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Pavlovian-instrumental transfer: Neurobehavioral and clinical findings

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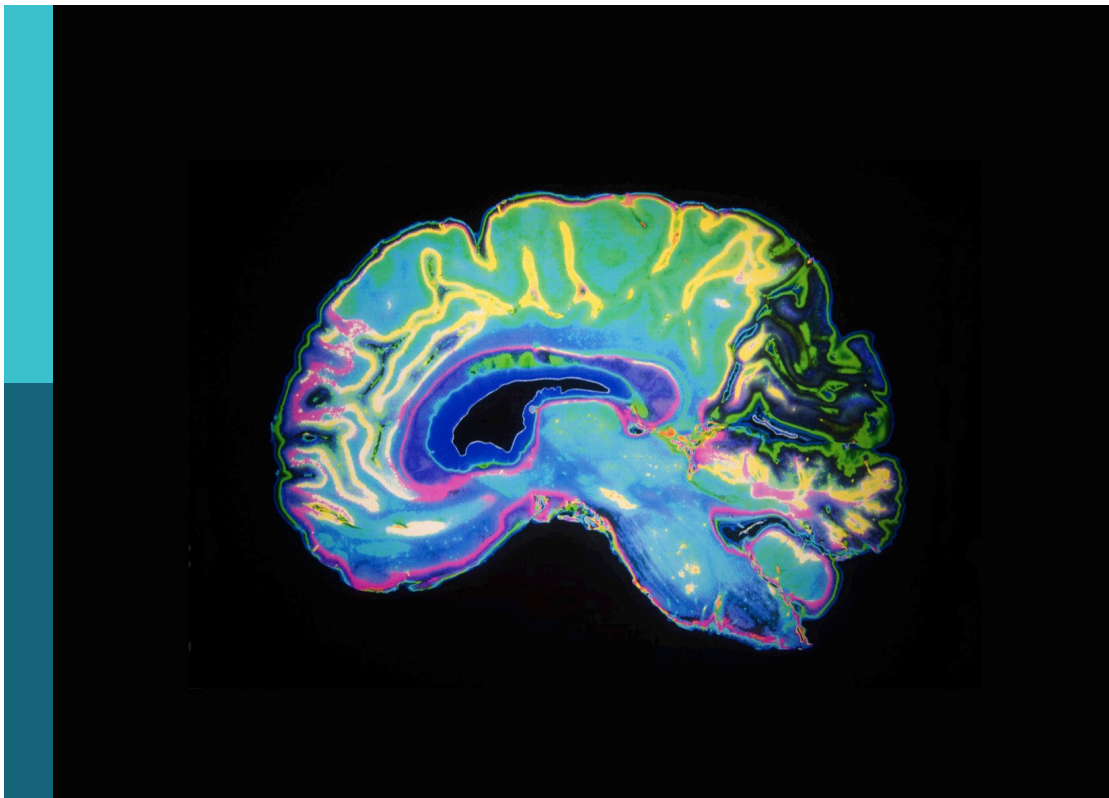

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Pavlovian-instrumental transfer: Neurobehavioral and clinical findings

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Editorial: Pavlovian-instrumental transfer: Neurobehavioral and clinical findings

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Editorial on the Research Topic
Pavlovian-instrumental transfer: Neurobehavioral and clinical findings

Pavlovian-instrumental transfer (PIT) examines how action learning (i.e., instrumental) and stimulus learning (i.e., Pavlovian) are integrated to control ongoing behaviors. It shows that this control can take at least two distinct forms (Holmes et al., 2010; Cartoni et al., 2016). In general PIT, a stimulus predicting an outcome of a particular motivational domain (i.e., appetitive or aversive) is shown to energize performance of actions associated with outcomes belonging to the same motivational domain. In specific PIT, a stimulus predicting a particular outcome is found to guide choice toward actions earning the same outcome and away from actions associated with different outcomes, even though these outcomes belong to the same motivational domain. PIT can therefore reflect a simple modulation of action performance (general PIT) or a more complex demonstrations of sensory-driven action selection (specific PIT). Although much progress has been made in describing the mechanisms underlying the two forms of PIT (Cartoni et al., 2016; Corbit and Balleine, 2016; Laurent and Balleine, 2011), much remains to be understood at both the psychological and neural level. The empirical and theoretical papers included in this Research Topic aimed to address this gap in knowledge and to demonstrate how PIT can provide insights into dysregulation and maladaptive behaviors.

A set of empirical papers used rodents to elucidate the psychological mechanisms and neural architecture underlying general and specific PIT. Specifically, Lingawi et al. manipulated the value of appetitive food outcomes to dissociate the control exerted by a predictive stimulus on action performance and action selection. They found that lowering outcome value abolished general PIT but left specific PIT mostly intact. However, Panayi and Killcross revealed that the latter can be disrupted when the manipulation lowering food value also reduces the capacity of a stimulus to retrieve information about its predicted outcome. Two other papers provide novel information about the neural circuity underlying general PIT (Halbout et al.) show that general PIT does not normally necessitate activity in the dorsomedial prefrontal cortex (dmPFC). However, they also show that the effect be blunted when dmPFC activity is artificially increased. In the other paper, Ge and Balleine provide the first evidence of the critical role played by the bed nucleus of stria terminalis (BNST) in supporting general PIT. The authors also offer an elegant model describing how the BNST may interact with other brain regions to achieve such function. Finally, Kim et al. demonstrates for the first time how general and specific PIT can be observed in the aversive domain and reveal their reliance on activity in the central amygdala.

In addition to rodent studies that attempt to dissect transfer effects from the psychological and neural perspective, our Research Topic includes work that explores the translation of PIT to humans in ways that relate to both basic and applied research. For example, a paper reporting an analysis of computational and representational elements that drive PIT in humans (Degni et al.) established procedures that isolate different forms of the transfer effect. Moreover, this study showed that general motivation effects are obtained under a variety of associative conditions. Non-specific, stimulus-enhanced responding was obtained when the outcome was either previously associated with an action, or not.

Other papers then underscore how PIT can provide insight into maladaptive behaviors and offer pathways to treatment. These include a rodent study exploring factors that influence craving for alcohol cues (Ginsburg et al.). This experiment used stimuli of varying length to show that cue duration inversely influences CS-elicited food-responding, while augmenting the transfer effect when longer duration ethanol-paired cues are tested. These data have profound implications for how we understand craving and provide an information processing framework from which to approach the issue.

The work reported here also includes a series of innovative studies showing how PIT can be used to investigate human disorders that directly or indirectly relate to emotion, including attention-deficit/hyperactivity (Geurts, den Ouden et al.), borderline personality (Geurts, Van den Heuvel et al.), and psychopathy (Geurts, von Borries et al.). In these studies, PIT was used to establish the efficacy of therapeutic treatments in patients with borderline disorder, while also providing psychopathy indicators in violent criminals. In studies examining ADHD patients across disorder subtypes, different degrees of inhibitory stimulus control were identified, and these differences were eliminated by combining traditional therapeutic approaches with cognition-based treatments.

This Research Topic convincingly demonstrates how PIT can be used to uncover the dynamics of different forms of learning and motivated behavior. It extends the utilization of the PIT task from a general exploratory task toward understanding the nature of dysregulation that underlies human disorder and the efficacy of relevant treatments. Together the studies reported in this collection provide new insights into the psychological and neural mechanisms supporting this intriguing phenomenon as well as these novel applications. However, these papers also underscore that much more work is required to obtain a clear understanding of the psychological and neural mechanisms that regulate PIT. Completing this work will be critical and there is great potential for the PIT task to provide increased sensitivity and subtlety to analyses in new and exciting areas across psychology and neuroscience. This will include the continued exploration of how PIT emerges from reward and other appetitive processes but also translating the task into new areas of motivation to identify parallels and idiosyncrasies.

Author contributions

VL and VC wrote the editorial letter, organized, and edited the submissions. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

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ORIGINAL RESEARCH
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Sensory-Specific Satiety Dissociates General and Specific Pavlovian-Instrumental Transfer

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Pavlovian conditioning enables predictive stimuli to control action performance and action selection. The present experiments used sensory-specific satiety to examine the role of outcome value in these two forms of control. Experiment 1 employed a general Pavlovian-instrumental transfer design to show that a stimulus predicting a food outcome energizes the performance of an instrumental action earning another food outcome. This energizing effect was removed when the stimulus-predicted outcome or a novel outcome was devalued by sensory-specific satiety. Experiments 2 and 3 employed a specific Pavlovian-instrumental transfer design to demonstrate that a stimulus predicting a particular food outcome promotes the selection of an instrumental action earning the same, but not a different, food outcome. Remarkably, this effect was maintained when all or just one of the stimulus-predicted outcomes were devalued by sensory-specific satiety. These results indicate that satiety alone removes the expression of general PIT. By contrast, satiety or outcome-specific devaluation does not regulate the expression of specific PIT, which is insensitive to changes in outcome value. This dissociation is consistent with the view that general and specific PIT are two separate phenomena driven by distinct psychological mechanisms.

Keywords: Pavlovian instrumental transfer, Pavlovian conditioning, instrumental conditioning, outcome value, sensory-specific satiety

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INTRODUCTION

Pavlovian enables predictive stimuli to control action performance and action selection. These two forms of control can be individually studied in general and specific appetitive Pavlovian-instrumental (PIT) tasks (Holmes et al., 2010; Cartoni et al., 2016; Corbit and Balleine, 2016). General PIT demonstrates that a stimulus predicting a food outcome energizes the performance of an instrumental action earning another food outcome. By contrast, specific PIT shows that a stimulus predicting a particular food outcome promotes the selection of an instrumental action earning the same food outcome over an instrumental action earning a different food outcome. Neural studies in humans and rodents suggest that general and specific PIT are separate phenomena, in the sense that they recruit activity in different brain regions (Corbit et al., 2001; Corbit and Balleine, 2005, 2011; Talmi et al., 2008; Prévost et al., 2012; Mendelsohn et al., 2014; van Steenbergen et al., 2017).

Yet, such studies fail to demonstrate that general and specific PIT are mediated by distinct psychological mechanisms. This evidence is more likely to be uncovered by assessing whether a particular behavioral manipulation affects one but not the other form of PIT.

Dissociating general and specific PIT with behavioral manipulations has proven to be quite challenging. For example, both appear to be relatively insensitive to changes in the predictive relationships initially established between the stimuli and their outcomes (Delamater, 1996; Hogarth et al., 2014; Laurent et al., 2016, 2021; Seabrooke et al., 2018). Nevertheless, rodent studies that manipulated primary motivational state showed a clear dissociation between general and specific PIT (Balleine, 1994; Corbit et al., 2007). One of these studies found that hungry rats display both general and specific PIT. However, sated rats failed to express general PIT but still showed specific PIT—although the size of the latter effect was severely reduced. Given that satiety reduces the desirability of the food outcomes, the authors proposed that general PIT requires stimuli to predict an outcome that is deemed valuable against current biological needs. By contrast, specific PIT is independent of the value requirement and instead relies on the capacity of the stimuli to predict the sensory-specific properties (e.g., odor, texture, smell) of their outcomes.

Studies employing outcome-specific devaluation have confirmed the insensitivity of specific PIT to changes in outcome value. These studies show that specific PIT survives devaluation of both, or one of the stimulus-predicted outcomes (Rescorla, 1994; Holland, 2004; Sommer et al., 2022). However, using a similar procedure, one of these studies found that outcome devaluation did not abolish general PIT (Holland, 2004), a finding clearly inconsistent with the view that the two forms of PIT can be dissociated based on their outcome value requirement. In that respect, the human literature adds further uncertainty, as some have reported that specific PIT is insensitive to changes in outcome value (Hogarth and Chase, 2011; Hogarth, 2012; Watson et al., 2014; Eder and Dignath, 2016; Seabrooke et al., 2017; van Steenbergen et al., 2017; Pritchard et al., 2018; Verhoeven et al., 2018) whereas others found the opposite (Allman et al., 2010; Eder and Dignath, 2016; Seabrooke et al., 2017, 2019; Hinojosa-Aguayo and Gonzalez, 2020). To the best of our knowledge, the role of outcome value in general PIT has yet to be determined in human subjects. Regardless, the current literature indicates that the role of outcome value in the expression of general and specific PIT remains elusive.

The present experiments used rats to revisit the relationship between outcome value and the capacity of predictive stimuli to control action performance and action selection. All experiments employed sensory-specific satiety to devalue the food outcomes. Experiment 1 examined whether the expression of general PIT is sensitive to changes in the value of the stimulus-predicted outcome or is more generally sensitive to changes in primary motivational states. The next experiments examined whether the expression of specific PIT is controlled by the value of the stimulus-predicted outcomes. In Experiment 2, both outcomes predicted by the two stimuli were devalued, whereas only one outcome was devalued in Experiment 3.

METHODS

Subjects

The subjects were 56 experimentally naive female and male Long-Evans rats obtained from the Rat Breeding Facility at the University of New South Wales (Sydney, Australia). The rats were at least 8 weeks old at the start of the experiment and were housed in plastic boxes (3–4 rats per box) located in a climate-controlled colony room maintained on 12 h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.). Four days prior to the start of the behavioral procedures, the rats were handled daily, and food intake was restricted to maintain them at ∼85% of their original weight. The Animals Ethics Committees at the University of New South Wales approved all experimental procedures. All procedures occurred between 7:00 a.m. and 7.00 p.m. Each experimental group included an equal number of female and male rats.

Apparatus

Training and testing took place in a set of 16 identical MED Associates operant chambers enclosed in sound- and light-resistant shells. Each chamber was equipped with two pumps fitted with a syringe that delivered 0.1 ml of a 20% sucrose solution or a 20% polycose solution into a recessed magazine in the chamber. The chambers were also equipped with two food pellet dispensers that delivered either grain (45 mg; # F0165, BioServ Technologies), purified (45 mg; # F0021, BioServ Technologies) or chocolate purified pellets (F0299; BioServ, Flemington, NJ, USA) when activated. Two retractable levers were located to the left and the right of the magazine. A 3 W, 28 V house light provided illumination of the operant chamber, and an infrared photo beam spanning across the magazine opening detected head entries. Chambers contained a 28 V DC mechanical relay that was used to deliver a 2 Hz clicker stimulus and a white noise generator (80 dB). Two computers running MED Associates proprietary software (Med-PC; Fairfax, VT, USA) controlled the equipment and recorded responses. To achieve outcome devaluation *via* sensory-specific satiety, each rat was placed in an individual box located in a separate feeding room from where training and testing took place.

Behavioral Procedures

Experiment 1: General Pavlovian-Instrumental Transfer

Experiment 1 examined whether the outcome value modulates the expression of general PIT. Rats received Pavlovian conditioning with one stimulus S1 predicting food outcome O1 and another stimulus S2 predicting nothing. Then, they were trained to perform a lever press action A to earn a distinct food outcome O2. A general PIT test was then administered and assessed the effects of S1 and S2 on A. In group No, this test occurred without prior outcome devaluation. By contrast, groups O1 and O3 underwent the test following outcome devaluation of O1 and O3, respectively. Devaluation was achieved through sensory-specific satiety. Finally, a consumption test was used to ensure rats could discriminate between the food outcomes.

Exposure to Food Outcomes

Prior to the start of training, rats were exposed to the three food outcomes (O1, O2, and O3) in the feeding boxes that were later used to conduct outcome devaluation *via* sensory-specific satiety. These exposures aimed to reduce neophobia to the food outcomes and provide habituation to the feeding cages where the devaluation procedure would take place.

Pavlovian Conditioning

Rats first received eight consecutive days of Pavlovian conditioning in which an auditory stimulus (S1; clicker or noise) was paired with a food outcome (O1; grain, purified, or chocolate pellets). All stimuli and outcome allocations were counterbalanced by experimental group and sex. Each training session lasted approximately 50 min and consisted of six reinforced S1 presentations of 2 min each with varying intertrial intervals ranging from 3 to 7 min (average 4 min). During each S1 presentation, O1 was delivered on a random-time 30 s reinforcement schedule. Magazine entries during S1 presentation and the 2-min prior to that presentation (pre-S1 period) were recorded by the MED-PC software.

Instrumental Conditioning

Rats then received 8 days of instrumental conditioning in which pressing the lever action (A) to the left of the magazine earned the delivery of a distinct food outcome (O2; grain, purified, or chocolate pellet). The left lever was continuously available, and each session lasted until 20 outcomes had been delivered, or 30 min had elapsed. The first day of training was conducted on a continuous reinforcement schedule where each lever press was reinforced by the delivery of one pellet. This was followed by 1 day of a random interval (RI)-15 s reinforcement schedule, where lever pressing was reinforced on average once every 15 s, and 1 day of RI-30 s training. The final five days of training were conducted on a RI-60 s schedule. Lever presses were recorded automatically by the MED-PC software.

Control Stimulus Exposure

Rats then received a single session of exposure to a control auditory stimulus (S2). S2 was a clicker if the noise had been used as S1 during Pavlovian conditioning, or a noise if the clicker had been used as S1. The session lasted approximately 50 min consisting of six, 2-min S2 presentations with the same intertrial intervals as those used during Pavlovian conditioning. No food outcomes were presented throughout this session. Magazine entries during presentations of S2 and during the 2-min pre-S2 period were recorded.

Instrumental Extinction

Rats then received one day of a 10-min instrumental extinction session. The left lever was present throughout the duration of the session; however, lever pressing was not reinforced by the delivery of any food outcome. Past research indicates stronger evidence for PIT following instrumental extinction, as it reduces the baseline response rate against which PIT is observed (Holmes et al., 2010).

Outcome Devaluation via Sensory-Specific Satiety

Immediately before the transfer test, outcome devaluation by means of sensory-specific satiety was administered to animals in groups O1 and O3. Rats in group No did not experience this devaluation. Each rat was placed in an individual box in a separate feeding room from where the test would take place. Rats in group O1 received free access to the food outcome predicted by S1 during Pavlovian conditioning sessions (O1; grain, purified, or chocolate pellets) for 1 h. Rats in group O3 received free access to the food outcome that had not been used during Pavlovian or instrumental conditioning (O3; grain, purified, or chocolate pellets).

General Pavlovian-Instrumental Transfer Test

The test was conducted in extinction immediately following the devaluation procedure. During this session, the left lever was continuously available, but no food outcomes were delivered. Responding was extinguished for 3 min at the commencement of the session to establish a low rate of baseline lever press performance. After this, the two auditory stimuli (white noise and clicker) were presented four times each in the following order: noise-clicker-clicker-noise-clicker-noise-noise-clicker. Each stimulus presentation lasted 2 min and each stimulus presentation occurred 3 min apart. The number of lever presses during each stimulus presentation and during the 2-min pre-S periods were recorded by the MED- PC software throughout the session.

Consumption Test

A consumption test was conducted to ensure that the rats distinguished the three food outcomes. Rats received free access to one of the three outcomes (grain, purified, or chocolate pellets) for 1 h. Immediately after, rats received a 10-min choice test whereby they could consume either the devalued outcome or a non-devalued outcome. Rats in group No received devaluation of either O2 or O3, rats in group O1 received devaluation of either O1 or O2, and rats in group O3 received devaluation of either O1 or O3. Devaluation was followed by a choice test between the two outcomes allocated to each group. The amount of food eaten during both the devaluation and choice test sessions was recorded for each subject. The success of the sensory-specific satiety manipulation in the devaluation of the outcome was inferred by comparing consumption between the devalued and valued outcome during the choice test.

Experiments 2 and 3: Specific Pavlovian-Instrumental Transfer

Experiments 2 and 3 examined whether the outcome value modulates the expression of specific PIT. Rats learned that two stimuli, S1 and S2, predicted two distinct food outcomes, O1 and O2. Next, rats were trained to earn O1 and O2 by performing two lever press actions A1 and A2, respectively. A specific PIT test then assessed the choice between A1 and A2 in the presence of either S1 or S2. In Experiment 2, this test was conducted following the devaluation of O1 and O2 or no devaluation. In Experiment 3, the test was conducted following the devaluation of O1 or no devaluation. In

both experiments, outcome devaluation was achieved through sensory-specific satiety.

Pavlovian Conditioning

Rats first received eight consecutive days of Pavlovian conditioning involving the pairing of two auditory stimuli (S1 and S2; clicker or noise) with two distinct food outcomes (O1 and O2; grain pellets and 20% sucrose solution). All stimuli and outcome allocations were counterbalanced by experimental group and sex. Each training session lasted approximately 60 min and consisted of four reinforced presentations of each stimulus that lasted 2 min. A varying intertrial interval ranging from 3 to 7 min (average 4 min) was used. During each S1 presentation, O1 was delivered on a random-time 30 s reinforcement schedule. During each S2 presentation, O2 was delivered on a random-time 30 s reinforcement schedule. Magazine entries during S1 and S2 presentations and the 2-min prior to these presentations (pre-S period) were recorded by the MED-PC software.

Instrumental Conditioning

Rats then received 8 days of instrumental conditioning with two daily sessions. In one session, a lever press action (A1; left or right lever) earned food outcome O1. In the other session, another lever press action (A2; right or left lever) earned food outcome O2. The order of the session was fully counterbalanced, and the action-outcome relationships were counterbalanced with respect to the stimulus-outcome relationships previously established. Each session lasted until 20 outcomes had been delivered, or 30 min had elapsed. The first 2 days of training were conducted on a continuous reinforcement schedule where each lever press was reinforced by the delivery of an outcome. This was followed by 3 days of a random ratio (RR)-5 reinforcement schedule, where an outcome was delivered after five lever presses on average. The final 3 days were conducted on an RR-10 schedule. In an attempt to minimize the reduction in instrumental performance produced by the subsequent devaluation of the food outcomes, rats received 3 days of RR-10 schedule with each action earning a third food outcome (O3; 20% polycose solution). The capacity of such a procedure to maintain instrumental performance after outcome devaluation has been confirmed in the past Rescorla (1994), and other studies have shown that training with the third outcome does not affect associations between the actions and their respective outcomes (Rescorla, 1991). The following day, rats were returned to the initial action-outcome relationships (A1-O1 and A2-O2) under an RR-10 schedule. Lever presses were recorded automatically by the MED-PC software.

Outcome Devaluation via Sensory-Specific Satiety

In both Experiments 2 and 3, rats underwent one specific PIT test after outcome devaluation and one test without prior outcome devaluation (order counterbalanced). Outcome devaluation was achieved by means of sensory-specific satiety. In Experiment 2, rats were placed in an individual box in a separate feeding room and were given O1 and O2 for 1 h. O1 and O2 were made available separately in alternating periods lasting 15 min (i.e., O1-O2-O1-O2 or O2-O1-O2-O1, counterbalanced) for a total duration of 1 h. In Experiment 3, rats were placed in an individual box in a separate feeding room and were given O1 for 1 h.

Specific Pavlovian-Instrumental Test

As explained, rats received two consecutive tests: one after outcome devaluation, one without outcome devaluation. During the tests, the two levers were continuously available, but no food outcomes were delivered. Responding was extinguished for 5 (first test) or 2 (second test) min at the commencement of the test to establish a low rate of baseline lever press performance. After this, the two auditory stimuli (white noise and clicker) were presented four times each in the following order: noise-clicker-clicker-noise-clicker-noise-noise-clicker. Each stimulus presentation lasted 2 min and each stimulus presentation occurred 3 min apart. The number of lever presses on each action during each stimulus presentation and during the 2-min pre-S periods were recorded by the MED-PC software throughout the session.

Data Analysis

Data were analyzed using a planned, orthogonal contrast procedure controlling the per contrast error rate (Hays, 1963). The rate of magazine entry was the behavioral measure for the Pavlovian stages. Lever presses rates were the behavioral measures for the instrumental stages. The amount of outcome consumed (grams) was used for the devaluation and consumption test. Magazine entry rates and lever press rates were analyzed for the transfer tests. These were recorded during the initial extinction period, the S1 and S2 presentations, and during the 2 min periods before a stimulus was presented which served as the measure of baseline responding. Due to significant instrumental extinction, only the first three trials of each stimulus during the general PIT test were used for analysis in Experiment 1. Instrumental extinction was more pronounced in Experiments 2 and 3, presumably due to repeated testing. The analyses, therefore, focused on the first two trials of each stimulus during the specific PIT tests. All analyses were carried out using the PSY statistical program (School of Psychology, The University of New South Wales, Australia) and significance was set at the 0.05 level to control the Type 1 error rate for each contrast tested.

RESULTS

Experiment 1: Satiety Alone Abolishes General PIT

Experiment 1 examined whether outcome value modulates the capacity of predictive stimuli to energize action performance. A general PIT design was used (**Figure 1A**). During Pavlovian conditioning, rats learned that stimulus S1 predicted food outcome O1 whereas stimulus S2 predicted nothing (i.e., it was neutral). During instrumental conditioning, rats were trained to perform a lever press action A to earn a distinct food outcome O2. A general PIT test then assessed the capacity of the two stimuli to energize action performance. This test was conducted under extinction and responding to the trained action A in the presence of either S1 or S2 was recorded. To assess the role of

outcome value on this response, we used sensory-specific satiety to devalue the Pavlovian outcome O1 in one group of rats (group O1; $n = 8$) or a novel outcome O3 in another group (group O3; $n = 8$). Performance in these groups was compared to that of a control group that did not receive outcome devaluation (group No; $n = 8$). To ensure that the rats discriminated between the various outcomes, consumption tests were conducted after the general PIT test.

Pavlovian and Instrumental Conditioning

Pavlovian conditioning (**Figure 1B**) was successful, and all rats entered the magazine more in the presence of stimulus S1 than in its absence (Period: S1 vs. pre; $F_{(1,21)} = 257.2$, $p < 0.001$), irrespective of group (lowest $p = 0.23$). The discrimination between the two periods grew as training progressed (Days × Period; $F_{(1,21)} = 32.5$, $p < 0.001$), regardless of group (lowest $p = 0.61$). Instrumental conditioning was similarly successful (**Figure 1C**), and lever press responding increased gradually across days (Days; $F_{(1,21)} = 119.6$, $p < 0.001$), irrespective of group (lowest $p = 0.11$).

Exposure to stimulus S2 (data not shown) revealed that this stimulus was treated as neutral, as it elicited low levels of magazine entries (Mean ± s.e.m; group No: 3.96 ± 0.62; group O1: 3.51 ± 1.49; group O3: 2.81 ± 0.84) that were equivalent to those recorded in its absence (Period: pre vs. S2; $p = 0.07$), regardless of group (lowest $p = 0.83$). Instrumental extinction occurred smoothly, as lever press responding decreased gradually across the session (Min: $F_{(1,21)} = 41.6$, $p < 0.001$; Mean ± s.e.m during the last minute; group No: 11.63 ± 2.60; group O1: 6.25 ± 1.06; group O3: 13.00 ± 4.18), irrespective of group (lowest $p = 0.16$).

Outcome Devaluation and General PIT Test

Outcome devaluation by sensory-specific satiety occurred without incident (**Figure 1D**). Groups O1 and O3 consumed an equivalent amount of O1 and O3, respectively ($p = 0.87$).

The data of most interest from the general PIT test are shown in **Figures 1E,F**. As expected, outcome devaluation severely reduced instrumental performance during the initial extinction period (Devaluation; $F_{(1,21)} = 36.5$, $p < 0.001$) and the baseline period (pre: 2 min before each stimulus presentation; Devaluation; $F_{(1,21)} = 34.4$, $p < 0.001$) of the test (**Figure 1E**). The two groups that received outcome devaluation displayed equivalent performance during these two periods (group O1 vs. O3; lowest $p = 0.79$). To minimize the impact of differences in baseline responding on our ability to detect a general PIT effect, we subtracted baseline responding (pre) from responding in the presence of the stimuli (S1 and S2). This approach allowed the detection of a general PIT effect by comparing performance triggered by the predictive S1 and the neutral S2 in each group (**Figure 1F**). Outcome devaluation abolished the expression of general PIT (Devaluation × Stimuli: $F_{(1,21)} = 12.7$, $p < 0.01$). Although S1 elevated responding on the action relative to S2 in group No ($F_{(1,7)} = 23.8$, $p < 0.001$), it failed to do so in groups O1 and O3 (lowest $p = 0.10$). Thus, outcome value is required for the capacity of predictive stimuli to energize action performance. This capacity is lost when either the outcome predicted by the stimulus, or a novel outcome is devalued by sensory-specific satiety prior to the test. This finding is consistent with previous research showing that a shift from hunger to satiety abolishes the expression of general PIT (Corbit et al., 2007).

We also analyzed magazine entries during the general PIT test (**Figures 1G,H**). Consistent with the data obtained with lever presses, outcome devaluation reduced magazine entries during the extinction and baseline periods (**Figure 1G**; Extinction: $F_{(1,21)} = 11.3$, $p < 0.01$; pre: $F_{(1,21)} = 6.3$, $p < 0.05$). This reduction was similar whether the devalued outcome was that predicted by S1 or was novel (group O1 vs. group O3; lowest $p = 0.44$). The analysis conducted on the net effect of the stimuli (**Figure 1H**) revealed that outcome devaluation decreased magazine entries in the presence of these stimuli (Devaluation: $F_{(1,21)} = 12.8$, $p < 0.01$) and the difference in magazine entries during S1 and S2 (Devaluation × Stimuli : $F_{(1,21)} = 16.6$, $p < 0.01$). This decrease did not depend on the identity of the devalued outcome (group O1 vs. group O3; $p = 0.39$). It is noteworthy, however, that outcome devaluation did not completely abolish the capacity of the predictive S1 to elicit more magazine entries than the neutral S2. Indeed, S1 triggered more magazine entries than S2 in groups No (Stimuli: $F_{(1,7)} = 65.7$, $p < 0.001$), O1 ($F_{(1,7)} = 57.9$, $p < 0.001$) and O3 ($F_{(1,7)} = 8.7$, $p < 0.05$).

Consumption Test

We next conducted consumption tests to ensure that the rats distinguished the various food outcomes. Each group was allocated to a set of two outcomes (group No: O2 and O3; group O1: O1 and O2; group O3: O1 and O3). Half of the rats in each group received outcome devaluation for one of the allocated outcomes by means of sensory-specific satiety. The other half received devaluation of the other outcome. Then, rats were offered a choice to consume either of the two outcomes allocated to their group (one devalued and one valued). During outcome devaluation (**Figure 1I**), all groups ate an equivalent amount of the freely available outcome (lowest $p = 0.52$). Within each group, rats consumed the same amount of the two possible outcomes (lowest $p = 0.08$). Critically, the consumption test (**Figure 1J**) showed that the rats consumed more of the valued outcome than the devalued outcome (Valued vs. Devalued: $F_{(1,21)} = 46.5$, $p < 0.001$), regardless of group (lowest $p = 0.25$). This confirmed that the rats were able to distinguish the various food outcomes.

Experiment 2: Devaluation of All Predicted Outcomes Spare Specific PIT

Experiment 2 examined whether outcome value modulates the capacity of predictive stimuli to guide action selection. A specific PIT design was used (**Figure 2A**). During Pavlovian conditioning, rats ($n = 16$) learned that two stimuli, S1 and S2, predicted two distinct food outcomes, O1 and O2. During instrumental conditioning, rats were trained to perform one lever press action A1 to earn O1 and another lever press action A2 to earn O2. Two consecutive specific PIT tests then assessed the capacity of the stimuli to guide the choice between the two actions. These tests were conducted under extinction and responding to the trained actions in the presence of either S1 or S2 was recorded. To assess the role of outcome value on this response, we used sensory-specific satiety before one of the tests

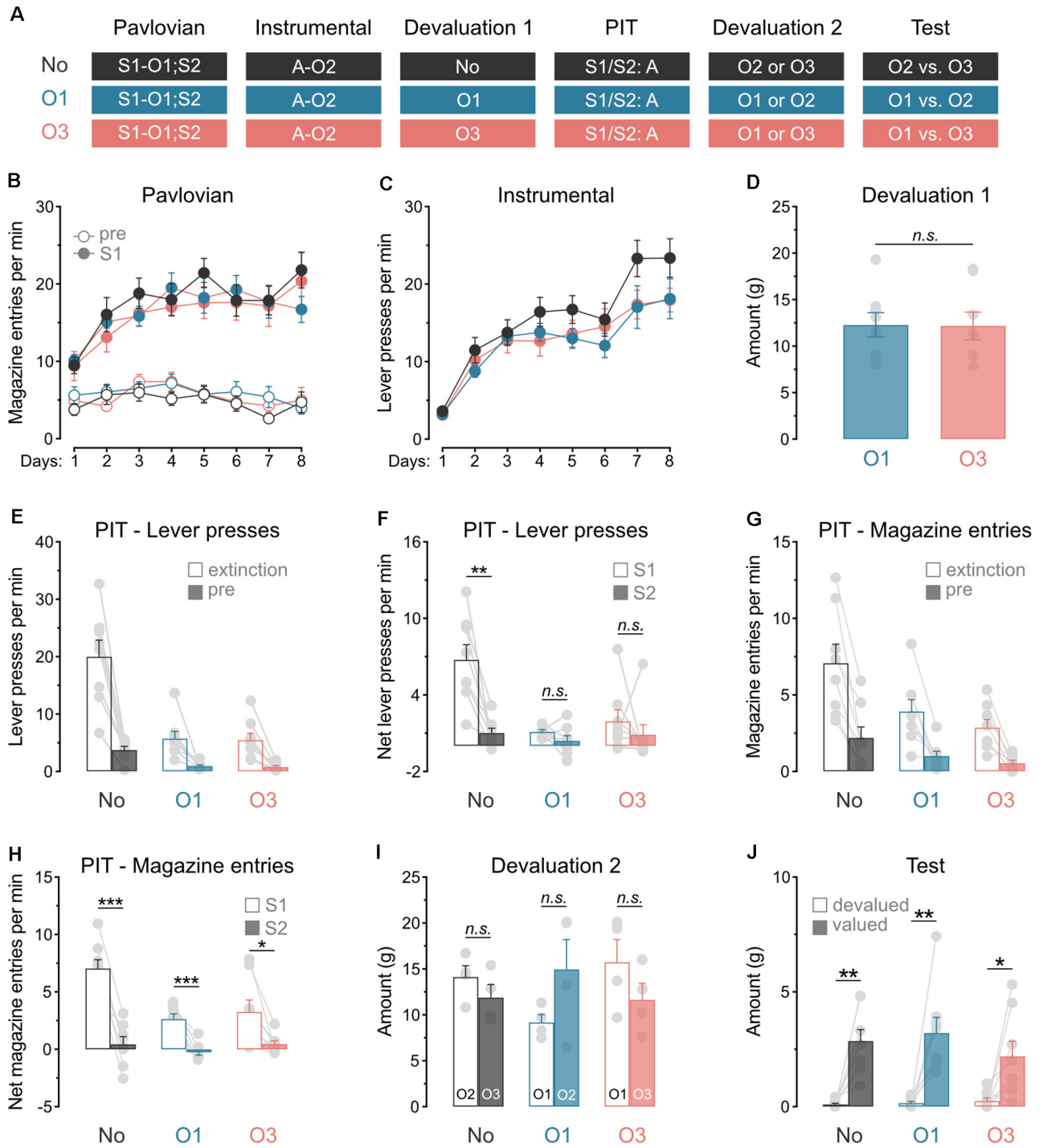

FIGURE 1 | Satiety alone abolishes general PIT. **(A)** Design of the first experiment; S1/S2: clicker or noise stimuli (counterbalanced); O1/O2/O3: grain, purified, or chocolate pellets outcomes (counterbalanced); A: left lever press action. **(B)** All rats learned that stimulus S1 predicted food outcome O1. **(C)** All rats learn to perform the left lever press action A to earn food outcome O2. **(D)** During outcome devaluation *via* sensory-specific satiety, rats in groups O1 and O3 consumed an equivalent amount of O1 and O3, respectively. **(E)** Outcome devaluation reduced lever press responding during the extinction and baseline (pre) period of the general PIT test. **(F)** Outcome devaluation abolished general PIT in groups O1 and O3. **(G)** Outcome devaluation reduced magazine entries during the extinction and baseline (pre) period of the general PIT test. **(H)** Although magazine entries were reduced after outcome devaluation, all groups showed higher magazine entries in the presence of S1. **(I)** During the second outcome devaluation *via* sensory-specific satiety, rats consumed an equivalent amount of the various outcomes. **(J)** All rats ate more of the valued outcome than the devalued outcome. Data are shown as mean ± SEM. Asterisks denote significant effect (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). n.s., nonsignificant.

to devalue the two Pavlovian outcomes, O1 and O2. Devaluation was omitted in the other test.

Pavlovian and Instrumental Conditioning

Pavlovian conditioning (**Figure 2B**) was successful, and all rats entered the magazine more in the presence of the stimuli than in their absence (Period: S1/S2 vs. pre; $F_{(1,15)} = 91.4$, $p < 0.001$) and the discrimination between the two periods grew as training progressed (Days × Period; $F_{(1,15)} = 61.7$, $p < 0.001$). Instrumental conditioning was similarly successful (**Figure 2C**), and lever press responding increased gradually across days (Days; $F_{(1,15)} = 351.4$, $p < 0.001$).

Rats also received instrumental conditioning during which the two lever press actions both earned a novel food outcome O3. Lever press responding was high since the beginning (Mean ± s.e.m; Day 1: 18.13 ± 1.49) and remained stable throughout (Days: $p = 0.25$; Mean ± s.e.m; Day 3: 20.07 ± 1.56). The following day, the animals were returned to the original instrumental conditioning arrangement and showed substantial lever press responding (Mean ± s.e.m; 29.14 ± 2.40).

Outcome Devaluation and Specific PIT Tests

Rats were given two consecutive PIT tests, one of which took place after the devaluation of O1 and O2 by sensory-specific satiety. Rats ate an equivalent amount of O1 and O2 during devaluation (**Figure 2D**; O1 vs. O2: $p = 0.53$).

The data of most interest from the general PIT tests are shown in **Figures 2E,F**. Training the two lever press actions with a third outcome did not prevent outcome devaluation from lowering overall instrumental performance. Responding during the initial extinction period and the baseline period of test was severely reduced by sensory-specific satiety of O1 and O2 (**Figure 2E**; Extinction: $F_{(1,15)} = 36.9$, $p < 0.001$; pre: $F_{(1,15)} = 151.1$, $p < 0.001$). To minimize the impact of differences in baseline responding on our ability to detect a specific PIT effect, we subtracted baseline responding (pre) from responding in the presence of the stimuli (S1 and S2). This approach allowed the detection of a specific PIT effect by comparing performance triggered by the two predictive stimuli S1 and S2. Thus, **Figure 2F** displays the net rate of responding to the action that earned the same outcome as the stimulus ("Same"; A1 during S1 and A2 during S2) and the action that earned a different outcome as the stimulus ("Different": A2 during S1 and A2 during S1). Outcome devaluation did not abolish specific PIT expression, it only attenuated the size of the effect ($F_{(1,15)} = 26.3$, $p < 0.001$). Indeed, the stimuli biased choice towards the action with which they shared the same outcome, whether this choice was preceded by outcome devaluation ($F_{(1,15)} = 8.1$, $p < 0.05$) or not ($F_{(1,15)} = 89.6$, $p < 0.001$). Thus, outcome value does not abolish the capacity of predictive stimuli to guide action selection.

We also analyzed magazine entries during the specific PIT tests (**Figures 2G,H**). Again, outcome devaluation reduced magazine entries during the extinction and baseline periods (**Figure 2G**; Extinction: $F_{(1,15)} = 76.6$, $p < 0.001$; pre: $F_{(1,15)} = 39.8$, $p < 0.001$). The analysis conducted on the net effect of the stimuli (**Figure 2H**) revealed that outcome devaluation decreased magazine entries in the presence of the stimuli (Devaluation: $F_{(1,15)} = 21.9$, $p < 0.01$). Inspection of the figure does indicate, however, that the stimuli were still able to elicit magazine entries despite outcome devaluation, a finding consistent with what was observed in the previous experiment.

Experiment 3: Devaluation of a Single Predicted Outcome Spares Specific PIT

The previous experiment revealed that specific PIT expression survives devaluation of the outcomes predicted by the stimuli. Experiment 3 aimed to extend this finding by showing that specific PIT is preserved in a situation where only one of the predicted outcomes is devalued. The design (**Figure 3A**) is identical to the one used for Experiment 2, except that rats ($n = 16$) received devaluation of only O1 by means of sensory-specific satiety.

Pavlovian and Instrumental Conditioning

Pavlovian conditioning (**Figure 3B**) was successful, and all rats entered the magazine more in the presence of the stimuli than in their absence (Period: S1/S2 vs. pre; $F_{(1,15)} = 123.5$, $p < 0.001$) and the discrimination between the two periods grew as training progressed (Days × Period; $F_{(1,15)} = 40.4$, $p < 0.001$). Instrumental conditioning was similarly successful (**Figure 3C**), and lever press responding increased gradually across days (Days; $F_{(1,15)} = 176.3$, $p < 0.001$).

Rats also received instrumental conditioning during which the two lever press actions both earned a novel food outcome O3. Lever press responding increased gradually as this training progressed ($F_{(1,15)} = 11.9$, $p < 0.05$). The following day, the animals were returned to the original instrumental conditioning arrangement and showed a substantial response (Mean ± s.e.m; 28.74 ± 1.73).

Outcome Devaluation and Specific PIT Tests

Rats were given two consecutive PIT tests, one of which took place after the devaluation of O1 by sensory-specific satiety. Rats ate a significant amount of O1 during devaluation (**Figure 3D**).

The data of most interest from the specific PIT tests are shown in **Figures 3E,F**. Training the two lever press actions with a third outcome did not prevent outcome devaluation from lowering overall instrumental performance. Responding during the initial extinction period and the baseline period of test was severely reduced by sensory-specific satiety of O1 (**Figure 3E**; Extinction: $F_{(1,15)} = 38.7$, $p < 0.001$; pre: $F_{(1,15)} = 34.5$, $p < 0.001$). A separate analysis revealed that this reduction was mostly driven by lower performance of the action that earned the devalued outcome (Extinction: $F_{(1,15)} = 55.1$, $p < 0.001$; pre: $F_{(1,15)} = 53.3$, $p < 0.001$), suggesting that instrumental behavior was goal-directed. As before, we assessed the presence of the specific PIT effect by comparing net lever press responding in the presence of either S1 or S2 (**Figure 3F**). Outcome devaluation did not abolish the expression of specific PIT effect or its size ($p = 0.17$), and the stimuli biased choice towards the action with which they shared the same outcome, whether this choice was preceded by outcome devaluation ($F_{(1,15)} = 4.8$, $p < 0.05$) or not ($F_{(1,15)} = 6.9$, $p < 0.05$). Thus, this experiment confirmed that outcome devaluation does not abolish the capacity of predictive stimuli to guide action selection.

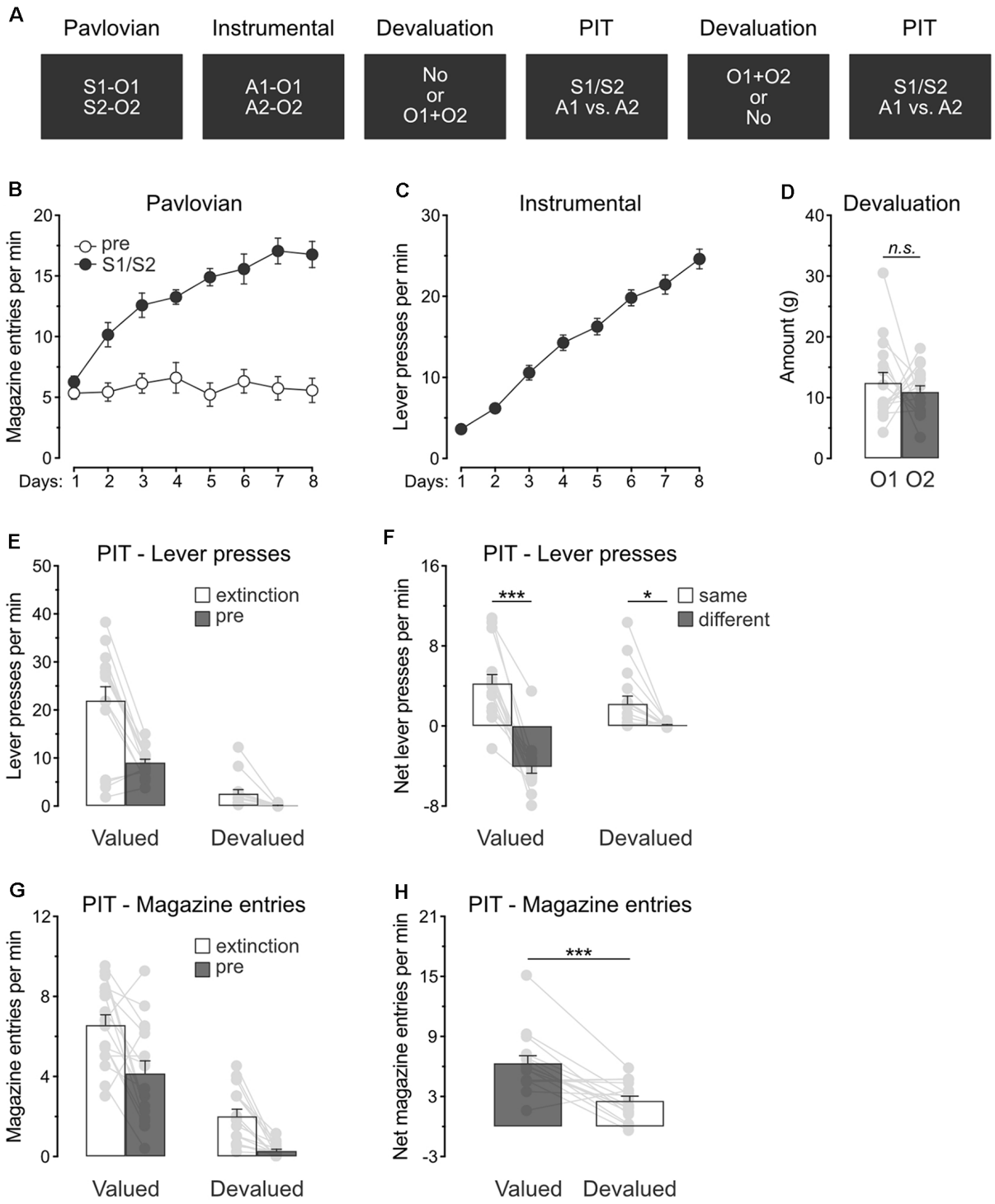

FIGURE 2 | Devaluation of all predicted outcomes spare specific PIT. **(A)** Design of the second experiment; S1/S2: clicker or noise stimuli (counterbalanced); O1/O2: grain pellet or sucrose solution outcomes (counterbalanced); A1/A2: left and right lever press actions. **(B)** All rats learned that the stimuli, S1 and S2, predicted food outcomes O1 and O2. **(C)** All rats learn to perform the left and right lever press actions, A1 and A2, to earn food outcomes O1 and O2. **(D)** During outcome devaluation *via* sensory-specific satiety, rats consumed an equivalent amount of O1 and O2. **(E)** Outcome devaluation reduced lever press responding during the extinction and baseline (pre) period of the specific PIT test. **(F)** Outcome devaluation spared specific PIT. **(G)** Outcome devaluation reduced magazine entries during the extinction and baseline (pre) period of the specific PIT test. **(H)** Although magazine entries were reduced after outcome devaluation, rats showed substantial levels of magazine entries in the presence of the stimuli. Data are shown as mean ± SEM. Asterisks denote significant effect (*$p < 0.05$; ***$p < 0.001$). n.s., nonsignificant.

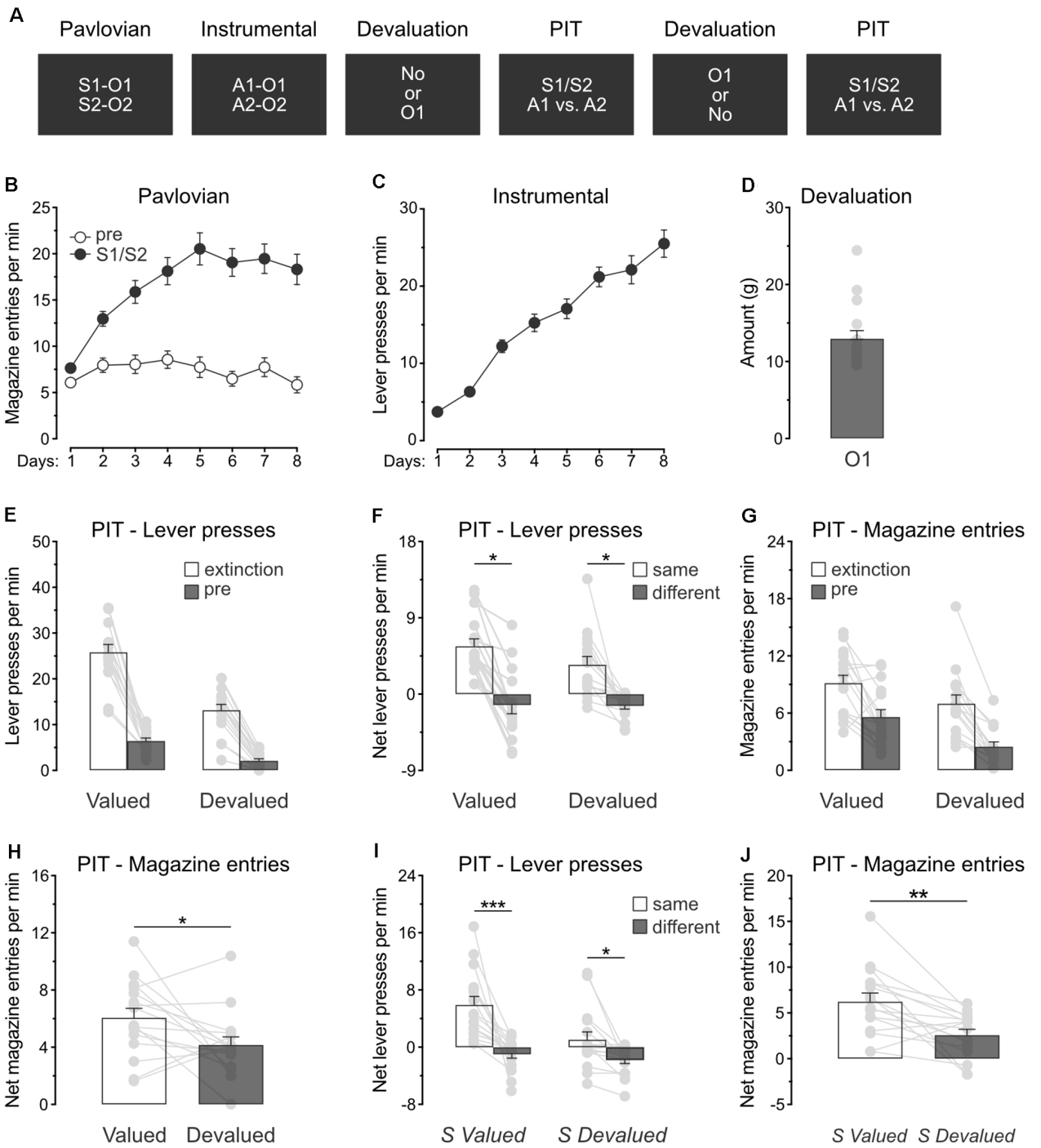

FIGURE 3 | Devaluation of a single predicted outcome spares specific PIT. **(A)** Design of the third experiment; S1/S2: clicker or noise stimuli (counterbalanced); O1/O2: grain pellet or sucrose solution outcomes (counterbalanced); A1/A2: left and right lever press actions. **(B)** All rats learned that the stimuli, S1 and S2, predicted food outcomes O1 and O2. **(C)** All rats learn to perform the left and right lever press actions, A1 and A2, to earn food outcomes O1 and O2. **(D)** During outcome devaluation *via* sensory-specific satiety, rats consumed a substantial amount of O1. **(E)** Outcome devaluation reduced lever press responding during the extinction and baseline (pre) period of the specific PIT test. **(F)** Outcome devaluation spared specific PIT. **(G)** Outcome devaluation reduced magazine entries during the extinction and baseline (pre) period of the specific PIT test. **(H)** Although magazine entries were reduced after outcome devaluation, rats showed substantial levels of magazine entries in the presence of the stimuli. **(I)** The stimuli promoted specific PIT whether they predicted a valued (S valued) or devalued (S devalued) outcome. **(J)** Although magazine entries were reduced after outcome devaluation, the stimuli elicited substantial levels of magazine entries whether they predicted a valued (S valued) or devalued (S devalued) outcome. Data are shown as mean ± SEM. Asterisks denote significant effect (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

We also analyzed magazine entries during the specific PIT tests (**Figures 3G,H**). As before, outcome devaluation reduced magazine entries during the extinction and baseline periods (**Figure 3G**; Extinction: $F_{(1,15)} = 10.5$, $p < 0.05$; pre: $F_{(1,15)} = 17.3$, $p < 0.01$). The analysis conducted on the net responding during the stimuli (**Figure 3H**) revealed that outcome devaluation decreased magazine entries in the presence of the stimuli (Devaluation: $F_{(1,15)} = 4.9$, $p < 0.01$). Once again, however, an inspection of the figure indicates that the stimuli remained capable of eliciting magazine entries despite outcome devaluation.

In this experiment, the specific PIT test conducted after outcome devaluation included a stimulus (S1) that predicted a now devalued outcome (O1) and another stimulus (S2) that predicted a valued outcome (O2). We conducted a separate analysis on this test to evaluate whether the two stimuli influenced action selection in a similar manner. Although it reduced the size of the effect (**Figure 3I**; $F_{(1,15)} = 9.3$, $p < 0.05$), outcome devaluation did not abolish the expression of specific PIT.Both stimuli biased choice towards the action with which they shared the same outcome, whether their predicted outcome had been devalued (S devalued; $F_{(1,15)} = 8.6$, $p < 0.05$) or not (S valued; $F_{(1,15)} = 20.5$, $p < 0.01$). Analysis of the net magazine entries rates (**Figure 3J**) confirmed a reduction as a result of outcome devaluation ($F_{(1,15)} = 14.0$, $p < 0.01$). Taken together, the results of Experiment 3 replicate those of Experiment 2 and confirmed that outcome value does not abolish the capacity of predictive stimuli to guide action selection.

DISCUSSION

The present experiments examined whether outcome value regulates the capacity of predictive stimuli to control action performance and action selection. Experiment 1 used a general PIT design to demonstrate that a stimulus predicting a food outcome energizes the performance of an instrumental action earning another food outcome. It found that this energizing effect is removed when the stimulus-predicted outcome is devalued by means of sensory-specific satiety. However, this removal was also observed when rats were sated on an entirely novel outcome prior to the test, suggesting that satiety alone abolishes the expression of general PIT. Experiments 2 and 3 used a specific PIT design to show that a stimulus predicting a particular food outcome promotes the selection of an action earning the same food outcome over an action earning a different outcome. Remarkably, both experiments demonstrated that devaluing all or just one of the outcomes predicted by the stimuli spared the expression of specific PIT, even though the size of the effect was attenuated. Collectively, these experiments indicate that outcome value regulates the capacity of predictive stimuli to energize action performance in general PIT but does not control the capacity of the same stimuli to guide the selection of actions in specific PIT.

To the best of our knowledge, Experiment 1 is the first investigation employing sensory-specific satiety to explore the role of outcome value in the expression of general PIT. It found that devaluation of the stimulus-predicted outcome abolishes the capacity of the stimulus to subsequently energize instrumental performance. This finding is at odds with a previous study in which the expression of general PIT was preserved following outcome-specific devaluation (Holland, 2004). One obvious difference between this and our study is that the former used a conditioned taste aversion procedure to devalue the outcome predicted by the stimulus. Residual instrumental responding has been observed following this procedure (Colwill and Rescorla, 1985a), raising the possibility that its impact on value-based behavior is not as strong as that produced by sensory-specific satiety (Colwill and Rescorla, 1985b). Consistent with this possibility, sensory-specific satiety severely reduced baseline instrumental responding in our experiments whereas this responding was minimally or not at all affected following conditioned taste aversion (Holland, 2004). Regardless, Experiment 1 also found that a stimulus lost its capacity to promote general PIT when an entirely novel stimulus had been devalued by sensory-specific satiety prior to the test. This finding was not due to a failure of the animals to distinguish between the various outcomes, as demonstrated by the consumption tests conducted in this experiment. Rather, it indicates that satiety alone disrupts the expression of general PIT, which agrees with previous work showing that a shift in primary motivational states abolishes this expression (Balleine, 1994; Corbit et al., 2007). Thus, our results are consistent with the view that a stimulus can only energize instrumental performance when it predicts an outcome that satisfies the current biological needs of the agent.

Experiments 2 and 3 examined the role of outcome value in the expression of specific PIT. They confirmed that a stimulus predicting a particular food outcome promotes the selection of an instrumental action earning that same, but not a different, outcome. They also confirmed that devaluation of the outcomes predicted by the stimuli spares the expression of specific PIT (Rescorla, 1994; Holland, 2004; Sommer et al., 2022). Unlike what is observed in general PIT, this preservation can be observed whether sensory-specific satiety or conditioned taste aversion is used to produce outcome-specific devaluation. Experiment 3 provides perhaps the best evidence that outcome value does not regulate the expression of specific PIT. In that experiment, only one outcome was devalued, allowing us to compare within-subjects whether a stimulus predicting a devalued outcome is less able to guide action selection than a stimulus predicting a valued outcome. The results clearly indicate that the two stimuli generated the specific PIT effect. It is noteworthy that these results faithfully reproduce those reported in a recent study that also used sensory-specific satiety to devalue one of the stimulus-predicted outcomes (Sommer et al., 2022). Our findings are therefore consistent with previous research showing that outcome value does not regulate the capacity of predictive stimuli to control action selection.

Although specific PIT was preserved in Experiments 2 and 3, the size of the effect was severely attenuated by sensory-specific satiety. This attenuation occurred despite efforts made to maintain substantial levels of baseline responding at test by training the instrumental actions with a third and common outcome. This approach has successfully been used previously in the context of conditioned taste aversion (Rescorla, 1994),

but it had no apparent effect in our experiments. This may underscore again that sensory-specific satiety drives a larger reduction of instrumental performance than conditioned taste aversion. Regardless, the attenuation of the specific PIT effect observed here agrees with previous reports assessing this effect following shifts in primary motivational states (Corbit et al., 2007) or sensory-specific satiety (Sommer et al., 2022). This attenuation of the specific PIT effect and its preservation following outcome devaluation is successfully predicted by a popular model developed by Balleine and Ostlund (2007). In this model, the presentation of a stimulus at test retrieves the sensory-specific properties of its outcome, which in turn allows retrieving the action with which it is associated during instrumental training. Thus, a stimulus guide action selection independently of the value assigned to its predicted outcome. However, the model also assumes that this assigned value gates the capacity of the stimulus to initiate and energize action performance. The model therefore successfully predicts our findings that outcome devaluation preserves but attenuates the specific PIT effect.

The general and specific PIT procedures employed in the present experiments involved obvious and necessary differences (e.g., number of predictive stimuli, instrumental actions) and diverged in terms of the instrumental schedules used to train the lever press actions. Random interval schedules were used for training the actions in general PIT and random ratio schedules were implemented in specific PIT. This raises the possibility that the use of these different schedules supported the opposite effects produced by outcome devaluation on the expression of general and specific PIT. However, this appears very unlikely, as sensory-specific satiety reduced baseline instrumental responding to a similar extent in all our experiments. Further, if anything, random interval schedules are more resistant to changes in outcome value than random ratio schedules (Dickinson et al., 1983). Yet, outcome devaluation removed general PIT but left specific PIT unaffected. It is also noteworthy that outcome devaluation diminished the ability of the stimuli to elicit magazine entries in all experiments. We are therefore confident that the contrasting effect of outcome devaluation on general and specific PIT reported here is not due to the use of different parameters across our experiments.

In summary, the present experiments demonstrate that changes in outcome value differentially regulate the capacity of predictive stimuli to control action performance and action selection. Using a general PIT design, we found that satiety alone removes the capacity of a predictive stimulus to energize action performance. By contrast, in a specific PIT design, we found that predictive stimuli can guide action selection regardless of the value of their associated outcomes. This dissociation is in line with neural studies (Corbit et al., 2001; Corbit and Balleine, 2005, 2011; Talmi et al., 2008; Prévost et al., 2012; Mendelsohn et al., 2014; van Steenbergen et al., 2017) showing that general and specific PIT recruit distinct brain regions and thereby, are likely to be two separate phenomena supported by distinct psychological mechanisms. Our findings are also consistent with the view that general PIT is driven by motivational processes whereas specific PIT involves cognitive processes that control action selection (Cartoni et al., 2016; Corbit and Balleine, 2016).

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 Animals Ethics Committees at the University of New South Wales.

AUTHOR CONTRIBUTIONS

VL designed the experiments. NWL, TB, and JB conducted the experiments. NWL, TB, JB, and VL analyzed the data. NWL, TB, and VL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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General and Specific Aversive Modulation of Active Avoidance Require Central Amygdala

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Three studies provide evidence that the central nucleus of the amygdala, a structure with a well-established role in conditioned freezing, is also required for conditioned facilitation of instrumental avoidance in rats. First, the immediate early gene c-Fos was measured following the presentation of a previously shock-paired tone in subjects trained either on an unsignaled avoidance task or not (in addition to tone only presentations in naïve controls). Significantly elevated expression of c-Fos was found in both the avoidance trained and Pavlovian trained conditions relative to naïve controls (but with no difference between the two trained conditions). In a subsequent study, intracranial infusions of muscimol into the central amygdala significantly attenuated the facilitation of shock-avoidance by a shock-paired Pavlovian cue relative to pre-operative responding. The final study used a virogenetic approach to inhibit the central amygdala prior to testing. This treatment eliminated the transfer of motivational control over shock-avoidance by both a shock-paired Pavlovian stimulus, as well as a cue paired with a perceptually distinct aversive event (i.e., klaxon). These findings provide compelling support for a role of central amygdala in producing aversive Pavlovian-instrumental transfer.

Keywords: avoidance, amygdala, transfer, motivation, instrumental

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INTRODUCTION

Studies of aversive Pavlovian learning have produced a rich understanding of the psychological mechanisms and neural circuitry responsible for conditioned defensive reactions. Adaptive behaviors (e.g., freezing) and cardiovascular responses come under the control of a previously neutral conditioned stimulus (CS; e.g., tone) through repeated pairings with an aversive unconditioned stimulus (US; e.g., footshock; LeDoux et al., 1988). At the neural level, this depends on signals along auditory and somesthetic pathways converging in the lateral nucleus of the amygdala (LA) and engaging Hebbian plasticity that potentiates the auditory input (Rogan and LeDoux, 1996; Rosenkranz and Grace, 2002). Following training, increased CS-elicited activity in LA produces conditioned responding (CR; e.g., freezing) via connections to the central amygdala

(CeA), which then projects to brainstem areas that directly stimulate the relevant behaviors (LeDoux, 2000). Indeed, much has been learned about the electrophysiological activity and various molecular processes that support this form of learning (Johansen et al., 2011; Herry and Johansen, 2014). However, aversive Pavlovian cues can serve other purposes beyond producing simple CRs, such as modulating ongoing goal-directed behavior (Bolles and Popp, 1964; Rescorla and Lolordo, 1965; Rescorla, 1968; Weisman and Litner, 1969; Overmier and Payne, 1971; Overmier and Brackbill, 1977; Patterson and Overmier, 1981). While studies of appetitive learning have elegantly examined the substrates for modulatory effects of the CS (see Cartoni et al., 2016), our understanding of how this is accomplished with aversive stimuli is very limited.

Different forms of Pavlovian-instrumental transfer (PIT; e.g., conditioned suppression and facilitation) demonstrate that an aversive Pavlovian CS can modulate ongoing instrumental behaviors (e.g., food-reinforced lever-press or shock-avoidance responding). While conditioned suppression has been studied extensively (LeDoux et al., 1990; Killcross et al., 1997; Cardinal et al., 2002; Lee et al., 2005; Elrich et al., 2012; also see Fernando et al., 2014), the neural mechanisms involved in aversive conditioned facilitation are not well understood. Using an aversive PIT task, where footshock-avoidance behavior (e.g., two-way shuttling) is enhanced by a separately trained shock-paired CS, we found that CeA is necessary for conditioned facilitation (Campese et al., 2013, 2015). However, these findings were obtained using electrolytic lesions, thus the possibility remains that nonspecific effects could have accounted for the behavioral results (but see Campese et al., 2017a). Given the role of CeA in conditioned freezing, further evidence for this opposing function (i.e., response facilitation) may prove valuable for understanding how CeA may regulate a variety of behavioral responses. Therefore, in the following studies, we sought to establish a role for CeA in aversive PIT using more selective means beyond lesions. Specifically, the immediate early gene c-Fos was quantified in CeA following PIT testing. This was compared to CeA in control subjects that had undergone Pavlovian conditioning but without shock-avoidance training. To follow this up, intracranial muscimol was used to inhibit CeA during PIT in a within-subjects design. The final study had a similar approach but used designer Kappa opioid receptors (KORD; Vardy et al., 2015; Marchant et al., 2016) controlled by the synthetic ligand salvinorin-B (Sal-B) to inhibit CeA. KORD was used because it provides a more accurate means to visualize the cells being targeted than muscimol. This study also extended the analysis to whether different forms of aversive conditioned facilitation (i.e., sensory-specific or general) depend on CeA. Appetitive procedures that isolate sensory-specific and general PIT have led to important discoveries about how distinct neural pathways process different elements of motivation (Cartoni et al., 2016). Identifying the degree of similarity in how the aversive analogs of these motivational substrates are generated at a neural level may prove similarly enlightening. The findings from these studies provide strong evidence that CeA is necessary for the modulation of avoidance behavior through both general and specific motivational mechanisms.

METHODS

Subjects

Fifty-four male Sprague-Dawley rats were used as subjects for the studies reported below. Rats were obtained from Hilltop Lab Animals (Scottsdale, PA) and weighed approximately 300 g at the start of behavioral training. Subjects were housed individually in standard paper bedding lined Plexiglass cages in a colony running a 12:12 h light-dark schedule with access to free food and water. Animal care and housing met the current standards of the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures reported herein were pre-approved by the New York University Animal Welfare Committee.

Apparatus

Subjects were trained in chambers manufactured by Coulbourn Instruments (Whitehall, PA) running Graphic State 3 (Actimetrics) software to control the sessions and measure responding. All chambers included stainless steel grid floors for shock delivery as well as an 8 ohm speaker for the presentation of the 5 kHz tone and white noise stimuli. Coulbourn precision animal shockers (model no. H13-15) and programmable audio generators (model no. A12-33) were used for stimulus delivery. For Pavlovian training, standard chambers (26 × 28 × 20 cm, length × width × height; model no. H10-11R-TC), each equipped with a klaxon horn (model no. 330; 114dB) made by Wolo (Deer Park, NY) were used. Footshock avoidance took place in two-compartment shuttlebox chambers divided by a panel with a threshold cutaway for passage (50.8 × 25.4 × 30.5 cm, length × width × height; model no. H10-11R-SC). Avoidance chambers were equipped with infrared emitter-detector arrays to capture responses automatically. All chambers were individually housed in light and sound attenuation cubicles (model no. H10-24C). Pavlovian training and transfer test sessions were recorded using a digital video recorder (model no. DVR814) purchased from CCTV Imports (Madisonville, LA) for quantification of freezing.

Procedure

Behavioral Training

The studies reported here involved a combination of aversive Pavlovian conditioning, active avoidance, and transfer testing where the effect of the Pavlovian stimuli on avoidance rates were evaluated. Because there were minor modifications to these procedures for reasons specific to each study, a general description of the tasks will be provided with details offered for the changes required in each study.

Pavlovian Threat Conditioning

Subjects received Pavlovian threat conditioning (PTC) in standard chambers (context A). PTC for a given cue was conducted in a single session where a 30 s 5 kHz tone co-terminated with a 1 s 0.6 mA footshock US. There were three

trials following a 5-min baseline separated by a 3-min intertrial interval (ITI) and sessions were 15-min in duration.

Fos Study
Subjects in both the PAV and PIT groups of this study received tone-shock training as described above (controls received no training) on the first day of the experiment. At the end of the session, subjects were removed from the chambers and returned to their home cages. The USAA phase of the study began 24 h later.

Muscimol Study
All subjects in this study received tone-shock training as described above and were returned to the colony for the rest of the day following training. The USAA phase began the following day.

KORD Study
Subjects in this study received two PTC training sessions over the first two days of training (one each day). Over these sessions, half of the subjects received tone-shock and noise-klaxon pairings, while these relationships were reversed for the other half. The order of these sessions was counterbalanced during training as were the specific stimuli. In other words, half of each counterbalanced subgroup had tone trained on day 1 and noise on day 2, while this was reversed in the other half of the subjects. For klaxon training, all other parameters were maintained, with the difference being the replacement of the footshock with a 5 s klaxon delivery.

Unsignaled Sidman Active Avoidance

Subjects underwent Unsignaled Sidman Active Avoidance (USAA) training in two-way shuttle chambers (context B) over the next 15 days of the study. During these sessions, 0.5 s footshocks (0.6 mA) were programmed for delivery every 5 s (Shock-Shock or S-S interval). However, each shuttling response delayed the delivery of the next shock by 30 s (Response-Shock or R-S) interval. Thus, rats could prevent the delivery of all shocks by shuttling at least once every 30 s. Avoidance responses were defined as shuttles during the R-S interval, while shuttles made during the S-S interval were defined as escape responses. Each shuttle response was accompanied by a brief 0.3-s blinking houselight to provide feedback to the subject (Sidman, 1953a,b). Daily sessions were 25-min in duration and concluded with the houselight turning off (see Lázaro-Muñoz et al., 2010).

Fos Study
Subjects in the PIT group received USAA training as described above. Subjects in the PAV group were not trained, but were instead, only placed in the avoidance chambers and received no shocks during this phase. For these subjects, shuttling still caused the house lights to blink, but this was not associated with safety. Box control subjects remained in their home cages during this phase.

Muscimol Study
All subjects in this study received USAA as described above.

KORD Study
All subjects in this study also received USAA as described above. For subjects given USAA training in all studies reported here, only those that made 20 or more avoidance responses in consecutive sessions within the first 10 days of training were tested for transfer. If a subject did not meet this requirement, they were excluded from further training and all analyses. In total, eight rats were excluded from analyses due to poor performance.

Pavlovian-Instrumental Transfer

To test for aversive transfer (i.e., PIT), shuttling responses during CS-free and CS periods were compared during extinction sessions (no shocks) in context B (Campese et al., 2013). Each test session began with a 15-min baseline period, after which, the CS presentation occurred once the shuttling rate dropped below two responses per minute for two full minutes. The CS then remained on until 10 shuttle responses were made, at which point, the houselight turned off and the session ended. Note that response-produced feedback stimuli were presented during tests as in training. For PIT tests, responses per minute data were used for both presentation and analysis.

Fos Study
In this study, there was a single PIT test using the procedure described above. While previous studies (and those below) involve multiple test sessions to evaluate transfer, a single test was used here to capture the first instance of transfer for the purpose of c-Fos measurements. It may be the case that as transfer tests accumulate, there could be learning effects that emerge, especially given that responding terminates test sessions. Given this possibility, differences in Fos expression could be seen as a function of whether one or multiple tests are used. The single test was followed by perfusion and brain removal for c-Fos analysis. Because subjects in the Fos study only had one test session, total time freezing to the CS during this test was measured. For all subjects, the tone remained on until 10 responses were made.

Muscimol Study
In this study, subjects received two PIT tests on consecutive days following USAA, but prior to cannula implantation surgery. Following a 1-week recovery from surgery, subjects were given additional testing with intracranial treatments. Two tests on consecutive days were conducted following vehicle or muscimol infusions. Then, 1 week later, another two tests were conducted on consecutive days using the other infusion treatment. The postoperative test rounds were separated by one-week to encourage response recovery. Two test videos from this study were lost due to hard drive errors. The rest were scored for freezing by a trained blind rater. Because multiple tests were conducted in this study, only the 1st min of the CS was scored for these test sessions.

KORD Study
For this phase, subjects underwent a total of eight individual transfer tests, arranged into four blocks with two tests in each block. The first four tests (i.e., blocks 1 and 2) were conducted with tone, and the last four tests (i.e., blocks 3 and 4) with the white noise CS. For all subjects, tone was tested on the first

two consecutive days of the test phase. This was followed by two additional tone test sessions 5 days later. The interpolated time between tests was meant to encourage response recovery. Noise testing began the day after the final tone test and was done in the same way (i.e., with 4 days off between tests involving different drug assignments). Subjects received a given IP treatment (i.e., Veh or sal-B) on consecutive days and were tested with the same CS on both days. This was counterbalanced for drug assignment over the test phase so that each subject produced a PIT score (comprised of a two-test average) for tone and noise under both vehicle and Sal-B treatments. Drug assignments were arranged to account for block order, so that if for example, a subject had vehicle treatment before tests 1 and 2, and sal-B for tests 3 and 4, this was reversed for the noise tests, resulting in sal-B treatment for tests 5 and 6, with vehicle prior to tests 7 and 8. It should be noted that this arrangement resulted in half of the subjects being tested for CS-shock while the other half were tested for transfer to CS-klaxon during each session. This scheme was chosen because previous findings suggest noise is more effective at driving transfer. Given the many sessions needed to produce within-subjects comparisons for KORD status, stacking noise sessions in the final blocks was aimed at avoiding a floor effect. A single video from this test phase was lost due to a recording error. Freezing was evaluated by a blind rater measuring the first min of each trial during each test.

Surgery

Cannulations and Intracranial Treatments

Following baseline PIT testing for subjects treated with muscimol, rats were anesthetized with a mixture of ketamine (100 mg/kg: Vedco) and xylazine (10 mg/kg) via intraperitoneal (IP) injection (0.1% bodyweight). Subjects were placed in a Kopf (David Kopf Instruments; Tujunga, CA) stereotaxic instrument, and an incision was made over the midsagittal line to reveal bregma and lambda on the surface of the skull. Stainless steel (22 gauge) guide cannula (Plastics One, Roanake VA) were fixed in place with jeweler's screws and dental cement 1.5 mm above CeA at -2.5 mm posterior, 4 mm lateral to the midline, and 6 mm ventral from the surface of the skull. Subjects recovered in the home cage for one week following surgery and then underwent further PIT testing. Prior to testing, muscimol (0.3 μl of 1 ng/nl solution) or deionized water was infused through (28 gauge) injectors extending 1.5 mm beyond the guides bilaterally at a rate of 0.15 μl/min with subjects connected to the infusion lines for an additional min for dispersal. This was accomplished using 10 μl Hamilton syringes (Model 701N) controlled by a Harvard Apparatus pump (PHD 22/2000) via polyethylene tubing connected to injectors extending 1.5 mm beyond the tip of their guide cannula. Subjects were tested 15–20 min after treatment.

Viral Injections

Prior to behavioral training subjects were anesthetized and prepared in the stereotaxic apparatus as described above. Through a 1 μl Hamilton Neuros syringe, 0.7 μl of AAV9 CamKII containing instructions for Gi-coupled modified kappa opioid receptor (KORD; Vardy et al., 2015; Marchant et al., 2016) was injected bilaterally into CeA over 5 min and allowed to spread for an additional 5 min before removing the needle. The incision was sutured and subjects were given 2 weeks to recover in the home cage prior to undergoing behavioral training. To engage KORD receptor-based neural inhibition, 20–30 min prior to PIT testing, subjects received a 0.1% bodyweight injection of 5 mg/kg IP salvinorin-B (Sal-B) or vehicle for the control treatment. Sal-B was purchased from Applepharms (Asheville, NC) and dissolved in 7% DMSO (Sigma Aldrich, St. Louis MO), then added to a 50–50 deionized water-polyethylene glycol (Sigma Aldrich, St. Louis, MO) mix at 40°C.

c-Fos Immunocytochemistry

Ninety minutes after the end of behavioral testing, animals were anesthetized with the ketamine and xylazine mixture and transcardially perfused with approximately 30 ml of phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4), followed by approximately 300 ml of 4% paraformaldehyde (PFA). The brains were removed from the skull, post-fixed in PFA, and were cut into 50 μm coronal sections on a Vibratome (Leica, Germany). Tissue sections containing the CeA were collected in PBS with 0.05% sodium azide and stored at 4°C. Tissue sections were incubated for 30 min in 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO) made in PBS to block nonspecific binding and then incubated overnight in polyclonal rabbit anti-c-Fos primary antiserum (Calbiochem; 1:10,000). Following the incubation, sections were rinsed, incubated for 30 min in biotinlyated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA), rinsed, and incubated for 30 min in the avidin–biotin–horseradish peroxidase complex (ABC; VECTASTAIN Elite Kit, Vector). Staining was visualized using the chromogen Very Intense Purple (VIP; Vector Laboratories). Primary and secondary antibody incubations were made in 1% BSA/0.05% sodium azide/PBS and the primary incubation contained 0.2% Triton-X. Sections were mounted on gelatinized slides, dehydrated briefly in 100% ethanol, defatted in xylene, and coverslipped with Permount (Fisher Scientific, Hampton, NH). High resolution, digital images were acquired at 10X using the VS120 Virtual Slide Microscope (Olympus) (see **Figure 1F** for examples). The CeA was defined according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2005) and sampled from the most posterior to the most anterior levels (Bregma −3.36 to −1.44). For each section, the surface area of the CeA was measured using FIJI (Image J software; National Institute of Health, Bethesda, MD) and an experimenter blind to the conditions manually counted the number of labeled Fos using the Image J "qwertyujk90-= cell counter" plug-in. For each experimental condition, the total number of Fos-positive cells were counted bilaterally and expressed as the number of Fos-positive cells per unit area.

Perfusions and Immunocytochemistry

Subjects were deeply anesthetized with the mixture of the ketamine/xylazine mixture and transcardially perfused. For cannulated subjects treated with muscimol prior to testing, 0.01 M PBS was followed by 10% formalin during perfusions

(Fischer Scientific). Brains were removed from the skull and postfixed in 10% formalin. Fluorescent muscimol (Sigma Aldrich, St. Louis, MO) infusions were made 10 min prior to perfusion using the same parameters described earlier. 50 μm coronal sections were made on a Vibratome and stained for thionin to identify injection sites or left untreated and coverslipped on a slide to visualize muscimol spread using fluorescence. For KORD expressing rats, subjects were perfused with 0.01 M PBS and then 4% PFA and brains post-fixed in PFA. Brains were cut into 50 μm coronal sections made on a Leica Vibratome and stored in PBS with 0.05% sodium azide and kept at 4°C until processing. Expression of KORDs was visualized using a rabbit anti-GFP antibody (1:2K; #A11122; Life Technologies), biotinylated goat anti-rabbit IgG (1:200; Vector Labs), avidin-biotin horseradish complex (Elite ABC Kit; Vector Labs) and Very Intense Purple (VIP Kit; Vector Labs). Sections were mounted, coverslipped, and viewed using a high-resolution digital camera microscope system.

RESULTS

Expression of Immediate Early Genes in Central Amygdala During Transfer of Motivational Control

To test for CeA activity in aversive transfer, the immediate early gene c-Fos was measured in relation to the presentation of a previously shock-paired tone on active avoidance behavior (i.e., two-way shuttling). Subjects underwent the sequence of training depicted in **Figure 1A** below involving aversive auditory Pavlovian conditioning in the first phase, where a tone CS was paired with a footshock US. This was followed by USAA training, where shuttle responding was negatively correlated with footshock. Finally, during the transfer test, the CS was presented during USAA performance (in the absence of shock) and the effect of the cue on shuttling rates was quantified. Following this session, subjects were perfused and c-Fos expression in CeA was measured in relation to this event. Based on our findings that CeA lesions impaired PIT (Campese et al., 2014, 2015) we anticipated that PIT testing would result in c-Fos expression in CeA. However, because CeA is well known for its role in freezing CRs, we evaluated this relative to control subjects that had undergone Pavlovian conditioning but did not have USAA training. This was done to determine whether USAA training quantitatively changes CS-processing-related CeA activity during PIT. Below, these rats are referred to as PAV subjects, whereas those that received avoidance are referred to as PIT subjects.

Avoidance Training

Two subjects were excluded from the PIT group due to poor USAA performance and one due to inadequate perfusion. The final sample size was $n = 4$ for the PAV group and $n = 5$ for the PIT group. Shuttling data from the USAA phase are presented in **Figure 1B** below. Avoidance responding increased over this phase but did so significantly more for subjects given USAA training compared to non-shocked USAA exposure. This was confirmed by a mixed repeated measures analysis of variance (rmANOVA) including *Day* (1–15) as a within-subjects factor and *Group* (PAV vs. PIT) as a between-subjects factor ($F_{\text{Day}\ (14,98)} = 10.85$, $p < 0.001$; $F_{\text{Group}\ (1,7)} = 4.1$, $p = 0.08$; $F_{\text{Interaction}\ (14,98)} = 7.08$, $p < 0.001$).

Transfer Test

Mean freezing during the CS is presented in **Figure 1C** below for the PAV and PIT groups. Significantly more freezing was seen in subjects that did not receive USAA training ($t_{2\text{-tailed}\ (7)} = 3.32$, $p = 0.01$). Rather than freeze to the CS, USAA trained subjects showed enhanced avoidance responding instead (see **Figure 1D**). This was confirmed by a mixed rmANOVA including *Interval* (Pre vs. CS) as a within-subjects factor and *Group* (PAV vs. PIT) as a between-subjects factor ($F_{\text{Interval}\ (1,7)} = 24.5$, $p < 0.01$; $F_{\text{Group}\ (1,7)} = 12.05$, $p = 0.01$; $F_{\text{Interaction}\ (1,7)} = 18.81$, $p < 0.01$). Follow-up Bonferroni corrected comparisons localized this effect to higher shuttle responding during the CS for PIT subjects compared to responding in all other intervals for both groups [Pre CS; $M_{\text{Pav}} = 1.3$, 95% CI(0.6 2.0), $M_{\text{PIT}} = 1.24$, 95% CI(0.6 3.2); CS; $M_{\text{Pav}} = 1.75$, 95% CI(0.3 1.87), $M_{\text{PIT}} = 5.26$, 95% CI(3.97 6.56)]. It should be noted that USAA training and the subsequent transfer effect in the PIT group enabled these subjects to end their transfer test earlier than the two control groups, by executing 10 responses more quickly. Means for CS duration during the test are 8.1, 8.6, and 2.0 min in the Pav, Tone (naïve control), and PIT groups respectively.

c-Fos Analysis

Preliminary analyses showed no effect of the Hemisphere, so data were collapsed across this factor. These data are presented in **Figure 1E** as percent labeling relative to the naïve control group (i.e., Tone) ($n = 4$) exposed to the tone CS in the avoidance arena during the test. There were no differences in c-Fos labeling in CeA between the PAV and PIT groups, $t_{(7)} = 0.71$, $p = 0.50$.

Intracranial Muscimol in CeA Eliminates Aversive PIT

Previous studies with electrolytic lesions have found that CeA is important for the aversive transfer effect (Campese et al., 2014, 2015). To provide converging evidence of this, in the current experiment we used reversible intracranial inhibition by the $GABA_A$ agonist muscimol to temporarily disrupt the activity of CeA neurons prior to PIT testing. Subjects were trained and given baseline PIT testing prior to cannula implantation and then tested again after recovery following infusion of muscimol or vehicle into CeA (see **Figure 2A**). Based on our previous work, we expected that muscimol treatment would impair the facilitative effect of an aversive CS on avoidance behavior.

Avoidance Training

Acquisition of footshock-avoidance responding proceeded normally over this phase. Three subjects were eliminated from the analyses due to poor USAA performance and 13 remaining subjects were included in the data reported for this study. Mean avoidance responding over training is summarized in **Figure 2B**. Over training, responding steadily increased, an impression

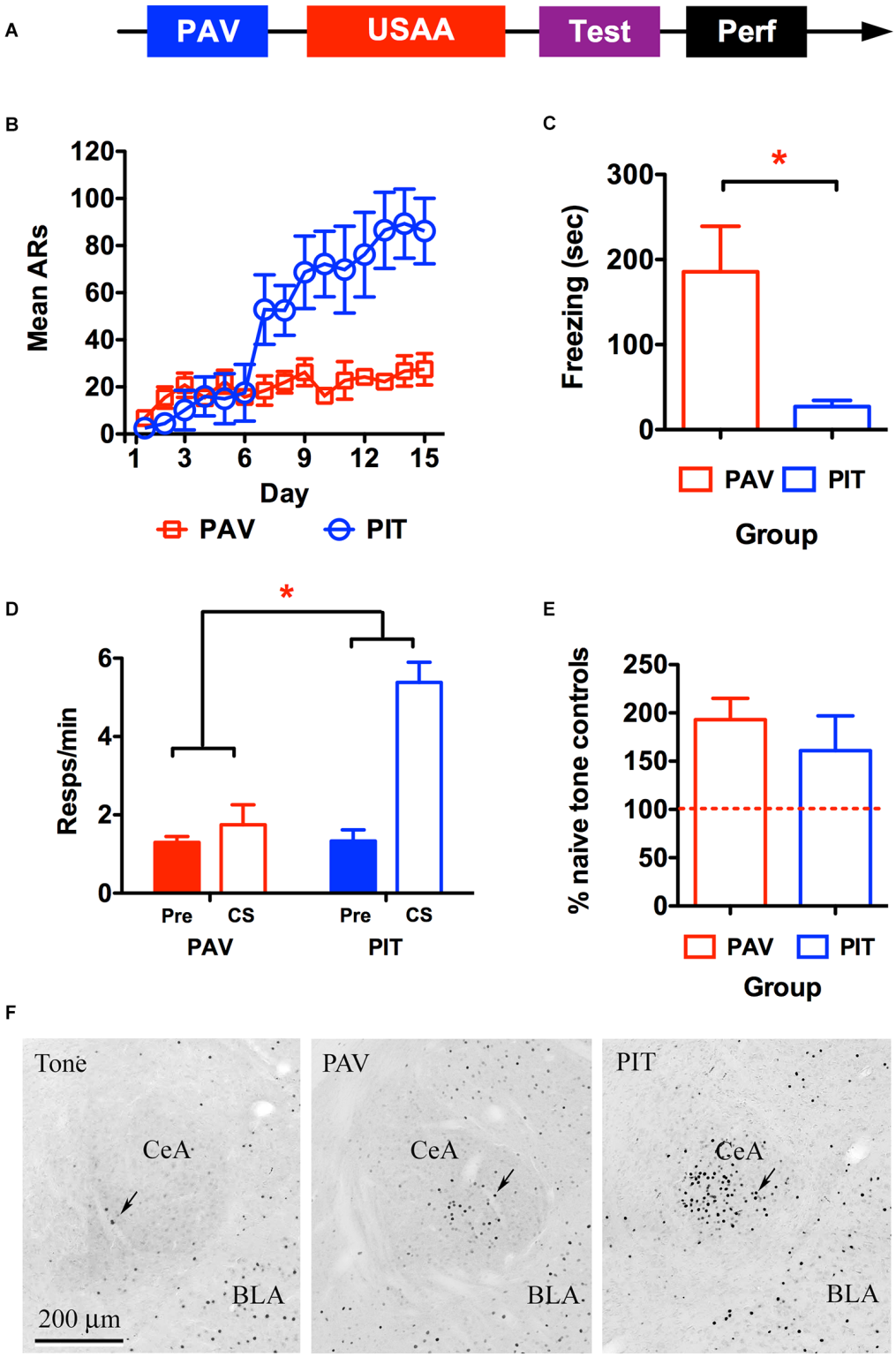

FIGURE 1 | Timeline for the behavioral procedure used to quantify c-Fos is shown in panel **(A)**. **(B)** Shuttling behavior depicts acquisition of avoidance responding during the Sidman (USAA) training phase for the avoidance-trained (PIT) and Pavlovian-only (PAV) control subjects. Freezing behavior **(C)** and shuttle responding **(D)** during the transfer test (Pre CS and CS) are presented for each group as well as average Fos counts per group expressed as a percentage of baseline Fos seen in the naïve control subjects exposed only to tone prior to perfusion **(E)**. Panel **(F)** shows representative images of amygdalae in Tone, PAV, and PIT groups from left to right. ARs, avoidance responses. Asterisks denote significance at the 0.05 alpha level.

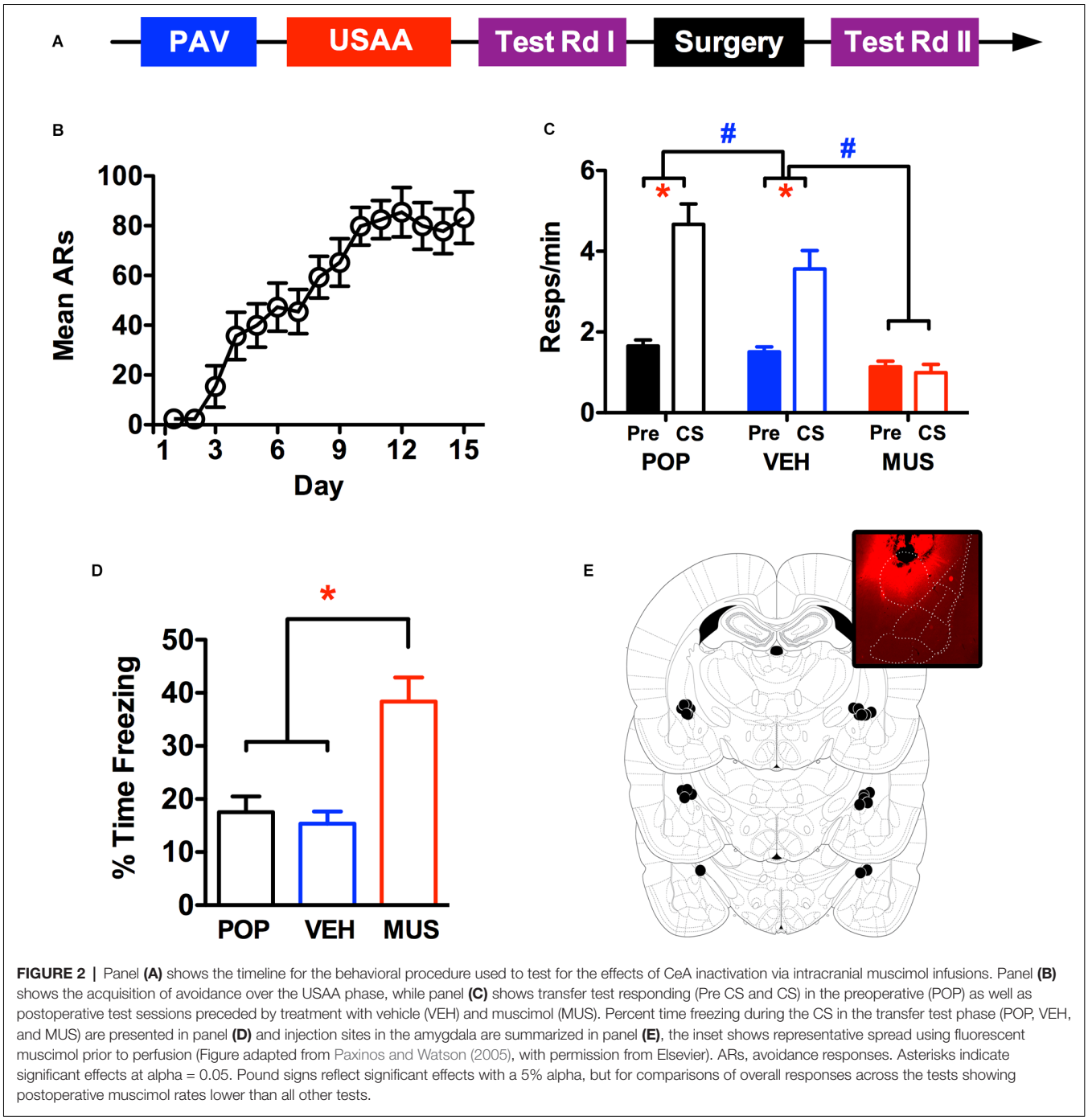

FIGURE 2 | Panel **(A)** shows the timeline for the behavioral procedure used to test for the effects of CeA inactivation via intracranial muscimol infusions. Panel **(B)** shows the acquisition of avoidance over the USAA phase, while panel **(C)** shows transfer test responding (Pre CS and CS) in the preoperative (POP) as well as postoperative test sessions preceded by treatment with vehicle (VEH) and muscimol (MUS). Percent time freezing during the CS in the transfer test phase (POP, VEH, and MUS) are presented in panel **(D)** and injection sites in the amygdala are summarized in panel **(E)**, the inset shows representative spread using fluorescent muscimol prior to perfusion (Figure adapted from Paxinos and Watson (2005), with permission from Elsevier). ARs, avoidance responses. Asterisks indicate significant effects at alpha = 0.05. Pound signs reflect significant effects with a 5% alpha, but for comparisons of overall responses across the tests showing postoperative muscimol rates lower than all other tests.

confirmed by a rmANOVA including *Day* as a within-subjects factor, $F_{(14,168)} = 18.31$, $p < 0.001$.

Transfer Testing

Shuttling data from the test phase are presented in **Figure 2C** for the preoperative (POP) as well as the postoperative vehicle (VEH) and muscimol (MUS) test waves. Preliminary analyses found no significant effects involving test order ($F_{\text{Test}(1,12)} = 0.45$, $p = 0.52$; $F_{\text{Test} \times \text{Interval}(1,12)} = 0.001$, $p = 0.97$) so data were collapsed across this factor and are presented as combined averages over the two tests in each wave. A rmANOVA including the within-subjects factors of *Wave* (POP, VEH, MUS) and *Interval* (Pre vs. CS) confirmed the impression that while PIT was normal following vehicle infusion, it was abolished by CeA muscimol infusions ($F_{\text{Wave}(2,24)} = 38.8$, $p < 0.001$; $F_{\text{Interval}(1,12)} = 23$, $p < 0.001$; $F_{\text{Interaction}(2,24)} = 17.9$, $p < 0.001$). Follow-up Bonferroni corrected comparisons showed that while overall responding was comparable between preoperative and vehicle tests ($p = 0.10$), responding after muscimol treatment was significantly lower ($p < 0.001$).

Inspection of the interaction effect revealed that responding during the CS was slightly reduced following surgery but much more so following muscimol than vehicle infusions [CS—M_{POP} = 4.67, 95% CI(3.57 5.77), M_{VEH} = 3.56, 95% CI(2.57 4.55), M_{MUS} = 0.99, 95% CI(0.55 1.44)]. While responding during the CS was significantly higher than pre CS responding for preoperative and vehicle testing, this was not the case for muscimol testing [Pre CS—M_{POP} = 0.15, 95% CI(1.33 1.98), M_{VEH} = 1.51, 95% CI(1.23 1.78), M_{MUS} = 1.14, 95% CI(0.84 1.44)]. This analysis confirms that muscimol inhibition of CeA eliminates PIT.

Percent time freezing over PIT testing during the first min of the CS is presented in **Figure 2D**. Freezing analysis was limited to the first min of CS testing because subjects received multiple tests with variable CS durations dependent on response rate. Two 30 s bins were analyzed because the CS duration during training was 30 s. However, since freezing was comparable in both intervals, they were ultimately collapsed across this factor for presentation. While freezing was generally low throughout testing, more freezing was seen following muscimol treatment independent of the interval. This impression was confirmed by a rmANOVA including *Wave* (POP, VEH, MUS) and *Interval* (1st 30 s vs. 2nd 30 s of CS) as within-subjects factors ($F_{Wave(2,22)}$ = 16.51, $p < 0.001$; $F_{Interval(1,11)}$ = 0.34, p = 0.57; $F_{Interaction(2,22)}$ = 1.47, p = 0.25). Follow-up Bonferroni corrected comparisons found that overall freezing was higher following muscimol treatment than vehicle treatment (p = 0.004) and prior to surgery (p = 0.001). Cannula placement and an example of infusion spread with fluorescence are presented in **Figure 2E**.

Chemogenetic Inhibition of CeA Impairs General and Sensory-Specific Aversive PIT

To extend the analysis of the role of CeA in PIT, different forms of aversive transfer were studied by using distinct aversive outcomes during Pavlovian conditioning. In studies of appetitive motivation, general and sensory-specific PIT have been found to depend on parallel pathways in the amygdala and striatum (Corbit and Balleine, 2005). However, very little is known about the neural basis of these different forms of *aversive* motivation. We have recently demonstrated that a CS paired with a klaxon can similarly augment footshock avoidance (Campese et al., 2017b). This effect is more dependent on general motivation than sensory-specific features of the shock outcome. In the current study, subjects had two different CSs (tone or white noise) paired with two distinct USs (footshock or klaxon) in separate PTC training sessions. Following USAA, both the shock-paired and klaxon-paired CSs were individually tested for their ability to augment footshock avoidance. To test subjects multiple times with the two stimuli over this phase, a chemogenetic approach was used to inhibit CeA. This was chosen over muscimol because repeated infusions can damage the area of interest and limit drug dispersal. KORD was surgically infused into CeA prior to any behavioral sessions, and after a 2-week recovery period, subjects underwent the training sequence depicted in **Figure 3A**. Prior to PIT testing subjects were systemically treated with vehicle and the designer ligand salvinorin-B using a fully counterbalanced within-subjects approach to examine the role of CeA in the different forms of PIT.

Avoidance Training

Data from the USAA phase are presented in **Figure 3B** as a function of the CS that predicted shock during Pavlovian training. Three subjects were excluded from the analysis due to poor USAA performance, leaving 13 subjects in the final sample (tone-shock n = 6, noise-shock n = 7). Overall acquisition proceeded successfully and similarly for both counterbalanced subsets. This was confirmed by a mixed rmANOVA with *Day* (1–15) as a between-subjects factor and *Group* (Tone vs. Noise) a between-subjects factor ($F_{Day\ (14,154)}$ = 11.9, $p < 0.001$; $F_{Group\ (1,11)}$ = 1.54, p = 0.24; $F_{Interaction\ (14,154)}$ = 1.58, p = 0.09).

Transfer Testing

Mean shuttling data from the PIT testing phase are presented in **Figure 3C** following vehicle and Sal-B treatment for all subjects. Overall, chemogenetic inhibition of CeA severely impaired the aversive transfer effect elicited by both the shock-paired and klaxon-paired stimuli. This was confirmed by a rmANOVA including *Treatment* (vehicle vs. Sal-B), *Stimulus* (CS-shock vs. CS-klaxon), *Interval* (Pre vs. CS) as within-subjects factors ($F_{Treatment\ (1,12)}$ = 25.34, $p < 0.001$; $F_{Stimulus\ (1,12)}$ = 0.5, p = 0.50; $F_{Interval\ (1,12)}$ = 18.4, p = 0.001). While the *Treatment* × *Interval* interaction was significant, $F_{(1,12)}$ = 18.28, p = 0.001, no other significant effects were found ($F_{Treatment\ \times\ Stimulus\ (1,12)}$ = 1.71, p = 0.22; $F_{Stimulus\ \times\ Interval(1,12)}$ = 0.3, p = 0.59; $F_{Treatment\ \times\ Stimulus\ \times\ Interval\ (1,12)}$ = 0.76, p = 0.40). Bonferroni corrected comparisons found that there was more responding overall following vehicle than Sal-B treatment ($p < 0.001$) and more CS than pre-CS responding (p = 0.001). Furthermore, examination of the *Treatment* × *Interval* interaction showed that while baseline responding was comparable ($M_{Vehicle}$ = 1.65, 95% CI(1.47 1.83), $M_{Sal\text{-}B}$ = 1.48, 95% CI(1.26 1.69)], Sal-B treatment significantly attenuated responding during the CS compared to control treatment with the vehicle ($M_{Vehicle}$ = 4.38, 95% CI(3.32 5.44), $M_{Sal\text{-}B}$ = 2.53, 95% CI(1.5 3.56)]. In this analysis, responding was collapsed over test order since preliminary analyses found no significant effects involving this factor alone or with the *Treatment* (vehicle vs. Sal-B) factor ($F_{Test\ (1,12)}$ = 3.55, p = 0.084; $F_{Test\ \times\ Treatment\ (1,12)}$ = 2.87, p = 0.12). A significant *Test* × *Interval* interaction was found ($F_{Test\ \times\ Interval\ (1,12)}$ = 6.491, p = 0.026), but this effect reflected differences in responding to the different CSs (tone vs. noise) and did not interact with treatment $F_{CS\ \times\ Test\ \times\ Treatment\ (1,12)}$ = 0.22, p = 0.89. Responding was also collapsed across stimulus identity and is presented as a function of the predicted outcome (i.e., CS-klaxon, or CS-shock). In agreement with previous findings (Campese et al., 2017b), preliminary analyses found that there was generally more responding elicited by the noise than the tone ($F_{CS\ (1,12)}$ = 10.23, $p < 0.01$; $F_{CS\ \times\ Interval\ (1,12)}$ = 14.34, $p < 0.01$), but this did not interact with treatment effects or the signaled outcome ($F_{CS\ \times\ Interval\ \times\ Treatment\ (1,12)}$ = 1.73, p = 0.21). To test whether non-specific effects of Sal-B caused this behavioral effect, a group of six non-operated controls were tested for PIT following Sal-B treatment (**Figure 3D**). Performance was normal in these subjects ($t_{(5)}$ = 3.69, p = 0.01), suggesting that effect

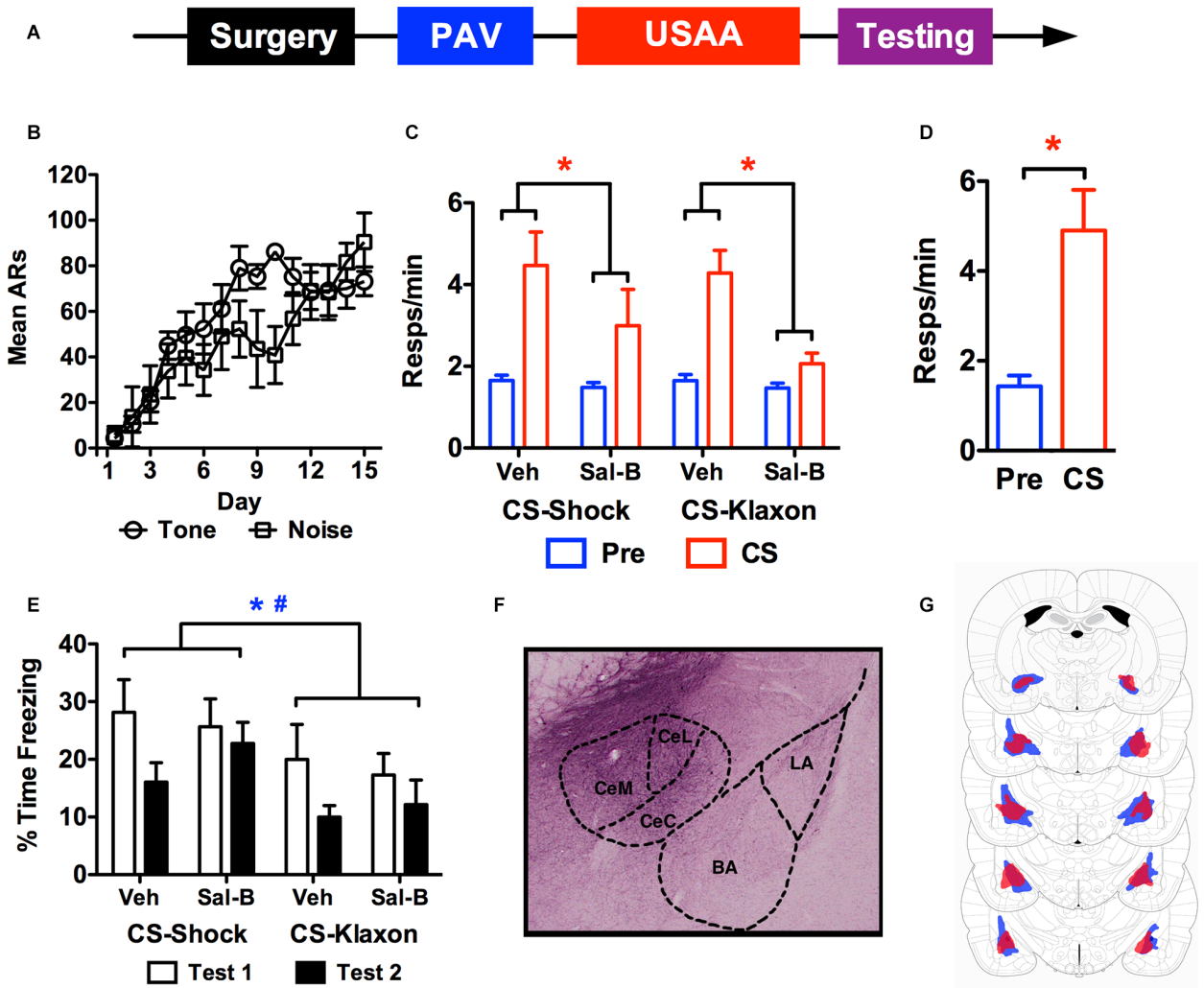

FIGURE 3 | The timeline used for studying the effects of chemogenetic inactivation of the central amygdala on aversive PIT is provided in panel **(A)**. Shock avoidance acquisition is presented in panel **(B)** as a function of whether the shock was trained with tone or noise during the Pavlovian phase. Panel **(C)** shows shuttling during the transfer test phase for each CS under each treatment condition. Panel **(D)** shows responses in control subjects treated with Sal-B in the absence of KORD expression. Freezing during the transfer test for KORD expressing subjects is presented in panel **(E)**. While a representative photo of KORD expression in CeA is presented in panel **(F)**, the minimum (red) and maximum (blue) extent of viral expression for KORD is seen in panel **(G)** (Figure adapted from Paxinos and Watson (2005), with permission from Elsevier). ARs, avoidance responses. Asterisks and pound signs indicate significant effects at alpha = 0.05 for *vehicle vs. Sal-B* comparisons and *CS-shock vs. CS-klaxon* comparisons respectively.

of the Sal-B ligand on KORDs in CeA specifically (i.e., neural inhibition) was responsible for the impaired transfer.

Freezing data from the test phase are presented in **Figure 3E** and extent of viral spread in **Figure 3G** (a representative histological image is presented in **Figure 3F**). While preliminary analysis of the transfer data showed no effect of test order, freezing data did show this effect. Therefore, the data are presented for tests 1 and 2 for each stimulus and treatment. While freezing was quantified the same way as described above, the data were again collapsed over the two 30 s intervals since freezing did not change on this basis. Overall, freezing was higher for CS-shock than CS-klaxon, and for test 1 than test 2. These impressions were confirmed by a rmANOVA including *Stimulus* (CS-shock vs. CS-klaxon), *Test* (1 vs. 2), *Interval* (1st 30 s vs. 2nd 30 s), and *Treatment* (vehicle vs. Sal-B) as within-subjects factors ($F_{\text{Stimulus (1,11)}} = 4.95$, $p = 0.048$; $F_{\text{Test (1,11)}} = 8.2$, $p = 0.015$). No other significant main effects ($F_{\text{Treatment (1,11)}} = 0.19$, $p = 0.67$; $F_{\text{Interval (1,11)}} = 1.06$, $p = 0.33$) or interactions were found ($F_{\text{Stimulus} \times \text{Treatment (1,11)}} = 0.11$, $p = 0.75$; $F_{\text{Stimulus} \times \text{Test(1,11)}} = 0.001$, $p = 0.98$; $F_{\text{Treatment} \times \text{Test(1,11)}} = 3.07$, $p = 0.11$; $F_{\text{Stimulus} \times \text{Interval(1,11)}} = 0.18$, $p = 0.68$; $F_{\text{Treatment} \times \text{Interval(1,11)}} = 0.53$, $p = 0.48$; $F_{\text{Interval} \times \text{Test(1,11)}} = 0.74$, $p = 0.41$; $F_{\text{Stimulus} \times \text{Treatment} \times \text{Test (1,11)}} = 0.66$, $p = 0.44$; $F_{\text{Stimulus} \times \text{Treatment} \times \text{Interval(1,11)}} = 1.38$, $p = 0.27$; $F_{\text{Stimulus} \times \text{Interval} \times \text{Test(1,11)}} = 0.48$, $p = 0.50$; $F_{\text{Interval} \times \text{Treatment} \times \text{Test(1,11)}} = 0.04$, $p = 0.85$; $F_{\text{Stimulus} \times \text{Treatment} \times \text{Interval} \times \text{Test(1,11)}} = 0.06$, $p = 0.80$). Thus,

while freezing reduced over the test phase, it did so comparably for both stimuli (CS-shock vs. CS-klaxon) and without any effect of Treatment (vehicle vs. Sal-B).

DISCUSSION

Together, the studies above extend previous lesion findings (Campese et al., 2014, 2015) and provide compelling evidence that CeA is important for the modulation of shock-avoidance behavior by an aversive CS. The findings from the study quantifying c-Fos clearly demonstrate the behavioral effects of USAA on subsequent aversive CS-elicited responding. Avoidance training reduced conditioned freezing to the CS and augmented footshock-avoidance shuttle responding compared to subjects that had only undergone Pavlovian conditioning; these subjects showed standard freezing CRs without any modulation of shuttling by the CS. While previous studies have shown that Pavlovian learning is required for the aversive transfer effect (Campese et al., 2013), the current findings show that successfully acquiring avoidance is also needed to produce the transfer effect.

While CeA is well known for its role in freezing CRs, there was no difference in c-Fos labeling between high-freezing/low-shuttling PAV subjects and low-freezing/high-shuttling PIT subjects. Thus, changes to CS-processing in CeA may be crucial for producing active rather than reactive CS-elicited behavior but they may not simply translate to changes in overall CeA activity. In a related finding, we have recently reported that neuromodulatory regulation of CeA by noradrenaline from the brainstem determines whether aversive PIT or conditioned freezing is expressed when the CS is tested (Campese et al., 2017a). If norepinephrine levels are increased, subjects revert to freezing CRs and PIT is reduced, suggesting that changes to CS-processing in this region may underlie the effect.

Sample sizes, while not large, were nevertheless comparable to other studies using c-Fos to evaluate the neural circuitry underlying active avoidance and provide an adequate basis for this purpose (Martinez et al., 2013). However, a more thorough analysis with larger sample sizes may reveal patterns within CeA insofar as to how responses may be distributed among the complex disinhibitory microcircuitry of this region as a function of avoidance training (Fadok et al., 2018). Whether the maintained activity in CeA drives responding after avoidance training cannot be assessed using the Fos approach. Therefore, the following study used muscimol to inactivate CeA prior to testing to determine whether this structure is necessary for aversive PIT, as previous lesion findings suggest (Campese et al., 2014, 2015).

These findings showed that aversive PIT was intact following CeA cannulations and not impaired by the surgical procedure itself. Responding to the CS was slightly attenuated 1 week following surgery, but this was likely due to extinction as the modulatory effect relative to the baseline period was preserved. This was not true following muscimol treatment, after which, the effect of the CS on shuttling was eliminated entirely. CeA is well-known for its role in freezing CRs, and it was surprising that muscimol treatment elevated freezing compared to preoperative and vehicle testing. Infusions were mostly restricted to CeA and did not spread significantly to LA or BA, providing further evidence that CeA is necessary for aversive PIT. However, it is also possible that elevations in freezing (and reductions in avoidance) were due to motor impairments and other non-specific effects of muscimol treatment. This was addressed by using KORD in the subsequent study, which also extended the examination of the involvement of CeA to different forms of aversive transfer.

The results from this study replicate previously reported transfer effects of comparable strength with both shock-paired and klaxon-paired stimuli using a within-subjects design (Campese et al., 2017b). Both shock-paired and klaxon-paired cues generated motivation to comparably augment footshock avoidance behavior. While shock-paired stimuli are associated with sensory features of footshock, so is shock-avoidance behavior. Thus, facilitation of shock avoidance by a shock-paired cue likely involves sensory-specific properties of the shock outcome. This cannot be the case for a klaxon-paired cue, which can only augment USAA through general processes. Using KORD to inhibit neurons in CeA impaired transfer to both klaxon-paired and shock-paired stimuli; PIT was intact following vehicle treatment, but chemogenetic inhibition of CeA via KORDs attenuated the transfer effect.

In contrast, different forms of appetitive motivation have been found dependent upon parallel circuits in the amygdala involving CeA and Basolateral amygdala (BLA). According to studies in appetitive PIT, CeA is more sensitive to general motivation, while BLA regulates behavior based on sensory-specificity (Corbit and Balleine, 2005). However, mixed results have been found when testing conditions are similar to those used in the current study, and only include a single instrumental response. In this case, behavior is heavily dependent upon CeA (Holland and Gallagher, 2003). Alternatively, the difference may have to do with motivational modality. More work would be needed to directly address this discrepancy.

The manipulation of CeA with KORDs in this study did not produce freezing effects that could interfere with the expression of PIT and, therefore, provides strong evidence that CeA is important for generating modulatory effects of the CS on USAA, regardless of the signaled outcome. It should be acknowledged that while more specific than electrolytic lesions used in previous work, KORD expression was often found to extend beyond CeA into the Basal amygdala (BA) and dorsal medial to CeA in the current study. However, because prior studies have shown that BA lesions do not impair aversive PIT, the possibility that this could influence the effects of KORD is low. Since CeA inactivation reliably impaired PIT, BA activation was likely incidental. There is insufficient data to speculate as to how the dorsal medial spread of KORD may impact aversive PIT, but data showing contributions of the extended amygdala to fear conditioning suggest more work is needed to determine possible roles for these regions in avoidance and related phenomena (Ravinder et al., 2013).

In summary, these data provide strong evidence that CeA is important for the facilitative effect of aversive conditioned

stimuli on active avoidance behavior. While avoidance itself is not dependent on CeA for acquisition or expression, the way acquired avoidance behavior may be integrated with prior experience appears to require changes to CS-processing in this region.

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 NYU University Animal Welfare Committee.

AUTHOR CONTRIBUTIONS

IK ran studies and did surgeries. CF processed tissue and quantified cell counts. MH processed tissue. SP, ET, and SC scored behavioral footage. VC designed the studies and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Aversive Pavlovian inhibition in adult attention-deficit/hyperactivity disorder and its restoration by mindfulness-based cognitive therapy

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Background: Control over the tendency to make or withhold responses guided by contextual Pavlovian information plays a key role in understanding impulsivity and hyperactivity. Here we set out to assess (1) the understudied relation between contextual Pavlovian inhibitory control and hyperactivity/impulsivity in adults with ADHD and (2) whether this inhibition can be enhanced by mindfulness based cognitive therapy (MBCT).

Methods: Within the framework of a randomized controlled trial 50 Adult ADHD patients were assessed before and after 8 weeks of treatment as usual (TAU) with (n = 24) or without (n = 26) MBCT. We employed a well-established behavioral Pavlovian-to-instrumental transfer task that quantifies Pavlovian inhibitory control over instrumental behavior.

Results: Task results revealed (1) less aversive Pavlovian inhibition in ADHD patients with clinically relevant hyperactivity/impulsivity than in those without; and (2) enhanced Pavlovian inhibition across all ADHD patients after TAU+MBCT compared with TAU.

Conclusion: These findings offer new insights in the neurocognitive mechanisms of hyperactivity/impulsivity in ADHD and its treatment: We reveal a role for Pavlovian inhibitory mechanisms in understanding hyperactive/impulsive behaviors in ADHD and point toward MBCT as an intervention that might influence these mechanisms.

KEYWORDS

ADHD, Pavlovian to instrumental transfer, mindfulness based cognitive therapy, inhibition, impulsivity

Introduction

Individuals diagnosed with attention-deficit/hyperactivity disorder (ADHD) have difficulties with controlling their behavior appropriately with respect to environmental demands. Two key cognitive systems that control our behavior with respect to the environment are the Pavlovian and instrumental systems (Dickinson and Balleine, 2002; Dolan and Dayan, 2013). Especially problems in Pavlovian control of goal-oriented instrumental behaviors are associated with a wide variety of psychiatric problems (e.g., Dayan et al., 2006; Heinz et al., 2016; Hallquist et al., 2018). This form of behavioral control might be key to adaptive inhibitory control which has since long been proposed to be central to understanding problems in ADHD (Barkley, 1997). Moreover, aberrant Pavlovian control over instrumental behavior can lead to maladaptive impulsivity in animals as well as in humans (Breland and Breland, 1961; Guitart-Masip et al., 2014; Hinojosa-Aguayo and González, 2020). This form of control has been shown to depend on monoaminergic transmission relevant for understanding ADHD (Dalley and Roiser, 2012; Geurts et al., 2013b; Salamone et al., 2015) and can specifically be modulated by methylphenidate (Swart et al., 2017). Nevertheless, it has received relatively little attention in human and animal ADHD research (Natsheh and Shiflett, 2015). To fill this gap in the literature, we tested whether Pavlovian control of instrumental behavior [i.e., Pavlovian to instrumental transfer (PIT)] is related to clinically relevant impulsivity/hyperactivity in ADHD. Therefore, we first compared Pavlovian control in adult ADHD patients diagnosed with and without clinically relevant impulsivity/hyperactivity. Second, we assessed the hypothesis that a mindfulness-based cognitive therapy (MBCT), i.e., an 8-week training program theoretically related to amending automatic tendencies (Segal et al., 2002) and shown to improve impulsivity/hyperactivity in ADHD (Janssen et al., 2018), should, accordingly, also modulate Pavlovian inhibitory control.

A wide range of animals, including humans, are endowed with mechanisms shaped throughout evolution that drive behavior (Dolan and Dayan, 2013). These drivers take advantage of environmental information carried by stimuli that predict motivationally salient future events or outcomes. The instrumental control system enables us to use specific actions when confronted with a certain stimulus to obtain a specific outcome (i.e., stimulus-action-outcome learning or operant conditioning). This system allows us to optimize our chances to achieve specific goals by learning when to exert specific actions and when not to act. Complementary to this instrumental control system, the Pavlovian control system regulates automatic, motivational responses in reaction to external and internal stimuli (Dolan and Dayan, 2013). This system enables us to associate neutral stimuli with motivationally salient outcomes in the environment (i.e., stimulus-outcome learning or classical conditioning). These neutral stimuli acquire part of the motivational properties of the outcome they are associated with (i.e., predict). When encountering these previously neutral, but now conditioned, stimuli (CS) again, the automatic preparatory reaction to the outcome will be elicited in response to these Pavlovian CSs. Critically, it has long been recognized that these two behavioral control systems do not act in separation, but interact. Pavlovian CS can (de) motivate ongoing instrumental behavior based on the valence (appetitive or aversive) of the Pavlovian CS (Rescorla and Solomon, 1967): Pavlovian CS that predict punishment (i.e., aversive Pavlovian CS) have the tendency to inhibit, whereas Pavlovian CS that predict reward (i.e., appetitive Palvovian CS) can activate instrumental behavior (Rescorla and Solomon, 1967; Huys et al., 2011; Geurts et al., 2013a). These interactions between instrumental and Pavlovian control of behavior are thought to be shaped by evolution and have adaptive properties in terms timing actions (i.e., when to make, and when not to make an action) to optimize gaining rewards and avoiding punishment at relatively low computational cost (Dolan and Dayan, 2013). However, too much or too little influence of the Pavlovian system on instrumental behavior has been proposed as a driver of several maladaptive behaviors (e.g., Dayan et al., 2006; Heinz et al., 2016; Hallquist et al., 2018). Too much potentiation of instrumental behavior by appetitive cues, or too little inhibition by aversive cues is linked to impulsive behavior in real life (Watson et al., 2014; Garbusow et al., 2016; Heinz et al., 2016; Hallquist et al., 2018). This latter source of disinhibition, i.e., disinhibition in the face of aversive affect, has been widely recognized to play a role in externalizing psychopathology, mainly under the umbrella of negative urgency (Whiteside and Lynam, 2001). Negative urgency has recently indeed been related to Pavlovian control of instrumental behavior in healthy controls (Hinojosa-Aguayo and González, 2020). However, whether the impact of appetitive activating and aversive inhibitory processes on instrumental behavior contributes to impulsivity/hyperactivity in ADHD remains an open question. We will test this specific hypothesis in ADHD patients diagnosed with and without clinically relevant impulsivity/hyperactivity symptomatology. Specifically, we compare patients diagnosed with the DSM-IV combined or hyperactive/impulsive subtype (both including relevant impulsive-hyperactive symptomatology) with those with the primarily inattentive subtype (without diagnosed impulsivity/hyperactivity).

The hypothesis that both increased appetitive PIT and decreased aversive PIT might drive impulsivity in ADHD can only be tested causally through an intervention study. A key prediction is that effective treatment of ADHD

should modulatethe effect of Pavlovian cues on instrumental behavior. One candidate for this is MBCT. MBCT has significant beneficial effects in ADHD (Cairncross and Miller, 2016; Gu et al., 2017; Hepark et al., 2017; Janssen et al., 2018), as well as impulsivity symptoms trans-diagnostically (Franco et al., 2016). It is a highly protocolled intervention that changes how patients deal with thoughts, emotions, bodily feelings and urges in reaction to both external and internal stimuli. Patients become more aware of internal and external triggers and consequent automatic patterns such as avoidance of aversive stimuli or attachment to appetitive stimuli, and learn to (initially) disengage from automatic reactivity (Segal et al., 2002, p. 217). Indeed, MBCT has been shown to reduce impulsivity/hyperactivity (Gu et al., 2017; Hepark et al., 2017; Janssen et al., 2018), improve self-reported adaptive inhibition (Hepark et al., 2017; Janssen et al., 2018) and increases experimentally measured behavioral inhibition (see for meta-analyses and critical notes; Lao et al., 2016; Vago et al., 2019). Moreover, previous findings from our group suggest that effects of MBCT on self-reported adaptive inhibition mediated the effects of MBCT on clinician rated ADHD symptoms (Geurts et al., 2020). Taken together, to test the hypothesis that aberrant PIT may drive impulsive responding in ADHD, we will assess whether MBCT changes the inhibitory or activating effects of Pavlovian cues. In line with the hypothesized relation between impulsivity and Pavlovian control, we expect MBCT to diminish the motivating effect of appetitive Pavlovian CS and to enhance the inhibiting effect of aversive Pavlovian CS on instrumental behavior, leading to more inhibition and less impulsivity/hyperactivity, respectively.

Materials and methods

Trial design and procedure

This behavioral intervention study was embedded in a multi-center randomized controlled trial (RCT) investigating the impact of MBCT in addition to TAU on adults with ADHD (NCT02463396) (Janssen et al., 2015, 2018). For this trial, a total of 120 adults with ADHD according to the criteria of Diagnostic and Statistical Manual of Mental Disorders—4th edition (DSM-IV-TR) (American Psychiatric Association, 2000) were randomized to either MBCT in addition to treatment as usual (MBCT + TAU) or TAU only. The eligibility criteria, study procedure and CONSORT diagram are described fully in the protocol paper (Janssen et al., 2015) and the main treatment outcome paper (Janssen et al., 2018) of the overarching RCT. Clinical outcome measures were assessed before (T0) and directly after (T1) and 3 months (T2) after MBCT or TAU. Behavioral data on the PIT task were collected before (T0) and after MBCT or TAU (T1). On each of these two test-days, patients were seated in front of a laptop and conducted the PIT computer task.

Patients

For the current study, behavioral data on the PIT task were collected from a subset of patients assessed at one site (RadboudUMC): 68 patients were asked to participate. One patient declined, which resulted in 67 patients participating in the pre-intervention test session. On the post-intervention test session 60 (90%) patients participated and 7 declined to participate. Unfortunately, there was a loss of 10 data sets on pre-intervention due to a technical (back-up) error, leaving 50 full data sets (MBCT+TAU: n = 24; TAU only n = 26) to be analyzed (for demographics see **Table 1**; see **Supplementary Data** for flow-chart of inclusions).

Intervention

Mindfulness-based cognitive therapy

MBCT (Segal et al., 2002) is an 8-week group-based intervention of 2.5 h each, plus a 6-h silent day between session 6 and 7. In short, the program included mindfulness practice (bodyscan, gentle yoga, sitting and walking meditation) combined with daily life practices, psycho-education, Cognitive Behavioral Therapy (CBT) techniques, group discussions, and inquiry into present moment experiences. By this procedure patients are taught to become more aware of dysfunctional automatic patterns, such as avoidance of aversive stimuli or grasping of appetitive stimuli and to consciously disengage from these patterns (Segal et al., 2002, for further detail see the protocol paper; Janssen et al., 2015).

Treatment as usual

TAU reflected the usual treatments of ADHD patients in various mental health centers across the Netherlands, consisting of pharmacotherapy and psychosocial treatment, such as psycho-education and cognitive behavioral therapy.

Assessments

Clinical assessments

Clinical assessments are presented in **Table 1**. The primary outcome measure was total ADHD symptoms according to the 30-item Conners' Adult ADHD Rating Scale (Conners et al., 1999), scored by a blinded clinician (CAARS-INV). In line with the findings of the overarching RCT (Janssen et al., 2018, n = 120), MBCT significantly reduced ADHD symptoms in our

subsample of the total patient group with a moderate effect size (Treatment (MBCT/TAU) × Day (pre/post) interaction on CAARS-IVNV ADHD score: $F(1, 48) = 5.2$, $p = 0.028$; independent sample t-test pre MBCT: $t(48) = 1.7$, $p = 0.098$; post MBCT: $t(48) = 2.8$, $p = 0.007$; paired sample t-test (pre vs. post): MBCT+TAU: $t(23) = 4.4$, $p < 0.001$; TAU: $t(25) = 3.7$, $p = 0.007$).

Overall functioning was measured using the 45-item Outcome Questionnaire 45 (OQ-45), which offers a comprehensive review of overall life functioning (Lambert et al., 1996). Items are scored on a five-point rating scale, ranging from never (0) to almost always (4), with a maximum score of 180 points (a higher score means worse overall functioning) (DE Jong et al., 2007).

Pavlovian to instrumental transfer task

We used a PIT task that allowed us to assess the influence of appetitive and aversive Pavlovian CS on instrumental approach actions. This task was identical to the approach blocks used in Huys et al. (2011, 2016) and Geurts et al. (2013b). In short, the task consisted of an instrumental conditioning, a Pavlovian conditioning and a PIT stage (**Figure 1**).

Instrumental conditioning

The instrumental task (**Figure 1A**) was a go/NoGo task, framed in terms of collecting "good" and "bad" mushrooms. Patients chose whether to collect the mushroom by moving the mouse toward and clicking on the mushroom (go) within a response-window of 1.5 s, or not collect the mushroom by abstaining from a response for 1.5 s (NoGo). The outcome (±5 cents) was then presented in the middle of the screen. Reinforcements were probabilistic, with the "correct" response for each mushroom leading to reward on 75% of the trials and to punishment otherwise. For the "incorrect" response these probabilities were reversed. Correct trials were those on which they collected a "good" mushroom or refrained from collecting a "bad" mushroom. Patients thus had to learn the better response

TABLE 1 Baseline demographic and clinical characteristics.

	MBCT+TAU (n = 24)		TAU (n = 26)		P (Phi/T statistics)
Demographic characteristics					
Female gender	13	54.2%	16	61.5%	0.28 (Phi = −0.075)
Age; *M* (*SD*)	42.6	12.4	39.0	10.5	0.26 (T_{48} = −1.1)
Clinical characteristics					
Subtype of ADHD, DSM-IV					
Inattentive type	13	54.2%	16	61.5%	0.87 (Phi = −0.28)
Hyperactive/impulsive type	0	0%	0	0%	
Combined type	10	41.7%	9	34.6%	
Not otherwise specified type	1	4.2%	1	3.8%	
ADHD symptoms (CAARS-INV)					
Subscales:					
Inattention	16.9	5.2	18.6	3.8	0.20 (T_{48} = 1.3)
Hyperactive/impulsive	11.7	6.5	14.2	5.9	0.15 (T_{48} = 1.5)
Total	28.6	9.4	32.8	8.4	0.10 (T_{48} = 1.7)
Use of ADHD medication	17	70.8%	14	53.8%	0.22 (Phi = 1.53)

TABLE 2 Baseline demographic and clinical characteristics compared between those patients diagnosed with subtypes with and without hyperactivity/impulsivity.

	Combined subtype including hyperactivity/impulsivity		Inattentive subtype		p
Demographic characteristics					
Female gender	19 /29	65.5%	9 /19	47.4%	0.21
Age; M(SD)	38.1	(9.9)	44.4	(12.7)	0.060
Clinical characteristics					
ADHD symptoms (CAARS-INV)					
Total score	33.6	(8.3)	26.9	(9.3)	0.012
Inattention subscale	18.2	(5.0)	17.4	(4.2)	0.6
Hyperactive/impulsive subscale	15.4	(4.8)	9.5	(6.6)	<0.001
Outcome questionnaire	55.7	(15.6)	63.7	(18.9)	0.12
Use of ADHD medication	17 / 29	(58.6%)	12 / 19	(63.2%)	0.8

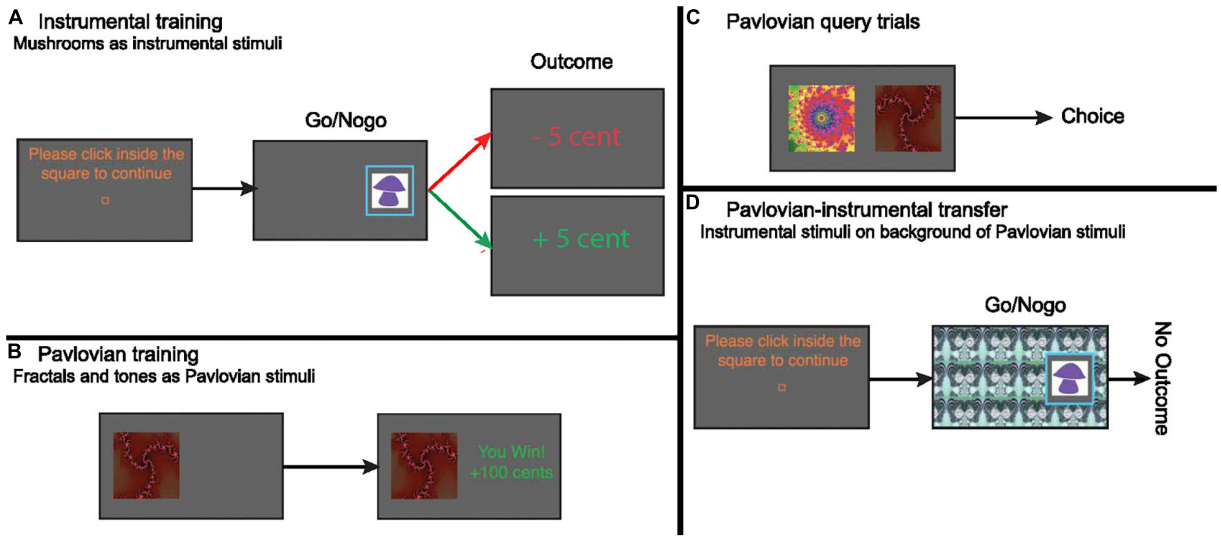

FIGURE 1
(A) Instrumental conditioning. To center the cursor, participants clicked in a central square. Participants needed to choose whether to move the cursor toward the mushroom and click inside the blue frame onto the mushroom (go), or do nothing (NoGo). Outcomes were presented immediately after go actions, or after 1.5 s (i.e., NoGo). There were 3 "good" (go) and 3 "bad" (NoGo) instrumental stimuli. Collecting a "good" (correct go) and not collecting a "bad" (correct NoGo) stimulus was rewarded most of the time (75% veridical outcome). Vice versa, collecting a "bad" (incorrect go) and not collecting a "good" (incorrect NoGo) stimulus was punished most of the time (75% veridical outcome). There were 60 trials in total. Instrumental stimuli were different for both days. **(B)** Pavlovian conditioning. Participants passively viewed stimuli and heard auditory tones, followed by wins (+10/+100), losses (−10/−100), or neutral outcomes (0). There were five fractal/tone combinations. Each combination was displayed 12 times. **(C)** On Pavlovian query trials, participants chose between two Pavlovian stimuli. Query trials were administered after every five Pavlovian conditioning trials. **(D)** Pavlovian-instrumental transfer. Participants responded to the instrumental stimuli trained during the instrumental conditioning stage, with Pavlovian stimuli tiling the background. No outcomes were presented, but participants were instructed that their choices counted toward the final total. No explicit instructions about the contribution of Pavlovian stimuli toward the final total were given. During this phase we assessed the impact of the Pavlovian CSs on instrumental choice (go/NoGo).

for each stimulus from the probabilistic, noisy reinforcement feedback. There were 3 "good" (go) and 3 "bad" (NoGo) mushrooms, meaning that the possible actions (i.e., collect or not collect) could be followed by both rewards and punishments.

Analyses and results on the instrumental conditioning stage are reported in **Supplementary Material**.

Pavlovian conditioning

The second part of the task consisted of a separate Pavlovian conditioning procedure. Five compound Pavlovian CS, consisting of a fractal visual stimulus (**Figure 1B**) and a tone, were deterministically paired with outcomes. The appetitive (S^P_{++}, S^P_{+}) and aversive (S^P_{-}, S^P_{--}) Pavlovian CSs predicted a gain/loss of 100 or 10 cents, respectively, while the neutral CS (S^P_0) was followed by an outcome of 0 cent. To ensure that patients paid attention, a query trial was presented on every fifth trial. Patients then had to choose between two different Pavlovian CS (**Figure 1C**) without any reinforcement. In addition, we asked patients to rate how much they liked the presented CS before and after the experiment on a visual analog scale (VAS).

Analyses and results on the Pavlovian conditioning stage are reported in **Supplementary Material**.

Pavlovian-instrumental transfer phase

This was the main phase of interest. Patients needed to choose whether to collect (go) or not collect (NoGo) the same mushrooms as in the instrumental training phase, while the Pavlovian CS now tiled the entire background (**Figure 1D**). No outcomes were presented during this phase to exclude further instrumental conditioning. Patients were instructed to continue performing the instrumental task; that choices were still earning them the same outcomes and were being counted; but that they would not be told about the outcomes during this phase. Thus, in this phase, we could assess the impact of the Pavlovian CS on the previously learned instrumental go/NoGo choices.

Data analysis

The primary effect of interest was the activating and inhibiting impact of the appetitive and aversive Pavlovian CSs on instrumental go/NoGo choices, respectively.

First, we assessed the relation between clinical impulsivity-hyperactivity and PIT: In the introduction we introduced two possible links between hyperactivity-impulsivity on the one hand and appetitive and aversive PIT on the other: Impulsivity-hyperactivity could theoretically be instantiated differentially

by (i) exaggerated appetitive PIT, i.e., too much instrumental potentiation in the face of an appetitive Pavlovian CS and (ii) diminished aversive PIT, i.e., too little inhibition in the face of an aversive Pavlovian CS (Watson et al., 2014; Garbusow et al., 2016; Huys et al., 2016; Hallquist et al., 2018). We assessed these differential associations at baseline by assessing differences in PIT between the combined subtype (including hyperactivity-impulsivity) and the inattentive subtype (not including hyperactivity/impulsivity) of ADHD. Thus, we employed two generalized linear mixed effects models (GLMM) with, respectively, Pavlovian CS Appetitive ($S^P{}_{++}$ /$S^P{}_n$) and Aversive ($S^P{}_n$/$S^P{}_{--}$ as within subject factor and ADHD subtype (combined/inattentive) as between-subject factor.

Second, to test whether MBCT modulated appetitive and aversive PIT we used a GLMM including the within-subject factors Pavlovian CS Valence (5 levels: $S^P{}_{++}$/$S^p{}_+$/$S^P{}_n$/$S^P{}_-$/$S^P{}_{--}$) and Day (Pre vs. Post treatment), and the between-subject factors Treatment Group (TAU+MBCT vs. TAU).

We used GLMMs to account for both between- and within-subject variability. We used the lme4 package in R (Bates et al., 2015; R Development Core Team, 2015). All GLMMs included all main effects and interactions as well as a full random effects structure to reduce inflation of Type I error (Barr et al., 2013).

Furthermore, to interpret the results of the above analyses as true changes in the interaction between Pavlovian and instrumental control, i.e., PIT, there should be no differences between the Treatment groups in task performance during the instrumental and Pavlovian training *per se* on Day 1 or a difference in change between the Groups from pre- to post treatment in these parts of the training. We assessed whether this was the case by using, where appropriate, *t*-tests and repeated measure ANOVA's with, respectively, average performance at the end of the instrumental training stage (mean correct after more than 5 stimulus presentations), average performance at the end of Pavlovian training (mean correct after more than 5 query trials) and VAS ratings from pre-to post Pavlovian conditioning as dependent variables.

We note, that we did not pursue analysis of reaction time, because previous reports (Huys et al., 2011; Geurts et al., 2013b) with this paradigm did not find any meaningful effects on this outcome measure.

Results

General Pavlovian to instrumental transfer effects

Across Treatment Group and Day we replicated the expected PIT effect: appetitive Pavlovian CS activated (i.e., appetitive PIT), whereas aversive Pavlovian CS inhibited (i.e., aversive PIT) instrumental approach actions [main effect of Pavlovian CS Valence: $\chi^2 = 17.4$, $p = 0.002$; simple contrast appetitive PIT ($S^P{}_n$/ $S^P{}_{++}$): $\chi^2 = 4.9$, $p = 0.026$); simple contrast aversive PIT ($S^P{}_n$/$S^P{}_-$): *FRS:* $\chi^2 = 7.4$, $p = 0.006$].

Aversive Pavlovian inhibition is related to clinical impulsivity-hyperactivity

Specific analyses, targeted at clinically diagnosed impulsivity/hyperactivity and its relation to aversive and appetitive PIT, respectively (see section "Introduction" and "Materials and methods"), revealed that aversive PIT was absent for those patients diagnosed with ADHD including impulsivity/hyperactivity (i.e., the combined subtype) compared with patients with ADHD with primarily inattentive symptoms [**Figure 2**, Subtype (combined/inattentive) × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{--}$): $\chi^2 = 4.6$, $p < 0.031$, **Table 2**]. More specifically, behavioral inhibition by aversive Pavlovian CS was not significant in patients diagnosed with the combined subtype ($\chi^2 = 1.5$, $p = 0.22$) and significant for the inattentive subtype ($\chi^2 = 12.77$, $p < 0.001$). No such effects were found for appetitive PIT [Subtype (combined/inattentive) × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{++}$): *FRS:* $\chi^2 = 0.1$, $p = 0.73$].

Mindfulness based cognitive therapy increased aversive Pavlovian inhibition over instrumental behavior

Notably, we found that MBCT modulated PIT as is revealed by a Treatment Group × Day × Pavlovian CS Valence interaction ($\chi^2 = 12.9$, $p = 0.011$, **Figure 3**). Simple contrast analyses showed that this interaction was driven by changes in aversive PIT [Treatment Group × Day × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{--}$): $\chi^2 = 7.4$, $p = 0.006$] and not appetitive PIT [Treatment Group × Day × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{++}$): $\chi^2 < 0.1$, $p = 0.86$]. Indeed, as revealed by the pattern in **Figure 3**, aversive PIT was enhanced post MBCT [Day (pre vs. post) × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{--}$): $\chi^2 = 5.6$, $p = 0.018$], but not post -TAU [Day (pre vs. post) × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{--}$): $\chi^2 = 3.3$, $p = 0.069$]. Moreover, there was no difference in PIT at baseline between the groups (Pre: Treatment Group × Pavlovian CS Valence: $\chi^2 = 5.3$, $p = 0.26$), but there was a difference after MBCT/TAU (Post: Treatment Group × Pavlovian CS Valence: $\chi^2 = 11.0$, $p = 0.027$), which was driven by enhanced aversive PIT for the MBCT compared to the TAU group [Post: Treatment Group × Pavlovian CS Valence ($S^P{}_n$/ $S^P{}_{--}$): $\chi^2 = 5.1$, $p = 0.023$].

Thus, MBCT increased the inhibitory effects of aversive Pavlovian CS on instrumental behavior and left unchanged the activating effect of appetitive Pavlovian CS.

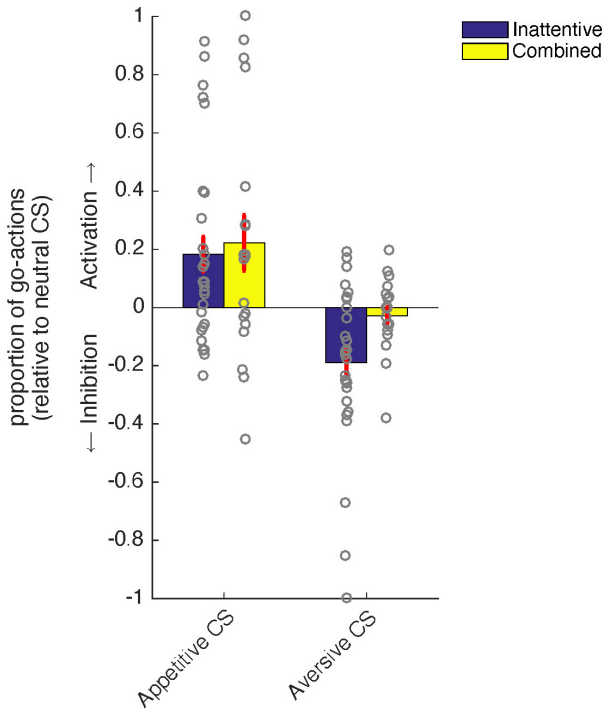

FIGURE 2
Relation between ADHD subtype [combined (yellow) vs. inattentive (blue)] and PIT. Patients with the inattentive subtype showed significant aversive inhibition of instrumental behavior in the context of an aversive Pavlovian conditioned stimulus (CS), while this was not the case for patients diagnosed with the combined subtype. There were no differences between ADHD subtypes in terms of appetitive activation of instrumental behavior. Error bars represent standard errors of the mean.

Instrumental and Pavlovian training

To interpret the above findings as true changes in the interaction between Pavlovian and instrumental control, i.e., PIT, there should be no (explanatory) differences between the Treatment groups at Day1 or a difference in change from pre- to post treatment between the groups in task performance at the end of instrumental and Pavlovian training. Indeed, we did not find evidence for such differences. Instrumental conditioning was successful as revealed by an above chance performance across the group at the end of the instrumental training on both days (one-sample *t*-test on mean correct choices after > 5 presentations vs. chance level (0.5 correct): Day1: $t_{(49)} = 4.2$, $p < 0.001$: 0.59, 95% CI 0.54–0.63; Day2: $t_{(49)} = 4.6$, $p < 0.001$; mean correct choices after > 5 presentations: 0.61, 95% CI 0.57–0.67). Moreover, performance did not differ between Treatment at Day 1 (two sample *t*-test on mean correct choices after > 5 presentations): $t_{(48)} = 0.8$, $p = 0.86$, 95% CI of difference: –0.08 to 0.09) nor was performance dependent on an interaction between Day and Treatment ($\chi^2 < 0.1$, $p = 0.99$). This was also the case for Pavlovian conditioning: Conditioning in terms of explicit associations between CS and outcomes resulted in above chance performance across Treatment group on both days (Day 1: one-sample *t*-test: mean = 0.88, 95% CI: 0.83–0.93, $t_{(49)} = 14.7$, $p < 0.001$: mean = 0.91, 95% CI: 0.87–0.96; Day2, $t_{(49)} = 19.5$, $p < 0.001$) and no group differences arose (Day 1: two sample *t*-test: $t_{(48)} = -0.25$, $p = 0.80$, 95% CI of difference: –0.12 to 0.09; interaction between Day and Treatment: $\chi^2 = 0.4$, $p = 0.53$). Moreover, VAS liking ratings from before to after conditioning showed the expected pattern (appetitive stimuli were judged appetitive and aversive stimuli as aversive after training: Time (2 levels: pre/post conditioning) × Pavlovian CS Valence (5 levels: $S^P_{++}/S^P_{+}/S^P_{n}/S^P_{-}/S^P_{--}$) at Day 1: $\chi^2 = 29.4$, $p < 0.001$) with again no difference in conditioning effects between the Treatment groups on Day1 [Group × Pavlovian CS Valence × Time (pre/post conditioning): $\chi^2 = 2.8$, $p = 0.093$] or as a function of change from pre- to post treatment [Group × Day × Pavlovian CS Valence × Time (pre/post conditioning): $\chi^2 < 0.1$, $p = 0.85$].

Discussion

Theory and data suggest that hyper(re)activity and impulsivity might be related to exaggerated appetitive Pavlovian activation and diminished aversive Pavlovian

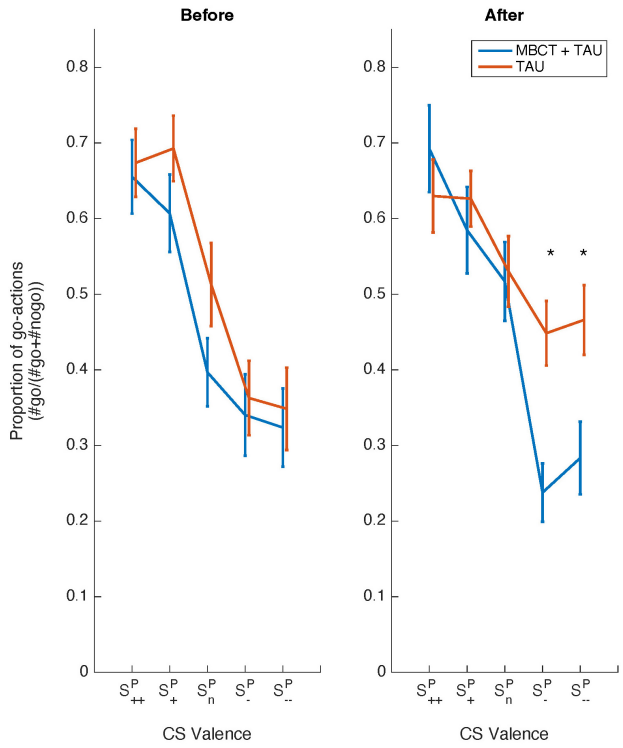

FIGURE 3
Behavioral data from the Pavlovian-instrumental transfer stage as a function of Treatment. Shown are choice data (proportion of go-actions) as a function of Pavlovian CS Valence ($S^P_{++}/S^P_{+}/S^P_{n}/S^P_{-}/S^P_{--}$) and Day (before vs. after) for a group receiving mindfulness based cognitive therapy and treatment as usual (MBCT+TAU, blue line) and a group receiving treatment as usual (TAU) only (red line). The group receiving MBCT shows increased aversive inhibition after MBCT ($p < 0.05$) compared to the TAU only group. Error bars represent standard errors of the mean.

inhibition (Watson et al., 2014; Garbusow et al., 2016; Heinz et al., 2016; Hallquist et al., 2018). This prediction, however, remained untested for ADHD. We present two key findings. First, an ADHD diagnosis with clinically relevant impulsivity/hyperactivity was accompanied by an *absence* of aversive Pavlovian inhibition, while an ADHD diagnosis without clinically relevant impulsivity/hyperactivity was accompanied by the expected aversive Pavlovian inhibition, akin to multiple healthy control studies (e.g., Huys et al., 2011; Geurts et al., 2013a). In contrast to our expectations, we did not find a relation between appetitive Pavlovian activation and impulsivity/hyperactivity. Second, within a randomized controlled setting, MBCT enhanced this aversive Pavlovian inhibition across the whole group of patients.

Both our findings, the relation between impulsivity/hyperactivity and aversive Pavlovian inhibition and the strengthening of this inhibition through MBCT in ADHD, are particularly interesting when considering the wide ranging, adaptive effects of Pavlovian inhibitory processes in more detail. Pavlovian conditioned reactions have long been recognized to help the organism prepare (in a fast and computationally efficient manner) for the predicted outcome (Dickinson and Balleine, 2002). In case of appetitive outcomes these "preparations" increase the chances to benefit from this outcome. In the case of aversive outcomes, the Pavlovian behavioral reactions (e.g., inhibition) might prevent damage to the organism. Allowing predictors of aversive outcome (i.e., aversive Pavlovian CS) to influence behavior thus might instigate adaptive behavior. Moreover, aberrant Pavlovian mechanisms, e.g., too much appetitive attraction and/or too little aversive inhibition, are thought to play a role in psychiatric disorders such as major depressive disorder, different anxiety disorders, addiction (Huys et al., 2015; Heinz et al., 2016; Mkrtchian et al., 2017) and personality disorders associated with impulsive behaviors (Ly et al., 2016; Hallquist et al., 2018). It has been proposed that not only actions are under the influence of Pavlovian inhibitory mechanisms, but also our thoughts (Huys et al., 2012; Mendelsohn et al., 2014). Indeed, Huys et al. (2012); and Lally et al. (2017) recently provided empirical evidence that Pavlovian inhibitory processes have a central place in planning action sequences. This warrants future studies on the Pavlovian inhibitory mechanisms in especially impulsivity/hyperactivity in ADHD and with respect to MBCT that might advance our understanding of the

neurocognitive mechanisms of both ADHD and the workings of MBCT, respectively.

One question that follows from the current study is why ADHD patients with clinically diagnosed impulsivity/hyperactivity lack the aversive Pavlovian inhibition we normally observe in healthy populations (Huys et al., 2011; Geurts et al., 2013a) and in ADHD patients without overt impulsivity/hyperactivity (this study). First, we note that instrumental and Pavlovian stimulus-outcome contingencies were learned by these patients as well as by the non-impulsive/hyperactive patients: Performance on query trials during conditioning nor (changes in) VAS-ratings of the Pavlovian stimuli across Pavlovian conditioning nor instrumental performance differed between these patient groups. Thus, the difference in aversive PIT cannot be readily explained by differences in learning. Moreover, it is not likely that within the PIT stage, these aversive Pavlovian CS were simply not noticed, because Pavlovian CS with appetitive valence exerted their normal (invigorating) effect (Huys et al., 2011). Thus, the absence of the inhibitory effect has to be searched downstream, in the interaction effect of Pavlovian and instrumental information itself. On a cognitive-psychological level this interaction effect might only surface when the aversive predictions are processed *and* used as guidance for steering instrumental behavior. Disturbances might thus come about through not processing the aversive information *as relevant* for behavioral procedures. The finding that patients showed increased effects of aversive Pavlovian stimuli post MBCT might be informative from this perspective. First, we note that the finding that MBCT increases the effect of aversive Pavlovian CS is in general accordance with a recent report on aversive Pavlovian conditioning (i.e., fear conditioning) before and after Mindfulness Based Stress Reduction (MBSR)(Hölzel et al., 2016). This study showed that through MBSR, healthy controls remained sensitive, as revealed by psychophysiological responses to the aversive Pavlovian CS (predictive of electrical shocks), whereas participants in the waitlist group lost this sensitivity. Our finding extends this result by showing that MBCT might potentiate the inhibitory effect of an aversive Pavlovian CS in adult ADHD patients. We speculate that this might be due to more openness to *guiding information* of contexts predicting adversity, instead of avoiding aversive information, in combination with an enhanced tendency to not immediately react, facilitated by the training. Moreover, on a neurophysiological level it has been shown that aversive Pavlovian inhibition depends on serotonergic signaling (Crockett et al., 2009; Geurts et al., 2013b; den Ouden et al., 2015) and is also influenced by methylphenidate suggesting catecholaminergic involvement (Swart et al., 2017). On a speculative account, we hypothesize that aberrant monoaminergic signaling related to Pavlovian control might be at the roots of this disinhibition, paralleling the psychological process by which aversive information guides instrumental behavior. Moreover, our data suggest that this process can be changed by MBCT.

Several limitations of this study should be noted: First we note that our relatively small sample size precluded us from assessing differential aspects of MBCT on the patients with the combined vs. the inattentive subtype of ADHD. This could have strengthened (or disproved) the suggestion that MBCT specifically remedies maladaptive aversive disinhibition. Moreover, including another active control treatment could have substantiated suggestions about the specificity of our result with regards to MBCT. With regard to the PIT paradigm, we think this might be improved by using a more naturalistic cover story (subjects informally reported that the game was boring) and more salient reinforcers (e.g., food, taste, shock, noise), which might make the task more ecologically valid and putatively more sensitive to change. Adding eye-tracking to this paradigm might also help to establish attentional components of the uncovered effect (e.g., more dwelling at the Pavlovian CS then at the instrumental stimulus) which might help to better understand the interindividual differences found here. Finally, because this paradigm has been shown to be sensitive to catecholaminergic modulation by methylphenidate and the current study suggests that it is also sensitive to change due to MBCT, it is interesting for future studies to assess whether this paradigm could have differential predictive properties in terms of treatment response for both pharmacological as well as psychotherapeutic interventions in ADHD.

In sum, our data suggests that the combined, but not the inattentive subtype of ADHD is associated with diminished aversive Pavlovian inhibition and that MBCT can enhance this inhibition. These findings offer new insights in the neurocognitive mechanisms of hyperactivity/impulsivity in the combined subtype of ADHD and point toward MBCT as an intervention that might influence these mechanisms.

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 the METC Oost-Nederland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DG, HO, RC, and AS contributed to conception and design of the study. DG, LJ, JS, and RC collected the data. DG performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.938082/full#supplementary-material

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Conditioned stimulus effects on paired or alternative reinforcement depend on presentation duration: Implications for conceptualizations of craving

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Conditioned stimuli (CS) associated with alcohol ingestion are thought to play a role in relapse by producing a craving that in turn increases motivation to drink which increases ethanol-seeking and disrupts other ongoing behavior. Alternatively, such CS may provide information indicating a likely increase in the density of the paired unconditioned stimulus and simultaneously elicit behavior that may be incompatible with other ongoing behavior, i.e., approach toward the CS. To explore these possibilities, rats were trained to respond for ethanol or food in two different components of the same session after which a light above the ethanol-lever was lighted twice during each component and each light presentation was followed by ethanol delivery. The duration of this CS was 10 s initially and then increased to 30 s, then to 100 s, and finally returned to 30 s. The change in responding for ethanol or food was compared to a matched period immediately preceding CS presentation. The CS presentation increased responding to ethanol, and this effect *increases* with longer CS presentations. In contrast, the CS presentation decreased responding to food, and this effect *decreases* with longer CS presentations. These results appear to support the informational account of CS action rather than simply a change in the motivation to seek and consume ethanol. This suggests that craving as it is commonly understood likely represents multiple behavioral processes, not simply increased desire for alcohol and that reports of craving likely reflect labeling based upon past experiences rather than a cause of future drug-taking.

KEYWORDS

alcoholism, craving, relapse, stimulus control, conditioned suppression, choice, Pavlovian, operant

Introduction

In this manuscript, we report the results of an experiment varying the duration of a stimulus preceding ethanol delivery on responding reinforced by food in the first component of a multiple schedule and by ethanol in the second component. This experiment was occasioned by a popular conceptualization of craving and its role in relapse and excessive drug intake. In this worldview, craving is the result of Pavlovian conditioning and is a manifestation of increased motivation to take drugs. This increased motivation is thought to increase the probability of relapse and excessive drug intake both directly through increased motivation and indirectly by distracting the individual from other tasks that might compete with drug use. In other experiments, we have already addressed whether increases in behavior might result from increased motivation that result from Pavlovian conditioning (Lamb et al., 2016a, 2017, 2019). In this experiment, we attempt to address whether increases in drug-seeking *and* decreases in other behavior during drug-paired stimuli are a result of these stimuli increasing motivation to consume drugs by examining whether drug-paired stimuli have comparable but opposing effects on behavior maintained by drug (increase) or an alternative reinforcement (decrease) across several different stimulus presentation durations.

There is ample evidence that at least under certain conditions, stimuli correlated with the delivery of the event that is reinforcing responding can increase that responding. For instance, food-paired stimuli can increase responding maintained by food. This has been shown for both animals responding to food (Lovibond, 1983) and in animals whose responding to food is in extinction (e.g., Estes, 1943). Similarly, ethanol-paired stimuli can increase responding to ethanol (Lamb et al., 2020), and ethanol- or cocaine-paired stimuli can increase responding to these drugs that are in extinction [Kruzich et al., 2001; Krank, 2003; Corbit and Janak, 2007, 2016; Krank et al., 2008; see Lamb et al. (2016c) for a review and critique of this literature].

Conversely, there is also ample evidence that stimuli paired either with significant negative events or significant positive events can disrupt ongoing behavior. Often, a stimulus paired with electric shock will disrupt food-maintained behavior [Estes and Skinner, 1941; Hunt and Brady, 1951; but see Waller and Waller (1963) for a counter-example]. Similarly, a stimulus paired with the delivery of food, water, or electrical brain stimulation will suppress responding maintained by food or water delivery (Azrin and Hake, 1969). Importantly, it has also been shown that cocaine-paired (Schindler et al., 2000), amphetamine-paired (Duncan et al., 1989; Watanabe, 1990), and pentobarbital-paired (Duncan, 1997) stimuli can disrupt food-maintained behavior. These increases or decreases in responding induced by paired stimuli may result from their effects on motivation, but other explanations are also possible.

Presumably, if both increased drug-seeking and disruption of other behavior result from increased motivation to take drugs then these should co-vary, i.e., disruption of other behavior should be positively correlated with increased drug-seeking. Thus, a manipulation that simultaneously changes the effectiveness of an ethanol-paired stimulus at increasing ethanol-maintained responding and decreasing food-maintained behavior might allow us to dissect whether these increases and decreases were resulting from the same mechanism, presumably motivation. One such manipulation is the duration of the paired stimulus. Stein et al. (1958) demonstrated that suppression of food-maintained responding by a shock-paired stimulus was greatest when the stimulus duration was short relative to the total session time without the stimulus. Henton and Brady (1970) showed that as the duration of a stimulus paired with food delivery increased, so too did the likelihood of increases in food-maintained responding during stimulus presentations. Meltzer and Brahlek (1970) also found that response increases were more likely with a longer stimulus duration and response suppression more likely with a shorter stimulus duration when the effects of a food-paired stimulus were studied on food-maintained behavior. Miczek and Grossman (1971) found that food-paired stimuli of shorter durations, but not a longer duration, suppressed food-maintained responding. Thus, it appears that short stimulus presentations are more likely to suppress responding, and relatively longer stimulus presentations are more likely to increase responding.

These findings argue that the suppression of behavior and the facilitation of behavior seen following the presentation of paired stimuli may not result from motivational changes *per se*, but rather from differences in the behavior elicited following presentations of stimuli differing in duration. However, these studies only looked at the effects of the paired stimulus on behavior maintained by a single event, yet it is the disruption of behavior other than that maintained by the (CS-paired) US that is hypothesized to result from increased motivation for the US. On the other hand, if the resulting effects of the CS on responding were a result of the information added to that context, then more nuanced results might be seen. The CS elicited goal approach decreases as CS length increases. CS also signal an increased density of US delivery. The first may disrupt ongoing behavior regardless of what is maintaining behavior. The latter may well increase behavior that is maintained by the US. Thus, behavior maintained by a reinforcer other than the US will be disrupted by shorter CS and relatively unaffected by longer CS. The response disruptive and response facilitating effects of short-duration CS may offset each other for behavior maintained by the US, while at longer CS, the response facilitating effects of the CS may be more apparent as the goal approach becomes less frequent. Therefore, the motivational and informational accounts of CS effects on responding make distinctly different predictions about what we should see as we

manipulate the duration of the ethanol-paired CS and examine its effects on food- and ethanol-maintained behavior. The results of this experiment could provide further support for the notion that craving-induced facilitation of drug-seeking and disruption of other behavior both result from increased motivation to seek drugs. Alternatively, it could provide support for an informational account, and the idea that craving is a subjective effect representing a self-assessment of one's likelihood of taking drugs when attempting not to take drugs [Tiffany, 1990; see Lamb et al. (1991) and Lamb and Henningfield (1994) for a discussion of subjective effects], as decreases in other behavior seen with short-duration CS and increases in drug-seeking seen with long-duration CS both increase the probability of future drug-taking by increasing the relative probability of drug-taking (see Lamb et al., 2016b; Lamb and Ginsburg, 2018).

People are said to crave a drink in two situations: The first is when stimuli associated with drinking increase their likelihood of wanting or seeking a drink, particularly when a drink might be unavailable or they are attempting not to drink. The second is when stimuli associated with drinking disrupt other ongoing behavior. Both effects are thought to be a result of stimuli associated with drinking increasing motivation to drink through Pavlovian conditioning. If this is the case, then manipulations that make stimuli predicting drink availability more effective at disrupting other ongoing behavior should also make stimuli more effective at increasing seeking an opportunity to drink; and conversely, manipulations that make stimuli more effective at increasing seeking an opportunity to drink should also make stimuli more effective at disrupting other behavior. Alternatively, if craving is simply a learned description of situations in which one's behavior has been altered by stimuli associated with drinking, then we would not necessarily expect a positive correlation between the disruption of other behavior and an increase in behavior that might lead to a drink. In this experiment, we examine whether changes in the duration of the CS associated with ethanol delivery similarly changes the effectiveness of this CS at disrupting other behavior and increasing ethanol-seeking.

Materials and methods

Subjects

All procedures conducted on the rats were approved by the local Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (2013). A total of six male Lewis rats weighing between 125 and 149 g were purchased from Envigo (Alice, TX, United States). The rats were individually housed, and for approximately 2 weeks were allowed unrestricted access to food and water. After this, food was restricted to 12–15 g per day, but water was freely available.

Apparatus

A total of six operant conditioning chambers were used (Gerbrands, Alderston, MA, United States), each equipped with a house light overhead, three response levers, three lever lights (one above each lever), a dipper mechanism capable of delivering 0.1 ml of ethanol solution, and a pellet magazine capable of delivering 45 mg food pellets. Each chamber was housed in a light and sound-attenuating cubicle (Gerbrands). The dipper mechanism was directly opposite the ethanol-associated lever, and the pellet magazine was directly opposite the food-associated lever. The third lever was located between the food magazine and the dipper mechanism and was not used in this experiment. Chambers were interfaced with an IBM-PC compatible computer. Commercially available software was programmed to coordinate light presentations, deliver reinforcers, and record lever responses (MedPC, MedAssociates, Georgia, VT, United States).

Ethanol self-administration

Ethanol drinking was induced over twenty-two sessions by giving rats access to 4s presentations of 0.1 ml 8% (w/v) ethanol, and 8% sucrose solution under a continuous reinforcement schedule (CRF). Under the CRF schedule each lever press when the 80 dB, 16 kHz tone sounded produced a 4-s dipper presentation and turned off the tone. Following the dipper presentation, the tone again sounded and lever presses were reinforced. Sessions lasted 4–5 h until lever pressing occurred reliably. This took from 8 to 16 sessions. Over the remaining sessions, the sucrose concentration was reduced to zero and the session length was reduced to 1 h. The complete training sequence is illustrated in **Figure 1**.

Following induction of ethanol drinking and the training of responding to ethanol, rats were trained to respond to ethanol in sessions in which each response on the lever was reinforced for 15 sessions. Over the first five sessions, the schedule was moved from every response being reinforced to a random interval (RI) 30 s schedule at which value it remained for the remaining 10 sessions. The sessions were 30 min long.

After training on the RI 30s schedule of ethanol presentation, rats were placed on a multiple schedule food delivery and ethanol presentation. Responding for food was reinforced on the lever opposite the food magazine and was signaled by an 8 kHz, 80 dB tone and each delivery of a 45-mg food pellet was accompanied by a 4s timeout during which the tone was turned off. The 30min food component preceded the 30min ethanol component. The tones were present for the duration of each session, except during post-delivery timeout periods as indicated. Over 16 sessions, the schedule for food presentation was changed from one in which every lever press

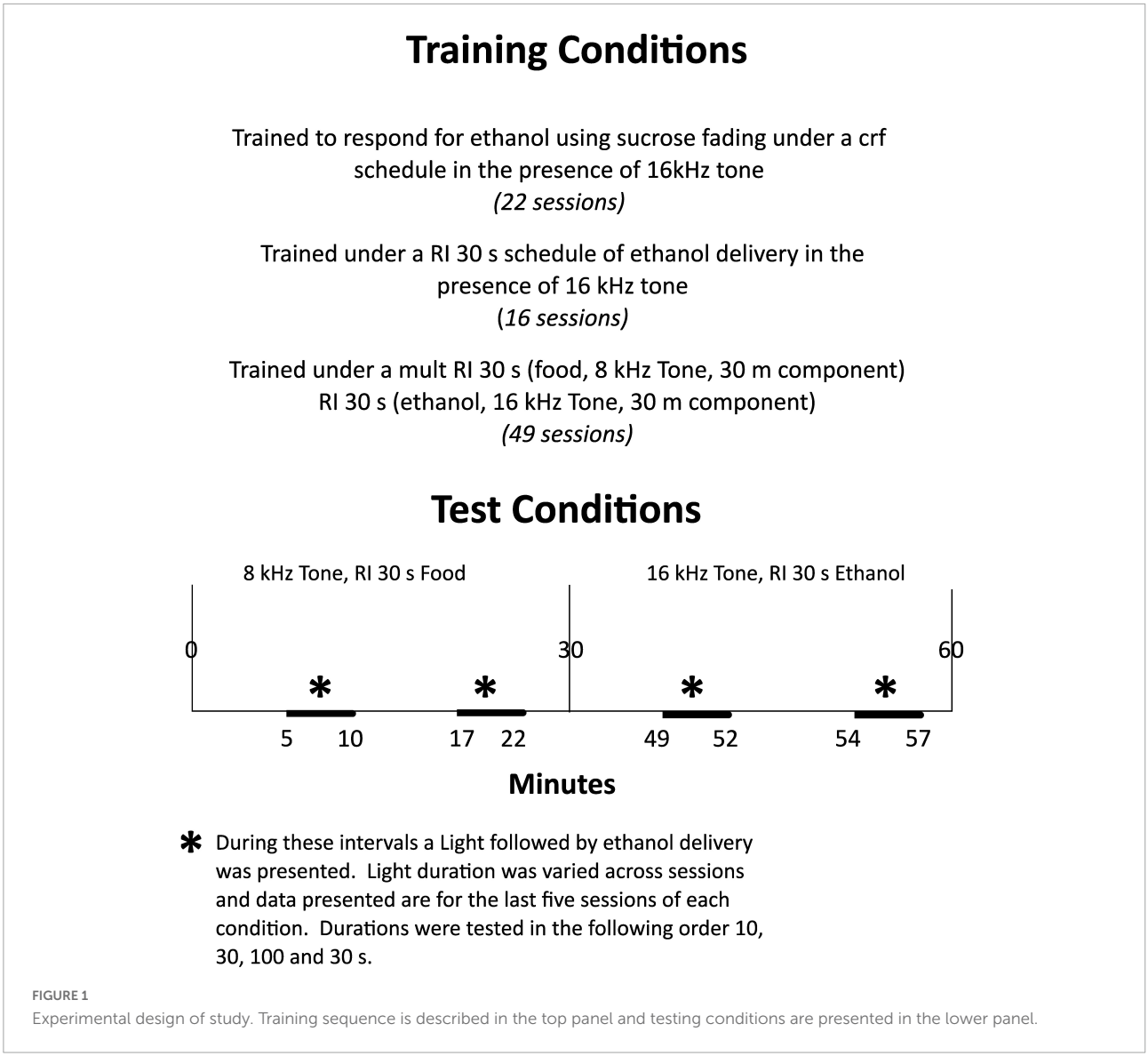

FIGURE 1
Experimental design of study. Training sequence is described in the top panel and testing conditions are presented in the lower panel.

was reinforced to an RI 30s schedule. The schedule for ethanol presentation remained at RI 30 s throughout this time.

After 49 sessions under this mult RI 30 s (food), RI 30s (ethanol) schedule, the stimulus light above the ethanol-lever was programmed to be illuminated twice for 10 s in each component. The timing of light illumination was random, but occurred between 5 and 11 min into the food component and again between 17 and 23 min into the food component. It occurred first in the ethanol component 19–22 min into the component and then again 24–27 min into the component. Each light illumination was followed immediately by a 4s ethanol presentation. As food-responding was at relatively high constant rates throughout the food component, light illuminations occurred when responding was at high levels in this component. As ethanol-responding declined over the duration of the ethanol component, light illuminations occurred when this responding was at relatively low levels. This condition was in effect for 83 sessions. Following this, the duration of the light illumination was increased to 30 s for 20 sessions and then to 100 s for 21 sessions, and then finally returned to 30s illuminations for 47 sessions. The testing sequence is illustrated in Figure 1.

Analysis

The main variable of analytic interest was the rate of responding during the light CS presentation compared to the period of the same duration preceding the light presentation during the last five sessions of each condition. Thus, the rate of responding for each across these sessions was calculated by dividing the number of responses during each period by the duration (s) of each period, excluding the time when the

dipper was presented. These data were calculated as responses/s. The difference between response rates during CS presentation and the matched period before CS presentation is thus the primary measure.

All analyses were performed using the R statistical program (R Core Team, 2022). Comparisons were made using a repeated measures analysis of variance (ANOVA) test of a linear mixed regression using the lme:nlme and anova:r-base packages (Pinheiro et al., 2022). Changes in response rates on each lever between periods where CS was present or absent were compared, with CS duration (10 s, 30 s, 100 s) and session number (1–5) as factors. Effects with $p < 0.05$ were considered significant and further analyzed utilizing pairwise comparisons performed with multiple *t*-tests corrected using the method of Benjamini and Hochberg (1995).

An ANOVA was performed as described above to compare changes in response rates when the CS duration was 30 s with replicate (1–2) as the factor during the last five sessions of each condition. Significant main effects and interactions were further analyzed using *t*-tests and again corrected for multiple comparisons using the method of Benjamin and Hochberg. Finally, an ANOVA was performed as described above on ethanol response rates in the food components and on food response rates in the ethanol components with CS duration (10 s, 30 s, and 100 s) and session number (1–5) as factors.

Results

Food-responding was suppressed more at shorter ethanol-paired CS durations than longer CS durations. As shown in **Figure 2** (open circles), shorter CS presentation duration resulted in a greater decrease in response rate compared to the response rate in the period immediately preceding CS presentation, and this change diminished as CS duration increased. This was evident from the positive slope of the linear regression on change in the food response rate as a function of CS duration (mean slope [95% CI] = 3.00×10^{-3} [$1.92 \times 10^{-3} - 4.04 \times 10^{-3}$]). An ANOVA on food response rates during food components with CS duration and session number as factors yielded a main effect of CS duration ($F_{[2,70]} = 5.49$, $p = 0.0060$). A complete ANOVA table is shown in **Table 1**. *Post hoc* analyses revealed that changes in food response rate from the period before stimulus presentation to the period of stimulus presentation were significant ($p < 0.05$ after correction for multiple comparisons) for all three CS durations tested, though, as noted above, the magnitude of the change decreased as a function of CS duration.

In contrast, ethanol-responding was facilitated more at longer CS durations than at shorter CS durations. As shown in **Figure 2** (closed circles), no change in response rate was observed during the 10s CS presentation, compared with the period immediately prior to CS presentation, but 30 and 100s

TABLE 1 ANOVA results for the effect of CS presentation duration and session number on responding for ethanol or food in each component type.

		Food component				Ethanol component				
		Numerator DF	**Denominator DF**	***F*-value**	***p*-value**		**Numerator DF**	**Denominator DF**	***F*-value**	***p*-value**
Food responses	(Intercept)	1	70	16.010	0.000	(Intercept)	1	70	0	1
	Conditioned stimulus duration	2	**70**	**5.499**	**0.006**	Conditioned stimulus duration	**2**	**70**	**4.737**	**0.012**
	Session	4	70	0.833	0.509	Session	4	70	0.249	0.909
	Conditioned stimulus × Session	8	70	0.365	0.936	Conditioned stimulus × Session	8	70	1.938	0.068
Ethanol responses	(Intercept)	1	70	0	1	(Intercept)	1	70	4.976	0.029
	Conditioned stimulus duration	2	70	1.129	0.329	Conditioned stimulus duration	**2**	**70**	**3.690**	**0.030**
	Session	4	70	0	1	Session	4	70	1.003	0.412
	Conditioned stimulus × Session	8	70	0.740	0.656	Conditioned stimulus × Session	8	70	0.813	0.594

Bolded values are those that are significant (excluding intercept values which simply imply mean is not equal to zero).

CS presentations resulted in elevated response rates compared with responding before CS presentation. This was evident from the positive slope of the linear regression on change in ethanol response rate as a function of CS duration (mean slope [95% CI] = 8.75×10^{-4} [$3.33 \times 10^{-4} - 1.42 \times 10^{-3}$]). ANOVA on ethanol response rates during ethanol components yielded a main effect of CS duration ($F_{[2,70]} = 3.68$, $p = 0.0300$). Changes in ethanol response rates were significant when the CS duration was 30 s or 100 s (see **Figure 2**). The session number was not a significant factor for response rate changes for food or ethanol.

To assess the replicability of the effects observed, separate ANOVA analyses were performed on food or ethanol response rates with the two replications of the 30s CS presentation conditions as the factor. In neither case did the change in response rate upon CS presentation depend on the replicate ($F_{[1,53]} = 0.66$ and $F_{[1,53]} = 1.43$, $p > 0.05$ for ethanol and food responses, respectively).

Off-target responding (e.g., food-responding during the ethanol component) was extremely low compared with on-target responding during each component (see **Table 2**), but tended to increase more during longer CS durations than shorter CS durations. However, this was only reliable during the ethanol component. No effect of CS duration or session was present for changes in ethanol response rate during food components. An effect of CS duration was significant for food responses in ethanol components ($F_{[2,70]} = 4.7$, $p < 0.05$). *Post hoc* comparisons revealed that a CS duration of 100 s resulted in a significant ($p < 0.05$) increase in food-lever responding during the ethanol component. No significant effect of session number was observed, nor was there an interaction between CS duration and session number in either analysis (see **Figure 2**).

Discussion

Here we report that an ethanol-paired CS can enhance ethanol-maintained responding and simultaneously disrupt food-maintained responding. The effect on ethanol-maintained responding is most pronounced at longer CS presentations, while the effect on food-maintained responding decreases as a function of CS length. This observation is consistent with previous studies showing that ethanol-paired stimuli can increase ethanol-seeking, perhaps by increasing craving or motivation to consume ethanol (Krank, 2003; e.g., Corbit and Janak, 2007; Lamb et al., 2016a). This observation is also consistent with the phenomenon of positive conditioned

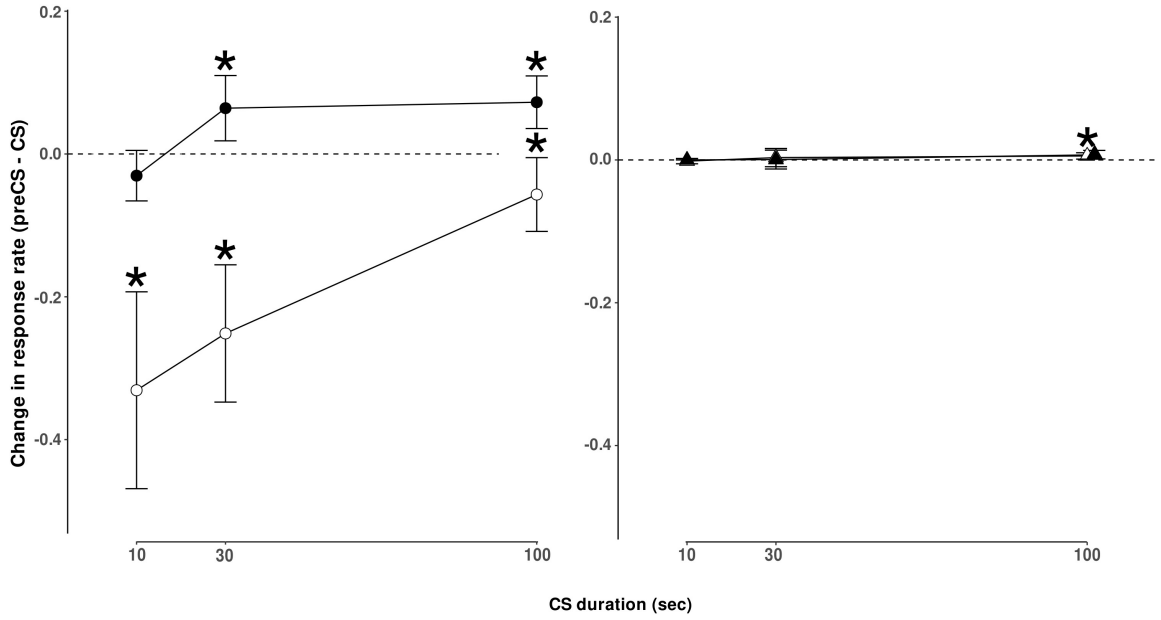

FIGURE 2
Left panel–On-target responding. Change in responding on the ethanol-lever during components where ethanol was available (•) or change in responding on the food-lever when food was available (○) during CS presentation. Results are presented as the number of responses observed during CS presentation minus responses observed during the period immediately before CS presentation. This measure reflects the relative increase or decrease in responding during CS presentation of varying durations. CS presentation duration varied as indicated, 10-s, 30-s, or 100-s. Points represent mean change ± S.E.M. for $n = 6$ rats. *Indicates points that differ significantly from zero (no change), $p < 0.05$ after correction for multiple comparisons. Right panel–Off-target responding. Change in responding on the ethanol-lever during components where food was available (▲) or on the food-lever when ethanol was available (Δ). Results are reported as responses observed during CS presentation minus responses observed during the period immediately before CS presentation. CS presentation duration varied as indicated, 10-s, 30-s, or 100-s. Points represent mean change ± S.E.M. for $n = 6$ rats. Points above 100-s CS presentation have been adjusted to show that responding for food was significantly increased ($p < 0.05$) in the ethanol component, but not responding for ethanol in the food component.

TABLE 2 Response rates (resp/sec) before (Pre-CS) and during (CS) CS presentation on each lever during each component.

	10-s						30-s						100-s					
	Food component		Ethanol component		Food component		Ethanol component		Food component		Ethanol component		Food component		Ethanol component		Food component		Ethanol component	
	Food		Ethanol		Ethanol		Food		Food		Ethanol		Food		Ethanol		Ethanol		Food	
Rat	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS	Pre-CS	CS
1	0.37	0.26	0.00	0.00	0.04	0.02	0.00	0.00	0.41	0.37	0.00	0.00	0.03	0.15	0.00	0.01	0.33	0.30	0.00	0.00	0.02	0.15	0.00	0.00
2	0.74	0.05	0.00	0.00	0.13	0.05	0.00	0.00	0.88	0.51	0.00	0.00	0.45	0.59	0.01	0.03	0.70	0.51	0.00	0.01	0.21	0.37	0.00	0.02
3	0.32	0.04	0.00	0.00	0.04	0.01	0.00	0.00	0.36	0.23	0.02	0.03	0.05	0.06	0.00	0.00	0.29	0.29	0.01	0.01	0.03	0.04	0.00	0.00
4	0.41	0.04	0.00	0.00	0.03	0.03	0.00	0.00	0.81	0.32	0.01	0.03	0.09	0.17	0.02	0.00	0.83	0.65	0.00	0.03	0.06	0.11	0.01	0.01
5	0.21	0.31	0.00	0.00	0.10	0.03	0.00	0.00	0.58	0.62	0.00	0.00	0.07	0.14	0.00	0.00	0.44	0.44	0.00	0.00	0.03	0.08	0.00	0.00
6	0.87	0.23	0.00	0.00	0.04	0.05	0.01	0.00	0.98	0.62	0.02	0.02	0.03	0.08	0.00	0.01	0.55	0.61	0.02	0.02	0.02	0.05	0.00	0.01
Mean	0.49	0.16	0.00	0.00	0.06	0.03	0.00	0.00	0.67	0.45	0.01	0.01	0.12	0.20	0.01	0.01	0.52	0.47	0.01	0.01	0.06	0.13	0.00	0.01
S.E.M.	0.10	0.05	0.00	0.00	0.02	0.01	0.00	0.00	0.11	0.07	0.00	0.01	0.07	0.08	0.00	0.00	0.09	0.06	0.00	0.00	0.03	0.05	0.00	0.00

suppression whereby a CS associated with a desirable event can reduce engagement in other activities (Azrin and Hake, 1969; Miczek and Grossman, 1971). Further, these observations are consistent with the change in operant behavior occurring because of the CR elicited by the CS, which depends upon the form of the CS, and its duration (Holland, 1977, 1980a,b). The implication of this work is that the effect of alcohol-paired CS presentation may have differential effects on ethanol-seeking or alternative behavior, depending on the duration, form, and timing of CS exposure.

Ethanol-paired-stimuli increase ethanol-responding and decrease food-responding (Krank, 2003; Corbit and Janak, 2007; Lamb et al., 2016a, 2020, this study). These outcomes are consistent with the idea that ethanol-paired-stimuli increase craving, which in turn is a result of increased motivation to drink ethanol, or more simply ethanol desire (Pomerleau et al., 1983). This increased ethanol desire may distract from the performance of other behavior. Both the increase in ethanol desire and the disruption of other behavior might be expected to promote excessive drinking and relapse (Lamb et al., 2016b; Lamb and Ginsburg, 2018). The procedure demonstrated here provides a means of examining both ethanol-paired-stimuli-induced increases in ethanol-seeking and ethanol-paired-stimuli-induced disruption of other behavior; and to the extent that these reflect craving, a means for studying craving using a steady-state procedure.

As already mentioned, craving is generally thought to result in decreases in other behavior and increases in ethanol-seeking (Lamb and Ginsburg, 2018; Bowen et al., 2022). However, it is equally possible that these two behaviors are what result in craving, i.e., that upon observing that one's routine behavior is disrupted by things that might signal opportunities to drink or that one is seeking ethanol, especially during recovery, when drinking is suppressed or unavailable, one learns this phenomenon called "craving a drink." We favor the latter viewpoint. Craving is a subjective effect, descriptive of a situation that cannot be objectively observed or measured, and subject to variability in meaning and reporting across individuals or social or cultural groups (Angel and Gronfein, 1988). Thus, one comes to use the term craving in situations and feelings associated with an increased likelihood of drinking or having one's behavior disrupted by thoughts of drinking and noting when you might seek alcohol rather than other activities, as this usage is reinforced by those around you (see Lamb et al., 1991; Lamb and Henningfield, 1994). If craving causes disruptions in other behavior and an increased propensity to drink, then measures of behavioral disruption and drinking are likely to be less sensitive than measures of craving. On the other hand, if behavioral disruption and an increased propensity to drink occasion reports of craving, behavioral disruption and propensity to drink are likely to be more sensitive measures. So far in other similar situations, direct behavioral measures have been more sensitive measures than subjective effects, e.g., lower

doses of morphine occasion drug-seeking than those needed to occasion reports of drug liking (Lamb et al., 1991).

Still, that craving reflects increased ethanol desire could be true no matter if it is a case of increased ethanol desire causing craving, which disrupts other behavior and increases drinking, or if increased ethanol desire increases ethanol-seeking and disrupts other behavior, which occasion reports of craving. However, the results of the present experiment are not consistent with the idea that ethanol-paired-stimuli increase ethanol-seeking *and* disrupt other behavior by increasing ethanol desire either directly or through an increase in craving. If increased ethanol desire was responsible for *both* the increase in ethanol-seeking and the disruption of other behavior then *both* should increase as ethanol desire increases. However, increasing CS duration increases ethanol-seeking, while decreasing the disruption of other behavior. Conversely, decreasing the CS duration increases disruption of other behavior, while attenuating the increase in ethanol-seeking. These observations are inconsistent with increases in ethanol-seeking *and* disruptions of other behavior seen during the presentation of ethanol-paired stimuli *both* being direct consequences of ethanol-paired-stimulus-elicited increases in ethanol desire.

These observations are more consistent with the change in operant behavior seen following CS presentation being a consequence of the CR elicited by the CS, which will depend upon the form of the CS and its duration (Holland, 1977, 1980a,b). Short CS frequently elicits orienting responses. In the case of food-responding in the present experiment, this involves looking and perhaps moving away from the food-lever. In the case of ethanol-responding, the ethanol-paired-stimulus was immediately above the ethanol-lever. Food-responding was decreased by the shorter CS, while ethanol-responding was essentially unaffected by the short CS. Results consistent with this hypothesis can be seen in experiments in which the CS location is varied. Karpicke et al. (1977) found suppression of food-responding when the CS was located away from the food-lever, but little effect of the CS on food-responding when the CS was located near the food-lever. Particularly germane to this argument, Krank et al. (2008) found that ethanol-paired-stimuli attracted approach and when these were located near the ethanol-lever, ethanol-paired-stimuli increased ethanol-responding. Such arguments are consistent with the roles of CS in drug addiction postulated by Tomie (1996) and Flagel et al. (2009) in which the attractive properties of the CS when appropriately situated help promote further drug-taking and addiction.

Conditioned stimuli not only elicit behavior that might promote addiction, CS also provide information. In the case of this experiment, the CS foretold the delivery of ethanol above and beyond that ordinarily available. This increased density of ethanol reinforcement might be expected to increase ethanol-responding under a random interval schedule, and to exert less effect on food-responding, with the increases resulting from generalization from the ethanol-lever to the food-lever or decreases resulting from rats responding on the ethanol-lever rather than the food-lever. It should be noted that stimulus control in this experiment was excellent and very few off-target responses were observed either in the presence or absence of the CS (though there were slightly more during the CS). The effect of a signaled increase in ethanol reinforcement density and the cue light approach elicited by the ethanol-paired-stimulus are likely in conflict. Thus, it is not surprising that increases in ethanol-responding are most readily observed at longer CS durations that appear to elicit fewer incompatible CRs.

In this study, rats were food-restricted. This allowed us to use food-maintained behavior as a comparison to ethanol-maintained behavior to determine the specificity of CS effects. While this condition may have affected our results, it is important to note that others have seen similar ethanol-associated CS effects on responding for ethanol in rats with no food restriction (Corbit and Janak, 2007) as well as food-restricted rats (Lamb et al., 2020). Additionally, others have shown that longer duration food-associated CS can increase food-maintained behavior in food-restricted animals (Meltzer and Brahlek, 1970; Miczek and Grossman, 1971), and when the CS-duration is shorter decreases in food-maintained behavior have been observed (Azrin and Hake, 1969). Further, in the present study, an ethanol-associated CS increased ethanol-maintained responding and not food-maintained responding in food-restricted rats (**Figure 2**). Thus, it is unlikely that these results are dependent on the food-restriction status of the subjects.

Conditioned stimuli are thought to play a role in relapse to alcohol or drug use disorders by producing craving, which is thought to reflect an increased desire for alcohol or drug. Craving, in turn, is thought to result in increased drug-seeking and the disruption of other ongoing behavior. While both outcomes upon exposure to an ethanol-associated CS might be considered "craving," they do not appear to occur solely as the result of increased ethanol motivation, or else they should co-vary. Instead, these results show that increased ethanol-responding and decreased food-responding can occur under different CS presentation conditions, suggesting other mechanisms beyond motivation for alcohol are involved. Specifically, ethanol-paired CS presentation increases responding to ethanol, and this effect *increases* with CS presentation duration. In contrast, CS presentation decreases responding to food, and this effect *decreases* with CS presentation duration. This outcome is inconsistent with an account of CS increasing drug-seeking and decreasing other ongoing behavior due to increased motivation for the CS-paired drug, due to the contrasting effect of longer CS presentation on ethanol-maintained and food-maintained behavior. This outcome is consistent with an informational account of CS action on drug-seeking, whereby the CS indicates

a likely increase in the density of the paired US (ethanol), while eliciting behavior toward the paired stimulus or US (ethanol)-delivery location that is incompatible with behavior maintained by the unpaired US (food) at shorter durations. These findings have two important implications for how craving might best be conceptualized. First, as it is commonly used craving refers both to an increased likelihood of future drug use and to a disruption of ongoing behavior resulting from stimuli and situations associated with past drug use. In this case, our results indicate craving likely represents multiple different behavioral processes, not simply increased motivation. Second, these results provide further evidence that the use of the term craving is likely as a subjective effect representing an assessment based upon past experiences, rather than reports about a causal mechanism that changes the likelihood of future 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 Institutional Animal Use and Care Committee, UT Health Science Center at San Antonio.

Author contributions

BG was involved in the analysis and authorship of this work. AN-M was involved in the analysis and visualization of the results. CS and RL were involved in study design, interpretation, and authorship. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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General Pavlovian-to-instrumental transfer in humans: Evidence from Bayesian inference

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When repeatedly paired with rewarding outcomes (i.e., Pavlovian conditioning), environmental cues may acquire predictive and motivational significance and later enhance instrumental responding for the same (i.e., outcome-specific transfer) or motivationally similar (i.e., general transfer) outcomes. Although outcome-specific and general Pavlovian-to-Instrumental Transfer (PIT) are characterized by different neural substrates and behavioral mechanisms, general transfer has never been studied in isolation from outcome-specific transfer in humans. The first aim of the present study was to test whether the general transfer effect could emerge in isolation and independently of outcome-specific transfer. Our results showed that general transfer can be elicited without the concurrent presence of outcome-specific transfer, supporting the idea that outcome-specific and general transfer can be studied independently of each other. The second aim of the present study was to clarify whether the affordance-like properties of the outcomes can affect the general transfer. In fact, a critical difference in current studies on general transfer concerns the use of cues associated with outcomes for which an action was previously learned (or not) during the instrumental training. This apparently minor difference affects the affordance-like properties of the outcome and may also be transferred to the cue, in turn impacting general transfer. Results revealed a general transfer of the same magnitude regardless of whether cues were associated with reward earned or not during instrumental conditioning. These findings increase the current knowledge on the incentive motivational mechanism behind general transfer, indicating that it is independent of the motor features of the outcome.

KEYWORDS

general Pavlovian-to-instrumental transfer, cue-guided choices, Bayesian statistics in neuroscience, motivation, decision-making

Introduction

Environmental cues (e.g., brand logos) exert a powerful influence on our daily choices. Although neutral in principle, such cues acquire a motivational value through their repeated pairing with a reinforcer (e.g., a chocolate bar), and may bias future choices, driving our reward-seeking behavior (Behrens et al., 2007; Doya, 2008; Watson et al., 2018). For example, a fast-food sign may lead us to that specific fast-food to purchase and eat a hamburger, or it may lead us toward the nearest restaurant to consume food in general.

In the laboratory, cue-guided choices have been investigated using an experimental paradigm called Pavlovian-to-Instrumental Transfer (PIT). The PIT paradigm has been extensively studied in animals (for review, see Dickinson and Balleine, 1994; Holmes et al., 2010; Cartoni et al., 2016). More recently, however, it has become an active area of research in humans as well (Paredes-Olay et al., 2002; for review, see Cartoni et al., 2016; Mahlberg et al., 2021), due to increasing interest in the role of predictive stimuli in guiding actions that are considered maladaptive and where, in general, there is a dysregulation of goal-directed control.

Pavlovian-to-instrumental transfer experiments typically involve three phases: the instrumental conditioning phase, in which participants learn outcome-response associations; the Pavlovian conditioning phase, in which participants learn stimulus-outcome associations; the transfer phase, which tests the ability of the Pavlovian stimulus to affect the instrumental response directed toward the same (outcome-specific transfer) or a similar (general transfer) outcome (Bray et al., 2008; Talmi et al., 2008; Garofalo and di Pellegrino, 2015; Garofalo et al., 2019).

General and outcome-specific transfer effects have most often been accounted for in terms of general or specific influences of Pavlovian cues on instrumental responding, depending on the ability of such stimuli to either enhance responding in general (general transfer), or cue a particular action that produces an outcome that had been previously paired with the stimulus (outcome-specific transfer). There is increasing evidence that each influence on instrumental responding is characterized by a different neural substrate (Corbit and Balleine, 2005, 2011; Prévost et al., 2012; Garofalo et al., 2021), and relies on a separate behavioral mechanism (Holland, 2004; Corbit et al., 2007; Garofalo and Robbins, 2017; Garofalo et al., 2019, 2020).

To date, the findings on human general transfer have been quite heterogeneous. For instance, while some studies report evidence for general transfer using response rate as a dependent variable (Lewis et al., 2013; Quail et al., 2017; Alarcón and Bonardi, 2020b), others failed to observe it (Meemken and Horstmann, 2019; Petrie et al., 2021), or found it only in aversive conditions (Nadler et al., 2011), or using vigor as a dependent variable (Watson et al., 2014; Garofalo and Robbins, 2017). Such heterogeneity in results may be at least partially explained by the lack of studies directly investigating this general transfer effect. Indeed, while most human studies have focused on outcome-specific transfer only (Rosas et al., 2010; Cartoni et al., 2015; Garofalo and di Pellegrino, 2015; Alarcón and Bonardi, 2016, 2020a), a small number has studied both the effects (Nadler et al., 2011; Prévost et al., 2012; Garofalo et al., 2020, 2021), or has investigated a form of transfer in which no distinction could be applied (Talmi et al., 2008; Huys et al., 2011; Geurts et al., 2013; Jeffs and Duka, 2019). To date, the general transfer effect has never been studied in isolation from outcome-specific transfer in humans.

Therefore, the first aim of the present study was to provide evidence about the general transfer effect, by devising a PIT task that did not involve the concurrent study of outcome-specific transfer. Specifically, we examined the ability of Pavlovian cues to enhance instrumental responses that were paired with outcomes (motivationally similar but sensorily) different from that paired with the CS.

The heterogeneity of results in general transfer studies reported above may also be related to several variations in testing procedures. A major difference in the experimental implementation of general transfer concerns the use of cues associated with outcomes for which an action was learned (or not) during the instrumental conditioning phase (**Table 1**). While many authors operationalized general transfer as the capacity of a Pavlovian cue to bias choice toward an outcome that was never obtained through an instrumental action (**Table 1**: General $PIT_{No-action}$) (Nadler et al., 2011; Lewis et al., 2013; Watson et al., 2014; Morris et al., 2015; Claes et al., 2016; Lehner et al., 2016; Garofalo and Robbins, 2017; Quail et al., 2017; Meemken and Horstmann, 2019; Alarcón and Bonardi, 2020b; Hinojosa-Aguayo and González, 2020; Krypotos and Engelhard, 2020; Soutschek et al., 2020; van Timmeren et al., 2020; Petrie et al., 2021), others used a different operationalization of general transfer, where the Pavlovian cue predicted an outcome previously earned by a specific instrumental action, which, however, was no longer available in the transfer phase (Prévost et al., 2012; Garofalo et al., 2019, 2020, 2021; Sennwald et al., 2020; **Table 1**: General PIT_{Action}).

TABLE 1 Two different operationalizations of general transfer used in the literature.

	General $PIT_{No-action}$	General PIT_{Action}
Instrumental	$Response_1 \rightarrow Outcome_1$ $Response_2 \rightarrow Outcome_2$	$Response_1 \rightarrow Outcome_1$ $Response_2 \rightarrow Outcome_2$ $Response_3 \rightarrow Outcome_3$
Pavlovian	$CS+ \rightarrow Outcome_3$ CS– → No Outcome	$CS+ \rightarrow Outcome_3$ CS– → No Outcome
Transfer	$CS+ = Response_1 + Response_2$ $CS- = Response_1 + Response_2$	$CS+ = Response_1 + Response_2$ $CS- = Response_1 + Response_2$

CS, conditioned stimulus.

Such a seemingly minor methodological difference actually affects the affordance-like properties of the outcome, i.e., its link with a motor program to obtain the outcome itself. According to some theories (Cisek, 2007), for instance, the presence of a response-outcome link may indeed enhance the motivational value of the outcome. Here, we ask whether such affordance-like properties may also be transferred to the CS associated with that outcome during Pavlovian conditioning and, in turn, impact its ability to elicit a general transfer effect. In other words, if we encounter the logo (i.e., a conditioned stimulus) of a food that we never purchased before (i.e., for which there is no motor program available in our past experience), will it prompt us to go get some food, or not?

Therefore, the second aim of the present study was to test whether response-outcome associations affect the general transfer effect. To address this aim, a modified version of the Pavlovian-to-Instrumental Transfer task was developed in order to allow us to directly compare, in each participant, the effect of a Pavlovian conditioned stimulus (CS) paired with an action-associated outcome ($CS+_{action}$), with a CS paired with an outcome that was never associated with an action ($CS+_{no-action}$).

Materials and methods

Participants

Thirty-eight volunteers (20 females; mean age = 23.18; sd = 4.97 years; mean education = 15.32; sd = 2.04 years) with no history of neurological or psychiatric diseases were recruited for the study. All participants gave their written informed consent to take part in the experiment. The number of participants was established based on a power analysis conducted on MorePower 6.0 (Campbell and Thompson, 2012), with the following parameters: RM design factors = 1 factor (3 levels); RM effect of interest = 1 factor (3 levels); effect size (η^2) = 0.12; significance level (Alpha 2-sides) = 0.05; power = 0.8. The effect size was estimated based on the average effect size of all previous studies conducted with a similar task (Garofalo and di Pellegrino, 2015; Garofalo and Robbins, 2017; Garofalo et al., 2019, 2020, 2021).

The study was conducted in accordance with institutional guidelines and the 1964 Declaration of Helsinki and was approved by the Bioethics Committee of the University of Bologna.

Pavlovian-to-instrumental transfer task

The PIT task was structured in three phases, described in detail below, which followed previously validated paradigms (Nadler et al., 2011; Prévost et al., 2012; Garofalo et al., 2021): (1) Instrumental conditioning phase, in which the participant learned a response-outcome association; (2) Pavlovian Conditioning phase, in which the participant learned a conditioned stimulus (CS)-outcome association; (3) Transfer phase, in which the influence of the conditioned stimulus (CS) on the instrumental response was tested. In all task phases, an image of a slot machine was presented in the middle of a computer screen on a white background (**Figure 1**). The slot machine had two black displays (one on the top and one on the bottom) and three buttons. The task was programmed using OpenSesame3.2 software (Mathôt et al., 2012).

Instrumental conditioning phase

In this phase, participants learned the association between the three possible responses (R1, R2, and R3) and their respective rewarding outcomes (O1, O2, and O3) (**Table 2**). The response consisted of pressing one of three computer keys, corresponding to one of the three buttons of the slot machine (**Figure 1A**). Each time a computer key was selected, visual feedback was provided such that the corresponding button on the slot machine appeared as illuminated and pressed.

For each response, there was a 50% to receive the associated reward. The rewarding outcomes consisted of three food snacks used as separate rewards and presented on the lower display. In non-reinforced trials, a non-rewarding outcome (white "X") was presented. After each response, the corresponding outcome appeared for 1 s, during which no response was possible. All trials lasted 6 s, during which participants were free to press the three buttons as many times as they wished. During the inter-trial interval (ITI) the slot machine was still visible, but the buttons disappeared, and response options were not available for a jittered duration ranging between 1 and 2 s. Before starting the task, three training trials with no rewards were presented.

The task was structured in a series of blocks to be repeated until a learning criterion was reached. Each block terminated after a total of 24 rewards (8 for each response type) were obtained, for an average duration of about 3 min. At the end of each block, the question "What food did you win by pressing this button?" appeared (one for each response) to test whether all response-outcome associations were correctly established. These blocks were repeated from a minimum of two times to a maximum of eight times. The learning criterion consisted in correctly reporting the response-outcome associations at least two times in a row. If the learning criterion was achieved, the task moved to the following phase. After four wrong answers, the task was aborted.

Pavlovian conditioning phase

In this phase, participants learned the association between three colored cues (red, blue, and yellow) serving as conditioned stimuli (CS) shown on the upper display of the slot machine, associated with three separate outcomes, respectively (**Figure 1B**). The $CS+_{action}$ was paired with the same outcome

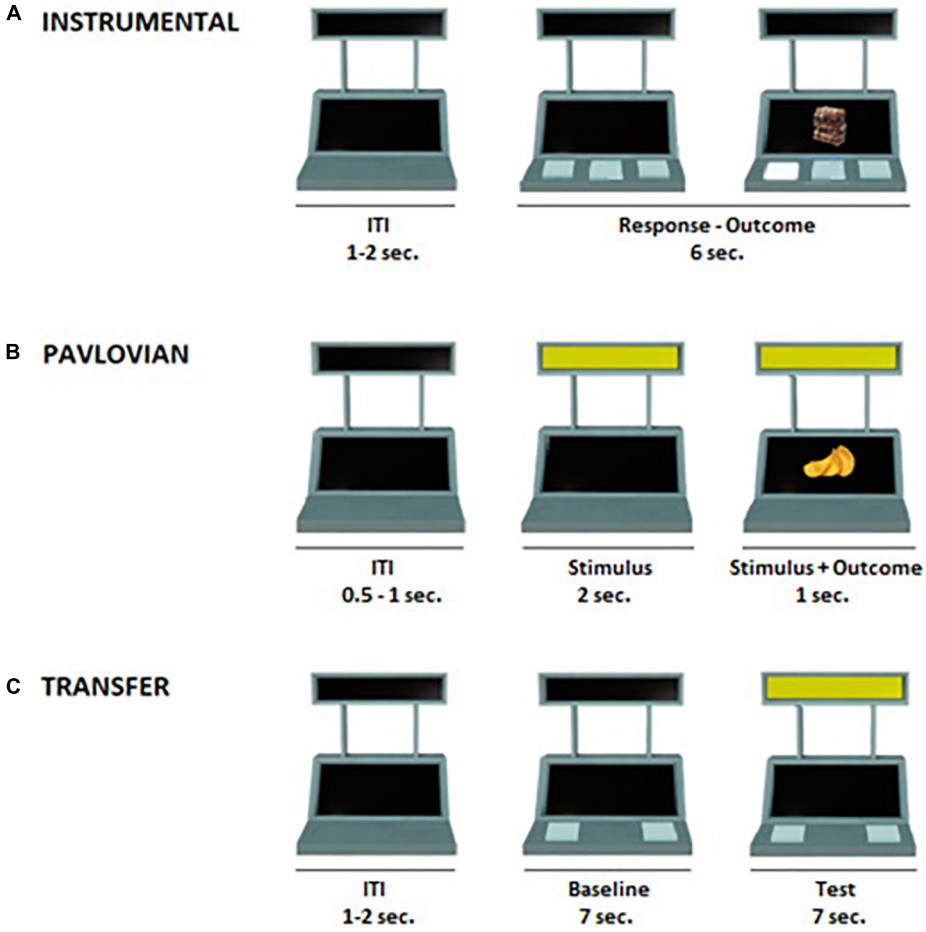

FIGURE 1
Illustration of the Pavlovian-to-Instrumental Transfer task. **(A)** Instrumental conditioning phase: participants learned the association between three distinct responses and three different food outcomes (e.g., $R_1 \rightarrow$ chocolate, $R_2 \rightarrow$ crackers, $R_3 \rightarrow$ candies). During each trial (6 s), participants were free to press several times the buttons. After each press, the corresponding outcome appeared for 1 second. The inter-trial interval (ITI) lasted 1–2 s. **(B)** Pavlovian conditioning phase: participants learned the association between three distinct colored cues and their respective outcomes. One cue was associated with the outcome corresponding to R_1 in the Instrumental conditioning phase ($CS+_{action}$; e.g., blue $\rightarrow$ chocolate), one cue was associated with a new outcome not previously available during the instrumental conditioning phase ($CS+_{no-action}$; e.g., yellow $\rightarrow$ chips) and a third cue was associated with a non-rewarding outcome (CS–; e.g., red $\rightarrow$ X). During each trial (3 s) one of the three cues appeared for 2 s, and the outcome was simultaneously presented with the cue during the last second of the trial. During this phase, no response buttons were available. The ITI lasted 0.5–1 s. **(C)** Transfer phase: during each trial (14 s), for the first 7 s (baseline) participants were free to press several times the two buttons as in the instrumental conditioning phase. In the following 7 s, participants were free to press several times the two buttons while the task-irrelevant CS was present. This phase was performed under nominal extinction. The ITI lasted 1–2 s.

(O1) associated with response 1 (R1) during the instrumental conditioning phase. The $CS+_{no-action}$ was paired with a food snack serving as new rewarding outcome (O4) not previously available during the instrumental conditioning phase (Table 2), and hence no corresponding response. These two stimuli were randomly rewarded with a 60% reinforcement rate. In the remaining trials, the non-rewarding outcome ("X") was presented. A third stimulus (CS–) was always paired with the non-rewarding outcome ("X"). Each trial consisted of variable ITI (0.5–1 s), in which the slot machine was "empty" (with no colors or rewards, as in the Pavlovian and instrumental conditioning phase), followed by the appearance of one of the CSs (3 s). The corresponding outcome appeared simultaneously to the CS during the last second. During this phase, no response buttons were represented and hence available.

The task was structured in a series of blocks to be repeated until a learning criterion was reached. Each block consisted of 45 trials (15 for each CS), for an average duration of about 3 min. At the end of each block, the question "What food did you win with this color?" appeared (one for each CS) to test whether all stimulus-outcome associations were correctly established. These blocks were repeated from a minimum of two times to a maximum of eight times. The learning criterion consisted in correctly reporting the stimulus-outcome associations at least

TABLE 2 Experimental design of the PIT task.

Instrumental conditioning	Pavlovian conditioning	Transfer
R1 → O1	$CS+_{action}$ → O1	$CS+_{action}$ → R2 + R3
R2 → O2	$CS+_{no-action}$ → O4	$CS+_{no-action}$ → R2 + R3
R3 → O3	CS− → No outcome	CS− → R2 + R3

R, Response; O, Outcome; CS, conditioned stimulus.

two times in a row. If the learning criterion was achieved, the task moved to the following phase. After four wrong answers, the task was aborted.

Transfer phase

This phase tested the influence of the Pavlovian conditioned stimuli (CSs) on the instrumental response. Each trial was structured as follows (**Figure 1C**): first, an empty slot machine (with no colors or rewards) appeared for a variable ITI length (1–2 s); then, two of the buttons previously trained during instrumental conditioning (R2 and R3) appeared for 7 s (baseline); finally, the task-irrelevant CSs ($CS+_{action}$; $CS+_{no-action}$; CS−) appeared for 7 s along with the two response options (test). During both baseline and test, participants were free to press the two buttons as many times as they wished (**Table 2**). This phase consisted of a total of 36 trials (12 for each CS), for about 10 min.

The whole phase was conducted under extinction, so no rewards were shown. Extinction is a standard procedure for assessing transfer, both in human and animal research, as it allows to test the influence of Pavlovian cues on instrumental responding without the confounding effects of the reward (Colwill and Rescorla, 1988; Bray et al., 2008; Talmi et al., 2008). Specifically, we employed a "nominal extinction" procedure in which participants were instructed that they were still winning but, since the lower display of the slot machine was malfunctioning, they would not be able to see the outcomes (Huys et al., 2011; Quail et al., 2017).

Procedure

The four rewards were tailored to each participant. Upon recruitment, participants rated the subjective liking of a set of 21 different food items (10 savory foods and 11 sweet foods). For each participant, the experimenter selected four highly and equally valued foods on a 5-point Likert scale ranging from 0 (Not at all) to 5 (Very much). These foods were later used as rewards for the experiment using the same images.

Participants were asked to refrain from eating for 3 h prior to the experiment. Before starting the experimental session, a new liking and wanting 9-point Likert scale, ranging from 0 (not at all) to 9 (very much), were presented for the four foods previously detected, to ensure comparable values between the four rewards. Specifically, we showed the picture of each food and asked to participants the following questions: "How much do you usually like to eat it?" and "How much would you like to eat it now?", respectively for investigating general liking and current wanting of the rewards. If the participant expressed a preference for one reward over the others, such reward would be substituted with a comparable one. Participants were also asked to rate their current level of hunger.

The experimental session lasted about 45 min and the participant could rest between the phases to prevent fatigue and loss of attention. After providing informed consent, participants were comfortably seated in a silent room and their position was centered relative to the computer screen at about 60-cm viewing distance. The experimenter placed on the table all the food previously chosen by the participant, to ensure a high level of motivation toward the food throughout the task. Participants were informed that, at the end of the experiment, they would receive an amount of food proportional to the number of food pictures visualized during all tasks. In each phase, participants were required to pay attention to the screen and follow the instructions reported at the beginning of the phase.

Statistical analysis

Analyses were performed with JASP 0.16 (Love et al., 2019) using a Bayesian inferential approach in order to get robust estimates of parameter values and their credible intervals, quantifying support in favor of the null hypothesis (corresponding to the possibility that action and no-action condition may be comparable), and use a model selection procedure (i.e., Bayesian informative Hypothesis) to compare and contrast a broader range of scientific expectations than the standard null and alternative hypotheses (Hoijtink, 2011; Kruschke, 2021).

For Bayesian analyses of variance (ANOVA), the Bayes Factor (BF_{10}) is reported as the probability associated with the alternative hypothesis (H_1) over the null hypothesis (H_0), along with its estimated proportional error (err%) (Kruschke, 2021). Bayes factor could be summarized in terms of discrete categories of evidential strength. Following the classification proposed in literature (Lee and Wagenmakers, 2013; Andraszewicz et al., 2015), the BF_{10} can be placed on a continuum from "no evidence" ($BF_{10} = 1$) to "extreme evidence" ($BF_{10} > 100$), including "anecdotal evidence" ($1 < BF_{10} \leq 3$), "moderate evidence" ($3 < BF_{10} \leq 10$), "strong evidence" ($10 < BF_{10} \leq 30$), "very strong evidence" ($30 < BF_{10} \leq 100$). Data are presented as model-averaged posterior distributions and the uncertainty is expressed by the credible interval around the median. Examination of the data distribution ensured that the assumptions for ANOVA were not violated.

In order to provide an assessment of the robustness of the Bayes factor under different prior specifications, a sensitivity

analysis was conducted. Sensitivity analysis shows how sensitive the posterior distribution is to the choice of prior distribution: if the qualitative conclusions do not change across a range of different plausible prior distributions, it means that the analysis is relatively robust (Kruschke, 2021; van Doorn et al., 2021).

Estimation plots were used to further illustrate relevant comparisons between conditions (Cumming, 2014; Ho et al., 2019). The web application available at https://www.estimationstats.com/ was used for this purpose. Estimation plots show individual data points for each condition and the paired difference with 95% bias-corrected accelerated confidence interval (CI) based on 5,000 bootstrap samples. Paired differences across conditions were estimated based on the mean (Δ_{mean}). The inference was based on the inspection of the estimated difference across conditions (Δ_{mean}) and the precision of such estimate (i.e., length of the CI): intervals including 0 were interpreted as indicative of no evidence of effect; intervals not including 0 were interpreted as indicative of weak, moderate, or strong evidence of effect based on the size of the estimated difference and its precision (the longer the CI, the lower the precision, and the weaker the evidence) (Cumming, 2014; Calin-Jageman and Cumming, 2019).

Bayesian Informative Hypotheses were used to provide a joint evaluation of three alternative models reflecting alternative expectations (Hoijtink, 2011; Kluytmans et al., 2012; Gu et al., 2019; Hoijtink et al., 2019b). Each model expresses a specific hypothesis that can be defined in terms of equality and/or inequality constraints among the parameters. For example, three equal parameters can be represented by an equality constrained hypothesis H_0: A1 = A2 = A3, and three ordered parameters can be represented by an inequality constrained hypothesis H_1: A1 > A2 > A3. The analysis can also include the unconstrained hypothesis (H_u), which is a hypothesis representing all possible sets of relationships between the parameters without constraints. The formulation of a model representing the null hypothesis is not mandatory and, as for any other model, should only be included if meaningful from a scientific point of view. For each hypothesis, the posterior model probability (PMP) is calculated *via* the Bayes theorem and expressed with a value between 0 and 1. This value can be interpreted as the relative amount of support for each hypothesis given the data and the set of competing hypotheses included (the sum of all posterior model probabilities adds up to 1). The model with the highest PMP reflects the best hypothesis, i.e., the hypothesis with the highest relative probability (Béland et al., 2012; Hoijtink, 2011; Kluytmans et al., 2012; Hoijtink et al., 2019a,b). To further support model selection, the PMPs can also be compared *via* Bayes factor to (a) that of the other hypotheses tested, (b) to its complement hypothesis (i.e., a model that contains any set of restrictions between the parameters except the one represented by the hypothesis tested), or (c) to the unconstrained hypothesis (Hu) (Hoijtink, 2011; Hoijtink et al., 2019a,b).

Results

Liking and wanting

Participants reported comparable liking (BF_{10} = 0.05; err% = 0.69) and wanting (BF_{10} = 0.05; err% = 0.51) values for the four rewarding outcomes, validating the methodological accuracy in the selection of foods. The descriptive statistics are reported in **Table 3**.

TABLE 3 Descriptive statistics for liking and wanting.

	Liking				Wanting			
	Mean	SD	95% credible interval		Mean	SD	95% credible interval	
			Lower	Upper			Lower	Upper
O1	7.39	1.26	6.98	7.81	6.71	1.83	6.11	7.31
O2	7.24	1.32	6.8	7.67	6.39	1.57	5.88	6.91
O3	7.29	1.27	6.87	7.71	6.71	1.90	6.09	7.34
O4	7.45	1.27	7.03	7.86	6.66	1.79	6.07	7.25

TABLE 4 Average response rates for each conditioned stimulus (CS) at baseline and test.

	Baseline				Test			
	Mean	SD	95% Credible Interval		Mean	SD	95% Credible Interval	
			Lower	Upper			Lower	Upper
CS−	3.32	1.07	2.97	3.68	3.21	1.23	2.803	3.61
$CS+_{action}$	3.35	1.06	3	3.70	3.54	1.05	3.199	3.89
$CS+_{no-action}$	3.40	1.11	3.03	3.76	3.58	1.13	3.208	3.95

Instrumental and Pavlovian conditioning phases

During the instrumental conditioning phase, all participants successfully achieved the learning criterion. Overall, 97.4% (37 participants) always answered correctly and did not require additional repetitions of the blocks other than the minimum two required, while 2.6% (1 participant) got a question wrong once and had to repeat the blocks for a total three times.

During the Pavlovian conditioning phase, all participants successfully achieved the learning criterion. Overall, 94.7% (36 participants) did not require additional repetitions of the blocks other than the minimum two required, while 5.3% (2 participants) got a question wrong once and had to repeat the blocks for a total of three times.

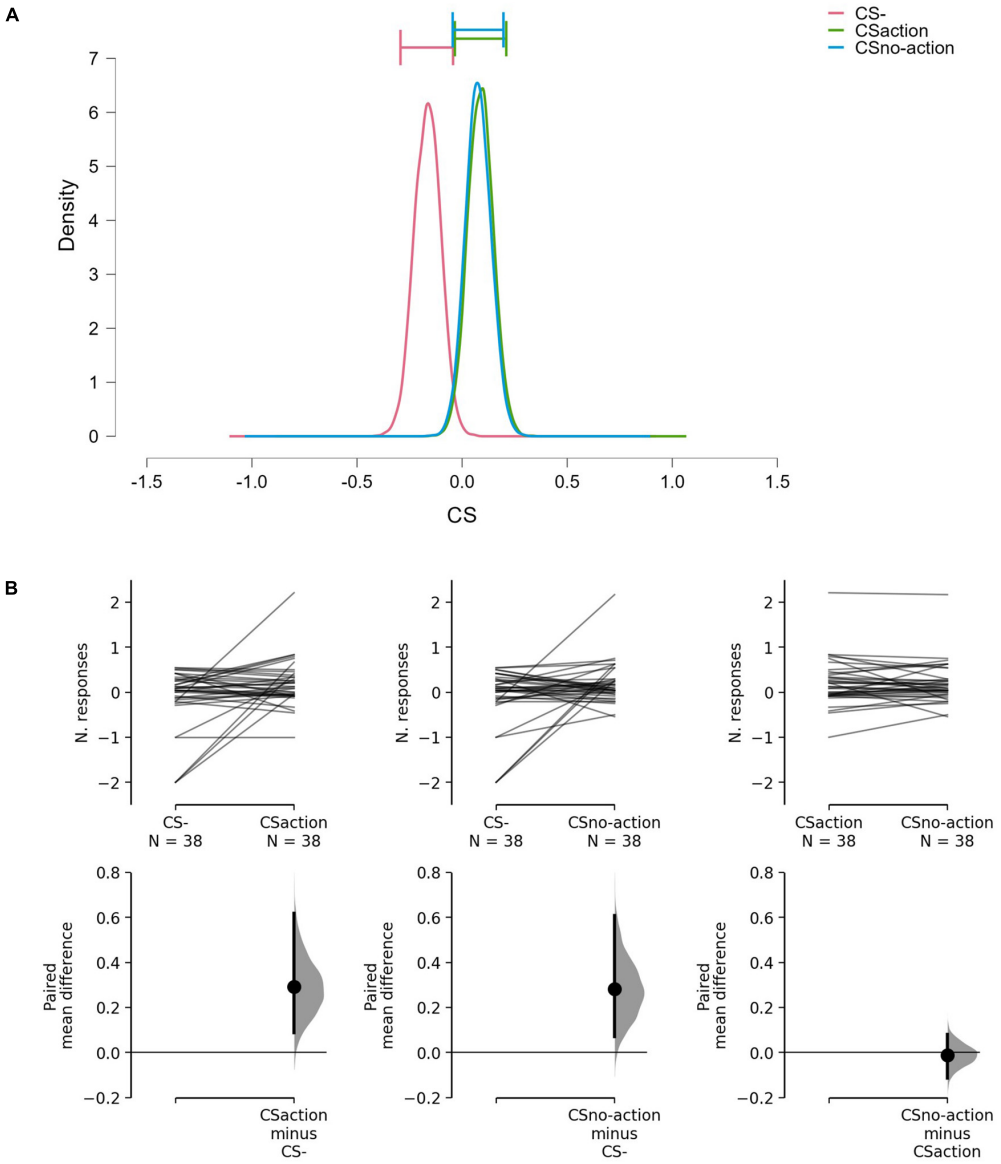

FIGURE 2
Model-averaged posterior distributions and estimation plots. **(A)** Model-averaged posterior distributions. Horizontal bars show 95% credible intervals around the median. **(B)** Estimation plots show raw data on the upper axes and paired mean difference between CS−, $CS+_{action}$, and $CS+_{no-action}$. On the upper axes, each paired set of observations is connected by a line. On the lower axes, 95% confidence intervals are indicated by vertical error bars, and mean differences, plotted as a bootstrap sampling distribution (5,000 samples), are depicted as dots. Data show increased response rate for $CS+_{action}$ and CS+no-action, compared to CS−, but no evidence of difference between $CS+_{action}$ and $CS+_{no-action}$.

Transfer phase

To test the presence of differences among the effect exerted by the three CSs on instrumental responding, we conducted a Bayesian one-way repeated measures Anova with the type of CS as the independent variable (3 levels: CS–, CS+$_{action}$, CS+$_{no-action}$) and the baseline-corrected average number of R2 + R3 in each trial as dependent variable. For baseline correction, the average number of R2 + R3 responses during baseline was subtracted from that performed at test, for each trial. Descriptive statistics for each CS separated for baseline and test are reported in Table 4.

Results showed differences between the CSs (BF_{10} = 3.16; err% = 0.79), suggesting that the alternative hypothesis (H_1: CS+$_{action}$ ≠ CS+$_{no-action}$ ≠ CS–) predicts the observed data 3.16 times better (moderate evidence) than the null hypothesis (H_0: CS+$_{action}$ = CS+$_{no-action}$ = CS–). The model-averaged posterior distributions (Figure 2A) show a clear separation between CS– and both CS+$_{action}$ and CS+$_{no-action}$. Estimation plots (Figure 2B) confirmed the presence of increased response rate for both CS+$_{action}$ (Δ_{mean} = 0.29, 95% CI [0.09 0.62]) and CS+$_{no-action}$ (Δ_{mean} = 0.28, 95% CI [0.07, 0.61]) as compared to the CS–, and no evidence of differences between CS+$_{action}$ and CS+$_{no-action}$ (Δ_{mean} = –0.01, 95% CI [–0.11, 0.08]).

Sensitivity analyses (Figure 3) showed that both evidence for CS+$_{action}$ and CS+$_{no-action}$ (Figures 3B,C), and evidence for the null hypothesis (CS = 0) for CS– (Figure 3A), were relatively stable across a wide range of prior distributions, supporting the robustness of the analysis.

Bayesian Informative Hypotheses were used to clarify whether CS+$_{action}$ and CS+$_{no-action}$ exert a different influence over response rate, relative to CS–. More specifically, we formulated three hypotheses about response rate, which were tested against each other. The first hypothesis posited that the CS+$_{action}$ exerted a stronger influence over response rates than CS+$_{no-action}$:

$$H_1 : CS+_{action} > CS+_{no-action}$$

The second hypothesis posited that the CS+$_{no-action}$ exerted a stronger influence over response rates than CS+$_{action}$:

$$H_2 : CS+_{no-action} > CS+_{action}$$

The third hypothesis posited that CS+$_{action}$ and CS+$_{no-action}$ exerted an equally stronger influence over response rates than CS–:

$$H_3 : (CS+_{action} = CS+_{no-action}) > CS-$$

The resulting posterior model probabilities showed that H_3 presented the highest relative probability, thus indicating this as the strongest hypothesis both when excluding (PMPa in Table 5 and Figure 4A) or including (PMPb in Table 5 and Figure 4B) the unconstrained hypothesis (H_u). In line with this, H3 also presented the highest Bayes Factor computed relative to its complement hypothesis (BFc in Table 5) and to the unconstrained hypothesis (BFu in Table 5). Overall, these analyses confirmed that CS+$_{action}$ and CS+$_{no-action}$ conditions exert a similar influence on response rates.

Discussion

The first aim of the present study was to test the ability of Pavlovian cues to enhance instrumental responses that were paired with outcomes (motivationally similar but sensorially) different from those paired with the CS. In particular, we aimed to test whether general transfer could emerge in isolation

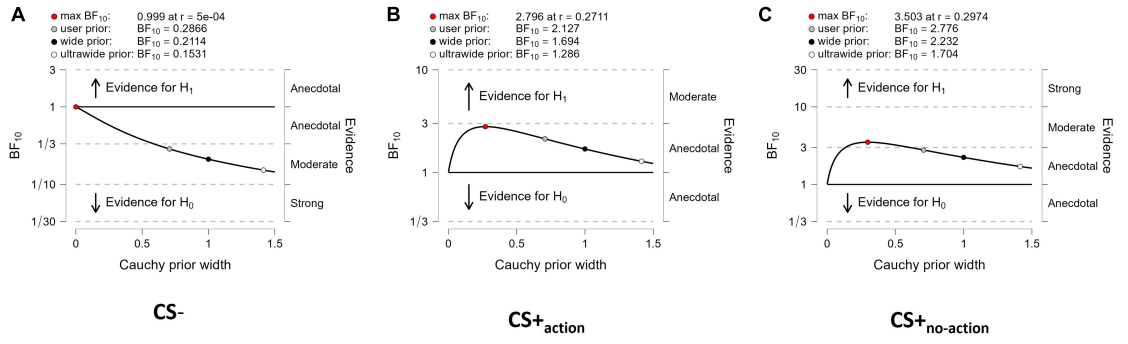

FIGURE 3
Sensitivity analysis. The Bayes factors are calculated over a range of prior width values from 0 to 1.5. The analysis provides BF10 values over a selection of four prior widths (max: maximum attainable Bayes factor, user: user-specified prior, wide: width of 1, and ultrawide: 1.4), for CS- **(A)**, CS+$_{action}$ **(B)** and CS+$_{no-action}$ **(C)**. The labels on the right of each panel show the robustness of the evidence for the alternative or null hypothesis.

TABLE 5 Bayesian informative hypothesis.

	BF.u	BF.c	PMP a	PMP b
H1	1.07	1.15	0.22	0.18
H2	0.93	0.87	0.19	0.16
H3	2.92	120.87	0.59	0.49
Hu				0.17

The table represents the results from the comparison of the three models (H_1, H_2, and H_3) *via* Bayesian Informative Hypothesis.

BFu, Bayes Factors of the hypothesis in the row vs. the unconstrained hypothesis and complement hypothesis; BFc, Bayes Factors of the hypothesis in the row vs. the complement hypothesis; PMPa, posterior model probability excluding the unconstrained hypothesis; PMPb, posterior model probability including the unconstrained hypothesis.

and independently of outcome-specific transfer, as studying general transfer in isolation may be crucial to disentangle the nature of the two transfer effects (Cartoni et al., 2016; Mahlberg et al., 2021). Indeed, Pavlovian cues can exert general motivational effects on behavior by increasing the likelihood of an instrumental response even when cue and response were previously associated with (motivationally similar but sensorially) different outcomes (e.g., Corbit and Balleine, 2005, 2011). This general (Pavlovian-to-instrumental) transfer effect can be differentiated from outcome-specific transfer in which a Pavlovian cue can increase the likelihood of a response associated with the same outcome as that signaled by the cue. Our results show, for the first time in humans, that general transfer can be elicited and therefore studied without the concurrent presence of outcome-specific transfer. Specifically, we found that the presence of cues previously associated with a rewarding outcome (CS+) increased the number of responses as compared to a cue that had been never associated with a reward (CS–).

It is well-established in literature that general transfer reflects a motivational process (Holland, 2004; Corbit and Janak, 2007). During a decision, when a Pavlovian cue cannot drive your choice toward one of two outcomes, it enhances the general vigor of your action, due to the motivational commonalities between the outcomes currently presented and the outcome that was previously associated with that cue (Dickinson and Dawson, 1987; Dickinson and Balleine, 1990). In other words, a representation of the outcome based on its motivational/affective value (value-based representation) is generated and leads to an association with the CS (independently from its sensory-specific characteristics). It allows to increase the general motivation toward similar outcomes, producing general transfer (Balleine, 1994; Dickinson and Balleine, 2002; Holland, 2004). Conversely, the nature of outcome-specific transfer is more debated. It seems to be mediated by a representation of the outcome based on its sensory-specific features (sensory-based representation), but also the involvement of motivational factors has been hypothesized in the emergence of that effect (Hinojosa-Aguayo and González, 2020). Specifically, together with the sensory-based representation of the outcome, also the value-based representation (Sommer et al., 2022) and the perceived outcome availability (Seabrooke et al., 2019) might drive outcome-specific transfer. Moreover, outcome-specific transfer has been reported to selectively require high-level cognitive abilities, such as working memory (Garofalo et al., 2019), and supraliminal (vs. subliminal) presentation of the reward-associated cues (Garofalo et al., 2020), as well as the involvement of the lateral prefrontal cortex (Garofalo et al., 2021). Within this supposedly hierarchical structure of cue-guided choices, which implies a continuum between low to high cognitive processes, studying general and outcome-specific transfer simultaneously does not allow to establish that the observed general transfer effect is due solely to motivational processes, as it may be influenced by higher cognitive strategies required for outcome-specific transfer, thus creating a possible confound.

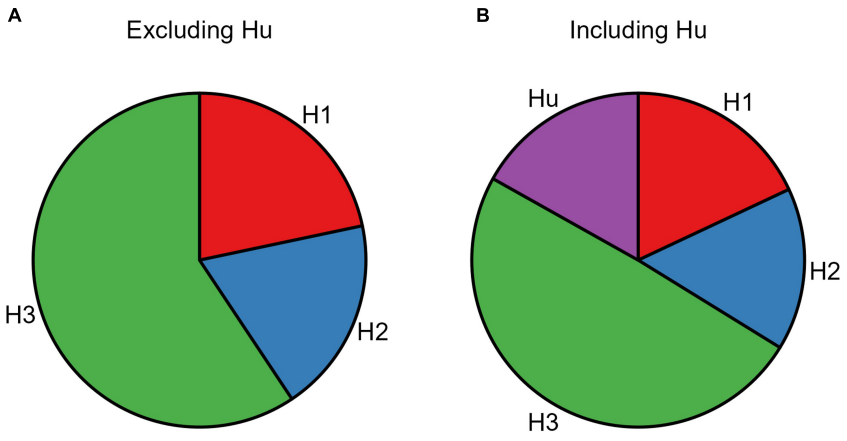

FIGURE 4
Pie chart of the posterior model probabilities. The two pie charts represent the posterior model probabilities associated with the three models (H1, H2, and H3), when excluding **(A)** or including **(B)** the unconstrained hypothesis (Hu).

Moreover, studying each effect in isolation can inform clinical practice by helping to understand which mechanism is at play in the maladaptive behavior and should, thus, be tackled. In line with this, several studies suggest that an alteration of general transfer contributes to relapse in maladaptive behaviors (for a review, see Doñamayor et al., 2021) like drug addiction (Corbit and Janak, 2007) and alcohol use disorder (Sommer et al., 2017, 2020). The selective involvement of general transfer in maladaptive cue-guided choice suggests that treatments should focus on modifying the motivational aspects of the outcomes involved in the maladaptive conduct. This hypothesis finds preliminary evidence in a study by Schad et al. (2019), which found that, in detoxified patients with alcohol use disorder, alcohol-related outcomes may acquire an aversive value and induce an inhibitory effect, reducing the general transfer effect and the probability of relapse. These results can be interpreted as a reduction of the general transfer due to a natural change in the motivational value of the outcome.

The second aim of the present study was to clarify whether response-outcome associations can affect the general transfer effect. More specifically, we aimed to contrast the effect of a Pavlovian conditioned stimulus paired with an action-associated outcome ($CS+_{action}$), with a CS paired with an outcome that was never associated with an action ($CS+_{no-action}$). In other words, we tested whether manipulating the affordance-like properties of two outcomes (one response-paired and one not) and in turn those of the two associated stimuli ($CS+_{action}$ and $CS+_{no-action}$, respectively), affected general transfer. Our results indicated that general transfer is found regardless of the affordance properties of the CS. Indeed, the number of responses to the $CS+_{action}$ was comparable to those of the $CS+_{no-action}$.

Together our results expand the current major theoretical accounts of the transfer effect, which state that general transfer is independent from the sensory-specific characteristics of the outcome, by adding that it is also independent from its motor-related characteristics. Indeed, the presence (or absence) of affordance-like information associated with the CS modulated neither the probability nor strength of the general transfer effect. This indicated that, at least behaviorally, previous experience with the action or the motor program that leads to the desired outcome does not impact the effect that an environmental cue can have on choice (Starita et al., 2022). To answer our initial research question, even seeing the logo of food that we never purchased before can drive us to get some food.

Crucially, these observations may not apply to outcome-specific transfer, which may be more sensitive to the motor properties of an outcome and thus possibly transferred to the associated CS. Furthermore, such absence or difference at the behavioral level may or may not be reflected at the neural level. Future studies may try to clarify that.

In conclusion, the present findings constitute the first evidence that general transfer can emerge independently of outcome-specific transfer in humans, supporting the idea that the incentive motivational mechanism behind general PIT is independent of the motor features of the outcome.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://osf.io/57uz6/.

Ethics statement

The studies involving human participants were reviewed and approved by the Bioethics Committee of the University of Bologna. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SG, LAED, FS, and GdP conceived and developed the main idea and study design. LAED and DD carried out testing and data collection. LAED and DD performed the analysis under the supervision of SG and GdP. LAED wrote the main manuscript text in collaboration and according to the critical revisions of DD, SG, GdP, FS, and MB. All authors read and approved the final version 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.

Publisher's note

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Amygdala response predicts clinical symptom reduction in patients with borderline personality disorder: A pilot fMRI study

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Borderline personality disorder (BPD) is a prevalent, devastating, and heterogeneous psychiatric disorder. Treatment success is highly variable within this patient group. A cognitive neuroscientific approach to BPD might contribute to precision psychiatry by identifying neurocognitive factors that predict who will benefit from a specific treatment. Here, we build on observations that BPD is accompanied by the enhanced impact of the aversive effect on behavior and abnormal neural signaling in the amygdala. We assessed whether BPD is accompanied by abnormal aversive regulation of instrumental behavior and associated neural signaling, in a manner that is predictive of symptom reduction after therapy. We tested a clinical sample of 15 female patients with BPD, awaiting dialectical behavior therapy (DBT), and 16 matched healthy controls using fMRI and an aversive Pavlovian-to-instrumental transfer (PIT) task that assesses how instrumental behaviors are influenced by aversive Pavlovian stimuli. Patients were assessed 1 year after the start of DBT to quantify changes in BPD symptom severity. At baseline, behavioral aversive PIT and associated neural signaling did not differ between groups. However, the BOLD signal in the amygdala measured during aversive PIT was associated with symptom reduction at 1-year follow-up: higher PIT-related aversive amygdala signaling before treatment was associated with reduced clinical improvement at follow-up. Thus, within the evaluated group of BPD patients, the BOLD signal in the amygdala before treatment was related

to clinical symptom reduction 1 year after the start of treatment. The results suggest that less PIT-related responsiveness of the amygdala increases the chances of treatment success. We note that the relatively small sample size is a limitation of this study and that replication is warranted.

KEYWORDS

borderline personality disorder, dialectical behavior therapy (DBT), fMRI, amygdala, Pavlovian-to-instrumental transfer

Introduction

Borderline personality disorder (BPD) is a prevalent and devastating psychiatric disorder associated with severe functional impairments and high mortality rates (American Psychiatric Association, 2000; Grant et al., 2008; Bolton and Robinson, 2010). Costs for society are high due to heavy use of expensive health care resources and persistent lack of productivity (Wunsch et al., 2014). Optimizing care for this patient group is of major importance (Gunderson, 2009).

Although several psychotherapeutic treatments exist for BPD, the response is highly variable and treatment effects are modest overall (Stoffers et al., 2012). For example, 27-35% of patients continue to have admissions, harm themselves, and conduct suicidal gestures (Lana and Fernández-San Martín, 2013). Only a few general predictors of outcome have been reported (Barnicot et al., 2012). The discovery of new outcome predictors is essential for the advancement of the field of personalized psychiatry. Neurocognitive mechanistic research might identify key predictors of available treatment outcomes and thus mitigate the large variability in treatment efficacy (Jones et al., 2015; Heinz et al., 2016; Huys et al., 2021). We report a proof-of-principle, pilot study focused on the relation between BPD symptom reduction over 1 year and affect-related neural processing, measured prior to the start of 1 year of dialectical behavior therapy (DBT).

Maladaptive and inflexible behavior in BPD has been argued to reflect derailed interaction between two principle controllers of human behavior, i.e., an instrumental and a Pavlovian controller (Hallquist et al., 2018). Instrumental control allows us to flexibly optimize our chances to achieve specific goals by learning what to do when (based on stimulus–action–outcome learning or operant conditioning). The Pavlovian system regulates inflexible, "automatic," motivational responses in reaction to external and internal emotional stimuli (based on stimulus–outcome learning or classical conditioning). In the context of BPD, the interaction between Pavlovian and instrumental control, the so-called Pavlovian-instrumental transfer (PIT), is particularly worth investigating, as dysregulation of this interaction has been related to heightened impulsivity (e.g., behavioral activation instead of inhibition by aversive contextual cues) (Breland and Breland, 1961; Guitart-Masip et al., 2014; Hinojosa-Aguayo and González, 2020; Geurts et al., 2022), increased influence of emotional/motivational states (e.g., hampering effective goal pursuit) (Dolan and Dayan, 2013; Watson et al., 2014) and interpersonal hypersensitivity (cf. Hallquist et al., 2018), and a combination of symptomatology lying at the core of BPD (Gunderson, 2009). Here, we will probe whether BPD is indeed characterized by an aberrant influence of the Pavlovian system by assessing PIT in BPD patients and healthy controls. Critically, we will explore within the group of BPD patients whether neurocognitive correlates of PIT are related to symptom reduction over 1 year of DBT.

Relying on the biosocial model of emotion regulation, DBT is one of the leading evidence-based psychotherapies for BPD with the main focus on skillfully regulating impulsive and emotion-driven behavior (Linehan, 1993). DBT teaches how aversive motivational tendencies can be accepted and dealt with skillfully through the training of skills like mindfulness, distress tolerance, emotion regulation, and interpersonal effectiveness. Thus, DBT might help optimize the interaction between aversive motivational (Pavlovian) influences and instrumental behavior. In this manuscript, we assess the 'vulnerability' of instrumental, goal-appropriate behaviors to disruptions by aversive Pavlovian conditioned stimuli (CS). For this purpose, we used a previously validated behavioral PIT task that allows us to quantify the impact of motivational cues on instrumental decision-making.

Specifically, we measure aversive PIT, which refers to the observation that aversive instrumental actions, such as inhibition and withdrawal, are potentiated in the context of aversive Pavlovian CS, i.e., stimuli that predict aversive outcomes. Thus, aversive Pavlovian CS have been shown to inhibit instrumental approach actions (i.e., aversive inhibition) and to enhance instrumental withdrawal actions (Huys et al., 2011; Geurts et al., 2013a). Accumulating evidence from experimental studies with animals and healthy humans (Talmi et al., 2008; Prevost et al., 2012; Geurts et al., 2013a) and patients (Garbusow et al., 2016; van Timmeren et al., 2020) demonstrates the involvement of (prefrontal) limbic circuitry in PIT, including the ventral striatum and amygdala (Cardinal et al., 2002; Talmi et al., 2008; Balleine and Doherty, 2009;

Prevost et al., 2012; Geurts et al., 2013a; Ly et al., 2014). The involvement of the amygdala is particularly relevant in the context of the current study, because the amygdala has also been central to neurocognitive theories and empirical research on BPD (Minzenberg et al., 2007; Hazlett et al., 2012; Soloff et al., 2017; Degasperi et al., 2021). For example, a recent meta-analysis reported functional hyperactivity of the left amygdala during aversive vs. neutral stimuli, as well as smaller gray matter volume of the amygdala in BPD (Schulze et al., 2016, 2019). This amygdala hyperactivation has been proposed to reflect the deviant salience of negative emotional stimuli and to be remediated by psychotropic medication (Schulze et al., 2016) and psychotherapy (Iskric and Barkley-Levenson, 2021) in BPD. We note that it is unclear whether remediation of amygdala hyperactivity is related to specific treatments or whether it is a general prerequisite for recovery from borderline symptomatology. Notwithstanding this ambiguity, evidence shows that effects of DBT are also associated with changes in blood oxygen level-dependent (BOLD) signal in the amygdala (Schnell and Herpertz, 2007; Krause-Utz et al., 2014; Salvador et al., 2016; Iskric and Barkley-Levenson, 2021). Here, we build on these previous findings by assessing the hypothesis that BPD is accompanied by abnormalities in aversive PIT and associated BOLD signal in the amygdala. Moreover, we ask whether aversive PIT and related amygdala signal before the start of therapy is associated with symptom reduction after treatment (Schmitt et al., 2016; cf. Schmitgen et al., 2019).

Thus, we hypothesize that borderline symptomatology might result from an imbalance between two major control systems of behavior: the motivational, reactive Pavlovian system on the one hand and a goal-oriented, instrumental system on the other. We explore this hypothesis by first investigating differences in baseline performance on a behavioral PIT task between healthy controls and BPD patients. Based on the above findings, we hypothesized that, relative to controls, BPD patients exhibit the enhanced impact of aversive Pavlovian CS on instrumental behavior, that is, greater aversive PIT (i.e., increased behavioral inhibition and withdrawal). Furthermore, we expect increased PIT-related BOLD signal in BPD relative to controls in the amygdala. Critically, we expect that the between-subject differences in amygdala response are related to symptom reduction across 1 year of DBT in the BPD group.

Materials and methods

Participants

To maximize external validity, we aimed for a patient sample that would represent patients treated in general mental health practice as closely as possible (Hoertel et al., 2015). Therefore, all patients who were enrolled in the pre-treatment phase of a 1-year DBT program at the Radboud University Medical Centre between March 2012 and March 2013 (n = 29) were invited to participate in this study. Twenty-three patients volunteered. Imaging datasets were obtained for 15 patients (all women), and clinical outcome measures after treatment were obtained for 14 of these patients (see **Supplementary materials** for details on inclusion). In addition, 16 healthy (MINI-plus) controls matched for gender and age were recruited per advertisement (for group demographics and questionnaire scores, see **Table 1**, and for comorbidity and medication use of the BPD group, see **Supplementary Table 1**). The local Medical Ethical Committee approved the study (NL36001.091.11), and consent was obtained from all participants.

Procedure

All patients enrolled in the pre-treatment phase of DBT were invited to attend three sessions: the first was a screening session, the second was a pre-treatment scan session just before treatment, and the third was a post-treatment assessment.

Screening session

During the screening session, participants received a full diagnostic structured interview, which included the MINI-plus international neuropsychiatric interview and the Structured Clinical Interview for DSM-IV Axis II disorders (SCID-II), administered by a senior resident in psychiatry (author DG). To familiarize subjects during the first visit with the scanning environment and procedures, we employed a short scan session of about 15 min during which a structural MRI scan was obtained and subjects were familiarized with the instructions and instrumental and Pavlovian training stages in the scanner.

Pre-treatment scan session

During the second visit, before treatment started, subjects completed several questionnaires (**Table 1**), of which the Borderline Personality Disorder Checklist (BPD47) measuring the symptom severity was of primary interest. Before entering the scanner, instructions on the computer task were repeated orally. After receiving the instructions for a third time, now projected on the scanner screen, they started the PIT paradigm (**Figure 1**). After a 15-min break, subjects performed a short neuropsychological test battery (**Table 1**).

Treatment

Participants received a 1-year group version of the standard DBT protocol (Linehan, 1993; Gutteling et al., 2012) divided into

the standard 4 weekly components (DBT group psychotherapy, groups skills training, 24/7 telephone coaching, and a therapist consultation team). The program differed from standard DBT only in that the weekly psychotherapy sessions were offered not individually but in groups. All DBT strategies (dialectics, behavior chain analysis, radical acceptance strategies of validation and mindfulness, contingency management, exposure, cognitive restructuring, and skills training) were used across all components addressing the five functions of DBT (increasing behavioral capabilities, improving motivation for skillful behavior, generalization of skills to the natural environment, reinforcement of functional over dysfunctional behavior, and enhancing therapist effectiveness) and were performed by well-trained DBT therapists and skill trainers. Although more elaborate research is needed to show that scaled versions as described above are as effective as standard DBT, Gutteling et al. (2012) demonstrated evidence that suggests that this scaled version of DBT is as effective as standard DBT for the treatment of borderline patients.

Post-treatment, follow-up session

The third and final follow-up session followed after treatment had ended, approximately 1 year after the pre-training scan session. Subjects completed the same questionnaires and participated in the same neuropsychological test battery as in the second session (Table 1). In addition, the MINI was administered once again to investigate whether axis I classifications had changed and the BPD47 to measure changes in borderline symptom severity.

Pavlovian-instrumental transfer paradigm

Participants performed a computerized PIT task to assess how instrumental approach and withdrawal actions are influenced by aversive Pavlovian CS, i.e., aversive PIT (Geurts et al., 2013a). The experiment consisted of three stages: (1) instrumental conditioning, (2) Pavlovian conditioning, and (3) PIT (see Figure 1 for a global overview and Table 2 for details on the experimental layout).

Stage 1. Participants performed an instrumental learning task to earn as much money as possible. There were two Action Contexts in this task: (i) One in which the active response led to an approach and (ii) another in which the active response led to a withdrawal. In each context, different instrumental stimuli (mushrooms/shells) were repeatedly presented to the participant (Figure 1A). In

TABLE 1 Demographical and clinical characteristics of the borderline personality disorder and healthy matched control participants.

	Healthy controls		Borderline personality disorder group			
	Baseline				1-year follow-up	
Size of group	***N* = 16**		***N* = 15**		***N* = 14**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age	29.5	8.8	28.5	8.8	–	–
IQ (NLV)	101.8	12.3	100.3	11.5	–	–
Right handedness	16	–	14		13	
BPD47	6.7	6.5	79.7***	33.2	64.8$^{\#}$	30.0
OQ – total	42.5	20.6	91.5**	19.2	79.1$^{\#\#}$	22.5
Sympt. distr.	19.1	10.6	56.7**	14.1	50.0$^{\#}$	16.5
Inter. pers.	8.8	4.9	20.2**	3.8	17.5	5.4
Social role	8.8	4.2	15.0**	4.5	11.6$^{\#}$	3.6
BDI-II	3.6	4.0	33.4***	14.3	28.4	14.0
BIS	18.4	7.1	23.5***	4.1	24.4	3.8
BAS	38.7	14.7	40.1***	5.8	41.4	5.1
Box Completion (s)	85.4	30.7	107.0**	20.9	96.8	27.4
Digit Span	13.2	2.5	16.2	4.0	15.2	4.1
Forward	7.1	1.6	8.3	1.9	7.7	2.2
Backward	6.0	1.3	7.9	2.4	7.5	2.4
Verbal Fluency	44.6	12.1	38.1	11.4	42.3	10.3

SD, standard deviation; NLV, Dutch reading test; BPD47, Borderline personality disorder checklist; OQ, outcome questionnaire; BDI-II, Beck depression index 2nd version; BIS, behavioral inhibition systems; BAS, behavioral activation system.

* Indicate significant differences between the groups (HC vs. BPD: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)), # indciate significant differences between baseline and follow-up measurement ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

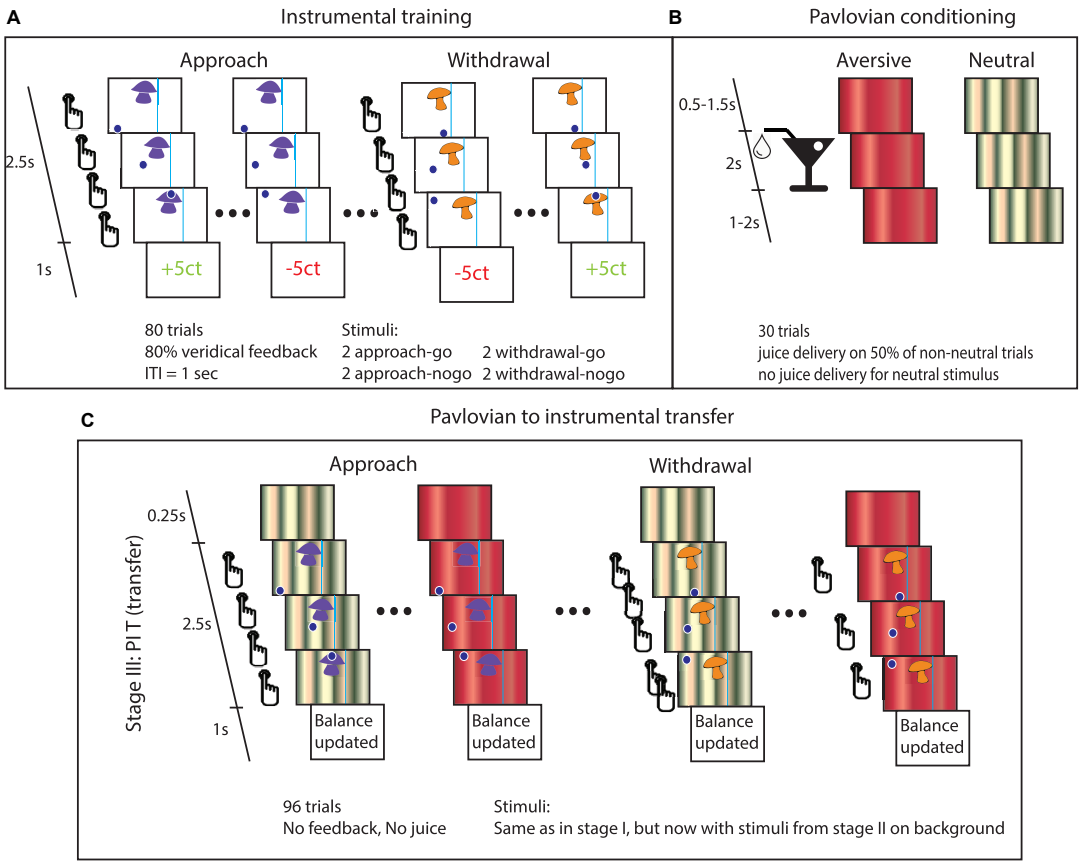

FIGURE 1
Task details. **(A)** Instrumental stage. Trials started with the appearance of the instrumental stimulus at the top center of the screen and a dot at the bottom of the screen. In approach trials, the dot appeared either on the left or on the right bottom of the screen. From left to right: Participants could choose to do nothing (approach-no-go), in which case the dot would move past the instrumental stimulus. Alternatively, they could press the button repeatedly to steer the dot through the instrumental stimulus (approach-go). In withdrawal trials, the dot started centrally beneath the instrumental stimulus. Participants could choose to press the button repeatedly to avoid moving through instrumental stimulus (withdrawal-go) or to do nothing (withdrawal-no-go). If the dot entered the target region, then the instrumental stimulus was 'collected'. The vertical line to one side of the instrumental stimulus could not be crossed by the dot. **(B)** Pavlovian conditioning. Participants were presented with different stimuli that were followed by juice delivery. **(C)** PIT stage. The PIT stage paralleled the instrumental training, except that Pavlovian CS tiled the background. The effect of interest is how the Pavlovian CS changed instrumental behavior (mean proportion of go-actions and the average number of button presses over the go-actions). Note that the trials involving the appetitive CS were omitted from this figure, because this particular paradigm has been shown to be insensitive to detecting appetitive PIT (see **Supplementary material**) and our hypotheses concern aversive PIT.

the approach, Action Context participants learned through monetary feedback (wins and losses) whether to 'collect' the instrumental stimulus (approach-go) or not (approach-no-go). In the withdrawal Action Context, they learned to avoid collecting instrumental stimuli (withdrawal-go) or not (withdrawal-no-go).

In both the approach and withdrawal Action Contexts, there were two go-stimuli, which yielded reward more often (i.e., 80% of the cases) after active responses (and punishment after not responding), and two no-go-stimuli, which yielded reward more often (i.e., also 80% of the cases) after not responding (and punishment after go-responding). Instrumental learning was assessed by calculating the proportion of correct responses (p(correct)) over time.

Stage 2. In this Pavlovian stage, different Pavlovian CS were conditioned (**Figure 1B**). During a classical conditioning procedure, three audiovisual stimuli were presented. The appetitive and aversive conditioned stimuli (CS) were followed, respectively, by appetitive or aversive juice (i.e., the unconditioned stimuli, USs) on 50% of trials. The neutral CS resulted in no outcome. The appetitive juice was based on subjective preference for apple, orange, or strawberry lemonade. The aversive juice was a bitter magnesium sulfate solution (0.3M).

Conditioning was assessed in two ways: (1) participants indicated the degree to which they liked each of the CS (and USs) by using a visual analog scale (VAS), before and after the experiment; and (2) participants chose one of the two presented

Pavlovian stimuli (presented for 2 s; ITI 0.5 s) in extinction on 12 interspersed query trials during the Pavlovian stage.

Stage 3. In the PIT stage, we tested how instrumental approach and withdrawal actions (trained in *stage 1*) are influenced by aversive Pavlovian CS (*conditioned in stage 2.)*. Therefore, stimulus presentation was the same as in the instrumental stage, except that Pavlovian CS from the Pavlovian stage 2 tiled the background from 250 ms before and during the trials, and this stage was run in nominal extinction, i.e., no juice or monetary outcomes were presented (**Figure 1C**). Participants were instructed that their choices counted toward the final monetary total and that the juices associated with the Pavlovian outcomes were collected outside the scanner for them to drink afterward. Whether instrumental approach and withdrawal actions were influenced by aversive Pavlovian CS was assessed per Action Context (approach/withdrawal) and CS stimulus (neutral/aversive).

There were two independent runs separated by a 2-min break (each including run-specific stimuli/CS), with each run including all three stages. Each instrumental stimulus was presented 12 times and each Pavlovian CS 32 times. These Pavlovian CS were counterbalanced over the eight instrumental stimuli.

Image acquisition

Whole-brain imaging was performed on a 1.5 Tesla MR scanner (Avanto, Siemens Medical Systems, Erlangen, Germany). Functional data were obtained using a multi-echo gradient T2*-weighted echo-planar (ME-EPI) scanning sequence (Poser et al., 2006) (see **Supplementary materials** for details).

Analysis

Our primary analysis was restricted to the PIT stage. Analysis and results of the instrumental and Pavlovian training data are presented in the **Supplementary materials**. The analyses presented below consist of two parts: First, we assessed the effects of the group on behavior and fMRI BOLD response during the PIT stage, measured at baseline. Here, we focus on both the behavioral and fMRI analyses in line with our hypothesis on aversive PIT. We discern two aspects of aversive PIT: Action Context-specific aversive PIT and aversive PIT that is independent of Action Context, i.e., aversive PIT across Action Contexts. Action Context-specific aversive PIT quantifies the differential effect of an aversive CS on approach and withdrawal behavior, whereas aversive PIT across Action Contexts quantifies general effects of the Pavlovian CS on instrumental behavior irrespective of whether it is approach or withdrawal behavior. Statistically, aversive PIT across Action Contexts is captured by the main effect of CS Valence (neutral vs. aversive across Action Contexts), while Action Context-specific aversive PIT is captured by the interaction between CS Valence and Action Context. These different aversive PIT effects have been associated in previous studies with different clinical outcomes and neural mechanisms (Geurts et al., 2013a,b; Garbusow et al., 2016; Huys et al., 2016). Specifically, while Action Context might arise from a vmPFC-dependent process (Geurts et al., 2013a) that is predictive of recovery from

TABLE 2 Experimental layout.

	# trials/time per trial(s)	# stimuli	Reinforcement
Instrumental training	80/2.5s		
Blocks of 8 trials per Action Context (approach/withdrawal)	20	2 stimuli requiring approach: $S^{I}_{1,2}$	80% reward/20% punishment for go
	20	2 stimuli requiring approach-nogo: $S^{I}_{3,4}$	80% reward/20% punishment for nogo
	20	2 stimuli requiring withdrawal: $S^{I}_{5,6}$	80% reward/20% punishment for go
	20	2 stimuli requiring withdrawal-nogo: $S^{I}_{7,8}$	80% reward/20% punishment for nogo
Pavlovian training	60/3s	3	
Each 10th trial a query trial to choose between two CS	20	1 stimulus followed by aversive juice → aversive CS: S^{P}_{1}	50% of trials are reinforced
	20	1 stimulus without reinforcement → neutral CS: S^{P}_{2}	No reinforcement
	20	1 stimulus followed by appetitive → juice appetitive CS: S^{P}_{3}	50% of trials are reinforced
Pavlovian to instrumental transfer	96/2.5s	4/3	
Blocks of 8 trials per Action Context (approach/withdrawal)	32	$S^{I}_{1,2,3,4,5,6,7,8} \mid S^{P}_{1}$	No direct reinforcement
	32	$S^{I}_{1,2,3,4,5,6,7,8} \mid S^{P}_{2}$	No direct reinforcement
	32	$S^{I}_{1,2,3,4,5,6,7,8} \mid S^{P}_{3}$	No direct reinforcement
2 Runs			

depression (Huys et al., 2016), the extent to which Pavlovian CS inhibit ongoing behavior across Action Contexts likely reflects amygdala/striatal activity and changes in serotonergic transmission (Geurts et al., 2013b), and is instead associated with the psychopathic tendency in a sample of violent offenders (unpublished findings, submitted to the current special issue of Frontiers in Behavioral Neuroscience).

Second, within the BPD group, we assessed whether aversive PIT and associated BOLD signals were associated with symptom reduction at the end of the 1-year DBT program.

We note in addition that our previous work in healthy controls, on which the current study builds, revealed that the current paradigm was not sensitive to (and therefore less valid to assess group effects on) appetitive PIT (Geurts et al., 2013a). We therefore only present the data on aversive PIT. In the **Supplementary material**, we confirm that, indeed, the current paradigm is not sensitive to appetitive PIT.

Pavlovian-instrumental transfer

Behavioral analyses

We focused our analyses on aversive PIT, i.e., the effect of aversive Pavlovian CS on instrumental behavior. The effects of Action Context (approach/withdrawal), CS Valence (neutral/aversive), and group (healthy controls/BPD patients) in the critical transfer test were assessed in terms of proportion of go-choices [p(go)] and the average number of button presses (BP, made during these go-choices). Note that our previous work in healthy controls, on which the current study builds, revealed that the current paradigm was not sensitive to (and therefore less valid to assess group effects on) appetitive PIT (Geurts et al., 2013a). We present behavioral data on appetitive PIT in the **Supplementary materials**.

Thus, analyses were targeted at the degree to which aversive CS influenced instrumental behavior. More specifically, we analyzed across Action Context (approach and withdrawal) how much the aversive Pavlovian CS (compared with the neutral CS) inhibited instrumental 'go' responding (i.e., the main effect of CS Valence). In addition, we also assessed the Action Context specificity of aversive PIT, i.e., to what extent the effect of the aversive Pavlovian CS is dependent on Action Context (i.e., interaction CS Valence X Action Context). The dependent variables were first averaged across runs and normality was assessed, before they were submitted to a repeated measures ANOVA (rmANOVA), with Action Context (approach/withdrawal) and CS Valence (neutral/aversive) as within-subject factors and group (healthy controls/BPD patients) as a between-subject factor. Due to non-normal distribution of p(go), we employed non-parametric tests to assess whether there was a significant aversive PIT effect across groups (related-samples Wilcoxon signed-rank test comparing the difference between p(go) for neutral Valence and p(go) for aversive as a function of Action Context) and whether there was a difference in aversive PIT between groups (independent samples median test comparing the compound measure of Action Context-specific aversive PIT, i.e., [(approach neutral- approach aversive – (withdrawal neutral - withdrawal aversive)] and aversive PIT across Action Contexts [(approach neutral + withdrawal neutral – (approach aversive - withdrawal aversive)] between groups).

fMRI analysis

An fMRI analysis was performed with SPM5 software (Wellcome Trust Centre for Cognitive Neuroimaging, London, United Kingdom). Pre-processing steps and first-level fMRI analysis were identical to those employed by Geurts et al. (2013a): First, realignment parameters were estimated for the images acquired at the first echo time and consequently applied to images resulting from the three other echoes. The echo images were combined by applying a PAID-weight algorithm assessing the signal-to-noise ratio as described by Poser et al. (2006). Thirty volumes, acquired before each instrumental training session, were used as input for this algorithm. Thereafter, the following preprocessing steps were applied: slice-time correction, co-registration, and a segmentation procedure using the tissue probability maps provided by SPM5 for gray matter, white matter, and CSF centered in MNI space to estimate normalization parameters based on the structural image. Structural and functional images were then normalized by applying these estimations. All normalized images were smoothed with an isotropic 8 mm full-width half-maximum Gaussian kernel (Worsley and Friston, 1995). The fMRI analysis was restricted to the PIT stage and was similar to our previous analyses (Geurts et al., 2013a). The general linear model (GLM, **Supplementary Figure 1**) at the participant level consisted of six main regressors representing the onset of the six different PIT trials [Action Context (approach/withdrawal) x CS Valence (appetitive/neutral/aversive)]. For each main regressor, an additional parametric regressor was added (Büchel et al., 1996): The PIT regressor (Talmi et al., 2008; cf. Geurts et al., 2013a) was a parametric modulator of BOLD responses by the number of button presses per trial. Contrasting this regressor between the different CS Valence measures thus reveals "PIT-related regions", i.e., regions where the BOLD signal is associated with valence-dependent coupling between amygdala BOLD signal and instrumental behavior on a trial by trial basis. Note that such a contrast goes beyond simple reactivity of a region to a CS or to instrumental behavior *per se*; it critically captures its interaction, i.e., PIT. A further parametric regressor contained the expectation associated with each instrumental stimulus (the *Q*-value) per trial as estimated from a model-based analysis of behavior (Huys et al., 2011) applied to the current data. This was done based on prior data showing that the BOLD signal in the prefrontal cortex and striatum, our regions of

interest, covaries with instrumental action value (O'Doherty, 2004; Valentin et al., 2007; Wunderlich et al., 2009; Smith et al., 2010; Jocham et al., 2011; see for meta-analysis, Chase et al., 2015). As such, this approach maximized the degree to which our GLM captured variability in the relevant BOLD signals. Furthermore, realignment parameters were added, high-pass filtering (128s) was applied, and parameter estimates were obtained by maximum-likelihood estimation (AR1).

The parameter estimates for the neutral and aversive parametric PIT regressors were used in a 2 × 2 × 2 rmANOVA at the group level (with random effects) with Action Context (approach/withdrawal) and Valence (neutral/aversive) as within-participant factors and group (healthy controls/BPD) as a between-participants factor. Within this rmANOVA, we assessed Action Context-specific aversive PIT and aversive PIT across Action Context for group differences. Moreover, we also assessed the main effect of Action Context. Based on Geurts et al. (2013a), we expected this analysis to reveal that the BOLD signal in the ventromedial prefrontal cortex would be Action Context-specific (approach > withdrawal). We did not expect a group effect on this contrast.

To capture additional PIT signals related to stable patterns of behavior beyond trial-by-trial variation in instrumental vigor, we contrasted the main regressors (**Figure 2**) at the participant level to calculate the main effect of Valence [(approach&neutral + withdrawal&neutral) - (approach&aversive + withdrawal&aversive)] (cf. Talmi et al., 2008; cf. Geurts et al., 2013a). The resulting SPM was then used in a two-sample *t*-test at the group level with aversive PIT in terms of the average number of button presses as a covariate for each group separately enabling comparison between groups. Based on Geurts et al. (2013a), we expected that behavioral aversive PIT across Action Contexts in terms of the average number of button presses [(BP| approach&neutral + BP| withdrawal&neutral) - (BP| approach&aversive + BP| withdrawal&aversive)] would be related to BOLD signal change (neutral-aversive) in the amygdala and nucleus accumbens and that this relationship would differ between the groups (i.e., a stronger correlation within the BPD group).

Treatment success and its prediction

Our primary measure of treatment success was the Borderline Personality Disorder Checklist, (BPD47, Bloo et al., 2017) a 47-item self-report questionnaire based on the Borderline Personality Disorder Severity Index (Arntz et al., 2003). Furthermore, as secondary measures, we also assessed the quality of life with the Outcome Questionnaire (OQ, Lambert et al., 1996) and depressive symptoms with the Beck Depression Inventory second edition (BDI-II, Beck et al., 1996). The treatment effect was computed by subtracting the post-treatment scores from those acquired during the first scan session.

Predictive relationship between aversive Pavlovian-to-instrumental transfer and symptom reduction

We assessed the association between aversive PIT and associated BOLD signal [at the whole-brain level and within the predefined amygdala region of interest (ROI)], measured pre-treatment, with clinical symptom reduction 1 year later. A second-level random-effects simple regression analysis was conducted to assess whether PIT-related neural signal was associated with symptom severity at baseline, and/or symptom reduction over 1 year. To this end, we computed Action Context-specific aversive PIT-related BOLD signal [(PITregressor| approach&neutral - PITregressor| approach&aversive) - (PITregressor| withdrawal&neutral - PITregressor| withdrawal&aversive)], aversive PIT-related BOLD signal across Action Contexts [PIT regressor| approach&neutral + PIT regressor| withdrawal&neutral – (PIT regressor| approach&aversive + PIT regressor| withdrawal&aversive)], as well as BPD47 scores at baseline and BPD47 change (before-after). These latter covariates of interest were tested in two simple regression analyses of the aversive PIT statistical parametric maps. Any relationship between the PIT-related BOLD contrasts and the BPD47 change (without a baseline relationship) would indicate that PIT-related signaling is predictive of symptom reduction. In addition, as a sensitivity analysis because of the small sample size, we also performed the non-parametric equivalent of this analysis with SnPM (Winkler et al., 2014) and we employed a leave-one-participant-out procedure (Esterman et al., 2010), in which a single participant is iteratively left out of the second-level correlational analysis. The resulting clusters within the anatomically defined bilateral amygdala (thresholded at $p < 0.001$ uncorrected) were then used to extract the mean beta weights of the left-out participant to calculate the aversive PIT contrast. This procedure was repeated for each participant. The GLM from the remaining participants thus serves as an independent localizer for the participant left out (Esterman et al., 2010).

Statistical thresholding

We report effects that survive family-wise error (FWE) correction for multiple comparisons across the whole brain ($P_{WB} < 0.05$, voxel-level) or in one of the following ROIs: The amygdala (automated anatomical labeling atlas, Tzourio-Mazoyer et al., 2002) was our primary ROI to assess the effect of symptom reduction. Both the amygdala and nucleus accumbens (same as in Geurts et al., 2013a) were chosen as ROIs for the analysis of the main PIT task effects (across and between groups) based on their key role in PIT (Corbit, 2005; Talmi et al., 2008; Corbit and Balleine, 2011; Prevost et al., 2012; Geurts et al., 2013a; Garbusow et al., 2016). Specifically, in our previous study, we found BOLD response in both these regions to be associated with behavioral PIT on a participant-by-participant basis. Following our prior work, we also assessed

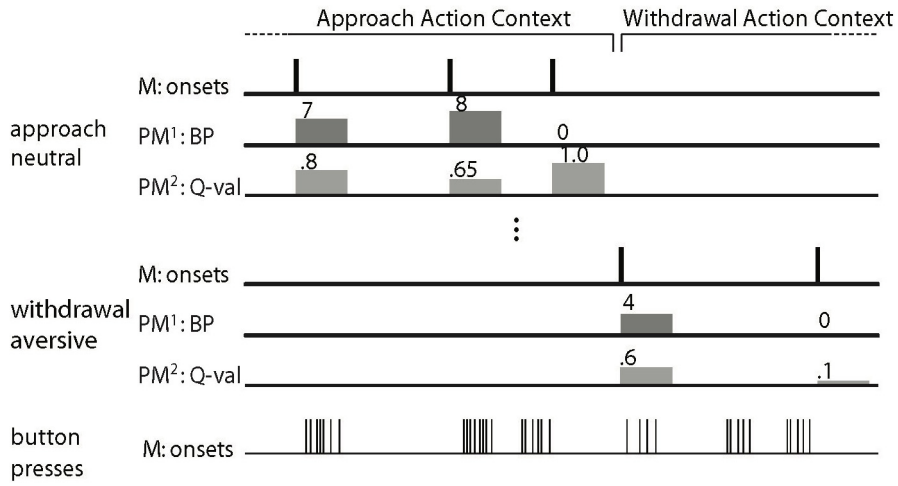

FIGURE 2
Schematic depiction of the general linear model to analyze the Pavlovian-instrumental transfer (PIT) data (Figure after Talmi et al., 2008; Geurts et al., 2013b). The main regressors (M) model the onset of a trial as a delta function. There is a main regressor for each of the six trial types. For all six main regressors, there are two parametric modulators (PM). The first parametric modulator (PM^1), the PIT regressor, consists of the number of button presses made per trial (0 for no-go). In the 7th main regressor (of no interest), every single button press is modeled by a delta function. For reasons of clarity, only two of the six trial types (approach neutral and withdrawal aversive) are depicted. The regressors of no interest are not shown (i.e., the movement nuisance regressors and the second parametric modulator).

action specificity in the ventromedial prefrontal cortex: The region shown to be sensitive to Action Context in our previous PIT study was used as ROI (MNI coordinates of ROI center: xyz = [-8 36-8]) (Geurts et al., 2013a). The left and right elements of each bilateral volume of interest were combined using Marsbar^{TM} (Brett et al., 2002).

Results

Baseline behavioral data

Pavlovian-instrumental transfer

Consistent with our previous studies using this paradigm, we observed opposite effects of the aversive Pavlovian CS on approach and withdrawal actions (in terms of choice p(go), **Figure 2**): aversive Pavlovian CS inhibited approach and activated withdrawal actions. Planned contrasts confirmed the statistical significance of this action specificity of the aversive PIT effect (related-samples Wilcoxon signed rank test [p(go| approach&neutral) - p(go| approach&aversive)] > [p(go| withdrawal&neutral)- p(go| withdrawal&aversive)]: p = 0.031, one-tailed). There were no differences between the groups (independent samples median test: p = 0.48), but we note that the action-specific PIT effect was present in healthy controls (p = 0.008), but not in patients (p = 0.860) when examined separately.

There were no main task effects except for the main effect of Action Context in terms of the average number of button presses ($F_{(1,29)}$ = 33.7, $p < 0.001$, all other $F < 1.8$ and $p > 0.2$, **Supplementary Table 2**). There were no group differences.

Performance on the instrumental task and assessments of Pavlovian training also did not differ between the groups (**Supplementary Results**). To be complete, we confirmed the already established insensitivity to detect appetitive PIT with the current paradigm (**Supplementary Results**).

Baseline imaging data

Consistent with our previous fMRI study using this paradigm, trial-by-trial instrumental action-related BOLD signal in the vmPFC varied as a function of Action Context. The BOLD signal was greater during approach than during withdrawal (small volume corrected results for the vmPFC ROI: peak voxel MNI-coordinates [−6 32 −12], k = 45, Z = 3.86, p_{FWE} = 0.021, **Figure 3**).

Conversely, we did not replicate the previously observed correlation between individual differences in behavioral aversive PIT and BOLD signals in the amygdala and nucleus accumbens. Moreover, we did not find significant main effects of or interactions with the factor group.

Aversive Pavlovian-to-instrumental transfer and symptom reduction

Symptom reduction

The 14 patients who were seen at follow-up, 1 year after the start of therapy, showed a significant reduction in symptom severity as measured with the BPD47 (mean difference = −17.3, t_{13} = 2.5, p = 0.027, reliable change index (Jacobson and Truax, 1991): 15.8), OQ (mean difference = −12.4, t_{13} = 3.1, p = 0.009),

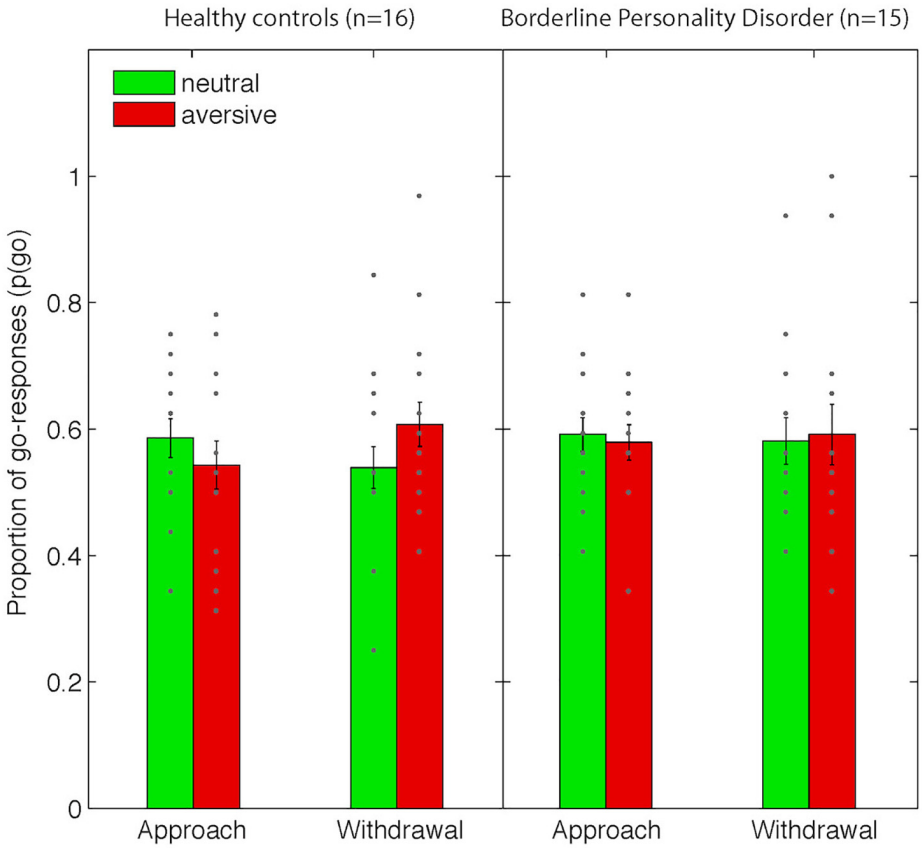

FIGURE 3
Behavioral data from the Pavlovian-instrumental transfer stage. Shown are mean proportions of go-responses [p(go)] as a function of Action Context (approach vs. withdrawal) and Valence (neutral/aversive). Error bars represent standard errors of the means and dots represent individual data points. Note that there were no significant differences between groups.

and in trend with the BDI-II (mean difference = −4.8, t_{13} = 1.8, p = 0.090).

None of the neuropsychological tests reported in Table 1 changed significantly from baseline to 1 year after treatment (all $-1.9 > t_{13} < 2.2$, all $p \geq 0.05$).

Pavlovian-to-instrumental transfer-related BOLD signal in the amygdala is related to symptom reduction 1 year later

Pre-treatment PIT-related BOLD signal in the bilateral amygdala was related to BPD symptom reduction after 1 year (Figure 4). Higher aversive PIT-related signals across Action Contexts were associated with less symptom reduction 1 year later. This observation was substantiated by using both parametric and non-parametric statistical analyses (small volume corrected effects in the amygdala; parametric tests with SPM: peak voxel MNI-coordinates [−24 0 −16], k = 22, Z = 3.79, p_{FWE} = 0.027; non-parametric test with SnPM: peak voxel MNI-coordinates [−24 0 −18], pseudo-t = 4.22, p_{FWE} = 0.013; and MNI-coordinates [22 4 −18], pseudo t = 3.19, p_{FWE} = 0.06). The robustness of these effects was confirmed by cross-validation ($r_{(14)}$: −0.655, p = 0.011) and by supplementary analyses on mean beta estimates extracted from the anatomically defined bilateral amygdala (Pearson $r_{(14)}$: −0.667, p = 0.009). Note, that no significant relation was observed between baseline BPD47 scores and PIT-related amygdala signal (Pearson $r_{(14)}$:0.33, p = 0.25).

Next, we explored the specificity of this predictive effect with respect to other (more easily acquired) baseline measures, including baseline BPD47, OQ, BDI-II, BIS, BAS, box completion time, verbal fluency, and digit span (Table 1). A stepwise linear regression analysis (with criteria probability of F to enter $\leq$ 0.05 and o F to remove $\geq$ 0.10) identified two predictors of symptom reduction. Indeed, pre-treatment PIT-related signal in the bilateral amygdala accounted for variance in symptom reduction over and above the other collected baseline measures. Verbal fluency was the only other selected predictor of symptom reduction (Final regression model including PIT-related amygdala signal and verbal fluency: $F_{(2,13)}$ = 11.6, p = 0.002, standardized coefficients beta for amygdala signal:0.64, p = 0.003; and for the verbal fluency: −0.48, p = 0.016). All other measures did not enter the model

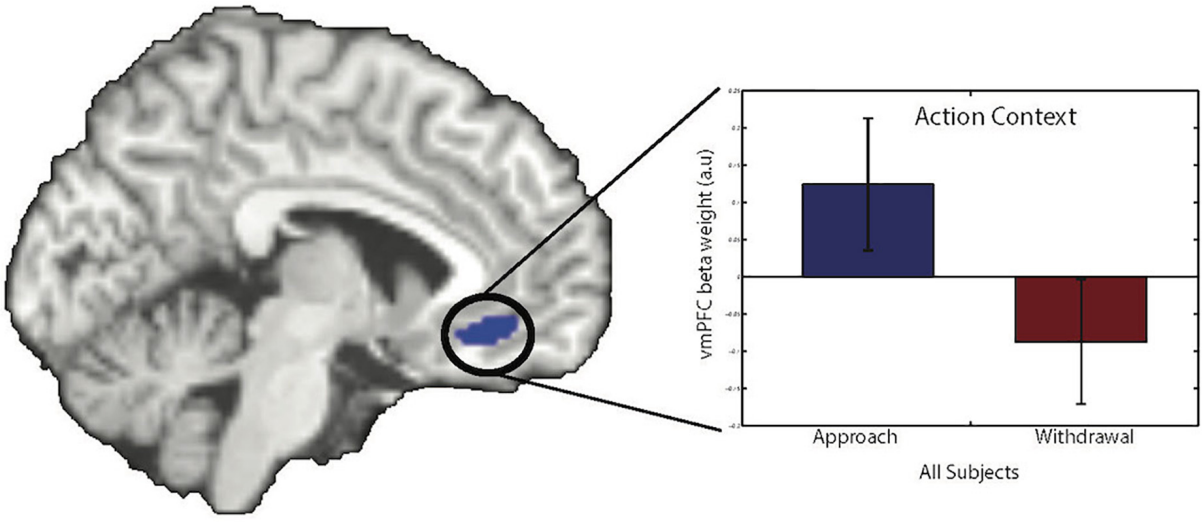

FIGURE 4
Action-specific BOLD response in the vmPFC. There was a main effect of Action Context in the vmPFC (peak voxel MNI-coordinates [−6 32 −12], $k = 45$, $Z = 3.86$, $p_{FWE} = 0.021$, small volume corrected). The bar graph shows parameter estimates from the peak voxel for the different Action Contexts (error bars show SEM). Images are displayed at a statistical threshold of $p < 0.001$ uncorrected.

(all $|t| \leq 1.5$, all $p > 0.2$). Next, we examined whether the predictive effect of pre-treatment PIT-related amygdala signal was specific to BPD47 change or whether it extended to other changes in clinical or neuropsychological measures. Indeed, stepwise multiple regression analysis with this amygdala signal as a dependent variable revealed that the association of this signal with BPD47 improvement ($F_{(1,12)} = 9.6$, $p = 0.009$) did not extend to any of the other changes in clinical or neuropsychological measures (all $|t| < 1.9$, all $p > 0.18$). This is relevant because improvement in borderline severity was accompanied by improvement in depressive symptoms as measured with the BDI-II ($r_{14} = -0.67$, $p = 0.008$), as well as improvement in verbal fluency ($r_{14} = 0.91$, $p < 0.001$).

Discussion

Results failed to confirm our prediction that patients with borderline personality disorder exhibit abnormal aversive PIT compared to healthy controls at the group level. However, on an individual level, the results demonstrate that the BOLD signal in the amygdala elicited during the aversive PIT task is related to symptom reduction in these patients across 1 year of follow-up. Greater PIT-related responsiveness of the (bilateral) amygdala was associated with reduced clinical improvement 1 year later. More specifically, this suggests that individual differences in the degree to which amygdala processing relates to trial-by-trial instrumental responding in the context of an aversive Pavlovian CS predict resistance to clinical improvement of (or slower recovery from) BPD. Thus, participants who showed increased coupling between the amygdala BOLD signal and instrumental behavior during aversive Pavlovian CS presentation showed less clinical improvement. In more general terms, this suggests that individual differences in amygdala response could predict clinical improvement of BPD.

Based on observations that BPD is associated with the abnormal impact of aversive stimuli on behavior (Soloff et al., 2017; Hallquist et al., 2018), we employed an aversive PIT task that measures the degree to which aversive Pavlovian CS alter instrumental behavior. We replicated the previously observed basic behavioral task effects, including the Action Context-specificity of aversive PIT (Huys et al., 2011; Geurts et al., 2013a), with an aversive Pavlovian CS suppressing approach, but potentiating withdrawal actions. These task effects were not modulated by BPD, although when analyzing the groups separately, we only found significant effects in the healthy controls. The absence of a group effect might be due to the relatively stressful scanner environment (Talmi et al., 2008; cf. discussion of Geurts et al., 2013a). Indeed, there are indications that stress reduces behavioral PIT effects (Quail et al., 2016; but see Pool et al., 2015) and patients with BPD might be more sensitive to this stress. It might also be a consequence of the use of psychotropic medication in about two-thirds of our patients, which has been associated with attenuated amygdalar hyperreactivity in BPD (Schulze et al., 2016) and is likely to change PIT through changing monoaminergic signaling (cf. Geurts et al., 2013b; Hebart and Gläscher, 2015; Swart et al., 2017). Moreover, given the small sample sizes, the absence of a group effect on action-specific PIT might also reflect insufficient statistical power to detect such a difference. However, we cannot

exclude that, as a group, BPD patients indeed do not exhibit abnormal aversive PIT.

The key observation of this study is that neural activity of the amygdala in BPD patients is associated with clinical symptom reduction. These results substantiate the promise of neurocognitive strategies for predicting treatment outcomes in various psychiatric disorders (Nitschke et al., 2009; Pizzagalli, 2010; Roiser et al., 2011; Månsson et al., 2015; Perez et al., 2016; Garbusow et al., 2016; Huys et al., 2016; Schmitgen et al., 2019; Westlund Schreiner et al., 2019; Sampedro et al., 2021). The considerable gap between cognitive neuroscience and clinical practice has been the subject of a fruitful ongoing debate (Paulus et al., 2016; Stephan et al., 2016; Huys, 2018). One major problem in the clinical relevance of neurocognitive research is that most studies have compared groups of patients, failing to address individual differences in the treatment efficacy. Future work is required to investigate whether an aversive PIT-related neural signal is associated selectively with DBT efficacy, or rather reflects general treatment efficacy or even BPD symptom change more irrespective of treatment.

Moreover, our results provide converging evidence for the validity of the PIT paradigm for predicting clinical symptom changes [in depression (Huys et al., 2016) and addiction (Garbusow et al., 2016)]. It should be noted that, here, amygdala signal across Action Contexts was the predictor, whereas in the study of Huys et al. (2016), it was the Action Context specificity of behavior that predicted recovery from depression. We did not find such an association for symptom reduction in patients with borderline personality disorder. Moreover, in the study of Garbusow et al. (2016), it was the PIT effect in the nucleus accumbens that predicted relapse in alcohol use. This suggests that different aspects of the neurocognitive mechanisms underpinning the transfer between Pavlovian CS and instrumental behavior might be disorder and/or treatment specific. We note that these studies, just like the current study, are relatively small in sample size. Nevertheless, these studies make concrete steps in translating hypotheses on mechanistic relevance for clinical treatments and as such are stepping stones for larger future studies making use of their methodology, which are already emerging (e.g., Chen et al., 2021).

The present results suggest that symptom reduction after DBT is greater in BPD patients who show lower amygdala signals during aversive PIT. The finding that the amygdala signal is predictive of symptom reduction in BPD after DBT concurs with empirical findings and neurocognitive theories, implicating a central role for the amygdala in BPD (Schulze et al., 2016, 2019) and DBT (Schnell and Herpertz, 2007; Goodman et al., 2014; Schmitt et al., 2016; Schmitgen et al., 2019). Several recent studies have shown changes in amygdala signaling after DBT (Schnell and Herpertz, 2007; Goodman et al., 2014; Schmitt et al., 2016; Niedtfeld et al., 2017; but see Winter et al., 2017). Schnell et al. employed a pilot study with six BPD patients who received several fMRI scans during 3 months of DBT. The four patients who responded to DBT all showed decreases in amygdala BOLD responses to emotional pictures. In keeping with this finding, Goodman et al. (2014) reported decreases in amygdala responses to emotional pictures and associated improvement in self-reported emotional regulation in 11 BPD patients after 1 year of DBT treatment. Moreover, Niedtfeld et al. (2017) showed in 28 patients with BPD that a scaled version of 12 weeks of DBT attenuated amygdala deactivation in response to pain. Schmitt et al. (2016) showed that patients who responded well to DBT exhibited reduced activation in, among other regions, the amygdala, during the reappraisal of negative stimuli after DBT.

These studies suggest that the association between amygdala signaling and symptom reduction, observed in the current study, might relate to treatment-induced changes in the amygdala. We stress, however, that we did not collect behavioral or fMRI data after therapy, which precludes us from assessing whether the amygdala signal indeed changed during this treatment, or whether it is a stable trait that indexes the susceptibility to the offered treatment. Moreover, due to the absence of a control condition in our design, we restrict our conclusions to the general case of clinical improvement. Thus, we cannot claim the specificity of our results to DBT. Moreover, PIT-related amygdala signal might also reflect more general, less treatment-specific, process underlying improvement like the ability to (emotionally) engage and/or commit oneself to treatment. We thus restrict our conclusion to the general *predictive* effect of amygdala signal on symptom change.

Although several studies, as mentioned above, assessed pre- to post-therapy changes in neural processing in BPD, so far, only two other studies assessed the value of selectively pre-treatment task-based fMRI signals for predicting treatment success (Perez et al., 2016; Schmitgen et al., 2019). In the study by Perez et al. (2016) including 10 patients with BPD, a greater pre-treatment BOLD signal in the right anterior cingulate cortex during an emotional go/no-go task was associated with reduced improvement after transference-focused psychotherapy (TFP) in terms of the factor 'constraint' of the multidimensional Personality Questionnaire. Moreover, a greater BOLD signal in the left posterior-medial OFC/ventral striatum was associated with reduced improvement in terms of the total score on the Affective Lability Scale. The study of Schmitgen et al. (2019) is of specific interest for the current study, because it explicitly addressed the prediction of clinical DBT effects based on, amongst others, task-based fMRI in a relatively large sample (n = 31) of BPD patients with a sophisticated cross-validation procedure to optimize a random forest prediction algorithm. They employed three emotion regulation tasks, fMRI, and structural MRI before 12 weeks of DBT. They showed that (left) amygdala (and parahippocampus) activation during a cognitive reappraisal task was particularly informative for treatment response prediction. Accuracy of predicting treatment response (base rate 52%) of the model based on solely these fMRI data

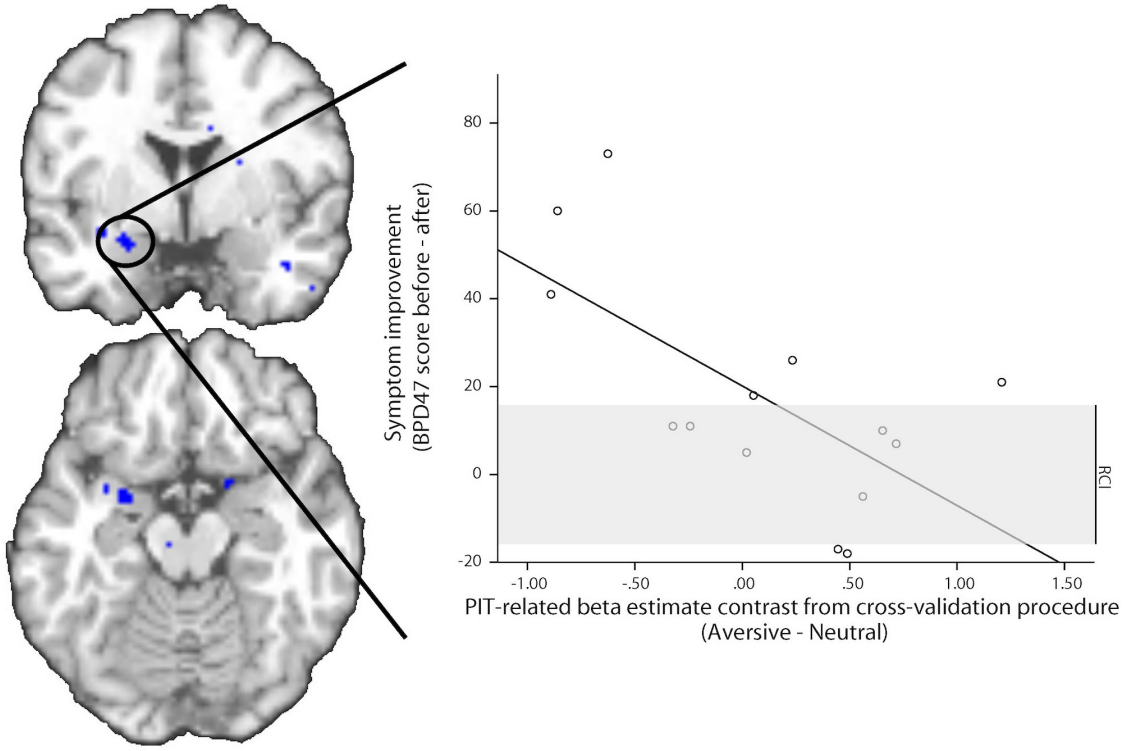

FIGURE 5
Association between amygdala BOLD signal change and symptom improvement. Pre-treatment PIT-related BOLD signal in the left amygdala predicts symptom improvement 1 year later. Images are displayed at a statistical threshold of $p < 0.001$ uncorrected. The scatter plot shows the PIT-related beta estimate contrast for aversive minus neutral CS trials before treatment in relation to symptom improvement, derived from a leave-one-participant-out cross-validation procedure. The regression line is the ordinary least square line. The gray area depicts the reliable change index (RCI) range; the changes outside this area are regarded as reliable (based on Jacobson and Truax, 1991).

reached 75%. Of note is that responders, while instructed to look at negative emotional pictures, showed lower left amygdala reactivity before therapy compared to non-responders. Together with these prior data, our findings strengthen the observation that particular limbic circuitry processing during affective action regulation renders BPD patients more resistant to clinical improvement after therapy. Moreover, differences between these studies employing two different treatment regimes (TFP vs. DBT) might speak to the future practical, clinical use of these findings. Future research should investigate how we can make treatment regimes more efficient by allocating specific patients to specific treatment modalities based on their functional neural signature. Thus, combining different neural predictors for treatment success specific to different treatment modalities might help us to reveal which patients should be allocated to which treatment. Before being able to implement this in clinical practice, more large-scale practice-based studies should be carried out to ensure the reliability and clinical usefulness of these predictions. Our data provide proof of principle of such a procedure within a practice-based convenience sample of BPD patients. We note, however, that only about half of the patients that were planned to follow during the DBT treatment did not volunteer or dropped out of this study. This observation is important for assessing the feasibility of employing these procedures broadly in clinical practice. Future qualitative, implementation research on facilitators and barriers to these procedures is warranted.

Further limitations of our study deserve special attention: First, our main result is based on a small sample size. Although we assessed the robustness of the effect extensively, for example, by cross-validation (leave-one-participant-out procedure) and by permutation-based analyses (SnPM), replication of our data is needed. Second, because we did not include a patient control group, we cannot assess whether the amygdala signal is a general predictor of positive change in symptomatology or whether it specifically moderates treatment outcomes. Third, our paradigm was insensitive to appetitive PIT (**Supplementary Material**) and therefore we cannot make any claims on the valence specificity of the presented results.

Fourth, we set out to include all the patients who were offered DBT during the inclusion period of this study. In this setting, all BPD patients were female, which thus precludes conclusions about male BPD patients. Moreover, the inclusion resulted in a 'real-life' BPD patient group with

the majority of patients being on psychotropic medication and having multiple comorbidities. This choice of patient selection was at the expense of internal validity [e.g., a recent meta-analysis shows that medicated compared with non-medicated patients with BPD show blunted amygdala responses (Schulze et al., 2016)], which we deliberately traded off against enhanced external validity (Hoertel et al., 2015). The majority of patients in normal clinical practice with BPD have multiple comorbidities and, although discouraged in many guidelines, take psychotropic medications, such as selective serotonin inhibitors. We acknowledge that we cannot exclude the possibility that differences in medication use contribute to the observed effect. With the low sample size and the diverse medication regimens of the included patients, we have no means to address this quantitively. To provide as much insight as possible, we report medication use in the **supplementary materials** for each patient (**Supplementary Table 1**) and also added a graph, similar to **Figure 5**, showing bilateral amygdala signals for those with and without medication use (**Supplementary Figure 1**). Choosing such a sample is in line with our ultimate aim to find useful biobehavioral markers to predict and optimize treatment success in real-life clinical practice.

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 METC Oost-Nederland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DG, QH, TV, and RC contributed to conception and design of the study. DG collected the data and performed the statistical analysis under supervision of RC. DG and RC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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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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Supplementary material

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Dorsomedial prefrontal cortex activation disrupts Pavlovian incentive motivation

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The dorsomedial prefrontal cortex (dmPFC) is known to make important contributions to flexible, reward-motivated behavior. However, it remains unclear if the dmPFC is involved in regulating the expression of Pavlovian incentive motivation, the process through which reward-paired cues promote instrumental reward-seeking behavior, which is modeled in rats using the Pavlovian-instrumental transfer (PIT) task. The current study examined this question using a bidirectional chemogenetic strategy in which inhibitory (hM4Di) or excitatory (hM3Dq) designer G-protein coupled receptors were virally expressed in dmPFC neurons, allowing us to later stimulate or inhibit this region by administering CNO prior to PIT testing. We found that dmPFC inhibition did not alter the tendency for a reward-paired cue to instigate instrumental reward-seeking behavior, whereas dmPFC stimulation disrupted the expression of this motivational influence. Neither treatment altered cue-elicited anticipatory activity at the reward-delivery port, indicating that dmPFC stimulation did not lead to more widespread motor suppression. A reporter-only control experiment indicated that our CNO treatment did not have non-specific behavioral effects. Thus, the dmPFC does not mediate the expression of Pavlovian incentive motivation but instead has the capacity to exert pronounced inhibitory control over this process, suggesting that it is involved in adaptively regulating cue-motivated behavior.

KEYWORDS

motivation, DREADD, anterior cingulate, behavioral flexibility, cognitive control

Introduction

Pavlovian reward-associated cues acquire potent motivational properties which allow them to instigate instrumental reward-seeking behavior, a phenomenon selectively modeled by the Pavlovian-instrumental transfer (PIT) task (Cartoni et al., 2016; Corbit and Balleine, 2016). This motivational influence is normally adaptive, promoting the

pursuit of goals like palatable food in situations where they are likely to become available. However, in substance use disorder and related conditions, cues can trigger intense cravings that motivate reward seeking even when efforts are made to abstain from such behavior (Unnithan et al., 1992; O'Brien et al., 1998; Sinha and Li, 2007; Tiffany and Wray, 2012; Fatseas et al., 2015; Vafaie and Kober, 2022). This maladaptive influence of cues is thought to be mediated, at least in part, by a loss of top-down inhibitory control over motivated behavior (Kober et al., 2010; Belin et al., 2013; Fatseas et al., 2015; Marshall and Ostlund, 2018; Antons et al., 2020).

The neural circuitry responsible for regulating cue-motivated behavior is not well understood, though the dorsomedial prefrontal cortex (dmPFC) is likely to be involved. The dmPFC, which refers here to the dorsal part of the prelimbic cortex as well as the anterior cingulate cortex, is richly connected with several brain regions known to mediate PIT (Cartoni et al., 2016; Corbit and Balleine, 2016), such as the nucleus accumbens, mediodorsal thalamus, and amygdala (Gabbott et al., 2005; Hoover and Vertes, 2007). Neural activity in the dmPFC is also strongly modulated by reward-predictive cues (Shidara and Richmond, 2002; Kennerley et al., 2011; Monosov, 2017), including during PIT testing (Homayoun and Moghaddam, 2009). Nevertheless, previous studies have found that disrupting dmPFC function does not alter PIT performance (Cardinal et al., 2003; Corbit and Balleine, 2003; Halbout et al., 2019), suggesting it may not be critical for the expression of Pavlovian incentive motivation.

However, the dmPFC has been strongly implicated in multiple aspects of behavioral flexibility including set-shifting (Ragozzino et al., 1999; Stefani et al., 2003; Floresco et al., 2006, 2008; Bissonette and Roesch, 2015; Powell and Redish, 2016; Brockett et al., 2020) and response inhibition (Bussey et al., 1996; Muir et al., 1996; Narayanan and Laubach, 2006; Jonkman et al., 2009; Terra et al., 2020; Hamel et al., 2022). Such studies have revealed that the dmPFC is important for withholding or otherwise modifying learned motor behaviors (e.g., instrumental habits), but do not directly address its role in negatively regulating Pavlovian incentive motivation as measured by PIT.

We hypothesized that the dmPFC is not required for the expression of PIT but does have the capacity to negatively regulate—or suppress—this motivational effect. To test this, we applied a bidirectional chemogenetic strategy. We reasoned that if the dmPFC is selectively involved in regulating Pavlovian incentive motivation, then activating this structure should attenuate PIT expression (i.e., it should dampen cue-motivated reward seeking). Furthermore, if one assumes that the PIT effect represents an adaptive motivational response to reward-paired cues, and is therefore normally expressed in an unregulated manner, then inhibiting the dmPFC should have little or no effect on this response. In contrast, if the dmPFC is more directly involved in mediating the expression of cue-motivated reward seeking, then inhibiting this structure should disrupt the PIT effect.

Materials and methods

Animals

Male Long-Evans rats ($N = 26$) were obtained from Charles River and weighed > 290 g at the start of the study. Female rats were not used here to minimize variability, as previous studies have observed significant sex differences in reward consumption (Marshall et al., 2017; Westbrook et al., 2018) and assays of incentive motivation (Pitchers et al., 2015; Reichelt et al., 2016; Madayag et al., 2017; Tapia et al., 2019) including PIT (Shields and Gremel, 2021; Derman and Lattal, 2022). Rats were paired-housed in transparent plastic cages in a temperature- and humidity-controlled vivarium. The rats were tested during the light phase of a standard 12:12 h light:dark schedule. Rats had *ad libitum* access to water in their home cages, and were food restricted (~13.5 g/day of home chow; Envigo) to maintain them at between 85 and 90% their free-feeding bodyweight throughout the experiment. All experimental procedures were approved by the UC Irvine Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Apparatus

Operant behavioral procedures were conducted in identical operant chambers (ENV-007, Med Associates, St Albans, VT, USA), each housed in a sound- and light-attenuated cubicle. A food-delivery port was located at the center of one end-wall of the chamber, 2.5 cm above the stainless-steel grid floor. A cup within the food port was used to receive 45-mg grain pellets (BioServ) via an automated pellet dispenser. A photobeam detector positioned across the food-port entrance was used to monitor head entries. A retractable lever was positioned to the right of the food port. A houselight (3 W, 24 V) at the top of the opposite end-wall provided general illumination and a fan mounted on the cubicle provided ventilation and background noise. Experimental events were controlled and recorded with a 10-ms resolution using MED-PC IV software.

Surgery

Rats were anesthetized using isoflurane and placed in a stereotaxic frame for microinjections of an adeno-associated virus (AAV) vectors to induce expression of the inhibitory DREADD [designer receptor exclusively activated by designer

drug (Armbruster et al., 2007)] hM4Di [pAAV5-CaMKIIa-hM4D(Gi)-mCherry, 1.1 × 10^{13} vg/mL; Addgene plasmid # 50477-AAV5] (n = 8) or the excitatory DREADD hM3Dq [CaMKIIa-hM3D(Gq)-mCherry 1.7 × 10^{13} vg/mL; Addgene plasmid # 50476-AAV5] (n = 8) fused with mCherry under the CaMKII promoter. DREADDs are genetically modified G protein coupled receptors that can be selectively activated by the designer drug Clozapine-n-oxide (CNO) (Armbruster et al., 2007). While the activation of hM4Di results in a general silencing of neurons through neuronal hyperpolarization and presynaptic inhibition of neurotransmitters release (Armbruster et al., 2007), the activation of hM3Dq leads to enhanced firing of neurons by facilitating their depolarization (Alexander et al., 2009). The use of the CaMKII promoter allows for DREADD expression in putative excitatory cortical neurons (Dittgen et al., 2004; Nathanson et al., 2009). An AAV expressing only the fluorescent reporter protein GFP (AAV5-CaMKIIa-EGFP, 3.6 × 10^{12} vg/mL; Addgene plasmid # 50469-AAV5) was used in the control group (n = 10). The AAV was injected bilaterally into the dmPFC (+3.2 mm AP, ± 0.7 mm ML, –2.8 mm DV from bregma; 0.7 μL/side). Animals were allowed at least 5 days of recovery before undergoing food restriction and behavioral training. Testing occurred at least 25 days after surgery to allow adequate time for viral expression of hM4Di, hM3Dq, or GFP throughout dmPFC neurons.

Pavlovian-instrumental transfer

Pavlovian conditioning

Rats first received 2 d of magazine training, during which 40 pellets were delivered into the food cup on a random 90-s intertrial interval (ITI). Rats then received 8 daily Pavlovian conditioning sessions. Each session consisted of a series of 6 presentations of a 2-min audio cue (CS+; either a pulsating 2 kHz pure tone (2 s at 80 db and 1 s at 90 db) or white noise; 80 dB), with trials separated by a 3 min variable ITI (range 2–4 min between CS onsets). During each CS+ trial, pellets were delivered on a 30-s random time schedule, resulting in an average of 4 pellets per trial. Rats were separately habituated to an unpaired audio cue (CS−; whichever cue was not used as CS+; 2-min duration). Rats were given 3 days of CS− only exposure (eight non-reinforced trials per session, 3 min variable ITI) following instrumental training (see below). Conditioning was measured by comparing the rate of food-cup approach between CS onset and the first pellet delivery (to exclude unconditioned feeding activity) to the rate of approach during the Pre-CS period.

Instrumental training

Rats then received 9 days of instrumental lever-press training. In each session, rats had continuous access to the lever, which could be pressed to deliver food pellets into the food cup. The schedule of reinforcement was adjusted over days from continuous reinforcement (CRF) to increasing random intervals (RI), such that reinforcement only became available once a randomly determined interval had elapsed since the last reinforcer delivery. Rats received 2 days of CRF, 1 day each of RI-15s and RI-30s, and 6 days of training with RI-60s. Each session was terminated after 30 min or after 20 rewards deliveries.

Pavlovian-instrumental transfer test

After the last instrumental training session, rats were given a session of Pavlovian (CS+) training, identical to initial training, and 3 sessions of CS− training. They were then given a 30 min extinction session, during which lever presses were recorded but had no consequence (i.e., no food or cues). On the next day, rats were given a PIT test, during which the lever was continuously available but produced no rewards. Following 8 min of extinction, the CS+ and CS− were each presented four times (2 min per trial) in pseudorandom order and separated by a fixed 3-min interval. Rats received CNO (5 mg/kg, i.p.) or vehicle (5% DMSO in saline) injections 30 min prior to testing. They underwent a second test following retraining, which consisted of two sessions of instrumental retraining (RI-60s), one session each of CS+ and CS− retraining, and one 30-min extinction session, as described above. The alternative drug pretreatment was administered prior to this second test (counterbalanced across groups).

Histology

Rats were deeply anesthetized with pentobarbital sodium and transcardially perfused with PBS, followed by 4% PFA. Brains were removed and postfixed overnight in 4% PFA at 4°C, transferred to 30% sucrose, and then sectioned into 40-μm-thick coronary brain slices that were stored in cryoprotectant solution. The expression of DREADD-mCherry or GFP were immunohistochemically amplified using antibodies directed against mCherry or GFP. Tissue was first incubated in 3% normal donkey serum PBS plus Triton X-100 (PBST; 1 h) and then in primary antibodies in PBST at 4°C for 48 h using rabbit anti-DsRed (mCherry tag; 1:1,000; Clontech; 632496), or mouse anti-GFP (1:1,500, Life Technologies; A-11120) antibodies. Sections were incubated for 2 h at room temperature in fluorescent conjugated secondary antibodies Alexa Fluor 594 goat anti-rabbit (DsRed; 1:500; Invitrogen; A11037) or (Alexa Fluor 488 goat anti-mouse (GFP; 1:500; Invitrogen; A10667). Sections were mounted with mounting medium with DAPI (Vectashield) and were imaged with a 10 × objective on a fluorescence microscope (Leica) to validate viral expression. Two rats (one hM4Di and one hM3Dq) were omitted from analysis due to inadequate viral expression in the dmPFC.

Data analysis

Statistical analysis was conducted in SPSS (v. 28.0.1), with alpha set at $p < 0.05$. Repeated measures ANOVAs were performed using relevant within-subjects factors such as Drug and Cue. For Pavlovian training and PIT testing, difference scores were computed by subtracting baseline response rates (responses per minute) during 2-min Pre-CS periods from response rates during CS periods. We focused on three distinct behavioral measures for PIT testing, the overall rate of lever pressing, the rate of initiating new bouts of lever pressing, and the rate of initiating new bouts of spontaneous (press-independent) food-port entry behavior. To identify new bouts of behavior, we assessed the distribution of inter-response times (IRTs) separating all responses performed during PIT testing (both full sessions), focusing on press-press, entry-entry, press-entry, and entry-press transitions with IRTs less than or equal to 10 s. Each distribution was normalized within-subject by dividing the number of IRTs in each 0.1-s bin by the total number of IRTs in the distribution.

Results

To investigate the contributions of the dmPFC to Pavlovian incentive motivation, AAV vectors were bilaterally injected into the dmPFC to locally express the inhibitory G-protein-coupled designer receptor hM4Di ($n = 7$) or the excitatory receptor hM3Dq ($n = 7$) in separate groups of rats (visualized with mCherry; **Figures 1A–C**). After recovering from surgery, rats were food deprived and trained on a standard PIT protocol (**Figure 1D**). Rats first received Pavlovian conditioning to associate an auditory cue (CS+) with a food-pellet reward. Both groups readily learned to enter the food-port during CS+ trials and withhold this response during CS− trials. By the final day of training with each cue, the CS+ increased food-port entries (CS – pre-CS) more than the CS− in groups hM4Di [$F_{(1,\ 6)} = 6.00$, $p < 0.05$] and hM3Dq [$F_{(1,\ 6)} = 11.67$, $p < 0.014$] (**Figures 1E,F**). Rats then received instrumental conditioning in the absence of the cue to learn that pressing a lever would earn the food-pellet reward. Both groups hM4Di [$F_{(9,\ 54)} = 9.83$, $p < 0.001$] and hM3Dq [$F_{(9,\ 54)} = 8.09$, $p < 0.001$] rapidly increased their rate of lever pressing over training days (**Figures 1E,F**).

PIT testing was then conducted to assess the motivational influence of the CS+ on instrumental performance. During each PIT test, the lever was available, but unrewarded, and each cue was presented in pseudorandom order. Rats received two tests, one following CNO and one following vehicle (counterbalanced for order) to determine the effects of dmPFC inhibition (CNO in hM4Di group) or stimulation (CNO in hM3Dq group) on PIT performance. While pre-CS press rates (**Figure 2A**) appeared to be slightly lower after CNO treatment, no reliable effect of drug was found for group hM4Di [$F_{(1,\ 6)} = 3.28$, $p = 0.12$] or group hM3Dq [$F_{(1,\ 6)} = 0.48$, $p = 0.52$], suggesting that these treatments did not induce gross alterations in baseline task performance. Difference scores (CS – pre-CS) were computed to isolate CS-related changes in responding (**Figure 2B**). While group hM4Di showed a tendency to increase their rate of lever pressing during CS+ relative to CS− trials, this effect did not reach significance [Cue: $F_{(1,\ 6)} = 3.053$, $p = 0.098$], making it difficult to determine whether their performance depended

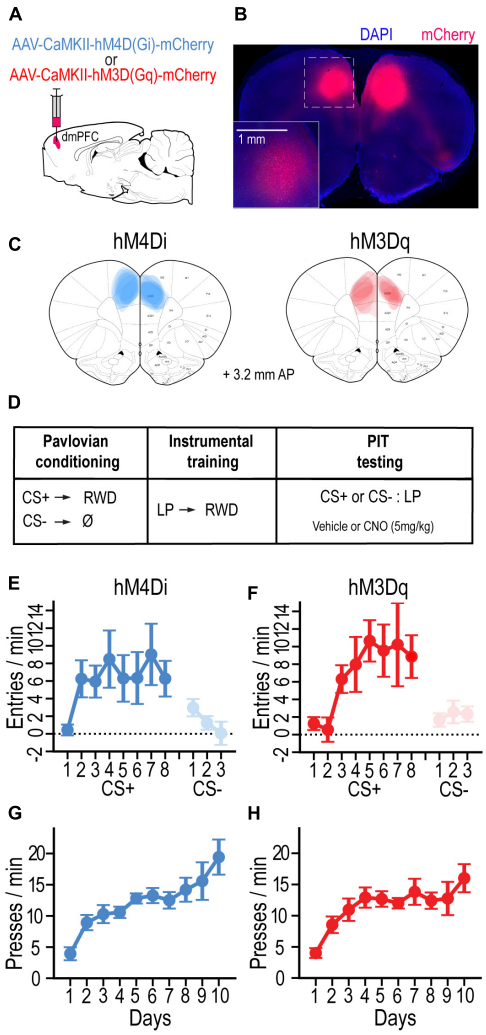

FIGURE 1
(A) Schematic of AAV strategy for expressing hM4Di or hM3Dq in dmPFC. **(B)** Coronal section showing representative mCherry-labeled DREADD expression. **(C)** Localization of DREADD expression for rats in hM4Di and hM3Dq groups (adapted from Paxinos and Watson, 2014). **(D)** Schematic of experimental design. **(E,F)** Mean cue-evoked food-port entry rate (entries/min; CS – Pre; ± SEM) during Pavlovian conditioning sessions with the CS+ and CS− for hM4Di **(E)** and hm3Dq **(F)** groups. **(G,H)** Mean rate of lever pressing (presses/min; ± SEM) during instrumental conditioning sessions for hM4Di **(G)** and hm3Dq **(H)** groups.

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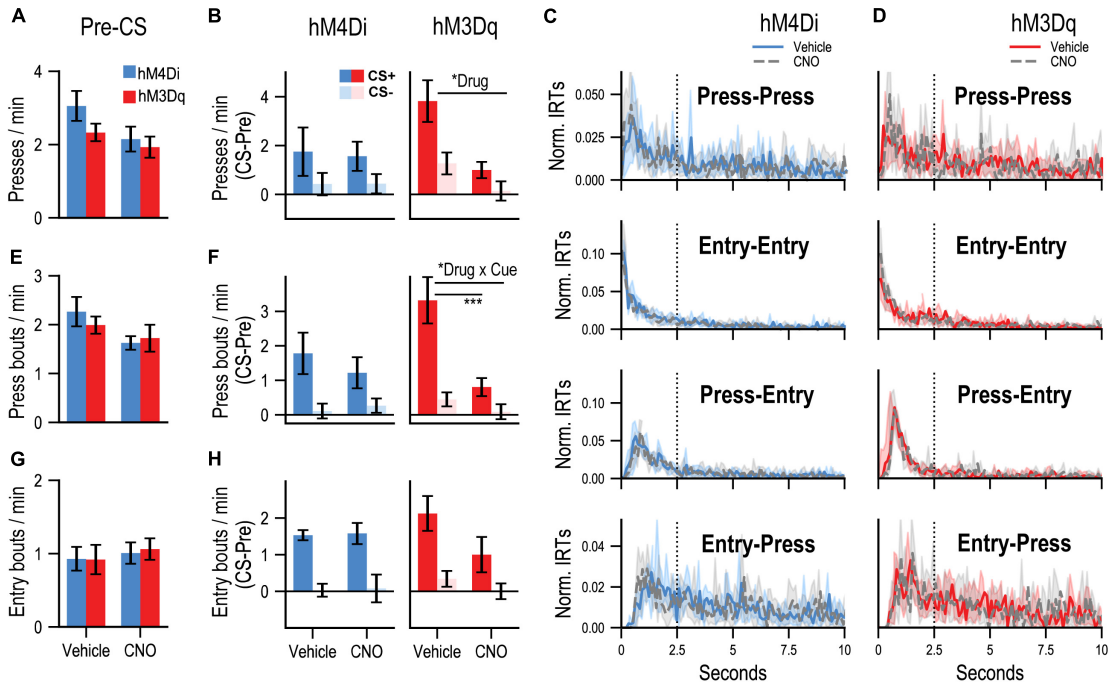

FIGURE 2

(A) Mean rates (responses/min; ± SEM) of lever pressing during Pre-CS (baseline) periods of the PIT test. **(B)** Mean rates (responses/min) of cue-evoked (CS − Pre; ± SEM) lever pressing at test. **(C,D)** Normalized inter-response time (IRT) distributions (<10 s; central tendency ± 95% confidence intervals; plotted over 0.1-s bins) for four possible response sequences: **Press-Press**, **Entry-Entry**, **Press-Entry**, and **Entry-Press**. Dotted vertical line indicates the 2.5-s cutoff used for bout analysis (refer to main text). Data are separately plotted for hM4Di **(C)** and hM3Dq **(D)** AAV group and drug condition as indicated. **(E)** Mean rates (responses/min; ± SEM) of press bouts during Pre-CS (baseline) periods of the PIT test. **(F)** Mean rates (responses/min) of cue-evoked (CS − Pre; ± SEM) press bouts at test. **(G)** Mean rates (responses/min; ± SEM) of spontaneous (press-independent) food-port entry bouts during Pre-CS (baseline) periods of the PIT test. **(H)** Mean rates (responses/min) of cue-evoked (CS − Pre; ± SEM) food-port entry bouts at test. "Drug" refers to significant main effects of Drug, and "Drug × Cue" refers to a significant Drug × Cue interaction. The bar below indicates a significant simple effect of Drug for the CS+ condition. *$p < 0.05$, ***$p < 0.001$.

on associative learning or a non-associative processes such as pseudoconditioning. Regardless, these cue-related changes in press rate were not reliably altered by CNO administration in group hM4Di [Drug: $F_{(1,\ 6)} = 0.003$, $p = 0.96$; Drug × Cue interaction: $F_{(1,\ 6)} = 0.003$, $p = 0.96$]. In contrast, group hM3Dq showed a preferential elevation in lever pressing during CS+ relative to CS− [Cue: $F_{(1,\ 6)} = 8.97$, $p = 0.008$] and were also sensitive to CNO treatment, which generally attenuated cue-related lever pressing [Drug: $F_{(1,\ 6)} = 12.01$, $p = 0.0028$]. However, this drug effect did not significantly interact with cue type [Drug × Cue interaction: $F_{(1,\ 6)} = 2.22$, $p = 0.15$], which further complicates data interpretation since it remains unclear if stimulating the dmPFC specifically interrupted the acquired motivational influence of the CS+ or whether it had a more general effect. It is also worth noting that although group hM3Dq appeared to show a more pronounced increase in pressing during the CS+ than group hM4Di under control (vehicle) conditions, this difference was not significant [t(12) = 1.41, $p = 0.18$, two-tailed independent *t*-test] and likely reflects random between-subjects variability.

Given these issues, we analyzed the microstructure of lever pressing to more directly assay the response-*instigating* influence of the reward-paired cue, which is a hallmark of Pavlovian incentive motivation (Rescorla and Solomon, 1967; Bindra, 1978). Instrumental behavior is organized into continuous bouts of lever pressing that are separated by occasional pauses, and previous work indicates that the rate of bout initiation provides a more selective readout of motivation than the overall rate of lever pressing, which is influenced by other learning and performance factors (Shull et al., 2001, 2004; Shull and Grimes, 2003; Shull, 2004; Johnson et al., 2009; Brackney et al., 2011). By plotting the distribution of inter-response-times in a given test it is possible to identify new bouts of lever pressing, which are initiated after long IRTs, and can be readily distinguished from higher-frequency, within-bout lever presses, which are separated by short IRTs. Inspection of the IRT distribution for all lever presses performed by groups hM4Di (**Figure 2C**, Press-Press) and hM3Dq (**Figure 2D**, Press-Press) during PIT test sessions confirmed that this behavior was indeed organized into bouts of high-frequency pressing, reflected by the distinct cluster IRTs in the 0.5–2 s range. We therefore defined bout-initiating presses as those occurring at least 2.5 s after the last press, to avoid misclassifying within-bout presses. Previous studies have used similar cut-off values

(Reed, 2011; Wassum et al., 2013; Smith et al., 2021) and indicate that such bout analyses are robust to variation in this parameter (Mellgren and Elsmore, 1991; Shull et al., 2002). Presses that occurred within 2.5 s of a food-port entry (**Figures 2C,D** and Entry-Press), regardless of the timing of the last lever press, were also categorized as bout-initiating presses as they represent a return to instrumental reward-seeking behavior.

During pre-CS periods, CNO administration did not significantly alter the rate at which new bouts of pressing were initiated (**Figure 2E**) in group hM4Di [Drug: $F_{(1,\ 6)} = 3.66$, $p = 0.10$] or group hM3Dq [Drug: $F_{(1,\ 6)} = .37$, $p = 0.57$]. Cue-elicited changes in bout initiation (CS – pre-CS, **Figure 2F**) were greater during CS+ trials in group hM4Di [Cue: $F_{(1,\ 6)} = 6.29$, $p = 0.046$] and group hM3Dq [Cue: $F_{(1,\ 6)} = 12.32$, $p = 0.013$], indicating that this measure was indeed more sensitive to the acquired motivational properties of the reward-predictive cue. CNO administration did not significantly alter cue-related bout initiation in group hM4Di [Drug: $F_{(1,\ 6)} = 0.16$, $p = 0.71$; Cue × Drug: $F_{(1,\ 6)} = 0.41$, $p = 0.55$]. In contrast, this behavior was strongly suppressed by CNO in group hM3Dq [Drug: $F_{(1,\ 6)} = 17.63$, $p = 0.006$; Cue × Drug interaction: $F_{(1,\ 6)} = 13.77$, $p = 0.01$], an effect that was limited to CS+ [$F_{(1,\ 6)} = 18.67$, $p = 0.005$] but not CS− [$F_{(1,\ 6)} = 1.98$, $p = 0.21$] trials.

We also examined how these treatments affected Pavlovian cue-elicited food-port entry behavior. Food-port entries were often performed in concert with ongoing instrumental behavior, as coordinated press-entry sequences (Marshall and Ostlund, 2018; Halbout et al., 2019; Marshall et al., 2020), which is reflected by the preponderance of short IRT Press-Entry sequences at test (**Figures 2C,D** and Press-Entry). The distribution of Entry-Entry IRTs (**Figures 2C,D** and Entry-Entry) indicated that these responses, like Press-Press sequences, were also clustered into discrete bouts of high frequency behavior (short IRTs). We therefore defined new bouts of spontaneous (press-independent) food-port entry behavior as entries that occurred at least 2.5 s after either the last entry or lever-press response.

We found that, during the pre-CS period, CNO administration did not alter the initiation of new food-port entry bouts in group hM4Di [Drug: $F_{(1,\ 6)} = 0.028$, $p = 0.87$] or group hM3Dq [Drug: $F_{(1,\ 6)} = .76$, $p = 0.42$; **Figure 2G**]. The CS+ was also more effective that the CS− at eliciting new entry bouts (**Figure 2H**) in groups hM4Di [Cue: $F_{(1,\ 6)} = 30.97$, $p = 0.001$] and hM3Dq [Cue: $F_{(1,\ 6)} = 24.27$, $p = 0.003$]. While cue-evoked food-port entry bouts appeared to be slightly attenuated by CNO in the hM3Dq group, this effect was not significant [Drug: $F_{(1,\ 6)} = 2.59$, $p = 0.16$; Drug × Cue: $F_{(1,\ 6)} = 1.31$, $p = 0.30$], nor was there a significant influence of CNO in group hM4Di [Drug: $F_{(1,\ 6)} = 0.17$, $p = 0.70$; Drug × Cue: $F_{(1,\ 6)} = 0.002$, $p = 0.96$].

To assess potential non-specific (DREADD-independent) behavioral effects of CNO administration, we conducted a separate experiment with GFP-only control rats ($n = 10$; **Figures 3A–C**). These rats readily learned to approach the food-port during Pavlovian conditioning (**Figure 3D**), such that by the last day of training with each cue, the CS+ elicited higher rates of entry than the CS− [$F_{(1,\ 9)} = 17.31$, $p = 0.002$]. They subsequently learned to press the lever for food pellets over instrumental training days [$F_{(9,\ 81)} = 36.75$, $p < 0.001$;

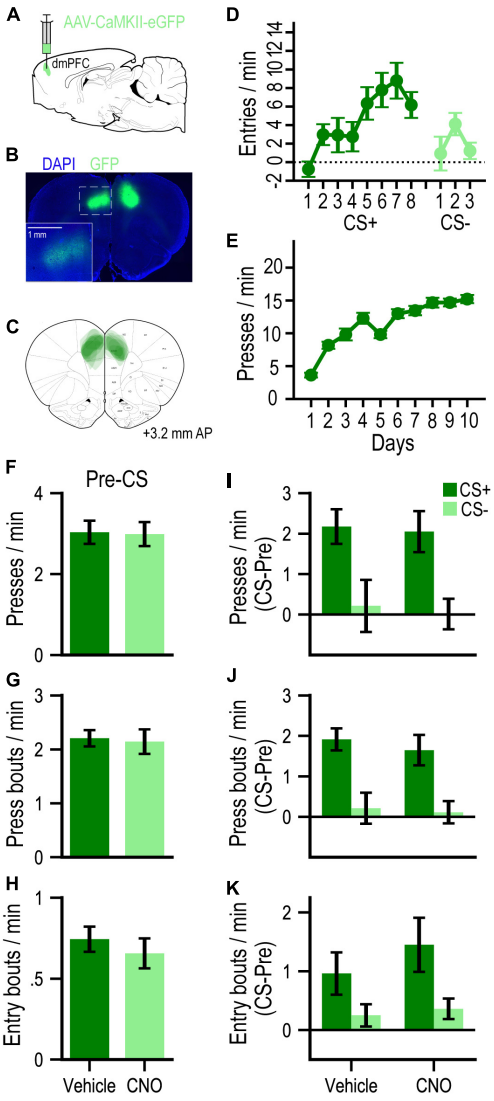

FIGURE 3
(A) Schematic of AAV strategy for expressing GFP in dmPFC. **(B)** Representative GFP expression. **(C)** Localization of expression for all rats in this experiment. **(D)** Mean cue-evoked food-port entry rate (entries/min; CS – Pre; ± SEM) during Pavlovian conditioning sessions with the CS+ and CS− for GFP group. **(E)** Mean rate of lever pressing (presses/min; ± SEM) during instrumental conditioning sessions for GFP group. **(F–H)** Mean rates (responses/min; ± SEM) of lever pressing **(F)**, press bouts **(G)**, and spontaneous (press-independent) food-port entry bouts **(H)** during Pre-CS (baseline) periods of the PIT test. **(I–K)** Mean rates (responses/min) of cue-evoked (CS – Pre; ± SEM) lever pressing **(I)**, press bouts **(J)**, and food-port entry bouts **(K)** at test.

Figure 3E]. During PIT testing, we found no effect of CNO on baseline (pre-CS) rates of pressing ($F < 1$, $p = 0.94$; Figure 3F), bouts of pressing ($F < 1$, $p = 0.86$; Figure 3G) or bouts of food-port entry ($F < 1$, $p = 0.57$; Figure 3H). These measures were all selectively elevated on CS+ vs. CS− trials [press rate: $F_{(1,\ 9)} = 21.83$, $p = 0.001$; press bout rate: $F_{(1,\ 9)} = 24.36$, $p < 0.001$; entry bout rate: $F_{(1,\ 9)} = 7.20$, $p = 0.025$; Figures 3I–K, respectively], in a manner that was not significantly affected by CNO administration (all Drug effects and Drug × Cue interactions, F's ≤ 1.25, p's ≥ 0.30).

Discussion

The current study examined the role of the dmPFC in Pavlovian incentive motivation. We found that stimulating the dmPFC (via CNO administration in the hM3Dq group) during PIT testing led to a pronounced disruption of cue-motivated lever pressing, whereas inhibiting the dmPFC (via CNO administration in the hM4Di group) had no reliable behavioral effects. These findings suggest that the dmPFC is capable of regulating Pavlovian incentive motivation but is not required for its expression. Moreover, no behavioral effects of CNO administration were observed in a reporter-only control group, confirming that this treatment did not have non-specific, DREADD-independent effects on PIT performance. Interestingly, dmPFC stimulation did not significantly alter Pavlovian cue-evoked food-port approach behavior, suggesting this structure is preferentially involved in regulating the motivational influence of reward-associated cues on instrumental reward seeking, rather than by exerting widespread control over all motor behavior.

The lack of effect of dmPFC inhibition on PIT expression would seem to be at odds with theories assigning this structure a motivational function (Paus, 2001; Stuss and Alexander, 2007; Holroyd and Yeung, 2012). Indeed, the dmPFC is closely connected with multiple brain regions implicated in PIT, including the ventral tegmental area, nucleus accumbens, dorsal striatum, mediodorsal thalamus, and basolateral amygdala (Cartoni et al., 2016; Corbit and Balleine, 2016). Moreover, a large proportion of dmPFC neurons responds to reward-predictive cues (Takenouchi et al., 1999; Otis et al., 2017), and encodes motivationally relevant parameters such as the magnitude, probability, and proximity of reward (Shidara and Richmond, 2002; Amiez et al., 2006; Kennerley et al., 2011; Toda et al., 2012). The dmPFC has also been implicated in other behavioral tests thought to engage Pavlovian incentive motivation, such as cue-induced reinstatement of drug-seeking behavior (Moorman et al., 2015; Feltenstein et al., 2021) and discriminative stimulus-elicited food-seeking behavior (Ishikawa et al., 2008).

However, our finding that the dmPFC is not critical for the expression of cue-elicited incentive motivation is consistent with previous PIT studies. For instance, Cardinal et al. (2003) found that rats with permanent excitotoxic lesions of the anterior cingulate were unimpaired on a simple (single-reward) PIT task similar to the one used in the current study. There is evidence that this version of the PIT task is predominantly driven by a non-specific or general appetitive arousal process that is capable of enhancing reward-seeking behavior broadly, regardless of which reward is predicted, though more direct measures of this so-called general PIT effect have been developed (Corbit and Balleine, 2016). Around the same time, Corbit and Balleine (2003) found that excitotoxic lesions of the nearby prelimbic cortex left intact the outcome-specific PIT effect, which measures a distinct influence of reward-predictive cues, namely their ability to bias action selection to promote the pursuit of a particular outcome (Corbit and Balleine, 2016). Interpreting such findings is complicated since permanent brain lesions may allow for functional compensation by neural circuitry that was intact during initial training sessions. The chemogenetic inhibition strategy used here, which was previously shown to reduce neuronal dmPFC neuronal activation and associated behaviors (Giannotti et al., 2018; Schmidt et al., 2019; Stolyarova et al., 2019), avoids this issue and bolsters the conclusion that the dmPFC is not a critical mediator of PIT expression.

While dmPFC inhibition did not impact PIT performance, stimulating the dmPFC attenuated this effect, which we suggest reflects this structure's capacity to exert inhibitory control over cue-motivated behavior. This is in line with previous findings that disrupting dmPFC function can weaken inhibitory control (Bussey et al., 1996; Muir et al., 1996; Broersen and Uylings, 1999; Narayanan and Laubach, 2006; Kamigaki and Dan, 2017; Hvoslef-Eide et al., 2018; Brockett et al., 2020; Li et al., 2020; Terra et al., 2020). However, these previous findings on their own do not address whether the dmPFC is specifically involved in regulating the expression of Pavlovian incentive motivation. This form of motivation, which is thought to drive impulsive and compulsive behaviors (Robinson and Berridge, 2008; Bari and Robbins, 2013), is not selectively probed in conventional tests of inhibitory control, which focus on measures such as premature, uncued responding (e.g., 5-choice serial reaction time task) or responding to inappropriate, non-reinforced cues (e.g., go/no-go or stop-signal tasks). While such responses may be motivated by prevailing reward-predictive cues, it is equally plausible that they are simply learned motor responses (e.g., conditioned reflexes or habits). This distinction is important as it is believed that behavioral/motor and emotional/motivational processes are regulated by separate neural systems (Bari and Robbins, 2013; Freeman et al., 2014).

Previous studies have shown that activating hM3Dq receptors on dmPFC neurons increases their spontaneous

and evoked activity (Hart et al., 2020). If neural activity in the dmPFC mediates a top-down inhibitory control function over Pavlovian incentive motivation, as hypothesized, then activating dmPFC neurons via hM3Dq-stimulation should suppress cue-motivated behavior, as reported here. However, it is also possible that stimulating dmPFC activity interfered with ongoing incentive motivational processing in downstream sites in a manner that may not reflect a normal function of that circuit. Further research will be needed to assess this possibility and determine if the dmPFC is in fact normally recruited to adaptively suppress maladaptive cue-motivated behavior. Importantly, while dmPFC inhibition did not alter PIT expression in the current study, the task used here was designed to assay an adaptive form of cue-motivated behavior and is therefore unlikely to engage of top-down control circuitry (Ostlund and Marshall, 2021).

In this context, it is useful to compare the current findings with a recent study examining the role of the nearby prelimbic cortex on regulating the expression of Pavlovian conditioned approach behavior (Campus et al., 2019), which focused on rats' tendency to sign-track (approach the reward-predictive cue) vs. goal-track (approach the food-port). A compelling case has been made that sign-tracking behavior represents a motivational response to the reward-predictive cue, whereas goal-tracking is the product of a cognitive, cue-evoked reward expectancy (Robinson et al., 2018). Using a bidirectional chemogenetic strategy similar to the one used here, Campus et al. (2019) found that inhibiting an anatomically defined subset of prelimbic neurons projecting to the paraventricular thalamus caused goal-trackers to sign-track (presumably by disinhibiting the Pavlovian incentive motivational system), and that stimulating these neurons caused sign-trackers to goal-track (presumably by inhibiting the motivational system). The findings are generally compatible with those reported here, though we targeted a more dorsal and less anatomically restricted population of medial prefrontal neurons for manipulation. Moreover, whereas Campus et al. (2019) conducted chemogenetic manipulations throughout training and test sessions, precluding conclusions about whether learning or performance processes were altered, our manipulations were restricted to test sessions to focus exclusively on performance processes.

The current study used response microstructure to isolate the tendency for reward-paired cues to instigate new bouts of lever pressing. There is growing evidence that reinforcement and motivational processes selectively influence the rate at which animals initiate new bouts of reward seeking (Shull et al., 2001, 2004; Shull and Grimes, 2003; Shull, 2004; Johnson et al., 2009; Brackney et al., 2011) and consumption (Marshall et al., 2018; D'Aquila et al., 2019). In the current study, the press bout rate measure proved to be useful for revealing associatively-mediated (i.e., CS+ specific) changes in lever press performance during PIT, which was critical for showing that the suppressive effect of dmPFC stimulation on cue-related lever pressing was specific to the CS+. This utility of bout analyses is also apparent in previous studies examining the neural mechanisms of PIT (Marshall and Ostlund, 2018; Halbout et al., 2019; Marshall et al., 2020). For instance, during cue presentations, bout-initiating lever presses are preceded phasic dopamine release in the nucleus accumbens (Wassum et al., 2013) and phasic glutamate release in the basolateral amygdala (Malvaez et al., 2015). Importantly, these neurochemical responses do not typically precede the execution of other (within-bout) lever presses and have an increased likelihood of occurring during motivationally-relevant, reward predictive cues.

The current findings reveal the dmPFC's capacity to regulate expression of Pavlovian incentive motivation. Since the current study used only male rats as subjects, further work will be needed to explore potential sex differences. Future studies will also be needed to determine whether and under which conditions the dmPFC is enlisted to flexibly suppress cue-motivated reward seeking, such as when this behavior might interfere with more adaptive reward retrieval activity (Marshall et al., 2020; Marshall and Ostlund, 2021; Ostlund and Marshall, 2021). It will also be important to identify the downstream circuitry through which the dmPFC exerts its suppressive influence over cue-motivated behavior, particularly as dysfunction in this circuitry may contribute to pathological forms of motivated behavior in addiction and other psychiatric disorders (Goldstein and Volkow, 2011; Bari and Robbins, 2013). The dmPFC may exert this influence by dampening incentive processes at projection sites or by recruiting additional components implicated in regulating motivated behavior including the paraventricular thalamus (Campus et al., 2019), subthalamic nucleus (Li et al., 2020), or striatal cholinergic interneuron system (Collins et al., 2016, 2019).

Data availability statement

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

Ethics statement

All experimental procedures were approved by the UC Irvine Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Author contributions

BH, SO, and KW contributed to the conception and design of the study and were responsible for writing the manuscript. BH and CH performed the experiments. BH and SO analyzed the data. All authors approved the submitted version 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.

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Psychopathic tendency in violent offenders is associated with reduced aversive Pavlovian inhibition of behavior and associated striatal BOLD signal

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Background: Violent offenders with psychopathic tendencies are characterized by instrumental, i.e., planned, callous, and unemotional (aggressive) behavior and have been shown to exhibit abnormal aversive processing. However, the consequences of abnormal aversive processing for instrumental action and associated neural mechanisms are unclear.

Materials and methods: Here we address this issue by using event-related functional magnetic resonance imaging (fMRI) in 15 violent offenders with high psychopathic tendencies and 18 matched controls during the performance of an aversive Pavlovian-to-instrumental transfer paradigm. This paradigm allowed us to assess the degree to which aversive Pavlovian cues affect instrumental action and associated neural signaling.

Results: Psychopathic tendency scores were associated with an attenuation of aversive Pavlovian inhibition of instrumental action. Moreover, exploratory analyses revealed an anomalous positive association between aversive inhibition of action and aversive inhibition of BOLD signal in the caudate nucleus of violent offenders with psychopathic tendencies. In addition, psychopathic tendency also correlated positively with amygdala reactivity during aversive versus neutral cues in Pavlovian training.

Conclusion: These findings strengthen the hypothesis that psychopathic tendencies in violent offenders are related to abnormal impact of aversive

processing on instrumental behavior. The neural effects raise the possibility that this reflects deficient transfer of aversive Pavlovian inhibitory biases onto neural systems that implement instrumental action, including the caudate nucleus.

KEYWORDS

psychopathy, Pavlovian-to-instrumental transfer, inhibition, fMRI, amygdala, caudate, putamen

Introduction

Instrumental decision making is susceptible to emotional/affective influences (Estes and Skinner, 1941; Damasio, 1997). Evidence suggests that this affective biasing of action selection can reflect an interaction between distinct behavioral control systems (Cardinal et al., 2002; Dayan et al., 2006; Kahneman and Frederick, 2007). For example, instrumentally controlled action selection is well established to be sensitive to biasing by a Pavlovian or hardwired "affective" system that regulates innately specified responses to aversive stimuli (Dayan and Seymour, 2013; Guitart-Masip et al., 2014). This Pavlovian system allows agents to control behavior through strategies that have been learnt across a lifetime and/or generations to be adaptive and thus to be generalizable to novel situations. Examples of such strategies are our tendencies to promote approach (and suppress withdarawal) actions when facing reward or to suppress approach (and potentiate withdrawal) actions when facing punishment. These strategies allow us to circumvent more expensive, rational instrumental (context-appropriate) calculations and to make judgments quickly and efficiently. However, they can also contribute to maladaptive behavior.

Anomalies in the interaction between these Pavlovian and instrumental control systems have been proposed to account for behavioral impairments seen in a wide variety of neuropsychiatric disorders (Dayan et al., 2006; Heinz et al., 2016; Huys et al., 2016; Hallquist et al., 2018; Nord et al., 2018; Chen et al., 2021). Here we focus on the high end of a psychiatric dimension (Patrick, 2022) that imposes a large burden on individual victims and society as a whole: psychopathic tendency. Specifically we study the interaction between Pavlovian and instrumental control systems in a group of violent offenders with high degrees of psychopathic tendency and a group of matched healthy controls. Psychopathic tendency is characterized by affective and behavioral anomalies (De Brito et al., 2021) and has been associated, in violent offenders, with "instrumental aggression" (i.e., callous and unemotional aggressive behavior) and high rates of recidivism even after prison sentences (Hare, 2003; Leistico et al., 2008; Warren and Burnette, 2013). Given our interest in psychopathic tendency, we employed the Psychopathy Checklist—revised (PCL-R) (Hare, 2003) as a psychological assessment tool to quantify, in each violent offenders, the degree of psychopathic tendency. The degree to which variation in Pavlovian-instrumental interaction varies with regard to individual differences in the PCL-R score were then assessed using correlational analyses.

A core feature of the crimes committed by violent offenders with psychopathic tendency is their "instrumentality," i.e., their planned, callous, and unemotional nature (Blair, 2001; De Brito et al., 2021). These crimes are premeditated and committed to achieve a desired goal at the expense of others. Despite the centrality of such callous and unemotional action in clinical observations and in elaborate cognitive models of psychopathic tendency [e.g., the violence inhibition model (Blair, 2005)], neuroscientific research on the mechanisms of instrumental action (i.e., actions planned to obtain a certain outcome) in the face of aversive cues is scarce. So far, the neuroscience of psychopathic tendency has focused mainly on reduced affective (primarily aversive) processing *per se* and associated neural signals, for example, in limbic circuitry (Brook et al., 2013). There is evidence (albeit in small samples) that people with psychopathic tendency respond normally to unconditioned aversive Pavlovian stimuli (US), but that their psychophysiological responses to conditioned aversive stimuli (CS) are compromised (Flor et al., 2002; Veit et al., 2002; Birbaumer et al., 2005; Rothemund et al., 2012; Schultz et al., 2016). However, it is unclear how such a deficiency in aversive information processing is related to the behavioral abnormalities of psychopathic tendency. Studies focusing on affective anomalies *per se* do not provide insight in the behavioral deficits that might stem from these affective anomalies. We set up the current study to test directly the hypothesis that psychopathic tendency is associated not only with abnormal aversive processing *per se*, but rather also with reduced transfer of aversive Pavlovian biases to instrumental behavior. We thus addressed one instance of the more general proposal that neuropsychiatric abnormality is associated with an absence of Pavlovian solutions to behavioral control.

The present study was conducted around the same time as another study we performed with violent offenders

(Ly et al., 2016) to test this same hypothesis. This prior study indeed demonstrated reduced potentiation of instrumental avoidance (versus approach) actions by aversive angry (versus appetitive happy) faces in a group of violent offenders compared with a group of matched controls. The added value of the present study is threefold. First, we provide a conceptual replication, thus increasing the construct validity of this prior finding by showing reduced impact of aversive Pavlovian cues on instrumental action in a different group of violent offenders with high levels of psychopathic tendencies, using a different paradigm. Notably, by including a neutral Pavlovian cue, this paradigm allowed us to establish that the altered impact of Pavlovian cues was due to reductions in aversive bias instead of increases in appetitive bias. Second, the present study addresses neural BOLD responses associated with aversive Pavlovian conditioning and the influence of aversive Pavlovian cues on instrumental behavior in violent offenders, showing a key role for the striatum in abnormal Pavlovian control of behavior. Finally, we demonstrate that the behavioral and neural changes are a function of individual differences in psychopathic tendency.

In our previous study, affective biases by facial cues were indexed during one and the same instrumental learning phase (Ly et al., 2016). By contrast, the paradigm employed here comprised three separate phases, allowing us to disentangle (i) instrumental action learning impairment, indexed during a first phase, from (ii) changes in the learning of, and responsiveness to Pavlovian cues themselves, indexed during a second Pavlovian conditioning phase, and (ii) changes in the key process of interest: Pavlovian-to-instrumental transfer, indexed during a final task phase. This key PIT process of interest was anticipated, based on prior work (Huys et al., 2011, 2016; Geurts et al., 2013a), to surface, across all participants, as potentiation of aversive instrumental withdrawal actions, but suppression of instrumental approach actions in the context of aversive Pavlovian cues, i.e., stimuli that predict aversive outcomes. Following our prior observation (Ly et al., 2016), violent offenders were expected to exhibit reduced impact of aversive cues on both types of instrumental action and we assess specifically whether this surfaces in a psychopathic tendency-dependent manner. Thus, we predicted that they exhibit reduced aversive inhibition of approach as well as reduced aversive potentiation of withdrawal actions.

Next, we assessed the neural mechanisms underlying the aversive PIT effects. Animal and human studies consistently implicate frontostriatal brain regions in instrumental action, especially the dorsomedial (caudate nucleus) and dorsolateral (putamen) parts of the striatum and the ventromedial regions of the prefrontal cortex (Tricomi et al., 2004; Valentin et al., 2007; Balleine and O'Doherty, 2009; Wunderlich et al., 2009; Dolan and Dayan, 2013). In addition, affective information is known to influence instrumental actions *via* the amygdala (Cardinal et al., 2002; Talmi et al., 2008; Balleine and O'Doherty, 2009; Prevost et al., 2012; Geurts et al., 2013a; Ly et al., 2014), and extensive evidence implicates dysfunction of the amygdala in people with psychopathic tendency (Veit et al., 2002; Birbaumer et al., 2005; Blair, 2008; Glenn and Raine, 2009; Moul et al., 2012). Thus, we anticipated, that violent offenders with psychopathic tendency would exhibit changes in aversive cue-related BOLD signal in the amygdala as well as differential aversive modulation of instrumental action-related signals in frontal and striatal brain regions. To this end, we focused our primary analyses on the striatum, ventromedial prefrontal cortex, and the amygdala.

Materials and methods

Subjects

Eighteen male violent offenders with psychopathic tendency (three left-handed) volunteered and were selected based on available information about clinical status and history from an in-patient population of a forensic hospital (**Supplementary material** and methods). All received a court-imposed placement under a hospital order with imprisonment for committing violence offenses repeatedly, including murder, slaughter, battery, rape, while suffering from psychiatric illness or disorder. The violent offenders all had a score of $\geq$26 on the Hare Psychopathy Check List-Revised (PCL-R) (Hare, 2003; **Table 1**). Additionally, twenty healthy men matched for age and IQ without criminal records or a history of psychiatric disorders were recruited from among the employees of the same hospital by advertisement. Participants in both groups were screened for drug use and for medical/neurological history (**Supplementary material** and methods and **Table 1**). Considering the particularities of the population, the testing environment, and the time period when testing was possible, these were the maximum numbers of inclusion.

Following previous studies (Brazil et al., 2009; von Borries et al., 2009), exclusion criteria were all major Axis-I and Axis-II disorders except for cluster B personality disorders in violent offenders, psychotropic medication, cannabis or other drug use 1 week before, alcohol or oxazepam use within 24 h before experiment, visual disorder, and neurological disorder. Furthermore, individuals not eligible for MRI scanning were excluded.

All participants received oral and written information about the experiment and gave written informed consent. They received payment as a reimbursement for participation. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethical committee (NL30545.091.09).

Two violent offenders withdrew from participation and one violent offender was excluded because of excessive head

TABLE 1 Group characteristics (mean, standard deviation) of the group of violent offenders with psychopathic tendency and healthy matched control subjects.

	Violent offenders with psychopathic tendency ($n = 15$)	Healthy controls ($n = 18$)	Statistics (P-value)
Age	40.2 (9.1)	41.2 (10.4)	0.78
IQ (NLV)	101.7 (8.8)	101.5 (8.7)	0.96
PCL-R total	30.7 (4.0)	–	–
PCL–R factor 1	11.9 (2.9)	–	–
PCL-R factor 2	13.9 (2.1)	–	–

Exclusion criteria for both groups were: (i) Use of alcohol more than 3 units/day during the week preceding the experimental measure and use of alcohol within 24 h of the measurement.
(ii) Use of cannabis or other illicit drugs within the week before measurement and use of psychotropic medication other than oxazepam during the 5 days before measurement.
(iii) Use of oxazepam within 12 h before measurement.
(iv) Smoking within 3 h before measurement.
(v) History of trauma capitis, visual and auditory disorders, neurological disorders, first degree relative with any relevant neurological disorders.

movement (more than twice the voxel size). Two non-criminal healthy controls were excluded because their behavioral data suggested they did not follow the instructions during the PIT stage [despite instructions to play the instrumental game (see paradigm) these participants determined their actions solely on the Pavlovian CS, but never on the instrumental stimuli in more than half of the trials: 58 and 83% resp., compared with on average 1%, range 0–17%, for all other participants]. Moreover, due to technical issues with the scanner and excessive head movement only one of two runs could be analyzed for one healthy control and two violent offenders. Thus, we analyzed datasets of 15 violent offenders and 18 healthy controls.

Pavlovian-instrumental transfer paradigm

Subjects performed a computerized task to assess aversive PIT (Geurts et al., 2013a; **Supplementary material**; **Figure 1**). The experiment consisted of three stages: (1) instrumental, (2) Pavlovian, and (3) PIT stage. The instrumental stage contained two Action Contexts: (i) a context in which the active response led to an approach action and (ii) another in which the active response led to a withdrawal action (**Figure 1A**). In the approach Action Context subjects learned through monetary feedback (wins and losses) whether to "collect" the instrumental stimulus (approach-go) or not (approach-no-go). In the withdrawal Action Context they learned to avoid collecting instrumental stimuli (withdrawal-go) or not (withdrawal-no-go). Instrumental stimuli were randomly assigned to one of the four trial types. Thus, in both the approach and withdrawal Action Contexts, there were two go-stimuli, which yielded reward more often (i.e., ∼85% of the cases) after active responses (and punishment after not responding), and two nogo-stimuli, which yielded reward more often (i.e., also ∼85% of the cases) after not responding (and punishment after go-responding).

The second, Pavlovian stage consisted of repeated presentation of three audiovisual stimuli (**Figure 1B**): The appetitive and aversive conditioned stimuli (CS) were followed, respectively, by appetitive or aversive juice (i.e., the unconditioned stimuli Uss) on 50% of trials. The neutral CS resulted in no outcome. The appetitive juice was based on subjective preference for apple, orange, or strawberry lemonade. The aversive juice was a bitter magnesium sulfate solution (0.3M). Conditioning was assessed in two ways: (1) subjects indicated the degree to which they liked each of the CSs (and USs) by use of visual analog scales (VAS), before and after the experiment; (2) subjects chose one of the two presented Pavlovian stimuli (presented for 2 s; ITI 0.5 s) in extinction on 12 interspersed query trials.

In the third (PIT) stage stimulus presentation was the same as in the instrumental stage, except that Pavlovian stimuli tiled the background from 250 ms before (Larson et al., 2013) and no outcomes were presented (**Figure 1C**). Subjects were instructed that their choices counted toward the final monetary total, and that the juices associated with the Pavlovian outcomes were collected outside the scanner for them to drink afterward. There were two independent runs which each comprised different stimuli/CSs. Both runs included all three stages, and were separated by a 2-min break.

Image acquisition

Whole-brain imaging was performed on a 3 Tesla MR scanner (Magnetrom Trio Tim, Siemens Medical Systems, Erlangen, Germany). Functional data were obtained using a multi-echo gradient T2*-weighted echo-planar scanning sequence (Poser et al., 2006; **Supplementary material**).

Analyses

Behavioral data analysis

In keeping with our research aims we assessed group differences as well as parametric associations with PCL-R score. These scores were only available for the violent offenders. The behavioral data were analyzed using the statistic software SPSS 16.0 and Matlab® 2009b.

Instrumental training

First, the proportion of correct responses was calculated for the first ten and last ten trials for each of the four trial types (covering all 80 instrumental trials). To assess whether subjects learned to make the correct choice, data were

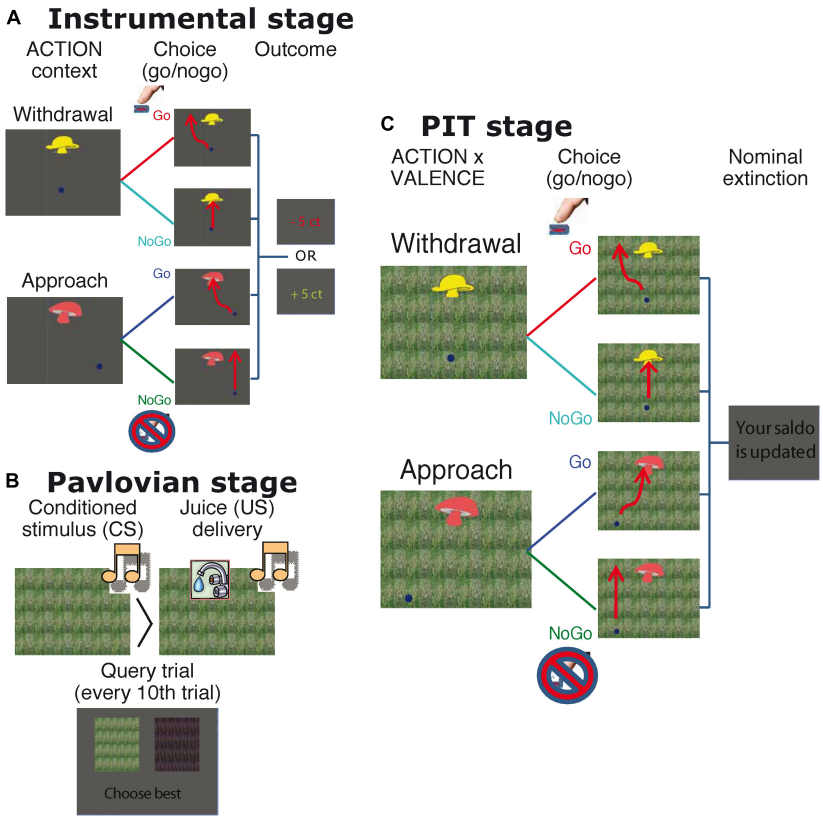

FIGURE 1
Task details. **(A)** Instrumental stage. Trials started with the appearance of the instrumental stimulus at the top center of the screen and of a dot at the bottom of the screen. In approach trials, the dot started either on the left or on the right bottom side of the screen. Participants could choose to do nothing (approach-no-go), in which case the dot would wiggle past the instrumental stimulus. Alternatively, they could push the button repeatedly to steer the dot through the instrumental stimulus (approach-go). In withdrawal trials, the dot started centrally at the bottom beneath the instrumental stimulus. Participants could choose to push the button repeatedly to avoid moving through instrumental stimulus (withdrawal-go) or to do nothing (withdrawal-no-go). The four possible trajectories are drawn in the figure (red lines). If the dot entered the goal region, then the instrumental stimulus was collected. After the dot moved outside the window feedback was provided. Thus, there were 2 ACTION contexts (approach and withdrawal), with each 4 different instrumental stimuli, with 2 stimuli resulting more often in reward after a go-action and 2 resulting more often in reward after a no-go. Each stimulus was presented 10 times, resulting in ($2 \times 4 \times 10$=) 80 instrumental trials (divided in mini blocks of 8 withdrawal or approach trials). The straight line just to one side of the instrumental stimulus was a reflecting boundary that the dot could not cross. Timings were as follows: Instrumental stimuli were presented for 2.5 s, during which responses were collected. After 2.5 s, feedback was presented for 1 s. The intertrial interval (ITI) was 1 s (blank screen). **(B)** Pavlovian stage. Each Pavlovian conditioned stimuli (CS) was presented 20 times, and for each session there was a separate set of three stimuli. Stimulus presentation order was fully randomized across participants. Stimulus duration was 4.5 s, and juice delivery (2 ml) occurred between 0 and 1.5 s after stimulus onset. The ITI was 1 s. Query trials were presented after every 10 Pavlovian trials. On these trials, participants chose one of the two presented Pavlovian (audiovisual) stimuli (presented for 2 s; ITI 0.5 s) without any feedback. **(C)** PIT stage. The PIT stage paralleled the instrumental training, except that Pavlovian CSs tiled the background. Each instrumental stimulus was presented 12 times and each Pavlovian CS was presented 32 times, counterbalanced across the different instrumental stimuli. No outcomes were presented, but participants were instructed that their choices counted toward the final total. Participants were explicitly instructed that the juices were collected outside the scanner, and they agreed before the start of the experiment to drink them afterward. Timing of one trial was as follows: 250 ms after the onset of the Pavlovian stimulus, the instrumental stimulus (and dot) was overlaid on top of this Pavlovian stimulus. Duration of the instrumental stimulus was 2.5 s; duration of the Pavlovian stimulus was 2.75 s. Upon offset of both stimuli, feedback was presented, which consisted only of the words "Balance is updated" (duration = 1 s, ITI = 1 s). Note that there were two runs in which all three stages (with new independent Pavlovian and instrumental stimuli) were assessed.

averaged across sessions and submitted to a repeated measures analysis of variance (rmANOVA) with Time (beginning/end of instrumental training), Action Context (approach/withdrawal) and Response (go/nogo) as within-subject and Group (healthy controls/violent offenders) as between-subject factor. Second, we assessed whether the learned behavior generalized to the PIT stage. Therefore, the factor Time was changed to include three levels: the end of the instrumental training and the beginning and the end of the PIT stage.

Pavlovian conditioning

Non-parametric tests were used to assess the proportion of correct responses on Pavlovian query trials and pre- and

post-conditioning VAS ratings of the CS, because data were not distributed normally.

Pavlovian-instrumental transfer

The behavioral outcome measure was proportion of go actions, p(go), as a function of trial type (i.e., Action Context and CS Valence). Effects of CS Valence and Action on p(go) reflect PIT effects on choice. This dependent variable was first averaged across runs before it were submitted to an rmANOVA with Action Context (approach/withdrawal), and CS Valence(neutral/aversive) as within-subject factors and Group (healthy controls versus violent offenders) as a between-subject factor. Note that we focused our analyses on aversive PIT, based on our hypothesis (see Section "Introduction") and on our previous work ($n = 33$) showing that the current paradigm was not sensitive to (and therefore not valid to assess) appetitive PIT [neutral vs. appetitive (Geurts et al., 2013a), **Supplementary results**]. The PCL-R-score was added as a covariate to assess its association with aversive PIT.

Functional magnetic resonance imaging analysis

Functional magnetic resonance imaging analysis was performed with SPM5 software (Wellcome Trust Centre for Cognitive Neuroimaging, London, UK). Pre-processing steps and first-level fMRI analysis were exactly as described by Geurts et al. (2013a): Pre-processing steps included applying a PAID-weight algorithm (Poser et al., 2006) to combine the different echoes, slice-time correction, coregistration, normalization based on parameters estimated through segmentation of the structural images, and smoothing.

The primary analysis was restricted to the PIT-stage. At the subject level a general linear model (GLM) was specified with 6 main regressors (4 of interest) representing the onset of the six different PIT trials of this paradigm [Action Context (approach/withdrawal) − Valence(appetitive/neutral/aversive)]. For each main regressor two additional parametric regressors were added (Büchel et al., 1996): The PIT-regressor (Talmi et al., 2008) was a parametric modulator of BOLD responses by the number of button presses per trial. A further parametric regressor contained the expectation associated with each instrumental stimulus (the Q-value) per trial as estimated from a model-based analysis of behavior (Huys et al., 2011). This was done based on prior data showing that BOLD signal in the prefrontal cortex and striatum, our regions of interest, covary with instrumental action value (e.g., Valentin et al., 2007; Wunderlich et al., 2009). As such, this approach maximized the degree to which our GLM captured variability in relevant BOLD signal. Furthermore, realignment parameters were added, high-pass filtering (128 s) was applied and parameter estimates were obtained by maximum-likelihood estimation (AR1).

The parameter estimates for the 4 parametric PIT-regressors were used in a 2 × 2 × 2 rmANOVA at the group-level (with random effects) with Action Context(approach/withdrawal) and Valence (neutral/aversive) as within-subject factors and GROUP (healthy controls/Vos) as between-subjects factor. Planned contrasts were the same as in Geurts et al. (2013a), but now assessed as a function of Group: [aversive-neutral] to reveal regions involved in aversive PIT across Action Contexts, and [(approach aversive-approach neutral)–(withdrawal aversive-withdrawal neutral)] to reveal regions involved in action-specific aversive PIT, and [approach–withdrawal] to reveal action-specific regions.

To capture group differences in brain-behavior associations, beyond those related to trial-by-trial variation, we contrasted the main regressors (Talmi et al., 2008; Geurts et al., 2013a) at the subject-level to calculate the main effect of Valence [(approach aversive + withdrawal aversive)–(approach neutral + withdrawal neutral)] and an interaction between Valence and Action Context [(approach aversive–approach neutral)–(withdrawal aversive–withdrawal neutral)]. The resulting individual contrasts were then used in a two-sample t-test at the group-level with behavioural aversive PIT-effects [p(go)] as a covariate for each group separately enabling comparison between groups. Thus, these analyses reveal regions, on a subject-by-subject basis, in which CS Valence-dependent BOLD signal change during the PIT stage was associated with aversive PIT. This association was assessed as a function of Action Context and Group. These analyses were repeated with PCL-R score (instead of behavioral PIT) as a covariate to assess whether CS Valence dependent BOLD signal change during the PIT stage was associated with psychopathy severity.

Next, additional analyses were performed to assess whether positive behavioral and fMRI findings from the PIT stage could be explained by BOLD signal change in the Pavlovian conditioning stage. Thus, we analyzed CS Valence-dependent BOLD signal change during the Pavlovian training phase as a function of individual differences in PCL-R score, aversive PIT and neural signaling in the caudate nucleus (see Section "Results") during the PIT stage (each inserted as a covariate in three separate whole-brain analyses of CS Valence-related signals during the Pavlovian training phase).

First, at the subject level, a GLM was specified with six main regressors of interest representing the onset of the CS trials (during which no US was presented) in the beginning and the end of the conditioning stage: Valence (appetitive/neutral/aversive) × Time (early/late). This latter distinction between early and late acquisition, was based on evidence of rapid habituation of the responses in the amygdala during conditioning (Birbaumer et al., 2005). Early trials were the first three trials following the first US presentation for aversive and appetitive CS trials. For the neutral CS, the early trials were the first three presentations and the late trials were all the remaining CS presentations thereafter. To capture the other parts of the Pavlovian training, four regressors were added: for appetitive and aversive US onsets, for juice delivery

onset (duration 2 s) and for the query trial onset (duration 2 s). Realignment and high-pass filtering was applied as before. Parameter estimates were obtained by maximum-likelihood estimation (AR1). We calculated the main effect of CS Valence (i.e., aversive-neutral) at the subject level for early, late and overall [early + late] conditioning and correlated the effects at the group-level (one sample *t*-test with covariate) with the PCL-R score; behavioral aversive PIT; and the extracted betas of the caudate nucleus as covariates of interest.

Regions of interest analysis

We report those effects that survive family wise error (FWE) correction for multiple comparisons across the whole brain ($P_{WB} < 0.05$, voxel-level) or regions of interest (ROIs, see Section "Introduction" for rationale): Following exactly the same procedure as in our previous work (Geurts et al., 2013a) the bilateral amygdala, caudate nucleus and putamen were defined using the automated anatomical labeling atlas in the WFU PickAtlas toolbox (Tzourio-Mazoyer et al., 2002). The bilateral nucleus accumbens was segmented for each participant using the FSL FIRST segmentation tool (Patenaude et al., 2011). These individual segments were then overlaid onto each other, generating one nucleus accumbens for the group (cf. Geurts et al., 2013a). Again following this prior work, we used the action-specific (approach > withdrawal) activation cluster [$p < 0.001$ uncorrected; peak voxel MNI-coordinates: $(-8, 36, -8)$] revealed by this previous PIT study to assess action specific signal in the vmPFC. The left and right elements of the bilateral volumes of interest were combined using Marsbar^{TM} (Brett et al., 2002). Masks of the ROIs can be found in the **Supplementary material**.

Results

Behavioral data

Instrumental and Pavlovian stage

Healthy controls tended to learn faster than the violent offenders during instrumental training {Group × Time $F_{(1,31)} = 4.3$, $p = 0.072$; note, however, that they did not differ from violent offenders in terms of instrumental performance during the PIT stage [main effect of Group: $F_{(1,31)} = 2.0$, $P = 0.17$]}. There were no other relevant group differences in the instrumental and Pavlovian stages of the PIT paradigm (**Supplementary material**).

Pavlovian-instrumental transfer

Data revealed the expected action-specific PIT effects across groups (Huys et al., 2011; cf. Geurts et al., 2013a): Aversive stimuli inhibited approach, but promoted withdrawal [**Figure 2A**; interaction Action Context(approach/withdrawal) × Valence(neutral/aversive): $F_{(1,31)} = 8.6$, $p = 0.006$; Valence during approach: $F_{(1,31)} = 4.7$, $p = 0.037$; Valence during withdrawal: $F_{(1,31)} = 4.3$, $p = 0.046$]. Subjects made more go-responses in the approach than in the withdrawal context overall [main Action Context effect: $F_{(1,31)} = 9.9$, $p = 0.004$]. However, in contrast to our expectation no significant main effect of or interaction with Group was found ($F < 2.4$, $p > 0.161$, **Supplementary Table 2**).

Regarding individual differences in psychopathic tendencies, we found that higher PCL-R scores were associated with less aversive inhibition, in a manner that was action-non-specific [interaction PCL-R score × Valence(neutral/aversive): $F_{(1,13)} = 12.6$, $p = 0.004$; **Figure 2B**]. Thus, higher PCL-R scores were associated with reduced aversive inhibition of approach actions [simple effects for approach block only: (p(go| aversive&approach)–p(go| neutral&approach)) × PCL-R score: $F_{(1,13)} = 5.6$, $p = 0.035$)] and a tendency toward increased aversively motivated withdrawal actions [simple effects for withdrawal block only: (p(go| aversive & withdrawal) – p(go| neutral & withdrawal)) × PCL-R score: $F_{(1,13)} = 4.3$, $p = 0.059$].

Imaging data

In contrast to our hypothesis, the neural responses during aversive PIT did not differ between groups [no significant Action Context (approach/withdrawal) × Valence (neutral/aversive) × Group (violent offenders/healthy controls) interactions]. Action-specific signals (approach vs. withdrawal) across CS Valence were found in the precuneus [(12, −78, 6), $k = 5453$, $Z = 7.02$, $p_{FWE} < 0.001$, whole brain corrected], lingual [(10, −52, 52), $k = 310$, $Z = 5.50$, $p_{FWE} = 0.001$, whole brain corrected] and middle occipital gyrus [(34, −88, 2), $k = 159$, $Z = 4.98$, $p_{FWE} = 0.016$, whole brain corrected].

Moreover, within the group of violent offenders there was no significant effect of psychopathic tendency on BOLD signal during aversive PIT at the whole brain or in the pre-specified regions of interest.

Individual differences in behavioral aversive PIT [p(go| aversive)–p(go|neutral)] correlated positively with BOLD signal (aversive main regressor–neutral main regressor) in the putamen across both groups: Greater aversive inhibition of behavior was associated with reduced BOLD signal during aversive PIT trials (versus neutral) in the left putamen [**Figure 3**, peakvoxel MNI-coordinates (−26, 2, 6), $k = 205$, $Z = 4.59$, $p_{FWE} = 0.004$, small volume corrected]. In other words, higher BOLD signal in the putamen during the presentation of the aversive (versus neutral) Pavlovian cue was accompanied by greater disinhibition of go-actions, in line with the hypothesis that putamen signal reflects motor execution. Conversely, there was a significant difference between groups in the caudate nucleus [peakvoxel MNI-coordinates (14, 20, 10), $k = 98$, $Z = 4.26$, $p_{FWE} = 0.006$, small volume corrected, **Figure 3**]: By contrast to the

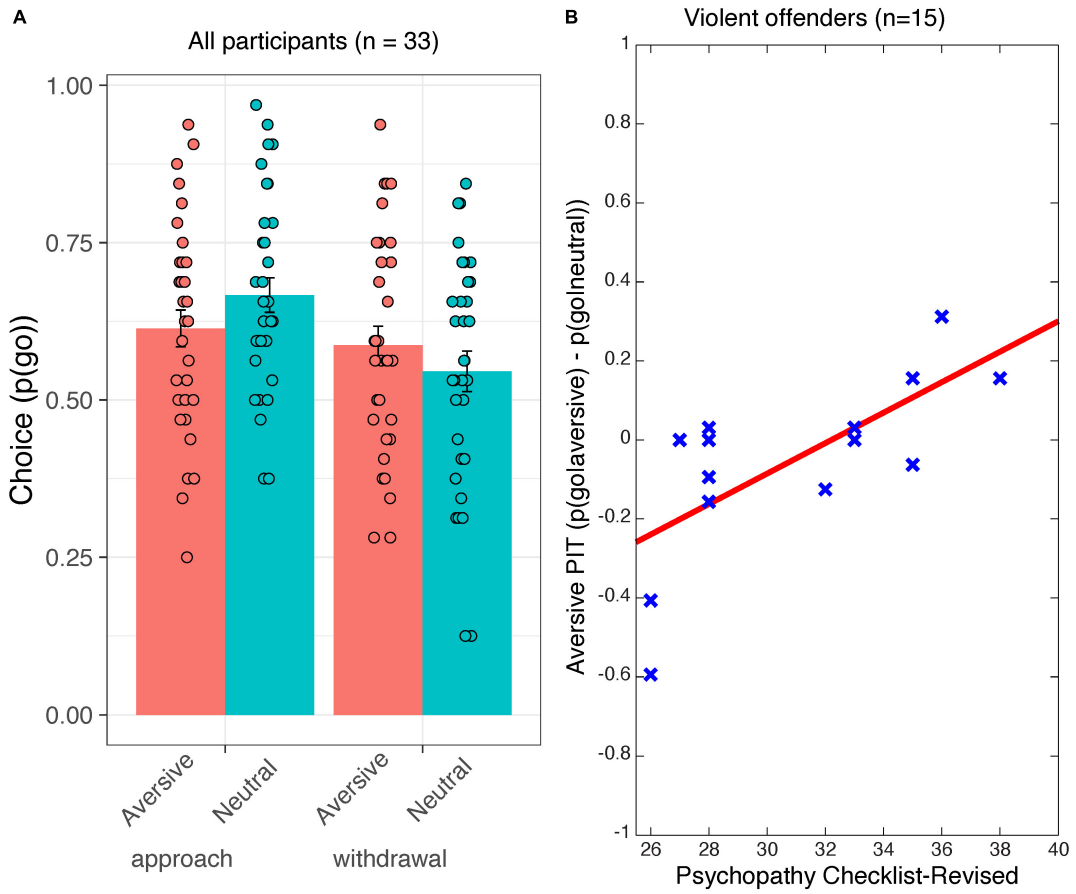

FIGURE 2
Behavioral data from the Pavlovian-instrumental transfer stage. Shown are **(A)** choice [*p*(go)] as a function of Action Context (approach and withdrawal) and Valence (aversive/neutral) for all participants. Error bars represent standard errors of the mean. **(B)** The correlation between aversive PIT [i.e., *p*(go| aversive)−*p*(go| neutral)] and psychopathy severity (in terms of the psychopathy checklist−revised total score). The red line is the ordinary least square trend line. Each cross represents an individual data point.

putamen, signal in the caudate nucleus of healthy controls (aversive versus neutral) correlated negatively with aversive PIT [$p_{\text{uncorrected}} = 0.012$ at peak voxel (14, 18, 10), **Figure 3**], so that greater aversive inhibition of behavior was associated with greater aversive BOLD signal during aversive (versus neutral) PIT trials. This concurs generally with the hypothesis that caudate nucleus signal reflects the operation of a more complex computational operation related to motor planning (Alexander et al., 1986; Herz et al., 2013; Provost et al., 2015), such as the transfer of affective information onto action. Critically, this positive relation between BOLD signal in the caudate during aversive trials and behavioral inhibition was completely reversed in the violent offenders so that greater aversive inhibition of behavior in the violent offenders was associated with reduced BOLD signal during aversive (versus neutral) PIT trials in the caudate nucleus [peak voxel MNI-coordinates (14, 18, 10), $k = 225$, $Z = 4.85$, $p_{FWE} = 0.039$, corrected for the whole-brain]. Thus, the caudate nucleus of violent offenders acted like their putamen, perhaps reflecting a failure to engage the computation that is required for translating aversive information into action suppression. Note, that we repeated this correlation analysis within the violent offender group by means of Kendall's tau analyses, because Kendall's tau analysis is preferred over parametric methods for small samples and is robust to outliers (Croux and Dehon, 2010). Correlation of the mean beta estimates from the caudate nucleus with behavior remained significant [$\text{tau}_{(15)} = 0.520$, $p = 0.008$]. Moreover, based on reviewer comments we also conducted this analyses without the two violent offenders that visually might seem to drive these findings and found that the correlation is robust to excluding these data points [$\text{Tau}_{(13)} = 0.450$, $p = 0.047$].

Next, we asked whether the psychopathic tendency-related behavioral PIT effects (**Figure 2B**) were accompanied by psychopathic tendency-related differences in Pavlovian conditioning. Psychopathic tendency in terms of PCL-R score was indeed related to CS-dependent BOLD signal change

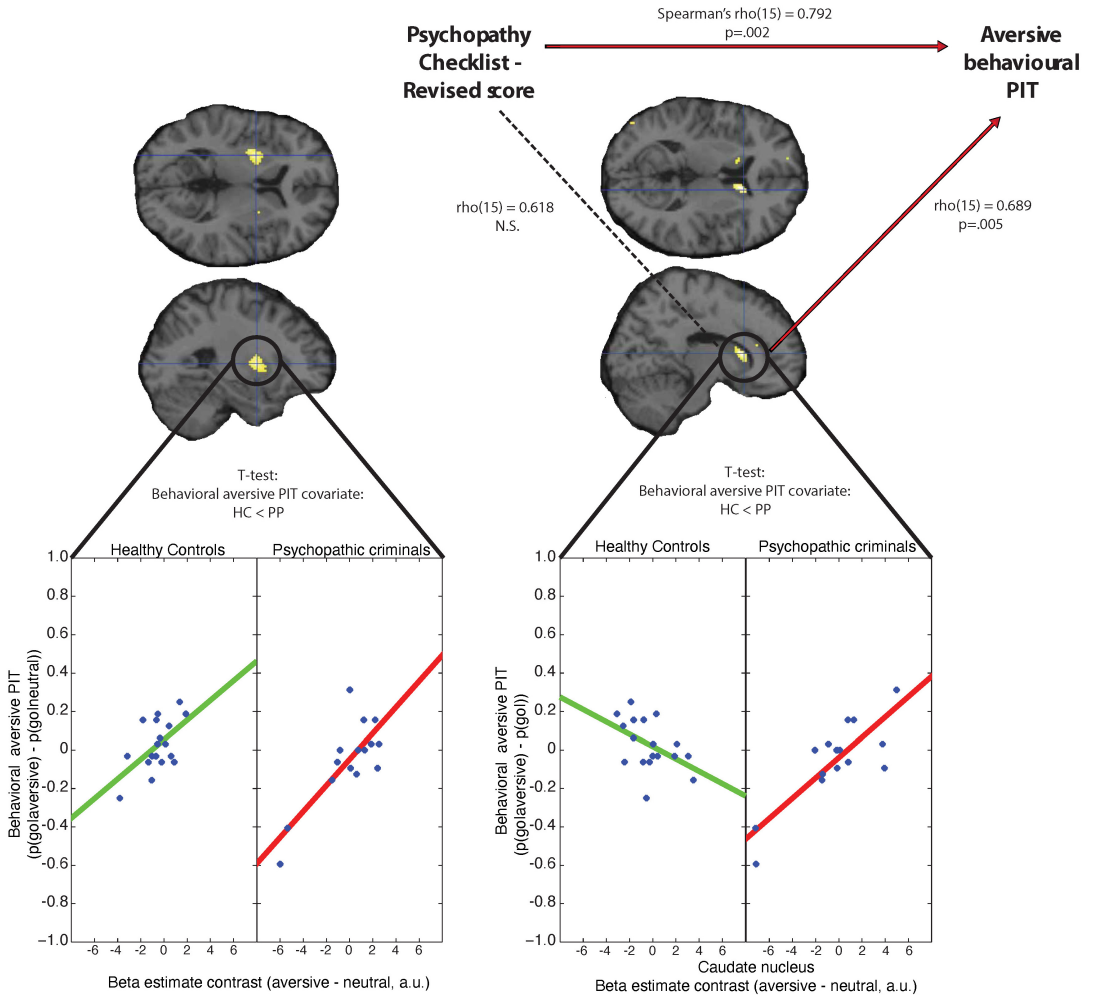

FIGURE 3
Association between aversive behavioral PIT, beta estimate contrasts (neutral–aversive trials) and psychopathy severity (in terms of the psychopathy checklist–revised total score) for the putamen and the caudate nucleus. **Left panel:** Beta estimate contrasts (neutral–aversive trials) within the putamen are positively correlated with behavioral aversive PIT [*p*(go| neutral)–*p*(go| aversive)] for violent offenders and healthy controls. **Right panel:** Beta estimate contrasts (neutral–aversive trials) within the caudate nucleus correlate positively with behavioral aversive PIT [*p*(go| neutral)–*p*(go| aversive)] for psychopathic criminals, but not for healthy controls. Correlations between the mean beta estimate contrasts and PCL-R and aversive behavioral PIT are calculated in terms of Spearman's rho. Scatterplots are for illustrative purposes only and were created by plotting the behavioral PIT effect against the extracted average beta estimate contrast from the $p < 0.001$ whole-brain uncorrected clusters within the putamen and caudate nucleus.

(aversive versus neutral) over the whole conditioning stage) in the bilateral amygdala [right amygdala: peakvoxel MNI-coordinates (24 2 −24), $k = 22$, $Z = 3.51$, $p_{FWE} = 0.033$, left amygdala: peakvoxel MNI-coordinates (−18 −2 −22), $k = 37$, $Z = 3.48$, $p_{FWE} = 0.036$, small volume corrected for the bilateral amygdala; Figure 4]. This psychopathy severity dependent effect was also accompanied by group differences in the left amygdala [left amygdala: peakvoxel MNI-coordinates (−12 −2 −22), $k = 5$, $Z = 3.65$, $p = 0.010$, small volume corrected for the bilateral amygdala], so that violent offenders exhibited greater amygdala signal during aversive versus neutral cues compared with healthy controls. Note, that we did not find a significant association between CS-dependent amygdala signal during Pavlovian conditioning and aversive PIT behavior ($p_{uncorrected} > 0.001$).

Discussion

The present study shows that, although we did not find behavioral group differences between healthy controls and violent offenders, higher psychopathic tendency within the violent offender sample was accompanied by attenuated inhibition of instrumental behavior in the presence of (non-consequential) Pavlovian aversive cues. In addition, while aversive PIT and caudate BOLD signal correlated negatively

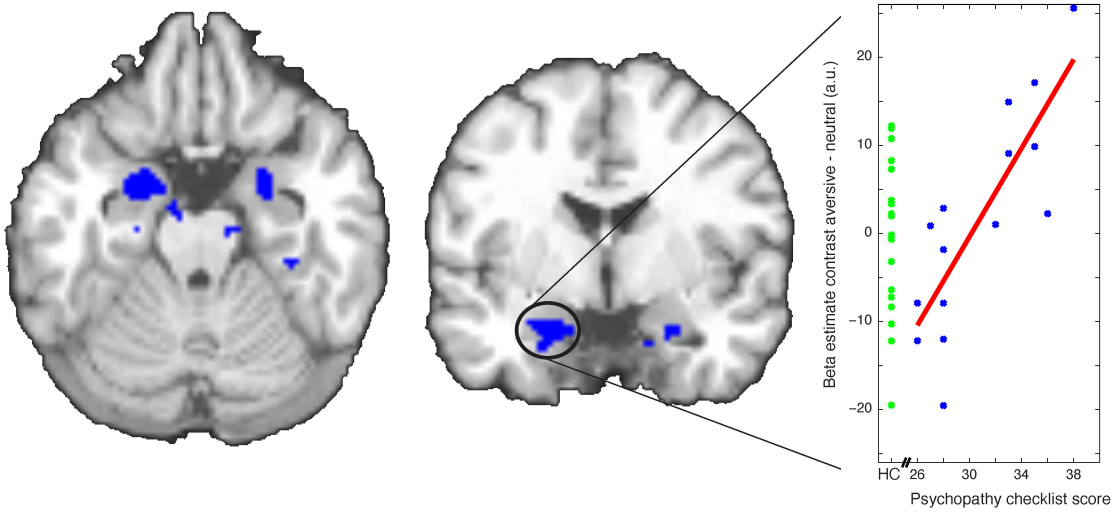

FIGURE 4
Association between psychopathy severity [in terms of Psychopathy Check List-Revised (PCL-R) total score] and beta estimate contrasts [conditioned stimuli (CS) aversive–CS neutral trials] during Pavlovian conditioning. Scatterplot depicts relation between PCL-R total score and the extracted average beta estimate contrast from the $p < 0.001$ whole-brain uncorrected cluster within the amygdala (for illustrative purpose only). Data is also displayed for healthy controls for whom no PCL-R score was available (green points).

in healthy volunteers, this correlation was completely reversed in the violent offenders with psychopathy (compared with non-criminal healthy controls). Moreover, within the group of violent offenders, we established a positive association between psychopathic tendency and amygdala reactivity to aversive cues during Pavlovian conditioning. Together, these data suggest that psychopathic tendency is associated with enhanced aversive Pavlovian cue reactivity (cf. Schultz et al., 2016), yet reduced aversive inhibition of instrumental behavior. In addition, these data suggest a link between psychopathic tendency, Pavlovian aversive inhibition, and striatal action selection.

The finding that increased psychopathic tendency was associated with reduced aversive inhibition is reminiscent of findings from our previous work with a comparable experimental task, showing that central serotonin depletion reduced aversive inhibition in healthy volunteers (Geurts et al., 2013b). This observation is remarkable considering the prior finding that psychopathic tendency in a violent offender sample is accompanied by reduced serotonin metabolites in the cerebrospinal fluid (Soderstrom et al., 2001, 2003) and that callous-unemotional traits in boys was related to lower serum levels of serotonin (Moul et al., 2013). In line with these observations, we found a strong correlation between the PCL-R-score and aversive inhibition in a PIT task. The next step will be to assess whether aversive Pavlovian disinhibition in psychopathy can be countered by serotoninergic drugs, such as selective serotonin reuptake inhibitors, consistent with findings that provoked aggression in primary psychopathy can be reduced by serotonin augmentation by paroxetine (Fanning et al., 2014; e.g., Butler et al., 2021).

Our finding that the amygdala of violent offenders with high psychopathic tendency was more responsive to aversive (versus neutral) CSs than those with lower psychopathic tendency (**Figure 4**) is inconsistent with the hypothesis that violent offenders with high psychopathy severity scores would be insensitive to aversive cues (Flor et al., 2002; Birbaumer et al., 2005). This generally concurs with other prior findings that challenge the view that violent offenders with psychopathic tendency are insensitive to punishment and/or lack fear (e.g., Gregory et al., 2015; Hoppenbrouwers et al., 2016). More specifically our findings are in line with the largest study on Pavlovian conditioning in psychopathy that shows increased rather than reduced amygdala BOLD signal (Schultz et al., 2016) as well as recent meta-analyses of task-based activation studies in psychopathy that challenge the predominant view of amygdala hypo-reactivity in psychopathy (Deming and Koenigs, 2020). Remarkably, as was the case for central serotonin depletion (Geurts et al., 2013a) increased psychopathic tendency was associated with reduced aversive inhibition of both the approach and withdrawal actions. This suggests that the aversive transfer computation that is disrupted operates at the level of action intensity rather than of action valence.

Both healthy volunteers and violent offenders exhibited a negative between-participant correlation between the degree to which instrumental actions were inhibited by aversive cues and the degree to which BOLD signal in the putamen was activated during aversive cues. This observation is generally in line with putamen signal reflecting motor execution (Alexander et al., 1986; Herz et al., 2013; Provost et al., 2015). By contrast, signal in the caudate nucleus of healthy volunteers correlated

positively with the degree to which instrumental actions were inhibited by aversive cues. Thus, greater aversive inhibition was associated with greater caudate nucleus signal during aversive cues. This raises the possibility that signal in the caudate nucleus of healthy controls does not reflect motor execution *per se*, but rather reflects the operation of the aversive Pavlovian inhibition computation of interest. The key finding is that this association was completely reversed in the violent offenders, so that the across-participant pattern of caudate nucleus signal resembled that in their putamen. In line with evidence from work with experimental rodents, evidence in humans (Balleine and O'Doherty, 2009) implicates the caudate nucleus [together with the ventromedial prefrontal cortex (Valentin et al., 2007)] in the instrumental control of behavior (Tanaka et al., 2008; de Wit et al., 2012) as well as response inhibition (Watanabe and Munoz, 2010; Schmidt et al., 2020). Accordingly, one might speculate, based on our neural findings, that aversive Pavlovian disinhibition in psychopathy is accompanied by a failure of the caudate nucleus to exhibit the Pavlovian inhibition computations that it exhibits normally, as suggested by the negative relation in healthy controls. Instead, the caudate nucleus of violent offenders exhibited the across-participant pattern of effects seen in their putamen, which instead reflects increases in motor execution: greater signal with more Go actions. We note that the group differences in the continuous brain-behavior associations were not accompanied by group differences in average BOLD signal. This suggests that the behavioral deficits in violent offenders do not reflect a failure to recruit the caudate nucleus *per se*, but rather that they reflect a failure to recruit the caudate nucleus as a function of the relevant aversive inhibition computation.

One puzzle is that the range of behavioral PIT scores in the violent offenders was comparable with that in the controls. Thus, we did not provide a conceptual replication of the expected group effect on behavior found in our previous study (Ly et al., 2016): While we did find a relation between psychopathic tendency and attenuated aversive inhibition within the group of violent offenders, we did not observe the impact of aversive cues on instrumental action to be altered in the group of violent offenders compared with the group of non-violent healthy controls. This complicates the interpretation of our results. One implication is that abnormal aversive PIT *per se* is not a sufficient prerequisite for developing criminal psychopathic tendency. Criminal psychopathy might surface only if abnormal aversive PIT is accompanied, for example, by excessive impact of reward on behavior and cognition (Buckholtz et al., 2010; Bjork et al., 2012; Yildirim and Derksen, 2015; Geurts et al., 2016). This would be in line with the literature on increased reward seeking and decreased sensitivity to punishment in psychopathic individuals (e.g., Newman et al., 1990). Thus, it might be that one factor or an interaction between multiple factors moderates the impact of differences in aversive PIT on clinical symptomatology (cf. Plichta and Scheres, 2014 for explanation of the moderator model). PCL-R-scores from the healthy controls were not available and therefore we cannot exclude that a similar association exists in healthy controls. However, we think this is unlikely, because there were no correlations between scores on the Psychopathic Personality Inventory and aversive PIT (**Supplementary results**).

We consider several explanations for this lack of a group effect. First, we have a relatively small sample size to detect such a group difference, which might have led to false negative findings. Second, our paradigm is not optimized for indexing appetitive PIT. As the main finding of Ly et al. (2016) is based on a contrast between aversive and appetitive cues, this effect might be due to aberrant effects of violent offenders to either appetitive, aversive or both cues. In the current study, focusing on aversive versus neutral cues, we did not find this group difference. This raises the possibility that the findings of Ly et al. (2016) reflect combined modulation of appetitive biasing as well as aversive biasing. Unfortunately, the insensitivity of our paradigm to appetitive PIT (cf. Geurts et al., 2013a; see **Supplementary results**) precludes strong conclusion about the valence-specificity of the effects. Third, where Ly et al. (2016) use happy and sad faces as affective cues that are presented during instrumental learning, we use neutral stimuli associated with appetitive and aversive juices that we present during already learned instrumental trials presented in nominal extinction. It might be that these latter Pavlovian CS sort more effect in violent offenders than (sad) faces (cf. Brennan and Baskin-Sommers, 2021). Future studies comparing effects of facial expressions with Pavlovian CS on instrumental behavior might elucidate these discrepancies.

Finally, we highlight the following limitations of the current study: First, although our sample size is comparable to that of other neuroimaging studies focusing on psychopathic criminals (cf. Brazil et al., 2009; von Borries et al., 2009; Rothemund et al., 2012; Contreras-Rodríguez et al., 2014), we recognize the limitation of such a small sample size especially when considering our parametric analysis (Button et al., 2013). We did find statistically significant, robust effects, but future studies with larger sample sizes are necessary to confirm the relationships uncovered in this study. Second, we were not able to replicate the strong action-specific BOLD signal found in the ventromedial prefrontal cortex as observed in our previous fMRI study with the same paradigm in healthy young volunteers (Geurts et al., 2013a). We have recently replicated this ventromedial effect in young women (both healthy and with borderline personality disorder) in another independent dataset (submitted to this issue, D. E. M. Geurts, T. J. van den Heuvel, R. Cools). One factor that might account for this discrepancy is that the average performance at the end of the instrumental task was significantly better in the latter studies (with mainly graduate students) compared with that of the healthy controls in the current study [mean accuracy (SEM): 2013 study = 0.76 (0.023), current = 0.64 (0.027), t-test: $t_{36} = 3.6$, $p = 0.001$]. Thus, it

is possible that the healthy controls and patients in the current study relied to a lesser degree on a goal-directed control strategy and to a greater degree on a habitual control strategy than did the subjects in our previous study. Although speculative, this might explain why the putamen was recruited as a function of aversive PIT in the current study in both the healthy control group and the psychopathy group, which was not the case in our previous study. Relevant in this context might also be the fact that the current study included only men, whereas the other studies mainly included women. Third, we should note that our group comparison is necessarily confounded by overt criminal history. As such, we cannot and do not claim specificity of our findings to violent offenders with psychopathic tendency compared with non-psychopathic violent offenders or psychopathic non-offenders (cf. "successful psychopaths").

In sum, our results strengthen the hypothesis that psychopathic tendency in violent offenders is associated with abnormal impact of aversive Pavlovian suppression of instrumental behavior. The neural results raise the possibility that this reflects deficits in neural computations involving the caudate nucleus.

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 Medisch Ethische Toetsing Commissie (METC) Oost-Nederland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DG and KB collected the data. DG performed the statistical analyses under supervision of RC. DG and RC wrote the first draft of the manuscript. All authors contributed to conception and design of the study and manuscript revision, read, and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.963776/full#supplementary-material

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Outcome devaluation by specific satiety disrupts sensory-specific Pavlovian-to-instrumental transfer

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Reward predictive cues can selectively motivate instrumental behaviors that predict the same rewarding outcomes, an effect known as specific Pavlovian-to-instrumental transfer (PIT). This selective effect is thought to be mediated by a representation of the sensory specific properties of an outcome, that has become associated with both the Pavlovian cue and the instrumental response during initial learning. Specific satiety is a common method of outcome devaluation that reduces an outcome's value but might also lead to the habituation of the outcome's sensory properties. Previous research has demonstrated that specific PIT is insensitive to changes in specific outcome value following taste aversion devaluation, as well as general satiety manipulations, and therefore specific satiety should not disrupt specific PIT by reducing outcome value. The present rodent experiments used a specific satiety devaluation procedure immediately prior to a specific PIT test to show that habituation of these outcome specific sensory representations can disrupt its efficacy as a stimulus and abolish the specific PIT effect. Experiment 1 employed a two-lever choice test to show that a non-devalued stimulus supports specific PIT, whereas a devalued stimulus abolished the specific PIT effect. Experiment 2 replicated this procedure while controlling for response competition by using a single-lever test to confirm that a devalued stimulus abolishes the specific PIT effect. These findings demonstrate that specific satiety can disrupt the ability of an outcome specific representation to support specific PIT. Given previous findings that specific PIT is insensitive to changes in outcome value by general satiety and taste aversion devaluation, this suggests that specific satiety devaluation might disrupt the use of sensory specific outcome representations to guide behavior *via* a mechanism that is independent of the outcome's current value.

KEYWORDS

Pavlovian, instrumental, transfer, specific satiety, devaluation, habituation, stimulus, motivation

Introduction

During learning in Pavlovian and instrumental conditioning procedures, outcome representations often form a complex part of the associative structure that is established (Rescorla, 1988; Hall, 2002; Urcuioli, 2005; Delamater and Oakeshott, 2007). For example, in a Pavlovian learning task, a rat learning that an auditory tone cue predicts sucrose reward (Stimulus-Outcome; S-O) will often represent multiple aspects of this outcome event such as its flavor and texture (i.e., unique sensory properties), spatial location, timing, motivational value etc. Similarly, during instrumental conditioning, i.e., when learning lever press response for a sucrose reward (Response-Outcome; R-O), these multiple aspects of the outcome can also form part of the associative relationship with the response (Colwill and Motzkin, 1994; Balleine and Killcross, 2006; Delamater and Holland, 2008).

Experimental research has focused mostly on the distinction between the unique sensory-specific properties vs. the general-motivational properties of outcome representations (Konorski, 1967; Dickinson and Dearing, 1979), in particular, using Pavlovian-to-instrumental transfer (PIT) procedures. In a typical rodent full PIT procedure (Corbit et al., 2007; Cartoni et al., 2016), rats are first trained on three unique stimulus-outcome relationships, e.g., S_1-O_1/ S_2-O_2/S_3-O_3, and independently trained on two unique lever response-outcome relationships, e.g. R_1-O_1/R_2-O_2. Finally, the stimuli (S_1/S_2) are presented in the presence of the instrumental responses (R_1/R_2) for the first time in a PIT transfer test conducted in extinction. The general PIT effect describes the ability for a Pavlovian stimulus to increase responding on an independently trained instrumental response i.e., S_3 will enhance responding on both R_1 and R_2. This PIT effect differentially biases responding when both the stimulus and response were trained with the same outcome i.e., S_1 will preferentially enhance R_1, and S_2 will preferentially enhance R_2. These distinct outcome specific and general PIT effects are strong evidence that the sensory-specific properties of the outcome (i.e., its identity) are an independent part of the associations formed during the initial Pavlovian and instrumental training.

Additional support for this distinction between the sensory-specific and general properties of outcomes in Pavlovian and instrumental learning, as well as PIT, comes from a growing body of neural evidence. For example, lesions or functional inactivation of the central amygdala (CeA), nucleus accumbens core (NAcc core), and ventral tegmental area disrupt general PIT while leaving specific PIT intact (Corbit et al., 2007), whereas targeting the basolateral amygdala (BLA), nucleus accumbens shell (NAcc shell), mediodorsal thalamus (MD), ventral pallidum, as well as frontal region such medial and lateral orbitofrontal cortex (mOFC, lOFC), selectively abolishes specific but not general PIT (Blundell et al., 2001; Corbit et al., 2001, 2003, 2007; Holland and Gallagher, 2003; Corbit and Balleine, 2005, 2011; Balleine and Killcross, 2006; Ostlund and Balleine, 2007a; Leung and Balleine, 2015; Balleine et al., 2011; Leung and Balleine, 2013; Ostlund and Balleine, 2008; Lichtenberg et al., 2017; Bradfield et al., 2018; Panayi and Killcross, 2018; Sias et al., 2021). While some of these neural pathways also underpin more general processes of Pavlovian and instrumental learning about the sensory specific and general properties of outcomes, there is compelling evidence to suggest neural processes that are unique to PIT. For example, successful expression of specific PIT uniquely depends upon the trafficking of delta-opioid receptors on cholinergic interneurons in the NAcc shell (but not NAcc core) during initial Pavlovian conditioning, which in turn modulate dopamine D1 (but not D2) receptor activity that is necessary for the learning and expression of specific PIT (Laurent et al., 2012, 2014; Bertran-Gonzalez et al., 2013).

The associative account of the specific PIT effect is that during the transfer test the Pavlovian stimulus will activate a representation of the expected outcome, including its sensory properties e.g., S_1-S_{O1}. This in turn will activate the associated instrumental response e.g., R_1, either by a backwards R_1-S_{O1} association, or by a forwards S_{O1}-R_1 association where the outcome has formed part of the discriminative stimulus for the response during acquisition (Trapold and Overmier, 1972; Colwill and Rescorla, 1988; Rescorla and Colwill, 1989; Rescorla, 1992; Colwill, 1994; for a discussion of this theoretical distinction see Ostlund and Balleine, 2007b; Gilroy et al., 2014). For simplicity, we will discuss the signaling properties of the outcome (S_O) in this associative chain as S_1-S_{O1}-R_1. Importantly, if the outcome is devalued by forming a taste aversion immediately before a PIT test, rats will show an intact specific PIT effect despite showing independent evidence of outcome specific devaluation on the underlying Pavlovian, instrumental, and consummatory responses (Colwill and Rescorla, 1988, 1990b; Rescorla, 1994; Holland, 2004). This suggests that the learned signaling properties of S_O can act independently of the current motivational value of the expected outcome (e.g., based on levels of hunger). A complementary piece of evidence for this dissociation is that reducing hunger (i.e., general satiety) abolishes the general, i.e., non-outcome specific, form of PIT but not specific PIT (Corbit et al., 2007; Lingawi et al., 2022; Sommer et al., 2022). Thus, specific PIT is argued to be unaffected by manipulating the current outcome value or general motivation for the outcome because these manipulations leave S_O, the sensory properties of the expected outcome, intact.

A prediction of this account is that the specific PIT effect should be reduced by a manipulation that reduces the ability to associatively activate a representation of S_O. Habituation by repeatedly presenting a stimulus has been shown to temporarily reduce the ability to associatively activate representations of the stimulus in a stimulus-specific manner (Wagner, 1981; Rankin et al., 2009; Lloyd et al., 2014). Indeed, during the course of standard instrumental training, McSweeney and Murphy

(2009) review a large body of work which demonstrates that instrumental responding often declines within-session because repeated deliveries of the outcome leads to sensory specific habituation of S_O rather than a general loss of hunger or motivation (see also Epstein et al., 2009; Bouton et al., 2013). Therefore, habituation of S_O by repeated pre-exposure to that outcome should reduce the likelihood of associatively activating S_O, thus impairing specific PIT for that outcome *via* an S-S_O-R pathway. Notably, extensive pre-exposure to an outcome before a test is used to induce outcome specific satiety (e.g., 1 h of unlimited access enabling the subject to voluntarily pre-expose themselves to the reinforcer to the greatest extent possible), another common method of outcome devaluation (Panayi and Killcross, 2018). A potential confound caused by specific satiety is that it also reduces the incentive value of the outcome (Balleine and Dickinson, 1998) as well as generally satiating the animal, however both of these factors do not disrupt specific PIT (Colwill and Rescorla, 1988, 1990b; Rescorla, 1994; Holland, 2004; Corbit et al., 2007; Lingawi et al., 2022). Here we tested this hypothesis that specific satiety will disrupt specific PIT.

Two recent studies have tested the effects of specific satiety on specific PIT in rodents and both reported that, similar to devaluation with taste aversion, specific satiety did not abolish specific PIT (Lingawi et al., 2022; Sommer et al., 2022). However, both studies employed two-lever choice tests, i.e., both the devalued and non-devalued lever were present during the PIT test, which complicates the interpretation of these findings. Differences in PIT can be the result of different baseline levels of responding on the devalued and non-devalued lever (Rescorla, 1994; Holland, 2004; Holmes et al., 2010; Cartoni et al., 2016), or competition between responses (Laurent and Balleine, 2015; Lovibond et al., 2015). For example, both studies report significantly lower responding on the devalued than the non-devalued lever during the baseline periods. Indeed, the specific PIT effect in the presence of a devalued stimulus reported by Lingawi et al. (Figure 3I in Lingawi et al., 2022) reflects a small but significant suppression in responding on the different outcome lever, but no evidence of elevated responding on the same outcome lever i.e., no specific PIT. The findings of (Sommer et al., 2022) show a small but significant specific PIT effect in the presence of the devalued stimulus but not the non-devalued stimulus (Figure 1D in Sommer et al., 2022). Therefore, the findings of these studies do not unambiguously disconfirm our prediction that specific satiety will disrupt specific PIT.

The present experiments were conducted prior to these two recent reports and were not specifically designed in response to these findings; however, the design of our PIT procedure overcomes some of the issues related to the two lever choice tests described above. Experiment 1 employed a two-lever choice test but preceded the test with a longer instrumental extinction period that eliminated difference in baseline responding on the levers. Experiment 2 replicated this design but employed a one-lever specific PIT test. Our findings supported our hypothesis such that specific satiety devaluation selectively abolished specific PIT for the devalued outcome but not for the non-devalued outcome.

Materials and methods

Animals

Rats were housed four per cage in ventilated Plexiglass cages in a temperature regulated (22 ± 1¬°C) and light regulated (12 h light/dark cycle, lights on at 7:00 AM) colony room. At least 1 week prior to behavioral testing, feeding was restricted to ensure that weight was ∼95% of *ad libitum* feeding weight, and never dropped below 85% (achieved by providing 15 g lab chow per rat per day, and monitoring weight at least twice a week). All animal research was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratories Animals (NIH publications No. 80-23, revised 1996) and approved by the University of New South Wales Animal Care and Ethics Committee. Subjects were and 32 male Wistar rats (BRC Laboratory Animal Service, University of Adelaide, South Australia, Australia) approximately 4 months old (Experiment 1, $N = 16$, weighing between 326 and 475 g, M = 386.0 g; Experiment 2, $N = 16$, weighing between 300 and 435 g, M = 373.4 g).

Apparatus

Test chambers

Behavioral testing was conducted in eight identical operant chambers (30.5 × 32.5 × 29.5 cm; Med Associates) individually housed within ventilated sound attenuating cabinets. Each chamber was fitted with a 3-W house light that was centrally located at the top of the left-hand wall. Food pellets could be delivered into a recessed magazine, centrally located at the bottom of the right-hand wall. Delivery of up to two separate liquid rewards *via* rubber tubing into the magazine was achieved using peristaltic pumps located above the testing chamber. The top of the magazine contained a white LED light that could serve as a visual stimulus. Access to the magazine was measured by infrared detectors at the mouth of the recess. Two retractable levers were located on either side of the magazine on the right-hand wall. A speaker located to the right of the house light could provide auditory stimuli to the chamber. In addition, a 5-Hz train of clicks produced by a heavy-duty relay placed outside the chamber at the back right corner of the cabinet was used as an auditory stimulus. The chambers were wiped down with ethanol (80% v/v) between each session. A computer equipped with Med-PC software (Med Associates Inc., St. Albans, VT, USA) was used to control the experimental procedures and record data. Throughout all stages of behavioral training in the test chambers, the house light and fan were always on.

Devaluation chambers

To provide individual access to reinforcers during the devaluation procedure, rats were individually placed into a clean mouse-sized home cage (33 x 18 x 14 cm clear Perspex cage with a wireframe top). Liquid reinforcers were presented in water bottles with a sipper tube. One day prior to the start of the devaluation period, all rats were exposed to the devaluation cages and given 30 mins of free access to home cage food and water to reduce novelty to the context and any potential neophobia to drinking from the water bottles.

Reinforcers

The reinforcers used were a single grain pellet (45 mg dustless precision grain-based pellets; Bio-serv, Frenchtown, NJ, USA), 20% w/v lemon flavored sucrose solution and 20% w/v peppermint flavored maltodextrin solution (Myopure, Petersham, NSW, Australia). Liquid reinforcers were flavored with either 0.4% v/v concentrated lemon juice (Berri, Melbourne, Victoria, Australia) or 0.2% v/v peppermint extract (Queen Fine Foods, Alderley, QLD, Australia) to provide unique sensory properties to each reinforcer. Liquids were delivered over a period of 0.33 s *via* a peristaltic pump corresponding to a volume of 0.2 mL. The volume and concentration of liquid reinforcers was chosen to match the calorific value of the corresponding grain pellet reward and have been found to elicit similar rates of Pavlovian and instrumental responding as a pellet reward in other experiments conducted in this lab. In all sessions involving liquids, the magazine was scrubbed with warm water and thoroughly dried between sessions to remove residual traces of the liquid reinforcer. To reduce neophobia to the reinforcers, 1 day prior to magazine training sessions all rats were pre-exposed to the reinforcers (10 g of pellets per rat and 25 ml of each liquid reinforcer per rat) in their home cage.

Behavioral procedures

The behavioral procedures for Experiment 1 and Experiment 2 were identical except for the number of levers extended during the final PIT test session. Experiment 1 employed a two-lever choice test, whereas Experiment 2 employed a single-lever test procedure (described in detail below).

Magazine training

All rats received three sessions of magazine training, one for each reinforcer with the following parameters: reward delivery was on a random time 60 s schedule (RT60s) for 16 rewards. Sessions occurred on three consecutive days with the order of reward identity counterbalanced between rats.

Lever training

Following magazine training, all rats were given 2 days of lever training on a continuous reinforcement schedule (each lever press was rewarded) with the same parameters as the instrumental training sessions described below.

Acquisition training

On each day all rats received either a single Pavlovian training session, or two instrumental training sessions. The order of Pavlovian and instrumental sessions alternated each day.

Pavlovian training

All rats received a total of 6 days of Pavlovian training. Pavlovian training sessions consisted of 3 stimuli (CS), a tone (2,800 Hz, 80 dB), white noise (78 dB), and a clicker (5 Hz). There were 4 presentations of each CS (i.e., a total of 12 cues presented within a session) each lasting 2 min with a variable ITI of 300 s. Reward was delivered throughout the cue period on a RT 30 s schedule. Each cue was paired with a unique outcome (grain pellet, lemon sucrose, and peppermint maltodextrin) and the identity of the cue-outcome relationship remained constant for each rat (counterbalanced between rats).

Instrumental training

All rats received a total of 6 days of instrumental training. Instrumental training involved two sessions per day, separated by at least 1 h. During the session a single lever was extended, and lever pressing was rewarded with a unique liquid outcome, either lemon sucrose or peppermint maltodextrin. During the second instrumental session of the day, a different lever was extended, and lever pressing was rewarded with the unique liquid outcome that was not paired with the previous lever. The identity of the lever outcome pairings was kept consistent within subjects and was counterbalanced between subjects. Training sessions lasted until a maximum of 20 rewards was earned or until 30 min had elapsed. On the first 2 days, reinforcement was delivered on a random ratio 5 schedule (RR5) such that on average a reward was delivered every 5 lever presses, followed by 4 days of RR10.

Devaluation

Satiety devaluation was achieved by providing rats with 1 h of free access to one of the liquid reinforcers in the devaluation chamber. At the end of the 1-h period, rats were removed from the devaluation chamber and put back in their home cage, and immediately transferred to the test chambers.

In experiment 1, devaluation occurred on two consecutive days with a different liquid reinforcer. In experiment 2,

devaluation occurred on two consecutive days with the same reinforcer to assess the effect of devaluation of the same outcome on both levers in separate sessions. Following 2 further days of Pavlovian and instrumental retraining, the alternative liquid reinforcer was then devalued for 2 days. This resulted in both liquid reinforcers being devalued and tested with each lever.

Extinction and PIT test

The PIT test started with lever extinction, both levers were extended in Experiment 1, and only a single lever was extended in Experiment 2. Lever pressing had no programmed consequences throughout the entire session. After 10 min, the CSs were presented (duration 2 min) with a fixed 2 min inter-stimulus interval. Each CS was presented three times (a total of 9 CS presentations) and the order of CS presentation was randomized. In Experiment 1 involving two levers, the PIT test was repeated on the following day (i.e., once per devalued outcome). In Experiment 2 involving only a single lever test session, a second test was repeated on the following day with the lever that had yet to be tested. Order of lever presentation was counterbalanced. This pattern of two single lever tests was then repeated after 4 days of retraining on Pavlovian and instrumental sessions (i.e., a total of 4 PIT test sessions, once per devalued outcome-lever combination).

Starting the PIT test with extinction of the levers served multiple purposes. First, it reduced lever pressing behavior to a low baseline response rate, which allows for clearer demonstration of the potential rate-enhancing effect of CS presentations on lever pressing i.e., the PIT effect. Secondly, the extinction period served as an instrumental outcome devaluation test to confirm the efficacy of the specific satiety devaluation manipulation. Thirdly, it minimizes any differences in baseline rates of responding between the levers associated with the devalued and non-devalued outcomes. Differences in baselines can limit the interpretation and expression of any differences in the PIT effect on each lever.

Data analysis

For the Pavlovian stage, a CS-PreCS elevation score was calculated by subtracting the rate of magazine entry during the 2 min immediately before each CS (PreCS) from the 2 min CS period. Lever pressing rates were analyzed for the instrumental stage. During the specific satiety devaluation stage, the amount of liquid reinforcer (in grams) consumed in 1 h was calculated. Both magazine entry and lever pressing rates were calculated for the PIT test. During the extinction period, lever pressing was analyzed during the first 9 min in time blocks of 3 min (note that the last minute of the 10-min extinction period was part of the baseline period for the PIT; Supplementary Figure 1A). During the PIT test, both lever pressing and magazine entries were analyzed as elevation scores during the CS presentation above baseline. Here, because activity during the CS persisted for a short time after the CS ended, baseline responding was defined as responding in the 1 min before each stimulus (i.e., the last minute of the inter-stimulus-interval period). All response rates were calculated as responses per minute.

Data were analyzed using repeated measures ANOVAs with R statistical software (R Core Team, 2021), using the afex package (Singmann et al., 2022) implementation of the aov_4 function, with a multivariate model for all follow up tests (setting: emmeans_model = "multivariate"). Simple effects were used to explore significant main effects and interactions using the emmeans package (Lenth, 2022), with a Tukey method of familywise error-rate correction. Simple effects for the analysis of rates of acquisition were conducted using the linear component of planned orthogonal trend contrasts. Repeated measures *t*-tests were used for analyses with only two conditions.

During the PIT test, three analysis strategies were planned to look at the relationship between the expected outcomes of the Pavlovian stimuli and instrumental actions (Outcome Specificity: Same, Different, General). (1) The relationship between outcome specificity and whether the outcome of the instrumental lever was devalued or non-devalued (Lever Devaluation). (2) Whether the stimuli increased lever pressing above baseline in each condition, indicating some form of PIT transfer. (3) The relationship between outcome specificity and whether the outcome of the Pavlovian stimuli was devalued or non-devalued (Stimulus Devaluation). Note that analysis options (1) and (3) do not change the underlying data (and are identical when only a single lever is present as in Experiment 2). Instead, both analyses were conducted to aid comparisons with earlier studies and provide a complete exploration of the complex experimental design (for a detailed discussion, see Supplementary Figure 2).

Exclusions

Two rats were excluded from Experiment 2 based on a substantial response bias to one cue. Responding was over 4x higher to one CS suggesting substantial cue or outcome preference.

Results

Experiment 1. Specific satiety abolishes specific PIT in a two-lever choice test

Experiment 1 assessed the impact of specific satiety on the ability of a Pavlovian CS to invigorate actions that produce the same outcome (Figure 1A). A full transfer paradigm was used (Cartoni et al., 2016). Rats ($n = 16$) were first trained with three unique Pavlovian CS-outcome relationships (i.e., S1-O1, S2-O2,

S3-O3) and two unique instrumental lever response-outcome relationships (i.e., R1-O1, R2-O2) on alternating days (see Figures 1A, 2A). Next, one of the instrumental outcomes (O1 or O2) was devalued by specific satiety immediately before a two-lever PIT choice test. The PIT test started with the presentation of both levers and an extinction period to reduce baseline lever pressing before CSs were presented (also in extinction) to examine their impact on instrumental lever pressing. O1 and O2 were always liquid reinforcers (sucrose and maltodextrin, counterbalanced), and O3 was always grain pellets. This ensured that devaluation of O1 or O2 was not confounded by any potential asymmetric effects when comparing liquid and solid food reinforcers.

During the PIT test there were three possible relationships between CSs and instrumental responses (1) Same: Pavlovian cues that predicted the same instrumental outcome (i.e. S1/R1, S2/R2), (2) Different Pavlovian cues that predicted a different instrumental outcome (i.e. S2/R1, S1/R2), and (3) General: the Pavlovian cue that predicted an outcome that was never an instrumental outcome (S3/R1, S3/R2). This test was repeated once following satiety devaluation with the instrumental outcome that was not presented before the first test.

Instrumental and Pavlovian conditioning

All rats successfully acquired instrumental lever responding (Figure 1A). Responding for both the sucrose and maltodextrin outcomes significantly increased over training days (main effect of Day, $F(5,75) = 63.39$, $p < 0.001$; significant positive linear trend over Day, $t(15) = 11.74$, $p < 0.001$), and at comparable rates for both rewards (no main effect of Reward, $F(1,15) = 0.36$, $p = 0.559$; or Day*Reward interaction, $F(5,75) = 1.47$, $p = 0.210$).

All rats successfully acquired increased Pavlovian magazine responding during the CS (CS-PreCS elevation scores) for sucrose, maltodextrin, and pellets (Figure 1B). Magazine responding significantly increased over training days (main effect of Day, $F(5,75) = 9.94$, $p < 0.001$; significant positive linear trend over Day $t(15) = 4.18$, $p = 0.001$), however overall responding was higher for pellets than for sucrose or maltodextrin (significant main effect of Reward $F(2,30) = 3.82$, $p = 0.033$; but no significant Day*Reward interaction $F(10,150) = 0.46$, $p = 0.911$). Simple main effects of Reward did not support this statistical difference after family-wise error rate correction (Maltodextrin vs. Pellet: $t(15) = -2.51$, $p = 0.059$, Sucrose vs. Pellet: $t(15) = -1.71$, $p = 0.236$, Maltodextrin vs. Sucrose: $t(15) = -1.18$, $p = 0.483$). The slightly elevated rate of magazine approach for pellets is likely to be due to the nature of the consummatory response (i.e., drinking liquid vs. chewing pellets) which are conflated with anticipatory approach in these response data. Importantly, there were no significant differences in the rate of instrumental and Pavlovian acquisition of the to-be-devalued rewards i.e., sucrose and maltodextrin.

Outcome devaluation and extinction

During 1 h of free access to one of the instrumental outcomes, rats readily consumed both sucrose and maltodextrin (Figure 1D). Consumption was marginally greater for sucrose than maltodextrin (Sucrose vs. Maltodextrin, $t(15) = 2.17$, $p = 0.046$).

Immediately following this specific satiety manipulation, instrumental responding was extinguished with both levers present for 9 min (Figure 1E). This extinction test confirmed that the devaluation manipulation was successful as it selectively reduced lever pressing for devalued reward. Responding on the devalued lever was significantly reduced compared to the non-devalued lever (significant main effect of Devaluation $F(1,15) = 31.63$, $p < 0.001$; main effect of Time $F(2,30) = 22.14$, $p < 0.001$; and Devaluation*Time interaction $F(2,30) = 7.13$, $p = 0.003$).

Specific PIT: Two lever choice test

The specific PIT test followed immediately after instrumental extinction. Baseline lever responding during the PreCS baseline period (1 min prior to stimulus presentation) was low and did not differ between non-devalued and devalued levers (Figure 1F; Non-Devalued vs. Devalued: $t(15) = -0.39$, $p = 0.705$). The absence of significant differences in baseline responding confirmed that it was appropriate to analyze PIT test responding during stimulus presentation as an elevation score (responding during stimulus–baseline) in subsequent analyses. In contrast, baseline responding on the devalued lever was reported as significantly lower than the non-devalued lever in previous studies testing the effect specific satiety on specific PIT (Lingawi et al., 2022; Sommer et al., 2022).

During the PIT test, the relationship between the expected outcomes of the Pavlovian stimuli and instrumental actions (Specific PIT: Same, Different, General) was first separated based on the devaluation status of the instrumental action (Lever Devaluation: Non-Devalued, Devalued; Figure 1G). Overall response levels in the presence of the cues, relative to baseline, were significantly higher on the non-devalued than the devalued lever (main effect of Lever Devaluation $F(1,15) = 15.85$, $p = 0.001$), which reflects a persistent effect of instrumental devaluation. Overall responding did not differ between the same and different cues [main effect of Specific PIT, $F(2,30) = 9.11$, $p = 0.001$; simple main effect of Same vs. Different, $t(15) = 0.25$, $p = 0.966$, $d = 0.24$, 95% CI (−2.35, 2.85)], but responding to both same and different cues was significantly elevated compared to responding for the general cue [Same vs. General, $t(15) = 3.16$, $p = 0.017$, $d = 3.16$, 95% CI (0.56, 5.76); Different vs. General, $t(15) = 4.44$, $p = 0.001$, $d = 4.44$, 95% CI

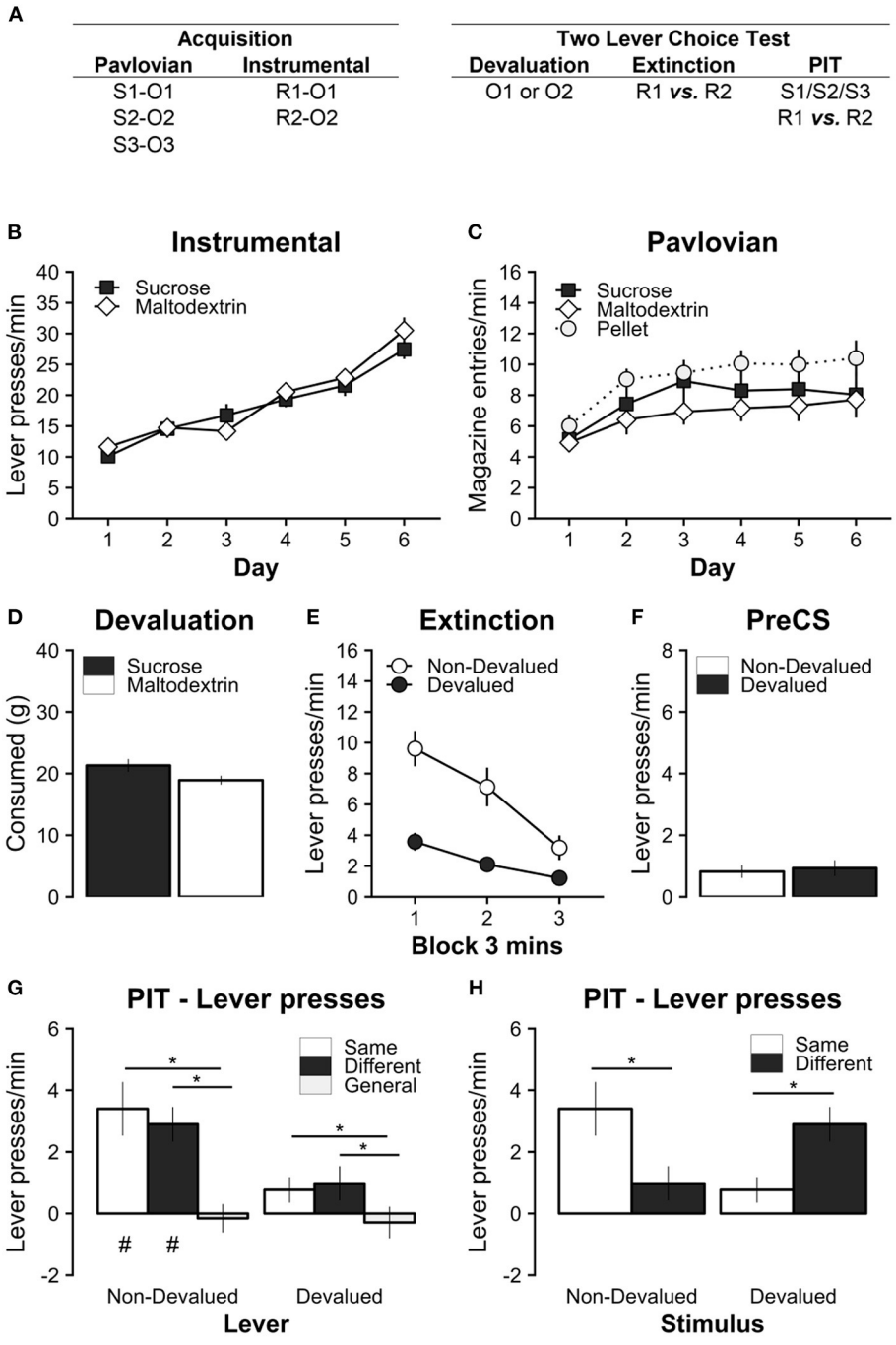

FIGURE 1
Experiment 1 tested the effect of outcome devaluation by sensory-specific satiety on specific PIT in a two-lever choice test. **(A)** Experimental design; S1/S2/S3: clicker, tone, and noise stimuli (counterbalanced); R1/R2: left and right lever press actions (counterbalanced); O1/O2: sucrose and maltodextrin liquid reinforcer outcomes (counterbalanced); O3: pellet reinforcer outcome. **(B)** All rats learned to perform the left and right lever responses for the sucrose and maltodextrin liquid reinforcers. **(C)** All rats learned that the Pavlovian stimuli uniquely predicted the sucrose, maltodextrin, and pellet outcomes. **(D)** During the 1-h specific satiety devaluation session, rats consumed a substantial amount of the reinforcer, but consumed slightly more when the reinforcer was sucrose. **(E)** Outcome devaluation successfully reduced the rate of lever pressing on the lever associated with the devalued outcome during the extinction period with both levers present. **(F)** Baseline responding during the PIT test was similar on the devalued and non-devalued levers. **(G)** Outcome devaluation abolished specific PIT when separating responses on the non-devalued (Left) and devalued (Right) levers. **(H)** Outcome devaluation reversed specific PIT when separating responses during the non-devalued (Left) and devalued (Right) stimuli. Note that the same data are presented in **(G)** and **(H)**. * Significant simple effects. $p < 0.05$. # Significant responding above baseline, $p < 0.05$. Full analysis details in main text. Data are presented as mean ± SEM.

A

Acquisition		Single Lever Test		
Pavlovian	Instrumental	Devaluation	Extinction	PIT
S1-O1	R1-O1	O1 or O2	R1 *or* R2	S1/S2/S3
S2-O2	R2-O2			R1 *or* R2
S3-O3				

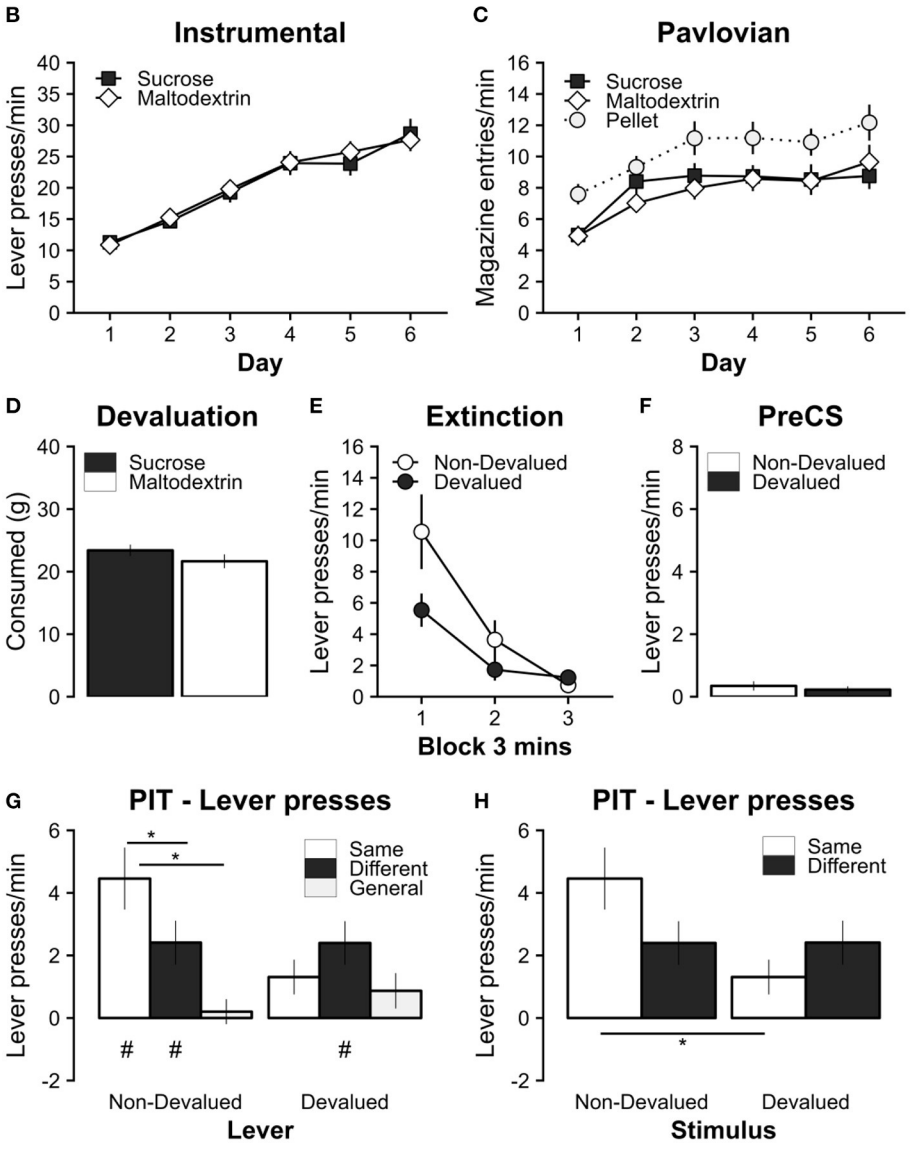

FIGURE 2
Experiment 2 tested the effect of outcome devaluation by sensory-specific satiety on specific PIT in a single-lever test. **(A)** Experimental design; S1/S2/S3: clicker, tone, and noise stimuli (counterbalanced); R1/R2: left and right lever press actions (counterbalanced); O1/O2: sucrose and maltodextrin liquid reinforcer outcomes (counterbalanced); O3: pellet reinforcer outcome. **(B)** All rats learned to perform the left and right lever responses for the sucrose and maltodextrin liquid reinforcers. **(C)** All rats learned that the Pavlovian stimuli uniquely predicted the sucrose, maltodextrin, and pellet outcomes. **(D)** During the 1-hour specific satiety devaluation session, rats consumed a substantial amount of the reinforcer, but consumed slightly more when the reinforcer was sucrose. **(E)** Outcome devaluation successfully reduced the rate of lever pressing on the lever associated with the devalued outcome during the extinction period with only a single lever present in each test. **(F)** Baseline responding during the PIT test was similar on the devalued and non-devalued levers. **(G)** Outcome devaluation abolished specific PIT when separating responses on the non-devalued (Left) and devalued (Right) levers. **(H)** Outcome devaluation reversed specific PIT when separating responses during the non-devalued (Left) and devalued (Right) stimuli. Note that the same data are presented in **(G)** and **(H)**. * Significant simple effects. $p < 0.05$. # Significant responding above baseline, $p < 0.05$. Full analysis details in main text. Data are presented as mean ± SEM.

(1.85, 7.05)]. While the pattern of results also suggests that the magnitude of the PIT effect was greater for the non-devalued than the devalued lever, this was not supported by a statistical interaction [Lever Devaluation*PIT interaction, $F(2, 30) = 1.95$, $p = 0.160$]. Therefore, sensory specific satiety abolished specific PIT on both the devalued and non-devalued lever. However, it should be noted that since this is a two-lever choice test, this analysis obscures the important effects of response competition between the levers within each trial (addressed below). There was also no evidence of any differences in magazine responding to the devalued, non-devalued, or general stimulus (main effect of Stimulus Devaluation $F(2, 30) = 0.02$, $p = 0.980$; Supplementary Figure 1B).

We performed a second analysis on these data to determine whether there was any evidence of PIT, i.e., the ability for the Pavlovian CS to invigorate instrumental responding, for each stimulus-lever-outcome relationship. This was done by testing whether responding was above baseline i.e., are baseline subtracted scores significantly above zero? This was tested using the individual parameter effect estimates from the ANOVA model above (Figure 1G). This analysis suggested that responding was significantly elevated above baseline on the non-devalued lever for both the same and different stimulus conditions [Non-Devalued: Same, $t(15) = 3.89$, $p = 0.009$, $d = 3.9$, 95% CI (0.87, 6.92); Different, $t(15) = 5.17$, $p = 0.001$, $d = 5.18$, 95% CI (2.14, 8.19); General, $t(15) = -0.34$, $p > 0.999$, $d = -0.35$, 95% CI (−3.37, 2.68)], but not on the devalued lever [Devalued: Same, $t(15) = 1.85$, $p = 0.408$, $d = 1.86$, 95% CI (−1.16, 4.89); Different, $t(15) = 1.77$, $p = 0.460$, $d = 1.77$, 95% CI (−1.26, 4.8); General, $t(15) = -0.57$, $p = 0.994$, $d = -0.56$, 95% CI (−3.6, 2.45)].

Another approach to analyzing these data is to compare the relationship between the expected outcomes of the Pavlovian stimuli and instrumental actions (Specific PIT: Same, Different) with the devaluation status of the Pavlovian cues (Stimulus Devaluation: Non-Devalued, Devalued; Figure 1H). This provides a different way of visualizing these data, consistent with earlier studies, that directly compares the two-levers present during each trial (Supplementary Figure 2). Again, the pattern of responding suggests that specific satiety devaluation abolished specific PIT. The non-devalued stimulus elicited a significantly greater response on the same than the different lever [Non-Devalued: Same vs. Different, $t(15) = 3.03$, $p = 0.009$, $d = 3.03$, 95% CI (0.89, 5.16)], whereas the devalued stimulus elicited the opposite pattern of responding [Devalued: Same vs. Different, $t(15) = 3.03$, $p = 0.009$, $d = 3.03$, 95% CI (0.89, 5.16); significant Stimulus Devaluation*Specific PIT interaction, $F(1, 15) = 28.93$, $p < 0.001$; no main effect of Stimulus Devaluation, $F(1, 15) = 0.18$, $p = 0.674$, or Specific PIT, $F(1, 15) = 0.06$, $p = 0.807$]. This suggests that devaluation not only abolished but reversed the specific PIT effect. However, it is important to interpret this finding with caution because it is possible that the reversal of the specific PIT effect is being driven by an overall reduction in approaching the devalued lever, and response competition with the non-devalued lever. Surprisingly, both the devalued and non-devalued stimulus elicited similar levels of responding on the non-devalued lever. This suggests that the devalued stimulus conferred some form of general transfer effect (or a counterfactual association e.g., Laurent and Balleine, 2015), in contrast to the general stimulus which did not increase responding on either lever.

Finally, we also included session number into the analysis to confirm that these effects were consistent across repeated test sessions (i.e., Figure 1H split by session; data not shown). While there was an overall reduction in total responding over repeated testing [main effect of Session $F(1, 15) = 9.45$, $p = 0.008$], there were no interactions between test session and any other factors [Session*Stimulus Devaluation, $F(1, 15) = 0.05$, $p = 0.822$; Session*Specific PIT $F(1, 15) = 0.12$, $p = 0.736$; Session*Stimulus Devaluation*Specific PIT $F(1, 15) = 0.42$, $p = 0.529$].

Experiment 2. Specific satiety abolishes specific PIT in a single lever test

The previous experiment revealed that the expression of specific PIT not only abolished but reversed by satiety devaluation in a two-lever choice test. To account for the potential effect of response competition between the devalued and non-devalued levers, Experiment 2 used an identical procedure but with only a single lever made available during the PIT test session (Figure 2A). Rats were first given two tests on one lever (e.g., R1) with a different outcome devalued before each session (i.e., O1 and O2), and then tested twice on the other lever (e.g., R2, devaluing O1 and O2). Rats were given brief reacquisition training on the Pavlovian and instrumental contingencies after the first two PIT sessions to minimize the effects of testing in extinction.

Instrumental and Pavlovian conditioning

As before, all rats successfully acquired instrumental lever responding (Figure 2B). Responding for both sucrose and maltodextrin outcomes significantly increased over training days [main effect of Day, $F(5, 65) = 46.35$, $p < 0.001$; significant positive linear trend over Day, $t(13) = 9.64$, $p < 0.001$], and at comparable rates for both rewards {no main effect of Reward, [$F(1, 13) = 0.02$, $p = 0.888$], or Day*Reward interaction, $F(5, 65) = 0.41$, $p = 0.83$).

All rats also successfully acquired robust Pavlovian magazine responding during the CS (CS-PreCS elevation scores) for sucrose, maltodextrin, and pellets (Figure 2C). Magazine responding for sucrose, maltodextrin, and pellets significantly increased over training days [main effect of Day, $F(5, 105) = 12.79$, $p < 0.001$; significant positive linear trend

over Day, $t(21) = 4.44$, $p < 0.001$], however overall responding was higher for pellets than for sucrose or maltodextrin [significant main effect of Reward, $F(2, 42) = 5.66$, $p = 0.007$, but no significant interaction Day*Reward interaction, $F(10, 210) = 0.69$, $p = 0.73$]. Simple main effects of Reward revealed that responding for pellets was significantly higher than for sucrose or maltodextrin [Maltodextrin vs. Pellet, $t(21) = -2.88$, $p = 0.024$; Sucrose vs. Pellet, $t(21) = -2.46$, $p = 0.056$], but no significant difference between maltodextrin and sucrose [Maltodextrin vs. Sucrose, $t(21) = -0.39$, $p = 0.920$]. This replicates the trend that was observed in Experiment 1 and suggests that the nature of magazine responding for the pellet reward was different to the two liquid reinforcers. However, once again, there were no significant differences in the rate of instrumental and Pavlovian acquisition of the relevant to-be-devalued rewards i.e., sucrose and maltodextrin.

Outcome devaluation and extinction

After 1 h of free consumption prior to each test session, consumption was marginally greater for sucrose than maltodextrin [Sucrose vs. Maltodextrin, $t(13) = 2.75$, $p = 0.017$; Figure 2D]. However, ignoring outcome identity, total consumption of liquids did not differ across the 4 days of testing [main effect of Test Number, $F(3, 39) = 2.52$, $p = 0.072$].

Immediately following the specific satiety manipulation, rats were given 9 min of instrumental extinction with only a single lever present (Figure 2E). This extinction test confirmed that the devaluation manipulation was successful. Responding on the devalued lever was significantly reduced compared to the non-devalued lever in the first 3 min [Non-devalued vs. Devalued: Time Block 1, $t(13) = -2.25$, $p = 0.042$; Time Block 2, $t(13) = -1.32$, $p = 0.210$; Time Block 3, $t(13) = 0.79$, $p = 0.444$; supported by a significant Devaluation*Time interaction $F(2, 26) = 4.03$, $p = 0.030$; main effect of Time $F(2, 26) = 25.20$, $p < 0.001$; but not Devaluation, $F(1, 13) = 3.77$, $p = 0.074$]. It is noteworthy that the magnitude of the devaluation effect is not as profound as that observed in Experiment 1 (Figure 1E), which is likely due to greater sensitivity to differences in value and response competition in a simultaneous choice test.

Specific PIT: Single lever test

The specific PIT test followed immediately after instrumental extinction. Baseline lever responding during the PreCS baseline period (1 min prior to stimulus presentation) was low and did not differ between non-devalued and devalued levers [Figure 2F; Non-Devalued vs. Devalued: $t(15) = -0.39$, $p = 0.705$]. Like Experiment 1, the absence of significant differences in baseline responding confirmed that it was appropriate to analyze responding during stimulus presentation as an elevation score.

There was no evidence of differences in magazine responding during the PIT test (Supplementary Figure 1C). Magazine entries during the PIT test did not differ between lever devaluation or stimulus conditions [no main effect of Lever Devaluation, $F(1, 13) = 0.81$, $p = 0.385$; Stimulus Devaluation, $F(2, 26) = 0.30$, $p = 0.740$; or Lever Devaluation*Stimulus Devaluation interaction, $F(2, 26) = 0.04$, $p = 0.959$]. The lack of difference in magazine responses during stimulus presentation suggests that any differences in lever pressing during the PIT test are not being driven by differential response competition with the magazine response.

During the PIT test sessions with the non-devalued lever extended (Figure 2G, left), a significant outcome-specific PIT effect was observed such that lever pressing was greatest in the presence of the Same cue [Non-Devalued: Same vs. Different, $t(13) = 2.85$, $p = 0.034$, $d = 2.85$, 95% CI (0.21, 5.49); Non-Devalued: Same vs General, $t(13) = 3.68$, $p = 0.007$, $d = 3.68$, 95% CI (1.04, 6.32)] but there was no significant difference in lever responding to the Different and General cues [Non-Devalued: Different vs. General, $t(13) = 2.39$, $p = 0.078$, $d = 2.39$, 95% CI (−0.25, 5.04)]. In contrast, in test sessions with the devalued lever extended (Figure 2F, right), there were no significant differences in lever responding in the presence of any of the cues [Devalued: Same vs. Different, $t(13) = -0.97$, $p = 0.605$, $d = -0.98$, 95% CI (−3.61, 1.6); Devalued: Same vs. General, $t(13) = 0.51$, $p = 0.867$, $d = 0.51$, 95% CI (−2.13, 3.15); Devalued: Different vs. General, $t(13) = 1.63$, $p = 0.267$, $d = 1.63$, 95% CI (−1, 4.27)]. This differential expression of this specific PIT effect on the devalued and non-devalued levers was supported by a significant Lever Devaluation*Outcome-Specific interaction [$F(2, 26) = 4.81$, $p = 0.017$; a main effect of Outcome-Specific $F(2, 26) = 6.18$, $p = 0.006$; but no main effect of Lever Devaluation $F(1, 13) = 3.93$, $p = 0.069$]. Therefore, outcome devaluation by specific satiety selectively abolished specific PIT.

As before, we tested whether lever responding was elevated above baseline in each condition as another metric of PIT. Responding was significantly above baseline on the non-devalued lever for the Same and Different cue conditions [Non-Devalued: Same $t(13) = 4.50$, $p = 0.004$, $d = 4.5$, 95% CI (1.4, 7.59); Non-Devalued: Different $t(13) = 3.44$, $p = 0.026$, $d = 3.44$, 95% CI (0.34, 6.54); Non-Devalued: General $t(13) = 0.51$, $p = 0.997$, $d = 0.5$, 95% CI (−2.58, 3.61)], but only above baseline on the devalued lever for the Different cue [Devalued: Same $t(13) = 2.35$, $p = 0.192$, $d = 2.35$, 95% CI (−0.74, 5.45); Devalued: Different $t(13) = 3.44$, $p = 0.026$, $d = 3.44$, 95% CI (0.34, 6.54); Devalued: General $t(13) = 1.54$, $p = 0.618$, $d = 1.54$, 95% CI (−1.56, 4.63)]. This analysis confirms again that specific PIT was abolished by outcome devaluation with specific satiety. It also suggests that there was a form of general PIT on the devalued lever in the presence of the Different, but not to the

General cue. Evidence of a PIT effect on the devalued lever also rules out the possibility that the strength of the devaluation on the instrumental lever prevented any form of PIT.

When considering whether the Pavlovian cue was devalued or non-devalued (Figure 2H), responding on the Same lever was significantly higher for the non-devalued than the devalued Pavlovian cue [Same: Non-Devalued vs Devalued, $t\,(13) = 3.17$, $p = 0.007$, $d = 3.17$, 95% CI (1.01, 5.33)], whereas responding on the different lever was almost identical for the Non-devalued and Devalued cue [Same: Non-Devalued vs. Devalued, $t\,(13) = -0.01$, $p = 0.991$, $d = -0.01$, 95% CI (−2.17, 2.15)]. Again, this pattern of differences in lever pressing was supported by a significant Pavlovian Devaluation*Outcome-Specific interaction [$F\,(1, 13) = 5.87$, $p = 0.031$; no significant main effect of Pavlovian Devaluation, $F\,(1, 13) = 4.33$, $p = 0.058$; or main effect of Outcome-Specific, $F\,(1, 13) = 0.73$, $p = 0.409$]. Therefore, specific PIT was abolished by specific satiety devaluation. This finding clarifies the findings of Experiment 1, and rules out alternative explanations of the effect being driven by differential baseline responding or response competition between the devalued and non-devalued levers.

Finally, we included session number into the analysis to confirm that these effects were consistent across repeated test sessions (i.e., Figure 2H split by session; data not shown). While there was an overall reduction in total responding over repeated testing [main effect of Session $F\,(1, 13) = 42.56$, $p < 0.00$]), there were no interactions between test session and any other factors [Session*Stimulus Devaluation, $F\,(1, 13) = 1.62$, $p = 0.225$; Session*Specific PIT $F\,(1, 13) = 0.02$, $p = 0.888$; Session*Stimulus Devaluation*Specific PIT $F\,(1, 13) = 0.18$, $p = 0.678$].

Discussion

The present studies tested the prediction that specific satiety would abolish specific PIT. This was based on the hypothesis that outcome devaluation by sensory specific satiety leads to habituation of an outcome's sensory representation, which disrupts the capacity for this representation to support specific PIT. Experiment 1 tested this prediction with a two-lever choice-PIT test, whereas Experiment 2 used a single lever PIT test design to control for potential response competition between levers. Specific PIT was assessed using two criteria (1) greater responding on the same than different lever to demonstrate outcome specificity, and (2) responding on the same lever above baseline to demonstrate a facilitative PIT transfer effect on response levels. Therefore, the findings of both experiments supported our prediction that specific satiety devaluation would abolish specific PIT.

Reports that specific satiety does not disrupt specific PIT

The present findings are in contrast to two recent reports of specific satiety leaving specific PIT intact, despite reducing the magnitude of effect (Lingawi et al., 2022; Sommer et al., 2022). Both studies used a two cue (S1-O1; S2-O2), two lever (R1-O1; R2-O2) design, where the outcomes were sucrose solution and grain pellets, a two-lever choice test, and a brief instrumental extinction period (between 2 and 6 min).

Sommer and colleagues report that a devalued stimulus is capable of supporting a significant specific PIT effect with responding on the same lever significantly above responding on the different lever and baseline (Figure 1D in Sommer et al., 2022). Surprisingly, the specific PIT effect they report was not as robust for the non-devalued stimulus, and baseline responding was significantly lower on the devalued than the non-devalued lever. Given the significant differences in baseline responding, it is unclear whether the magnitude of the specific PIT effect was reduced by specific satiety devaluation.

Lingawi and colleagues reported a significant reduction in the magnitude of the specific PIT effect for the devalued stimulus compared to the non-devalued stimulus (Figure 3I in Lingawi et al., 2022). However, the magnitude of the specific PIT effect was assessed by comparing baseline subtracted responding on the same and different levers. Close inspection of the data suggests that during the devalued stimulus responding on the same lever was not elevated above baseline, and instead the same-different effect is predominantly driven by a reduction in responding on the different lever (i.e., the non-devalued lever) relative to its baseline. Given that baseline responding was reported as significantly higher on the non-devalued lever, it is possible that an equivalently low response rate on both levers could produce this difference once different baseline response rates are subtracted. (Lingawi et al., 2022) tried to minimize differences in baseline responding by providing additional training on both levers with a common separate outcome (i.e., R1-O3; R2-O3), however this did not prevent robust differences in baseline responding. It is also possible that the different levels of baseline responding are the consequence of using the entire inter-stimulus period during the PIT test. In contrast, in the present experiments only the minute immediately before the stimulus was used as the baseline to remove the influence of any responding that persisted immediately after the previous stimulus ended.

The importance of having reasonably matched baseline responding has also been a point of focus for earlier work looking at the effect of taste aversion devaluation on specific PIT (Rescorla, 1994; Holland, 2004). This confound is particularly relevant when interpreting the magnitude of the specific PIT effect. In the present experiments, baseline lever responding was extinguished for significantly longer prior to the stimuli being

presented, which successfully abolished significant differences in baseline levels that were used for calculating elevation scores. However, the present findings are qualitatively different from these two reports (Lingawi et al., 2022; Sommer et al., 2022) in that the magnitude of the specific PIT effect (i.e., comparing same vs. different) was not only abolished, but reversed for the devalued stimulus (supported statistically in Experiment 1, and numerically in Experiment 2). Therefore, it is unlikely that different baselines sufficiently account for these contrasting findings. Overall, the findings of the present study, and the results reported by (Lingawi et al., 2022) demonstrate a consistent sensory specific impact of satiety on the magnitude of the specific PIT effect.

Another consideration is that the mixed evidence of specific PIT observed in Experiment 1, unlike in Experiment 2, is the result of additional cognitive demands placed (e.g., conflicting response cues) on the animals during a two-lever choice test, which may also account for the differences observed with the reported findings. It is also possible that these conflicting findings are the result of methodological differences between each study, such as the specific rewards used or the relative amount of Pavlovian and instrumental training, which have been shown to influence the nature of the observed PIT effect (Holmes et al., 2010). Indeed, this is also evident in distinct nature of the basic specific PIT effect (i.e., in non-devalued sessions) reported by these studies. Specifically, while all procedures produced a robust and significant elevation in responding on the same lever, responding on the different lever was significantly elevated [present studies, (Panayi and Killcross, 2018)], significantly suppressed (Lingawi et al., 2022), or not different to baseline (Sommer et al., 2022). It is unclear whether these three different forms of specific PIT are differentially sensitive to specific satiety devaluation. However, it is also possible that the precise impact of specific satiety devaluation on specific PIT may be less clear without robust responding on the different lever (Supplementary Figure 2).

Surprisingly, both of these studies (Lingawi et al., 2022; Sommer et al., 2022) report a robust effect of specific satiety devaluation on magazine responding during the Pavlovian cues, whereas this effect was not found in the present experiments. This may reflect some differential sensitivity to Pavlovian and instrumental devaluation as a consequence of the specific training and testing parameters between procedures. Indeed, establishing different Pavlovian outcome expectancies (e.g., different outcome probabilities, uncertainty, and magnitude) have recently been shown modulate magazine-lever response competition at test in general PIT (Ostlund and Marshall, 2021). It is possible that similar factors may account for the differential impact of specific satiety devaluation on Pavlovian magazine responding between the present results and these recent reports (Lingawi et al., 2022; Sommer et al., 2022). Further work is needed to establish whether the magnitude of specific PIT is also influenced by these outcome expectancy properties.

The effect of specific satiety on general PIT

A unique finding in the present results is that we observed robust elevation of responding on the Different lever. In Experiment 1, the devalued stimulus (but not the non-devalued stimulus) generated a robust increase in responding on the different lever i.e., both the devalued and non-devalued stimuli increased responding on the non-devalued lever (Figure 1G, Left). In Experiment 2, with only a single lever present at test, both devalued and non-devalued stimuli were capable of invigorating responding above baseline on the different lever at roughly similar levels. Elevated responding on the different lever has been predicted by theories of PIT, however responding on the different lever is usually reported to be no different to baseline responding (Cartoni et al., 2016; Lingawi et al., 2022; Sommer et al., 2022). Elevated responding on the different lever is hypothesized to be driven by a form of general PIT i.e., a non-specific energizing of the instrumental response driven by an association between the stimulus and the general motivational properties of the expected outcome (Balleine and Killcross, 2006; Corbit et al., 2007; Delamater and Oakeshott, 2007; Corbit and Balleine, 2015; Ostlund and Marshall, 2021).

Surprisingly, in both Experiment 1 and Experiment 2, the general stimulus (S_3) did not drive any general PIT behavior. However, (Lingawi et al., 2022) have convincingly demonstrated that satiety manipulations with any outcome (either relevant or irrelevant to the Pavlovian or Instrumental conditions) abolishes general PIT. The absence of a general PIT effect with this stimulus in the present findings is therefore consistent with this result. However, we have previously reported an absence of general PIT with S_3 in hungry/non-sated rats using the same single lever test design employed in Experiment 2 (appendix 1-Figure 1 in Panayi and Killcross, 2018). Therefore, despite the original intent of the experimental design, the specific parameters used do not generate general PIT with this stimulus S_3. Furthermore, the elevated responding on the different lever (Experiment 2) might not be considered a general PIT effect either, as it was insensitive to non-specific satiety. However, we have not directly tested this assumption.

The role of habituation processes in specific satiety

Our findings are consistent with evidence that specific satiety devaluation can lead to the habituation of an outcome's sensory representation (S_O) and impact associated behaviors. Habituation describes the phenomenon where an initial response to a stimulus will decrease over repeated presentations of the stimulus (Thompson, 2009). Habituation is commonly used to describe sensory adaptation, for example, the perceived

loss of flavor of the same food over the course of a meal. Indeed, sensory specific habituation is a mechanism that underlies how specific satiety changes food choice behavior (Epstein et al., 1992, 2009, 2010; Rolls, 2004). Beyond immediate consumption, sensory specific habituation has also been shown to be one key process driving the phenomenon of decreasing within-session instrumental lever pressing behavior in rats (McSweeney and Murphy, 2009). For example, the decline in within-session instrumental responding can be increased by introducing different or unexpected outcomes, even if these lead to increased effort or levels of satiety, which is consistent with dishabituation manipulations that disrupt habituation (Epstein et al., 2009; McSweeney and Murphy, 2009; Bouton et al., 2013). Therefore, there is empirical and theoretical support to suggest that habituation can play a role in how specific satiety impacts consummatory and appetitively motivated instrumental behaviors. A within-session decline in instrumental responding was also observed in the present experiments despite the relatively small number of outcomes (20) per session and short session length (10 min on average; Supplementary Figure 3).

Sensory specific habituation after satiety also appears to impact instrumental choice behavior over long periods of time. (Parkes et al., 2016) provide strong evidence for this showing that specific satiety devaluation in rats can affect instrumental choice behavior up to 2 h later, and even up to 5 h later when satiety and test occur in the same context. The long time course and context sensitivity of satiety devaluation on instrumental behavior suggests that behavioral control is being influenced by an associative recall process and retrieval interference mechanisms (Bouton, 1993). These findings are also consistent with accounts of long-term habituation where cues and contexts can prime representations of stimuli into working memory and modulate their ability to be recalled and influence learning and behavior (Wagner, 1981; Wagner and Brandon, 1989; Epstein et al., 2009; Robinson and Bonardi, 2015).

Implications for devaluation

The effect of specific satiety reported here is important when contrasted with the consistent finding that outcome devaluation does not disrupt the expression of specific PIT when the outcome is devalued by taste aversion, often by pairing an outcome with LiCl induced illness (Colwill and Rescorla, 1990a; Rescorla, 1994; Holland, 2004). Indeed, these studies report that the size of the specific PIT effect is not reduced by taste aversion devaluation. In contrast, supporting our hypothesis, specific satiety devaluation either reversed (experiment 1), abolished (Experiment 2), or significantly reduces the size of the specific PIT effect (Lingawi et al., 2022).

Taste aversion and specific satiety methods of devaluation are often used interchangeably to probe an organism's ability to update behavior when the value of an outcome changes, and to establish learning about associations between cues and specific outcome identities and their neural substrates (Killcross and Blundell, 2002; Balleine et al., 2003). However, the present findings suggest that specific satiety might not engage the same associative mechanisms as taste aversion devaluation. These differences are of particular importance when using devaluation to probe the neural substrates of learning paradigms (Ostlund and Balleine, 2007c). However, it is important to note that the habituation mechanism being proposed is likely to be only one of multiple potential contributors to underlying a robust outcome devaluation effect experimentally.

Limitations and future directions

An important limitation of the present study is that habituation was not directly manipulated independently of other satiety processes that may account for these findings. Instead, these alternative processes are ruled out by the results of other published experiments. It is therefore important to clearly identify each of these assumptions explicitly, their limitations, and to propose future experimental evidence required to overcome these limitations.

First is the issue of manipulating general levels of satiety. Is it the case that specific satiety reduces hunger and general motivation for appetitive outcomes, and therefore disrupts all forms of PIT, including specific PIT? It is reasonable to assume that specific satiety might generally disrupt specific PIT through a non-specific reduction in hunger and general motivation for appetitive outcomes. However, in the present experiments, the non-devalued stimulus and directly control for the impact of this general reduction in hunger. Furthermore, explicit general satiety manipulations have been shown to disrupt general but not specific PIT. This was first demonstrated by Corbit et al. (2007) (i.e., independently of whether rats were hungry or sated, responding to the Same but not the Different stimulus above baseline; a direct comparison of Same and Different conditions was not tested), and has been successfully replicated by multiple researchers (Lingawi et al., 2022; Sommer et al., 2022).

The second issue is that of manipulating specific outcome value, which we refer to as the hedonic value of the outcome (in contrast to general motivational value). Is it the case that during specific satiety, the hedonic value of the outcome is reduced, and specific PIT is sensitive to the current hedonic value of specific outcomes? While specific satiety does reduce hedonic value (Berridge, 1991), changing the hedonic value of an outcome using conditioned taste aversion has been shown to leave specific PIT effects intact (Colwill and Rescorla, 1988, 1990b; Rescorla, 1994; Holland, 2004). To what extent can the change in hedonic value induced by conditioned taste aversion be compared to specific satiety? Both specific satiety and taste aversion reduce positive hedonic responses to reinforcers (e.g., sucrose) in measures of taste reactivity, however only taste

aversion (but not specific satiety) increases negative hedonic responses (Berridge et al., 1981; Berridge, 1991; Breslin et al., 1992). This suggests that both taste aversion and specific satiety reduce the hedonic value of an outcome. Furthermore, these changes in outcome value following specific satiety and taste aversion both depend upon incentive learning (Balleine, 1994; Balleine and Dickinson, 1998; Dickinson and Balleine, 2002). Taken together, it is therefore reasonable to conclude that the specific PIT effect is not sensitive to changes the current hedonic value of an outcome.

However, it is also possible that there is some fundamental difference in the nature of the shift in hedonic value produced by these two devaluation procedures. The effects of satiety involve a temporary reduction in positive hedonic value, whereas taste aversion produces a long lasting positive-to-negative change in hedonic value. It is therefore important to test the extent to which specific PIT is sensitive to changes in the hedonic value of the specific outcomes that do not involve aversion. This could be achieved by either reducing or inflating the hedonic value of a specific outcome immediately before a PIT test using incentive learning/contrast effects (e.g., Experiment 3 and 4 in Balleine and Dickinson, 1998), or by pharmacologically inducing specific nutrient deprivation states relevant to specific outcomes (Krieckhaus and Wolf, 1968; Fudim, 1978; Davidson et al., 1997).

Further experiments are still needed to directly test the role of sensory habituation in specific PIT following specific satiety. One approach would be to test whether the ability for specific satiety to disrupt specific PIT is context specific in the same manner, and over the same long time scales, as reported for instrumental outcome devaluation (Parkes et al., 2016). Another prediction of this habituation account is that it should be disrupted by a dishabituation manipulation. For example, briefly presenting a novel taste immediately after the specific satiety manipulation should recover specific PIT. Similarly, presenting multiple outcomes during outcome devaluation should also disrupt sensory habituation during a specific satiety devaluation. Indeed, Lingawi and colleagues have shown, using a S_1-O_1/S_2-O_2 and R_1-O_1/R_2-O_2 specific PIT design, that devaluing both O_1 and O_2 during the same specific satiety consumption period attenuates but does not completely abolish specific PIT (Figure 2 in Lingawi et al., 2022).

More generally, the present discussion also highlights the importance of testing multiple aspects of the specific PIT effect. Tests of specific PIT should assess (1) outcome specificity: greater responding on the same than different lever, and (2) a facilitative PIT transfer effect: responding on the same lever above baseline. Consistent statistical reporting of both of these tests would enhance comparisons made between studies, and may reveal important psychological and neural distinctions between the signaling and the response invigorating properties of stimuli in PIT (Delamater and Holland, 2008; Holmes et al., 2010; Laurent and Balleine, 2015; Marshall et al., 2022).

Given the ubiquity of interactions between stimuli and instrumental actions in the daily lives of human and non-human animals, further research is required to improve our current understanding of the nature of PIT effects. Significant progress has been made recently with an increasing interest in PIT research such as, establishing the relevant contents of learning involved in PIT (Gilroy et al., 2014; Laurent and Balleine, 2015; Lingawi et al., 2016; Laurent et al., 2021), identifying the boundary conditions of general PIT effects (Holmes et al., 2010; Ostlund and Marshall, 2021), understanding the role of PIT in substance use (Ostlund and Marshall, 2021), and revealing fine grained neural circuits underlying PIT and their signaling processes (Laurent et al., 2014; Lichtenberg et al., 2017; Bradfield et al., 2018; Sias et al., 2021).

Data availability statement

Data are available at DOI 10.17605/OSF.IO/NHT6A.

Ethics statement

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

Author contributions

MP and SK designed the experiments and wrote the manuscript. MP conducted the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.983480/full#supplementary-material

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The role of the bed nucleus of the stria terminalis in the motivational control of instrumental action

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We review recent studies assessing the role of the bed nucleus of the stria terminalis (BNST) in the motivational control of instrumental conditioning. This evidence suggests that the BNST and central nucleus of the amygdala (CeA) form a circuit that modulates the ventral tegmental area (VTA) input to the nucleus accumbens core (NAc core) to control the influence of Pavlovian cues on instrumental performance. In support of these claims, we found that activity in the oval region of BNST was increased by instrumental conditioning, as indexed by phosphorylated ERK activity (Experiment 1), but that this increase was not due to exposure to the instrumental contingency or to the instrumental outcome *per se* (Experiment 2). Instead, BNST activity was most significantly incremented in a test conducted when the instrumental outcome was anticipated but not delivered, suggesting a role for BNST in the motivational effects of anticipated outcomes on instrumental performance. To test this claim, we examined the effect of NMDA-induced cell body lesions of the BNST on general Pavlovian-to-instrumental transfer (Experiment 3). These lesions had no effect on instrumental performance or on conditioned responding during Pavlovian conditioning to either an excitory conditioned stimulus (CS) or a neutral CS (CS_0) but significantly attenuated the excitatory effect of the Pavlovian CS on instrumental performance. These data are consistent with the claim that the BNST mediates the general excitatory influence of Pavlovian cues on instrumental performance and suggest BNST activity may be central to CeA-BNST modulation of a VTA-NAc core circuit in incentive motivation.

KEYWORDS

bed nucleus of the stria terminalis (BNST), central amygdala (CeA), instrumental conditioning, Pavlovian conditioning, incentive motivation, Pavlovian-instrumental transfer (PIT)

Introduction

Contemporary analyses of instrumental conditioning suggest that a variety of learning and motivational processes can affect instrumental performance (Balleine, 2019). The focus in recent years has been on the learning processes contributing to the goal-directed and habitual control of such actions, i.e., the

relative strength of the response-outcome and stimulus-response associations that support these forms of learning process (Balleine and O'Doherty, 2010; Balleine, 2019). At least as important, however, is the role of various incentive processes, that can modulate performance either through their effects on the *experienced value* of rewarding or reinforcing events directly, or indirectly by modifying the degree to which reward is anticipated or predicted in the environment (Corbit and Balleine, 2016; Balleine, 2019). These latter *predicted values* can exert quite selective effects on action selection through the anticipation of specific events or outcomes. Alternatively, predictions can be more general, being based, not on specific features of rewarding events but on their motivational and emotional effects, something that can alter the state of arousal and so the degree of vigour with which responses are performed (Cartoni et al., 2016; Corbit and Balleine, 2016).

Of these sources of predicted value, there have been several recent reviews of those controlling the influence of identity-specific reward predictions on instrumental performance, focussing mostly on their function in action selection in outcome-specific Pavlovian-instrumental transfer (Holmes et al., 2010; Cartoni et al., 2016; Watson et al., 2018; Balleine, 2019; Eder and Dignath, 2019; Laurent and Balleine, 2021). The current article is instead concerned with the contribution of general incentive processes to performance, i.e., those that induce their effects through a form of energetic shift in motivational or affective arousal. There have been numerous assessments of arousal on instrumental performance over many years of research and any broad attempt to review these issues is beyond the scope of this article (Lang and Davis, 2006; Bradley, 2009; Berridge et al., 2010). It is worth noting here that previous reviews have documented the motiving influence of reward-related contexts and other diffuse predictors (Salamone, 1994; Ikemoto and Panksepp, 1999; Everitt et al., 2003). But, of course, the most thoroughly researched phenomenon demonstrating the influence of affective arousal induced by general reward predictions on instrumental performance comes from assessments of what has come to be called "general" Pavlovian-instrumental transfer (PIT; Dickinson and Dawson, 1987; Dickinson and Balleine, 2002; Corbit and Balleine, 2005, 2011). Here we first provide background to the behavioural assessment of general transfer before consideration of its neural bases.

General transfer—behavioural factors

The pairing of conditioned stimuli (CSs) with complex multi-faceted unconditioned stimuli (USs) has been demonstrated to produce a similarly complex array of conditioned responses, including those that are US-specific or consummatory in nature, e.g., licking for fluidic outcomes vs. chewing for dry food, and those that are associated with a general appetitive motivational or affective state, which include general search, arousal, and approach responses (Konorski, 1967; Bindra, 1974, 1978; Hearst and Jenkins, 1974; Toates, 1986; Rescorla, 1988; Delamater and Oakeshott, 2007). The consummatory and motivational effects of CSs have been delineated in a number of ways; for example, in the report of distinct signtracking and goal-tracking phenotypes (Jenkins and Moore, 1973; Hearst and Jenkins, 1974; Boakes, 1977), presentation of a localised localized CS, such as light or illuminated lever causes animals variously to approach and contact the CS and to approach the location of impending US delivery. Evidence suggests the former reflects the motivational/emotional and the latter the consummatory influence of the CS: Sign tracking conditioned responses (CRs) are often relatively imprecise or diffuse and less sensitive to changes in US value than goal-tracking CRs (Davey et al., 1989; Chang and Smith, 2016). Other examples have similarly involved manipulations of US "proximity," either in space or time, with spatial or temporal distance reducing the precision of US-specific CRs and increasing the performance of more general exploratory or activity-related CRs (Konorski, 1967; Vandercar and Schneiderman, 1967; Gast et al., 2016). These kinds of data suggest that Pavlovian CSs can convey distinct forms of information, providing the basis for their differential motivational influence on instrumental performance.

Given this perspective, whereas outcome specific transfer must require sufficiently specific predictions to allow CSs to select actions based on the identity of their consequences, such predictions should not be required for general transfer. Nevertheless, both forms of Pavlovian-to-instrumental transfer evaluate the effects of the interaction between Pavlovian and instrumental conditioning in a test phase in which the effect of the Pavlovian cues on instrumental performance are assessed for the first time (Cartoni et al., 2016). Unlike specific transfer, which typically involves training on two action-outcome associations, general transfer is often demonstrated by examining the excitatory effects of Pavlovian cues on a single action, whether it is trained with the same or a different outcome to that paired with the cue (Estes, 1943; Lovibond, 1983; Hall et al., 2001; Dickinson and Balleine, 2002; Holland and Gallagher, 2003). The Pavlovian phase can establish either different conditioned stimuli paired with distinct outcomes, as has been the case in assessing motivational influences on transfer (Dickinson and Dawson, 1987), or, more frequently, examine the effect of a stimulus paired with an appetitive outcome on instrumental performance against an unpaired control stimulus; one to which the animal has been exposed but not sufficiently for it to become inhibitory (CS_0). Under these conditions the paired cue typically invigorates the performance of the action relative both to periods without cue presentation and to the unpaired control cue (Cartoni et al., 2016). Importantly, this effect is usually of comparable magnitude regardless of the similarity of the instrumental and Pavlovian outcomes (providing they

are similarly valued) and so is usually interpreted as being a product of the appetitive arousal induced by the cue (Rescorla and Solomon, 1967; Dickinson and Balleine, 2002).

This source of appetitive arousal is both in addition to the reward value of the outcome earned by instrumental performance and is gated by primary motivational state. An appetitive cue's invigoration of single-lever responding can still be observed when the predicted reward is delivered on test (Lovibond, 1983). Furthermore, a cue paired with liquid food or liquid salt when thirsty can elevate performance when animals are subsequently hungry or in a sodium appetite (Dickinson and Nicholas, 1983; Dickinson and Balleine, 1990; Balleine, 1994). Conversely, a cue paired with liquid food when animals are hungry can increase instrumental responding on a pellet-associated lever when tested thirsty (Dickinson and Dawson, 1987). These kinds of data, indicative of what has been called the irrelevant incentive effect (Krieckhaus and Wolf, 1968; Dickinson and Balleine, 2002), demonstrate the motivational control of these forms of general transfer. This degree of control is not observed in specific transfer, which is more strongly regulated by US-specific information than the influence of appetitive motivation. Thus, specific transfer remains largely unaffected by shifts in primary motivation, e.g., from hunger to satiety, whereas this shift can abolish general transfer (Corbit et al., 2007).

General transfer—neural bases

Despite older claims that general and specific transfer are mediated by a common incentive process, it is clear from the behavioural evidence above and from experiments investigating their neural bases that they are subserved by quite distinct psychological and brain processes. Thus, whereas specific transfer depends on the integrity of basolateral amygdala (BLA; Blundell et al., 2001; Corbit and Balleine, 2005), nucleus accumbens shell (Corbit et al., 2001; Shiflett and Balleine, 2010; Corbit and Balleine, 2011), and their interconnecting pathway (Morse et al., 2020), general transfer has been found to depend on the central nucleus of the amygdala (CeA) and nucleus accumbens core (NAc core; Balleine and Killcross, 1994; Hall et al., 2001; Holland and Gallagher, 2003; Lingawi and Balleine, 2012).

General PIT depends on intact dopamine (DA) transmission: it is abolished by systemic application of D_1/D_2 dopamine receptor antagonist flupenthixol in rats (Dickinson et al., 2000; Wassum et al., 2011; Ostlund and Maidment, 2012), and reduced by D_2/D_3 receptor antagonist amisulpride in humans (Weber et al., 2016). DA's role in general transfer is thought to be mediated by NAc core. Bilateral pre-training lesions of NAc core (Hall et al., 2001) or local application of the D_1 dopamine receptor antagonist SCH-23390 in NAc core on test abolishes general transfer (Lex and Hauber, 2008) whereas the DA agonist amphetamine enhances it (Wyvell and Berridge, 2000). More, direct measurement of DA concentration in NAc core with microdialysis has found that DA level is increased in response to food or drug conditioned cues (Bassareo and Di Chiara, 1999; Ito et al., 2000). Notably, using fast-scan cyclic voltammetry to detect DA release in real time, it has been shown that reward predicting cues induce an increase in phasic dopamine release in NAc core, the amplitude of which positively correlates with lever-pressing rate (Wassum et al., 2013; Aitken et al., 2016). Given the NAc core receives a heavy dopamine innervation from VTA (Beier et al., 2015), mesolimbic DA released into core is considered to underlie the conditioned cue's general excitatory effect on instrumental actions. Supporting this view, pre-training lesions of VTA reduce general transfer (El-Amamy and Holland, 2007) whereas inactivation of VTA on test abolishes (Murschall and Hauber, 2006) or suppresses it (Corbit et al., 2007).

An important question concerning the neural circuitry underlying general transfer is the brain regions that contribute to the encoding of the cue's motivational properties. A starting point to address this question is to locate areas that trigger VTA release of DA into NAc core in this effect. Some reports indicate the NAc itself provides one of the heaviest inputs onto VTA DA neurons (Watabe-Uchida et al., 2012), as well as VTA GABA neurons (Xia et al., 2011; Bocklisch et al., 2013; Beier et al., 2015) and so it cannot be ruled out that NAc functions as a controller of DA release into its core division. Apart from NAc core and VTA, a structure that has been repeatedly shown to be indispensable for general transfer is the central nucleus of the amygdala (CeA). Bilateral lesions of the CeA abolish general transfer in rodents (Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005; Lingawi and Balleine, 2012); and in humans the CeA region is active during a general PIT task in a fMRI study (Prevost et al., 2012). As the CeA lacks direct connections with NAc core (Zahm et al., 1999), it has been proposed that CeA regulates DA release in NAc core through CeA→VTA projections to mediate general transfer (Hall et al., 2001).

The CeA→VTA→NAc core sequential link has also been hypothesised to account for CeA and NAc core's similar involvement in cue-directed conditioned approach behaviours (Everitt et al., 2000). However, no evidence documenting the functional involvement of this circuit has been published and, indeed, some tracing studies have described CeA's projection to VTA as light to negligible (Zahm et al., 1999). This picture has, however, been clouded by studies using a rabies strategy to map inputs to VTA showing that CeA sends a moderate input to both VTA DA and GABA neurons, although mostly onto GABAergic neurons (Watabe-Uchida et al., 2012; Beier et al., 2015). Supporting this latter finding is a rather puzzling piece of evidence showing that contralateral lesions of CeA rescued the impairment of general PIT induced by a unilateral VTA lesion whereas an ipsilateral lesion of CeA had no restorative effect

(El-Amamy and Holland, 2007). This result suggests that CeA's direct influence on VTA DA neurons is inhibitory, implying that it interacts with a structure other than the VTA to generate general transfer. In fact, the CeA's close neighbour within the extended amygdala, the bed nucleus of stria terminalis (BNST), is well positioned to undertake this role.

The extended amygdala: an anatomical and functional unit

The BNST is a heterogeneous limbic structure that joins the caudal part of the nucleus accumbens shell anteriorly and posteriorly connects with CeA through the fibre tract of the stria terminalis. The parcellation or nomenclature of the BNST is rather inconsistent in the literature. According to the prevailing view, the BNST can be generally divided into medial–lateral and anterior–posterior portions when ontogeny, cytoarchitecture, chemoarchitecture, input, and output connections are taken into considerations (Ju and Swanson, 1989; Ju et al., 1989; Dong et al., 2001a). Because the anterior portion is the area that receives the projection terminals from the CeA, and has been highly implicated in reward processing, our focus is primarily on this area. The anterior BNST can be further subdivided into dorsal and ventral regions based on their positions in relation to the anterior commissure. Anterodorsal (ad), oval (ov), and fusiform (fu) subnuclei within the anterior BNST have received the most attention in recent years following influential studies demonstrating their abilities to shift emotional or motivational state (Tye et al., 2011; Jennings et al., 2013a,b; Kim et al., 2013; Janak and Tye, 2015). As the adBNST and ovBNST make up the majority of the dorsal division, they are often referred to together as dorsal BNST (dBNST). In contrast, the fuBNST is the only nucleus located in the ventral division and is, therefore, referred to as the ventral BNST (vBNST) in most studies. Importantly, both dBNST and vBNST project to the VTA (Silberman and Winder, 2013).

Studies investigating regional or whole BNST's role in emotional or motivational processes have demonstrated that its functional profile spreads over a wide-range of physiological or pathological behaviours from food intake, mating, arousal, fear, to anxiety (Kalin et al., 2005; Waddell et al., 2006; Davis et al., 2009; Fox et al., 2015), depression-like behaviours (Stout et al., 2000; Hammack et al., 2004), substance abuse disorders (Erb and Stewart, 1999; Aston-Jones and Harris, 2004; Koob, 2008, 2015; Buffalari and See, 2011; Pleil et al., 2015), obsessive-compulsive disorder (van Kuyck et al., 2008; Kohl et al., 2016; Wu et al., 2016; Raymaekers et al., 2017), anorexia (Roman et al., 2012), and pain (Tran et al., 2014). The growing body of evidence on BNST's functions highlights its potential as a therapeutic target for various maladaptive reward-seeking behaviours and has attracted considerable interest in the mechanism of its regulation over affective or motivational states.

Importantly, in the current context, evidence suggests that the CeA and BNST maintain strong connections; indeed, traditionally, the BNST has been thought of as a downstream output of the CeA (de Olmos and Heimer, 1999) and receives more substantial afferents from CeA than CeA receives from BNST (Oler et al., 2017). Swanson and colleagues view BNST as the pallidal output to CeA's striatal-like structure (Swanson, 2000; Dong et al., 2001b). In contrast, de Olmos and Heimer (de Olmos and Heimer, 1999) propose that, instead of a simple striatal-pallidal sequential relationship, CeA and BNST maintain multiple symmetrical pairings between sub-nuclei (McDonald, 1983; Holstege et al., 1985; Shammah-Lagnado et al., 2000; Alheid, 2003) with a strong resemblance of cell type and neurochemical makeup within each pair of structures (Alheid and Heimer, 1988; McDonald, 2003) The strong implication is, therefore, that this pair of structures function together as two aspects of a circuit. Considering the less explored status of the BNST relative to the voluminous literature on CeA, this view is particularly helpful in formulating hypotheses with respect to the role of the BNST in emotional or motivated learning. Overall, there is general agreement that the CeA and BNST have similar cortical afferents and subcortical efferents (Gray and Magnusson, 1987; Gray and Magnuson, 1992; McDonald et al., 1999; McDonald, 2003; Nagy and Paré, 2008; Bienkowski and Rinaman, 2013), and strong reciprocal connections (Krettek and Price, 1978; Sun et al., 1991; Sun and Cassell, 1993; Dong et al., 2001b; Dong and Swanson, 2004). Thus, it is safe to assume that BNST should also be functionally linked with CeA, exhibiting a similar functional profile to that of CeA.

In appetitive Pavlovian conditioning, the CeA is involved in assigning conditioned motivation to food predicting cues. CeA lesions impair the acquisition of a visual CS-directed conditioned orienting response, without affecting unconditioned orienting responses to the visual cue (Gallagher et al., 1990). This result has been interpreted as suggesting that the CeA mediates an attentional response to cues (Holland and Gallagher, 1999). The CeA is also involved in the acquisition of conditioned approach responses directed to a localised cue (CS directed sign-tracking CR; Parkinson et al., 2000). Although CeA may not be *necessary* for the expression of a sign-tracking CR, post-training intra-CeA infusion of a dopamine D_3 receptor agonist enhances CS potentiated food-cup approach behaviours (Hitchcott and Phillips, 1998). In contrast, CeA lesions have no effect over Pavlovian conditioned food-cup approach before the delivery of food (Gallagher et al., 1990), and these US-directed conditioned responses remain sensitive to devaluation (Hatfield et al., 1996). This suggests that the CeA is not involved in the CS's access to the sensory or incentive value components of the US representation (Cardinal et al., 2002; Everitt et al., 2003). CeA lesions have also been reported to disrupt increments, but not decrements, in conditioned stimulus processing (Holland and Gallagher, 1993a) induced in an unblocking paradigm. Although the processing of a cue is usually blocked when it is

presented with a cue that has already been conditioned, if the value of the US is increased or decreased when a second neutral cue is added to the already conditioned CS, processing, and so conditioning, of the second cue is increased. However, in rats with CeA lesions, conditioning of the second cue will only occur when the US value is increased, so called "upshift" unblocking (Holland and Gallagher, 1993a,b). This result suggests that the CeA mediates increases in the associability of the CS (Cardinal et al., 2002). The concept of associability in learning theory denotes a CS's ability to form associations with the US during conditioning (Pearce and Hall, 1980). In other words, from an error-correction theory perspective of Pavlovian conditioning, the CeA appears to be involved in attributing a positive reward prediction error to the CS.

In contrast to the wide-ranging studies involving CeA, the literature on the BNST's involvement in appetitive learning is mostly concentrated on its mediation of conditioned place preference (CPP) to natural rewards or drugs of abuse whereas this task has not been the focus of research into CeA function (Jennings et al., 2013a). Nevertheless, CPP is an appetitive contextual conditioning effect (Bardo and Bevins, 2000; Cunningham et al., 2006) supporting the suggested involvement of the BNST in incentive motivation. Nevertheless, the involvement of the BNST in the motivational control of instrumental action and particularly in general transfer effects remains unknown.

The BNST→VTA pathway

Despite their overall striking similarities, the CeA and BNST maintain dissimilar strengths of connectivity with several key downstream effectors—the paraventricular nucleus of hypothalamus (PVN), substantia nigra pars compacta (SNc), and the VTA. Projections to the PVN from the ventral BNST are particularly massive, whereas few projections from CeA are seen (Gray et al., 1989; Prewitt and Herman, 1998). CeA and BNST have distinct innervation of mid-brain dopamine rich regions like the VTA and SNc. CeM sends considerable efferents to lateral SNc, whereas only few terminalis from BNST end in SNc. In return, the SNc appears to be the only brain region that provides inputs to CeM but not to ventral BNST (Bienkowski and Rinaman, 2013). The CeA's connections with the SNc are known to be functional and mediate conditioned orienting (Han et al., 1997). Disconnecting CeA from SNc significantly impairs the acquisition of conditioned orienting to auditory cues but preserves food-cup responses (Lee et al., 2005), whereas disconnection of CeA and VTA has no effect on the acquisition of conditioned orienting (El-Amamy and Holland, 2007).

More pertinently, BNST sends prominent projections to VTA. The BNST→VTA pathway has been rigorously demonstrated in rodents in studies utilizing a variety of techniques, including traditional tracing, channel rhodopsin assisted mapping, and a Cre-dependent double-virus strategy (Georges and Aston-Jones, 2002; Dumont and Williams, 2004; Deyama et al., 2007; Jennings et al., 2013a; Kudo et al., 2014; Kaufling et al., 2017; Pina and Cunningham, 2017). Most importantly, manipulations of BNST→VTA pathway potently alter motivational state and reward-seeking behaviours; optogenetic activation of VTA-projecting glutamatergic cells produce real-time place aversion and anxiogenic effects, whereas activation of VTA-projecting GABAergic cells produces place preference and anxiolytic effects (Jennings et al., 2013a). These demonstrations reveal the capacity of BNST→VTA pathway to shift motivational appetitive contextual conditioning. Evidence suggests that CPP largely depends on VTA dopamine transmission. Genetic NMDA receptor knockout on DA neurons dampens burst firing to appetitive cues and induces deficits in CPP (Zweifel et al., 2008). Moreover, direct photo-inhibition of VTA DA neurons supports conditioned place aversion whereas, conversely, phasic activation of VTA DA neurons leads to transient DA release and establishes a place preference in the absence of other rewards (Tsai et al., 2009).

BNST has also been found to mediate the expression of drug CPP, and this effect is likely not induced by BNST's projection to lateral hypothalamus orexin cells. Instead, disconnection of BNST and VTA impairs the expression of cocaine CPP (Sartor and Aston-Jones, 2012). VTA projecting BNST cells show enhanced c-Fos immunoreactivity during expression of cocaine CPPs (Mahler and Aston-Jones, 2012) whereas inhibition of VTA-projecting BNST cells blocks the expression of CPP to ethanol (Pina and Cunningham, 2017). Adding the fact that BNST can positively regulate VTA DA activity through its dual innervation of VTA GABA and DA neurons, the BNST→VTA pathway appears critical for appetitive contextual conditioning. In addition, the BNST→VTA pathway plays an important role in cue- or stress-induced drug seeking behaviour. Inactivation of BNST attenuates cue- or stress-induced relapse of cocaine-seeking behaviours (Buffalari and See, 2011). VTA-projecting BNST cells show enhanced c-Fos immunoreactivity during cue-induced reinstatement of cocaine seeking (Mahler and Aston-Jones, 2012) whereas disconnection of BNST and VTA reduces stress-induced cocaine seeking (Vranjkovic et al., 2014). Overall, evidence from drug CPP studies suggests that the BNST responds to external and internal cues and regulates drug motivated behaviour through its innervations of VTA (see also Tian et al., 2022).

These various lines of evidence suggest, therefore, that the BNST, the CeA's close neighbour within the extended amygdala, is a promising candidate structure as a relay of the CeA's involvement in general transfer. First, the CeA and BNST are tightly interconnected, receiving largely overlapping cortical and amygdala inputs and innervate similar downstream targets, albeit to different degrees. It is, therefore, tempting to speculate that they are involved in similar neurobiological processes. Reports of their roles in appetitive and aversive Pavlovian

conditioning provide support for this idea. Second, compared to the CeA, the BNST sends robust projections to VTA, which is a critical locus for general transfer. And optogenetic manipulations of the BNST→VTA pathway potently flip motivational state in real time. Collectively, these studies raise the possibility that the BNST regulates the motivational aspects of general transfer. Given that it remains unclear how CeA interacts with VTA to mediate general transfer, BNST could serve as the missing link for the hypothesised CeA-VTA circuitry. However, whether BNST mediates general transfer has not been assessed.

The role of the BNST in the motivational control of instrumental performance

Given the claims above, it is tempting to speculate that BNST also regulates the influence of other sources of arousal on the performance of instrumental actions, whether due to Pavlovian cues or *via* other Pavlovian processes embedded in the instrumental conditioning situation (Rescorla and Solomon, 1967). For example, instrumental acquisition can take place in the presence of an explicit discriminative stimulus or an implicit stimulus-outcome relationship between situational stimuli and the reinforcer and in both kinds of situation these stimuli have been found to modulate the vigour of instrumental performance (Bindra, 1978; Colwill and Rescorla, 1988). Furthermore, a context paired with alcohol (Ostlund et al., 2010) or with methamphetamine (Furlong et al., 2017) alters the control of instrumental actions trained in a different context. As discussed previously, evidence suggests that the BNST mediates appetitive contextual conditioning and, therefore, the BNST could theoretically modulate instrumental motivation through its mediatory role in contextual conditioning.

At present there is very little evidence with which to evaluate the role of the BNST in instrumental performance; it is not known whether: (i) instrumental conditioning engages the BNST; (ii) whether any such engagement reflects the conditioned anticipatory or unconditioned features of exposure to the instrumental outcome; and so (iii) whether the BNST is involved in the motivational control of instrumental performance by predictive cues in the general transfer situation.

To address these questions, the current study sought first to examine whether any changes were induced in the activity of neurons in the BNST as a consequence of instrumental conditioning, i.e., as a consequence of mice learning to press a lever for food pellets. We contrasted these changes against those in a yoked control that received matched exposure to reward delivery but for whom lever pressing and rewards were unpaired. There have been reports of robust pERK (phosphorylated extracellular signal-regulated kinase) expression in the dorsal BNST in response to various drugs of abuse (Valjent et al., 2004) and pERK is widely considered as a cellular activity marker for learning and memory (Shiflett and Balleine, 2011a,b). Therefore, pERK was used as the marker of cellular activity for this experiment. The above evidence suggested to us that dorsal BNST was the more likely target of CeA afferents and so of CS-related activity—which turned out to be the case—and so we also used PKC-δ as a marker to delineate both the ovBNST and the lateral region of the CeA within which boundaries pERK+ cells were counted (Wang et al., 2019).

Experiment 2 investigated: (i) the degree to which any changes in activity reflected the amount of instrumental training; and (ii) the anticipation of, vs. exposure to, the instrumental outcome, which we addressed by examining pERK activity in the BNST after a brief period of training, more extended training, and after a brief period of extinction during which the reward was anticipated but no reward exposure was given.

Finally, Experiment 3 examined the functional effects of a lesion of the BNST on Pavlovian conditioning, instrumental conditioning and on the influence of Pavlovian cues on instrumental performance in a general transfer design.

Materials and methods

Animals

Seven to 10-week old male C57B16 mice were acquired from the Australian Research Council (Perth). They were housed in a holding room maintained at 21°C on a 12-h lightdark cycle (lights off at 7 pm). Throughout behavioural experiments the mice were foodrestricted to 85%–90% of their initial weight by giving them 1.5–2.5 g of their maintenance chow each day. They were fed after training each day and had *ad libitum* access to tap water while in the home cage. All procedures were approved by the Animal Ethics Committee of UNSW Sydney.

Apparatus

All behavioural training and testing was conducted in eight identical mouse operant chambers (ENV-307A, Med Associates, Vermont, USA). Chambers were housed in light and sound resistant shells. Each chamber has a house light on one side of the box and a recessed food magazine and two retractable levers on the opposite side with the magazine located in the center and two levers positioning symmetrically on the left and right of the magazine. The reward for all behavioural manipulations was 20 mg grain pellets (Bioserve Biotechnologies, Flemington, NJ, USA), delivered by pellet dispensers into the magazine. The house light and a ventilating fan were turned on throughout all behavioural procedures. Each chamber was also equipped with generators of 3-kHz tone or white noise (∼70 dB, Med Associates, Burlington, VT, USA). All chambers were connected to a computer that controlled the equipment and recorded

behavioural responses during training using custom codes programmed and run in Med-PC software (Med Associates, Burlington, VT, USA).

Experimental designs

Experiment 1: pERK expression in the BNST and CeA induced by instrumental conditioning

Eighteen mice at 8-weeks of age were evenly assigned to instrumental or yoked training. Mice in the yoked group served as controls for exposure to the various stimulus- and context-reward associations. Each instrumentally trained animal had a corresponding yoked control which had a pellet delivered to the magazine at the same interval as its trained counterpart regardless of whether it pressed the lever or not. All mice were trained for nine daily sessions, including three on continuous reinforcement (CRF), two on random interval (RI)15, one on RI30 and three on RI60. Immediately after the third RI60 session, mice were sacrificed and pERK expression in the BNST and CeA was examined to establish the number of cells displaying pERK immunofluorescence. Sections were also counterstained for PKC-δ as a marker to delineate both the ovBNST and the lateral region of the CeA.

Experiment 2: pERK expression at BNST and CeA following extended instrumental training

Eighteen mice were divided to three groups: Trained ($n = 5$), Longer trained ($n = 5$), and Longer trained + test ($n = 8$). Mice in the Trained group underwent an identical instrumental training procedure to Experiment 1. Animals in the other two groups had three more sessions of training on RI60 compared to the Trained group. Animals in Trained and Longer trained groups were immediately sacrificed after the 2nd and 5th RI60 session respectively, whereas the Longer trained + test group were given an additional 5-min extinction test on the day after the 5th RI60 session followed by immediate euthanasia. Again pERK expression in the BNST and CeA was examined in sections counterstained for PKC-δ.

Experiment 3: effects of pre-training BNST lesions on general transfer

Surgery was conducted in 20 mice, groups of Lesion ($n = 12$) and Sham ($n = 8$) mice received either bilateral NMDA (10 mg/ml) or vehicle (sterile 0.9% normal saline) injections, respectively, into BNST, 55 nl per side. One week after the surgery, mice were given nine daily instrumental training (3 CRF, 2 RI15, 2 RI30, 2 RI60) sessions before six daily Pavlovian conditioning sessions. Tone and noise were used as the CS and CS_0 in Pavlovian conditioning. Assignment of auditory stimuli was counterbalanced with lever side and experimental group. On the day after the last Pavlovian session, lever-press performance was tested in extinction in a Pavlovian-instrumental transfer test.

As in a typical PIT paradigm, therefore, the procedure consisted of three phases: instrumental training, Pavlovian conditioning and a transfer test. The procedure adopted a well-established single-lever design (Dickinson et al., 2000; Hall et al., 2001; Holland and Gallagher, 2003) to elicit general transfer, in which performance on one instrumental lever press action was assessed during a CS, a CS_0 neutral stimulus, and in the absence of both stimuli.

Instrumental training

Training started with two sessions of magazine training with the outcome delivered on a variable time (VT)-60 schedule during which all mice were familiarised with the chamber environment and learned to retrieve pellets from the magazine. Then they were given 12 daily sessions of instrumental training, in which one lever (left or right) was presented and reinforced with grain pellets. Half of the animals in each group were trained on the left lever and half on the right lever. In the initial 3 days of training, reinforcement was delivered on a CRF schedule, that is, one lever-press lead to the immediate delivery of one pellet. Training sessions ended after 50 pellet deliveries or 60 min, whichever came first. When most mice earned all 50 pellets in a CRF session, they were shifted onto a RI schedule, where the interval between lever-press and reward delivery was random, with an average of 15 s. The training followed a serial progression of increasing interval schedules: three CRF, two RI15, two RI30, and five RI60 sessions. Lever-press and magazine entry events were recorded by the MEDPC program.

Pavlovian conditioning

After instrumental training, mice went on to receive daily Pavlovian conditioning sessions for a total of 6 days. In each of the first five sessions, there were eight trials of 2-min stimulus (CS), during which pellets were delivered on a random time 30 s schedule. CS trials were spaced with an inter-trial interval (ITI) that averaged 5 min, which included a fixed 2-min period before CS presentation (Pre-CS) serving as baseline. No pellets were given during ITI or baseline periods. On the 6th session, a neutral stimulus (CS_0) was introduced into the trial sequence. This stimulus was presented twice during the session and so designated as a neutral stimulus or CS_0. It also lasted for 2 min, but no pellets were delivered. Magazine entries during the stimuli and pre-stimuli periods were recorded.

Transfer test

Prior to the transfer test, all mice were given one instrumental reminder session where their actions were reinforced with 50 pellets on RI60 as in the last instrumental session before the Pavlovian phase. During the transfer test, their lever-press performance was assessed in extinction with 2-min CS and CS_0 stimuli presented periodically. The first 9 min of the test was free of stimuli, which was inserted to reduce baseline lever responding. Then four 2-min CS and four 2-min CS_0 were presented in a pseudorandom order, interlaced with fixed 5-min ITIs, including 2-min Pre-CS or Pre-CS_0 baseline periods. Stimuli were presented in an S1, S2, S1, S2, S2, S1, S1, S2 order. Lever responding and magazine entries were recorded throughout the session.

Stereotactic surgery

Mice underwent surgeries at 8–12 weeks of age. They were anaesthetised with 5% isoflurane gas in 100% oxygen (1 L/min) and placed onto the stereotactic frame (Kopf Instruments). Their anaesthetic state was maintained with continuous 0.5%–1.5% isoflurane gas provided by an anaesthetic vaporiser (Ohmeda Tec 5 Anaesthetic Vaporiser Isoflurane). First, the scalp was shaved and disinfected with betadine and 70% ethanol. Then local infiltrative bupivacaine (0.25%, 5 mg/kg) was applied before a small incision was made in the middle of the scalp. Next, a small burr hole was opened with a micromotor drill (Volvere i7), through which a thin glass pipette attached on a nanoliter injector (Nanoject II, Drummond Scientific) was lowered slowly to target coordinates. Last, NMDA was released into targets in 4.6 nl boluses, timed at a rate of approximately 2.3 nl/s. Upon completion of injections, the pipette remained in place for 8 min before removal to minimise track spread. After the surgery, carprofen (1 mg/ml, 5 mg/kg) was given subcutaneously for postoperative analgesia.

Coordinates relative to bregma used for injections were (in mm): anterodorsal BNST (AP +0.14, ML ±1.13, DV −4.20). Coordinates were determined based on a standard mouse brain atlas: The Allen Reference Atlas (Lein et al., 2007) was further adjusted based on the results of pilot surgery. 10 mg/ml NMDA (Sigma-Aldrich, St Louis, MO, USA) was used to create lesions in BNST. NMDA was freshly dissolved in sterile 0.9% normal saline before intracranial injection.

Tissue processing

Upon completion of the last training session, mice were removed from the chambers and anaesthetised with Lethabarb (300 mg/kg; i.p.). Next, they were transcardially perfused with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 4 min, brains extracted and post-fixed in the same solution at 4°C overnight. Over the following couple of days, brains were cut into 30-μm coronal sections with Vibratome (VT1000, Leica Microsystems) and stored at −20°C in cryoprotectant (30% ethylene glycol, 30% glycerol, and 0.1 M PB) until they were further processed for immunofluorescence.

Immunofluorescence

Sufficient sections were taken to cover the ovBNST and CeA regions and were processed to detect of pERK and PKC-δ. Free-floating sections were rinsed in Trisbuffered saline (TBS: 0.25 M Tris, 0.5 M NaCl, 0.1 mM NaF, pH 7.6) three times for 10 min each, followed by 5 min in TBS containing 3% H_2O_2 and 10% methanol. After immersed in blocking buffer (0.2% Triton X-100 and 10% normal horse serum in TBS) for 1 h, sections were probed with rabbit anti-phospho-p44/42 MAPK (ERK1/2; 1:1,000; Cell Signaling Technology) and mouse anti-PKC-δ (1:1,000; BD Biosciences) diluted in blocking buffer at 4°C overnight. Next, after three washes in TBS for 10 min each, sections were incubated in blocking buffer containing donkey anti-rabbit Alexa Fluor 546 (1:1,000, Invitrogen), donkey anti-mouse Alexa Fluor 647 IgG (1:1,000, Invitrogen), and Nissl Green (1:1,000, Invitrogen) at 4°C overnight. Then they were washed in TBS for three times, mounted on slides (microscope plain slides, Thermo Scientific) and coverslipped in medium (0.17 mm thickness, Thermo Scientific; Fluoromount-G, SouthernBiotech). For lesion verification in Experiment 3, rabbit anti-GFAP (1:1,000, Sapphire Bioscience) was used as the primary antibody and donkey anti-rabbit Alexa Fluor 488 (1:1,000, Invitrogen) as the secondary antibody. Rest of the procedures were the same as described above.

Imaging and cell quantification

Image stacks from both dorsal BNST and CeA were collected from all subjects using a sequential laser scanning confocal microscopy (Olympus FV1000, BX61WI microscope) with 10× (NA 0.40) or 20× objective (NA 0.75). Scan settings of the objective (pinhole size, pixel/μm, laser intensity, and gain) were adjusted following the same procedure for different batches of immunofluorescence and kept the same within the same batch. Donkey anti-rabbit Alexa Fluor 488 and Nissl Green were excited by laser at the wavelength of 473 nm; donkey anti-rabbit Alexa Fluor 546 was excited by 559 nm laser; donkey anti-mouse Alexa Fluor 647 was excited by 635 nm laser. Images in single-slices (10×) or stacks consisting of 2–4 consecutive slices (20×, step size 1.16 μm) were acquired at the dorsal BNST or CeA region respectively. Images taken from both hemispheres of each subject were included for visual inspection and cell quantification. All images were processed and

quantified with Open Source Fiji imageJ. Quantification of pERK immunoreactive neurons (pERK+) contained in a stack adhered to the same automatic processing algorithm that projected all cells in a stack onto a 2D image and minimally processed for counting. Size filter was set at 80 μm^2 for BNST and at 60 μm^2 for CeA. Results were represented as the number of cells per mm^2 in the ROI (ovBNST or CeL) within a slice of 1-μm thickness. Numbers of pERK+ neurons in stacks were first averaged within subjects, subject means were then analysed with statistical tests.

Statistical analyses

Behavioural and cell count data were analysed in Prism (version 7.0 and 9.0). For comparison of means between two groups, unpaired Student's t-test, was used. For comparison of means among groups, One-way ANOVA with Brown-Forsythe test of homogeneity of variances or Two-way ANOVA were used, and Tukey test or Sidak's multiple comparisons test were used for *post-hoc* multiple comparisons. Correlations between independent variables were tested with Pearson's correlation. $P < 0.05$ was considered as statistically significant in all analyses.

Results

Experiment 1

To search for evidence of the extended amygdala's involvement in instrumental motivation, we first looked at the expression of pERK in the BNST and CeA following instrumental training and compared the quantity of pERK labelled neurons between trained and yoked groups. The trained group successfully acquired the lever-press action (**Figure 1A**); lever presses per minute in the final training session was 24.9 ± 3.7 (Mean ± SEM), whereas the Yoked group did not learn the lever action (two-way ANOVA, group and session, group $F_{1,16} = 40.74$, $p < 0.0001$). Both groups exhibited comparable magazine entry rates during the final session (Trained: 8.7 ± 1.2, Yoked: 7.7 ± 1.5 entries/min, **Figure 1B**).

pERK expression was mostly restricted within ovBNST and ovBNST was clearly demarcated by PKC-δ expression. Only pERK+ neurons within ovBNST were quantified. The trained group had significantly higher pERK expression than the yoked group (**Figure 1C**), demonstrated in their respective 131.4 ± 11.61 and 88.5 ± 14.31 pERK+ neurons per mm^2 in ovBNST (unpaired t-test, two-tailed, $t = 2.287$, $df = 15$, $p = 0.037$). As for CeA, mean of pERK+ cells in CeA was 181.5 ± 15.84 in Trained and 144.9 ± 18.61 in Yoked (**Figure 1D**); however the difference was not significant (unpaired t-test, $t = 1.467$, $df = 11$, $p = 0.170$). Nevertheless, there was a significant correlation between the expression of pERK in the BNST and in the CeA (**Figure 1E**, Pearson's correlation, $R^2 = 0.6118$, $p = 0.0026$). Representative images of pERK, PKC-δ, and Nissl Green staining in the Trained and Yoked groups were shown in **Figure 1F**. In general, these data demonstrate that pERK activity was increased in the ovBNST by instrumental training and that this increase was over and above that induced by Pavlovian conditioning to any incidental stimuli or to the context or through exposure to the reward alone.

Experiment 2

To examine how BNST's activity changes with extended instrumental training, under rewarded vs. unrewarded conditions, we next compared pERK expression in BNST and CeA in mice given instrumental training (group Trained = group T), extended instrumental training (group Longer-Trained = group LT), and those with extended training plus a brief additional test during which the outcome was withheld (group Longer Trained on Test = group LTT). All three groups successfully learnt the lever-press action (**Figure 2A**). Press rate was transiently lower in LTT and LT vs. T groups on the 2nd RI60 session (two-way ANOVA, group and session, interaction $F_{16,120} = 1.789$, $p = 0.0401$), however all three groups showed comparable press rates on their final session of training: 29.7 ± 4.7 in T; 24.3 ± 4.6 in LT and 30.4 ± 3.7 in LTT (one-way ANOVA, $F < 1$). The groups showed similar rates of magazine entry across acquisition (**Figure 2B**).

Quantification suggested that the number of pERK+ neurons in the three groups differed.

The average pERK+ neurons in ovBNST was 98.8 ± 8.05 in group T, 82.4 ± 5.25 in group LT, and 134.4 ± 10.95 in group LTT (**Figure 2C**). The LTT group showed significantly higher pERK expression than the other two groups (one way ANOVA, $F = 8.041$, $p = 0.0042$; Tukey's test: LT vs. LTT difference = −51.94, $p = 0.0045$; T vs. LTT difference = −35.58, $p = 0.0486$). On the other hand, the number of pERK+ neurons in the CeL was not significantly different between the group T and LT, or between the group LT and LTT (one-way ANOVA, $F = 4.338$, $p = 0.0326$; Tukey's multiple comparisons, T vs. LTT, difference = 47.56, $p = 0.0275$), with a mean number of 149.3 ± 17.57 in the group T, 113.0 ± 8.58 in the group LT and 101.7 ± 9.16 in the group LTT (**Figure 2D**). Additionally, as in Experiment 1 we found a significant positive linear relationship between the number of pERK+ neurons in the CeA and pERK+ neurons in the ovBNST in Group T (Pearson's test, $R^2 = 0.9653$, $P = 0.0028$) but not in either Group LT or LTT, suggesting that any relationship between CeA and BNST declines with overtraining (**Figure 2E**). Representative images of pERK, PKC-δ staining in the ovBNST for each of the groups in Experiment 2 are

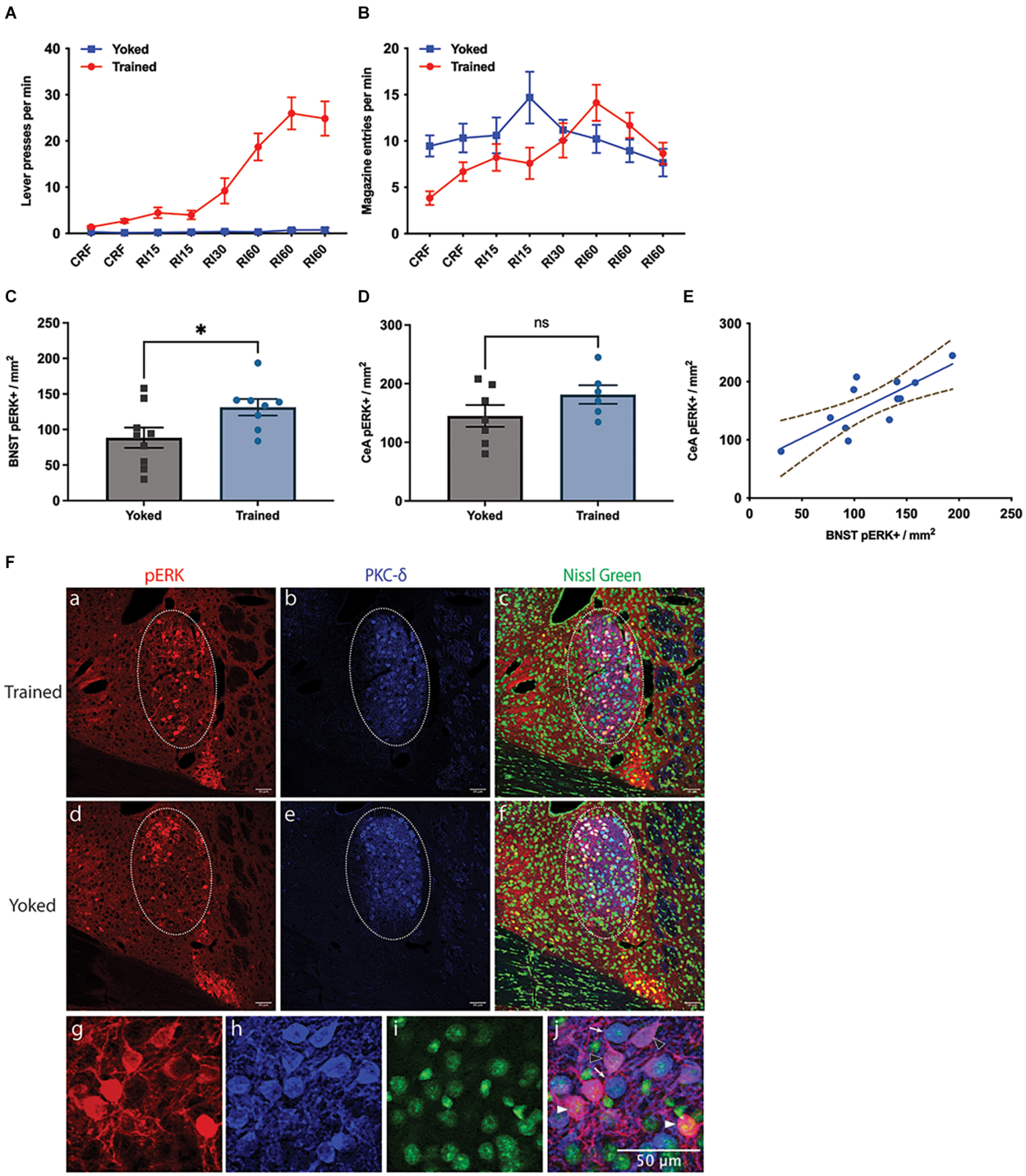

FIGURE 1
Effect of instrumental training on BNST activity. **(A,B)** Changes in instrumental lever press performance **(A)** and magazine entry **(B)** across the course of instrumental acquisition; **(C–F)** changes in pERK activity in the Oval BNST **(C)** and the CeA **(D)** induced by instrumental training relative to a yoked control; Trained showing significantly higher pERK labelling than Yoked (unpaired t-test, two-tailed, $t = 2.287$, $df = 15$, $P = 0.037$). **(E)** The relationship between pERK activity in the BNST and CeA; significant correlation found between the expression of pERK at ovBNST and at CeL, regardless of training (Pearson's correlation, $R^2 = 0.6118$, $P = 0.0026$); **(F)** representative changes in the labelling of pERK (a,d), PKC-d (b,e), Nissl Green (c,f), and colocalisation of pERK and PKC-d (c,f) in the BNST from Trained (top panel) and Yoked (middle panel) animals; PKC-d expression marking the area of ovBNST (b,c,e,f), encircled by dotted line (a–f); (g–j) three subpopulations identified at ovBNST based on the expression of pERK or PKC-d: PKC-d+ /pERK+ (magenta, arrowhead outline), PKC-d+ /pERK- (blue, open arrowhead), and PKC-d-/pERK+ (red, filled arrowhead). All lines and bars presented values as Mean ± SEM. Dotted lines **(E)** represented 95% confidence bands. $^*P < 0.05$. Scale bar: 50 µm, ns: number of subjects in a group.

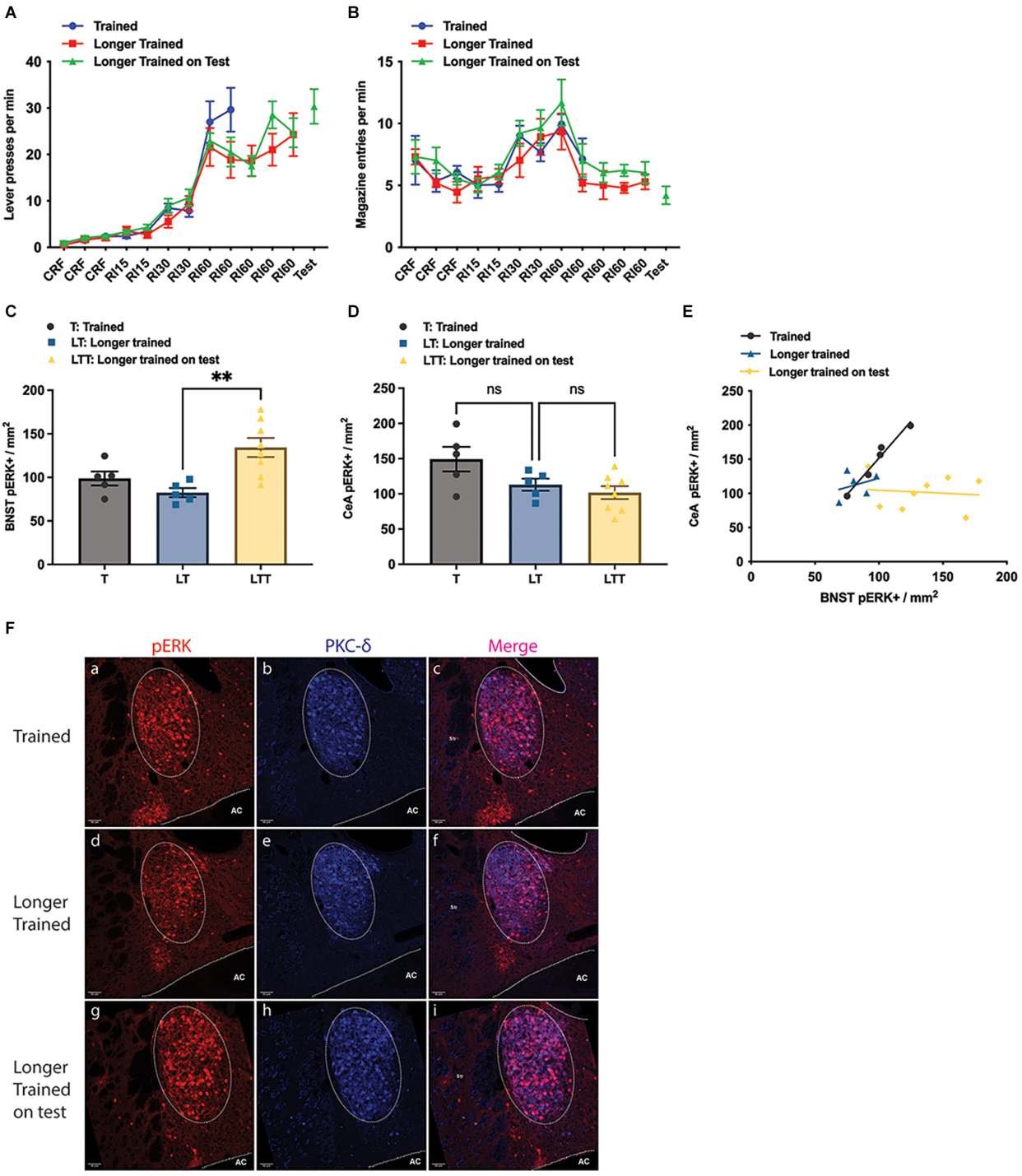

FIGURE 2
Effect of training and longer training, with and without a brief test in which the outcome was withheld. **(A)** Lever presses per minute across the training phase; **(B)** magazine entries per minute across training; **(C,D)** pERK activity in the Oval BNST **(C)** and in the CeA **(D)** as a consequence of instrumental training (T), longer instrumental training (LT) and longer instrumental training plus an unrewarded test (LTT); LTT exhibiting significantly higher pERK labelling than LT (One-way ANOVA, $F = 8.041$, $P = 0.0042$; *post-hoc* Tukey's test, LT vs. LTT difference −51.94, $P = 0.0046$). **(E)** The relationship between pERK activity in the BNST and CeA; significant correlation was only found in the Trained (Pearson's correlation, $R^2 = 0.9653$, $P = 0.0028$); **(F)** representative images taken from group T, LT, and LTT (top, middle, and bottom panel respectively) showing labelling of pERK (a,d,g), PKC-d (b,e,h), and colocalisation of pERK and PKC-d (c,f,i); PKC-d expression (b,c,e,f) marking the area of ovBNST, encircled by dotted line (a–f). All lines and bars presented values as Mean ± SEM. AC, anterior commissure. $^{**}P < 0.01$. Scale bar: 50 μm, ns: number of subjects in a group.

shown in Figure 2F. Generally, these data confirm that BNST was highly activated during instrumental performance but that this activity was greater during a test in which the outcome was anticipated but not delivered. This is consistent with the argument that the BNST is activated by the influence of incentive processes associated with the prediction of reward on instrumental performance.

Experiment 3

Lesion assessment

Representative images from the lesioned and sham groups and reconstruction of BNST lesions in the lesioned group are shown in Figures 3A,B. BNST lesions were confirmed by visual inspection using GFAP immunofluorescence on three coronal sections (bregma +0.245, +0.145, +0.020) in each subject. Three subjects from the Lesion and one from the Sham group were excluded from behavioural analyses due to either faint GFAP signals or to major spread of the signal into the striatum meaning nine and six mice remained in Lesion and Sham groups, respectively.

Behavioural results

Figures 3C–E present the data from the instrumental training and Pavlovian conditioning phases of this experiment. Both BNST Lesion and Sham groups showed rapid acquisition of lever-pressing over instrumental sessions (Two-way ANOVA, lesion and session, session $F_{9,117} = 31.83$, $p < 0.0001$, lesion $F_{1,13} = 0.2054$). Press rate on final RI60 training session was 16.9 ± 3.8 (Mean ± SEM) presses/min and 18.7 ± 2.8 presses/min in Lesion and Sham groups, respectively (Figure 3C). Corresponding magazine entry rates during the final session were 7.8 ± 1.4 and 4.2 ± 0.8 entries/min. Although entry rates appeared to be higher in the lesion vs. Sham group across sessions, this difference was not significant (Figure 3D, Two-way ANOVA, lesion and session, lesion $F_{1,13} = 3.502$, $p = 0.084$).

Again, during Pavlovian conditioning the Lesion Group showed a slightly higher entry rate during the Pre-CS period compared to the Sham Group (Two-way ANOVA, lesion and session, lesion $F_{1,13} = 5.076$, $p = 0.0422$), with an average of 1.89 ± 0.6 and 1.06 ± 0.35 entries/min (Figure 3E). During CS presentation, however, both groups entered the magazine at a similar rate across sessions (two-way ANOVA, lesion and session, lesion $F < 1$), with 7.9 ± 2.3 entries/min in Lesion and 7.7 ± 2.3 entries/min in Sham. Entry rate was significantly higher during the CS than the Pre-CS period, in both the Lesion and Sham groups (two-way ANOVA, session and CS presentation in Lesion, CS presentation $F_{1,16} = 12.18$, $p = 0.0030$; two-way ANOVA, session and CS presentation in Sham, CS presentation $F_{1,10} = 16.85$, $p = 0.0021$). As such, despite the slight increase in baseline magazine entries in the lesion group, there was no evidence that Pavlovian conditioned responding differed in the two groups.

Results of the PIT test are plotted in Figures 3F,G. During this test, the Lesion group had a lever-press rate of 5.3 ± 0.9 presses/min during CS and 3.6 ± 0.4 during the Pre-CS baseline, relative to Sham's 6.9 ± 2.0 during CS and 2.0 ± 0.5 during Pre-CS. Both groups had comparable lever-press rate during CS_0 (Lesion 3.6 ± 0.6, Sham 3.8 ± 1.2) or Pre-CS_0 (Lesion 3.8 ± 0.8, Sham 4.2 ± 0.8). Transfer was measured as the ratio of lever-presses during CSs to total lever presses during the CSs plus the preceding Pre-CS period (Figure 3F). The Lesion Group had response ratios of 0.57 ± 0.05 during CS and 0.50 ± 0.02 during CS_0, whereas the Sham group had ratios of 0.75 ± 0.05 during CS and 0.43 ± 0.08 during CS_0. Two-way ANOVA found that the CS, relative to CS_0, significantly elevated the response ratio (stimulus and lesion as two factors, stimulus $F_{1,13} = 18.87$, $p = 0.0008$), demonstrating successful generation of general PIT with the current experimental procedure. Importantly, a significant interaction between stimulus and lesion was found ($F_{1,13} = 7.868$, $p = 0.0149$). Furthermore, whereas the response ratio during CS did not differ from that during CS_0 in the Lesion Group (Sidak's multiple comparison test, $t = 1.217$, $df = 13$, adjusted $p = 0.4305$), it was significantly increased from that during CS_0 in the Sham Group ($t = 4.615$, $df = 13$, adjusted $p = 0.001$), suggesting transfer was impaired in Lesion while preserved in Sham. Comparable results were found when we subtracted the pre-CS baseline from responding during the CS and CS_0 (Figure 3G). Lever presses were increased during CS compared to CS_0 (two-way ANOVA, stimulus and lesion, stimulus: $F_{1,13} = 9.517$, $p = 0.0087$). A transfer effect was observed in the Sham Group (Sidak's multiple comparison test, $t = 2.960$, $df = 13$, adjusted $p = 0.0220$) but not the Lesion Group ($t = 1.252$, df = 13, $p = 0.411$).

This experiment assessed the functional effects of dBNST lesions on general Pavlovian-instrumental transfer. Although no effects of the lesion were found on baseline instrumental performance or on the influence of CS_0 on that performance, lesions of dBNST significantly reduced the excitatory effect of a CS on that performance and so significantly attenuated the general transfer effect. As anticipated by our presentation of the literature above, therefore, these results suggest that the BNST mediates the influence of incentive motivation on instrumental performance.

Discussion

This series of studies was developed based on a review of the literature on the function of the BNST in

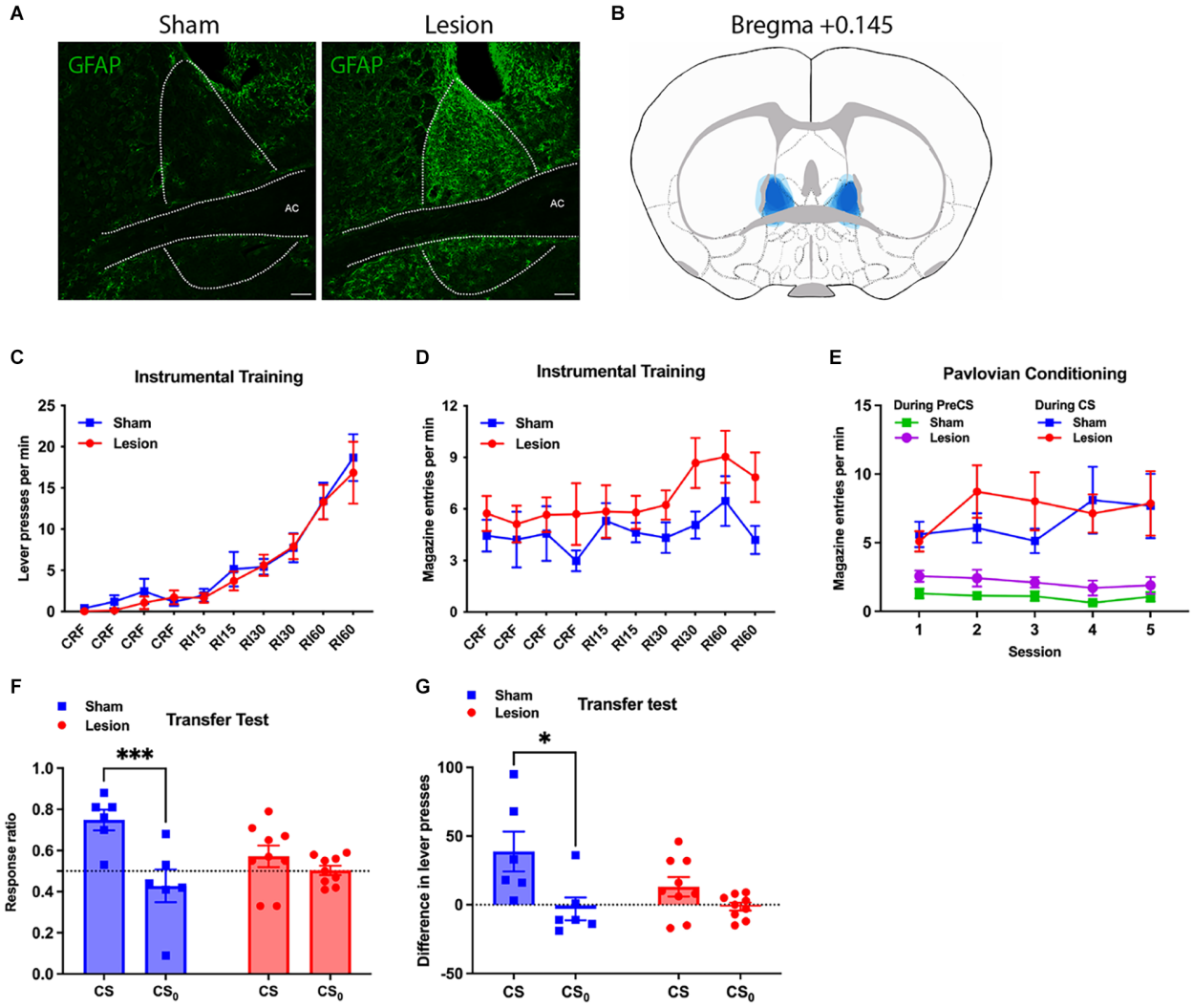

FIGURE 3
Effect of NMDA-induced lesion of BNST on general Pavlovian-instrumental transfer. **(A)** Representative images of the BNST showing GFAP activity in the Lesion and Yoked groups; **(B)** reconstruction of the lesion in BNST by overlapping lesion placement of all subjects in Lesion group (in blue); **(C,D)** performance during the instrumental training phase showing lever presses per minute **(C)** and magazine entries per minute **(D)** across sessions; **(E)** conditioned magazine entry responses performed during the Pavlovian conditioning sessions in the Lesion and Sham Groups showing pre-CS baseline performance and performance during the CS; **(F)** responding during the test of general Pavlovian-instrumental transfer showing the effects of BNST lesions on the elevation in response vigour during the CS and CS_0 relative to baseline using an elevation ratio: (responding during the CS)/(responding during CS+ responding during the baseline); transfer effect found impaired in Lesion (Two way ANOVA, CS × Lesion interaction, $F_{1,13} = 7.868$, $P = 0.0149$); **(G)** shows the same data during the transfer test except using a straight subtraction of CS—baseline responding; transfer effect observed only among the Sham (Sidak's multiple comparison test, $t = 2.960$, $df = 13$, adjusted $P = 0.0220$). All lines and bars presented values as Mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$. AC, anterior commissure. Scale bar: 100 μm.

incentive motivation. Current evidence suggests that the BNST plays a significant role in the way Pavlovian cues alter the vigour of instrumental actions. To assess this we examined three questions: (i) what impact does instrumental training vs. yoked exposure to the instrumental outcome have on activity in the BNST? (ii) are any changes in BNST activity increased by longer training or are they merely related to the degree of outcome anticipation? and (iii) is the influence of Pavlovian cues on instrumental performance sensitive to lesion-induced damage to the BNST?

Experiment 1 found increased pERK+ cells in the ovBNST in instrumentally trained compared to yoked controls. A straightforward interpretation of this finding is that this ovBNST activity reflects added processes in the trained relative to the yoked condition. Trained mice differed from yoked mice in processes related to instrumental learning, which includes but is not limited to initiation and execution of

the action, and evaluation of the outcome. Given the BNST's broad involvement in motivated behaviour and our previous conclusion regarding the role of the BNST in the influence of conditioned motivation on instrumental actions, this result suggests that ovBNST's activity likely indicates the motivational control of instrumental action. A number of studies link the dorsal division of BNST to the modulation of instrumental vigour. For example, Dumont et al. (2005) found an elevated NMDAR/AMPAR ratio in dorsal BNST following instrumental learning for cocaine reward. Importantly, the NMDAR/AMPAR ratio, which reflects neuroplasticity, positively correlated with instrumental vigour for cocaine reward. This report suggests that dorsal BNST could be an important locus that psychostimulants modify to generate heightened or sensitised responding. Also, because pERK expression follows activation of NMDARs, as seen in striatum, increased pERK expression in ovBNST among instrumentally trained animals, as observed in our experiment, was likely a product of a similar process of NMDAR upregulation. It is worth noting, however, that Dumont et al. (2005) failed to observe a change in NMDAR/AMPAR ratio in subjects who were trained to press for a natural reward of sucrose. There are few reports of BNST's involvement in instrumental conditioning, which is in stark contrast to the bulk of the literature which focusses on its role in the effects of stress or drugs of abuse on various reward-seeking behaviours. This discrepancy raises the possibility that the BNST is especially vulnerable to influences from neuromodulators or psychoactive agents. Overall, ovBNST's activity in instrumental learning, as indexed by increased pERK expression, can be reasonably interpreted as evidence of BNST's role in instrumental motivation.

Next, in Experiment 2, ovBNST showed a higher degree of pERK activity after instrumental performance had been tested in the training context when reward was anticipated but withheld (Group LTT) than when reward was actually delivered during training (Group LT). The final press rates in the T vs. LT vs. LTT Groups did not differ significantly in this experiment, suggesting that the increased proportion of pERK in ovBNST with reward withheld had little to do with instrumental vigour. Furthermore, pERK expression was, if anything, slightly reduced in mice in the LT Group (i.e., 5× RI60 sessions vs. 2× RI60 sessions) and so changes induced by training itself or extended access to reward appear to have had little impact in themselves. Instead, and particularly given the brevity of the extinction test, it seems likely that it was the prediction of reward *in the absence of its delivery* that provoked the considerable increase in pERK activity in the LTT Group. Nevertheless, it is unclear precisely what role the absence of reward played in this finding: i.e., whether withholding reward enhanced its anticipation or increased the saliency of reward predictors by increasing ambiguity or uncertainty, something that has recently been linked to BNST in aversive situations (Figel et al., 2019; Goode et al., 2019; Naaz et al., 2019).

Given this finding and from the perspective of our analysis of the literature on the extended amygdala, particularly BNST's highly interconnected and mirrored relationship with CeA, we hypothesised that BNST plays a similar role as CeA in general transfer. It is well established that pre-training lesions of CeA abolish general transfer. Therefore, pre-training lesions of BNST were predicted to disrupt general transfer and, indeed, we found just this effect. Although the lesion was aimed at dorsal BNST, and the majority of the damage was localised there, there was some invasion of ventral BNST and so the precise source of the effect remains unclear. Nevertheless, this result adds weight to the view that the two structures are functionally linked and increases the likelihood that BNST relays CeA's influence on general transfer. Indeed, CeA participates in the encoding of the CS's motivational properties and is essential for the acquisition of CS-directed conditioned approach (sign-tracking CR; Cardinal et al., 2002). Furthermore, as discussed above, the motivational properties attributed to the CS are likely to constitute the invigorating power supporting general transfer and, if CeA lesions undermine general transfer by preventing the establishment of this CS-elicited motivation, then our result suggests that the effects of BNST lesions may have also been mediated by the CS's acquisition of motivational properties.

Aside from the CeA, the NAc core has been recognised as a key correlate for the expression of general transfer. In the same manner as CeA and NAc core (Hall et al., 2001), we found that pre-training lesions of BNST did not significantly affect Pavlovian conditioning or instrumental acquisition but attenuated general transfer. The BNST's remarkable similarity to the CeA and NAc core in terms of selective involvement in general transfer encourages the view that the BNST belongs to a functional circuit that includes CeA and NAc core to modulate the general transfer effect. Importantly, there is no evidence in any of these studies to conclude that the BNSTs effects or those of any of its affiliated structures are involved in Pavlovian conditioning *per se*. Rather it appears that this circuit mediates a specific aspect of appetitive motivation; the arousal generated by Pavlovian predictors. Thus, conditioned responding during Pavlovian conditioning was unaffected by BNST lesions whereas, in contrast, the influence of that conditioning on instrumental performance was strongly attenuated.

On the other hand, in instrumental training, the press rate of subjects with BNST lesions was numerically—if not significantly—lower than that of sham controls, as has been previously reported with NAc core lesions (Hall et al., 2001). It has been proposed that the minor reduction in instrumental responding seen in animals with NAc core lesions results from impaired context conditioning (Balleine and Killcross, 1994; Aberman and Salamone, 1999). Since there is considerable evidence showing the BNST plays an important role in appetitive context conditioning, it is likely that BNST lesions mildly affect instrumental vigour in the same way and for the same reason as those of the NAc core.

Our result positions BNST in the encoding of CS's motivational properties, and such a role is likely to be amplified by BNST's descending connections with VTA. BNST both sends and receives robust projections to and from VTA GABA and DA neurons, which enable BNST to exert a direct influence over DA release (Melchior et al., 2021; Yu et al., 2021). VTA-projecting BNST neurons are overwhelmingly GABAergic and these neurons preferentially synapse onto VTA GABA neurons. About 70% of VTA GABA neurons are responsive to stimulation of GABAergic terminals from BNST and optogenetic stimulation of BNST GABAergic inputs to the VTA is rewarding and anxiolytic, effects similar to those resulting from optogenetic inhibition of VTA GABA neurons (Jennings et al., 2013a). Therefore, activation of BNST projection neurons to VTA likely disinhibits VTA DA neurons leading to increased DA activity in its targets including NAc core.

Implications

Our results provide the first evidence to our knowledge of BNST's contribution to general transfer and encourage positioning BNST within the theoretical circuit mediating transfer. In particular, the results are in line with our argument that the CeA mediates general transfer through its connections with BNST, implying a place for the extended amygdala in the acquisition of the motivational properties of a conditioned stimulus. Future research is needed to flesh out the BNST's role in general transfer and shed light on the neural mechanisms underlying its influence. As BNST takes part in a wide array of motivated behaviours, understanding its role in the neural bases of conditioned motivation will have broad implications in elucidating the pathogenesis of the dysfunctional responding commonly seen in psychological disorders, and will be fruitful in developing strategies to restore normal motivational control.

For example, as noted previously, general transfer is thought to underlie maladaptive behavioural responding in various psychiatric conditions, such as stress and anxiety (Pool et al., 2015; Quail et al., 2017), drug addiction (Belin et al., 2009; Hogarth et al., 2013; Ostlund et al., 2014), alcohol use disorder (Corbit and Janak, 2007; Garbusow et al., 2016), and bipolar disorder (Hallquist et al., 2018). Given our conclusion that BNST mediates general transfer and possibly regulates instrumental motivation, there should be evidence indicating that the BNST plays a role in these same conditions. And, indeed, there are reports that the BNST plays a crucial role not only in the regulation of anxiety (Tye et al., 2011; Yassa et al., 2012; Grupe and Nitschke, 2013; Jennings et al., 2013a; Kim et al., 2013), drug-seeking behaviours (Avery et al., 2016; Daniel and Rainnie, 2016; Gungor and Pare, 2016; Mantsch et al., 2016), but also in binge-drinking (Pleil et al., 2015; Rinker et al., 2017), binge-eating (Jennings et al., 2013b; Micioni Di Bonaventura et al., 2014), anorexia (Sweeney and Yang, 2015), excessive water drinking-related compulsive behaviours (van Kuyck et al., 2008; Wu et al., 2016), and OCD (Kohl et al., 2016; Luyten et al., 2016; Raymaekers et al., 2017). Many of these conditions arguably share a basis in maladaptive instrumental responding. A deeper understanding of the BNST's role in instrumental processes is therefore of the highest importance and may prove fruitful in elucidating the pathological mechanisms underlying these conditions.

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 Sydney Animal Ethics Committee.

Author contributions

MG conducted the research and wrote the manuscript. BB wrote the manuscript and supervised the research project. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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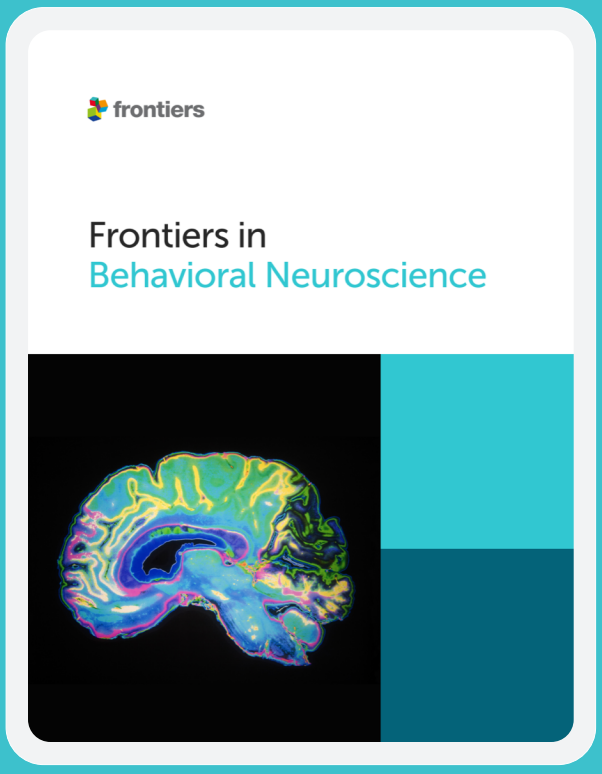

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