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Striatal dopamine D1 receptor is essential for contextual fear conditioning

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Fear memory is critical for animals to trigger behavioural adaptive responses to potentially threatening stimuli, while too much or inappropriate fear may cause psychiatric problems. Numerous studies have shown that the amygdala, hippocampus and medial prefrontal cortex play important roles in Pavlovian fear conditioning. Recently, we showed that striatal neurons are required for the formation of the auditory fear memory when the unconditioned stimulus is weak. Here, we found that selective ablation of striatal neurons strongly diminished contextual fear conditioning irrespective of the intensity of footshock. Furthermore, contextual fear conditioning was strongly reduced in striatum-specific dopamine D1 receptor knockout mice. On the other hand, striatum-specific dopamine D2 receptor knockout mice showed freezing responses comparable to those of control mice. These results suggest that striatal D1 receptor is essential for contextual fear conditioning.

Fear is one of the most potent emotional experiences. Learning about fearful experiences is critical for animals to trigger a set of defensive mechanisms for adapting to dangerous environmental threats. The fear system has been most systematically investigated using a Pavlovian fear-conditioning paradigm[1]. In a typical fear conditioning protocol, animals receive pairing of an initially neutral conditioned stimulus (CS), such as tone or the context of the conditioning chamber, and an aversive unconditioned stimulus (US), such as a footshock. After learning this association, the CS elicits a set of defensive responses that typically occur when an animal encounters a threating stimulus.

Numerous studies have shown the importance of the amygdala, hippocampus and medial prefrontal cortex for Pavlovian fear conditioning. The amygdala is critical for learning about both contextual and discrete stimuli and the hippocampus has a selective role in fear to contextual stimuli[2–4]. In addition, cortical areas including the medial prefrontal cortex is involved in the extinction of contextual fear memories[5–7].

Dopamine is one of the neurotransmitters most potently modulating the mechanisms underlying states of fear[8,9]. Correspondingly, dopamine D1 receptor (D1R) and D2 receptor (D2R) are expressed in the hippocampus, amygdala and prefrontal cortex that are involved in fear memory formation[10], while there are high levels of D1R and D2R in the striatum[11]. Systemic administration of antagonists for D1-like receptors reduced fear conditioning[12–15]. Systemic or amygdala-selective injections of antagonists for D2-like receptors were reported to block expression or retention of fear conditioning, whereas others reported that these drugs exerted little effect on fear conditioning[12,13,15]. There are analogous discrepancies among studies using agonists or antagonists for D2-like receptors[12,15–17]. Since dopamine receptor antagonists vary widely in their selectivity among D2R, D3R and D4R[18], differences in the dose and choice of pharmacological agents or behavioural methodology may account for these discrepancies.

We found that striatal neurons play roles in the formation of auditory fear memory when the unconditioned stimulus is weak[19]. Furthermore, NMDA receptors and *de novo* protein synthesis in the striatum are crucial for the consolidation of auditory fear memory formed with a low-intensity unconditioned stimulus[20]. Here, we examined the role of striatal neurons in contextual fear conditioning. Selective ablation of striatal neurons in the adult brain impaired contextual fear conditioning irrespective of the intensities of US (footshock). Since D1R and D2R are highly expressed in the striatum[11], we then generated striatum-specific D1R and D2R knockout mice to investigate the contributions of these receptors in contextual fear conditioning. Striatum-specific D1R knockout mice showed significantly reduced freezing responses in contextual fear conditioning. On the other hand, striatum-specific D2R knockout mice showed freezing responses comparable to those of control mice. These results suggest that striatal D1R but not D2R is required for contextual fear conditioning. Our results provide

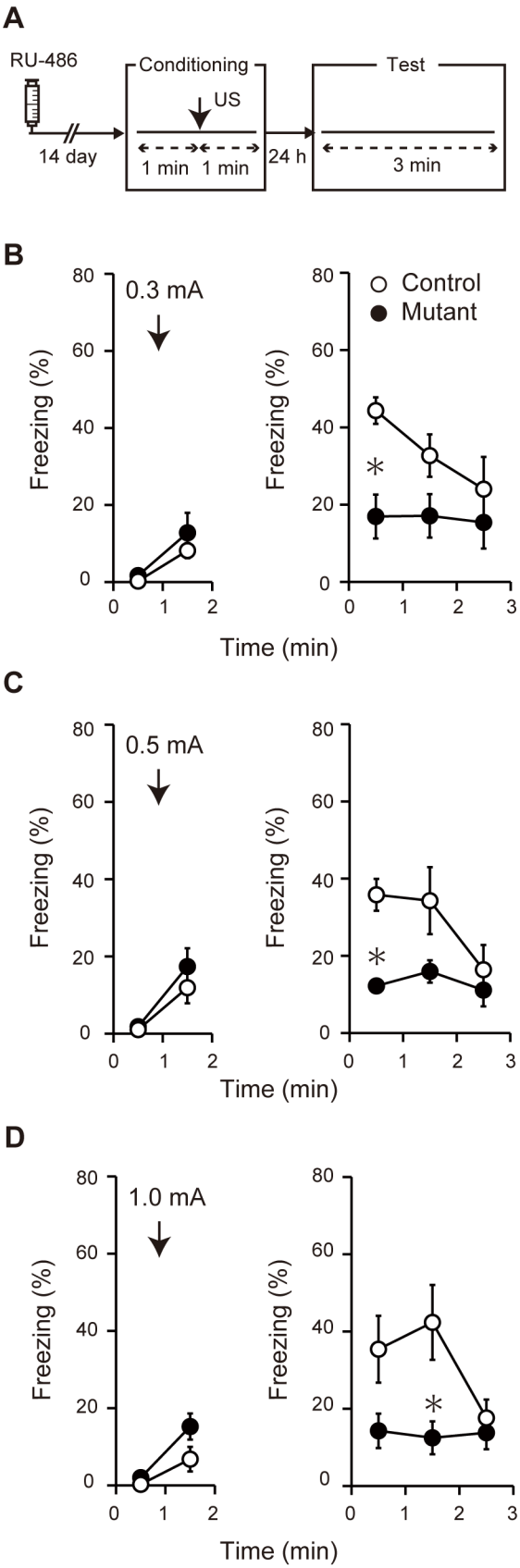

Figure 1 | Effect of striatal neuron ablation on contextual fear conditioning. (A) Experimental design to examine the acquisition of contextual fear memory. Doubly transgenic mice were injected with RU-486 or vehicle. Fourteen days after treatment, the animals were subjected to contextual fear conditioning. (B) Freezing responses of control (open, $n = 5$) and mutant (filled, $n = 6$) mice to fear conditioning with 0.3-mA footshock. (C) Freezing responses of control (open, $n = 7$) and mutant (filled, $n = 6$) mice to fear conditioning with 0.5-mA footshock. (D) Freezing responses of control (open, $n = 6$) and mutant (filled, $n = 7$) mice to fear conditioning with 1.0-mA footshock. *, $p < 0.05$; ANOVA Tukey's test.

evidence for the importance of the striatum as a key component of brain systems controlling contextual fear memory.

Results

Impairment of contextual fear conditioning by ablation of striatal neurons. We previously developed an inducible ablation system of striatal neurons in a transgenic mouse line carrying *Gng7*-promoter-driven Cre recombinase-progesterone receptor fusion (CrePR) and Cre-dependent Diphtheria toxin A (DTA) genes[19]. Induction of CrePR-mediated DTA expression by RU-486 injection successfully ablated almost completely the medium-sized spiny neurons (MSNs) that comprise approximately 90% of the NeuN-positive striatal neurons within 13 days. In the present investigation, we examined the effect of striatal neuron ablation on contextual fear conditioning using transgenic mice treated with RU-486 as striatal neuron-ablated mutant mice and corresponding mock-injected littermates as controls. Fourteen days after RU-486 or mock treatment, mice were placed in the conditioning chamber for 1 min and then given a scrambled electrical footshock (0.3, 0.5 or 1.0 mA for 1 s) (Fig. 1A). One minute after footshock, the mice were returned to their home cage. On the next day, mice were placed in the chamber for 3 min. There were significant differences in the freezing responses of control and mutant mice to fear conditioning with 0.3-mA footshock (control, 33.7 ± 3.9%, $n = 5$; mutant, 16.5 ± 3.3%, $n = 6$; $F_{(1,27)} = 11.9$; $p < 0.01$, repeated measures ANOVA) (Fig. 1B). Freezing responses to fear conditioning with 0.5-mA footshock were also significantly reduced in mutant mice than in control mice (control, 28.8 ± 4.1%, $n = 7$; mutant, 13.1 ± 1.8%, $n = 6$; $F_{(1,33)} = 12.2$; $p < 0.01$, ANOVA) (Fig. 1C). Furthermore, there were significant differences in freezing responses of control and mutant mice even to fear conditioning with 1.0-mA footshock (control, 31.8 ± 5.0%, $n = 6$; mutant, 13.5 ± 2.4%, $n = 7$; $F_{(1,33)} = 13.2$; $p < 0.01$, ANOVA) (Fig. 1D). There was no significant difference in the pain sensitivity between control and striatal neuron-ablated mutant mice[19]. Thus, contextual fear conditioning was strongly diminished by the selective ablation of striatal neurons irrespective of the intensity of footshock.

We further examined whether the ablation of striatal neurons affects the retention of previously acquired contextual fear memory. Mice were first trained with 1.0-mA footshock and placed back in the home cage. Twenty-four hours after conditioning when long-term memory was formed, the animals were treated with RU-486 to induce the ablation of striatal neurons (Fig. 2A). When tested 14 days after the drug treatment, freezing responses were comparable between mutant and mock-injected control mice (control, 43.1 ± 4.3%, $n = 8$; mutant, 38.4 ± 4.5%, $n = 9$; $F_{(1,45)} = 0.54$; $p = 0.47$, ANOVA) (Fig. 2B). These results suggest that striatal neurons are dispensable for the retention of contextual fear memory.

Generation of striatum-specific D1R and D2R knockout mice on the pure C57BL/6 genetic background. The striatal projection neurons are MSNs that are classified into two subpopulations, i.e., striatonigral neurons in the direct pathway and striatopallidal neurons in the indirect pathway[21–23]. The striatonigral neurons selectively express D1R, while the striatopallidal neurons show the confined expression of D2R[23–25]. To examine whether the direct or indirect pathway is responsible for contextual fear conditioning, we generated striatum-specific D1R and D2R knockout mice.

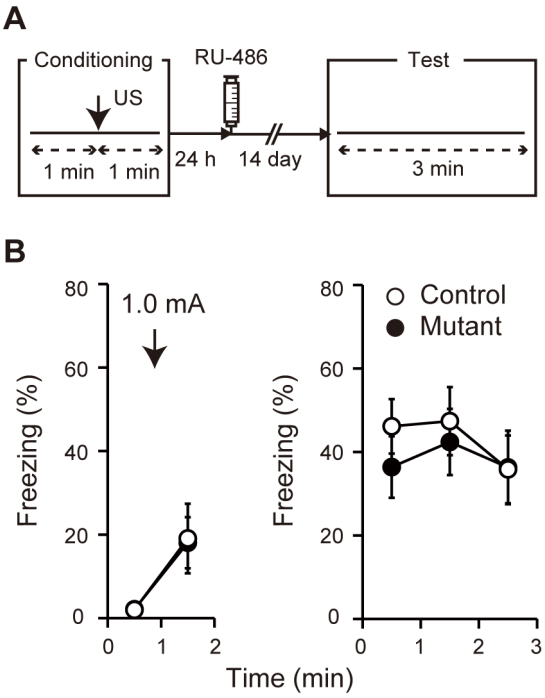

Figure 2 | Effect of striatal neuron ablation on the retention of contextual fear memory. (A) Experimental design to examine the retention of contextual fear memory. Mice were subjected to contextual fear conditioning with 1.0-mA footshock. Twenty-four hours after conditioning, the conditioned mice were injected with RU-486 or vehicle. Their freezing responses were measured 14 days after drug treatment. (B) Freezing responses of control (open, $n = 8$) and mutant (filled, $n = 9$) mice.

To investigate the roles of striatal D1R and D2R in the formation of fear memory, we constructed targeting vectors in which two Cre recombinase recognition (*loxP*) sites were inserted into the mouse dopamine receptor D1A and D2 (*Drd1a* and *Drd2*) genes. The first *loxP* site was in the upstream region of exon 2, and the second one linked to the neomycin phosphotransferase (*neo*) gene flanked by two Flp recognition target (*frt*) sites were in the downstream of exon 2. Using embryonic stem (ES) cells derived from the C57BL/6 strain[26], we obtained recombinant *Drd1a*$^{+/flox}$; +/*neo* and *Drd2*$^{+/flox}$; +/*neo* mice. Crossing to B6-Tg (CAG-FLPe) 36 mice of the C57BL/6 strain[27] successfully eliminated the *neo* gene to yield *Drd1a*$^{+/flox}$ and *Drd2*$^{+/flox}$ mice with the *Drd1a* and *Drd2* genes flanked by two *loxP* sites (Fig. 3A–D). We crossed *Drd1a*$^{flox/flox}$ and *Drd2*$^{flox/flox}$ mice with striatal MSN-selective Cre (*Gng7*$^{+/cre}$) mice[19] to yield *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ and *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice, respectively (Fig. 3E and F). *Drd1a*$^{flox/flox}$; *Gng7*$^{+/+}$ and *Drd2*$^{flox/flox}$; *Gng7*$^{+/+}$ littermates were served as controls. These mutant and control mice were fed with food pellets on the floor and grew to adulthood.

We examined the expression levels of the *Drd1a* and *Drd2* mRNAs by RT-PCR analysis. The amount of the *Drd1a* mRNA in the striatum of *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice was decreased to 7.9% of that of control mice ($t_{(4)} = -8.4$; $p < 0.01$) (Fig. 4A). Similarly, the amount of the *Drd2* mRNA in the striatum of *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice was decreased to 15.4% of that of control mice ($t_{(22)} = -5.4$; $p < 0.01$) (Fig. 4B). On the other hand, the amounts of the *Drd1a* and *Drd2* mRNAs in the cerebral cortex and hippocampus were comparable between control and *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice and between control and *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice (Fig. 4A and B). Thus, the expression of the *Drd1a* and *Drd2* mRNAs was abolished selectively in the striatum of *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ and *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice, respectively. Based on these results, *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ and *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice were named as striatum-specific D1R and D2R knockout mice, respectively.

Immunohistochemical analysis with anti-D1R and anti-D2R antibodies using coronal and sagittal brain sections showed that immunostaining signals for D1R were diminished in the striatum of *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice and those for D2R were significantly reduced in the striatum of *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice (Fig. 5A and B). Western blot analysis showed that the amount of D1R protein in the striatum of *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice was decreased to 16.6 ± 5.1% of that of control mice ($t_{(4)} = 4.3$; $p = 0.01$) (Fig. 5C). The amount of D2R protein in the striatum of *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice was decreased to 41.6 ± 5.2% of that of control mice ($t_{(10)} = 2.9$; $p = 0.02$) (Fig. 5D). On the other hand, the amounts of D1R and D2R proteins in the cerebral cortex and hippocampus were comparable between control and *Drd1a*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice and between control and *Drd2*$^{flox/flox}$; *Gng7*$^{+/cre}$ mice, respectively (Fig. 5C and D). D1R is expressed at excitatory postsynapses[28,29], while D2R is expressed in both postsynaptic sites of MSNs and presynaptic terminals of neurons projecting to the striatum[30,31]. Thus, most of residual D2R protein in the striatum of the mutant mice can be ascribed to presynaptic D2R protein in afferent terminals.

Reduced pain could result in less freezing[32]. We first tested whether the striatum-specific ablation of D1R or D2R might alter nociceptive reactions to electric shock, namely flinch, vocalization and jump[33]. We measured current thresholds for these three reactions of mice. There were no significant differences between control and striatum-specific D1R knockout mice in current thresholds for flinch (control, 0.11 ± 0.01 mA, $n = 8$; striatum-specific D1R KO, 0.11 ± 0.01 mA, $n = 8$; $t_{(14)} = 0.61$; $p = 0.55$), vocal (control, 0.13 ± 0.01 mA, $n = 8$; striatum-specific D1R KO, 0.14 ± 0.01 mA, $n = 8$; $t_{(14)} = 0.72$; $p = 0.48$) and jump reactions (control, 0.23 ± 0.01 mA, $n = 8$; striatum-specific D1R KO, 0.24 ± 0.02 mA, $n = 8$; $t_{(14)} = 0.85$; $p = 0.41$) (see Supplementary Fig. S1A online). There were also no significant differences between control and striatum-specific D2R knockout mice in pain thresholds for flinch (control, 0.10 ± 0.01 mA, $n = 8$; striatum-specific D2R KO, 0.09 ± 0.01 mA, $n = 8$; $t_{(14)} = -0.42$; $p = 0.68$), vocal (control, 0.11 ± 0.01 mA, $n = 8$; striatum-specific D2R KO, 0.12 ± 0.02 mA, $n = 8$; $t_{(14)} = 0.31$; $p = 0.76$) and jump reactions (control, 0.21 ± 0.02 mA, $n = 8$; striatum-specific D2R KO, 0.23 ± 0.02 mA, $n = 8$; $t_{(14)} = 1.1$; $p = 0.31$) (see Supplementary Fig. S1B online). These results suggest that the striatum-specific ablation of D1R and D2R exerted little effect on the pain sensitivities of respective mutant mice.

Effect of striatum-specific ablation of D1R and D2R on contextual fear conditioning. To examine whether D1R and D2R in the striatum are involved in the formation of contextual fear memories, we first tested the freezing responses of striatal D1R knockout mice. Striatum-specific D1R knockout and control mice were given a foot shock (US; 0.5 mA) on the conditioning day. Twenty-four hours after the conditioning, striatum-specific D1R knockout mice showed much smaller freezing responses than control mice (control, 35.7 ± 2.5%, $n = 13$; striatum-specific D1R KO, 11.7 ± 1.7%, $n = 13$; $F_{(1,144)} = 67.3$; $p = 0.001$, ANOVA) (Fig. 6A). There was no significant difference in the locomotor activity measures as a total distance travelled during the 3-min preshock period between control and striatum-specific D1R knockout mice (control, 204 ± 9.9 cm, $n = 13$; striatum-specific D1R KO, 240 ± 18.7 cm, $n = 13$; $t_{(24)} = -1.68$; $p = 0.11$) (see Supplementary Fig. S2 online). Since freezing responses of wild-type and *Gng7*$^{+/cre}$ mice were comparable (wild type, 44.4 ± 2.3%, $n = 13$; *Gng7*$^{+/cre}$, 41.0 ± 2.4%, $n = 14$; $F_{(1,150)} = 1.1$; $p = 0.29$, ANOVA) (Fig. 6B), *Gng7*^{cre} exerted little effect on the fear responses. These results suggest that striatal D1R is required for contextual fear conditioning.

We next examined the contextual fear responses of striatum-specific D2R knockout mice. Twenty-four hours after the conditioning, the mutant mice showed freezing responses comparable to those of

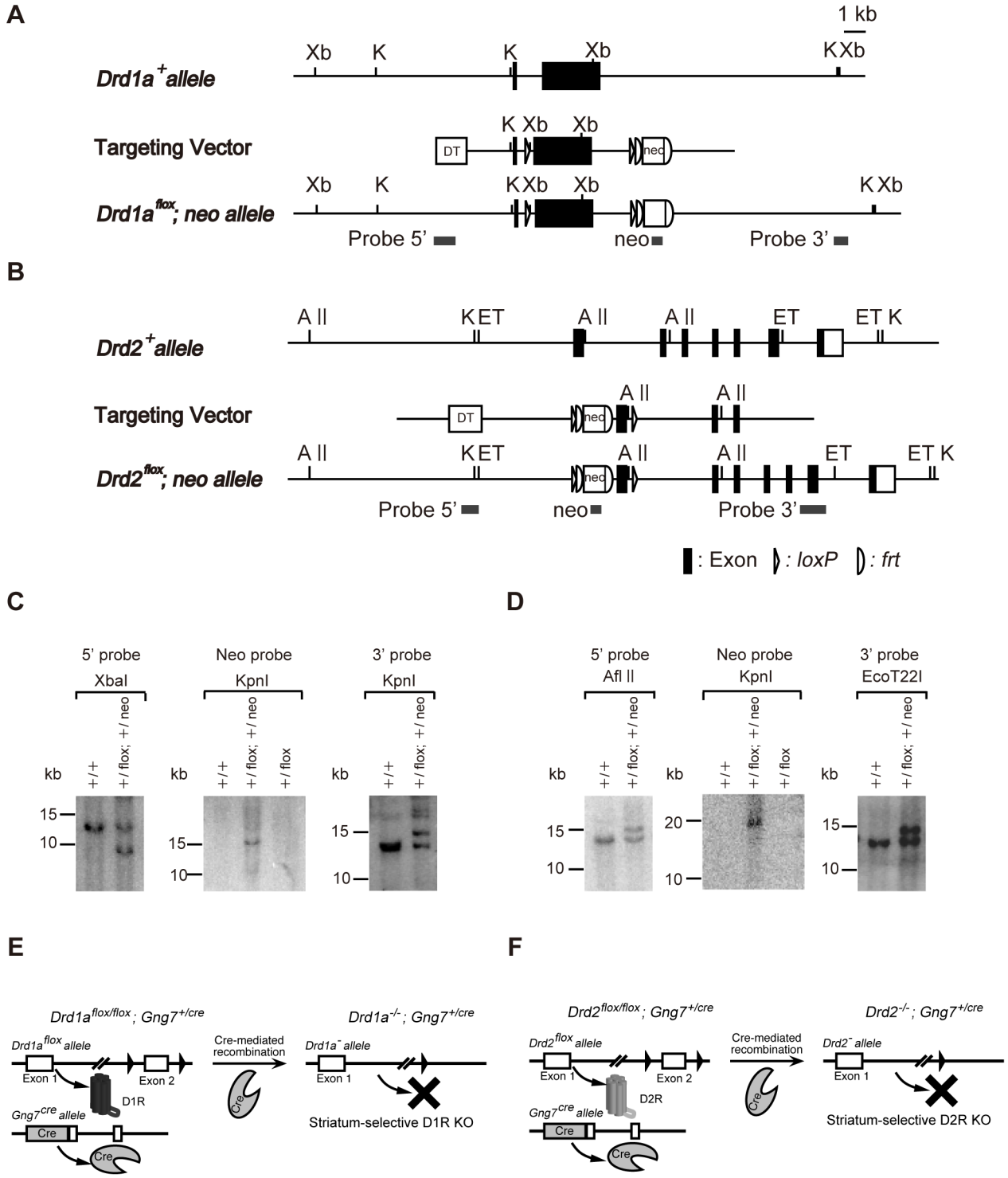

Figure 3 | Generation of striatum-specific D1R or D2R KO mouse. (A) Schematic representation of the *Drd1a* gene, targeting vector and floxed *neo*-inserted allele (*Drd1a*^{flox}; *neo*). (B) Schematic representation of the *Drd2* gene, targeting vector and floxed *neo*-inserted allele (*Drd2*^{flox}; *neo*). Gray bars indicate the location of probes for southern blot analysis (Probe 5′, neo and 3′). Abbreviations: DT, diphtheria toxin gene; neo, neomycin phosphotransferase gene; AII, *Afl*II; ET, *Eco*T22I; K, *Kpn*I; Xb, *Xba*I. (C) Sourthern blot analysis of genomic DNA from *Drd1a*$^{+/+}$, *Drd1a*$^{+/flox}$; +/*neo* and *Drd1a*$^{+/flox}$ mice. Left, *Xba*I-digested DNA hybridized with 5′ probe; middle, *Kpn*I-digested DNA hybridized with neo probe; right, *Kpn*I-digested DNA hybridized with 3′ probe. (D) Sourthern blot analysis of genomic DNA from *Drd2*$^{+/+}$, *Drd2*$^{+/flox}$; +/*neo* and *Drd2*$^{+/flox}$ mice. *Afl*II-digested DNA hybridized with 5′ probe; middle, *Kpn*I-digested DNA hybridized with neo probe; right, *Eco*T22I-digested DNA hybridized with 3′ probe. (E) Schema for striatum-specific D1R ablation. (F) Schema for striatum-specific D2R ablation.

control mice (control, 52.4 ± 2.4%, $n = 9$; striatum-specific D2R KO, 55.6 ± 2.1%, $n = 12$; $F_{(1,114)} = 1.0$; $p = 0.31$, ANOVA) (Fig. 6C). There was no significant difference in the locomotor activity during the 3-min preshock period between control and striatum-specific D2R knockout mice (control, 192 ± 19.5 cm, $n = 9$; striatum-specific D2R KO, 163 ± 13.7 cm, $n = 12$; $t_{(19)} = 1.29$; $p = 0.21$) (see Supplementary Fig. S2 online). Thus, striatal D2R is dispensable for contextual fear conditioning.

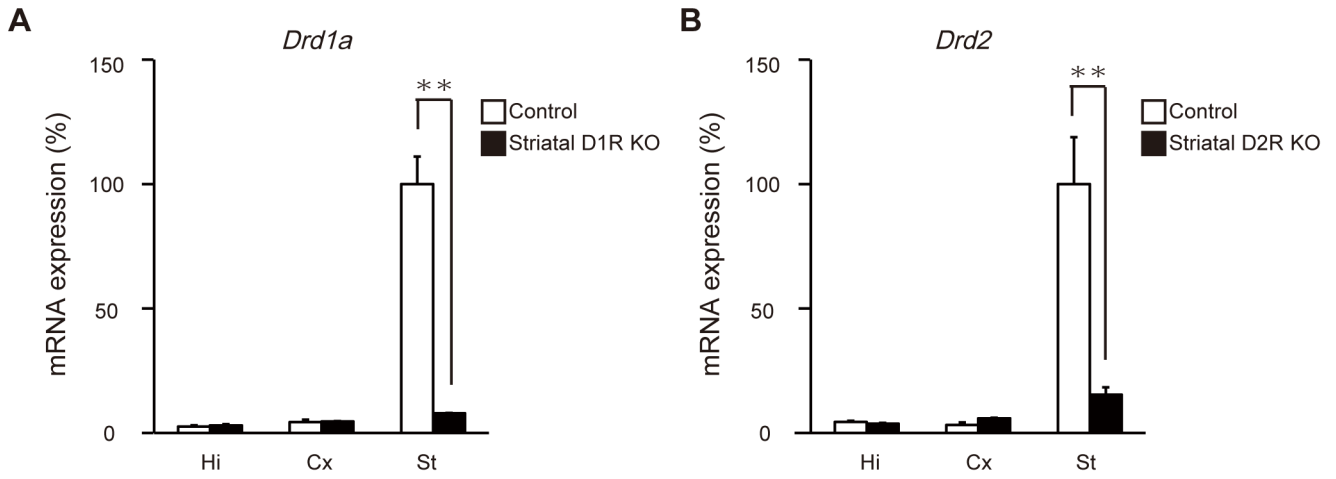

Figure 4 | Expression of *Drd1a* and *Drd2* mRNAs in the cerebral cortex, hippocampus and striatum of D1R (A) and D2R (B) KO mice. Each mRNA expression level is expressed as a ratio of that in the control striatum (flox D1R or flox D2R). All values represent mean ± SEM. **, $p < 0.01$; Student's *t* test. Abbreviations: Cx, cortex; Hi, hippocampus; St, striatum.

Differential effect of striatum-specific ablation of D1R on short- and long-term contextual fear memories. We then further examined the role of striatal D1R in the formation of short- and long-term contextual fear memories (Fig. 7). Striatum-specific D1R knockout and control mice were given a foot shock at 0.5 mA on the conditioning day. During conditioning, striatum-specific D1R knockout mice showed significantly smaller freezing responses than control mice (control, 8.1 ± 1.5%, $n = 12$; striatum-specific D1R KO, 1.3 ± 0.3%, $n = 13$; $F_{(1,92)} = 21.5$; $p < 0.001$, ANOVA). When tested 10 min after conditioning, striatum-specific D1R knockout mice hardly showed freezing responses, the magnitudes of which were much small than those of control mice (control, 37.4 ± 2.3%, $n = 12$; striatum-specific D1R KO, 5.8 ± 0.7%, $n = 13$; $F_{(1,138)} = 221$; $p < 0.01$, ANOVA). Twenty-four hours after the conditioning, striatum-specific D1R knockout mice showed significant freezing responses, although the amplitudes were significantly smaller than those of control mice (control, 30.0 ± 2.4%, $n = 12$; striatum-specific D1R KO, 12.5 ± 1.7%, $n = 13$; $F_{(1,138)} = 40.9$; $p < 0.01$, ANOVA). Thus, the formation of short-term contextual fear memory was more severely affected by the striatum-specific ablation of D1R than that of long-term contextual fear memory.

Multiple US evoked fear responses of striatum-specific D1R knockout mice. To examine the effect of strong US on contextual fear conditioning, mice were given five consecutive footshocks at 3, 4, 5, 6 and 7 min after placement in the conditioning chamber (Fig. 8). Mice were returned to the home cage 1 min after the last foot shock. On the conditioning day, the freezing responses of striatum-specific D1R knockout mice increased according to the number of footshocks, but were much smaller than those of control mice (control, 30.6 ± 2.7%, $n = 11$; striatum-specific D1R KO, 5.6 ± 1.3%, $n = 7$; $F_{(1,80)} = 66.7$; $p < 0.01$, ANOVA). Twenty-four hours after conditioning, striatum-specific D1R knockout mice showed significant freezing responses, which were much smaller than those of control mice (control, 56.2 ± 2.6%, $n = 11$; striatum-specific D1RKO, 35.3 ± 2.4%, $n = 7$; $F_{(1,96)} = 33.4$; $p < 0.001$, ANOVA). Thus, stronger US enhanced the freezing responses of striatum-specific D1R knockout mice but did not restore them to the level of control mice.

Discussion

Cumulative evidence indicates that the amygdala, hippocampus and medial prefrontal cortex are critically involved in contextual fear learning and extinction[5–7]. Here, we showed that contextual fear conditioning was impaired by the ablation of striatal MSNs induced in the adult brain. Furthermore, contextual fear conditioning was diminished in striatum-specific D1R knockout mice but not in striatum-specific D2R knockout mice. Our results provide strong evidence that striatal neurons in the direct pathway play a role in contextual fear conditioning.

Impairment of contextual fear conditioning by the ablation of striatal MSNs is consistent with classical lesion studies suggesting the importance of the ventral striatum for the conditioning. Electrolytic lesions of the nucleus accumbens (NAc) of rats impaired context but not cue fear conditioning[34]. Furthermore, lesions of the NAc shell of rats reduced contextual fear conditioning[35]. When RU-486 was administrated to induce the ablation of striatal MSNs 24 h after contextual fear conditioning, the drug treatment hardly affected the freezing responses of mice. Thus, it is likely that striatal MSNs are involved in the acquisition of contextual fear memory, but are dispensable for its retention. Consistently, an infusion of the local anaesthetic bupivacaine into the NAc of rats impaired the acquisition but not the expression of contextual fear conditioning[36]. These results suggest that MSNs in the ventral striatum play a role in the acquisition of contextual fear memory.

Recently, we showed that striatal MSNs play roles in the formation of auditory fear memory only when US is weak and NMDA receptors and *de novo* protein synthesis in the striatum are required for the auditory fear memory formation[19]. In contrast, we here found that striatal neurons are essential for contextual fear conditioning irrespective of the strength of US. It is to be noted, however, that multiple footshocks (US) partly restore contextual fear conditioning in striatum-specific D1R knockout mice. Furthermore, striatal neurons are required for the retention of the auditory fear memories but are dispensable for that of contextual fear memories. Thus, striatal MSNs play differential roles in auditory and contextual fear conditioning. Interestingly, dorsal striatal lesions reduced tone fear conditioning in rats, while contextual fear conditioning was hardly affected by the lesions[37].

It is thought that two subpopulations of striatal MSNs forming the direct and indirect pathways exert complementary and sometimes opposing actions on behaviours that are controlled by the cortico-striatal system[38]. Our results suggest that striatal neurons in the direct pathway play a role in contextual fear conditioning since freezing responses to the context were diminished in striatum-specific D1R knockout mice but not in striatum-specific D2R knockout mice. Systemic administration of D1-like receptors antagonists reduced fear conditioning[12–15]. Consistently, freezing responses in contextual fear conditioning were impaired in conventional global D1R knockout mice[39], while it was reported that contextual freezing responses of

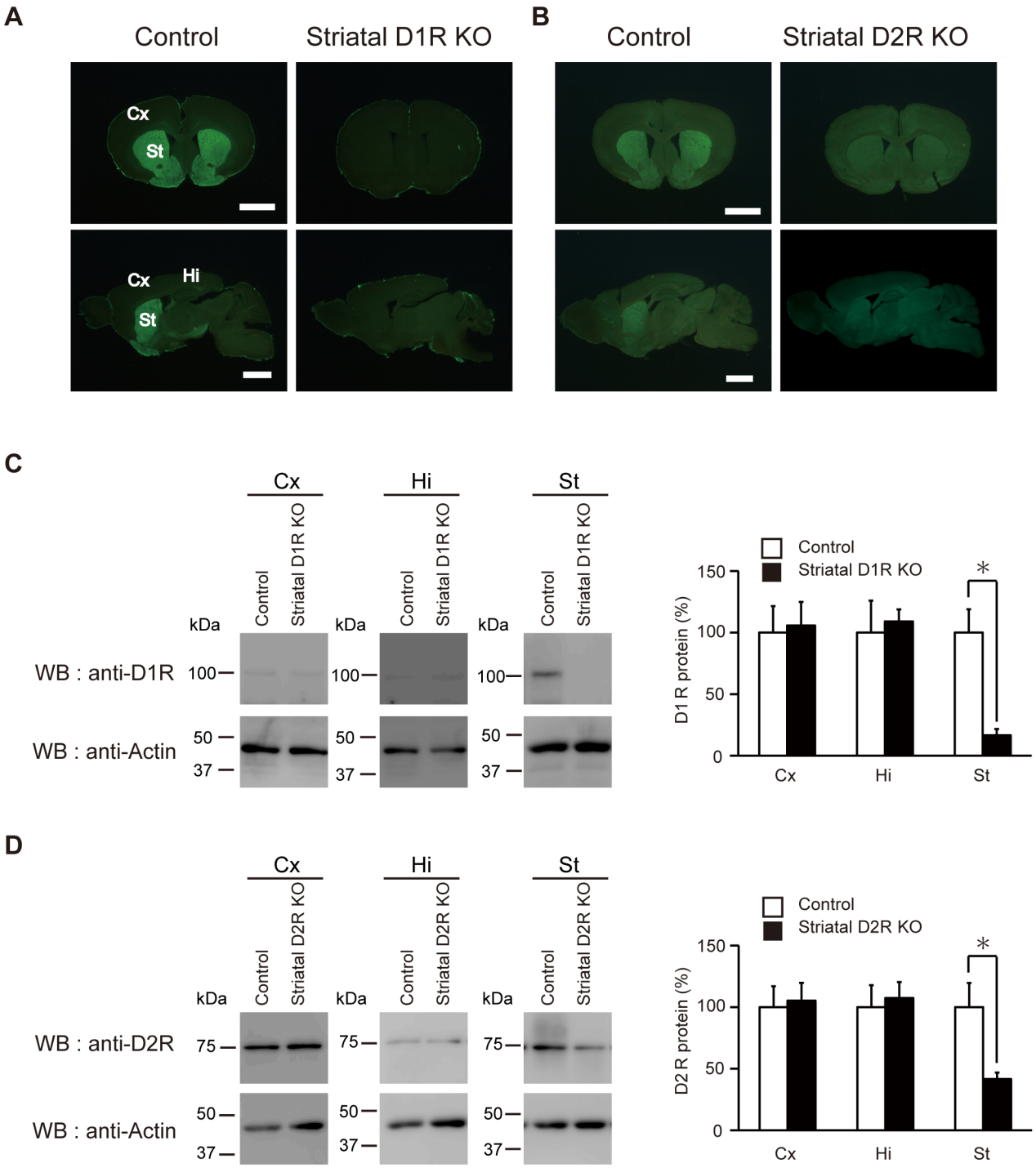

Figure 5 | Expression of D1R and D2R in the forebrain. (A) Sections from control (left column) and striatum-specific D1R KO mice (right column) were immunostained with anti-D1R antibody ($n = 3$, each). (B) Sections from control (left column) and striatum-specific D2R KO mice (right column) were immunostained with anti-D2R antibody ($n = 3$, each). Scale bars represent 1 mm. Abbreviations: Cx, cortex; Hi, hippocampus; St, striatum. (C) S1 fractions of cerebral cortex, hippocampus and striatum from control and striatum-specific D1R KO mice were separated by SDS-PAGE, followed by western blot analysis with anti-D1R and anti-actin antibodies ($n = 3$ each) (left). Relative expression levels of D1R in striatum-specific D1R KO mice (right). (D) S1 fractions of cerebral cortex, hippocampus and striatum from control and striatum-specific D2R KO mice were separated by SDS-PAGE, followed by western blot analysis with anti-D2R and anti-actin antibodies ($n = 6$ each) (left). Relative expression levels of D2R in striatum-specific D2R KO mice (right). The gels were run under the same experimental conditions. Cropped blots are shown (full-length blots are presented in Supplementary Figure S3). All values represent mean ± SEM. *, $p < 0.05$; Student's t test. Abbreviations: Cx, cortex; Hi, hippocampus; St, striatum.

global D1R knockout mice were comparable to those of wild-type mice when conditioning was repeated several times[40]. Since D1R is expressed in the brain regions essential for contextual fear conditioning, the amygdala, hippocampus and prefrontal cortex[10], the gloval D1R knockout should affect multiple phases of contextual fear conditioning. Our results together with previous pharmacological studies[36] suggest that D1R in ventral striatal MSNs plays a role in the acquisition of contextual fear memory.

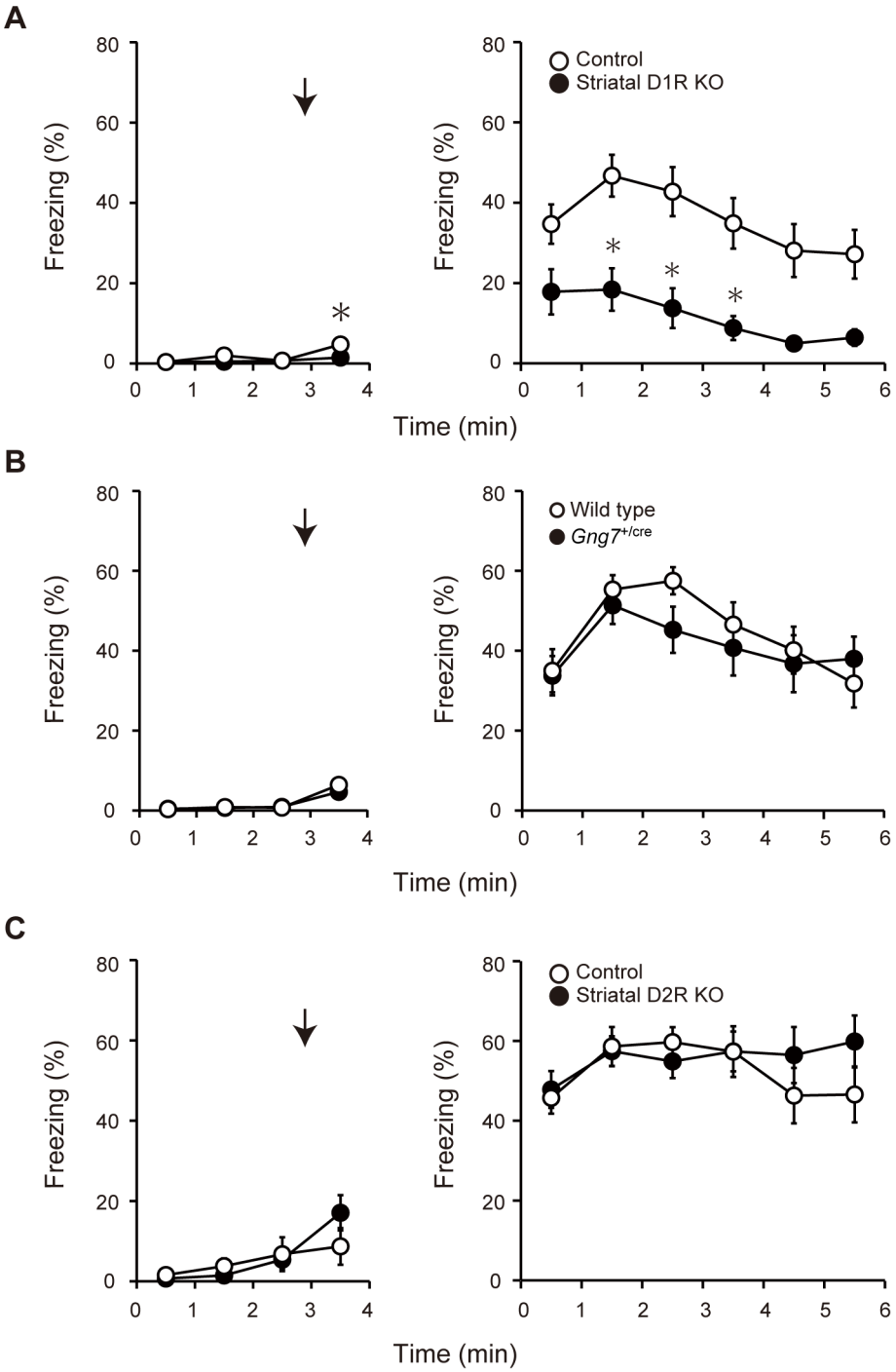

Figure 6 | Impairment of freezing responses of striatum-specific D1R KO mice. (A) Contextual fear conditioning with 0.5-mA footshock (an arrow) was carried out. Percentage freezing of control and mice ($n = 13$ each) on the conditioning (left) and test (right) days was determined by time sampling at 60 s intervals. Freezing responses immediately after shock were small for both types of mice (control, 2.0 ± 0.4%, $n = 13$; striatum-specific D1R KO, 0.8 ± 0.2%, $n = 13$; $F_{(1,96)} = 8.9$; $p < 0.01$, ANOVA). (B) Insertion of Cre recombinase gene into the *Gng7* gene has no effects on freezing responses. Contextual fear conditioning with 0.5-mA footshock (an arrow) was carried out. Percentage freezing of wild-type ($n = 13$) and $Gng7^{+/cre}$ ($n = 14$) on the conditioning (left) and test (right) days was shown at every minute. (C) No impairment of freezing responses in striatum-specific D2R KO mice. Contextual fear conditioning with 0.5-mA footshock (an arrow) was carried out. Percentage freezing of control ($n = 9$) and mutant ($n = 12$) on the conditioning (left) and test (right) days was shown at every minute. *, $p < 0.05$; ANOVA Tukey's test.

It is well established that midbrain dopamine neurons are activated by reward or sensory stimuli predicting rewards[41–43]. However, there is evidence that the mesolimbic dopamine system carries valuation signals not only for appetitive or gain-related stimuli but also for aversive and loss-related stimuli[44]. We have shown that fear conditioning enhances c-Fos expression in the stratum of mice in a US strength-dependent manner[20]. In humans, fMRI studies showed that the ventral striatum is directly activated in anticipation of aversive stimuli[45,46]. A group of dopamine neurons in monkey are excised by aversive or aversive event-predicting stimuli, suggesting that two types of dopamine neuron convey positive and negative motivational signals[47]. Thus, the striatum plays a central role in integrating neural

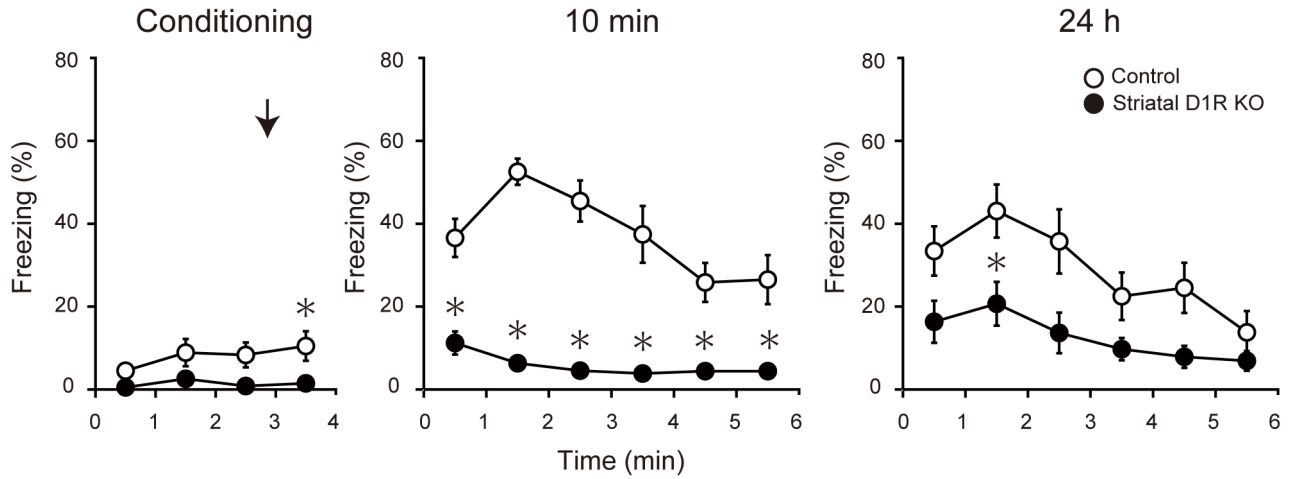

Figure 7 | Freezing responses of striatum-specific D1R KO mice 10 min and 24 h after conditioning. Contextual fear conditioning with 0.5-mA footshock (an arrow) was carried out. Percentage freezing of control ($n = 12$) and striatum-specific D1R KO mice ($n = 13$) during conditioning (left), 10 min (middle) and 24 h (right) after conditioning was shown at every minute. *, $p < 0.05$; ANOVA Tukey's test.

information from the cerebral cortex and thalamus to facilitate selection of actions that achieve reward-seeking outcomes and avoid aversive outcomes[48]. In mice, synaptic transmission in the indirect pathway is involved in aversive behaviour, while that in the direct pathway is necessary for reward learning and cocaine sensitization[49]. On the other hand, the development of highly motivated and perseverative toward cocaine is associated with synaptic plasticity in MSNs expressing D2R in the NAc of mice[50]. Studies of Pavlovian fear conditioning and extinction in rodents and humans suggest that a neural circuit including the hippocampus, amygdala and medial prefrontal cortex is involved in the learning and memory processes of that enable context-dependent behaviour[51]. Our results suggest that striatal MSNs expressing D1R also play an important role in contextual fear conditioning. Thus, the striatum is a key component of brain systems governing contextual fear memory.

Methods

Induction of striatal neuron ablation. To examine the role of striatal neurons in contextual fear conditioning, we employed doubly transgenic mice carrying the progestin-inducible CrePR gene and the Cre recombinase-dependent DTA gene[19]. RU-486 (Sigma, St. Louis, MO) was suspended at a concentration of 50 mg/ml in water containing 0.25% carboxymethyl cellulose (Sigma) and 0.5% Tween 80 (Sigma). In order to induce CrePR recombinase activity, we injected 1 mg per g body weight of RU-486 into the peritoneum of the transgenic mice at postnatal day 42.

Generation of striatum-specific D1R and D2R knockout mice on pure C57BL/6 genetic background. We identified bacterial artificial chromosome (BAC) clones RP24-266B7 and RP23-232J20 prepared from the C57BL/6 strain (BACPAC Resources Center, Oakland, CA) as those carrying the entire coding sequences of the *Drd1a* and *Drd2* genes using basic local alignment search tool searches against the mouse genome sequence database. The 11.8-kb genomic DNA fragment carrying exon 2 of the *Drd1a* gene was introduced into the pMC1DTpA[52]. The *loxP* site was inserted into 495 bp-upstream of exon 2 and the 1.8-kb DNA fragment carrying the *loxP* site and *phosphoglycerate kinase 1* (*Pgk-1*) promoter-driven *neo* gene flanked by two *frt* sites was inserted into immediately after exon 2. Targeting vector pTV-Drd1a contained exon 2 of the *Drd1a* gene flanked by *loxP* sites, *neo* gene flanked by two *frt* sites, the 9.0-kb upstream and 3.0-kb downstream genomic sequences, and 4.3-kb pMC1DTpA. The 11.0-kb genomic DNA fragment carrying exon 2 of the *Drd2* gene was introduced into the pMC1DTpA. The 1.8-kb DNA fragment carrying the *loxP* site and *Pgk-1* promoter-driven *neo* gene flanked by two *frt* sites was inserted into the 380-bp upstream of exon 2 containing the translational initiation site of the *Drd2* gene and the *loxP* site was inserted into 379-bp downstream of exon 2. Targeting vector pTV-Drd2 contained exon 2 of the *Drd2* gene flanked by *loxP* sites, *neo* gene flanked by two *frt* sites, the 3.4-kb upstream and 6.5-kb downstream genomic sequences, and 4.3-kb pMC1DTpA. Targeting vectors pTV-Drd1a and pTV-Drd2 were linearized and electroporated into ES cell line RENKA derived from C57BL/6 strain[26] as described previously[53]. G-418 (150 μg/ml)-resistant clones were picked, and *Drd1a* recombinant clones were identified by Southern blot hybridization analysis of *Xba*I- or *Kpn*I-digested genomic DNA using neo[54], 5′ and 3′ probes. The 5′ and 3′ probes

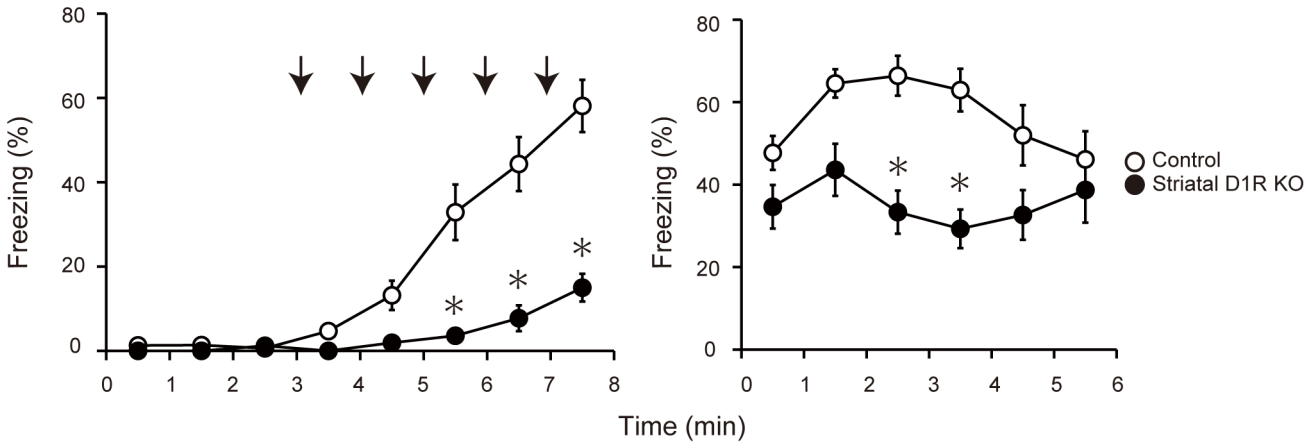

Figure 8 | Freezing responses to multiple US of striatum-specific D1R KO mice. Contextual fear conditioning with five consecutive footshocks at 0.5 mA (arrows) was carried out. Footshocks were given at 3, 4, 5, 6 and 7 min after placement in the conditioning chamber. Percentage freezing of control ($n = 11$) and striatum-specific D1R KO mice ($n = 7$) on the conditioning (left) and test (right) days was shown at every minute. *, $p < 0.05$; ANOVA Tukey's test.

were prepared by PCR with primers 5′-CCTGACTTCTTGATATCAAGC-3′ and 5′-CCAGGGTCCTGTTAAGCTAC-3′ and with primers 5′-CTTTAGGGGCTC GGTCTATTC-3′ and 5′-CTAAGGGCTGTCACCTGAGG-3′ using BAC clone RP24-266B7 as a template, respectively. *Drd2* recombinant clones were identified by Southern blot hybridization analysis of *Afl*II-, *Kpn*I or *Eco*T22I-digested genomic DNA using neo, 5′, and 3′ probes, respectively. The 5′ and 3′ probes were prepared by PCR with primers 5′-TGAATACTGGGAACAGATGA-3′ and 5′-ACTGAAATG GAAGGGAGGCC-3′ and with primers 5′-CAAGGTCCCTACAATTGGCT-3′ and 5′-ACATACCTAGAACACAGGCT-3′ using BAC clone RP23-232J20 as a template, respectively. Recombinant ES cells were injected into eight-cell stage embryo of CD-1 mouse strain. Resulting chimeric mice were mated to B6-Tg(CAG-FLPe)36 mice of the C57BL/6 strain[27] to eliminate the *neo* gene from the genome through *Flp*/*frt*-mediated excision. Striatum-specific D1R and D2R knockout mice were generated by crossing $Drd1a^{+/flox}$ and $Drd2^{+/flox}$ mice with $Gng7^{+/cre}$ mice[19], respectively. Littermates derived from $Drd1a^{flox/flox}$ and $Drd1a^{flox/flox}$; $Gng7^{+/cre}$ mice, and from $Drd2^{flox/flox}$ and $Drd2^{flox/flox}$; $Gng7^{+/cre}$ mice on the pure C57BL6 genetic background were used for subsequent studies, respectively. The $Drd1a^{flox}$, the $Drd2^{flox}$, and the $Gng7^{+/cre}$ alleles were identified by PCR with primers 5′-CACTCTGCCTGTCAA GCTCAGC-3′ and 5′-CCTGTCTGAGGAAGCCCAGCTC-3′, with primers 5′-CTATATGATCCTCACAGCAG-3′ and 5′-GGAAAGGGCTACAGCATGG-3′, and with primers 5′-TATAGGTACCCAGAAGTGAATTCGGTTCGC-3′, 5′-GGCGACGTTGTTAGTACCTGAC-3′ and 5′- ATCCCTGAACATGTCCAT CAGGTTC-3′, respectively. Subsequent analyses were carried out with 8-week-old mice unless otherwise specified. Breeding and maintenance of mice were carried out under institutional guidelines. Mice were fed with food pellets on the floor *ad libitum* with standard laboratory chow and water in standard animal cages under a 12 h light/dark cycle. All animal procedures were approved by the Animal Care and the Use Committee of Graduate School of Medicine, the University of Tokyo (Approval #1721T062).

Histochemistry. Under deep pentobarbital anaesthesia (100 mg/g of body weight, i.p.), mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Sections (50 μm in thickness) were prepared with a microslicer (VT1000S, Leica Microsystems, Wetzlar, Germany). After blocking with 10% normal goat serum, sections were incubated with guinea pig anti-D1R and rabbit anti-D2R antibodies[55] at 4°C over night, and incubated with Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) at room temperature for 2 h. Stained samples were mounted in slides using Vectashield mounting medium containing DAPI (H-1500; Vector Laboratories, Burlingame, CA). Photographs were taken by a fluorescence stereomicroscope (M165FC; Leica Microsystems).

Immunoblot. Homogenates of mouse cerebral cortex, hippocampus and striatum were prepared essentially as described[56]. Proteins (3 μg per lane) were separated by SDS-PAGE and analysed by immunoblot with guinea piganti-D1R, rabbit anti-D2R or rabbit anti-actin (Sigma) antibody. The signals were analysed quantitatively by LAS-4000 mini image analyzer using ImageQuant TL software (GE Healthcare, Buckinghamshire, UK) and normalized against the signals for actin.

RT-PCR. Total RNAs from each brain sample were prepared using TRIzol (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with LightCycler 480 (Roche Diagnostics, Mannheim, Germany) and EXPRESS Two-Step SYBR GreenER (Invitrogen) using primers 5′-ATCGTC ACTTACACCAGTATCTACAGGA-3′ and 5′-GTGGTCTGGCAGTTCTTGGC-3′ for *Drd1a*, 5′-CTGGAGAGGCAGAACTGGAG-3′ and 5′-TAGACGACCCAG GGCATAAC-3′ for *Drd2*, and 5′-CATGGCCTTCCGTGTTCCTA-3′ and 5′-GCGGCACGTCAGATCCA-3′ for *Gapdh*. Thermocycling parameterswere as follows: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amounts of the *Drd1a and Drd2* mRNAs were normalized with the amount of the *Gapdh* mRNA as an internal standard.

Contextual fear conditioning. Mice were housed individually for 1 week before behavioural testing and were handled for 30 s everyday. A computer-controlled fear conditioning system (CL-M2; O'Hara and Co., Tokyo, Japan) was used in the fear conditioning experiments. A clear conditioning chamber (10 × 10 × 10 cm) with polyvinyl chloride boards and a stainless steel rod floor that was composed of 14 stainless steel rods (2 mm in diameter spaced 7 mm apart) was surrounded by a sound-attenuating white chest (74 lux). The conditioning chamber was cleaned with 70% ethanol between sessions. Masking noise of 52 dB was provided by a ventilation fan. Mice were placed in the conditioning chamber for 1 or 3 min and then given a scrambled electrical footshock (0.3, 0.5 or 1.0 mA for 1 s). Freezing responses were monitored for 1 min more after the foot shock, and then the animals were returned to their home cages. On the pretest and test day, mice were placed in the conditioning chamber and freezing was scored for 3 or 6 min. Pretests and tests day were performed 10 min and 24 h later after conditioning day, respectively. The test chamber was cleaned with benzalkonium (Ecolab Inc., St. Paul, MN) between tests. All behaviours were monitored by a CCD camera (WAT-902B; Watec Co. Ltd., Yamagata, Japan) attached to the ceiling of the chest. Images were captured at a rate of two frames per second and freezing behaviour was automatically analysed as an index of fear using IMAGE FZC software (O'Hara and Co.). Freezing behaviour was defined as the absence of any visible movement of the body and vibrissae except for movement necessitated by respiration. Freezing time was summated and the percentage of freezing was calculated per minute.

Statistics. Data are expressed as mean ± SEM. The statistics significance was evaluated using Student's *t* test or repeated measures ANOVA. When the interaction was significant, Tukey's test was employed. The criterion for statistical significance was $p < 0.05$.

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Author contributions

M.I., T.U., A.K. and M.M. designed the study. M.I., T.U. and A.K. performed experiments. K.S. provided research tools. M.I. and A.K. analysed the data. M.I., T.U., A.K. and M.M. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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