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ORIGINAL ARTICLE

Dopamine D1 receptor subtype mediates acute stress-induced dendritic growth in excitatory neurons of the medial prefrontal cortex and contributes to suppression of stress susceptibility in mice

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Dopamine in prefrontal cortices is implicated in cognitive and emotional functions, and the dysfunction of prefrontal dopamine has been associated with cognitive and emotional deficits in mental illnesses. These findings have led to clinical trials of dopamine-targeting drugs and brain imaging of dopamine receptors in patients with mental illnesses. Rodent studies have suggested that dopaminergic pathway projecting to the medial prefrontal cortex (mPFC) suppresses stress susceptibility. Although various types of mPFC neurons express several dopamine receptor subtypes, previous studies neither isolated a role of dopamine receptor subtype nor identified the site of its action in mPFC. Using social defeat stress (SDS) in mice, here we identified a role of dopamine D1 receptor subtype in mPFC excitatory neurons in suppressing stress susceptibility. Repeated social defeat stress (R-SDS) reduces the expression of D1 receptor subtype in mPFC of mice susceptible to R-SDS. Knockdown of D1 receptor subtype in whole neuronal populations or excitatory neurons in mPFC facilitates the induction of social avoidance by SDS. Single social defeat stress (S-SDS) induces D1 receptor-mediated extracellular signal-regulated kinase phosphorylation and c-Fos expression in mPFC neurons. Whereas R-SDS reduces dendritic lengths of mPFC layer II/III pyramidal neurons, S-SDS increases arborization and spines of apical dendrites of these neurons in a D1 receptor-dependent manner. Collectively, our findings show that D1 receptor subtype and related signaling in mPFC excitatory neurons mediate acute stress-induced dendritic growth of these neurons and contribute to suppression of stress susceptibility. Therefore, we propose that D1 receptor-mediated dendritic growth in mPFC excitatory neurons suppresses stress susceptibility.

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INTRODUCTION

Severe or persistent stress often causes harmful consequences, including emotional and cognitive abnormalities as well as neural changes, that promote systemic diseases.[1,2] Epidemiologic studies have suggested that stress during development and in adulthood increases the risk and severity of mental illnesses, such as schizophrenia and mood disorders.[3,4] Rodent studies have revealed that dopamine in the medial prefrontal cortex (mPFC) has a crucial role in suppressing stress susceptibility. Social defeat stress (SDS) activates dopaminergic pathway projecting to mPFC, and repetition of this stress attenuates dopaminergic function in mPFC,[5] leading to social avoidance. Pharmacological ablation[5] or optogenetic inhibition[6] of this dopaminergic pathway facilitates the induction of social avoidance by SDS in mice. Further, transgenic mice with neuronal expression of mutant DISC-1, a risk factor for major mental illnesses, show social isolation-induced deficit in the dopaminergic pathway projecting to mPFC,[7] suggesting reduced dopaminergic function in the mPFC associated with gene–environment interaction for mental illnesses. Therefore, dopaminergic signaling in the mPFC may represent an attractive target for the development of drugs and functional biomarkers for stress-related pathophysiology in mental illnesses.

Dopamine exerts its functions through multiple dopamine receptor subtypes, namely, D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors.[8] Pharmacological studies have implicated roles of D1-like receptors in prefrontal cortices in cognitive functions, such as working memory and cognitive flexibility, in both primates[9,10] and rodents.[11,12] These studies have led to pharmaceutical development of D1-like receptor agonists to normalize cognitive dysfunction in mental illnesses.[13] In addition, positron emission tomography (PET) studies have shown that reduced binding potential of D1-like receptors in the prefrontal cortices is correlated to the levels of cognitive deficits and negative symptoms of schizophrenia.[14] However, the therapeutic

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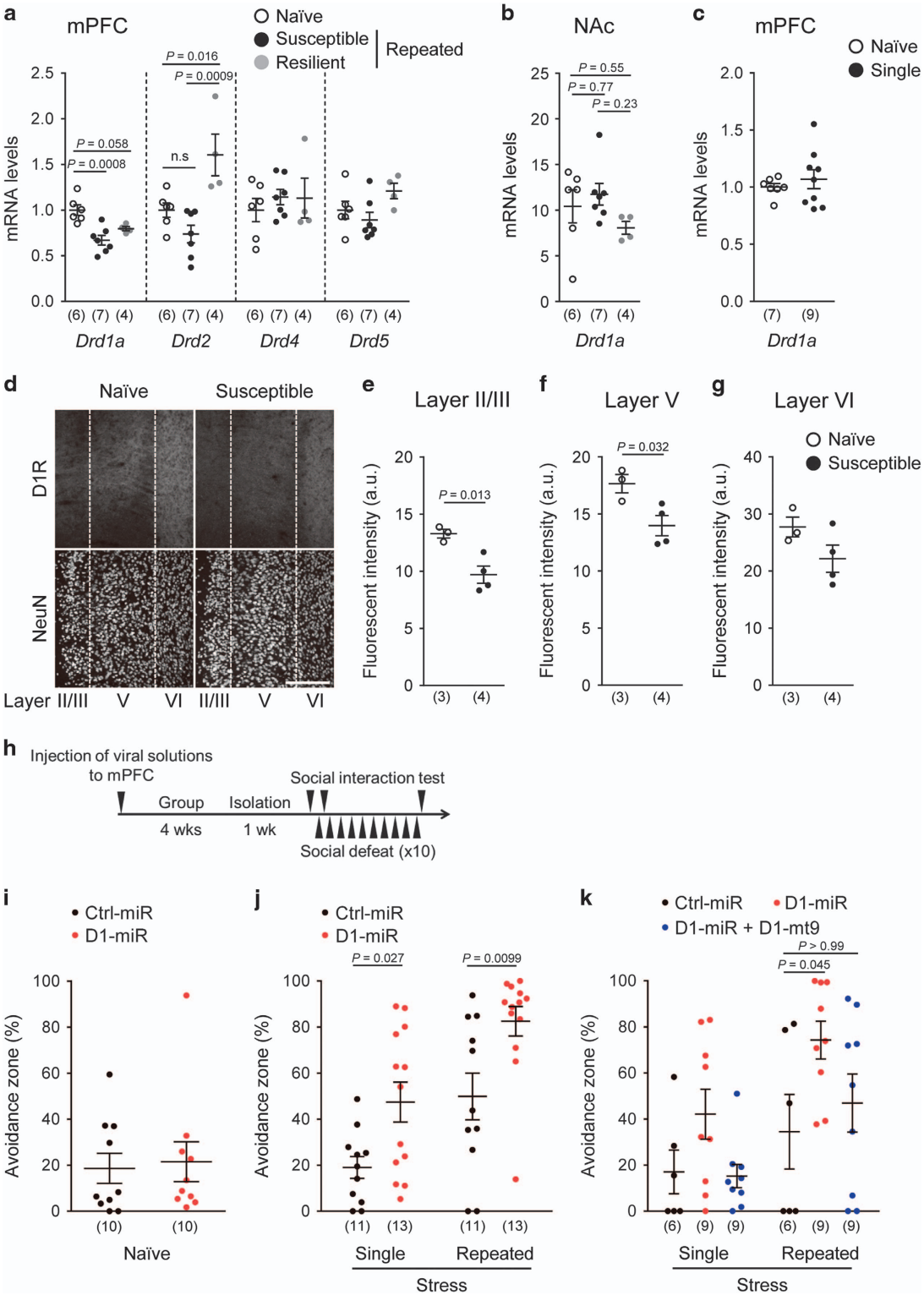


effects of D1-like receptor agonists are varied among different measures of cognitive functions,[15,16] and the altered D1-like receptor binding in schizophrenic patients described above is not consistently found across previous studies.[17,18] Rodent studies have shown that pharmacological manipulation of D1-like receptors in the mPFC affects spatial working memory and cognitive flexibility in different dose–effect relationships,[19] suggesting that dopamine receptor subtypes in mPFC mediate multiple cognitive functions through distinct mechanisms. It should be noted that several dopamine receptor subtypes, including D1 and D5, are expressed in various types of neurons in the mPFC.[20–22] Owing to lack of subtype-selective pharmacological probes,[23] pharmacological studies cannot isolate a role of each receptor subtype. Pharmacological studies cannot

Figure 1. Dopamine D1 receptor subtype in medial prefrontal cortex (mPFC) neurons contributes to suppression of repeated social defeat stress-(R-SDS) induced social avoidance. (**a**) mRNA levels of D1 (*Drd1a*), D2 (*Drd2*), D4 (*Drd4*) and D5 (*Drd5*) subtypes of dopamine receptors in mPFC tissues obtained from naive, susceptible and resilient mice. The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Tukey's *post hoc* test following one-way analysis of variance (ANOVA; for *Drd1a*, $F_{(2,\ 14)} = 11.44$, $P = 0.0011$; for *Drd2*, $F_{(2,\ 14)} = 11.25$, $P = 0.0012$; for *Drd4*, $F_{(2,\ 14)} = 0.3839$, $P = 0.6881$; for *Drd5*, $F_{(2,\ 14)} = 2.718$, $P = 0.1006$). Results are shown as mean ± s.e.m. (**b**) mRNA levels of D1 receptor subtype (*Drd1a*) in nucleus accumbens (NAc) tissues obtained from naive, susceptible and resilient mice. The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Tukey's *post hoc* test following one-way ANOVA ($F_{(2,\ 14)} = 1.471$, $P = 0.2631$). Results are shown as mean ± s.e.m. (**c**) mRNA levels of D1 receptor subtype (*Drd1a*) in mPFC tissues obtained from mice without or with single social defeat stress (S-SDS) (Naive or Single, respectively). The numbers of mice in respective groups are shown below bars. Results are shown as mean ± s.e.m. (**d**) Representative images of immunofluorescent staining for D1 receptor subtype and NeuN, a neuronal marker, in mPFC of naive and susceptible mice. Dashed lines indicate the boundaries of respective layers. Scale bar, 200 μm. (**e**–**g**) Quantification of the immunofluorescent signals for D1 receptor subtype in the layer II/III (**e**), layer V (**f**) and layer VI (**g**) in mPFC of naive and susceptible mice, as shown in panel (**d**). The numbers of mice in respective groups are shown below bars. *P*-values shown in the graphs were calculated by unpaired *t*-test. Results are shown as the mean ± s.e.m. (**h**) A schedule of behavioral experiments to test the effect of D1 knockdown in mPFC neurons on social interaction without stress (naive) or after S-SDS or R-SDS. Four weeks after injection of adeno-associated virus (AAV) solutions to mPFC, the mice were isolated for a week and then were subjected to S-SDS or R-SDS followed by the social interaction test. For naive mice, the mice that received AAV injection to mPFC were isolated for a week and then were subjected to the social interaction test without SDS. (**i**) The effect of D1 knockdown in mPFC neurons on social avoidance without prior stress. Mice without or with D1 knockdown in mPFC neurons (Ctrl-miR mice or D1-miR mice, respectively) were subjected to the social interaction test without stress (Naive), and the proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in the respective groups are shown below bars. Results are shown as mean ± s.e.m. (**j**) The effect of D1 knockdown in mPFC neurons on the induction of social avoidance by SDS. Ctrl-miR mice or D1-miR mice were subjected to S-SDS or R-SDS, and the proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in the respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way repeated-measure ANOVA. ($F_{(1,\ 22)} = 10.44$, $P = 0.0038$ for the main D1 knockdown effect). Results are shown as mean ± s.e.m. (**k**) The lack of the behavioral effect of D1 knockdown in mPFC neurons in the presence of D1 microRNA (miRNA)-resistant D1 mutant. Mice without D1 knockdown (Ctrl-miR mice) or mice with D1 knockdown in mPFC neurons in the absence (D1-miR mice) or presence (D1 miRNA+D1-mt9) of D1 miRNA-resistant D1 mutant were subjected to S-SDS or R-SDS. The proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in the respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way repeated-measure ANOVA ($F_{(2,\ 21)} = 3.617$, $P = 0.0447$ for the main D1 knockdown effect). Results are shown as mean ± s.e.m. NS, not significant.

manipulate dopamine receptor subtypes in a specific neuronal population in the mPFC that mediates each cognitive function either.

To identify a subtype-specific role, systemic knockout mice of each dopamine receptor subtype have frequently been used.[24] However, this technique cannot identify the site of action of dopamine receptor subtypes and cannot rule out a possible role of dopamine receptor subtypes during brain development.[25] To overcome these issues, conditional knockout mice of dopamine receptor subtypes using mice expressing Cre recombinase in a specific brain region and cell type of interest have been developed and used for behavioral studies.[26,27] However, mice expressing Cre recombinase specifically in the mPFC are not available so far. The advent of recombinant viral vectors with high infection efficiency to various types of neurons offers a novel strategy to manipulate gene expression in any specific brain region of interest,[28] including mPFC. In the present study, using mPFC injection of recombinant viral vectors combined with mice expressing Cre recombinase specifically in excitatory or inhibitory neurons or with a viral vector expressing Cre recombinase specifically in mPFC excitatory neurons, we successfully manipulated expression of D1 receptor subtype selectively in each of these neurons in the mPFC and revealed that D1 receptor subtype and related signaling in mPFC excitatory neurons contribute to suppression of susceptibility to repeated social defeat stress (R-SDS). We also found the evidence suggesting that single social defeat stress (S-SDS) increases dendritic arborization and dendritic spines through activation of D1 receptor subtype. To the best of our knowledge, these morphological changes of mPFC excitatory neurons are a novel neuronal correlate of suppressing stress susceptibility through D1 receptor signaling in the mPFC.

MATERIALS AND METHODS

All procedures for animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Kyoto University Graduate School of Medicine and Kobe University Graduate School of Medicine. Materials and methods in detail are described in Supplementary Materials and Methods.

SDS and social interaction test

SDS and social interaction test were performed as previously described[29] with minor modifications.[5] In this experiment, male C57BL/6N mice aged 13–14 weeks, that had received adeno-associated virus (AAV) injection to the mPFC at the age of 8 weeks were used for all experiments, except in Figures 1a–c and 3d and e and Supplementary Figure 1, in which the mice aged 8–9 weeks that had not received AAV injection were used. C57BL/6N mice aged 8–9 weeks and 13–14 weeks showed similar degrees of social avoidance after R-SDS. After isolation for a week, these mice were put to the home cage of a male ICR mouse for 10 min daily for every day in the 10 day defeat. After the 10-min defeat episode, the mice were returned to the home cages and kept isolated without any contact to the ICR mice until SDS on the next day.

The social interaction test was performed 18 h after the first (S-SDS) and tenth exposure to SDS (R-SDS). The second exposure to SDS was applied about 2 h after the first social interaction test. Behaviors in the open field chamber with a novel male ICR mouse enclosed in a metal meshwork were recorded. The proportions of the time that each mouse spent in the social avoidance zone and the social interaction zone (Supplementary Figure 1a) were measured *post hoc*. Stress-induced social avoidance from an ICR mouse can be detected as an increase in the time for the social avoidance zone and a decrease in the time for the social interaction zone. As R-SDS induces social avoidance in only half of the defeated mice,[30] we chose susceptible and resilient mice as follows: Susceptible mice refer to those in which the durations for both the interaction zone and the avoidance zone were beyond respective s.d. from their averages of naive mice. Resilient mice refer to those in which the durations for both the interaction zone and the avoidance zone were kept within respective s.d. from their averages of naive mice.

Viral vectors

The AAVs generated in this study are as follows: AAV (serotype 2/rh10) expressing artificial microRNA (miRNA) targeting dopamine D1 receptor or control miRNA with Emerald Green Fluorescent Protein (EmGFP) only in the presence of Cre recombinase (AAV-DIO-EmGFP-D1miR and AAV-DIO-EmGFP-CtrlmiR, respectively); AAV2/rh10 expressing D1 miRNA-resistant D1 receptor mutant fused with FLAG tag at its N terminus (FLAG-D1-mt9)

only in the presence of Cre recombinase (AAV-DIO-D1-mt9); AAV2/rh10 expressing Cre recombinase fused with nuclear localization signal (NLS) at its N terminus (NLSCre) only in the presence of Cre recombinase (AAV-DIO-Cre); and AAV2/rh10 expressing NLSCre under the CaMKIIα promoter (AAV-CaMKIIα-Cre). AAV2/rh10 particles were produced, as previously described[31] with minor modifications. A lentiviral vector expressing mCherry only in the presence of Cre recombinase (Lenti-DIO-mCherry) was produced, as described previously.[32]

Stereotaxic surgery for viral injection to the mPFC

Stereotaxic surgeries for viral injection to mPFC were performed as previously described[5] with minor modifications. The stereotaxic coordinates were targeted to the ventral portion of mPFC (1.8 mm anterior from the bregma, 0.4 mm lateral from the midline and 2.8 mm ventral from the skull surface at the bregma) based on the Paxinos and Franklin mouse brain atlas.[33] Mice were recovered for 4 weeks after the surgery, during which the expression of miRNA or a protein of interest was fully induced before behavioral experiments.

Immunofluorescent staining

Immunofluorescent staining in brain sections and AAV293 cells, except for analyses of dendritic morphologies (shown below), was performed as previously described[34,35] with minor modifications.

Analysis of dendritic morphology of mPFC neurons

For morphometric analyses of mPFC neurons, coronal brain sections of 100 μm thickness were made by cryostat (Leica, Wetzlar, Germany). Layer V/VI was identified by the presence of Ctip2-positive neurons, and mPFC pyramidal neurons at layer II/III or layer V/VI were separately analyzed. Semiautomated tracing of dendrites was performed, and the lengths and branching points of dendrites were measured, using the IMARIS software (Bitplane, Zurich, Switzerland). The Sholl analysis was also performed as previously described[36] with minor modifications. As the depth of the cell body from the midline varied considerably across neurons, the interval of the concentric circles was normalized to the depth of the cell body for each neuron.

Spine classification was determined by the parameters of the spine length and the diameter of spine neck and head. Spines with head/neck diameter ratios >1.7 were classified as 'Type 1' spines, and the remains were classified as 'Type 2' spines. Within spines with head/neck diameter ratio >1.2, spines with the spine length <3 μm and the spine head >0.6 μm were categorized into mushroom spines, and the remains were categorized into thin spines. Within spine with head/neck diameter ratio no more than 1.2, spines with the spine length <1 μm were categorized into stubby spines, and the remains were into filopodia. This classification of mushroom spines, thin spines, stubby spines and filopodia was performed as previously described[37] with minor modifications.

RNA extraction and quantitative reverse transcriptase PCR (RT-PCR)

RNA extraction from brain tissues and quantitative RT-PCR were performed as previously described[30] with minor modifications.

Statistical analysis

All data are shown as mean ± s.e.m. Statistical analyses were performed using unpaired two-tailed Student's *t*-test, one-way analysis of variance followed by Tukey's *post hoc* test and two-way analysis of variance followed by Bonferroni's *post hoc* test, as described in the figure legends. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Repeated SDS reduces the expression of dopamine D1 receptor subtype in the mPFC

As dopaminergic projections in mPFC are critical for suppressing the induction of social avoidance by SDS,[5] we examined whether R-SDS alters the mRNA levels of dopamine receptor subtypes in mPFC in a manner correlated to social avoidance. For this purpose, we punched out mPFC tissues after R-SDS or without stress (naive mice) and measured the mRNA levels of five subtypes of dopamine receptors in mPFC. Quantitative RT-PCR analyses detected the mRNA expression levels of D1, D2, D4 and D5 receptor subtypes (Figure 1a) but not of D3 receptor subtype (data not shown). R-SDS reduced the mRNA expression of D1 receptor subtype in susceptible mice, and this reduction was smaller in resilient mice (Figure 1a). Consistently, immunofluorescent signals for D1 receptor subtype were significantly reduced in mPFC layer II/III and layer V of susceptible mice compared with naive mice (Figures 1d–g). In contrast to R-SDS, S-SDS did not alter the mRNA expression of D1 receptor subtype in mPFC (Figure 1c). R-SDS did not significantly change the mRNA expression of D2 receptor subtype in susceptible mice, although D2 mRNA was significantly elevated in resilient mice (Figure 1a). mRNA expression levels of D4 and D5 receptor subtypes were not significantly changed in either susceptible or resilient mice (Figure 1a). We also measured the mRNA levels of D1 receptor subtype in the nucleus accumbens, a brain region that is also involved in the induction of social avoidance by R-SDS.[30] In contrast to mPFC, R-SDS did not significantly change the mRNA expression of D1 receptor subtype in the nucleus accumbens (Figure 1b).

D1 receptor subtype in mPFC neurons contributes to suppression of the induction of social avoidance by SDS

To examine a role of D1 receptor subtype in R-SDS, we generated AAV (AAV2/rh10) expressing artificial microRNA targeting D1 receptor subtype (D1 miRNA) together with EmGFP under the constitutive EF1α promoter (Supplementary Figure 2a). To achieve neuron type-specific expression of D1 miRNA later, AAV was designed to enable the expression of D1 miRNA and EmGFP only in the presence of Cre recombinase (AAV-DIO-EmGFP-D1miR). To express D1 miRNA and EmGFP in all types of mPFC neurons, we injected this viral vector to mPFC with AAV expressing Cre recombinase under the constitutive CMV promoter (AAV-Cre) (Supplementary Figures 2b and c). Immunofluorescent staining for NeuN, a marker for neurons, showed that EmGFP is expressed selectively in the neurons in mPFC (Supplementary Figure 2d). This neuronal selectivity is probably due to the preference of this AAV serotype for infecting neurons.[31] mRNA expression of D1 receptor subtype, but not of the other subtypes, was reduced with AAV expressing D1 miRNA in the mPFC, compared with AAV expressing negative control miRNA (Supplementary Figure 2e). Immunofluorescent signals for D1 receptor subtype in mPFC were decreased with D1 miRNA, compared with the signals with control miRNA (Supplementary Figures 4d and e). These findings confirmed that the expression of D1 miRNA induces knockdown of D1 receptor subtype.

We then examined whether knockdown of D1 receptor subtype in mPFC neurons could affect the induction of social avoidance by SDS (Figure 1h). Without SDS, either male mice expressing control miRNA in mPFC neurons (Ctrl-miR mice) or male mice expressing D1 miRNA in mPFC (D1-miR mice) similarly showed social interaction with a novel ICR mouse enclosed in a metal meshwork (Figure 1i and Supplementary Figure 3a), suggesting that D1 receptor is not involved in social interaction without prior stress. In contrast, D1-miR mice showed the facilitated induction of social avoidance by SDS, compared with Ctrl-miR mice (Figure 1j and Supplementary Figure 3b). On the other hand, Ctrl-miR and D1-miR mice showed similar durations of submissive posture, a behavioral sign of social defeat,[38] during the first and tenth SDS (Supplementary Figure 3c). Therefore, D1 receptor in mPFC neurons is not critical for the perception of SDS.

To exclude a potential off-target effect of D1 miRNA, we generated a D1 receptor mutant with nine synonymous substitutions in DNA sequence named D1-mt9, which are expected to encode the same amino-acid sequence as wild-type D1 receptor subtype (Supplementary Figure 4a). In cell lines, D1 miRNA reduced immunofluorescent signals for overexpressed wild-type

D1 receptor subtype but not the signals for the D1-mt9 mutant (Supplementary Figures 4b and c). These findings indicate that the D1-mt9 mutant is resistant to D1 miRNA. We then injected AAV expressing the D1-mt9 mutant (AAV-DIO-D1-mt9) with AAV-DIO-EmGFP-D1miR and AAV-Cre and examined the level of social avoidance induced by SDS. Simultaneous expression of the D1-mt9 mutant restored D1 receptor expression (Supplementary Figures 4d and e) and normalized the level of social avoidance induced by SDS even in the presence of D1 miRNA in mPFC neurons (Figure 1k and Supplementary Figure 3d).

Taken together, these results indicate that D1 receptor subtype in mPFC neurons contributes to suppression of the induction of social avoidance by SDS.

mPFC excitatory neurons are the site of action of D1 receptor subtype in suppressing the induction of social avoidance by SDS

Given that D1 receptor subtype is expressed in both excitatory and inhibitory neurons in the mPFC,[20] we performed neuron type-specific knockdown of D1 receptor subtype. To generate male mice expressing D1 miRNA selectively in mPFC excitatory neurons (CaMKIIα-D1-KD mice), we injected AAV-DIO-EmGFP-D1miR to mPFC of CaMKIIα-CreERT2 mice (Figure 2a), which express tamoxifen-inducible Cre recombinase (CreERT2) under the CaMKIIα promoter.[39] We amplified Cre expression by simultaneously injecting AAV expressing Cre recombinase only in the presence of Cre recombinase (AAV-DIO-Cre) (see Supplementary Materials and Methods for the reason why we had to amplify Cre expression). After postsurgical recovery for 3 weeks, tamoxifen was administrated for 5 consecutive days to induce Cre-mediated recombination. EmGFP (simultaneously expressed with D1 miRNA) was induced in at least half of the mPFC neurons (Supplementary Figure 5, right) and was largely restricted to CaMKIIα-expressing excitatory neurons in the mPFC (Figure 2b, lower panels). As a negative control, AAV-DIO-EmGFP-D1miR and AAV-DIO-Cre were injected to mPFC of wild-type littermates. EmGFP was not detectable in these control mice (Figure 2b, upper panels). CaMKIIα-D1-KD mice showed the facilitated induction of social avoidance by S-SDS, compared with the control mice (Figure 2c and Supplementary Figure 3e).

To equalize Cre expression and its potential toxicity in the comparison groups, we performed mPFC injection of AAV-DIO-EmGFP-D1miR or AAV expressing control miRNA only in the presence of Cre recombinase (AAV-DIO-EmGFP-CtrlmiR) with AAV expressing Cre recombinase under the CaMKIIα promoter (AAV-CaMKIIα-Cre) (Figure 2d). This experiment showed that knockdown of D1 receptor subtype, rather than Cre expression, selectively in mPFC excitatory neurons facilitates the induction of social avoidance by SDS (Figure 2e and Supplementary Figure 3f). In this experiment, the facilitated induction of social avoidance was observed after R-SDS. This effect was not evident in CaMKIIα-D1-KD mice after R-SDS in Figure 2c, as intraperitoneal injection of tamoxifen appears to delay the induction of social avoidance in both CaMKIIα-D1-KD mice and their control mice, compared with Figure 2e. By contrast, the facilitated induction of social avoidance after S-SDS was observed in CaMKIIα-D1-KD mice in Figure 2c, whereas this effect was not evident in the experiment with AAV-CaMKIIα-Cre in Figure 2e, perhaps due to a low expression level of D1 miRNA induced by Cre recombinase. Despite these subtle differences, both of the two experiments described above suggest that mPFC excitatory neurons are the site of action of D1 receptor subtype for contributing to suppressing stress susceptibility.

We next generated mice expressing D1 miRNA selectively in mPFC inhibitory neurons (Viaat-D1-KD mice) by injecting AAV-DIO-EmGFP-D1miR to mPFC of Viaat-Cre mice (Figure 2d), which express Cre recombinase under the vesicular inhibitory amino-acid transporter (Viaat) promoter.[40] EmGFP fluorescent signals were restricted to GABA-positive and GAD67-positive neurons in Viaat-Cre mice (Figure 2f). The signal intensities in individual EmGFP-expressing neurons were comparable to those in CaMKIIα-D1-KD and D1-miR mice (see Figure 2b and Supplementary Figure 2d). In contrast to CaMKIIα-D1-KD mice, the response to SDS of Viaat-D1-KD mice was comparable with the control mice (Figure 2g and Supplementary Figure 3g).

S-SDS activates dopamine D1 receptor signaling in mPFC excitatory neurons

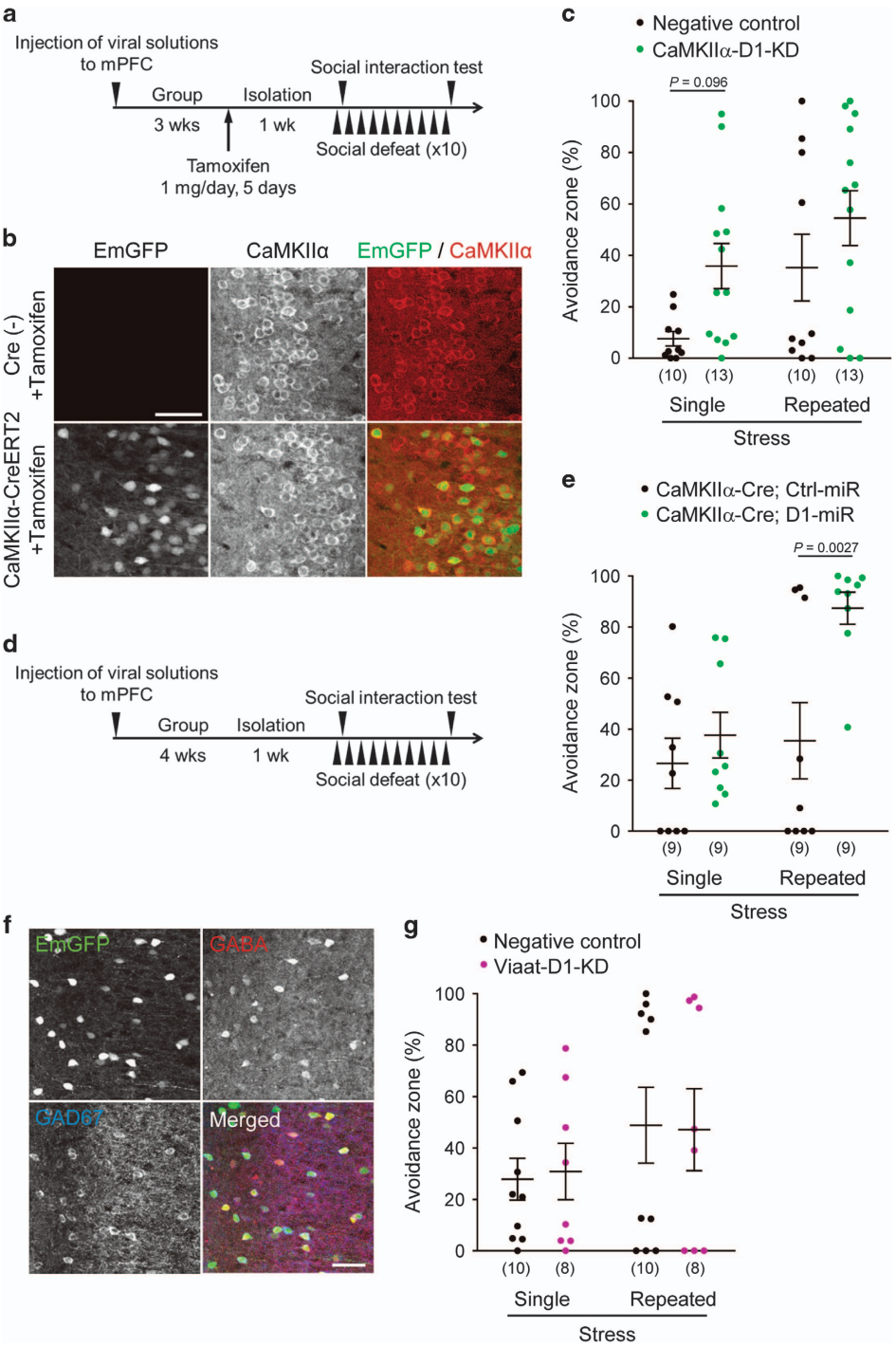
As phosphorylation of extracellular signal-regulated kinase (ERK) is coupled to dopamine receptor activation in many brain areas,[41,42] we examined whether SDS induces ERK phosphorylation in mPFC neurons through D1 receptor subtype. We first examined the effect of systemic injection of a D1-like receptor agonist SKF81297 on ERK phosphorylation in mPFC neurons. Ten minutes after intraperitoneal injection of SKF81297 or saline, the mice were killed for immunostaining for phosphorylated ERK (pERK) and NeuN. The injection of SKF81297 increased not only the intensity of pERK in neurons but also the proportion of pERK-positive neurons in multiple layers of mPFC compared with injection of saline (Supplementary Figure 6), suggesting that the activation of D1-like receptors induces ERK phosphorylation in mPFC neurons.

We next examined whether SDS induces D1 receptor-dependent ERK phosphorylation in mPFC neurons. Immediately after S-SDS, Ctrl-miR and D1-miR mice were killed for immunostaining for pERK and NeuN (Supplementary Figure 7). In Ctrl-miR mice, S-SDS increased the intensity of pERK in mPFC neurons (Figures 3a and b) and the proportion of pERK-positive mPFC neurons (Figure 3c). These changes induced by S-SDS were attenuated in mPFC neurons in D1-miR mice (Figures 3a–c). Double immunostaining with CaMKIIα or GABA, a marker for excitatory or inhibitory neurons, respectively, showed that S-SDS significantly increases the number of pERK- and CaMKIIα-positive excitatory neurons in the mPFC, and pERK was rarely observed in GABA-positive inhibitory neurons without or with the stress (Figures 3d and e). D1 knockdown in mPFC neurons attenuated S-SDS-induced increase in the number of pERK- and CaMKIIα-positive excitatory neurons (Figure 3f). These results indicate that S-SDS induces ERK phosphorylation selectively in mPFC excitatory neurons at least partly through D1 receptor subtype.

We also examined whether SDS induces D1 receptor-dependent expression of c-Fos and Zif268, immediate early genes which have frequently been used as neuronal activation markers (for example, Jungenitz *et al.*[43]), in mPFC neurons. Ninety minutes after S-SDS, the mice were killed for immunostaining for c-Fos or Zif268 (Supplementary Figures 8 and 9). In Ctrl-miR mice, S-SDS slightly increased the number of c-Fos-positive neurons in mPFC layer II/III (Figures 3g and h), and this increase was less evident in mPFC layer V/VI neurons (Figures 3g and i). In D1-miR mice, the induction of c-Fos expression by S-SDS was abolished in mPFC layer II/III neurons (Figures 3g and h), suggesting that D1 receptor subtype and related signaling contribute to the response of these neurons to S-SDS. The number of Zif268-positive mPFC neurons was not affected by either S-SDS or D1 knockdown in mPFC neurons (Supplementary Figure 9).

S-SDS increases dendritic arborization and spine density selectively in apical dendrites of mPFC layer II/III pyramidal neurons in a D1 receptor-dependent manner

Repeated restraint stress and chronic mild stress induce dendritic shortening and reduced spine density in mPFC pyramidal neurons,[44,45] and these morphological changes are associated with stress-induced behavioral changes.[46–48] We examined whether such dendritic shortening could be induced by R-SDS and whether D1 receptor subtype could be involved in this process. To visualize dendritic morphology of mPFC pyramidal



neurons, we sparsely labeled these neurons by mPFC injection of low-titer lentivirus expressing mCherry in the presence of Cre recombinase (Lenti-DIO-mCherry) with AAV-Cre (Supplementary Figure 10). Ctrl-miR and D1-miR mice were subjected to R-SDS, and the level of social avoidance was measured at 18 h later. Then at 72 h after R-SDS, the mice were killed for morphological analyses of mPFC neurons (Figure 4a). R-SDS significantly reduced dendritic lengths, but not dendritic branching points or spine density, of apical dendrites of mPFC layer II/III pyramidal neurons in the susceptible group, but not the resilient group, of Ctrl-miR mice (Figures 4b and c and Supplementary Figures 11a–c), suggesting that R-SDS induces shortening of apical dendrites in mPFC layer II/III pyramidal neurons in a manner correlated with stress susceptibility. Such dendritic shortening appears to be much less in basal dendrites of mPFC layer II/III pyramidal neurons (Figures 4b and d) and apical and basal dendrites of mPFC layer V/VI pyramidal neurons (Supplementary Figures 12a and b). In D1-miR mice, the shortening of apical dendrites of mPFC layer II/III neurons was not potentiated (Supplementary Figure 13a and b), whereas the induction of social avoidance was facilitated (see Figure 1j), suggesting the dissociation between the dendritic shortening and the social avoidance in D1-miR mice. D1 knock-down in mPFC neurons did not apparently affect spine density of mPFC layer II/III pyramidal neurons (Supplementary Figures 11d–f)

Figure 2. Medial prefrontal cortex (mPFC) excitatory neurons are the site of action of D1 receptor subtype for contributing to suppression of repeated social defeat stress (R-SDS)-induced social avoidance. (**a**) A schedule of behavioral experiments to test the effect of D1 knockdown in mPFC excitatory neurons on social interaction after single social defeat stress (S-SDS) or R-SDS. Three weeks after injection of adeno-associated virus (AAV) solutions to mPFC, the mice were administered with 1 mg of tamoxifen daily for 5 days. The mice were isolated for a week and then were subjected to S-SDS or R-SDS followed by the social interaction test. (**b**) Representative images of Emerald Green Fluorescent Protein (EmGFP) fluorescence and immunofluorescent staining for CaMKIIα in mPFC without or with D1 knockdown ('Cre (−) +Tamoxifen' or 'CaMKIIα-CreERT2+Tamoxifen', respectively) in mPFC excitatory neurons. In the merged images, EmGFP and CaMKIIα are shown in green and red, respectively. Scale bar, 50 μm. (**c**) The effect of D1 knockdown in mPFC excitatory neurons on the induction of social avoidance by SDS. Mice without or with D1 knockdown in mPFC excitatory neurons (Negative control mice or CaMKIIα-D1-KD mice, respectively) were subjected to S-SDS or R-SDS, and the proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in the respective groups are shown below bars. A *P*-value shown in the graph was calculated by Bonferroni's *post hoc* test following two-way repeated-measure analysis of variance (ANOVA) ($F_{(1,\ 21)} = 4.348$, $P = 0.0495$ for the main D1 knockdown effect). Results are shown as mean ± s.e.m. (**d**) A schedule of behavioral experiments to test the effect of D1 knockdown in mPFC excitatory neuron using AAV-CaMKIIα-Cre (**e**) or mPFC inhibitory neurons using Viaat-Cre mice (**g**) on social interaction after S-SDS or R-SDS. Four weeks after injection of AAV solutions to mPFC, the mice were isolated for a week and then were subjected to S-SDS or R-SDS followed by the social interaction test. (**e**) The effect of D1 knockdown in mPFC excitatory neurons using AAV-CaMKIIα-Cre on the induction of social avoidance by SDS. Mice without or with D1 knockdown in mPFC excitatory neurons ('CaMKIIα-Cre; Ctrl-miR' or 'CaMKIIα-Cre; D1 miR', respectively) were subjected to S-SDS or R-SDS, and the proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in respective groups are shown below bars. A *P*-value shown in the graph was calculated by Bonferroni's *post hoc* test following two-way repeated-measure ANOVA ($F_{(1,\ 16)} = 6.637$, $P = 0.0203$ for the main D1 knockdown effect). Results are shown as mean ± s.e.m. (**f**) Representative images of EmGFP fluorescence and immunofluorescent staining for GABA and GAD67 in mPFC of a Viaat-Cre mouse that was injected with AAV-DIO-EmGFP-D1miR. In the merged image, EmGFP, GABA and GAD67 are shown in green, red and blue, respectively. Scale bar, 50 μm. (**g**) The effect of D1 knockdown in mPFC inhibitory neurons on the induction of social avoidance by SDS. Mice without or with D1 knockdown in mPFC inhibitory neurons (Negative control mice or Viaat-D1-KD mice, respectively) were subjected to S-SDS or R-SDS, and the proportion of time that each mouse spent in the avoidance zone was measured. The numbers of mice in respective groups are shown below bars. No significant changes are observed ($F_{(1,\ 16)} = 0.001491$, $P = 0.9697$ for the main D1 knockdown effect by two-way repeated-measure ANOVA). Results are shown as the mean ± s.e.m.

or the lengths of basal dendrites of mPFC layer II/III pyramidal neurons (Supplementary Figure 13c) or apical or basal dendrites of mPFC layer V/VI pyramidal neurons (Supplementary Figures 12c and d) either.

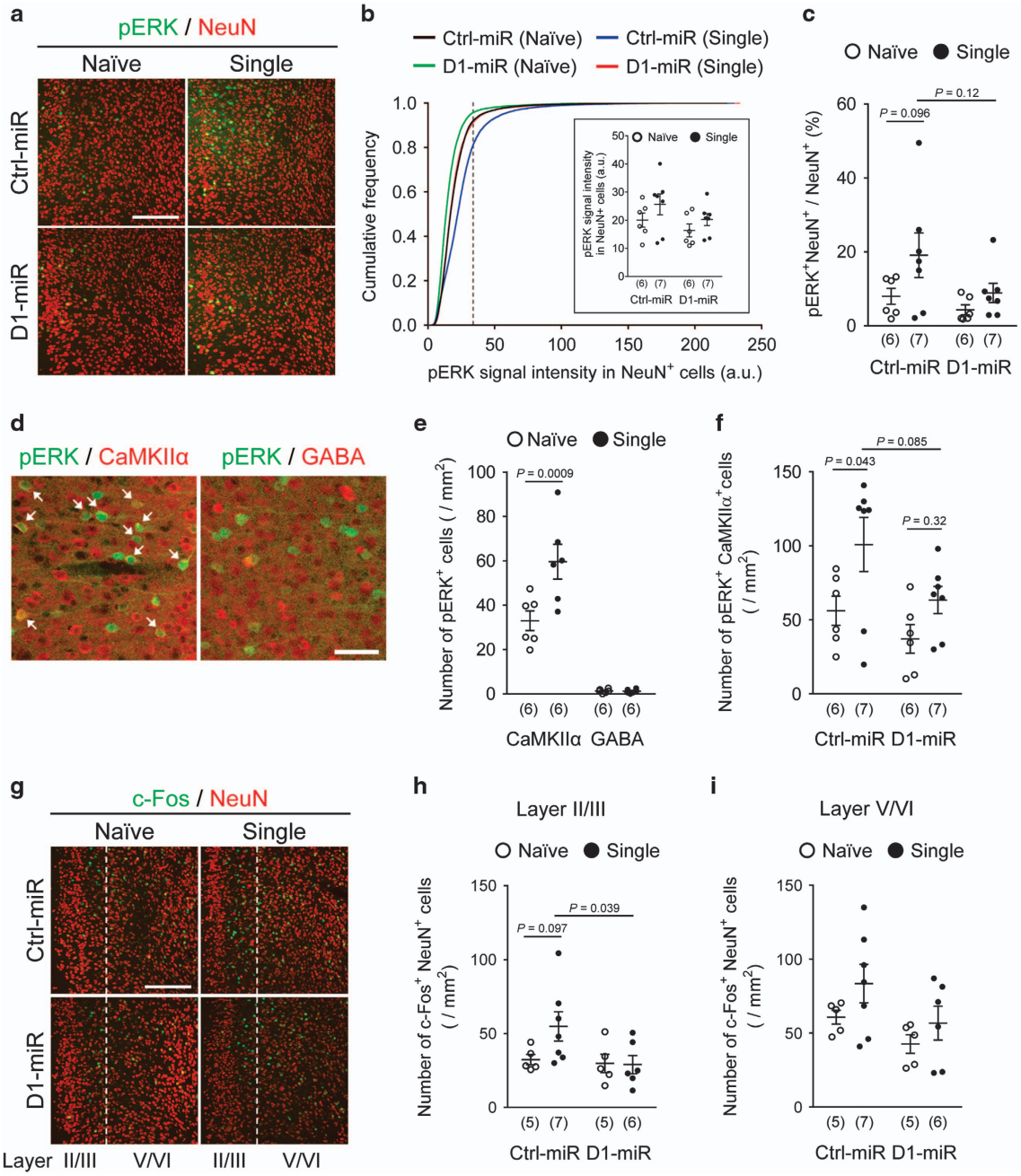
As D1 receptor signaling in mPFC layer II/III excitatory neurons is activated by S-SDS, we examined whether and how S-SDS could affect neuronal morphology of mPFC pyramidal neurons and whether D1 receptor subtype could be involved in this process. At 72 h after S-SDS, the mice were killed for morphological analyses of mPFC neurons (Figure 4e). S-SDS increased the lengths and branching points of apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice (Figures 4f–h). By contrast, these morphological changes were not seen in D1-miR mice, and the lengths and branching points of apical dendrites of these neurons were comparable to those of naive Ctrl-miR mice with or without prior stress (Figures 4f–h). Using the Sholl analysis, we analyzed in which region(s) of apical dendrites S-SDS induces dendritic growth through D1 receptor subtype. S-SDS appears to increase the complexity of apical dendrites within layer I and layer II/III in Ctrl-miR mice but not in D1-miR mice (Supplementary Figure 15a). By contrast, the morphological changes were not induced by S-SDS in basal dendrites of mPFC layer II/III pyramidal neurons, where D1 knockdown slightly increased the lengths and branching points of these dendrites without or with S-SDS (Supplementary Figures 14 and 15b). In mPFC layer V/VI pyramidal neurons of Ctrl-miR and D1-miR mice, S-SDS did not induce dendritic growth in either apical or basal dendrites (Supplementary Figure 16). We also examined whether S-SDS affects the density of dendritic spines of mPFC neurons in a manner dependent on D1 receptor subtype. S-SDS increased the density of dendritic spines on both distal and proximal apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice but not in D1-miR mice (Figures 5a–c). The increase in spine density after S-SDS was not evident on basal dendrites of mPFC II/III pyramidal neurons in either Ctrl-miR or D1-miR mice (Figure 5d).

We next examined whether and how S-SDS and D1 knockdown could affect the morphology of dendritic spines. S-SDS appears to slightly increase the diameter of spine neck in Ctrl-miR mice but not in D1-miR mice (Supplementary Figure 17a). In contrast, the diameter of spine head and the length of dendritic spine were less affected by S-SDS or knockdown of D1 receptor subtype (Supplementary Figures 17b and c). As there are several types of dendritic spines according to their morphologies,[49] we suspected that S-SDS might affect dendritic spines of a specific type through D1 receptor subtype. According to the diameters of spine head and neck, we were able to identify two evident clusters of dendritic spines: dendritic spines in which the diameter of spine head is larger than that of a spine neck and those with a head and a neck of similar diameters (Supplementary Figures 18a and b). For the purpose of statistical analyses, we separated these two clusters by classifying spines with head/neck diameter ratios > 1.7 as 'Type 1' spines and the remains as 'Type 2' spines. S-SDS significantly increased the density of Type 2 spines on distal apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice, whereas this increase was less apparent in Type 1 spines (Figures 5e and f). S-SDS-induced increase in the density of Type 2 spines were not observed in D1-miR mice (Figure 5f). We also performed a conventional classification of dendritic spines, in which dendritic spines have been classified to mushroom spine, thin spine, stubby spine and filopodia, and the latter two types of spines are prominent during the early stage of synaptic formation.[50] S-SDS significantly increased the densities of stubby spines and filopodia in a D1 receptor-dependent manner, and this increase was not evident in the other types of spines (Supplementary Figures 18c–f).

Collectively, these results show that S-SDS activates D1 receptor signaling and increases dendritic arborization and spine density, especially that of immature types of spines, selectively in apical dendrites of mPFC layer II/III pyramidal neurons, whereas the shortening of these dendrites induced by R-SDS is not potentiated by D1 knockdown in mPFC neurons.

DISCUSSION

Roles for D1-like receptors in prefrontal cortices have been extensively investigated in cognitive functions,[19,51,52] and impaired D1-like receptor signaling in the prefrontal cortices has been implicated in cognitive impairments associated with mental illnesses.[53] However, as these studies relied on drugs or ligands targeting D1-like receptors, they did not discriminate D1 and D5



receptor subtypes and did not identify the site of action of dopamine receptor subtypes in prefrontal cortices. Using mPFC- and neuron type-specific manipulation of gene expression, here we elucidated a novel role for D1 receptor subtype in mPFC excitatory neurons, that is, suppressing stress susceptibility in mice. This newly described function of D1 receptor subtype may underlie stress-related pathophysiology of various mental illnesses and represent an attractive target for the development of therapeutic drugs and biomarkers of molecular imaging in mental illnesses. Furthermore, we found that stress-evoked D1 receptor signaling increases dendritic arborization and dendritic spine density in mPFC excitatory neurons. This finding is, to the best of our knowledge, the first to demonstrate that D1 receptor signaling induces dendritic growth of mPFC neurons in adult brains and possibly exerts the long-lasting effect of D1 receptor signaling in the mPFC. We propose that these morphological changes of mPFC excitatory neurons mediate the effect of D1 receptor subtype in suppressing stress susceptibility.

Reduced expression of D1 receptor subtype in mPFC after R-SDS

R-SDS reduces the mRNA level of D1 receptor subtype, but not those of the other subtypes, in mPFC. As the immunoreactivity of D1 receptor subtype is reduced broadly in the mPFC of the susceptible mice compared with the naive mice, it is plausible that the protein level of D1 receptor subtype in mPFC is reduced after R-SDS. Given the reduced mRNA level of D1 receptor subtype, R-SDS could suppress its protein synthesis in mPFC neurons either through reduced mRNA synthesis or reduced mRNA stability or both. Although the mechanism underlying reduced expression of D1 receptor subtype in the mPFC remains unclear, this reduction is associated with the induction of social avoidance by R-SDS,

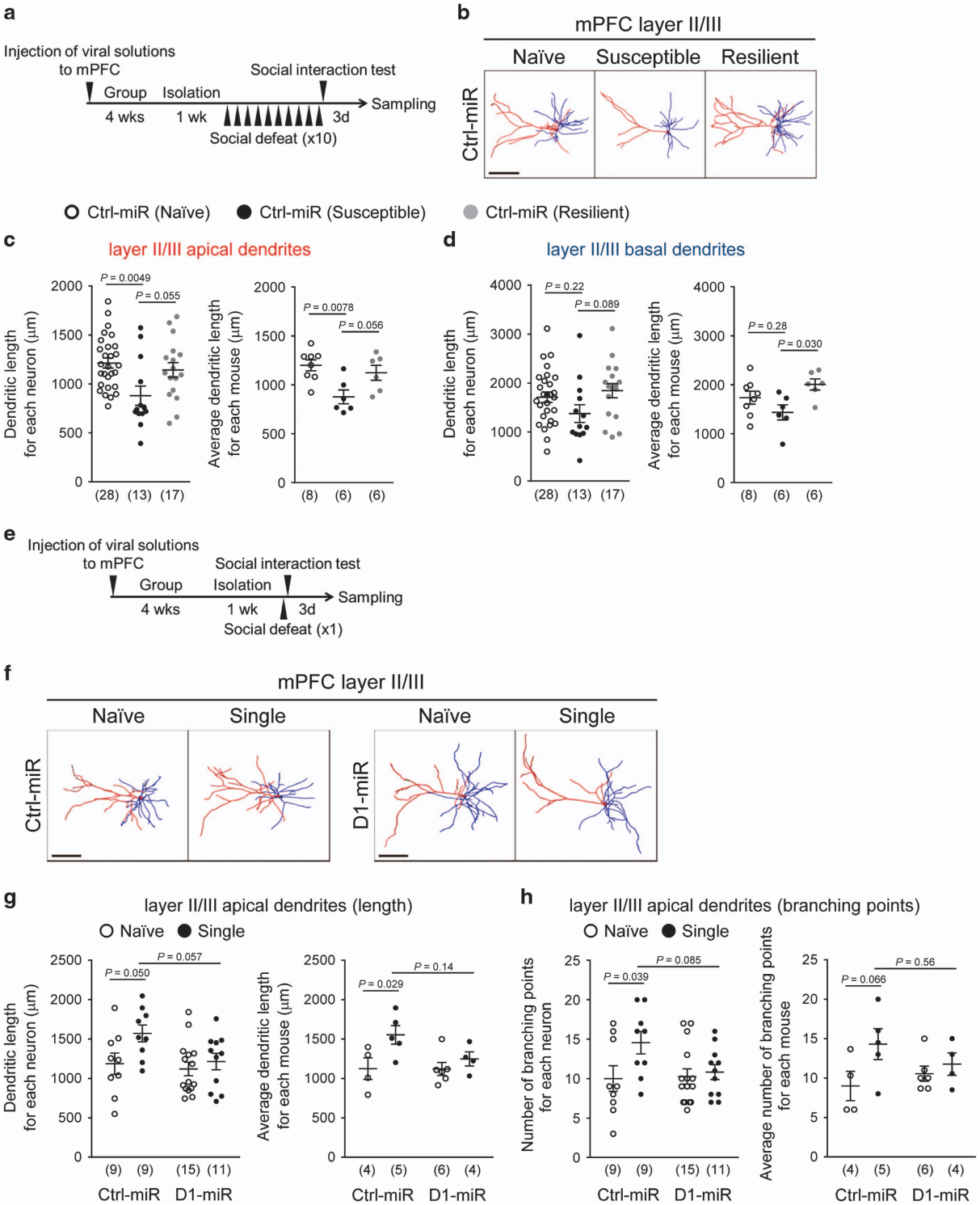
Figure 3. Single social defeat stress (S-SDS) induces extracellular signal-regulated kinase (ERK) phosphorylation and c-Fos expression in medial prefrontal cortex (mPFC) neurons through D1 receptor subtype. (**a**) Representative images of immunofluorescent staining for phosphorylated ERK (pERK) in mPFC of mice without or with S-SDS (Naive or Single, respectively) and without or with D1 knockdown in mPFC neurons (Ctrl-miR mice or D1-miR mice, respectively). NeuN was co-stained to identify neurons in mPFC. The signals for pERK and NeuN are shown in green and red, respectively. The same images of respective fluorescent channels and their magnified images are shown in Supplementary Figure 7. Scale bars, 200 μm. (**b**) Cumulative frequency of immunofluorescent signals for pERK in mPFC neurons of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). $N = 35\,345$–$41\,283$ neurons from 6–7 mice for each group. The inset shows the averages of the immunofluorescent signals for pERK shown in the cumulative frequency across neurons for respective mice. Each dot represents the averaged values for an individual mouse. The numbers of mice in respective groups are shown below bars. Results are shown as mean ± s.e.m. (**c**) The proportions of pERK-positive neurons in mPFC of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). Those neurons that showed pERK signals higher than the averaged values for naive Ctrl-miR mice by the s.d. (a dashed line in Figure 3b) are defined as pERK-positive neurons. The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way analysis of variance (ANOVA) ($F_{(1,22)} = 3.406$, $P = 0.0785$ for the main RNAi effect, $F_{(1,22)} = 4.359$, $P = 0.0486$ for the main Stress effect). Results are shown as mean ± s.e.m. (**d**) Representative images of immunofluorescent staining for pERK in mPFC of mice without or with S-SDS (Naive or Single, respectively). CaMKIIα or GABA was co-stained as a marker for excitatory or inhibitory neurons in the mPFC, respectively. In the merged images, the signals for pERK and CaMKIIα or GABA are shown in green and red, respectively. Arrows represent pERK- and CaMKIIα-positive neurons in mPFC. Scale bar, 50 μm. (**e**) The densities of pERK-positive excitatory or inhibitory neurons in the mPFC without or with S-SDS (Naive or Single, respectively). The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1,20)} = 8.638$, $P = 0.0081$ for the main Stress effect, $F_{(1,20)} = 99.48$, $P < 0.0001$ for the main Neuron-type effect). Results are shown as mean ± s.e.m. (**f**) The densities of pERK-positive excitatory neurons in the mPFC of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1,22)} = 4.864$, $P = 0.0382$ for the main RNAi effect, $F_{(1,22)} = 7.683$, $P = 0.0111$ for the main Stress effect). Results are shown as mean ± s.e.m. (**g**) Representative images of immunofluorescent staining for c-Fos in mPFC of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). NeuN was co-stained to identify neurons in the mPFC. The signals for c-Fos and NeuN are shown in green and red, respectively. The same images of respective fluorescent channels and their magnified images are shown in Supplementary Figure 8. Scale bars, 200 μm. (**h**) The densities of c-Fos-positive neurons in the mPFC layer II/III of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). The numbers of mice in respective groups are shown below bars. *P*-values shown in the graph were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1,19)} = 3.438$, $P = 0.0793$ for the main RNAi effect, $F_{(1,19)} = 2.000$, $P = 0.1735$ for the main Stress effect). Results are shown as mean ± s.e.m. (**i**) The densities of c-Fos-positive neurons in the mPFC layer V/VI of Ctrl-miR mice or D1-miR mice without or with S-SDS (Naive or Single, respectively). The numbers of mice in respective groups are shown below bars. No significant changes are observed ($F_{(1,19)} = 4.381$, $P = 0.0500$ for the main RNAi effect, $F_{(1,19)} = 2.942$, $P = 0.1026$ for the main Stress effect by two-way ANOVA). Results are shown as mean ± s.e.m.

because the reduction of mRNA was seen in susceptible mice, but much smaller in resilient mice. Importantly, reduced mRNA expression of D1 receptor subtype was not observed in the nucleus accumbens, suggesting that the expression of D1 receptor subtype is regulated in a brain region-specific manner. Further, R-SDS did not affect the mRNA expression of D5 receptor subtype in both susceptible and resilient mice, despite its expression in mPFC neurons.[22] These findings provide a rationale for the use of D1 receptor subtype in the mPFC as a functional biomarker of stress-related pathophysiology in PET. PET studies of D1 receptors have thus far relied on the use of D1/D5 mixed ligands,[14,17,18] because no subtype-specific D1 receptor ligand is available so far. Given the importance of D1 receptor subtype in suppressing stress susceptibility, it is desirable to develop a PET radioligand specific to D1 receptor subtype rather than D5 receptor subtype. Previous PET studies have investigated the relationship between D1-like receptor subtypes in prefrontal cortices and cognitive impairments in patients with mental illnesses.[14,18,54] However, the results appear to be inconsistent across studies, and the reason for this inconsistency remains elusive.[55] As the expression of D1 receptor subtype in mPFC is susceptible to R-SDS in mice, the history of stressful life events of individuals should be taken into consideration to decipher and analyze the data of PET studies of D1 receptors in the prefrontal cortices.

In contrast to D1 receptor subtype, we found that R-SDS increases the mRNA expression of D2 receptor subtype in the mPFC only in resilient mice. Consistently, it has recently been reported that deep brain stimulation of the medial forebrain bundle increases the expression of D2 receptor subtype in the mPFC along with antidepressant-like effects.[56] In mPFC excitatory neurons, expression levels of D1 and D2 receptor subtypes are partially overlapped, and it has been suggested that D1 and D2 receptor subtypes form a D1–D2 heteromer coupled to G_q signaling rather than G_s/G_{olf} or G_i/G_o signaling activated by D1 or D2 receptor subtype independently.[23] Thus increased expression of D2 receptor subtype, but not of D1 receptor subtype, in the mPFC of resilient mice would increase the proportion of D2 receptor subtype uncoupled from D1 receptor subtype. It has been reported that pharmacological disruption of the D1–D2 heteromer in the mPFC induces antidepressant-like effects in the forced swim test and the learned helplessness test in rats.[57] Therefore, whether D2 receptor subtype uncoupled from D1 receptor subtype is involved in stress resilience warrants future investigation.

A role for D1 receptor subtype in mPFC excitatory neurons in suppressing stress susceptibility

In most of the previous studies, pharmacological agents have been used to examine the roles for D1 receptors in prefrontal cortices.[9–12] However, the pharmacological studies cannot discriminate respective functions of D1 and D5 receptor subtypes and cannot identify their sites of action. Therefore, in this study, we generated AAV expressing D1 miRNA and injected this viral vector to mPFC. We confirmed that the expression of D1 miRNA in mPFC neurons selectively reduced the mRNA expression of D1 receptor subtype, but not those of the other subtypes, including D5 receptor subtype. We also developed neuron type-specific expression of D1 miRNA in mPFC, using AAV expressing D1 miRNA only in the presence of Cre recombinase and transgenic mice expressing Cre recombinase in specific neuronal populations or using AAV expressing Cre recombinase selectively in excitatory neurons, and showed that D1 receptor subtype and related signaling in mPFC excitatory neurons contribute to suppression of stress susceptibility. By contrast, D1 receptor subtype in mPFC inhibitory neurons appears not to be critical for suppressing stress susceptibility, although we cannot fully exclude the possibility that D1 knockdown in mPFC inhibitory neurons may not be sufficient to cause its behavioral phenotype. These results represent, to the best of our knowledge, the first to demonstrate a role for a



dopamine receptor subtype in a specific neuronal population in prefrontal cortices *in vivo*. Therefore, this study provides a novel method to investigate the roles of individual dopamine receptor subtypes in a specific brain area of interest with advantage over classical pharmacological agents.

Distinct groups of mPFC excitatory neurons project to different brain areas[58] and may exert opposing actions on depression-like behaviors. It has been suggested that mPFC neurons projecting to the raphe nucleus[59] and those projecting to the nucleus accumbens[60] suppress depression-like behaviors, whereas those projecting to the lateral habenula increases these behaviors.[59] Distinct groups of mPFC excitatory neurons may be susceptible to stress in various degrees, as repeated restraint stress induces dendritic shortening in mPFC layer II/III pyramidal neurons projecting to the entorhinal cortex but not in those projecting to the basolateral amygdala.[61] Therefore, it is critical to identify a brain region projected by mPFC excitatory neurons in which D1 receptor subtype and related signaling contribute to suppression of stress susceptibility.

Figure 4. Single social defeat stress (S-SDS) induces dendritic growth selectively in apical dendrites of medial prefrontal cortex (mPFC) layer II/III pyramidal neurons through D1 receptor subtype. (**a**) A schedule of behavioral experiments to test the effect of repeated social defeat stress (R-SDS) on dendritic morphology of mPFC pyramidal neurons without or with D1 knockdown in mPFC neurons. Four weeks after injection of viral solutions to the mPFC, the mice were isolated for a week and then were subjected to R-SDS followed by the social interaction test. Three days after R-SDS, the brains were collected and subjected to morphological analysis of mPFC neurons. (**b**) Three-dimensional reconstruction of mPFC layer II/III pyramidal neurons in naive, susceptible and resilient mice without D1 knockdown in mPFC neurons (Ctrl-miR mice). Apical and basal dendrites are shown in red and blue, respectively. Scale bar, 100 μm. (**c**) The effects of R-SDS on the lengths of apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice. Each dot represents the value from an individual neuron (left graph) or its average across neurons from an individual mouse (right graph). The numbers of neurons (left graph) or mice (right graph) in respective groups are shown below bars. *P*-values shown in the graphs were calculated by Tukey's *post hoc* test following one-way analysis of variance (ANOVA) ($F_{(2, 55)} = 5.490$, $P = 0.0067$ for the left graph and $F_{(2, 17)} = 6.288$, $P = 0.0090$ for the right graph). Results are shown as mean ± s.e.m. (**d**) The effects of R-SDS on the lengths of basal dendrites of the same neurons analyzed in panel (**c**). The values were analyzed and are shown similarly. The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. *P*-values shown in the graphs were calculated by Tukey's *post hoc* test following one-way ANOVA ($F_{(2, 55)} = 2.408$, $P = 0.0994$ for the left graph and $F_{(2, 17)} = 3.974$, $P = 0.0384$ for the right graph). Results are shown as mean ± s.e.m. (**e**) A schedule of behavioral experiments to test the effect of S-SDS on dendritic morphology of mPFC pyramidal neurons without or with D1 knockdown in mPFC neurons. Four weeks after injection of viral solutions to mPFC, the mice were isolated for a week and then were subjected to S-SDS followed by the social interaction test. Three days after S-SDS, the brains were collected and subjected to morphological analysis of mPFC neurons. (**f**) Three-dimensional reconstruction of mPFC layer II/III pyramidal neurons without or with S-SDS (Naive or Single, respectively) and without or with D1 knockdown in mPFC neurons (Ctrl-miR mice or D1-miR mice, respectively). Apical and basal dendrites are shown in red and blue, respectively. Scale bar, 100 μm. (**g**) The effects of S-SDS on the lengths of apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice or D1-miR mice. Each dot represents the value from an individual neuron (left graph) or its average across neurons from an individual mouse (right graph). The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. *P*-values shown in the graphs were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1, 40)} = 4.895$, $P = 0.0327$ for the left graph and $F_{(1, 15)} = 6.646$, $P = 0.0210$ for the right graph for the main Stress effect). Results are shown as mean ± s.e.m. (**h**) The effects of S-SDS on the numbers of branching points of apical dendrites of the same neurons analyzed in Figure 4g. The numbers of neurons (left graph) or mice (right graph) in respective groups are shown below bars. *P*-values shown in the graphs were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1, 40)} = 4.355$, $P = 0.0433$ for the left graph and $F_{(1, 15)} = 4.312$, $P = 0.0554$ for the right graph for the main Stress effect).

A novel neuronal correlate for the action of D1 receptor subtype in mPFC excitatory neurons in suppressing stress susceptibility

In this study, we found that S-SDS increases dendritic arborization and spine density of apical dendrites of mPFC layer II/III pyramidal neurons. This study is, to the best of our knowledge, the first to show that brief stress exposure induces dendritic growth of mPFC pyramidal neurons. As this dendritic growth is abolished by D1 knockdown in mPFC neurons, D1 receptor subtype and related signaling contribute to this morphological change. Indeed, S-SDS induces ERK phosphorylation selectively in mPFC excitatory neurons, which depends, at least in part, on D1 receptor subtype. It has been reported that stimulation of D1-like receptors enhances *N*-methyl-D-aspartate (NMDA) receptor-mediated excitatory synaptic inputs[62] and that NMDA receptor activation can induce dendritic growth.[63,64] ERK phosphorylation occurs upon NMDA receptor stimulation[65] and is required for NMDA receptor-mediated dendritic growth.[66,67] Therefore, D1 receptor-mediated enhancement of glutamatergic inputs and consequent ERK phosphorylation in mPFC excitatory neurons may be involved in dendritic growth of mPFC pyramidal neurons induced by S-SDS.

By contrast, we found that R-SDS induces dendritic shortening of apical dendrites of mPFC layer II/III pyramidal neurons only in susceptible mice. This finding is consistent with previous reports that repeated restraint stress and chronic mild stress induce dendritic shortening of mPFC pyramidal neurons in a manner correlated to behavioral changes.[44,45] However, as D1 knockdown in mPFC neurons did not potentiate the dendritic shortening but facilitated the induction of social avoidance, the dendritic shortening of mPFC pyramidal neurons after R-SDS does not account for the action of D1 receptor subtype in mPFC in suppressing stress susceptibility. This finding all the more highlights the link of S-SDS-induced dendritic growth of mPFC layer II/III pyramidal neurons through D1 receptor subtype with suppressing stress susceptibility.

Our finding that S-SDS-evoked signaling of D1 receptor subtype induces dendritic growth in mPFC neurons suggests the presence of long-lasting effects of D1 receptor activation in mPFC. It has been reported that a rapid-acting antidepressant such as ketamine rapidly increases glutamatergic synaptic connections of mPFC pyramidal neurons,[68] thereby producing the long-lasting antidepressant effects.[69] In primates, repeated treatments with a D1 receptor agonist can reverse working memory deficits induced by chronic haloperidol treatment, and this improvement is sustained for more than a year after cessation of treatment with D1-like receptor agonist.[70] These pieces of evidence, including ours, suggest that activation of D1 receptor subtype provides long-lasting effects on prefrontal functions. Studies using *in vivo* proton magnetic resonance spectroscopy have shown reduced glutamate levels in the anterior cingulate cortex of depressive patients,[71] and successful electroconvulsive therapy improves these levels.[72] Therefore, glutamate levels in prefrontal cortices may be a promising biomarker to estimate long-term therapeutic effects of drugs targeting D1-like receptors in patients with mental illnesses.

As S-SDS and R-SDS induce dendritic growth and dendritic shortening, respectively, of mPFC pyramidal neurons, the pro-adaptive dendritic growth switches into the maladaptive dendritic shortening with repetition of SDS. As D1 knockdown in mPFC neurons did not potentiate R-SDS-induced dendritic shortening of mPFC pyramidal neurons, reduced D1 expression in mPFC cannot account for R-SDS-induced dendritic shortening. Rodent studies have shown that repeated stress increases the levels of inflammation-related molecules in the brain, and these molecules or their receptors are critical for repeated stress-induced behavioral changes.[5,73–75] Therefore, it is intriguing to examine whether inflammation-related molecules are involved in repeated stress-induced dendritic shortening and, if so, how D1-mediated dendritic growth switches into dendritic shortening mediated by inflammation-related molecules.

In conclusion, our findings show that D1 receptor subtype and related signaling in mPFC excitatory neurons mediate S-SDS-induced dendritic growth and contribute to suppression of stress susceptibility. In contrast to previous pharmacological studies, this study has identified a dopamine receptor subtype contributing to suppression of stress susceptibility and its site of action in mPFC. This study also substantiates the presence of long-term effects of D1 receptor subtype on structures and functions of mPFC at least in this behavioral model. As expression of D1 receptor subtype in

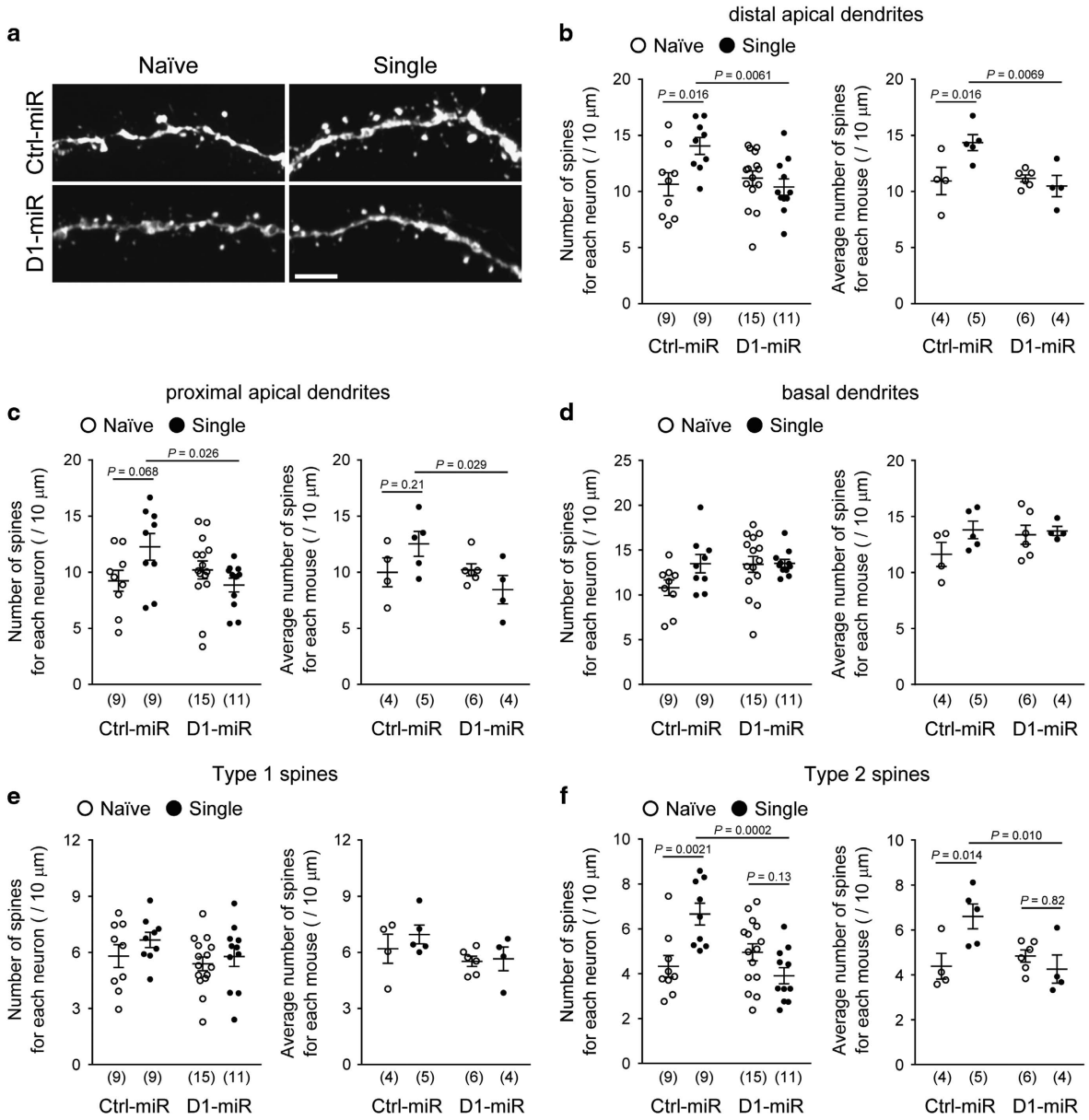


Figure 5. Single social defeat stress (S-SDS) increases spine density selectively on apical dendrites of medial prefrontal cortex (mPFC) layer II/III pyramidal neurons through D1 receptor subtype. (**a**) Representative images of dendritic spines on distal apical dendrites of mPFC layer II/III pyramidal neurons without or with S-SDS (Naive or Single, respectively) and without or with D1 knockdown in the mPFC neurons (Ctrl-miR mice or D1-miR mice, respectively). Scale bar, 5 μm. (**b**) The effects of S-SDS on spine densities on distal apical dendrites of mPFC layer II/III pyramidal neurons in Ctrl-miR mice or D1-miR mice. Each dot represents the averaged value across multiple images from an individual neuron (left graph) or the averaged value across multiple neurons from an individual mouse (right graph). The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. *P*-values shown in the graphs were calculated by Bonferroni's *post hoc* test following two-way analysis of variance (ANOVA) ($F_{(1,\ 40)} = 2.712$, $P = 0.1074$ for the left graph and $F_{(1,\ 15)} = 3.110$, $P = 0.0982$ for the right graph for the main Stress effect). Results are shown as mean ± s.e.m. (**c**) The effects of S-SDS on spine densities on proximal apical dendrites of the same neurons analyzed in panel (**b**). The values were analyzed and are shown similarly. The numbers of neurons (left graph) or mice (right graph) in respective groups are shown below bars. *P*-values shown in the graphs were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1,\ 40)} = 0.8648$, $P = 0.3580$ for the left graph and $F_{(1,\ 15)} = 0.1428$, $P = 0.7108$ for the right graph for the main Stress effect). (**d**) The effects of S-SDS on spine densities on basal dendrites of the same neurons analyzed in panel (**b**). The values were analyzed and are shown similarly. The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. No significant changes are observed ($F_{(1,\ 40)} = 2.609$, $P = 0.1141$ for the left graph and $F_{(1,\ 15)} = 2.223$, $P = 0.1567$ for the right graph for the main Stress effect by two-way ANOVA). (**e**) The effects of S-SDS on the densities of Type 1 spines on distal apical dendrites of the same neurons analyzed in panel (**b**). The values were analyzed and are shown similarly. The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. No significant changes are observed ($F_{(1,\ 40)} = 1.688$, $P = 0.2013$ for the left graph and $F_{(1,\ 15)} = 0.7160$, $P = 0.4107$ for the right graph for the main Stress effect by two-way ANOVA). (**f**) The effects of S-SDS on the densities of Type 2 spines on distal dendrites of the same neurons analyzed in panel (**b**). The values were analyzed and are shown similarly. The numbers of neurons (left graph) or mice (right graph) in the respective groups are shown below bars. *P*-values shown in the graphs were calculated by Bonferroni's *post hoc* test following two-way ANOVA ($F_{(1,\ 40)} = 2.210$, $P = 0.1449$ for the left graph and $F_{(1,\ 15)} = 2.719$, $P = 0.1199$ for the right graph for the main Stress effect).

mPFC is reduced after R-SDS, prefrontal functions to cope with stress and therapeutic effects of D1 receptor agonists on prefrontal dysfunctions may be limited in susceptible subjects after repeated stress. Furthermore, as R-SDS attenuates the activity of dopamine neurons projecting to mPFC,[5,6] the recovery in D1 expression in mPFC excitatory neurons alone may not be sufficient to restore the action of D1 receptor subtype in suppressing stress susceptibility. Therefore, the molecular pathway downstream of D1 receptor subtype that is maintained after repeated stress may offer a novel and more promising target for therapeutic interventions for prefrontal dysfunctions of mental illnesses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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