Distinct effects of ventral tegmental area NMDA and acetylcholine receptor blockade on conditioned reinforcement produced by food-associated cues.

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1 **Abstract**

2 Stimuli paired with rewards acquire reinforcing properties to promote reward-seeking behavior.
3 Previous work supports the role of ventral tegmental area (VTA) nicotinic acetylcholine
4 receptors (nAChRs) in mediating conditioned reinforcement elicited by drug-associated cues.
5 However, it is not known whether these cholinergic mechanisms are specific to drug-associated
6 cues or whether VTA cholinergic mechanisms also underlie the ability of cues paired with
7 natural rewards to act as conditioned reinforcers. Burst firing of VTA dopamine (DA) neurons
8 and the subsequent phasic DA release in the nucleus accumbens (NAc) plays an important role in
9 cue-mediated behavior and in the ability of cues to acquire reinforcing properties. In the VTA,
10 both AChRs and NMDA receptors (NMDARs) regulate DA burst firing and phasic DA release.
11 Here, we tested the role of VTA nAChRs, muscarinic AChRs (mAChRs), and NMDARs in the
12 conditioned reinforcement elicited by a food-associated, natural reward cue. Subjects received 10
13 consecutive days of Pavlovian conditioning training where lever extension served as a predictive
14 cue for food availability. On day 11, rats received bilateral VTA infusion of saline, AP-5 (0.1 or
15 1 μ g), mecamylamine (MEC: 3 or 30 μ g) or scopolamine (SCOP: 3 or 66.7 μ g) immediately
16 prior to the conditioned reinforcement test. During the test, nosepoking into the active
17 (conditioned reinforced, CR) noseport produced a lever cue while nosepoking on the inactive
18 (non-conditioned reinforced, NCR) noseport had no consequence. AP-5 robustly attenuated
19 conditioned reinforcement and blocked discrimination between CR and NCR noseports at the 1
20 μ g dose. MEC infusion decreased responding for both CR and NCR while 66.7 μ g SCOP
21 disrupted the subject's ability to discriminate between CR and NCR. Together, our data suggest
22 that VTA NMDARs and mAChRs, but not nAChRs, play a role in the ability of natural reward-
23 associated cues to act as conditioned reinforcers.

Keywords: conditioned reinforcement, ventral tegmental area, nicotinic receptor, muscarinic receptor, NMDA receptor, incentive salience

1. Introduction

The ability to make informed decisions about food availability is critical for an animal's survival and ultimate reproductive fitness. When an environmental stimulus is paired with food availability, animals learn to utilize this conditioned stimulus (CS) to guide their ongoing behavior. Conditioned stimuli have been demonstrated to powerfully promote both food-craving and food-seeking (Nicola et al., 2005, Ball et al., 2011, Grimm et al., 2011, Guy et al., 2011, Jastreboff et al., 2013) and drug-craving and drug-seeking (Sinha et al., 2000, Caggiula et al., 2001, Fuchs et al., 2006, Bossert et al., 2007, Liechti et al., 2007, Chaudhri et al., 2010) in both humans and in preclinical rat models. During conditioned reinforcement, subjects readily learn to perform a new operant action in order to receive a CS, which serves as a conditioned reinforcer (CR), in the absence of primary reward (Wolterink et al., 1993, Parkinson et al., 1999). It has also been proposed that reward-associated stimuli can acquire "incentive salience," where the cues themselves become wanted or desired (Robinson and Berridge, 1993), and these incentive properties may further facilitate the ability of these cues to act as reinforcers. Thus, understanding the neurobiological mechanisms of conditioned reinforcement can provide important insights into the underlying processes that allow cues to powerfully guide motivated behavior.

Dopaminergic activity in the ventral tegmental area (VTA) to nucleus accumbens (NAc) pathway has been shown to play an important role in several cue-related behaviors (Wolterink et

al., 1993, Schultz, 1998, Nicola et al., 2005, Bossert et al., 2007, Saunders and Robinson, 2012). The presentation of reward-associated cues leads to increased burst firing of dopamine (DA) neurons in the VTA (Schultz, 1998, Pan et al., 2005) and increased phasic DA release in the NAc (Phillips et al., 2003, Day et al., 2007, Sombers et al., 2009). Previous work has also demonstrated that DA release in the NAc is both necessary and sufficient for cue-mediated reward-seeking behavior (Nicola et al., 2005). Specifically, the concentration of cue-evoked phasic DA release is proportional to the ability of these cues to predict reward (Sugam et al., 2012) and also proportional to the magnitude of the predicted reward (Beyene et al., 2010). Dopaminergic activity in the NAc is also a critical mechanism underlying the ability of drugs of abuse to enhance conditioned reinforcement for natural reward-associated cues (Taylor and Robbins, 1984, Wolterink et al., 1993, Parkinson et al., 1999). Given the evidence that cue presentation is encoded by burst firing activity of VTA DA neurons and downstream phasic DA release in the NAc (Schultz, 1998, Phillips et al., 2003, Pan et al., 2005, Day et al., 2007, Sombers et al., 2009), it is likely that VTA mechanisms are also important for regulating the ability of cues to act as reinforcers. Indeed, recent studies have revealed VTA mechanisms involved in cue-induced drug-seeking behavior (Zhou et al., 2007, Lu et al., 2009, Solecki et al., 2013). However, there is limited understanding of the VTA mechanisms that underlie the ability of reward-associated cues to act as conditioned reinforcers.

In the VTA to NAc circuit, DA burst firing and phasic DA release is mediated by NMDA receptor (NMDAR) and acetylcholine receptor (AChR) mechanisms (Forster and Blaha, 2000, 2003, Grace et al., 2007, Sombers et al., 2009, Wickham et al., 2013) and recent evidence points towards a role for VTA NMDARs and AChRs in cue-dependent behavior. In particular, the mesopontine tegmentum (MPT), which contains the laterodorsal tegmentum (LDTg) and

pedunculopontine tegmentum (PPTg), sends both cholinergic and glutamatergic projections to the VTA (Clements et al., 1991, Honda and Semba, 1995, Oakman et al., 1995, Takakusaki et al., 1996). PPTg inactivation has been shown to impair stimulus-reward learning, conditioned reinforcement (Inglis et al., 2000) and also impairs the ability of VTA dopamine neurons to burst fire in the presence of reward-predictive cues (Pan et al., 2005). Within the VTA, NMDARs have been shown to regulate the magnitude of phasic DA release to reward-associated cues (Sombers et al., 2009, Zweifel et al., 2009), and to alter cue-dependent reward learning (Zweifel et al., 2009). However, it is unclear whether VTA NMDARs play a role in the ability of cues to act as conditioned reinforcers. Importantly, a previous study demonstrated an important role for VTA AChRs in conditioned reinforcement for drug cues, as antagonism of VTA nAChRs decreased conditioned reinforcement for ethanol-associated cues (Lof et al., 2007). However, no studies have determined whether similar mechanisms also underlie the ability of natural reward-associated cues to act as conditioned reinforcers or whether AChR mechanisms are specific to drug-associated cues. Here, we tested the hypothesis that VTA AChR and NMDAR mechanisms mediate the ability of a natural reward-associated cue to act as a reinforcer.

2. Experimental Procedures

2.1 Subjects

Male Sprague Dawley rats (250-350 g) were acquired from Charles River Laboratories (Wilmington, MA, USA) and were placed on *ad libitum* food and water. Animals were housed 2 to 3 per cage on a 12 hour light/dark cycle (lights on at 7 am) and were allowed 1 week to acclimate to the facility prior to any surgical procedures. Animals were handled every other day

during acclimation and every day after surgery. After surgery, all animals were fed *ad libitum* and were allowed to recover for 1 week prior to Pavlovian conditioning training. During training and subsequent testing for conditioned reinforcement, animals' body weights were maintained at 100% of their pre-surgical weight (usually between 300 and 320 grams). After each session, rats were immediately given food (~15 grams), which was consumed within 1 hour. Thus, animals were trained and tested in a non-sated state, consistent with previously published methodology (Robinson and Flagel, 2009). All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Yale University Institutional Animal Care and Use Committee.

2.2 Drugs

Mecamylamine (3 or 30 μ g, MP Biomedicals, Solon, OH, USA), AP-5 (0.1 or 1 μ g, Sigma Aldrich, St. Louis, MO, USA), or scopolamine (3 or 66.7 μ g, Sigma Aldrich, St. Louis, MO, USA) were dissolved into 0.9% saline and infused into the VTA in a 0.5 μ L volume at a rate of 0.5 μ L/min using a Hamilton 25 gauge syringe. After infusion, the internal cannula was left in place for one additional minute to allow adequate absorption of the drug. The doses for all experiments were calculated based on previous work from our laboratory and others' demonstrating the ability of VTA administration of these doses to modulate reward-related behavior and to modulate phasic DA release in the NAc (Yeomans and Baptista, 1997, Sombers et al., 2009, Solecki et al., 2013, Wickham et al., 2013). Importantly, we have previously determined that VTA administration of these drugs at these doses does not alter locomotor activity (Solecki et al., 2013). Animals were tested for conditioned reinforcement immediately

after local drug micro-infusion. The 0.5 μ L infusion volume was selected based on our previous histological verification, where we observed that infusion of 0.5 μ L Chicago Blue Dye led to staining that was restricted to the VTA (Solecki et al., 2013).

2.3 Surgery

Rats were anesthetized with ketamine HCl (100 mg/kg, i.m., Sigma Aldrich, USA) and xylazine (10 mg/kg, i.m., Sigma Aldrich, USA) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for cranial implantation of cannula. All coordinates were obtained from the rat brain atlas (Paxinos and Watson, 2007) with anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) positions referenced from Bregma. A bilateral cannula (26 gauge) spaced 1 mm apart (Plastics One, Roanoke, VA, USA) was placed into the VTA (AP -5.2 mm, ML -0.5 to +0.5 mm, DV 7 mm below dura) and secured using screws (Gexpro, High Point, NC) and dental cement (Dentsply, Milford, DE, USA). Animals were allowed one week to recover prior to behavior training. During infusion, a bilateral 33 gauge internal cannula was inserted into the bilateral guide cannula and extended 1 mm past the tip of the guide cannula to infuse drug into the VTA (DV -8.0).

2.4 General Procedures

2.4.1. Magazine Training

Eight MED Associates test chambers (21.6 x 17.8 cm floor area, 12.7 cm high; MED Associates, St. Albans, VT) were used for magazine training, where each rat received 2 sessions (1 h each), on 2 consecutive days, where 20 banana flavored sucrose pellets were delivered into a food receptacle (located in the center of a 21.6 cm-wide wall) on a variable interval-90 (VI-90) schedule, where a food pellet was administered into the food receptacle (with replacement) on a variable schedule, that randomly used intervals of 30, 45, 60, 75, 90, 105, 120, 135, or 150, with an average interval of 90 s over the entire session. After these two sessions, all animals learned to retrieve food from the food receptacle.

2.4.2. *Pavlovian Conditioning*

After magazine training, the same chambers were used for Pavlovian training. The chambers were equipped with a retractable levers (MED Associates, St. Albans, VT) located 2.5 cm to the left or right of the central food receptacle, 3 cm above the floor. Animals received 25 lever-pellet pairings during each training session (approximately 45 minutes) and were trained for 10 consecutive days. On a VI-75 schedule (30, 45, 60, 75, 90, 105, or 120 second intervals), a lever was extended for 8 s and then retracted during simultaneous delivery of a food pellet. Thus, lever presentation was predictive of pellet delivery that was delivered in a non-contingent manner, independent of lever pressing activity. In a separate, non-paired, group of animals, the lever presentation occurred randomly throughout the training session and did not predict food availability. In this non-paired group, the pellet was administered on a separate VI-75 schedule that minimized pellet delivery during the lever presentation. For all animals, head entries into the food receptacle were monitored by infrared photobeam breaks across the receptacle during both

the cue and inter-trial interval. Additionally, lever presses were monitored during the cue presentation. Previous studies have shown that rats exhibit different approach strategies, either approaching the cue (sign tracking) or approaching the food receptacle during the cue (goal tracking). Furthermore, the evidence suggests that sign and goal tracking approach strategies are mediated by different underlying neurochemical mechanisms (Flagel et al., 2011, Saunders and Robinson, 2012) and produce different levels of conditioned reinforcement (Flagel et al., 2007, Flagel et al., 2011). In the current work, to account for the potential influence of differential approach strategy on conditioned reinforcement, animals were grouped as either "sign" trackers, "goal" trackers, or "intermediate" based on the Pavlovian Conditioning Approach (PCA) score (Meyer et al., 2012). This approach score has previously been shown to be highly predictive of conditioned reinforcement and effectively quantifies the degree of "sign" and "goal" tracking behavior (Meyer et al., 2012). The following equations were used to calculate the PCA score:

(1) *Response Bias: (Lever Presses - Cue Head Entries) / (Lever Presses + Cue Head Entries).*

The response bias score is an index of the preference an animal has for making a lever press or a head entry.

(2) *Latency Score: (Cue Head Entry Latency - Lever Press Latency) / 8*

The latency score is an index of how fast an animal makes a response for a lever press and a head entry. Thus, it indexes which response occurs first.

(3) *Probability Difference: p/Lever Press - p/Cue Head Entry*

The probability difference is an index of the likelihood that an animal makes a lever press or a head entry during the cue.

$$(4) \quad PCA = (Response\ Bias) + (Latency\ Score) + (Probability\ Difference) / 3.$$

Scores of -1 to -0.5 identified an animal as a goal tracker, scores of -0.5 to +0.5 identified an animal as an intermediate, and scores from +0.5 to +1 identified an animal as a sign tracker.

2.4.3 Conditioned reinforcement

24 h after the last Pavlovian conditioning session, each rat underwent a conditioned reinforcement test (1 h). For this test, the food receptacle was removed and the lever was placed in the center of wall, above the food receptacle. Additionally, two noseports (MED Associates, St. Albans, VT) were placed on left and right side of the lever. Nosepokes into one of the active noseports produced the presentation of the CS (extension of the lever for 2-4 seconds) as a measure of conditioned reinforcement (CR) while nosepokes into the inactive noseport had no programmed outcome, here termed no conditioned reinforcement (NCR).

2.5 Histological verification

Brains were fixed in 4% formalin for 3 days then allowed to wash in 30% sucrose solution for 1 week prior to slicing using a cryostat (Leica Microsystems, Buffalo Grove, IL, USA). All sections were 30 μ m and animals that had cannula outside of the VTA were excluded from analysis (**Fig. 1A**). In order to visually examine the regional specificity of our infusion method, we infused 0.5 μ L of Chicago Blue dye (0.5 μ L) into the VTA of a rat that had

1 previously received drug infusion (**Fig. 1B**). The dye was infused in a manner identical to
2 previous drug infusion. Immediately after dye infusion, the rat was sacrificed with a lethal
3 injection of pentobarbital (150 mg/kg, i.p.) and the brain was fixed in 4% formalin, washed in
4 30% sucrose and sliced on a cryostat.

6 2.6 Data Analysis

7 Data collected from the conditioned reinforcement experiments were analyzed using a
8 two-way ANOVA (with noseport and drug as factors) with repeated measures due to within
9 subject sampling of CR and NCR, unless otherwise stated. Bonferroni post-hoc analysis was
10 performed for additional statistical comparisons. In order to maintain a homogeneity of variance
11 and meet the requirements of ANOVA analysis, the conditioned reinforcement data was square
12 root transformed, as performed and described previously by others (Lof et al., 2007). Paired t-
13 tests were performed for comparing CR versus NCR within specific groups. Data collected from
14 the Pavlovian conditioning phase was analyzed using a two-way ANOVA (with group and time
15 as factors) with repeated measures over time. Post-hoc Tukey's multiple comparison tests were
16 used to compare effects between groups.

18 2.7 Determining treatment cohorts

19 Animals were selected for different pharmacological manipulations based on PCA score,
20 such that the average PCA score was similar for each drug group. Therefore, any differences
21 observed in CR were attributed to VTA pharmacological manipulations and not a result of

1 difference in Pavlovian training. To verify and ensure similar CR levels across all experimental
2 groups (in experiments that were performed over a 1 year period), each experimental cohort of
3 animals included at least 3-4 control rats received that received VTA saline microinfusion.
4 Together, there were seven treatment groups: SAL (n = 19), 0.1 μ g AP-5 (n = 12), 1 μ g AP-5 (n
5 = 8), 3.0 μ g MEC (n = 11), 30 μ g MEC (n = 13), 3.0 μ g SCOP (n = 11), and 66.7 μ g SCOP (n =
6 12). In order to demonstrate that paired presentation of the cue with the lever was required for
7 conditioned reinforcement, an initial experiment was performed in non-VTA cannulated animals
8 (n=23). Moreover, this experiment allowed for a comparison of conditioned reinforcement in
9 non-VTA cannulated versus VTA cannulated rats.

11 **3. Results**

12 **3.1 Conditioned reinforcement test**

13 Non-VTA cannulated animals which received paired presentations during Pavlovian
14 training, where the cue (lever) preceded sucrose pellet delivery, were compared to non-paired
15 animals who received random cue presentations, where cues were not predictive of sucrose pellet
16 delivery (See Materials and Methods). Analysis across all animals revealed a main effect of both
17 noseport ($F(1,21) = 18.98$, $p < 0.0001$, **Fig. 2**) and group ($F(1,21) = 21.56$, $p < 0.001$, **Fig. 2**).
18 Paired animals had more CR (active) responses compared to non-paired animals (**Fig. 2**,
19 noseport $\times$ group $F(1,21) = 3.93$, $p < 0.05$, post-hoc test $p < 0.001$) with no difference in NCR
20 responses between paired and non-paired subjects ($p > 0.05$). Rats from the paired group
21 displayed higher CR in comparison to NCR (**Fig. 2**, $p < 0.001$). In contrast, rats from the non-
22 paired group did not differ between CR and NCR responding ($p > 0.05$). This data demonstrates

that predictive cues, as opposed to non-predictive cues, supported the development of robust conditioned reinforcement.

3.2 VTA NMDAR blockade during the conditioned reinforcement test

VTA NMDARs mediate specific cue-mediated behaviors (Sombers et al., 2009, Zweifel et al., 2009), thus we tested the hypothesis that acute VTA NMDAR blockade on test day would decrease conditioned reinforcement. Blockade of NMDARs with AP-5 decreased conditioned reinforcement (**Fig. 3**, treatment $\times$ noseport interaction $F(2,36) = 5.31$, $p < 0.01$; treatment $F(2,36) = 3.347$; $p < 0.05$; noseport $F(1,36) = 46.48$ $p < 0.0001$). Additionally, there were no differences in NCR between the treatment groups ($p > 0.05$ for 1.0 μg AP-5 vs. SAL, $p > 0.05$ for 0.1 μg AP-5). Subsequent post-hoc analysis revealed that the SAL and 0.1 μg AP-5 animals were able to discriminate between CR and NCR (**Fig. 3**, SAL; $t(18) = 7.765$; $p < 0.0001$, 0.1 μg AP-5: $t(11) = 5.354$; $p < 0.001$). However, 1 μg AP-5 subjects did not discriminate between CR and NCR (**Fig. 3**, $t(7) = 1.596$; $p = 0.15$). Thus, AP-5 infusion into VTA attenuated conditioned reinforcement across doses, and at the highest dose blocked conditioned reinforcement, as reflected by a lack of discrimination between CR and NCR.

3.3 VTA AChR blockade during the conditioned reinforcement test

Like NMDARs, VTA AChRs have been shown to alter cue-mediated behavior (Lof et al., 2007) and phasic DA release in the NAc (Forster and Blaha, 2000, Wickham et al., 2013) which plays an important role in specific cue-mediated behaviors (Flagel et al., 2011, Saunders and

Robinson, 2012). Here we tested the hypothesis that VTA nAChR inhibition would decrease conditioned reinforcement, similar to what we observed with NMDAR inhibition. VTA infusion of MEC non-specifically decreased responding, irrespective of CR and NCR responding (**Fig. 4**, main effect of treatment $F(2,40) = 3.584$; $p < 0.05$, noseport: $F(1,40) = 79.77$; $p < 0.001$), with no interaction between treatment and noseport (treatment $\times$ noseport interaction: $F(2,40) = 1.662$; $p = 0.2$). In addition, both MEC groups (3 or 30 μ g) showed higher CR than NCR responses (**Fig. 4**; $t(10) = 4.982$; $p < 0.001$ for 3 μ g MEC; $t(12) = 3$; $p < 0.01$ for 30 μ g MEC), indicating that intra-VTA MEC infusion did not disrupt the ability of the subjects' to discriminate between CR and NCR.

In contrast to the non-specific effects of MEC, blockade of VTA mAChRs disrupted conditioned reinforcement (**Fig. 5**, treatment $\times$ noseport interaction, ($F(2,40) = 4.884$; $p < 0.05$; noseport $F(1,40) = 63.49$, $p < 0.0001$; treatment $F(2,40) = 4.94$, $p < 0.05$). Administration of 3 μ g SCOP into the VTA had no effect on CR or NCR noseport responding (**Fig. 5**, $p > 0.05$ for SAL vs. 3 μ g SCOP CR; $p > 0.05$ for SAL vs. 3 μ g SCOP NCR). Interestingly, post-hoc analysis revealed that intra-VTA infusion of 66.7 μ g SCOP led to significant increase in NCR (**Fig. 5**, $t(10) = 4.921$; $p < 0.01$ vs SAL) with no difference in CR (**Fig. 5**, $p > 0.05$ vs SAL), and a lack of discrimination between CR and NCR (**Fig. 5**, $t(11) = 2.130$; $p > 0.05$). Thus, the data revealed that intra-VTA infusion of high dose of the mAChR antagonist, SCOP, disrupts conditioned reinforcement by elevating NCR responding.

3.4 Examination of sign vs. goal tracking approach strategy on conditioned reinforcement

To examine whether approach strategy was correlated with the degree of conditioned reinforcement, we determined whether subjects that had been tested for CR showed previous differences in approach behavior, as indexed by PCA score (Materials and Methods: **Fig 6A-C**). As detailed in the methods section, PCA score grouped animals into three groups: sign tracking (a score between +0.5 to 1), intermediate tracking (-0.5 to +0.5), and goal tracking (-1 to -0.5). Subsequent analysis revealed significant differences in lever presses between sign, goal and intermediate groups as revealed by a main effect of group (**Fig. 6A**, $F(2,109) = 57.73$, $p < 0.0001$) and time ($F(9,981) = 59.98$, $p < 0.0001$), and a significant group x time interaction ($F(18, 981) = 24.05$, $p < 0.0001$). Post hoc analysis revealed greater lever pressing in sign trackers compared to intermediate (Tukey's multiple comparison test, $p < 0.01$) and goal trackers ($p < 0.0001$, **Fig. 6A**). Moreover, intermediate trackers had greater lever pressing than goal trackers ($p < 0.0001$, **Fig. 6A**). Analysis of head entries during cue presentation also showed differences among PCA categorized groups, as we observed a main effect of group ($F(2,109) = 52.84$, $p < 0.0001$) and a significant interaction of group x time ($F(18,981) = 14.98$, $p < 0.0001$), but no effect of time ($F(9,981) = 1.701$, $p = 0.08$, **Fig. 6B**). Post hoc tests revealed greater head entries for goal trackers compared to intermediate ($p < 0.0001$) and sign trackers ($p < 0.0001$) as well as a greater head entries by intermediate trackers compared to sign trackers ($p < 0.001$, **Fig. 6B**). Differences in head entries during the ITI also emerged, as indicated by a main effect of group ($F(2,109) = 6.362$, $p < 0.01$, **Fig. 6C**), a main effect of time ($F(9,981) = 57.73$, $p < 0.001$), and a significant interaction of group x time ($F(18,981) = 2.654$, $p < 0.001$). Post hoc tests revealed that sign trackers had less head entries overall during the ITI period compared to goal trackers ($p < 0.01$) but not compared to intermediate trackers ($p > 0.05$).

To determine whether approach strategy influenced the degree of reinforcement, we examined the potential correlation between the PCA score with the amount of conditioned reinforcement, indexed by the number of CR nosepokes minus the NCR nosepokes (as previously performed (Robinson and Flagel, 2009, Saunders and Robinson, 2012). Analysis of VTA-cannulated subjects, that received intra-VTA saline on CR test day, revealed no significant correlation between PCA score and conditioned reinforcement (Pearson $r=0.17$, $p=0.49$, **Fig. 6D**). In addition, analysis of non-surgerized animals, that did not receive any pharmacological manipulation on CR test day, also revealed no significant correlation between PCA score and conditioned reinforcement (Pearson $r = 0.15$; $n = 24$; $p = 0.48$, data not shown). Despite this lack of correlation between PCA score and CR, AP-5, MEC or SCOP infusion could have differential effects on the presence or absence of a correlation between PCA score and conditioned reinforcement. However, our experimental design, utilizing a dose response analysis of AP-5, MEC and SCOP effects on conditioned reinforcement, did not provide sufficient power to independently determine whether PCA score correlated with conditioned reinforcement across each AP-5, MEC and SCOP doses.

4. Discussion

Cues, that have become wanted, or desired, powerfully influence motivated behaviors. Here, we tested whether VTA NMDAR and AChR mechanisms play a role in conditioned reinforcement for a cue previously paired with food reward. Indeed, it has been previously shown that VTA NMDAR mechanisms play an important role in reward processing (Sombers et al., 2009, Cacciapaglia et al., 2011) and reward-seeking behavior (Yeomans et al., 1993, Sharf et

al., 2006a, Sharf and Ranaldi, 2006, Sombers et al., 2009), and we found that VTA NMDAR blockade disrupted conditioned reinforcement. In contrast, our results revealed that VTA nAChR antagonism non-selectively decreased both CR and NCR responding, whereas VTA mAChR antagonism elevated NCR, but not CR, responding and impaired the subjects' ability to discriminate between CR and NCR. Together, these results suggest VTA NMDARs and mAChRs, but not VTA nAChRs, mediate conditioned reinforcement.

The findings of our study, which is the first to examine VTA NMDAR and AChR mechanisms in conditioned reinforcement for natural reward-associated cues, provide new understanding that builds upon previous examinations of the role of VTA NMDARs in cue-mediated and reward-related behavior. In the VTA, both NMDARs and AChRs are critical for elevating DA release (Forster and Blaha, 2003, Wickham et al., 2013). Despite the evidence for these VTA receptor mechanisms in mediating DA signaling, the precise role of VTA NMDARs and AChRs in cue-dependent behavior is still being determined. Here, we observed that acute VTA NMDAR inhibition dose-dependently blocked conditioned reinforcement. There are at least two underlying processes that could have contributed to this result. First, VTA NMDAR blockade may have impaired the subjects' ability to learn that the new operant response, nose-poking, led to cue presentation during the test. A second possibility is that VTA NMDAR blockade decreased the incentive salience of the cue, making it less reinforcing to the animal. While we cannot conclude which process contributed to the AP-5 findings in our current study, evidence from previous studies suggests that VTA NMDARs play a role in the reward-related learning. Previous work has demonstrated that blockade of NMDARs with AP-5 prevents the acquisition, but not maintenance, of both Pavlovian and operant conditioning for food (Zellner et al., 2009, Ranaldi et al., 2011). These previous findings highlight impairments in acquiring

1 stimulus-outcome (Pavlovian) and action-outcome (operant) behaviors, suggesting that VTA
2 NMDARs play a role in reward-related learning rather than in the maintenance of reward-
3 dependent behavior. Thus, we propose that our observed AP-5 mediated blockade of conditioned
4 reinforcement is likely due to a disruption of reward-related learning. However, we cannot
5 discount the possibility that VTA NMDAR inhibition altered the incentive value of the cue.
6 Indeed, previous work suggests a role for VTA NMDARs in encoding the hedonic valence of
7 primary food rewards as VTA antagonism of NMDARs leads to decreased food-evoked DA
8 release in the NAc (Westerink et al., 1997). However, the role of VTA NMDARs in encoding
9 hedonic value has not been well defined to date.

10 With respect to cholinergic mechanisms, previous evidence suggests a role of the VTA
11 AChRs in mediating primary reward. Specifically, VTA acetylcholine levels rise during eating
12 and drinking (Rada et al., 2000) while VTA infusion of mAChR antagonists, but not nAChR
13 antagonists, decrease feeding behavior (Yeomans et al., 1993, Sharf et al., 2006b). Furthermore,
14 pharmacological activation of VTA AChRs leads to the development of a conditioned place
15 preference (Yeomans and Baptista, 1997, Ikemoto and Wise, 2002) and is sufficient to produce
16 self-administration behavior (Ikemoto and Wise, 2002), suggesting an important role of VTA
17 AChRs in primary reward processing. VTA mAChR mechanisms have also been implicated as
18 important mediators of reward-related learning. Specifically, repeated VTA micro-infusion of
19 SCOP, but not MEC, prevents rats from acquiring operant behavior for food delivery (Sharf et
20 al., 2006a). Similar to our AP-5 findings with conditioned reinforcement, the ability of mAChR
21 blockade to prevent acquisition of correct operant responding could be due either to a disruption
22 in learning or to the decreased incentive value of the cue (or both). Interestingly, after high dose
23 SCOP infusion, subjects no longer discriminated between CR and NCR noseports, due to an

1 increase in NCR responding. This effect was in contrast to our nAChR findings where MEC did
2 not disrupt the ability of subjects' to discriminate between CR and NCR noseports. Thus, we find
3 a dissociation between the ability of mAChR and nAChR to disrupt discrimination during
4 conditioned reinforcement, similar to the dissociation observed between mAChRs and nAChRs
5 in operant responding for food (Sharf et al., 2006a).

6 We observed that while VTA NMDAR or mAChR blockade both disrupt CR, this
7 disruption occurs in different ways. VTA NMDAR blockade decreased CR responding compared
8 to saline, suggesting that the CR is less reinforcing. Naturally, if the CR is only weakly
9 reinforcing, the animal will have a much more difficult time discriminating the CR from the
10 NCR. In contrast to VTA NMDAR blockade, VTA mAChR blockade at the high dose of SCOP
11 did not disrupt CR responding. Indeed, CR responding in the SCOP infused rats was comparable
12 to CR responding in saline infused rats. However, NCR responding in SCOP infused rats was
13 elevated to near CR levels. Thus, it is possible that animals receiving the high dose of SCOP
14 found the cue reinforcing, yet were unable to properly assign credit to the action that produced
15 the cue. Therefore, we propose that rats in the high dose SCOP group found the cue reinforcing,
16 but could no longer discriminate between the CR and NCR - and thus were indiscriminately
17 nose-poking at both CR and NCR ports. In addition to our observed dissociation between
18 NMDAR, mAChR and nAChR mechanisms, it also possible that these receptors play a
19 differential role in conditioned reinforcement in subjects that show different approach strategies
20 during Pavlovian conditioning. Indeed, previous findings suggest that sign trackers develop more
21 robust conditioned reinforcement than goal trackers (Robinson and Flagel, 2009) and these
22 differences in sign and goal tracking have been linked to phasic DA mechanisms in the NAc
23 (Flagel et al., 2007, Flagel et al., 2011). Given the role of VTA NMDARs and AChRs in

1 mediating phasic DA release in the NAc (Forster and Blaha, 2000, Solecki et al., 2013, Wickham
2 et al., 2013) examining how VTA NMDAR and AChR receptor blockade alters conditioned
3 reinforcement in sign and goal trackers could provide new insight of potential role of VTA
4 mechanisms. However, our analysis revealed no significant correlation between approach
5 strategy and conditioned reinforcement in our saline control animals. It is possible that
6 differences in training may have contributed to this lack of correlation - our animals received 10
7 daily session (consistent with previous work, (Lof et al., 2010)) with 25 pairings per session,
8 whereas others have observed a positive correlation after 5 training sessions using 25 pairings
9 per session (Meyer et al., 2012). Thus, it is possible that the correlation between approach
10 strategy and conditioned reinforcement is strongest during a specific time-window of training.
11 Such considerations can be further explored in future work. In addition, a remaining important
12 question is whether VTA AChR and NMDAR blockade differentially alters conditioned
13 reinforcement in sign trackers, goal trackers, and intermediates. In the current study, our
14 experimental design, which included an important dose-response analysis for VTA AP-5, MEC,
15 and SCOP effects on conditioned reinforcement, did not provide sufficient power to analyze drug
16 effects across sign, goal and intermediate groups. However, such analyses will be important in
17 future studies and will require larger sample sizes in order to achieve sufficient power.

18 Our results, in the context of previous studies, also highlight important differences in
19 VTA nAChR mechanisms that mediate the reinforcing properties of food-associated versus drug-
20 associated cues. In the conditioned reinforcement test for natural reward-associated cues, we find
21 that VTA nAChR inhibition leads to an overall decrease in activity that is not specific to CR or
22 NCR nosepokes. MEC administration did not prevent the development of conditioned
23 reinforcement, as MEC subjects still discriminated between CR and NCR on test day. Such

findings are in contrast to those observed with drug-associated cues, where inhibition of nAChRs disrupts conditioned reinforcement for ethanol-associated cues (Lof et al., 2007). This conditioned reinforcement for ethanol cues was mediated by VTA $\alpha 6\beta 2^*$ nAChR (Lof et al., 2007), the same VTA nAChR subtype that we recently identified as a critical regulator of phasic DA release in the NAc (Wickham et al., 2013). In addition, we have previously demonstrated a role for VTA nAChRs in cue-induced cocaine-seeking, which measures the ability of cocaine-associated cues to maintain drug-seeking behavior in the absence of drug delivery (Solecki et al., 2013). Together the current and previous findings, suggest that VTA nAChRs mediate the reinforcing properties of drug-associated, but not food-associated, cues. One possible contribution for this differential role of nAChRs of natural cue versus drug cue-mediated behavior is drug-induced plasticity at nAChRs in the VTA. Indeed, exposure to drugs of abuse enhances synaptic plasticity in the VTA (Saal et al., 2003, Chen et al., 2008, Mameli et al., 2011). Furthermore, chronic nicotine exposure leads to increased conditioned reinforcement for food cues – an effect which is dependent upon $\beta 2$ containing nAChRs (Brunzell et al., 2006). These findings raise the possibility that drug-mediated plasticity may recruit the involvement of VTA nAChRs mechanisms in conditioned reinforcement whereas, in the absence of drug exposure, VTA nAChR mechanisms do not mediate the reinforcing value of natural reward cues. There is, however, a lack of evidence or understanding of the mechanisms that mediate these differences. In future work, the potential mechanisms underlying this dissociation could be investigated by examining whether VTA nAChRs and mAChRs differentially regulate dopamine responses to food and drug-related cues.

5. Conclusion

1 Here, we provide evidence that VTA NMDARs and mAChRs, but not nAChRs,
2 modulate the ability of a natural reward-associated cue to act as a reinforcer. In the context of
3 previous work examining VTA mechanisms of the reinforcing properties of cues paired with
4 drug, our data suggests that different VTA receptor mechanisms mediate conditioned
5 reinforcement for drug-associated versus natural reward-associated cues. This possibility is
6 interesting in light of identifying clinical targets, such as $\alpha 6\beta 2^*$ nAChRs, that can may be able
7 to selectively attenuate the motivational properties of drug-cues while not interfering with the
8 receptor mechanisms mediating the motivational properties of natural reward cues.

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Figure Legends

Fig. 1 A. VTA cannula infusion sites, based on histological verification of cannula tip placements (adapted from (Paxinos and Watson, 2007)). Black circles indicate correctly placed VTA cannula tips. Grey circles indicate misplaced cannula from rats that were excluded from analysis. B. Photomicrograph depicting localized spread of Chicago blue dye within the VTA. Abbreviations: VTA-ventral tegmental area; ml-medial lemniscus; SNR-substantia nigra reticulata.

Fig. 2. Conditioned reinforcement after training in non-cannulated animals receiving a cue predictive of food (paired group; $n = 16$) versus animals receiving a cue that is not explicitly paired with food (unpaired group; $n = 7$). **** $p < 0.001$; Bonferonni post-hoc test; CR = conditioned reinforced response. NCR = non-conditioned reinforced response.

Fig. 3. VTA infusion of AP-5 attenuates, but does not block, conditioned reinforcement. Comparison of nosepoke responses in subjects that received SAL ($n = 19$) or AP-5 ($0.1 \mu\text{g}$ ($n=12$) or $1.0 \mu\text{g}$ ($n=8$)) VTA microinfusion immediately prior to the conditioned reinforcement test. The saline group is the same for Figs. 3, 4 and 5. Data shown as mean $\pm$ SEM. ** $p < 0.01$; Bonferonni post-hoc test; SAL = saline.

Fig. 4. VTA infusion of MEC does not alter conditioned reinforcement. Comparison of conditioned reinforcement immediately after SAL ($n = 19$) or MEC ($3.0 \mu\text{g}$ ($n = 11$); or $30 \mu\text{g}$ ($n = 13$)) infusion into the VTA. The saline group is the same for Figs. 3, 4 and 5. Data shown as mean $\pm$ SEM. ** for $p < 0.01$; Bonferonni post-hoc test. MEC = mecamlamine.

Fig. 5. VTA infusion of high dose SCOP elevates NCR responding. Comparison of conditioned reinforcement in subjects receiving SAL ($n = 19$) or SCOP ($3.0 \mu\text{g}$ ($n = 11$) or $66.7 \mu\text{g}$ ($n = 12$)) infusion into the VTA immediately prior to test. The saline group is the same for Figs. 3, 4 and 5. Data shown as mean $\pm$ SEM. ** for $p < 0.01$; Bonferonni post-hoc test. SCOP = scopolamine.

Fig. 6. Lever presses (A) and head entries (B) during the cue presentation, and head entries during the inter-trial interval (ITI; C) for sign trackers, goal trackers, and the intermediate group. Data shown as mean with standard error from the mean (SEM.). (D) Correlation between PCA score and CR nose pokes minus NCR nose pokes. Tukey's post-hoc test comparing main effects of group (for A-C). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.00001$.

Figure 1

A

SAL (n = 19)

0.1 μ g AP-5 (n = 12)

1.0 μ g AP-5 (n = 8)

3.0 μ g MEC (n = 11)

30.0 μ g MEC (n = 13)

3.0 μ g SCOP (n = 11)

66.7 μ g SCOP (n = 12)

B

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6