Supplemental Figures

Figure S1

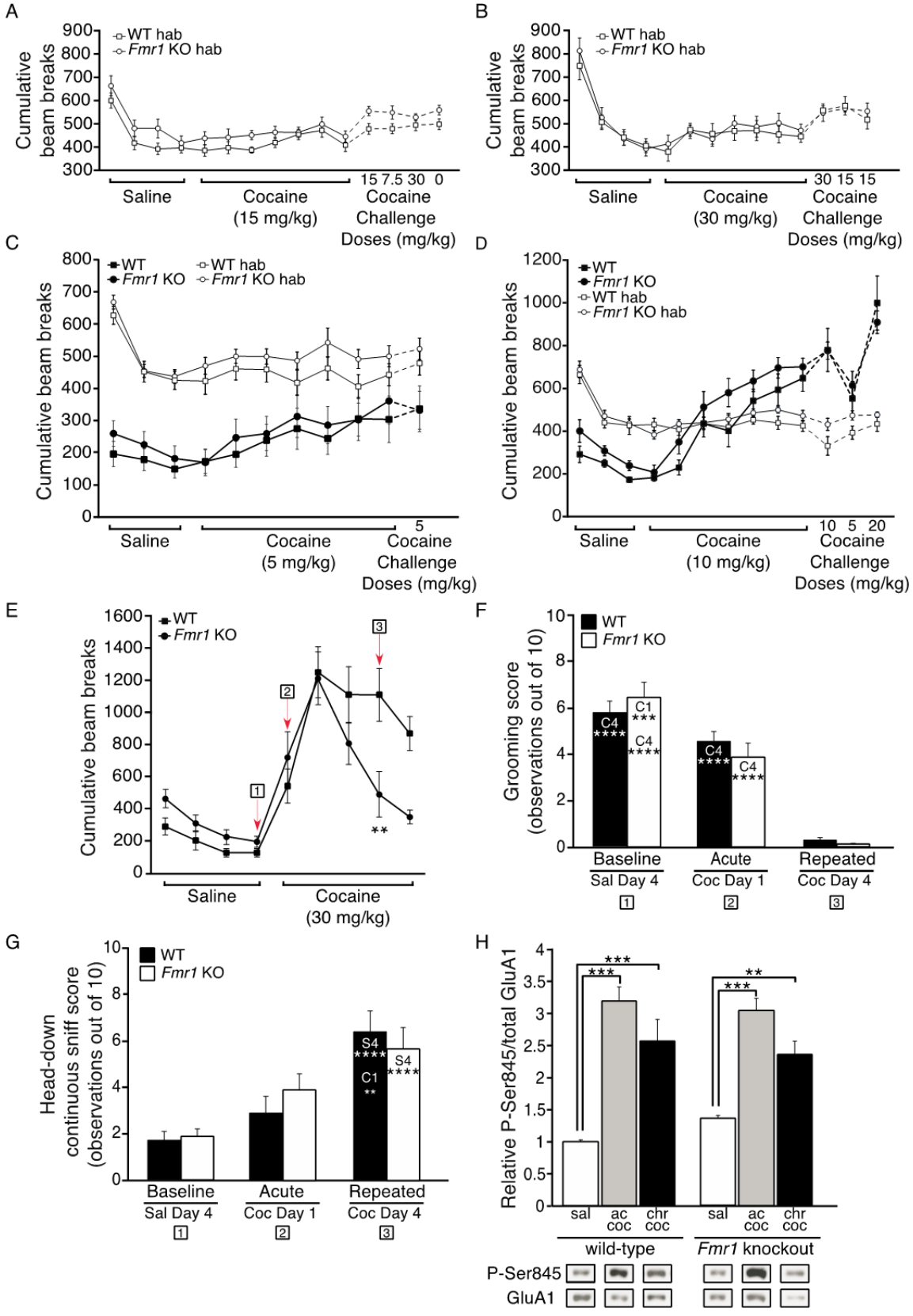

Fig. S1, Related to Figure 1. During locomotor sensitization, no significant differences between genotypes were observed in the habituation trials (daily trials prior to injection) for either the (A) 15 mg/kg (n=11 KO, 13 WT) or (B) 30 mg/kg cocaine dosing paradigms (n=10 KO, 10 WT). (C) Repeated low-dose cocaine (5 mg/kg) induced similar locomotor sensitization in both *Fmr1* KO ($p<0.01$) and WT ($p<0.05$) littermates (Bonferroni comparisons, COC Day 1 vs 7; n=12 KO, 17 WT), and there was no difference between genotypes at challenge, suggesting *Fmr1* KO mice are not hypersensitive to cocaine. Habituation activity (thin lines) also did not differ between genotypes. (D) Similarly, *Fmr1* KO and WT littermates did not differ in locomotor response to repeated or acute cocaine at 10 mg/kg (n=11 KO, 8 WT). Neither did activity on habituation trials during this test differ. (E) Cumulative beam breaks during saline and 30 mg/kg stereotypy analysis (first 30 min of injection trials, including time points 1, 2 and 3, as indicated). Locomotion did not differ between groups at time points 1 and 2, but was significantly reduced in *Fmr1* KO mice at time point 3, as expected (n=12 KO, 12 WT; see also Fig. 1D). (F) Grooming observed after injection each day decreased with cocaine exposure similarly in each genotype. (G) The stereotyped behavior of head-down sniffing (>5 sec continuously) increased similarly in each genotype over cocaine exposures. (H) Absence of FMRP did not alter cAMP-dependent phosphorylation of the GluA1 S845 site 20 min following either acute (1 d) or chronic (5 d) exposure to cocaine at a behaviorally relevant dose (20 mg/kg; n=14 KO SAL, 7 KO AC, 8 KO CHR, 14 WT SAL, 6 WT AC, 8 WT CHR). In F & G, significant Bonferroni post-hoc tests are indicated. In H, significant follow-up Tukey HSD post hoc analyses are indicated. Column abbreviations denote within-group differences of the labeled bar compared to noted day (S4=SAL day 4, C1=COC day 1, C4=COC day 4). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; data shown are mean±S.E.M.)

Figure S2

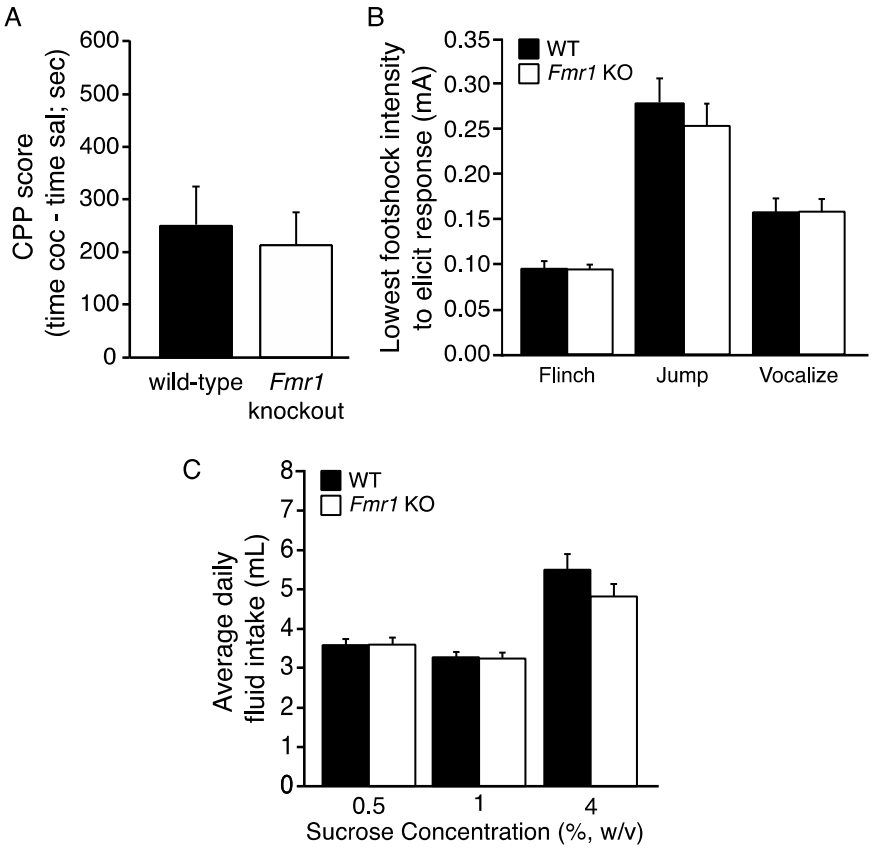

Fig. S2, Related to Figure 2. (A) For CPP, *Fmr1* KO and WT littermates did not differ in preference shown to a lower dose of cocaine (5 mg/kg; n=12 KO, 9 WT). (B) Lack of differences in fear conditioning were not due to disparate pain thresholds, as the lowest foot shock intensities to elicit common behavioral responses were not different between genotypes. (C) They also did not differ in overall fluid intake in two-bottle choice preference for three different concentrations of sucrose solution. (Data shown are mean ± S.E.M.)

Figure S3

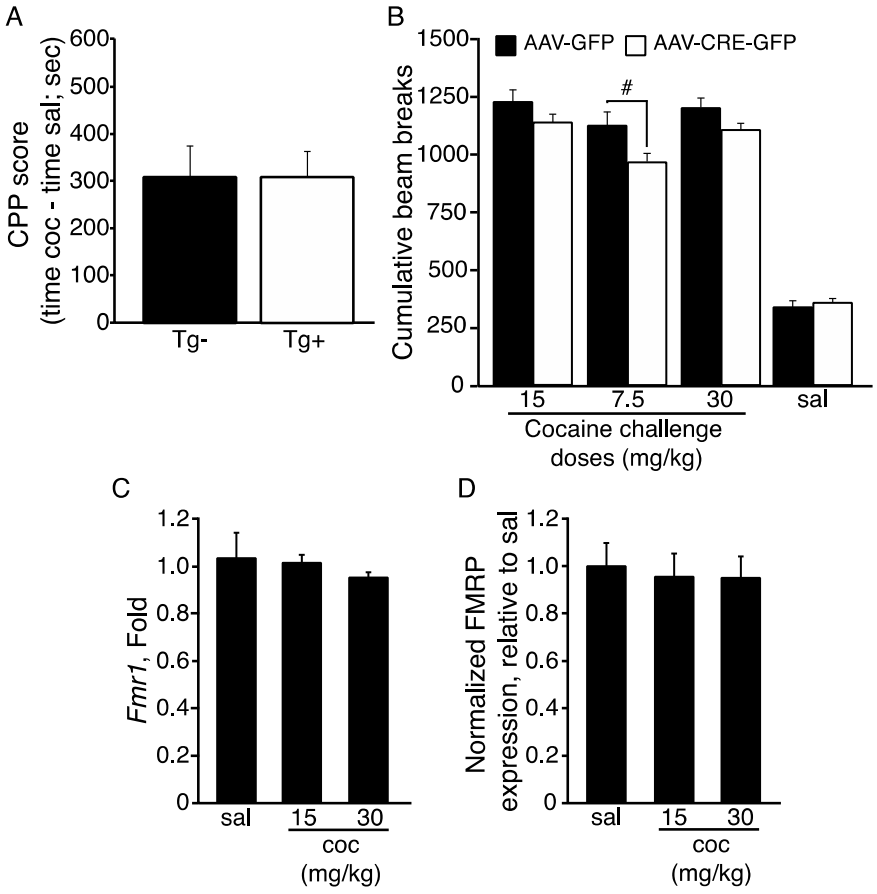

Fig. S3, Related to Figure 3. (A) Floxed-*Fmr1* conditional KO mice that were either positive (Tg+) or negative (Tg-) for cre-recombinase in neurons expressing the dopamine transporter (DAT-Cre) show equivalent cocaine CPP (7.5 mg/kg; n=18 Tg+, 18 Tg-). (B) Responses of Floxed-*Fmr1* conditional KO mice injected with AAV-CRE-GFP and AAV-GFP to cocaine (15, 7.5, 30 mg/kg) and saline challenges following cocaine sensitization (15 mg/kg; n=18 CRE, 22 GFP). When we examined WT mice administered five (1x/daily) injections of either 15 or 30 mg/kg cocaine compared to those injected with saline there were no differences in (C) *Fmr1* (n=9 SAL, 10 “15”, 10 “30”) or (D) FMRP expression (n=8 SAL, 8 “15”, 8 “30”) in the NAc 24 hours later. (Data shown are mean ± S.E.M.)

Figure S4

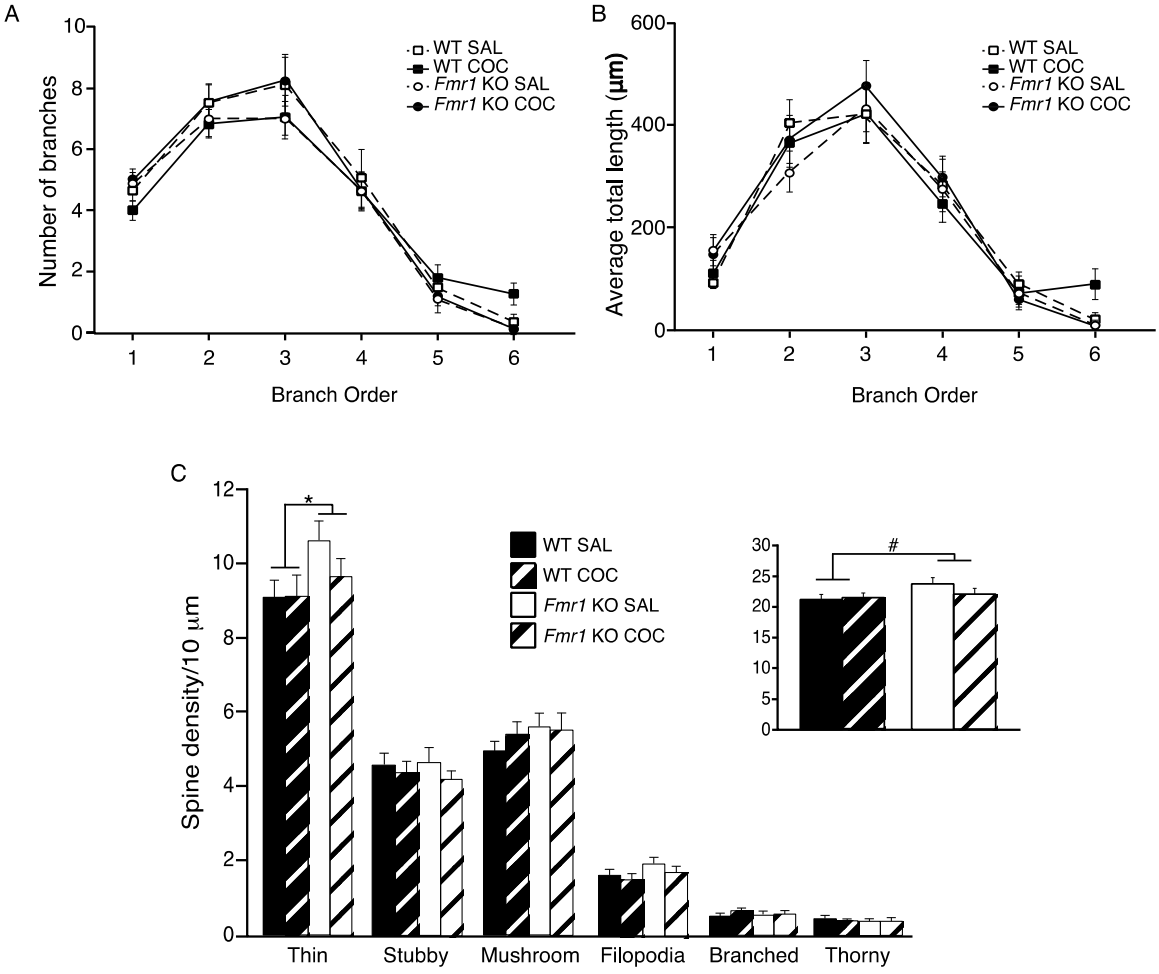

Fig. S4, Related to Figure 4. (A) For NAc shell MSNs, the number of branches was not altered significantly by genotype or treatment. (B) Similarly, the total length of NAc shell MSN branches was not altered by these factors. (C) *Fmr1* KO mice showed a trend toward greater overall spine density of NAc core MSNs compared to WT littermates 24 hrs after the last of seven 1x/daily doses of 15 mg/kg cocaine (figure inset). This effect of genotype was only evident in the thin spine subcategory, where *Fmr1* KO mice showed an increase over WT mice, regardless of treatment. Asterisk n C indicates follow-up MV ANOVA genotype simple main effect. ($^{\#}$ $p<0.10$, * $p<0.05$; data shown are mean ± S.E.M.)

Figure S5

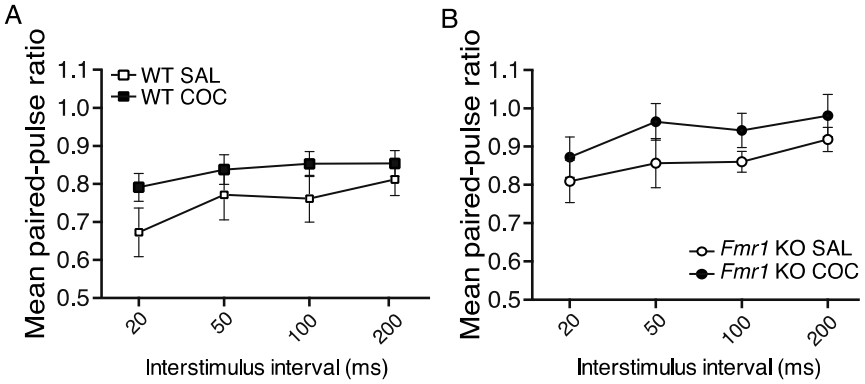

Fig. S5, Related to Figure 5. Paired-pulse ratios for (A) WT and (B) *Fmr1* KO NAc shell MSNs after treatment with saline or cocaine (5 d, 15 mg/kg + 24 hrs). Analysis over interstimulus intervals showed presynaptic release to be altered significantly by genotype (n, cells=9 KO SAL, 14 KO COC, 7 WT SAL, 11 WT COC), where *Fmr1* KO MSNs had significantly enhanced PPRs (reduced presynaptic release) compared to WT MSNs. There was also a trend towards a significant main effect of treatment, where cocaine mildly reduced presynaptic release compared to saline treatment. (Data shown are mean ± S.E.M.)

Table S1. Summary of Statistical Details & Results
See Supplementary Excel file.

Table S1. Summary of Statistical Details and Results. Description of statistical tests and results for all figures. Abbreviations: BO=branch order, br=branch, CHAL=challenge, dens=density, gen=genotype, HAB=habituation, HD=head down, IS=independent samples, ISI=interstimulus interval, LOC=locomotor, OS=orofacial stereotypy, PC=planned comparison, PPR=paired-pulse ratio, pts=points, sp=spine, treat=treatment, T-S=tone-shock. Brackets [ ] denote isolated levels of independent variables in follow-up analyses; prime (’) indicates use of Greenhouse-Geisser correction.

Supplemental Experimental Procedures:

Animals and drugs

Unless otherwise noted, mice were group-housed with same-sex littermates and maintained on *ad libitum* food/water under a 12-hr light cycle (lights on at 0700). All testing was performed in adult males (≥10 wks of age). For total KO studies, *Fmr1* heterozygous (HET) KO females were crossed with C57BL/6N males, and male *Fmr1*$^{-/y}$ and WT littermate offspring were used. For conditional KO (cKO) studies, floxed-*Fmr1* cKO females (homozygous or HET; kindly provided by David L. Nelson, Baylor College of Medicine) (Mientjes et al., 2006) were bred with C57BL/6N males, and *Fmr1*$^{fl/y}$ offspring were used. For the *DAT-Cre:Fmr1* cKO study, *Fmr1*$^{fl/fl}$ females were bred with *DAT-Cre* (Tg+) males, and *DAT-Cre(tg+):Fmr1*$^{fl/y}$ and *DAT-Cre(tg-):Fmr1*$^{fl/y}$ littermates were used. All behavioral tests, including different doses, were performed in separate cohorts of animals, except 1) NAc *Fmr1* conditional KO mice underwent CPP and sensitization (see timeline, Fig. 3B), and 2) mice used in food CPP were subsequently used for 5 mg/kg sensitization. Cocaine HCl (Sigma or Mallinckrodt) was dissolved in 0.9% NaCl (vehicle), and injection (i.p.) volume equaled 0.1 mL/10 g body weight.

Locomotor sensitization

Following 30 min to 1 hour of acclimation (inside home cages) to the behavioral ante-room, mice were placed individually into clean housing cages with a thin layer of fresh bedding placed inside of black Plexiglas chambers (22.5" x 14.5" x 9.4") equipped with photobeam arrays (San Diego Instruments, San Diego, CA). Activity was collected in five-minute bins via five beams evenly spaced across the cage length for two to three hours/day. After the first 30 minutes to 1 hour (habituation trial), an injection (i.p.) was given, and mice were immediately returned to the chamber. Mice received saline for the first 3-4 days, and cocaine (5, 10, 15 or 30 mg/kg) for the next seven days. After one (10, 15 and 30 mg/kg) or two weeks (5 mg/kg), a cocaine challenge was given at the same dose, followed by various additional challenges (see timelines). For the floxed conditional KO study, challenge withdrawal periods were 7d, 6-7d, 8-13d, 3-11d for the 15, 7.5, 30 and 0 mg/kg challenges, respectively. Data shown are the sum of total beam breaks over the first 20 or 30 minutes of each trial.

Stereotypy analysis

Stereotypy was assessed in two different experiments. Time points 1, 2, and 3 correspond to locomotor data shown in Fig. S1E. Time point 4 was the 15 mg/kg challenge given after 10 mos of cumulative withdrawal (Fig. 1D). During the indicated locomotor testing trials, mice were observed directly or video recorded for later observation. Observations took place in 10-30 sec bins at regular intervals over 30 minutes following injection, and the predominant behavior during each bin was scored using a published stereotypy scale (Spangler et al., 1997) (modified so that 6=intense sniffing or orofacial stereotypy in one location). Median scores for each mouse across bins were used to calculate group averages. In order to better define the increased stereotypy observed at the higher dose of cocaine, additional time-sampling was performed for a number of individual behaviors during these trials, including inactivity, grooming, locomotion, rearing, head-up and head-down sniffing, and orofacial

stereotypies (taffy pulling and paws-to-mouth). For these analyses, mice were observed in 1-minute bins in the same intervals as previously and received a score of 0 or 1 per behavior per bin, depending on the behavior's absence/presence. "Inactivity," head-up sniffing," "head-down sniffing" and "paws-to-mouth" had to be observed continuously for at least five seconds to be scored as present. "Locomotion" was defined as crossing the lengthwise midline of the cage. Scores for each individual behavior were summed over bins per mouse and used to calculate group averages.

Cocaine conditioned place preference

Mice were acclimated each day $\geq$1 hour (inside home cages) to the behavioral ante-room. Three-chambered, unbiased CPP apparatuses were used under dim white lighting. Chambers were homemade, having two large conditioning chambers (one gray with large wire grid flooring and one black-and-white striped with small wire grid flooring) connected by a smaller distinct chamber (white with metal bar flooring), and used commercial software and photobeams (Med-Associates, St. Albans, VT). A change in institutions meant that one experiment was performed with commercial chambers (Med-Associates, St. Albans, VT), also having two large conditioning chambers (one black with bar flooring and one white with wire grid flooring) connected by a smaller distinct chamber (gray with Plexiglas flooring). These chambers were carefully piloted to ensure pretest preferences were not biased toward one chamber. At pretest (Day 1), mice were placed into the center and allowed to explore all three chambers for 20 minutes. Groups were balanced so that they had a similar pre-existing preference score for the cocaine-paired chamber and so that cocaine was paired with each chamber similarly across groups. On conditioning days, partitions were lowered and mice were given an injection (i.p.) before placement into one of the large chambers for 30 minutes. Conditioning occurred over four days, where cocaine (7.5 mg/kg) was paired with one chamber (Day 2), then saline with the opposite chamber (Day 3), etc. On Day 6 (posttest), the pretest protocol was repeated. Data are expressed as time spent in the cocaine-paired chamber minus time spent in the saline-paired chamber (CPP or preference score) during the posttest. Food CPP differed in that mice were food restricted (free access to food for only four hrs each day) starting two days prior to the experiment and continuing throughout the test. Mice were also given two days of exposure to the high fat diet (Days 1 & 2; Teklad Custom Diet; 42% from fat; TD.88137) in their home cage prior to the pretest (Day 3). Food conditioning occurred over 12 days, where high fat diet and regular chow were paired with a chamber on alternating days, as described for cocaine and saline above. The food CPP posttest took place on Day 16.

Fear conditioning

On Day 1 (training), mice were placed in a fear conditioning chamber inside an opaque sound-attenuating box for six minutes, where they received three 30 second white-noise tones that each co-terminate with a two second (0.5 mA) shock and which were separated by one minute intervals. The "shock+" context included presence of shock-grid flooring, fan noise, house light, while specific odors were omitted. On Day 2 (contextual testing), mice were returned to the same context for five minutes, without shock or tone, and freezing was scored every 4-5 sec. On Day 3 (cued testing), mice were placed in the chamber after contextual alterations were made, and freezing was measured over the course of six minutes, the first three minutes without tone and the last three with

continuous tone. Contextual alterations included insertion of a smaller plastic box inside the chamber (which covered the shock-grid flooring and the walls), vanilla scent and fluorescent lighting, while fan noise was omitted. Freezing on all days was hand-scored by an observer blind to genotype. After all tests were concluded, mice were placed in the original conditioning chamber in order to assess foot shock sensitivity. Intermittent foot shocks of increasing intensity were given, and the lowest shock intensities to elicit flinching, jumping and vocalizations for both groups were measured.

Sucrose preference

Animals were individually housed starting five days prior to the beginning of the experiment. Two plastic centrifuge tubes (50 mL) capped with small double-balled sipper tube stoppers were placed on each cage. Prior to the first test, animals underwent an acclimation period, where both tubes were filled with water for two days, then with 1% sucrose for two days. Testing started the next day, and each test consisted of four 24-hour periods of access to water in one tube and a sucrose solution in the other. Animals underwent three total testing phases (1%, 4% and then 0.5% sucrose solutions), with breaks of 5-10 days of normal water access between each test. The positions of the tubes were swapped daily throughout acclimation and testing. Fluid levels were marked on the tubes at the same time each day and distance between marks was measured; consumption was calculated based on a conversion of 1 mm = 0.54 mL.

Stereotaxic surgery

Floxed-*Fmr1* mice were anesthetized with ketamine (100 mg/kg; Fort Dodge, Fort Dodge, IA) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, IA) and received either AAV2-GFP or AAV2-CRE-GFP (1 uL/hemisphere; Vector Biolabs, Philadelphia, PA) bilaterally (D/L -4.4, M/L +1.5, A/P +1.6). Injections were given over 10 min (0.05 uL/30 sec) with an additional 10 min before needles were retracted. CPP conditioning began 20-21 days following surgery (Fig. 3B).

Tissue collection and processing

For protein and mRNA studies, mice were decapitated, and brains were rapidly removed onto a culture plate on ice. Coronal slices (1 mm) were prepared using a mouse brain matrix. Bilateral punches were taken from 1-2 slices containing the NAc (16 gauge for rostral, starting Bregma ~1.94 mm, and 14 gauge for caudal, starting Bregma ~1.10 mm) using the anterior commissure as a guide. Dorsal striata were hand-dissected bilaterally from a single section (starting Bregma ~1.10). The entire dissection was performed on ice using chilled equipment, then samples were snap frozen in a dry ice/ethanol bath and stored at -80°C. Tissue from one hemisphere was used for assessment of RNA and the other for protein. For Western blotting, tissues were sonicated (30% amplitude) in a small amount of sucrose lysis buffer containing inhibitors (11% sucrose, 0.005 M HEPES, 1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 0.1 uM cyclosporin A, 0.1 uM okadaic acid, 1 mM PMSF, 1 mM EDTA, 1X Roche EDTA-free protease inhibitor tablet), boiled at 98°C for 10 min, centrifuged briefly and frozen at -80°C until protein quantification and SDS-PAGE. Tissue for RT-PCR was processed as previously described (Hale et al., 2011). For Golgi staining, whole brains were incubated for two weeks in Solution A+B of the FD Rapid Golgi Staining Kit following decapitation; manufacturer's instructions were followed for all staining steps (FD

Neurotechnologies, Catonsville, MD). Slices (150 um) were prepared on a cryostat (Leica CM3050 S, Leica Microsystems) and mounted on 1.0 mm gelatin-subbed slides (SouthernBiotech, Birmingham, AL). For immunohistochemistry, mice were deeply anaesthetized with chloral hydrate (~60 mg/mouse; Sigma) and perfused intracardially (5 mL/min) with 1X PBS (7 min), then 4% paraformaldehyde (PFA) prepared in 0.2 M PB (15 min). Brains were postfixed in 4% PFA overnight with gentle shaking at 4°C, then transferred to 30% sucrose + 0.02% NaN_3 for cryoprotection until sectioning (performed after brains had sunk). Serial sections (30 um) were prepared on a microtome (Leica SM2000 R, Leica Microsystems), with every 6^{th} section collected in the same well of a 24-well plate containing 1X PBS + 0.02% NaN_3 and were protected from light at 4°C until staining.

cAMP signaling

Fmr1 KO and WT male littermates were given five (1X/daily; i.p.) injections. Exposure was considered either acute (saline on days 1-4 and either saline or cocaine on day 5; 20 mg/kg) or chronic (either saline or cocaine on days 1-5; 20 mg/kg), and mice were sacrificed 20 minutes after the last dose. Tissue was collected as described and prepared for Western blotting.

Western blotting

Protein quantification was assessed by modified-Lowry using the DC Protein Assay Kit (Biorad, Hercules, CA). SDS-PAGE gels (8-10%) were loaded with equal amounts of total protein/well (for P-GluR1 experiments, 8-9 ug/well; for FMRP regulation experiments, 40 ug/well). Proteins were transferred to PVDF membrane overnight at 40V at 4˚C. PVDF membranes were blocked with 5% nonfat milk in 1X TBS-Tween 20 (0.05%; TBS-T), then rinsed briefly in 1X TBS-T (except TBS without Tween was used prior to FMRP antibody incubation). Membranes were incubated with shaking either overnight at 4°C or 2 h at room temperature in 5% bovine serum albumen (BSA; unless noted otherwise) plus 0.02% NaN_3 with primary antibodies: P-Ser845 1:5000 (EPR2148, Millipore, Billerica, MA), GluR1 1:1000 (ab31232, Abcam, Cambridge, MA), beta tubulin 1:10,000 prepared in 3% BSA (Millipore, Billerica, MA; 05-661), FMRP 1:1500 or 1:2500 prepared in 0.1% nonfat milk in 1X TBS (no Tween) (Millipore; MAB2160). Membranes were washed in TBS-T, incubated with shaking for 1 hr at room temperature with HRP-conjugated anti-rabbit or mouse IgG (1:10,000; Calbiochem), washed and developed using standard enhanced chemiluminescence (ECL) and X-ray film.

Quantitative real-time PCR

RNA isolation, reverse transcription, and quantitative real-time PCR were carried out, as previously described (Hale et al., 2011). *Fmr1* expression was determined by running reactions of 5 ng of cDNA. Fold changes relative to *GAPDH* were determined using the ΔΔCt method, in which mean fold change ($2^{-\Delta\Delta CtAVE}$) and S.E.M. ($abs(((2^{-\Delta\Delta CtAVE} \times 2^{-\Delta\Delta CtSEM}) - (2^{-\Delta\Delta CtAVE} / 2^{-\Delta\Delta CtSEM})) / 2)$) were determined. The primers used to amplify *Fmr1* RNA were 5'-TGTTTTGGTCCACTTTTCCAG-3' (forward) and 5'-CTTCCCTGAACTCTGCATCC-3' (reverse). The primers used to amplify *GAPDH* were 5'-AGGTCGGTGTGAACGGATTTG-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse).

Dendritic analyses

Slide-mounted Golgi-stained sections were viewed on an Olympus B51 upright microscope outfitted with a Z encoder (Heidenhain, Schaumburg, IL) and Neurolucida software Version 9 (MBF Bioscience, Williston, VT). Unobstructed MSNs of the NAc core or shell (2-6/sub-region/animal) were traced bilaterally using a 40X objective. Branch order was assigned using the centrifugal method, and a single third-order or higher, complete terminal tip ($\geq$20 uM) was chosen from each neuron for spine analysis. Spines were categorized according to type (thin, mushroom, stubby, filopodial, branched, or thorny) using a 100X objective beginning at the last branch node and ending at the branch tip. Thin ($\leq$0.55 um) and mushroom (>0.55 um) spines were distinguished from stubby spines by the presence of a neck thinner in diameter than a head; thin and mushroom types were categorized based on previously established criteria (Jedynak et al., 2007). The stubby spine category included spines that are elsewhere considered "chubby" or "wide." Spines with multiple heads emerging from a single contact with the dendritic shaft were considered "thorny," as previously described (Velázquez-Zamora et al., 2011). Quantitative assessment of branching and spines was performed using NeuroExplorer software (MBF Bioscience).

Immunohistochemistry/injection placement

Free-floating sections were incubated in blocking solution (3-5% normal goat serum, 3% Tween-20, 1X PBS) with shaking for 1 hr at room temperature. Primary antibody (rabbit α-GFP; 1:200; Invitrogen) incubation was done overnight at 4°C with shaking. Sections were incubated in secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG; A-11008, Invitrogen) for 1 hr at room temperature with shaking while shielded from light. Sections were counterstained with DAPI (1:5000), mounted on SuperFrost Slides (Fisherbrand, Houston, TX), and coverslipped after air-drying using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). Data from mice with GFP staining outside of the NAc or that had little or no GFP staining in the NAc were removed from analysis.

Electrophysiology

After two daily injections of saline plus five daily injections of cocaine (15 mg/kg) or saline**,** mice were anesthetized with isofluorane and sagittal slices containing the NAc shell were prepared as previously described (Thomas et al., 2001). Slices recovered for 30 min in aCSF solution saturated with 95% O_2/5% CO_2 containing (in mm) 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$ and 11 glucose. For electrophysiological recordings, picrotoxin (100um) and lidocaine (0.7 mM) were added to aCSF to block GABAergic neurotransmission and prevent action potentials. Cells were visualized using infrared-differential contrast (IR-DIC) microscopy and medium spiny neurons (MSNs) were identified by their morphology and typical hyperpolarized resting potential (-70 to -80mV). Using an Axon Instruments Multiclamp 700A, MSNs were voltage clamped at -80 mV using electrodes (3-5 MΩ) containing (in mm) 117 cesium gluconate, 2.8 NaCl, 20 HEPES, 0.4 EGTA, 5 TEA-Cl, 2 MgATP, and 0.3 MgGTP, pH 7.2–7.4 (265–275 mOsm). Data were filtered at 2kHz by Axonclamp amplifier and digitized at 10 kHz via custom Igor Pro software (Wavemetrics, Lake Oswego, OR). At the beginning of each sweep,

a depolarizing step (4 mV for 100 ms) was generated to monitor series (10-40 MΩ) and input resistance (>400 MΩ). Data were collected in series of traces until >300 events were recorded. Synaptic events were detected via custom parameters in MiniAnalysis software (Synaptosoft, Decatur, GA) and subsequently confirmed by observer. For each event, amplitude and frequency was measured and used to determine average mean and construct probability plots. For AMPA/NMDA ratio measurements, afferents were stimulated at 0.1 Hz by glass monopolar microelectrodes placed at the border between the prelimbic cortex and NAc. Next, EPSC amplitudes were generated at +40 mV in the presence and absence of D-AP5. Evoked EPSCs in the presence of D-AP5 (AMPAR-EPSCs) were digitally subtracted from EPSCs generated in the absence of D-AP5 (AMPAR+NMDAR-EPSCs) via Igor Pro to generate NMDAR-EPSCs. The peak amplitude of AMPAR-mediated EPSCs was divided by the peak amplitude of NMDAR-mediated EPSCs to produce the AMPAR/NMDAR ratio. For Paired-Pulse Ratio (PPR) measurements, two EPSC amplitudes were generated at -80mV with the following inter-stimulus intervals: 20, 50, 100, and 200 msec. The peak amplitude of the second EPSC (P2) was divided by the peak of the first amplitude (P1) to generate the PPR ratio (P2/P1).