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Dampened dopamine-mediated neuromodulation in prefrontal cortex of fragile X mice

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Key points

- Activation of D1 receptors produces an initial suppression followed by facilitation of evoked inhibitory postsynaptic currents (IPSCs) in layer II pyramidal neurons of mouse prefrontal cortex.
- In *Fmr1* knockout (KO) mice, the D1-mediated facilitation of evoked IPSCs is absent whereas the initial suppression is unaltered.
- Downstream mechanisms of the D1-mediated facilitation (i.e. cAMP-dependent facilitation) persists in *Fmr1* KO neurons; however, there is a decrease in D1 receptor protein in the *Fmr1* KO tissues.
- These results indicate the dopamine-dependent modulation of inhibition is dampened in *Fmr1* KO animals, which could produce a relative hyperexcitability of neural circuitry within decision-making regions of the prefrontal cortex in fragile X syndrome.

Abstract Fragile X syndrome (FXS) is the most common form of inheritable mental retardation caused by transcriptional silencing of the *Fmr1* gene resulting in the absence of fragile X mental retardation protein (FMRP). The role of this protein in neurons is complex and its absence gives rise to diverse alterations in neuronal function leading to neurological disorders including mental retardation, hyperactivity, cognitive impairment, obsessive-compulsive behaviour, seizure activity and autism. FMRP regulates mRNA translation at dendritic spines where synapses are formed, and thus the lack of FMRP can lead to disruptions in synaptic transmission and plasticity. Many of these neurological deficits in FXS probably involve the prefrontal cortex, and in this study, we have focused on modulatory actions of dopamine in the medial prefrontal cortex. Our data indicate that dopamine produces a long-lasting enhancement of evoked inhibitory postsynaptic currents (IPSCs) mediated by D1-type receptors seen in wild-type mice; however, such enhancement is absent in the *Fmr1* knock-out (*Fmr1* KO) mice. The facilitation of IPSCs produced by direct cAMP stimulation was unaffected in *Fmr1* KO, but D1 receptor levels were reduced in these animals. Our results show significant disruption of dopaminergic modulation of synaptic transmission in the *Fmr1* KO mice and this alteration in inhibitory activity may provide insight into potential targets for the rescue of deficits associated with FXS.

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Abbreviations ANOVA, analysis of variance; D1R, D1 receptor; DA, dopamine; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; HRP, horseradish peroxidase; KO, knockout; mIPSC, miniature inhibitory postsynaptic current; mPFC, medial prefrontal cortex; NMDA, *N*-methyl-D-aspartate; PFC, prefrontal cortex; TTX, tetrodotoxin; WT, wild-type.

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Introduction

The prefrontal cortex (PFC) plays a role in higher-level cognitive functions that engage working memory and attention (Seamans & Yang, 2004; O'Grada & Dinan, 2007). Disruptions of normal processing within the PFC are associated with neurological disorders such as fragile X syndrome (FXS), autism and schizophrenia. The mesocortical pathway from the ventral tegmental area consists of dopamine (DA)-containing neurons that project to the medial prefrontal regions of the neocortex which are associated with working memory, planning, motivation and cognition (Egan & Weinberger, 1997; Seamans & Yang, 2004). The modulatory actions of dopaminergic processes are thought to influence neurotransmission and intrinsic properties of PFC neurons differentially, thereby playing an influential role in prefrontal output.

FXS is an inherited form of mental retardation due to the lack of fragile X mental retardation protein (FMRP) (Brown, 1996; Turner *et al.* 1996) and has been associated with neurological conditions including autism, attention deficit disorder and epilepsy (Hagerman, 1996; Berry-Kravis *et al.* 2002). FMRP is localized in the nucleus (Verheij *et al.* 1993; Eberhart *et al.* 1996; Feng *et al.* 1997) and one function of FMRP is to escort mRNAs out of the nucleus and into the cytoplasm (Bagni & Greenough, 2005). In the cytoplasm, both FMRP and *Fmr1* mRNA are found in dendrites and spines and the mRNAs that are regulated or bound by FMRP are involved in synaptic transmission, neuronal maturation, and dendritic structure and function (Brown *et al.* 2001; Miyashiro *et al.* 2003).

A number of behavioural studies suggest the involvement of DA in FXS. Discrimination learning, attentional set formation and working memory appear to be affected in both FXS patients and FXS mouse models (Keenan & Simon, 2004; Ventura *et al.* 2004; Moon *et al.* 2006; Hooper *et al.* 2008; Casten *et al.* 2011). Spontaneous blink rates, a clinical measure of DA function, was significantly greater in FXS boys compared to controls (Roberts *et al.* 2005). FXS males performed significantly worse than controls on aspects of selective attention, divided attention, sustained attention and inhibition, all of which involve prefrontal processing (Munir *et al.* 2000*b*). Similarly, *Fmr1* knockout (KO) mice displayed greater premature responses in basic and sustained attention tasks compared to wild-type (WT) littermates, suggesting impaired inhibitory control and impulsivity (Moon *et al.* 2006). FXS patients and animal models also exhibit behavioural abnormalities such as hyperactivity and anxiety (Fryns, 1984; Largo & Schinzel, 1985; Veenema *et al.* 1987; Pieretti *et al.* 1991; Munir *et al.* 2000*a*; Dockendorff *et al.* 2002; Yan *et al.* 2004). Overall, these behavioural studies suggest that normal functioning of the PFC, and its modulation by DA, are probably altered in FXS.

Few studies have focused on the involvement of DA-mediated alterations associated with FXS. In the drosophila model of FXS, DA levels are elevated in the knockout model (Zhang *et al.* 2005). In the mammalian FXS model, DA-mediated facilitation of long-term potentiation of excitatory synaptic transmission via D1 receptors (D1Rs) was impaired in the medial PFC (mPFC; Wang *et al.* 2008). In the current study, we investigated the modulation of inhibitory synaptic transmission in WT and *Fmr1* KO mice by DA in mPFC pyramidal neurons. DA-mediated enhancement of inhibitory synaptic activity via D1Rs was absent in *Fmr1* KO mice and this was accompanied by decreased D1R levels in the mPFC.

Methods

All experimental procedures were carried out in accordance with the National Institute of Health guidelines, approved by the University of Illinois Animal Care and Use Committee, and are similar to those previously described (Govindaiah & Cox, 2004).

Male and female *Fmr1* KO and WT mice (FVB, postnatal age 12–20 days) were deeply anaesthetized with pentobarbital sodium (50 mg kg^{-1}) and decapitated. The brain was quickly removed and placed into cold, oxygenated slicing medium containing (in mM): 2.5 KCl, 10.0 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, 11.0 glucose and 234.0 sucrose. Tissue slices (300 μm thickness) were cut in the coronal plane using a vibrating tissue slicer, transferred to a holding chamber and incubated for $\geq$1 h before recording. Individual slices were transferred to a submersion-type recording chamber on a modified microscope stage and continuously superfused with oxygenated physiological saline at 32°C. A low-power objective ($\times$5) was used to identify layers II/III of mPFC and a high-power water-immersion objective ($\times$63) was used to visualize individual pyramidal neurons. The physiological solution used in the experiments contained (in mM): 126.0 NaCl, 2.5 KCl, 1.25 $MgCl_2$, 2.0 $CaCl_2$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$ and 10.0 glucose. This solution was gassed with 95% O_2/5% CO_2 to a final pH of 7.4.

Electrophysiology

Intracellular recordings, using the whole-cell configuration, were obtained with the visual aid of a modified Axioskop 2FS equipped with infrared differential interference contrast optics (Zeiss Instruments, Thornwood NY, USA). Recording pipettes had tip resistances of 3–6 MΩ when filled with an intracellular solution containing (in mM): 117.0 potassium gluconate, 13.0 KCl, 1.0 $MgCl_2$, 0.07 $CaCl_2$, 0.1 EGTA, 10.0 Hepes,

2.0 Na_2-ATP and 0.4 Na-GTP. Pyramidal neurons in layer II of mPFC were identified by their typical soma shape, apical dendrite oriented towards layer I. For recordings of miniature inhibitory postsynaptic currents (mIPSCs), Cs^+ was substituted for K^+ in the intracellular solution and the bath contained 1 μM tetrodotoxin (TTX). The pH was adjusted to 7.3 and the osmolarity was adjusted to 290 mosmol l^{-1}. The initial access resistance ranged from 10 to 20 MΩ and remained stable during the recordings included for analyses.

A Multiclamp 700 amplifier (Molecular Devices, Foster City, CA, USA) was used in voltage clamp mode for current recordings. Voltage and current protocols were generated using pClamp software (Molecular Devices) and data stored on computer. In current-clamp recordings, an active bridge circuit was continuously adjusted to balance the drop in potential produced by passing current through the recording electrode. The apparent input resistance was calculated from the linear slope of the *I–V* relationship obtained by applying constant current pulses ranging from −100 to +40 pA (500 ms duration).

Stimulating electrodes were placed in layer I and deep layers (layer V/VI), and synaptic responses were evoked with constant current pulses (50–400 μA, 100 μs). All evoked and spontaneous IPSCs were recorded in the presence of *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor antagonists, (RS)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid CPP (10 μM) and 6,7-Dinitroquinoxaline-2,3-dione DNQX (20 μM), respectively. Evoked IPSCs were recorded at a holding potential of −50 mV whereas mIPSCs were recorded at 0 mV. To obtain isolated layer I stimulation, a surgical knife was used to make an incision perpendicular to the pial surface just below the layer I border down through the underlying white matter (Cauller & Connors, 1994). The stimulating electrode is then placed in layer I on the opposite side of the transection from the recording electrode. This preparation, in conjunction with pharmacological agents, ensures that a monosynaptic pathway is obtained between the stimulating electrode and the recording electrode via layer I.

Agonists were applied by injecting a bolus into the input line of the chamber using a motorized syringe pump. Based on the rate of drug injection and rate of chamber perfusion, the final bath concentration of the agonist was estimated to be one-quarter of the concentration introduced into the flow line (Cox *et al.* 1995). All concentrations mentioned in this study are the final concentrations in the bath. DA was administered with 0.08% ascorbic acid to prevent oxidation and this mix was made fresh every 2–3 h. Control injections of physiological saline produced no changes in membrane potential or input resistance, indicating that the temporary increase in flow rate during bolus injection had no effect on the recordings. All antagonists were bath applied at final concentration. All chemicals were obtained from Tocris Bioscience (Ellisville, MO, USA).

All data are presented as mean ± standard error of the mean (SEM) unless noted otherwise. Statistical analyses consist of two-sample *t* test and, when appropriate, a paired *t* test was used. A two-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test was used for comparisons of population data between WT and *Fmr1* KO mice over a 10 min time period. In these comparisons, individual data points were averaged in 1 min bins. *P* values <0.05 were considered statistically significant.

Western blotting

Punch biopsies of mPFC from WT and *Fmr1* KO cortical slices were processed for Western blotting as previously described (Schafe *et al.* 2000). Tissue samples were sonicated in buffer containing (in mM): 50 Tris-HCl, pH 7.4, 150 NaCl, 50 NaF, 5 $Na_4P_2O_7$ and 1% SDS. The sonicated lysates were boiled for 10 min, spun down to remove cell debris and stored at −80°C until immunoblotting. Equal amounts of protein (25 μg), as determined by bicinchoninic acid protein assay, were separated on 10% SDS-PAGE gels, and transferred onto nitrocellulose membrane. The blots blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 and probed with antibodies to D1R (1:1000, Abcam, Cambridge, MA, USA, N-terminal epitope), FMRP (1:200, Abcam) and GAPDH (1:750, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight. The blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA) and the HRP signal detected using SuperSignal Western blot kit (Pierce Biotechnology, Rockford, IL, USA). The images were captured on a FluorChem 8900 Alpha imager (Alpha Innotech, Santa Clara, CA, USA) and densitometric analyses of band intensities were performed using ImageJ (v. 1.45i, NIH). D1R levels were normalized using total protein and loading control (GAPDH) levels, and the difference between two groups was analysed using Student's *t* test. The normalized percentages of receptor levels are expressed as mean ± SEM and a *P* value of less than 0.05 was considered statistically significant.

Results

Whole-cell recordings were obtained from 89 pyramidal neurons from layer II/III of the mPFC (47 WT, 42 *Fmr1* KO), which included both prelimbic and cingulate regions. The resting membrane potential of the neurons for the WT and *Fmr1* KO groups was −79.4 ± 1.0 mV ($n = 39$) and −78 ± 0.9 mV ($n = 36$), respectively. The apparent input resistance of WT neurons was 262 ± 19.4 MΩ ($n = 39$)

and $304.3 \pm 15.7\,\text{M}\Omega$ ($n = 36$) for *Fmr1* KO mice. In recordings obtained with Cs^+ in the recording pipette (mIPSC recordings), the resting membrane potentials and input resistances were not determined.

Differential actions of DA on IPSCs in WT and *Fmr1* KO mice

IPSCs were evoked by either superficial layer I (S1) or deep layer/white matter (S2) stimulation, and obtained from both WT and *Fmr1* KO mice. We used both S1 and S2 stimuli to control for possible differences in the IPSCs evoked by different laminar sources. In a representative pyramidal neuron recorded from a WT animal, bath application of DA (50 μM, 4 min) produced an early reversible suppression of the IPSC evoked by S1 stimulation that returned to baseline levels within 10 min (Fig. 1*A*). In contrast, in a neuron from an *Fmr1* KO animal, DA produced a similar early suppression of the IPSC, but a persistent, smaller magnitude suppression continued through the duration of the recording (>20 min, Fig. 1*A*, *Fmr1* KO). Overall, during the early phase (<5 min) DA significantly reduced the IPSC response by $27.8 \pm 1.3\%$ ($P < 0.05$, $n = 9$, paired t test) and $26 \pm 1.9\%$ ($P < 0.05$, $n = 10$, paired t test) in WT and *Fmr1* KO mice, respectively. However, during the later phase (14–23 min post DA), the IPSC was significantly suppressed in *Fmr1* KO animals (Fig. 1*Ab*, $P < 0.0005$, $F_{1,9} = 53.96$, two-way ANOVA).

With deep layer stimulation (S2), DA produced an initial suppression that was followed by a longer lasting facilitation of the IPSC in WT neurons (Fig. 1*Ba*, WT). In contrast, DA produced an initial suppression of the IPSC but failed to facilitate the IPSC in *Fmr1* KO neurons (Fig. 1*Bi*, *Fmr1* KO). In WT neurons, DA produced a significant suppression of the IPSC from baseline levels ($17.6 \pm 3.9\%$, $n = 9$, $P < 0.05$, paired t test) that was followed by a significant increase in IPSC amplitude ($22.8 \pm 8.1\%$, $n = 9$, $P < 0.05$, paired t test). However, in *Fmr1* KO animals, while DA produced a significant early suppression of the IPSC ($27.3 \pm 4.9\%$, $n = 10$, $P < 0.05$, paired t test), there were no lasting alterations in IPSC amplitude ($2.4 \pm 4.6\%$, $P > 0.5$, $n = 10$, paired t test) as observed in WT neurons. During the late phase (14–23 min post DA), IPSC amplitudes were significantly greater in WT neurons than in *Fmr1* KO neurons ($P < 0.05$, $F_{1,9} = 50.85$, two-way ANOVA). These results indicate that normal DA facilitation of IPSCs in layer II/III pyramidal neurons evoked by deep layer stimulation is disrupted in *Fmr1* KO mice.

D1R-mediated facilitation of IPSCs is absent in *Fmr1* KO

We next tested if a specific receptor subtype mediates the facilitatory actions of DA on the IPSCs. In a WT neuron,

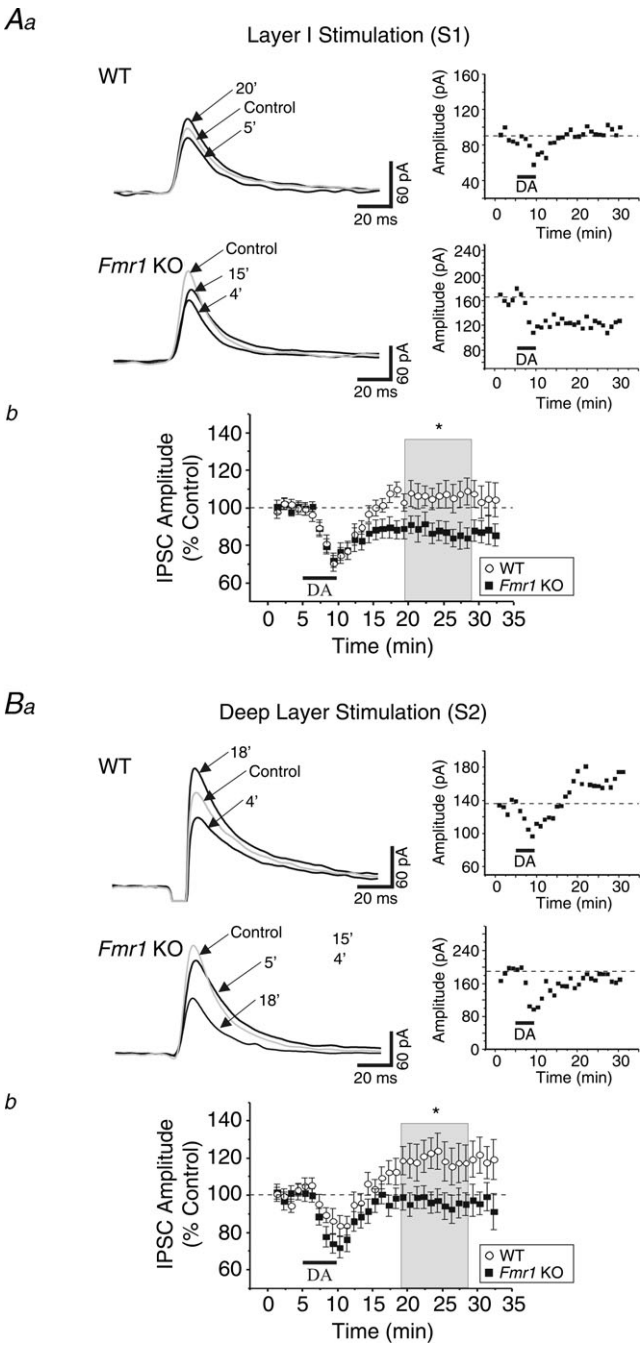

Figure 1. DA modulation of IPSCs is altered in *Fmr1* KO mice
Aa, average of five consecutive IPSCs evoked by layer I stimulation prior to (grey) and following DA (50 μM) application in neurons from WT and *Fmr1* KO mice. Corresponding graphs depict the temporal response to DA for these neurons. *Ab*, population data indicate that the initial IPSC suppression was unchanged in *Fmr1* KO mice, but late facilitation was significantly attenuated in the *Fmr1* KO animals (*$P < 0.0005$, $n = 9$ WT, $n = 10$ *Fmr1* KO). *Ba*, averaged traces and corresponding timeline of representative neurons show a similar attenuation of the late phase facilitation of IPSC in *Fmr1* KO mice in response to deep layer stimulation (S2). *Bb*, population data show that the late phase facilitation in WT is significantly greater than that in *Fmr1* KO mice (*$P < 0.05$, $n = 9$ WT, $n = 10$ *Fmr1* KO).

the D1R selective agonist SKF38393 (10 μM) produced a robust increase in IPSC amplitude (Fig. 2*Aa* WT); however, in a neuron from an *Fmr1* KO mouse, SKF38393 did not alter the IPSC amplitude (Fig. 2*Aa*, *Fmr1* KO). Overall, SKF38393 significantly increased IPSC amplitude by $39.5 \pm 7.9\%$ in WT neurons ($P < 0.05$, $n = 7$, paired t test at 20 min post SKF38393), but did not alter IPSC amplitude in neurons from *Fmr1* KO mice ($2.4 \pm 5.8\%$, $P > 0.5$, $n = 7$, paired t test). When comparing conditions, IPSCs from WT neurons were significantly greater than those in *Fmr1* KO neurons during the late phase (Fig. 2*Ab*, $P < 0.0005$, $F_{1,9} = 169.48$, two-way ANOVA).

The IPSCs evoked by S2 stimulation were similarly affected by SKF38393. As illustrated in Fig. 2*Ba*, SKF38393 produced a robust facilitation of IPSC amplitude in neurons from WT animals, and a smaller enhancement in neurons from *Fmr1* KO animals. In the overall population, SKF38393 significantly increased IPSC amplitude in neurons from WT animals by $36.7 \pm 7.4\%$ ($P < 0.0005$, $n = 7$, paired t test, 20 min post drug). The response in the *Fmr1* KO mice peaked at $10.4 \pm 6.1\%$, which did not significantly differ from control levels ($P > 0.05$, $n = 7$, paired t test). As with S1 stimulation, SKF38393 produced a significantly larger enhancement of the IPSC in neurons from WT animals compared to *Fmr1* KO animals (Fig. 2*Bb*, $P < 0.0005$, $F_{1,9} = 54.25$, two-way ANOVA).

mIPSCs are unaltered in *Fmr1* KO mice

To test whether the disruption of delayed facilitation of inhibitory response by DA in *Fmr1* KO may involve a presynaptic mechanism, we isolated mIPSCs by recording in the presence of TTX (1 μM) and bath applied DA (100 μM, 4 min). Under these conditions, DA produced an increase in both mIPSC frequency and amplitude in neurons from WT and *Fmr1* KO animals (Fig. 3*A*). Overall, DA significantly increased mIPSC frequency in WT neurons to $25.7 \pm 5.6\%$ ($P < 0.05$, $n = 8$, paired t test) and $31.1 \pm 7.8\%$ in *Fmr1* KO neurons ($P < 0.05$, $n = 6$, paired t test). The increase in frequency in both WT and *Fmr1* KO neurons recovered to baseline levels within 30 min and did not significantly differ from each other. DA produced a statistically significant increase in mIPSC amplitude in WT neurons ($7.1 \pm 1.6\%$, $n = 8$, $P < 0.05$, paired t test) but not in *Fmr1* KO neurons ($6.5 \pm 3.7\%$, $n = 6$, $P > 0.05$, paired t test); however, the physiological significance of this small increase is unclear. The magnitude of the increase did not differ significantly between WT and *Fmr1* KO neurons ($F = 0.1$, $P > 0.5$, $n = 8$ WT, $n = 6$ *Fmr1* KO, two-way ANOVA). These data suggest that the alteration in IPSC produced by DA in *Fmr1* KO mice is probably not due to presynaptic effects such as release probabilities, but perhaps a result of postsynaptic modifications.

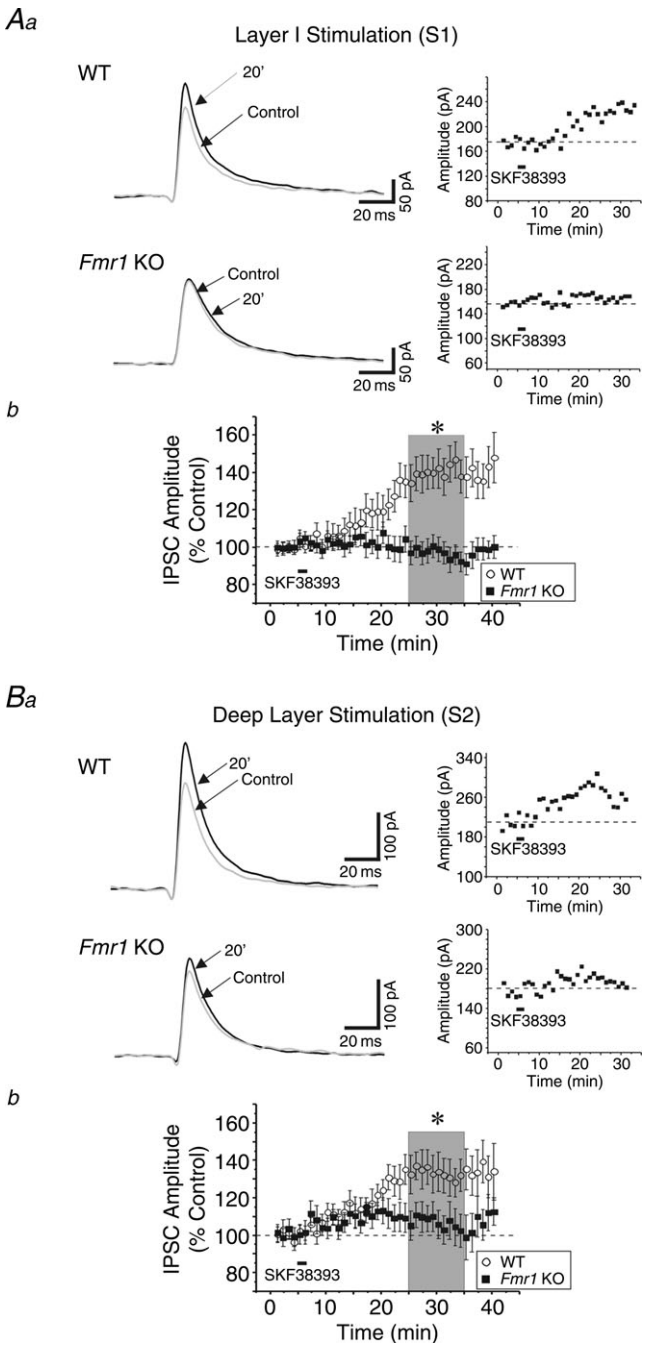

Figure 2. D1R agonist modulation of evoked IPSC is abolished in *Fmr1* KO mice
Aa, evoked IPSCs (average of five consecutive responses) to layer I stimulation prior to (grey) and following SKF38393 (10 μM) in WT and *Fmr1* KO mice. Corresponding figures in the right panel show the timeline response to SKF38393 for these neurons. *Ab*, population data timeline shows that the facilitation of evoked IPSCs by SKF38393 in WT is absent in *Fmr1* KO (*$P < 0.0005$, $n = 7$ WT, $n = 7$ *Fmr1* KO). *Ba*, averaged traces and corresponding timeline of representative neurons show a similar attenuation of facilitation of evoked IPSCs in *Fmr1* KO mice in response to deep layer stimulation (S2). *Bb*, population data show that the facilitation in WT is significantly greater than that in *Fmr1* KO mice (*$P < 0.0005$, $n = 7$ WT, $n = 7$ *Fmr1* KO).

D1 receptor signalling pathway is unaffected in *Fmr1* KO mice

We next evaluated whether the signalling pathway triggered by D1R activation is disrupted in *Fmr1* KO mice. As stimulation of D1Rs in mPFC is known to activate adenylyl cyclase leading to increased cAMP levels, we tested the effect of cAMP activator forskolin on evoked IPSCs. Short application of forskolin (10 μM, 90 s) produced a lasting facilitation of IPSCs evoked by S1 stimulation in neurons from both WT and *Fmr1* KO animals (Fig. 4*Aa*). Overall, forskolin enhanced the IPSC amplitude by $51.2 \pm 1.5\%$ ($n = 5$) and $29.5 \pm 0.7\%$ ($n = 5$) in neurons from WT and *Fmr1* KO mice, respectively. With S2 stimulation, forskolin (10 μM) produced maximal increases of $60.5 \pm 2.0\%$ ($n = 4$) and $53.4 \pm 1.7\%$ ($n = 5$) in WT and *Fmr1* KO mice, respectively. The increase in the IPSC amplitude between WT and *Fmr1* KO animals was significantly different for S1 stimulation ($P < 0.0005$, $F_{1,9} = 57.2$, two-way ANOVA) but not for S2 stimulation ($P > 0.5$, $F_{1,9} = 0.32$, two-way ANOVA). The similar action of forskolin in both

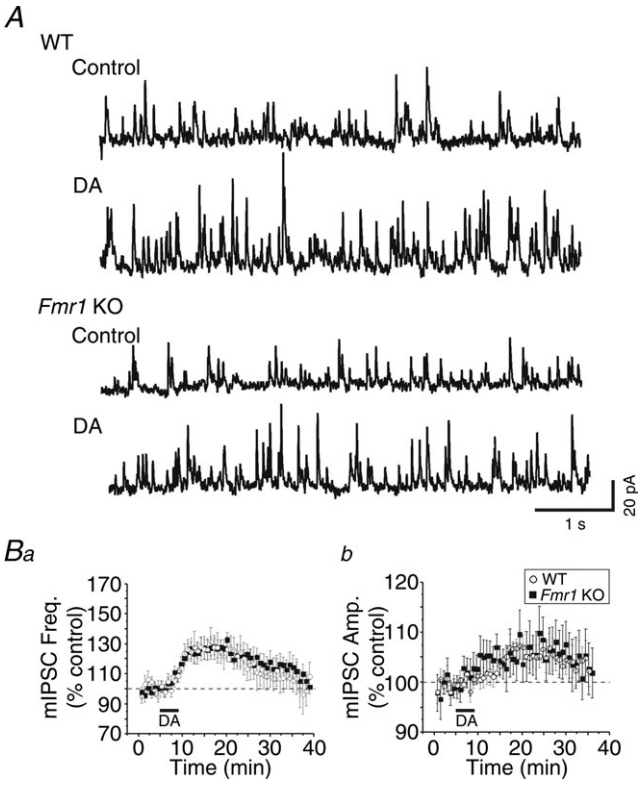

Figure 3. DA increases mIPSC frequency and amplitude in WT and *Fmr1* KO mice
A, current traces from a layer II pyramidal neuron from a WT animal in control and in DA (100 μM, 4 min) show an increase in both frequency and amplitude. A similar increase in frequency and amplitude is observed in mIPSC recordings from an *Fmr1* KO mouse. *B*, population data from eight WT and six *Fmr1* KO neurons show the effect of DA on the frequency and amplitude of mIPSCs.

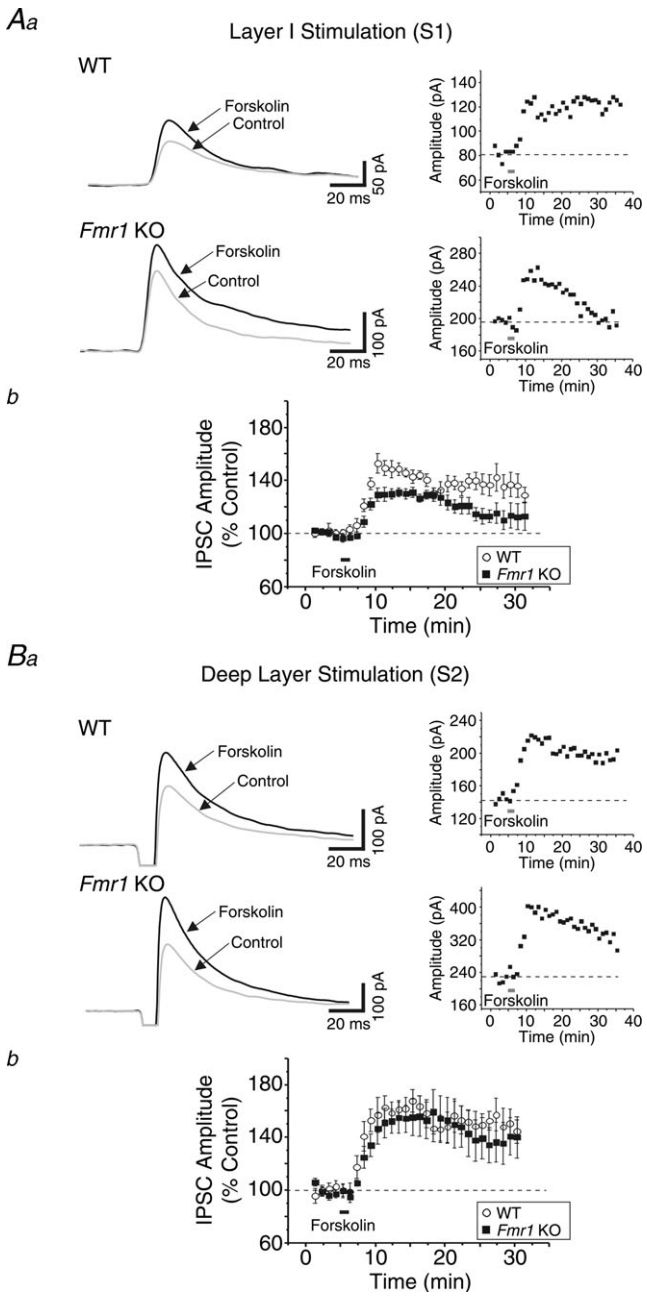

Figure 4. Inhibition due to direct cAMP activation is preserved in *Fmr1* KO mice
Aa, averaged traces of evoked IPSCs to layer I stimulation in control (grey) and forskolin (20 μM) for WT and *Fmr1* KO mice. Corresponding figures in the right panel show the timeline response to forskolin for these neurons. *Ab*, population data timeline shows that the facilitation of evoked IPSCs by forskolin in WT ($n = 5$) is preserved in *Fmr1* KO mice ($n = 5$). *Ba*, averaged traces and corresponding timeline of representative neurons show a similar facilitation of evoked IPSCs in WT and *Fmr1* KO mice in response to S2 stimulation. *Bb*, population data show that the facilitation in *Fmr1* KO is not significantly different from that in WT mice.

conditions indicates that the second messenger machinery underlying the facilitatory action is intact in the neurons from *Fmr1* KO animals.

Although activation of cAMP led to an enhanced IPSC, it is unclear if this is downstream of the D1R-mediated increase in IPSC that we observed. To test this, we initially applied forskolin to maximally activate cAMP in the neuron, and subsequently applied SKF38393 to test if the forskolin pretreatment could occlude the SKF38393-mediated enhancement. As illustrated in Fig. 5*A*, forskolin (20 μM, 90 s) produced a robust increase in IPSC amplitude in WT neurons. At the point of maximal increase (approximately 7 min following forskolin application), SKF38393 (10 μM, 90 s) was applied and did not produce any additional increase in IPSC amplitude with either S1 or S2 stimulation (Fig. 5*A*). In the overall population, forskolin increased IPSC amplitude by 56.9 ± 13.7% ($n = 3$) with S1 stimulation and 54.0 ± 16.0% ($n = 3$) with S2 stimulation (Fig. 5*B*). Subsequent application of SKF38393 (10 μM) produced no additional increase in IPSC amplitude and did not alter the gradual decay in the time course of the forskolin-mediated facilitation. As the increase in inhibition produced by D1R stimulation is occluded by cAMP activation, our data strongly indicate that the intracellular messenger pathway cascade initiated by D1R stimulation is unaltered in the *Fmr1* KO mice.

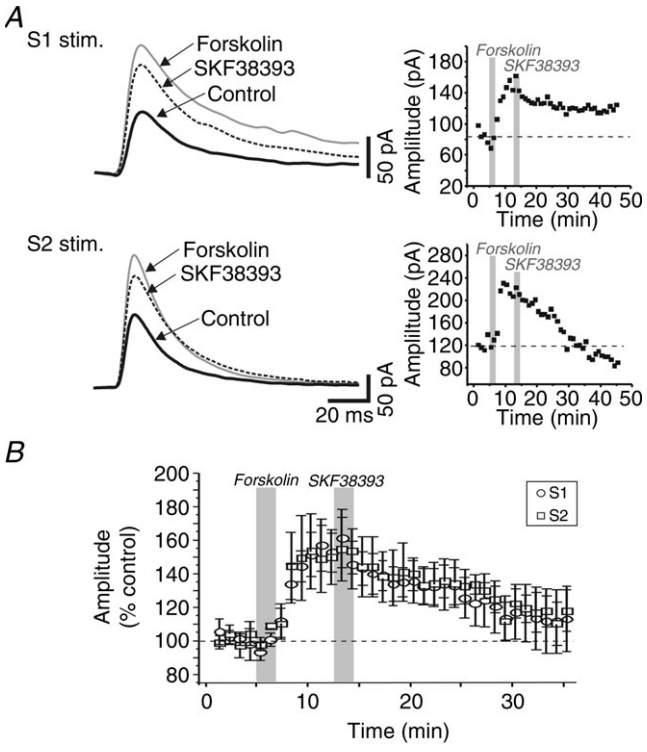

Figure 5. D1 facilitation of IPSCs is occluded by cAMP stimulation
A, in neurons from WT mice, subsequent application of SKF38393 (10 μM) after the peak forskolin (20 μM) facilitation of the IPSC failed to produce additional increase in IPSC amplitude in both S1 and S2 stimulation. SKF38393 was applied during the peak of the forskolin response. *B*, in population data, forskolin produced an increase of 156.9 ± 13.7% ($n = 3$) with S1 stimulation and 154.0 ± 16.0% ($n = 3$) with S2 stimulation. The effect of subsequent application of SKF38393 is occluded within the decay of the peak response to forskolin.

Reduced D1R levels in *Fmr1* KO mice

As the downstream intracellular actions of D1R activation are intact in *Fmr1* KO mice, we next determined whether the levels of D1R protein may account for attenuated enhancement of IPSCs in *Fmr1* KO neurons. We isolated mPFC from both WT and *Fmr1* KO animals, and using Western blotting measured D1R protein levels. Compared to WT controls (100.0 ± 9.0%, $n = 7$), the level of D1R protein was significantly lower in *Fmr1* KO mice (65.0 ± 6.1%, $n = 9$, $P < 0.01$, unpaired t test, Fig. 6).

Discussion

We have provided evidence that the lasting enhancement of inhibitory synaptic transmission by DA via D1R activation in the mPFC is strongly attenuated or abolished in *Fmr1* KO mouse. Furthermore, our data suggest that this lack of DA-mediated actions on inhibition is due to decreased D1Rs in mPFC of the *Fmr1* KO animal, and not an alteration of downstream intracellular mediators following DA receptor activation.

Previous studies have shown that micromolar DA evokes a complex, temporally biphasic effect on inhibitory synaptic responses in the PFC (Seamans *et al.* 2001; Seamans & Yang, 2004; Trantham-Davidson *et al.* 2004): an initial suppression, followed by a long-lasting facilitation of the evoked IPSC. The initial suppression of IPSC is D2R mediated and is thought to occur via post-synaptic mechanisms. The longer latency enhancement of the IPSC is mediated by D1Rs that elicit an increased

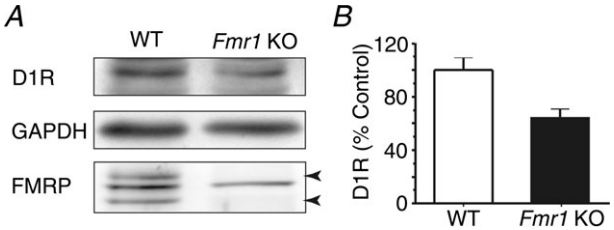

Figure 6. Decreased DA receptor protein in *Fmr1* KO mice
A, representative Western blots showing the expression of D1R, GAPDH and FMRP. *B*, the level of D1R protein was significantly lower in *Fmr1* KO mice compared to WT controls (*$P < 0.01$, $n = 7$ WT, $n = 9$ *Fmr1* KO).

excitability of presynaptic interneurons and, depending on the composition of postsynaptic $GABA_A$ receptors, may involve postsynaptic modulation (Trantham-Davidson *et al.* 2004). These biphasic effects of DA on inhibitory synaptic transmission were studied in young rats (postnatal age 14–28 days) (Seamans *et al.* 2001; Seamans & Yang, 2004; Trantham-Davidson *et al.* 2004). In light of those studies, we have investigated alterations associated with the *Fmr1* KO mice within a similar age range. In our current study, having controlled for the same age range for WT and *Fmr1* KO mice, our data show that the D1 mediated long-lasting facilitation of IPSC is attenuated in *Fmr1* KO mice but not in WT mice.

Our electrophysiological and molecular biological results are consistent with the hypotheses that the significant reduction in D1R-mediated enhancement of the IPSC in *Fmr1* KO mice is associated with decreased D1R levels in mPFC and not a disruption of the downstream intracellular machinery (e.g. cAMP pathway). This contrasts with a previous study in which the authors found that DA-mediated facilitation of excitatory synaptic currents produced by a long-term potentiation induction protocol was reduced in *Fmr1* KO mice (Wang *et al.* 2008). They concluded that there was an alteration in the coupling between the Gs protein and D1R, and found no alteration in cAMP machinery nor D1R levels, the latter being different from our findings. To address this inconsistency, we evaluated the differences in experimental paradigm. A major difference was the use of N-terminal D1R antibody in this study as compared to C-terminal D1R antibody by Wang and colleagues. Interestingly, a recent study found alteration in D1R levels in the amygdala during cocaine withdrawal only with the N-terminal D1R antibody, but not with C-terminal D1R antibodies (Krishnan *et al.* 2010). This suggested that the C-terminal epitope could be masked, thereby preventing exact quantification of D1R protein levels. Hence, the decrease in D1R levels in *Fmr1* KO mice detected here can be attributed to the use of N-terminal D1R antibody.

Direct stimulation of cAMP by forskolin evoked a prolonged facilitation of evoked IPSC in neurons from both WT and *Fmr1* KO animals, indicating that the basic intracellular cascade machinery involving D1→cAMP→protein kinase A activation remains intact in these neurons. Interestingly, our data indicate that while forskolin-induced facilitation of IPSCs evoked by stimulation of deep layer inputs were similar in WT and *Fmr1* KO mice (Fig. 4*B*), the forskolin-induced facilitation of IPSCs evoked by layer I stimulation was reduced in *Fmr1* KO neurons (Fig. 4*A*). It is possible that a localized region of layer II pyramidal neurons receiving inputs from layer I may have reduced cAMP levels in *Fmr1* KO mice but dendritic regions of the neuron that receive inputs from deep layers have normal cAMP levels. A few studies have demonstrated reduced cAMP production levels in FXS brains of drosophila, mouse cortex and human neural cell lines (Kelley *et al.* 2007; Bhattacharyya *et al.* 2008). Further studies are required to determine a direct functional synaptic consequence of reduced cAMP levels that may be localized to different parts of a neuron that receive inputs from different origins.

Our results show that the D1R-mediated facilitation of inhibition in pyramidal neurons is attenuated in the *Fmr1* KO mice and this effect is due to reduced D1R levels in the mPFC. Anatomical studies have shown that D1R and D2R are present on pyramidal neurons as well as on GABAergic interneurons, which project onto the pyramidal neurons. In addition, interneurons receive excitatory input from pyramidal neurons and inhibitory inputs from neighbouring interneurons. Therefore, the overall balance of excitation/inhibition in these principal cells and its DA-dependent modulation may be altered due to the lack of FMRP. *In vivo* recordings using optogenetic tools which allow for selective excitation of populations of excitatory pyramidal neurons or a class of inhibitory interneurons in the mPFC have shown that the elevation of excitation/inhibition ratio but not its reduction produces impaired social deficits seen in diseases such as autism (Yizhar *et al.* 2011).

Our results suggest that the normal modulation of excitation/inhibition balance by DA is increased in *Fmr1* KO mice due to a loss of functionality of D1Rs that leads to reduced inhibition and consequently would increase the excitation/inhibition ratio. Future investigations will reveal whether dopaminergic modulation of different classes of inhibitory interneurons is also altered by the lack of FMRP.

Implications for PFC functionality and spatial tuning

Behavioural and imaging studies indicate that FXS patients show deficits in attention, working memory and inhibitory control (Munir *et al.* 2000*c*; Cornish *et al.* 2001; Keenan & Simon, 2004; Hooper *et al.* 2008; Ornstein *et al.* 2008; Lanfranchi *et al.* 2009; Scerif *et al.* 2009). FXS patients have been shown to have difficulty with the Wisconsin Card Sort Test as well as other tasks that require inhibition of a previously learned rule or target and shift in attention to a new rule or target. In normal rats, DA depletion in the PFC produced impairment in performance of serial intradimensional set shift such that the learning of an initial visual discriminatory task was unaffected, but the capacity for learning a dimensional rule over several subsequent discriminatory tasks was impaired (Crofts *et al.* 2001; Robbins & Roberts, 2007; Robbins & Arnsten, 2009). In behavioural studies with *Fmr1* KO mice, performance is impaired, which coincides with an increase in premature responses after a change in task characteristics or an

intradimensional shift in reward cues (Moon *et al.* 2006; Casten *et al.* 2011). Therefore, in both patients and animal models, impaired performance in a dimensionally varying task is a key feature of FXS and is also present in rats depleted of DA in the PFC.

In vivo recordings in dorsolateral PFC in non-human primates have shown that both regular spiking pyramidal neurons and fast spiking interneurons are spatially tuned such that during delayed oculomotor tasks, specific groups of these neurons are activated in preferred directions, but not in others during the delay period (Rao *et al.* 1999, 2000; Vijayraghavan *et al.* 2007). Blockade of $GABA_A$ receptors resulted in loss of directional selectivity in neuron firing due to disinhibition that resulted in increased activity in all directions (Rao *et al.* 2000). Normal GABAergic activity in the PFC may help in tuning or sharpening the directional selectivity to a specific direction/location or 'memory field' and reduced GABAergic activity results in the increase in activity in non-preferred directions. Therefore, D1R-mediated enhancement of GABAergic inhibition may sharpen the tuning of pyramidal neurons in the PFC to focus activity on task-related items (Seamans *et al.* 2001). Furthermore, Vijayraghavan *et al.* (2007) showed that low-level D1R stimulation enhances spatial tuning by suppressing responses to non-preferred directions. Our data suggest that in FXS, the absence of long-lasting D1R-mediated inhibition may lead to disinhibition within the microcolumnar organization in PFC, which may result in a lack of focus of prefrontal cortical mechanisms to the task at hand (Rao *et al.* 2000; Seamans *et al.* 2001). A weak 'D1 state' may result in a lack of a dominant network representation or memory field during mnemonic tasks, allowing a multiple network representation that is easy to disrupt (Durstewitz *et al.* 2000; Seamans & Yang, 2004).

Overall, we speculate that the impairment in PFC functioning in FXS patients will depend in part on the attenuation of DA-mediated modulation of inhibitory processes and this results in an overall dampening of DA-mediated neuromodulation of inhibitory synaptic transmission.

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Author contributions

K.P., C.L.C. and D.V. designed the study. K.P. conducted all electrophysiological experiments and analysed the data. D.V. did the Western Blots and data analysis. K.P., D.V. and C.L.C. wrote the manuscript. The study was undertaken in the laboratory headed by C.L.C.

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