

Nucleus accumbens neurons encode Pavlovian approach behaviors: evidence from an autoshaping paradigm

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Abstract

Environmental stimuli predictive of appetitive events can elicit Pavlovian approach responses that enhance an organism's ability to track and secure natural rewards, but may also contribute to the compulsive nature of drug addiction. Here, we examined the activity of individual nucleus accumbens (NAc) neurons during an autoshaping paradigm. One conditioned stimulus (CS+, a retractable lever presented for 10 s) was immediately followed by the delivery of a 45-mg sucrose pellet to a food receptacle, while another stimulus (CS-, a separate retractable lever presented for 10 s) was never followed by sucrose. Approach responses directed at the CS+ and CS- were recorded as lever presses and had no experimental consequence. Rats ($n = 9$) selectively approached the CS+ on more than 80% of trials and were surgically prepared for electrophysiological recording. Of 76 NAc neurons, 57 cells (75%) exhibited increases and/or decreases in firing rate (i.e. termed 'phasically active') during the CS+ presentation and corresponding approach response. Forty-seven percent of phasically active cells (27 out of 57) were characterized by time-locked but transient increases in cell firing, while 53% (30 out of 57) showed a significant reduction in firing for the duration of the CS+. In contrast, the same excitatory subpopulation exhibited smaller increases in activity relative to CS- onset, while the inhibitory subpopulation showed no change in firing during the CS- period. The magnitude and prevalence of cue-related neural responses reported here indicates that the NAc encodes biologically significant, repetitive approach responses that may model the compulsive nature of drug addiction in humans.

Introduction

Environmental stimuli that predict rewards can gain biological salience and elicit automatic approach actions, thus helping organisms track and secure food. An animal model of this phenomenon, termed 'autoshaping', allows the presentation of one conditioned stimulus (CS+) to predict an appetitive event, while the presentation of another (CS-) is never followed by this event. In this paradigm, animals will selectively approach the CS+ while remaining unresponsive to the CS- (Bussey *et al.*, 1997). Such conditioned approach responses are believed to be under Pavlovian control because they are generally unaltered by the introduction of new contingencies and lack the malleability of instrumental behavior (Williams & Williams, 1969; Jenkins & Moore, 1973; Locurto *et al.*, 1976; Bussey *et al.*, 1997). Autoshaping is demonstrated by a variety of species (Brown & Jenkins, 1968; Sidman & Fletcher, 1968; Wilcove & Miller, 1974), and is thought to lend direction and movement to automatically acquired stimulus–outcome associations (Everitt *et al.*, 2001). In addition, approach behaviors may be linked to impulsive choice and could provide an interesting analog to compulsive behaviors that develop following repeated drug use (Tomie *et al.*, 1998, 2000).

Several lines of research converge in suggesting that the nucleus accumbens (NAc) mediates goal-directed behaviors for both drug and natural (e.g. water, sucrose and food) rewards (Carelli & Deadwyler,

1994; Wise, 1998; Kelley, 1999; Carelli *et al.*, 2000; Robbins & Everitt, 2002; Di Chiara *et al.*, 2004; Roitman *et al.*, 2004). Moreover, conditioned cues associated with primary reinforcement can evoke patterned responses among NAc neurons and elicit dopamine release in this structure (Carelli, 2000; Phillips *et al.*, 2003; Nicola *et al.*, 2004a; Roitman *et al.*, 2005). The NAc (especially the core subregion) also plays an important role in the development and expression of Pavlovian autoshaping behaviors. Cardinal *et al.* (2002) demonstrated that excitotoxic lesions of the NAc core impaired the ability of rats to discriminate between a CS+ predictive of reward and a CS- with no predictive value. Furthermore, depletion of dopamine in the NAc resulted in similar deficits in the acquisition and expression of approach behaviors (Parkinson *et al.*, 2002). These results echoed an earlier finding that dopamine antagonists infused into the NAc core impaired approach behavior in ways dissociable from glutamate antagonists (Di Ciano *et al.*, 2001).

Although previous studies identified important roles for specific NAc subnuclei and neurotransmitter systems in mediating the Pavlovian approach response, the firing patterns of NAc cells during this process remain poorly understood. Here, we tested the hypothesis that individual NAc neurons encode both the predictive value of a stimulus and Pavlovian approach actions elicited by conditioned stimuli. *In vivo* electrophysiological methods were employed to characterize the activity of single NAc neurons during an autoshaping paradigm, where cues either predicted or did not predict sucrose delivery. Thus, the present study assessed the responsiveness of NAc neurons relative to a fundamental aspect of learning (prediction of reward), but also examined the relationship

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between learned cue–reward associations and biologically significant approach responses.

Materials and methods

Animals

Male, Sprague–Dawley rats ($n = 9$, Harlan Sprague–Dawley, Indianapolis, IN, USA) aged 90–120 days and weighing 260–330 g were used as subjects and individually housed with a 12 : 12 h light : dark cycle. All experiments were conducted between 09.00 and 13.00 h. Bodyweights were maintained at no less than 85% of pre-experimental levels by food restriction (10–15 g of Purina laboratory chow each day, in addition to approximately 1 g of sucrose consumed during daily sessions). This regimen was in place for the duration of behavioral testing, except during the postoperative recovery period when food was given *ad libitum*. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Autoshaping procedure

Experimental sessions occurred in $43 \times 43 \times 53$ cm Plexiglas chambers (Medical Associates, St. Albans, VT, USA) housed within sound-attenuating boxes (Fibrocrete, Crandall, GA, USA). Two symmetrically located retractable levers (Colburn Instruments, Allentown, PA, USA) were placed 17 cm apart on one wall of the chamber. Cue lights were positioned above each lever, but were not active in this experiment. A food receptacle was centered between the levers, 2.5 cm from the floor. A house light was centrally located on the wall opposite the food receptacle and levers, 2 cm from the ceiling.

A session began by placement of an animal into the chamber with the house light illuminated and white noise present. Daily 1 h sessions consisted of 50 experimental trials. On 25 trials, one retractable lever (the CS+) was inserted into the chamber for 10 s and then retracted, immediately followed by delivery of a 45-mg sucrose pellet (Sucrose Reward Formula F; Noyes, Lancaster, NH, USA) into the food receptacle. On the other 25 trials, a separate lever (the CS–) was inserted into the chamber for 10 s and then retracted. Importantly, the retraction of the CS– lever was never followed by the unconditioned stimulus (UCS, sucrose). Thus, the CS+ was a positive predictor of sucrose, whereas the CS– was not. Trials were initiated on a variable schedule every 45–75 s; the average intertrial interval was 60 s. Trial type (i.e. CS+ or CS–) was chosen semi-randomly, with no more than two trials of either type occurring in sequence. Additionally, the CS+ lever was counterbalanced left–right across animals and across experimental chambers. Contact with each CS, registered as a lever press, was recorded during every trial. However, lever pressing had no programmed consequences, as sucrose delivery was not dependent upon contact with the CS+. As in previous studies, repeated contact with either cue was interpreted as approach behavior (Di Ciano *et al.*, 2001). After 10 training sessions, all rats exhibited approach responses on more than 80% of CS+ trials, and were surgically prepared for electrophysiological recording.

Surgery

Animals were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (20 mg/kg), and microelectrode arrays were implanted with the NAc, using established procedures (Carelli

et al., 2000). Electrodes were custom-designed and purchased from a commercial source (NB Laboratories, Dennison, TX, USA). Each array consisted of eight microwires (50 μ m diameter) arranged in a 2×4 bundle that measured ~ 1.5 mm anteroposterior and ~ 0.75 mm mediolateral. Arrays were targeted for permanent, bilateral placement in the core and shell subregions of the NAc (AP, +1.3 to 1.7 mm; ML, ± 0.8 or 1.3 mm; DV, -6.2 mm; all relative to bregma on a level skull; Paxinos & Watson, 2005). Ground wires for each array were coiled around skull screws and placed 3–4 mm into the ipsilateral side of the brain, ~ 5 mm caudal to bregma. After implantation, both arrays were secured on the skull using surgical screws and dental cement. All animals were allowed at least 6 postoperative recovery days before being reintroduced to the autoshaping paradigm. Thereafter, animals underwent a final autoshaping training session to ensure stable approach responding before the commencement of electrophysiological recordings during the final test day.

Electrophysiological recordings

Electrophysiological procedures have been described in detail previously (Carelli *et al.*, 2000; Carelli, 2002b; Hollander & Carelli, 2005). Before the start of the recording session, the subject was connected to a flexible recording cable attached to a commutator (Medical Associates) that allowed virtually unrestrained movement within the chamber. The headstage of each recording cable contained 16 miniature unity-gain field effect transistors. NAc activity was recorded differentially between each active and inactive (reference) electrode from the permanently implanted microwires. The inactive electrode was examined before the start of the session to verify the absence of neuronal spike activity and served as the differential electrode for other electrodes with cell activity. Online isolation and discrimination of neuronal activity was accomplished using a neurophysiological system commercially available (multichannel acquisition processor, MAP System, Plexon, Dallas, TX, USA). Multiple window-discrimination modules and high-speed analog-to-digital (A/D) signal processing in conjunction with computer software enabled isolation of neuronal signals based on waveform analysis. The neurophysiological system incorporated an array of digital signal processors (DSPs) for continuous spike recognition. The DSPs provided a continuous parallel digital output of neuronal spike events to a Pentium computer. Another computer controlled behavioral events of the experiment (Medical Associates) and sent digital outputs corresponding to each event to the MAP box to be time stamped along with the neural data. The neurophysiological system has the capability of recording up to four neurons per microwire using real-time discrimination of neuronal action potentials. However, in the present study one–two neurons were typically recorded per active microwire (Chang *et al.*, 1994; Nicolelis *et al.*, 1997; Roitman *et al.*, 2005). Principal component analysis (PCA) of continuously recorded waveforms was performed prior to each session and aided in the separation of multiple neuronal signals from the same electrode. This sophisticated analysis generates a projection of waveform clusters in a three-dimensional space, enabling manual selection of individual waveforms. Before the session, an individual template made up of many ‘sampled’ waveforms was created for each cell isolated using PCA. During the behavioral session, waveforms that ‘matched’ this template were collected as the same neuron. Cell recognition and sorting was finalized after the experiment using the Offline Sorter program (Plexon), when neuronal data were further assessed based on PCA of the waveforms, cell firing characteristics, and interspike intervals.

Data analysis

Pavlovian approach responses directed at conditioned stimuli were recorded as lever presses. These data are presented here as the probability of approach for each CS, or the number of trials in which the animal made at least one press on that lever divided by 25 (the total number of trials). The differential acquisition of stimulus-selective conditioned appetitive responses was evaluated using a within-subjects cue (two levels, CS+ and CS-) \times session (12 levels) repeated-measures ANOVA (Statistica, StatSoft, Tulsa, OK, USA). Newman-Keuls *post hoc* tests were used to elucidate time-specific differences between the cues (CS+ and CS-). Further analyses compared cue-specific contact (mean lever presses during each second of cue presentations) during the test session using a within-subjects cue (two levels) \times time (10 levels), repeated-measures ANOVA. Statistical significance was assessed using an alpha level = 0.05.

Neuronal firing patterns in the NAc were characterized using raster displays and peri-event histograms (PEHs) constructed with commercially available software (NeuroExplorer, Plexon). These displayed the activity of each cell during three time epochs: (1) a 'baseline' (-10 to 0 s) period prior to CS presentation; (2) the 10-s cue presentation (0-10 s); and (3) the 10 s following the CS presentation (10-20 s). The result was a histogram that represented cell firing throughout a 30-s window surrounding CS+ and CS- presentations and sucrose delivery (CS+ only). Consistent with other reports from this laboratory (e.g. Carelli *et al.*, 2000; Roitman *et al.*, 2005), NAc cells were described as 'phasically active' during a given epoch if rates of cell firing increased or decreased by 40% from baseline rates. Here, cells were classified as having either an 'excitatory' or 'inhibitory' response to the CS+ or CS- if they exhibited a 40% rise or fall in activity during any of the first three 1-s time bins of the CS presentations. In cases where a cell showed both an excitation and inhibition during the initial 3 s of CS onset, the first response was used for classification. Cellular responses to the UCS were similarly assessed during the 5-s period that followed CS+ retraction/sucrose delivery. Cells that exhibited no phasic response to the CS+ were classified as CS- specific (displaying a change in activity in the first 3 s of the CS- presentation only), sucrose specific (displaying a change in firing rate during the 5 s following the sucrose delivery, but no response to either cue) or non-phasic (no change in firing rate relative to any experimental event).

The primary goal of this study was to compare cellular responsiveness to cues that did and did not elicit approach responses. Therefore, neuronal data for the excitatory and inhibitory subpopulations of CS+ responsive cells were separately averaged. Then, the mean firing rate for these populations during the CS- presentation was plotted, allowing for a direct comparison between the changes in neural activity evoked by these cues. The effect of a cue's predictive value (and ability to evoke approach responses) on neuronal firing rates was assessed independently for CS+ excitatory and inhibitory cell populations using repeated-measures cue (two levels) \times time (11 levels, including baseline) ANOVAs that compared mean baseline firing rates with mean activity during each 1-s bin of the cue periods. Newman-Keuls *post hoc* tests of the interaction terms were used to determine the time bins at which firing rate differed significantly from baseline or at which a cue difference existed. Signal-to-baseline (S : B) ratios for CS+ excitatory and CS+ inhibitory populations were computed by dividing the 'signal' (firing rate for an individual cell during a single second of the cue period) by the baseline (averaged activity for that cell during the 10 s prior to cue presentation). This analysis controlled for the heterogeneity in baseline firing rates among cells, providing a more

conclusive and substantive examination of cue-induced changes in neuronal activity.

Histology

Upon completion of the experiment, rats were deeply anesthetized with a ketamine and xylazine mixture (100 mg/kg and 20 mg/kg, respectively). In order to mark the placement of electrode tips, a 15 μ A current was passed through each microwire electrode for 5 s. Transcardial perfusions were then performed using physiological saline and 10% formalin, and brains were removed. After postfixing and freezing, 50- μ m coronal brain sections were mounted and stained with thionin and potassium ferricyanide to reveal a blue reaction product corresponding with the location of an electrode tip. The specific position of individual electrodes was assessed by visual examination of successive coronal sections. Placement of an electrode tip within the NAc core or shell was determined by examining the relative position of observable reaction product to visual landmarks (including the anterior commissure and the lateral ventricles) and anatomical organization of the NAc represented in a stereotaxic atlas (Paxinos & Watson, 2005). Statistically significant differences in the distribution of excitatory and inhibitory cells in the core and shell subregions were assessed by performing a Chi-square test on the frequency of observed cell types.

Results

Pavlovian approach behavior

Animals rapidly acquired approach behaviors directed at reward-predictive stimuli. Figure 1A displays the mean probability of approach for all rats across sessions for each cue. On the initial day of testing, naïve rats rarely approached either cue. However, as the number of CS-UCS pairings increased, the probability of approach behaviors directed at the CS+ also increased. In contrast, approaches directed at the CS- did not change. By the final test session, approach probability was stable (mean CS+ approach probability, 0.97 ± 0.02 ; mean CS- approach probability, 0.08 ± 0.05). A repeated-measures ANOVA revealed a main effect of cue ($F_{1,8} = 151.14$; $P < 0.01$) and a main effect of session ($F_{11,88} = 14.23$; $P < 0.01$) on approach probability. Furthermore, a cue-session interaction was present ($F_{11,88} = 28.81$; $P < 0.01$), demonstrating that the variance in approach responding was session-specific. *Post hoc* comparisons revealed that rats approached the CS+ more than the CS- at all time points after the third session (all $P < 0.05$). The probability of approach towards the CS- never increased from levels achieved in the initial session ($P > 0.05$ for all *post hoc* comparisons). The approach response towards the CS+ typically occurred within seconds of its onset. Figure 1B displays an average histogram of the latency to approach the lever (measured as the first press following CS+ extension).

In addition to discriminated approach behavior, rats also exhibited more contact with the CS+, as measured by the number of lever presses during cue periods. This contact typically took the form of biting, grasping and pawing at the lever. Figure 1C shows the mean number of lever presses for all rats during each second of the CS+ and CS- presentations on the test day. A repeated-measures ANOVA revealed a significant effect of cue on lever contact ($F_{1,8} = 98.09$; $P < 0.01$), but no significant effect of time and no interaction between cue and time. The CS+ engendered significantly more lever presses than the CS- during each time bin. Moreover, lever contact was

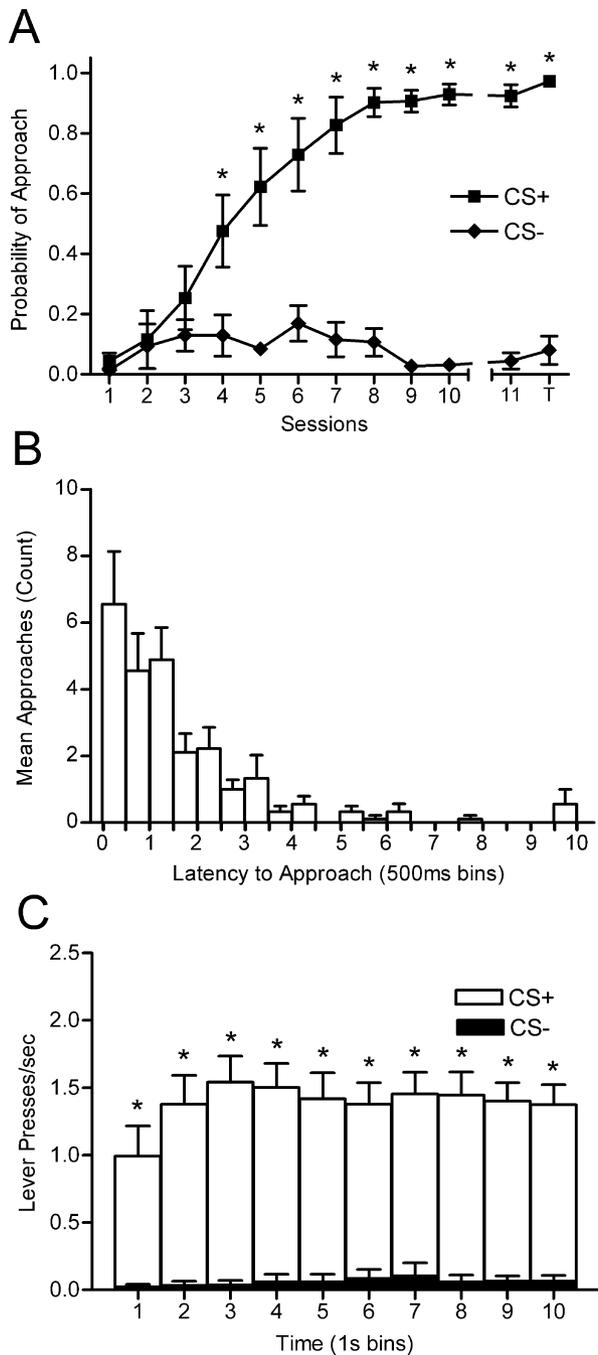


FIG. 1. Behavioral performance during autoshaping paradigm. (A) Acquisition of Pavlovian approach responses. Animals ($n = 9$) approached the predictive conditioned stimulus (CS+) significantly more than the non-predictive conditioned stimulus (CS-) after session 3 ($*P < 0.05$ stimulus comparison). After 10 training sessions animals received surgical implantation of microelectrode arrays (indicated by break in graph) followed by one post-surgical recovery session (session 11). Electrophysiological data were collected during the test session completed the next day (indicated by 'T'). (B) Averaged histogram of latency to approach the CS+ on the test day. The majority of approach responses occurred within seconds of the CS+ presentation. (C) Lever presses per second on the test day. Animals made more contact with the CS+ than the CS- in every 1-s time bin. After the initial approach, contact with the CS+ was maintained for the duration of the cue period.

relatively stable during the CS+ presentation; the rate of lever presses was not significantly increased or decreased across the 10-s presentation.

NAC neurons selectively encode aspects of the autoshaping paradigm

Our primary goal was to characterize the activity of NAC neurons during the presentation of cues that either predicted or did not predict sucrose. Thus, although we observed phasic cellular responses to the CS+, CS- and the UCS, we first sought to characterize response types based on firing rates relative to the CS+. Of 76 NAC neurons isolated and recorded during the autoshaping task, 57 (75%) showed a phasic change (increase or decrease) in firing rate within seconds of the CS+ onset. Of these neurons, 27 cells responded with excitations in firing rate, while 30 responded with inhibitions. Nearly half of all CS+ responsive cells (28 out of 57, or 49%) exhibited opposite or non-phasic responses during the CS- period. Only 19 neurons did not show a phasic change in firing pattern relative to the CS+, but the majority (15/19 cells) were responsive during other aspects of the task. Specifically, some neurons ($n = 4$) responded to the CS- alone, while another population of cells ($n = 11$) responded to sucrose delivery alone. Finally, the remaining four cells exhibited no phasic patterned activity during the autoshaping task, classified as non-phasic. A more detailed description of each type of neuronal pattern during the autoshaping procedure is presented below.

Responses to sucrose – predictive cue (CS+)

The rasters/PEHs in Fig. 2 show the activity of a single NAC neuron that was excited during CS+ presentation, across baseline, cue and post-cue epochs. This neuron exhibited a baseline firing rate of 0.28 Hz, but peaked to 10.72 Hz during the first second of the CS+ presentation (top panel). The firing rate then declined monotonically during the remainder of the CS+ period, spiked again upon lever retraction and sucrose delivery, and remained elevated during sucrose consumption. In contrast, during the CS- presentation (bottom panel) the same cell exhibited no substantial changes in activity compared with baseline firing.

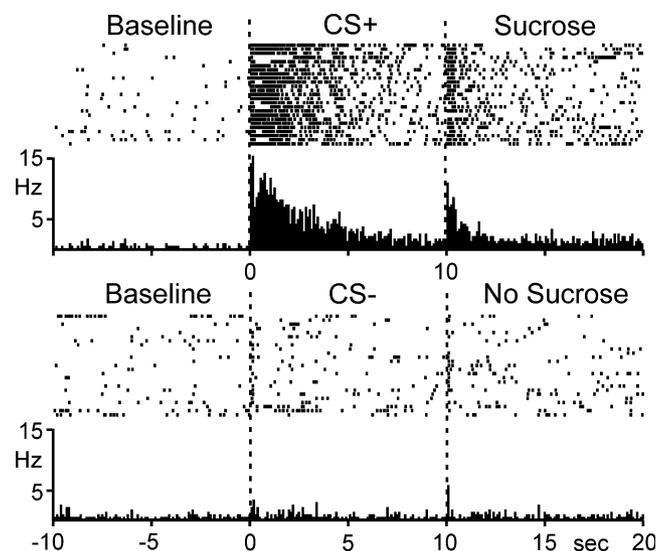


FIG. 2. Single NAC neuron showing a characteristic increase in firing rate within seconds of the CS+ (top panel) but not the CS- (bottom panel) presentation. Top, raster plot and PEH in a 30-s window relative to CS+ extension (first dashed line) and lever retraction/sucrose delivery (second dashed line). Bottom, raster plot and PEH for the same neuron during a 30-s window surrounding the CS- period, after which no sucrose was delivered.

The mean activity of all CS+ excitatory cells ($n = 27$; mean baseline firing rate \pm SEM = 2.62 ± 0.93) is illustrated in the composite PEHs in Fig. 3. Interestingly, the majority of CS+ excitatory cells (19 out of 27, or 70%) also responded to the CS- with excitations. However, as is evident in Fig. 3, the magnitude of the composite response for the CS- was relatively blunted as compared with the CS+ response. A repeated-measures ANOVA on mean cell firing during these periods revealed a main effect of time ($F_{10,260} = 4.26$; $P < 0.01$), as well as a significant interaction between cue and time ($F_{10,260} = 1.87$; $P < 0.05$). There was no main effect of cue ($F_{1,26} = 2.40$; $P = 0.13$). Newman-Keul *post hoc* comparisons revealed that firing rate was significantly increased over baseline rates during each 1-s time bin of the CS+ period (all $P < 0.05$). In contrast, firing rate was only significantly increased during the first second of the CS- presentation. Additionally, firing rate was significantly higher in the first, second, third, fifth and sixth seconds of the CS+ period than during the same seconds of the CS- period. Thus, this population of neurons responded to the sucrose predictive cue with robust excitations that were strongest during the first few seconds of the cue presentation. The same cells responded to the non-predictive cue with transient, less substantial excitations.

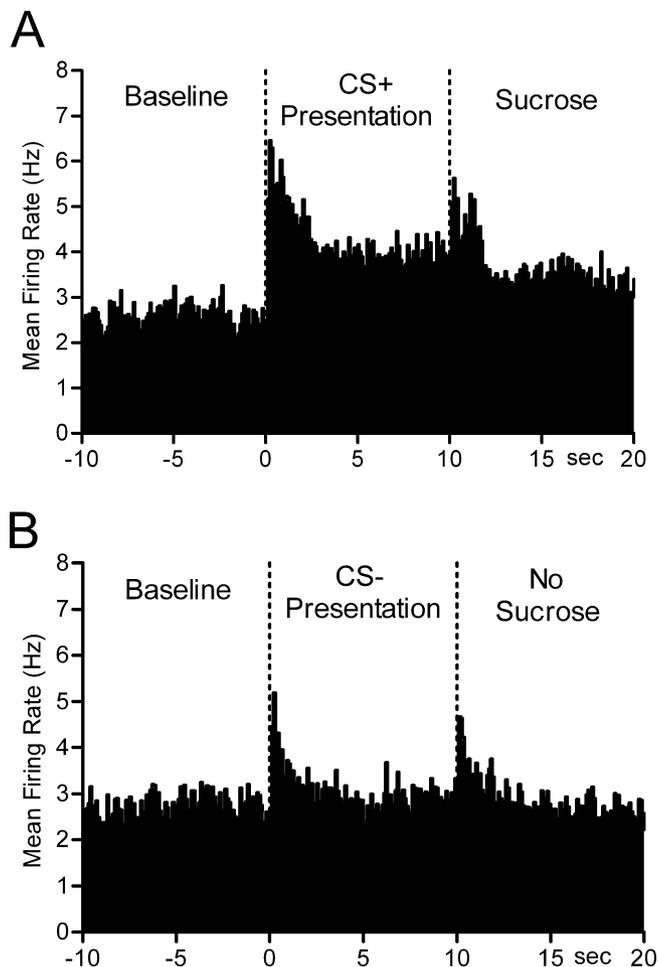


FIG. 3. Composite PEHs showing average activity of CS+ excitatory NAC neurons ($n = 27$). (A) NAC neurons exhibit excitatory responses to a sucrose predictive cue. Composite PEH of NAC neurons during a 30-s window surrounding the CS+ presentation (first dashed line) and lever retraction/sucrose delivery (second dashed line). (B) Composite PEH showing the same neurons during the CS- extension and retraction. Excitations were of lesser magnitude and shorter duration.

Figure 4 shows an example of a representative CS+ neuron that displayed an inhibition in cell firing during the CS+ period. This neuron had a baseline firing rate of 3.8 Hz, but fired at 1.44 Hz or lower during each second of the CS+ presentation. Cell activity returned to baseline levels following cue retraction, and then increased steadily until roughly 8 s after sucrose delivery. Conversely, no changes were observed during the CS- presentation. The inhibitory response of this neuron was therefore selective for the predictive cue.

Composite PEHs showing the average activity of all CS+ inhibitory neurons ($n = 30$; mean baseline firing rate \pm SEM = 1.909 ± 0.51) relative to CS+ and CS- presentations are displayed in Fig. 5. The mean firing rate of CS+ inhibitory cells was significantly affected by cue ($F_{1,26} = 23.09$; $P < 0.01$) and time ($F_{10,260} = 3.59$; $P < 0.01$), as assessed by a repeated-measures ANOVA. In addition to these main effects, there was a significant interaction between cue and time ($F_{10,260} = 6.82$; $P < 0.01$). *Post hoc* comparisons revealed that mean firing rate was significantly decreased from baseline at all time points during the CS+ presentation (all $P < 0.05$). Furthermore, neuronal activity during the CS+ period was lower than activity during the CS- period for every 1-s time bin ($P < 0.05$ for each second). On average, these cells exhibited no patterned phasic response to the cue that did not precede sucrose.

S : B ratios were calculated for CS+ excitatory and CS+ inhibitory neurons to control for differences in baseline firing rates across cells, and provide more detailed information regarding second-to-second changes in cell firing during each cue presentation. S : B ratios across time for CS+ excitatory and CS+ inhibitory neurons are shown in Fig. 6A and B, respectively. The mean S : B ratio of CS+ excitatory neurons ($n = 27$) increased to over 3 during the first second of the CS+, signifying a 200% increase in cell firing relative to baseline. In contrast, the same cells exhibited more modest increases ($\sim 70\%$) during the first second of the CS- period. The excitatory action of these cells was evident for the duration of the CS+ period, but returned to baseline levels for the majority of the CS- period. The mean S : B ratio of CS+ inhibitory cells was even more striking (Fig. 6B). At each time point during the presentation of the sucrose predictive cue, the S : B ratio was close to

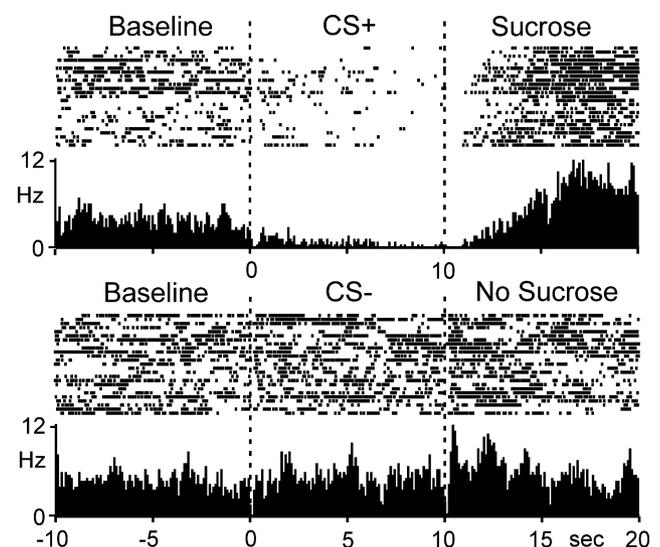


FIG. 4. Example NAC neuron showing a decrease in firing rate during the CS+ but not the CS- presentation. Top, raster plot and PEH in a 30-s window relative to CS+ extension (first dashed line) and lever retraction/sucrose delivery (second dashed line). Bottom, raster plot and PEH for the same neuron during a 30-s window surrounding the CS- period, after which no sucrose was delivered.

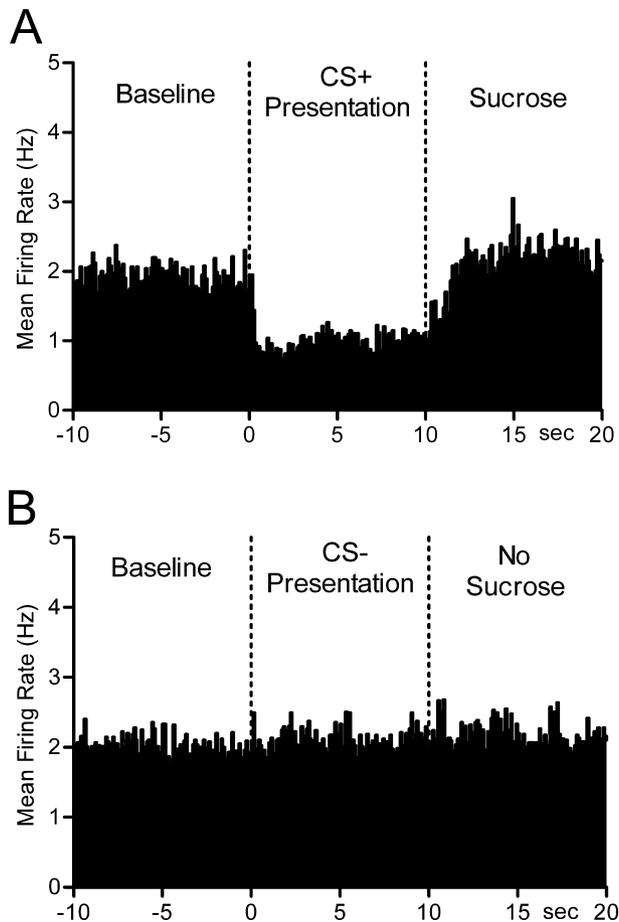


FIG. 5. Composite PEH showing average activity of CS+ inhibitory NAc neurons ($n = 30$). (A) Composite PEH of NAc neurons during a 30-s window surrounding the CS+ presentation (first dashed line) and lever retraction/sucrose delivery (second dashed line). NAc neurons exhibit robust inhibitions during the presentation of a sucrose-predictive cue. (B) Composite PEH showing no change in cell firing for the same neurons during a 30-s window surrounding the CS- presentation.

0.5, meaning that the firing rate of these cells was reduced by half. Conversely, the mean S : B ratio of the same cells during the CS- presentation was never below 1, indicating that activity of this subpopulation did not change relative to the non-predictive cue.

Responses to non-predictive cue (CS-)

Fifty-five percent of all NAc neurons were phasically active during the CS- period, with 29 cells exhibiting an excitatory response and only 13 displaying an inhibitory response. However, of 76 neurons recorded in this study, only 4 (5%) responded to the CS- but not the CS+. Three of these cells exhibited an excitation when the CS- was presented. A representative example of this cell type is displayed in Fig. 7A. During the baseline period for the CS+, this cell fired at an average of 2.95 Hz. The activity of this cell did not deviate by more than 40% from this rate during any 1-s bin of the sucrose-predictive cue. The baseline rate preceding the CS- period was 2.92 Hz, but increased to 5.0 Hz during the first second of the CS- presentation, a clear excitatory response. Firing rate returned to baseline levels during the remainder of the CS- presentation, and exhibited another excitation (peaking at 6.28 Hz) upon lever retraction. As the occurrence of this cell type (CS- specific) was infrequent, composite data are not presented here.

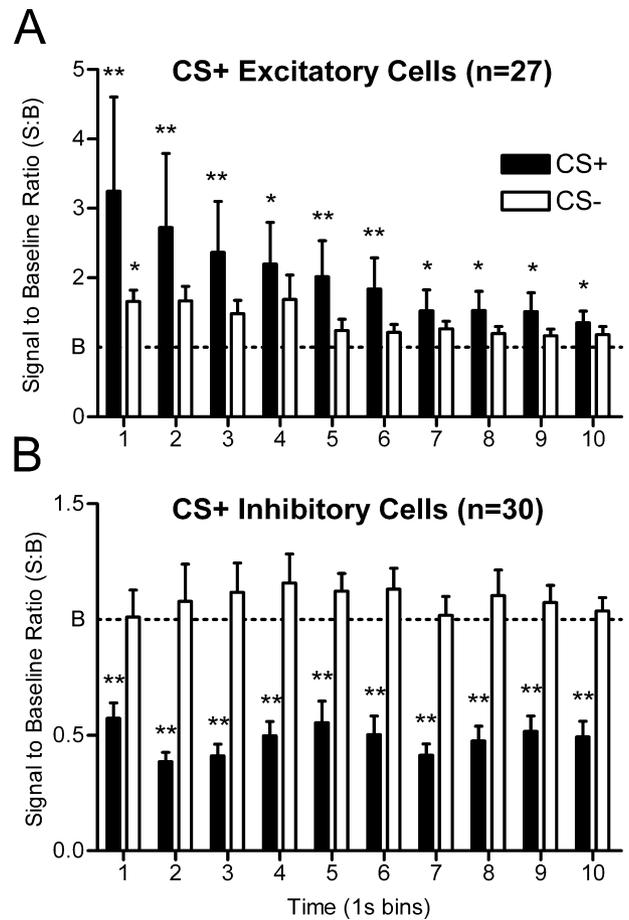


FIG. 6. Signal-to-baseline ratios (S : B) for CS+ excitatory and CS+ inhibitory neurons shows differential responsiveness during sucrose predictive vs. non-predictive cues. (A) Average S : B ratios for CS+ excitatory cells during each second of the CS+ and CS- presentations (see Materials and methods for details). While the CS- elicited significant excitations only in the initial second, the CS+ produced significant increases in firing rate for each time point. (B) Average S : B ratios for CS+ inhibitory cells during each second of the CS+ and CS- presentations. The mean firing rate during the CS+ period was significantly decreased from baseline rates and was lower than activity during the corresponding time points of the CS- period. *Significant ($P < 0.05$) change in mean firing rate from baseline levels; **significant ($P < 0.05$) difference in mean firing rate between stimuli for that second and in mean firing rate from baseline levels.

Responses to sucrose

Sixty-eight NAc units (89%) exhibited changes in firing rate within 5 s following sucrose delivery. Eleven (14%) of these cells were not responsive to either cue, but exhibited phasic changes in cell firing only during sucrose delivery and consumption. Of this group, nine cells displayed robust excitations in response to sucrose, whereas two responded with inhibitions. Figure 7B shows an example of a single representative neuron that displayed an excitatory response during sucrose availability alone. Cell activity was stable during the baseline periods (CS+ baseline, 3.97 Hz; CS- baseline, 3.72 Hz) and did not deviate during the presentation of either cue. However, 4 s after the retraction of the CS+, the firing rate was elevated to 14.4 Hz. There was no excitatory response during the same period following the retraction of the CS-. Indeed, the phasic response of this cell was selective for sucrose, and was not influenced by predictive or non-predictive cues. Again, because this and similar cells accounted for a relatively small

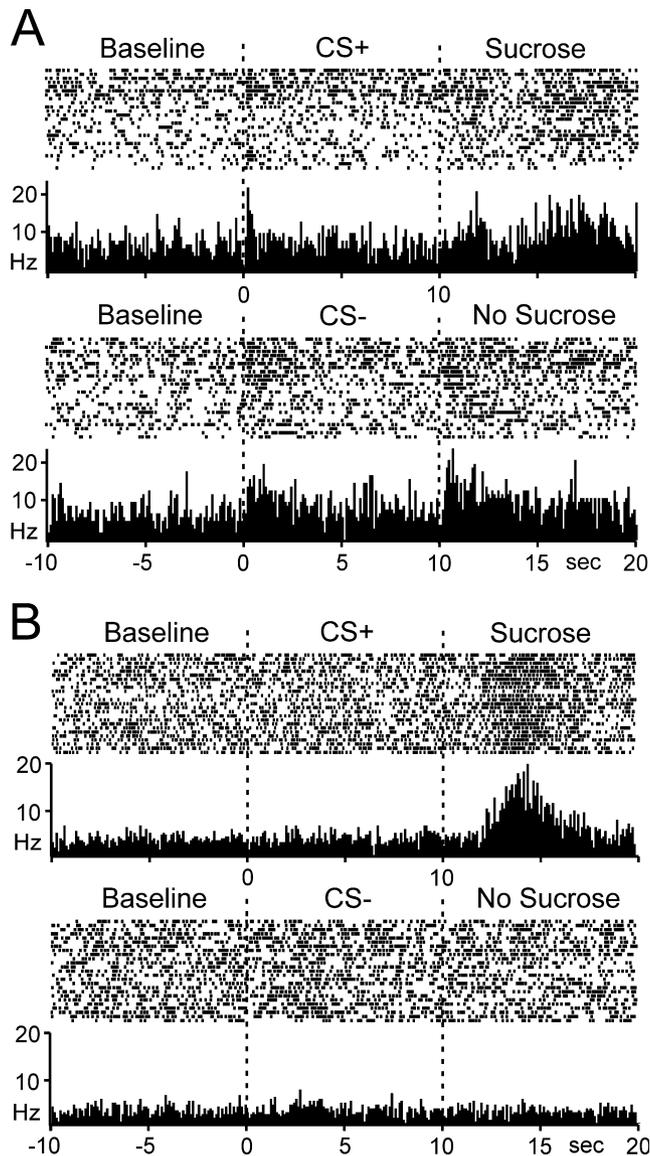


FIG. 7. A minority of NAc neurons exhibited phasic responses to events other than the CS+. (A) Raster and PEH for single NAc unit exhibiting a 40% increase in firing rate when the CS- was presented, but no significant change when the CS+ was presented. Only 5% of neurons recorded here responded to the CS- but not the CS+. (B) Raster and PEH for individual sucrose-responsive NAc cell. This cell showed no changes in activity when either cue was presented, but was specific to the period after sucrose delivery. Only 14% of cells responded to sucrose alone.

percentage of overall patterned responding, composite data are not shown here.

Histology

A total of 144 microwire electrodes (16 per animal) were implanted in the brains of nine animals. Forty-nine neurons were recorded from 72 wires histologically verified to be in the NAc core. Likewise, 27 neurons were recorded from 44 wires located in the shell. Thus, not every wire yielded neural data and, in some cases, more than one cell was recorded per wire (i.e. there was not a one-to-one correspondence between each wire and neural data). Additionally, 17 individual neurons were recorded on the remaining 28 electrodes placed outside

of the NAc. Because those wires were distributed randomly across multiple neural structures, resulting data were excluded from this study. Across all animals, bilateral electrode placements in the NAc ranged from 0.96 to 2.52 mm anterior to bregma and 0.5–2.5 mm lateral to the midline. Figure 8A shows the distribution of marked electrode tip placements across all animals according to the stereotaxic atlas of Paxinos & Watson (2005). Only electrodes that recorded at least one neuronal waveform are included in this figure.

Figure 8B shows the observed frequency of cellular response types across stimuli (as a percentage of total neurons recorded in the core or shell). There was a differential distribution of neurons that exhibited excitations to the CS+ or CS- during the task. Specifically, a significantly greater percentage of neurons in the NAc core displayed excitations to conditioned stimuli ($\chi^2 = 6.503$, $P = 0.01$ for CS+ $\chi^2 = 5.615$, $P = 0.017$ for CS-). However, no significant differences were observed between excitatory responses in core and shell neurons following the UCS ($P = 0.39$). Additionally, there were no significant core/shell differences in the proportion of neurons that displayed inhibitory responses to the conditioned cues or the UCS (all $P > 0.05$). It is important to note that there was overlap in populations of neurons that were classified across the dimensions shown in Fig. 8B. In other words, the CS+, CS- and UCS categories constructed in this figure are not mutually exclusive. For example, an individual neuron could be both inhibitory relative to the CS+ presentation and excitatory relative to UCS delivery.

Discussion

The present study offers the first evidence of patterned NAc activity during the execution of simple Pavlovian approach behaviors toward stimuli that predict rewards. Consistent with other reports (Bussey *et al.*, 1997; Di Ciano *et al.*, 2001; Cardinal *et al.*, 2002), rats rapidly acquired approach responses directed almost exclusively at a CS+ that predicted sucrose delivery. A series of studies have demonstrated that such repetitive approach behaviors are subserved by key interactions between the NAc and associated nuclei (Parkinson *et al.*, 2000, 2002; Di Ciano *et al.*, 2001; Cardinal *et al.*, 2002). The present results confirm those reports and provide insight into cellular mechanisms involved in autoshaping. Specifically, the vast majority of NAc cells recorded here exhibited robust, phasic changes in firing rate within milliseconds of CS+ onset. However, several subpopulations were observed. One subset was characterized by sustained increases in firing rate during CS+ presentation. This population also responded to the seldom-approached CS-, but excitations were significantly smaller in magnitude than those observed for the CS+. Another group of neurons showed significant inhibitions in firing rate for the duration of the CS+ period. The same cells exhibited little or no change in activity during the CS- period, indicating a relative specificity for stimuli that predict sucrose and elicit approach responses. The finding that NAc neurons demonstrate two distinct patterned responses during autoshaping corroborates evidence suggesting that functional ensembles – or coordinated neuronal networks – in the NAc act to integrate diverse signals and carry out different tasks during reward-related events (Carelli & Wightman, 2004). Additionally, our observation that the firing patterns reported here were differentially distributed in the NAc core and shell suggests that these subregions play different roles during autoshaping.

A cue predicting a natural appetitive stimulus (sucrose) elicited striking excitations in a considerable percentage (36%) of NAc neurons (classified as CS+ excitatory cells). Recent investigations have reported similar excitations evoked by cues signaling both drug and food rewards (Nicola *et al.*, 2004a). These excitations may originate from

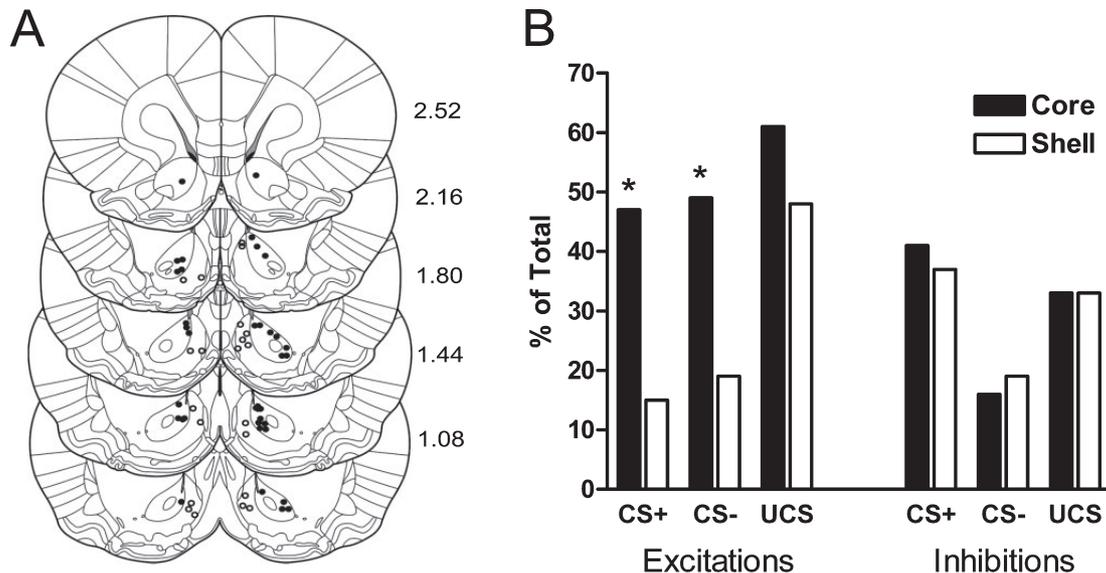


FIG. 8. Anatomical distribution of electrode placements and cell response types. (A) Coronal diagrams displaying marked electrode tip locations across all nine animals. Marked locations are limited to electrodes that contributed neuronal data. Filled circles indicate wires located in the NAc core subregion; open circles indicate wires located in the NAc shell. Electrode tip locations were marked by the presence of a blue dot reaction product. Numbers to the right indicate the anteroposterior coordinates (in mm) rostral to bregma. Coordinates were taken from the stereotaxic atlas of Paxinos & Watson (2005). (B) Percentage of phasic neurons in the core and shell subregions of the NAc, separated by response type and stimulus. Significantly more neurons in the NAc core responded to conditioned stimuli with an excitation ($*P < 0.05$). There were no core/shell differences with respect to unconditioned stimuli (UCS) excitations or inhibitions relative to any stimulus.

glutamatergic inputs from cortical and limbic structures (Pennartz *et al.*, 1994; Nicola *et al.*, 2000, 2004a; Wilson & Bowman, 2004). However, temporally similar cue-evoked excitations have also been observed in dopamine neurons in rodent and primate striatum (Schultz *et al.*, 1993; Pan *et al.*, 2005). The degree of these responses vary based on the nature, size and certainty of the reward being predicted (Hassani *et al.*, 2001; Cromwell & Schultz, 2003; Fiorillo *et al.*, 2003). The dopaminergic innervation from the ventral tegmental area (VTA) is required for cue responses in the NAc (Yun *et al.*, 2004). Additionally, NAc dopamine concentration rapidly increases in response to predictive cues (Phillips *et al.*, 2003; Roitman *et al.*, 2004), supporting the hypothesis that NAc dopamine modulates the ability of glutamatergic inputs to drive NAc cell firing (Cepeda & Levine, 1998; Nicola *et al.*, 2000; Wise, 2004; Goto & Grace, 2005). Thus, while cortical areas could excite NAc neurons during autoshaping, dopaminergic signals may gate the nature and function of those excitations.

The magnitude of CS+ excitatory responses identified here may only partially depend on the predictive value of the cue. The non-predictive CS- also elicited significant (but relatively blunted) excitations in the same cells, but did not produce conditioned approach responses. Thus, the excitatory signal may not encode conditioned movements per se, but may simply reflect orienting responses directed at dynamic events in a changing environment, or rapid movements in general. While this is certainly a possibility, several studies suggest otherwise. Using detailed videotape analysis, Woodward and colleagues (Chang *et al.*, 1994) compared NAc cell activity relative to lever presses and orienting motor responses in a cocaine self-administration paradigm with similar movements that occurred during the intertrial interval. Those authors demonstrated that phasic patterns of cell firing in the NAc were specific to goal-directed events. NAc neurons did not exhibit patterned discharges (excitations or inhibitions) during the execution of similar actions that did not produce cocaine. Other investigations have corroborated these findings (Schultz *et al.*, 1992; Peoples *et al.*, 1998), suggesting that the

firing patterns of NAc neurons are specifically altered by reward-related events, and not movement alone.

It is possible that the excitations observed for the CS- may also be the result of conditioning history. Both NAc and dopamine neurons respond to predictive and non-predictive stimuli, and this effect had been attributed to stimulus generalization (Mirenowicz & Schultz, 1996; Nicola *et al.*, 2004a). In the present study, the CS+ and CS- were alike in every way except spatial location, leaving open the possibility that generalization occurred. As a predictive cue, the CS+ may produce a response among NAc neurons as its incentive value increases (Berridge & Robinson, 1998). Likewise, other stimuli that share its physical properties may develop their own salience and come to elicit similar cellular responses. The observation that excitatory responses were generally much larger relative to the CS+ onset seems to support the latter explanation. Furthermore, significant differences between CS+ and CS- excitations tended to occur early in the cue period (see Fig. 6), when spatial distinction between cues would be necessary to direct an approach response. Another intriguing possibility is that the CS- elicits a conditioned avoidance response in well-trained animals. If so, it seems likely that such a response would also be encoded by the NAc.

Another subset of NAc neurons (39%) exhibited strong inhibitions in cell firing for the duration of the CS+ (termed CS+ inhibitory cells). The same cells showed no change in activity when the CS- was presented, indicating that NAc inhibitions may be critical to the execution of approach responses. As discussed by Nicola *et al.* (2004b) and supported elsewhere (Kelley, 2004; Roitman *et al.*, 2005), NAc inhibitions may be linked to motor actions leading to or associated with food consumption. Decreases in the firing rate of NAc neurons are tightly correlated with oro-motor behavior directed at sucrose consumption (Roitman *et al.*, 2005), and also during operant responding for sucrose, water, cocaine and heroin (Carelli *et al.*, 1993; Carelli & Deadwyler, 1994; Chang *et al.*, 1994, 1996, 1997; Ghitza *et al.*, 2004; Nicola *et al.*, 2004a). Here, approach responses usually occurred less than 2 s after the CS+ presentation, and animals

maintained contact with the CS+ for the remainder of the cue period. The finding that inhibitory responses were time-locked to the initial approach as well as subsequent contact suggests that inhibitory signals may encode appetitive reactions to reward-predictive cues. A major output of the NAc is a γ -aminobutyric acid (GABA)ergic connection with the ventral pallidum (VP) (Usuda *et al.*, 1998). The inhibition of this projection would result in the disinhibition of VP neurons, which can elicit feeding behavior (Stratford & Kelley, 1999) and increase locomotion (Gong *et al.*, 1997). Thus, the NAc inhibitions observed here may act through the VP to sustain appetitive contact with the CS+. These inhibitions may originate from reciprocal connections between NAc neurons (Nicola *et al.*, 2004a). This hypothesis accounts for the observation that excitatory and inhibitory subpopulations tend to be of equal sizes, as reported here. Alternatively, an inhibitory response could arise from the GABAergic projection back from the VP, in which case the inhibition of NAc neurons would reflect (but not mediate) Pavlovian approach behaviors.

The magnitude of cue-related neural responses reported here, coupled with the prevalence of cells exhibiting such changes, provides compelling evidence that the NAc encodes aspects of the autoshaping paradigm. Electrophysiological investigations in the NAc typically report that less than half of recorded neurons exhibit a phasic change in firing rate relative to behavioral or environmental events (Chang *et al.*, 1996; Carelli *et al.*, 2000; Carelli, 2002b; Nicola *et al.*, 2004b). Here, 89% of NAc neurons exhibited robust changes in activity when either sucrose or a sucrose-predictive cue was presented. Some evidence suggests that NAc neurons can develop responses to an event even when they are not initially activated by that event (Carelli, 2002a; Hollander & Carelli, 2005). Interestingly, responses to cues in a subset of NAc neurons do not appear to be innate (i.e. they are not present in naïve animals), but emerge when cues predict appetitive or aversive events (Setlow *et al.*, 2003; Roitman *et al.*, 2005).

The high percentage of phasically active cells in the present study may indicate that the NAc becomes increasingly engaged after repetition of a behavior or a learned association. One theory holds that predictive stimuli promote a specific behavioral action by competing for motor resources controlled by striatal circuits (Pennartz *et al.*, 1994; Plenz, 2003). The response of NAc neurons to predictive cues may originate from this competition and produce the relatively inflexible approach responses observed here. Indeed, the NAc may add a compulsive element to the stimulus–outcome associations present in this design, allowing them to dominate behavioral output. Interestingly, approach responses persist even when they cancel reward delivery (Brown & Jenkins, 1968), supporting the idea that impulsivity and autoshaping may be linked phenomena that are both controlled by the NAc (Tomie *et al.*, 1998; Cardinal *et al.*, 2001; Winstanley *et al.*, 2005).

Several researchers have proposed that conditioned approach responses may be a central component of the drug-addicted state (Newlin, 1992; Tomie *et al.*, 2002; Everitt & Robbins, 2005). However, such assertions are not without controversy. Kearns & Weiss (2004) recently employed cocaine as a UCS in an autoshaping paradigm and found that, unlike sucrose, neither high nor low doses of cocaine are able to promote approach responses to stimuli that predict reward delivery. Additionally, requiring an artificial consummatory response before the cocaine infusion did not increase approach responding. This conclusion seems to suggest that cocaine alone cannot support Pavlovian approach responses. However, it is also possible that differences between cocaine and sucrose delivery, such as the timing of the cocaine infusion, may have contributed to the results. Indeed, a previous investigation demonstrated that an ingested ethanol/saccharine combination can support Pavlovian approach responses to spatial predictors (Krank, 2003). Clearly, future parametric studies will be required to determine

precise similarities and/or differences between natural and drug reinforcers with respect to conditioned approach liability.

In addition to describing the phasic activity of NAc neurons during autoshaping, our findings may also highlight potential differences between NAc subregions. Significantly fewer neurons in the NAc shell exhibited excitatory responses to conditioned cues, regardless of whether those cues elicited approach responses. While this discrepancy is based on relatively small sample sizes, and should be interpreted carefully, it is perhaps not surprising that we found differences between these two regions. Lesions and pharmacological manipulations in the NAc core disrupt selective approach towards predictive stimuli, indicating that this subregion may help organisms discriminate between biologically relevant and irrelevant cues (Di Ciano *et al.*, 2001; Cardinal *et al.*, 2002). Furthermore, recent electrophysiological studies have also unmasked distinctions between neural responses in the core and shell (Ghitza *et al.*, 2004, 2005). However, it should also be noted that there were no differences in the present study between the proportion of core and shell neurons that responded with inhibitions. While the NAc core and shell subregions have some overlapping outputs, they also send separate projections to functionally dissimilar regions. For example, the core sends outputs to the substantia nigra and dorsal globus pallidus, while the shell sends more axons to the VTA (Heimer *et al.*, 1991; Everitt & Wolf, 2002). At this time, the precise downstream significance of NAc excitations and inhibitions with respect to autoshaping are unclear.

The present results provide support for the hypothesis that limbic information concerning predictive stimuli is integrated by the NAc, which promotes the motor behaviors necessary to obtain rewards (Mogenson *et al.*, 1980). A recent review posited that critical interactions between the NAc core, central nucleus of the amygdala (CeN), and anterior cingulate cortex (ACC) underlie the proper acquisition and performance of the Pavlovian approach response (Robbins & Everitt, 2002), as lesions to the CeN or ACC also impair the acquisition of approach responding. Indeed, glutamatergic inputs from the ACC may disambiguate the CS+ and CS– during autoshaping, while the CeN may invigorate approach responding through control of midbrain dopaminergic signals to the NAc (Robbins & Everitt, 2002). In the context of drug addiction, conditioned cues may precipitate relapse by promoting impulsive, engrained approach responses via this circuitry (Robbins & Everitt, 2002). Understanding the functional interactions between limbic, cortical and striatal networks may elucidate the processes by which predictive cues come to exert powerful control over behavioral output.

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Abbreviations

ACC, anterior cingulate cortex; CeN, central nucleus of the amygdala; CS, conditioned stimulus; DSP, digital signal processor; GABA, γ -aminobutyric acid; NAc, nucleus accumbens; PCA, principal components analysis; PEH, peri-event histogram; S : B, signal-to-baseline; VP, ventral pallidum; VTA, ventral tegmental area; UCS, unconditioned stimulus.

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