nature neuroscience

Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens

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The ability to predict favorable outcomes using environmental cues is an essential part of learned behavior. Dopamine neurons in the midbrain encode such stimulus-reward relationships in a manner consistent with contemporary learning models, but it is unclear how encoding this translates into actual dopamine release in target regions. Here, we sampled dopamine levels in the rat nucleus accumbens on a rapid (100 ms) timescale using electrochemical technology during a classical conditioning procedure. Early in conditioning, transient dopamine-release events signaled a primary reward, but not predictive cues. After repeated cue-reward pairings, dopamine signals shifted in time to predictive cue onset and were no longer observed at reward delivery. In the absence of stimulus-reward conditioning, there was no shift in the dopamine signal. Consistent with proposed roles in reward prediction and incentive salience, these results indicate that rapid dopamine release provides a reward signal that is dynamically modified by associative learning.

Organisms forage and survive in demanding environments by learning about the events surrounding them and adapting their behavioral strategies accordingly. One simple, yet biologically critical, form of learning involves the ability to link environmental stimuli with favorable outcomes that they predict. Recent investigations indicate that midbrain dopamine neurons encode such stimulus-reward associations[1], and current hypotheses suggest that dopamine may act as a teaching signal, consistent with contemporary models of animal learning[2–5]. In these models, phasic changes in the firing rate of dopamine neurons are thought to provide a 'prediction error' signal that compares expected outcomes with actual outcomes[1,2,6]. Unexpected rewards produce brief synchronous bursts among dopamine neurons[1], whereas fully predicted rewards typically evoke little or no phasic activity. Moreover, the events that serve as predictors come to elicit brief dopamine bursts even though they often possess no inherent biological value, and the magnitude of these conditioned neuronal responses is correlated with the certainty of the reward being predicted[7]. This and other information provided by dopamine neurons may not only influence reward learning, but also affect decision-making strategies[8].

Dopamine neurons are not alone in processing reward-related information. In fact, expanding research has identified a distributed network of brain nuclei involved in this process. At the center of this network is the nucleus accumbens (NAc), which receives convergent glutamatergic input from the prefrontal cortex, hippocampus and basolateral amygdala, as well as a dopaminergic projection from the ventral tegmental area (VTA). The NAc projects to motor areas such as the ventral pallidum, making it an ideal location for detailed reward information to be turned into motivated action[9]. NAc neurons strongly encode stimuli that predict rewarding events[10,11], and dopaminergic input is required for NAc neurons to show such responses[12]. Furthermore, pharmacological manipulations in this region markedly affect the acquisition and expression of pavlovian conditioned responses[13–15], indicating that dopamine signaling in the NAc likely has a critical role in stimulus-reward learning.

Although dopamine neurons evidently provide a reward-prediction signal, it is unclear how this translates into dopamine release in target regions such as the NAc. This is a key concern for several reasons. First, there is not always a one-to-one relationship between dopamine cell firing and dopamine release, which is subject to active facilitation and depression by a number of terminal factors[16–18]. Indeed, direct stimulation of dopamine cell bodies can produce remarkably different release profiles depending on the recent history of release events[19]. Moreover, there are times at which such stimulation produces no detectable change in dopamine concentration ([DA]) at target areas[20]. Second, midbrain dopamine neurons project to multiple targets with different functional roles, including the prefrontal cortex, amygdala, dorsal striatum (caudate and putamen) and NAc. It is uncertain if these heterogeneous regions receive the same or even overlapping information from dopamine neurons, although several microdialysis studies suggest otherwise[14,21]. Finally, although the majority of electrophysiological examinations distinguish dopamine neurons based on waveform properties[7,22], it is unclear whether the neuronal population isolated using this method is limited to dopamine neurons[23,24]. Measuring dopamine release directly avoids all of these concerns, and the ability to do so on a subsecond timescale ensures that measurements are both physiologically and behaviorally relevant.

The present study investigated how stimulus-reward learning effects subsecond dopamine release in the NAc. *In vivo* detection of dopamine

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Received 9 March; accepted 15 May; published online 1 July 2007; doi:10.1038/nn1923

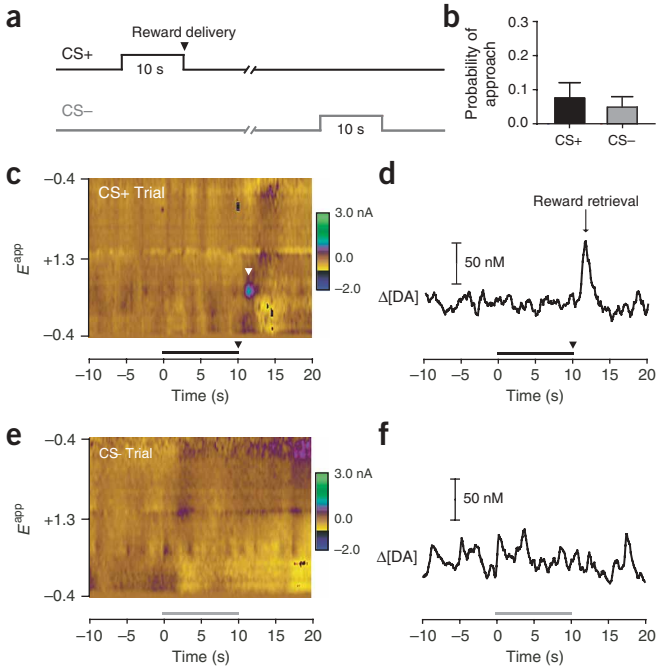

Figure 1 Early in associative learning, rapid elevations in NAc [DA] were time-locked to receipt of reward, but not to conditioned stimuli. (**a**) Conditioning procedure. Conditioned stimuli were semi-randomly presented to naive rats in a single conditioning block of 50 trials. The appearance of one stimulus (the CS+) predicted reward delivery (45-mg sucrose pellet), whereas the other stimulus (the CS−) did not. Each 10-s conditioned stimulus was presented 25 times. (**b**) Mean (± s.e.m.) approach probability. There was no cue difference in approach probability, indicating that rats made no behavioral distinction between stimuli. (**c**) Two-dimensional representation of electrochemical data collected during a single CS+ trial. The applied voltage (E^{app} ordinate) is plotted during a 30-s window surrounding CS+ presentation (horizontal black bar beginning at time-point zero, abscissa). Changes in current at a carbon-fiber electrode located in the NAc are indicated in color. The inverted black triangle denotes reward delivery, whereas the inverted white triangle marks reward retrieval. Dopamine is visible as a green-encoded spike in current at reward retrieval. (**d**) Differential [DA] obtained from representative example in **c**. Data are plotted relative to CS+ presentation (horizontal black bar) and reward delivery (inverted black triangle). On this trial, a robust increase in [DA] corresponded to reward retrieval. (**e**) Two-dimensional representation of electrochemical data during a CS− trial. The horizontal gray bar denotes cue presentation. (**f**) Differential [DA] obtained from representative example in **e**. No robust changes in [DA] were observed at any time point.

was accomplished using fast-scan cyclic voltammetry (FSCV), an electrochemical technique that permits rapid sampling on a timescale analogous to extracellular activity[25–27]. We first examined dopamine release characteristics in naive rats during a single conditioning block that paired an experimental stimulus with natural rewards. Next, we characterized dopamine signals in rats that received either many stimulus-reward pairings or unpaired stimuli and rewards. Consistent with its role as a reward-prediction error signal, our observations demonstrate that phasic dopamine release events in the NAc initially marked primary rewards, but shifted to a predictive cue following pavlovian conditioning. However, when the same stimulus was presented in an explicitly unpaired manner, primary rewards still evoked rapid dopamine release. Thus, midbrain dopamine reward signals are transmitted to the NAc and are dynamically modified as a result of pavlovian learning.

RESULTS

Phasic dopamine release during initial conditioning

Primary rewards produce bursts in the firing rate of dopamine neurons unless animals have learned to predict rewards using experimental cues[6]. However, important questions about this signal remain unanswered. For example, the majority of existing studies have only assessed dopamine signaling in well-trained or experienced animals, making it difficult to resolve dopamine's function when an organism is foraging and learning associations in novel environments. To address this issue, we carried out FSCV in experimentally naive rats ($n = 6$) during a single conditioning block that consisted of 50 discrete trials. On 25 trials, one conditioned stimulus (the CS+, a retractable lever and cue light) was presented for 10 s and then retracted. On retraction, a reward (45 mg sucrose pellet) was immediately delivered to a food receptacle (**Fig. 1a**). Thus, the CS+ predicted reward delivery on each trial, which was independent of any behavioral response. On the other 25 trials, another conditioned stimulus (the CS−, a spatially separate retractable lever and cue light) was presented for 10 s, but was not followed by a reward. Trial type was selected semi-randomly, with a variable intertrial interval (45−75 s; see Methods for details). Previous research using a similar conditioning design has shown that approach responses toward reward-predictive cues develop as a function of conditioning[10,13]. Termed 'sign-tracking' or 'autoshaping', these responses are believed to reflect pavlovian learning and the incentive salience of predictive cues[28–30]. These responses were therefore recorded and interpreted as a behavioral measure of the strength of stimulus-reward associations. We chose the NAc core as a dopamine detection site for FSCV in all experiments because this subregion receives input from dopamine axons and has a critical role in this form of associative reward learning[29,31,32].

Approach behaviors directed at the CS+ and CS− during the initial conditioning block were not statistically distinguishable from zero (both 95% confidence intervals contained 0) or from each other ($t = 0.933$, degrees of freedom (d.f.) $= 5$, $P = 0.39$; **Fig. 1b**), indicating that the animals did not behaviorally discriminate between the cues. To determine how conditioning and rewarding stimuli altered subsecond [DA] in the NAc core, we evaluated electrochemical data as single-trial traces (see **Fig. 1c–f** for representative CS+ and CS− traces from a single animal). Notably, a brief yet robust elevation in NAc [DA] occurred when this animal retrieved a sucrose reward from the food dish (**Fig. 1c,d**; timing of retrieval determined using detailed videotape analysis). In contrast, there were no phasic changes in NAc [DA] when the CS+ (**Fig. 1c,d**) and CS− (**Fig. 1e,f**) were presented. Realignment of the averaged electrochemical data with respect to reward retrieval for all animals (**Fig. 2a,b**) revealed a significant increase in extracellular [DA] at the precise time of retrieval ($F_{40,200} = 5.272$, $P < 0.001$; Tukey *post hoc* comparisons versus baseline $P < 0.05$ at −0.1 to 0.4 s surrounding sucrose retrieval). Thus, the phasic increase in NAc [DA] began before rewards were actually procured or consumed (**Fig. 2a**), indicating that visual, auditory, or even olfactory information may contribute to the initiation of this signal. Pooled across trials and animals, peak [DA] during sucrose retrieval was 42.9 ± 6 nM. Additionally, this reward-related increase in dopamine was not altered by conditioning, but was steady throughout the experimental session ($F_{1,111} = 0.08$, $P = 0.77$; test for linear trend between trial number and [DA] at sucrose retrieval; see **Fig. 2b**).

To determine whether dopamine signals gradually became time-locked to experimental cues as conditioning progressed (as electrophysiological findings would suggest[3]), we divided the initial

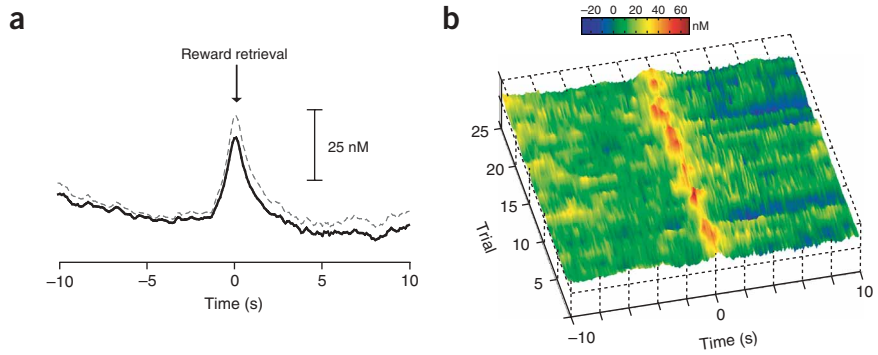

Figure 2 Rapid increase in NAc dopamine relative to reward retrieval during initial conditioning block. (**a**) Mean [DA] (solid line) ± s.e.m. (dashed line) relative to reward retrieval (time zero). At retrieval, [DA] was significantly higher than baseline levels. (**b**) Trial-by-trial mean [DA] relative to reward retrieval (at time zero). A reward-related increase in dopamine signal was observed early, and did not change throughout the conditioning session. Negative concentrations are considered because measurements are differential rather than absolute (see Methods for details).

conditioning session into blocks of five trials for both the CS+ and CS−. Neither cue produced an increase in NAc [DA] in the first block of trials ($P > 0.05$ for all comparisons; **Fig. 3a**, top traces), suggesting that cues did not initially evoke an increase in NAc dopamine. Visual inspection of mean [DA] from the final five trials revealed an apparent (but statistically insignificant; $P > 0.05$ for all comparisons) increase in [DA] within seconds of both CS+ and CS− onset (**Fig. 3a**, bottom traces). As the CS+ and CS− did not evoke significantly different changes in [DA] ($P > 0.05$) or approach probability (**Fig. 1b**), dopamine recordings were collapsed across cue type and examined in chronological order. Although cues did not produce a significant increase in [DA] on average, there was remarkable between-animal variability. NAc [DA] was significantly increased after cue presentation in four of six animals during the last ten trials ($P < 0.05$ in at least one time bin within 2 s of cue onset), whereas two animals showed no cue-evoked increase. Notably, the time interval between cue offset and reward retrieval during the entire session predicted the existence of a cue-related dopamine signal by the end of the session (**Fig. 3b**). Animals that retrieved the reward quickly after the CS+ elapsed showed a phasic dopamine response to cue (CS+ and CS−) onset by the end of the session, whereas those with a more delayed retrieval response did not show a significant cue-evoked response ($r^2 = 0.72$, $P < 0.03$; **Fig. 3b**; individual examples are provided in **Supplementary Fig. 1** online). For animals that showed relatively rapid (<5 s) retrieval responses during the session, cue-related dopamine signals increased in strength as a function of conditioning (positive linear relationship between the maximal change in [DA] produced by cues and trial number, $r^2 = 0.27$, $P < 0.001$; **Fig. 3c,d**). Even when cue responses developed, there was no significant difference in the magnitude of dopamine signals after the CS+ and CS− (comparison between CS+ and CS− maximum change in [DA] on last five trials, $P > 0.05$). Moreover, the development of a cue-evoked dopamine signal was not linked to a difference in general cue approach behavior or CS+/CS− discrimination ($P > 0.3$ for both t-tests). Thus, we observed no behavioral or electrochemical differences between the CS+ and CS− for this group during the first conditioning session.

Transition in dopamine release after associative learning

To further determine how pavlovian learning modified NAc dopamine signaling, we subjected another group of rats ($n = 6$) to a total of 12 conditioning sessions on 12 separate days. As above, each conditioning session consisted of 50 trials (25 CS+/reward and 25 CS−), and FSCV was carried out during the final conditioning session. A repeated measures ANOVA revealed a significant cue-session interaction in approach responding ($F_{11,110} = 21.57$, $P < 0.001$). Consistent with

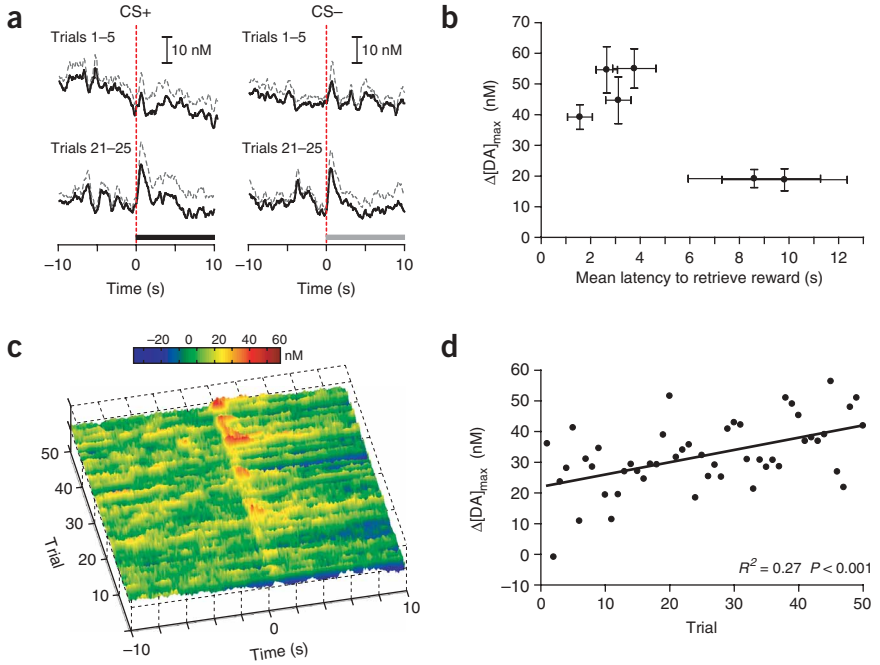

Figure 3 Dopamine signaling in response to conditioned stimuli during the initial conditioning block. (**a**) On average, neither the CS+ (horizontal black bar, left traces) nor the CS− (horizontal gray bar, right traces) elicited a significant increase in NAc [DA] during the first five or last five conditioning trials (mean ± s.e.m.). (**b**) Cue-evoked peak Δ[DA] (± s.e.m.) during the last ten trials (collapsed across cues) as a function of mean (± s.e.m.) latency to retrieve sucrose reward after CS+ offset for individual animals. Animals that retrieved the reward at shorter latency after CS+ offset showed a greater cue-evoked dopamine signal. (**c**) Trial-by-trial mean [DA] in response to cue onset (time zero) for the four animals with relatively short (<5 s) retrieval latencies. Again, negative concentrations are considered because of differential measurements. For these animals, cue-evoked dopamine signals emerged as conditioning progressed. Trial-by-trial electrochemical data for individual animals with differing retrieval latencies are shown in **Supplementary Fig. 1**. (**d**) Cue-related dopamine signals (peak Δ[DA]) taken from the mean traces in **c**. Peak [DA] evoked by cue onset became significantly stronger during the course of the experimental session.

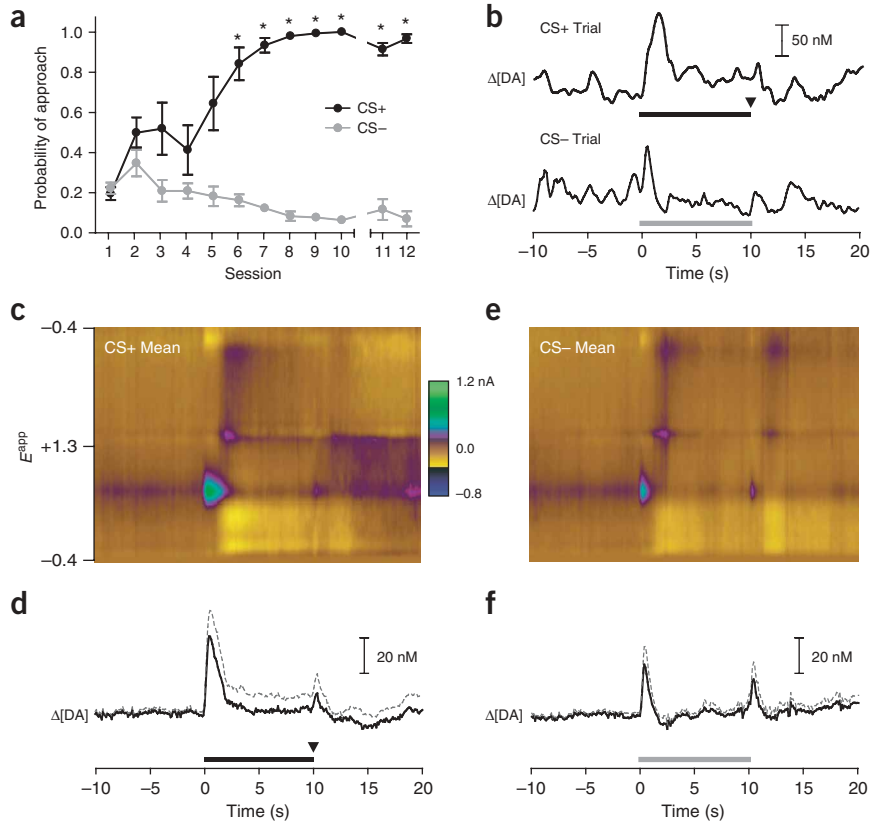

Figure 4 After extended conditioning, rapid dopamine release events in the NAc shift to conditioned stimuli and no longer signal primary rewards. (**a**) Behavioral discrimination (mean ± s.e.m. of approach probability) between conditioned stimuli based on predictive value. Rats approached the predictive CS+ significantly more than the nonpredictive CS− in sessions 6−12. After ten conditioning sessions, animals underwent surgery for implantation of the voltammetric recording apparatus (indicated by break in graph). (**b**) Representative changes in dopamine signaling during individual CS+ (top) and CS− (bottom) trials. (**c**) Three-dimensional representation of mean electrochemical data collected during reward-predictive CS+ trials. CS+ presentations evoked an immediate rise in signal that returned to baseline levels in seconds. Conventions are the same as **Figure 1c**. (**d**) Mean (± s.e.m.) increase in [DA] evoked by CS+ onset was significantly greater than baseline [DA] at 0.3−1.4 s after CS+ onset. No increase in signal was observed relative to reward delivery. (**e**) Three-dimensional representation of mean electrochemical data collected during CS− trials. CS− presentations evoked relatively smaller increases in signal. (**f**) Mean (± s.e.m.) [DA] also changed after CS− onset. *Post hoc* comparisons revealed a rapid increase in dopamine at 0.4−0.5 s after CS− onset (see **Supplementary Fig. 3** for precise CS+/CS− differences). The CS− also produced a significant increase in NAc [DA] at 0.4 s following cue offset.

previous reports on autoshaping[10,13], approach responses directed at the CS+ increased as a function of conditioning, whereas CS− approaches did not (**Fig. 4a**). CS+ approach probability was greater than that for CS− for conditioning sessions 6−12 (Bonferroni *post hoc* tests, all P values < 0.05), which indicated that animals could discriminate behaviorally between the conditioned stimuli and that the CS+ possessed enhanced incentive-motivational salience as a cue that signaled reward. During the final conditioning session, the majority of CS+ approaches occurred very rapidly after cue onset (**Supplementary Fig. 2** online).

After extended pavlovian conditioning, both conditioned stimuli evoked changes in NAc [DA] in seconds of cue onset (CS+, $F_{40,200} = 10.12$, $P < 0.001$; CS−, $F_{40,200} = 4.635$, $P < 0.001$). Consistent with previous reports that visual and auditory cues can excite dopamine neurons at very brief latency[33,34], we observed that conditioned increases in NAc [DA] were typically of short onset and short duration (see **Fig. 4b** for examples). The CS+ (**Fig. 4c,d**) produced robust increases in NAc [DA] from 0.3−1.4 s following cue onset ($P < 0.05$). Peak [DA] (53.9 ± 15.0 nM) occurred at 550 ± 56 ms after CS+ onset. Despite their close temporal proximity, there was no indication that the rapid rise in [DA] preceded or caused the pavlovian approach response. Indeed, although approach responses were generally completed during the seconds surrounding the peak [DA] response (**Supplementary Fig. 2**), the timing of these variables was not significantly correlated ($r^2 < 0.01$, $P = 0.76$; **Supplementary Fig. 2**). Additionally, there was no relationship between the magnitude of the dopamine signal observed on a given CS+ trial and the vigor (number of lever presses) after the approach response on that trial ($r^2 = 0.014$, $P = 0.21$; **Supplementary Fig. 2**). Unlike early in learning, reward delivery did not evoke a significant increase in NAc [DA] ($P > 0.05$ for all comparisons; **Fig. 4d**).

CS− presentation evoked an increase in [DA] at 0.4−0.5 s after cue onset ($P < 0.05$; **Fig. 4e,f**). Peak [DA] occurred at 383 ± 31 ms after CS− onset and reached 37.3 ± 11.2 nM. Peak dopamine responses to the CS− were significantly smaller than those produced to the CS+ ($t = 2.917$, d.f. = 5, $P = 0.033$). Additionally, the dopamine response evoked by the CS− was significantly lower than that evoked by the CS+ at 0.5−0.8 s following cue onset (95% confidence interval of difference score does not include zero; **Supplementary Fig. 3** online). In addition

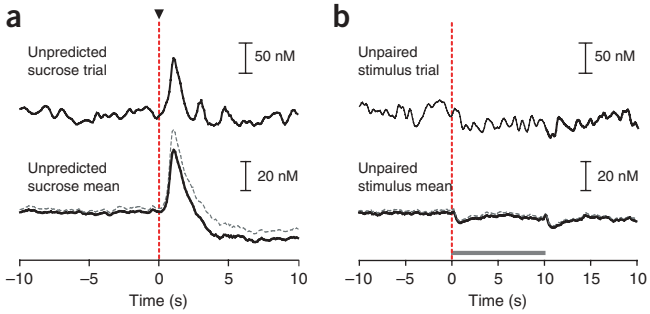

Figure 5 For another group of animals, phasic dopamine signals remained time-locked to reward delivery in the absence of a predictor. (**a**) Single-trial and mean (± s.e.m.) dopamine signals during the final session. Unpredicted reward delivery (vertical dashed line) evoked significant increases in NAc dopamine levels at 1.0−1.3 s after delivery. (**b**) Single-trial and mean (± s.e.m.) [DA] relative to presentation of an explicitly unpaired stimulus (horizontal gray line at time-point zero). This cue produced decreases in NAc [DA] at 1.0−1.2- and 10.5−13.0-s time bins relative to cue onset.

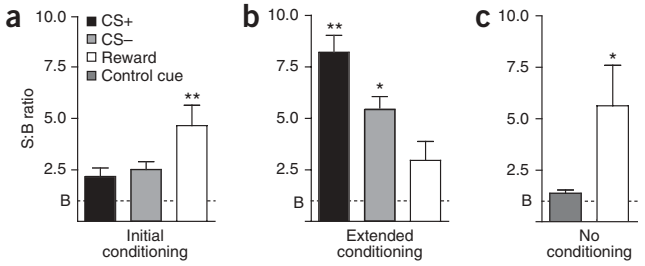

Figure 6 Comparison of dopamine changes relative to cue and reward stimuli using S:B transformation. (**a**) For the initial conditioning group, the reward signal (mean ± s.e.m.) was significantly greater than the signals for either conditioned stimulus (**Tukey multiple-comparisons test, $P < 0.05$ for both reward versus cue comparisons). (**b**) After extended conditioning, dopamine signals were significantly greater for both conditioned stimuli than for reward delivery. Additionally, the S:B ratio for the CS+ was greater than that for the CS− (*Tukey multiple-comparisons test, $P < 0.05$ for CS− versus reward; **Tukey multiple-comparisons test, $P < 0.05$ for CS+ versus CS− and CS+ versus reward). (**c**) In the absence of a predictive cue, the reward signal was significantly greater than the unpaired cue signal.

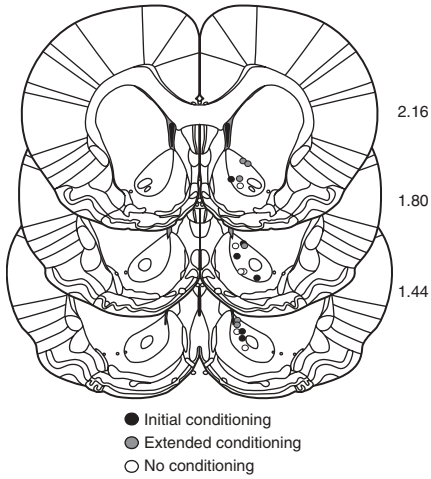

Figure 7 Anatomical distribution of carbon-fiber electrode placements in the NAc core. Coronal diagrams show electrode tip locations for 18 animals (6 per experimental group). Numbers to the right indicate anteroposterior coordinates (± 0.2 mm) rostral to bregma. Coordinates were taken from a stereotaxic atlas[50].

to the phasic response at cue onset, a significant increase in [DA] occurred at 0.4 s following CS− offset ($P < 0.05$; **Fig. 4f**).

NAc dopamine and unpredicted reward

Previous investigations in nonhuman primates indicate that phasic activation of dopamine neurons signals reward when there is no predictor available, even after repeated exposure[1]. To determine how unpredicted reward delivery affected NAc [DA], we exposed another group of rats ($n = 6$) to 12 nonconditioning sessions. During each session, 25 sucrose rewards were delivered at random to a food dish. Additionally, 10-s cues (identical to those used above) were presented 50 times in an explicitly unpaired design.

FSCV was carried out during the final (12^{th}) session. In this group, reward delivery produced a significant increase in NAc [DA] (**Fig. 5a**; $F_{40,200} = 7.27$; $P < 0.001$; $P < 0.05$ for specific comparisons at 1.0−1.3 s after reward delivery). Peak reward-related [DA] across animals was 54.3 ± 13.7 nM. The explicitly unpaired stimulus (**Fig. 5b**) also produced a change in [DA] ($F_{40,200} = 3.073$, $P < 0.001$). However, the onset and offset of this cue produced decreases in [DA] ($P < 0.05$ at 1.0−1.2-s and 10.5−13.0-s time bins).

Differential dopamine signals and conditioning history

To compare the relative magnitude of dopamine signals in response to cue and reward stimuli within each experimental group, electrochemical data were converted to signal-to-baseline (S:B) ratios (defined as peak differential [DA] during event/average baseline differential [DA]). In the early conditioning group, the CS+ and CS− evoked relatively small S:B ratios (2.18 ± 0.42 and 2.52 ± 0.38, respectively), indicating that phasic dopamine signals were only weakly modified by the presentation of these cues (**Fig. 6a**). Conversely, the maximal dopamine signal during reward retrieval was a nearly fivefold increase over baseline (actual S:B = 4.65 ± 0.99), significantly greater than that produced by either CS ($F_{2,17} = 8.089$, $P = 0.008$; Tukey multiple-comparisons test, $P < 0.05$ for both reward versus cue comparisons).

After extended conditioning (12 experimental sessions) of a second group of animals, peak dopamine signals were greatest in response to conditioned stimuli and smallest when rewards were delivered ($F_{2,17} = 28.538$, $P < 0.0001$; **Fig. 6b**). Specifically, mean peak [DA] increased over eightfold from baseline levels during CS+ presentation. Peak dopamine signals relative to CS− presentation and reward delivery were significantly smaller (Tukey multiple-comparisons test, $P < 0.05$ for each comparison; CS+ > CS− > reward). This result suggests that NAc dopamine signals were no longer time-locked to reward delivery or retrieval, but instead corresponded to the presentation of a reward-predictive cue and (to a lesser extent) a separate, but similar, cue that did not predict rewards.

In the group that received no conditioning (that is, stimuli and rewards were explicitly unpaired), the maximal S:B ratio during reward delivery was significantly greater than that for the cue period ($t = 2.618$, d.f. = 5, $P = 0.047$; **Fig. 6c**). Thus, nonconditioning sessions did not produce a shift in the phasic dopamine signal. Moreover, unlike the CS− from the previous experiment, the unpaired cue in this condition did not produce increases in [DA].

DISCUSSION

The use of environmental cues to predict impending outcomes is a fundamental part of learned behavior. By sampling NAc dopamine concentration at different stages of conditioning, our experimental design enabled us to determine how such associative learning alters real-time NAc dopamine signaling in response to predictive cues and rewarding stimuli. Here, we demonstrated that subsecond dopamine release in the NAc core signals reward in naive rats. However, when animals were trained to associate an experimental cue with the delivery of a reward, the dopamine signal shifted to this predictor and was no longer present when the reward was made available. In the absence of a predictor, phasic elevations in NAc [DA] remained time-locked to reward delivery. Taken together, these findings show that associative learning dynamically alters NAc dopamine responses to both predictive cues and primary rewards.

The present results are highly consistent with prediction error models of dopamine function[2,3]. Early in learning, reward delivery was not yet associated with the CS+ and therefore occurred unpredictably. In this condition, phasic dopamine release events were time-locked to the receipt of a reward, but not to the CS+. As conditioning progressed, both the CS+ and CS− came to evoke increases in NAc [DA] in some animals, but not in others. Individually, this development was predicted by the duration between the CS+ and reward;

animals that obtained the reward sooner after cue offset showed a phasic cue-evoked dopamine signal by the end of the behavioral session. Thus, the acquisition of dopamine signals during conditioning corresponds to the temporal proximity of the cue and reward, providing an early link between associative strength[4] and NAc dopamine signaling. Furthermore, the emergence of an acquired dopamine response at cue onset was not selective for the reward-predictive CS+, but also occurred when the CS− was presented. This finding may underscore the limits of the dopamine system. Faced with the task of successfully predicting reward delivery in a novel environment, rapid increases in dopamine may signal not only predictive cues, but also similar cues which may turn out to provide valuable information. Such a function could prove beneficial in natural environments where food could be predicted by spatially separate, but physically similar, cues.

After many conditioning sessions, the animals developed a behavioral discrimination between the CS+ and CS−, indicating that they had learned the existing predictive relationships. Consistent with dopamine cell recordings in primates[6,22], rapid dopamine release events shifted to the cue that predicted future rewards. In contrast, predicted reward delivery lost the ability to elicit increases in NAc [DA]. This change in dopamine signaling was only present in animals that underwent stimulus-reward pairings; dopamine release events still signaled reward delivery in animals that received equal exposure to rewards without a predictor. Although stimulus-reward learning clearly altered dopamine signaling in the NAc, it should be noted that not all cues paired with rewards produce phasic dopamine responses. In previous work that used a blocking procedure, reward-predictive cues did not produce an increase in dopamine cell firing when an earlier predictive cue was provided during conditioning[22]. Thus, prediction errors (and not stimulus-reward associations alone) are the determining factor in the generation of phasic cue-related dopamine responses.

Even after extended conditioning, a CS− that predicted the absence of rewards evoked a brief increase in NAc [DA] (**Fig. 4f**). Although this response may seem paradoxical, it should be noted that electrophysiological studies have reported similar patterns in burst firing among a subset of dopamine neurons when CS− cues are presented[22], and that these responses have also been modeled using temporal difference algorithms[35]. One interpretation suggests that this response reflects a form of stimulus generalization[22,35]. The initiation of both CS+ and CS− dopamine signals likely begins with the audio component of cue onset, as reward-predictive audio stimuli evoke increases in dopamine cell firing at shorter latency than do visual cues[33]. However, as the cues used here generated highly similar sounds (and were only spatially distinct), audio information alone may not enable adequate discrimination. Accordingly, cue onset may produce a rapid increase in dopamine cell firing that corresponds to the expected value predicted by both cues, which is half of a reward (average of 0 for CS− and 1 for CS+). When the identity of the cue is fully ascertained through visual input, the dopamine response may adjust to reflect the updated prediction. Thus, the CS+ signals a better-than-expected outcome and the increase in dopamine continues, whereas the CS− signals a worse-than-expected outcome and [DA] rapidly decreases in a manner consistent with electrophysiological results from dopamine neurons[22]. A similar phenomenon may occur at CS− offset, when the existing prediction is the absence of a reward. Here, the sound of cue offset is associated with reward on 50% of trials, and so a small positive prediction error may be generated on CS−, but not CS+, trials. This position is further strengthened by the observation that no phasic increases in dopamine were produced by an unpaired cue when animals did not have concurrent exposure to a predictive cue (**Fig. 5b**). Here, cue onset and offset produced decreases in NAc [DA] even though this cue and the CS− carry highly similar information with respect to reward delivery. This result highlights the potential impact of learning environment, and especially the presence of other cues, in the promiscuity of the dopamine signal.

Behavioral discrimination between reward-predictive cues and other stimuli likely requires concerted activity in a distributed network of brain structures that includes the NAc and its dopaminergic innervation, the anterior cingulate cortex (ACC) and the central nucleus of the amygdala (CeA)[29]. Conditioned approaches toward a predictive CS+ are impaired by D1/D2 dopamine receptor antagonism and dopamine depletion in the NAc core[13,36]. Moreover, excitotoxic lesions to the ACC or CeA also significantly alter the allocation of conditioned approach responses[31]. In this circuit, it has been proposed that excitatory ACC input into the NAc facilitates discrimination between sensory cues, whereas the CeA augments the firing of dopamine cells that project to the NAc[29]. However, the precise behavioral role of phasic dopamine release in the NAc remains unclear. One possibility is that these signals are responsible for the generation of approach responses toward predictive stimuli[37]. Although recent reports suggest that dopamine can actively produce or modulate operant reward-seeking behaviors[25,38], several results argue against this interpretation with respect to the pavlovian approach responses observed in the present context. First, the CS+ and CS− both evoked brief increases in NAc [DA] in animals that received extended conditioning, but the same animals approached the CS− on only 6% of trials, whereas they approached the CS+ on over 95% of trials (**Fig. 4a**). It is uncertain how this clear behavioral discrimination could be made on the basis of a phasic dopamine signal that is highly similar for the CS+ and CS− immediately after cue onset (see **Supplementary Fig. 3**). Second, the timing and magnitude of the dopamine signal on CS+ trials was unrelated to the timing or degree of behavioral activation (**Supplementary Fig. 2**). We therefore hypothesize that dopamine-related reward-prediction information may be processed by the NAc and used to instruct or to strengthen[39] (but not to generate) certain motor responses as they occur or after they occur. A related and intriguing explanation posits that rapid dopamine release may reflect the incentive value of the CS+ and reward[40,41]. Early in conditioning, the sight or sound of a reward may signal an 'incentive' to retrieve the reward and to produce a phasic increase in NAc [DA]. During learning, the CS+ comes to predict the reward in the same manner, thereby acquiring its own incentive value and evoking a similar dopamine response.

The ability of the NAc and other striatal regions to influence behavioral output on the basis of pavlovian associations almost certainly involves the modification of individual synaptic inputs during learning. Indeed, recent studies have demonstrated that although the majority of NAc neurons do not innately respond to neutral environmental cues, responses quickly emerge when cues begin to predict rewarding events[11,42]. Moreover, the majority of NAc neurons show robust changes in activity when reward-predictive cues are presented after an extended conditioning design similar to the one used here[10]. It has been suggested that dopamine-glutamate interactions in the NAc may be important in this cellular plasticity, with dopamine gating the efficacy of NAc glutamatergic inputs from limbic and cortical structures[43]. Consistent with this hypothesis, blockade of dopamine D1 receptors inhibits long-term potentiation in corticostriatal slices[44] and prevents the proper expression and consolidation of learned stimulus-reward relationships[15,45]. We propose that the phasic dopamine signals observed here possess a special role with respect to D1 receptor activation during stimulus-reward learning. Recent no-net-flux microdialysis studies have placed the basal concentration of dopamine at

levels far below those needed to activate low-affinity D1 receptors[46,47]. However, by rapidly increasing the local concentration of dopamine, phasic release events are capable of providing a signal that can stimulate D1 receptors on a timescale commensurate with behavioral events and environmental stimuli. In turn, D1 receptors could act through well-described signaling cascades[48] to prolong recent memory traces and allow fast synaptic communications to interact with those traces. Understanding the complexities of this interplay in brain regions such as the NAc may provide critical insight into the neurobiology of both natural and aberrant stimulus-reward learning.

METHODS

Animals. Male Sprague-Dawley rats (Harlan Sprague-Dawley) aged 90–120 d and weighing 260–330 g were used as subjects and individually housed with a 12 h/12 h light/dark cycle. All experiments were conducted between 9:00 a.m. and 5:00 p.m. Body weights 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 at Chapel Hill Institutional Animal Care and Use Committee.

Conditioning procedures. Naive rats ($n = 6$) were surgically fitted for voltammetric recordings using methods described below for the first experiment, early conditioning. After full recovery, rats were placed in a standard experimental chamber (Med Associates) and received a magazine training session in which single sucrose pellets (45 mg) were delivered at random intervals to a food dish. This served to acquaint the animal with the location and taste of sucrose before conditioning began. On the next day, electrochemical data were collected in the NAc core during a conditioning session that consisted of 50 individual trials. On 25 trials, a compound stimulus (extension of a retractable lever and illumination of cue light above the lever) was presented to the animal for 10 s. At the end of the stimulus presentation, a sucrose pellet was immediately delivered to a food dish. On the other 25 trials, another compound stimulus (extension of a separate retractable lever and illumination of associated cue light) was presented for 10 s, but was not followed by sucrose delivery (**Fig. 1a**). Thus, the first stimulus (termed the CS+) provided a positive predictor of sucrose delivery, whereas the second stimulus (the CS−) was a negative predictor of sucrose delivery (that is, the cue signaled the absence of sucrose). The order of CS+ and CS− trials was semi-random, with no more than two of either trial type occurring in sequence. Individual trials were initiated on a variable schedule every 45–75 s; the average intertrial interval was 60 s. Additionally, the lever and cue light that served as the CS+ were counterbalanced across animals. The CS+ and CS− stimuli were symmetrically located on the same wall as the food receptacle with a horizontal separation of 17 cm. Contact with conditioned stimuli (registered as lever presses) was recorded during each trial. However, sucrose delivery was independent of contact with the CS+. Manual frame-by-frame videotape analysis of the entire conditioning session was used to pinpoint the timing of each sucrose retrieval for each animal. For this analysis, a Sony video cassette recorder received video input from a camera fastened to the ceiling of the experimental chamber, allowing a complete view of the subject and experimental setup. This input was recorded on VHS tapes along with time-stamped session information from a Video Character Generator (University of North Carolina Electronics Facility), which enabled the electrochemical data to be realigned with respect to the actual recovery of the sucrose pellet. Sucrose retrieval was operationally defined as the first 100-ms bin after sucrose delivery in which the rat's nose and mouth were lowered into the food receptacle.

A second group of naive rats ($n = 6$) underwent ten conditioning sessions that were nearly identical to that described above (50 total trials, 25 CS+ and 25 CS− presentations; 1 session per day) for the second experiment, extended conditioning. After the fifth conditioning session, cue lights were no longer illuminated during the CS+ and CS−, making the retractable levers the only stimuli with predictive value. After the tenth conditioning session, animals were fed *ad libitum* and surgically prepared for voltammetric recordings. Following a 1-week recovery period, rats were subjected to another 50-trial conditioning session. On the test day, electrochemical data were collected in the NAc core during the 12th and final conditioning session. Thus, at the start of the recording session, rats had received 275 pairings between the CS+ and sucrose delivery, as well as 275 CS− presentations that were not followed by sucrose delivery.

Another group of rats ($n = 6$) underwent ten sessions in which sucrose delivery and stimulus presentations occurred in an explicitly unpaired manner for the third experiment, unexpected reward. Each session consisted of 50 unpaired stimulus presentations (extension of right or left retractable lever and associated cue light for 10 s) and the unpredicted delivery of 25 sucrose pellets to the food dish. Here, unpaired stimulus trials were initiated on a variable time interval every 60–90 s (mean of 75 s). Sucrose deliveries were timed to occur sporadically between lever presentations, but never occurred within 15 s of stimulus onset or offset. Again, after five sessions the cue lights were no longer illuminated with lever extension. After the tenth session, animals were fed *ad libitum* and surgically fitted for electrochemical recordings. After full recovery, rats received another session of unpredicted reward delivery. The result was that, before the test session, rats had experienced 550 cue presentations that did not predict sucrose and 275 unsignaled sucrose deliveries. On the experimental test day, [DA] was measured in the NAc core during the 12th and final session.

FSCV. Rats were surgically prepared for voltammetric recordings as described previously[49]. After establishing an anesthetic plane with ketamine hydrochloride (100 mg per kg of body weight, intramuscular) and xylazine hydrochloride (20 mg per kg, intramuscular), rats were placed in a stereotaxic frame. A guide cannula (Bioanalytical Systems) was positioned dorsally to the core subregion of the NAc (1.3 mm anterior, 1.3 mm lateral from bregma). An Ag/AgCl reference electrode was placed contralateral to the stimulating electrode in the left forebrain. Stainless steel skull screws and dental cement were used to secure all items. A bipolar stimulating electrode was placed dorsally to the VTA (5.2 mm posterior, 1.0 mm lateral from bregma, and 7 mm ventral from the dural surface). A detachable micromanipulator containing a glass-sealed carbon-fiber electrode (75–100-μm exposed tip length, 7-μm diameter, T-650, Amoco) was inserted into the guide cannula, and the electrode was lowered into the NAc core. The bipolar stimulating electrode was then lowered in 0.2-mm increments until electrically evoked dopamine release was detected at the carbon-fiber electrode in response to a stimulation train (60 biphasic pulses, 60 Hz, 120 μA, 2 ms per phase). The stimulating electrode was then fixed with dental cement and the carbon-fiber electrode was removed.

Following surgery, animals were allowed 1 week to recover presurgery body weight. Food intake was then reduced to ensure motivation during conditioning. To collect electrochemical data on the test day, a new carbon-fiber electrode was placed in the micromanipulator and attached to the guide cannula. The carbon-fiber electrode was then lowered into the NAc core. The carbon-fiber and Ag/AgCl electrodes were connected to a head-mounted voltammetric amplifier attached to a commutator (Crist Instrument Company) at the top of the experimental chamber. All electrochemical data were digitized and stored using computer software written in LabVIEW (National Instruments). To minimize current drift, the carbon-fiber electrode was allowed to equilibrate for 30–45 min before the start of the experiment.

The potential of the carbon-fiber electrode was held at −0.4 V versus the Ag/AgCl reference electrode. Voltammetric recordings were made every 100 ms by applying a triangular waveform that drove the potential to +1.3 V and back at a rate of 400 V s^{-1}. The application of this waveform causes oxidation and reduction of chemical species that are electroactive in this potential range, producing a change in current at the carbon fiber. Specific analytes (including dopamine) are identified by plotting these changes in current against the applied potential to produce a cyclic voltammogram[26]. The stable contribution of current produced by oxidation and reduction of surface molecules on the carbon-fiber was removed by using a differential measurement (that is, background subtraction) between times when such signals were present, but dopamine was not. For data collected during the behavioral session, this background period (500 ms) was obtained during the baseline window (10 s before cue onset). This practice does not subtract the presence of phasic dopamine events during the baseline because the background was explicitly selected for the absence of fast dopamine signals. Following equilibration,

dopamine release was electrically evoked by stimulating the VTA (24 biphasic pulses, 60 Hz, 120 μA, 2 ms per phase) to ensure that carbon-fiber electrodes were placed close to release sites. The position of the carbon fiber was secured at the site of maximal dopamine release. Experiments began when the signal-to-noise ratio of electrically evoked dopamine release exceeded 30. During conditioning sessions, experimental and behavioral data were recorded with a second computer, which translated event markers to be time-stamped with electrochemical data. VTA stimulation was repeated following the experiment to verify electrode stability and ensure that the location of the electrode could still support dopamine release.

Signal identification and separation. After *in vivo* recordings, dopamine release evoked by VTA stimulation was used to identify naturally occurring dopamine transients using methods described previously[26,27]. Stimulation of the VTA leads to two well-characterized electrochemical events: an immediate, but transient, increase in [DA] and a delayed, but longer-lasting, basic pH shift. To separate these signals, a training set was constructed from representative, background-subtracted cyclic voltammograms for dopamine and pH. This training set was used to perform principal component regression on data collected during the behavioral session. Principal components were selected such that at least 99.5% of the variance in the training set was accounted for by the model. All data presented here fit the resulting model at the 95% confidence level. After use, carbon-fiber electrodes were calibrated in a solution of known [DA] to convert observed changes in current to differential concentration.

Statistical analysis. Significant changes in NAc [DA] were evaluated using a one-way repeated measures ANOVA with Tukey *post hoc* tests for multiple comparisons of 100-ms time bins and a baseline window (mean [DA] during 10 s preceding cue onset or reward delivery (unpaired group only)). To determine whether cue-related dopamine responses emerged for each animal in the early conditioning group, data were divided into blocks of five trials and a one-way repeated measures ANOVA was carried out for the first and final blocks. The differences between CS+ and CS− cues were evaluated using paired *t*-tests on peak [DA]. In a separate analysis, the S:B ratio was computed by dividing the maximal differential [DA] observed during an event (signal) by the average differential [DA] observed during the 10s baseline window preceding cue onset (or preceding reward delivery in cases where sucrose was not signaled by a cue). Differences in S:B ratio relative to CS+, CS−, reward and control cue presentations within groups were assessed by conducting one-way repeated measures ANOVAs (early and extended conditioning groups) or one-tailed paired Student's *t*-tests (unpaired group). Tukey *post hoc* tests for multiple comparisons were employed following ANOVAs to determine S:B differences between individual events.

Pavlovian approach responses directed at conditioned stimuli were recorded as lever presses. For each behavioral session, the probability of approach was calculated for the CS+ and CS− by dividing the total number of approaches (lever presentations in which at least one lever press occurred) by the number of opportunities for approach. For the initial conditioning group, approach probabilities for the CS+ and CS− were compared using a paired Student's *t*-test. For the extended conditioning group, differential acquisition of stimulus-selective approach behavior was evaluated using a within-subjects cue (two levels) × session (12 levels) repeated measures ANOVA. Bonferroni *post hoc* tests were employed to identify sessions in which approaches directed at the CS+ and CS− differed. The relationship between the latency or vigor of approach responses and dopamine release was evaluated using linear regression analysis. Statistical significance was designated at $\alpha = 0.05$. All statistical analyses were carried out using InStat version 3.0 for Windows (Graphpad Software) and SPSS version 12.0 for Windows (SPSS). Three-dimensional graphical analyses were carried out using Matlab software (MathWorks).

Histological verification of electrode placement. On completion of each experiment, rats were deeply anesthetized with a ketamine/xylazine mixture (100 mg per kg and 20 mg per kg, respectively). To mark the placement of electrode tips, a 50−500-μA current was passed through a stainless steel electrode for 5 s. Transcardial perfusions were then carried out using physiological saline and 10% formalin, and brains were removed. After postfixing and freezing, 50-μm 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 in the NAc core 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[50] (precise electrode placements for each experiment can be found in **Fig. 7**).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

The authors would like to thank R.A. Wheeler, B.J. Aragona, P.E.M. Phillips and J.L. Jones for helpful comments on this manuscript, and the University of North Carolina Department of Chemistry Electronics Facility for technical expertise. This research was supported by the US National Institute on Drug Abuse (DA 017318 to R.M.C., DA to 10900 R.M.W., DA 021979 to J.J.D. and DA 018298 to M.F.R.).

AUTHOR CONTRIBUTIONS

J.J.D. and M.F.R. conducted the behavioral and electrochemical experiments. J.J.D. wrote the manuscript and conducted data and graphical analyses. M.F.R. contributed to the writing of the manuscript. R.M.C. and R.M.W. supervised the project and the writing of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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