Dopamine release and its control over early Pavlovian learning differs between the NAc core and medial NAc shell

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Running title: Regional differences in dopamine release during early learning

Abstract:

Dopamine neurons respond to cues to reflect the value of associated outcomes. These cue-evoked dopamine responses can encode the relative rate of reward in rats with extensive Pavlovian training. Specifically, a cue that always follows the previous reward by a short delay (high reward rate) evokes a larger dopamine response in the nucleus accumbens (NAc) core relative to a distinct cue that always follows the prior reward by a long delay (low reward rate). However, it was unclear if these reward rate dopamine signals are evident during early Pavlovian training sessions and across NAc subregions. To address this, we performed fast-scan cyclic voltammetry recordings of dopamine levels to track the pattern of cue and reward-evoked dopamine signals in the NAc core and medial NAc shell. We identified regional differences in the progression of cue-evoked dopamine signals. However, reward rate encoding was not evident in either the NAc core or NAc shell during early training sessions. Pharmacological experiments found that dopamine-sensitive conditioned responding emerged in the NAc core before the NAc shell. Together, these findings illustrate regional differences in NAc dopamine release and its control over behavior during early Pavlovian learning.

Introduction:

Learning to associate cues with rewarding outcomes is a fundamental process that underlies reward-driven behaviors. The mesolimbic dopamine system regulates behavioral responses toward reward-predictive cues [1,2]. In particular, dopamine neurons respond to cues to encode the value of the outcome in well-trained animals. These cue-evoked dopamine responses can convey prospective reward-related information, such as reward preference, reward size, and reward probability [3-7]. However, the dopamine response to cues can also signal retrospective reward-related information. Specifically, a cue that always follows the previous reward by a short delay (high reward rate) evokes a larger dopamine response relative to a distinct cue that always follows the prior reward by a long delay (low reward rate) [8]. While dopamine release in the nucleus accumbens (NAc) core encodes the reward rate in rats with extensive Pavlovian training (>24 sessions), it is unclear when these signals first emerge and if they are broadcast uniformly throughout the medial NAc.

Dopamine's role in reward learning has been primarily studied using Pavlovian tasks with a *single* cue-reward relationship [9,10]. In contrast, the difference in the dopamine response between cues has been primarily studied in well-trained animals [3,4,6,7]. As such, it is unclear how dopamine signals emerge when learning multiple cue-reward relationships simultaneously. Cue-evoked dopamine release could acquire value-related information through a multi-step process or a single-step process. For example, in a multi-step process cue-evoked dopamine release first signals an upcoming reward (independent of value), and over training conveys the relative difference in value between cues. Alternatively, in a single-step process the cue-evoked dopamine response will reflect differences in reward value as these signals first emerge during training.

To examine this, we performed voltammetry recordings of NAc dopamine release during a Pavlovian conditioning task that incorporates two distinct cue-reward relationships. In this task, one cue always followed the previous reward with a short delay (high reward rate) while another cue always followed the previous reward with a long delay (low reward rate). By recording dopamine levels during early training sessions, we could determine if reward rate encoding arises from a multi-step or a single-step process. Dopamine recordings were performed in the NAc core

and the medial NAc shell, as cue-evoked dopamine responses are present in both NAc subregions [11-13]. Furthermore, we pharmacologically inhibited dopamine receptors to determine if conditioned responding requires dopamine in the NAc core and/or medial NAc shell.

Methods:

Subjects and surgery:

The University of Texas at San Antonio Institutional Animal Care and Use Committee approved all procedures. Male CD IGS Sprague Dawley rats (Charles River Laboratories, RRID:RGD 734476) were pair-housed upon arrival, allowed *ad libitum* access to water and chow, and maintained on a 12 h light/dark cycle. Voltammetry electrodes were surgically implanted under isoflurane anesthesia in rats weighing 300 –400 g. Carbon fiber electrodes were placed bilaterally targeting the nucleus accumbens core or medial shell (relative to bregma: 1.3 mm anterior; ± 1.3 mm lateral; 7.0 mm ventral or 1.5 mm anterior; ± 0.6 mm lateral; 7.3 mm ventral, respectively), along with an Ag/AgCl reference electrode placed under the skull. Bilateral stainless steel guide cannulae (InVivo One) were implanted 1 mm dorsal to the nucleus accumbens core or medial shell. Following surgery, rats were single-housed for the duration of the experiment and allowed to recover for 1-3 weeks before behavioral procedures.

Behavioral procedures:

At ≥ 7 days post-surgery, rats were placed on mild dietary restriction to 90% of their free feeding weight, allowing for a weekly increase of 1.5%. Rats were handled regularly before behavioral testing commenced. All behavioral sessions occurred during the light cycle in operant boxes (Med Associates) with a grid floor, a house light, a recessed food tray equipped with an infrared beam-break detector, and auditory stimulus generators (white noise and 4.5 kHz tone). To familiarize the animals with the operant chamber and food retrieval from the tray, rats first received 1-2 magazine training sessions in which 20 unsignaled food pellets (45 mg, BioServ) were delivered at a 90 s variable interval. Rats underwent 6 Pavlovian reward conditioning sessions as described previously [8]. Pavlovian sessions consisted of 50 trials where the

termination of a 5 s audio CS (tone or white noise, counterbalanced across animals) resulted in the delivery of a single food pellet and illumination of the food port light for 4.5 s. Each session contained 25 Short Wait trials and 25 Long Wait trials delivered in pseudorandom order. The Short Wait CS was presented after a 20 ± 5 s ITI, and the Long Wait CS was presented after a 70 ± 5 s ITI. We monitored head entries into the food tray across training sessions. Conditioned responding was quantified as the change in the rate of head entries during the 5 s CS relative to the 5 s preceding the CS delivery [8,14].

Pharmacology:

Flupenthixol dihydrochloride (Tocris) was dissolved in sterile 0.9% NaCl. Rats received bilateral 0.5 µl microinjections of flupenthixol (10 µg/side) or vehicle into the nucleus accumbens core or shell at 0.25 µl/min. The injectors were removed 1 min after the infusion ended. Behavioral sessions commenced 30 min after the microinjections [14,15].

Voltammetry recordings and analysis:

Indwelling carbon fiber microelectrodes were connected to a head-mounted amplifier to monitor dopamine release in behaving rats using fast-scan cyclic voltammetry [8,14,16-19]. During voltammetric scans, the potential applied to the carbon fiber was ramped in a triangular waveform from -0.4 V (vs. Ag/AgCl) to +1.3 V and back at a rate of 400 V/s. Scans occurred at 10 Hz with the electrode potential held at -0.4 V between scans. Dopamine was chemically verified by obtaining high correlation of the cyclic voltammogram during a reward-related event with that of a dopamine standard (correlation coefficient $r^2 \geq 0.75$ by linear regression). Voltammetry data for a session were excluded from analysis if the detected voltammetry signal did not satisfy the chemical verification criteria [8,14,17]. Dopamine was isolated from the voltammetry signal using chemometric analysis [20] with a standard training set accounting for dopamine, pH and drift. The background for voltammetry recording analysis was set at 0.5 s before the CS onset. Trials were excluded if chemometric analysis failed to identify dopamine on > 25% of the data points. The change in dopamine concentration was estimated based on the average post-implantation electrode sensitivity (34 nA/µM)[16].

All quantification of dopamine responses was performed on signals that had been smoothed via a 3-point moving average. CS-evoked dopamine release was quantified as the mean dopamine level during the 5 s CS relative to the 5 s prior to the CS delivery [8,14]. The slope of the CS response was calculated by taking the difference in the mean dopamine levels during the peak (1.5 - 2 s) and the end of the CS (4.5 – 5 s). US-evoked dopamine was quantified as the mean dopamine level during the 2 s following the pellet delivery relative to the 0.5 s preceding the pellet delivery.

Experimental design and statistical analysis:

We performed all statistical analyses in Graphpad Prism 8. All data are plotted as mean ± SEM. A mixed-effects model fit (restricted maximum likelihood method) was used to analyze effects on behavioral measures and dopamine responses. Data were analyzed in 5 trial bins for within-session analyses or averaged within session for full training analyses. The significance level was set to $\alpha = 0.05$ for all tests. The full list of statistical analyses is presented in **Supplementary Table 1**.

Histology:

Rats with were anesthetized, electrically lesioned via the voltammetry electrodes, and perfused intracardially with 4% paraformaldehyde. Brains were extracted and post-fixed in the paraformaldehyde solution for a minimum of 24 hrs, then were transferred to 15 % and 30 % sucrose in phosphate-buffered saline. Tissue was cryosectioned and stained with cresyl violet. Implant locations were mapped to a standardized rat brain atlas [21].

Results:

Dopamine in the NAc does not encode reward rate in early training sessions

Rats were trained on a Pavlovian delay conditioning task in which 5 s audio conditioned stimuli (CSs) signaled the delivery of a food reward (US). This task involved two trial types with distinct CSs. Both CSs resulted in the identical outcome (a single food pellet) but differed in the time elapsed since the previous reward (**Fig. 1A**). In Short Wait trials, the CS was presented 15 -

25 s following the previous reward delivery (high reward rate). In Long Wait trials, the CS was presented 65 - 75 s following the previous reward delivery (low reward rate). Training sessions consisted of 25 Short Wait trials and 25 Long Wait trials presented in a pseudorandom pattern so that the identity of the upcoming trial could not be predicted. Rats with extensive training on this task (> 24 sessions) exhibit a larger NAc core dopamine response to the Short Wait CS relative to the Long Wait CS [8]. While dopamine encodes reward rate in well-trained rats, it is unclear when this signal first emerges. Furthermore, it is unknown whether dopamine signaling uniformly reflects reward rate throughout the medial NAc.

To address this, we performed voltammetry recordings of NAc dopamine levels in the NAc core and medial NAc shell during the first 6 Pavlovian training sessions. Rats significantly increased conditioned responding to both CSs over all conditioning trials (two-way mixed effects analysis: trial effect $F_{(29,\ 493)}$=14.1, p<0.0001, n = 18 rats; **Fig. 1B**). The magnitude of conditioned responding did not differ between the Short and Long Wait CSs (reward rate effect $F_{(1,\ 17)}$=0.281, p=0.603), consistent with prior work [8]. We first assessed dopamine signals from electrodes in the NAc core (n = 10 electrodes; **Fig. 1C**). Short and Long Wait CS presentation and US delivery evoked time-locked phasic dopamine responses (**Fig. 1D-E**). The CS response increased over conditioning trials but did not differ between trial types (two-way mixed effects analysis: trial effect $F_{(5.0,\ 44.9)}$=5.54, p=0.0005; reward rate effect $F_{(1.0,\ 9.0)}$=1.71, p=0.223; **Fig. 1F**). The US response decreased over all conditioning trials but likewise did not differ between trial types (trial effect $F_{(3.6,\ 32.0)}$=10.5, p<0.0001; reward rate effect $F_{(1.0,\ 9.0)}$=0.208, p=0.659; **Fig. 1G**). While the overall pattern of CS- and US-evoked dopamine release is consistent with prior studies, we found no evidence of reward rate encoding during early training sessions in the NAc core.

Next, we examined dopamine signals from electrodes in the medial NAc shell (n = 14 electrodes; **Fig. 2A**). Similar to the NAc core, CS and US presentation evoked time-locked phasic dopamine responses (**Fig. 2B**). The CS dopamine response increased over conditioning trials but did not differ between trial types (two-way mixed effects analysis: trial effect $F_{(5.0,\ 65.1)}$=4.93, p=0.0007; reward rate effect $F_{(1.0,\ 13.0)}$=0.000192, p=0.989; Figure 2c). The US dopamine response decreased over all conditioning trials but did not differ between trial types

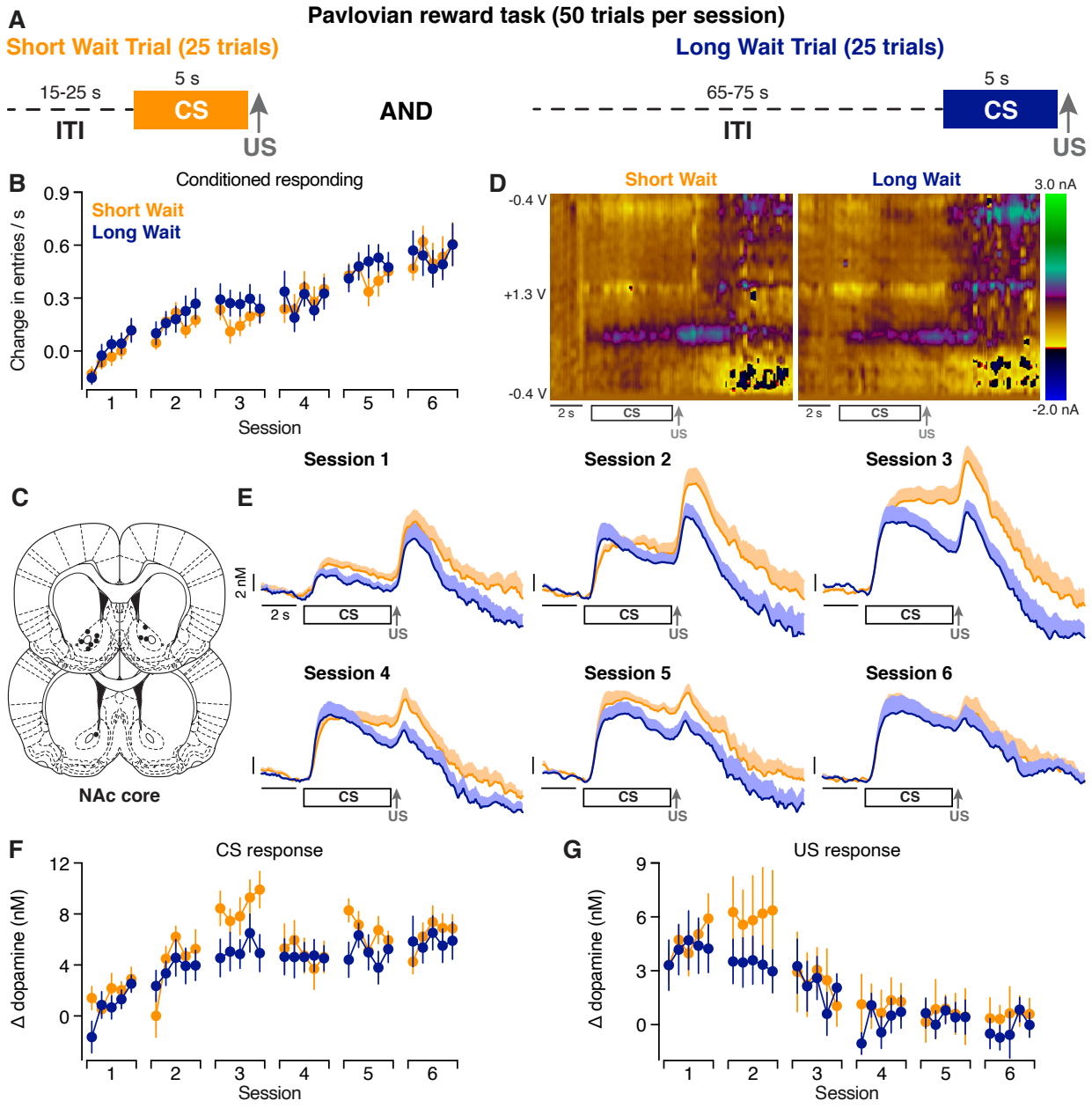

Figure 1. Dopamine release in the NAc core during early training sessions. (A) Pavlovian task. Short and Long Wait trials were presented in a pseudorandom pattern. (B) Conditioned responding across sessions in 5 trial bins. (C) Representative color plots of voltammetry recordings in the NAc core. (D) Location of voltammetry electrodes. (E) Average dopamine signals across training sessions. (F) Dopamine response to the CS across sessions in 5 trial bins. (G) Dopamine response to the US across 5 trial bins.

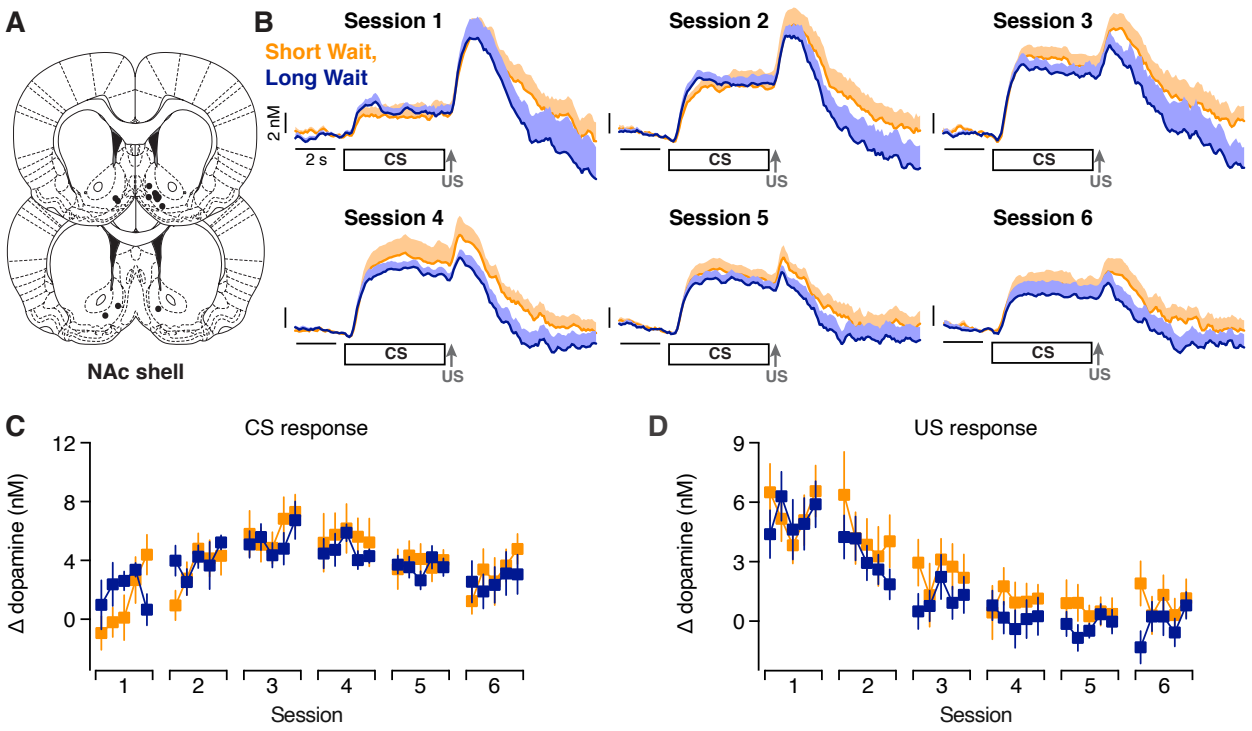

Figure 2. Dopamine release in the medial NAc shell during early training sessions. (A) Location of voltammetry electrodes. (B) Average dopamine signals across training sessions. (C) Dopamine response to the CS across sessions in 5 trial bins. (D) Dopamine response to the US across 5 trial bins. trial bins.

(trial effect $F_{(3.8,\ 49.0)}$=9.83, p=<.0001; reward rate effect $F_{(1.0,\ 13.0)}$=2.33, p=0.151; Figure 2D). Collectively, these results demonstrate that reward rate is not encoded by CS-evoked dopamine signals in either the NAc core or the medial NAc shell in early training sessions.

Transient changes in the slope of the CS-evoked dopamine response

While there were no gross differences in the amplitude of CS-evoked dopamine release between Short and Long wait trial types, the temporal dynamics of the response varied between trial types and across training sessions in the NAc core (**Fig. 1E**). To quantify these dynamics, we calculated the difference in dopamine levels from when average peak CS response occurred relative to the end of the CS presentation (**Fig. 3A**). In the NAc core, the slope of the CS response was significantly decreased in Long wait trials in sessions 3, 4, and 5 (two-way mixed effects analysis: reward rate effect in session 3 $F_{(1.0,\ 9.0)}$=8.31, p=0.018; session 4 $F_{(1.0,\ 8.0)}$=11.0, p=0.0086; session 5 $F_{(1.0,\ 9.0)}$=6.80, p=0.028; **Fig. 3B**). This transient effect was no longer

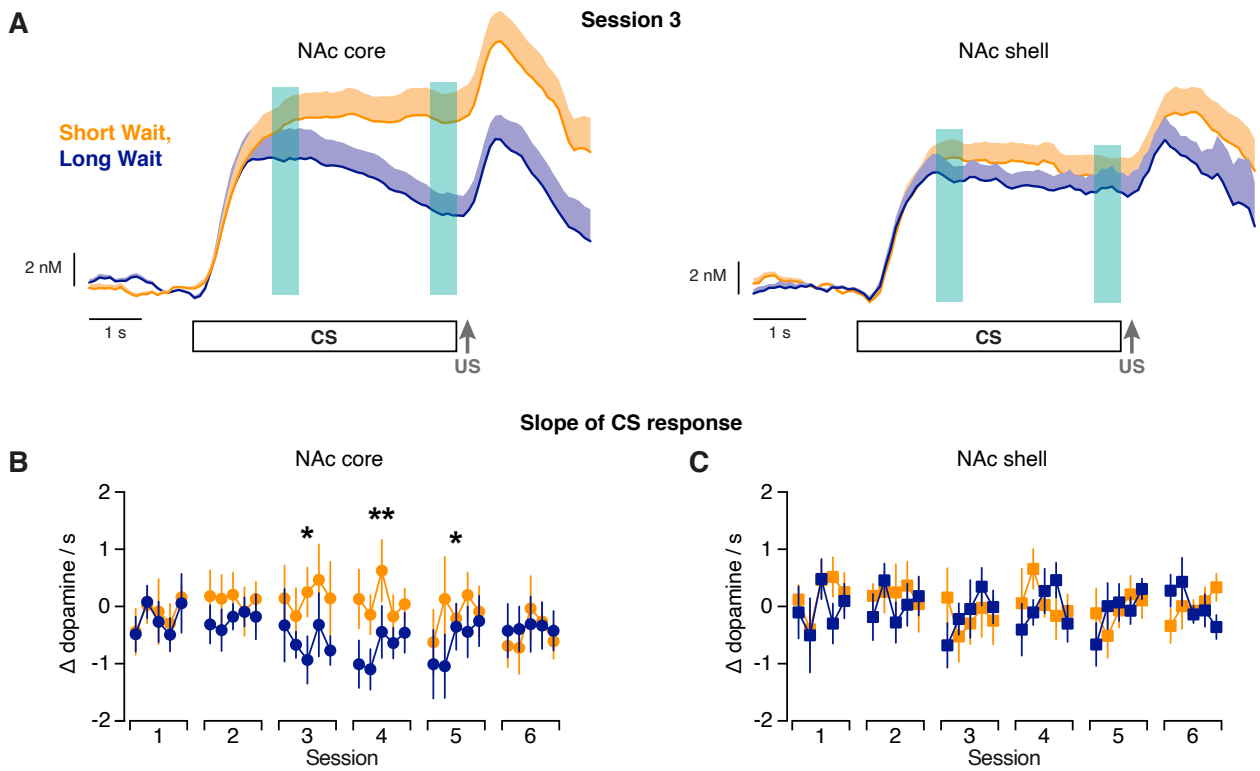

Figure 3. Differential dynamic of the CS dopamine response between trial types in the NAc core. (A) Slope of the CS dopamine response is calculated as the difference between the regions of interest denoted by the teal overlay. (B) Slope of the CS dopamine response in the NAc core. (C) Slope of the CS dopamine response in the medial NAc shell. * $p < 0.05$, ** $p < 0.01$ main effect of trial type.

observed by session 6 (reward rate effect: $F_{(1.0, 9.0)}$=0.131, p=0.725). In contrast to the NAc core, the slope of the CS-evoked dopamine response did not differ between Short and Long wait trial types in the medial NAc shell (reward rate effect for all trials: $F_{(1.0, 13.0)}$=0.00984, p=0.923; **Fig. 3C**). Together, these results highlight trial-type and region-specific changes in the dynamics of the CS-evoked dopamine during early Pavlovian training sessions.

Regional differences in CS-evoked dopamine release

Prior studies have noted differences in dopamine release between the NAc core and NAc shell in reward-based tasks [11-13,22,23]. As such, we directly compared CS- and US-evoked dopamine release between the NAc core and medial NAc shell during early Pavlovian training sessions. This analysis identified a significant interaction of brain region and training sessions on

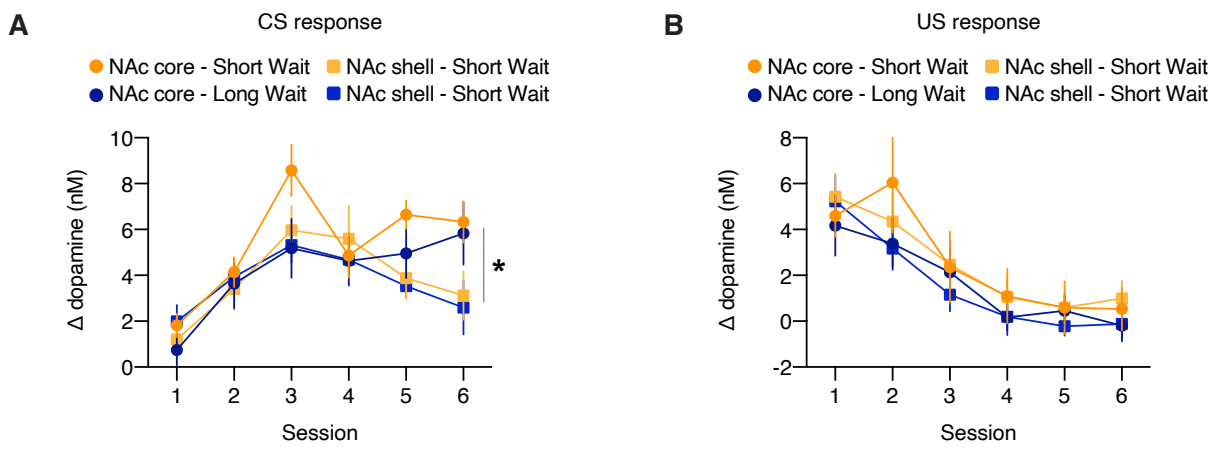

Figure 4. Comparing the dopamine response between the NAc core and medial NAc shell. (A) CS dopamine response. (B) US dopamine response. * $p < 0.05$ interaction effect of session x NAc core x NAc shell.

the CS-evoked dopamine response (three-way mixed effects analysis: region effect $F_{(1, 84)}$=2.075, p=0.154; session effect $F_{(2.0, 43.3)}$=12.15, p<0.0001; region x session $F_{(5, 84)}$=2.93, p=0.0174; reward rate effect $F_{(1.0, 22.0)}$=0.989, p=0.331; **Fig. 4A**). This interaction effect was driven by a smaller dopamine response in the NAc shell during later training sessions. In contrast, US-evoked dopamine release did not differ between the NAc subregions (three-way mixed effects analysis: region effect $F_{(1, 84)}$=0.00396, p=0.950; session effect $F_{(1.60, 35.2)}$=28.3, p<0.0001; reward rate effect $F_{(1.0, 22.0)}$=1.41, p=0.248; **Fig. 4B**). Therefore, the trajectories of CS-evoked dopamine release in the NAc core and medial NAc shell diverge over the course of associative learning.

Emergence of dopamine-sensitive conditioned responding in the NAc core and medial NAc shell

NAc dopamine signaling is necessary for conditioned behavioral responses to rewarding cues [15,24]. However, the differential development of the CS-evoked dopamine responses between the NAc core and medial NAc shell suggests that these regions may not contribute equally to behavior throughout training. To address this, rats were implanted with bilateral cannulae targeting the NAc core or the medial NAc shell for local pharmacological manipulations (**Fig. 5A**). The D1/D2 dopamine receptor antagonist flupenthixol (10 µg/side) or

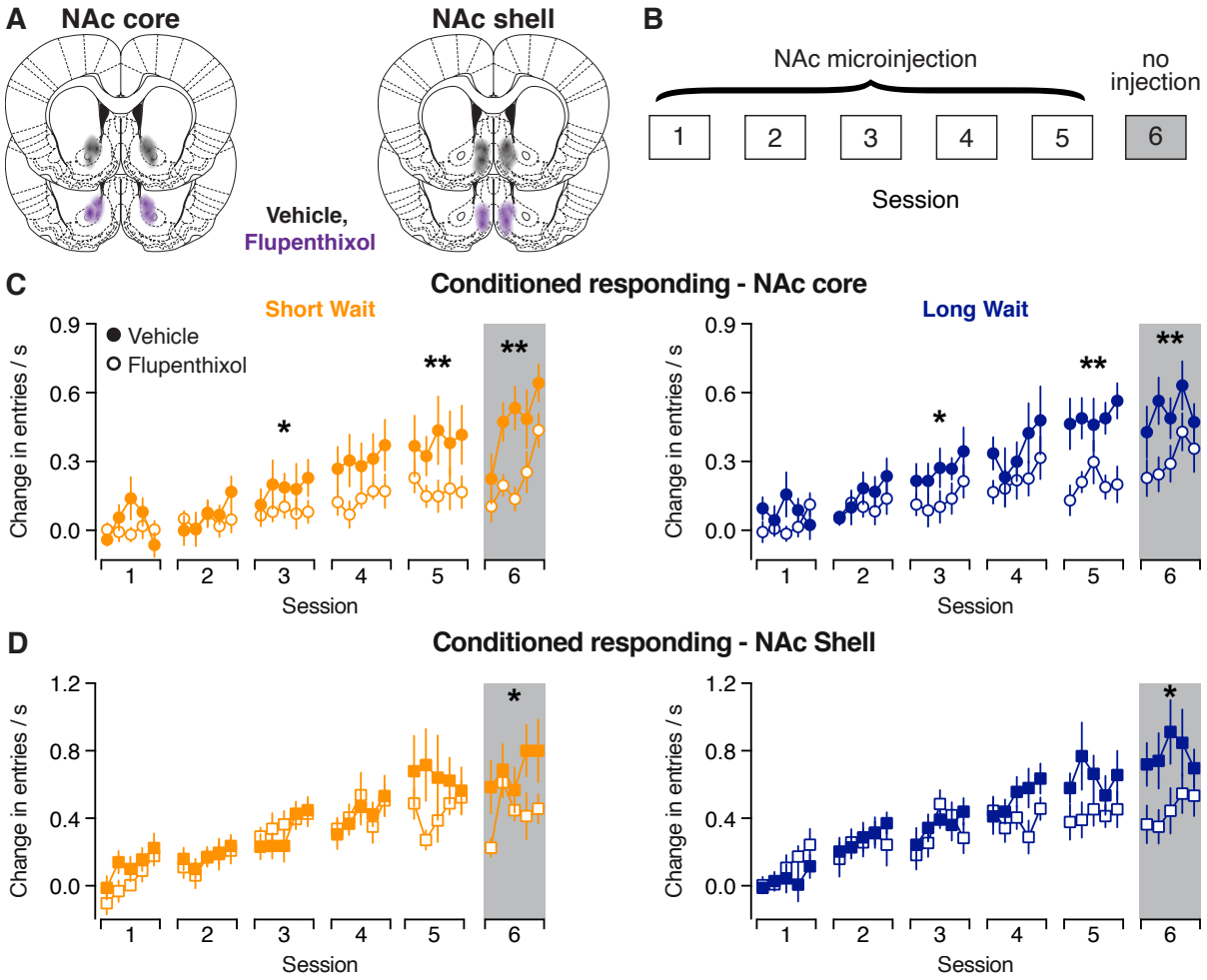

Figure 5. Emergence of dopamine-dependent conditioned responding in the NAc core and medial NAc shell. (A) Training paradigm. Flupenthixol or vehicle was infused into the NAc core or NAc shell before the first five training sessions. Training continued for an additional session without injections. (B) Location of the injector tips and infusion area. (C) Conditioned responding in rats receiving injections into the NAc core. (D) Conditioned responding in rats receiving injections into the NAc shell. * $p < 0.05$, ** $p < 0.01$ main effect of drug injection.

vehicle was infused 30 min before the first 5 sessions. Rats were trained without microinjections for an additional session to differentiate acute versus sustained behavioral effects of the drug treatment (**Fig. 5B**). Flupenthixol microinjections in the NAc core significantly disrupted conditioned responding in the third and fifth conditioning session, and the behavioral impairment persisted in the sixth session in which no injection was administered (three-way mixed effects analysis: drug effect session 1 $F_{(1,76)}$=1.97, p=0.165; session 2 $F_{(1,76)}$=0.878, p=0.352; session 3 $F_{(1,76)}$=4.16, p=0.0449; session 4 $F_{(1,76)}$=3.95, p=0.0503; session 5 $F_{(1,76)}$=10.3, p=0.00190; prior

drug treatment effect session 6 $F_{(1,76)}$=11.5**,** p=0.00110; n= 10 vehicle, 11 flupenthixol; **Fig. 5C**). Flupenthixol application in the NAc core selectively reduced the number of CS-evoked head entries without altering head entries during the ITI or the latency to approach the food cup from cue onset (**Supplementary Fig. 1**). Disruption of NAc core dopamine transmission therefore selectively impairs cue-driven appetitive behavior without altering motor function or response initiation.

In rats with cannulae in the medial NAc shell, conditioned responding was not acutely affected by flupenthixol treatment; however, a behavioral deficit was evident on the sixth session in which no injection was given (three-way mixed effects analysis: drug effect session 1 $F_{(1,68)}$=0.0765, p=0.783; session 2 $F_{(1,68)}$=0.221, p=0.634; session 3 $F_{(1,68)}$=0.0185, p=0.892; session 4 $F_{(1,68)}$=0.750, p=0.389; session 5 $F_{(1,68)}$=2.29, p=0.135; prior drug treatment effect session 6 $F_{(1,68)}$=5.06, p=0.0277; n= 10 vehicle, 9 flupenthixol; **Fig. 5D**). Flupenthixol treatment in the NAc shell caused a subsequent non-significant reduction of cue-evoked head entries and significantly increased latency to approach the food cup during the sixth training session (**Supplementary Fig. 2**). This effect on response latency suggests that dopamine transmission in the NAc shell regulates the speed of response initiation in addition to the magnitude of the conditioned response. Collectively, these results demonstrate that dopamine signals in the NAc core and medial shell contribute to Pavlovian appetitive behavior at distinct phases of training.

Discussion:

Dopamine neurons respond to cues to convey reward-related information in Pavlovian tasks [3,7,8]. In particular, cue-evoked dopamine release in the NAc core signals the relative reward rate in rats with extensive Pavlovian training (>24 sessions) [8]. However, it was not known if reward rate dopamine signals rapidly emerged during early learning. To address this, we recorded dopamine release during the first 6 Pavlovian training sessions. Our data demonstrates that the reward rate is not reflected in cue-evoked dopamine responses in the NAc core or the medial NAc shell during early training. Coupled with our prior work, these findings suggest that NAc dopamine encodes value-related information via a sequential, multi-step process [8]. Specifically, cue-evoked dopamine release initially signals that a reward is

forthcoming. However, over training these cue-evoked dopamine responses then signal the relative value of the reward.

Dopamine neurons projecting to the striatum are genetically and functionally diverse [25-27]. While rewards and reward-predictive cues elicit dopamine release in the NAc core and medial NAc shell, regional differences in the dynamics of the dopamine response are evident in some tasks [11-13,22,23]. Our data demonstrates that reward-evoked dopamine release decreased over Pavlovian training sessions, with no difference in dopamine between the NAc core and medial NAc shell. Dopamine release to cues initially increased in both regions, though there was a selective attenuation in the cue-evoked dopamine response in the NAc shell during later sessions. Although there was no difference in the average dopamine response between Long and Short Wait cues, we identified transient differences in the dynamics of the dopamine response between trial types in the NAc core. Specifically, the decay of the cue-evoked dopamine response was more pronounced for Long Wait trials relative to Short Wait trials during sessions 3-5, though this effect was absent in session 6. The dynamics of the cue-evoked dopamine response could be controlled by dopamine neuron firing patterns and/or regulation of dopamine release at the terminals [28-30]. Regardless, future studies are needed to determine if these endogenous patterns of cue-evoked dopamine release are related to learning and performance in appetitive tasks.

Conditioned responding can be modulated by the dopamine response to cues and rewards, though this can depend on task parameters and prior training [14,26,31,32]. We previously found that conditioned responding updates with cue-specific changes in dopamine release in well-trained animals. Rats trained to experience Short and Long Wait trials in separate sessions exhibited a selective elevation in dopamine release and conditioned responding to the Short Wait cue upon experiencing both trials together for the first time [8]. Based on these findings, we anticipated the emergence of cue-evoked dopamine release would parallel emergence of dopamine-sensitive conditioned responding during early training sessions. Our voltammetry recordings and dopamine receptor antagonist experiments instead illustrate that the increase in cue-evoked dopamine release precedes the emergence of dopamine-mediated conditioned responding. In the NAc core, flupenthixol treatment reduced conditioned

responding starting in the third training session. These impairments persisted during the sixth training session with no drug treatment, which demonstrates NAc core dopamine is involved with Pavlovian learning, as reported previously [10,15]. In contrast, flupenthixol injections into the medial NAc shell failed to alter conditioned responding during the first five training sessions. However, this prior treatment with flupenthixol impaired conditioned responding during the sixth training session with no drug treatment. These results highlight a potential role for NAc shell dopamine in consolidation. In support, local injections of amphetamine into the NAc shell after the Pavlovian training sessions elevated conditioned responses [33]. Future studies will be needed to identify the specific temporal window during the trial or after the session when dopamine signaling contributes to conditioned responding. Collectively, our results highlight region-specific critical periods during training when dopamine signaling regulates conditioned responding.

Dopamine is thought to mediate distinct functions in the NAc core and NAc shell, with NAc core dopamine primarily involved with reward learning and NAc shell dopamine regulating learned behavioral actions [10,12,15,26]. However, it is important to note that these general roles may not be applicable to all behavioral tasks [34]. Indeed, our results highlight that dopamine in both the NAc core and medial NAc shell contribute to Pavlovian learning when cues convey distinct reward rates, albeit at different points during training. Future studies are needed to determine if dopamine in the NAc core and shell is similarly required for Pavlovian learning when cues signal differences in other reward-related parameters, such as reward size or probability. Research on dopamine's role in behavior has largely focused on the 'what' and the 'where': what task elements increase dopamine release and where in the brain is dopamine released. Our results collectively highlight that it is also important to consider 'when' during training dopamine is capable of regulating behavioral actions.

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Author Contributions: CES, KSG, and MJW designed the experiments. CES, KSG, MJL, KMF, and MJW performed the experiments and analyzed the data. CES and MJW wrote the manuscript.

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