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Differential roles of the dopamine 1-class receptors, D1R and D5R, in hippocampal dependent memory

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Activation of the hippocampal dopamine 1-class receptors (D1R and D5R) are implicated in contextual fear conditioning (CFC). However, the specific role of the D1R versus D5R in hippocampal dependent CFC has not been investigated. Generation of D1R- and D5R-specific in situ hybridization probes showed that D1R and D5R mRNA expression was greatest in the dentate gyrus (DG) of the hippocampus. To identify the role of each receptor in CFC we generated spatially restricted KO mice that lack either the D1R or D5R in DG granule cells. DG D1R KO mice displayed significant fear memory deficits, whereas DG D5R KO mice did not. Furthermore, D1R KO but not D5R KO mice, exhibited generalized fear between two similar but different contexts. In the familiar home cage context, c-Fos expression was relatively low in the DG of control mice, and it increased upon exposure to a novel context. This level of c-Fos expression in the DG did not further increase when a footshock was delivered in the novel context. In DG D1R KO mice, DG c-Fos levels in the home cage was higher than that of the control mice, but it did not further increase upon exposure to a novel context and remained at the same level upon a shock delivery. In contrast, the levels of DG c-Fos expression was unaffected by the deletion of DG D5R neither in the home cage nor upon a shock delivery. These results suggest that DG D1Rs, but not D5Rs, contribute to the formation of distinct contextual representations of novel environments.

T he hippocampus is crucial for aversive Pavlovian conditioning, such as contextual fear conditioning (CFC) (1, 2). In CFC, the conditioned stimulus (context) is paired with the unconditioned stimulus (footshock), and after pairing, the context serves as a cue to predict a potential footshock (3, 4). Although the role of dopamine has been studied in the context of reward learning (5), evidence suggests that midbrain dopaminergic neurons are also important for aversive Pavlovian conditioning (6–9). In line with this evidence, hippocampal encoding of novel and contextual information is linked to dopamine release via excitation of dopamine neurons of the midbrain (5, 10, 11). Additionally, delivery of aversive stimuli, such as a footshock, results in increased dopaminergic neuron activity (12). Moreover, inactivation of hippocampal D1Rs and D5Rs attenuates contextual fear memory (13). Thus, it follows that delivery of an aversive stimulus activates midbrain dopamine neurons that project to the hippocampus, which is crucial for encoding novel contextual cues (12, 14, 15). Activation of hippocampal D1Rs and D5Rs may then strengthen the encoding of novel contextual information during CFC.

The precise role of subregion-specific D1R or D5R activation in hippocampal-dependent learning and memory is unknown. This is in part due to the inability to discriminate between and spatially restrict D1R from D5R function (16–18), which is an important caveat because each receptor is involved in modulating distinct neuronal processes (19–22). Indeed, there is a lack of consensus of D1R and D5R expression patterns in the rodent hippocampus (23–27). Moreover, pharmacological findings are at odds with D1R and D5R global KO studies, which show that neither D1Rs nor D5Rs are required for fear conditioning (16, 17). Therefore, to reconcile these disparate findings and to test the necessity of D1R and D5R activation for CFC, it is necessary to functionally isolate and spatially restrict hippocampal D1R and D5R activity.

In this study, we found that D1Rs and D5Rs exhibit overlapping expression in dentate gyrus (DG) granule cells. DG D1R activation is necessary to increase c-Fos expression in the DG and CA3 to enhance novel contextual encoding. Moreover, DG D1R activation decreases generalization of the conditioned fear response to novel contexts. However, we found no role for DG D5Rs in modulating DG c-Fos expression or contextual fear learning and memory. In using our subregion-specific KO mice, we show that the hippocampal dopamine signal plays a definitive role in CFC.

Results

D1R and D5R Expression Exhibits Greatest Overlap in Dentate Gyrus Granule Cells. The expression patterns of D1Rs and D5Rs have remained unclear, making it difficult to identify the function of these receptors in the modulation of hippocampal dependent learning and memory. Because D1Rs and D5Rs carry a high level of similarity in their amino acid sequence (18, 24), the use of antibodies to identify region specific D1R and D5R expression has not been effective (28). To overcome this issue, we have created D1R- and D5R-specific mRNA probes for in situ hybridization (ISH) experiments. We validated the specificity of each riboprobe by generating D1R−/− and D5R−/− mouse lines and tested each probe on brains slices of these constitutive KO mice (Fig. S1 B–E). Using the D1R-specific probe, we have

Significance

Pharmacological and global KO studies have worked to elucidate the function of dopamine 1-class receptors (D1Rs and D5Rs) in hippocampal-dependent learning and memory. Yet, these manipulations are unable to restrict D1R from D5R activity within hippocampal subregions. We generated mice that lack D1Rs or D5Rs in dentate gyrus (DG) granule cells of the hippocampus. This allowed us to characterize the precise role of D1Rs and D5Rs in modulating c-Fos activity in the hippocampus and in Pavlovian fear conditioning. We demonstrate that DG D1R deletion, but not D5R deletion, increases DG granule cell baseline c-Fos activity, decreases DG and CA3 c-Fos activity in response to contextual exposure and to contextual fear conditioning, impairs contextual memory formation, and enhances generalization of the conditioned fear response.

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The authors declare no conflict of interest.

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shown that D1R mRNA is present throughout the cortical layers and expressed strongly in the striatum, olfactory tubercle (OT), olfactory bulb, and the DG (Fig. 1 A and B). The D5R mRNA is expressed strongly in the DG, CA3, and CA1 (Fig. 1D) and weakly in the paraventricular nucleus of the thalamus (Fig. S1D), cortex, the cerebellum, the striatum, and OT (Fig. 1C). These data are consistent with previous ISH studies (23, 24, 29). We have revealed, to our knowledge for the first time, that in the hippocampus, the DG is the area where both D1Rs and D5Rs are robustly expressed.

Comparison of Forebrain Restricted D1R and D5R KO Mice to Pharmacological Antagonist Studies. Prior studies investigating the role of D1Rs and D5Rs in learning and memory have relied on pharmacological antagonists (13, 30). However, these antagonists block both D1Rs and D5Rs by crossing CaMKII-Cre mice (31) with floxed (flx) D1R/DSR mice, which resulted in forebrain-wide D1R and D5R deletion (D1R/DSR KO) (Fig. 1 E and G). Receptor deletion in the D1R/DSR KOs was quantified by measuring the grayscale gradient of the ISH image within each region of interest (32). Significant reduction of the D1R mRNA occurred in the DG (unpaired t-test, P < 0.0001) and striatum (unpaired t-test, P < 0.0001) (Fig. 1 E, F, and J) in the mutant mice. The DG D1R mRNA signal was nearly absent by 16 wk of age. The DG (unpaired t-test, P < 0.0001) and CA1 (unpaired t-test, P < 0.0001) D5R mRNA signal was significantly reduced in D1R/DSR KO mice, with near complete deletion by 28 wk of age (Fig. 1 G, H, and J). The levels of D5R mRNA expression were unaltered in the CA3 area of the KO animals compared with control mice (unpaired t-test, P = 0.362) (Fig. L).

D1R/DSR deletion in the DG was also confirmed by observed deficits in synaptic plasticity at the medial perforant path (mPP)-DG synapse in KO animals. Previous studies have shown that pharmacological blockade of D1R/DSR5Rs inhibits mPP-DG late phase long term potentiation (L-LTP) (33); therefore, we assessed synaptic transmission and L-LTP at the mPP-DG synapse using an anesthetized in vivo preparation. An input–output curve was generated by stimulation of the mPP, with the recording electrode placed at the hilus of the DG. There was no significant difference between genotypes [two-way ANOVA, F(1,13) = 0.4413, P = 0.5181] (Fig. 1K). A theta burst protocol was used to induce t-LTP at the mPP-DG synapse in flx and mutant mice (Fig. L). D1R/DSR KO mice showed initial potentiation (paired t-test, t25–30 min, 113.1% ± 3.3%, P < 0.05). However, the slope of the tEPSP returned to baseline (paired t-test, 175–180 min, 95.9% ± 4.7%, P = 0.492), whereas flx D1R/DSR mice exhibited robust t-LTP (paired t-test, 175–180 min, 125.4% ± 10.0%, P < 0.05).

D1R/DSR KO mice show deficits in CFC. When given a 24-h fear memory test, D1R/DSR KO mice exhibited fear memory deficits as measured by reduced freezing levels in KO animals (unpaired t-test, P < 0.05) (Fig. 1 M and N). The observed phenotype in mutant mice was not due to differences in pain sensitivity, gross motor dysfunction, or altered anxiety levels (Fig. S2 A–D). Because the amygdala is pivotal in processing fear memory, we trained mice on an amygdala-dependent cue fear conditioning protocol where a discrete auditory cue predicts and terminates with an aversive foot shock. D1R/DSR KO mice showed normal conditioning to tone (unpaired t-test, P = 0.5533), suggesting that contextual fear memory deficits in the KO mice are not due to altered amygdalar function (Fig. S3A).

Identification of DG-Specific D1R and D5R Function in Contextual Fear Conditioning and Subregion-Specific c-Fos Activation. We generated DG specific D1R KO and D5R KOs by crossing POMC-Cre mice (34) with flx D1R mice or flx D5R mice, respectively. D1R gene recombination was restricted to DG granule cells of the hippocampus. The D1R mRNA signal was nearly absent by 16 wk of age in D1R mutants (Fig. 2 A and B). Quantification of the D1R ISH signal in flx and KO mice showed significant reduction of D1R mRNA expression in the DG (unpaired t-test, P = 6.27 × 10−4) with normal DG D5 mRNA expression (unpaired t-test, P = 0.309) (Fig. 2 C–E). D5R KO animals exhibited maximum deletion of D5R mRNA (unpaired t-test, P = 3.86 × 10−6) by 15 wk of age and showed normal D1R mRNA expression in the DG (unpaired t-test, P = 0.250) (Fig. 2 F–J).

D1R KO deletion impairs CFC, whereas DG D5R deletion does not. When mice were trained on a CFC paradigm, D1R KOs exhibited impaired fear memory compared with control mice [two-way ANOVA (time × genotype), F(9, 801) = 9.963, P < 0.0001; F(1, 89)genotype = 20.36, P < 0.0001]. Bonferroni multiple comparisons test also shows deficits at multiple time points (Fig. 2K). DG D5R KO mice exhibited normal contextual fear memory when average freezing levels were compared between the mutant and control mice (unpaired t-test, P = 0.908) (Fig. 2N). We also tested the necessity of DG D1Rs for short-term memory (1 and 3 h after training) and observed no fear memory deficits (unpaired t-test, 1 h, P = 0.5584; 3 h, P = 0.3337) (Fig. S3B). DG D1R KOs showed normal cued fear conditioning during tone presentation (unpaired t-test, P = 0.4877) (Fig. S3C). Moreover, neither the DG D1R KO nor the DG D5R KO mice exhibited gross differences in pain sensitivity, motor function, or anxiety (Fig. S2 E–J).

D1R KO, but not D5R, deletion impairs DG and CA3 c-Fos expression to novel contextual exposure and CFC. We used c-Fos expression levels to measure hippocampal subregion encoding of novel contextual information (35–38). We measured c-Fos+ neurons of the DG and CA3 in response to the animals’ home cage, exposure to a novel context or novel contextual exposure plus footshock (i.e., mice receiving CFC). Flx D1R controls showed a robust increase in the number of c-Fos+ neurons when exposed to a novel context (unpaired t-test, P < 0.0005) or given CFC (unpaired t-test, P < 0.0005), in comparison with the home cage group. Moreover, there was no significant difference in DG c-Fos+ neurons in flx D1R mice, when the context and CFC groups were compared (flx: unpaired t-test, P < 0.599) (Fig. 2L). In contrast, compared with the control mice, D1R KOs showed significantly reduced c-Fos+ expression in the DG in response to both the novel context exposure (unpaired t-test, P < 0.01) and CFC (unpaired t-test, P < 0.01) (Fig. 2L). Interestingly, in the home cage group, D1R KOs showed significantly enhanced c-Fos+ DG granule cells, compared with flx control (unpaired t-test, P < 0.01) (Fig. 2L). No significant difference was found in the number of c-Fos+ neurons between the home cage group and novel context exposure (unpaired t-test, P = 0.148) or between the home cage group and fear-conditioned groups in the D1R KOs (unpaired t-test, P = 0.058) (Fig. 2L). However, a noticeable trend toward significance was observed between the home cage and fear-conditioned groups.

In both flx control and DG D1R KOs, the presentation of a footshock significantly increased the number of c-Fos+ neurons in CA3, compared with novel context exposure (flx: unpaired t-test, P < 0.05; D1 KO: unpaired t-test, P < 0.01) (Fig. 2M). Nevertheless, DG D1R deletion results in a significant decrease of c-Fos+ CA3 neurons in both the novel context exposure (unpaired t-test, P < 0.05) and CFC groups (unpaired t-test, P < 0.05), compared with control mice (Fig. 2M). Fear-conditioned DG D5R KOs did not exhibit any significant differences in c-Fos expression in the DG (unpaired t-test, P = 0.406) or CA3 (unpaired t-test, P = 0.568) compared with control mice (Fig. 2 O and P).

D1R KO deletion results in contextual generalization. Given the deficits in DG c-Fos expression in DG D1R KOs (Fig. 2L) and the observed CFC deficits in these mice (Fig. 2K), we hypothesized that the KOs have impaired encoding of contextual...
In this study we found that the overlapping expression of 
P

**Discussion**

**DG D1Rs Contribute to the Formation of Novel Contextual Representations.** In this study we found that the overlapping expression of D1Rs and D5Rs is greatest in the DG of the hippocampus among all brain regions. The strong expression of DG D1Rs and D5Rs highlights the significance of the dopamine signal in hippocampal dependent memory processing. Additionally, we have shown that the loss of either both receptors or one of the two receptors in the DG results in an impairment of i-LTP at the mPP-DG synapse. Furthermore, we found that DG D1R deletion, but not D5R deletion, increases DG c-Fos expression in a familiar environment (i.e., home cage). However, D1R deletion decreases DG and CA3 c-Fos expression when the animal was exposed to a novel context or when they are fear conditioned in a novel context. Moreover, DG D1R KO deletion impairs contextual fear conditioning. The lack of enhanced c-Fos levels in DG D1R KO to novel but not familiar contextual exposure supports the specific role of DG D1Rs in encoding novel contextual information. In addition, the observed enhancement of context generalization in the KO animals further supports the role of DG D1Rs in accurately encoding novel contextual information. It is likely that the freezing deficit observed in DG D1R KO is due to this impairment in forming contextual representation.

Recent work, however, showed that DG-to-CA3 transmission is absent: There are no observed deficits in contextual fear conditioning (39). Our present results (Fig. 2K) seemingly go against these earlier findings. However, given that the DG D1R KOs exhibit a significant enhancement of baseline c-Fos activity, which does not significantly increase to novel context exposure or to fear conditioning (Fig. 2L), we propose that KO mice may transmit corrupt contextual information from the DG to CA3. Thus, CA3 is likely processing abnormal information from the DG resulting in disrupted encoding of novel contextual information leading to contextual fear memory deficits in DG D1R KO mice. Our reasoning is in line with hippocampal c-Fos activation and hippocampal lesion studies. First, hyperactivation of c-Fos expression in the DG results in impaired contextual fear memory (40). Second, hippocampal lesions result in the disruption of encoding contextual information and not the context–footshock association (1, 2, 41, 42). However, unlike the lesions studies, our DG D1R deletion does not simply lead to loss of function, but rather to a gain of impairment. That is, DG D1R deletion impairs the ability to build a distinct contextual representation of similar contexts because D1R deletion increases the transfer of inaccurate contextual information to CA3, which in turns results in CFC deficits.

The 24-h contextual fear memory test (fix, n = 10; KO, n = 11). (M) Average of L, CP, caudate/putamen; CTX, cortex; NAcc, nucleus accumbens; OLB, olfactory bulb; OT, olfactory tubercle.

**Fig. 1.** Characterization of forebrain D1R/5R KO mice. (A and B) D1R mRNA expression in fix mice. (C and D) D5R mRNA expression in fix mice. (E and F) D1R mRNA expression in D1R/5R KO mice. (G and H) D5R mRNA expression in D1R/5R KO mice. (I and J) Quantification of the D1R (DG fix and KO, n = 8; basal ganglia fix and KO, n = 5; J) and D5R mRNA (fix and KO, n = 8 for all subregions; J) signal in D1R/5R KO mice. (K) mPP-DG input–output curve (fix, n = 6; KO, n = 9). (L) i-LTP at the mPP-DG synapse (fix, n = 6; KO, n = 8). (M)
Spatially Restricted KO Animals Clarify the Discrepancies Between Pharmacological and Global KO Studies. Until now, experimental manipulations have been unable to adequately address the individual functions of D1Rs and D5Rs in hippocampal dependent memory. Pharmacological studies have provided evidence that both D1Rs and D5Rs or one of the two, are necessary for CFC, which has been at odds with D1R−/− and D5R−/− mutant studies that showed that neither D1Rs nor D5Rs were required for fear conditioning (13, 16, 17). There are several potential reasons for these discrepancies. First, the D1R/D5R antagonists used can alter other neuromodulatory receptor activity known to affect synaptic plasticity (43–47). Because there are functional differences between D1Rs and D5Rs, the pharmacological data may not accurately depict the necessity of D1R/D5Rs in hippocampal memory formation. Second, the lack of an observed fear conditioning phenotype in the D1R−/− and D5R−/− mice, may be due to compensatory mechanisms (e.g., receptor up-regulation) of D1Rs and D5Rs in D5R−/− and D1R−/− mice, respectively. We have observed that D1R−/− mice generated in our laboratory lack motivation to seek food and water. These mice died soon after weaning unless food was directly and easily accessible, which is in agreement with previous studies reporting similar behavioral abnormalities (16, 48). Therefore, the global KO studies may not have provided data that is a true function of D1Rs or D5Rs in hippocampal dependent memory formation. Given these issues, it has been difficult to reconcile pharmacological and global KO studies, hindering the ability to accurately determine D1R from D5R function. Nonetheless, our current findings are in agreement with some of the pharmacological data, because we find that forebrain D1Rs or D5Rs, or both are required for fear memory and L-LTP at the mPP-DG synapse (Fig. 1 L and M). The genetic tools used in our current study have provided a unique advantage over the pharmacological studies by allowing us to investigate the precise role of each receptor subtype in the DG.

Experimental Procedures

Animal Sex. All experimental animals in this manuscript are male.

Generation of the D1/D5 Receptor KO Mice. To generate inbred C57BL/6 mice lines with the dopamine D1R and D5R flanked by loxP sites, we made targeting

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**Fig. 2.** Characterization of DG-specific D1R and D5R KO lines. (A and B) D1R mRNA expression in D1 KO line. (C) D5R mRNA expression in D1 KO line. (D and E) Quantification of D1R (flx, n = 4; KO, n = 4; D) and D5R mRNA (flx, n = 4; KO, n = 3; E) expression in the DG. (F and G) D5R mRNA expression in D5 KO line. (H) D1R mRNA expression in D5 KO line. (I and J) Quantification of D5R (flx and KO, n = 4 for all subregions; I) and D1R mRNA (flx, n = 4; KO, n = 4; J) expression in the DG. (K) D1R KO line, contextual fear memory test (flx, n = 51; KO, n = 40). (L and M) D1R KO line, quantification of c-Fos+ neurons in the DG (L) and CA3 (M) neurons (DG and CA3; HC flx and KO, n = 3; CTX flx and KO, n = 5; FC flx and KO, n = 4). (N) DG D5R KO line, contextual fear memory test (flx, n = 9; KO, n = 11). (O and P) D5R KO line, quantification of c-Fos+ neurons in the DG (O) and CA3 (P) neurons (DG and CA3; HC flx, n = 3; KO, n = 2; FC flx and KO, n = 3). CTX, context exposure; FC, fear conditioning; HC, home cage.

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**Fig. 3.** Contextual shift design task. (A) Schematic of training and testing schedule. (B) Contextual fear memory test in training context and non-training context (fix = 20, KO = 17).
constructs from BAC clones obtained from a mouse C57BL/6 genomic library (Fig. 5A1). For both loci, the entire coding regions were flanked by two loxP sites, and a third loxP site was inserted at each step and averaged. A population spike of at least 5 mV was necessary for experimentation. c-LTP was induced by a theta burst protocol where six trains were given at 5 Hz, each train consisted of six pulses at 400 Hz, this was repeated six times with a 30-s interval.

c-Fos Experiments. Mice were anesthetized with an overdose of avertin 60 min after contextual fear conditioning. The fully anesthetized animals were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS. Brains were stored in fixative (4% [wt/vol] PFA in PBS) overnight at 4 °C, and then incubated overnight in 30% [vol/vol] sucrose. A cryostat was used to collect sagittal sections of 60-μm thickness. Sagittal sections were blocked with PBST (PBS with 0.3% Triton X-100) with 5% [vol/vol] normal goat serum for 1 h and then incubated with primary antibody at 4 °C for 2 h (rabbit anti-c-Fos 1:500, Santa Cruz; mouse anti-NeuN 1:1,000, Millipore). Slices then underwent three wash steps at 15 min each in PBST, followed by 3-h incubation with secondary antibody (1:200 AlexaFluor 488 anti-mouse, Invitrogen; 1:200 AlexaFluor 546 anti-rabbit, Invitrogen). Slices were then incubated for 30 min with NeuroTrace 640/660 for Deep-Red Fluorescent Nissl Stain (Invitrogen), and underwent three more wash steps of 15 min each in PBS, followed by mounting and coverslipping on microscope slides. For quantification analysis of number of c-Fos-positive cells in DG and CA3 region in mice, sampling of c-Fos-positive cells was conducted throughout the dorsal DG. Ten sagittal hippocampal sections were used to count c-Fos-positive cells in DG and hippocampal CA3 region in a genotype- and treatment-blinded manner.

Behavioral Batteries. Pain sensitivity. A heating block with high walls was set to 50 °C. Mice were placed onto the heating block one at a time. The time from being set onto the heating block to the time the mouse ribs its paws was used as the index for pain sensitivity.

Open Field Activity. Mice were housed alone for 3 consecutive days for 2 min per cage before the first day of open field test. Activity was measured by IR beam interruption and recorded in 1-min intervals over a 10-min period in a novel chamber (Digiscan apparatus, Accuscan Instruments). This was conducted for 3 consecutive days.

Rotated. Mice were placed on a rotating platform that increases in the rate of rotation over a 300-s window. The time from when the mice were placed onto the apparatus to the time they fell off was recorded.

Fear Conditioning. All experiments were conducted using FreezView software. Animals received either a single 0.5-mA or 0.75-mA unsignaled footshock at 118 s with a 2-s duration in a novel chamber. Mice remained in the chamber for 60 s after footshock. One, 3, or 24 h after CFC, mice were returned to same conditioning chambers where freezing was assessed for 5 min. This contextual fear memory was then tested using a contextual shift paradigm where mice were placed into context B 24 h after training or in a novel context (context B). Twenty-four hours after the first test, mice placed in the training context were then placed in context B and vice versa. In cue feared conditioning, mice received a single paired conditioned stimulus tone and unconditioned stimulus (30 s, 5 kHz, 75 dB, 0.75 mA, 2 s duration). Twenty-four hours after training, mice were placed into a similar context as compare with the training context and explored the context for 2 min, after which a tone presentation was given for 3 min.

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