Dopamine Release and Uptake Impairments and Behavioral Alterations Observed in Mice that Model Fragile X Mental Retardation Syndrome

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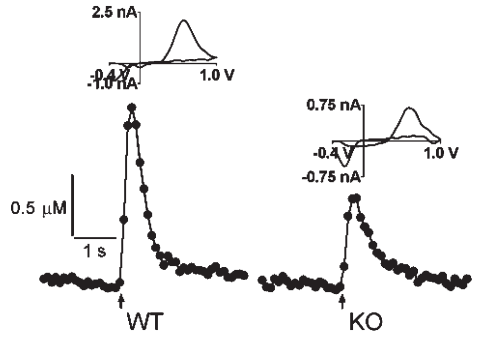
Abstract



In this study, we evaluated the relationship between amphetamine-induced behavioral alterations and dopamine release and uptake characteristics in *Fmr*1 knockout (*Fmr*1 KO) mice, which model fragile X syndrome. The behavioral analyses, obtained at millisecond temporal resolution and 2 mm spatial resolution using a force-plate actometer, revealed that *Fmr*1 KO mice express a lower degree of focused stereotypy compared with wild-type (WT) control mice after injection with 10 mg/kg (ip) amphetamine. To identify potentially related neurochemical mechanisms underlying this phenomenon, we measured electrically evoked dopamine release and uptake using fast-scan cyclic voltammetry at carbon-fiber microelectrodes in striatal brain slices. At 10 weeks of age, dopamine release per pulse, which is dopamine release corrected for differences in uptake, was unchanged. However, at 15 (the age of behavioral testing) and 20 weeks of age, dopamine per pulse and the maximum rate of dopamine uptake was diminished in *Fmr*1 KO mice compared with WT mice. Dopamine uptake measurements, obtained at different amphetamine concentrations, indicated that dopamine transporters in both genotypes have equal affinities for amphetamine. Moreover, dopamine release measurements from slices treated with quinpirole, a D2-family receptor agonist, rule out enhanced D2 autoreceptor sensitivity as a mechanism of release inhibition. However, dopamine release, uncorrected for uptake and normalized against the corresponding predrug release peaks, increased in *Fmr*1 KO mice, but not in WT mice. Collectively, these data are consistent with a scenario in which a decrease in extracellular dopamine levels in the striatum result in diminished expression of focused stereotypy in *Fmr*1 KO mice.

Keywords: Fragile X syndrome, dopamine, amphetamine, focused stereotypy, striatum, voltammetry

Fragile X syndrome (FXS) is the most common known genetic cause of mental retardation with an occurrence of approximately 1 in 1250 males and 1 in 2500 females (*1*). Moreover, an estimated 33% of individuals with FXS are formally diagnosed with autism (*2*). The underlying genetic cause of FXS is an expansion of a CGG repeat in the promoter region of the fragile X mental retardation 1 (*Fmr1*) gene, which resides on the X chromosome. This expansion silences the expression of the fragile X mental retardation protein (FMRP) (*3*, *4*) thereby resulting in subsequent impairment of brain development.

The most common behavioral manifestations of FXS, particularly in males, who tend to be more severely affected by the full mutation, include ADHD-like symptoms as well as communication and visuomotor control deficits (*5*−*8*). Furthermore, FXS commonly shares several behavioral features with autism, including stereotypic, repetitive behaviors that resemble those of obsessive-compulsive disorder (OCD), amplified sensitivity to various types of sensory stimuli, and social anxiety (*5*, *8*, *9*). The development of the *Fmr*1 KO (knockout) mouse (*10*), in which the *Fmr*1 gene is inactivated, has provided an animal model that can be used to investigate underlying pathophysiological mechanisms

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associated with FXS as well as evaluate potential therapeutic treatments. Like humans with FXS, *Fmr*1 KO mice have shown an increased susceptibility for seizures (*11*−*13*). In regard to cognitive and affective domains, however, correlations with human FXS are not as well-defined. For example, only mild deficits at most were observed in spatial learning tasks measured using the Morris water maze or radial arm maze (*10*, *14*−*18*). It has been suggested that this discrepancy arises from the fact that these tasks evaluate learning and memory rather than impaired attention and inhibitory control, which make up the most prominent cognitive deficits in FXS (*19*).

Several different mechanisms, acting individually or in concert, may influence brain development in FXS. Recently, it has been suggested that excessive induction of hippocampal (*20*) and cerebellar (*21*) long-term depression (LTD) results in impaired neural development of *Fmr*1 KO mice. In the striatum, the activation of D2 family dopamine (DA) receptors, acting in combination with group I mGluRs, L-type calcium channels, and CB1 cannabinoid receptors, is a required step for the induction of one form of LTD (*22*−*25*). Thus, given the high abundance of DA in the striatum (*26*), it is not surprising that alterations in DA regulation may influence striatal synaptic plasticity and subsequent brain development (*27*, *28*). Moreover, DA receptor blockers (antipsychotics) have been used to treat stereotypy and hyperactivity (*29*), behaviors that have been associated with FXS (*30*, *31*) and autism (*32*−*35*). Therefore, DA signaling abnormalities can potentially influence the expression of anomalous behaviors.

Amphetamine (AMPH), a DA uptake inhibitor and releaser (*36*), has often been used as a pharmacological tool to probe for behavioral phenotypic differences between knockout mice and their full genome counterparts (*37*−*41*). Use of this drug to induce dopamine-related behavioral changes in mice is not straightforward because lower doses (e.g., 1−2 mg/kg) produce substantial locomotor stimulation but higher doses (8−10 mg/kg) inhibit locomotion and at the same time induce an excited state characterized by repetitive small-to-medium very rapid movements of the head, forelimbs, and trunk, a syndrome referred to as focused stereotypy (*38*, *42*). After still higher doses (15 mg/kg and above, especially with repeated dosing), self-mutilation of forepaws or digits may be seen in some mouse strains (*43*, *44*). In the context of using an AMPH probe in *Fmr*1 KO mice, to our knowledge only two studies are available in the literature (Ventura et al. (*40*), 2 mg/kg; Zupan and Toth (*41*), 2 and 4 mg/kg), and neither used a dose in the focused stereotypy range. Our experiment reported here used both locomotion-increasing doses of AMPH and a focused stereotypy-inducing dose of AMPH (but below the self-mutilation threshold) along with a behavioral measurement methodology (a force-plate actometer) that can distinguish between and quantify both types of behavior.

There is broad acceptance that the expression of AMPH-induced locomotion is caused in part by elevation of DA levels in the ventral striatum while AMPH-induced stereotypy results from increased levels of it in the dorsal striatum (*45*, *46*). Microdialysis studies in ambulatory *Fmr*1 KO mice in which AMPH was administered to *Fmr*1 KO mice reveals differential neurochemical effects on the brain: in the prefrontal cortex, DA outflow is *greater* in *Fmr*1 KO mice than WT (wild-type) control mice, while in the striatum DA outflow is *less* in *Fmr*1 KO mice compared with WT mice (*40*). Thus, DA regulation in the striatum also appears to be altered in *Fmr*1 KO mice; however, the origin of these alterations and their impact on behavioral expression in response to AMPH are not well characterized.

In this work, fast-scan cyclic voltammetry at carbon-fiber microelectrodes (FSCV) was used to investigate the electrically evoked release and uptake of DA in striatal brain slices taken from *Fmr*1 KO mice and WT mice. These neurochemical measurements were compared with behavioral measurements of locomotion and focused stereotypy obtained using a force-plate actometer, a behavioral device that measures variation in behavior-induced reactive forces even while the subject is not locomoting (*47*).

Results and Discussion

Behavior: Locomotor Activity

In regard to locomotor activity, expressed as distance traveled (see Figure 1), a three-way ANOVA indicated the following: no *main effect* genotype, $F(1,40) = 0.17$, $p > 0.05$; a main effect of AMPH dose, $F(3,40) = 46.77$, $p < 0.01$; a genotype-by-dose interaction, $F(3,40) = 3.09$, $p < 0.05$; a repeated injection effect, $F(1,40) = 37.51$, $p < 0.01$; a genotype-by-injection interaction, $F(3,40) = 7.33$, $p < 0.01$; a dose by injection interaction, $F(3,40) = 9.06$, $p < 0.01$; and a genotype-by-dose-by-injection interaction, $F(3,40) = 5.80$, $p < 0.01$. The significant interactions between genotype and the other two independent variables show that there were significant differences between the two genotypes but those differences depended on the dose administered or prior experiences with AMPH or both. To clarify where in the distance-traveled data space the differences between WT and KO mice occurred, post hoc tests on cell means were calculated (see symbols accompanying data points in panels A and B in Figure 1 and statistics in the caption).

Distance Traveled in the Absence of AMPH. The *Fmr*1 KO mice traveled significantly more distance than the WT mice after both vehicle injections

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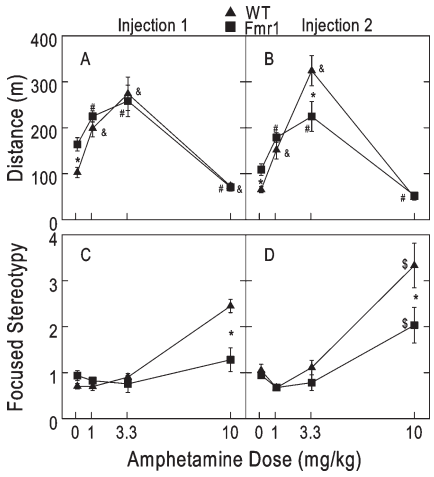

Figure 1. Induction of locomotor activity and focused stereotypy by *d*-amphetamine sulfate in *Fmr*1 knockout and WT control mice. Separate groups of mice ($n = 6$) were used at each dose. Data points are group means, and the brackets indicate ± SEM. The four symbols, asterisk (∗), ampersand (&), pound sign (#), and dollar sign ($), reflect significant differences between means as determined by *t* tests conducted after initial three-way ANOVA. The ∗ indicates significant differences between wild-type (WT) and *Fmr*1 knockout mice at the indicated doses. The & designates significant differences between vehicle and each dose of amphetamine for WT mice for a given injection, while # marks differences between vehicle and each amphetamine dose administered to the *Fmr*1 mice. The $, used in panel D only, designates differences between injection 1 and injection 2 for a given genotype. (A) Distance traveled−Injection 1: (∗) WT vs *Fmr*1KO at dose 0, $t_{10} = 4.16$, $p < 0.01$; (&) WT 0 vs 1.0 mg/kg, $t_{10} = 4.42$, $p < 0.01$; 0 vs 3.3 mg/kg, $t_{10} = 4.50$, $p < 0.01$; and 0 vs 10.0 mg/kg, $t_{10} = 2.34$, $p < 0.05$ (a decrease); (#) *Fmr*1 0 vs 1.0 mg/kg, $t_{10} = 3.24$, $p < 0.01$; 0 vs 3.3 mg/kg, $t_{10} = 2.53$, $p < 0.05$; and 0 vs 10.0 mg/kg, $t_{10} = 5.50$, $p < 0.01$. (B) Distance−Injection 2: (∗) WT vs *Fmr*1 KO at dose 0, $t_{10} = 2.98$, $p < 0.01$; WT vs *Fmr*1 at dose 3.3 mg/kg, $t_{10} = 6.29$, $p < 0.001$. (&) WT 0 vs 1.0 mg/kg, $t_{10} = 4.12$, $p < 0.01$; 0 vs 3.3 mg/kg, $t_{10} = 7.69$, $p < 0.01$; (#) *Fmr*1 0 vs 1.0 mg/kg, $t_{10} = 4.25$, $p < 0.01$; 0 vs 3.3, $t_{10} = 3.32$, $p < 0.01$; 0 vs 10.0 mg/kg, $t_{10} = 4.25$, $p < 0.01$ (a decrease). (C) Focused Stereotypy−Injection 1: (∗) WT vs *Fmr*1 at dose 10.0 mg/kg, $t_{10} = 2.897$, $p < 0.05$. (D) Focused Stereotypy−Injection 2: (∗) WT vs *Fmr*1 at dose 10.0 mg/kg, $t_{10} = 3.33$, $p < 0.01$; ($) injection 2 vs injection 1, WT, $t_{10} = 2.60$, $p < 0.05$; *Fmr*1, $t_{10} = 3.04$, $p < 0.05$.

(panels A and B, dose = 0, in Figure 1). A sampling of the pertinent literature reporting on locomotor activity differences in WT and *Fmr*1 KO mice is generally consistent with our results (*40*, *41*, *48*−*54*). Methods included multiple brands of photobeam actometers, manual counting of line crossings, and computer scored video tracking. Some authors reported nonsignificant differences between WT and KO mice (*51*−*53*), but the graphic data presented in the reports indicated that the *Fmr*1 KO mice were numerically higher than the WT mice. From the nine papers referenced above, we used the published graphic data to estimate the *Fmr*1 KO distance traveled as a proportion of WT performance for 12 independent experiments involving the first exposure to the apparatus. The average proportion of WT control was 1.23 (95% CI; 1.098−1.353). With our data for injection 1 (proportion of control = 1.60) included in the computation, the mean was 1.25 (CI; 1.23−1.39). Lesser differences between WT and KO appeared to be associated with shorter recording sessions (as short as 5 in.) or with the C57BL/6 mouse as a major contributor (50% or entirely) to the background strain in which the *Fmr*1 mutation was expressed (*51*−*53*). An exception to the latter statement is work that used a C57BL/6 background and a 10-min assessment session (*48*), which yielded a proportion of WT control = 1.35.

Distance Traveled after AMPH Treatment. For injection 1, each dose group, regardless of genotype, exhibited a significant AMPH effect such that the two lower doses increased and the highest dose decreased distance traveled (Figure 1, panel A). At none of the three AMPH doses was there a statistically reliable difference. The dose effect pattern after injection 2 was similar to that of injection 1, with two exceptions. At the 3.3 mg/kg AMPH dose, *Fmr*1 KO mice were significantly lower than the WT mice, and at the 10.0 mg/kg dose, WT mice were not significantly lower than the WT mice under vehicle conditions, probably owing to the low distance traveled by the WT mice at the vehicle dose after injection 2. Two previously published reports (*40*, *41*) examined the effects of 2.0 mg/kg AMPH on locomotor activity in *Fmr*1 KO mice (on an FVB background), and neither study found a significant difference between KO and WT mice. This discrepancy (i.e., both injections of 1.0 mg/kg significantly increased activity in the current study) may be the result of the enhanced temporal and spatial resolution of our force-plate method. The control mice were established from the line of *Fmr1* KO mice used for this study and are recommended by the vendor. Therefore, differences in response to AMPH caused by differences in environmental conditions or by genetic drift cannot be ruled out completely. However, it is worth noting that the significantly lower activity by the *Fmr*1 KO mice compared with the WT mice at the 3.3 mg/kg AMPH dose (Figure 1, panel B) is similar to the results obtained previously at 4.0 mg/kg (*41*).

Behavior: Focused Stereotypy

AMPH dose−effect data for focused stereotypy are shown in Figure.1 (panels C and D). The three-way ANOVA of focused stereotypy measurements indicated significant effects as follows: a main effect of genotype, $F(1,40) = 8.94$, $p < 0.01$; a main effect of dose, $F(3,40) = 46.77$, $p < 0.01$; a genotype-by-dose interaction, $F(3,40) = 7.53$, $p < 0.01$; a main effect of repeated injections, $F(1,40) = 10.19$, $p < 0.01$, and an AMPH dose-by-injection effect, $F(3,140) = 5.92$, $p < 0.01$. Genotype-by-injection and genotype-by-dose-by-injection interactions were not significant. Post hoc comparisons

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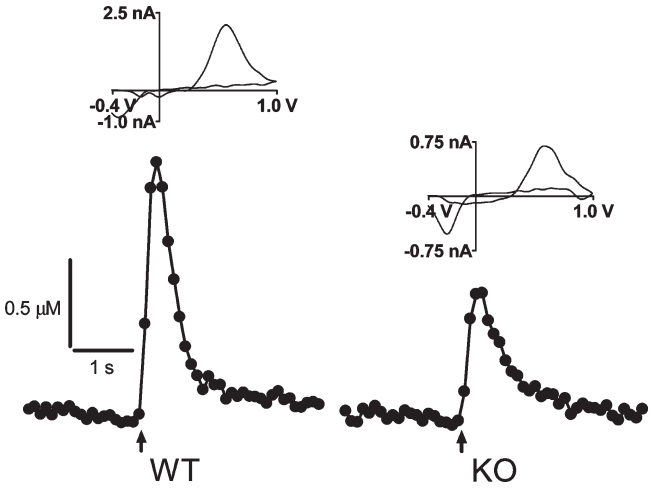

Figure 2. DA release is impaired in 15 week-old *Fmr*1 KO mice. Representative plots of electrically stimulated DA release obtained in brain slices from a 15 week-old *Fmr*1 KO mouse and an age-matched WT control mouse. The application of a single, biphasic electrical stimulus pulse (4 ms total duration, 350 μA current) is denoted by the arrow under each plot. Cyclic voltammograms, provided above each plot, confirm the presence of DA.

(see Figure 1, panels C and D and corresponding caption information) indicated significantly greater focused stereotypy in the WT than in the *Fmr*1 KO mice on both injection days at the 10.0 mg/kg dose, with the group mean of the WT mice falling about 50% higher than the KO mice. Moreover, additional comparisons showed that for both mouse types, focused stereotypy was significantly higher after injection 2 relative to injection 1. Although injection 2 produced more focused stereotypy than was induced by injection 1, the 1−2 rise in stereotypy was about equal in both mice types. These data suggest that the *Fmr*1 KO mice have a diminished high-dose response to AMPH. For the focused stereotypy measure, AMPH dose effects were not detected when the 10.0 mg/kg dose groups were removed from the analysis (three-way post hoc ANOVA, statistics not shown), showing that the WT−KO difference in focused stereotypy was primarily at the 10.0 mg/kg dose. To our knowledge, there are no prior reports describing the effect of a stereotypy-appropriate dose of AMPH on *Fmr*1 KO mice. However, one report (*48*) that relied on consecutive beam breaks in a photobeam actometer as a measure of "stereotypy" showed that undrugged *Fmr*1 KO mice displayed more such beam breaks than WT mice. It is unlikely that the behavior underlying the "stereotypy" reported by Hayashi et al. (*48*) is related to amphetamine-induced focused stereotypy, and some contemporary authorities (*55*) say that photobeam breaks poorly measure stereotypy "and should never be used."

DA Release in Dorsal Striatum

Previous evidence suggests that focused stereotypy expression is strongly influenced by increased extracellular DA levels in the dorsal striatum (*46*). Therefore, the differences in AMPH-induced stereotypic behavior between KO and WT mice prompted us to determine DA release and uptake parameters in striatal brain slices from KO and WT mice. FSCV was used to generate plots of stimulated DA release and uptake (Figure 2). Curve modeling software was then used to calculate DA released per stimulus pulse ($[DA]_p$), which is peak DA release corrected for uptake and electrode performance, and V_{max}, the maximum rate of DA uptake (Figure 3). Values obtained from *Fmr*1 KO mice were normalized against corresponding WT values. At 10 weeks of age, no significant difference in $[DA]_p$ was noted between KO mice ($n = 7$) and WT mice ($n = 7$). This finding is consistent with voltammetric measurements obtained previously in *Fmr*1 KO mice (*56*). However, at 15 (KO $n = 6$ and WT $n = 6$) and 20 (KO $n = 10$ and WT $n = 10$) weeks of age, $[DA]_p$ was diminished in KO mice compared with WT mice ($p < 0.01$ and $p < 0.001$, respectively). Similarly, V_{max} was the same in KO and WT mice at

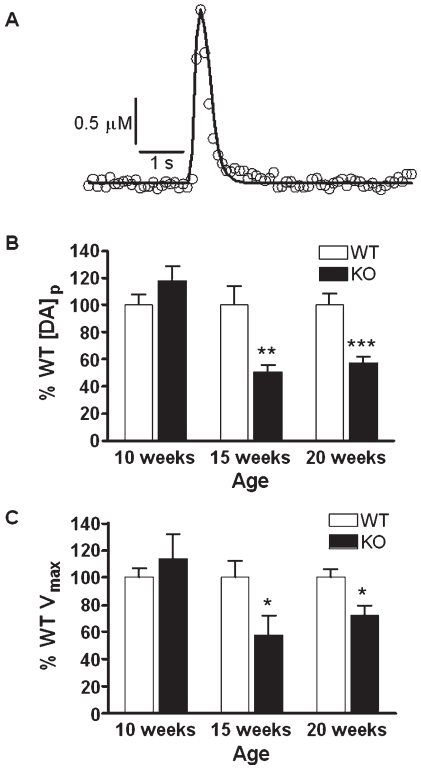

Figure 3. $[DA]_p$ and V_{max} are impaired in 15- and 20-week old KO mice compared with aged-matched WT control mice. (A) The open circles represent the experimental data, while the line shows the model fit to the data. (B) When the stimulated DA release of the KO mice were compared with that of the WT mice, a significant difference in the release was seen at 15 and 20 weeks of age. (C) When the data were modeled, V_{max} was also determined and the KO were compared with the WT. A significant decrease in V_{max} was seen in the 15 and 20 week old mice. Statistics: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (10 weeks, $n = 7$ WT and 7 KO mice; 15 weeks, $n = 6$ WT and 6 KO mice; 20 weeks, $n = 10$ WT and 10 KO mice).

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Table 1. Catacholamine Content in Striatal Lysates from KO and WT Mice[a]

	KO			WT		
age (weeks)	DA (ng/g)	DOPAC (ng/g)	HVA (ng/g)	DA (ng/g)	DOPAC (ng/g)	HVA (ng/g)
10	10166 ± 623 (n = 5)	1077 ± 91 (n = 5)	1336 ± 79 (n = 5)	9529 ± 329 (n = 7)	1154 ± 91 (n = 7)	1536 ± 81 (n = 7)
15	9934 ± 1051 (n = 5)	764 ± 81 (n = 5)	1177 ± 113 (n = 5)	8796 ± 637 (n = 6)	805 ± 38 (n = 6)	1483 ± 186 (n = 6)
20	9375 ± 624 (n = 5)	1294 ± 382 (n = 5)	2546 ± 596 (n = 5)	9653 ± 967 (n = 4)	1419 ± 250 (n = 4)	1481 ± 111 (n = 4)

[a] No significant differences in the content of DA, DOPAC, or HVA were noted between KO and WT. The n-values in parentheses indicate number of mice.

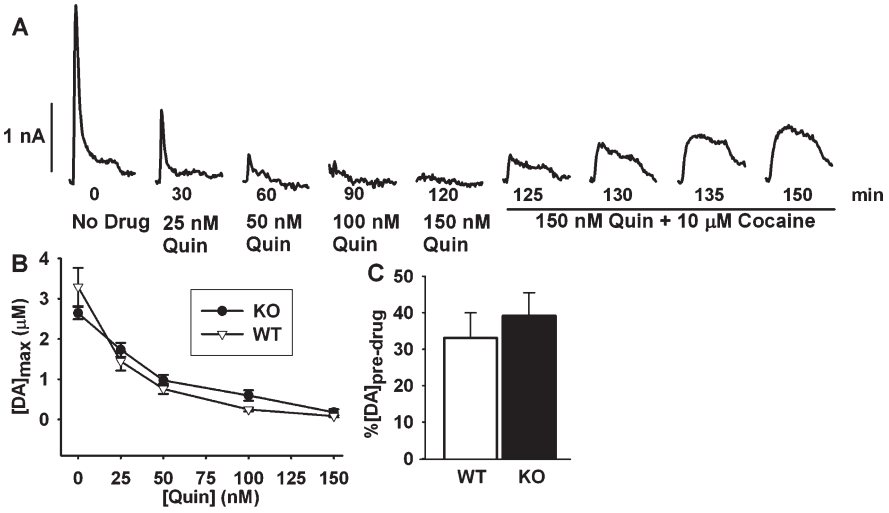

Figure 4. D2 autoreceptor activation and cocaine-induced reserve pool activation were similar in WT and KO mice. (A) Representative data collected in a striatal brain slice from a 20 week old WT mouse. The slice was exposed to cumulatively increasing concentrations of quinpirole while electrically evoked DA release was measured every 5 min. After DA release disappeared, cocaine was added to the slice with the quinpirole to mobilize reserve pool DA. (B) Quinpirole inhibited DA release similarly in WT and KO mice (ANOVA). (C) The percent DA recovery post quinpirole and cocaine showed no difference in the KO and WT mice.

10 weeks of age but was decreased in KO mice at 15 and 20 weeks of age. It is not known why impairments in release and uptake develop later in life in KO mice; however, this finding may imply that neurochemical changes occur into adulthood in humans with fragile X syndrome.

A potential underlying cause for the decrease in $[DA]_p$ noted in the older KO mice is a diminishment in the amount of DA available for release. Therefore, striatal catecholamine content was measured using high-performance liquid chromatography (HPLC) with electrochemical detection (*57*). Total DA content was the same between age groups and genotype (n = 4−7 mice; Table 1). Moreover, no differences were noted in the striatal content of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), two metabolites of DA. Therefore, even though sufficient DA stores are present in the KO striatum, these stores are not released as effectively as in the WT mice.

D2 Autoreceptor Function

Presynaptic D2 autoreceptors are G-protein coupled receptors that inhibit DA synthesis and release upon activation (*58*). To determine whether D2 autoreceptor overactivation causes decreased $[DA]_p$ in KO mice, striatal brain slices from 20-week old mice were treated with increasing concentrations of quinpirole, a D2 agonist (*59*). After treatment with the maximum quinpirole concentration of 150 nM, DA release disappeared (Figure 4A,B). No significant interactions between drug concentration and genotype were found (ANOVA, $p > 0.05$, KO n = 4 and WT n = 6); therefore, overactivation of D2 autoreceptors is not a likely underlying cause of decreased release in KO mice.

An important aspect of D2 autoreceptor function is inhibition of DA synthesis. Continuous treatment with quinpirole and the concurrent application of periodic electrical stimulus pulses should deplete terminals of releasable DA, leaving only reserve pool DA. To identify potential differences in reserve pool mobilization and content, cocaine (final concentration of 10 μM) was added to the slices in addition to the quinpirole (150 nM) already present, and the stimulation regimen of a series of 50 pulses applied at 10 Hz every 5 min was continued. The purpose of using the extended pulse sequence as

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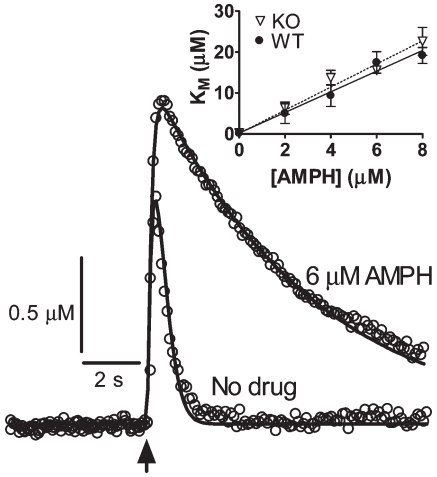

Figure 5. DA uptake is similarly inhibited in the KO and WT striatum. The stimulated release plots (○) were modeled (—) to determine K_M for DA uptake during a cumulative treatment regimen. Average values of K_M at each AMPH concentration were determined and plotted (inset). There was no significant interaction between genotype and K_M or between genotype and AMPH concentration (ANOVA).

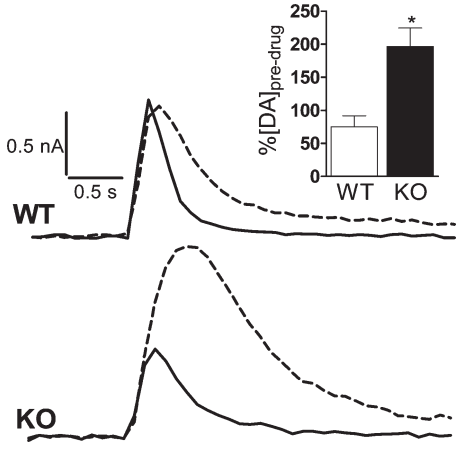

Figure 6. AMPH enhances stimulated DA release in brain slices from KO mice but not in those from WT mice. Representative plots of stimulated DA release are shown for WT and KO mice before (—) and after (– – –) treatment with 6 μM AMPH. After AMPH administration, stimulated DA release increased, relative to predrug values, in KO mice but not in WT mice (inset). Statistics: ∗, $p < 0.01$, t test, $n = 4$ WT and $n = 4$ KO mice.

opposed to single pulses was to facilitate the depletion of releasable DA. Cocaine has been shown to mobilize a synapsin-dependent DA reserve pool (*60*, *61*). Therefore, as expected, within 5 min, the reserve pool vesicles were mobilized, and the stimulated release peak reappeared. After 30 min of cocaine treatment, KO and WT stimulated release peaks, expressed as a percentage of the predrug peak, were compared (Figure 4C). These values did not differ significantly (student's t test, KO $n = 4$ and WT $n = 6$, $p > 0.05$), indicating that similar amounts of reserve pool DA were mobilized in KO and WT mice.

Neurochemical Response to AMPH

To compare the DA uptake efficiencies between KO and WT mice, brain slices from both genotypes were treated with successively increasing concentrations of AMPH, which inhibits DA uptake through the DA transporter (DAT). Drug response curves were then obtained by plotting the average K_M values against AMPH concentration. Linear regression analyses reveal that the slopes for the KO and WT curves both deviate significantly from zero ($p < 0.001$, $n = 4$ KO and $n = 4$ WT mice), and analyses by two-way ANOVA show that there is no significant interaction between genotype and AMPH concentration (Figure 5). Therefore, differences in behavioral activation due to AMPH administration likely do not arise from differences in the affinity of AMPH for DAT.

Further analyses of these data revealed no difference in $[DA]_p$ between KO and WT mice at any given concentration of AMPH (data not shown); however, a concern with these release values was that genotype-related differences in $[DA]_p$ could be masked by the high degree of heterogeneity of dopaminergic innervation within the striatum. To account for this variability, we used a separate experimental approach in which DA release was sampled from four locations in the dorsolateral caudate and averaged, both before and after treating the slice with 6 μM AMPH (a concentration that provided a large increase in K_M but did not appear to cause diminished release). However, DA release plots obtained in this way were not amenable to modeling because V_{max} changes when the working electrodes are moved to other locations within the striatum. Because curves generated from these measurements were not modeled, the DA release values were uncorrected for uptake. For each slice, the peak DA release value after AMPH treatment was expressed as a percentage of average release prior to treatment (% no drug release). Slices were averaged to obtain a value for each mouse. These data revealed that % no drug release was significantly greater in KO mice than in WT mice ($p < 0.01$, t test, $n = 4$ WT and $n = 4$ KO mice; Figure 6) and indicate that AMPH treatment enhanced DA release in slices from KO mice but not in slices from WT mice. The raw values of DA release following AMPH treatment were 1.50 ± 0.31 μM in KO mice and 0.58 ± 0.46 μM in WT mice ($p < 0.05$, t test, $n = 4$ WT and $n = 4$ KO mice).

AMPH empties DA from presynaptic terminals and vesicles. To do this, it first enters terminals either through membrane-bound DAT protein molecules or by lipophilic diffusion through the membrane. AMPH that enters through the DAT causes allosteric translocation of the protein and induces the reverse transport of DA from the cytosol to the extracellular space (*62*–*64*).

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Additionally, AMPH displaces vesicular DA into the cytoplasm where it can then be released by reverse transport (*65*, *66*). Thus, the availability of DAT protein molecules for both AMPH entrance and DA reverse transport should impact extracellular DA levels in AMPH-injected mice.

Our data show a decrease in V_{max} in KO mice at 15 and 20 weeks of age. According to Michaelis−Menten kinetics, V_{max} is directly proportional to the number of functioning DAT protein molecules (*67*) ($V_{max} = k_{cat}[E]_t$ where k_{cat} is a rate constant and $[E]_t$ is the total enzyme concentration). This means that less transporter protein molecules function properly in KO mice compared with WT mice. Logically, our voltammetric data are consistent with a scenario in which AMPH cannot enter and DA cannot leave terminals in the *Fmr*1 KO striatum as readily as in the WT striatum. Therefore, DA is able to reside within terminals and vesicles for a longer period of time in the KO striatum and is, therefore, available for release in the event of an action potential. These neurochemical results are consistent with those of Ventura et al. (*23*), who found that AMPH induces a smaller increase in extracellular striatal DA levels in KO mice compared with WT mice. More importantly, however, these neurochemical results agree with our behavioral data in the case of 15-week old KO mice, which showed diminished expression of focused stereotypy compared with aged-match WT mice.

Conclusion

To our knowledge, this is the first published study to utilize the high-resolution force-plate actometer to provide quantitative measurements of behavior in *Fmr*1 KO mice in comparison to WT control mice, and it is the first study to evaluate a dose of AMPH (10.0 mg/kg) that previous literature has shown to be appropriate for inducing focused stereotypy in WT mice. This behavior measurement method has the utility of being able to identify and quantify rodent behavior even when the animal is not locomoting. In addition to confirming that *Fmr*1 KO mice are significantly hyperactive compared with WT mice under vehicle conditions, our results reveal a significant difference in behavioral response to AMPH injection. In particular, *Fmr*1 KO mice exhibited muted expression of AMPH-induced focused stereotypy compared with WT mice. Our DA release plots support the concept that this resistance is potentially influenced not only by an impairment in the ability of these mice to release DA but also by decreased AMPH-induced efflux of DA from terminals in the *Fmr*1 KO striatum. Moreover, because these neurochemical differences were found only in mice 15 weeks of age or older, it may be that changes in DA release and uptake occur into adulthood of individuals with fragile X syndrome. Collectively, these findings suggest that altered striatal DA regulation influences behavioral expression in mature *Fmr*1 KO mice. Therefore, these results lend support to the idea that modulating selected aspects of dopamine signaling may be therapeutically useful in controlling FXS symptoms.

Methods

Animals

All animal procedures were conducted in accordance with protocols approved by the Animal Care and Use Committee of the University of Kansas. Fragile X knockout mice, FVB.129P2-*Fmr1*^{tm1Cgr}/J (*10*), and wild-type control mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The wild-type mice were derived from FVB.129P2-*Fmr1*^{tm1Cgr}/J mice and are recommended by The Jackson Laboratory for use as controls. These mice were received at approximately 8 weeks of age and were housed in the University of Kansas Animal Care Unit (ACU) under a temperature-regulated (70 ± 2 °C), humidity-controlled (50% ± 25%) environment with a 12 h light/dark cycle (lights on at 6:00 a.m., lights out at 6:00 p.m.). The mice had unrestricted access to food and water, except during behavior assessment procedures. Behavioral testing was conducted between 10:30 a.m. and 5:00 p.m.

Drugs

d-Amphetamine sulfate, quinpirole, and cocaine were purchased from Sigma-Aldrich (St. Louis, MO).

Behavior

Behavioral Apparatus. Behavioral comparisons between KO and WT mice were conducted with eight force-plate actometers (*47*) having 28 cm × 28 cm square floors. Each actometer was located in a sound-attenuating chamber (about 30 dB) equipped with a silent, vibration-free ventilation system. The enclosure prevented ambient light from entering so that the behavioral measurements were conducted with the mice in the dark. Center of force was tracked 100 times/s with a spatial resolution of less than 2 mm. The vertical force associated with both locomotor and nonlocomotor movements was also recorded and analyzed. For determination of distance traveled (a commonly used metric for locomotor activity in rodents), the distances between consecutive center of force coordinates 0.5-s apart were summed. This measure accentuates locomotion and reduces the contribution of small, high-frequency movements to the distance variable. Because the force-plate method actively senses the behavior of a mouse even while the animal is "staying in one place", the instrument has been used to quantitate focused stereotypy (*42*) induced by central nervous system stimulants, such as AMPH (*68*) or cocaine (*69*). The FS score was a combination of spatial confinement (staying in one place, not ambulating) and vigor of activity (in mice especially head, limb, and trunk movements) while confined (initially labeled "focused stereotypy" by Rebec and Segal, *42*). The first step in obtaining the FS score was to divide the 28 × 28 cm^2 floor area into 256 equal-size square sectors. The percent of time within a 1-min interval that a mouse occupied each of the 256 sectors was calculated.

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The FS score was computed in the following way: (1) the percent time in a given sector was multiplied by the variance in the vertical force component for the same sector; (2) these products were summed across all 256 sectors (note that if no time was spent in a given sector, then the product was zero and did not contribute to the sum of the products); and (3) the sum of the products was then divided by the number of sectors occupied (i.e., number of squares with percent time >0) in the 1-min period to yield the FS score. The FS score for a 60-min period was the mean of the 60 1-min scores. By concurrently measuring the locomotion of the mouse on the actometer floor and the forcefulness of the mouse's movements at that location, this methodology provides data that can circumvent the tendency of some investigators to erroneously label near complete suppression of rodent locomotor activity by psychostimulants as a "calming" or sedative-like effect (*37*). During the expression of psychostimiulant-induced focused stereotypy, locomotion is greatly inhibited but the drugged rodent is nevertheless "excited" or highly "aroused", which is confirmed by our measurements here and by direct observation (*38*).

Behavioral Procedures. Fifteen-week-old *Fmr*1 KO mice and age-matched WT control mice were placed in the actometers for two 1 h recording sessions conducted 10 days apart. A few seconds before sessions, separate groups of $n = 6$ mice each received ip injections of saline vehicle or 1.0, 3.3, or 10.0 mg/kg of *d*-amphetamine sulfate. In experimental design terminology, this was an analysis of variance (ANOVA) with two genotypes, four doses, and two injections as a repeated measures variable. Therefore, the procedure dictated the use of 24 WT and 24 KO mice. Doses of AMPH were selected to produce locomotor stimulation at the two lower doses and focused stereotypy at the highest dose in WT mice. The 1.0 and 3.3 mg/kg doses of AMPH were chosen to be near the doses previously used to probe for differences in behavioral responses of WT and *Fmr*1 KO mice (*40*, *54*). The 10.0 mg/kg dose of AMPH was chosen because it is near a dose reported to induce focused stereotypy in mice (*38*, *44*) but avoids the higher doses that are associated with self-injurious behavior in some mouse strains (*43*, *44*)

Brain Slice Preparation

Striatal brain slices were prepared as described previously (*70*). Briefly, mice were anesthetized by inhalation of isoflurane gas and decapitated. The brain was removed immediately and placed into ice-cold artificial cerebrospinal fluid (aCSF) saturated with 95%O_2/5%CO_2 The aCSF solution consists of (in mM) 126 NaCl, 22 HEPES, 1.6 NaH_2PO_4, 2.5 KCl, 25$NaHCO_3$, 2.4 $CaCl_2$, 1.2 $MgCl_2$, and 11 D-glucose and was adjusted to a pH of 7.4. For support while slicing, a 1 cm^3 block of agar gel was glued to the support block. The cerebellum was removed with a razor blade and the brain was bisected. Next, the brain was glued to the support block with Roticoll 1 (Carl Roth, Karlsruhe, Germany), a quick-setting glue, and the buffer tray was then filled with ice-cold aCSF. The remaining half of the brain was dissected and the striatum was removed, placed on dry ice, and stored at -80 °C for HPLC analysis. Coronal slices 300 μm thick were made using a Leica VT1000 S vibrating-blade microtome (Leica Microsystems, Nussloch, Germany). Striatal slices obtained between 0.4 mm and 1.60 mm anterior to bregma were used for voltammetry analyses. The brain slices were stored in ice-cold aCSF saturated with 95%O_2/5%CO_2 until needed for use. Prior to experimentation, slices were placed in a perfusion chamber with aCSF continuously flowing (1 mL/min) and kept at a temperature of 34 °C. Slices were allowed to equilibrate in the perfusion chamber for at least 90 min before data collection. Drugs were introduced to slices through a three-way valve that allowed aCSF containing the chosen drug to flow from a separate reservoir to the slice chamber.

Fast-Scan Cyclic Voltammetry

Microelectrodes, with the exposed carbon-fiber tip having a diameter of 7 μm and a length of 30 μm, were constructed as previously described (*71*). Electrodes were calibrated before and after use by exposure to known DA concentrations in a flow cell. The stimulation electrodes were made by gluing two tungsten electrodes (A-M Systems Inc., Carlsborg, WA, USA) and adjusting the distance between the two tips using heat shrink (3 M Electronics, Austin, TX) until they were 200 μm apart. Electrochemical measurements were obtained using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) interfaced with a computer through a locally constructed breakout box and custom designed software written by M. L. A. V. Heien and R. M. Wightman, Department of Chemistry, University of North Carolina, Chapel Hill, NC.

For brain slice experiments, carbon fiber microelectrodes were inserted 100 μm below the surface of the brain slice between the two stimulus electrodes, situated in the dorsolateral caudate. To obtain most measurements, DA release was evoked by the application of a single, biphasic, electrical pulse (4 ms duration, 350 μA current). A series of 50 stimulus pulses applied at 10 Hz was used when evaluating the effects of quinpirole and cocaine. To detect released DA, a triangular waveform was applied to the carbon fiber microelectrode in which the potential was linearly scanned from a holding potential of -0.4 V to $+1.0$ V and back to -0.4 V at a scan rate of 300 V/s and an update rate of 10 Hz. A Ag/AgCl reference electrode was used. Currents measured prior to stimulation were subtracted from those measured during and immediately after stimulation to yield a cyclic voltammogram for DA. To measure DA release in the absence of drugs, recordings were collected from four different locations within the dorsolateral caudate region of each slice and averaged (*70*). This averaging approach decreased variability caused by the heterogeneous nature of dopaminergic innervation in the striatum.

Stimulated release plots were modeled using software written by R. M. Wightman, University of North Carolina, Chapel Hill, NC. From this modeling operation, kinetic parameters associated with dopamine release and uptake were calculated. These parameters include dopamine per pulse, $[DA]_p$, which is peak dopamine release corrected for electrode performance and reuptake, K_M, and V_{max}. The approach used here is similar to that applied previously to FSCV data obtained using brain slices (*70*).

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) experiments were carried out using a Shimadzu model LC 20 AD,

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(Shimadzu Corporation, Kyoto, Japan) with a dual reciprocating pump and a DGV 20A3 degasser. The system controller was a model CBM 20A. The detector was an ESA Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA) consisting of a guard cell and two analytical cells. The guard cell was set at 350 mV. The analytical cellswere set at −150 mV for the first detector and +220 mV for the second detector. Mobile phase consisted of 10% organic phase (acetonitrile) and 90% aqueous phase (containing 75 mM sodium dihydrogen phosphate monohydrate, 100uL/L triethylamine, 25 μM EDTA tetrasodium tetrahydrate, and 1.7 mM 1-octanesulfonic acid sodium salt) adjusted to a pH of 2.8 with 80% phosphoric acid. Chromatographic separations were carried out isocratically using a 150 mm ×3.2 mm ESA MD-150 column. The flow rate was set to 0.4 mL/min.

Animals were euthanized, and the striatum was removed and stored at −80 °C until analysis. For analysis, tissue was prepared by homogenizing in 0.500 mL of 0.2 N perchloric acid containing 200 ng/mL DHBA as internal standard. Homogenate was centrifuged at 14 000 rpm for 20 min at 4 °C. Supernatant was removed and filtered through a 0.2 μm syringe filter prior to injection in the HPLC instrument. Standard curve solutions were prepared immediately prior to each HPLC run by dissolving pure substance in 0.2 N perchloric acid containing 200 ng/mL internal standard and diluting by serial dilution. Nine concentrations were used for each compound (1000, 500, 200, 100, 50, 25, 10, 5, and 1 ng/mL).

Statistical Analyses

Statistical analyses were performed using either student's *t* test or ANOVA. A value of $p < 0.05$ was considered significant. Values were expressed as average ± SEM. The behavioral data consisted of two dependent variables: distance traveled and focused stereotypy score. As a first step in the statistical analysis of each variable, a three-way ANOVA was performed (genotype [WT vs KO] by AMPH dose [four levels] by injection number [first or second], with repeated measures on the injection factor). When significant interaction effects were obtained in the three-way analyses, post hoc *t* tests were used to compare individual cell means (*72*).

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Author Contributions

M.A.J. conceived of the project; M.A.J., S.C.F., and J.L.F. designed the research approach; J.L.F., B.E.O., S.K.W., and A.N.O. performed electrochemical experiments; M.A.J and J.L.F. analyzed electrochemical data and performed statistical analyses on these data; J.L.F and E.V. performed behavioral experiments; S.C.F. analyzed behavioral data and performed statistical analyses of these data; M.A.J., J.L.F., and S.C.F. wrote the manuscript.

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Abbreviations

aCSF, artificial cerebral spinal fluid; AMPH, amphetamine; COC, cocaine; DA, dopamine; $[DA]_p$, dopamine per pulse; DAT, dopamine transporter; FMRP, fragile X mental retardation protein; FSCV, fast scan cyclic voltammetry; FXS, fragile X syndrome; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; KO, knockout; LTD, long-term depression; OCD, obsessive-compulsive disorder; WT, wild-type.

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