

NUCLEUS ACCUMBENS NEURONAL ACTIVITY DURING A SENSORY
PRECONDITIONING TASK

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ABSTRACT

DOMENIC CERRI: Nucleus accumbens neuronal activity during a sensory preconditioning task

(Under the direction of Regina M. Carelli)

The nucleus accumbens (NAc) is involved in associative learning and motivated behavior but its role in Sensory Preconditioning (SPC) remains unknown. Here, electrophysiological recordings were taken from the NAc core while rats performed three phases of a SPC task. During Preconditioning (phase 1), the NAc was more activated by a stimulus that was preceded by another stimulus, versus the same stimulus presented in isolation, suggesting that the NAc may encode information about neutral cue associations. During First-Order Conditioning (FOC) (phase 2), animals readily acquired the association between the conditioned and unconditioned stimulus, and this information was processed by distinct populations of NAc neurons. At SPC Test (phase 3), animals showed behavioral evidence of SPC; however, NAc activity did not track this behavior. These findings show that although the NAc encodes associations between neutral stimuli in Preconditioning, and processes information about FOC, SPC expression is not encoded by the NAc core.

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CHAPTER 1

INTRODUCTION

In Pavlovian First-Order Conditioning (FOC), an initially neutral stimulus (e.g. tone or light) is paired with a biologically salient, unconditioned stimulus (US) (e.g. food or drugs) that normally elicits an unconditioned response (UR) such as an approach behavior. After repeated CS-US pairings, the once neutral stimulus becomes a conditioned stimulus (CS) capable of evoking a conditioned response (CR) (Rescorla, 1988). In this fundamental form of learning the CS does not just evoke an automatic CR but is endowed with motivational value much like the US, such that the CS can support new learning (Gewirtz & Davis, 2000; Rizley & Rescorla, 1972). Consequently, there are circumstances where a neutral stimulus can become a CS despite never being directly paired with a US. For example, in Second-Order Conditioning (SOC) a novel neutral stimulus repeatedly paired with a first-order CS will also become a CS capable of evoking its own CR. Alternatively, in Sensory Preconditioning (SPC), if two neutral stimuli are repeatedly paired before one of them is turned into a CS via FOC, then the other stimulus will also become a CS capable of eliciting a CR.

The nucleus accumbens (NAc), particularly the core subregion, is well-known to be involved in associative learning and the acquisition and expression of the motivational value of cues. For example, while lesions of the core do not impair food-directed responses to a CS, they have been shown to selectively impair the ability of first-order CSs to elicit cue-oriented responses (Cardinal et al., 2002; Chang, Wheeler, & Holland, 2012), and to impair

enhancement of instrumental responding in a Pavlovian-to-Instrumental transfer task (Hall, Parkinson, Connor, Dickinson, & Everitt, 2001). Indeed, animals with NAc lesions, even if made after FOC, are unable to use the motivational value of a CS to acquire and express new responses in SOC (McDannald, Setlow, & Holland, 2013). Further, disconnection lesions of the basolateral amygdala (BLA), a major limbic input to the NAc, also impair cue-oriented responses (Chang et al., 2012), and SOC (Setlow, Holland, & Gallagher, 2002). Unlike lesions of the NAc however, BLA lesions made after FOC do not disrupt SOC (Setlow, Gallagher, & Holland, 2002); thus, it appears that BLA-NAc connectivity is necessary for the acquisition, while the NAc alone is sufficient for the expression of the motivational value of cues.

Interestingly, SPC appears to utilize different brain areas than SOC. For example, unlike in SOC, bilateral lesions of the BLA are without effect on SPC or the ability to make associations between neutral stimuli (Blundell, Hall, & Killcross, 2003; Dwyer & Killcross, 2006). Instead, it has been found that lesions of the perirhinal cortex, a major medial temporal lobe source of polysensory information produces large deficits in SPC (Nicholson & Freeman, 2000). Further, lesions of the orbitofrontal cortex, a prefrontal area important for value estimations, also disrupt SPC (Jones et al., 2012). Clearly the neural processing of the acquisition and expression of SPC is not identical to that of SOC; however, the involvement of the NAc core in SPC has yet to be directly investigated.

Therefore, the present study was designed to directly examine the activity of NAc neurons in the core during the acquisition and expression of SPC. Specifically, NAc neurons were recorded during three stages of an SPC task: preconditioning of neutral stimuli, first-order conditioning, and test presentations of preconditioned stimuli (test for SPC). We

hypothesize that if the NAc plays a role in the acquisition of information about preconditioned stimulus relationships, then differential firing should be observed to the cues in the Preconditioning phase. Alternatively, if the NAc core is critical for expression of SPC, we expect enhanced cell firing to the SPC cue during test. Thus, this approach enabled a determination of whether or not associations between neutral stimuli are encoded, and if the NAc can later use information about those stimuli to evoke a CR.

CHAPTER 2

METHODS

Subjects

Experimentally naive male Sprague-Dawley rats ($n = 27$; Charles River Laboratories), aged 8-12 weeks and weighing approximately 300 g at the time of arrival were used. The individually-housed rats habituated to their homecages for approximately 1 wk, during which time they had ad-libitum access to food and water and were maintained on a 12 h light / dark schedule. Following habituation, a subset of rats ($n = 19$) were implanted with indwelling electrophysiological recording arrays in the core of the NAc (see below). After 2 wks recovery (or 3 wks after arrival for animals that did not undergo surgery), rats were food restricted (unlimited water access) to 15 g chow/d to maintain their weight. Rats remained on this restricted diet for the duration of all behavioral procedures. Animal procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the guidelines of the University of North Carolina at Chapel Hill Institutional Care and Use Committee.

Surgical methods

Prior to all behavioral testing, rats were anesthetized with ketamine (100 mg / kg) and xylazine (10 mg / kg), and then secured in a stereotaxic apparatus (Kopf Instruments, Tijuana, CA, USA). The scalp was incised and retracted, and the skull was adjusted to level in all planes. Holes were drilled in the skull above the NAc core (AP: +1.8 mm, ML: ± 1.4

mm, relative to Bregma) in both hemispheres. An eight-microwire recording array (NB Labs, Denison, TX, USA) was slowly lowered into the NAc core at a depth of 6.2 mm from the brain surface. The arrays consisted of two parallel rows of four stainless steel Teflon-coated, 50 μ m-diameter wires, tips spaced evenly 0.5 mm apart. A ground wire for each array was placed in the brain distal to the recording location in the same hemisphere. The apparatus was permanently secured with dental acrylic attached to screws embedded in the skull surface. Animals were given an oral dose of 1.0 mg / kg meloxicam (Metacam, Boehringer Ingelheim Vetmedica, St Joseph, MO, USA) as a post-operative analgesic for 2 d, and at least 1 wk to recover from surgery before beginning food restriction and behavioral training.

Apparatus

All training and testing took place in a custom-built behavioral chamber (43 x 43 x 53 cm; MED associates, St Albans, VT, USA) housed in a sound-attenuating cabinet. The interior walls of the cabinet were covered in metal mesh to provide insulation from external electrical signals. Chambers were illuminated by a houselight located on the ceiling. Masking noise and ventilation were provided by a wall mounted fan. A centrally-located foodcup (4 cm above the floor), equipped with photobeams to automatically detect head entries, was mounted on the right wall of the chamber. Auditory stimuli were delivered by a speaker 18 cm above the floor, and consisted of either a tone (800 Hz) or white noise, calibrated to 90 and 65 dB, respectively, to account for differences in auditory sensitivity to the different frequencies (Kelly & Masterton, 1977). Visual stimuli were presented at a pair of 2.5 cm-diameter cue lights (flanking the food cup 22 cm apart and 12 cm above the floor). Visual

stimuli consisted of a solid light and a flashing light (4 Hz), delivered at the right and left cue lights, respectively.

Electrophysiological recordings were taken during all behavioral sessions. Details on electrophysiological recording procedures have been reported previously (Carelli & Ijames, 2000). Briefly, rats were connected to a recording cable that terminated in a headstage (Plexon Inc., Dallas, TX, USA). The cable was connected at the other end to a commutator (MED Associates and Crist Instruments) allowing free movement throughout the chamber during sessions. Amplified neural signals were then passed to a Multichannel Acquisition Processor (MAP) system (Plexon Inc.) where they were captured by a neural analysis program (Sort Client, Plexon Inc.). A separate computer controlled external stimuli and captured behavioral events (TRANS IV, MED Associates). Neural data were acquired using techniques and apparatus similar to those described elsewhere (Roitman, Wheeler, & Carelli, 2005). Briefly, software was employed to sort neural waveforms by principal components analysis (Offline Sorter, Plexon Inc.). Finally, the resulting timestamps for valid waveforms were further analyzed in relation to behavioral markers and events of interest using NeuroExplorer software (NEX Technologies, Littleton, MA, USA).

Behavioral task

Before training, rats were given a brief session in which they received 8 noncontingent, pseudorandomly delivered 45 mg sucrose pellets (Purina, Richmond, IN, USA) in order to familiarize them with reward delivery and the food cup. Rats with recording arrays were also connected to the recording apparatus during this session to habituate them to the tether. An overview of the sensory preconditioning task is shown in

Table 1. The same cue types were used for each animal throughout training; thus, the noise, tone, flashing light, and solid light stimuli (described above) were made to correspond to cues *A*, *B*, *X*, and *Y*, respectively. All cues were always presented for 10 s.

Preconditioning. Rats were divided into Paired and Unpaired groups; each received 2 consecutive days of Preconditioning (days 1 and 2). Animals in the Paired group ($n = 19$, 11 with microwire arrays) received 2 blocks of 12 cue pairings per day; in one, presentation of *A* co-terminated with the onset of *X*, while in the other, *B* co-terminated with the onset of *Y*. A variable inter-trial interval (ITI) of 90-270 s separated each set of cue pairings. Blocks were separated by a 10 m timeout without fan or houselight, and block order was counterbalanced between animals and reversed on the second day of training. Animals in the Unpaired group ($n=8$, all with microwire arrays) received 12 trials of each of the 4 cues, presented in isolation, on both days of Preconditioning. Cues were presented in pseudorandom order, following a variable ITI of 50-140 s. Note that animals in the Paired and Unpaired group only differed in whether or not preconditioned stimuli were paired, and received identical behavioral manipulations on all subsequent days of SPC task.

First-Order Conditioning. After Preconditioning, all animals received 3 consecutive days of Pavlovian First-Order Conditioning (FOC) (days 3-5). Each day, the preconditioned cues *X* and *Y* were presented in pseudorandom order to serve as Pavlovian conditioned stimuli (CSs). Trials were separated by a variable ITI of 90-270 s. Three 45 mg sucrose pellets were delivered immediately after each termination of *X* (*the CS+*), while *Y* (*the CS-*) was never followed by reinforcement during these sessions. On the first two days of FOC, 21 trials of *X* and 20 of *Y* were given, with the US omitted on 3 and 4 of the total cue *X* trials, respectively, in order to increase resistance to extinction effects during later testing. On the

third day, 20 trials of *X* and 21 of *Y* were given, with the US omitted on just 2 of the trials with *X*.

Test session. Once FOC training was completed, the Sensory Preconditioning (SPC) effect was assessed during a final Test session (day 6). Rats in both the Paired and Unpaired groups were pseudorandomly presented with preconditioned cue *A*, for 19 trials, and *B*, for 21 trials, featuring each cue in isolation. In addition, 3 reminder trials for both of the CSs used during FOC (*X* followed by the US, and *Y* without the US) were interspersed into the session to prolong behavior under extinction conditions. Again, each cue trial was separated by a variable 90-270 s ITI.

Histology

Histological verification of electrode placements was accomplished using established procedures (e.g. Day et al., 2006). Briefly, after the experiments, animals were heavily anesthetized with ketamine (100 mg / kg) and xylazine (10 mg / kg). A 14.4 μ A current was then passed through each stainless-steel microwire for 5 seconds to leave an iron deposit in the tissue. To identify the wire tips, rats were perfused transcardially with saline (10 m, 20 mL / m), followed by a 3% potassium ferricyanide in 10% formalin solution. The brain was removed, frozen to -20 °C and coronally sliced (40 μ m thick) throughout the extent of the NAc. Slices were mounted on slides, documented with high-resolution photomicrographs, and electrode placement was confirmed within the NAc using a standard atlas (Paxinos & Watson, 1997).

Analysis of behavior

Behavioral conditioning was assessed during each session as the mean number of ‘cue’ head entries made during 10 s presentations of a particular stimulus across trials minus the ‘baseline’ mean head entries made during the 10 s period preceding all stimuli, for each animal. This number, hereon just ‘head entries’, was used for comparative statistical analyses.

Analysis of neural firing

Cells were examined and classified as ‘phasic’ if they exhibited significant increases and/or decreases in firing rate during cue presentation compared to their baseline rates. Specifically, during a given session, the mean firing rate across trials during the 10 s baseline period was compared to the mean firing rate following the cue period (paired t-tests). If a neuron exhibited phasic activity to just one of the preconditioned cues (*A* or *B*) and/or one of the CSs (*X* or *Y*), it was counted as ‘selective’ for the cue(s). If a neuron exhibited activity to both of the preconditioned cues or CSs, we then determined whether encoding for one cue was more selective than the other cue. To determine this, the mean firing rate during *A* and *B* was directly compared using unpaired t-tests. If significantly different, the neuron was deemed selective to the cue that evoked the largest absolute value deviation from baseline; however, if not significant, the cue was deemed non-selective. Comparisons were computed likewise for *X* and *Y*. Neurons displaying firing rates uncharacteristic of medium spiny neurons were excluded from the analysis (Berke, Okatan, Skurski, & Eichenbaum, 2004; Cameron & Carelli, 2012).

To probe the involvement of the NAc core in each component of the task, it was necessary to determine how selective cells were distributed within the overall population of cells recorded. To this end, the proportion of cells that were selective to a given stimuli (or combination of preconditioned cues and CSs) during each session was calculated by dividing the number of selective cells by the total number of cells recorded for each animal during the same session. Only animals with greater than 3 total cells recorded were used for analysis; however, when 3 days were being compared and a rat had more than 3 total cells on only 2 of those days, then the % selective data for that animal for the day with 3 or fewer cells recorded was still included in analysis. The ‘% selective’ data for each animal was in turn used for all subsequent comparative statistical analyses.

Statistical Analysis

All statistical analyses were conducted using STATISTICA version 10 (StatSoft, Inc., 2011). Repeated measures Analysis of Variance (ANOVA) were used to compare conditioned behavior (i.e. head entries) or neural encoding (i.e. % selective data) for all animals, with cue and day (where applicable) as within-subjects factors and group (Paired vs. Unpaired) as a between subjects factor. Significant main effects and interactions were further investigated using Tukey’s HSD pairwise comparisons. Finally, linear regressions were used to determine whether or not neural activity was correlated with behavior. The critical value for each comparison was determined at $\alpha = 0.05$.

CHAPTER 3

RESULTS

Behavior

Preconditioning. The number of head entries during each cue in the Preconditioning phase are shown in Figures 1A and B. Since no food was delivered during this phase, neither group of animals were expected to show significant behavioral conditioning. A repeated measures 3-way ANOVA confirmed no significant main effects of day ($F_{1, 25} = 0.86, P = 0.36$), cue ($F_{3, 75} = 1.79, P = 0.16$), group ($F_{1, 25} = 0.85, P = 0.37$), or interactions among those factors on head entries (all P values > 0.2). These results reflect a uniform absence of any conditioned behavior during this phase.

First-Order Conditioning. To determine whether animals successfully acquired the Pavlovian discrimination between X and Y by the end of FOC training, a repeated measures 3-way ANOVA (day, cue and group) was conducted. Analysis indicated main effects of day ($F_{2, 50} = 60.87, P < 0.0001$) and cue ($F_{1, 25} = 15.53, P = 0.0006$). Indeed, there was also a significant cue by day interaction ($F_{2, 50} = 28.50, P < 0.0001$), illustrating that while rats failed to discriminate between cues on the first and second days of FOC (Tukey, all P values > 0.7), they performed more head entries in the presence of the CS+ (cue X) than the CS- (cue Y) on the final day of training ($P = 0.0001$) (Fig. 1C). Critically, cue exposure during Preconditioning had no differential effects on the acquisition of Pavlovian discriminations, as indicated by no significant main effect of group ($F_{1, 25} = 1.85, P = 0.19$), or interactions between group and FOC day or cue type on conditioned behavior (all P values > 0.2).

Test. During the Test session, we assessed the ability of the preconditioned cues (*A* and *B*) to elicit head entries. Note that *A* in the Paired group was the only preconditioned cue that was associated, indirectly via the CS+, with the food reinforcer during FOC. As such, we predicted that SPC during test should be selective to *A* in the previously Paired but not Unpaired group. In support, a repeated measures 2-way ANOVA revealed that the number of head entries to preconditioned cues were different between groups ($F_{1,25} = 4.95, P = 0.04$). There was also a main effect of cue ($F_{1,25} = 22.09, P < 0.0001$), and as predicted, a significant cue by group interaction ($F_{1,25} = 4.98, P = 0.03$). Indeed, post hoc tests revealed that animals in the Paired group (Tukey, $P = 0.0002$), but not the Unpaired group ($P = 0.47$), exhibited more head entries to *A* than *B*, and activity to *A* was greater in the Paired versus Unpaired group ($P = 0.01$) (Fig. 1D).

Neural Data

Preconditioning. Distinct subsets of NAc neurons exhibited phasic activity (i.e., increases or decreases in firing rate) relative to the cues during the Preconditioning phase. One set of cells exhibited phasic activity to presentation of *A* and/or *B*. A representative example neuron that was activated by *A* (but not *X*) is shown in Figure 2A, demonstrating an increase in firing rate immediately after onset of *A*. In other cases, cells exhibited phasic activity to presentation of *X* and/or *Y*. A representative example of a neuron that was activated by *X* (but not *A*) is shown in Figure 2B. Finally, other neurons displayed changes in activity in response to presentation of both *A* and *X* (or both *B* and *Y*); importantly, these cues were presented together in the Paired group. A representative example of a neuron that was activated by both *A* and *X* is shown in Figure 2C. In this case, the neuron showed an

increase in firing rate during *A* with a similar increase in activity during *X*. Note that phasic changes in cell firing were in some cases manifested as decreases in firing rate (i.e., inhibitions) relative to onset of various cues (raster/PEH data not shown). Phasic neurons that also met criteria for selectivity to either *A* or *B*, and/or *X* or *Y* (see *Analysis of neural firing*) were used in the quantitative analysis of neural activity during Preconditioning.

A repeated measures 3-way (group, day, cue) ANOVA was used to quantify differences in % selective activity during Preconditioning. The ANOVA revealed no main effects of group ($F_{1, 13} = 0.67$, $P = 0.42$) or day ($F_{1, 13} = 0.66$, $P = 0.43$) across both days of Preconditioning. However, a main effect of cue ($F_{3, 39} = 26.34$, $P < 0.0001$) indicated a significant difference between the encoding of individual cues during the Preconditioning phase. This observation was illuminated by a significant cue by group interaction ($F_{3, 39} = 4.43$, $P = 0.008$). To explore this further, we first compared the % selective encoding for *A* and *B* (i.e., preconditioned cues). There were similar proportions of cells encoding *A* (Tukey, $P = 0.92$) and *B* ($P = 1.00$) during Preconditioning between the Paired and Unpaired groups, with substantially greater % selective activity to *A* compared to *B* within each group (all P values < 0.0012) (Fig. 2D). However, when we looked at *X* and *Y* (i.e., cues that were later used as CSs), we saw a significant difference between both cues and groups. Specifically, there were significantly greater proportions of cells encoding *X* in the Paired compared to the Unpaired group ($P = 0.03$), but the % selective activity to *Y* did not differ between groups ($P = 1.00$). Further, % selective activity to *X* was greater than *Y* in the Paired ($P = 0.03$), but not Unpaired group ($P = 0.99$) (Fig. 2E). These findings indicate that NAc neurons encode information about cues prior to FOC.

Another analysis was completed to determine if the activation of neurons by X as shown in Figure 2E was associated with activation of the same neurons to A . A 2-way ANOVA examined the proportion of neurons selective for both A and X between groups and across days. There was a main effect of group ($F_{1, 13} = 9.33, P = 0.009$), but not day ($F_{1, 13} = 0.38, P = 0.55$) on % selective activity to both A and X , with significantly more neurons displaying this type of encoding in the Paired versus Unpaired group (Fig. 2F). This finding indicates that neurons are more likely to encode associated pairs (i.e., A and X) instead of explicitly unpaired stimuli.

First-Order Conditioning. Distinct subsets of NAc neurons exhibited phasic activity relative to X and/or Y cues during FOC (here referred to as CS+ and CS- during the first-order conditioning sessions, when they were paired with food, to differentiate them from Preconditioning sessions when they were not reinforced and acted as paired associates of the preconditioned cues). An example of a representative neuron showing phasic activity to the CS+ (but not food) on the final day of FOC is shown in the raster and PEH in Figure 3A. Note that phasic activity to the cues in FOC was not specific to excitatory firing in that in some cases neurons exhibited a significant decrease in firing rate to either the CS+ or CS- (data not shown).

In order to determine whether the NAc differentially encoded information about the CS+ and CS- during FOC, a repeated measures 3-way (day, cue, group) ANOVA was conducted. The ANOVA revealed a main effect of day ($F_{2, 30} = 17.56, P < 0.0001$), and cue ($F_{1, 15} = 8.81, P = 0.009$), as well as a significant day by cue interaction ($F_{2, 30} = 5.85, P = 0.007$) for the % selective neurons recorded for each animal. As predicted, there was a significant increase in % selective activity to the CS+ between the first and last days of FOC

(Tukey, $P = 0.0005$), but not to the CS- ($P = 0.99$). Further, while % selective encoding between conditioned stimuli did not differ on the first or second day of FOC (all P values > 0.8), neurons were more selective for the CS+ compared to the CS- on the final day of training ($P = 0.002$) (Fig. 3B). Thus neurons become more selective to the CS+ as FOC training progresses. Indeed, NAc neural activity during FOC is closely related to behavior, as the % selective activity to the CS+ for each animal was correlated with their respective head entries to the CS+ on each day of FOC training ($r_{42} = 0.58$, $P < 0.0001$). Critically, like behavior, cue exposure during Preconditioning had no differential effects on the encoding of CSs during FOC, as indicated by no significant main effect of group ($F_{1, 15} = 0.09$, $P = 0.77$), or interactions between group and FOC day or cue (all P values > 0.25).

Test. Distinct subsets of NAc neurons exhibited phasic activity (increased or decreased cell firing) relative to the preconditioned cues during Test. An example of a representative neuron with an excitatory response to *A* is shown in Figure 4A. To determine whether neural encoding was meaningful to SPC, we compared % selective activity to *A* and *B* during Test with activity to those cues during Preconditioning days 1 and 2 in a repeated measures 3-way ANOVA. While there was a main effect of cue ($F_{1, 13} = 59.22$, $P < 0.0001$), with greater activity to *A* compared with *B* as reported previously for Preconditioning (see Figure 2D), training did not create a main effect between groups ($F_{1, 13} = 1.23$, $P = 0.29$). Indeed, there was actually a significant main effect of day ($F_{2, 26} = 3.44$, $P = 0.047$), but no interactions involving the aforementioned factors (all P values > 0.52), demonstrating a uniform decrease in % selective activity to *A* and *B* between Preconditioning and Test days (Fig. 4B). These findings indicate that information encoded about SPC by NAc core neurons is not necessary for conditioned responding during SPC.

Histology

Histological reconstruction of electrode positions revealed that the neurons recorded during the SPC task sessions were located in the core subregion of the NAc, as defined by Paxinos and Watson (1997). Electrode placements spanned a rostral–caudal distance of ~2.5 mm, ranging from 3.1 to 0.7 mm rostral to bregma, with a medial-lateral range from 0.8 to 3.8 mm lateral to midline, and a dorsal-ventral range of 6.0 to 8.2 mm ventral to the skull surface at bregma. Cases in which wires were not positioned in the NAc were excluded from the data analysis.

CHAPTER 4

DISCUSSION

The results of this experiment suggest neural encoding in the NAc core may have a general role in encoding associative relationships, even those between neutral stimuli; however, it does not appear that this structure encodes the expression of SPC. Behaviorally, rats in this study showed a pattern of responding comparable to that observed in other SPC tasks using an appetitive US, as in Jones et al. (2012). As expected, animals in both the Paired and Unpaired groups exhibited a uniform absence of head entries to the neutral stimuli during Preconditioning. However, during Preconditioning, it was also found that neurons in the core are more likely to encode information about neutral stimuli when they are presented in a predictive relationship with another stimulus. Following Preconditioning, both groups of animals readily acquired the association between X and the US during FOC. This was supported by a greater number of head entries elicited by the CS+ compared the CS-, and a correlated increase in NAc core encoding of the CS+ over days of training, as is typical in FOC (Day, Wheeler, Roitman, & Carelli, 2006). Finally, animals in the Paired group, but not Unpaired controls, performed more head entries to lone presentations of the preconditioned cue A than B during Test, with the relative robustness of the CR comparable to that of other SPC studies (Jones et al., 2012; Nicholson & Freeman, 2000). Despite the clear development of SPC, cell firing during both preconditioned cues A and B was no different between groups, and actually decreased from Preconditioning levels, suggesting that SPC expression

was not encoded by NAc core neurons. The activity of NAc neurons across the 3 phases of the task and its implication for encoding aspects of learning is discussed in detail below.

NAc activity during preconditioning

An interesting point from our study is that, even during Preconditioning where all stimuli should be neutral, considerably more NAc neurons encoded information about the white-noise, preconditioned cue A, than the corresponding tone, preconditioned cue B. This effect does not appear to arise from the formation of neutral cue associations, as the relative levels of encoding for cues A versus B were no different between the Paired and Unpaired group of animals. That is, lone presentations of A were sufficient to elicit strong neural responses. Further, there were virtually no head entries made to either cue during Preconditioning, nor were their differences in head entries between A and B in the Unpaired group during Test, so the differential encoding should not reflect differences in inherent motivational value between the cues.

A possible explanation for this phenomenon is that the white-noise was sufficiently salient (i.e. arousing) to engage the attention of animals, while the other cues were not. There is some evidence from human and animal literature that value-neutral, but highly salient events can activate BOLD responses and DA release in the NAc, particularly in the absence of distracting or reinforcing stimuli (Horvitz, 2000; Zink, Pagnoni, Martin, Dhamala, & Berns, 2003). Further, Cole and Robbins (1989) used a loud burst of white noise to disrupt conditioned responding, and found that animals with dopaminergic lesions of the NAc were less likely to be disrupted by the highly salient stimuli. There is also evidence that the NAc may control some responses to novelty (Burns, Annett, Kelley, Everitt, & Robbins, 1996).

Given that the neural encoding of white-noise remained high, even after decreasing significantly between Preconditioning and Test, it may be that animals had difficulty habituating to the stimulus (perhaps due to its complex tonal structure) and continued to perceive it with some degree of novelty.

Assuming that the relatively high level of NAc core activity to cue A is due to heightened attentional processing, then the observation that cue X in this study evoked more core activity when paired with A during Preconditioning, but cue Y paired with B did not, is not surprising. Models of Pavlovian learning have shown stimulus salience to be an influential factor in the acquisition of learned associations for quite some time (see *alpha* in Rescorla and Wagner (1972), and *S* in Pearce and Hall (1980)). Indeed, while the scope of these models is too limited to encompass the stimulus-stimulus associations made during Preconditioning in SPC, Schmajuk, Lam, and Gray (1996) proposed a model that directly suggests that SPC learning can be facilitated by higher-salience stimuli. Further, Salzman and Newsome (1994) have shown that more salient cues do in fact support more learning, and do so at the expense of less-salient cues. Nonetheless, the elevated levels of neural activity to cue X in the Paired group suggest that the NAc core serves a role in the acquisition of associations between neutral stimuli.

NAc activity during FOC

While NAc core encoding during Preconditioning was clearly unrelated to conditioned responding (i.e., it occurred before any conditioning was acquired), this was not the case during FOC. Here, the relative neural encoding of cues tracked the development of conditioned head entries in the presence of the CS+ over CS- across days of FOC. This

observation fits previous work from our lab, illustrating that conditioned responding is positively correlated with neural encoding in the core (Day et al., 2006). Indeed, others present evidence that the level of core activity coincides with the current predictive/motivational value of a CS (Setlow, Schoenbaum, & Gallagher, 2003), and that core activity is related to execution of a given CR in the presence of a CS (Nicola, Yun, Wakabayashi, & Fields, 2004). Further, studies have also shown that, like NAc core cell firing, dopamine (DA) release in the core is also involved in the acquisition of a Pavlovian CR. Specifically, CS-evoked DA is correlated with the development of the CR over days of FOC training (Clark, Collins, Sanford, & Phillips, 2013; Day, Roitman, Wightman, & Carelli, 2007; Roitman, Stuber, Phillips, Wightman, & Carelli, 2004). However, both NAc core cell-firing and DA, while indicative of all Pavlovian CRs, may only play a necessary role for cue-directed, but not food-directed CRs. Evidence for this functional specificity comes from studies employing general (Cardinal et al., 2002; Chang et al., 2012), as well as DA specific NAc core lesions (Parkinson et al., 2002), performed either before or after conditioning. In this case, each manipulation impaired the ability of animals to make discriminative cue-oriented but not food-oriented CRs in the presence CSs. Thus, the food-oriented head entries to the CSs observed in this study are clearly associated with, but may not necessarily be dependent upon NAc core activity.

Does the NAc core play a role in SPC?

A primary goal of the present study was to examine the role of the NAc core in the expression SPC. While animals in the Paired group displayed differential encoding of cues compared to the Unpaired group during the acquisition of neutral associations at

Preconditioning, Paired animals did not differ from Unpaired animals in the encoding of Preconditioned cues at Test. This lack of neural encoding occurred despite Paired, but not Unpaired, animals demonstrating a clear SPC behavioral effect. Again, it appears that unlike FOC, conditioned responding and NAc core activity are not directly related during the acquisition or expression of SPC.

While the involvement of the NAc core in SPC has never been directly investigated prior to the present study, one report by Young and colleagues (1998) revealed a potential role for NAc DA in SPC. Using microdialysis, the authors first showed that DA concentrations were higher for two neutral stimuli presented simultaneously (paired) versus when presented in an unpaired fashion. Thus, these results seem to be in accordance with the heightened encoding of cue X observed in the present study during Preconditioning. Second, they found elevated levels of DA to the first-order CS+, which again corresponds to the increases in cell-firing observed to the CS+ over FOC training in the present study. Finally however, they found that during Test, DA increased for the previously paired, but not unpaired preconditioned cue; by contrast, we did not find differences in encoding for cues between Paired and Unpaired groups during Test. There are several differences between this study and the present study which may account for the discrepancies during Test. Notably, Young et al. (1998) presented neutral stimuli at the same time instead of sequentially, and did not include a CS- or an associated preconditioned cue. In addition, microdialysis has a very limited temporal and spatial resolution compared to electrophysiology, so unlike the present study, measurements were taken from the entire NAc, and across all trials of a particular cue including the time before and after cue presentations. Further, microdialysis cannot account for different DA release dynamics, such as phasic versus tonic DA release; two states which

may have opposing effects on cell firing (Dreyer, Herrik, Berg, & Hounsgaard, 2010). Future investigations using fast-scan cyclic voltammetry should be conducted to more directly relate NAc DA to neural activity during SPC (Phillips, Robinson, Stuber, Carelli, & Wightman, 2003).

Given that the NAc seems to have a role in acquiring neutral cue associations, but does not appear to be necessary for the expression of SPC, it is plausible that other brain areas are instead responsible for allowing animals to act upon preexisting cue associations. A very likely candidate is the OFC, as Jones et al. (2012) provide compelling evidence that in SPC the OFC is responsible for giving once neutral paired stimuli the motivational value necessary to produce a CR during SPC. In situations when animals have to exhibit a response in the absence of any valuable reinforcers, they instead must rely on their knowledge of the environment and preexisting “models” of the relationship between stimuli in their world to infer what the most valuable outcome to act upon may be. In SPC, animals infer the value of the preconditioned cues in this fashion and are able to act accordingly. Lesions of the OFC disrupt SPC, and therefore the OFC is said to be important for inferring value when more obvious reinforcers are absent. Further, Jones and colleagues (2012) note that there is no evidence that the OFC actually stores the relationship between stimuli. As such, it is plausible that the OFC actually retrieves the putative associative information from other neural regions such as the NAc core. Future studies looking at OFC neural encoding or disconnecting the NAc and OFC should help shed light on any potential interactions of these structures in the use of neutral cue associations during SPC.

Concluding Remarks

The results of this study suggest that while the NAc core may serve some role in forming associations between neutral stimuli, it is not necessary for the expression of SPC. Thus unlike FOC, neural encoding during SPC is not correlated with conditioned responding. Indeed, recent investigations from our lab on SOC have shown that, although the necessary information for conditioned responding may ultimately be encoded by the NAc shell subregion, neural encoding in the core is correlated with conditioned responding to the second-order cue (Saddoris & Carelli, In Press). These findings indicate that the neural representations underlying SPC are fundamentally different from both simple and other forms of higher-order Pavlovian learning.

	Preconditioning	First-Order Conditioning	Test
	Days 1-2	Days 3-5	Day 6
Paired	A → X B → Y	X → US Y	A B
Unpaired	A B X Y	X → US Y	A B

Table 1. Behavioral design for SPC training.

A, noise; B, tone; X, flashing light; Y, solid light; US, 3 sucrose pellets.

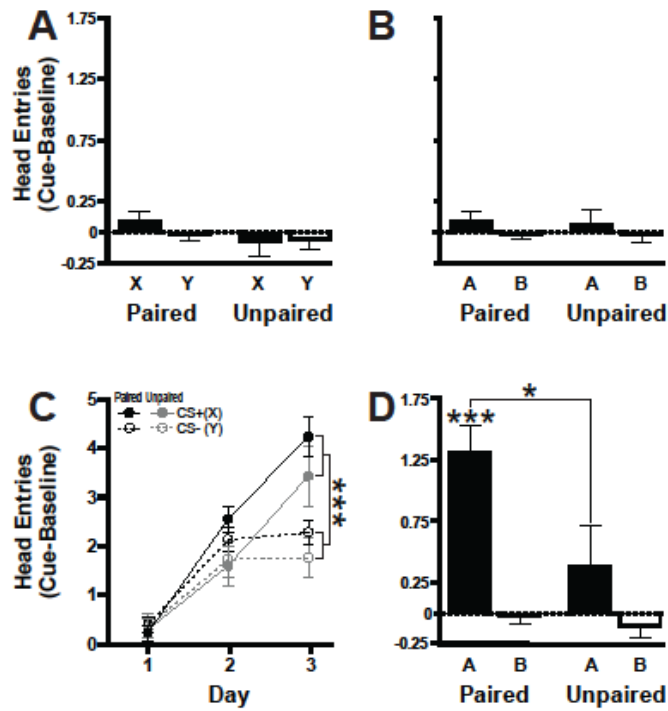


Figure 1. Behavior during the 3 phases of the SPC task. (A & B) There was a uniform absence of any conditioned head entries across both days of Preconditioning to all cues. (C) Rats in both Paired and Unpaired groups (during Preconditioning) successfully acquired the Pavlovian first-order discrimination by day 3 of FOC, showing more conditioned behavior in the presence of the CS+ than the CS- (***) $P < 0.001$). (D) Animals in the Paired, but not Unpaired group, exhibited more head entries to cue A than B during Test indicative of SPC. *** $P < 0.001$, Paired A vs. B; * $P < 0.05$, Paired A vs. Unpaired A.

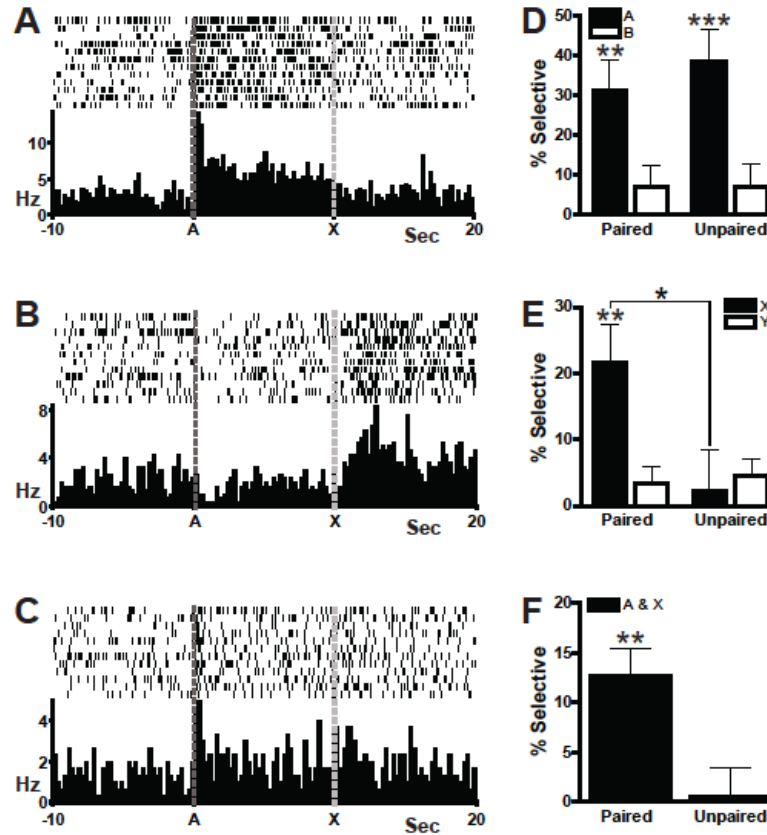


Figure 2. Preconditioning phase: NAc core neurons showed selective activity in the presence of neutral stimuli across both days of Preconditioning. (A) Raster plot and PEH (250 ms bins) of the activity of a representative ‘phasic’ neuron that increased firing to cue A (and not cue X). (B) Example of a neuron that increased firing to cue X (and not cue A) from baseline. (C) Example of a neuron that increased firing to both cue A and cue X from baseline. (D) % selective encoding was significantly greater for cue A than for cue B in both groups. ** $P < 0.01$, Paired A vs. B; *** $P < 0.001$, Unpaired A vs. B. (E) % selective encoding was significantly greater for cue X as compared to cue Y in the Paired, but not Unpaired group. ** $P < 0.01$, Paired X vs. Y; * $P < 0.05$, Paired X vs. Unpaired X. (F) There was a significantly greater % selective population for both cues A and X in the Paired group (when X followed A) compared to the Unpaired group (when X and A were independent) (** $P < 0.01$).

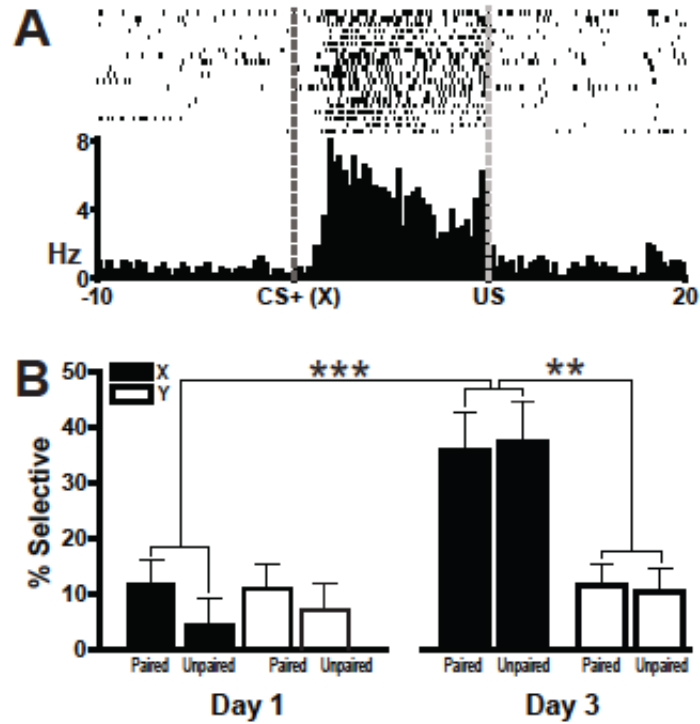


Figure 3. First-order conditioning phase: the % selective encoding of CSs during FOC. (A) Raster plot and PEH (250 ms bins) of an example phasic neuron showing an increase in firing to the CS+ (and not the US) on day 3 of FOC. (B) % selective encoding in both groups (Paired and Unpaired during Preconditioning) for the CS+ (cue X) on day 3 of FOC was greater than that to the CS- (cue Y) on the same day (** $P < 0.01$), and the CS+ on day 1 of FOC (***) $P < 0.001$).

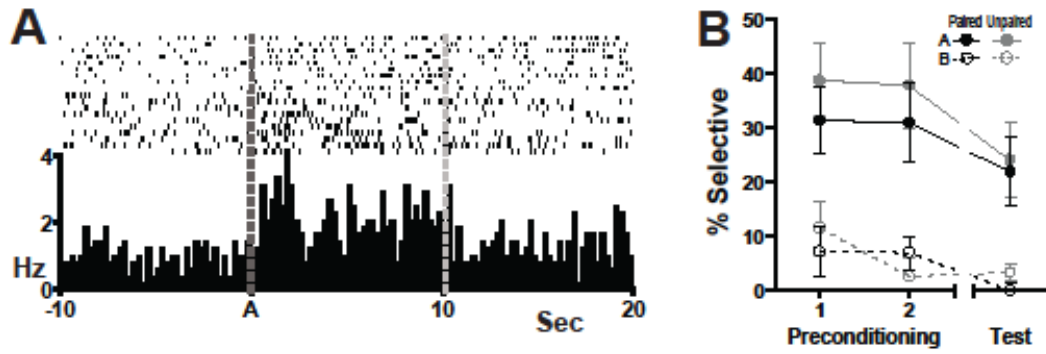


Figure 4. Sensory Preconditioning Test: the % selective encoding of preconditioned cues during the Preconditioning phase and the Test session. (A) Raster plot and PEH (250 ms bins) of an example phasic neuron showing an increase in firing to preconditioned cue A on Test. (B) % selective activity to the preconditioned cues A and B uniformly decreased between Preconditioning and Test sessions for the Paired and Unpaired groups. These findings indicate that information encoded about SPC by NAc core neurons are not necessary for conditioned responding during SPC

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