains intact but animals show much reduced performance of motivated behaviours (e.g., Roughley and Killcross, 2019). In all experiments, the vehicle solution for control injections was physiological saline (0.9% w/v).

Behavioural Procedures

Pretraining

Rats were handled daily in the week preceding the onset of experimental procedures to familiarise them with the experimenter and basic protocols. In Experiments 1, 3, and 4, on two days prior to the start of training, rats were familiarised with the food pellets they would receive during context exposure (45 mg grain pellets; Bio-Serve, Frenchtown, NJ, USA).

Context Exposure

A summary of the experimental timeline from context exposure onwards can be found in (**Figure 1**). Experimental protocols are based on those used by Holmes and colleagues (e.g., Holmes et al., 2013). On each of the first two days of the

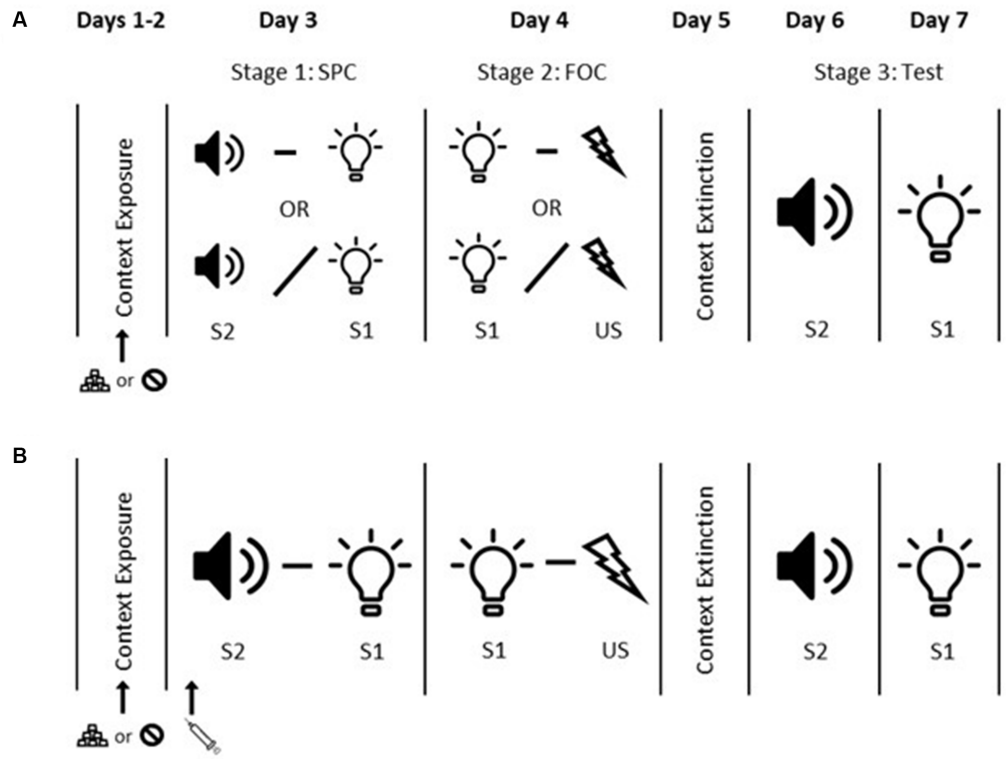

FIGURE 1 | Sensory preconditioning experimental timeline. Panel **(A)** shows the timeline for Experiment 1. Days 1 and 2 = context exposure. On Day 2 rats in the Appetitive condition received food pellet exposure during context exposure while rats in the Neutral condition did not. Day 3 = sensory preconditioning (SPC). Rats in Groups P-P and P-UP received paired S2 and S1 presentations (tone and light; counterbalanced). S2 and S1 were presented in an unpaired fashion for Group UP-P. Day 4 = first-order conditioning (FOC). Rats in Groups P-P and UP-P received paired S1-US (shock) presentations. S1 and US were presented in an unpaired fashion for Group P-UP. Day 5 = context extinction. Days 6 and 7 = tests of S2 alone and S1 alone. Panel **(B)** shows the timeline for Experiments 2–4. Days 1 and 2 = context exposure. On Day 2 all rats in Experiment 3 and rats in the Appetitive condition in Experiment 4 received food pellet exposure during context exposure. Rats in Experiment 2 and rats in the Neutral condition in Experiment 4 did not. Day 3 = SPC. All rats received paired S2-S1 presentations. Injections of dopamine antagonist or vehicle (according to the group) were administered prior to the SPC session. Day 4 = FOC. All rats received paired S1-US presentations. Day 5 = context extinction. Days 6 and 7 tests of S2 alone and S1 alone.

experimental protocol, rats received two 30-min sessions of exposure to the conditioning chamber. These were separated by a minimum interval of 3 h. For experiments/groups in which sensory preconditioning took place in a neutral context (1, 2, 4), no programmed events took place during exposure sessions. In other experiments (1, 3, 4), in order to establish the conditioning chamber as an appetitive context, food pellets were delivered periodically to the food magazine in the chamber throughout the final two exposure sessions (variable time 60 s; approximately one pellet per minute). In all cases rats reliably came to retrieve pellets from the magazine. These were the only sessions in which rats received any food in the chambers.

Sensory Preconditioning

Stage 1 sensory preconditioning (SPC) occurred over a single session approximately 46 min in duration. The session involved eight presentations each of the visual and auditory stimuli, designated S1 or S2 (fully counterbalanced). S2 was presented on a fixed time schedule with 5 min 10 s between each presentation, and each presentation lasting 30 s. In groups receiving paired (contingent) S2-S1 presentations, S1 began at the termination of S2. S1 presentations lasted 10 s. In groups receiving unpaired (non-contingent) S2 and S1 presentations, S1 was presented in the middle of the interval between S2 presentations. Rats remained in the conditioning chamber for 1 m following the final stimulus presentation. For experiments involving drug manipulations (Experiments 2, 3 and 4), animals were randomly assigned to receive an injection of either saline or dopamine antagonist prior to this session.

First-Order Conditioning

Stage 2 first-order fear conditioning (FOC) took place the next day over a single session of approximately 22 min duration. The session involved four 10 s presentations of the stimulus previously designated S1 (either tone or light, counterbalanced) and four presentations of the US (a 0.5-s, 0.8-mA foot-shock). S1 presentations were separated by an interval of 5 min. For groups receiving paired S1-US presentations, the shock was delivered in the final 0.5 s of each S1 presentation. For groups receiving unpaired S1 and US presentations, the shock was delivered in the middle of the interval between S1 presentations.

Rats remained in the conditioning chamber for 1 min following the final stimulus presentation.

Context Extinction

On the day following FOC, rats received two 30-min sessions of context extinction in which they were placed in the conditioning chamber but no programmed events occurred. These sessions were separated by a minimum interval of 3 h. The purpose of these sessions was to extinguish any freezing elicited by the context, in order to better observe freezing specifically elicited by conditioned stimuli on subsequent tests.

Testing

On test days rats first received an additional 10 min of context extinction, followed 2 h later by the test session. Test sessions involved 8 presentations of the S2 stimulus alone (Test 1) or S1 stimulus alone (Test 2; 24 h later). In each test stimulus durations were as in training (S2 = 30 s; S1 = 10 s) and presentations were separated by 3-min intervals.

Data Analysis

Freezing was the measure of conditioned fear, defined as the absence of all movement (except breathing) in an awake animal. For each rat, observations were made every 2 s during stimulus presentations and a baseline period (2 min at the start of session), where the rat was scored as either "freezing" or "not freezing." Scoring was conducted by an observer blind to experimental condition. A proportion of observations (∼10%) were cross-scored by a second independent observer (also blind to experimental conditions) to ensure observer reliability. In all cases, there was a high degree of agreement between primary observer and cross-scorer (<90%). Overall freezing scores were expressed as a percentage of total observations and used to calculate a difference score (% freezing during stimulus presentations − % freezing during baseline) to be used in statistical analysis. Data were analysed using between-subjects univariate analysis of variance (ANOVA) with *post hoc* pairwise comparisons (Scheffe correction for multiple comparisons) where relevant. Significance was set at $\alpha = 0.05$.

RESULTS

Experiment 1: Sensory Preconditioned Fear in a Neutral vs. Appetitive Context

Experiment 1 comprised a control study based on Holmes et al. (2013), designed to demonstrate that fear of S2 at test (indexed by freezing) is a function of the learned associations between both S2 and S1, and S1 and shock, and that changing the valence of the context (neutral vs. appetitive) does not in and of itself change the basic associative processes in sensory preconditioning.

Forty-eight rats were randomly allocated to receive training in either a neutral or appetitive context. For the Appetitive condition, rats were placed on a food restriction schedule during handling. Context exposure occurred on Days 1 and 2, and for the Appetitive condition Day 2 exposure involved the delivery of food rewards throughout both sessions in order to establish the training context as a positive environment. This manipulation was shown to be successful, as indicated by a significant increase in entries to the food magazine per min from the first to the second session of context exposure with food presentation [t-test; $t_{(23)} = 3.989$, $p = 0.001$, where mean (±SD) for session 1 = 9.919 (±3.834) and session 2 = 12.892 (±4.629)]. For the Neutral condition, context exposure proceeded without any programmed events.

Rats in each condition were randomly divided into three further groups ($n = 8$; 4M and 4F) that received either paired or unpaired stimulus presentations during SPC and/or FOC sessions. In the experimental group, Group P-P (paired-paired condition), a contingent relationship was established between both S2-S1 and S1-US; rats received contingent S2-S1 presentations during the SPC session and contingent S1-Shock presentations during the FOC session. In the control groups, Group P-UP (paired-unpaired condition) and Group UP-P (unpaired-paired condition), a contingent relationship was established for only one of the stimulus pairs (either S2-S1 or S1-US). Group P-UP received paired S2-S1 presentations during the SPC session, but explicitly unpaired S1 and shock presentations during the FOC session. In reverse, Group UP-P received explicitly unpaired S2 and S1 presentations in the SPC session and paired S1-Shock presentations during the FOC session. Context extinction was carried out for all rats on Day 5, and tests of freezing to S2 and S1 were conducted on days 6 and 7, respectively.

Figure 2A shows average levels of freezing in response to S2 during the test for the Neutral context and Appetitive context conditions (stimulus-baseline; averaged across eight S2 presentations). In both Neutral and Appetitive conditions, freezing was significantly higher in Group P-P than either Group P-UP or UP-P. A two-way ANOVA with between subject factors of Group (P-P, P-UP, and UP-P) and Context (Appetitive and Neutral) revealed a significant main effect of Group ($F_{(2,42)} = 12.465$; $p < 0.001$) but no main effect of Context or Group by Context interaction (both $F < 1$). *post hoc* pairwise comparisons indicate that averaging across context, freezing was significantly higher in Groups P-P than either P-UP ($p < 0.001$) or UP-P ($p = 0.003$).

Figure 2B shows average levels of freezing to S1 during the test for the Neutral and Appetitive conditions. Freezing was higher in the P-P and UP-P groups than the P-UP groups, irrespective of context condition. A two-way ANOVA revealed a significant main effect of Group ($F_{(2,42)} = 37.126$; $p < 0.001$) but no main effect of Context or Group by Context interaction (both $F < 1$). *Post hoc* pairwise comparisons indicate that averaging across context, freezing was significantly higher in Groups P-P and UP-P compared to P-UP (both $p < 0.001$).

These results demonstrate that freezing to S2 was dependent on its contingent relationship to S1 and also the contingent relationship between S1 and shock. Furthermore, this did not differ as a function of context valence. Thus, whether SPC occurred in a Neutral or Appetitive context, fear to S2 reflected learned associations between S2 and S1, and S1 and shock, rather than being a function of an inherent ability of S1 to condition fear (which would be indicated by high responding in Group P-UP)

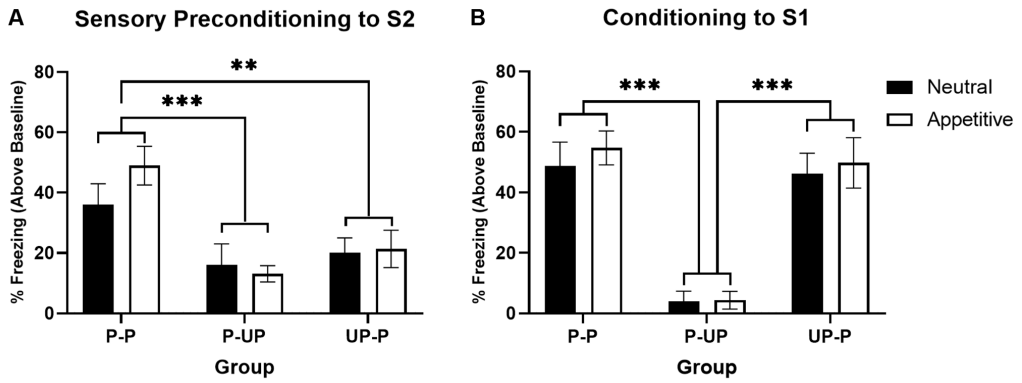

FIGURE 2 | Sensory preconditioning in a neutral vs. appetitive context. Panel **(A)** shows the average per cent time spent freezing in response to S2 (stimulus—baseline) on the test of sensory preconditioning when groups were trained in a neutral context vs. appetitive context. Groups P-P show significantly more freezing than either Groups P-UP or UP-P, irrespective of context valence. Panel **(B)** shows the average per cent time spent freezing to S1 (stimulus—baseline) on the test of first-order conditioning when groups were trained in a neutral context vs. appetitive context. Groups P-UP show significantly less freezing than either Groups P-P or UP-P, irrespective of context valence. Black bars indicate groups trained in a neutral context, while white bars indicate groups trained in an appetitive context (n = 8 for all groups). Groups P-P experienced paired S2-S1 and S1-US training; Groups P-UP experienced paired S2-S1 and unpaired S1 and US training; Groups UP-P experienced unpaired S2 and S1 and paired S1-US training. Error bars represent ±SEM. Significant comparisons are indicated by: ** where $p < 0.01$; *** where $p < 0.001$.

or of fear generalisation to S2 from S1 (which would be indicated by high responding in Group UP-P).

Experiment 2: Blocking Dopamine D1 or D2 Receptors Impairs Learning of Neutral S2-S1 Associations

The aim of Experiment 2 was to investigate dopamine's involvement in learning associations between neutral stimuli. Specifically, we aimed to determine whether systemic blockade of activity at dopamine D1R and/or D2R impaired S2-S1 learning during a sensory preconditioning procedure, as indexed by subsequent impairments in freezing to S2 at test. In addition, to further the broader aim of improving the generalisability of findings in behavioural neuroscience, male and female rats were used in this experiment and sex was assessed as a factor.

All rats underwent exposure sessions on Days 1 and 2 in a neutral context. Immediately prior to the SPC session, rats were randomly allocated to receive an injection of either saline (Group VEH), one of three doses of the D1R antagonist SCH39166 (Groups SCH-Low, SCH-Mid and SCH-High), or one of three doses of the D2R antagonist eticlopride (ETI-Low, ETI-Mid, or ETI-High). In this experiment, all rats received paired S2-S1 presentations during the SPC session on Day 3 and paired S1-shock presentations during the FOC session on Day 4. Context extinction was conducted on Day 5, and tests of S2 and S1 were carried out on Days 6 and 7, respectively.

Due to a camera malfunction, behaviour was not recorded during the test of S1 for one rat and so it was excluded from analysis. Final group sizes (N = 91) were as follows: VEH, n = 16 (8F; 8M); SCH-Low, n = 14 (7F; 7M); SCH-Mid, n = 12 (6F; 6M); SCH-High, n = 13 (6F; 7M); ETI-Low, n = 12 (6F; 6M); ETI-Mid, n = 12 (6F; 6M); ETI-High, n = 12 (6F; 6M).

Figures 3A,C show the average levels of freezing in response to S2 during the test in Female and Male rats, respectively (stimulus-baseline; averaged across eight S2 presentations). In both male and female rats, freezing to S2 was significantly higher for those in the vehicle-treated group than SCH39166- or eticlopride-treated groups. However, no dose-dependent effect was apparent for either SCH39166 or eticlopride. For illustrative purposes, **Figure 3E** shows comparisons between VEH, SCH, and ETI conditions collapsed across dose and sex.

A two-way ANOVA with between-subjects factors of Drug Type (VEH, SCH, and ETI; averaging across dose) and Sex (male and female) revealed a significant main effect of Drug Type ($F_{(2,85)}$ = 8.802, $p < 0.001$), but no main effect of Sex ($F_{(1,85)}$ = 2.160, p = 0.145) or Sex by Drug Type interaction ($F < 1$). *post hoc* pairwise comparisons indicate that averaging across sex, freezing was significantly higher in vehicle-treated rats than either SCH39166- (p = 0.040) or eticlopride-treated rats ($p < 0.001$). Follow-up two-way ANOVAs with between-subjects factors of Dose (Low, Mid, and High) and Sex (male and female) did not reveal any significant main effects of Dose or Sex, or Dose by Sex interaction in either the SCH groups (all $F < 1$) or ETI groups ($F < 1$; $F_{(1,30)}$ = 1.526, p = 0.226; $F < 1$, respectively). Together, this indicates that irrespective of dose, blockade of dopamine D1R or D2R impaired learning during sensory preconditioning.

Figures 3B,D show average levels of freezing to S1 during the test in Female and Male rats, respectively. **Figure 3F** shows comparisons between VEH, SCH, and ETI conditions collapsed across dose and sex. There were no significant drug effects, irrespective of sex. A two-way ANOVA revealed no significant main effect of Drug Type ($F < 1$), Sex ($F < 1$), nor Sex by Drug Type interaction ($F_{(2,85)}$ = 1.188, p = 0.310). This indicates that the deficits in S2 freezing observed above in drug-treated groups was not a function of impaired conditioning to S1. Together these results demonstrate that

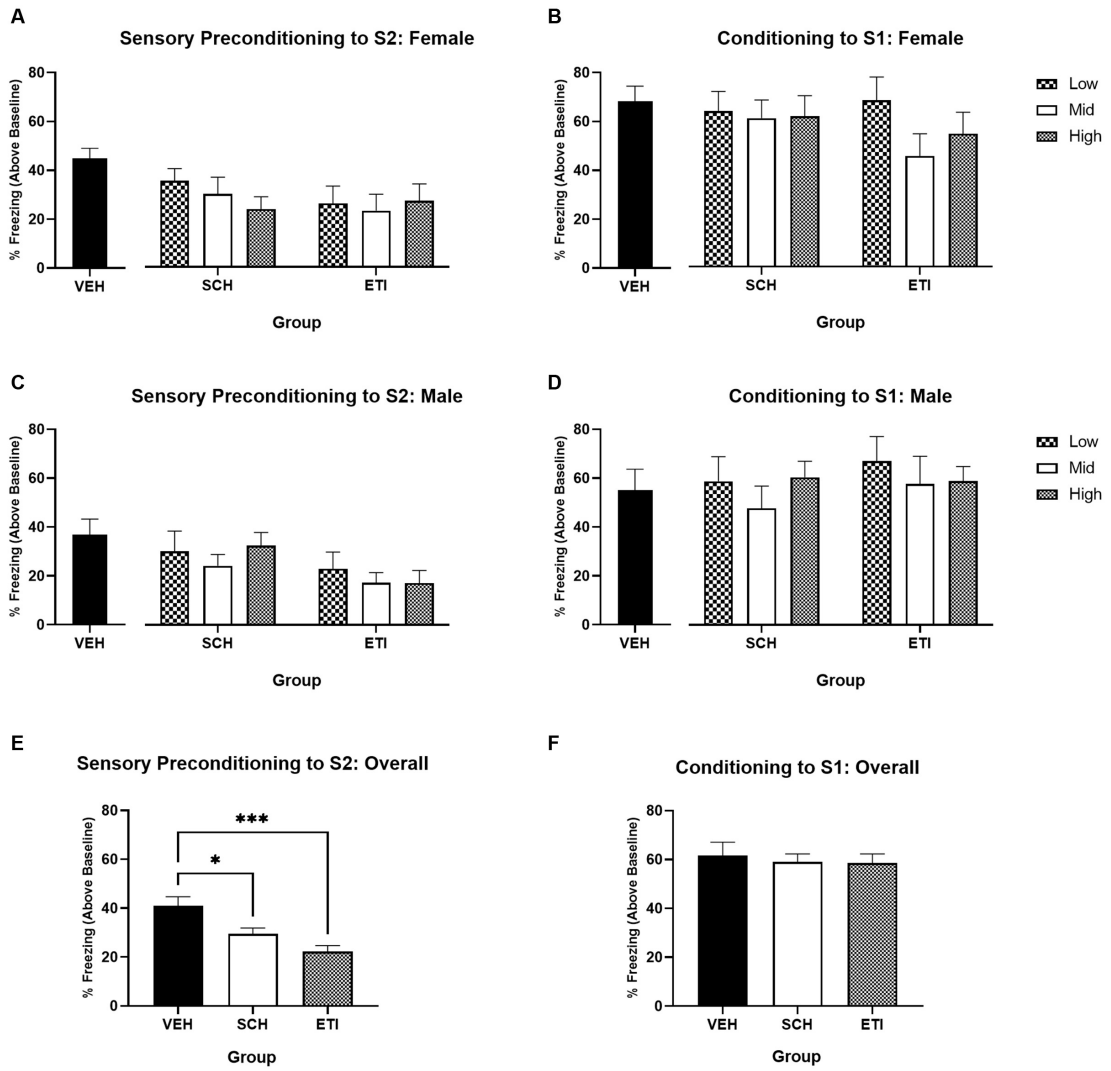

FIGURE 3 | Sensory preconditioning under conditions of dopamine receptor antagonism in male and female rats. Training in a neutral context. Panels **(A,C)** show average per cent time spent freezing in response to S2 (stimulus—baseline) on the test of sensory preconditioning in groups of female vs. male rats, respectively. Panels **(B,D)** show average per cent time spent freezing to S1 (stimulus—baseline) on the test of first-order conditioning in groups of female vs. male rats, respectively. In Panels **(A–D)**, black bars indicate treatment with saline vehicle, black and white chequered bars indicate treatment with the low-dose antagonist, white bars indicate treatment with the mid-dose antagonist, and grey hashed bars indicate treatment with the high-dose antagonist. Panels **(E,F)** show the average per cent time freezing (stimulus—baseline) during tests of S2 and S1 respectively, collapsed across dose and sex. Vehicle-treated rats (VEH; $n = 16$) froze significantly more than SCH39166- (SCH; $n = 39$) or eticlopride-treated rats (ETI; $n = 36$). Error bars represent ±SEM. Significant comparisons are indicated by: * where $p < 0.05$; *** where $p < 0.001$.

activity at dopamine D1 and D2 receptors is important for learning the association between S2 and S1, in both male and female rats.

Experiment 3: Dopamine D1 vs. D2 Receptor Involvement in Learning S2-S1 Associations in an Appetitive Context

Experiment 2 highlighted dopamine's importance in the learning of neutral associations between sensory cues, confirming and extending previous work by demonstrating the integral role of activity at both dopamine D1- and D2-receptors. However, although it is not without precedent that D1R and D2R are found to subserve similar or cooperative roles, the failure to find any evidence for differential function in dopamine receptor subtypes in this context was somewhat surprising given the existing literature. Specifically, the hypothesis that it is D1R, but not D2R, that are particularly implicated in the formation of conditioned associations. Accordingly, it is of interest to further investigate the conditions under which D1R and D2R are recruited for learning.

The aim of Experiment 3 was to assess whether differences in D1R vs. D2R involvement may be observed if otherwise neutral S2-S1 learning took place in a motivationally significant environment. The rationale behind this is two-fold. Firstly, most of the related background literature examines dopamine's role in reward-learning (e.g., appetitive Pavlovian conditioning and reinforcement learning), which always takes place in an appetitive context. Together with Experiment 2, this study will provide a valuable comparison that may be able to shed more light on the precise function(s) of dopamine in the more typical appetitive conditioning preparations.

Secondly, there is existing evidence to indicate that the neural structures and mechanisms recruited for innocuous stimulus-stimulus learning are influenced by the nature of the context in which that learning takes place (Holmes et al., 2013, 2018; Holmes and Westbrook, 2017). For the more general purpose of enhancing our understanding of the underlying processes involved in sensory preconditioning, it is of interest to assess whether the role of dopamine in S2-S1 learning is similarly sensitive to the motivational significance of the context.

Since no differential effects of dose (for either SCH39166 or eticlopride) were observed in Experiment 2, only the mid dose of each antagonist was used in this study (0.025 mg/kg and 0.0125 mg/kg, respectively). Furthermore, since there was no evidence from Experiment 2 that S2-S1 learning, or the impact of dopamine antagonism on S2-S1 learning, differed as a function of sex, male and female rats in this experiment were grouped together.

All rats were placed on a food restriction schedule during handling, and context exposure occurred on Days 1 and 2. Day 2 involved exposure to food rewards throughout both sessions in order to establish the training context as a positive environment. There was a significant increase in entries to the food magazine per min from the first to the second session of context exposure with food presentation [*t*-test; $t_{(35)} = 4.320$, $p < 0.001$, where mean (±SD) for session 1 = 10.848 (±3.551) and session 2 = 14.070 (±4.603)], indicating that the manipulation was successful. Immediately prior to the SPC session, rats were randomly allocated to receive an injection of either saline (Group VEH), SCH39166 (Group SCH), or eticlopride (Group ETI). In this experiment, all rats received paired S2-S1 presentations during the SPC session on Day 3 and paired S1-shock presentations during the FOC session on Day 4. Context extinction was conducted on Day 5, and tests of S2 and S1 were carried out on Days 6 and 7, respectively. One rat did not receive a shock during the FOC session and was excluded from the analysis. Final group sizes ($N = 35$) were as follows: Group VEH, $n = 11$ (5F; 6M); Group SCH, $n = 12$ (6F; 6M); Group ETI, $n = 12$ (6F; 6M).

Figure 4A shows the average levels of freezing in response to S2 at the test (stimulus-baseline; averaged across eight S2 presentations). Freezing was significantly impaired in both drug-treated groups relative to the saline-treated group. A one-way univariate ANOVA revealed a significant effect of Drug Type (Saline, SCH, and ETI; $F_{(2,32)} = 5.957$, $p = 0.006$), with *post hoc* pairwise comparisons confirming that freezing in Group Saline was significantly higher than in Group SCH ($p = 0.035$) or Group ETI ($p = 0.011$). Thus, when exposure occurs in an appetitive context, dopamine antagonism at either D1R or D2R impairs learning of the S2-S1 association.

Figure 4B shows the average levels of freezing in response to S1 at test. There was no evidence to suggest responses differed between groups, with a one-way univariate ANOVA revealing no significant effect of Drug Type (Saline, SCH, and ETI; $F_{(2,32)} = 1.287$, $p = 0.290$). This shows that the deficits in S2 freezing observed in drug-treated groups was not a function of impaired conditioning to S1. These results complement those of Experiment 2, demonstrating that when sensory preconditioning occurs in a motivationally attractive context (just as for when it occurs in a neutral context) activity at dopamine D1 and D2 receptors is important for learning an association between S2 and S1.

Experiment 4: Blocking Dopamine D1 and D2 Receptors Together Does Not Appear to Impact Learning of S2-S1 Associations

Results of Experiment 3 further confirm those of Experiment 2, demonstrating that both D1R and D2R activity is important in the learning of neutral-stimulus S2-S1 associations. Together with Experiment 2, the findings of Experiment 3 also suggest that the involvement of D1R and D2R in S2-S1 learning does not appear to differ as a function of the motivational significance of the context in which learning occurs, although this cannot be directly compared across the two experiments. The aim of Experiment 4 was to assess whether the detrimental effects of dopamine antagonism on S2-S1 conditioning are additive when D1- and D2-receptors are blocked together, as well as to provide a direct comparison of these effects when learning takes place in a motivationally neutral vs. motivationally attractive context.

Fifty-six male rats were randomly allocated to receive training in either a neutral or appetitive context. For the appetitive condition, rats were placed on a food restriction schedule during handling and context exposure occurred on Days 1 and 2. Day 2 involved exposure to food rewards throughout both sessions. Magazine entry data was unfortunately recorded incorrectly for eight of 24 rats in the appetitive condition. In the remaining 16, there was a significant increase in entries to the food magazine per min from the first to the second session of context exposure with food presentation [*t*-test; $t_{(15)} = 4.695$, $p < 0.001$, where mean (±SD) for session 1 = 8.400 (±3.493) and session 2 = 11.400 (±2.650)], which is at least suggestive that the manipulation was successful overall. For the Neutral condition, context exposure proceeded without any programmed events.

Rats in each condition were further randomly allocated to receive an injection of either saline (Groups Neutral-VEH and Appetitive-VEH) or α-flupenthixol (Groups Neutral-FLU and Appetitive-FLU) immediately prior to the SPC session. In this experiment, all rats received paired S2-S1 presentations during the SPC session on Day 3 and paired S1-shock presentations during the FOC session on Day 4. Context extinction was conducted on Day 5, and tests of S2 and S1 were carried out on Days 6 and 7, respectively. Final group sizes were as follows: Group Neutral-VEH, $n = 16$; Group Neutral-FLU, $n = 16$;

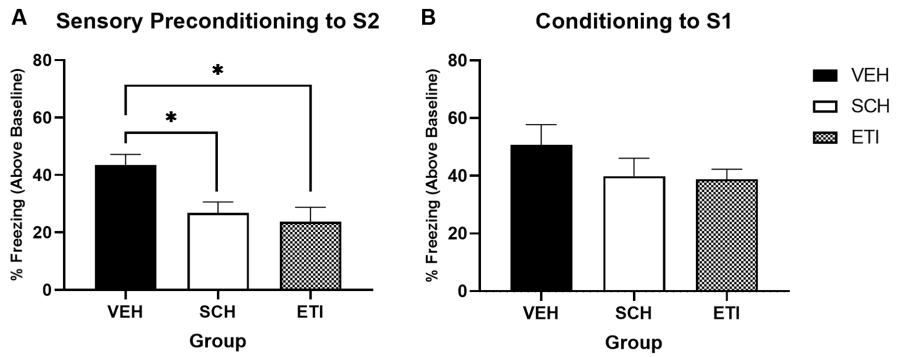

FIGURE 4 | Sensory preconditioning under conditions of dopamine receptor antagonism when trained in an appetitive context. Panel **(A)** shows the average per cent time spent freezing in response to S2 (stimulus—baseline) on the test of sensory preconditioning. Freezing was significantly higher in vehicle-treated rats (VEH; $n = 11$) compared to either SCH39166- (SCH; $n = 12$) or eticlopride-treated rats (ETI; $n = 12$). Panel **(B)** shows the average per cent time spent freezing to S1 (stimulus—baseline) on the test of first-order conditioning. Black bars indicate treatment with saline vehicle, white bars indicate treatment with (mid-dose) D1R antagonist SCH39166, and grey hashed bars indicate treatment with (mid-dose) D2R antagonist eticlopride. Error bars represent ±SEM. Significant comparisons are indicated by: * where $p < 0.05$.

Group Appetitive-VEH, $n = 12$; Group Appetitive-FLU, $n = 12$. The neutral condition was slightly overpowered as previous experiments suggest there is more variance in the data when animals are trained in a neutral vs. appetitive context.

Figure 5A shows average levels of freezing in response to S2 at test (stimulus-baseline; averaged across eight S2 presentations) for groups that were training in a neutral vs. an appetitive context. No groups differences in levels of freezing to S2 were observed. Despite numerically higher levels of freezing in the α-flupenthixol-treated group trained in the appetitive context, a two-way univariate ANOVA revealed no main effect of Drug Type (VEH vs. FLU; $F < 1$) or Context (Neutral vs. Appetitive; $F_{(1,52)} = 1.138$, $p = 0.291$), or Drug Type by Context interaction ($F < 1$).

Figure 5B shows the average levels of freezing in response to S1 at the test for groups that were training in a neutral vs. an appetitive context. There was no evidence to suggest responses differed between groups, with a two-way univariate ANOVA revealing no significant effect of Drug Type (Saline vs. FLU; $F_{(1,52)} = 1.292$, $p = 0.261$), Context (Neutral vs. Appetitive; $F < 1$) or Drug Type by Context interaction ($F < 1$).

Thus, there is no evidence in this experiment to suggest that non-selective dopamine antagonism has any impact on learning S2-S1 associations, irrespective of the motivational significance of the context. This stands in explicit contrast to the findings of both Experiments 2 and 3 above, which demonstrate when either D1- or D2-receptors are blocked independently (in a neutral or appetitive context), S2-S1 learning is impaired.

DISCUSSION

In a preliminary set of experiments, we first established a sensory preconditioning procedure modelled on that used by Holmes and colleagues (e.g., Holmes et al., 2013). This procedure produced reliable sensory preconditioning that depended upon both the pairing of sensory cues S2 and S1 and the subsequent pairing of S1 with a mild foot-shock US. The magnitude of the sensory preconditioning effect was similar regardless of whether the conditioning context was neutral, or had previously been paired with a non-contingent appetitive outcome. These experiments provide a baseline for the investigation of the role of dopaminergic receptors in the development of sensory preconditioning.

We then examined the impact of dopamine D1R (SCH39166) and D2R (eticlopride) selective antagonists on the development of sensory preconditioning (in an affectively neutral context), when administered immediately prior to the critical SPC phase in which neutral cues S2 and S1 were paired. Primarily, we found that treatment with either the D1R antagonist or the D2R antagonist decreased the level of S2-S1 learning, as observed in the subsequent test session. In both instances, the degree of sensory preconditioning observed in drug-treated animals, as indexed by conditioned freezing to S2, was significantly lower than that seen in vehicle-injected control animals (and numerically lower than the levels of sensory preconditioning seen in the initial behavioural studies). Thus, both D1R and D2R activity appears important for learning an association between two innocuous sensory cues.

Experiment 2 also presented several other findings of note. In neither instance (D1R or D2R) did we find an effect of dopamine receptor antagonism on the level of conditioned freezing observed in the presence of the directly trained cue, S1; that is, conditioned freezing to this cue at test did not differ between drug-treated groups and vehicle-injected control groups, and reflected the relatively high level of freezing expected in a cue paired directly with mild foot-shock. This is unsurprising given the antagonists were administered only prior to S2-S1 pairings, but nevertheless provides evidence that the observed deficit in freezing to S2 was not secondary to some long-lasting impact of the drug-treatment on first-order conditioning.

We also did not observe any effect of the sex of the subjects on the level of sensory preconditioning or the level

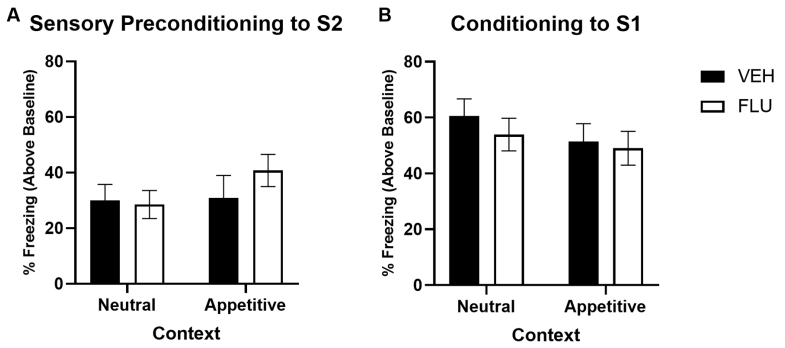

FIGURE 5 | Sensory preconditioning under conditions of non-selective dopamine receptor antagonism when trained in a neutral vs. appetitive context. Panel **(A)** shows the average per cent time spent freezing in response to S2 (stimulus—baseline) on the test of sensory preconditioning for groups in the Neutral vs. Appetitive context condition. Panel **(B)** shows the average per cent time spent freezing to S1 (stimulus—baseline) on the test of first-order conditioning for groups in the Neutral vs. Appetitive context condition. Black bars indicate groups treated with saline vehicle (VEH; Neutral condition $n = 16$, Appetitive condition $n = 12$), and white bars indicate groups treated with α-flupenthixol (FLU; Neutral condition $n = 16$; Appetitive condition $n = 12$). Error bars represent ±SEM.

of first-order conditioning, and there were also no observed interactions between sex and the impact of dopaminergic receptor antagonism on these measures. Therefore, we did not find any evidence of differences in sensory preconditioning *per se*, nor in the relative importance of D1 and D2 dopamine receptors in sensory preconditioning, between male and female rats.

Thirdly, we did not find any evidence of a relationship between the dose of dopamine receptor antagonist administered and the degree of deficit observed in sensory preconditioning. Overall, low, middle and higher doses of either SCH39166 or eticlopride produced equivalent deficits in sensory preconditioning, and the magnitude of the deficit also did not significantly differ between D1R and D2R antagonism. The doses selected for SCH39166 and eticlopride were selected on the basis of several previous experiments, both in our lab and elsewhere, where dose-response effects have been observed (e.g., Nelson and Killcross, 2013; Hosking et al., 2015; Roughley, 2017; Roughley and Killcross, 2019) This suggests that dopamine D1R or D2R function is important in sensory preconditioning, but may fulfil a basic permissive role, rather than one governing level of stimulus-stimulus learning.

We would also note that sensory preconditioning was reduced by dopamine D1R or D2R antagonism to levels numerically similar to those seen in Groups P-UP and UP-P in Experiment 1 (though these cannot be directly compared). As such, it appears that the levels of sensory preconditioning observed following D1R and D2R antagonism are reduced to levels seen in groups in which S2 and S1 pairings (or S1-US pairings) had not occurred. Whilst this does not necessarily indicate that sensory preconditioning was completely abolished, it was reduced to a level which would not have been detectable compared to unpaired groups. As such, residual levels of freezing to S2 in the case of D1R and D2R antagonist-treated groups could well be unrelated to the formation of S2-S1 associations and may be due to non-associative processes such as stimulus generalisation at test (between S1 and S2), or cue-related priming of residual contextual fear. However, further experiments with appropriate within-experiment controls would be needed to fully explore these possibilities.

In a third experiment, we examined the impact of D1R or D2R antagonism on the formation of S2-S1 associations in a sensory preconditioning study conducted in a context which had been rendered affectively appetitive by prior presentations of food reward in that context. We did this to examine a number of issues. First, whilst we found no evidence for differential involvement of D1R and D2R in sensory preconditioning in our initial study, previous work has suggested that D1- and D2-related systems may be involved in dissociable aspects of learning about rewards; for example, it has been proposed that D1R systems may be more selectively involved in the encoding of prediction error in learning, whereas D2R systems may be more involved in the assignment of motivational significance to cues paired with reward (Roughley and Killcross, 2019). Second, previous work (Holmes et al., 2013, 2018; Holmes and Westbrook, 2017) has indicated that the neural bases of sensory preconditioning may vary depending on the motivational significance of the environment in which neutral S2-S1 pairings occur. In short, however, we failed to observe any influence of the motivational status of the training context on the impairment in sensory preconditioning observed following dopamine receptor antagonism. Replicating the previous studies, both D1R and D2R antagonism produced significant deficits in freezing to S2 to at test, whilst no deficits were seen in freezing to the directly trained cue S1.

In the final experiment, we sought to extend these findings in two ways. First, we sought to examine whether deficits in sensory preconditioning following D1R or D2R blockade were enhanced when both D1R and D2R antagonism was imposed simultaneously, following treatment with the non-selective dopamine D1R/D2R antagonist α-flupenthixol. Second, we sought to further examine the potential that the motivational status of the learning environment might have an impact under this increased breadth of dopamine receptor antagonism. Hence, we examined the impact of treatment with α-flupenthixol prior to sensory preconditioning S2-S1 pairings in either a neutral or

appetitive learning environment. Surprisingly, though the dose of α-flupenthixol used was known to be behaviourally effective (see below), we found that combined D1R/D2R antagonism failed to produce deficits in freezing to S2 (or S1) in either a neutral or appetitive learning environment. Potential explanations for this finding are discussed below, including the possibility that at these doses the different antagonists have distinct loci of action, or that perhaps performance relies less on absolute levels of activity at D1/D2 receptors, but rather the balance of activity between the two.

In summary, we employed a procedure for sensory preconditioning in which we demonstrated that the pairing of neutral cues S2 and S1 is required for the development of conditioned responding to S2 following S1-US pairings. This sensory preconditioning effect was markedly reduced following administration of dopamine D1R or D2R antagonists prior to S2-S1 pairings. This effect did not vary across male and female rats and was uninfluenced by the motivational status of the training environment. Unexpectedly, these deficits in sensory preconditioning were not seen following administration of the D1/D2R antagonist α-flupenthixol at an otherwise effective dose.

The overall aim of this set of experiments was to use the sensory preconditioning protocol to examine dopaminergic involvement in associative learning processes (e.g., prediction error) whilst controlling for the motivational processes that typically accompany learning in a reward setting (and are also believed to be dopamine-dependent). Broadly speaking, these data confirm and extend previous findings indicating a role for dopamine systems that goes beyond reward prediction error (Sharpe et al., 2017; Gardner et al., 2018). Whilst the evidence for a role for dopamine in reward prediction error is strong, there is also mounting evidence that the role of dopamine extends to learning about stimulus-related prediction errors, as well as aversive events. This adds to the basic initial observation that midbrain dopamine neurons fire strongly to unanticipated sensory cues (Ljungberg et al., 1992; Schultz and Dickinson, 2000). For example, Sharpe et al. (2017) have demonstrated that dopamine transients are sufficient and necessary for the formation of stimulus-stimulus associations, using a version of a sensory preconditioning task. This has led some to suggest a role for dopamine in subserving a generalised prediction error term that signals errors in both sensory and reward predictions (Suri, 2001; Gershman, 2017; Gardner et al., 2018).

Our findings are in line with these models in demonstrating a role for dopaminergic systems in stimulus-stimulus learning. However, rather than concentrating on the role of dopaminergic signalling of error terms, our experiments also demonstrate the downstream role of dopamine binding to D1R and D2R. Our initial experiments suggest that antagonism of either D1R or D2R is capable of attenuating the formation of S2-S1 associations. This constitutes an important next step in identifying the neural pathways recruited for this learning and thereby being able to determine commonalities and differences with reward-based learning that can inform our understanding of the basic mechanisms underpinning both. For example, the absence of any dose-response curve in the effects of systemic D1R or D2R antagonism perhaps suggests a permissive, rather than graded, role—a possibility that invites further experimentation in the future. Although higher systemic doses of these antagonists have general motor and arousal effects that would preclude a clear interpretation of findings, other approaches are possible, including localised receptor inactivation by the antagonist and chemogenetic or optogenetic inhibition.

That the independent role of D1R and D2R appear similar in this task is in contrast to some findings where opposing effects of D1R and D2R manipulation are seen (Eyny and Horvitz, 2003; Yue et al., 2004; Nelson and Killcross, 2013), but there is also substantial evidence where the impact of D1 and D2 systems has been shown to be complementary (Ikemoto et al., 1997; Smith et al., 1997; Capper-Loup et al., 2002; Iordanova et al., 2006; Cerri et al., 2014; Perreault et al., 2014). The effect we have reported here falls into this latter category, and also provides a fruitful opportunity for further investigation. For example, our findings may suggest an impact of these systemic treatments in areas of the brain (such as the nucleus accumbens) where D1R and D2R systems are less clearly in opposition than has been reported in other areas (e.g., dorsal striatum; Gerfen and Surmeier, 2011; Kravitz et al., 2012; Kupchik et al., 2015). Future experiments employing anatomically specific dopamine receptor manipulations to identify the neural circuitry involved will be important for describing the precise nature of the functions performed by D1R and D2R in this context.

A further extension of previous work is the present finding that the importance of D1R and D2R in sensory preconditioning does not appear to differ as a function of the motivational status of the learning environment. One limitation of investigating dopamine's role in the processes of learning associative relationships has been the fact that this research has primarily been conducted using reward-based learning procedures in which the learning context is established as attractive prior to learning as a typical part of an experimental protocol (i.e., pre-training involves several sessions of exposure to reward in the training context to familiarise the animal with both before training begins). Although research has demonstrated that the motivational significance of the training environment can have a significant impact on the neural mechanisms recruited for learning (Holmes et al., 2013, 2018), this has not been explicitly examined or controlled for in the context of dopamine despite established effects of dopaminergic systems in appetitive contextual conditioning (Spyraki et al., 1982a,b; Beninger and Hahn, 1983; Spyraki et al., 1987). In doing so, the present findings further confirm not only that dopamine is important for learning relationships between neutral stimuli, but that this is equally true in a familiar but neutral environment as it is in an attractive one.

Our assumption that the training context was rendered attractive is evidenced by the observation that entries into the food magazine increased with exposure to food within the context. However, one could argue that this was not a particularly strong manipulation and that it only addresses the appetitive,

not aversive, domain. Whilst this is true, and more powerful (appetitive or aversive) manipulations could be employed, the present manipulation was designed to be in keeping with procedures typically employed in experiments of reward-based learning.

Intriguingly, despite having found clear evidence for independent effects of D1R and D2R antagonism across two experiments, we failed to observe any impact of combined D1/D2R antagonism on sensory preconditioning. Firstly, this is unlikely to be because the antagonist was simply without effect. The dose of the α-flupenthixol (0.5 mg/kg) was the same as (or higher than) doses used in our lab and elsewhere, which have established impacts on learning and performance (Killcross et al., 1994; Dickinson et al., 2000; Dunn and Killcross, 2007). Further, we used the same dose from the same batch of α-flupenthixol to examine the impact of D1R/D2R antagonism on appetitive Pavlovian conditioned responding (something we have replicated several times in our lab). Here we found that 0.5 mg/kg α-flupenthixol, administered prior to an appetitive conditioning session in a manner identical to the administration prior to S2-S1 pairings in Experiment 4, drastically reduced the level of appetitive conditioned responding relative to control animals [magazine entries per min; *t*-test, $t_{(14)} = 7.210$, $p < 0.001$, where mean (±SD) for saline-treated rats = 11.767 (±1.309) and α-flupenthixol-treated rats = 4.213 (±2.660)]. There are also numerous examples (again, from our lab and elsewhere) where this dose of α-flupenthixol has been shown to be capable of mimicking the impact of more selective dopamine D1R and D2R antagonists at the doses used in our earlier experiments (e.g., Roughley and Killcross, 2019).

Accordingly, we have good reason, both within this series of studies and from others, to believe this dose of α-flupenthixol would have been effective. And whilst we would note caution around drawing strong conclusions on the basis of a null result finding, the failure to find an effect of combined D1/D2 antagonism does stand in contrast to the findings of the previous experiments, following the same methodology, in which clear effects of D1R and D2R antagonism alone were observed. Future studies may benefit from a within-experiment comparison of the effects of selective vs. non-selective D1R/D2R antagonists. Nevertheless, if we take this result at face value, there would be few studies that fail to find similar effects of combined D1/D2R antagonism when both D1R and D2R antagonism has been shown to be effective. Although speculative, there are some potential routes for future investigation outlined below.

One possibility might lie in the locus of action of the selective D1R and D2R antagonists, and the combined antagonist, particularly at the doses used in these experiments. A more discriminatory impact on inhibitory D2 autoreceptors (as opposed to post-synaptic D2R) could, for example, play a role if these proved to be differentially sensitive to selective and non-selective dopamine receptor antagonists. Similarly, given what we know about other neural substrates of sensory preconditioning (Ward-Robinson et al., 2001; Coutureau et al., 2002; Holmes et al., 2018; Fournier et al., 2021; Kahnt and Schoenbaum, 2021), as well as the different roles of dopamine in striatal regions (e.g., Young et al., 1998; Li and McNally, 2015; Yee et al., 2020), there could be a more complicated interplay of dopaminergic involvement than can be teased out with systemic drug administration studies. Accordingly, central administration studies will be needed to help clarify this in the future.

Given the potential that functioning of D1R and D2R systems in attenuating sensory preconditioning appears to operate as a logical NAND gate, then another possibility to explain the differential impact of selective vs. non-selective dopamine antagonists is that it might be the relative balance of D1R and D2R activity that is critical. That is, it may be that blocking either D1R or D2R activity and disrupting the relative balance between the two results in impaired sensory preconditioning. In contrast, blocking both D1R and D2R together reduces dopamine signalling overall, but preserves the relative balance of D1R and D2R activity and allows sensory preconditioning to proceed unimpaired. This notion is not without precedent. For example, Furlong et al. (2017) demonstrated that methamphetamine sensitisation reduced activity in D1R-expressing direct pathway neurons in the dorsomedial striatum (relative to D2R-expressing indirect pathway neurons), and that behavioural deficits observed following this impact could be reduced by pre-test administration of the adenosine 2A receptor antagonist ZM241385 into the dorsomedial striatum to reduce activity in D2R-expressing neurons. They hypothesised that this additional reduction in activity restored the balance of D1- and D2-related activity in the striatum and hence restored normal behavioural control. It is also the case that the potential heterogeneity of dopamine responses has been highlighted by recent theoretical models that seek to broaden the role of dopamine beyond reward prediction error (Suri, 2001; Gardner et al., 2018). Additional experiments, for example investigating sensory preconditioning under conditions of D1R and D2R agonism, would be needed to address this possibility.

In summary, both dopamine D1R and D2R activity appear to be independently important for sensory preconditioning in a manner which is also independent of the motivational status of the learning environment. This confirms and extends previous findings indicating a role for dopamine signalling beyond reward prediction error, and lays the groundwork for further investigation of the downstream mechanisms supporting this function in dopamine systems. Moreover, these findings underscore the utility of the sensory preconditioning procedure as a contrast to more typical reward-based learning procedures in further delineating the range of dopaminergic function in learning. However, the potential that combined dopamine D1R and D2R blockade does not impact sensory preconditioning further highlights the complexity of signalling in dopaminergic systems in relation to learning. In decoding this complexity, future studies are well-placed to employ neuroanatomically targeted approaches to identify the brain regions in which D1R and D2R activity exerts an influence in sensory preconditioning and undertake more direct and controlled manipulations, for example in the balance of activity in D1R- and D2R-expressing neural populations. Furthering our understanding of dopamine function in this manner has important clinical implications

for our understanding of psychological disorders involving dysfunctional associative and motivational processes, such as in addiction, schizophrenia, depression, and ADHD.

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 UNSW Animal Care and Ethics Committee.

AUTHOR CONTRIBUTIONS

SR and SK conceived and designed the experiments. SR and AM collected and analysed the data. SR and SK interpreted the data and wrote the manuscript, with input from AM. All authors contributed to the article and approved the submitted version.

FUNDING

Some of the experiments presented here were funded by ARC Discovery Grant DP170103952 to SK.

ACKNOWLEDGEMENTS

We thank Alexandra Ross for her contribution to data collection.

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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.

frontiers in Behavioral Neuroscience

REVIEW
published: 09 September 2021
doi: 10.3389/fnbeh.2021.726218

Higher-Order Conditioning: What Is Learnt and How it Is Expressed

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Pairing a neutral conditioned stimulus (CS) with a motivationally significant unconditioned stimulus (US) results in the CS coming to elicit conditioned responses (CRs). The widespread significance and translational value of Pavlovian conditioning are increased by the fact that pairing two neutral CSs (A and X) enables conditioning with X to affect behavior to A. There are two traditional informal accounts of such higher-order conditioning, which build on more formal associative analyses of Pavlovian conditioning. But, higher-order conditioning and Pavlovian conditioning have characteristics that are beyond these accounts: Notably, the two are influenced in different ways by the same experimental manipulations, and both generate conditioned responses that do not reflect the US *per se*. Here, we present a formal analysis that sought to address these characteristics.

Keywords: association, behavior, Pavlovian conditioning, similarity, timing

OPEN ACCESS

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

Received: *16 June 2021*
Accepted: *19 July 2021*
Published: *09 September 2021*

Citation:
Honey RC and Dwyer DM (2021) Higher-Order Conditioning: What Is Learnt and How it Is Expressed. Front. Behav. Neurosci. 15:726218. doi: 10.3389/fnbeh.2021.726218

INTRODUCTION

Pavlov observed that dogs given pairings of light with food came to salivate during the light, but also during a tone that was later paired with the light. In his terms, the light (a conditioned stimulus, CS) had become a substitute for food (an unconditioned stimulus, US), evidenced both through the capacity of the light to elicit salivation (the conditioned response, CR) and to support a "*reflex of the second order*" to the tone. In fact, Pavlov described such second-order CRs as "*in most cases very weak*," indicating that there were substantial individual differences in their size and transience (Pavlov, 1927; pp. 104–105). We will return to the important issue of individual differences towards the end of this article. For now, it is sufficient to note that second-order conditioning is a well-established phenomenon across a range of preparations (e.g., *appetitive conditioning*: Rashotte et al., 1977; *aversive conditioning*: Rizley and Rescorla, 1972; *sexual conditioning*: Crawford and Domjan, 1995), and so too is another example of higher-order conditioning, sensory preconditioning (e.g., *appetitive conditioning*: Allman and Honey, 2006; *aversive conditioning*: Brogden, 1939; *flavor-aversion learning*: Rescorla and Cunningham, 1978). For sensory preconditioning, the tone and light in the opening example are paired before the light is conditioned, whereupon the tone also elicits conditioned responding (see **Table 1**).

Higher-order conditioning procedures have become a popular means of examining the neurobiology of learning and memory (for a review, see Gewirtz and Davis, 2000; see also, e.g., Lin and Honey, 2011; Gilboa et al., 2014; Holland, 2016; Lin et al., 2016; Lay et al., 2018; Maes et al., 2020; Mollick et al., 2020). This popularity reflects the relevance of higher-order conditioning to clinical domains (e.g., Davey and Arulampalan, 1982; Davey and McKenna, 1983; Wessa and Flor, 2007; see also, Field, 2006; Haselgrove and Hogarth, 2011), but also the practical advantages of the procedures, and the potential insights that their use enables: The procedures allow the complex

TABLE 1 | Higher-order conditioning procedures.

	Stage 1	Stage 2	Test
Second-order conditioning:	X→US	A→X	A?
Sensory preconditioning:	A→X	X→US	A?

Note: A and X are conditioned stimuli and the US denotes an unconditioned stimulus.

effects generated by the presentation of a motivationally significant US, on X→US trials, to be separated from the associative processes operating on A→X trials; and they also allow the nature of different acquisition and performance processes to be separately probed. But, what is learned during higher-order conditioning and how is that learning expressed? These two related questions have not been addressed in an integrated fashion by traditional accounts of higher-order conditioning. In fact, a recent critical review of evidence relating to these accounts suggested that they leave many important issues unresolved, which motivated the development of a new computational model of higher-order conditioning (Honey and Dwyer, under review). This model was built on a recent analysis of Pavlovian conditioning and performance: HeiDI (Honey et al., 2020a). Here, we first present a synthesis of extant informal accounts of higher-order conditioning together with the evidence that they fail to address, before presenting the new computational model of higher-order conditioning.

TRADITIONAL ACCOUNTS OF HIGHER-ORDER CONDITIONING

Mackintosh (1974; pp. 85–91; see also Gewirtz and Davis, 2000) identified two accounts of higher-order conditioning that have enjoyed an enduring appeal. One is closely aligned to conventional accounts of Pavlovian conditioning, wherein an association is held to form between the CS representation and either the US representation (i.e., a stimulus-stimulus association) or the processes responsible for responses that it generates (i.e., a stimulus-response association). For higher-order conditioning, it has been argued that an association forms between stimulus A and the US (or processes involved in generating the CR) through a process of representation mediated learning. Thus, for second-order conditioning, the X→US trials might allow A to become linked to the representation of the US that is retrieved by X on A→X trials (Konorski, 1948, p. 68) or to processes more directly responsible for the CR to X (Pavlov, 1927, p. 105; Rizley and Rescorla, 1972). Whereas for sensory preconditioning, the A→X trials might allow the representation of A retrieved by X on X→US trials to be linked to the US (e.g., Ward-Robinson and Hall, 1996, Ward-Robinson and Hall, 1998; see also, Holland, 1981; Hall, 1996; Iordanova et al., 2011). Accounts based upon representation mediated learning are often contrasted with the simpler possibility that a (directional) associative chain underpins higher-order conditioning (e.g., Gewirtz and Davis, 2000). Here, X→US pairings allow an association to form between representations of X and the US, or those processes responsible for the UR, while A→X pairings enable an association to develop between representations of A and X. The efficacy of the associative chains, A→X→US or A→X→UR, will then determine the propensity for A to elicit conditioned responding. However, the accounts described above and depicted in **Figure 1** are challenged by the conditions under which higher-order conditioning is observed and how it is evident in behavior.

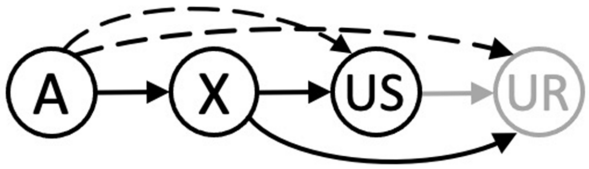

FIGURE 1 | An integrated schematic for higher-order conditioning: The associative chains (black continuous arrows) and retrieval mediated associations (black dashed arrows) resulting from separate A→X and X→US trials. The gray solid arrow is an (unconditioned) link between the US its UR. The A, X, and US nodes are held to be activated by their corresponding stimuli, with the UR generated by the presentation of the US.

A SYNTHESIS OF UNRESOLVED ISSUES

The Conditions Under Which Higher-Order Conditioning Is Observed

When there is a trace interval between a CS and US (i.e., X→trace→US), conditioned responding during the CS is normally less evident than when there is no interval (see Mackintosh, 1983, pp. 86–89). The accounts of higher-order conditioning outlined above seem constrained to predict that when there is a trace interval between X and the US the CR to A should also be less marked: X→trace→US trials will be an ineffective basis for X to retrieve the US (or evoke the UR) on A→X trials in second-order conditioning procedures, and X→trace→US will be an ineffective vehicle for the retrieved representation of A to become linked to the US in sensory preconditioning procedures. Similarly, the final X→US or X→UR link in any associative chain will be less effective (in both procedures) after X→trace→US trials. However, trace conditioning with X enhances conditioned responding to A in both sensory preconditioning (Ward-Robinson and Hall, 1998; Lin and Honey, 2011; see also, Kamil, 1969) and second-order conditioning procedures (Lin and Honey, 2011; see also, Cole et al., 1995; Barnet and Miller, 1996). Another simple observation is similarly problematic: Extinguishing first-order conditioned responding to X, before test trials with A, does not (always) reduce the capacity of A to generate responding in sensory preconditioning (Ward-Robinson and Hall, 1996) or second-order conditioning procedures (e.g., Rizley and Rescorla, 1972; Cheatle and Rudy, 1978; Amiro and Bitterman, 1980; Nairne and Rescorla, 1981; Archer and Sjödén, 1982; but see Rescorla, 1982). These results are inconsistent with an associative chain account to the extent that the efficacy of the final link in the chain should have been reduced by extinguishing X, and they have been taken to support the view that A has an association with the US (or its UR) that is independent of the association of X with the US (or its UR). A final intriguing observation about

sensory preconditioning is that when A is presented together with X during the test, the resulting AX compound provokes more conditioned responding than when X is either presented alone or with a control stimulus (e.g., Ward-Robinson et al., 2001; Lin et al., 2013). By default, and ignoring the results from the trace conditioning procedure, these results have been taken to support a retrieval mediated learning account since it supposes that A has a basis to elicit conditioning responding independently of X. However, these results could also reflect the fact that the directly activated representation of a stimulus (X), and its trace or retrieved representations (X*; see Lin and Honey, 2011, 2016; Lin et al., 2013) can be discriminated from one another, and enter into separate associations that affect performance in distinct ways (Lin and Honey, 2010). For example, enhanced higher-order conditioning with trace conditioning could reflect the fact that the representation of X that is retrieved by A is more similar to the representation of X that enters into association with the US during trace conditioning than during standard conditioning. Also, whether the extinction of X does or does not affect responding to A could be determined by the similarity of the representation of X retrieved by A during the test to the representation of X that was subject to extinction (see Rescorla, 1982). Later, we will develop a more formal analysis of this suggestion, which relies on representations of X, its trace and retrieved forms being dynamically coded in terms of the dimension of perceived intensity, and forming part of what is learned about a given stimulus.

How Higher-Order Conditioning Is Evident in Behavior

Higher-order conditioning procedures include two types of trial, A→X and X→US, and there has been an understandable focus on how X→US trials enable responding to A. However, A→X trials can—in and of themselves—generate behavior. For example, when an auditory stimulus is paired with a localized visual stimulus (i.e., A→X), A comes to elicit an orienting response that reflects the location in which X is presented (e.g., Honey et al., 1998a,b; see also, Narbutovich and Podkopayev, 1936; cited in Konorski, 1948, p. 91; Silva et al., 2019). Any complete analysis of higher-order conditioning needs to address the fact that A will come to elicit behaviors that reflect the nature of both the US and X (see Lin and Honey, 2011, 2016; Lin et al., 2013). Not considering how the nature of the retrieved X might affect behavior to A is a pervasive issue with both informal accounts of higher-order conditioning and more formal models of Pavlovian conditioning: How do the proposed associative structures generate different forms of behavior? This process has been left underspecified by both formal models of Pavlovian conditioning (e.g., Mackintosh, 1975; Rescorla and Wagner, 1972; Pearce and Hall, 1980; Wagner, 1981) and informal accounts of higher-order conditioning.

The accounts of higher-order conditioning that we have considered assume that the associations responsible for performance are directional. For accounts based on representation mediated learning, the association is from A to the US (i.e., A→US), whereas for those based on an associative chain they are from A to X (i.e., A→X) and from X to the US (i.e., X→US). The requisite additional assumption is that performance is (ordinally) related to either the strength of the association between A and the US (i.e., $V_{A\text{-}US}$), or the product of the links in the associative chain (i.e., $V_{A\text{-}X\text{-}US} = V_{A\text{-}X} \times V_{X\text{-}US}$; see Rescorla and Wagner, 1972). But, we know that accounts based on such assumptions are, at best, incomplete: The conditioned behavior generated by X→US trials reflects both the properties of the US and of the CS (e.g., Timberlake and Grant, 1975; see also, Holland, 1977; Patitucci et al., 2016; Iliescu et al., 2018). In fact, following Holland (1977, 1984), we can broadly distinguish between CS-oriented conditioned responding (e.g., sign-tracking; Hearst and Jenkins, 1974; see also, Davey and Cleland, 1982; Flagel et al., 2009) and US-oriented responding (e.g., goal-tracking; Boakes, 1977). Directional associations or chains of such associations from a CS to the US provide no foundation for CS-oriented conditioned behaviors[1]. Similarly, behaviors generated through Pavlovian conditioning (e.g., X→US) are not (quantitatively or qualitatively) the same as those generated by higher-order conditioning trials (e.g., A→X). This should be so if higher-order conditioned behavior is generated solely by associative activation of the US representation (see Holland and Rescorla, 1975; see Pavlov, 1927). Two examples from quite different preparations will suffice.

Stanhope (1992) gave hungry and thirsty pigeons training where keylight X was paired with food and keylight Y was independently paired with water. As a result, the pigeons directed pecks to X and Y, but those to X (the food keylight) were of greater force than those to Y (the water keylight; see Jenkins and Moore, 1973). The pigeons were then given trials where keylight A was paired with X while B was paired with Y. As a result, A and B came to elicit keypecking (see Rashotte et al., 1977), but the force of the keypecks to A and B did not differ in force (see also, e.g., Holland, 1977). Dwyer et al. (2012) gave thirsty rats separate access to two flavor compounds containing two flavors (A with X and B with Y); and then rats received access to X paired with illness and access to Y that was not. This procedure resulted in a reluctance to consume X relative to Y, and also A relative to B (see Rescorla and Cunningham, 1978). An important further finding was that while the first-order aversion was also evident in how rats consumed X (i.e., as a reduction in lick cluster size, indicative of a reduction in hedonic responses; see Dwyer, 2012), the second-order aversion to A was not. Neither a mediated A→US association nor an A→X→US associative chain provides a principled basis for the dissociations observed by Stanhope (1992) and by Dwyer et al. (2012; see also Holland and Rescorla, 1975).

A MORE FORMAL ANALYSIS

The model that we now describe builds on the assumption that learning involves the development of reciprocal associations: a central feature of the HeiDI model (see Honey et al., 2020a,b,c). This assumption provides a basis for the fact that conditioning

[1]Some combinations of stimuli might activate response units that generate behaviors that do not closely resemble those observed when the same stimuli are presented individually (e.g., conditioned freezing).

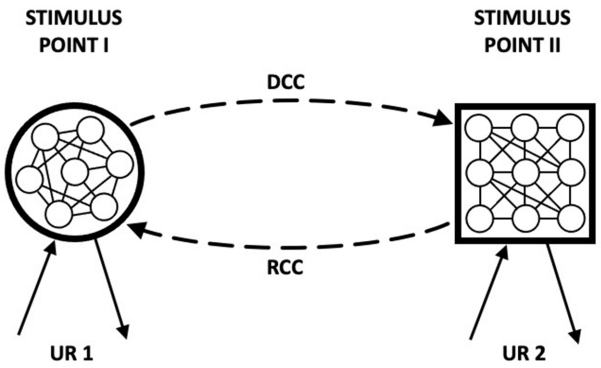

FIGURE 2 | "Figure 8 Scheme of the conditioned reflex with bidirectional connection. DCC, direct conditioned connection; RCC, reverse conditioned connection; UR 1, unconditioned reflex No. 1; UR 2, unconditioned reflex No. 2." Adapted from Asratian (1965). Note that while Stimulus Points I and II are reciprocally connected, the elements within Stimulus Points I and II are neither fully interconnected with one another and nor is their nature made explicit.

can result in both an increase in CS-oriented and US-oriented behaviors to a CS, and was foreshadowed by Asratian (1965). **Figure 2** is an adaptation of Figure 8 (Asratian, 1965; p. 179) where standard conditioning trials are held to result in a directly conditioned connection (DCC) and reverse conditioned connection (RCC) between Stimulus Points I and II (e.g., A and X, or X and the US). UR 2 can be generated both through direct activation of Stimulus Point II and through DCC by activation of Stimulus Point I, and UR 1 can be generated through activation of Stimulus Point 1 and by activation of Stimulus Point II through RCC. There is evidence to support the idea that reciprocal associations are formed during CS→US pairings (e.g., Asch and Ebenholtz, 1962; Heth, 1976; Tait and Saladin, 1986; Zentall et al., 1992; Gerolin and Matute, 1999; Arcediano et al., 2005).

The model described here and developed in Honey and Dwyer (under review), has three components: (1) Learning rules together with the associative structures that they generate; (2) performance rules that determine how those structures generate different behaviors; and (3) a function that specifies the similarity between a CS, its trace, and retrieved forms, in terms of their perceived intensities. Schematics for the associative structures generated by higher-order conditioning trials (i.e., A→X and X→A) are depicted in **Figure 3**. We assume that the unconditioned structure has existing links of differing strengths from A, X, and the US to a set of response units (r1-r6; left panel), and that reciprocal (excitatory) links form between A and X, and between X and the US during both sensory preconditioning (middle panel) and second-order conditioning (right panel). In the case of sensory preconditioning conditioning, the X→US trials will also result in the formation of an accompanying inhibitory US→A link, whereas in the case of second-order conditioning, the A→X trials result in the formation of an inhibitory A→US link (see next paragraph).

In general terms, the formation of reciprocal links between the components of higher-order conditioning trials (A, X, and the US) provides a mechanism by which conditioned responding (to X) and higher-order conditioning (to A) are affected by the properties of the components of any given trial. In the case of higher-order conditioning, performance during A will reflect its properties (e.g., Holland, 1977; Patitucci et al., 2016; Iliescu et al., 2018), and those of the stimuli with which it is associated: X (Honey et al., 1998a,b; Silva et al., 2019; see also, Narbutovich and Podkopayev, 1936; cited in Konorski, 1948, p. 91) and the US (e.g., Holland and Rescorla, 1975; Holland, 1977; Stanhope, 1992; Dwyer et al., 2012). Similarly, performance to X will reflect the stimulus itself as well as its associations with A and the US. The issue then becomes one of specifying how the combined associative strengths within the extended associative structures (see **Figure 3**) is distributed to reflect the properties of A through the response units it is connected to and those of the retrieved representations of X and US. Following HeiDI (Honey et al., 2020a), we assume that they do so in proportion to their perceived intensities: for example, if the perceived intensity of A is higher than that of the retrieved memories of X or the US then a greater proportion of the combined associative strength would generate responses that are linked to A. Finally, we assume that this process is modulated by the similarity between the perceived intensities of the stimuli presented at the test (e.g., the associatively retrieved memory of X) to their perceived intensities on the conditioning trials (see Ward-Robinson and Hall, 1996, 1998; Ward-Robinson et al., 2001; Lin and Honey, 2011, 2016; Lin et al., 2013; see also, Kamil, 1969; see also, Cole et al., 1995; Barnet and Miller, 1996). We now give formal expression to these general ideas.

Learning Rules

The formation of reciprocal associations between stimulus 1 and stimulus 2, having perceived intensities of α_1 and α_2, is determined by two equations: $\Delta V_{1\text{-}2} = \alpha_1(c.\alpha_2 - \Sigma V_{TOTAL\text{-}2})$; and $\Delta V_{2\text{-}1} = \alpha_2(c.\alpha_1 - \Sigma V_{TOTAL\text{-}1})$[2]. These rules underpin the HeiDI model (Honey et al., 2020a). For both equations, associative changes on a given trial ($\Delta V_{1\text{-}2}$ and $\Delta V_{2\text{-}1}$) are influenced by pooled error terms (i.e., $c.\alpha_2 - \Sigma V_{TOTAL\text{-}2}$ and $c.\alpha_1 - \Sigma V_{TOTAL\text{-}1}$) in which $\Sigma V_{TOTAL\text{-}2}$ and $\Sigma V_{TOTAL\text{-}1}$ are the summed associative strengths of stimuli present on that trial to the subscripted stimulus ($_1$ or $_2$). The maximum possible associative strengths are given by c (which is 1 in units of V) multiplied by the perceived intensities of the stimuli (α_2 and α_1)[3]. Otherwise, the learning rules are simplified extensions to the one developed by Rescorla and Wagner (1972; see also, McLaren et al., 1989)[4]. Equations 1 and 2 reference these generic equations to the critical A→X and X→A associations, and Equations 3 and 4 reference them to the X→US and

[2]The constant (c = 1 in units of V) is required to balance the equations in terms of the dimensions/units involved (see Honey et al., 2020a).

[3]The fact that the asymptotes and the rates at which they are reached are determined by α_1 and α_2 creates computational advantages when specifying the similarity of (1) the retrieved values of α_2 and α_1 (given by the numerical values of $V_{1\text{-}2}$ and $V_{2\text{-}1}$, respectively), and (2) their conditioned values α_2 and α_1.

[4]The rules have no independent lambda (λ) parameter to determine the asymptote for the $V_{1\text{-}2}$ association (or for the $V_{2\text{-}1}$ association). There is also no need to have separate learning rate parameters for when the target for the association (1 or 2) is present (e.g., β_E) and absent (e.g., β_I; see Honey et al., 2020b). β_I was required by the Rescorla-Wagner model $-\Delta V_{CS\text{-}US} = \alpha\beta(\lambda - \Sigma V)$ — to enable learning to occur when the US was absent and β would otherwise = 0.

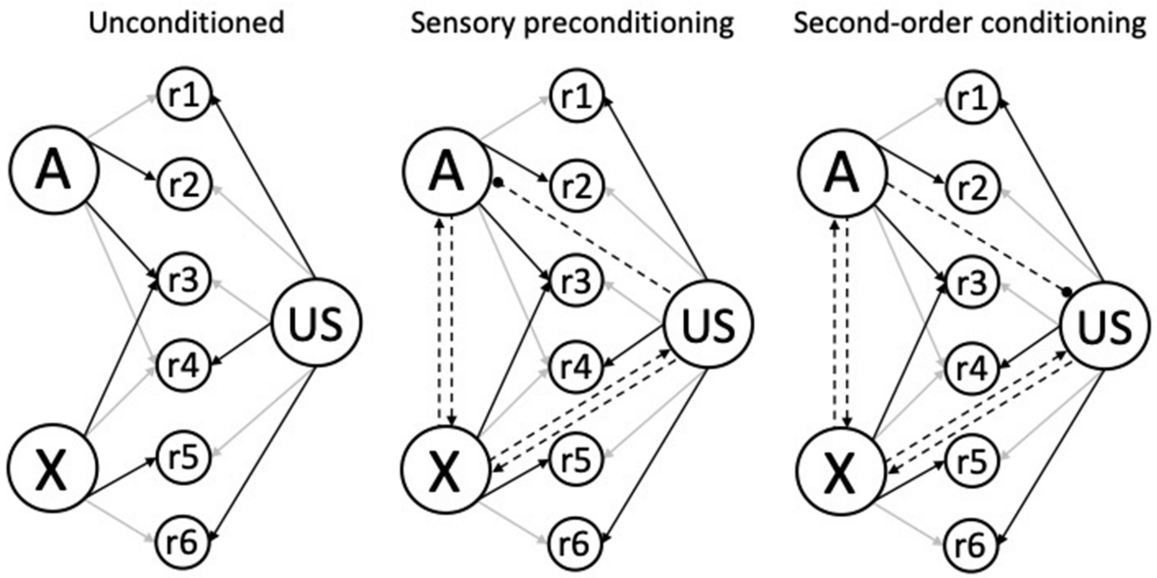

FIGURE 3 | Schematic associative structures for higher-order (excitatory) conditioning. The left structure shows the unconditioned links from the CSs (A and X) and the US to response-generating units (r1-r6) before conditioning. The darkness of the arrows indicates link strength: A is strongly linked to r2 and r3, B is strongly linked to r3 and r5; and the US is strongly linked to r1, r4, and r6; and the remaining unconditioned links are weak or absent. The central and right structures show the reciprocal associations between the A and X, and between the X and US nodes (denoted by the dashed lines with arrowheads), acquired during higher-order trials (e.g., A→X and X→US); with a directional inhibitory US→A association for sensory preconditioning (center panel) and an inhibitory A→US association in second-order conditioning (right panel; denoted by the dashed line with the circular end; based upon one interpretation of inhibitory learning). Adapted from Honey and Dwyer (under review).

US→X associations (analogous equations can be specified for the reciprocal links between A and the US). The maximum associative strength in Equation 3 is set by $\beta_{US,}$ which is the learning rate parameter in Equation 4.

$$\Delta V_{A\text{-}X} = \alpha_A(c.\alpha_X - \Sigma V_{TOTAL\text{-}X}) \quad (1)$$

$$\Delta V_{X\text{-}A} = \alpha_X(c.\alpha_A - \Sigma V_{TOTAL\text{-}A}) \quad (2)$$

$$\Delta V_{X\text{-}US} = \alpha_X(c.\beta_{US} - \Sigma V_{TOTAL\text{-}US}) \quad (3)$$

$$\Delta V_{US\text{-}X} = \beta_{US}(c.\alpha_X - \Sigma V_{TOTAL\text{-}X}) \quad (4)$$

This analysis already affords additional explanatory power in the context of demonstrations of higher-order conditioning. For example, the analysis provides a simple explanation for (so-called) backward sensory preconditioning (Ward-Robinson and Hall, 1996, 1998). In this case, the fact that X→A pairings replace the typical A→X pairings has been taken to mean that an A→X→US chain cannot be constructed upon which to generate conditioned responding to A. The suggestion that X→A pairings enable reciprocal associations to form between X and A means that an A→X→US associative chain is generated. The same form of argument can be applied to the fact that when the usual X→US trials are replaced with US→X trials, subsequent presentations of A provoke marked (US-oriented) responding in a sensory preconditioning procedure (for an alternative analysis, see Miller and Barnet, 1993; see also, Cole and Miller, 1999). Finally, it has been demonstrated that second-order conditioning to A is reduced if the US is presented on the A→X trials (i.e., A→X→US; see Holland, 1980). This result is predicted to the extent that the US competes with A to become associated with X (because it is more intense; Mackintosh, 1976) and with X to become associated with A; and that this reduction in the strength of the A→X association outweighs the fact that X continues to be paired with the US.

Performance Rules

Having specified the learning rules that generate the associative structures depicted in **Figure 3**, we now need to specify how these structures give rise to different conditioned behaviors. Our analysis is again based on HeiDI (Honey et al., 2020a,b,c). HeiDI separates the associative strengths of the CS→US and US→CS associations (Hebb, 1949) from the influence on performance of the intensities of the (presented) CS and (retrieved) US (see Hull, 1949). Thus, when the CS is presented the combined strength of the reciprocal associations [$V_{COMB} = V_{CS\text{-}US}$ + (numerical value of $V_{CS\text{-}US} \times V_{US\text{-}CS}$)] is distributed into CS- and US-oriented components (R_{CS} and R_{US}, respectively).[5] With this distribution being determined by the perceived intensity of the CS (α_{CS}) relative to the (retrieved) US (β_{US}, as retrieved by the CS; see Holland, 1977; Patitucci et al., 2016). In general, this means that when α_{CS} is higher than β_{US}, the CS-oriented component

[5]The reciprocal associations are combined in this way, rather than being simply mapped onto CS-oriented (US→CS) and US-oriented (CS→US) responding, to reflect the interactive nature of the reciprocal associations, but also to avoid the prediction that extinction of the CS would leave CS-oriented responding unaffected because it would only impact the CS→US association (see Iliescu et al., 2020).

is greater than the US-oriented component, and when β_{US} is higher than α_{CS} the reverse is true. Individual differences in α_{CS} and β_{US} would be reflected in both CS-oriented and US-oriented responding and learning through the error-correcting learning rules. It is now time to consider how the extended associative structures depicted in **Figure 3** and generated through Equations 1–4, affect behavior.

First, we should specify how the excitatory links in the middle and right panels of **Figure 3** are integrated when either A or X is presented. When A is presented, we can assume that its associative influence (denoted $V_{CHAIN\ A\text{-}X\text{-}US}$) is the product of the numerical value of $V_{A\text{-}X}$ and $V_{COMB\ X\text{-}US}$; where $V_{COMB\ X\text{-}US}$ is calculated in the manner described in the context of combining the reciprocal associations between a CS and US. To capture the additional effect of the inhibitory link between A and the US (in the right-hand panel of **Figure 3**) the influence of $V_{COMB\ A\text{-}US}$ needs to be added. $V_{COMB\ A\text{-}US}$ has a negative value in second-order conditioning and a value of zero in sensory preconditioning (see the bracketed terms in Equations 5–7). In contrast, should X be presented, $V_{COMB\ X\text{-}US}$ would be combined with the $V_{CHAIN\ X\text{-}A\text{-}US}$.

Now, these combined values can be separated into three components that influence the links from A, X, and the US to r1-r6 in proportion to their (perceived) intensities (see Equations 5–7). Upon presentation of A at test, its intensity would be directly given (i.e., by α_A; unless one was assessing test performance during its trace; see Lin et al., 2013); while that of the (retrieved) X would be given by the absolute numerical value of $V_{A\text{-}X}$ (for sensory preconditioning), and the sum of the absolute numerical values of $V_{A\text{-}X}$ and $V_{A\text{-}US\text{-}X}$ (for second-order conditioning). This allows the perceived intensity of a retrieved stimulus to exceed its α value, in much the same way as the Rescorla-Wagner model (see Kremer, 1978). β_{US} would be given by the absolute numerical value of $V_{A\text{-}X\text{-}US}$ for sensory preconditioning, while for second-order conditioning it would be given by the absolute numerical value of the sum of $V_{A\text{-}X\text{-}US}$ + $V_{A\text{-}US}$. The fact that the link from A to the US is indirect and weak, in contrast to the direct link between X and the US, will result in a greater bias toward CS-oriented (R_A) than US-oriented (R_{US}) behaviors during A than during X (see Dwyer et al., 2012; Holland and Rescorla, 1975; Stanhope, 1992).

$$R_A = \frac{\alpha_A}{\alpha_A + \alpha_X + \beta_{US}}(V_{CHAIN\ A\text{-}X\text{-}US} + V_{COMB\ A\text{-}US}) \quad (5)$$

$$R_X = \frac{\alpha_X}{\alpha_A + \alpha_X + \beta_{US}}(V_{CHAIN\ A\text{-}X\text{-}US} + V_{COMB\ A\text{-}US}) \quad (6)$$

$$R_{US} = \frac{\beta_{US}}{\alpha_A + \alpha_X + \beta_{US}}(V_{CHAIN\ A\text{-}X\text{-}US} + V_{COMB\ A\text{-}US}) \quad (7)$$

The influence of R_A, R_X, and R_{US} on the response-generating units (r1-r6 in **Figure 3**) will reflect the strengths of the unconditioned links between A, X and the US and r1-r6; for example, through multiplying R_A, R_X, and R_{US} by the weights from A, X and the US to r1-r6 (see Honey et al., 2020a). **Figure 4** presents some indicative simulations of the values of R_A, R_X, and R_{US}.

The upper panels of **Figure 4** depict simulations of sensory preconditioning, while its lower panels depict simulations of second-order conditioning. In both cases, $\alpha_A = \alpha_X = \beta_{US} = 0.80$. The left-hand panels show the values of R_A, R_X, and R_{US} for the presentation of A, which were calculated after 10 A→X trials and 2 X→US trials (sensory preconditioning) and after 10 X→US trials and 2 A→X trials (second-order conditioning). The right-hand panels show the corresponding values for the presentation of X. Values that are positive indicate the presence of higher-order conditioning. In the upper left panel, R_A and R_X output values are positive and similar, with both being higher than R_{US}. The similar output values for R_A and R_X reflect that they have the same α value and $V_{A\text{-}X}$ (the numerator in Equation 6) $\approx \alpha_X$ because it has approached asymptote over the course of 10 A→X trials. R_{US} has a lower value since the numerator in Equation 7 derives from the (absolute) numerical value of $V_{A\text{-}X} \times V_{X\text{-}US}$; which aligns to the perceived intensity of the US as retrieved by A through X. The upper right-hand panel shows the corresponding values for X[6]. R_A is lower than R_X and R_{US} because the value of $V_{X\text{-}A}$ declines over the course of X→US pairings. These simulations reveal that while R_A and R_X (aligned to CS-oriented responding) are similar whether A or X is tested, R_{US} (aligned to US-oriented responding) takes a higher value during X than A.

The lower panels of **Figure 4** show output values for simulations of second-order conditioning, generated with the same parameters as sensory preconditioning, and after the same number of trials in the first and second stages (10 X→US trials and 2 A→X[7]). Comparing first the upper and lower panels (noting their different scales), R_A and R_X output values were relatively similar during A (and X) for simulations of sensory preconditioning and second-order conditioning (see Barnet et al., 1991). However, R_{US} values were far lower for second-order conditioning than for sensory preconditioning. Indeed, if α_A and α_X are set to lower values, it results in the components of the excitatory chain becoming less effective with the consequence that there is now no second-order conditioning. In any case, the fact that R_{US} is particularly low for second-order conditioning (relative to R_A and R_X) reflects the influence of the inhibitory $V_{A\text{-}US}$ on the calculated value of β_{US}: When A is tested, the value of β_{US} = numerical values of $V_{A\text{-}US}$ (inhibitory) + $V_{A\text{-}X} \times V_{X\text{-}US}$ (excitatory); and when X is tested, β_{US} = numerical values of $V_{X\text{-}A} \times V_{A\text{-}US}$ (inhibitory) + $V_{X\text{-}US}$ (excitatory). A further difference from sensory preconditioning is that during the test with A the output value for R_A is greater than for R_X. This difference derives from the fact that in sensory preconditioning $V_{A\text{-}X}$ (the numerator in Equation 6) $\approx \alpha_X$, whereas in second-order conditioning $V_{A\text{-}X}$ does not

[6]Here, α_A = the (absolute) numerical value of $V_{X\text{-}A}$ (i.e., $1/c|V_{X\text{-}A}|$), $\alpha_X = \alpha_X$ and β_{US} = the (absolute) numerical value of $V_{X\text{-}US}$.

[7]Maintaining the number of trials of the two types (10 A→X and 2 X→US), rather than the number of trials in the two stages (10 for stage 1 and 2 for stage 2), results in extinction of the X→US association over the course of the 10 A→X trials.

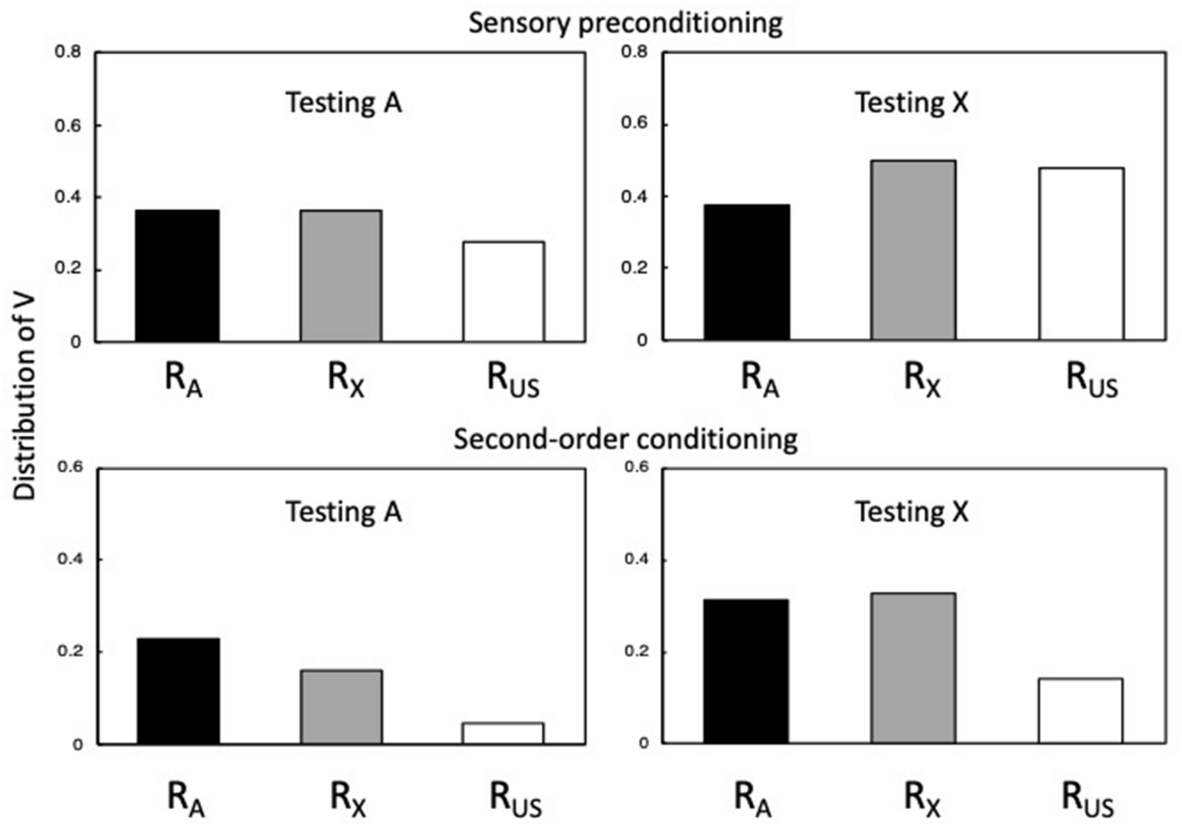

FIGURE 4 | Simulations of sensory preconditioning and second-order conditioning. The output values for R_A, R_X, and R_{US} were generated for A and X using Equations 1–7 with $\alpha_A = \alpha_X = \beta_{US} = 0.80$. There were 10 A→X trials and 2 X→US trials for the sensory preconditioning simulation, and 10 X→US trials and 2 A→X for the second-order conditioning simulation. For both simulations, the values of a R_A, R_X, and R_{US} were then computed for A. Adapted from Honey and Dwyer (under review).

reach asymptote as a consequence of two A→X trials, and is further constrained by $V_{A\text{-}US\text{-}X}$ being negative. The simulations in **Figure 4** can be aligned with results reported by Stanhope (1992) using an autoshaping procedure in pigeons, and Dwyer et al. (2012) using a flavor-aversion procedure in rats: If pecking a keylight (in pigeons) and fluid consumption (in rats) is equated to CS-oriented responding (generated by R_A and R_X), and the force of pecks and lick cluster size is equated with US-oriented responding (generated by R_{US}).

Similarity Function

The central idea captured in Equations 5–7 is that the relative intensities of components of the test pattern (some present and others retrieved) determine how the associative structures depicted in **Figure 3** generate behaviors aligned to those components (A, X, and US). What they do not capture is how differences in the intensities of a given component between test and conditioning influences R_A, R_X, and R_{US}. In Equations 5–7 identity is simply assumed. There are three reasons why this needs to be addressed: First, Equations 5–7 have no (internal) mechanism for restricting conditioned behavior to stimuli that have been present on conditioning trials or to those associated with them: Associatively neutral stimuli might well influence the distribution of associative strength, but without necessarily eliciting anything other than unconditioned responses (see Pavlov, 1927, p. 44; see also, Honey et al., 2020a). Second, animals can learn discriminations in which the effective stimuli involve: (a) whether the same stimulus is presented at one intensity or a different intensity (e.g., Inman et al., 2016; for a review, see Inman and Pearce, 2018), and (b) whether the same stimulus has been presented more or less recently (e.g., Lin and Honey, 2010; see also, Pavlov, 1927; Mackintosh, 1974, p. 104; Staddon and Higa, 1999; Staddon, 2005). The latter observation reducing to the former once different components of a decaying trace are equated with different stimulus intensities; both observations suggest that different intensities of a given stimulus can enter into different associations, but also that there is generalization between those intensities. Third, the idea that the representation of the CS includes the intensity at which it is presented affords an account for when higher-order conditioning is observed: As already noted, trace conditioning might enhance higher-order conditioning because when A retrieves X at test (i.e., X*) it is more similar in perceived intensity to the stimulus that became linked to the US during trace conditioning (X*) than

standard conditioning (X; Ward-Robinson and Hall, 1998; Lin and Honey, 2011; see also, Kamil, 1969; Cole et al., 1995; Barnet and Miller, 1996). It would also help to explain the fact that higher-order conditioning to A can be left unaffected by the extinction of responding to X (e.g., Rizley and Rescorla, 1972; Cheatle and Rudy, 1978; Amiro and Bitterman, 1980; Nairne and Rescorla, 1981; Archer and Sjödén, 1982; Ward-Robinson and Hall, 1996; but see, Rescorla, 1982): Because X (rather than the trace, X*) would undergo extinction when X is presented (see Kamin, 1969; Mackintosh, 1976). Finally, when A is presented with X at test, A will retrieve X*, which has strength independently of X itself (e.g., Ward-Robinson et al., 2001; Lin et al., 2013). This analysis is plausible, but without a function that specifies the similarity between the perceived intensities of stimuli, their traces, and retrieved representations it remains tendentious (see Lin and Honey, 2011, 2016; see also, Lin et al., 2013). However, one such function is presented below in the context of how the retrieved memory of X affects performance during the presentation of A (i.e., in a modification of Equation 6).

$$R_X = \frac{\alpha_X}{\alpha_A + \alpha_X + \beta_{US}}\left(\left(\alpha_{X\text{-}R}S\alpha_{X\text{-}C} \times V_{CHAIN\ A\text{-}X\text{-}US}\right) + V_{COMB\ A\text{-}US}\right)$$

Where: (8)

$$\alpha_X = \alpha_{X\text{-}R} = \left|\tfrac{1}{C}V_{A\text{-}X}\right| \text{ and } \alpha_{X\text{-}C} = \alpha \text{ of X upon delivery of the US}$$

$$\alpha_{X\text{-}R}S\alpha_{X\text{-}C} = \frac{\alpha_{X\text{-}R}}{(\alpha_{X\text{-}R}+|\alpha_{X\text{-}C}-\alpha_{X\text{-}R}|)} \times \frac{\alpha_{X\text{-}C}}{(\alpha_{X\text{-}C}+|\alpha_{X\text{-}C}-\alpha_{X\text{-}R}|)}$$

The function ($\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$) introduced in Equation 8 (in the gray boxes) determines the similarity (S) of two values: The numerical value of $V_{A\text{-}X}$ (denoted $\alpha_{X\text{-}R}$) and its conditioned counterpart or trace (denoted $\alpha_{X\text{-}C}$). It is worth remembering that when $V_{A\text{-}X}$ reaches asymptote, its numerical value $\approx \alpha_X$, which means that $\alpha_{X\text{-}R} \approx \alpha_{X\text{-}C}$. This function is also applied to modify the bracketed term in Equations 5 and 7 when A is presented. Its basic properties are simple: When the values of $\alpha_{X\text{-}R}$ and $\alpha_{X\text{-}C}$ are close together then $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ approaches 1, but as they diverge then $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ approaches 0. Applying these ideas to how $\alpha_{X\text{-}R}$ affects performance is also simple. Because the asymptote for $V_{A\text{-}X}$ during A→X training is α_X, when A is presented at test $\alpha_{X\text{-}R}$ will have approached α_X over the A→X trials. If A→X training had proceeded until $V_{A\text{-}X}$ reached asymptote then $\alpha_{X\text{-}R}$ and $\alpha_{X\text{-}C}$ would be maximally similar, provided α_X during X→US conditioning trials was the same as during A→X trials (as it usually is). Now, we can appreciate how $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ varies when α_X has one value for A→X trials (e.g., 0.50) and is then reduced for X→US trials (e.g., 0.45); this reduction in $\alpha_{X\text{-}C}$ is intended to mimic the effect of introducing a trace interval between X and the US (see Lin and Honey, 2011, 2016; Lin et al., 2013). It should be clear that before $V_{A\text{-}X}$ has reached asymptote during A→X trials, its numerical value can match more closely 0.45 than 0.50; and that as $V_{A\text{-}X}$ tends to 0.50 for A→X trials the numerical value of $V_{A\text{-}X}$ will become closer to 0.50 than 0.45. The accuracy of this analysis was confirmed by simulations.

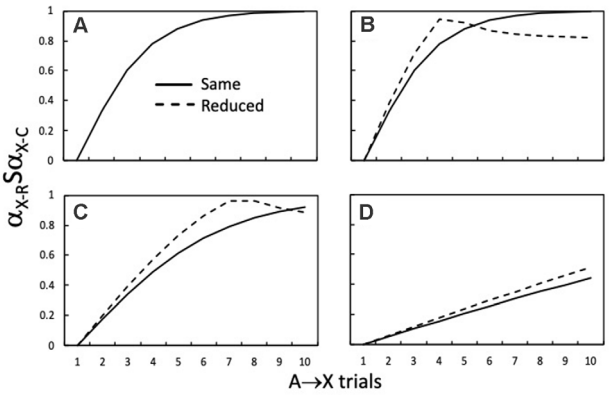

FIGURE 5 | How the similarity ($\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$) of the retrieved X ($\alpha_{X\text{-}R}$) to the conditioned X ($\alpha_{X\text{-}C}$) during a test with A varies with the number of initial A→X trials. The continuous lines denote $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ output values when the α_X value (0.50) used to compute changes in $V_{A\text{-}X}$ (i.e., $\alpha_{X\text{-}R}$) was the *same* as that for $\alpha_{X\text{-}C}$ on X→US conditioning trials. α_A was 0.50 in panels **(A,B)**, 0.30 in panel **(C)**, and 0.10 in panel **(D)**. The dashed lines denote $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ output values when the α_X value used to compute changes in $V_{A\text{-}X}$ (0.50; i.e., $\alpha_{X\text{-}R}$) was *reduced* to 0.45 for $\alpha_{X\text{-}C}$ to calculate $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$. This manipulation is akin to using trace conditioning for X→US trials. Adapted from Honey and Dwyer (under review).

Figure 5 shows how $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ varies as a function of the number of A→X training trials during the first stage of training. The continuous lines show $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ when $\alpha_{X\text{-}R}$ and $\alpha_{X\text{-}C}$ are generated by the same α_X value (e.g., 0.50), as in standard higher-order conditioning procedures. Comparison of the continuous lines across **Figures 5A–D** shows that the rate at which maximum similarity is approached, across a series of A→X trials, decreases as α_A is reduced from 0.50 (**Figures 5A,B**), to 0.30 (**Figure 5C**), and then 0.10 (**Figure 5D**). Turning now to the dashed lines in **Figures 5B–D**, it is clear that there is a period of initial A→X training when reductions in $\alpha_{X\text{-}C}$ increase $\alpha_{X\text{-}R}S\alpha_{X\text{-}C}$ compared to when $\alpha_{X\text{-}C}$ is the same (i.e., 0.50 for the continuous lines). With more extended A→X training this pattern reverses as $\alpha_{X\text{-}R}$ (i.e., $V_{A\text{-}X}$) approaches 0.50 and consequently deviates from the reduced value of $\alpha_{X\text{-}C}$ (i.e., 0.45). This reversal is apparent in **Figures 5B,C**, but not within 10 trials in **Figure 5D**.

The take-home message from these simulations is that trace conditioning will have the potential to enhance higher-order conditioning if A is tested when A→X training has left $V_{A\text{-}X}$ within the range where the dashed line has higher values than the continuous line. The influence of such increases in similarity on higher-order conditioning will be contingent on them more than counteracting any direct effect of reducing $\alpha_{X\text{-}C}$ on the efficacy of the X-US component of the chain (i.e., $V_{CHAIN\ A\text{-}X\text{-}US}$). In fact, simulations reveal that increases in R_A, R_X, and R_{US} of between 10% to 20% are produced by reducing $\alpha_{X\text{-}C}$ by 10%, which is in the range where reducing $\alpha_{X\text{-}C}$ has little effect on the rate at which $V_{X\text{-}US}$ approaches the asymptote determined by β_{US}. These effects of similarity are more marked for sensory preconditioning

than second-order conditioning (see Honey and Dwyer, under review).

Our formal analysis assumes that the value of α on a conditioning trial is encoded and is one basis for generalization between a CS presented at one intensity and the same CS but delivered at a different intensity. It also assumes that there is a (computational) equivalence between different α (and β) values generated by changing a stimulus physically (e.g., Inman et al., 2016) and the values generated through the central processes of decay and retrieval (e.g., Lin and Honey, 2010; see also Iliescu et al., 2020). In addition to providing an analysis for how trace conditioning can enhance higher-order conditioning, it can also explain related observations: the facts that extinction of X is not always reflected in responding to A, and the compound AX generates more responding than X in sensory preconditioning procedures. As already noted, the effects of extinction treatments involving the presentation of X will be more likely to impact its directly activated α value as opposed to its decaying value through a process of overshadowing (Mackintosh, 1976); and whether this affects higher-order conditioning will depend on whether the representation of X that supports responding to A (which is determined by the strength of the A→X association; see Rescorla, 1982) is similar to its directly activated or decaying forms. Equation 8 provides a formal example of how test performance is affected by the similarity between the value of X retrieved by A as a consequence of A→X trials and its encoded value during conditioning trials. According to our analysis, AX will generate more responding than X because the associative chain can exert an independent influence on the US representation (for further details, see Honey and Dwyer, under review).

To close the theoretical loop, the learning rules (e.g., $\Delta V_{1\text{-}2} = \alpha_1(c.\alpha_2 - \Sigma V_{S\text{-}TOTAL\text{-}2})$) can be modified to reflect the fact that the associative strengths of stimuli contributing to $\Sigma V_{S\text{-}TOTAL\text{-}2}$ (including $V_{1\text{-}2}$) need to be scaled by their similarity (subscript s) to their intensities when conditioned (see Pearce, 1994). For instance, Equation 3 can be re-cast as Equation 9, where the subscript s denotes this scaling process. The similarity function is as before, but $\alpha_{X\text{-}R}$ is the perceived intensity of the CS on previous trials, while $\alpha_{X\text{-}C} = \alpha_X$ of the same CS on the current trial. In this way, the perceived intensity of a CS is encoded as one component of what is learned on a conditioning trial (if α_X changes from one trial to the next then new learning occurs), which reflects the generalization of associative strength between a stimulus conditioned at one intensity and later presented at another intensity (i.e., $\Sigma V_{S\text{-}TOTAL\text{-}US}$ is reduced because $\alpha_{X\text{-}R}S\alpha_{X\text{-}C} < 1$). It should be recognized, however, that increases and reductions in intensity have different effects on behavior through the proportion terms in the equations that determine the distribution of associative strength (e.g., in Equation 8). Finally, it is worth noting that the effect of changing α_X from one trial to the next on the US→X association will be that $V_{US\text{-}X}$ homes in on the new α_X (see Equation 4), which parallels the fact that changes in US intensity across trials affects the asymptote of the X-US association.

$$\Delta V_{X\text{-}US} = \alpha_X(c.\beta_{US} - \Sigma V_{S\text{-}TOTAL\text{-}US}) \quad (9)$$

DISCUSSION: SOME CONCLUDING CONSIDERATIONS

Understanding higher-order conditioning has theoretical and translational value, but traditional (informal) accounts of this phenomenon are poorly equipped to address two fundamental issues: What is learned and how it is expressed. The analysis described here and developed in Honey and Dwyer (under review) borrows from HeiDI, which is a model of Pavlovian learning and performance (Honey et al., 2020a). The learning and performance rules are derived from HeiDI, but their influence is modulated by a similarity function. This function specifies the similarity between the same nominal stimulus, which can take different perceived intensities as a result of manipulating the intensity at which it is delivered and through processes of retrieval or trace decay. The resulting analysis has clear implications for behavioral neuroscience, where group-level differences in higher-order conditioning should be interpreted with caution: Changes in a given behavioral measure of higher-order conditioning consequent on a manipulation might have a variety of origins. For example, differences in learning or performance might not reflect differences in the underlying learning mechanisms but rather changes to: α (for A and X), β (for the US), or their associated decay functions (see Honey and Good, 2000); or indeed the requisite (neural) computations involving the processes represented by these parameters.

In developing this more formal analysis of higher-order conditioning, no appeal has been made to any process of retrieval mediated learning or stimulus-response learning. This is not intended to suggest that such forms of learning are without consequence, but simply that they are not required by the available evidence. For example, the model presented here could accommodate retrieval mediated learning between A and the US in a sensory preconditioning procedure by substituting the numerical value of $\Sigma V_{TOTAL\text{-}A}$ for α_A: $\Delta V_{A\text{-}US} = 1/c.\Sigma V_{TOTAL\text{-}A}(c.\beta_{US} - \Sigma V_{S\text{-}TOTAL\text{-}US})$; recall that multiplying $\Sigma V_{TOTAL\text{-}A}$ by 1/c transforms it into a dimensionless scalar like α_A. In this way, a retrieved stimulus, or stimulus trace, might acquire associative strength while limiting that acquired by other stimuli present on a conditioning trial. As we have noted, retrieved stimuli will also affect performance through the proportion terms in Equations 5–8 (see Holland, 1983). This analysis joins others that have attempted to provide a more specific account of the process of retrieval mediated learning, albeit that they do not apply as readily to higher-order conditioning as they do to other phenomena (e.g., Van Hamme and Wasserman, 1994; Dickinson and Burke, 1996; see also, Dwyer et al., 1998).

We should briefly comment on the complexity of the model. While the model has three components (relating to learning, performance, and similarity) it only has two free parameters: α (for A and X) and β (for the US); and their associated decay functions. It can also be summarized in two simple statements: 1. The perceived intensities of stimuli present during a test affect how learning represented within an extended associative structure affects performance; and 2. The similarity of the

perceived intensities of the tested stimuli to conditioned stimuli within that structure modulates the translation of learning into performance.

Our use of the term *perceived intensity* clearly affords a potential analysis of individual differences in both Pavlovian conditioning and higher-order conditioning at the level of learning and performance (see Honey et al., 2020a,b,c), but also now in terms of the similarity between directly activated representations, their decaying traces, and retrieved forms. Pavlov (1927; p. 105) noted that there were marked individual differences in the strength of second-order reflexes: "*Among the experimental dogs one finds special types of nervous systems; in particular there are dogs with weak nervous systems in which this phenomenon is clearly expressed.*" The fact that there are significant individual differences in how learning is evident in behavior has been neglected by general-process models of learning. The model upon which our analysis is based, HeiDI, represents a prosaic approach to accommodating both quantitative and qualitative individual differences in conditioned behavior.

AUTHOR CONTRIBUTIONS

RH and DD contributed to the ideas presented in this article and to its preparation for publication. Both authors contributed to the article and approved the submitted version.

FUNDING

The development of the ideas, together with our research that informed them, was supported by a BBSRC grant (UK; BB/T004339/1; PI: RH).

ACKNOWLEDGMENTS

We thank A. F. Iliescu for comments on a draft of this article.

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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.

frontiers in Behavioral Neuroscience

REVIEW
published: 04 October 2021
doi: 10.3389/fnbeh.2021.745388

Higher-Order Conditioning and Dopamine: Charting a Path Forward

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Higher-order conditioning involves learning causal links between multiple events, which then allows one to make novel inferences. For example, observing a correlation between two events (e.g., a neighbor wearing a particular sports jersey), later helps one make new predictions based on this knowledge (e.g., the neighbor's wife's favorite sports team). This type of learning is important because it allows one to benefit maximally from previous experiences and perform adaptively in complex environments where many things are ambiguous or uncertain. Two procedures in the lab are often used to probe this kind of learning, second-order conditioning (SOC) and sensory preconditioning (SPC). In second-order conditioning (SOC), we first teach subjects that there is a relationship between a stimulus and an outcome (e.g., a tone that predicts food). Then, an additional stimulus is taught to precede the predictive stimulus (e.g., a light leads to the food-predictive tone). In sensory preconditioning (SPC), this order of training is reversed. Specifically, the two neutral stimuli (i.e., light and tone) are first paired together and then the tone is paired separately with food. Interestingly, in both SPC and SOC, humans, rodents, and even insects, and other invertebrates will later predict that both the light and tone are likely to lead to food, even though they only experienced the tone directly paired with food. While these processes are procedurally similar, a wealth of research suggests they are associatively and neurobiologically distinct. However, midbrain dopamine, a neurotransmitter long thought to facilitate basic Pavlovian conditioning in a relatively simplistic manner, appears critical for both SOC and SPC. These findings suggest dopamine may contribute to learning in ways that transcend differences in associative and neurological structure. We discuss how research demonstrating that dopamine is critical to both SOC and SPC places it at the center of more complex forms of cognition (e.g., spatial navigation and causal reasoning). Further, we suggest that these more sophisticated learning procedures, coupled with recent advances in recording and manipulating dopamine neurons, represent a new path forward in understanding dopamine's contribution to learning and cognition.

Keywords: dopamine, sensory preconditioning, second order conditioning, reinforcement learning, basolateral amygdala, hippocampus, orbitofrontal cortex

OPEN ACCESS

Edited by:
Nathan Holmes, University of New South Wales, Australia

Reviewed by:
Shauna L. Parkes, Centre National de la Recherche Scientifique (CNRS), France
Heidi Catherine Meyer, Weill Cornell Medicine Psychiatry, United States

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

Received: *22 July 2021*
Accepted: *06 September 2021*
Published: *04 October 2021*

Citation:
Seitz BM, Blaisdell AP and Sharpe MJ (2021) Higher-Order Conditioning and Dopamine: Charting a Path Forward. Front. Behav. Neurosci. 15:745388. doi: 10.3389/fnbeh.2021.745388

DOPAMINE AND HIGHER-ORDER COGNITION: CHARTING A PATH FORWARD

Introduction

To understand their worlds, humans and other animals learn to predict outcomes that are important to them, like food or pain. This is adaptive; if you can predict these outcomes, you can learn to increase or decrease your chances of encountering them depending on current needs. Sometimes this process is simple. The sight of a burrito predicts calories. But often it is more complex. Perhaps you have to remember the name of the restaurant that sells the burrito, or even recall the route you previously took to get there. This more complex learning process is referred to as higher-order conditioning and involves the combining of information that allows one to navigate cognitively or spatially to their goals. Higher-order conditioning likely accounts for many of our learned experiences; learning how to predict the consequences of our environment is rarely a more simplistic encounter with direct predictors of food or pain (Gewirtz and Davis, 2000).

In the lab, we mimic this process of higher-order conditioning through the use of the second-order conditioning (SOC) and sensory preconditioning (SPC) procedures. SOC was first described by Pavlov (1927) and refers to instances in which a neutral stimulus (e.g., a tone) is paired with something important, like food. After this, another novel stimulus (e.g., a light) is paired with the tone. SOC occurs when the light elicits an appetitive response by virtue of being paired with the food-predictive tone (see **Figure 1A**). Thus, because the tone has been directly paired with reward, it can now reinforce associations between itself and stimuli that predict it (i.e., the light). On the other hand, SPC involves first pairing the light and tone together when they are both neutral and then presenting the tone with something significant (e.g., food). SPC refers to the finding that humans and other animals will now show an appetitive response to the light, even though they have never experienced the light directly paired with food (Brogden, 1939; see **Figure 1A**). These procedures indicate that we can learn complex mental routes to something biologically significant, even if what we are learning about has not been directly paired with those significant outcomes.

At first glance, the phenomena of SOC and SPC might seem similar. Indeed, the only difference in their procedures is the order of training (**Figure 1A**). That is, both procedures involve pairing two neutral stimuli together, the light and the tone, and separately pairing the tone with food. Yet in SOC the pairing of the neutral stimuli occurs after pairings of the tone with food, and in SPC the pairing of the neutral stimuli occurs before pairings of the tone with food. Despite this seemingly minor difference, SOC and SPC differ in their associative structure and neural substrates. SOC appears to rely on the transfer of affective value from the food-predictive tone to the light, facilitated by amygdala circuits (Gewirtz and Davis, 1997; Parkes and Westbrook, 2010). In contrast, SPC relies on forming a more complex association between all three elements

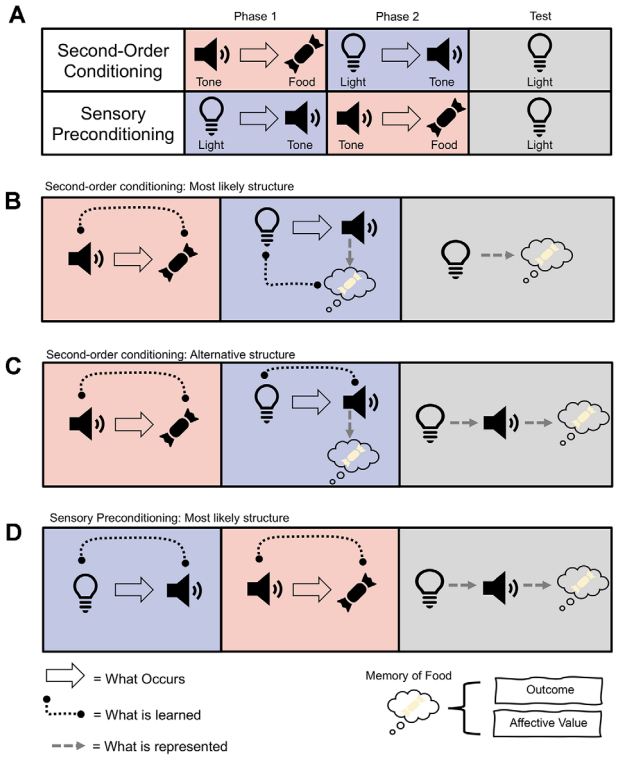

FIGURE 1 | (A) Example procedures for second-order conditioning (SOC) and sensory preconditioning (SPC). **(B)** Most likely associative structure underlying SOC, whereby the light becomes directly associated with a memory of the food and its affective value or sensory-specific representation. **(C)** Alternative associative structure of SOC whereby light is associated with the tone, which is associated with the memory of the food and induces a response. **(D)** Most likely associative structure of SPC whereby the light is associated with the tone, which is associated with a memory of the food and involves the hippocampus and orbitofrontal cortex (OFC).

(i.e., light→tone→food), with help from the hippocampus and orbitofrontal cortex (Rizley and Rescorla, 1972; Jones et al., 2012; Barron et al., 2020; Hart et al., 2020). Phasic dopamine activity in the midbrain, however, has recently been shown to be necessary for both phenomena to occur (Sharpe et al., 2017a; Maes et al., 2020). This places midbrain dopamine (and the dopamine prediction error, described below) at the heart of more complex learning and cognition. Herein, we review how dopamine unites the contrasting processes of SOC and SPC, and the implications this has for conceptualizing dopamine as a teaching signal that transcends associative structure.

Associative Structure and Neural Substrates of Higher-Order Conditioning

The finding that a stimulus (e.g., tone) paired with reward (e.g., food) can on its own come to elicit a response (e.g., a rat making a nose poke into the location where food is delivered) seems a straightforward and obvious phenomenon. But over a century of research in Pavlovian conditioning has revealed that diagnosing the associative basis of behavior is not necessarily straightforward. For instance, the tone might be associated with

the specific response to nose poke. Here, the presentation of the tone automatically causes the animal to nose poke in a reflexive manner (i.e., a tone-response association). On the opposite side of the spectrum, the tone might be associated with a detailed and rich sensory-specific memory of the food. In this scenario, the presentation of the tone makes the animal think about the food outcome and its various features (e.g., texture, odor, and taste), and thinking about this specific outcome drives the response to nose poke towards the location where food is usually delivered (i.e., a tone-outcome association). Somewhere in between these two accounts, the tone may become associated with the general affective value of the food reward, and this appetitive value may drive the nose poking response (i.e., a tone-value association). One tool learning theorists have used to differentiate between these accounts involves manipulating the representation or desirability for the outcome (Rescorla and Solomon, 1967; Dickinson, 1985; Dolan and Dayan, 2013). To use the example above, if the food was paired with illness (referred to as "devaluation"), an animal that has associated the tone with a detailed representation of the outcome will recall that they no longer find the food rewarding and will not nose poke at the location. However, if the animal has learned a more reflexive association between the tone and nose poking, or the tone and the appetitive value, devaluation of the outcome should not influence nose poking. This is because the association involves the tone and the response or value, not tone and the specific food outcome it predicts. Effectively, the ability of a stimulus to drive a behavioral response may originate from many different associations. Indeed, these associations may even drive the response at the same time (Rescorla, 1988). Accordingly, we need to adequately test and probe the associative basis of any given association to understand its underlying structure and neural substrates.

Below, we review the associative and neural basis of SOC and SPC. For simplicity, we will continue to use our example where the tone is directly paired with the outcome (food or shock), and the light predicts the tone. However, this does not necessarily reflect the stimuli used in the procedures discussed. Indeed, SOC and SPC are not limited to conditioning with food or pain, nor do they require such simplistic stimuli to occur. SOC and SPC have been observed repeatedly using a number of different procedures, complex stimuli (e.g., spatial landmarks), and species ranging from sea slugs (Hawkins et al., 1998) to pigeons (Sawa et al., 2005) to humans (Wimmer and Shohamy, 2012; Craddock et al., 2018). We also note that some of the to-be-reviewed structures have only had their involvement tested in one phenomenon (e.g., orbitofrontal cortex in SPC) and may or may not be involved in the other.

Second-Order Conditioning

SOC allows for predictive stimuli to facilitate further learning to neutral stimuli that precede it. For example, once the tone has been established as predictive of food, it can reinforce the development of an appetitive association with the light that now predicts its occurrence. There are several associative structures that could support this learning (see **Figures 1B,C**). The first possibility is an association between the light and tone, whereby the presentation of the light elicits a representation of tone, which then elicits a memory of the food resulting in the conditioned response (Rizley and Rescorla, 1972; Barnet et al., 1997). This is said to be a more cognitive account because it relies on the light evoking a representation of the tone. The second possibility is a more direct association between light and the food (Konorski, 1967). According to this view, the tone evokes a representation of the food, and so when the light is paired with the tone, it too becomes associated with the representation of food.

To test these two accounts, researchers have manipulated the status (i.e., memory) of the tone after it is paired with the light (i.e., tone→food, light→tone), but before the light is tested alone to assess the magnitude of SOC. For example, Rizley and Rescorla (1972) repeatedly presented the tone without consequence after establishing the tone-light association. This process of extinction reduced responding to tone. However, the light still elicited the same magnitude of SOC (for a recent replication in rats, see Holmes et al., 2014; and in humans see Jara et al., 2006; but for failed replications in humans and discussion, see Craddock et al., 2018; Lee, 2021). Similarly, Holland and Rescorla (1975) devalued the food outcome after establishing the tone→food and light→tone associations. Here, devaluation also attenuated responding the tone, while responding to the light remained intact. These results suggest responding to light does not rely on an evoked representation of tone, or a sensory-specific representation of food.

Further insight into the associative structure of SOC is provided by the fact that light and tone can exhibit different types of responses. For example, when pigeons learn that a tone predicts food, presentation of the tone elicits general food-seeking behavior towards the location of where the food is delivered. However, when light is paired with food, pigeons will peck at the source of the light (i.e., a key; Nairne and Rescorla, 1981). In SOC, when the light is paired with the food-predictive tone, the light will still evoke the key peck. Thus, SOC does not seem to be supported by an association between the light and the conditioned response evoked by the tone (Gewirtz and Davis, 2000). A more conservative summary of the data, therefore, is that responding to the light in SOC is associated with an affective state—or valence—but it does not evoke a representation of the tone or the response associated with the tone (see **Figure 1B**; Holland, 1977; Gewirtz and Davis, 2000).

The neural regions that are involved in SOC make understanding of the associative nature more complex. In particular, studies (e.g., Holmes et al., 2013) have shown that glutamatergic signaling in the basolateral amygdala (BLA), likely facilitated by BLA pyramidal neurons, is necessary for SOC in an aversive setting. That is, infusion of an NMDA antagonist (AP5 or ifenprodil) prior to the pairing of the light with a shock-predictive tone, prevents the ability of the light to support SOC. This is contradictory to the hypothesis that SOC relies on the transfer of general valence to the light as the BLA is known to be critical for the development of sensory-specific associations between stimuli and outcomes, and explicitly not associations between stimuli and general value (Corbit and Balleine, 2005; Balleine and Killcross, 2006; Prévost et al., 2012). Thus, it is surprising that BLA is necessary for the development of SOC in this phenomenon. This may suggest either SOC is not the

result of the transfer of general valence, or indicate the presence of multiple associations driving SOC, with some aspects and/or procedures being supported by the BLA.

In support of this, the BLA appears less critical in SOC with appetitive reinforcement. For example, lesions to the BLA before the tone is paired with food will prevent the development of SOC when the light is subsequently paired with the food-predictive tone. However, if similar lesions are made after the tone is established as food-predictive and before the light is paired with the tone, SOC is spared (Setlow et al., 2002) and in some instances, enhanced (Holland, 2016). The reason for the discrepancy in BLA involvement between aversive and appetitive SOC might rest in the amount of training that supports learning in aversive and appetitive procedures. Aversive procedures generally use few pairings of stimuli and aversive outcomes, while appetitive procedures involve many pairings of stimuli and outcomes, across days or even weeks. Holland has shown that if the tone is paired with food across few pairings, the tone will be able to serve as a "substitute" for the food. For example, if the tone is devalued (i.e., paired with LiCl) the food will now also be devalued (i.e., mediated conditioning). However, if the tone is paired many times with food, it will no longer substitute as the food in mediated conditioning, despite the tone still producing an appetitive response (Holland, 1998). This could suggest that the number of pairings of the tone and outcome might influence the nature of the association that is supported during SOC. Accordingly, the general value may be sufficient to support SOC in appetitive procedures, which generally utilize many pairings of the tone and outcome, making the BLA unnecessary. In contrast, the associations driving SOC in aversive conditioning may be more based on associations between stimuli and detailed representations of outcomes and require the BLA, which encodes these forms of associations (Balleine and Killcross, 2006; Wassum and Izquierdo, 2015). Of course, this hypothesis is yet to be tested and it is possible that other differences between appetitive and aversive SOC procedures could underlie this discrepancy. However, it is unlikely to be the general appetitive or aversive nature of the task *per se*, as many researchers have found BLA plays a similar role in learning about food and shocks (Balleine and Killcross, 2006; Wassum and Izquierdo, 2015).

Similarly, to the role of the BLA in SOC, the role of the hippocampus in SOC is mixed. Lin and Honey (2011) found SOC was unaffected by pre-training lesions encompassing the dorsal and ventral hippocampus. On the other hand, Gilboa et al. (2014) found that these pre-training lesions prevented SOC, while the response to the food-predictive tone remained intact. However, their SOC procedure was a bit unorthodox in that after pairing the tone with food, they then paired the tone with the light (typically light is paired with tone). According to most accounts of value transfer [e.g., Temporal Difference Reinforcement Learning, see Sutton and Barto (1981)], this procedure is likely to occlude the transfer of value from the tone to the light because the value in these models is thought to back propagate to earlier predictors of reward. Thus, presenting the food predictive tone followed by light may have "forced" a more cognitive associative structure of SOC and thus relied on the hippocampus.

Interestingly, the retrosplenial cortex, a brain region that projects to (and receives information from) the hippocampus and that is known to be involved in learning and memory processes (Bucci and Robinson, 2014), also does not appear necessary for SOC (Todd et al., 2016). Ultimately, the fact that SOC may in some instances be reliant on the hippocampus but in other instances be hippocampal-independent, may again reflect the fact that SOC can be supported by several different types of associations (see **Figures 1B,C**). Findings of hippocampal involvement in SOC might depend on certain SOC procedures that encourage associations between the light and tone, or light and food, whereas those that suggest the hippocampus and retrosplenial cortex are not involved in SOC might derive from procedures that favor the light and valence of the outcome.

Sensory Preconditioning

SPC involves first presenting the light and tone together and then pairing the tone with food (or another outcome), which results in an appetitive response being elicited by both the light and tone. In this way, SPC can be taken as the strongest evidence in favor of animals learning associations between truly neutral stimuli, as neither stimulus was motivationally significant prior to their pairing. Unlike the mixed data that investigates the associative basis of SOC, it is reasonably well accepted that SPC entailed a cognitive representation between the light, tone, and outcome, which have been chained together by the inference that the light is likely to lead to food as its associate, the tone, is food predictive (i.e., light→tone→food; see **Figure 1D**; Rizley and Rescorla, 1972; Wikenheiser and Schoenbaum, 2016; Hart et al., 2020). This is because responding to the light in SPC is devaluation sensitive (Hart et al., 2020). Further, responding to the light in SPC is dependent on the status of the food-predictive tone (Rizley and Rescorla, 1972). That is, if responding to the tone is extinguished after the light and tone are presented together, the light will no longer support SPC. Thus, in contrast to much of the literature that has examined the associative structure underlying SOC, it is generally accepted that SPC produces a more complex cognitive representation of the relationships between the stimuli and outcome.

Recently, Sharpe et al. (2017a) demonstrated that SPC can fall prey to the blocking effect (see **Figure 2**; see also Denniston et al., 1996; Blaisdell et al., 1998). In a blocking procedure (see **Figure 2A**), a stimulus (e.g., tone) is established as food predictive. Subsequently, the tone is presented in compound with a novel stimulus (e.g., light) and followed by food. In this example, responding to the light on a subsequent test is believed not to occur because during the compound trials the animal is already expecting food after the presentation of the tone, and so there is no violation when the tone-light compound leads to the same food. As a violation of expectations (or prediction error) is thought to be required for learning to take place (Rescorla and Wagner, 1972), learning about the light is "blocked" because it does not coincide with a prediction error. Sharpe et al. employed an SPC procedure but added an additional blocking phase (see **Figure 2B**). That is after the light had been paired with tone (light→tone), the light and an additional novel stimulus (e.g., noise) were paired with the tone (light+noise→tone). Again, the

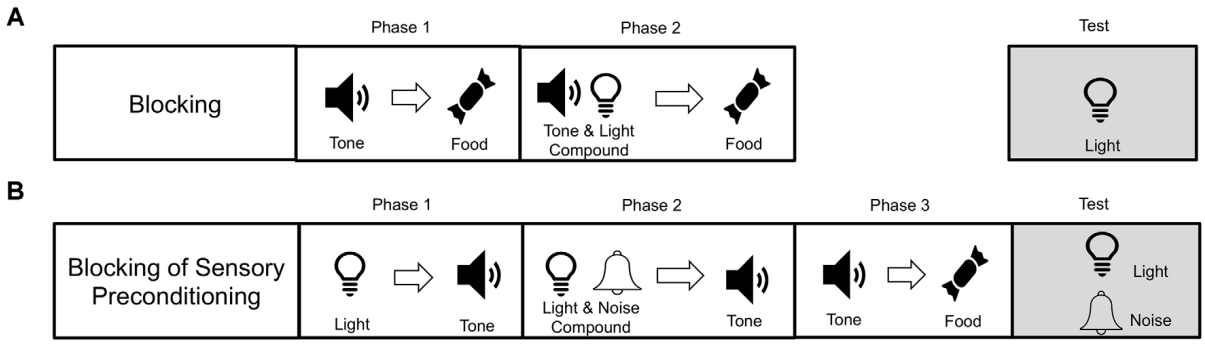

FIGURE 2 | Procedural paradigms for blocking and blocking of sensory preconditioning. **(A)** Blocking involves first pairing a stimulus (e.g., a tone) with an outcome (e.g., food). Then the tone is paired in compound with another novel stimulus (e.g., light), which leads to the same food outcome (light+tone→food). Blocking is said to occur when responding to the light is reduced as a consequence of the blocking procedure. **(B)** Blocking of sensory preconditioning is when subjects first learn that two neutral stimuli are related in time (e.g., light→tone). Then the light is presented in compound with another neutral stimulus (e.g., noise), and this leads again to the tone (i.e., light+nois→tone). Like blocking with food rewards, this procedure also reduces the sensory preconditioning effect. This demonstrates that the tone can serve as a sensory-specific prediction, which can be blocked, much like a food reward that has inherent value. This supports the idea that SPC is mediated by a representation of a sensory-specific relationship between the tone and light.

noise is redundant in predicting the tone. This is because the light already predicts the tone. Then, like in normal SPC, the tone is paired with food. Finally, Sharpe et al. (2017a) tested response to noise and found that it was successfully blocked, unable to promote appetitive responding. This demonstrates that neutral sensory stimuli (the light) can be used to block predictions of other neutral sensory stimuli (the noise), in a manner that transcends scalar value inherent in an outcome like food. Again, this supports the idea that training during SPC is supported by the development of sensory-specific representations between specific stimuli.

It has also been demonstrated that SPC explicitly does not involve the transfer of general value. Using a standard SPC design, where the light and tone are paired together, and then the tone is paired with food, Sharpe et al. (2017b) demonstrated that rats will not perform an instrumental response to receive presentations of the light (i.e., conditioned reinforcement). That is, the light would promote the appetitive response to go to the location where food is usually delivered, however, they would not press a lever that produced the light. This showed that the light was able to predict food, but did not become valuable in and of itself, supporting the view that SPC involved an association between the light and food, and not the light and general value, which could be achieved by virtue of the cognitive inference light→tone→food. Thus, SPC provides strong evidence that animals are capable of learning associations between various neutral stimuli which they can use to build internal models and help navigate towards rewards.

Compatible with the idea that SPC promotes the development of complex internal models of stimulus relationships, SPC recruits neural circuits that are known to play a role in these types of inferential processing, including the hippocampus and orbitofrontal cortex. For example, hippocampal neurons in CA1 increase in excitability during the pairing of the light and tone in SPC, and this excitability correlates with future response to the light after its pairing with the food-predictive tone. Further, subsequent lesions to those same stimulus-responsive neurons in CA1 disrupts responding to the light, but not the food-predictive tone (Port et al., 1987). The role of the hippocampus is also supported by studies in humans; neural activity in the hippocampus that is observed to the light during SPC is re-evoked when the tone is paired with reward, suggesting the development of the cognitive framework that supports SPC in the hippocampus (Wimmer and Shohamy, 2012). Recently, Barron et al. (2020) found that the hippocampus is not only important during light-tone pairings, but also, at the time of test, helping to support appetitive responding to the light. Specifically, optogenetic inhibition of CA1 neurons at test reduces responding to the light. Finally, areas adjacent and heavily connected to the hippocampus (e.g., retrosplenial cortex and perirhinal cortex) have been found to be necessary for the learning of stimulus-stimulus associations in SPC (Nicholson and Freeman, 2000; Robinson et al., 2011; Holmes et al., 2018; Fournier et al., 2020). Indeed, Wong et al. (2019) found that temporarily inactivating the perirhinal cortex while the tone was paired with the motivationally-significant outcome later disrupted motivated responding to the light, but not the tone. One interpretation of these data is that while the tone was paired with the outcome, the perirhinal cortex recruited a representation of the light, which was then associated with the outcome (Doll and Daw, 2016; Sharpe et al., 2017b). Thus while SPC is often thought to rely on a chain-like-association between light-tone-outcome, the perirhinal cortex might be critical in SPC procedures that promote mediated conditioning (i.e., resulting in a direct light-outcome association), and this appears to be dependent on the perirhinal cortex. In any event, these studies establish the hippocampus and several adjacent regions as critical to the development of SPC, often supporting a cognitive account of SPC but in other cases supporting the mediated account.

Similar to the role of the hippocampus in SPC, the orbitofrontal cortex is also critical to SPC. Specifically, neurons in the orbitofrontal cortex acquire responses to the light and

tone during SPC in a manner that reflects the development of a sensory-specific association between the light and tone (Sadacca et al., 2018). Further, optogenetic inhibition of these neurons prevents the development of the association between the light and tone, while pharmacological inactivation of orbitofrontal cortex at test also reduces responding. This strongly implicates the orbitofrontal cortex in the stimulus-stimulus associations at play in SPC, consistent with the core function of the orbitofrontal cortex in representing and navigating through the structure of our environments (Schuck et al., 2016; Wikenheiser and Schoenbaum, 2016; Wikenheiser et al., 2017; Sharpe et al., 2019). Given the role of both the hippocampus and orbitofrontal cortex in the SPC, and their complementary roles in learning, it becomes of interest to examine how these two regions might interact to produce the complex associations that drive behavior in SPC in future research.

Dopamine's Role in Pavlovian and Higher-Order Conditioning

One of the modern success stories of neuroscience has been the discovery that dopamine neurons in the midbrain serve as a neural substrate for reward prediction errors that drive appetitive Pavlovian conditioning (Waelti et al., 2001; Schultz, 2016). Schultz et al. (1997) famously showed that phasic activity in midbrain dopamine neurons increases following an unexpected reward, but not when a reward is expected. For example, these neurons will exhibit a phasic response if an animal is given a reward in an unpredictable manner, but not if they have learned that a stimulus reliably predicts the delivery of the reward. This also works in the reverse. If a reward was expected but not delivered, dopamine neurons show a phasic decrease in firing from baseline. Thus, these neurons follow the mathematical patterns described in error-reduction models of associative learning (e.g., Bush and Mosteller, 1951; Rescorla and Wagner, 1972), which conceptualize learning as a process that allows our expectations to meet reality and facilitates adaptive behavior.

The content of information that can be endowed by the phasic dopamine signal has been the topic of much debate. Initially, Schultz and colleagues described the increase in dopamine firing to reflect the transfer of scalar value inherent in the reward back to a stimulus that predicts its occurrence (Schultz, 1998). This conceptualization of phasic dopamine firing is consistent with that described by the model-free temporal difference reinforcement learning (TDRL) algorithm described by Sutton and Barto (1981). Critical to this proposal is that the reward-predictive stimulus has now been endowed with value inherent in reward, and not that the stimulus is associated with a sensory-specific representation of that reward. In other terms, the reward-predictive stimulus becomes "good" but does not evoke a representation of the reward. While this value is sufficient to alter behavior to the reward-predictive stimulus (i.e., induce an appetitive response), it constrains the role that the dopamine prediction error can have in learning to value-based associations that do not comprise detailed representations between stimuli (rewarding or otherwise).

Using Higher-Order Conditioning to Understand Dopamine's Contribution to Learning

A number of studies have now challenged the "value hypothesis" of the dopamine prediction error (Chang et al., 2017; Sharpe et al., 2017a, 2020; Takahashi et al., 2017; Howard and Kahnt, 2018; Keiflin et al., 2019). SPC and SOC are two procedures that have helped us understand how the dopamine prediction error contributes to learning and behavior. Of course, central to the narrative that dopamine represents reward prediction error is the idea that the dopamine signal continues to back-propagate to the earliest predictor of reward. This begs the question of whether the presence of the dopamine error at the onset of a reward can support conditioning in its own right. Maes et al. (2020) confirmed this by optogenetically inhibiting dopamine neurons in the ventral tegmental area (VTA) during SOC. Rats were first trained that a tone predicted food. Then, the light was paired with the tone, and dopamine neurons in VTA were inhibited across the transition between the light and tone, to prevent a prediction error from occurring. Maes et al. (2020) found that this reduced the subsequent ability of the light to support the appetitive response, demonstrating that the dopamine prediction error can function to support the development of the light-tone pairings in SOC.

The involvement of the prediction error in SOC is consistent with it acting either as a teaching signal that facilitates the development of associations between stimuli or acting as a value signal. However, examining the role of the prediction error in SPC can dissociate between these possibilities. In fact, all error correction models of learning that rely on value to drive learning [e.g., TDRL (Sutton and Barto, 1981)], or directly-experienced outcomes (Rescorla and Wagner, 1972), have historically struggled with explaining SPC because during preconditioning there is no expectation of reward with which to generate a reward prediction error (Miller et al., 1995). Sharpe et al. (2017a) used the novel blocking of SPC described above (see **Figure 2B**), in combination with optogenetics, which would allow a test of whether stimulating VTA dopamine neurons could drive the sensory-specific associations present in SPC. Specifically, Sharpe et al. first paired two neutral stimuli together (e.g., light→tone; A→X), and then presented the light in compound with another novel tone stimulus, followed by the tone (AB→X). Under normal circumstances, learning about the B→X relationship is blocked because A already predicts X. However, at the transition between AB and X, they briefly stimulated VTA dopamine neurons to produce a prediction error to see whether they could unblock the B→X relationship. Consistent with this, rats receiving a prediction error during AB→X trials showed higher levels of appetitive response to the B stimulus (after × has been paired with food), relative to rats that did not receive stimulation of VTA dopamine neurons. Sharpe et al. also found that the increased appetitive response to unblocked B was sensitive to goal devaluation, demonstrating that the presence of the dopamine prediction error endowed rats with a sensory-specific association between B→X that allowed B to become predictive of the specific food reward predicted by X.

The nature of SPC also facilitates an examination of whether dopamine can "add" value to an antecedent stimulus, as well as endowing a cognitive representation of stimulus transitions (e.g., light→tone). Recall, SPC does not endow the neutral, "preconditioned cue" (e.g., the light, A, or B) with a general value that supports conditioned reinforcement. Sharpe et al. (2020) used this premise to test whether optogenetic stimulation of dopamine neurons would allow the preconditioned cue to gain value that would promote conditioned reinforcement. That is, rats first experienced A and X paired together (A→X), and then the compound AB was paired with X, during which a prediction error was produced using optogenetics to unblock the B→X association. Here, rats showed higher levels of response into the food port when B was presented, showing dopamine unblocked the B→X association as previously demonstrated, but they would not press a lever to receive B. This demonstrates that stimulation of dopamine neurons facilitated the sensory-specific associations present in SPC, without adding value to these associations. These data are consistent with a role for the dopamine prediction error in acting as a teaching signal to drive associations between stimuli, and not as a signal that makes antecedent stimuli valuable.

DISCUSSION

Extended Role of Higher-Order Conditioning (and Potentially Dopamine) in Cognition

Midbrain dopamine neurons have now been causally implicated in both SOC and SPC (Sharpe et al., 2017a; Maes et al., 2020). While their involvement in SOC is not unexpected, that they're critical to the formation of the stimulus-stimulus association in SPC is surprising. This is because it positions dopamine to facilitate Pavlovian conditioning in a more flexible manner than previously conceptualized. Further, that these higher-order phenomena are associatively and neurologically distinct, and yet both fundamentally driven by dopamine, demonstrates that the role of dopamine prediction errors in learning need not be constrained by specific associative or neurological structures. Put another way, while dopamine was once thought to act as a value signal, which restricts the role it can play in associative learning, its involvement in higher-order conditioning processes suggests a much broader role for dopamine as a critical driver of Hebbian plasticity in many regions of the brain.

What are the implications of dopamine being involved in learning in such a broad way? To understand this, we need to think about the more general role of higher-order stimulus relations play in complex behavior and cognitive processes. For instance, Blaisdell and colleagues have explored the role of SPC in forming cognitive maps for spatial search (Blaisdell and Cook, 2005; Sawa et al., 2005; Bouchekioua et al., 2021). In one experiment, pigeons were taught a consistent relationship between visual landmarks on a 4 × 4 grid of gravel-filled cups (e.g., Landmark 2 is always two cups to the left of Landmark 1). Then, pigeons were separately taught a relationship between Landmark 1 and the hidden location of food (e.g., food is always one cup below Landmark 1). At the test, pigeons were presented with Landmark 2, and they were able to locate the food despite never having experienced the relationship between Landmark 2 and the food cup (Blaisdell and Cook, 2005). Similar results were obtained with pigeons using a modified version of this task using an operant touchscreen (Sawa et al., 2005), a computer version in humans (Molet et al., 2010), and the Morris water maze with rats (Chamizo et al., 2006). At present, there has been little investigation of the neural basis of the integration of these separately learned spatial maps, but it is exciting to think that dopamine may be critical for such sophisticated cognitive processes. Indeed, mice lacking D_1 dopamine receptors showed deficits in several spatial learning tasks without showing deficits in visual or motor performance (El-Ghundi et al., 1999).

There is also evidence for the integration of temporal maps in higher-order conditioning procedures. The temporal coding hypothesis describes the role time plays in associative learning experiments (Miller and Barnet, 1993; Savastano and Miller, 1998; Arcediano et al., 2003). Analogous to the role of higher-order conditioning in the integration of spatial maps, temporal maps acquired during Pavlovian conditioning can be integrated as a result of higher-order conditioning procedures. In one example, Leising et al. (2007) presented rats with a long (60 s) light paired with a short (10 s) tone[1]. However, one group of rats had the tone onset soon after the onset of the light ("group early"), thus it terminated well before the light terminated. The tone for the other group onset toward the end of the light presentation ("group late"). The tone was then paired with food, and appetitive response was examined to the light. Appetitive response was higher at the beginning of the light in the group early, relative to the group late. Similar results have been reported using fear conditioning procedures in rats (Savastano and Miller, 1998) and appetitive procedures in humans (e.g., Arcediano et al., 2003). This research demonstrates that rats had not only encoded the relationships between the light and tone but that they encoded these relationships into a temporal map. Again, it would be interesting to think about how dopamine might contribute to the inferred temporal relationships that can be formed during the SPC procedure.

Higher-order associative processes even appear to be involved in learning causal models of events. In a study using appetitive SPC, Blaisdell et al. (2006) showed rats can infer different causal models by integrating associations between the light, tone, and food (see also Leising et al., 2008). For instance, if rats are taught to encode a causal chain model whereby light→tone→food[1], they will expect the delivery of food: (1) if they press a lever to receive presentations of a light, or (2) if the light is presented alone without a lever press. However, if they are taught that the

[1]Note in the studies conducted by Sawa et al. (2005) and Blaisdell et al. (2006), tone and light were used in the opposite manner. That is, in Sawa et al., the tone was the longer 60 s cue and in Blaisdell et al., the tone was the common cause of light and food. We have revised our descriptions of these studies so that tone remains the cue directly paired with food as is consistent with all other examples in this article.

tone produces both the light and the food (i.e., rats learn that tone→light and also that tone→food), they will show appetitive response to the light when presented without a lever press, but not when the light was caused by a lever press. This is because they reason that, in the latter case, the light was caused by their own action and not by the tone, as it was in the former case. Thus, they did not expect the light to produce a food reward. This sophisticated reasoning process exhibited by these rats is akin to that observed in adults (e.g., Waldmann and Hagmayer, 2005) and children (e.g., Gopnik et al., 2004). These results and others (e.g., Dwyer et al., 1998) illustrate the far-reaching involvement of higher-order conditioning processes in many aspects of cognition. However, there is a dearth of research on the role of dopamine—or other neural substrates—in these domains.

What is next for those interested in understanding how dopamine and higher-order processes give rise to more complex cognition? One direction is that these sophisticated learning procedures could be coupled with recently developed technologies to record from and manipulate dopamine and related circuits. Because these techniques (e.g., optogenetics, calcium imaging) allow access to specific neuronal cell types and their projections and have a high degree of temporal specificity, they can be used to understand how distinct neuronal populations contribute to higher-order conditioning, as well as identify circuits between various regions that are involved in these processes, over very short timescales (Deisseroth, 2011; Patriarchi et al., 2018; Sych et al., 2019). This increase in specificity is critical to understanding the anatomical and associative basis of SOC and SPC. Similarly, while the circuits that support learning of neutral stimuli in SPC are ongoing, there is also recent evidence that some regions (e.g., Lateral Hypothalamus) might actively oppose the development of neutral associations that underlie SPC (Hoang and Sharpe, 2021; Sharpe et al., 2021). This brings to bear the possibility that there is more than one system at play in the forming of these associations. More generally, future research utilizing these tools in combination with higher-order tasks would help to elucidate how we make sense of the world around us, and how this may go awry in psychological disorders.

AUTHOR CONTRIBUTIONS

All authors contributed to the synthesis of research and writing of the article. All authors contributed to the article and approved the submitted version.

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frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
published: 03 November 2021
doi: 10.3389/fnbeh.2021.746161

Manipulating Memory Associations Minimizes Avoidance Behavior

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Memories of the past can guide humans to avoid harm. The logical consequence of this is if memories are changed, avoidance behavior should be affected. More than 80 years of false memory research has shown that people's memory can be re-constructed or distorted by receiving suggestive false feedback. The current study examined whether manipulating people's memories of learned associations would impact fear related behavior. A modified sensory preconditioning paradigm of fear learning was used. Critically, in a memory test after fear learning, participants received verbal false feedback to change their memory associations. After receiving the false feedback, participants' beliefs and memories ratings for learned associations decreased significantly compared to the no feedback condition. Furthermore, in the false feedback condition, participants no longer showed avoidance to fear conditioned stimuli and relevant subjective fear ratings dropped significantly. Our results suggest that manipulating memory associations might minimize avoidance behavior in fear conditioning. These data also highlight the role of memory in higher order conditioning.

Keywords: memory, sensory preconditioning, false feedback, avoidance, subjective fear ratings

OPEN ACCESS

Edited by:
Nathan Holmes,
University of New South Wales, Australia

Reviewed by:
Hugo Lehmann,
Trent University, Canada
Julieta Ramos-Loyo,
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Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *23 July 2021*
Accepted: *13 October 2021*
Published: *03 November 2021*

Citation:
Wang J, Smeets T, Otgaar H and Howe ML (2021) Manipulating Memory Associations Minimizes Avoidance Behavior. Front. Behav. Neurosci. 15:746161. doi: 10.3389/fnbeh.2021.746161

MANIPULATING MEMORY ASSOCIATIONS MIMIMIZES AVOIDANCE BEHAVIOR

In the early 1930s, one of Pavlov's dogs demonstrated the sensory preconditioning effect (see Kimmel, 1977). A whistle and a light were paired together several times, after which the dog was conditioned to flex its limb (using electric shock) upon presentation of the light. This resulted in the whistle also eliciting limb flexion, even though the whistle had never brought the dog harm (see also Brogden, 1939). Sensory preconditioning illustrates the generalization of fear responses from conditioned stimuli to neutral stimuli, which is a common symptom in anxiety disorders such as specific phobia, generalized anxiety disorder, and post-traumatic stress disorder (Dymond et al., 2015). Hence, it is crucial to understand the underlying mechanisms of the sensory preconditioning effect, such as why such fear generalization happens and how it can be interrupted.

In Pavlov's sensory preconditioning experiment, the dog obviously formed the "whistle-light" association as well as the "light-shock" association, and somehow integrated these two memory associations to guide its reaction toward the whistle. This reaction implies that memory plays a central role in sensory preconditioning learning because if either of the memory associations was not properly remembered, the dog should not fear the whistle. Surprisingly, the question how memory plays a role in sensory preconditioning has long been neglected (e.g., Shohamy and Daw, 2015), probably due to the fact that animal subjects were mostly used in sensory

preconditioning studies and it is not possible to ask animals what they remember about their fear experiences.

Recently, by testing human participants in conditioning paradigms, researchers have discovered the close link between explicit episodic memory and Pavlovian conditioning. On the one hand, fear conditioning can selectively prioritize fear related memories in long-term episodic memory (Dunsmoor and Kroes, 2019). For example, using a trial-unique fear conditioning paradigm, researchers found that people remembered the fear conditioned stimuli (CS +) better compared to the non-conditioned stimuli (CS-), and even memories of CS + related stimuli that were not conditioned got strengthened (Dunsmoor et al., 2015). On the other hand, memory has been found to play a role in various Pavlovian conditioning paradigms. Wimmer and Shohamy (2012) examined the neural mechanisms underlying human sensory preconditioning and observed that the preconditioning effect was predicted by activity in the hippocampus, where associated memories are usually formed. Other studies have found that forgetting or priming a specific memory can impact conditioned decision making (Murty et al., 2016; Bornstein et al., 2017). However, these studies were limited in using a reward learning task but did not examine the role of memory in fear conditioning. More recently, Bernstein et al. (2021) tested memory abilities of patients with anxiety disorders and found that poor mnemonic discrimination predicted overgeneralization of fear.

Taken together, the above studies suggest the possibly important role of memory in guiding (pre)conditioned behavior. Based on this observation, we wondered if fear related memories were to be manipulated, would fear conditioned behavior be impacted as well? It has been well established that human memory is a highly adaptive and constructive system where its elements can be easily manipulated *via* false feedback (Loftus, 2005; Frenda et al., 2011; Schacter, 2012). A classical study showed that participants misremembered seeing a "stop" sign after they received a verbal misleading information while in fact there was a yield sign (Loftus, 1975). More recent studies showed that encoded memories could be undermined or weakened after receiving false (verbal) feedback (Mazzoni et al., 2014; Otgaar et al., 2014; Wang et al., 2017, 2019; Li et al., 2020). For example, after participants performed actions such as clapping their hands in front of a video camera, their memories of the performed actions were tested a few days later, and false feedback was provided telling participants that their memories were wrong and some actions were never performed (Mazzoni et al., 2014). Participants' beliefs in their memories dropped significantly and some recollective aspects of their memories such as spatial and temporal clarity became weaker after receiving false feedback.

In a recent study, false feedback was provided regarding learned associations in a reward preconditioning task, and participants' learned memory associations were successfully undermined (Wang et al., 2019). In the study, participants learned that a picture (S1 +) was always paired with a patterned circle (S2 +) and the S2 + stimulus was later rewarded with money (US). Participants normally preferred the S1 + stimulus because the monetary value could be transferred to S1 + *via* S2 + in the memory network. However, after telling participants that their memories were wrong (e.g., the S1 + was not paired with S2 +), their associative memories between S1 + and S2 + were weakened significantly, leading to no preference to the S1 + any more. According to the spreading activation account of memory (Anderson, 1983; Roediger et al., 2001; Howe et al., 2009), S1-S2 association as well as S2-US association could be established in the memory network after learning. Attenuating the S1-S2 memory association thus could have interrupted the value transfer from S2 to S1 while the value transfer from US to S2 remained intact. This study again demonstrated the malleability of memory as well as the crucial role of memory in sensory preconditioning. Based on the reviewed results, we reasoned that fear related behavior could be modulated by providing false feedback to fear related memory associations.

To our knowledge, no research has been conducted concerning the manipulation of fear related memories and its consequences on fear conditioned behavior. By using a modified sensory preconditioning paradigm, the current study aimed to investigate the impact of manipulating memory associations on fear avoidance behavior and subjective fear ratings. Specifically, participants first learned associations between S1 + pictures and S2 + circles and then learned that S2 + stimuli led to noise. In a memory test later, participants were falsely told that the S1 + picture was not paired with the S2 + circle, but was associated with another non-conditioned circle. Based on the spreading activation theories (Anderson, 1983; Roediger et al., 2001; Howe et al., 2009), participants would be conditioned to form "picture—circle—noise" associations in the memory network. Thus fear of noise could be spread to the preconditioned picture *via* the conditioned circle. By providing false feedback to weaken the "picture—circle" association, the transfer of fear to the picture should be reduced. Therefore we expected that fear avoidance and subjective fear of S1 + pictures should be impacted by receiving false feedback.

METHODS

Participants

Before recruiting participants, we used G*Power 3.1 (Faul et al., 2007) to calculate the required sample size. With an estimated medium effect ($d = 0.4$) based on previous research (Wang et al., 2019), an *a priori* power analysis revealed that 52 participants were required to achieve a power of 0.80 (selecting *t test, matched pairs* in G. Power). Fifty-two students from Maastricht University, Netherlands, participated in our study either for course credits or a financial reward of €7.5. The sample consisted of 16 males and 36 females, with age ranging from 18 to 57 years old (M_{age} = 23.56, SD = 6.9). The study was approved by the ethical committee of the Faculty of Psychology and Neuroscience, Maastricht University. This study was pre-registered on the Open Science Framework[1].

[1]https://osf.io/zahu4

Design and Procedure

The study adhered to a within-subject design in which we provided either false feedback or no feedback in the memory test in order to manipulate memory associations. During the memory test, half of the associations received false feedback to break their established associations and the other half received no feedback (i.e., the control condition). The procedure basically followed the same steps as in previous sensory preconditioning research but with a memory feedback phase inserted before measuring fear (e.g., Wimmer and Shohamy, 2012; Wang et al., 2019). A loud blust of white noise served as the unconditioned stimulus (US) as a large body of research has validated the effectiveness of noise to induce conditioned fear responses (see Mueller et al., 2014; Sperl et al., 2016; Lonsdorf et al., 2017). The US intensity (75–105 dB, with 5 dB intervals) was calibrated for each participant before the experiment so that the noise as was perceived as unpleasant, but not painful by each participant. For instance, participants heard the lowest noise first and each time the noise was increased by 5 dB until it reached the participant's threshold. The experiment contained the following four phases.

Preconditioning Phase 1: Association Phase

As **Figure 1** shows, in the first phase, neutral pictures were paired with neutral patterned circles. Participants were only instructed to view some pictures on screen but were not explicitly told to memorize associations. A picture always appeared before a particular patterned circle. Each stimulus was presented for 1.5 s. The interval between the picture and the circle was 1 s and the interval between separate pairs was 3.5 s. Each pair was presented ten times, in randomized order. There were four categories of pictures (scene, furniture, body part or vehicle) and each category contained two pictures, a S1 + picture that was paired with a later fear conditioned circle and a S1- picture that was paired with a non-conditioned circle. Materials were counterbalanced in that each picture had equal chance to be a S1 + or S1- picture. Four filler pairs were also presented so that there were not too few items tested in the upcoming memory test and fear measurement phase. After all pairs were presented, participants rated their anxiety, arousal, pleasantness and liking for each stimulus on a 1–7 Likert scale to measure their baseline subjective affect ratings (Sperl et al., 2016; Lonsdorf et al., 2017).

Preconditioning Phase 2: Fear Conditioning Phase

During this phase, half of the circles (S2 +) that had been presented in the association phase were followed by a loud burst of white noise (US). The other half of the circles, labeled as S2- stimuli, were never paired with the aversive noise. Noise was administered *via* over-ear headphones. Each S2 + stimulus was conditioned 16 times, with 100% contingency rate while each S2- stimulus was presented 16 times but not conditioned (Wimmer and Shohamy, 2012).

Memory Feedback Phase

After the preconditioning phase, participants put down the headphones to receive instructions from the experimenter and to avoid any potential learning in the memory test. They completed an incidental memory test for learned associations in the first phase. Participants had to recognize which circle was paired with a particular S1 picture (two choices were provided: a correct one and a wrong one). Four associations (two S1 + and two S1- associations) were provided with false feedback after their recognition to undermine their memories. The computer program falsely indicated that the other (actually incorrect) association was the correct answer. Additionally, the experimenter verbally informed the participant that their memory was wrong and that the experimenter had clearly seen that the image was actually paired with the other, incorrect circle. Four other associations and four filler picture pairs received no feedback (i.e., no memory manipulation). After each recognition, participants were asked to rate their recollection ("Do you actually remember that the two items were paired together?") and belief ("Do you believe that the two items were paired together?") for the original memory association on an 8-point scale (1 = no memory or belief at all, 8 = complete memory or belief; Scoboria et al., 2014).

Fear Response Measurement Phase

Finally, participants went through the fear response phase to measure their avoidance behavior. For each trial, two pictures or two circles appeared left and right on screen. Participants were asked to choose a picture to avoid noise by pressing the F (left) or J (right) button, and choosing a wrong picture would bring a noise lasting 2 s. Such operant responses have been used in previous research to measure the preconditioning effect (Wimmer and Shohamy, 2012), which mimicked operant fear measurement in rodents (e.g., choosing between two chambers to avoid shock; Krypotos et al., 2015). Headphones were put up again so that they could receive the noise. Each trial consisted of a S1 + picture and a S1- picture from the same category (e.g., beach vs. lake or leg vs. arm). The S2 + and S2- circles were presented in another trial to assess fear learning. The same two stimuli were presented for four times, with each stimulus randomly appeared on the left or right side. To avoid re-learning in the fear measurement phase, noise was not administered immediately after each trial, but participants were told that noise would be accumulated if they made the wrong choice and they would receive a certain amount of noise in the end of each block. S1 pairs and S2 pairs were intermixed in each block. There were a practice block and two official blocks. There were 32 critical trials in total. After all trials, participants were asked again to provide subjective affect ratings for each stimulus.

RESULTS

Memory Data and Manipulation Check

Participants were asked to choose the S2 circle that they recalled was associated with a S1 picture. Memory accuracy for associations pre-false feedback [$M = 0.60$, 95%CI (0.49, 0.70)] did not differ significantly from the memory accuracy for associations in the no feedback condition [$M = 0.67$, 95%CI (0.58, 0.77)], $t(52) = -1.16$, $p = 0.25$, indicating equivalent levels of associative memories formed in the two conditions.

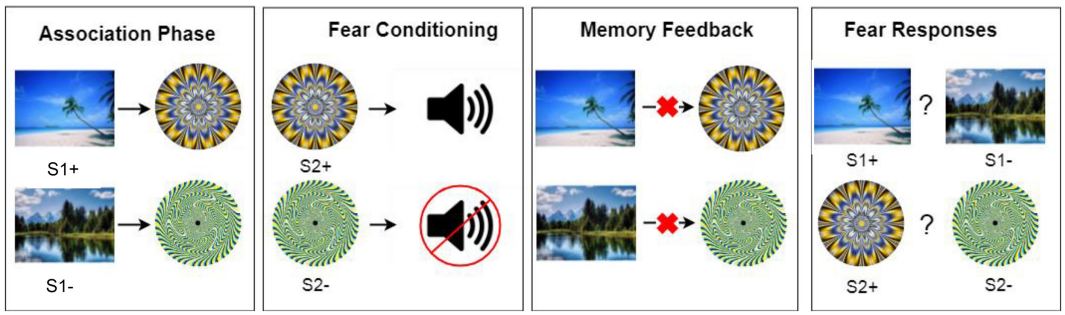

FIGURE 1 | A brief illustration of the procedure. Here illustrates one of the four picture categories (i.e., the scenery pictures). E-prime was used to present all stimuli. All S1 and S2 materials were generated from Wang et al. (2019).

After false feedback was provided in the memory test, participants rated their recollections and beliefs for the associations. A 2 Memory component (Recollection vs. Belief) × 2 Feedback (False vs. No) repeated measures ANOVA was conducted to examine participants' memory ratings. As **Figure 2** shows, there was a significant main effect of Feedback, F (1, 51) = 24.20, $p < 0.001$, partial $\eta^2 = 0.32$, and a significant main effect of Memory component, F (1, 51) = 42.63, $p < 0.001$, partial $\eta^2 = 0.46$. No interaction effect between Memory component and Feedback was found, F (1, 51) = 0.77, $p = 0.38$, suggesting that false feedback weakened both recollection and belief ratings of learned memory associations. Specifically, as **Figure 2** shows, false feedback has lowered recollection rating at the magnitude of Cohen's $d = 0.71$, $p < 0.001$ and lowered the belief rating with a size of Cohen's $d = 0.58$, $p < 0.001$.

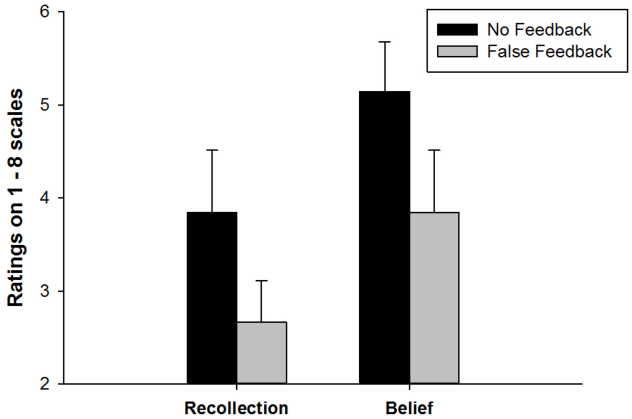

FIGURE 2 | Recollection and belief ratings in False feedback and No feedback conditions. Error bars represent 95%CI.

Avoidance Behavior

Avoidance of S2 +

First, we needed to make sure that participants learned fear for S2 + stimuli in the fear conditioning phase in the form of avoiding S2 + later. Avoidance was operationalized as the choosing rate of a fear conditioned image in the fear response phase. Hence, the lower choosing rate of a stimulus, the more avoidance to that stimulus; and 50–50 chance of choosing a stimulus in a pair suggests no avoidance or preference. For directly fear conditioned stimuli (S2 +), participants chose overall 16.23% of the times S2 + but 83.77% of the times chose S2- to avoid noise, suggesting successful fear learning of S2 + in the form of avoiding S2 +. The mean choosing rate of S2 + in the false feedback condition [M = 20.19%; 95%CI (0.12, 0.28)] did not statistically differ from that in the no feedback condition [M = 12.26%; 95%CI (0.05, 0.19); $p = 0.06$], both of which were significantly below 50% chance level ($ps < 0.001$). These data suggest that participants learned fear of S2 + to the same extent in the two conditions.

Avoidance of S1 +

Next, we analyzed how fear transferred to S1 + stimuli. The key dependent variable we were interested in was the avoidance of S1 + relative to S1-, that is the choosing rate of S1 + vs. S1- stimulus in different feedback conditions. Participants again showed preconditioned fear responses in the no feedback condition. That is, they avoided choosing S1 + [M = 33.65%; 95%CI (0.24, 0.43)] but chose S1- more often [M = 66.35%; 95%CI (0.57, 0.76)], demonstrated by the significant lower choosing rate of S1 + than 50%, $t(51) = -3.44$, $p = 0.001$, Cohen's $d = 0.48$. However, participants did not exhibit the fear preconditioning effect in the false feedback condition, that is, participants showed no avoidance to the S1 + stimuli but exhibited a choosing rate of S1 + [M = 43.99%; 95%CI (0.36, 0.52)] not different from chance level (50%), $t(51) = -1.51$, $p = 0.14$. Thus, false feedback decreased an absolute number of 10.34% fear avoidance choosing rate and relative 30.73% of the original fear avoidance compared to no feedback. More detailed analyses on the direct comparison between these two conditions will be discussed now.

To visualize participants' avoidance behavior regarding the preconditioned stimuli (S1 +), the net avoidance score of S1 + for each participant was calculated, which was the times of choosing S1 + stimuli minus times of choosing S1- stimuli over four rounds (see Wang et al., 2019). As **Figure 3** shows, a negative value indicates that participants avoided choosing S1 + stimuli over S1- stimuli; a positive value indicates participants preferred

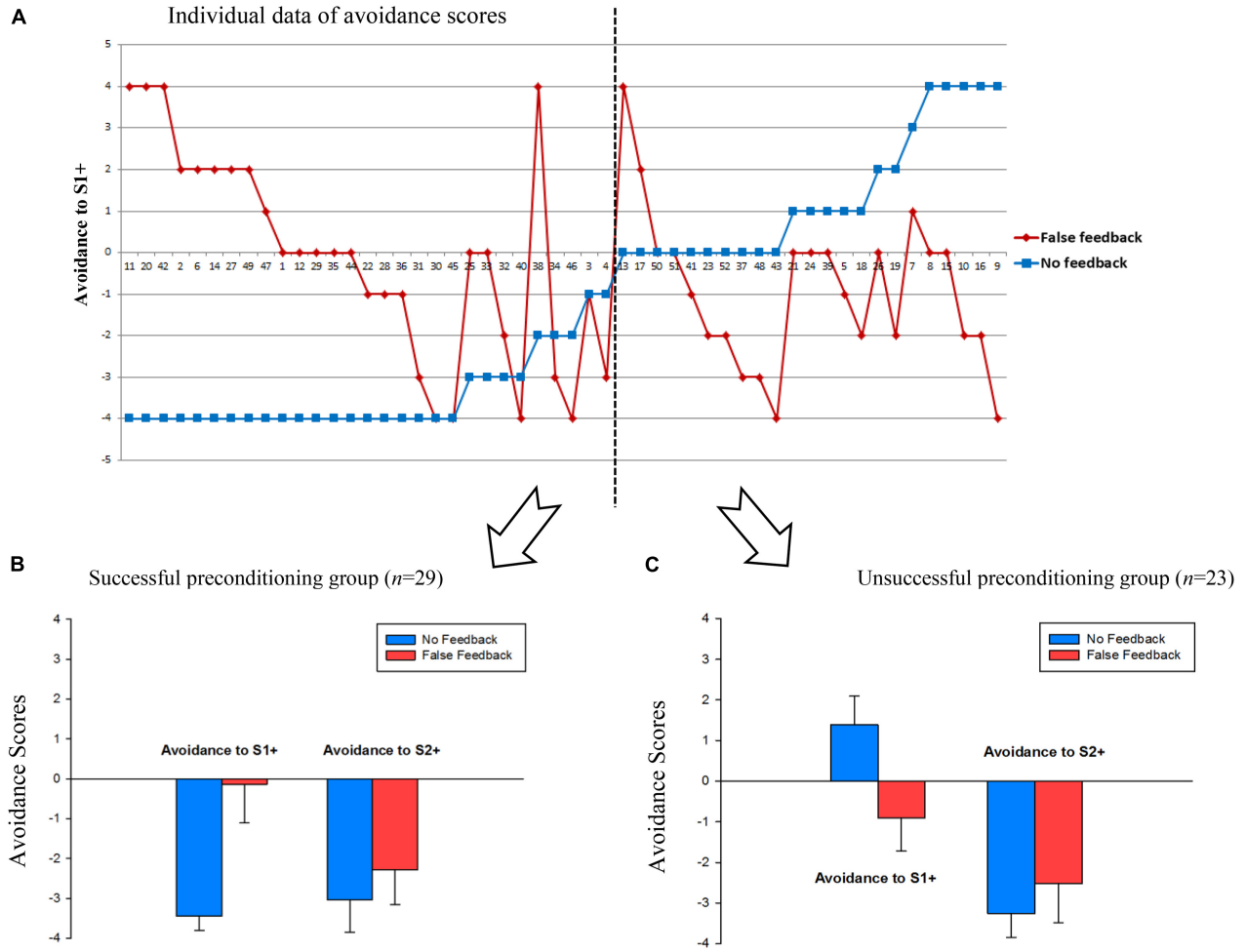

FIGURE 3 | (A) Individual data of avoidance scores in false and no feedback conditions. Avoidance score = (Times of choosing S1 +)—(Times of choosing S1-). The smaller the value, the more avoidance to S1 +. Each number on the X-axis represents one participant. Error bars represent 95%CI. **(B)** Avoidance scores to S1 + and S2 + stimuli in the successful preconditioning group. **(C)** Avoidance scores to S1 + and S2 + stimuli in the unsuccessful preconditioning group.

S1 + stimuli; 0 value means 50% chance level. The avoidance score ranged from −4 to 4. **Figure 3A** shows individual data on avoidance scores. Before analyzing the avoidance scores, it is crucial to check whether participants had been successfully preconditioned to fear S1 + stimuli in the control condition.

We found that in the control condition, there were 29 people who successfully learned the fear preconditioning (i.e., < 50% chance of choosing S1 +) and there were people (n = 23) who failed to learn the fear preconditioning (i.e., no avoidance or even preference of S1 +). Thus, we split participants into two groups: the successful fear preconditioning group and the unsuccessful fear preconditioning group. In the successful fear preconditioning group (n = 29), false feedback [M = −0.14, 95%CI (−1.11, 0.84)] eliminated avoidance behavior significantly relative to the no feedback condition [M = −3.45, 95%CI (−3.81, −3.09)], $t(28)$ = 5.94, p < 0.001, Cohen's d = 1.16; in the unsuccessful fear preconditioning group (n = 23), false feedback [M = −0.91, 95%CI (−1.73, −0.10)] still reversed the avoidance/preference behavior compared to the no feedback condition [M = 1.39, 95%CI (0.69, 1.09)], $t(22)$ = 4.29, p < 0.001, Cohen's d = 0.89. The individual data in **Figure 3A** shows that fear-avoidance behavior was impacted by false feedback at an individual level. Meanwhile, fear learning of S2 + stimuli was not impacted in both groups, i.e., participants in either feedback condition have successfully learned avoidance to S2 + (ps > 0.05).

Subjective Affect Ratings

Before conditioning, there was no significant difference between S1 + and S1- stimuli for baseline ratings of anxiety, $t(51)$ = 0.34, p = 0.73, arousal, $t(51)$ = −0.63, p = 0.53, pleasantness, $t(51)$ = −1.79, p = 0.08, or liking, $t(51)$ = −0.96, p = 34. To examine whether false feedback affected participants' affect ratings after the feedback phase in the successful preconditioning group, a 2 Feedback (False feedback vs. No feedback) × 2 Stimulus (S1 + vs. S1-) repeated measures ANOVA was conducted on the mean scores for each rating (anxiety, arousal, pleasantness and liking). Results showed a significant

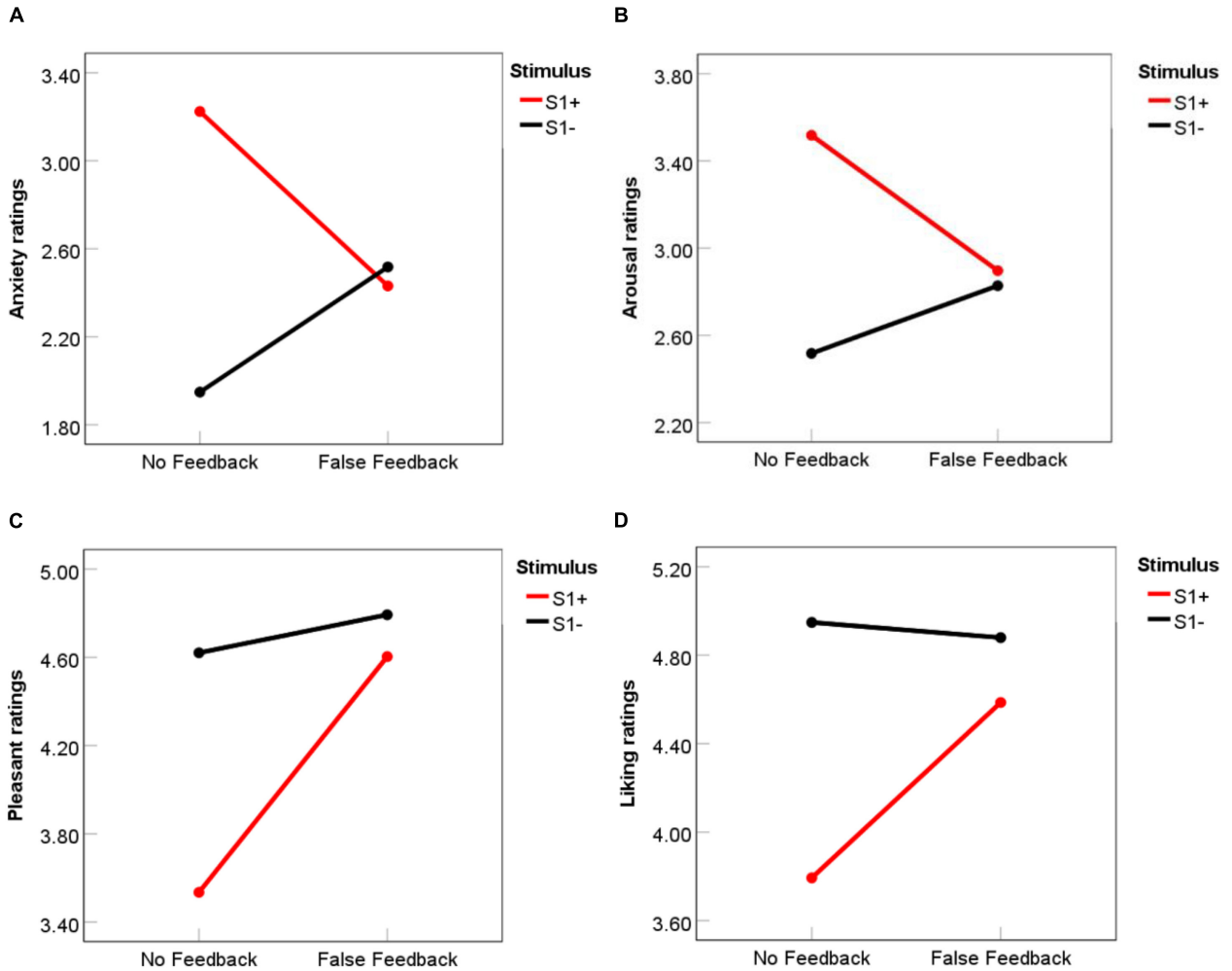

FIGURE 4 | Mean ratings of anxiety **(A)**, arousal **(B)**, pleasantness **(C)**, and liking **(D)** for S1 + and S1- in false feedback and no feedback conditions. Ratings ranged from 1 to 7. For Anxiety, 1 = not anxious at all, 4 (middle point) = moderate anxiety, 7 = very anxious; For Arousal, 1 = very calm, 4 = neutral, 7 = very aroused; for Pleasantness, 1 = very unpleasant, 4 = neutral, 7 = very pleasant; for Liking, 1 = very disliked, 4 = neutral, 7 = very liked.

Feedback × Stimulus interaction effects on both anxiety ratings, F (1, 28) = 8.81, p = 0.006, $\eta^2_{partial}$ = 0.24, and arousal ratings, F(1, 28) = 4.73, p = 0.038, $\eta^2_{partial}$ = 0.14. As demonstrated in **Figures 4A,B**, in the no feedback condition, participants had significant higher anxiety and arousal ratings for S1 + than S1- stimuli, (for anxiety, $M_{difference}$ = 1.28, p < 0.001, d = 0.78, for arousal, $M_{difference}$ = 1.00, p = 0.003, d = 0.60). However, false feedback eliminated the discrepancies between S1 + and S1- stimuli (for anxiety, p = 0.78, for arousal, p = 0.83), making participants no longer fear S1 + stimuli.

For pleasantness ratings (**Figure 4C**), a similar Feedback × Stimulus interaction pattern was observed, F (1, 28) = 4.04, p = 0.05, $\eta^2_{partial}$ = 0.13. Pleasant ratings for S1 + was significantly lower than S1- in the no feedback condition, $M_{difference}$ = 1.09, p = 0.001, d = 0.66, but no difference was found in the false feedback condition, p = 0.50. Liking ratings (**Figure 4D**) showed similar patterns but the interaction between feedback and stimulus did not reach significance, F (1, 28) = 3.27, p = 0.08, $\eta^2_{partial}$ = 0.11; only a main effect of stimulus was found that participants in general liked S1- more than S1 + , F (1, 28) = 14.92, p = 0.001, $\eta^2_{partial}$ = 0.35.

DISCUSSION

This is the first study that examined the impact of manipulating memory associations on fear avoidance behavior using a sensory preconditioning task. We found that false feedback directed at participants' memories resulted in decreased recollection and belief ratings for their learned associations, which demonstrates the malleability of memory and is consistent with previous research (Loftus, 2005; Schacter, 2012; Wang et al., 2019). More importantly, false feedback eliminated avoidance behavior and eased participants' subjective fear ratings relative to the control condition.

Our results support the role of explicit or episodic memory in fear learning. Episodic memory is the conscious recollection of learned experiences, including time, space or other contextual

details (Tulving, 2002). The current study measured participants' recollections of paired circles and pictures by asking them whether they actually remembered these events instead of asking them whether they knew such events (i.e., semantic), which is a common way to measure episodic memories. For a long time, episodic memory and Pavlovian fear conditioning were two isolated research fields (see a review by Dunsmoor and Kroes, 2019). The current study connects these two fields by manipulating learned associative memories in a fear preconditioning task. We found that undermining associative memories canceled avoidance behavior to the preconditioned stimuli and it reduced anxiety and arousal ratings compared to the control condition. We also measured liking and pleasantness ratings, which are opposite affects of subjective fear (Lonsdorf et al., 2017), but we only found significant changes on pleasant ratings induced by memory feedback. The reason might be that liking ratings is not directly related to fear, although it showed a similar pattern at a descriptive level, albeit not significant (p = 0.08). Overall, the current study points out that episodic memory might be one crucial mechanism underlying sensory preconditioning and it highlights the potential of using memory manipulation techniques to reduce fear. As we only measured avoidance behavior and subjective affect ratings, further research is needed to investigate how false feedback on memory associations may impact physiological fear responses such as skin conductance and startle responses.

The current results can be readily explained by the spreading activation account of memory (Roediger et al., 2001; Howe et al., 2009). According to this account, memory consists of mental representations of stimuli (i.e., "nodes" in a memory network) and associations between stimuli that participants have remembered from experience. For example, when a S1 + picture was paired with a S2 + circle, a "picture—circle" memory association could be encoded in the memory network; when the S2 + circle was paired with noise, a "circle—noise" could be encoded in the memory network as well. The key principle in the spreading activation account is that activation of one memory node spreads automatically to other memory nodes along the memory network. Thus, when participants saw a S1 + picture, activation was spread to a S2 + circle and then spread to noise, resulting in activation of noise when seeing a S1 + picture. As a consequence, participants should avoid S1 + pictures. In our study, false feedback attenuated the "picture—circle" memory association, so the activation spread to noise was to some extent interrupted and participants' fear responses to S1 + pictures were reduced.

The present results also support the memory-chaining account of sensory preconditioning relative to the online-integration account (see Rizley and Rescorla, 1972; Sharpe et al., 2017; Wong et al., 2019). The online-integration account, suggests that during the S2-noise fear conditioning phase, S1-S2 associations are activated and thus S1 is associated with noise already in the fear conditioning phase (Shohamy and Daw, 2015; Wong et al., 2019). If this is the case, manipulating the S1-S2 memory associations after the fear conditioning phase should not impact the preconditioning effect because S1 has been linked with fear already during the fear conditioning phase. However, our results showed that memory manipulation *after* the fear conditioning phase minimized the preconditioning effect, which is consistent with the memory-chaining account. That is, the transfer of fear might happen at the time of testing when presence of S1 stimulus activates the S1-S2 memory association, which in turn activates the S2-noise association, so participants showed avoidance to the S1 stimulus (Rizley and Rescorla, 1972; Sharpe et al., 2017). Thus, disrupting the S1-S2 memory association can cancel the preconditioning effect. The memory-chaining account of sensory preconditioning is intriguingly similar to the spreading activation account of memory, which deserves more investigation into the role of memory in sensory preconditioning.

Previous research on the neural mechanisms of the sensory preconditioning effect showed that the medial temporal lobe (e.g., hippocampus and its surrounding regions) are responsible for the S1-S2 phase of the preconditioning effect in both rodents and humans (Wimmer and Shohamy, 2012; Holmes et al., 2018), with also the amygdala being involved in the S2-US fear conditioning phase (Gewirtz and Davis, 2000). Coincidentally, the hippocampus/parahippocampal cortex, as well as regions in the anterior prefrontal cortex and medial parietal cortex, have been found to support the encoding and retrieval of episodic memory (Squire et al., 2000; Eichenbaum and Cohen, 2001). The hippocampus is mostly involved in forming associative memories while the prefrontal cortex is related to the monitoring or evaluation of memory traces (Mitchell and Johnson, 2009). Studies found that misinformation can impact activations in the hippocampus and prefrontal cortex, resulting in possible reconstruction of memory (Okado and Stark, 2005). The present findings imply that false feedback to learned associations may involve activities in the hippocampus and prefrontal cortex, which might lead to interruption of the S1-S2 memory associations, and that the integration between these regions and the amygdala may be important in both episodic fear memory and sensory preconditioning. Future research may look at the neural structures involved in memory-based fear learning.

This study might have certain clinical implications regarding how to interrupt the overgeneralization of fear without affecting the original fear learning memories. In our study, we did not manipulate memory associations in the fear learning phase (i.e., the "circle—noise" association), but we manipulated participants' learned associations in the preconditioning phase (i.e., the "picture—circle" association). Results showed that fear of conditioned S2 + circles remained intact but only fear of preconditioned S1 + pictures was reduced after our false feedback manipulation. This means that fear generalization to S2 + stimuli was stopped without affecting fear learning. In clinical settings, fear (over)generalization is a pathogenic marker of anxiety disorders (Lissek et al., 2010). Our study implies that cognitive methods or techniques targeting at patients' memories might be a fruitful future direction (see Phelps and Hofmann, 2019).

To conclude, the present research showed that false feedback to participants' learned associations minimized avoidance behavior and reduced subjective fear ratings of preconditioned

stimuli. These results suggest that episodic memory might be one of the mechanisms underlying sensory preconditioning. The time has come now to investigate how principles of memory may impact fear learning and fear generalization.

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 Ethical Committee of the Faculty of Psychology and Neuroscience, Maastricht University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JW, HO, and MH conceived the idea. JW and TS designed the experiment. JW collected the data and analyzed the data. All authors contributed to the writing of the manuscript.

FUNDING

This study was supported by a grant from the Ministry of Education in China (MOE) Project of Humanities and Social Sciences (No. 21YJC190015) to JW.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.746161/full#supplementary-material

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frontiers in Behavioral Neuroscience

MINI REVIEW
published: 11 November 2021
doi: 10.3389/fnbeh.2021.749517

Higher-Order Conditioning With Simultaneous and Backward Conditioned Stimulus: Implications for Models of Pavlovian Conditioning

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In a new environment, humans and animals can detect and learn that cues predict meaningful outcomes, and use this information to adapt their responses. This process is termed Pavlovian conditioning. Pavlovian conditioning is also observed for stimuli that predict outcome-associated cues; a second type of conditioning is termed higher-order Pavlovian conditioning. In this review, we will focus on higher-order conditioning studies with simultaneous and backward conditioned stimuli. We will examine how the results from these experiments pose a challenge to models of Pavlovian conditioning like the Temporal Difference (TD) models, in which learning is mainly driven by reward prediction errors. Contrasting with this view, the results suggest that humans and animals can form complex representations of the (temporal) structure of the task, and use this information to guide behavior, which seems consistent with model-based reinforcement learning. Future investigations involving these procedures could result in important new insights on the mechanisms that underlie Pavlovian conditioning.

Keywords: backward conditioning, higher-order conditioning, reinforcement learning, reward prediction error, simultaneous conditioning

OPEN ACCESS

Edited by:
Nathan Holmes, University of New South Wales, Australia

Reviewed by:
Melissa J. Sharpe, University of California, Los Angeles, United States
Michael Todd Allen, University of Northern Colorado, United States
Belinda Lay, Concordia University, Canada

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

Received: *29 July 2021*
Accepted: *18 October 2021*
Published: *11 November 2021*

Citation:
Prével A and Krebs RM (2021) Higher-Order Conditioning With Simultaneous and Backward Conditioned Stimulus: Implications for Models of Pavlovian Conditioning. Front. Behav. Neurosci. 15:749517. doi: 10.3389/fnbeh.2021.749517

INTRODUCTION

When being exposed to a new environment, humans and other animals can detect and learn that cues or contextual stimuli predict the prospect of meaningful events. This learning process and the behavioral change associated are classically named Pavlovian conditioning (Hollis, 1997; Fanselow and Wassum, 2015). Not limited to the pairing between a stimulus and an outcome, Pavlovian conditioning is also observed for stimuli that predict outcome-associated cues. This second type of conditioning, in which a cue predicts another predictive stimulus, is referred to as higher-order Pavlovian conditioning (Gewirtz and Davis, 2000). Higher-order conditioning is particularly interesting as it is an excellent way to understand how humans and other animals form complex representations of the structure of the environment, and how they use these representations to guide flexible responses (Jones et al., 2012; Sadacca et al., 2016, 2018; Wang et al., 2020; Chandran and Thorwart, 2021). In the lab, higher-order conditioning is studied by second-order conditioning or sensory preconditioning (e.g., Gewirtz and Davis, 2000; Parkes and Westbrook, 2011). In second-order conditioning, a stimulus (CS1) is first paired with an unconditioned stimulus (US) until CS1 evokes a conditioned response (CR). Then, in a subsequent phase, a second stimulus (CS2) is paired with CS1 but without the US. At the end of the second phase, and despite the absence

of direct pairing with the US, the presentation of CS2 alone is sufficient to evoke a CR (see **Figures 1A–C**; Rizley and Rescorla, 1972; Rashotte et al., 1977). The pairing procedure used in sensory preconditioning is similar to second-order conditioning except that the order of phases 1 and 2 is inversed (i.e., CS2 → CS1 pairings, then CS1 → US pairings; Rescorla and Cunningham, 1978).

Traditionally, investigations on higher-order conditioning involve forward CS2 → CS1 and CS1 → US pairings. However, far less investigated are procedures involving simultaneous or backward pairings (e.g., Prével et al., 2019). In this mini-review, we will argue that these procedures are actually particularly relevant for the understanding of Pavlovian conditioning. Results from these experiments are indeed difficult to interpret in terms of the Reward Prediction Error (RPE) hypothesis (Schultz and Dickinson, 2000) and for models that implement this learning-rule like Temporal Difference (TD) learning models (Sutton and Barto, 2018). On the opposite end, the results seem to be conceptually consistent with model-based reinforcement learning systems (Daw et al., 2005; Gläscher et al., 2010; O'Doherty et al., 2017) and call for new investigations on the underlying computational mechanisms. After a presentation of the RPE hypothesis and a description of how a TD approach can account for higher-order conditioning, we will present results from higher-order conditioning studies that used simultaneous and backward pairing. We will discuss how far they are difficult to interpret from a reward prediction error perspective and how they seem to support model-based reinforcement learning systems. We will conclude this mini-review by discussing the perspectives offered by follow-up studies on higher-order conditioning with simultaneous and backward pairing.

REWARD PREDICTION ERROR AND HIGHER-ORDER CONDITIONING

Historically, one of the most dominant hypotheses about Pavlovian acquisition has been the RPE hypothesis (Schultz and Dickinson, 2000; Niv and Schoenbaum, 2008). This hypothesis states that a change in the value of a CS is driven by the discrepancy between the outcome expected from that stimulus, and the outcome actually received. Quantitative formulations of the RPE hypothesis are now largely based on TD learning (Niv and Schoenbaum, 2008; Ludvig et al., 2012; Sutton and Barto, 2018). Close to the Rescorla and Wagner (1972) model in terms of learning rule, TD models present the advantage of solving some of its important failures. The TD approach makes notably successful predictions about second-order conditioning, a phenomenon difficult to explain in terms of the Rescorla and Wagner model (Miller et al., 1995). In TD models, RPE (δ) is defined by:

$$\delta_{t+1} = R_{t+1} + \gamma V_{t+1} - V_t$$

Where R_{t+1} is the observed reward at t+1, V_{t+1} and V_t are the predicted value at t+1 and t, and γ is a discount factor (with $0 < \gamma \leq 1$). δ is used to update the prediction made at t by:

$$V_t = V_t + \alpha\,(\delta_{t+1})$$

Where α is a learning rate parameter (with $0 < \alpha \leq 1$).

Using this learning rule the TD models of Pavlovian conditioning can successfully explain second-order conditioning (Seymour et al., 2004; Sutton and Barto, 2018; Maes et al., 2020; see **Figures 1D,E**). In the first phase of the procedure, the pairing between CS1 and the US results in a positive δ and the acquisition of predicted value from CS1 (i.e., positive V_{CS1}). Then, this predicted value can be used to drive learning on CS2 in the second phase of the procedure. Despite the absence of reward during the second-order conditioning phase (i.e., $R_{CS1} = 0$), the positive value of V_{CS1} is sufficient to produce a positive δ (i.e., $\gamma V_{CS1} - V_{CS2} > 0$) and to increase the predicted value from CS2 (V_{CS2}). Interestingly, at the neural level, it has been found that the activity of dopaminergic neurons in a similar task moves backward from the US to the first predictive stimulus cue (i.e., CS2), as it would be predicted by TD models (Schultz, 2015).

Thus, TD learning seems particularly relevant to understanding the acquisition of higher-order predictive values, both at a behavioral and a neural level. The approach, however, is not without limitations. Particularly, the model fails to explain the acquisition of predictive value by CS2 in sensory preconditioning tasks: Due to the absence of reward in phase 1 and the predicted value of zero for CS1 (i.e., $R_{CS1} + \gamma V_{CS1} = 0$), a change in V_{CS2} is not expected according to TD models. However, the evidence from measuring responses to CS2 suggests the acquisition of predicted value from the stimulus. This challenge to TD learning has been repeatedly highlighted in the literature, and it becomes one of the arguments against the hypothesis that Pavlovian conditioning is only driven by RPE (Niv and Schoenbaum, 2008; Sadacca et al., 2016). Much less considered is the challenge posed by results from higher-order conditioning studies that involve a simultaneous or a backward CS1. Here, we believe that these results are particularly relevant for our understanding of higher-order learning. The next section will be dedicated to these findings.

HIGHER-ORDER CONDITIONING WITH SIMULTANEOUS AND BACKWARD PAIRING

In Pavlovian conditioning, the classic pairing procedure used to study the acquisition of new stimulus-outcome associations is the forward procedure in which the CS precedes the presentation of the US. Contrasting with this, in simultaneous and backward pairing the CS is presented simultaneously and after the US, respectively (**Figures 2A,B**). Experiments that used these procedures classically showed low response rates to the CS, or even the development of conditioned inhibition (Spooner and Kellogg, 1947; Fitzwater and Reisman, 1952; Moscovitch and LoLordo, 1968; Siegel and Domjan, 1974; but see

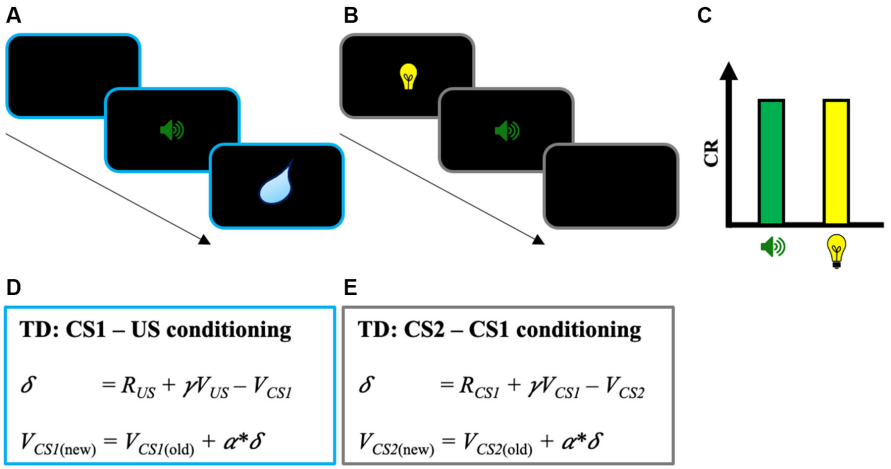

FIGURE 1 | Illustration of the second-order conditioning procedure. **(A)** Phase 1: First-order conditioning between a stimulus (CS1—sound) paired with an unconditioned stimulus (US—water). **(B)** Phase 2: Second-order conditioning between a second stimulus (CS2—light) paired with the previously paired stimulus CS1. **(C)** Classic results found in the second-order conditioning task with the conditioned response (CR) evoked both by CS1 and CS2. In sensory preconditioning, the procedure is similar except that phases 1 and 2 are inversed. **(D)** TD learning for the first-order conditioning phase with change in CS1's predicted value V_{CS1}. Note that V_{US} is zero because of the absence of predicted value at the time of the US. Because R_{US} is positive, the pairing between CS1 and the US results in a positive δ (i.e., $R_{US} - V_{CS1} > 0$), and the acquisition of predicted value from CS1 through the update of V_{CS1} ($V_{CS1(new)} = V_{CS1(old)} + \alpha^*\delta$). **(E)** TD learning for the second-order conditioning phase with change in CS2's predicted value V_{CS2}. Note that R_{CS1} is zero because of the absence of reward at CS1. Here, the positive V_{CS1} learned during the first-order conditioning phase is sufficient to produce a positive δ (i.e., $\gamma V_{CS1} - V_{CS2} > 0$) and to increase the predicted value from CS2 (V_{CS2}). TD, Temporal Difference.

Spetch et al., 1981; Prével et al., 2016). These observations suggest that simultaneous and backward pairings are not appropriate procedures to produce a robust CR, which is consistent with TD models: When a simultaneous or a backward CS is presented, the stimulus is never followed by a reward at $t + 1$. Thus, a change in V_{CS} is consequently not expected from those pairing procedures. In addition, a higher-order cue (CS2) that precedes a simultaneous or a backward CS1 should not produce robust responding because V_{CS1} is zero at the end of the first-order conditioning phase.

From a functional perspective, the absence of a robust CR in simultaneous and backward pairing is not surprising if we consider that the function of the response is to prepare the organism for the US (Hollis, 1997). Because the CS is not predictive of the US, there is a priori no reason to expect a preparatory response evoked by that stimulus. However, what is not clear is whether the absence of a CR measured to the simultaneous or backward CS really means that subjects did not learn anything from these pairing procedures due to the RPE of zero. Alternatively, it is possible that subjects in these experiments learned an association between the simultaneous or backward CS and the US, but these associations are simply not overtly expressed due to the absence of predictive value of the CS (Arcediano and Miller, 2002). In what follows, we will discuss the results from higher-order conditioning studies that support this interpretation.

For example, Barnet et al. (1991) tested whether a first-order stimulus CS1 paired simultaneously with a US can support the conditioning of a second-order stimulus CS2 (see **Figure 2C** for an illustration). Consistent with common findings in simultaneous conditioning studies, the authors reported low responses evoked by CS1 in comparison to a forward first-order stimulus, supporting the idea that the procedure is not efficient to produce a robust CR. However, when in a subsequent phase a second-order stimulus CS2 was paired with CS1 using a forward pairing (i.e., CS2 → CS1 pairings), the authors found a substantial level of CR evoked by CS2, despite the low response measured on CS1. These results by Barnet et al. (1991) seem difficult to explain in terms of TD learning. According to the account described above, a change in CS2 value (V_{CS2}) depends directly on CS1's own value (V_{CS1}). Thus, a second-order pairing with a first-order stimulus CS1 that evokes low response (and with presumably a low predicted value) should result in low response to CS2. However, the evidence of substantial response to that stimulus challenges this interpretation. Later, Barnet and Miller (1996) extended their investigations to backward conditioning. In phase 1 of a second-order conditioning task, a first-order stimulus CS1 was paired to a US using backward pairing. This resulted in the development of conditioned inhibition, a classic result of this pairing procedure. Interestingly, when in phase 2 a second-order stimulus CS2 was paired with CS1 using forward pairing, this resulted in substantial CR to CS2 despite the inhibitory status of CS1. Again, the result is problematic for the TD account of second-order conditioning. It is not clear why a first-order stimulus CS1 with an acquired inhibitory status (and presumably, a negative predicted value V_{CS1}) can support the conditioning of a second-order stimulus CS2.

These results by Barnet and Miller (1996) were replicated by Cole and Miller (1999), who found that the effect varied

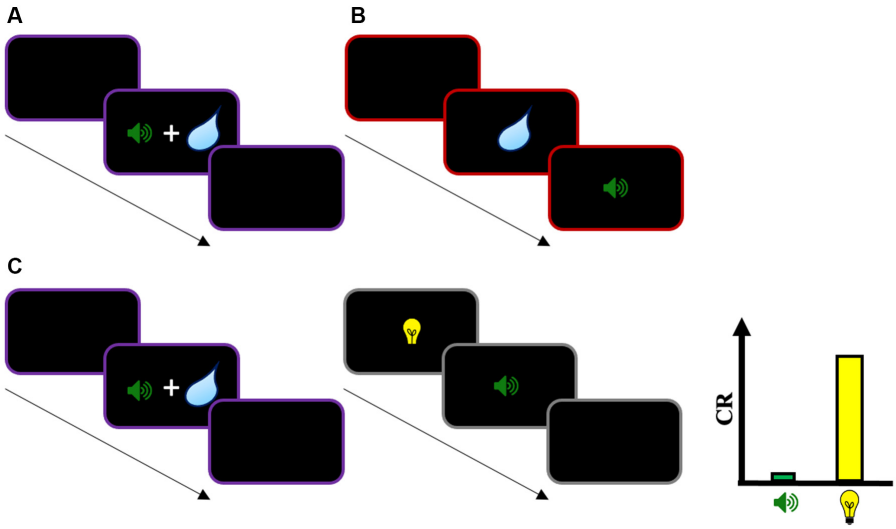

FIGURE 2 | Illustration of simultaneous, backward, and second-order conditioning with simultaneous CS1. **(A)** Simultaneous conditioning with a stimulus (CS1—sound) presented simultaneously with an unconditioned stimulus (US—water). **(B)** Backward conditioning with CS1 presented after a US. **(C)** Second-order conditioning with simultaneous CS1: In phase 1, a stimulus CS1 is presented simultaneously with a US. In phase 2, a second stimulus CS2 is paired with CS1 through forward pairing. During the test, while CS1 will evoke low conditioned response (CR), CS2 will evoke substantial CR. According to the TD account, a low CR evoked by CS1 is expected because CS1 is not followed by the US (i.e., $R_{US} = 0$) in phase 1. In addition, a change in CS2 value (V_{CS2}) depends directly on CS1's own value (V_{CS1}). Thus, a second-order pairing with a first-order stimulus CS1 that evokes a low CR level (and with presumably a low predicted value) should result in low responding to CS2. The evidence of substantial response to that stimulus challenges the TD account. The same holds for a model-based account of higher-order learning if the change in V_{CS2} depends on CS1's own predicted value V_{CS1}. Instead, it seems necessary for CS2's predicted value to be based on US expectations to account for this finding. Note that the same pattern of results is observed for second-order conditioning with backward CS1, and for sensory preconditioning with simultaneous and backward CS1.

with the number of backward pairing trials in phase 1. More exactly, the authors reported that a backward CS1 supports second-order conditioning only when the number of backward pairing trials is low or high, but the CR to CS2 decreases at an intermediate number of trials. Parallel to these investigations, Barnet et al. (1997) demonstrated that a backward CS1 can support stronger second-order conditioning compared to a forward first-order CS1, despite a lower CR to that backward stimulus. More recent observations by Prével et al. (2019) are consistent with these findings. Specifically, the authors demonstrated that a second-order stimulus CS2 can function as an efficient conditioned reinforcer for instrumental response in the test phase, even when that stimulus was paired with a backward CS1 that did not evoke CR during phase 2. Finally, similar findings were reported using sensory preconditioning. For example, Matzel et al. (1988) found evidence of substantial sensory preconditioning with simultaneous and backward first-order paired stimuli. Barnet et al. (1997) reported results similar to their observations in a second-order conditioning task with sensory preconditioning. Finally, Arcediano et al. (2003) found successful sensory preconditioning with backward first-order CS. In summary, it seems clear from all these experiments that a simultaneous or backward first-order CS can support higher-order learning, even if that same stimulus shows a low CR level or conditioned inhibition. As we have seen, the evidence is difficult to explain based on TD models, and particularly with regard to sensory preconditioning due to the additional absence of RPE in phase 1. In the next section, we will describe the model-based reinforcement learning account as a valuable alternative to TD learning.

MODEL-BASED REINFORCEMENT LEARNING AND HIGHER-ORDER CONDITIONING

Because of the challenges posed by effects like sensory preconditioning, the last 10–20 years have seen the development of another class of models termed model-based reinforcement learning (Daw et al., 2005; Gläscher et al., 2010; O'Doherty et al., 2017). In this approach, human subjects and animals can learn a model of the environment to guide appropriate responding. This model includes the states encountered by the subjects, as well as the transition probabilities between states and the available rewards. This contrasts with (model-free) TD models in which the subjects merely learn the predicted value of each state, but not the potential transition between states. Another characteristic of the model-based approach resides in the fact that the subjects can use the learned-transitions between states to update the states' value through a (mental) simulation mechanism. This second aspect is particularly interesting because it can be used to account for goal-directed phenomena like devaluation (e.g., Wilson et al., 2014), but certainly also sensory preconditioning: Here, during phase 1 participants would learn the transition probability between CS2 and CS1, before learning during phase

2 the positive predictive value of CS1 based on its direct pairing with the US. Then, through a simulation mechanism, the learned transition between CS2 and CS1 and the expected value from V_{CS1} could be used to update V_{CS2}, i.e., subjects could (mentally) assign a new value to CS2 based on the learned-transition between CS2 and CS1 (i.e., CS2 is followed by CS1), and the learned predicted value from CS1. For example, if we adapt the model-based mechanism proposed by Wilson et al. (2014) to sensory preconditioning, at the end of training the (model-based) value of CS2 could be updated through:

$$V_{CS2} = V_{CS1} \times p\,(\text{CS1} \parallel \text{CS2})$$

Where p(CS1 ‖ CS2) is the estimated learned probability of CS2 leading to CS1, and V_{CS1} is the predicted value from CS1. Because p(CS1 ‖ CS2) and V_{CS1} are positive due to the pairings in phases 1 and 2, this would result in a positive V_{CS2} and the ability of the stimulus to evoke CR.

In addition to sensory preconditioning, the model-based learning approach seems also very promising to account for the findings presented in the previous section. The assumption that humans and animals can learn a model representing the structure of the environment, and that they use this model to flexibly update the value of states (stimuli) and guide responding, seems remarkably consistent with the results described above. In these experiments, it is as if subjects learned the (temporal) structure of the task and used this structure to infer a predictive value from CS2 and guide responding: Participants first learned that CS1 is presented simultaneously or after the US, but the absence of predictive value of CS1 prevented the development of a robust CR. However, through the integration of the associations learned in phases 1 and 2, the forward pairing between CS2 and CS1 conferred a predictive value between CS2 and (the representation of) the US, which resulted in the CR measured in response to this stimulus (see Arcediano and Miller, 2002). Interestingly, multiple results in the literature suggest the acquisition of such temporal maps (e.g., Cole et al., 1995; Arcediano et al., 2005; Thrailkill and Shahan, 2014). However, it must be noted that it is not clear what the exact computational mechanism is that supports the temporal integration and the acquired predicted value on CS2 observed in these studies. If we consider for example the model-based mechanism described above, because a change in V_{CS2} depends in this formulation on CS1's own predicted value V_{CS1}, the problem remains that it is difficult to understand why a stimulus that shows low CR or conditioned inhibition supports substantial CR to CS2. Instead, it seems necessary for CS2's predicted value to be based on US expectation to explain the results presented in the previous section. More investigations will be necessary to propose a complete account of higher-order learning, and particularly a mechanism that allows the temporal integration of the task structure to guide flexible and adaptive responses.

CONCLUSION AND OUTLOOK

The evidence from sensory preconditioning and higher-order conditioning with simultaneous and backward pairing pose a challenge to the assumption that Pavlovian conditioning is driven only by RPE. Rather, these observations suggest that subjects were able to learn a representation of the (temporal) structure of the task and to use this representation to guide their responses, which seems consistent with the assumptions of model-based reinforcement learning. However, the exact nature of the computational mechanisms is still missing. Here, we are highlighting three fruitful directions for future investigations on higher-order conditioning with simultaneous and backward CS. First, it must be noted that model-free reinforcement learning approaches such as TD models are not necessarily dismissed by these results. To the best of our knowledge, the consensus in the literature seems to assume a co-existence of both model-free and model-based reinforcement learning systems, representing habitual and goal-directed behaviors, respectively (Gläscher et al., 2010; Wilson et al., 2014; O'Doherty et al., 2017). Additional investigations on higher-order conditioning with simultaneous and backward pairing could provide new insights regarding the computational mechanisms that underly model-based reinforcement learning and temporal integration in higher-order conditioning, as well as how model-free and model-based reinforcement learning computations are integrated in that context. Second, an important research question in the study of higher-order conditioning concerns the nature of the associations learned (Gewirtz and Davis, 2000). New investigations using the procedures described in this mini-review could give new insights into what is learned by subjects in these tasks, which in turn could have important implications on the underlying computational mechanisms. Finally, an important hypothesis in the neuroscientific domain is that the phasic activity of dopaminergic neurons represents the RPE teaching signal in the context of model-free reinforcement learning (Schultz, 2015). However, recent results suggest instead that this activity could reflect model-based computations (Sadacca et al., 2016; Sharpe et al., 2017; Langdon et al., 2018; Sharpe and Schoenbaum, 2018). Here, it might be interesting to study how this activity changes during the presentation of CS1 and CS2 depending on the pairing conditions and to test which neural structures are subserving task representations and value updates.

AUTHOR CONTRIBUTIONS

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

FUNDING

This study was supported by a starting grant of the European Research Council (ERC) under the Horizon 2020 framework (grant No. 636116 awarded to RK).

ACKNOWLEDGMENTS

We would like to thank the three reviewers for their helpful comments on previous versions of the manuscript.

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frontiers in Behavioral Neuroscience

PERSPECTIVE
published: 23 November 2021
doi: 10.3389/fnbeh.2021.766767

Higher-Order Conditioning in the Spatial Domain

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Spatial learning and memory, the processes through which a wide range of living organisms encode, compute, and retrieve information from their environment to perform goal-directed navigation, has been systematically investigated since the early twentieth century to unravel behavioral and neural mechanisms of learning and memory. Early theories about learning to navigate space considered that animals learn through trial and error and develop responses to stimuli that guide them to a goal place. According to a trial-and error learning view, organisms can learn a sequence of motor actions that lead to a goal place, a strategy referred to as response learning, which contrasts with place learning where animals learn locations with respect to an allocentric framework. Place learning has been proposed to produce a mental representation of the environment and the cartesian relations between stimuli within it—which Tolman coined the cognitive map. We propose to revisit some of the best empirical evidence of spatial inference in animals, and then discuss recent attempts to account for spatial inferences within an associative framework as opposed to the traditional cognitive map framework. We will first show how higher-order conditioning can successfully account for inferential goal-directed navigation in a variety of situations and then how vectors derived from path integration can be integrated via higher-order conditioning, resulting in the generation of higher-order vectors that explain novel route taking. Finally, implications to cognitive map theories will be discussed.

Keywords: higher-order conditioning, cognitive map, spatial memory, associative learning, inference, spatial integration, navigation

OPEN ACCESS

Edited by:
Arnau Busquets-Garcia, Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Spain

Reviewed by:
Siobhan Robinson, Hamilton College New York, United States
John Pearce, Cardiff University, United Kingdom

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

Received: *30 August 2021*
Accepted: *25 October 2021*
Published: *23 November 2021*

Citation:
Bouchekioua Y, Kosaki Y, Watanabe S and Blaisdell AP (2021) Higher-Order Conditioning in the Spatial Domain. Front. Behav. Neurosci. 15:766767. doi: 10.3389/fnbeh.2021.766767

INTRODUCTION

O'Keefe and Nadel (1978) elaborated on the separate systems that utilize bottom up vs. top-down processes for navigation, each having a separate neural basis. The taxon system is bottom up and utilizes processes of path integration and associative learning (e.g., beacon homing). The locale system is top down and involves the allocentric representation of space as the cognitive map. One important tenet of the cognitive map theory is that its manifestation should not be explained by path integration (O'Keefe and Nadel, 1978; Bennett, 1996; Singer et al., 2006), the process by which animals go back to a home nest using a direct vector after a random journey, first reported by Darwin (1873) as dead-reckoning. This basic and automatic ability to compute a direct home vector can be solely based on internal information such as vestibular and proprioceptive perception (Collett et al., 1998; Müller and Wehner, 1988; Schatz et al., 1999; Etienne and Jeffery, 2004).

Constantly updating distance and direction from the current position to the start place during a journey enables direct return to the start place simply by following the last computed vector, even if this goal-vector points toward a path never experienced. This can be achieved through vector arithmetic where a journey is decomposed into vectors, with the first vector taking origin at the start place, and with any change of direction triggering the calculation of a new vector. Path integration, along with other egocentric based navigation strategies, is part of the taxon system, as opposed to the map-based locale system responsible for allocentric navigation strategies (O'Keefe and Nadel, 1978).

Contrary to the traditional view of cognitive map theory that a detailed spatial map is necessary for spatial inferences, higher-order conditioning is a bottom-up associative process that provides an alternative means to learn relationships between events without direct experience, and that enables spatial inferences without a detailed spatial representation connecting all spatial locations visited during prior navigation. Higher-order conditioning was first discovered by Pavlov (1927) and consists of the conditioning that can occur to a cue (e.g., a conditional stimulus or CS) even when that CS had not been directly paired with a rewarding outcome (e.g., an unconditional stimulus or US). For instance, in a sensory preconditioning (SPC) procedure, first discovered by Pavlov (Kimmel, 1977) and later confirmed by Brogden (1939), a CS (CS2) is first paired with another CS (CS1) in stage 1, followed by a second stage during which CS1 is paired with an unconditional stimulus (US). When testing CS2 in stage 3, a conditional response is observed even though CS2 had not been directly paired with the US. Higher-order conditioning has been observed in a broad range of species, including, but not limited to, crickets (Matsumoto et al., 2013), molluscs (Kojima et al., 1998), drosophila (Heisenberg et al., 2001), honeybees (Muller et al., 2000), pigeons (Sawa et al., 2005), rodents (Brogden, 1939), and humans (Brogden, 1947), and suggests that it is a fundamental mechanism of learning in organisms possessing a central nervous system.

SPATIAL INTEGRATION

The following is a representative overview of studies specifically designed to assess higher-order conditioning in the spatial domain, in different species, namely, pigeons, rats and humans, and with spatial information of various nature (intra-maze cues, extra-maze cues and boundaries).

In Pigeons With Intra-Maze Cues

Assessment of the role of higher-order conditioning in goal-directed navigation was instigated by Blaisdell and Cook (2005) in a study that involved pigeons navigating in an open area with intra-maze cues as CSs (**Figure 1A**). During the first phase, pigeons were trained to find a hidden food reward (G1) located between two intra-maze cues, L and T, with their spatial relationship with respect to each other kept constant across trials. However, their position in the arena was changed stochastically between trials, thus neutralizing the use of spatial information provided by room cues to find G1. During the second phase, L was removed, and pigeons learned to find the hidden food reward (G2) in a new location with which T kept a constant spatial relationship. The animals were then tested in the presence of L alone, which had never directly been paired with G2. If we consider that pigeons learned the L→T vector in Phase 1, and the T→G2 vector during Phase 2, integrating these two vectors through the common element they share (i.e., T) should enable pigeons to infer vector L→G2. Consistent with a strategy based on the integration of these spatial relationships, pigeons spent more time searching for food at the location based on the inferred L→G2 vector than at other locations (with the exception of the location of G1 acquired during Phase 1). While the procedure used in this experiment does not strictly follow the sensory preconditioning procedure, where neutral stimuli are originally paired in the absence of a US, it recapitulates the critical feature of higher-order conditioning, being that separately learned associations can be integrated if they share a common element or event. Overcoming this limitation, Sawa et al. (2005) replicated this finding in a 2D version of the task using a touchscreen panel, where the US (i.e., food reward) was not present during the first phase. Spatial integration has been confirmed in humans, using a 3D virtual reality version of the task, where the reward was presented only during Phase 2 (Molet et al., 2011).

In Rats With Extra-Maze Cues

In a similar study, the ability to integrate a set of extra-maze cues in a Morris-water maze task was assessed in rats (Chamizo et al., 2006) by training the animals to find a hidden platform using a set of three extra-maze cues (e.g., A, B and C) during a first phase (**Figure 1B**). In a second phase, a second set of cues including C (e.g., C, D E) was paired with the hidden platform at the same location. When tested with one cue from each Phase (e.g., A and E), without the presence of the common landmark C, rats searched more for the platform in the quadrant where its location would be inferred if the missing cues were retrieved by an integration of both set of cues separately learned. Moreover, the animals performed as well as rats trained with all cues present on all trials. Rats trained without a common cue C between the two sets of cues (e.g., A, B and C in Phase 1 and D, E and F in Phase 2) failed to search for the platform in the correct quadrant more than a chance amount of time.

In Humans With Boundaries

Consistent with the studies presented above, it has been shown in humans that boundaries of an environment can be used as CSs and follow the rules of higher-order conditioning (Bouchekioua et al., 2013). In a 3D virtual maze, human participants were trained to explore two paths connecting a starting room with two separate adjoining boxes ("Common middle boxes," see **Figure 1C**). During a second Phase, the participants directly started from one of the common middle boxes on half of the trials, and from the other common middle box on the second half of the trials. Each common middle box was connected to an end box via a pathway. The participants were allowed to navigate from a common middle box to an end box during Phase 2 and found a reward (a virtual treasure Chest) in only one of the end boxes,

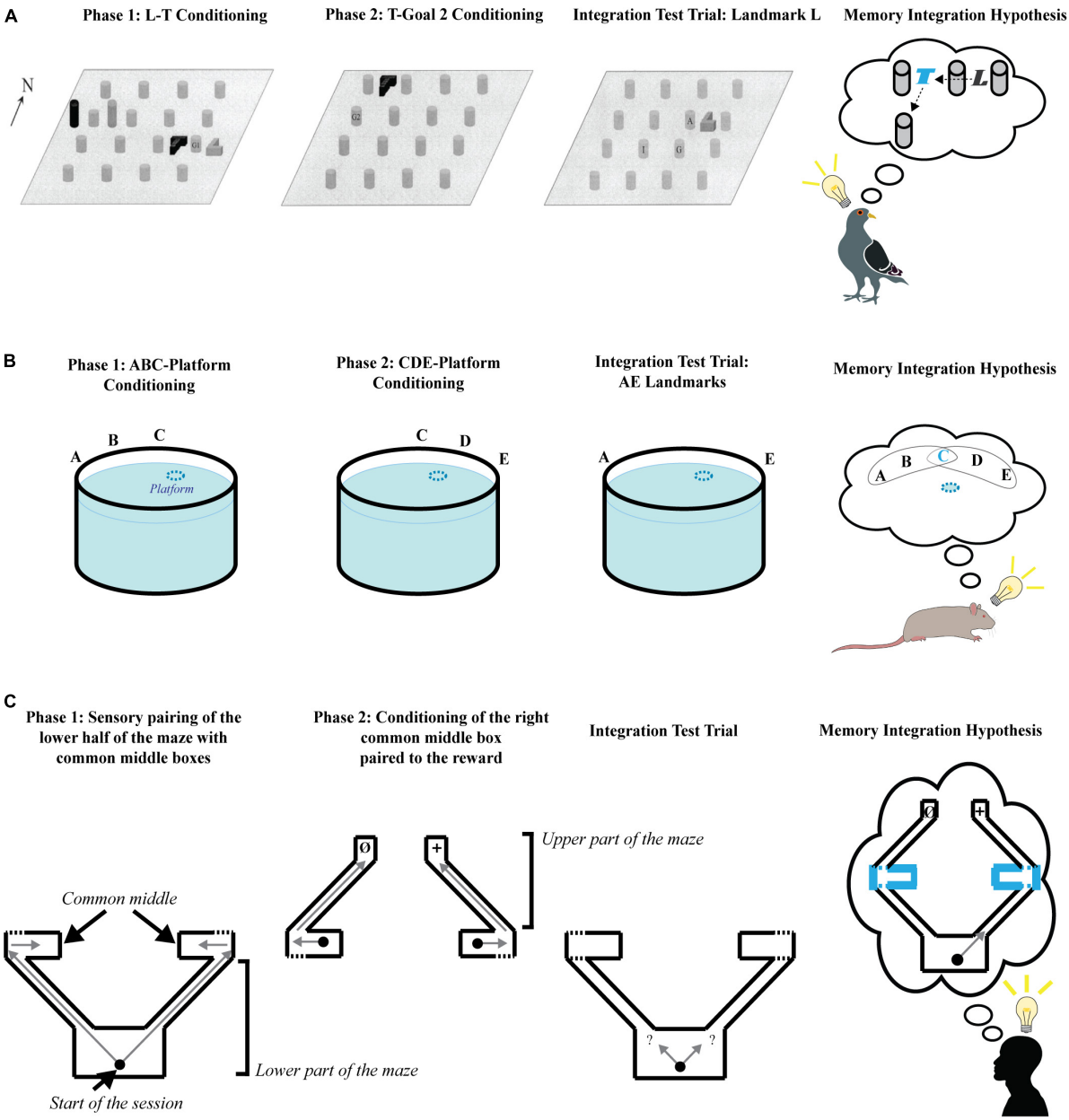

FIGURE 1 | Schematic representation of spatial integration tasks following the sensory preconditioning procedure. Each experiment consists of two learning phases followed by a test phase. A schematic representation of the hypothetical result of memory integration is proposed for each study. **(A)** Diagram of the experimental design used by Blaisdell and Cook (2005), showing the configuration of the 4 × 4 grid of gravel-filled cups, the hidden food (G), and the landmarks (T, L, and two foils). The left panel show the spatial arrangement of the consistent landmarks (T and L), goal 1 (G1), and inconsistent landmarks (cylindrical foils) during Phase 1. The second panel (from the left) shows the spatial arrangement of landmark T to goal 2 (G2) during Phase 2. The third panel (from the left) shows the spatial arrangement of landmark L and the potential locations of search during the integration test. Letters on bottom panel: I = predicted cup for choices guided by the L → T goal 2 hierarchical map, A = predicted cup for choices guided by the phase 1 L→ goal 1 vector, and G = predicted cup for choices guided by a generalization to L of the T→ goal 2 vector. Reprinted with permission of the authors. The last panel (from the left) represents the hypothetical integration of separately formed memories, where the common element (i.e., landmark T) appears in cyan color. **(B)** Schematic representation of the pool and the configuration of the landmarks used by Chamizo et al. (2006) in each phase of the task. The blue dashed-line circle represents the hidden platform under opaque water. A, B, C, D, and E are extra-maze visual cues. The last panel (from the left) represents the hypothetical integration of separately learned memories, where the common element (i.e., landmark C) appears in cyan color. **(C)** Schematic representation of the 3D virtual task used by Bouchekioua et al. (2013) for each phase of the task. The black dot represents the start place in all panels, and the gray arrows of the two first panels (from the left) show the journeys experienced by the participants. In the second panel (from the left), Ø = no reward is present in the end box, + = a reward is present in the end box. The gray arrow question marks in the third panel (from the left) show the two possible choices during the test. The last panel (from the left) represents the hypothetical integration of separately formed memories, where the common elements (i.e., the common middle boxes) are in cyan bold lines, and the gray arrow represents the choice leading to the reward.

while the remaining end box was left empty. In a subsequent one-shot test, the participants were placed in the starting room and asked to find the treasure. Most participants chose the pathway that led to treasure, suggesting that they were able to infer that this pathway leads to the goal-place, even though they had never directly experienced the reward from this pathway. This suggests that they had integrated each of the separately learned sets of geometrical information that shared a common element. A control group directly started outside of the common middle boxes in Phase 2 and were not allowed to associate the common middle boxes to their respective end box. While they had equal experience of the end boxes, and found the treasure in only one of them, they performed at chance level at test.

Taken together, these studies suggest that higher-order conditioning enables the use of various types of spatial CS (intra-maze, extra-maze, boundaries) for flexible goal-directed navigation where the complete route leading to a goal place is explored in a piecemeal fashion. The mental integration of these spatial routes using higher order conditioning processes supports spatial inference, such as the selection of the shortest route or a correct route that leads to a reward and suggests that the taxon-system is sufficient for supporting flexible goal-directed navigation. The traditional view of the cognitive map is that it enables the linking "together conceptually parts of an environment which have never been experienced at the same time" (O'Keefe and Nadel, 1978; Poucet, 1993), and can be used to mentally retrieve parts of the environment that are outside of the field of perception (Pick and Rieser, 1982). Contrasting with this view, a recent study found that rats flexibly adapted to changes in connectivity between four familiar rooms, while CA1 hippocampal place-cells did not respond to changes in connectivity (Duvelle et al., 2021). Thus, associative learning and cognitive map theories provide satisfactory accounts for inferential goal-directed navigation supported by mental integration of separately explored, but familiar routes. The ability of taking a novel route never fully explored either within a single session, or in a piecemeal fashion during separate sessions, has been predicted by the cognitive map theory but not by associative learning theories. It is indeed difficult to conceive how a stimulus that had never been perceived could be associated with a goal-place. While the cognitive map theory predicted the ability of novel route taking, it did not provide any mechanism for it. How could places never explored be integrated into a mental representation of the environment? We present next a study that implies both spatial integration and novel route taking in rats within the same experiment (Roberts et al., 2007), and show how a combination of two strategies from the taxon-system, namely higher-order conditioning and path integration, can explain these results.

NOVEL ROUTE TAKING

Several criteria have to be met when assessing novel route taking: (1) The novel shortcut should be performed in a one-shot trial; (2) It should not result from a behavior previously reinforced (e.g., response learning); (3) Extra-maze and intra-maze cues directly paired with a goal-place should not be available during the novel shortcut test (i.e., beacon homing must be prevented); and (4) Taking a novel route should not be explained by simple path integration. Roberts et al. (2007) tested the ability of rats to a take novel route in an experiment designed to meet all the above criteria. To that aim, the authors used an enclosed maze covered by a ceiling and the maze was rotated to a random orientation (north, south, east, or west) before each trial, thus neutralizing extra-maze cues, and no distinct intra-maze cue was available during the test session. To ensure that the animals could not associate any room cues with the goal-place while being moved from their cage to the maze, they were transported in an opaque box, and released directly inside the maze. White noise was played to cover any sound that could serve as a directional or positional cue, and the maze was washed with a vinegar and water solution after each trial to eliminate any olfactory trace that may have been left by the rat. The first phase of training took place in a restricted area of the entire maze, composed of only three boxes (A, B, and D; see **Figure 2A**) and their connecting alleys. Rats were first allowed to consume a small portion of food in one box before being placed in one of the two remaining boxes. Across the first phase of training, rats learned to directly find the food reward from the two remaining boxes, with all possible combinations of boxes serving as a starting place or goal-place. During the second phase of training, all rats consumed a small amount of food in the new box C, but only half of them, constituting the experimental group, were then placed in B and allowed to go back to C. Blocks were introduced to prevent rats from exploring any of the maze beyond the internal alleys that directly connected B to C. During the test, all rats were pre-fed in goal place C and transported to D. The access to internal alleys of the maze were blocked, and the animals were given a choice between two new adjacent paths never previously explored or perceived, only one of them leading to goal place C. Even though animals had no chance to directly connect D to C during previous training, only those of the experimental group successfully chose the correct path leading from D to C by making a right turn. It is worth noting that reinforcement of a left turn in Phase 2 neutralizes a simple egocentric account, as the correct turn during the test trial was to the right side. As predicted by cognitive map theory, the results provide unequivocal evidence that rats were able to take a novel route in goal-directed navigation, without using simple path integration, response learning, extra-, or intra-maze cues, and cannot be explained by trial-and-error learning. It is unclear how referring to a spatial representation of the maze could help in solving the novel route task. Place cells, neurons in the hippocampus that have the property of manifesting a maximal firing rate for a specific area of a familiar environment, have been proposed to support prospective planning of spatial navigation. Serial activations of place cells coding for adjacent places and covering a familiar environment has thus been proposed as a mechanism of cognitive mapping for flexible goal-directed navigation (O'Keefe and Nadel, 1978; Pfeiffer and Foster, 2013). Rats had, however, no occasion to form a map-like representation of the correct route in the experiment of Roberts et al. (2007) using place cells, as they explored none of

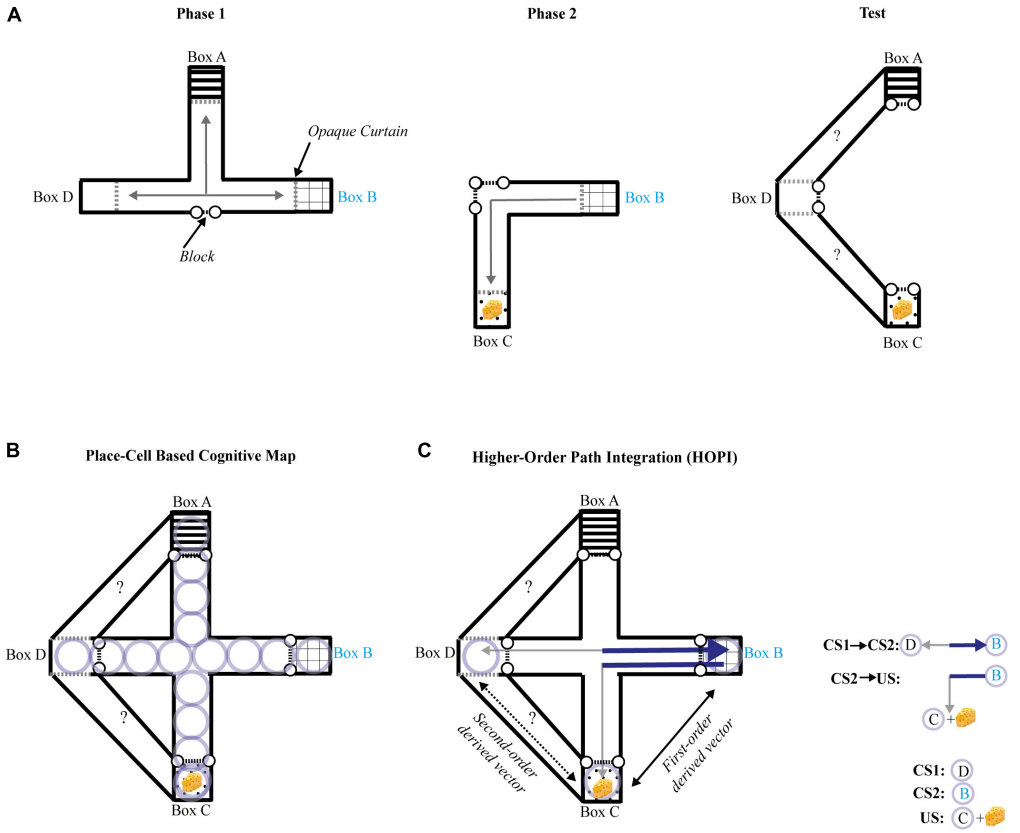

FIGURE 2 | (A) Schematic representation of the maze and procedure used by Roberts et al. (2007) seen from above during each phase of the experiment. Black dashed lines with dots represent barriers, and gray arrows represent the paths traveled by rats during training phases 1 and 2 (left and middle panels). The right panel shows the configuration of the maze during the test phase, where two new adjacent paths were opened, while access to the rest of the maze was blocked. The question marks represent the possible choices during the test. Dashed gray lines represent opaque curtains, the common element (box B) is annotated in cyan characters. The yellow cheese represents the reward. **(B)** Schematic representation of the maze used by Roberts et al. (2007) seen from above during the test phase. Light purple circles represent place-cell representations formed during the training phases. **(C)** Schematic representation of the maze used by Roberts et al. (2007) seen from above during the test phase. The underlying strategy based on higher-order path integration (HOPI, see Bouchekioua et al., 2021) is represented as follows: solid gray arrows represent direct vectors formed during the training phases 1 and 2; solid black arrow represents a first-order derived vector computed by vector arithmetic between direct vectors. The dark blue section of the direct vectors in bold lines represents the common segment they share; dashed black arrow represents a second-order derived vector resulting from vector arithmetic between a direct vector and a first-order derived vector.

the two new adjacent paths available during the test (**Figure 2B**)[1]. Recent studies suggest that place-cells are not involved in route planning, but rather play a role in discriminating alternative routes (Grieves et al., 2016) irrespective of their relationship with the reward (Duvelle et al., 2019). We recently proposed a model called higher-order path integration (HOPI) to explain spatial inferences such as that shown by the rats in Roberts et al. (Bouchekioua et al., 2021). HOPI combines two strategies that O'Keefe and Nadel (1978) assign to the taxon system, path integration and higher-order conditioning, and presumes that place-cell activity reflects known routes rather than inferred, unfamiliar ones. HOPI can explain novel shortcut behavior if we consider the possibility that vectors derived from path integration can be stored in reference memory as direct vectors. Separate direct vectors can be integrated into first-order derived vectors if they share a common segment or connecting point. Furthermore, separate first-order derived vectors that share a common segment or connecting point can be further integrated into second-order derived vectors through vector arithmetic (Etienne et al., 1998). Such integrated first-order and second-order derived vectors could then be used to navigate along novel routes to reach goals (**Figure 2C**). HOPI explains how vectors, whether direct or derived from path integration, can be integrated the same way other types of cues are, that is, following rules higher-order conditioning processes. Specifically, associations sharing a common element can be mentally connected. In addition, HOPI applies vector arithmetic (i.e., addition and/or subtraction) to vectors sharing a common element, which results in the generation of higher-order vectors that connect places with approximated distance and directional information, even

[1]We should also consider the possibility of path integration being performed virtually via a sequential activation of place cells covering the internal paths connecting D to C, resulting in a bidirectional vector that informs the direction and distance between D and C. While purely hypothetical, this explanation violates one of the criteria according to which cognitive mapping should not be explained by path integration.

if the places covering these mentally computed vectors have never been explored. In other words, HOPI informs the shortest direction and distance between two places, without requiring prior exploration of this novel shortcut. Importantly, unlike the explicit map-like mental representation envisioned by Tolman (1948) with neural underpinnings proposed by O'Keefe and Nadel (1978), spatial relationships of the environment are built up from bottom-up associative and path integration processes (i.e., the taxon system) to form an implicit knowledge of cartesian space and the objects, events, and places within it that can be accessed at any time to derive navigation choices, even for unfamiliar routes. To clarify this subtle difference imagine the following scenario. A person is placed in a familiar location with which they have had a lot of prior experience, such as a city street in their home town. Let's imagine the person was asked where a building is located in comparison to their current position, and that they had never previously traveled from their current location to the target building, thus, the route connecting them is unfamiliar. According to cognitive map theory, the person could draw with pen and paper a top down map of the route directly connecting the two. According to HOPI, however, the person would not be able to draw a map of the route, but would be able to point in the general direction of the target building and provide an approximate distance to reach it.

DISCUSSION

The limitations attributed to conditioning when it comes to explaining apparently complex spatial behaviors is often due to a simplistic conception of associative learning processes that are reduced to S-R learning and/or first-order CS-US associations. We demonstrated how higher-order conditioning enables flexible goal-directed behavior, even in a one-trial test consisting of a new situation never encountered during the learning phase. Higher-order conditioning (Pavlov, 1927) results in the association between a CS2 and a US that have never been physically paired and can thus lead to new adaptive behaviors in the absence of directly experiencing a CS2 and the US together. Associative learning theories involve the acquisition and retrieval of CS-US associations. Higher-order conditioning extends this process to associations between neutral stimuli or routes, thus enabling larger connected networks of associations. These interconnected associative networks in turn support inferences of novel relations between any two points or bits of information within the network, thereby enabling rapid and flexible navigation even through unfamiliar territory. Spatial integration and novel route taking are no longer the sole purview of cognitive map theories (i.e., the locale system), but now can be accounted for by bottom-up associative processes (i.e., the taxon system). Specifically, we showed how a combination of higher-order conditioning and path integration processes can result in the formation of a novel goal-directed vector without requiring a representation of this route. Furthermore, a place-cell based cognitive mapping strategy may fail on its own to generate novel routes (Duvelle et al., 2021). O'Keefe and Nadel (1978) proposed that the taxon system is involved in non-flexible navigation, such as response learning, while the locale system supports flexible behaviors such as taking a detour, a shortcut, or a novel route. We propose that the taxon/locale systems dichotomy is not realistic and should be abandoned, and that an allocentric map-like representation of the environment as formulated by cognitive map theories is not necessary nor sufficient for flexible navigation that involves taking novel routes. Place coding, however, does retain an important function in discriminating parts of familiar environments. Our analysis reveals that associative learning and path integration processes can play a much larger role in flexible navigation. The next challenge consists of experimentally addressing the hypotheses proposed in the present article, for example by adapting strategies where the use of goal-directed vectors would be neutralized (for a review, see Wehner, 2020) in tasks testing spatial integration and novel route taking, and to determine the underlying neural processes that support the processes elucidated by HOPI.

AUTHOR CONTRIBUTIONS

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

FUNDING

Support for this research was provided by the National Science Foundation Grant BCS-1844144 (AB).

ACKNOWLEDGMENTS

We are grateful to Toma-Izaiah Uchimoto and Paul Craddock for their insightful and constructive comments.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 06 December 2021
doi: 10.3389/fnbeh.2021.771767

The Opioid Receptor Antagonist Naloxone Enhances First-Order Fear Conditioning, Second-Order Fear Conditioning and Sensory Preconditioning in Rats

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The opioid receptor antagonist naloxone enhances Pavlovian fear conditioning when rats are exposed to pairings of an initially neutral stimulus, such as a tone, and a painful foot shock unconditioned stimulus (US; so-called first-order fear conditioning; Pavlov, 1927). The present series of experiments examined whether naloxone has the same effect when conditioning occurs in the absence of US exposure. In Experiments 1a and 1b, rats were exposed to tone-shock pairings in stage 1 (one trial per day for 4 days) and then to pairings of an initially neutral light with the already conditioned tone in stage 2 (one trial per day for 4 days). Experiment 1a confirmed that this training results in second-order fear of the light; and Experiment 1b showed that naloxone enhances this conditioning: rats injected with naloxone in stage 2 froze more than vehicle-injected controls when tested with the light alone (drug-free). In Experiments 2a and 2b, rats were exposed to light-tone pairings in stage 1 (one trial per day for 4 days) and then to tone-shock pairings in stage 2 (one trial per day for 2 days). Experiment 2a confirmed that this training results in sensory preconditioned fear of the light; and Experiment 2b showed that naloxone enhances sensory preconditioning when injected prior to each of the light-tone pairings: rats injected with naloxone in stage 1 froze more than vehicle-injected controls when tested with the light alone (drug-free). These results were taken to mean that naloxone enhances fear conditioning independently of its effect on US processing; and more generally, that opioids regulate the error-correction mechanisms that underlie associative formation.

Keywords: naloxone, pavlovian fear conditioning, second-order fear conditioning, sensory preconditioning, mediated conditioning, prediction error

OPEN ACCESS

Edited by:
Mathieu Wolff, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Anne-Marie Mouly, INSERM U1028 Centre de Recherche en Neurosciences de Lyon, France
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Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *07 September 2021*
Accepted: *14 October 2021*
Published: *06 December 2021*

Citation:
Michalscheck RML, Leidl DM, Westbrook RF and Holmes NM (2021) The Opioid Receptor Antagonist Naloxone Enhances First-Order Fear Conditioning, Second-Order Fear Conditioning and Sensory Preconditioning in Rats. Front. Behav. Neurosci. 15:771767. doi: 10.3389/fnbeh.2021.771767

INTRODUCTION

One of the central ideas in the study of learning is that of error correction. The idea is that organisms compare a new experience with existing knowledge, evaluating the degree to which the experience is discrepant from what is already known. When the evaluation yields a discrepancy, knowledge is updated to bring it into line with the new experience. This idea originated in the demonstrations

of blocking, contingency and signal validity effects reported in the classic experiments by Kamin (1968); Rescorla (1968), and Wagner et al. (1968), respectively. These experiments differed in several ways but were alike in showing that the normally effective relation for conditioning was rendered ineffective when the target conditioned stimulus (CS) was accompanied by a better predictor of the unconditioned stimulus (US). These results led to the Rescorla-Wagner model which held that conditioning was regulated by prediction error: by the difference between the amount that could be learned about the US and the amount that had already been learned by all the stimuli present (Rescorla and Wagner, 1972). This was formalized in the equation, $\Delta V = \alpha \times \beta \times (\lambda - \Sigma V)$, where ΔV is the change in the strength of the association between a CS and US, α and β denote the effectiveness of the CS and US, respectively, and the bracketed term, $\lambda - \Sigma V$, reflects the discrepancy between the presence or absence of the US (λ) and its current expectancy (ΣV).

One of the many lines of investigation initiated by this model concerned the neural mechanisms that mediate error correction. The discovery of endogenous opioids (Hughes, 1975; Hughes et al., 1975; for review see McNally and Akil, 2002) and their activation by a CS paired with an aversive (e.g., shock) US led to the proposal that the error signal that regulates associative formation in Pavlovian fear conditioning is instantiated through the endogenous opioid system (Fanselow, 1984, 1998; for review see McNally, 2009). Evidence for this proposal was provided by demonstrations that a systemic injection of the opioid receptor agonist morphine given before CS-US presentations impairs the acquisition of fear to the CS; while a systemic injection of the opioid receptor antagonist naloxone before CS-US presentations enhances the acquisition of fear to the CS (e.g., Westbrook et al., 1991; McNally et al., 2004). An explanation for these findings is that opioid receptor agonists and antagonists alter the functional or perceived intensity of the shock US through opioid-mediated pain regulation (Madden et al., 1977; Harris, 1996). That is, opioid receptor agonists reduce pain sensitivity and US intensity (i.e., they decrease λ and hence the λ-ΣV quantity), thereby impairing fear acquisition; while opioid receptor antagonists enhance pain sensitivity and US intensity (they increase λ and hence the λ-ΣV quantity), thereby enhancing fear acquisition (e.g., Westbrook et al., 1991; Young and Fanselow, 1992).

However, naloxone has also been shown to affect the extinction of Pavlovian conditioned fear. McNally and Westbrook (2003) conditioned two groups of rats to fear a CS through its pairings with shock and then exposed them to a series of CS alone presentations to extinguish this fear. Rats received either a systemic injection of naloxone or vehicle before the CS alone presentations. Naloxone-treated rats exhibited an equivalent level of CS-elicited freezing as vehicle-controls at the start of the extinction session. However, unlike the controls, naloxone-treated rats failed to exhibit any significant decline in freezing across the first extinction session (i.e., they showed no evidence of within-session extinction learning) and exhibited a slower decline in freezing across subsequent extinction sessions. The contrasting effects of naloxone on the acquisition and extinction of conditioned fear suggests that opioid receptor activity does more than just affect the processing of the US. If naloxone only affected the processing of an aversive shock US (Fanselow and Bolles, 1979a,b), it should not have affected the extinction of conditioned fear which occurs in the absence of the US. Thus, in addition to regulating the functional intensity of the US, it has been suggested that naloxone reduces the contribution of prior conditioning experiences to the expectancy of an aversive event, and thereby, interferes with the error correction processes that underlie the acquisition and extinction of conditioned fear (McNally and Westbrook, 2006; for review see McNally, 2009). Expressed in terms of the Rescorla-Wagner model, naloxone may effectively block ΣV (i.e., ΣV remains zero), causing the discrepancy between $\lambda - \Sigma V$ to persist for longer in acquisition, resulting in enhanced fear conditioning; and to diminish more rapidly in extinction, resulting in impaired fear extinction (for review see McNally, 2009; for further evidence in support of these ideas see Fanselow and Bolles, 1979a,b; McNally et al., 2004).

The proposal that naloxone acts on error-correction mechanisms implies that it may enhance fear conditioning independently of its effects on US processing (i.e., by maintaining ΣV at zero rather than by increasing λ). This implication can be tested through the use of protocols that produce fear conditioning in the absence of US exposure. One such protocol is second-order fear conditioning which is typically produced by first pairing a neutral stimulus (S1) with an aversive US and then pairing a second neutral stimulus (S2) with the already conditioned, fear-eliciting S1. As far as we are aware, only one previous study has examined the role of endogenous opioids in the acquisition of second-order conditioned fear. Cicala et al. (1990) injected rats with either naloxone or vehicle before second-order fear conditioning. At test, naloxone-injected rats exhibited more fear to S2 (as indexed by conditioned lick suppression) compared to vehicle-injected controls. However, the vehicle-injected rats in this experiment showed no evidence of having acquired second-order conditioned fear to S2: they exhibited as little fear of S2 as rats in control groups that received either unpaired presentations of S1 and the US in stage 1 or of S2 and S1 in stage 2. This leaves open the possibility that, rather than enhancing acquisition of second-order conditioned fear, naloxone simply altered the generalization of fear from S1 to S2. That is, the Cicala et al. (1990) study leaves open the question of whether naloxone enhances second-order fear conditioning, and more generally, whether naloxone facilitates the formation of associations between stimuli that are not innately aversive, as seen in second-order fear conditioning and sensory preconditioning.

The present study addressed this gap in knowledge. It had two specific aims. The first was to identify the effect of naloxone on the acquisition of second-order conditioned fear. To this end, Experiment 1a established a one-trial-per-day second-order conditioning protocol; and Experiment 1b used this protocol to assess the effect of naloxone on the acquisition of both first- and second-order conditioned fear. The second aim was to identify the effect of naloxone on the acquisition of sensory preconditioned fear. To this end, Experiment 2a established a one-trial-per-day sensory preconditioning protocol;

and Experiment 2b used this protocol to assess the effect of naloxone on the acquisition of first-order and sensory preconditioned fear.

EXPERIMENT 1A

The aim of this experiment was to demonstrate second-order conditioned fear using a protocol in which rats received a single conditioning trial each day across successive days. Such a protocol was used previously to show that rats given a single CS-US trial each day under a systemic injection of naloxone froze more across successive trials than control rats injected with vehicle (McNally et al., 2004). This protocol has the advantage of ensuring that the effects of naloxone are equivalent across every trial of conditioning: i.e., it alleviates any concern that the effects of naloxone may dissipate across a longer conditioning session that includes multiple trials. The successful demonstration of second-order conditioned fear in this protocol would then allow us to examine whether rats given a single second-order conditioning trial each day under naloxone would also freeze more across successive trials than vehicle-treated controls. The protocol involved exposing one group of rats (labeled PP) to a single pairing (P) of an auditory stimulus (S1) and foot shock each day across four successive days (stage 1) and, after extinction of any freezing elicited by the context, exposing them to a single pairing (P) of a visual stimulus (S2) and the conditioned S1 each day across four successive days (stage 2). Finally, rats were tested for levels of freezing elicited by S2. A second group (PU) was included to assess whether the test levels of freezing to S2 in Group PP were due to the associations produced by its pairings with the conditioned S1 rather than to generalization from the conditioned S1. Rats in this group were also exposed to a single S1-shock pairing each day in stage 1 and to single presentations of S2 and S1 each day in stage 2, but these presentations were unpaired. A final group (UP) was included to assess whether the levels of freezing elicited by S2 in Group PP were due to its pairings with the conditioned S1. Rats in this group received unpaired presentations of S1 and the shock US in stage 1 and daily pairings of S2 with S1 in stage 2 (see **Table 1**).

TABLE 1 | Design of Experiments 1a, 1b, 2a and 2b.

Group	Stage 1	Stage 2	S2 test	S1 test
Experiment 1a				
PP	S1+	S2–S1	S2−	S1−
PU	S1+	S1/S2		
UP	+/S1	S2–S1		
Experiment 1b				
NAL-NAL	(NAL) S1+	(NAL) S2–S1	S2−	S1−
NAL-VEH	(NAL) S1+	(VEH) S2–S1		
VEH-NAL	(VEH) S1+	(NAL) S2–S1		
VEH-VEH	(VEH) S1+	(VEH) S2–S1		
Experiment 2a				
PP	S2–S1	S1+	S2−	S1−
PU	S2–S1	+/S1		
UP	S1/S2	S1+		
Experiment 2b				
NAL-NAL	(NAL) S2–S1	(NAL) S1+	S2−	S1−
VEH-NAL	(VEH) S2–S1	(NAL) S1+		
NAL-VEH	(NAL) S2–S1	(VEH) S1+		
VEH-VEH	(VEH) S2–S1	(VEH) S1+		

A plus sign (+) following one event indicates that it was co-terminated with shock; a minus sign (–) between events indicates that they were paired; a forward-stroke sign (/) indicates that they were explicitly unpaired; and a minus sign (–) following one event indicates it was presented alone. NAL = a subcutaneous injection of naloxone (2.5 mg/ml) and VEH = a subcutaneous injection of vehicle only. All injections were administered 5 min before the start of the training/test session.

Materials and Methods

Subjects

Subjects were 23 (7 males, 16 females) experimentally naive, adult Long Evans rats (250–450 g) obtained from the breeding facility maintained by the School of Psychology at the University of New South Wales. The rats were housed by sex in plastic tubs (67 cm length × 40 cm width × 22 cm height) with 3–4 rats per tub. The tubs were kept in an air-conditioned colony room whose temperature was maintained at 20 degrees Celsius and whose lights were on between 07:00 and 19:00. All rats had *ad libitum* access to water and food throughout the experiment.

Apparatus

Training and testing occurred in a set of eight identical chambers (30 cm length × 26 cm width × 30 cm height). The front and rear walls of each chamber were clear Plexiglas, the side walls and ceiling were aluminum, and the floor was constructed of stainless-steel rods, each 7 mm in diameter and spaced 1.8 mm apart. A shock could be delivered through the rods via a custom-built generator located in another room in the laboratory. Each chamber was located in its own light- and sound-attenuating wooden cabinet. A 2 × 3 array of white LEDs, a speaker, and a camera were mounted on the back wall of each cabinet and an infrared light was mounted on its ceiling. The LEDs and the speaker were used to present the auditory and visual stimuli. The camera was connected to a monitor and DVD recorder that were located in another room in the laboratory and used to record the behavior of each rat.

Stimuli

The two stimuli were a 1,000 Hz, 72 dB tone and a 3 Hz, 57 lux flashing light measured at the center of each chamber. These stimuli were used as the S1 and S2 stimuli, respectively. Each presentation of S1 lasted for 10 s and each presentation of S2 lasted for 30 s. The US was a 0.8 mA 1 s foot shock. Stimuli were programmed and presented using MATLAB software.

Scoring

Freezing, defined as the absence of all movement except that required for breathing, was the measure of conditioning (Fanselow, 1980). A time sampling procedure was used in which each rat was observed once every 2 s and its behavior scored as "freezing" or "not freezing." A percentage score was calculated to determine the proportion of total observations each rat

spent freezing on each trial. All test data were scored by the experimenter and an experienced observer who was blind to the group allocations and purpose of the experiment. The Pearson product-moment correlation between the experimenter's and observer's scores was > 0.9 in all experiments. Any discrepancies between the experimenter's and observer's scores were resolved in favor of the blind observer.

Statistical Analyses

The principal data obtained in Experiment 1a were acquisition of freezing to S1 in stage 1, acquisition of freezing to S2 and retention of freezing to S1 in stage 2, and test levels of freezing to both S2 and S1. The freezing data for S1 and S2 were analyzed separately in acquisition and testing using mixed model ANOVAs with a between-subject factor of group (Groups PP, PU, and UP), and a within-subject factor of trial (in acquisition) or block-of-trials (in testing). For all statistical analyses, the criterion for rejection of the null hypothesis was set at alpha = 0.05. With 1 and 20 degrees of freedom (df), the F critical (F_c) was 4.35. Partial eta-squared (η_p^2) was calculated as a measure of the effect size for all statistically significant differences (η_p^2 of 0.14 is considered a large effect size).

Procedure

On each of days 1–4 (stage 1), rats in Groups PP and PU received a single presentation of the 10 s S1 which co-terminated with the foot shock. The onset of S1 occurred 2 min after placement in the chamber and rats remained in the chambers for an additional 1 min after the foot shock. Rats in Group UP received the foot shock ∼10 s after placement in the chambers and S1 approximately 3 min later. They were then removed from the chamber a few seconds later. On each of days 5–8, all rats received a 20 min exposure to the chambers in the absence of any scheduled events. This was done to extinguish any freezing elicited by the chambers; freezing that would obscure the subsequent detection of second-order conditioning.

On each of days 9–12 (stage 2), rats in Groups PP and UP received a single presentation of the 30 s S2 which co-terminated in the onset of the 10 s S1. The onset of S2 occurred 4.5 min after placement into the chamber and rats remained in the context for an additional 2 min after offset of the S1. Rats in Group PU received a presentation of the 10 s S1 a few seconds after placement in the chambers and approximately 5.5 min later a presentation of the 30 s S2. They were removed from the chambers a few seconds later. On each of days 13 and 14, all rats were exposed to the chambers in the absence of any scheduled events to extinguish any freezing elicited by the context alone.

Rats were tested with S2 and S1 on days 15 and 16, respectively. There were eight presentations of the 30 s S2 and 16 presentations of the 10 s S1. We doubled the number of S1 presentations because short duration stimuli typically require a greater number of trials to extinguish and we wanted to avoid any potential ceiling effects in the test of the S1. Onset of the first stimulus presentation occurred 3 min after placement in the chambers, the interval between stimulus presentations was fixed at 3 min, and rats remained in the chambers for a further 2 min after the final stimulus presentation.

Results

Figure 1A shows the mean levels of freezing to S1 across the 4 days of stage 1 (left panel) and the mean levels of freezing to S2 and S1 across the 4 days of stage 2 (right panel). They suggest that freezing increased in all groups across stage 1; however, only Groups PP and PU froze to S1 in stage 2 and only Group PP acquired freezing to S2. The statistical analyses supported these impressions. The analysis of freezing to S1 in stage 1 confirmed that there was a significant linear increase in freezing, $F_{(1,20)} = 19.27$, $p = 0.0003$, $\eta_p^2 = 0.49$, CI [0.58, 1.63] and that there were no significant between-group differences in the rate of this increase or the overall levels of freezing, Fs < 1. The analysis of freezing to S1 in stage 2 revealed that it remained stable across the S2–S1 pairings (no significant linear trend, $F < 1$) and there was no trend × group interaction, $F < 1$. However, there were between-group differences such that rats in Groups PP and PU that had received S1-shock pairings in stage 1 froze more to S1 than those in Group UP given unpaired presentations of S1 and shock in stage 1, $F_{(1,20)} = 59.03$, $p = 0.0001$, $\eta_p^2 = 0.75$, CI [2.12, 3.71]. It is worth noting that the freezing by rats in UP across stage 1 likely reflected context conditioning which, of course, had been extinguished before stage 2, revealing the absence of conditioning to the unpaired S1. The statistical analysis of freezing to S2 revealed a significant linear increase, $F_{(1,20)} = 37.40$, $p < 0.0001$, $\eta_p^2 = 0.65$, CI [0.99, 2.02] and a significant trend × group interaction, $F_{(1,20)} = 55.72$, $p < 0.0001$, $\eta_p^2 = 0.74$, CI [2.78, 4.94], which, from inspection of the figure, was due to the increase in freezing by rats in Group PP. Finally, rats in this group froze significantly more to S2 than those in Groups PU and UP, $F_{(1,20)} = 58.35$, $p < 0.0001$, $\eta_p^2 = 0.75$, CI [1.74, 3.04].

Figure 1B shows the mean levels of freezing in each group during the drug-free tests of S2 (left panel) and S1 (right panel). They suggest that Group PP froze more to S2 than Groups PU and UP, and that Groups PU and PP froze more to S1 than Group UP. The statistical analysis again supported these impressions. The analysis of freezing to S2 confirmed that Group PP froze significantly more to S2 than Groups PU and UP, $F_{(1,20)} = 75.40$, $p < 0.0001$, $\eta_p^2 = 0.79$, CI [1.51, 2.47]. There was no statistically significant linear trend in freezing across the S2 alone presentations or trend × group interaction (**Figure 1B**, left panel), largest $F < 4$. The analysis of freezing to S1 confirmed that Groups PP and PU froze significantly more to S1 than Group UP, $F_{(1,20)} = 10.88$, $p < 0.0036$, $\eta_p^2 = 0.35$, CI [0.43, 1.90]. There was no significant linear trend in freezing across the S1 alone presentations or trend × group interactions (**Figure 1B**, right panel), Fs < 1.

Discussion

This experiment has shown that rats exposed to S1-shock pairings in stage 1 and then to S2–S1 pairings in stage 2 (Group PP) froze more when tested with S2 than rats in two control groups: one exposed to S1-shock pairings but unpaired presentations of S2 and S1 (Group PU), and the other exposed to unpaired presentations of S1 and shock but S2–S1 pairings (for similar demonstrations, see Rizley and Rescorla, 1972; Yin et al., 1994; Parkes and Westbrook, 2010; Witnauer and Miller, 2011;

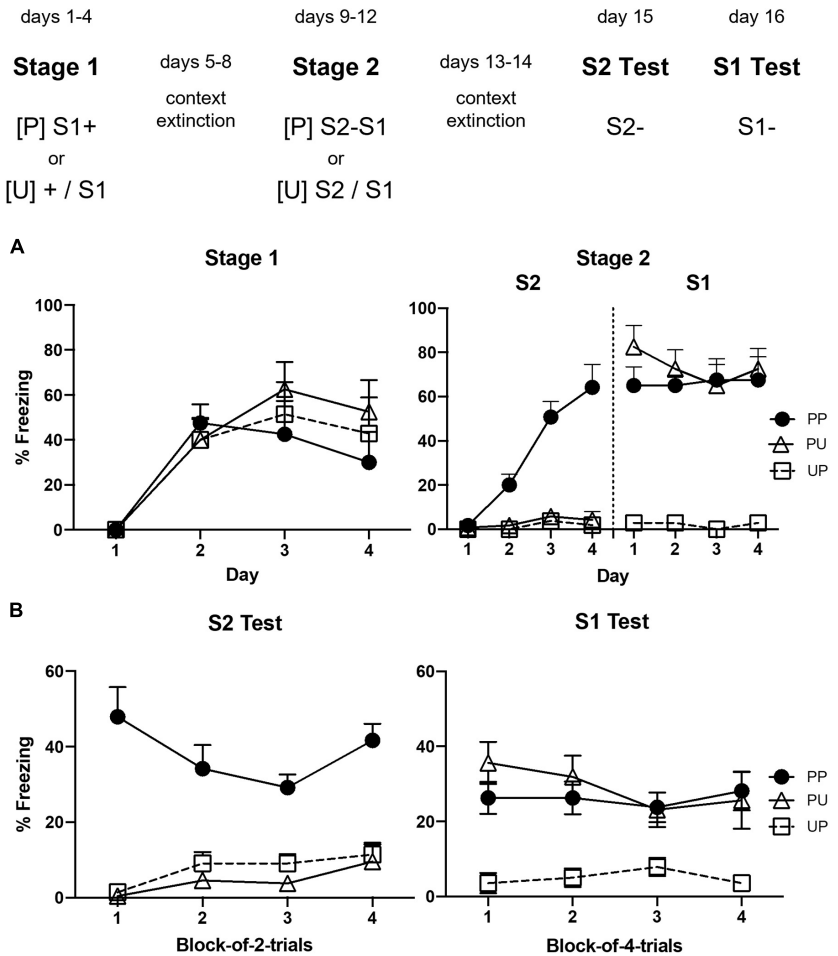

FIGURE 1 | Results of Experiment 1a showing that freezing to S2 is due to second-order conditioning. **(A)** Shows the mean (+ SEM) levels of freezing to S1 in stage 1 (left panel) and to S2 and the previously conditioned S1 in stage 2 (right panel). **(B)** Shows the mean (+ SEM) levels of freezing during the final drug-free tests across blocks of two S2 alone trials (left panel) and four S1 alone trials (right panel). The numbers of rats in each group were: Group PP, $n = 8$; Group PU, $n = 8$; and Group UP, $n = 7$.

Holmes et al., 2013). Thus, these results show that freezing to S2 in Group PP was associatively mediated, due to the S1-shock and S2–S1 pairings rather than generalization from the conditioned S1 or to any unconditioned ability of S1 to condition freezing to S2. Critically, this demonstration of second-order conditioned fear was obtained in the single trial per day protocol previously used to demonstrate that naloxone enhances first-order conditioned fear (McNally et al., 2004). The next experiment used this protocol to assess whether naloxone also enhances second-order conditioned fear.

EXPERIMENT 1B

This experiment had two aims. The first was to replicate previously reported findings that naloxone enhances acquisition of first-order conditioned fear (McNally et al., 2004). The second aim was to determine the effect of naloxone on acquisition of second-order conditioned fear. The conditioning protocol was the same as that used for Group PP in the previous experiment: rats received a single S1-shock pairing on each of days 1–4, context alone exposures (to extinguish context-elicited freezing) across days 5–8, and a single S2–S1 pairing on each of days 9–12. Two groups received an injection of naloxone prior to each of the S1-shock pairings in stage 1 (Groups NAL-VEH and NAL-NAL), while the remaining two groups received an injection of vehicle only prior to these pairings (Groups VEH-NAL and VEH-VEH). One group in each of these pairs received an injection of naloxone prior to each of the S2–S1 pairings in stage 2 (Group NAL-NAL and VEH-NAL), while the other received an injection of vehicle only prior to these pairings (Groups NAL-VEH and VEH-VEH). Finally, all rats received extinction of any context-elicited freezing on days 13 and 14; and were tested with S2 on day 15 and S1 on day 16 (see **Table 1**).

We expected to replicate previous findings that naloxone enhances acquisition of first-order conditioned fear: that is, we expected rats injected with naloxone prior to each of the single S1-shock pairings (Groups NAL-VEH and NAL-NAL) to exhibit

faster acquisition of freezing to S1 as well as higher levels of freezing to S1 across subsequent second-order conditioning and testing. The second question of interest concerned the effect of naloxone on acquisition of second-order conditioned fear. If naloxone enhances fear conditioning independently of its effect on an aversive US, then rats that received naloxone injections prior to the S2–S1 pairings in stage 2 (Groups VEH-NAL and NAL-NAL) will freeze more to S2 than vehicle-treated rats (Groups NAL-VEH and VEH-VEH) across its pairings with S1 and on the subsequent drug-free S2 alone test.

Materials and Methods

Subjects and Apparatus

Subjects were 32 (17 males, 15 females) experimentally naive, adult Long Evans rats (250–450 g). They were sourced, housed and handled as described for Experiment 1a. The apparatus and stimuli were those used in Experiment 1a.

Drugs

Naloxone hydrochloride (Sigma Aldrich, Sydney, Australia) was dissolved in 0.9% (wt/vol) non-pyrogenic saline to obtain a concentration of 2.5 mg/ml (McNally and Westbrook, 2003). Non-pyrogenic saline was also used for control injections (i.e., vehicle). All injections were administered subcutaneously (s.c.) into the dorsal neck region at a volume of 1 ml/kg. Past research that used this same dose and route of administration did not report any non-specific effects of naloxone on freezing or locomotor activity in rats (e.g., Fanselow and Bolles, 1979a; McNally and Westbrook, 2003).

Scoring and Statistical Analyses

The method of scoring was identical to that described in Experiment 1a. The principal data were acquisition of freezing to S1 in stage 1, acquisition of freezing to S2 and retention of freezing to S1 in stage 2, and test levels of freezing to S2 and S1. The data for S1 and S2 were analyzed separately in acquisition and testing using a mixed model ANOVA with between-subject factors of stage 1 treatment (naloxone or vehicle) and stage 2 treatment (naloxone or vehicle); and a within-subject factor of trial (in acquisition) or block-of-trials (in testing). For all analyses, the criterion for rejection of the null hypothesis was set at alpha = 0.05. With 1 and 28 df, this yielded an F_c of 4.2. Partial eta-squared (η_p^2) was calculated as a measure of the effect size for all statistically significant differences (η_p^2 of 0.14 is considered a large effect size).

Procedure

On each of days 1–4 (stage 1), rats received an injection of naloxone (Groups NAL) or vehicle (VEH). Five min later, they were placed in the conditioning chambers and exposed to a single S1-shock pairing in the manner described for Group PP in Experiment 1a. On each of days 5–8, all rats received an injection of vehicle and, 5 min later, were placed in the context for one 20 min session of context extinction. These sessions were intended to extinguish any freezing elicited by the chambers prior to the S2–S1 pairings in stage 2.

On each of days 9–12 (stage 2), rats received an injection of naloxone (Groups NAL-NAL and VEH-NAL) or vehicle (Groups NAL-VEH and VEH-VEH). Five min later, they were placed in the chambers and exposed to a single S2–S1 pairing in the manner described for Group PP in Experiment 1a. On each of days 13 and 14, all rats received an injection of vehicle and, after 5 min, were placed in the chambers for 20 min in the absence of any scheduled events. This was done to extinguish any such freezing that could obscure detection of the freezing elicited across the testing of S2 and S1.

On days 15 and 16, all rats received an injection of vehicle and, 5 min later, were tested for levels of freezing to S2 (day 15) and S1 (day 16). The details for these test sessions were identical to those described for Experiment 1a.

Results

Figure 2A shows the mean levels of freezing to S1 across its pairings with shock in stage 1 (left panel) and to S2 and S1 across their pairings in stage 2 (right panel). It suggests that naloxone enhanced acquisition of both forms of conditioning but did not affect retrieval/expression of the already conditioned fear to S1. These impressions were confirmed by the statistical analyses. During stage 1, averaged across all groups, there was a significant linear increase in freezing across the daily S1-shock pairings, $F_{(1,28)} = 71.91$, $p < 0.0001$, $\eta_p^2 = 0.72$, CI [1.19, 1.94]. The rate of this increase differed between the naloxone- and vehicle-treated groups, $F_{(1,28)} = 14.52$, $p = 0.0007$, $\eta_p^2 = 0.34$, CI [0.65, 2.16]. Groups NAL-VEH and NAL-NAL acquired freezing more rapidly and froze more to S1 than Groups VEH-VEH and VEH-NAL, $F_{(1,28)} = 29.39$, $p < 0.0001$, $\eta_p^2 = 0.51$, CI [0.81, 1.80]. The remaining main effects and interactions were not statistically significant, Fs < 1.

The analysis of freezing to S2 across its pairings with S1 revealed a similar pattern of results. Averaged across all groups, there was a significant linear increase in freezing to S2, [$F_{(1,28)} = 125.68$, $p < 0.0001$], $\eta_p^2 = 0.82$, CI [1.48, 2.14]. The rate of this increase differed between groups injected with naloxone or vehicle, $F_{(1,28)} = 6.67$, $p = 0.0153$, $\eta_p^2 = 0.19$, CI [0.17, 1.49]. Groups VEH-NAL and NAL-NAL acquired freezing more rapidly and froze more to S2 than Groups VEH-VEH and NAL-VEH, $F_{(1,28)} = 18.10$, $p = 0.0002$, $\eta_p^2 = 0.39$, CI [0.61, 1.75]. The remaining main effects and interactions were not statistically significant, largest $F < 3$. The analysis of freezing to the conditioned S1 revealed no significant linear trend, $F < 4$; and no significant trend × group interactions, largest $F < 3$. The overall level of freezing to S1 did not differ between groups exposed to the S2–S1 pairings under naloxone or vehicle, $F < 1$, but did differ between groups that had been injected with naloxone or vehicle across the prior S1-shock pairings: those that received naloxone in stage 1 (Groups NAL-VEH and NAL-NAL) froze more to S1 than those that received vehicle in stage 1 (Groups VEH-NAL and VEH-VEH), $F_{(1,28)} = 14.68$, $p = 0.0007$, $\eta_p^2 = 0.34$, CI [0.51, 1.69].

Figure 2B shows the mean levels of freezing in each group during drug-free testing with S2 (left panel) and S1 (right panel). It suggests that rats that had been injected with naloxone prior to each of the S2–S1 pairings (Groups VEH-NAL and NAL-NAL)

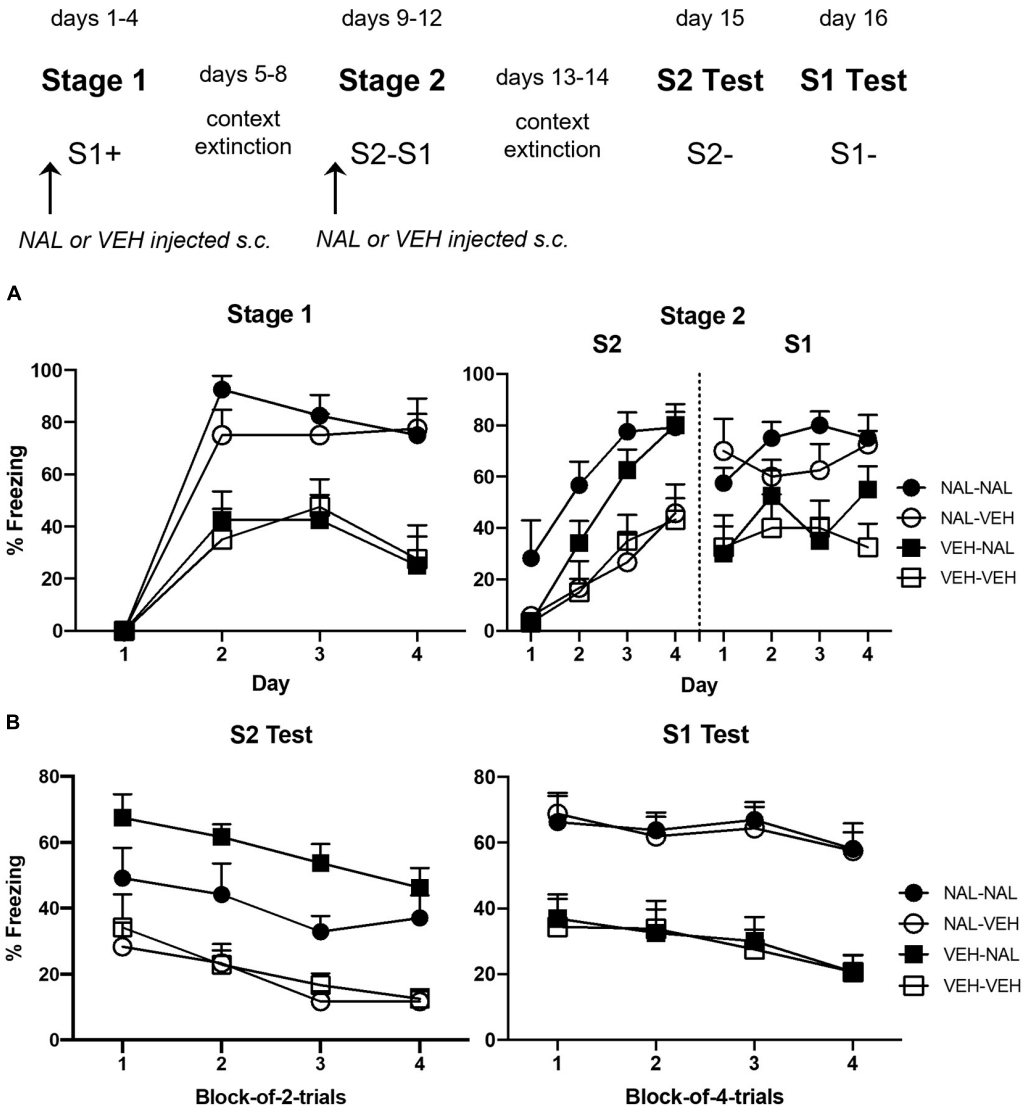

FIGURE 2 | Results of Experiment 1b showing that naloxone enhances the acquisition of first- and second-order conditioned fear. **(A)** Shows the mean (+ SEM) levels of freezing to S1 in stage 1 (left panel) and to S2 and the previously conditioned S1 in stage 2 (right panel). **(B)** Shows the mean (+ SEM) levels of freezing during the final drug-free tests across blocks of two S2 alone trials (left panel) and four S1 alone trials (right panel). The numbers of rats in each group were: Group NAL-NAL, $n = 8$; Group NAL-VEH, $n = 8$; Group VEH-NAL, $n = 8$; and Group VEH-VEH, $n = 8$.

froze more to S2 than rats that had been injected with vehicle before these pairings (Groups VEH-VEH and NAL-VEH); and rats that had been injected with naloxone prior to each S1-shock pairing (Groups NAL-VEH and NAL-NAL) froze more to S1 than rats that had been injected with vehicle (Groups VEH-VEH and VEH-NAL) before these pairings. These impressions were confirmed by the statistical analyses. In the S2 test, averaged across all groups, there was a significant linear decline in freezing across the S2 alone presentations, $F_{(1,28)} = 20.31$, $p = 0.0001$, $\eta_p^2 = 0.42$, CI [−1.29, −0.49]. Overall, groups injected with naloxone prior to each of the daily S2–S1 pairings (VEH-NAL and NAL-NAL) froze more to S2 than groups injected with vehicle (VEH-VEH and NAL-VEH), $F_{(1,28)} = 45.70$, $p < 0.0001$, $\eta_p^2 = 0.62$, CI [1.14, 2.12]. Moreover, groups injected with naloxone prior to each of the daily S1-shock pairings (NAL-VEH and NAL-NAL) froze less to S2 than groups injected with vehicle (VEH-NAL and VEH-VEH) before these pairings, $F_{(1,28)} = 5.08$, $p = 0.0322$, $\eta_p^2 = 0.15$, CI [−1.04, −0.05]. There was no significant difference in freezing to S2 between Groups NAL-VEH and VEH-VEH ($F < 1$), indicating that the naloxone injections prior to S1-shock pairings did not automatically increase second-order freezing to S2. There were no significant trend × group interactions, largest $F < 3$. In the S1 test, averaged across all groups, there was a significant linear decline in freezing across the S1 alone presentations, $F_{(1,28)} = 7.81$, $p = 0.0093$, $\eta_p^2 = 0.22$, CI [−0.80, −0.12]. Overall, groups injected with naloxone prior to the S1-shock pairings (NAL-NAL and NAL-VEH) froze more to S1 than groups injected with vehicle (VEH-NAL and VEH-VEH),

$F_{(1,28)} = 43.19$, $p < 0.0001$, $\eta_p^2 = 0.61$, CI [1.35, 2.57]. However, there was no significant difference in freezing to S1 between rats that had been injected with naloxone or vehicle in stage 2, and no significant interactions between linear trend and groups, $Fs < 1$.

Discussion

This experiment has revealed three major findings. First, naloxone acutely enhanced the acquisition of first-order fear to S1 and second-order fear to S2: rats injected with naloxone prior to each S1-shock pairing in stage 1 (administered one per day) froze more to S1 than rats injected with vehicle; and rats injected with naloxone prior to each S2–S1 pairing in stage 2 (again administered one per day) froze more to S2 than rats injected with vehicle. Second, during the S2–S1 pairings in stage 2, freezing to the already conditioned S1 was unaffected by the naloxone injection: that is, rats injected with naloxone prior to each S2–S1 pairing in stage 2 froze to S1 at the same level as rats injected with vehicle. Finally, in the drug-free tests of S2 and S1, the enhancing effect of naloxone on first- and second-order fear conditioning persisted such that rats that had received naloxone in stage 2 froze more to S2 than rats that had received vehicle in stage 2; and rats that had received naloxone in stage 1 froze more to S1 than rats that had received vehicle in stage 1. The implication of these findings will be explored in section "General Discussion."

EXPERIMENT 2A

The aim of this experiment was to demonstrate sensory preconditioned fear using a one-trial-per-day protocol that could then be used to assess the effect of naloxone on that form of learning. The design was the same as that used in Experiment 1a, except that the order of the training stages was reversed (see **Table 1**). Rats in Group PP were exposed to a single S2–S1 pairing each day in stage 1 and then to a single S1-shock pairing each day in stage 2; rats in Group PU were exposed to a single S2–S1 pairing each day in stage 1 but to an unpaired presentation of S1 and shock each day in stage 2; and, finally, rats in Group UP were exposed to unpaired presentations of S2 and S1 each day in stage 1 but to a single S1-shock pairing each day in stage 2. Finally, all rats were tested for freezing to S2 and S1. The rationale for such a design was that described previously. To show that any freezing elicited by S2 in Group PP was due to the associations produced by the pairings in each stage, it was necessary to assess whether: the pairings of S2 and S1 in stage 1 were sufficient to imbue S2 with the ability to elicit freezing in the absence of any fear conditioning of S1 (Group PU); and the degree to which freezing conditioned to S1 generalized to S2 (Group UP).

Materials and Methods

Subjects and Apparatus

Subjects were 25 (9 males, 16 females) experimentally naive, adult Long Evans rats (250–450 g). They were sourced, housed and handled as described in Experiment 1a. The stimuli and apparatus were the same as those used in previous experiments.

Scoring and Statistical Analyses

The method of scoring was identical to that used in previous experiments. The principal data obtained were acquisition of freezing to S1 in stage 2 and test levels of freezing to S2 and S1. The data for S1 and S2 were analyzed separately using mixed model ANOVAs with a between-subject factor of group (PP, PU, and UP) and a within-subject factor of trial (in acquisition) or block-of-trials (in testing). For all analyses, the criterion for rejection of the null hypothesis was set at alpha = 0.05. With 1 and 22 df, this yielded a $\boldsymbol{F_c}$ of 4.30. Partial eta-squared ($\boldsymbol{\eta_p^2}$) was calculated as a measure of the effect size for all statistically significant differences.

Procedure

On each of days 1–4 (stage 1), rats in Groups PP and PU were placed in the chambers and 4.5 min later exposed to a 30 s S2 which co-terminated in the onset of the 10 S1. They were removed from the chambers 2 min later. Rats in Group UP were exposed to the 10 s S1 a few seconds after placement in the chambers and 5.5 min later to the 30 s S2. They were removed from the chambers a few seconds later.

On each of days 5 and 6 (stage 2), rats in Groups PP and UP received a single presentation of the 10 s S1 which co-terminated with the 1 s foot shock. We reduced the number of S1-shock pairings (two in total) relative to the number used in Experiments 1a and 1b (four) as we wanted to increase the sensitivity of the sensory preconditioning protocol to any potential effect of naloxone. The onset of S1 occurred 2 min after placement in the chamber and rats remained in the chambers for an additional 1 min. On each of these days, rats in Group PU were shocked a few seconds after placement in the chambers, presented with S1 3 min later, and removed from the chambers a few seconds later. On each of days 7 and 8, all rats were exposed to the chambers for 20 min in the absence of any scheduled events to extinguish any freezing elicited by the chambers; freezing that would obscure detection of the freezing elicited by S2 and S1.

On day 9, rats were tested with S2 and on day 10 with S1. Testing consisted in 16 S2 alone presentations, each 30 s, and 16 S1 alone presentations, each 10 s. The first stimulus presentation occurred 3 min after placement in the chambers, the interval between presentations was fixed at 3 min, and rats remained in the chambers for 2 min after the final stimulus presentation.

Results

Figure 3A shows the mean levels of freezing to S1 across sessions in which it was presented with shock in stage 2. Inspection of the figure indicates little or no freezing during the first presentation of S1 but substantial freezing in all groups during its second presentation. The statistical analysis confirmed that there was a significant increase in freezing to S1 across the two trials, $F_{(1,22)} = 22.50$, $p = 0.00098$, $\eta_p^2 = 0.51$, CI [0.63, 1.61], but no significant trend × group interaction or between-group differences in the overall levels of freezing, largest $F < 3$.

Figure 3B shows the mean levels of freezing in each group during the tests of S2 (left panel) and S1 (right panel). It suggests that rats in Group PP froze more to S2 than rats in Groups PU and UP; and that rats in Groups UP and PP froze more to

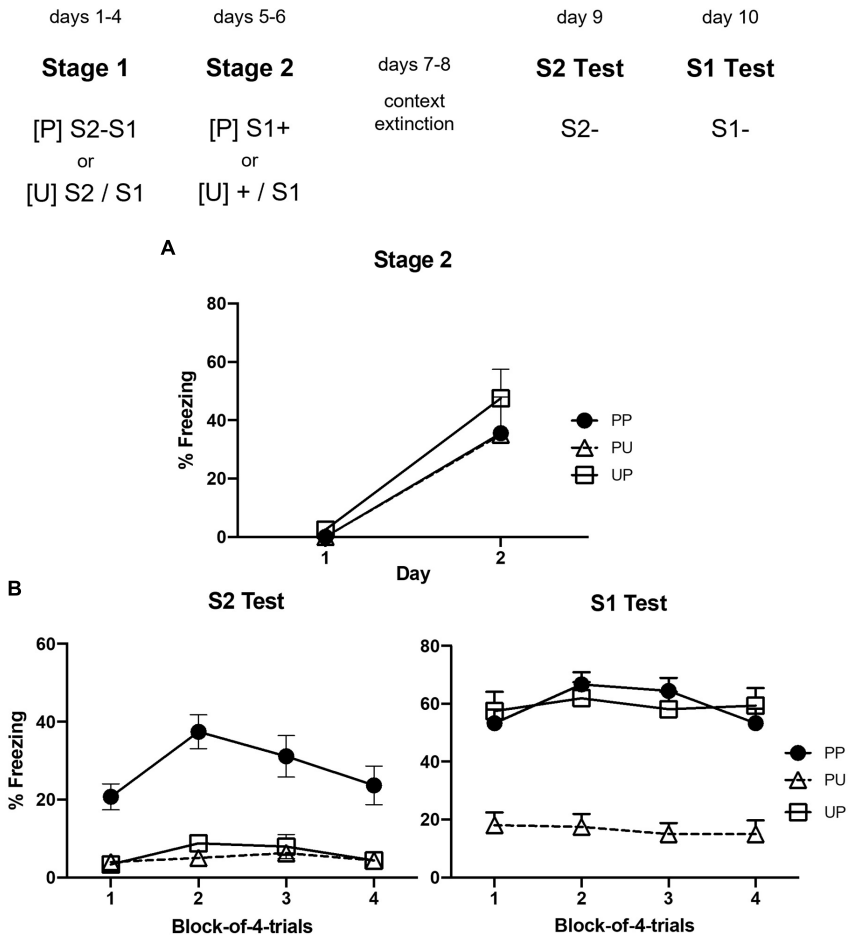

FIGURE 3 | Results of Experiment 2a showing that freezing to S2 is due to sensory preconditioning. **(A)** Shows the mean (+ SEM) levels of freezing to S1 in stage 2. **(B)** Shows the mean (+ SEM) levels of freezing during the final drug-free tests across blocks of four S2 alone trials (left panel) and four S1 alone trials (right panel). The numbers of rats in each group were: Group PP, $n = 9$; Group PU, $n = 8$; and Group UP, $n = 8$.

S1 than rats in Group PU. These impressions were confirmed by the statistical analyses. Group PP froze significantly more to S2 than Groups PU and UP, $F_{(1,22)} = 114.47$, $p < 0.0001$, $\eta_p^2 = 0.84$, CI [1.68, 2.49], who did not differ from each other, $F < 1$. Averaged across all groups, there was no significant linear decline in freezing to S2 or significant interaction between linear trend and grouping, Fs < 1. Groups PP and UP did not differ from each other, $F < 1$, but froze significantly more to S1 than Group UP, $F_{(1,22)} = 27.11$, $p < 0.0032$, $\eta_p^2 = 0.55$, CI [1.12, 2.61]. Averaged across all groups, there was no significant linear decline in freezing to S1 or significant interaction between linear trend and grouping, Fs < 1.

Discussion

This experiment exposed rats in Group PP to a single S2–S1 pairing each day and then to a single S1-shock pairing each day. It found that these rats froze more when tested with S2 than control rats exposed to either S2–S1 pairings but unpaired presentations of S1 and shock (Group PU) or to unpaired presentations of S2 and S1 but pairings of S1 and shock (Group UP). These results show that a single pairing each day produces an association between S2 and S1 in stage 1; that a single pairing each day produces an association between S1 and shock in stage 2; and that the integration of these associations results in freezing when rats are tested with S2. The next experiment used this protocol to assess the effect of naloxone on the acquisition of sensory preconditioned fear.

EXPERIMENT 2B

This experiment had two aims. The first was to replicate the finding that naloxone enhances acquisition of first-order conditioned fear (Experiment 1b; McNally et al., 2004). The second aim was to determine whether naloxone enhances the acquisition of sensory preconditioned fear just as it enhanced the acquisition of second-order conditioned fear (Experiment 1b). The protocol was the same as that used for Group PP in Experiment 2a (see **Table 1**). Rats in two groups received an injection of naloxone prior to each S2–S1 pairing in stage 1

(Groups NAL-VEH and NAL-NAL), while rats in another two groups received an injection of vehicle prior to each of these pairings (Groups VEH-NAL and VEH-VEH). One group in each pair then received an injection of naloxone prior to each S1-shock pairing in stage 2 (Groups VEH-NAL and NAL-NAL), while the other group received an injection of vehicle only prior to these pairings (Groups NAL-VEH and VEH-VEH). Finally, all rats were injected with vehicle and tested with S2 and then with S1. The questions of interest concerned the levels of freezing among rats that received naloxone relative to those that received vehicle prior to stage 1 and stage 2. We expected that naloxone would enhance the acquisition of first-order conditioned fear and, hence, that rats in Groups NAL-NAL and VEH-NAL would freeze more to S1 across its acquisition and testing than Groups VEH-VEH and NAL-VEH. If naloxone also enhanced the acquisition of the S2–S1 association in stage 1, then Groups NAL-VEH and NAL-NAL would freeze more to S2 across its testing than Groups VEH-NAL and VEH-VEH.

Materials and Methods

Subjects and Apparatus

Subjects were 32 female, experimentally naive, adult Long Evans rats (250–300 g), sourced, housed and handled as described for Experiment 1a. The apparatus and stimuli were the same as those used in previous experiments. The details for drug/vehicle preparation and administration were the same as used in Experiment 1b.

Scoring and Statistical Analyses

The method of scoring was identical to that used in previous experiments. The principal data were acquisition of freezing to S1 in stage 2 and test levels of freezing to S2 and S1. The test data for S1 and S2 were analyzed separately using a mixed model ANOVA with between-subject factors of stage 1 treatment (naloxone or vehicle) and stage 2 treatment (naloxone or vehicle), and a within-subject factor of trial (in acquisition) or block-of-trials (in testing). For all analyses, the criterion for rejection of the null hypothesis was set at alpha = 0.05. With 1 and 28 df, this yielded a F_c of 4.20. Partial eta-squared (η_p^2) was calculated as a measure of the effect size for all statistically significant differences.

Procedure

On each of days 1–4 (stage 1), rats received an injection of either naloxone or vehicle. Five minutes later, they were placed in the chambers and exposed to a single S2–S1 pairing in the manner described for Group PP in Experiment 2a.

On each of days 5 and 6 (stage 2), half of the rats that had been injected with naloxone in stage 1 were again injected with naloxone (Group NAL-NAL), while the remainder were injected with vehicle (Group NAL-VEH). Similarly, half of the rats that had been injected with vehicle in stage 1 were now injected with naloxone (Group VEH-NAL), while the remainder were again injected with vehicle (Group VEH-NAL). Five min after the injection, rats were placed in the chambers and exposed to a single S1-shock pairing in the manner described for Group PP in Experiment 2a. On each of days 7 and 8, all rats received an injection of vehicle and, after 5 min, were placed in the chambers for 20 min in the absence of any scheduled events. This was done to extinguish any freezing elicited by the chambers.

On days 9 and 10, rats were tested with S2 and S1, respectively. On each day, they received an injection of vehicle and, 5 min later, were placed in the chambers where they were tested with S2 (day 9) or S1 (day 10) in the manner described for Experiment 2a.

Results

Figure 4A shows the mean level of freezing to S1 across its parings with shock in each of the four groups. The statistical analysis confirmed that naloxone enhanced conditioning. There was a significant linear increase in freezing across the pairings, $F_{(1,28)} = 130.60$, $p < 0.0001$, $\eta_p^2 = 0.82$, CI [2.41, 3.47], and a significant trend × drug interaction, $F_{(1,28)} = 13.37$, $p = 0.0011$, $\eta_p^2 = 0.32$, CI [0.83, 2.94]. Importantly, there was a significant drug effect such that rats injected with naloxone prior to each S1-shock pairing (Groups VEH-NAL and NAL-NAL) froze more to S1 than rats injected with vehicle (Groups VEH-VEH and NAL-VEH), $F_{(1,28)} = 17.00$, $p < 0.0003$, $\eta_p^2 = 0.38$, CI [0.50, 1.50]. There was no significant interaction between the treatments in stages 1 and 2: naloxone or vehicle treatment in stage 1 did not affect freezing to S1 among naloxone- or vehicle-treated rats in stage 2 (Fs < 1).

Figure 4B shows the mean levels of freezing in each group during drug-free testing with S2 (left panel) and S1 (right panel). Inspection of the left panel suggests that rats that had been injected with naloxone prior to each of the S2–S1 pairings in stage 1 (Groups NAL-NAL and NAL-VEH) froze more to S2 than rats injected with vehicle (Groups VEH-VEH and VEH-NAL). Inspection of the right panel suggests that rats injected with naloxone prior to each of the S1-shock pairings in stage 2 froze more to S1 (Groups VEH-NAL and NAL-NAL) than rats that had been injected with vehicle before these pairings (Groups VEH-VEH and NAL-VEH). The statistical analyses confirmed these impressions. The analysis of the S2 test data revealed a significant linear decline in freezing across the stimulus presentations, $F_{(1,28)} = 6.27$, $p = 0.0184$, $\eta_p^2 = 0.18$, CI [−0.09, −0.94]. It also showed that, overall, groups injected with naloxone in stage 1 (NAL-VEH and NAL-NAL) froze more to S2 than groups injected with vehicle in stage 1 (VEH-VEH and VEH-NAL), $F_{(1,28)} = 14.58$, $p = 0.0007$, $\eta_p^2 = 0.34$, CI [0.42, 1.41]. There was no significant difference in the level of freezing between rats that had been injected with naloxone or vehicle in stage 2, including between Groups VEH-NAL and VEH-VEH, $F < 1$, indicating that the naloxone injections prior to S1-shock pairings did not automatically increase sensory preconditioned freezing to S2. There was no significant interaction between linear trend and grouping, Fs < 1.

The analysis of the S1 test data also revealed a significant linear decline in freezing across the stimulus presentations, $F_{(1,28)} = 6.85$, $p = 0.0144$, $\eta_p^2 = 0.20$, CI [−0.64, −0.08]. Overall, groups injected with naloxone in stage 2 (NAL-NAL and VEH-NAL) froze more to S1 than groups injected with vehicle in stage 2 (NAL-VEH and VEH-VEH), $F_{(1,28)} = 14.42$, $p < 0.0007$, $\eta_p^2 = 0.34$, CI [0.55, 1.83]. However, there was no significant difference in the level of freezing between rats that had been

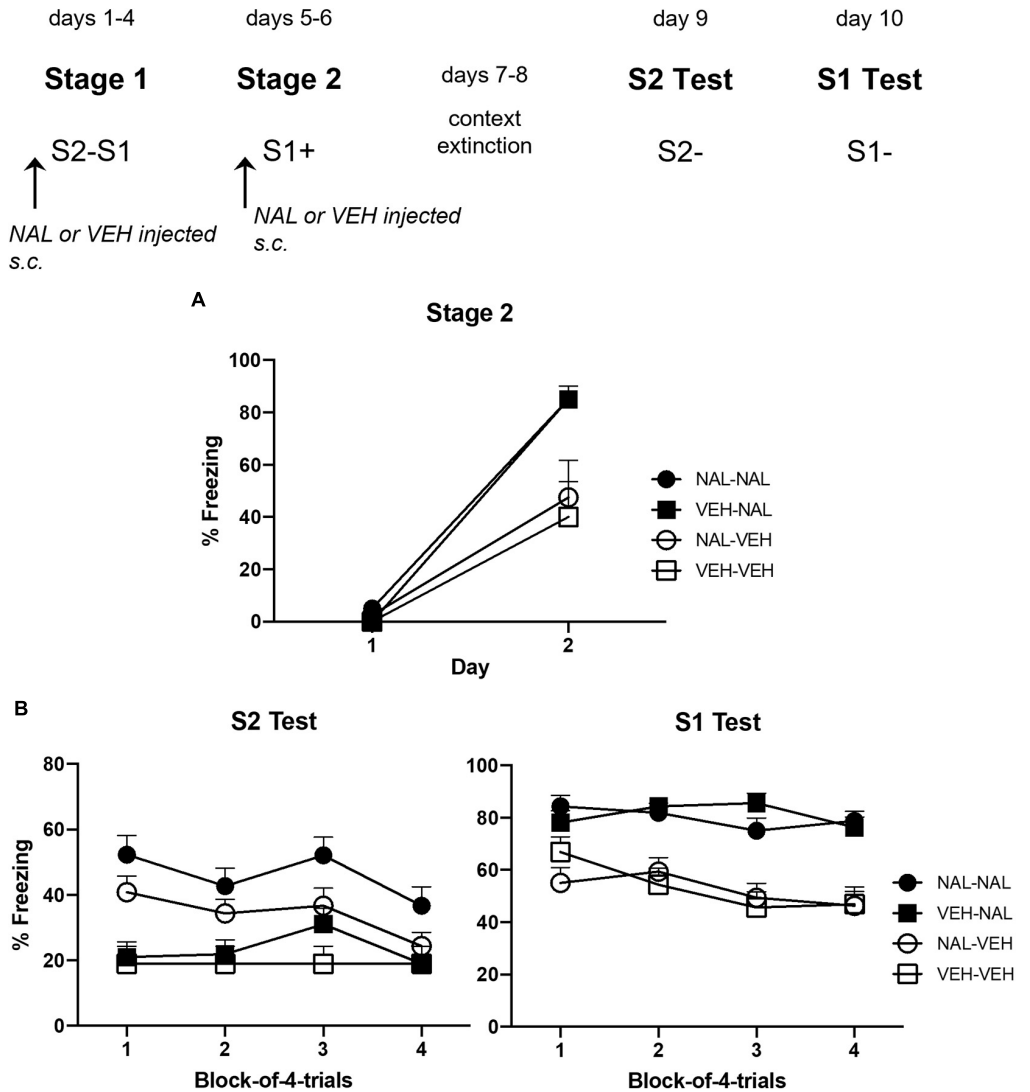

FIGURE 4 | Results of Experiment 2b showing that naloxone enhances the acquisition of sensory preconditioning and first-order conditioned fear. **(A)** Shows the mean (+ SEM) levels of freezing to S1 across its pairings with shock in stage 2. **(B)** Shows the mean (+ SEM) levels of freezing during the final drug-free tests across blocks of four S2 alone trials (left panel) and four S1 alone trials (right panel). The numbers of rats in each group were: Group NAL-NAL, $n = 8$; Group VEH-NAL, $n = 8$; Group NAL-VEH, $n = 8$; and Group VEH-VEH, $n = 8$.

injected with naloxone or vehicle in stage 1, and no significant interactions between linear trend and grouping, largest $F < 3$.

Discussion

This experiment has again confirmed that naloxone enhances the acquisition of first-order fear to S1. Rats injected with naloxone prior to the single S1-shock pairing on each of days 5 and 6 froze more to S1 on day 6 and across subsequent drug-free testing than rats conditioned under vehicle. It has also shown for the first time that naloxone enhances the acquisition of sensory preconditioned fear to S2: rats injected with naloxone prior to each S2–S1 pairing in stage 1 froze more when tested drug-free with S2 than rats injected with vehicle prior to these pairings. Importantly, the effects of naloxone on first-order fear to S1 and sensory preconditioning to S2 did not interact: in the final drug-free tests, rats that had received naloxone in stage 2 froze more to S1 than rats that had received vehicle in stage 2, regardless of the injection that rats had received in stage 1; and rats that had received naloxone in stage 1 froze more to S2 than rats that had received vehicle in stage 1, regardless of the injection that rats had received in stage 2. The implication of these findings will be explored in the section "General Discussion".

GENERAL DISCUSSION

This series of experiments examined whether naloxone can enhance conditioning independently of its effect on US processing. It did so by examining the effect of naloxone on two

forms of conditioning that occur in the absence of US exposure: second-order fear conditioning and sensory preconditioning. The initial experiments examined second-order conditioning. Experiment 1a established second-order conditioned fear using a protocol in which rats were exposed to a single S1-shock pairing on each day of stage 1 and a single S2–S1 pairing on each day of stage 2. Rats trained in this way froze more when tested with S2 alone than controls that had been exposed to explicitly unpaired presentations of the relevant stimuli in training, confirming that the freezing to S2 is due to second-order conditioning. Experiment 1b then used this one-trial-per-day protocol to assess the effect of naloxone on both first- and second-order conditioned fear. Relative to vehicle-injected controls, rats injected with naloxone prior to each S1-shock pairing exhibited faster acquisition of freezing to S1 and more freezing when it was tested drug-free; similarly, rats injected with naloxone prior to each S2–S1 pairing exhibited faster acquisition of freezing to S2 and more freezing when it was tested drug-free. Thus, naloxone enhances second-order fear conditioning just as it enhances first-order fear conditioning, thereby showing that it influences Pavlovian fear conditioning independently of its effect on US processing (e.g., Young and Fanselow, 1992): i.e., it enhances fear conditioning to a stimulus paired with danger regardless of whether the source of the danger is an aversive US, as in first-order conditioning, or a learned source of danger, as in second-order conditioning.

The remaining experiments examined whether the effects of naloxone on Pavlovian conditioning are specific to learning about danger. They did so by examining whether naloxone also enhances sensory preconditioning. Experiment 2a established sensory preconditioned fear using a protocol in which rats were exposed to a single S2–S1 pairing on each day of stage 1 and a single S1-shock pairing on each day of stage 2. Rats trained in this way froze more when tested with S2 alone than controls that had received explicitly unpaired presentations of the relevant stimuli in training, confirming that the freezing to S2 was associative in nature, due to the pairings of S2 and S1 in stage 1 and of S1 and foot shock in stage 2. Experiment 2b then used this one-trial-per-day protocol to assess the effect of naloxone on both first-order conditioned fear and sensory preconditioned fear. It replicated the finding that naloxone enhances first-order fear to S1 and showed, for the first time, that naloxone also enhances sensory preconditioning: relative to vehicle-injected controls, rats injected with naloxone prior to each S2–S1 pairing exhibited more freezing to S2 when it was tested drug-free. These results show that the effects of naloxone are not specific to learning about danger: rather, naloxone enhances associative formation between stimuli that are presented together, including associative formation between neutral stimuli in sensory preconditioning.

The common effect of naloxone on the different types of conditioning suggests that, just as opioids encode the error signal that underlies first-order fear conditioning (e.g., McNally and Westbrook, 2006), an opioid-dependent error signal also underlies second-order fear conditioning and sensory preconditioning. This, in turn, raises two immediate questions: what is learned in second-order conditioning and sensory preconditioning; and how is this learning regulated by error? An obvious possibility is that, in both cases: (1) animals learn to predict S1 when S2 is present and the error in this prediction drives formation of an S2–S1 association; and (2) test presentations of the S2 then retrieve this association, which is "chained" with the S1-shock association to generate fear to the S2. However, the available evidence suggests that this rarely occurs in protocols of the sort used in this study (forward serial pairings of a visual and auditory stimulus); and two aspects of the present findings suggest that this was not the case here. In Experiments 1b and 2b, naloxone enhanced first-order fear conditioning to S1 but this did not automatically increase second-order or sensory preconditioned fear to S2, as predicted by the chaining account: e.g., rats injected with vehicle prior to the S2–S1 pairings exhibited the same test level of freezing to S2 regardless of whether they had been injected with naloxone or vehicle prior to the S1-shock pairings. Therefore, we take these findings to mean that second-order conditioning and sensory preconditioning are not due to chaining of the S2–S1 and S1-shock associations at the time of testing with S2; and by extension, that the naloxone-induced enhancements of second-order conditioning and sensory preconditioning reflect a broader role for prediction error in different types of associative formation.

What then is learned in second-order conditioning and sensory preconditioning; and how is this learning affected by naloxone? The available evidence suggests that, in protocols like the ones used here, the learning that underlies second-order and sensory preconditioned fear is not the same. When S2 is paired with S1 in second-order conditioning, it associates with the central state of fear elicited by the S1: i.e., animals form an S2-fear association that exists independently of the already-conditioned S1-shock association (for further discussion, see Rizley and Rescorla, 1972; Rescorla, 1973, 1982; Holmes et al., 2014). In contrast, when S2 is paired with a neutral S1 in sensory preconditioning, animals *do* form an S2–S1 association; but this is not chained with the S1-shock to generate fear of S2 at testing. Rather, when S1 is conditioned in stage 2, it calls to mind its past associate, the S2, and thereby, mediates an association between the memory of S2 and the foot shock US (Holland, 1981; for supporting data, see Wong et al., 2019). Therefore, we take the present findings to mean that prediction error differentially regulates the S2-fear, S2–S1, and mediated S2-shock associations that form in second-order conditioning and sensory preconditioning. Naloxone preserves error in relation to the S2 and fear, thereby enhancing second-order fear conditioning across the S2–S1 pairings. Similarly, naloxone preserves error in relation to the S2 and S1 events in sensory preconditioning, resulting in stronger S2–S1 associative formation in stage 1, and thereby, retrieval-mediated conditioning of S2 in stage 2. In contrast, naloxone does not affect the mediated S2-shock association that forms when animals are exposed to S1-shock pairings in sensory preconditioning, suggesting that this association is not regulated by prediction error. We propose that the mediated S2-shock association differs from the others in this respect because it involves learning about a retrieved stimulus representation, which may be governed by a different set of rules (e.g., Bae et al., 2015; Lingawi et al., 2018). This hypothesis will be tested in future studies.

Finally, the opioid-dependent error signal that underlies Pavlovian conditioning was inferred from the contrasting effects of naloxone on the acquisition and extinction of first-order fear (McNally, 2009); and has been identified with activity in midbrain circuits including the amygdala and periaqueductal gray (McNally and Cole, 2006; Johansen et al., 2010; Yau and McNally, 2018). At present, the effects of naloxone on extinction of second-order and sensory preconditioned fear are unknown, as are the neural substrates of its effects on second-order conditioning and sensory preconditioning more generally. However, it seems reasonable to predict that naloxone will impair extinction of second-order and sensory preconditioned fear in the same way as it has been shown to impair extinction of first-order fear; and further, that the neural substrates of its effects on second-order fear conditioning and sensory preconditioning will involve the same regions that have been shown to regulate its effects on first-order fear conditioning and its extinction. Specifically, given the critical involvement of the basolateral amygdala complex (BLA) in acquisition and extinction of first-order, second-order and sensory preconditioned fear (Gewirtz and Davis, 1997; Parkes and Westbrook, 2010; Holmes et al., 2013, 2018; Lay et al., 2018; Lingawi et al., 2021), it is likely that an opioid-dependent error signal regulates associative formation in each of these cases via its effects in this region of the brain.

In summary, the present series of experiments has shown that the endogenous opioid system regulates associative formation whenever two events are paired and independently of their affective content. They thus confirm that endogenous opioids do not only affect US processing in Pavlovian fear conditioning: they also encode an error signal that reflects the discrepancy between observed and expected events. Endogenous opioids do not, however, regulate conditioning to a retrieved stimulus representation, presumably because it occurs independently of prediction error. Future work will test this hypothesis, the effects of naloxone on extinction of second-order and sensory preconditioned fear, and finally, the neural substrates of these effects in the BLA. Specifically, it will examine whether naloxone impairs extinction of second-order and sensory preconditioned fear in the same way as it has been shown to impair the extinction of first-order fear and other forms of learning produced by CS alone exposure (e.g., latent inhibition; Leung et al., 2013); and whether naloxone achieves its effects on second-order conditioning and sensory preconditioning via its effects in the BLA.

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 University of New South Wales Animal Care and Ethics Committee.

AUTHOR CONTRIBUTIONS

NH and RW designed the study. RM and DL conducted the experiments, collected, and analyzed the data. RM, NH, and RW wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by an Australian Research Council Discovery Project Grant (DP190100747) awarded to NH and RW and the University of New South Wales Scientia Ph.D. Scholarship Award to DL.

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frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
published: 11 February 2022
doi: 10.3389/fnbeh.2022.751274

Early Auditory Event Related Potentials Distinguish Higher-Order From First-Order Aversive Conditioning

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Stimuli in reality rarely co-occur with primary reward or punishment to allow direct associative learning of value. Instead, value is thought to be inferred through complex higher-order associations. Rodent research has demonstrated that the formation and maintenance of first-order and higher-order associations are supported by distinct neural substrates. In this study, we explored whether this pattern of findings held true for humans. Participants underwent first-order and subsequent higher-order conditioning using an aversive burst of white noise or neutral tone as the unconditioned stimuli. Four distinct tones, initially neutral, served as first-order and higher-order conditioned stimuli. Autonomic and neural responses were indexed by pupillometry and evoked response potentials (ERPs) respectively. Conditioned aversive values of first-order and higher-order stimuli led to increased autonomic responses, as indexed by pupil dilation. Distinct temporo-spatial auditory evoked response potentials were elicited by first-order and high-order conditioned stimuli. Conditioned first-order responses peaked around 260 ms and source estimation suggested a primary medial prefrontal and amygdala source. Conversely, conditioned higher-order responses peaked around 120 ms with an estimated source in the medial temporal lobe. Interestingly, pupillometry responses to first-order conditioned stimuli were diminished after higher order training, possibly signifying concomitant incidental extinction, while responses to higher-order stimuli remained. This suggests that once formed, higher order associations are at least partially independent of first order conditioned representations. This experiment demonstrates that first-order and higher-order conditioned associations have distinct neural signatures, and like rodents, the medial temporal lobe may be specifically involved with higher-order conditioning.

Keywords: EEG, ventromedial prefrontal cortex (vmPFC), second-order conditioning, value, learning, model-based choice, hippocampus, Pavlovian (classical) conditioning

OPEN ACCESS

Edited by:
Arnau Busquets-Garcia, Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Spain

Reviewed by:
Lluís Fuentemilla, University of Barcelona, Spain
Nathan Holmes, University of New South Wales, Australia

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

Received: *31 July 2021*
Accepted: *03 January 2022*
Published: *11 February 2022*

Citation:
Dhamija P, Wong A and Gilboa A (2022) Early Auditory Event Related Potentials Distinguish Higher-Order From First-Order Aversive Conditioning. Front. Behav. Neurosci. 16:751274. doi: 10.3389/fnbeh.2022.751274

INTRODUCTION

Stimuli in the environment can acquire positive or negative value, if they appear in direct association with primary rewards or punishment (e.g., classical conditioning), however, this rarely occurs as an isolated process. Instead, it is thought that value is often inferred through complex higher-order associations. Higher-order associations form when intrinsically neutral stimuli that

have acquired value through direct association with primary rewards or punishments, thus known as conditioned stimuli, are then associated with novel stimuli (Pavlov, 1927; Gewirtz and Davis, 2000). Through this process, higher-order associations enable the representation of important environmental stimuli and their inferred value, promoting flexible and adaptive behavior.

The formation of first-order associations between neutral and inherently valued stimuli is a pre-requisite for inference of value in higher-order relationships. In first-order conditioning, an unconditioned stimulus (US) with an intrinsic value elicits a natural behavior, unconditioned response (UR). The US is preceded by an initially neutral stimulus, known as the conditioned stimulus (CS). The CS is thought to acquire the value of the US (Pavlov, 1927; Gewirtz and Davis, 2000) and motivates behavior even in the absence of the US, which is known as the conditioned response (CR). In higher-order conditioning, this process is extended by one step as the CS with acquired value are associated with novel stimuli without value. These novel stimuli acquiring value are known as higher-order stimuli (HO) and can elicit a CR despite never being directly associated with the US (Pavlov, 1927).

While the formation of higher-order associations depends on the strength of their corresponding first-order associations, once robust higher-order associations are formed, higher-order associations are not simply an extension of first-order learning. Neural structures that represent reward value and motivation cannot independently support representations of intrinsic higher order relations (Wimmer and Shohamy, 2012; Gilboa et al., 2014; Gilboa and Moscovitch, 2021). Lesion studies suggest that first-order and higher-order associations are supported by partially overlapping, but distinct, neuroanatomical structures that seem to differ in their contributions to first-order and higher-order associations. The basolateral amygdala (BLA) and hippocampus are two such structures. Holland (2016) demonstrated that if both forward conditioned first-order training (CS ⟶ US) and forward conditioned (HO ⟶ CS) higher-order training occurred after lesion to the BLA, higher-order learning was impaired. However, if first-order training occurred with an intact BLA and only higher-order training occurred after lesion, then enhanced higher-order associations were formed (Holland, 2016). This suggests that the BLA is critical for first-order conditioning, but that once first-order conditioning is formed, higher-order associations can develop independently of the BLA. Furthermore, the absence of the BLA may enhance higher-order learning because it slows the extinction of first-order CSs during higher-order training (Lindgren et al., 2003; Holland, 2016). Conversely, in a study by Gilboa et al. (2014) hippocampal lesions did not affect forward auditory first-order conditioning (CS ⟶ US) but did severely impair both acquisition and retention of backward serial unimodal auditory higher-order conditioning (CS ⟶ HO). In addition to these neuroanatomical dissociations revealed by lesion studies, higher-order associations can become functionally independent of their corresponding first-order association, as demonstrated by persisting higher-order associations after extinction of the underlying first-order associations (Pavlov, 1927; Rizley and Rescorla, 1972; Rashotte et al., 1977; Rescorla, 1979, 1982; Cole et al., 1995). These studies utilize either appetitive or aversive USs with a a cross-modal presentation of CSs and HOs, with most studies utilizing a forward conditioning paradigm (CS ⟶ US; HO ⟶ CS), though some have used a backward conditioning paradigm (CS ⟶ US; CS ⟶ HO; Cole et al., 1995).

The above-described work suggests that first-order and higher-order can be dissociated by both behavioral responses and the neuroanatomical structures in rodents, consistent with a neural-psychological correspondence view of memory (NPRC; Gilboa and Moscovitch, 2021; cf. Hebscher et al., 2019). However, relatively few studies have investigated higher-order conditioning in humans (Pauli et al., 2019; Prével et al., 2019; Luettgau et al., 2021; see Honey and Dwyer, 2021; Lee, 2021 for a review). Even fewer have examined the neural processes of first-order and higher-order learning in humans. Using functional resonance magnetic imaging (fMRI) to examine sequential learning paradigms (CS1⟶ CS2 ⟶ US), the striatum and orbitofrontal structures have been implicated in learning distally predictive sitmuli (Pauli et al., 2019). These paradigms, however, present both the first-order and higher-order stimuli within the same training trials as the US, tapping gradual learning of complex temporal relationships among conditioned and unconditioned stimuli rather than transfer of value that had been acquired previously by the CS, in the absence of a US. Moreover, fMRI provides excellent spatial resolution but lacks high temporal resolution to examine differences between short timescale temporal features of first-order and higher-order learning. Electroencephalogram (EEG) provides such temporal resolution and has been used previously to examine higher-order conditioning in smokers using pre-established first-order visual stimuli as appetitive CS (e.g., cigarette packs) and simple geometric figures as higher-order stimuli (Littel and Franken, 2012). Higher order visual-visual associations in smokers led to increased evoked response potentials (ERPs) as early as 200–280 ms. over fronto-central electrodes whereas smoking-related first order conditioned stimuli produced a larger P3 component similarly distributed but starting later, from 300 ms. The earlier components of the ERP in which significant differences were elicited by higher-order conditioned stimuli is surprising. However, comparison of well-established, addictive first-order associations to novel higher-order associations may differ from higher-order learning that occurs soon after first-order learning has been established. Moreover, naturalistic smoking related cues are more visually complex than the simple figures used as CS2 by Littel and Franken (2012), which may partially account for the temporal difference.

In the current study, we aimed to determine if aversive auditory higher-order conditioning could be established in humans, by adapting our rodent paradigm where we demonstrated that backwards higher-order associations could be dissociated from first-order associations (Gilboa et al., 2014). Furthermore, we explored the accompanying electrophysiological activity to determine if the neural responses to first-order and higher-order associations were dissociable in the temporal and spatial domains. Participants were conditioned

using consecutive (non-overlapping) auditory stimuli. First-order associations were formed between a neutral tone (CS+) and an aversive burst of white noise (US+; addition of aversive value is indicated by + and neutral value is indicated by –). Implicit anticipation of aversive stimuli (intrinsic or acquired) was measured by pupillary dilation (Korn et al., 2017). To compare these conditioned responses to a control, participants associated two neutral tones as the neutral conditioned stimulus (CS–) and neutral unconditioned stimulus (US–). Following the establishment of first-order conditioned associations, we paired the CS tones that had acquired value with distinct novel auditory stimuli (HO+/HO–) to form higher-order associations.

METHOD

Participants

A total of 16 healthy middle-aged adults were recruited for the study. Of these, one participant withdrew from the study and one participant's data were lost due to technical error, leaving data from 14 individuals (9 males, 5 females, average age = 54.71 years, average education = 16.54 years). Participants were recruited using Baycrest Hospital research participant database, had no history of substance abuse, neurological or significant psychiatric disorders, had normal hearing and normal or corrected to normal vision and were between 40 and 65 years old. Participants also completed a questionnaire that included health questions and questions on age, gender, and education level. This study was approved by the Research Ethics Board at the Rotman Research Institute/Baycrest Hospital. All participants provided written and informed consent before the experiment and were monetarily compensated at rate of $15 per hour plus travel expenses.

Stimuli and Stimulus Presentation

We generated seven distinct auditory stimuli by varying frequency (350, 500, 750, and 1000 hz) and waveform (sawtooth and sine) to establish within sensory modality conditioning effects. These were similar to stimuli we have used in our previous rodent studies examining higher-order conditioning (Gilboa et al., 2014, 2019). In addition, a 500 ms burst of 100 dB white noise was used as the aversive unconditioned stimulus (US+). The peak amplitude of the US+ was 40 dB higher than that of the conditioned tones to ensure that it was sufficiently aversive. The stimuli were randomly assigned to each condition[1].

To ensure that stimuli were not initially different, we compared naïve ratings of the stimuli. As expected, the US+ stimulus was rated as significantly more aversive than US–, whereas no other stimuli (CS+ compared to CS– or HO+ compared to HO–) were rated significantly different (see **Supplementary Analysis 1** and **Supplementary Figure 1**).

Furthermore, after data collection, we compared participants' pupil responses in the first seven presentations of the stimuli to test whether there were pre-learning differences in responses to the physical characteristics of the auditory tones. We observed significantly larger pupil dilation in response to US+ compared with US– (**Supplementary Figure 2**), consistent with the aversive nature of these stimuli, but no significantly larger pupil responses for CS+ compared with CS– (**Supplementary Figure 3**) or for HO+ compared with HO– (**Supplementary Figure 4**). This suggests that unlike US+, larger pupil dilation for CS+ and HO+ found later in the experiment are likely acquired through training rather than inherent to the physical characteristics of the stimuli.

The experiment and cover task were deployed using E-prime 1.2 (Psychology Software Tools, PA, United States). Visual cues appeared on an LCD monitor with a refresh rate of 60 hz. E-prime delivered meta-trial information to the EEG and eye tracker when initiating each trial. The experiment was conducted in a sound isolated room. Auditory stimuli were delivered using ER-3A insert earbuds (Etymotic Research, Elk Grove, IL, United States). Acoustic tubing was used to avoid electromagnetic artifacts caused by stimulus delivery, similar to previous auditory EEG experiments (Aiken and Picton, 2008; Campbell et al., 2012). Participants were seated in a comfortable chair with cushions for support.

Experimental Design

The current study proceeded over 2 days in five phases with participants completing phase one (first-order conditioning) on the first day and the remaining four phases (first-order reminder, higher-order conditioning, first-order testing, and higher-order testing) on the second day.

Participants completed a tone rating task at the start and end of phase one, phase two, and phase three to examine the prior and post rating of stimuli. We examined this information for three reasons: to test reactivity to the US, to test if conditioned stimuli were inherently aversive, and to test if there were shifts in explicit ratings of stimuli's aversiveness after each conditioning phase. Given the non-declarative nature of Pavlovian conditioning (Squire and Zola, 1996) we did not necessarily expect to observe changes in ratings that would correspond with autonomic reactivity changes, and in fact used a perceptual cover task to maximize attention to the stimuli and their relationships but minimize intentional encoding of the conditioned associations.

In phase one, first-order conditioning, participants were conditioned while performing a cover task to form first-order associative relationships, pairing CS+ with US+ and CS– with US–. This cover task was used for phases one through three. In phase 1 (1st day) participants responded to the cover task using the left and right buttons on the mouse to indicate if the tones originated from the same direction (left) or different directions (right). In phase 2 (2nd day), first-order reminders, participants repeated a shorter version of phase one as a reminder of the first-order CS± and US± pairs. In phase 3, higher-order conditioning, participants established higher-order conditioning relationship between CS± and the HO±. This was similar to phase one except that the US± tones were replaced with novel neutral tones intended to become the HO±. In phase four, we tested participants' reactivity to the CS± tones and in phase five, we examined participants' reactivity to the HO± tones.

[1]We had planned to test neurological patients for this study before the COVID-19 pandemic, and we would not have enough patients for counterbalancing, and therefore, we did not counterbalance stimuli in this study.

We measured participants' pupil dilation to evaluate their evoked physiological responses throughout all phases of the study. Pupillometry has been shown to be an effective measure of Pavlovian conditioned responses in humans (Reinhard and Lachnit, 2002) and has been suggested to be one of the best methods to discriminate CS+/CS– conditioned responses (Ojala and Bach, 2020; see Finke et al., 2021 for review and meta-analysis). To measure evoked neural responses, continuous EEG was recorded throughout the experiment (more detail below).

Cover Task and Trial Overview

Participants were informed that the task was perceptual in nature, and their goal was to determine the directional origin of the stimuli. They completed this cover task throughout the study to avoid intentional learning of the associations, and at the same time to ensure they remain engaged with the stimuli and, crucially, the relationships between them. On each trial, participants viewed a fixation cross for a random duration between 3000 and 6000 ms (across all phases, M = 4718.04 ms; SD = 862.29 ms) before the onset of the two consecutive auditory stimuli to collect a stable baseline. Participants continued to fixate on the cross while the auditory stimuli were presented. In the conditioning and reminder phases (phases 1, 2, and 3), two consecutive, no gap, non-overlapping stimuli were presented. Whereas for testing phases (phases 4 and 5), a single stimulus was presented. At the end of each trial, participants were asked to indicate if the two tones originated from congruent or incongruent directions, or, in the testing phases, if the single tone had originated from the left or right. After both stimuli had been presented, a decision screen appeared prompting participants to indicate if the stimuli originated from congruent or incongruent directions with a mouse. A reminder of the left/right response mapping appeared and remained on screen until they made their decision. Participants had unlimited time to make their decision.

Day One

Tone Rating Task

Participants performed a tone-rating task before and after phase 1, 2, and 3. Participants rated four 3-s CS± and US± tones on unpleasantness on a Likert-like scale from 1 to 9 (1 = Neutral, 9 = Extremely Unpleasant). In Phase 1 and Phase 2, tones were presented in the following order: CS–, CS+, US–, US+. In phase 3, tones were presented in the following order: CS–, CS+, HO+, US–.

Phase One: First-Order Conditioning

Following the tone-rating task, participants incidentally learned first-order associative relationships between the CS± and US±. To ensure that participants continued to pay attention to the task while being unaware of the conditioning procedure, they were given the cover task described above. On each trial, participants viewed a fixation cross, heard two consecutive auditory stimuli while still fixating, followed by a decision screen for the tone direction cover task until they indicated their choice by key press. Participants were presented with 80 trials (32 CS+: US+, 32 CS–: US–; 16CS+: CS+), in randomized order for each participant. The duration of the CS± varied between 3500, 4500, 5500, or 6500 ms and was counterbalanced to ensure that each length and congruency of the stimuli were presented equally for CS+: US+ and CS–: US– trials. On 16 of the trials, CS+ stimuli were presented twice consecutively instead of the CS+ being followed by presentation of the US+. This partial reinforcement schedule resulted in reduced predictability of the CS±: US± associations which had three benefits: reduction of explicit learning of the associations, prevention of habituation to the US+ and enhanced acquisition of first-order associations. Partial reinforcement schedules have been suggested to produce more robust higher-order conditioning (Gewirtz and Davis, 1997, 2000; Kamil, 1969). The duration of the US± stimuli were fixed at 500 ms.

Day Two: Phase Two to Five

Phase Two: First-Order Conditioning Reminder

Participants were presented with a total of 20 trials from phase one to reactivate memories of the first-order conditioning pairs from the previous day. This consisted of 8 CS+: US+ trials, 8 CS–: US– trials and 4 CS+: CS+ trials.

Phase Three: Higher-Order Conditioning

Following the tone-rating task, the higher-order relationships between the CS± and HO± were presented to the participants. On each trial, participants viewed a fixation cross, heard the CS and HO pair and followed by a decision screen, indicating whether the pair of tones originated from congruent or incongruent directions. Participants were presented with 48 trials (24 CS+: HO+ trials and 24 CS–: HO– trials) in randomized order for each participant (intertrial interval M = 4767.63 ms, SD = 861.45 ms). To prevent expectancy and maintain stimulus salience, the duration of the CS± were varied between 3000 and 6000 ms. The duration of the HO± was fixed at 4000 ms.

Phase Four: First-Order Stimuli Testing

Participants completed 50 trials presented in random order to examine their response to the CS± stimuli (24 CS+, 24 CS–, 2 CS+: US+). On each trial, participants heard either the CS+ or CS– for 4000 ms (intertrial interval M = 4698.93, SD = 885.27 ms). A similar cover task to the one used in phases one to three was given to participants. Participants were asked to indicate the origin of the tone (left or right) using left or right mouse clicks. On two of the 50 trials, participants were presented with first-order reminder trials; the US+ (100 db burst of white noise) was presented immediately after the CS+. These reminder trials were employed because training on high-order conditioning is known to lead to extinction of the first-order associations (Gewirtz and Davis, 2000) and we expected reminder trials to mitigate behavioral extinction of CS+ responding. The reminder trials were excluded from pupillometry and EEG analyses.

Phase Five: Higher-Order Stimuli Testing

Similar to phase four, participants completed 48 trials (24 HO+; 24 HO–) presented in random order to examine their response to the HO± stimuli (intertrial interval M = 4728.15, SD = 865.65). On each trial, participants heard either the HO+ or HO– for 4000 ms. A similar cover task used as the one used in phase four

was given to participants where they were asked to indicate the origin of the tone (left or right) using the mouse.

Data Analysis

There was one case where a participant's EEG data were corrupted and were therefore not included in the analysis of phase 3. The participant's data were included for analysis in the other phases.

There were three cases where a participant's pupillometry was corrupted in phase 1 and one case in phase 2. Those participant's data were removed from analysis of the affected phase.

Pupillometry Apparatus and Analysis

Measurements of the size of participants' left pupil were acquired using Eyelink 1000 (SR Research; Ottawa, ON, Canada) with a sampling rate of 500 hz. Prior to each phase of the experiment, calibration and drift correction were performed. Cohen and Hershman Analysis Pupil (CHAP version 1.5), a MATLAB (ver. R2020a) open-source software, was used for pre-processing and analysis of pupillometric data (Hershman et al., 2019).

Preprocessing of pupil data using CHAP included four steps to ensure that data were viable for analysis. The first was the exclusion of outlier samples with Z-scores exceeding $\pm$ 3. Z-scores were calculated for each trial using the mean and standard deviation of the 1500 ms baseline period prior to stimuli presentation. Second, outlier trials were excluded if $>$25% samples were missing. We excluded 14.18% of trials in this way. Third, blinks were detected by an algorithm which identifies sharp decreases and increases that precedes and follows a missing pupil during blinking (Hershman et al., 2018). Missing data that were caused by blinks were corrected using linear interpolation. The fourth pre-processing step was the exclusion of participants who were missing 50% or more of trials in either condition. Based on these four preprocessing steps, one participant was excluded from phase 3, two participants were excluded in phase 2, and three participants were excluded from phase 5. This resulted in the following participants included in the analysis for pupillometry: 11 participants in phases 1 and 5, 12 participants in phases 3 and 4, and 13 participants in phase 2. Prior to analysis, trials were aligned using the onset of the first stimulus for each trial and converted to change scores based on each trials baseline (1500 ms pre-stimulus onset). Each trial's data was converted to a z-score by using the 1500 ms pre-stimulus onset period as the expected mean and standard deviation.

Analysis was conducted by comparing the relative z-scored pupil size change between the two conditions during the post-stimulus onset period of interest (220 ms post-stimulus onset to the end of the trial; Hershman et al., 2018). For each phase, a series of Bayesian paired sample t-tests were conducted over the post-stimulus period of interest. This meant that each sample, taken every 2 ms, was compared between the two conditions using a Bayesian paired-samples t-test. We used a default Cauchy prior width of r = 0.707 for effect size on the alternative hypothesis over the null hypothesis (Rouder et al., 2012). The Bayes Factor (BF) threshold was 3, a value that represents substantial evidence (Jeffreys, 1961). In this case, this means that the measured pupil sizes from two conditions are not the same. This type of analysis has been used in recent studies comparing pupil dilation (Papesh and Pinto, 2019; Hershman et al., 2021).

Evoked Response Potentials Recordings and Analysis

Continuous EEG was recorded using the Biosemi Active Two acquisition system (BioSemi V.O.F., Amsterdam, Netherlands) and a montage of 72 electrodes, with a Common Mode Sense (CMS) active electrode and Driven Right Leg (DRL) passive electrode serving as ground. In addition to the 64-channel scalp electrode cap based on the 10/20 system, we used eight facial electrodes placed below the hairline (both mastoid points, both pre-auricular points, outer canthus of each eye, and inferior orbit of each eye) to measure ocular movement and ensure even coverage of the whole scalp. Equal scalp coverage ensured that we were able to use an average of all scalp EEG channels as a reference for each channel for ERP analysis. Neural activity was digitized continuously at a rate of 512 Hz with a bandpass of 0.16 hz–100 Hz and stored for offline analysis. Brain Electrical Source Analysis software (BESA, version 6.1; MEGIS GmbH, Gräfelfing, Germany) was used to perform analysis.

The continuous EEG were visually inspected for channels displaying faulty recordings and these were either interpolated or ignored (if they were around the rim of the cap) and large muscle artifacts were tagged. An independent component analysis (ICA) was then performed on a 40 s time window to parse any spatial topographies of artifact-related patterns of activity (e.g., horizontal or vertical eye movements, eyeblinks, EKG activity, etc.). These were identified and subtracted from the continuous EEG. A 0.53 high bandpass digital filter (forward, 6 dB/octave) was applied. The continuous EEG files were segmented into 900 ms epochs (including a 100 ms pre-stimulus window and the first 800 ms. of the stimulus presentation) and re-referenced to a common average reference. Trials were sorted by phase of experiment and stimulus type: phase 3 first-order tones (CS+, CS–), phase 4 first-order tones (CS+ or CS–) and phase 5 higher-order testing (HO+ or HO–). Note that ERPs corresponded to the initial time window of each trial and so evoked responses during conditioning were always to the first stimulus in each pair, before the HO appeared in phase 3. ERPs were digitally low-pass filtered to attenuate frequencies of $>$20 Hz and averaged for each condition. ERP amplitudes were measured relative to the mean amplitude over the pre-stimulus interval. Statistical analyses of ERP waveform differences were performed for 0–800 ms. using BESA Statistics 2.0, which includes a spatio-temporal permutation-based correction for multiple comparisons. We used a cluster alpha of 0.05, 1000 permutations, with clusters defined using the default channel distance of 4 cm.

An iterative 3D source imaging method, CLARA (Classical Low-Resolution Electromagnetic Tomography Analysis Recursively Applied), was used for source estimation of surface-level evoked response components that showed

significant difference in amplitude. The CLARA approach applies the LORETA algorithm iteratively localizes activity to the constrained regions identified from the previous solution. Three iterations were computed using the default voxel dimension of 7 mm^3 and 1% regularization constant. The solution was computed using an adult realistic head model in BESA 6.1 and registered against the standardized BESA finite element model, which was created from the average of 24 individual anatomical magnetic resonance imaging (MRIs) in the Talairach-Tournoux coordinate space. Condition differences in source solution of the evoked responses were tested using a parameter-free permutation paired *t*-test in combination with data clustering to correct for multiple comparisons of the averaged source across the time window of significant surface-level component difference, implemented in BESA statistics 2.0.

Correlation of Pupil Size and Neural Responses

To examine if greater pupil responses were associated with a more extreme electrophysiological response, the *z*-score of mean pupil size from segments that were found to be significantly different were extracted and correlated with individual cluster scores from the ERP scalp analysis for each participant using Jamovi, a GUI for R (R Core Team, 2014; The Jamovi Project, 2020).

RESULTS

Below we first describe participants' pre-task subjective ratings of each stimulus. We then describe the autonomic pupil and neural responses to first-order stimuli followed by the autonomic pupil and neural responses to higher-order stimuli.

Cover Task and Subjective Tone Rating of Stimuli

Participants' responses to the cover task suggested that participants remained engaged with the task as they had responded on every trial for all tasks. Furthermore, participants' response accuracy during conditioning phases (phase 1, phase 2, and phase 3) suggest that they were attending to the relation between the two stimuli and that the task was sufficiently challenging so as to avoid ceiling effects, and not too challenging so as to avoid floor effects (proportion correct, $M = 0.72$, $SD = 0.42$).

To ascertain whether the US+ tone was intrinsically more aversive than other stimuli, we compared participants' ratings of each tone prior to the start of the experiment. A one-way repeated measures ANOVA revealed a main effect of tone type, *Greehouse–Geisser* $F(5,50) = 10.10$, $p < 0.001$, $\eta^2 = 0.503$. Follow up tests revealed that participants rated the US+ tone as significantly more aversive than all other tones, $p < 0.001$. All other tones were not rated as significantly different than each other, all *p's* > 0.05. Thus, participants found the US+ more aversive than the other stimuli prior to any conditioning, and the other tones were not inherently different subjectively.

In addition, comparison of subjective ratings of tones before or after the task revealed no significant differences (all *p's* > 0.05). Collapsing pre-and post- tone ratings, participants rated the CS– tone as more aversive than the HO– tone in phase 3, though stimuli were never rated significantly different than their complementary tone in valence (i.e., no difference between CS+ and CS– and HO+ and HO–; see **Supplementary Analysis 1** and **Supplementary Figure 1** for more detail).

Behavioral Responses to First-Order Stimuli: Pupillometry

We predicted that participants would demonstrate greater pupil dilation for the stimuli associated with the acquired aversive value, i.e. greater pupil dilation in the CS+ and HO+ conditions than the CS– and HO– respectively.

Pupil Responses to First-Order Stimuli During Phase 1: First-Order Conditioning

Our analysis suggests that there is substantial evidence ($BF_{10} \geq 3$) for meaningful differences in mean relative pupil dilation between the aversive and neutral tones from approximately 1100 to 1800 ms, and from 2000 to 3000 ms during the CS only presentations (see **Figure 1A**). Note that these differences reflect the gradual acquisition of value by the CS (see **Supplementary Figure 3**), but despite this there was evidence for conditioning. US onset was variable and occurred at the offset of the CS. There were differences from 3000 to 4750 ms during a time window where either the CS continued or a US may have appeared (mixed), and between approximately 6500 and 7000 ms during a time only a US was present (see **Supplementary Figure 2** for onset aligned responses to US stimuli only early in the phase 1).

Pupil Responses to First-Order Stimuli During Phase 2: First-Order Reminders

Participants underwent a block of additional first-order conditioning trials after returning for the second day of the experiment. Our analysis suggests that there was substantial evidence ($BF_{10} \geq 3$) for meaningful differences in mean relative pupil dilation between the CS+ and CS–, which arose from approximately 330 ms and remained for over 5 s throughout the CS only and CS/US mixed time window until 5420 ms post onset of the CS, as well as throughout the US only time window (**Figure 1B**). This suggests robust maintenance of the value acquired the previous day, as this block was much shorter than phase 1.

Pupil Responses to First-Order Stimuli During Phase 3: Higher-Order Conditioning

Our analysis suggests that there was substantial evidence ($BF_{10} \geq 3$) for meaningful differences in mean relative pupil dilation from 1050 to 2700 ms during presentation of the CS (**Figures 1C**, **2A**).

Pupil Responses to First-Order Stimuli During Phase 4: First-Order Testing

Conditioned pupil dilation to CS+ pupil dilation responses appear to have been extinguished as there are no longer

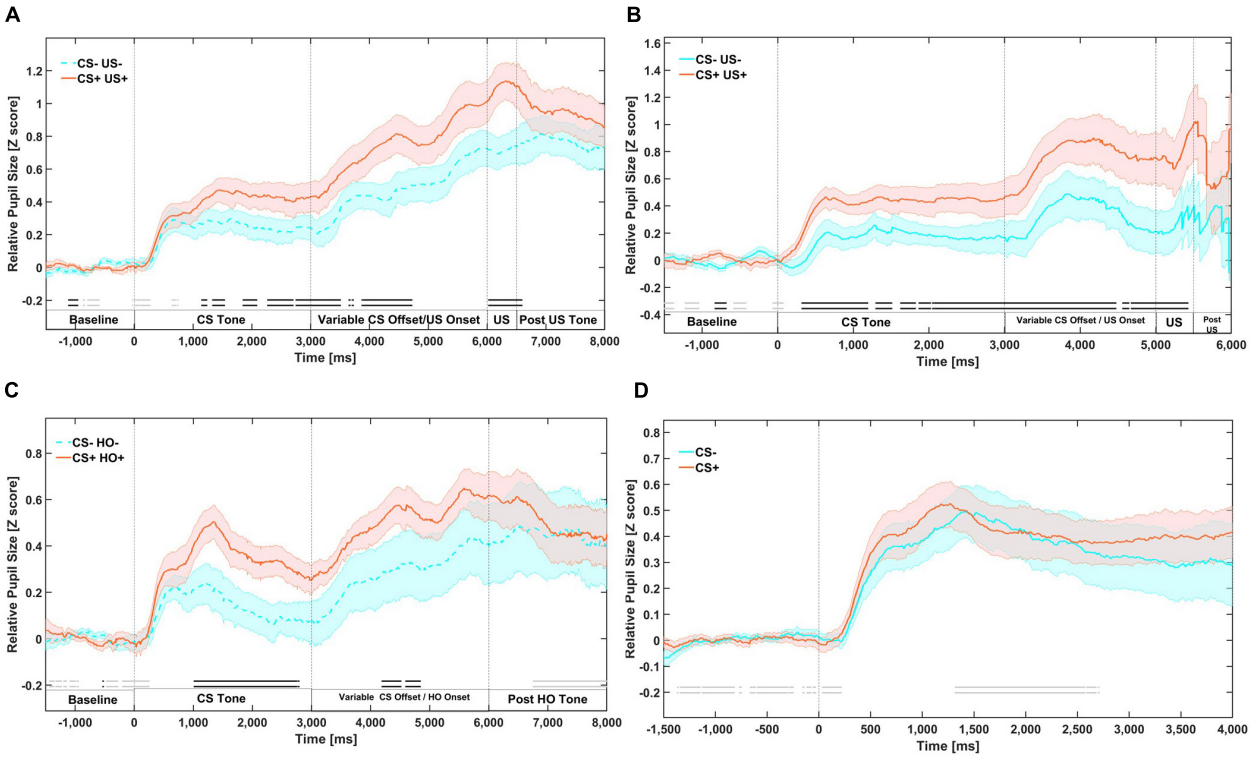

FIGURE 1 | Mean relative pupil dilation (*z*-score) responses during first order tones in four different phases. **(A)** Phase 1: First-order conditioning on day 1. **(B)** Phase 2: First-order reminders on day 2. **(C)** Phase 3: Higher-order conditioning. First-order stimuli are paired with higher-order stimuli. **(D)** Phase 4: First-order testing. First-order stimuli presented alone. Meaningful differences (BF10 $\geq$ 3) are indicated by an opaque double line along the X axis of each panel of the figure, above the segment labels. Evidence in favor of the null hypothesis is indicated by faded double line along the X axis of each panel of the figure. The dark line in color represents the mean of that condition (orange for CS+/US+ trials and blue for CS–/US– trials). The lighter color bands surrounding the darker colored lines represent Standard Error of the Mean.

meaningful differences ($BF_{10} \geq 3$) in mean relative pupil dilation between the CS+ and CS– throughout the presentation of the CS+ (**Figure 1D**).

Neural Responses to First-Order Stimuli Presented During Phase 3: Evoked Response Potentials

Evoked responses to auditory cues for both conditions revealed the typical well-established cortical auditory components described in the literature (Winkler et al., 2013; Remijn et al., 2014). These include a P1, N1 and an early P3 obligatory early response complex, which in this study peaked at roughly at 75, 125, and 275 ms, respectively (Winkler et al., 2013). The P1 is thought to reflect initial pre-attentive arousal to the auditory stimulus (Winkler et al., 2013), the N1 is thought to reflect pre-attentive representations of auditory stimulus features, including mnemonic characteristics (e.g., mismatch negativity; Molholm et al., 2005). Finally, the P3 is a marker of attentional capture (early) and of task-relevant stimulus identity processing (late; Winkler et al., 2013). As mentioned previously, we examined neural responses to first-order stimuli during presentation of first-order stimuli during phase 3: higher-order conditioning. The two reasons for this decision were: (1) first-order responses had extinguished by phase 4 (first-order testing) and (2) there were not adequate number of trials in phase 2 (first-order reminders), for fully powered bootstrap cluster analyses, however, see **Supplementary Analysis 2** and **Supplementary Figure 5** for phase 4 analyses.

Scalp Analysis

Permutation based analyses correcting for temporal and spatial extents of the ERP waveforms revealed a significant difference from 240 to 300 ms, with a greater positive peak in response to CS+ tones. This cluster encompassed electrodes bilaterally in frontal (F1, Fz, F2, F4, FC1, FCz, FC2, FC4), central (C3, C1, Cz, C2, C4), and parietal areas (CP3, CP1, CPz, CP2, CP4 P1, Pz). Within the temporal extension of the significant cluster, the peak response was centered on frontal, and central electrodes (Fz, FCz, Cz) throughout the response, cluster-based statistics, $p < 0.001$ (**Figures 2B,C**).

There was a significantly greater negative peak, peaking primarily – in the parietal areas (CP3, CP1, CPz, P3, Pz, P2, PO3) from 665 to 750 ms, cluster-based statistics, $p = 0.038$.

Source Analysis

Source estimation analysis was conducted on the ERP segment where significant greater positive modulation was identified

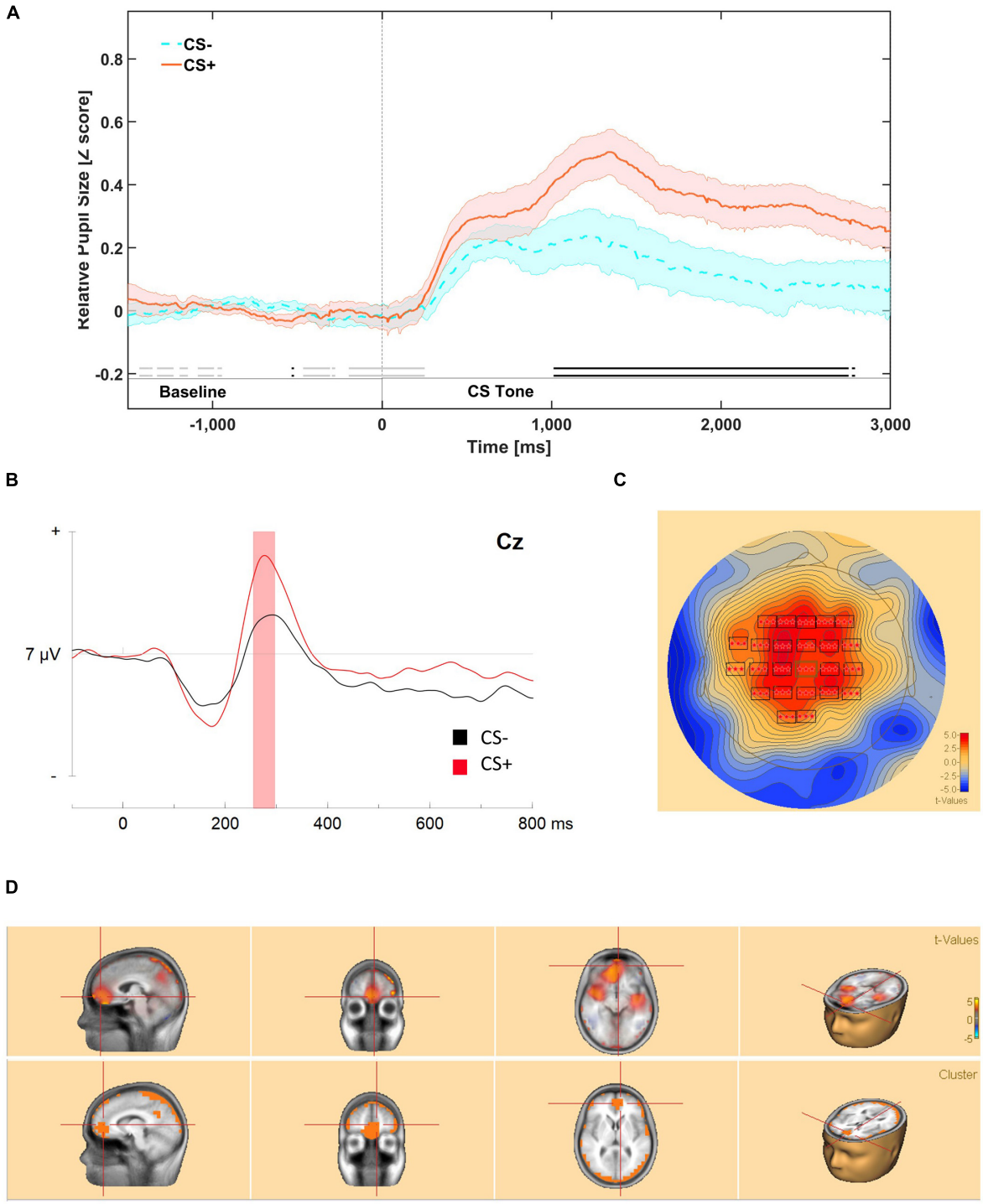

FIGURE 2 | (A) Mean relative pupil dilation (*z*-score) responses during first order tones in phase 3, between –1500 ms (pre-stimulus onset) and 3000 ms (post-stimulus onset). Meaningful differences ($BF_{10} \geq 3$) are indicated by an opaque double line along the X axis of the figure, above the segment labels. Evidence in favor of the null hypothesis is indicated by faded double line near the bottom of the figure. The dark line in color represents the mean of that condition (orange for CS+/US+ trials and blue for CS–/US– trials). The lighter color bands surrounding the darker colored lines represent Standard Error of the Mean. **(B)** ERP response to first-order stimuli presented during phase 3: higher-order conditioning at Cz. Bootstrap cluster analyses revealed a significant difference from 240 to 300 ms. **(C)** Mean potential distribution maps average across participants (n = 14 at scalp level. The significant positive modulation peaks bilaterally in frontal-parietal areas. **(D)** Source estimation analysis was conducted from 245 to 300 ms encompassing the significant greater modulation identified in source estimation. A positive significant source was identified right medial prefrontal cortex.

in scalp analysis (240–300 ms). These cluster based bootstrap analyses revealed one significant positive cluster in the dorsal anterior cingulate area (BA 32) encroaching into the left anterior medial prefrontal cortex (BA 10), and onto other medial frontal areas including right amygdala cluster-based statistics, $p < 0.0001$ (**Figure 2D**).

Correlations Between Pupil and Neural Responses to First-Order Stimuli During Phase 3

We conducted an exploratory correlation analysis of behavioral and neural responses to first-order tones presented in phase 3. There was a significant correlation between mean pupil dilation z-score during the maximal CS+/CS– difference (1050–2700 ms) and the significant cluster from the bootstrap analysis of evoked responses (240–300 ms) for CS+, $r(12) = 0.643$, $p = 0.002$, but not CS–, $r(12) = 0.419$, $p = 0.175$.

Behavioral Responses to Higher-Order Stimuli During Phase 5: Pupillometry

We predicted that participants would demonstrate greater pupil dilation for the stimuli associated with the acquired aversive value. We predicted that participants would demonstrate greater pupil dilation in the HO+ conditions than the HO– respectively.

Our analysis suggests that there was substantial evidence ($BF_{10} \geq 3$) for meaningful differences in mean relative pupil dilation between the HO+ and HO– from approximately 320 to 650 ms and approximately 1300 to 1400 ms during the presentation of the tone (**Figure 3A**).

Neural Responses to Higher-Order Stimuli During Phase 5: Evoked Response Potentials

Scalp Analysis

Evoked responses to auditory cues for both conditions matched the pattern of a P1, N1, and early P3 early response complex described in the literature (Woodman, 2010; Winkler et al., 2013) at roughly 75, 125, and 225 ms, respectively.

Permutation based analyses correcting for temporal and spatial extents of the ERP waveforms revealed a significant difference reflecting greater negative modulation in response to HO+ tones that encompasses N100, peaking predominantly in the left hemisphere in frontal area (FC3, FC1, FCz, FC2) central area (C3, C1, Cz, C2, C4, CP3, CP1, CPz, CP4) from 100 to 145 ms; cluster-based statistics $p = 0.04$ (**Figures 3B,C**).

Source Analyses of Evoked Response Potentials to Higher-Order Stimuli During Phase 5

Source estimation analysis was conducted from 100 to 145 ms encompassing the significant greater negative modulation identified in scalp analysis.

A significant source cluster reflecting greater positivity for HO+ compared to HO– encompassed the right hippocampus, while also encroaching onto the left Parahippocampal area (BA 36), $p = 0.004$ (**Figure 3D**).

Correlations Between Pupil and Evoked Response Potentials Responses to Higher-Order Stimuli During Phase 5

We conducted an exploratory correlational analysis to determine if mean pupil dilation and significant clusters from ERPs were correlated. We did not find significant correlations between mean pupil dilations and evoked responses for either HO+, $r(11) = 0.013$, $p = 0.696$, or HO–, $r(11) = -0.181$, $p = 0.594$ (**Figure 4**).

Correlation Between First-Order and Higher-Order Behavioral Responses

To determine if the strength of responses to higher-order stimuli were dependent on strength of responses to first-order stimuli. We conducted a correlation between mean pupil dilation responses for first-order stimuli during phase 3, and higher-order stimuli during phase 5. To control for potential different baseline pupil responses, we took a difference score between the stimuli associated with an aversive outcome and the stimuli associated with a neutral outcome ($CS^{difference} = CS^{+} - CS^{-}$and $HO^{difference} = HO^{+} - HO^{-}$). We did not find significant correlations between mean $CS^{difference}$ and $HO^{difference}$, $r(13) = 0.370$, $p = 0.213$.

DISCUSSION

In the current study, we demonstrated that distinct behavioral and neural responses to higher-order and first-order stimuli could be identified in human participants. Participants acquired and retained first-order and higher-order associations, indexed by greater pupil dilation in response to CS+ and HO+ stimuli when compared to CS– and HO– stimuli, respectively. Evoked responses to first-order and higher-order stimuli shared the typical well established cortical auditory components including a P1, N1 and early P3, however, first-order and higher-order neural responses could be differentiated by the auditory component that was responsive to their acquired value. The later auditory component (early P3) uniquely discriminated acquired value for first-order stimuli (CS+ from CS–) whereas an earlier auditory component (N1) uniquely discriminated acquired value for higher-order stimuli (HO+ from HO–). First-order acquired aversive associations revealed greater positivity bilaterally over the central parietal scalp area during early P3, specifically from 240 to 300 ms. Conversely, higher-order acquired associations displayed an earlier greater negativity, the N1 component, specifically from 100 to 145 ms over the central scalp area. Source estimation models revealed distinct sources for these two components associated with first-order and higher-order conditioning. While the CS+ early P3 likely originated from left anterior mPFC, and amygdala, the HO+ N1 was estimated to arise from the parahippocampal cortex and the hippocampus. Interestingly, behavioral and evoked responses to higher-order stimuli were detectable even after responses to first-order stimuli no longer elicited a conditioned response during phase 4 testing. Persisting behavioral and neural responses suggest that while

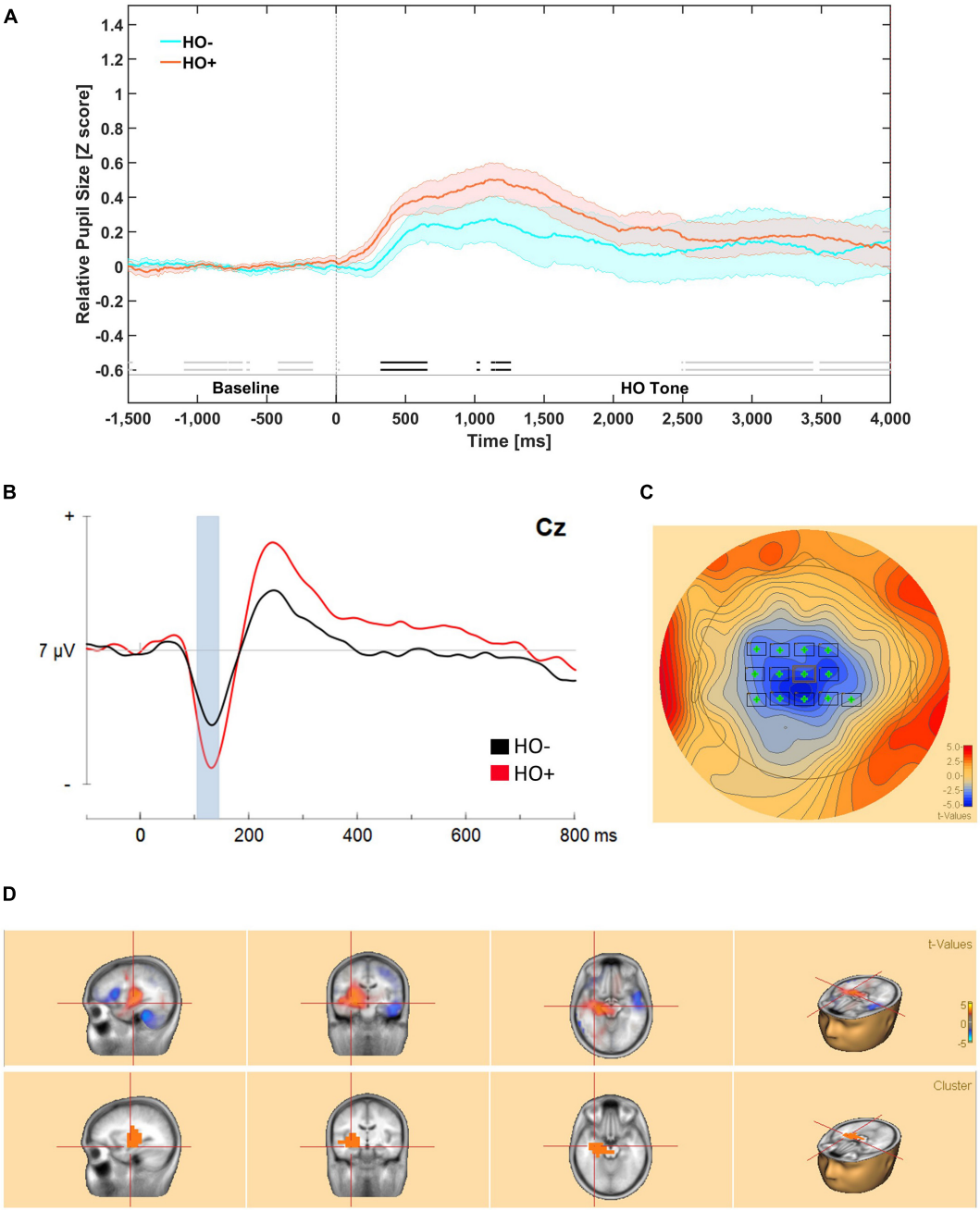

FIGURE 3 | (A) Mean relative pupil dilation (*z*-score) responses during higher-order tone presentation in phase 5. Meaningful differences ($BF_{10} \geq 3$) are indicated for by an opaque double line near the bottom of the figure above the segment labels. Evidence in favor of the null hypothesis is indicated by faded double line near the bottom of the figure. Meaningful differences are observed from 400 to 1500 ms post-stimulus onset. The dark line in color represents the mean of that condition (orange for CS+/US+ trials and blue for CS–/US– trials). The lighter color bands surrounding the darker colored lines represent Standard Error of the Mean. **(B)** Evoked responses to higher order tones revealed from bootstrap cluster analysis. Evoked responses waveform measured at Cz to higher order tones. Bootstrap cluster analyses revealed a significant difference from 100 to 155 ms. **(C)** Mean potential distribution map averaged across participants ($n = 14$) at scalp level. The significant negative modulation peaks bilaterally in central-parietal electrodes. **(D)** Source estimation from 100 to 135 ms revealed two significant clusters. A significant negative source encompassed the left parahippocampal area (BA 36) and the left Hippocampus (BA 54).

higher-order associations appear to be affected by the strength of first-order associations, they can withstand the concomitant extinction of first-order tones during higher-order training, suggesting at least partial independence of value representations. These findings are consistent with our previous animal studies (Gilboa et al., 2014, 2019) and with the NPRC model that argues

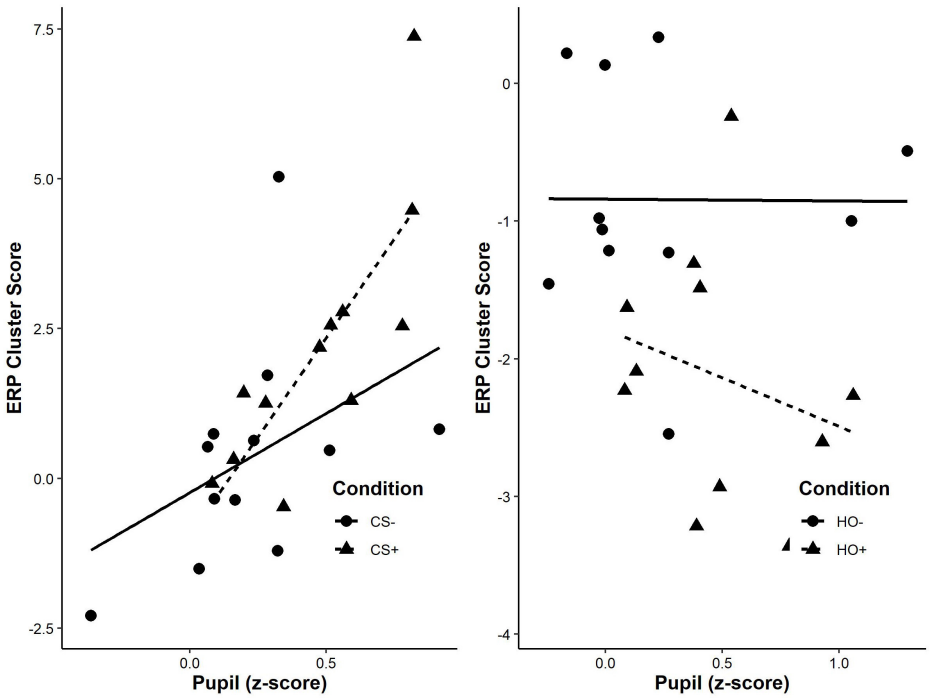

FIGURE 4 | Correlation of mean pupil *z*-score and ERP cluster score for each participant comparing the valence of the effect (+: aversive represented by triangles; −: neutral represented by circles) for first-order (left) and higher-order stimuli (right). A significant positive correlation between mean pupil dilation z-score during the maximal CS+/CS− difference (1050–2700 ms) and the significant cluster from the bootstrap analysis of evoked responses (240–300 ms) for CS+, $r(12) = 0.643$, $p = 0.002$, but not CS−, $r(12) = 0.419$, $p = 0.175$. No such correlation was detected in response to higher-order stimuli.

for a correspondence between the type of memory representation and the neural substrate that supports it (Hebscher et al., 2019; Gilboa and Moscovitch, 2021). We discuss the implications of these findings to memory representations of value in relation to the animal and human research on higher-order conditioning.

Previously neutral tones acquired value following both first-order and higher-order conditioning, as reflected by pupil dilation and by larger amplitudes of the auditory ERPs. However, the specific temporal window within which significant amplitude increases occurred differed across conditions. Significant CS+/CS− differences occurred later, during early P3, whereas the HO+/HO− difference occurred earlier, during N1. The different auditory components suggest that first-order and higher-order responses can be dissociated by distinct pre-attentive or peri-attentive neural responses because these auditory components are believed to reflect discrete processes. The early P3 component is thought to reflect peri-attentive mechanisms that are responsive to stimulus frequency and intensity whereas the N1 component is thought to reflect pre-attentive sensory elements of auditory selective attention that are responsive to sudden sound changes in the environment (Winkler et al., 2013; Remijn et al., 2014). These characterizations of the P3 and N1 components suggest that responses to CS+ in our study reflect the highly aversive associated intensity of the US+, whereas responses to higher-order stimuli are driven by an early attentional orientation to the auditory features of the response. This early orientation may reflect learning in which stimuli can become represented as contextual stimuli, helping the animal determine in which context a CS:US contingency is active (Honey and Watt, 1999). The earlier response to higher-order than first-order acquired values is reminiscent of findings in smokers (Littel and Franken, 2012) in whom visual ERPs for smoke-related stimuli was later (300 ms) than ones to recently acquired higher-order conditioned stimuli (200–280 ms). Note that while the order is similar, observed ERPs in our study appear much earlier for both first-order and higher-order responses. This may be related to a combination of differences in modality (visual vs. auditory), valance (appetitive vs. aversive), significance (meaningful vs. arbitrary) and strength of first order associations (years of addiction vs. experimental session). Nonetheless, it is noteworthy that in both cases responsivity to higher-order conditioned stimuli appeared earlier than first-order conditioned stimuli. Animal electrophysiology may shed more light on this finding.

First-order and higher-order conditioned responses also differed with respect to the estimated neuroanatomical structures that generated the significant ERP clusters. First-order ERP's were source estimated to the amygdala, and prefrontal cortex, aligning with human and rodent models which have implicated the amygdala and prefrontal cortex in acquisition and extinction of first-order learning (Lindgren et al., 2003; Holland, 2016; Ebrahimi et al., 2019). Higher-order ERP's were source estimated the anterior temporal lobe, hippocampus and parahippocampus. These findings align with our previous rodent study that the hippocampus is critical for higher-order but not first-order

conditioning (Gilboa et al., 2014). It is consistent with models of memory that posit that the nature of the representation determines the neural substrates engaged during encoding and retrieval (Hebscher et al., 2019; Gilboa and Moscovitch, 2021), contrary to dichotomous memory systems views (e.g. Squire and Zola, 1996). It should be noted that source estimation in our study is limited and thus should be interpreted with caution. Nonetheless, these findings are useful to inform future studies that examine higher-order conditioning using methods with high spatial resolution.

We based our ERP analysis of first-order conditioned associations on phase 3 (higher-order conditioning) because pupil dilation effects for CS+ were still detectable, which were no longer present during phase 4. Interestingly, despite the lack of differentiated pupil responses, CS+/CS– differences in ERPs in phase 4 were still observed in the same ERP component with a broadly similar scalp distribution as in phase 3 (**Supplementary Figure 5**). The early P3 occurred slightly earlier at 200–255 ms and was source estimated to the right insula and right putamen, consistent with prior research on aversive conditioning (Seymour et al., 2004). While ERPs in phase 3 were measured purely during CS+/CS– tone presentation, the trial as a whole nonetheless entailed other processes as well, such as HO acquisition and possibly CS+ extinction, which may account for these differences.

The current experiment used aversive conditioning, whereas our previous study with rodents used appetitive conditioning with a conflicting aversive contingency. In that previous study, if rodents entered the reward chamber they would receive very mild shock creating incentive to approach only under high certainty (Gilboa et al., 2014). This highlights two important questions that future work would need to examine: what differences we might see when comparing appetitive and aversive higher-order conditioning and what role might conflicting contingencies play in higher-order learning. Previous work in both humans and animal models has shown the importance of the hippocampus for approach-avoidance conflict decision making (Ito and Lee, 2016) consistent with hippocampal involvement in representing complex associations between stimuli (Olsen et al., 2012).

Higher-order associations had been acquired and recalled as shown by a greater pupil response to HO+ compared to HO- in phase 5. We infer that, once acquired, higher-order responses may be at least partially independent of first-order responses to value, because the pupil responses were present in phases 2 and 3 but were no longer observable during phase 4. It appears that despite our efforts to strengthen first-order associations by intensive phase 1 training, (i.e. a night of sleep-enhanced consolidation, phase 2 re-training, and 2 US reminders in phase 4), first order responses had been extinguished during training of higher-order associations, a typical concomitant response to higher-order conditioning (Gewirtz and Davis, 2000). ERP effects during phase 4 suggest that first-order memory engrams were still present but were probably not sufficient to produce detectable pupil responses. Expression of independent first-order and higher-order associations align with previous studies in which independent higher-order associations are expressed after extinction of first-order associations (Pavlov, 1927; Rizley and Rescorla, 1972; Rashotte et al., 1977; Rescorla, 1979; Cole et al., 1995), and in studies where elevated skin conductance responses to higher-order stimuli remained even after extinction of first-order associations (Davey and Arulampalam, 1982). It may be that similar to our findings, residual neural engrams were also present in rodents and humans in these earlier studies, but that it was insufficient to drive overt behavioral responses.

The sequence of stimulus presentation during higher-order conditioning may partially account for the dissociations between higher-order and first-order associations in our experiment. First-order association pairs were arranged in a forward sequence so that the CS precedes the US, whereas the higher-order association pairs were arranged in a backward sequence so that the HO follows the, now value carrying, CS. In other words, the HO was presented when participants expected the US to appear and this may have contributed to the persistence of higher-order associations. While several human higher-order conditioning studies use classical Pavlovian training procedures in which HO predicts a previously conditioned CS (see Lee, 2021 for review), different conditioning procedures have been used including sequential conditioning where distal (HO) and proximal (CS) both precede the US (Seymour et al., 2004; Pauli et al., 2019) and also different combinations of backward conditioning as in our study (Prével et al., 2016, 2019). Higher-order learning in our paradigm was modeled after an animal higher-order conditioning study that used the same presentation structure (Gilboa et al., 2014). That study also found a dissociation between first-order and higher-order behavioral responses as well as the neuroanatomical structures involved in both processes. Other rodent work has used backwards higher-order conditioning with a forwards first-order trace conditioning and found a dissociation between higher-order and first-order associations (Cole et al., 1995) although the authors suspected predictable stimulus durations provided temporal information that may have confounded CS2 responses. We varied CS duration, as recommended by Cole et al. (1995), avoiding this potential confound. In line with our study, previous human higher-order conditioning studies have used backwards sequence higher-order associations and showed that expression of higher-order CRs was maintained after first-order stimuli no longer elicit the CR (Prével et al., 2019). Note however, that in the Prével et al. (2019) study, first order associations were also trained with backward conditioning. These authors suggest that backwards conditioning may lead to an associative structure that is resistant to extinction of first-order associations and that individuals may learn to associate stimuli to form flexible representations of their environment as participants' responses appear to index bidirectional relationships rather than linear chains (Honey and Watt, 1999; Arcediano et al., 2005; Molet et al., 2010; Prével et al., 2016, 2019) consistent with representational characteristics of hippocampal memory traces (Gilboa and Moscovitch, 2021).

Results from this study should be interpreted with caution and may not be generalizable due to the small sample size of participants that were included. Further studies should be conducted to examine this phenomenon. Moreover, we cannot completely rule out that some participants may have developed awareness of the associative nature of the experiment. While this

is unlikely given the early nature of the ERP differences and lack of change in explicit ratings of tone valence, further studies should probe the issue of awareness more extensively.

CONCLUSION

In conclusion, this experiment demonstrates that memory for first-order and higher-order conditioned associations reflect distinct pre-attentive and peri-attentive electrophysiological responses. These likely originate from distinct sources. The findings are consistent with literature implicating the amygdala and prefrontal cortex in first-order conditioning and extinction. They are also in line with recent animal studies that have implicated the hippocampus, specifically in higher-order conditioning and suggest its involvement may be rapid.

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 Rotman Research Institute/Baycrest Hospital Research Ethics Board. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AG designed the experiment. AW collected the data and conducted the cleaning and basic analysis of EEG data. PD conducted the data cleaning, pre-processing, analysis of pupillometry, and EEG data. AG and PD conducted the analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Science Engineering Council of Canada Discovery Grant #378291 to AG. PD is supported by the Natural Sciences and Engineering Research Council of Canada's Canada Graduate Scholarship Doctoral Program.

ACKNOWLEDGMENTS

We would like to thank Elynor Solomon, Kyle Nealy, George He, and Saambavy Shan for help with data collection and Avishai Henik's lab for making the CHAP software available.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.751274/full#supplementary-material

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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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MINI REVIEW
published: 18 April 2022
doi: 10.3389/fnbeh.2022.845616

Understanding Associative Learning Through Higher-Order Conditioning

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Associative learning is often considered to require the physical presence of stimuli in the environment in order for them to be linked. This, however, is not a necessary condition for learning. Indeed, associative relationships can form between events that are never directly paired. That is, associative learning can occur by integrating information across different phases of training. Higher-order conditioning provides evidence for such learning through two deceptively similar designs – sensory preconditioning and second-order conditioning. In this review, we detail the procedures and factors that influence learning in these designs, describe the associative relationships that can be acquired, and argue for the importance of this knowledge in studying brain function.

Keywords: associative learning, second-order conditioning, sensory preconditioning, memory integration, extinction

OPEN ACCESS

Edited by:
Nathan Holmes, University of New South Wales, Australia

Reviewed by:
Aaron P. Blaisdell, University of California, Los Angeles, United States

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

Received: *30 December 2021*
Accepted: *04 March 2022*
Published: *18 April 2022*

Citation:
Gostolupce D, Lay BPP, Maes EJP and Iordanova MD (2022) Understanding Associative Learning Through Higher-Order Conditioning. Front. Behav. Neurosci. 16:845616. doi: 10.3389/fnbeh.2022.845616

INTRODUCTION

Understanding how stimuli occur relative to other stimuli in our environment is fundamental to making accurate predictions about the future and adapting behaviour accordingly. One way for such learning to ensue is to present stimuli together in time. For example, the painful experience of having been bitten by a dog can result in the development of fear of dogs causing one to avoid places whether dogs can be encountered. In the laboratory, this learning (i.e., first-order conditioning; Pavlov, 1927) is modeled using Pavlovian conditioning, which consists of pairings between a neutral sensory cue (or stimulus) such as a tone with an event of biological significance. While this form of learning accurately captures the formation of many associative relationships, it misses many others. Indeed, one need not directly experience event relationships in order to infer the likelihood of their occurrence in novel situations. To return to the example of the dog bite, one will likely not only avoid dogs (the stimulus directly associated with the aversive event) but also places where dogs frequent (e.g., parks, trails, your next-door neighbour's yard) even though the bite had not occurred there. Here, the knowledge of where dogs can be encountered is integrated with the knowledge that dogs can cause painful bites. In other words, information acquired across different episodes or time points can be linked, thus offering an opportunity to infer unique event relationships and make novel predictions about the environment. Such integration is an example of the dynamic nature of memories, how memories become linked and how flexible behaviour is orchestrated [Holland, 1990; Gewirtz and Davis, 2000; Blaisdell, 2009; Seitz et al., 2021; for more on discontinuous events see Wallenstein et al. (1998) and Cai et al. (2016)].

Integration of distinct associative memories is elegantly captured in higher-order conditioning (e.g., Pavlov, 1927; Brogden, 1939). This learning consists of two conditioning episodes–one that leads to associative links between two neutral stimuli (i.e., S2→S1 where S2 could be an auditory cue such as a tone and S1 could be a visual cue such as a light) and another that links one of these stimuli (S1) with a biologically significant outcome (an appetitive or aversive unconditioned stimulus [US], i.e., S1→US). Subsequent presentations of S2 reveal its ability to invigorate conditioned responses

(CRs) indicative of expectation of the US. This form of learning is termed higher-order because S2 is never directly paired with the US. Rather, it engages conditioned responding by virtue of its pairing with S1 which was directly associated with the US. That is, S2 acquires value through an intermediary. This learning requires integration of the sensory learning phase with the fear conditioning phase and provides a mechanism through which value (be it aversive or appetitive) can propagate across the memory network through higher-order associative links.

PROCEDURES AND FACTORS THAT INFLUENCE HIGHER-ORDER CONDITIONING

There are two classic designs of higher-order conditioning, namely, sensory preconditioning and second-order conditioning. While both types of designs consist of the same learning phases, that is, sensory training and appetitive or aversive conditioning (as outlined above), the order of the phases is reversed. In sensory preconditioning, S2→S1 pairings precede S1→US pairings, whereas in second-order conditioning S1→US pairings precede S2→S1 pairings. Although, the order of the learning phases may seem like a minor difference in experimental design, it is of tremendous importance because it governs what is learned during these distinct forms of higher-order conditioning (see below). Accounts of higher-order learning were originally reported by Pavlov (1927) where cues directly paired with an appetitive or aversive outcome could support the acquisition of secondary conditioned reflexes when paired with novel cues in the absence of the associated outcome (i.e., second-order conditioning). In both humans and animals, Prokofiev and Zeliony (1926) reported that sensory pairings between two cues followed by aversive conditioning of one of those cues led to fear of the other (indirectly paired) sensory cue (i.e., sensory preconditioning). This was subsequently investigated more thoroughly by Brogden (1939), coining the term "sensory preconditioning." While these forms of higher-order learning have been replicated numerous times across species including drosophila, goldfish, pigeons, mice, rats, rabbits, monkeys, and humans (e.g., Reid, 1952; Rizley and Rescorla, 1972; Rashotte et al., 1977; Pfautz et al., 1978; Rescorla, 1979; Amiro and Bitterman, 1980; Cook and Mineka, 1987; Beauchamp and Gluck, 1988; Gibbs et al., 1991; Müller et al., 2000; Brembs and Heisenberg, 2001; Mead and Stephens, 2003; Tabone and de Belle, 2011; Lee and Livesey, 2012; Busquets-Garcia et al., 2017; Renaux et al., 2017; Craddock et al., 2018; Wong and Pittig, 2022), the precise design parameters employed can easily influence the strength and content of learning. Below, we enumerate a list of design factors that have been reported in the literature along with their associated influence on higher-order conditioning. This information has also been summarized in **Table 1**.

Stimulus Type

Various stimuli have been used in higher-order conditioning experiments including colour (Rashotte et al., 1977), shape (Rescorla, 1980a), odour (Holland, 1981), flavour (Holland, 1981, 1983), auditory cues such as tone (Rizley and Rescorla, 1972), white noise (Holland and Ross, 1983), clicker (Ward-Robinson and Hall, 1998) and visual cues such as key light (Rashotte et al., 1977), flashing light (Parkes and Westbrook, 2010; Wong et al., 2019), and context (Archer and Sjöden, 1982; Helmstetter and Fanselow, 1989; Iordanova et al., 2008). The types of USs used in higher-order designs are similar to those used in first-order conditioning studies including footshocks, rewards such as food to a hungry rat, lithium chloride (LiCl)—induced illness (e.g., Rizley and Rescorla, 1972; Holland and Rescorla, 1975a; Archer and Sjöden, 1982; Ward-Robinson and Hall, 1998). Other aspects of stimulus type such as the intensity of the US with which S1 is paired, and the physical similarity between S2 and S1 (Garcia and Koelling, 1966; Rescorla and Furrow, 1977; Rescorla, 1980a) influence the strength of higher-order conditioning.

Stimulus Similarity

An important contributing factor to learning in higher-order conditioning is stimulus similarity. Specifically, when similar stimuli are used in the roles of S2 and S1, higher-order conditioning is facilitated compared to using dissimilar stimuli. Rescorla and Furrow (1977) showed that second-order conditioning proceeded more rapidly when S1 and S2 belonged to the same, compared to different, class of stimuli (e.g., colour: blue or green; orientation: horizontal or vertical lines). These effects were not due to stimulus generalization or pseudo-conditioning (Rescorla and Furrow, 1977). Cue similarity also facilitates second-order conditioning when the cues form a part-whole relationship. For example, in a pigeon autoshaping design, Rescorla (1980a) used achromatic shapes (triangle or square) as S2 and red shapes (triangle or square) as S1. Congruency in the shape, that is, when the achromatic shape was the same as the coloured shape, resulted in better second-order conditioning. Similar effects were reported in sensory preconditioning (Holland and Ross, 1983) and in appetitive second-order conditioning (Holland, 1977) using same cue modality or spatial similarity (Rescorla and Cunningham, 1979).

Stimulus Arrangement

The sensory cues used in higher order conditioning designs can be presented simultaneously or serially. Simultaneous presentations of S1 and S2 refer to instances when the cues are presented in compound such that they overlap. In serial presentations, S1 tends to follow S2 such that S2 offset often coincides with S1 onset. Although learning accrues to S2 in both scenarios, the temporal arrangement influences the association acquired by the higher-order S2. In sensory preconditioning, simultaneous presentation of stimuli during sensory training results in superior learning compared to serial S2→S1 pairings (Thompson, 1972; Rescorla, 1980b; Holland and Ross, 1983). This effect can be explained when considering the associations that form between the cues during sensory training. Simultaneous presentations facilitate associations between the sensory characteristics of S2 and S1 rather than a predictive relationship between them (i.e., S2 predicts S1 presentation). The latter is favored by a serial arrangement. Second-order conditioning is also achieved using both simultaneous and serial

TABLE 1 | Procedures and factors that influence higher-order conditioning.

Factors	Examples	Influence on learning
Stimulus type	Auditory (e.g., tone, white noise, clicker) Visual (e.g., flashing light, key light, context) Odour (e.g., almond, vanilla) Flavour (e.g., sucrose, saline) Shape (e.g., rectangle, triangle) Appetitive US (e.g., food pellets, sucrose pellets) Aversive US (e.g., footshock, illness)	Associability and similarity between S2 and S1 influence the strength of higher-order conditioning.
Stimulus arrangement	Serial (i.e., S2 offset coincides with S1 onset) Simultaneous (i.e., S2 and S1 presented at the same time)	Simultaneous arrangement results in superior sensory preconditioning effect relative to serial arrangement (Thompson, 1972; Rescorla, 1980b; Holland and Ross, 1983). Both serial and simultaneous S2-S1 pairings produce robust second-order learning, however, the arrangement has a differential effect on the content of learning.
Stimulus similarity	S2 and S1 chosen from the same stimulus type S2 and S1 chosen from different stimulus type	Pairing of similar stimuli proceed more rapidly relative to dissimilar stimuli in second-order conditioning (Rescorla and Furrow, 1977; Rescorla and Cunningham, 1979). Spatial similarity and using same cue modality promote sensory preconditioning (Holland, 1977; Rescorla and Cunningham, 1979)
Stimulus order	Forward serial order (i.e., S2 precedes S1) Backward serial order (i.e., S1 precedes S2, US precedes S1)	Higher-order conditioning designs classically use forward serial pairings (Pavlov, 1927). However, backward serial pairings of S1 and S2; US and S1 also support learning (Barnet et al., 1997; Ward-Robinson and Hall, 1998)
Trial number	Conditioned aversion: Single S2-S1 trial Aversive: 4 serial S2-S1 trials, 8 serial S2-S1 trials (Parkes and Westbrook, 2010) Appetitive: 100 trials (Rashotte et al., 1977) 40 trials (Holland and Rescorla, 1975a) 200 trials (Reid, 1952) 2 trials (Jones et al., 2012; Sadacca et al., 2018)	Sensory preconditioning and second-order conditioning can be obtained in single S2 and S1 pairing in conditioned aversion preparation (Archer and Sjöden, 1982). Aversive higher-order learning proceeds in four trials for second-order conditioning and eight trials for sensory preconditioning (Parkes and Westbrook, 2010). In contrast, appetitive designs may require more training trials (Reid, 1952; Rashotte et al., 1977; Jones et al., 2012; Sadacca et al., 2018).
Reinforced presentations	S2-S1 pairing followed by US delivery	Second-order learning can be obtained by reinforced S2→S1 pairings following S1 training (Leidl et al., 2018; Williams-Spooner et al., 2019).

S2 and S1 presentations. Rescorla (1982) showed that both serial and simultaneous arrangements result in similar levels of second-order conditioning, but the arrangement has a differential effect on what is learned (see below).

Stimulus Order

In studies where the cues have been presented serially in sensory preconditioning or second-order conditioning, it is common for S2 to precede S1. However, instances of S1 preceding S2 (i.e., S1→S2) are also effective in supporting learning. In an aversive design, sensory preconditioning was successfully obtained using such a serial *backward* order (i.e., S1→S2; Ward-Robinson and Hall, 1998). Reversing the order during first-order conditioning (i.e., the US preceded S1) also resulted in robust sensory preconditioning and second-order conditioning, in a lick suppression preparation with rats (Barnet et al., 1997).

Trial Number

The number of trials used to establish higher-order conditioning depends on various factors including the nature of the design (e.g., fear, reward, taste aversion), cue modality, stimulus arrangement, the model organism (e.g., rat, pigeon, rabbit), and the response measure (e.g., magazine approach, freezing, conditioned suppression). Higher-order fear conditioning progresses fairly rapidly: four trials of serial S2→S1 pairings is sufficient to obtain second-order learning (Rizley and Rescorla, 1972; Parkes and Westbrook, 2010; Lay et al., 2018) and sensory preconditioning can be achieved in eight serial S2→S1 trials (Rizley and Rescorla, 1972; Parkes and Westbrook, 2011; Wong et al., 2019). Higher-order conditioning designs involving rewards require more extensive S2→S1 training. In particular, second-order conditioning is successful using 100 trials across 10 days in pigeons (Rashotte et al., 1977), or 40 trials across four days in rats (Holland and Rescorla, 1975a) whereas sensory preconditioning has been obtained with 200 trials across 10 days in pigeons (Reid, 1952), but with as few as 12 trials across two days in rats (Jones et al., 2012; Sadacca et al., 2018).

The large number of trials often required for second-order conditioning can have unintended effects. As the number of S2→S1 trials increase in second-order conditioning, responding to S2 decreases, which is in contrast with the increase in responding to S1 across S1→US pairings. When S2→S1 pairings are alternated with continued S1→US pairings, the S2 can become a signal for the absence of the US (Herendeen and Anderson, 1968; Rescorla et al., 1973; Holland and Rescorla, 1975b; Yin et al., 1994). That is, conditioned inhibition to

S2 accrues, competing with its ability to exhibit second-order conditioning (Gewirtz and Davis, 2000; Parkes and Westbrook, 2010). In a lick suppression study in rats, 20 simultaneous S2→S1 pairings favored conditioned inhibition over second-order conditioning and a hundred such trials rendered S2 a conditioned inhibitor regardless of whether S2 and S1 were paired simultaneously or serially (Stout et al., 2004). The transition of S2 from a second-order excitor to a conditioned inhibitor was quicker when S2 and S1 were presented in compound (Stout et al., 2004). To limit the development of conditioned inhibition in second-order conditioning, fewer S2→S1 pairing should be employed. This is possible in conditioned taste aversion. Indeed, a single pairing between a gustatory S2 and a contextual S1 was sufficient to obtained sensory preconditioning and second-order conditioning provided the US used to conditioned S1 was very salient (i.e., LiCl; Archer and Sjöden, 1982). These data, among others, reveal the importance of the strength of S1→US association on higher-order conditioning (Bond and Harland, 1975; Bond and Di Giusto, 1976).

Reinforced Presentations

Some instances of second-order fear conditioning consist of reinforced serial S2→S1 pairings following S1 training [i.e., S2→S1→US; Williams-Spooner et al., 2019; see also Mahmud et al. (2019)]. This design, like the standard non-reinforced design, results in robust learning about the second-order stimulus relative to an unpaired control (Leidl et al., 2018; Williams-Spooner et al., 2019). In reward learning, reinforced serial S2→S1 presentations lead to higher level of responding during training compared to non-reinforced S2→S1 presentations (Holland, 1980). This effect, however, was likely due to the development of S2→US associations (Holland, 1980). To show this, Holland (1980) tested S2 under conditions that reveal the strength of second-order associations (i.e., under food satiation) and reported lower level of responding to S2 when trained in the reinforced serial case. Holland (1980) further showed that surprising food presentations or omissions were more detrimental to second-order conditioning than when such events were expected. This was taken as evidence for the role of outcome interference in the development S2→S1 associations, which was successfully alleviated by delaying outcome delivery (Holland, 1980).

RESPONSE MEASURES IN HIGHER-ORDER CONDITIONING

In first-order conditioning, an aversive US (e.g., a mild electric shock) conditions species-specific defensive behaviours (e.g., freezing, Blanchard and Blanchard, 1969; Bolles, 1970; Fanselow, 1980) or conditioned suppression (e.g., Rescorla and Furrow, 1977; Bouton and Bolles, 1980), whereas an appetitive US (e.g., sucrose pellets) supports conditioned approach (e.g., Holland, 1977). The US, however, is not the only determinant of conditioned responses. Auditory and visual cues can support cue-based responses including rearing, head jerk, perambulation, and general activity (Holland and Rescorla, 1975a,b; Holland, 1977, 1984). While auditory stimuli elicit startle and head jerk, visual stimuli elicit rearing (Holland, 1977). Startle and rearing are considered orienting responses (OR) and are seen to novel but not familiar non-reinforced cues and maintained or augmented to cues that have undergone conditioning. Head jerk is specific to conditioned auditory cues. ORs and CRs are differentially distributed across the duration of a conditioned stimulus, with ORs occurring mostly during the beginning of visual cues and food-cup CRs following afterwards, while CRs and ORs elicited by auditory cues are more evenly distributed (Holland, 1977; Hatfield et al., 1996). In second-order conditioning, pairing an auditory S2 with either a visual or auditory S1 leads to similar proportions of CRs and ORs, with head jerk being the predominant response to S2 in both cases (Holland, 1977).

CONTENT OF HIGHER-ORDER CONDITIONING

The associative links that govern sensory preconditioning and second-order conditioning differ depending on the procedural details. As different designs are often used to study the neural substrates of higher-order learning, it is imperative that one is aware that procedural differences can lead to differences in associative content (i.e., what is learned; see also Gewirtz and Davis, 2000; Parkes and Westbrook, 2011; Gostolupce et al., 2021). We cover these below.

Extinction of S1

The first evidence to highlight the differences in learning between sensory preconditioning and second-order conditioning came from Rizley and Rescorla (1972). In a fear conditioning procedure with footshock as the US, the authors showed that reduction in responding to S1 *via* repeated presentations of this cue in the absence of the US (i.e., S1 extinction training) consequently reduced responding to S2 in sensory preconditioning but not in second-order conditioning [see also Parkes and Westbrook (2010), Holmes et al. (2014)]. Similar findings have been reported in higher-order reward conditioning by Holland and Rescorla (1975a) as well as in a conditioned taste aversion design (Archer and Sjöden, 1982). These data provide convincing evidence that the association between S2 and S1 is key to regulating sensory preconditioning but not second-order conditioning (Rizley and Rescorla, 1972; Holland and Rescorla, 1975a).

It turns out, however, that the arrangement of stimulus presentation or stimulus similarity can influence the nature of associative learning in second-order conditioning. Rescorla (1982) showed that simultaneous S2→S1 pairings produce second-order responding that is sensitive to S1-extinction. This may be because simultaneous presentations lead to within compound associations or the development of an S2S1 configural unit (Pearce, 1994, 2002), meaning that the sensory cues can activate representations that contain one another.

It is also possible to obtain second-order responding that is sensitive to extinction of S1 when S2 and S1 belong to the

same cue modality (i.e., both S2 and S1 as auditory cues such as tones of different frequencies or as visual cues such as a flashing houselight or a jeweled signal light; Rescorla and Furrow, 1977). In an autoshaping procedure, pigeons were first trained to peck a white key light S1 by pairing it with grain delivery (Rashotte et al., 1977). A blue key light S2 was trained with S1 in a serial manner to achieve second-order conditioning to S2. Extinction of S1 resulted in disruption of second-order key peck responding. These findings demonstrate that second-order conditioning is sensitive to manipulations of S1 when S2 and S1 belong to the same cue modality (but see Experiment 4 using different modalities in which S1 is an operant discriminant).

Determining what is learned during second-order conditioning is further informed by the behaviours that are measured. While extinction of a visual S1 after second-order conditioning leaves intact food-cup approach and head jerk CRs to an auditory S2 (Holland and Rescorla, 1975a), rear ORs to S2 are abolished (Setlow et al., 2002; McDannald et al., 2013). Given that rearing is generally only evoked by either visual cues or S2s by virtue of them being paired with a visual S1, this OR is thought to represent the behavioural readout of a S2→S1 (i.e., stimulus→stimulus) association (Setlow et al., 2002). This suggests that an S2→S1 association may be formed, but such associations are unlikely to drive the conditioned responses normally measured in second-order conditioning.

Finally, in a series of clever studies that used the nature of responding to determine the nature of the associations between events in second-order conditioning, Holland (1977) revealed that S2 is likely linked to the affective or motivational state induced by the US. Specifically, he examined whether an auditory S2 would acquire auditory ORs or visual ORs when paired with either an auditory or visual S1 in a second-order design. The data confirmed that an auditory S2 elicits an auditory-specific response and does not become associated with the cue-based response elicited by S1, eliminating the likelihood of a S2→CR (i.e., stimulus→response) association. Holland's interpretation was further confirmed by Winterbauer and Balleine (2005) who showed that second-order cues enter into associations with the specific motivational aspects of the US in water- and food-deprived rats and that this learning was not dependent on the motivational state at the time of training.

Devaluing the US

Although S2 is never directly paired with the US in higher-order designs, some evidence suggests that a S2→US association must not be discounted. Indeed, the development of associations between actual and associatively evoked stimuli are well-supported by the literature (e.g., Holland, 1981, 1983; Holland and Forbes, 1982; Iordanova et al., 2008; Lin and Honey, 2010, 2011, 2016; Wong et al., 2019). To account for such learning, Holland (1981, 1983) proposed a modification to Wagner's Standard Operating Procedures (SOP; Wagner, 1981). Briefly, according to SOP, stimuli can be in three states of activation, a focal A1 state, a working memory A2 state and an inactive I state. Excitatory conditioning occurs when events are concurrently in an A1 state of activity whereas inhibitory conditioning occurs when a cue is in an A1 state and the US in an A2 state. In a series of conditioned taste aversion experiments, Holland (1981, 1983) provided evidence that excitatory learning also occurs between two events (e.g., food and LiCl) when the food is in A2 (i.e., associatively activated) and the LiCl in A1 (physically present). This proposal accounts for the development of S2→US associations in sensory preconditioning designs because S1 would place its associate S2 into an A2 state during S1→US training while the US is in an A1 state, thereby allowing for S2→US learning in this phase.

To determine whether the representation of the US is linked to S2, Holland and Rescorla (1975a) used a devaluation procedure. They showed that reducing the value of an appetitive US led to a corresponding reduction in responding to S2 in sensory preconditioning but not second-order conditioning. This was also confirmed by Rescorla (1973) who devalued an aversive US (loud noise) using habituation. In other words, S2 is not linked to the US in second-order conditioning, at least early on in S2→US training. The lack of devaluation effects in second-order conditioning is consistent with the original stipulation of SOP (Wagner, 1981), which holds that during S2→S1 pairings, S2 would be in an A1 state whereas the US would be associatively evoked by S1 and therefore in an A2 state, resulting in the development of S2 as a conditioned inhibitor for the US. As mentioned, conditioned inhibition can accrue to a second-order S2 when the number of S2-S1 pairings increases and these trials are alternated with continued S1-US pairings (Herendeen and Anderson, 1968; Rescorla et al., 1973; Holland and Rescorla, 1975b; Yin et al., 1994), lending support for the SOP proposal. Intriguingly, this inhibitory association can co-exist with the excitatory second-order association (Holland and Rescorla, 1975b) and is greater when stimuli are similar compared to dissimilar (Rescorla, 1980a).

In summary, the studies reviewed above show that higher-order conditioning can be obtained using a variety of stimuli under diverse conditions. While sensory preconditioning is supported by associations between S2 and S1 as well as between S2 and the associatively evoked US, those that drive second-order conditioning are parameter-dependent. In the somewhat classic serial design that uses cues of different modalities, second-order conditioning is dependent on S2→motivational state associations and not on S2→S1 (evidenced using S1 extinction), nor S2→US (evidenced using US devaluation), nor S2→CR (evidenced in the inability of S2 to acquire cue-specific responses indicative of S1 expectation) associations. Altering the cue arrangement or modality, shifts the content of what is learned.

IMPLICATIONS FOR NEUROSCIENCE

The quest for uncovering the neural mechanisms of higher-order learning is gaining momentum [e.g., Iordanova et al., 2009, 2011a,b; Horne et al., 2010; Jones et al., 2012; Wimmer and Shohamy, 2012; Holmes et al., 2013; Holland and Hsu, 2014; Holland, 2016; Lin and Honey, 2016; Sadacca et al., 2018; Wong et al., 2019; Hart et al., 2020; for reviews

see Parkes and Westbrook (2011), Fournier et al. (2021), Gostolupce et al. (2021), and Holmes et al. (2021)]. Indeed, dissociations in the neural mechanisms of sensory preconditioning and second-order conditioning have been reported (Parkes and Westbrook, 2010; Holmes et al., 2013; Holland and Hsu, 2014; Holland, 2016) as have been nuances in the regulation of cue- vs. outcome-based responses elicited by higher-order stimuli (Gallagher et al., 1990; Hatfield et al., 1996; McDannald et al., 2013). These lines of evidence suggest that different neural areas regulate different types of associations despite the similarity in training, and that parallel systems drive subsets of behavioural responses established under the same training conditioning.

Our understanding of the functional role of distinct neural substrates can be greatly advanced by the study of higher-order learning. These preparations expand the conditions under which learning occurs, extending our study of how the brain learns. In addition, they provide important information into the associative structures that control behaviour, thereby offering particular insight into the function of brain areas that regulate this learning. This manuscript reviews the distinct procedures and parameters that supports higher-order learning and how this affects the corresponding associative architecture, which we hope will bolster the field's analysis of the corresponding neural architecture.

AUTHOR CONTRIBUTIONS

All authors contributed to the article by conceiving the idea for the manuscript and developing the narrative. All authors approved the submitted version.

FUNDING

This work was supported by a CIHR Project Grant (to MI Grant: PJT-173226), an excellence award by the Canada Research Chairs Program (to MI Grant: 950-230456), a FRQS post-doctoral fellowship (to BL #276908), and Concordia International Tuition award of excellence (to DG).

ACKNOWLEDGMENTS

We thank Alexandra Usypchuk for her insightful comments on the manuscript.

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