Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior

Larry S. Zweifel[a,b], Jones G. Parker[a,b], Collin J. Lobb[c], Aundrea Rainwater[a,b], Valerie Z. Wall[a,b], Jonathan P. Fadok[a,b], Martin Darvas[a,b], Min J. Kim[d], Sheri J. Y. Mizumori[d], Carlos A. Paladini[c], Paul E. M. Phillips[e,f], and Richard D. Palmiter[a,b,1]

Departments of [d]Psychology, [e]Psychiatry and Behavioral Sciences, [f]Pharmacology, and [a]Biochemistry and [b]Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195; and [c]Department of Biology, University of Texas, San Antonio, TX 78249

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Midbrain dopamine (DA) neurons fire in 2 characteristic modes, tonic and phasic, which are thought to modulate distinct aspects of behavior. However, the inability to selectively disrupt these patterns of activity has hampered the precise definition of the function of these modes of signaling. Here, we addressed the role of phasic DA in learning and other DA-dependent behaviors by attenuating DA neuron burst firing and subsequent DA release, without altering tonic neural activity. Disruption of phasic DA was achieved by selective genetic inactivation of NMDA-type, ionotropic glutamate receptors in DA neurons. Disruption of phasic DA neuron activity impaired the acquisition of numerous conditioned behavioral responses, and dramatically attenuated learning about cues that predicted rewarding and aversive events while leaving many other DA-dependent behaviors unaffected.

cue-dependent learning | mouse behavior | electrophysiology | cyclic voltammetry

Dopamine (DA) neurons of the ventral midbrain project to the dorsal and ventral striatum, as well as to other corticolimbic structures such as the hippocampus, amygdala, and prefrontal cortex. Differential DA release (tonic or phasic) is thought to activate distinct signal transduction cascades through the activation of postsynaptic inhibitory and excitatory G protein coupled receptors. Phasic DA is proposed to activate excitatory, low-affinity DA D1-like receptors (Rs) (1, 2) to facilitate long-term potentiation of excitatory synaptic transmission and enhance activity of the basal ganglia direct pathway facilitating appropriate action selection during goal-directed behavior. Conversely, tonic DA release is proposed to act on inhibitory, high-affinity DA D2Rs to facilitate long-term depression of cortico-striatal synapses and suppress activity of medium spiny neurons (MSNs) of the basal ganglia indirect pathway (1, 3–5). Thus, coordinate D1R and D2R activation modulates motor and cognitive function, and facilitates behavioral flexibility by a dichotomous control of striatal plasticity (5).

During reinforcement learning shifts in phasic DA neuron responses from primary rewards, to reward predicting, stimuli are thought to reflect the acquisition of incentive salience for the predictive conditioned stimuli (6–10). Coincident DA and glutamate release onto MSNs during conditioned-stimulus response learning facilitates long-term potentiation of excitatory synapses that is thought to underlie reinforcement learning (1, 2, 11). Pharmacological or genetic disruption of D1R signaling impairs learning in numerous behavioral paradigms (2, 11); thus, phasic DA acting through D1R is thought to facilitate memory acquisition by "stamping-in" stimulus-response associations.

Although considerable correlative electrophysiological evidence, as well as pharmacological and genetic evidence, supports an important role of phasic DA in stamping-in cue-reward associations, other evidence suggests that DA is not necessary for learning conditioned-stimulus responses. Mice genetically modified to be hyperdopaminergic do not learn faster than normal mice. However, they do demonstrate increased motivation to work for food reward (12, 13). Also, mice that lack the ability to synthesize DA (DA-deficient mice) can develop conditioned reward associations, but lack the motivation to obtain the reward (14–16). These findings suggest that DA provides an incentive motivational signal to engage in goal-oriented tasks in response to learned conditioned stimuli, but is not necessary for learning conditioned-stimulus associations (17).

Burst firing by DA neurons is mediated, in part, by large amplitude, slow inactivating excitatory postsynaptic currents (EPSCs) from NMDARs that allow for the temporal summation of synaptic inputs (18–20). Iontophoretic administration of NMDAR antagonists, but not AMPAR-selective antagonists, attenuates burst firing. Also, NMDAR antagonists attenuate burst frequency without altering the frequency of nonburst events (19), suggesting that inactivation of NMDAR signaling in DA neurons could provide the selectivity necessary to asses the contribution of phasic DA to DA-dependent behaviors without producing a complete DA-deficient state.

Results

Genetic Inactivation of NMDAR in DA Neurons Impairs Burst Firing. Genetic inactivation of the essential NR1 subunit (*Grin1*) of the NMDAR selectively in neurons expressing the dopamine transporter gene (*Slc6a3*) is sufficient to inactivate NMDAR currents in these cells (21, 22). To determine whether burst firing depends on functional NMDAR signaling, we monitored DA neuron activity in freely moving control ($Slc6a3^{+/Cre}$;$Grin1^{+/lox}$) and knockout (KO, $Slc6a3^{+/Cre}$;$Grin1^{\Delta/lox}$) mice, chronically implanted with recording electrodes in the ventral tegmental area/substantia nigra pars compacta. Putative DA neurons were identified by action potential waveform and inhibition by the D2R autoreceptor, which is present in most, but not all, DA neurons (23, 24), as described (Fig. S1) (25). In Fig. 1 *A* and *B* we show that the wave forms were similar, whereas in

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[1]To whom correspondence should be addressed. E-mail: palmiter@u.washington.edu.

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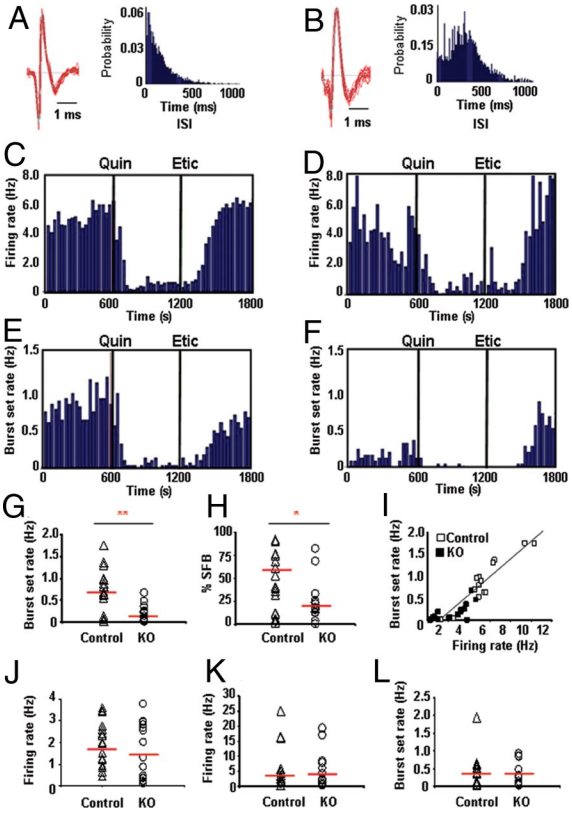

Fig. 1. Burst firing by DA neurons is impaired in KO mice. (*A* and *B*) Waveform of a DA neuron recorded from a control (*A*) and KO (*B*) mouse, and corresponding ISI histogram (10-ms bins). (*C* and *D*) Firing rate histogram (30-s bins) of the DA neurons in *A* and *B*, from control (*C*) and KO (*D*) mouse, demonstrating sensitivity to D2R agonist, quinpirole (quin), and D2R antagonist, eticlopride (etic). (*E* and *F*) Burst-rate histogram (30-s bins) of the DA neurons in *A* and *B*, from control (*E*) and KO (*F*) mice. (*G*) Burst set-rate (burst sets/s) by DA neurons from control and KO mice. (*H*) Percentage spikes fired in bursts (percentage SFB) by DA neurons from control and KO mice. (*I*) Correlation between burst set rate and firing rate. (*J*) Frequency of nonburst spikes. (*K*) Firing frequency of non-DA neurons. (*L*) Burst set-rate of non-DA neurons. (*G* and *H*) Mann–Whitney *U* test; *, $P < 0.05$; **, $P < 0.01$.

Fig. 1 *C* and *D* that quinpirole had similar inhibitory effects in bothcontrol and KO mice; controls: 81.8 ± 2.08% inhibition, n = 17 cells from 3 mice vs. KO 78.3 ± 3.81% inhibition, n = 18 cells from 4 mice. Phasic activity was defined as bursts of spikes occurring with an interspike interval (ISI) of ≤80 ms and terminating with an ISI of ≥160 ms (26). NMDAR inactivation had a significant effect on the pattern of activity reducing the median frequency of burst events by >6-fold (median burst sets/s = 0.63 Hz control vs. 0.10 Hz, KO, Mann–Whitney *U* test $P < 0.01$; see Fig. 1 *E–G* and *I*). The percentage of spikes fired in bursts (percentage SFB) were similarly reduced (median percentage SFB = 61.4, control vs. 17.3, KO, Mann–Whitney *U* test $P < 0.05$; see Fig. 1*H*). We also observed a small reduction in burst duration (147.5 ± 18.0 ms, control, vs. 96.2 ± 17.0 ms, KO; Student's *t* test $P < 0.05$). Total firing rate was reduced in KO mice, and it correlated with reduced burst set rate (4.86 ± 0.61 Hz, control, vs. 2.17 ± 0.44 Hz, KO; $r = 0.82$, Student's *t* test $P < 0.01$; see Fig. 1*I*). However, the frequency of nonburst spikes was unaffected (1.66 ± 0.24 Hz, control, vs. 1.40 ± 0.35, KO; see Fig. 1*J*), indicating that NMDAR inactivation in DA neurons does not affect tonic activity. Firing rate and bursting activity of cells that did not fulfill the criteria for DA neurons were similar between the 2 groups (Fig. S1; average percentage quinpirole inhibition: 16.5 ± 7.8; control n = 13 vs. 9.1 ± 7.6, KO n = 11; median frequency = 3.62 Hz, control, vs. 4.04 Hz, KO; median burst sets/s = 0.32 Hz, control, vs. 0.29 Hz, KO; see Fig. 1 *K–L*).

Burst firing by DA neurons is modulated, in part, by excitatory (glutamatergic and cholinergic) afferents from the pedunculopontine tegmental nucleus (PPTg), which is thought to relay cue-related sensory information to these cells (27–29). To confirm that burst firing is impaired in KO mice, we assessed stimulus-evoked burst activity in antidromically-identified DA neurons from anesthetized control and KO mice (see *SI Materials*). The success rate of PPTg-evoked burst firing was higher in control mice than KO mice (10/19 vs. 5/17 cells). Of those cells in which bursts were evoked, the percentage of stimulus-evoked bursts and the number of spikes/burst were reduced in KO mice (35.0 ± 9.5%, control, vs. 12.1 ± 3.8%, KO; Mann–Whitney *U* test $P < 0.05$; median spikes/burst = 3.78, control, vs. 3.00, KO; Mann–Whitney *U* test, $P < 0.05$; see Fig. S2). These findings confirm that NMDARs contribute significantly to burst firing by these cells.

Phasic DA Release Is Impaired in KO Mice. Bursts of DA neuron activity are thought to facilitate neurotransmitter release, resulting in transient increases in synaptic DA (2). To determine whether DA release associated with burst firing is altered in KO mice, we measured PPTg-evoked DA release in the dorsal striatum using fast-scan cyclic voltammetry (30). Hindbrain stimulation (0.15 mA at 60 Hz for 1 s) corresponding to the stereotaxic coordinates for the PPTg reliably evoked DA release in the dorsal striatum (Fig. 2 *A* and *B*; n = 9 stimulation electrode tracts from 6 control, and n = 5 stimulation tracts from 4 KO mice). Similar to PPTg-evoked burst firing, the success rate of PPTg-evoked DA release was twice as high in control mice compared with KO mice (n = 14/24 stimulation sites from 12 control vs. n = 6/21 stimulation sites from 8 KO mice); only stimulation sites that evoked release were used in subsequent analysis. Varying PPTg stimulus intensity and duration had a significant effect on DA release in control mice that was greatly reduced in KO mice (2-way repeated measures ANOVA, genotype × stimulus, $F_{(7, 84)} = 4.37$; $P < 0.001$, and $F_{(6, 72)} = 3.26$; $P < 0.01$, respectively; see Fig. 2 *C–F*). To determine whether the releasable pool of DA that can be evoked by electrical stimulation is altered in KO mice, after PPTg stimulation, we measured DA release evoked by direct stimulation of DA neuron fibers in the medial forebrain bundle. There was no significant difference in DA release, with >92% of the stimulation experiments producing detectable responses in both groups (Fig. 2 *G–H*; n = 14/15 stimulation sites, control, vs. n = 11/12 stimulation sites, KO). Deficits in PPTg-evoked DA release confirm our electrophysiology results, and demonstrate that NMDAR inactivation in DA neurons significantly impairs DA neuron burst firing and subsequent DA release.

Many DA-Dependent Behaviors Are Unaffected in KO Mice. To assess whether disruption of phasic DA leads to generalized behavioral impairment, we performed an extensive analysis of DA-dependent behaviors (summarized in Table 1). Lack of NMDAR in DA neurons does not affect 24-hour locomotor activity during light or dark phase, the locomotor response in a novel environment, or acute responses to cocaine, amphetamine, morphine, or D1R agonists (22). Because DA neurons are directly and indirectly modulated by hormones that regulate feeding behavior (31), we monitored daily ad libitum food consumption and the latency of calorie-restricted (85% body weight) control and KO mice to eat freely available food pellets. We did not observe significant differences between control and KO mice in either parameter (Fig. S3). Progressive DA deficiency, as observed in Parkinson's disease (PD), is associated with impaired motor and cognitive function (3). To examine motor function, we assessed the ability of mice to improve their performance on an accelerating rotating rod and their latency to escape to a visible

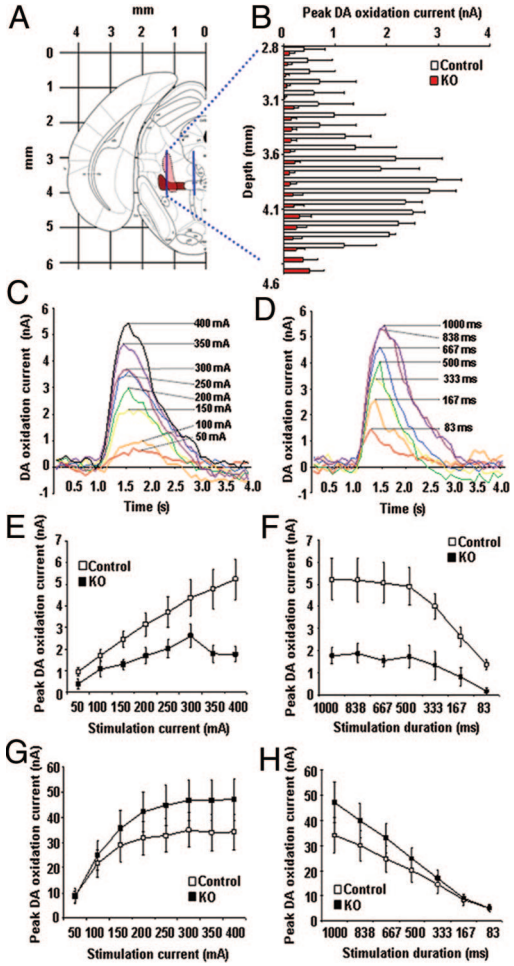

Fig. 2. PPTg-evoked DA release is attenuated in KO mice. (*A*) Schematic representation of stereotaxic coordinates of stimulating electrode placement (blue) into caudal (red) and rostral PPTg (red check), representation adapted from (54). (*B*) Peak DA oxidation currents from PPTg stimulation at different depths (mean ± SEM). (*C*) Representative DA oxidation current in response to increasing stimulus intensity (50–1,000 μA at 60 Hz for 1 s). (*D*) Representative DA oxidation currents in response to decreasing stimulus duration (400 μA at 60 Hz for 83–1,000 ms). (*E*) Average peak DA oxidation currents in response to increasing stimulus intensity are reduced in KO compared with control mice (mean ± SEM, 2-way, repeated measures ANOVA; $P < 0.01$). (*F*) Average peak DA oxidation currents in response to decreasing stimulus duration are reduced in KO compared with control mice (mean ± SEM, 2-way, repeated measures ANOVA; $P < 0.01$). (*G* and *H*) Peak DA oxidation currents after increasing medial forebrain bundle stimulus intensity or decreasing stimulus duration is unaltered in KO mice.

platform in a straight-alley, water-escape task, behaviors that are significantly impaired in DA-deficient mice (32, 33). KO mice were not significantly impaired in either task (Fig. 3 *A* and *B*). In addition to modulating sensorimotor function, DA also facilitates sustained cortical network activity during working memory (4), which is impaired in PD (3). We monitored working memory in control and KO mice in a water-based, T-maze, in which the arms are bent such that the goal cannot be observed at the choice point. The mice are presented with a forced choice trial leading to an escape platform in one arm followed 10 s later by a free choice, in which the escape platform was located in the opposite arm. KO and control mice demonstrated equivalent improvement in this task (Fig. 3*C*; 2-way repeated measures ANOVA, day: $F_{(17,324)} = 11.611$; $P < 0.01$). Also, KO mice performed, as well as control mice, in a novel-object recognition task (Fig. S3).

Table 1. Summary of behavioral analysis of mice with impaired phasic DA neuron activity

Food consumption, ad libitum*	↔
Latency to eat, free access*	↔
Body weight	↔
Rotarod	↔
Water-escape latency	↔
Working memory	↔
Novel object recognition*	↔
Sociability*	↔
Forced-swim test*	↔
Elevated plus maze*	↔
Prepulse inhibition	↔
Locomotor activity, novelty (ref. 22)	↔
Locomotor activity, drugs of abuse (refs. 21, 22)	↔
Sensitization, cocaine	
Acquisition (refs. 21, 22)	↔
Withdrawal (ref. 22)	↓
Cocaine CPP (refs. 21, 22)	↓ ↔
Extinction (ref. 21)	↔
Reinstatement (ref. 21)	↓
Food CPP	↓
Cued water maze	
Acquisition	↓
Recall	↔
T-maze	↓
FPS	↓
Operant conditioning	↓

Increase (↑, $P < 0.05$), decrease (↓, $P < 0.05$), or no change (↔) relative to control mice. CPP is impaired after 3 days of intermittent cocaine injections (22), but not after 8 consecutive days (21).
*See *SI Methods*.

Altered DA signaling is associated with numerous psychiatric disorders, including schizophrenia (34). Also, modified behavior associated with anxiety, sociability, stress, and drug-seeking behavior are correlated with altered DA neuron activity in mice (35). To assess anxiety, we monitored the time spent in the open arm of an elevated, plus maze; however, we did not observe any difference between KO and control mice (Fig. S3). Likewise, social interaction did not differ between groups, and the latency to immobility in a forced-swim test did not differ from controls (Fig. S3). Alterations of tonic DA signaling in mice are associated with disruptions of sensory motor gating in reflexive startle paradigms (36). To assess sensory motor gating, we monitored prepulse inhibition (PPI) of the acoustic startle reflex; 120-dB startle pulses were preceded by varying prepulse intensities above background noise (65 dB), KO mice demonstrated equivalent PPI compared with controls (Fig. S3). Thus, KO mice can perform many DA-dependent tasks without any apparent impairment. These findings suggest that tonic firing by DA neurons is sufficient for execution of most behaviors, and that disruption of phasic DA does not impact performance of these tasks.

Acquisition of Conditioned-Place Preference (CPP) and Learning in a Water Maze Are Deficient in KO Mice. Drug seeking behavior, as monitored by acquisition of cocaine CPP, is impaired in these KO mice during the first 3 days of training (22), but an association can eventually be formed after 8 context-reward presentations (21). To assess whether phasic DA facilitates reinforcement learning for natural rewards, we monitored the acquisition of food CPP. Food-restricted mice (85% of normal body weight) were presented with food in 1 of 2 contextually distinct compartments of a CPP box, and without food in the other compartment. Pairings of food with context were performed every other day. On intermittent days, mice were tested

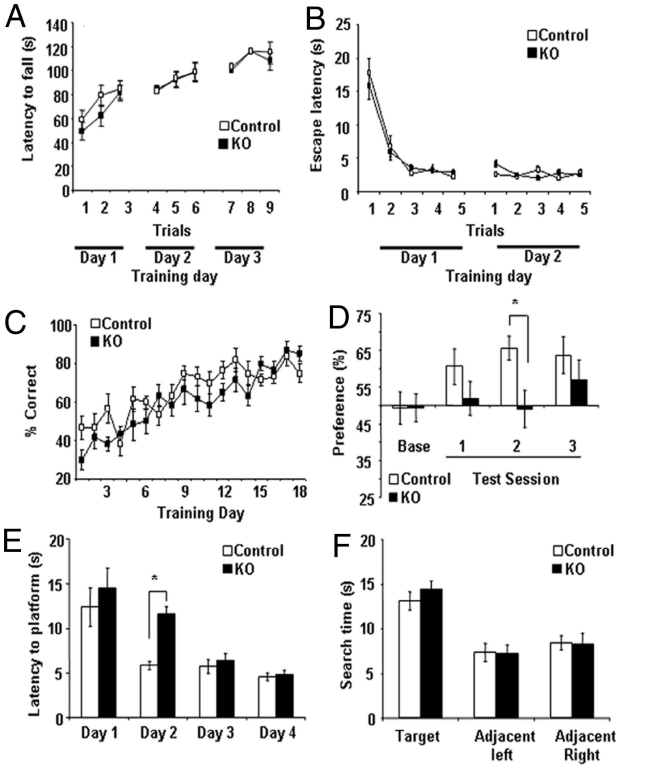

Fig. 3. Selective behavioral impairments in KO mice. (*A*) Rotarod performance during 3 trials per day for 3 consecutive days is not different between the 2 groups control ($n = 19$) and KO mice ($n = 13$). (*B*) Latency to escape to a visible platform in a straight-alley water-escape task is not different between control ($n = 13$) and KO mice ($n = 14$). (*C*) Performance (percentage correct choice) in a working-memory task is not impaired in KO mice. (*D*) CPP for food is impaired in KO ($n = 12$) vs. control ($n = 10$) mice (mean ± SEM, 2-way, repeated measures ANOVA, Fisher's LSD; *, $P < 0.05$). (*E*) The acquisition phase of a cue-dependent Morris water maze is impaired in KO mice (mean ± SEM, 2-way, repeated measures ANOVA, Fisher's LSD; *, $P < 0.05$). (*F*) Time spent searching in the area where the hidden platform was located in the cue-dependent Morris water maze is not different between groups.

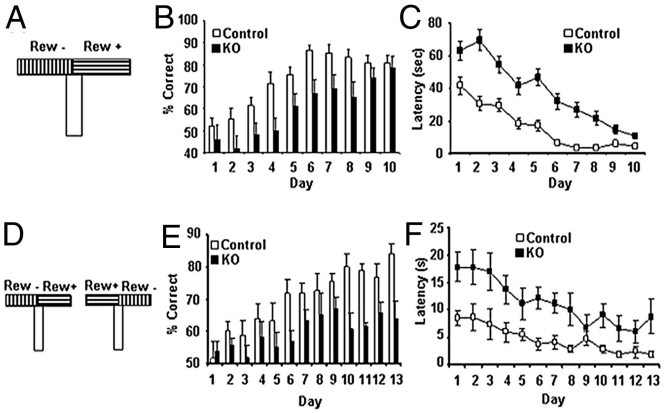

Fig. 4. Cue-dependent reward learning is impaired in KO mice. (*A*) Schematic representation of T-maze task in which Rew+ and Rew− location did not change. (*B*) KO mice were significantly delayed in learning the task (percentage correct arm entries) compared with control mice (2-way repeated measures ANOVA; $P < 0.05$). (*C*) Latency to make a choice is significantly longer in KO mice compared with controls (2-way repeated measures ANOVA; $P < 0.05$). (*D*) Schematic representation of T-maze in which Rew+ and Rew− cues were presented in pseudorandom order. (*E*) Learning, measured as percentage correct arm entries was significantly impaired in KO mice compared with control mice (2-way repeated measures ANOVA; $P < 0.05$). (*F*) Latency to choice was also significantly impaired in KO mice relative to control mice (mean ± SEM, 2-way, repeated measures ANOVA; $P < 0.05$).

for the development of a preference for the food-paired compartment. Preference for the food paired compartment was significantly impaired in KO mice ($n = 12$) relative to controls ($n = 10$) (2-way repeated measures ANOVA, genotype, $F_{(1, 20)} = 4.29$, $P < 0.05$; see Fig. 3*D*), although food consumption during the training sessions was equivalent, indicating that the mice were equally hungry (Fig. S3).

Dopamine signaling has also been demonstrated to modulate learning in a cue-dependent, Morris water maze, and it is thought to reflect a disruption of synaptic plasticity within the forebrain (37, 38). We measured memory acquisition in a modified, Morris water maze. Mice were given 5 trials per day for 4 days to learn the location of a hidden platform using cues located within the maze. KO mice ($n = 8$) were significantly slower to learn the task, as measured by latency to find the hidden platform, compared with controls ($n = 9$) (2-way repeated measures ANOVA, genotype × day, $F_{(3,45)} = 2.88$; $P < 0.05$; see Fig. 3*E*); however, they demonstrated equivalent recall (time spent in zone where hidden platform was located) once the task was learned (Fig. 3*F*). These behavioral analyses suggest selective impairments in cue-dependent learning.

Phasic DA Neuron Activity Facilitates Learning in T-Maze Tasks. To further explore whether phasic DA facilitates learning, mice were trained in an appetitive T-maze task, in which arms were baited with an accessible food pellet (horizontal stripes, Rew+) in one arm, and an inaccessible food pellet (vertical stripes, Rew−) in the other arm (Fig. 4*A*), as done previously with DA-deficient mice (16). Two independent groups of food-restricted control ($n = 15$) and KO mice ($n = 12$) were given 10 trials per day for 10 days. Performance of this task (percentage correct arm choices) was significantly impaired in KO mice compared with control mice (2-way repeated measures ANOVA, genotype, $F_{(1,25)} = 11.15$, $P < 0.01$; see Fig. 4*B*); however, they eventually made a similar percentage of correct arm choices in the final 2 days of training. Both KO and control mice consumed all rewards after a correct arm entry. However, KO mice appeared slower to make a choice of arms to enter than control mice. Reduced latency to choice was confirmed in the second group of mice by quantifying the latencies to choice (2-way repeated measures ANOVA, genotype, $F_{(1,13)} = 6.63$, $P < 0.05$; $n = 6$ KO, and $n = 9$ control; see Fig. 4*C*).

Although KO mice eventually learned the T-maze task with repeated training, the reward location did not change. Thus, it is possible that the mice learned the task in a response-dependent manner, rather than a cue-dependent manner. To directly assess the ability of the mice to use the cues to predict reward availability, mice were trained with cues presented in pseudorandom order (horizontal stripes, Rew+; vertical stripes, Rew−), such that the reward was located in each arm half of the time for a total of 20 trials per day (Fig. 4*D*). Maximal time allotted to make a choice before a forced choice was given was reduced from 2 to 1 min to facilitate learning. Control mice ($n = 9$) demonstrated significant improvement in the task; however, KO mice ($n = 8$) were significantly impaired relative to controls (percentage correct choices, 2-way, repeated measures ANOVA, genotype × day, $F_{(13,185)} = 1.81$; $P < 0.05$; see Fig. 4*E*). KO mice were again significantly slower to make a choice relative to controls (2-way, repeated measures ANOVA, genotype × day, $F_{(13,185)} = 1.86$; $P < 0.05$; see Fig. 4*F*).

Phasic DA Is Unnecessary for Motivation to Work for Food Rewards. Increased latencies to choice in T-maze tasks may reflect deficits in learning, motivation, or both. To determine whether motiva-

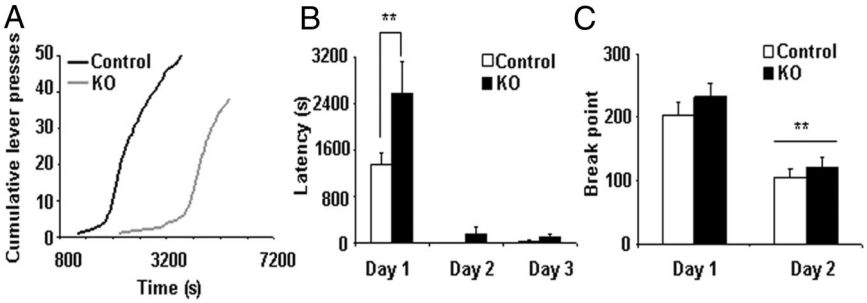

Fig. 5. Motivation to work for food reward is not impaired in KO mice. (*A*) Cumulative lever presses in an instrumental task during day 1 is significantly delayed in KO mice compared with control mice ($P < 0.05$). (*B*) Latency to initiate lever pressing is also significantly delayed on day 1 of preconditioning (mean ± SEM, 2-way repeated measures ANOVA, Fisher's LSD; **, $P < 0.01$). (*C*) Break point in a progressive ratio task is equivalent in KO and control mice (day 1), and is equally reduced after 24 h ad libitum food access (day 2, 2-way repeated measures ANOVA, Fisher's LSD; **, $P < 0.01$).

tion is impaired in KO mice, we measured their willingness to work for food in a progressive ratio, instrumental conditioning task similar to that previously described (39). After 1 week of pretraining (noncontingent reward pellets delivered coincident with a lever extension-retraction), instrumental conditioning was established by using a simple fixed ratio schedule, in which a lever press delivered a single food pellet (FR1). All KO ($n = 10$) and control ($n = 10$) mice reached criterion within 3 days (50 lever presses within 2 h). However, KO mice were significantly slower to reach criterion on the first day (2-way repeated measures ANOVA, genotype × day: $F_{(2,36)} = 3.66$, $P < 0.05$; Fisher's LSD day 1: $P < 0.05$; see Fig. 5*A*), but not on subsequent days. Also, KO mice were significantly slower to initiate lever pressing on the first day (2-way repeated measures ANOVA, genotype × day: $F_{(2,36)} = 3.95$, $P < 0.05$, Fisher's LSD: $P < 0.01$ day 1; see Fig. 5*B*), but not on subsequent days (day 2, $P < 0.20$ and day 3, $P = 0.10$). Assessment of break-point (maximal lever presses to achieve a single reward pellet) revealed no significant difference between the 2 groups (Fig. 5*C*), indicating KO mice were equally motivated to work for food. When mice were retested after overnight ad libitum food access to devalue the food rewards, both groups demonstrated a significant decline in break-point (2-way repeated measures ANOVA, day: $F_{(1,18)} = 54.01$, $P < 0.01$; see Fig. 5*C*). These findings demonstrate that phasic DA is necessary for cue-dependent reward learning, and suggest that phasic DA also has a more general role in facilitating learning about conditioned stimulus-responses (S-R), but not motivation to work once the S-R is learned.

Phasic DA Neuron Activity Also Facilitates Cue-Dependent Fear Learning. Some DA neurons are phasically activated by aversive stimuli, acute stressors, and cues associated with aversive events (40–42). However, the majority of DA neurons are inhibited by these stimuli (43). Because DA neurons segregate anatomically, pharmacologically, and electrophysiologically (23), it is difficult to generalize their function, which could explain the equivocal results related to DA neuron activity in response to aversive stimuli (40–43). To determine whether phasic DA is important for cue-dependent fear, we assessed learning in a pavlovian fear-potenitated acoustic startle (FPS) paradigm. Because the acoustic startle response is reflexive, it can be examined independently of motivation (44). Fear-conditioning was assessed the day after a conditioning session (10 presentations of a cue that coterminated with a 0.2-mA footshock) by measuring acoustic startle responses in the presence or absence of the cue; training and testing were repeated on subsequent days. After the second and third conditioning days, control mice ($n = 15$) developed FPS that was absent in KO mice ($n = 16$) (2-way, repeated measures ANOVA, genotype × day, $F_{(3,87)} = 5.57$, $P < 0.01$; see Fig. 6*A*). KO mice showed significantly elevated startle responses, compared with controls, in the absence of the cue on all test days after a conditioning session (2-way, repeated measures ANOVA, genotype × day, $F_{(3,87)} = 8.52$, $P < 0.01$) that was the same as their startle responses in the presence of the cue (Fig. 6*B*). Thus, KO mice manifested generalized fear responses, but did not learn to discriminate the cue that predicted the footshock. Control mice also had potentiated responses to acoustic startle in the absence of the cue after a single training session. However, this response diminished with further training (Fig. 6*B*; control: baseline no cue vs. no cue test 1; $P < 0.01$; vs. test 2, $P > 0.5$; test 3, $P > 0.1$), indicating a learned association of the cue that predicted the footshock. These findings demonstrate that phasic DA neuron activity is also important for learning about cues that predict fearful events.

Discussion

Here, we show that NMDARs in DA neurons modulate burst firing and DA release in postsynaptic brain regions. Remarkably, the absence of burst firing leaves many DA-dependent behaviors intact (body weight regulation, working memory, and motor performance); however, selectively impairs learning in cue-dependent learning tasks (see Table 1). Some of the behavioral tasks involve food rewards (CPP, T-maze, instrumental learning), some involve escape from an unpleasant environment (Morris water maze), whereas some learning situations are clearly aversive (FPS paradigm). A unifying interpretation of these results is that bursts of DA neuron activity in response to important events provide generalized salience signals that facilitate learning associations of environmental cues with these events, whereas increased tonic DA, such as those measured by microdialysis, is independent of burst firing, and provides sufficient DA to engage most behaviors.

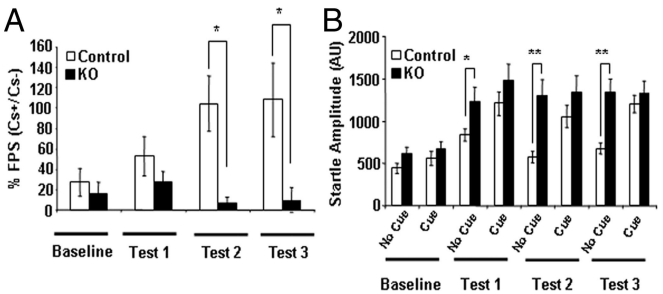

Fig. 6. Cued fear is attenuated in KO mice. (*A*) FPS is significantly attenuated in KO mice (mean ± SEM, 2-way repeated measures ANOVA; $P < 0.01$). (*B*) Acoustic startle responses in the absence of the cue is significantly elevated in KO mice compared with controls (mean ± SEM, 2-way, repeated measures ANOVA; $P < 0.01$, Tukey's HSD; **, $P < 0.01$; *, $P < 0.05$).

Lack of NMDARs in DA neurons not only impairs burst firing, but also precludes LTP of synaptic AMPARs (21, 22). AMPAR currents are transiently potentiated in DA neurons after exposure to cocaine, stress, or during learning paradigms (21, 22, 45–47). However, the role of AMPAR LTP in DA neurons is unclear. Synaptic scaling after NMDAR inactivation in DA neurons leads to chronically elevated AMPAR currents in KO mice, similar to levels normally observed after exposure to cocaine, stress, or during learning (21, 22, 45–47). Despite the enhanced level of AMPAR in DA neurons of KO mice, which one might suspect would enhance firing rate (20), burst firing was dramatically attenuated in KO mice, and the tonic firing rate was unaffected. This result is consistent with reports that AMPAR do not potently modulate burst firing by DA neurons (20), and observations that numerous behaviors thought to be dependent on tonic DA signaling [acute locomotor responses to drugs such as cocaine, amphetamine and morphine (22), rotarod performance, working memory, and others; see Table 1] are unaltered in KO mice. Transient increases in AMPAR currents that have been demonstrated during conditioned-stimulus reward associations may provide an important gate for NMDAR-mediated burst firing by DA neurons. We suggest that spike-timing-dependent plasticity, in which local dendritic calcium influx through NMDAR, together with elevated global calcium generated by NMDAR-dependent burst firing work synergistically to increase synaptic AMPARs (48). Increased synaptic AMPAR currents, in turn, facilitate removal of the magnesium block from the NMDAR; thus, increasing the probability of burst firing.

Previous studies have demonstrated that both hyper and hypodopaminergic mice can learn various tasks. However, motivation to engage in the tasks is significantly altered in these mice, suggesting that DA mediates "wanting" rewards, rather than learning (17). For example, DA-deficient mice appear to be unmotivated and will not engage in most tasks (49), whereas hyperdopaminergic mice perform some tasks more rapidly with less meandering, suggesting enhanced motivation (13). We observed longer latencies by the KO mice to make choices and engage in goal-directed behavior in many learning paradigms, suggesting that they may be less motivated in the absence of burst firing. Burst-firing increases the probability of neurotransmitter release (50); thus, bursting by DA neurons likely increases extracellular DA in target areas that are necessary to engage in reward-based tasks. Consistent with this idea, elevated synaptic DA associated with burst firing is proposed to activate D1R (1), and D1R antagonists decrease the probability of cue-elicited approach responses in the early stages of S-R training (51). We observed several cases in which learning was significantly delayed in KO mice (water-maze, instrumental conditioning, T-maze with stationary cue; also, see Table 1). However, performance often became equivalent or nearly equivalent with further training. Impaired acquisition of some learned behaviors in KO mice is consistent with a role for phasic DA in facilitating the acquisition of incentive value for environmental cues that in turn facilitate engagement of goal-directed behavior (52). However, with repeated training, responses become habitual, and phasic DA is no longer necessary. Thus, during early stages of S-R conditioning, phasic DA facilitates learning. Delays in the acquisition of the S-R association would in turn manifest as increased latencies to engage the behavior, making it appear as if the mice were less motivated. Despite the observation that KO mice may be slower to make a choice in the appetitive T-maze tasks, food rewards were always eaten once found. Another aspect of motivation is the willingness to work for food rewards (53). We examined this aspect of motivation using the progressive ratio strategy, and found that, although KO mice were delayed in the acquisition of the S-R association, they were as motivated as controls once the response was acquired; they would both press a lever ≈200 times for a single food pellet. Also, when the value of the food reward was devalued by prior feeding, lever pressing declined by a similar amount in both groups.

To generalize, we propose that exposure to a salient event stimulates excitatory inputs onto DA neurons, and perhaps reduces inhibitory inputs, which facilitates activation of NMDARs, allowing calcium influx and promoting burst firing activity. The bursts of activity by DA neurons result in transient spikes of extracellular DA in synapses within striatum, prefrontal cortex, amygdala, and/or hippocampus. The transient elevation in DA concentration would preferentially activate the lower-affinity D1R, and facilitate LTP in brain regions involved in spatial memory. Subsequent exposure to cues within the environmental context would evoke phasic DA release that would facilitate engagement of goal-directed behavior. However, with extended conditioning, these responses would become habitual, and bursts of DA release would become less important for the learned response. Conversely, the requirement of phasic DA in making accurate choices based on discrete cues, such as choosing to enter 1 of 2 arms of a T-maze to acquire a food reward, or predict a footshock, does not significantly diminish with repeated conditioning. Thus, phasic DA remains essential in more complex processes such as 2-choice discrimination.

Materials and Methods

Animals. All behavioral and electrophysiology experiments were approved by the University of Washington and University of Texas, San Antonio Institutional Animal Care and Use Committees. The KO and control mice were generated by the breeding scheme described (22). The genetic background of the mice was almost completely C57BL/6 as a consequence of extensive backcrossing. Experiments were performed on 8- to 12-week-old male and female mice, except for electrophysiology, which was conducted in 10- to 12-week-old male mice. During calorie restriction, mice were individually housed in environmentally enriched cages, and maintained on high-energy chow (LabDiet 5IJ5) to 85% body weight for a minimum of 1 week.

Electrophysiology. Electrophysiology in freely moving mice was performed by using HS-16, 4-tetrode microdrives (Neuralynx). Microdrives were implanted in anesthetized mice by using stereotaxic coordinates for the VTA (3.5 mm A-P, 0.5 mm M-L, and 4.0 mm D-V). Two weeks after surgery, mice were connected through an HS-16 headstage preamplifier to an ERP27 patch panel, signals were amplified (200- and 8,000-fold) and filtered (600–6,000 Hz) by using a Lynx-8 programmable amplifier, and data were acquired by using Cheetah acquisition software (Neuralynx). Tetrodes were lowered by ≈50-μm increments each day until putative DA neurons were identified by action potential waveform and sensitivity to quinpirole (Sigma; 0.2 mg mL^{-1} i.p.; $>$ 70% inhibition of baseline frequency) and eticlopride (Sigma; 0. mg mL^{-1}i.p.; return to $>$70% baseline frequency). Baseline DA neuron firing properties were recorded for 10 min, followed by treatment with confirmation drugs for 10 min each. Tetrode placement was confirmed postmortem by cresyl violet staining of midbrain sections. Neurons were isolated by cluster analysis using Offline Sorter software (Plexon). Clustered waveforms were subsequently analyzed by using MATLAB software (Mathworks). Baseline activity was used to calculate burst sets (burst onset, ISI of $\leq$80 ms; burst offset, ISI of $\geq$160 ms), burst set rate (burst sets/s), percentage spikes fired in bursts (burst spike/total spikes), spikes/burst, burst duration, and firing frequency (total spikes/s). Data were analyzed by *t* test unless normality tests failed, in which case Mann–Whitney *U* tests were performed by using Statistica software (Statsoft).

Fast-Scan Cyclic Voltammetry. Fast-scan cyclic voltammetry was performed using glass-encased, carbon-fiber microelectrodes. A 0.15-mm diameter bipolar stimulating electrode (Plastics One) was used with an analog stimulus isolator (Model 2200, A-M Systems, Inc.). Stimulation patterns were generated using Tarheel CV (National Instruments). Mice were anesthetized with 1.5 g/kg urehane (i.p.) (Sigma), and electrodes were placed based on stereotaxic alignment. All anterior-posterior (AP) and medial-lateral (ML) coordinates are reported in millimeter distance from Bregma unless otherwise noted; all dorsal-ventral (DV) coordinates are millimeter from dura. The carbon-fiber microelectrode was placed in the dorsal striatum (AP = 1.1, ML = 1.2, and DV = −2.35). The carbon-fiber was cycled at 60 Hz to allow the electrode to equilibrate and switched to 10 Hz for data acquisition. The reference electrode was placed AP = 4.9 and ML = 0.0. For pedunculopontine tegmental

nucleus (PPTg) stimulations, the stimulating electrode was lowered in 0.1-mm increments at AP = −0.68 from lambda and ML = 0.7 until dopamine release was observed. The working electrode was lowered in 0.1-mm increments from DV = −1.5 until dopamine release was observed. The average DV coordinate for maximal PPTg-stimulated dopamine release was DV = −2.69. Following PPTg stimulation, the stimulating electrode was lowered into the median forebrain bundle at AP = −2.4 and ML = 1.1. As described for PPTg stimulations, the electrode was lowered in 0.1-mm increments from DV = −3.0 until dopamine was recorded at the working electrode. The electrode was positioned where maximum stimulation evoked dopamine was observed, which, on average, occurred at DV = −4.89. With the stimulating electrode in place, a stimulation-response pattern was obtained by increasing stimulation current at 60 Hz and 60 pulses from 50 mA-400 mA and then decreasing the stimulation duration at 60 Hz and 400 mA from 60 to 5 pulses. For each stimulation parameter 2 stimulations were conducted, and the current response was recorded as the average of the peak dopamine oxidation current in response to each stimulation. Following surgeries, stimulating electrode placement was confirmed by cresyl violet staining of hindbrain sections. Data were analyzed by repeated-measures ANOVA using Statistica software (Statsoft).

Behavioral Testing. ***CPP.*** Food CPP was performed by using the same procedure used for cocaine CPP (22).

Water-escape task. Mice were tested in this task essentially as described (32); latency to reach the platform was scored.

Rotarod performance. Mice were testing on an accelerating rotarod as described (33); latency to fall was scored.

Working memory. Before discrimination testing in a water based, T-maze, in which the arms are bent so that the goal cannot be seen at the choice point, mice were given 5 trials in a water escape task (60 s) in the pool to acclimate them to swimming. Mice received 6 trials per day, consisting of a sample run, in which mice were forced to choose one arm by the presence of a door blocking the entrance to the other arm, according to a pseudorandom sequence (equal number of left and right turns per day and with no >2 consecutive turns in the same direction). After completion of the forced choice, the animal was allowed to rest for 10 s, the door was then removed, the animal placed at the start position, and a free choice was given with both arms available and the escape platform located in the alternate arm of the forced run. Entry into the wrong arm resulted in the mouse being locked in that arm for 10 s, then allowed to swim to the escape platform and rest for 10 s. The intertrial interval (ITI) between pairs was 10 min.

Morris water maze. Water maze performance was assessed by using a small, 3-lobe pool (90-cm diameter) filled to 10 cm with tepid water containing nonfat dry milk. The hidden platform (weighted white plastic box) was submerged 1 cm below the pool surface next to 1 of 3 cues. On the first day, mice were placed on the hidden platform for 30 s, followed by 5 conditioning trials separated by 10 min. For each trail, mice were placed at new start location within the pool; 5 conditioning trails were given each day for a total of 4-consecutive days. On the fifth day, the hidden platform was removed, mice were placed in the center of the pool, and time spent in the area around the 3 cues was monitored for 30 s by using a video acquisition system (Canopus MediaCruise), data were acquired by using Ethovision software (Noldus Information Technology). Data were analyzed by repeated-measures ANOVA by using Statistica software (Statsoft).

T-maze. Performance in cue-dependent T-maze was measured as total correct arm entries over 20 trials for 13 consecutive days. Mice were allotted 60 s to leave the start box and make a choice (> 50% of body across the plane separating the center chamber from the arm), after which a forced choice to either the Rew+ or Rew− arm was given and scored as incorrect. Mice were given 60 s to consume the reward pellet after a correct choice (all reward pellets were consumed). After reward consumption, mice were immediately returned to the start box. After an incorrect choice, mice were retained in the Rew− arm for 60 s before being returned to the start box. To assess blocking, mice were presented with a second set of distinct cues paired with the original Rew+ or Rew− cue during the last 60 trials, followed by a 20 trial test session, in which only the second set of cues were presented. Each trial was separated by an average ITI of 20 s. Data were analyzed by repeated-measures ANOVA by using Statistica software (Statsoft).

Instrumental conditioning. Instrumental conditioning was measured in sound-attenuated, operant chambers (ENV-300; Med Associates). Calorie-restricted mice received 7 consecutive days of preconditioning, in which 15 food pellets (20 mg, Bio-Serv) were delivered immediately after a lever-extension for 8 s, followed by a lever retraction with an average ITI of 90 s. For instrumental conditioning, sessions began with the simultaneous illumination of the houselight and extension of both levers. Sessions lasted for 2 h or until 50 lever presses were recorded. Noncontingent food pellets were delivered randomly (≈1 per minute) during the first 15 min of each session. At the end of each session, the houselight and fan were extinguished, and both levers retracted. Each subject was then placed back in their home-cage. Food pellets left uneaten in the food hopper were recorded and removed. To assess break point, a progressive ratio schedule was used by increasing a nonarithmetic fixed ratio/reinforcement schedule. Data were analyzed by repeated-measures ANOVA using Statistica software (Statsoft).

FPS and PPI. FPS and PPI were measured by using sound-attenuated acoustic startle boxes (San Diego Instruments). A 7-day FPS paradigm was used. For PPI experiments, mice were given a 10-min habituation period before the test begun. Throughout the entire test, the background noise level was maintained at 65 dB. After the habituation, mice were presented with 5, 40-ms duration 120 dB, pulse-alone trials to obtain baseline startle responses. Mice were then presented with 50 trials of either a startle pulse-alone trial, 1 of 3 prepulse trials, or a null trial, in which there was no acoustic stimulus. The ITI averaged 15 s, (range of 5 to 25 s). A startle trial consisted of a 40 ms, 120-dB pulse of white noise. The 3 types of prepulse trials consisted of a 20-ms prepulse of 70-, 75-, or 80-dB intensity (5, 10, and 15 dB above background) that preceded the 40 ms, 120-dB pulse by 100 ms. Peak amplitude of the startle response, occurring in the first 65 ms after pulse onset, was used as the measure of startle response magnitude. For FPS, day 1 (baseline) consisted of a 5-min habituation period, followed by a series of 20 trials, split evenly between 2 trial types. The trial types were startle pulse alone, or startle pulse in the presence of the cue. On startle pulse alone trials, animals were presented with a 40 ms, 105-dB acoustic pulse. On cue trials, the animals were presented with a 10 s light cue, which coterminated with a 40 ms, 105-dB acoustic pulse. These trials were presented in pseudorandom order. The ITI ranged from 60 to 180 s, (average of 120 s). Throughout the experiment, the background sound level was maintained at 65 dB. Peak amplitude of the startle response occurring in the 65 ms after pulse onset was used as the measure of the acoustic startle response. On days 2, 4, and 6, animals were placed into the chambers and, after a 10-min habituation period, were given 10 presentations of the cue light, which coterminated with a 0.2 mA, 0.5-s footshock. The ITI ranged from 60 to 180 s (average of 110 s). Peak responses occurring during the 500-ms footshock were recorded and averaged for each animal. The test sessions occurred on days 3, 5, and 7, and were identical to the baseline session described above. Percentage of FPS was calculated for each animal. Data were analyzed by repeated-measures ANOVA by using Statistica software (Statsoft).

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