Absence of NMDA receptors in dopamine neurons attenuates dopamine release but not conditioned approach during Pavlovian conditioning

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During Pavlovian conditioning, phasic dopamine (DA) responses emerge to reward-predictive stimuli as the subject learns to anticipate reward delivery. This observation has led to the hypothesis that phasic dopamine signaling is important for learning. To assess the ability of mice to develop anticipatory behavior and to characterize the contribution of dopamine, we used a food-reinforced Pavlovian conditioning paradigm. As mice learned the cue–reward association, they increased their head entries to the food receptacle in a pattern that was consistent with conditioned anticipatory behavior. D1-receptor knockout (D1R-KO) mice had impaired acquisition, and systemic administration of a D1R antagonist blocked both the acquisition and expression of conditioned approach in wild-type mice. To assess the specific contribution of phasic dopamine transmission, we tested mice lacking NMDA-type glutamate receptors (NMDARs) exclusively in dopamine neurons (NR1-KO mice). Surprisingly, NR1-KO mice learned at the same rate as their littermate controls. To evaluate the contribution of NMDARs to phasic dopamine release in this paradigm, we performed fast-scan cyclic voltammetry in the nucleus accumbens of awake mice. Despite having significantly attenuated phasic dopamine release following reward delivery, KO mice developed cue-evoked dopamine release at the same rate as controls. We conclude that NMDARs in dopamine neurons enhance but are not critical for phasic dopamine release to behaviorally relevant stimuli; furthermore, their contribution to phasic dopamine signaling is not necessary for the development of cue-evoked dopamine or anticipatory activity in a D1R-dependent Pavlovian conditioning paradigm.

D1 receptors | fast-scan cyclic voltammetry | nucleus accumbens | reinforcement learning | reward-prediction

During an appetitive Pavlovian conditioning paradigm, a discrete cue (CS) is repeatedly paired with the delivery of a reward (US). As an animal learns to associate CS presentation with US delivery, a number of conditioned responses (CR) may occur in anticipation of reward delivery (1). The emergence of these anticipatory responses has served as a behavioral proxy by which the neural processes that underlie stimulus–reward learning may be studied. One commonly observed CR in rodents is the development of conditioned approach (CA) behavior; as the CS becomes predictive of US delivery, an animal will physically approach the location of the CS or US with increased frequency in the presence of the CS (2–5). Recent studies using electrophysiological and pharmacological manipulations have specifically implicated the dopamine system in the acquisition of CA behavior.

The presentation of behaviorally relevant stimuli elicits burst-firing by the dopamine neurons of the ventral midbrain (6) and transient dopamine release in terminal regions including the nucleus accumbens core (AcbC) (7). During Pavlovian conditioning, a previously neutral stimulus (CS) elicits a phasic dopamine response as it becomes a predictor of the reward (US) (2, 4, 5, 8, 9), a process that has been postulated to represent the acquired incentive value of the stimulus (10). As a CS-elicited dopamine response develops, the dopamine response to the US diminishes such that when the reward becomes completely predicted it no longer evokes dopamine release (4, 8). In formal reinforcement-learning models, learning occurs only when outcomes are not fully predicted by environmental cues and is driven by "prediction-error" signals that represent differences between expected and received rewards (11). Because phasic dopamine transmission correlates well with a prediction-error signal under several learning paradigms (12), it is thought to play an important role in reinforcement learning.

Consistent with the hypothesis that dopamine release in the AcbC contributes to learning a Pavlovian association are the observations that local dopamine receptor antagonism (13), 6-hydroxydopamine–induced depletion of dopamine projections to this nucleus (14), or excitotoxic lesions of the AcbC (15, 16) attenuate both the development and expression of learned CA behavior. Specifically, learning is thought to require the activation of "low-affinity" D1 receptors (D1Rs) in the AcbC (17–19), consistent with a requirement for the high extracellular concentrations of dopamine that result from phasic transmission (20).

The cellular mechanisms through which phasic activation and subsequent dopamine release occur is thought to involve NMDA-type glutamate receptor (NMDAR)-dependent burst-firing in dopamine neurons (21–23). In addition to burst-firing, NMDAR signaling is required for long-term potentiation (LTP) in dopamine neurons (24, 25). LTP within dopamine neurons may contribute to the development of phasic dopamine release to reward-predicting stimuli (5). Indeed, LTP has been shown to occur in dopamine neurons at the time when the CS begins to elicit anticipatory behaviors during Pavlovian conditioning (5). However, whether NMDAR-dependent LTP in dopamine neurons is required for the development of phasic dopamine release to reward-predicting stimuli and whether phasic dopamine release directly facilitates learning is not known.

Previous work has shown that mice lacking functional NMDAR signaling in dopamine neurons through conditional genetic inactivation of the essential NMDAR subunit NR1 (NR1-KO) have attenuated dopamine neuron burst-firing and transsynaptically driven phasic dopamine release with no difference in tonic dopamine neuron activity (23). In addition, dopamine neurons in slices from NR1-KO mice were unable to undergo LTP (25). Although many behaviors in NR1-KO mice appear to be normal, including their behavioral responses to drugs that are known to increase extracellular dopamine (25), these mice take longer to reach the same level of performance as

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their littermate controls in several learning tasks (23). These observations suggest that NMDAR signaling in dopamine neurons contributes to a pattern of dopamine signaling that is important for modulating goal-directed behavior.

To facilitate the use of these and other genetic mouse models to probe the neural circuitry underlying the acquisition of Pavlovian CA, we have adapted a commonly used Pavlovian conditioning paradigm to mice. Once we were able to observe a stable measure of CA, we took advantage of genetic and pharmacological manipulations to characterize the contribution of specific aspects of dopamine signaling to the development of this behavior in mice. Finally, we report the first use of fast-scan cyclic voltammetry (FSCV) in awake mice during Pavlovian conditioning. We used this method to simultaneously assess the contribution of NMDARs in dopamine neurons to phasic dopamine release and Pavlovian CA.

Results

C57BL/6 Mice Learn to Anticipate Reward Delivery During Pavlovian Conditioning. Our Pavlovian conditioning paradigm consisted of an 11-s CS presentation paired with the delivery of a food reward at t = 10 s with an intertrial interval (ITI) of 60 ± 20 s (Fig. 1*A*). To verify that this adaptation of a commonly used Pavlovian conditioning paradigm was suitable for use in mice, we tested a cohort of WT C57BL/6 mice in both the paired (CS^+) and unpaired (CS^-) conditions. WT mice selectively elevated their head-entry (HE) rate to the food receptacle during the CS relative to the ITI in the paired but not in the unpaired condition (Fig. 1 *B* and *C*). We quantified this discrimination in HE activity as a CA score:

$$\text{CA Score} = CS\ \ HE\ \ rate - ITI\ \ HE\ \ rate$$

As predicted, WT mice in the paired but not in the unpaired experiment increased their CA score, indicating that the increase in HE rate was specific for a reward association with the cue. When HE rates during CS presentation were analyzed in 2-s bins, we found that the paired group of animals had a uniform increase in HE rate throughout the CS (Fig. S1). This pattern of responding indicates that although the animals anticipated reward delivery, they were unable to precisely time the delivery event. This observation is in contrast to mice trained in a similar paradigm in which CS presentation terminated just before the US; in these experiments, HE rate peaked at the time of reward delivery (26, 27). In the latter case, CS termination may have allowed the mice to more precisely predict reward delivery. Nonetheless, the observation of a cue-selective increase in approach behavior in the paired but not in the unpaired condition validates this paradigm for assessing Pavlovian reinforcement learning in mice.

D1R Signaling Is Critical for the Acquisition and Expression of CA. To determine whether the development of CA in this paradigm was dependent on dopamine, we tested D1R-KO mice and their littermate controls. Control mice performed similarly to WT mice and selectively increased their CS-elicited HE rate, resulting in an elevation in their CA score (Fig. 2*A* and *C*). In contrast, D1R-KO mice showed no apparent learning in this paradigm (Fig. 2 *B* and *C*).

The lack of learning in D1R-KO mice could be caused by a deficit in either learning or the expression of learning. To dissociate the contribution of D1R signaling to the acquisition and expression of Pavlovian CA, we tested mice with two systemic doses of the selective D1R antagonist, SCH23390. Similar to D1R-KO mice, WT mice treated with the higher dose of SCH23390, but not those treated with the lower dose or saline, failed to selectively increase their CS-elicited HE rate after six training sessions (Fig. 3 *A*–*C*). This failure corresponded with a lack of elevation in the CA score of these mice (Fig. 3*D*). Although mice treated with the higher dose of SCH23390 had a significant decrease in their overall HE rate, their rate was similar to that observed in the D1R-KO mice during training (Fig. S2*A*), suggesting that this effect was D1R specific. Despite variable rates of HE to the food receptacle, all groups of mice on all days (including D1R-KO mice) consumed all of their reward pellets, indicating that neither pharmacological nor genetic manipulation dramatically affected the ability of these mice to engage in this learning paradigm.

When the mice treated with the higher dose of SCH23390 were tested in the absence of the drug on day 7, there was an increase in their overall HE rate compared with their rate in the presence of the drug on day 6 (Fig. S2*B*). However, this increase was independent of the presence or absence of the cue and resulted in a pattern of responding that was similar to that of the saline-treated animals on day 1 (Fig. 3 *A* and *C*). The CA score in the mice trained on the higher dose of D1R antagonist was significantly attenuated on day 7 and no different from controls on day 1 (Fig. 3*D*). These findings indicate that no learning took place during the sessions where SCH23390 was present and that D1R signaling is critical for acquisition of the cue-reward association.

To assess the contribution of D1Rs to the performance of previously learned CA, we tested the saline-treated mice in the presence of the high dose of SCH23390 on day 8. There was a significant decline in the overall HE rate of these mice (Fig. S2*B*) that was preferential for their CS-elicited HE rate, because the ITI HE rate remained unchanged (Fig. 3*A*). This selective decrement in the learned response resulted in a significant reduction in their CA score on day 8 (Fig. 3). These results suggest that, in addition to being critical for acquisition, D1R signaling is necessary for the expression of CA.

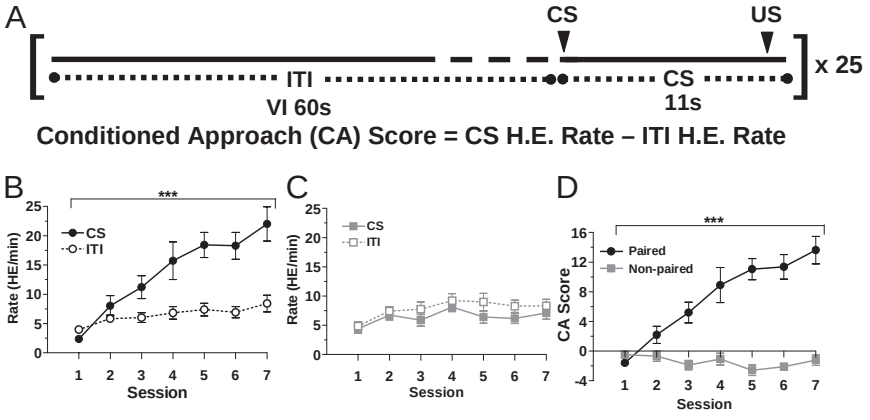

Fig. 1. Pavlovian conditioning elicits reward-associated CA behavior in C57BL/6 mice. (*A*) Our paradigm consisted of an 11-s lever presentation (CS) paired with the delivery of a 20-mg food pellet at t = 10 s. Mice received 25 CS–US pairings at a variable ITI averaging 60 s. (*B* and *C*) Mice selectively increased their HE rate during CS presentation relative to baseline (ITI) when it was paired (*B*) but not when it was unpaired (*C*) with US delivery (mean ± SEM, two-way ANOVA, rate x session; $F_{(5,110)} = 7.7$; ***$P < 0.001$). (*D*) Only the mice in the paired group increased their CA score during training (mean ± SEM, two-way ANOVA, group x session; $F_{(5,110)} = 18.9$; ***$P < 0.001$).

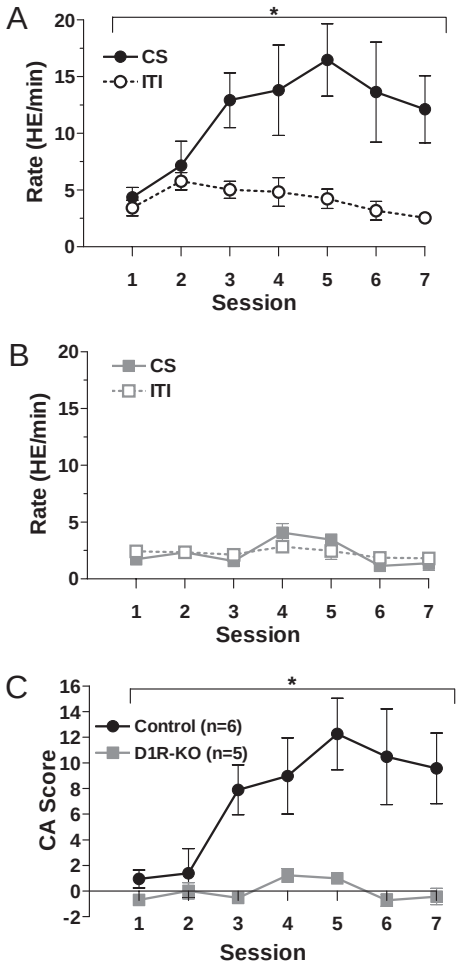

Fig. 2. D1R-KO mice were unable to learn in this Pavlovian conditioning paradigm. (*A* and *B*) Control (*A*) but not D1R-KO mice (*B*) increased their CS-elicited HE rate relative to baseline (mean ± SEM, two-way ANOVA, rate x session; $F_{(6,60)} = 3.0$; $^{*}P < 0.05$). (*C*) This trend resulted in increase in CA score only in the control mice (mean ± SEM, two-way ANOVA, genotype x session; $F_{(6,54)} = 3.1$; $^{*}P < 0.05$).

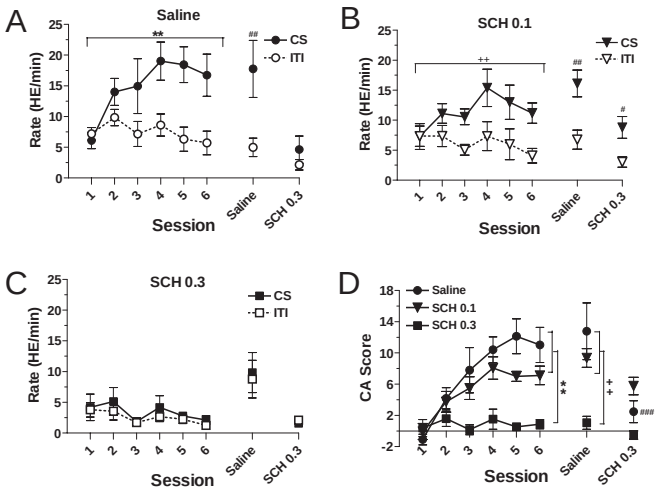

Fig. 3. D1R antagonism blocks both the acquisition and performance of Pavlovian CA. (*A*) Saline-treated C57BL/6 mice significantly increased their HE rate during the CS relative to baseline (mean ± SEM, two-way ANOVA, rate x session; $F_{(5,60)} = 4.0$; $^{**}P < 0.01$). On day 7, the selective increase in CS-elicited HE rate remained (Fisher post hoc analysis; $^{\#\#}P < 0.01$); however, in the presence of the D1R antagonist on day 8, there was no difference between CS and ITI HE rate (Fisher post hoc analysis; $P = 0.52$). (*B*) Mice treated with 0.1 mg/kg D1R antagonist had an intermediate level of increased HE rate during CS relative to baseline (mean ± SEM, two-way ANOVA, CS vs. ITI; $F_{(1,10)} = 19.9$; $^{++}P < 0.01$). On day 7, the selective increase in CS-elicited HE rate remained (Fisher post hoc analysis; $^{\#\#}P < 0.01$); these mice still showed an elevation in HE rate during the CS when tested in the presence of the high dose of D1R antagonist (Fisher post hoc analysis; $^{\#}P < 0.05$). (*C*) Mice treated with 0.3 mg/kg D1R antagonist had no increase in HE rate during the CS. When tested in the absence of the antagonist on day 7, there still was no difference between CS and baseline HE rate (mean ± SEM). (*D*) CA score increased in saline- and low-dose–, but not high-dose–treated mice [mean ± SEM, days 1–6, two-way ANOVA; treatment vs. session (saline vs. SCH0.3), $F_{(5,50)} = 6.7$; treatment vs. session (SCH0.1 vs. SCH0.3), $F_{(5,45)} = 7.1$; $^{**}P < 0.01$]. Only the group treated with the highest dose of D1R antagonist still had no CA on day 7 when all groups were tested in the absence of the antagonist (Fisher post hoc analysis; $^{++}P < 0.01$). Saline-treated mice had a significant decrease in CA score in the presence of the high dose of D1R antagonist on day 8 (Fisher post hoc analysis, day 7 vs. day 8; $^{\#\#\#}P < 0.01$).

Genetic Inactivation of NMDAR Signaling Exclusively in dopamine Neurons Does Not Affect the Acquisition of CA. Emerging evidence suggests that D1Rs are activated specifically by the high extracellular dopamine concentrations that result from phasic dopamine release (17–19). Because phasic dopamine transmission is thought to be driven by the activation of NMDARs in dopamine neurons, we tested mice lacking these receptors exclusively in dopamine neurons. Both NR1-KO and control mice selectively increased their CS-elicited HE rates relative to the ITI (Fig. 4 *A* and *B*), resulting in a similar pattern of elevation in CA score (Fig. 4*C*). These results indicate that NMDARs in dopamine neurons are not required for the acquisition of Pavlovian CA.

NR1-KO Mice Have Attenuated Phasic dopamine Release During Pavlovian Conditioning as Measured by Fast-Scan Cyclic Voltammetry. Although we previously have shown that spontaneous burst-firing in dopamine neurons is attenuated in NR1-KO mice (23), we did not monitor dopamine signaling in response to behaviorally relevant events such as the delivery of a food reward or the presentation of a conditioned stimulus. To determine whether the NR1-KO mice had attenuated phasic dopamine transmission during this paradigm, we used fast-scan cyclic voltammetry (FSCV) to monitor phasic dopamine release at chronically implanted carbon-fiber microelectrodes in the AcbC (Fig. 5*A*). In response to the presentation of an unexpected food pellet before the start of each conditioning session, phasic dopamine release was observed in both NR1-KO and control mice (Fig. 5 *B* and *C*), but the amplitude was approximately 3-fold lower in the NR1-KO mice on all days tested (Fig. 5*D*).

After confirming a reward-elicited phasic dopamine response, we subjected these mice to our Pavlovian conditioning paradigm. The mean phasic dopamine release also was 3-fold lower in the KO mice in response to reward retrieval on day 1 or to US delivery in subsequent training sessions (Fig. 6 *A* and *D*, *Inset*). Within both groups, US-evoked dopamine remained stable throughout training (Fig. 6*A*).

To determine whether the residual dopamine release in NR1-KO mice could develop to a reward-predictive cue, we analyzed the changes in dopamine release to the lever extension (CS) during training. Similar to the US-elicited response, CS-evoked dopamine release was attenuated in the NR1-KO mice; however, the decreased phasic dopamine release in NR1-KO mice did not affect their ability to develop significantly increased CS-evoked dopamine during learning, because the rate of increase in this response was equivalent in control and NR1-KO mice (Fig. 6 *B* and *D*). In agreement with this observation, the two groups of mice developed Pavlovian CA at same rate (Fig. 6*C*).

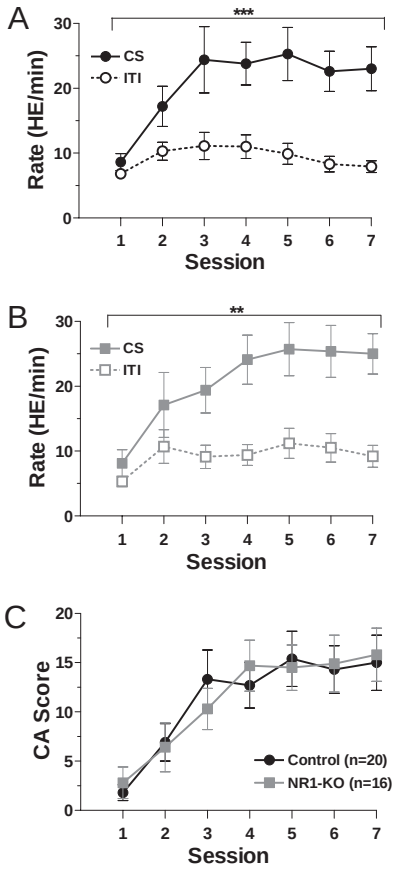

Fig. 4. NMDARs in dopamine neurons are not necessary to acquire Pavlovian CA. (*A*) Control mice selectively increased their CS-elicited HE rate during training (mean ± SEM, two-way ANOVA, rate x session; $F_{(6,228)} = 4.6$; $^{***}P < 0.001$). (*B*) NR1-KO mice also increased their CS-elicited HE rate relative to baseline (mean ± SEM, two-way ANOVA, rate x session; $F_{(6,180)} = 3.2$; $^{**}P < 0.01$). (*C*) CA scores increased comparably in control and KO mice (mean ± SEM).

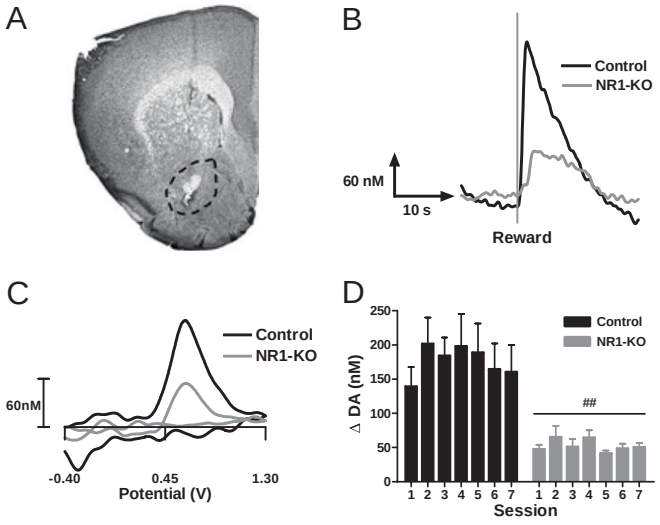

Fig. 5. Phasic dopamine release in response to an unexpected food reward is attenuated in NR1-KO mice. (*A*) Cresyl violet-stained 30-μm brain section revealing electrode track and site of postexperimental electrolytic lesion. Dashed black line indicates the AcbC. (*B*) Representative dopamine traces from control and NR1-KO mice in response to the delivery of an unexpected food reward. (*C*) Representative cyclic voltammograms from control and NR1-KO mice obtained from a current in response to unexpected reward delivery. (*D*) Peak phasic dopamine release elicited by the delivery of the first unexpected food pellet each day was attenuated in NR1-KO ($n = 5$) relative to control ($n = 6$) mice (mean ± SEM, two-way ANOVA, control vs. NR1-KO; $F_{(1,9)} = 14.7$; $^{\#\#}P < 0.01$).

The US-evoked phasic dopamine response did not diminish despite clear evidence of learning by both groups (Fig. 6*A*). Moreover, US-evoked dopamine was greater than the CS-evoked signal on all training days in both groups (Fig. 6 *E* and *F*). Because these observations were inconsistent with the reward-prediction hypothesis, we tested the possibility that the animals had not completely learned the association by subjecting a subset of control mice to three additional training sessions (250 total pairings). There was no increase in CA score after the additional training, demonstrating that these mice had reached asymptotic performance by session 7 (Fig. S3*A*). During this additional training period, the US-evoked dopamine release remained robust and larger than the CS response (Fig. S3*B*). This result shows that the persistence of the US response was not an artifact of incomplete learning.

Discussion

We have described an appetitive Pavlovian conditioning paradigm in which mice reliably elevate their CA behavior as they acquire the CS–US association. Using this pattern of responding as a metric of learning, we showed that D1R signaling is critical for both the acquisition and performance of Pavlovian CA. This observation is in agreement with previous studies in rats (13–16) and is presumably the consequence of removing D1R-dependent neuroplasticity in the medium spiny neurons of striatal target regions (28, 29). Because D1Rs are postulated to be activated specifically by high extracellular concentrations of dopamine (17, 18), we predicted that disrupting dopamine neuron burst-firing and phasic dopamine release would attenuate the acquisition of Pavlovian CA.

Surprisingly, disrupting phasic dopamine signaling by removing NMDARs from dopamine neurons had no effect on the development of Pavlovian CA. This result is at odds with our previous finding that NR1-KO mice are slower to learn conditioned place preference, T-maze, and instrumental conditioning (23). This observation is also is contrary to what would be expected if NMDAR-dependent synaptic plasticity within dopamine neurons contributes significantly to the acquisition of Pavlovian CA (5). Because there are no previous reports in which phasic dopamine has been monitored while simultaneously manipulating NMDAR signaling during the acquisition phase of learning, we sought to reconcile our results using FSCV in NR1-KO mice during training.

In agreement with the proposed role of NMDAR signaling in phasic dopamine transmission (22, 23), we found that NR1-KO mice had a 3-fold decrease in phasic dopamine release for unexpected rewards and for CS and US presentations, as compared with controls. However, even with attenuated phasic dopamine release, the rate at which CS-elicited dopamine increased during training was not altered. Because we have shown previously that the dopamine neurons in NR1-KO mice were unable to undergo LTP in slice preparations (25), we conclude that the increase in the phasic dopamine response to the CS is the result of plasticity within neurons that project onto dopamine neurons rather than in the dopamine neurons themselves. However, we cannot rule out that NMDAR-independent plasticity in dopamine neurons occurs in vivo. Nonetheless, the comparable rate of increase in CS-elicited dopamine release is consistent with the observation that NR1-KO mice learned at the same rate as controls. Our results confirm that although NMDARs in dopamine neurons contribute to phasic dopamine release in response to behaviorally relevant stimuli, removing the contribution of these receptors does not alter the

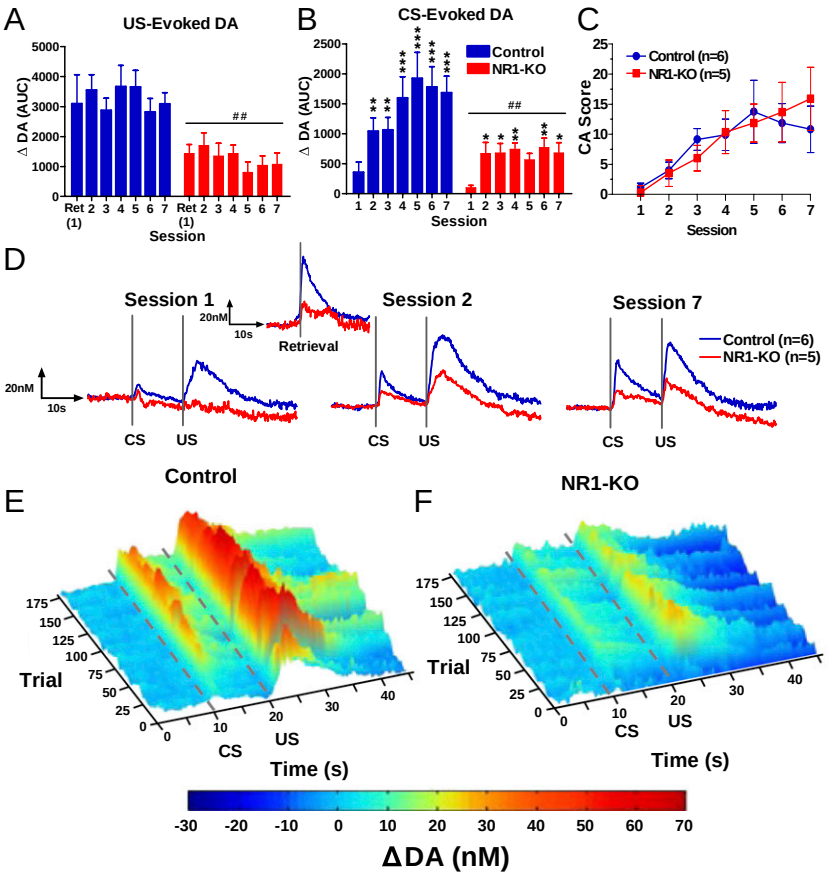

Fig. 6. Phasic dopamine release develops to the CS and persists to the US during Pavlovian conditioning in control and NR1-KO mice. (*A*) US-evoked dopamine responses were decreased in NR1-KO (n = 5) compared with control (n = 6) mice across all seven training sessions [mean area under the curve (AUC) ± SEM, two-way ANOVA, control vs. NR1-KO; $F_{(1,9)}$ = 10.8; $^{\#\#}P < 0.01$]. dopamine release at the time of reward retrieval rather than delivery was used for session 1. (*B*) CS-evoked dopamine responses were decreased in NR1-KO mice but increased significantly during training in both groups (mean AUC ± SEM, two-way ANOVA, genotype x session; $F_{(6,54)}$ = 3.4; $^{\#\#}P < 0.01$; Fisher post hoc analysis; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (*C*) CA scores increased similarly in the control and NR1-KO mice used in the FSCV experiment (mean ± SEM). (*D*) Average dopamine traces form control and NR1-KO mice in response to CS and US presentations on days 1, 2, and 7. *Inset* shows mean dopamine traces at the time of reward retrieval during session 1. (*E* and *F*) Three-dimensional representations summarize the phasic dopamine release to the CS and US for control (*E*) and NR1-KO (*F*) across all training days (five-trial blocks).

ability of mice to associate a previously neutral stimulus with reward in a D1R-dependent learning paradigm.

Despite having significantly attenuated phasic dopamine release, NR1-KO mice still had some event-related dopamine release throughout training. Although the NMDAR-independent mechanism underlying the remaining dopamine release is unknown, it could be mediated by glutamate acting on AMPA receptors (25), other excitatory neurotransmitters such as acetylcholine (30), changes in GABA-mediated inhibition (31, 32), or some combination of these influences. Because we anticipate that dopamine release in NR1-KO mice should be no more efficacious at postsynaptic receptors than in controls because D1R signaling appears to be normal in these mice (25), we conclude that the residual dopamine release in the NR1-KO mice was sufficient to facilitate learning in the paradigm used here. Furthermore, it remains possible that phasic dopamine release is not required at all for the activation of D1Rs and the development of Pavlovian CA.

Because NR1-KO mice have impairments in other learning paradigms (23), their normal performance in this Pavlovian task highlights potentially important interactions between phasic dopamine and task parameters. For example, tasks requiring animals to learn contextual cues, such as in a T-maze or place conditioning, may require greater levels of phasic dopamine release than Pavlovian tasks using temporally discrete cues. This discrepancy suggests that the associability of the cue and reward used in our paradigm was below the threshold required to observe differences in learning between control and NR1-KO mice. Nonetheless, our results indicate that as long as a particular stimulus is sufficiently salient, attenuating phasic dopamine release does not affect the rate at which the subject assigns incentive value to that stimulus.

Unlike in previous reports that also used a CS that was spatially separated from the location of US delivery (4, 9), the mice in our paradigm exclusively directed their CA toward the food receptacle and did not interact with the CS (lever). This observation is consistent with recent results using C57BL/6 × 129Sv mice (26, 33) and probably reflects the CR strategy used by these strains of mice. This pattern of conditioned behavioral response has been described as "goal-tracking" as opposed to "sign-tracking" (3) and may reflect species, strain, or even individual differences in the manner in which values are assigned to environmental cues (1). The persistence of the dopamine response to the US and the observation that it remains larger than the response to the CS is inconsistent with some previous reports (4, 8, 9) and correlates with the propensity of these mice to engage in goal-tracking as opposed to sign-tracking behavior (1, 4, 9). However, task parameters also can influence the pattern of CA behavior (34) and phasic dopamine transmission (35). For example, unlike in our task in which the US delivery occurred during the last second of CS presentation, the CS in previous studies terminated shortly before reward delivery (4, 9); thus, CS termination just before reward delivery could provide a proximal cue that may have allowed the rats to predict the time of reward delivery more precisely, resulting in a smaller error in reward prediction. Likewise, in studies using monkeys in which the US occurred during the last second of CS presentation, US-elicited activation of dopamine neurons was maintained after training and remained larger than CS responses (35, 36). This interpretation agrees with the observation that the mice in our paradigm were unable to time reward delivery as well as the same strain of mice trained in a paradigm with no overlap between the CS and US (26, 27). Taken together, these findings suggest that the maintenance of the US signal throughout training may be a physiologically rele-

vant phenomenon that correlates with task or behavioral distinctions during learning.

In conclusion, we show that D1R signaling is required for the development of CA behavior in mice. Furthermore, pharmacologically blocking D1Rs in mice that had already learned the cue–reward association preferentially attenuated their CS-elicited HE rate, resulting in the diminishment of their CA score. Taken together, these findings demonstrate that D1R signaling is critical for the acquisition and expression of Pavlovian CA. Although D1R signaling is required for the development of CA, and although these receptors are thought to be specifically activated by phasic dopamine release, attenuating phasic dopamine signaling by selectively removing NMDARs from dopamine neurons did not affect the rate at which these animals developed CS-elicited dopamine release or CA behavior. These findings suggest that NMDAR-independent dopamine signaling was sufficient to activate D1Rs and enable learning in this paradigm.

Materials and Methods

Animals. All behavioral testing and voltammetry experiments were approved by the University of Washington Animal Care and Use Committee. The generation and maintenance of all mouse lines is described in *SI Text*.

Pavlovian Conditioning. Behavioral testing was conducted in operant conditioning chambers (ENV-307W; Med Associates, Inc.). Mice were trained to retrieve food pellets in a single magazine training session in which 10–20 food pellets (20 mg; BIO-SERV) were delivered randomly. On subsequent days, mice underwent Pavlovian conditioning in which an 11-s lever presentation (CS) was paired with delivery of a 20-mg food pellet (US). Although a lever was used as the CS in this paradigm, there was no instrumental contingency for food delivery. US delivery occurred 10 s after CS onset. Animals received 7–10 sessions with 25 trials per session and a variable ITI of 60 s. In the unpaired group the CS was never paired with US delivery. Learning was assessed by comparing HE rate during CS presentation with HE rate during the ITI. HEs were detected by an infrared photobeam within the food dispenser. Med-PC software (Med Associates, Inc.) was used for all behavioral programs and all data acquisition. SCH23390 (Sigma) was prepared in 0.9% saline and injected i.p. (0.01 mL/g) 30 min before each training session.

FSCV in Vivo. FSCV procedures were modified from those described by Clark et al. (9). A carbon-fiber microelectrode was implanted into the AcbC (Anteroposterior = 1.52, Mediolateral = 1.15, and Dorsoventral = −3.75). After the animals had recovered form surgery, recordings were obtained in a modified operant chamber using a custom-built headstage and an electric commutator (Dragonfly, Inc.). Before each training session, the cyclic voltammogram (CV) obtained after the reward delivery of a random reward was compared with a CV obtained by electrical stimulation of the median-forebrain bundle in an anesthetized mouse. The success rate for observing phasic dopamine release in response to food delivery before all seven sessions was comparable in the control (6/13) and KO (5/11) groups. Dopamine concentrations were extracted from voltammetric signals using principle component regression with a training set based upon stimulated dopamine release and a calibration factor determined from electrode calibration in vitro (37). Electrode placement was confirmed by electrolytic lesion after applying 300 V for 30 s to the recording electrode upon termination of the experiment. A more detailed description of these procedures may be found in *SI Text*.

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