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β-arrestin2 couples metabotropic glutamate receptor 5 to neuronal protein synthesis and is a potential target to treat fragile X

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Summary

Synaptic protein synthesis is essential for modification of the brain by experience and is aberrant in several genetically-defined disorders, notably fragile X, a heritable cause of autism and intellectual disability. Neural activity directs local protein synthesis via activation of metabotropic glutamate receptor 5 ($mGlu_5$), yet how $mGlu_5$ couples to the intracellular signaling pathways that regulate mRNA translation is poorly understood. Here, we provide evidence that β-arrestin2 mediates $mGlu_5$-stimulated protein synthesis in the hippocampus and show that genetic reduction of β-arrestin2 corrects aberrant synaptic plasticity and cognition in the *Fmr1*$^{-/y}$ mouse model of fragile X. Importantly, reducing β-arrestin2 does not induce psychotomimetic activity associated with full $mGlu_5$ inhibitors, and does not affect G_q signaling. Thus, in addition to identifying a key requirement for $mGlu_5$-stimulated protein synthesis, these data suggest that β-arrestin2-biased negative modulators of $mGlu_5$ offer significant advantages over first-generation inhibitors for the treatment of fragile X and related disorders.

eTOC Blurb

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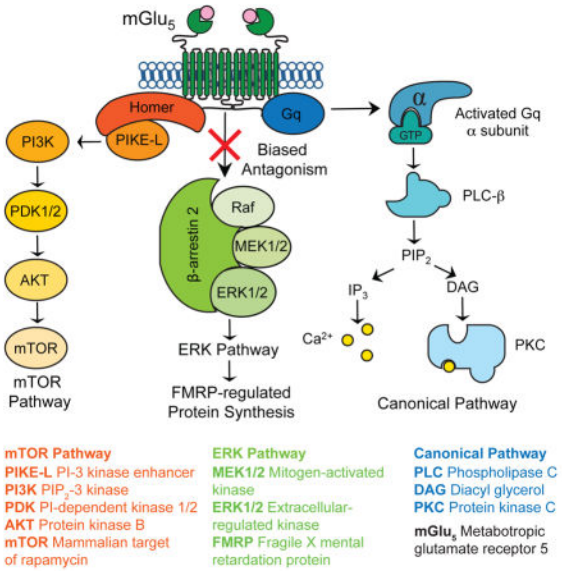
Author Contributions:

M.F.B. and R.J.L. conceived the project. R.J.L. provided the *Arrb* mutant mice and critical input. M.F.B. directed and coordinated the experiments. L.J.S. designed and performed biochemistry and behavioral experiments. B.D.A. and R.K.S. designed and performed electrophysiological recordings. R.K.S. designed and performed fluorescence-based calcium imaging experiments. A.R.P. designed analysis code in MATLAB to analyze hyper-locomotion experiments and assisted with behavioral experiments.

Stoppel et al. find that β-arrestin2 is a critical link between $mGlu_5$ and activity-dependent neuronal protein synthesis. Reducing β-arrestin2 levels corrects many synaptic and cognitive deficits in a mouse model of fragile X.



Keywords

metabotropic glutamate receptors; mGlu5; arrestins; β-arrestin2; fragile X; extracellular regulated kinase; ERK; synaptic protein synthesis; biased ligands; long-term depression; autism; intellectual disability

Introduction

Numerous genetic and molecular studies have demonstrated that poorly regulated synaptic protein synthesis downstream of metabotropic glutamate receptor 5 ($mGlu_5$) contributes to the pathophysiology of fragile X (FX), a genetic cause of intellectual disability (ID) and autism spectrum disorder (ASD) (Pop et al., 2014). This work suggests that targeting $mGlu_5$ or its downstream effectors may be a fruitful approach for improving the course of FX and other genetic syndromes with shared pathophysiology (Aguilar-Valles et al., 2015; Auerbach et al., 2011; Barnes et al., 2015; Bozdagi et al., 2010; Tian et al., 2015; Wenger et al., 2016). Indeed, $mGlu_5$-based therapies have been immensely successful at correcting FX in animal models (Bhakar et al., 2012). To date, however, the results of human clinical trials in FX using $mGlu_5$ negative allosteric modulators (NAMs) have been disappointing (Berry-Kravis et al., 2016; Scharf et al., 2015).

Although many factors contribute to the challenge of translating findings from animal models to humans, one factor that is common to all drug trials is the therapeutic window—the range of doses that can treat disease pathophysiology without causing negative side effects. In humans, for example, it has been reported that inhibition of $mGlu_5$ produces dose-limiting psychotomimetic effects (Abou Farha et al., 2014; Pecknold et al., 1982; Porter et

al., 2005). The first-generation $mGlu_5$ NAMs were identified based on their ability to inhibit G_q signaling mediated by phosphoinositide hydrolysis and release of Ca^{2+} from intracellular stores (Cosford et al., 2003; Gasparini et al., 1999; Lindemann et al., 2011). However, available data suggest alternative signaling pathways are central to the regulation of protein synthesis by $mGlu_5$ (Bhakar et al., 2012; Osterweil et al., 2010; Richter et al., 2015). Thus, it is possible that therapeutic effects can be enhanced and separated from side effects by selectively targeting the coupling of $mGlu_5$ to disease-relevant signaling pathway(s).

One pathway that is known to be central to $mGlu_5$-stimulated protein synthesis and FX pathophysiology culminates in activation of ERK1/2 and the phosphorylation of proteins involved in the regulation of cap-dependent mRNA translation (Banko et al., 2006; Osterweil et al., 2013; Osterweil et al., 2010). Activation of this pathway by $mGlu_5$ can occur independently of G-protein signaling, but how this is achieved has remained a mystery. As is the case for many seven-transmembrane domain receptors, G-protein signaling of ligand-bound $mGlu_5$ is terminated by recruitment of β-arrestin to the carboxyl tail of the receptor. In recent years it has become clear that β-arrestin recruitment can also trigger activation of alternative signaling cascades. Of particular relevance is the observation that β-arrestin2 recruitment to the angiotensin II receptor (which, like $mGlu_5$, is also G_q-coupled) stimulates the ERK1/2 pathway and increases mRNA translation rates in both human embryonic kidney 293 and rat vascular smooth muscle cells (Ahn et al., 2009; DeWire et al., 2008). We therefore hypothesized that β-arrestin2 comprises a crucial link between $mGlu_5$ and protein synthesis in neurons.

Results

Heterozygous deletion of β-arrestin2 disrupts $mGlu_5$ stimulated ERK activation and protein synthesis without affecting Gq-signaling

To determine the role of β-arrestin2 in $mGlu_5$-mediated protein synthesis, we stimulated hippocampal slices from male *Arrb2*$^{+/-}$ and wild-type (WT) littermates with a selective agonist and positive modulator of $mGlu_5$, 3-Cyano-N- (1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB,10 μM, 30 minutes), and measured the incorporation of ^{35}S-methionine/cysteine into new protein as described previously (Henderson et al., 2012; Osterweil et al., 2010). We found that $mGlu_5$ activation caused a parallel increase in protein synthesis (Fig. 1A) and ERK1/2 phosphorylation (Fig. 1B) in WT slices, which were both absent in slices from *Arrb2*$^{+/-}$ mice. This blunted response to $mGlu_5$ stimulation occurred in the absence of differences in basal protein synthesis rates or ERK phosphorylation levels (Fig. 1A, B). We failed to observe a comparable effect on stimulated protein synthesis in *Arrb1*$^{+/-}$ mice (Fig. S1), suggesting that β-arrestin2 is the relevant isoform for $mGlu_5$ signaling. From a therapeutic standpoint, it is noteworthy that $mGlu_5$-stimulated protein synthesis is abrogated in mice lacking a single allele of *Arrb2*; a full knockout is not required to see an effect.

β-arrestins have also been shown to participate in additional signaling cascades (DeWire et al., 2007), including the Akt-mTOR pathway that has been implicated in the regulation of protein synthesis (Hou and Klann, 2004) and the pathophysiology of FX (Gross et al., 2015; Sharma et al., 2010). However, in agreement with previous studies in the hippocampal slice

(Osterweil et al., 2010), we found that $mGlu_5$ activation failed to increase phosphorylation of Akt or ribosomal protein S6, a readout of mTOR activity, in WT mice. These measures of mTOR pathway activity were also unaffected in slices prepared from *Arrb1*$^{+/-}$ and *Arrb2*$^{+/-}$ mice (Fig. S2).

To assay the integrity of G_q signaling, we examined calcium mobilization in hippocampal slices from WT and *Arrb2*$^{+/-}$ animals using the cell-permeable calcium fluorescent dye Fluo4-AM. We found that a brief application of the agonist S-3,5-dihydroxyphenylglycine (DHPG, 25 μM, 1 min) to slices resulted in a rapid increase in Ca^{2+}-mediated fluorescence in area CA1 that was not significantly different between WT and *Arrb2*$^{+/-}$ slices (Fig. 1C–D). DHPG was employed in these experiments because it activates both of the G_q-coupled metabotropic glutamate receptors, $mGlu_1$ and $mGlu_5$. These DHPG-induced changes in calcium fluorescence were completely blocked by pretreatment with the phospholipase C inhibitor, U73122 (data not shown). These results indicate that a partial reduction in β-arrestin2 does not result in aberrant G_q signaling in response to $mGlu_5$ activation. Moreover, they suggest that modulation of $mGlu_5$ receptor-mediated protein synthesis can be dissociated from G-protein dependent signaling via manipulation of β-arrestin2.

Deficient $mGlu_5$-mediated translation impairs synaptic plasticity in the hippocampus of Arrb2$^{+/-}$ mutants

Activation of $mGlu_5$ results in a form of synaptic long-term depression (LTD) in the hippocampus that requires rapid *de novo* synaptic protein synthesis (Huber et al., 2000). We therefore investigated the functional relevance of the observed biochemistry by determining if genetic reduction of *Arrb2* also alters the expression and/or protein synthesis-dependency of LTD induced with DHPG (25 μM, 5 min) (Huber et al., 2001). Basal synaptic transmission was normal (Fig. S3), but LTD magnitude was significantly reduced in *Arrb2*$^{+/-}$ slices compared to WT (Fig. 1E–F). Consistent with previous observations, LTD in WT slices was significantly diminished in the presence of the protein synthesis inhibitor cycloheximide (CHX, 60 μM). In contrast, the residual LTD in slices from *Arrb2*$^{+/-}$ animals was unaffected by CHX (Fig. 1E–F). Therefore, we conclude that the protein synthesis-dependent component of $mGlu_5$-mediated LTD is absent in the *Arrb2*$^{+/-}$ hippocampus.

In WT mice, the LTD that remains when DHPG is applied in the presence of CHX is expressed via a presynaptic mechanism, revealed by a change in the paired-pulse ratio (Auerbach et al., 2011). This change in paired-pulse ratio after DHPG was similar in *Arrb2*$^{+/-}$ mice, indicating that this presynaptic, protein synthesis-independent mechanism of LTD is unaffected by reducing signaling through β-arrestin2 (Fig. S3). Another, mechanistically distinct form of hippocampal LTD can be induced by stimulating NMDA receptors. This type of LTD is expressed postsynaptically, but does not require ERK1/2 or immediate translation of mRNA. We found that it is also unaffected by genetic reduction of β-arrestin2 in the hippocampus (Fig. S3). Taken together, these results suggest that the diminished LTD magnitude observed in *Arrb2*$^{+/-}$ animals is likely a specific consequence of impaired $mGlu_5$-stimulated mRNA translation at the synapse.

Decreasing β-arrestin2 levels reverses synaptic and behavioral deficits in a mouse model of fragile X

Our results indicate that β-arrestin2 couples $mGlu_5$ activation to ERK-dependent protein synthesis and LTD. Aberrantly increased $mGlu_5$-dependent protein synthesis observed *in vivo* (Qin et al., 2005), brain slices (Dolen et al., 2007; Osterweil et al., 2010) and synaptoneurosomes (Henderson et al., 2012) is believed to be pathogenic in *Fmr1*-null mice (Bhakar et al., 2012; Dolen et al., 2007). Therefore, we investigated whether a genetic reduction of *Arrb2* in *Fmr1*-null mice could correct FX phenotypes. We crossed $Arrb2^{+/-}$ male mice to $Fmr1^{+/-}$ female mice and found that both the increased protein synthesis (Fig. 2A–B) and exaggerated mGlu-LTD (Fig. 2C–D) characteristic of $Fmr1^{-/y}$ mice were restored to WT levels in $Arrb2^{+/-}$ x $Fmr1^{-/y}$ mice.

We next investigated the possibility that restoration of normal protein synthesis and $mGlu_5$-dependent synaptic plasticity could lead to improvements in cognitive and behavioral assays previously shown to be impaired in $Fmr1^{-/y}$ mice. We assayed inhibitory avoidance, a hippocampus-dependent behavior known to be disrupted in $Fmr1^{-/y}$ mice (Dolen et al., 2007; Qin et al., 2002) (Fig. 3A). Memory strength was measured as the latency to enter the dark side of a box that was associated with a foot shock. We discovered that $Arrb2^{+/-}$ as well as $Fmr1^{-/y}$ mice failed to form a strong association between the context and foot shock (between time 0 and 6 hours) indicating impaired memory acquisition. This is consistent with previous results showing that both excessive and deficient hippocampal protein synthesis can manifest similarly at the behavioral level (Auerbach et al., 2011). Remarkably, however, $Arrb2^{+/-}$ x $Fmr1^{-/y}$ mice were indistinguishable from WT and exhibited normal memory acquisition and extinction over the course of 48 hours (Fig. 3B).

We also investigated non-aversive object recognition memory. Mice were first allowed to explore an arena with two identical objects for two sessions. The following day, one of the familiar objects was replaced with a novel object (Fig. 3C). While $Fmr1^{-/y}$ mice explored both the novel and familiar objects to an equal degree, indicating a severe impairment in novelty detection, $Arrb2^{+/-}$ x $Fmr1^{-/y}$ mice as well as $Arrb2^{+/-}$ single mutants showed a strong preference for the novel object similar to WT mice (Fig. 3D).

In an additional series of behavioral experiments, we investigated audiogenic seizures (AGS), as increased susceptibility to AGS is a hallmark phenotype in $Fmr1^{-/y}$ mice. Genetic reduction of *Arrb2* in *Fmr1*-null mice significantly attenuated seizure incidence (Fig. 3E), very similar to what has been observed using $mGlu_5$ and ERK-pathway inhibitors (Dolen et al., 2007; Osterweil et al., 2010; Yan et al., 2005).

Unlike first generation $mGlu_5$ NAMs, β-arrestin2 reduction does not exacerbate MK-801-induced hyperlocomotion

Our data suggest that the $mGlu_5$ signaling relevant to FX pathophysiology passes through β-arrestin2 to activate ERK and protein synthesis. If this conclusion is correct, then modulators that specifically target $mGlu_5$ coupling to β-arrestin2 might avoid side effects that arise from inhibition of G_q and/or mTOR pathway signaling. First-generation $mGlu_5$ NAMs were all identified based on inhibition of G_q signaling, and in humans one adverse side effect

reported following treatment with these compounds is derealization and visual hallucinations (Abou Farha et al., 2014; Pecknold et al., 1982; Porter et al., 2005). Similarly in mice, $mGlu_5$ NAMs exacerbate hyperlocomotion in response to treatment with the potent pyschotomimetic MK801 (Homayoun et al., 2004; Pietraszek et al., 2005). Therefore, we examined the effect of genetic reduction of β-arrestin2 and $mGlu_5$ NAM treatment on MK801-induced hyperlocomotion in mice. We confirmed that pretreatment with the selective $mGlu_5$ inhibitor 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-pyridine (MTEP) (Cosford et al., 2003) significantly potentiates MK801-induced hyperlocomotion in WT mice. However, we found that baseline locomotor activity was the same in *Arrb2*$^{+/-}$ and WT mice, as was the synergistic effect of MTEP pretreatment on MK801-induced hyperlocomotion (Fig. 4). The fact that MTEP continues to exacerbate hyper-locomotion in *Arrb2*$^{+/-}$ mice that lack $mGlu_5$-regulated protein synthesis suggests that the psychotomimetic effects of the NAM are mediated by inhibition of pathways unrelated to FX pathophysiology.

Discussion

It has been established previously that $mGlu_5$-stimulated protein synthesis and LTD are blocked by inhibitors of MEK and ERK (Banko et al., 2006; Gallagher et al., 2004; Osterweil et al., 2010; Schnabel et al., 1999), but are unaffected by inhibitors of PKC and PLC (Fitzjohn et al., 2001; Rush et al., 2002; Schnabel et al., 1999). It was also known that the ERK pathway is recruited even in the presence of PLC inhibitors (Fitzjohn et al., 2001; Gallagher et al., 2004; Huber et al., 2001). However, it was unknown how $mGlu_5$ can stimulate ERK and protein synthesis independently of G_q/PLC activation. We show here that reducing β-arrestin2 completely blocks $mGlu_5$-stimulated ERK activation, protein synthesis, and protein synthesis-dependent LTD, but has no effect on G_q-dependent mobilization of intracellular Ca^{2+} via PLC. Thus, β-arrestin2 couples $mGlu_5$ to the ERK signaling pathway and protein synthesis in neurons. This conclusion is in accordance with data on ERK pathway activation and the stimulation of protein synthesis by other G_q-coupled receptors in non-neuronal cells (Ahn et al., 2009; DeWire et al., 2008).

Our findings also are in general agreement with a contemporaneous investigation of β-arrestin involvement in $mGlu_1$ and $mGlu_5$ signaling in the hippocampus (Eng et al., 2016). This study confirmed our finding of impaired $mGlu_5$-dependent synaptic LTD and ERK pathway activation in mice lacking β-arrestin2. One difference is their finding that LTD induced with DHPG (unlike synaptic stimulation) was unaffected in *Arrb2*$^{-/-}$ mice. However, this discrepancy is likely accounted for by the fact that their slices were not sufficiently rested to observe the protein synthesis-dependent component of agonist-induced LTD (see (Osterweil et al., 2010)). In any case, both studies are in agreement that protein synthesis-independent DHPG-LTD, expressed by a presynaptic modification, is unaffected by reducing β-arrestin2 (see Fig. 1E–F).

The discovery that $mGlu_5$ stimulates protein synthesis via β-arrestin2 has clinical as well as basic biological significance. The core pathophysiology of FX is believed to be excessive synaptic protein synthesis downstream of $mGlu_5$ (Bhakar et al., 2012). In animal models of FX, it has been shown that inhibition of $mGlu_5$ can correct a wide array of mutant phenotypes. This work led directly to human FX clinical trials with $mGlu_5$ inhibitors but,

unfortunately, the results of these trials to date have disappointed (Berry-Kravis et al., 2016). With all drug trials, the maximum allowable dosage is determined by the occurrence of adverse side effects. In the case of first generation $mGlu_5$ drugs, a potentially serious dose-limiting psychiatric side effect is derealization and visual hallucinations. To separate the therapeutic effect of $mGlu_5$ inhibition (suppression of protein synthesis) from the unwanted side effects, it is essential to understand the mechanism that specifically couples $mGlu_5$ to the ERK signaling pathway. The correction of multiple FX phenotypes, including excessive basal protein synthesis, in *Fmr1*$^{-/y}$ mice crossed with β-arrestin2 heterozygous mice indicates that β-arrestin2 is a key component of a pathogenic pathway. Further, the fact that MK-801 induced hyperlocomotion is still augmented in the *Arrb2*$^{+/-}$ mice by MTEP, a first-generation NAM with high selectivity for $mGlu_5$ (Cosford et al., 2003), indicates that this undesirable effect of inhibiting $mGlu_5$-G_q signaling is likely to be pharmacologically separable from the therapeutic effect of inhibiting $mGlu_5$-β-arrestin2 signaling.

G-protein coupled receptors respond to a wide variety of signals and initiate a large number of distinct cellular signaling pathways. This versatility has made these receptors attractive targets for pharmacological therapies, and over 50% of the current drugs used clinically target these receptors (Insel et al., 2007). The finding that β-arrestin- and G protein-dependent cellular signaling are pharmacologically separable has opened a new vista for the treatment of disease. For some disorders, modulation of only one of these signaling pathways may be therapeutically beneficial, while the other(s) could mediate undesirable and possibly conflicting outcomes (Whalen et al., 2011). Our findings suggest that $mGlu_5$ modulators for the treatment of FX is a case in point. There is little doubt that β-arrestin-biased allosteric modulators of mGlu receptors are feasible (Hathaway et al., 2015; Iacovelli et al., 2014; Sheffler et al., 2011), and their development could lead to the next generation of drugs for the treatment of FX and several other genetically defined causes of ID and ASD (Aguilar-Valles et al., 2015; Auerbach et al., 2011; Barnes et al., 2015; Bozdagi et al., 2010; Tian et al., 2015; Wenger et al., 2016).

Because it is a monogenic disorder, FX has emerged in recent years as a bellwether for the utility of developing medicines for psychiatric diseases by reproducing genetic etiologies in animal models to identify pathophysiology and therapeutic targets. The current study is important because it reveals some of the previously unappreciated limitations of targeting $mGlu_5$ signaling via G_q, and suggests an exciting alternative approach.

Experimental Procedures

Arrb2$^{+/-}$ male and female mutant mice on the C57Bl/6J clonal background were mated to produce the WT and *Arrb2*$^{+/-}$ offspring used in this study. *Fmr1*$^{-/+}$ female mice (Jackson Labs) were crossed with *Arrb2*$^{+/-}$ male mice to generate double mutant animals. All experimental animals were age-matched male littermates, and were studied with the experimenter blind to genotype and treatment condition. All experimental techniques were approved by The Institutional Animal Care and Use Committee at MIT and all animals were treated in accordance with NIH and MIT guidelines. Hippocampal slice preparation, electrophysiological recordings, metabolic labeling, immunoblotting as well as inhibitory avoidance and audiogenic seizure assays were performed as previously described (Auerbach

et al., 2011; Dolen et al., 2007; Osterweil et al., 2010) and are detailed in Supplemental Experimental Procedures. Slices were stimulated with the selective agonist/positive modulator of $mGlu_5$ CDPPB for metabolic labeling and immunoblotting, or the group 1 mGluR agonist (S)-DHPG for electrophysiology and calcium imaging experiments. Calcium mobilization was assessed using the cell-permeable calcium fluorescent dye Fluo4-AM in the presence of TTX and AP-5. The effect of MTEP on MK801-induced hyperlocomotion was assessed using Plexon's *CinePlex*® Studio and custom written MATLAB software. Two-way ANOVAs with post-hoc two-tailed *t* tests or Bonferroni's test for multiple comparisons were used to determine differences between genotypes and drug treatments unless stated otherwise. All data shown represent mean ± SEM. A full description of the Experimental Procedures can be found in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- β-arrestin2 is required for stimulated protein synthesis downstream of $mGlu_5$
- β-arrestin2 reduction disrupts $mGlu_5$-mediated ERK activation but not G_q-signaling
- Decreasing β-arrestin2 in *Fmr1*-null mice reverses synaptic and behavioral phenotypes
- No psychotomimetic effects are associated with β-arrestin2 deletion

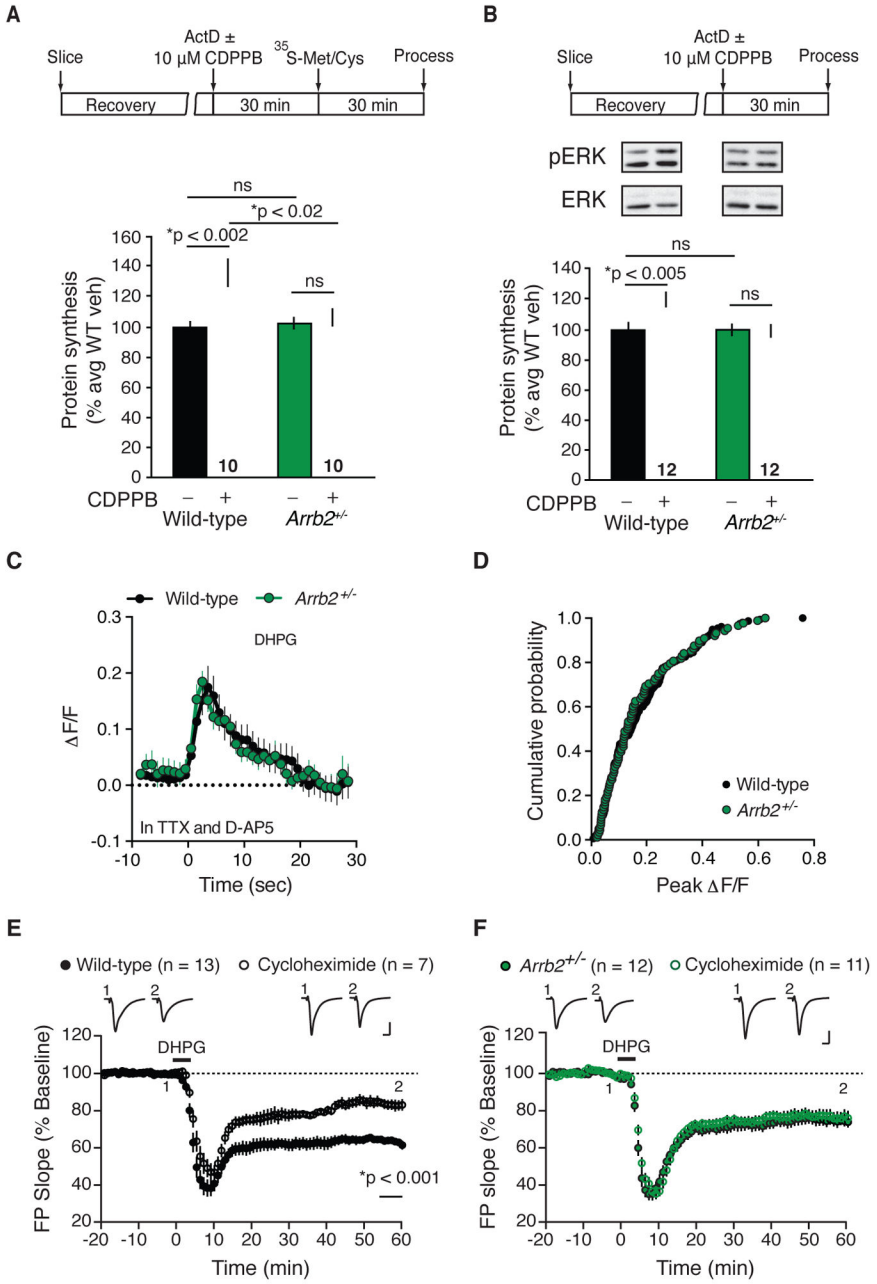

Figure 1. β-arrestin2 is necessary for protein synthesis-dependent mGlu-LTD and ERK1/2 activation

(**A)** Schematic illustrates experimental timeline. Protein synthesis was elevated in WT slices stimulated with CDPPB compared with vehicle (two-tailed *t* test, t = 3.6928, *p = 0.0017, n = 10 animals per group) whereas treatment had no effect in *Arrb2*$^{+/-}$ slices (two-tailed *t* test, t = 0.654, p = 0.5214, n = 10 animals per group). Two-way ANOVA, genotype vs. treatment, F = 7.081; *p = 0.012. Mean ± SEM ^{35}S incorporation (%CPM/μg): WT + vehicle = 3.3057 ± 0.2441; WT + CDPPB = 4.4417 ± 0.3196; *Arrb2*$^{+/-}$ + vehicle = 3.4463 ± 0.3004; *Arrb2*$^{+/-}$ + CDPPB = 3.3940 ± 0.3397. **(B)** Representative immunoblots of ERK1/2 phosphorylation and total ERK protein from hippocampal slices ± CDPPB stimulation from WT and

Arrb2$^{+/-}$ mice. WT slices stimulated with CDPPB show elevated ERK1/2 phosphorylation compared with vehicle (two-tailed *t* test, t = 3.1421, *p = 0.0047, n = 12 animals per group), whereas no change was observed in *Arrb2*$^{+/-}$ mice (two-tailed *t* test, t = 0.1826, p = 0.8568, n = 12 animals per group). Two-way ANOVA, genotype vs. treatment, F = 6.458, *p = 0.015. Full and uncropped versions of blots underlying the figures are collected in Fig. S4. (**C**) Quantification of calcium fluorescence over time in WT and *Arrb2*$^{+/-}$ slices. Data are normalized as ΔF/F as discussed in materials and methods. There is no significant difference in the peak calcium fluorescence measured between WT and *Arrb2*$^{+/-}$ slices (two-tailed Mann-whitney test, p = 0.7959, Mann-whitney U= 46, n = 10 animals per group). (**D**) The cumulative probability of peak fluorescence for all cells analyzed is not different between WT and *Arrb2*$^{+/-}$ slices (Kolmogorov-Smirnov test, p = 0.8334, n = WT, 155 cells, *Arrb2*$^{-/+}$, 88 cells). (**E, F**) DHPG-LTD (25 μM, 5 min) is reduced and unaffected by pretreatment with the protein synthesis inhibitor cycloheximide (CHX, 60 μM) in hippocampal slices from *Arrb2*$^{+/-}$ animals. Two-way ANOVA, genotype vs. treatment, F = 9.678,*p = 0.003.). Bonferroni multiple comparisons shows a significant effect of genotype under control conditions (*p = 0.005, WT = 13 animals, *Arrb2*$^{+/-}$ = 12 animals) but not in the presence of CHX (p = 0.125; WT = 9 animals, *Arrb2*$^{+/-}$ = 11 animals). CHX treatment significantly reduced LTD magnitude in WT slices (*p < 0.001, t = 4.676; control n = 13 animals, CHX n = 9 animals) but not *Arrb2*$^{+/-}$ slices (p = 0.646, t = 0.463; control n = 12 animals, CHX n = 11 animals). Representative field potential (FP) traces (average of 10 sweeps) were taken at times indicated by numerals. Scales bars equal 0.5 mV, 5 ms. For this and all subsequent figures, data is plotted as mean ± s.e.m. Statistics were performed using each animal as one “n”, with each animal represented by the mean of 1–4 slices, unless otherwise noted. See also Figures S1, S2, and S3.

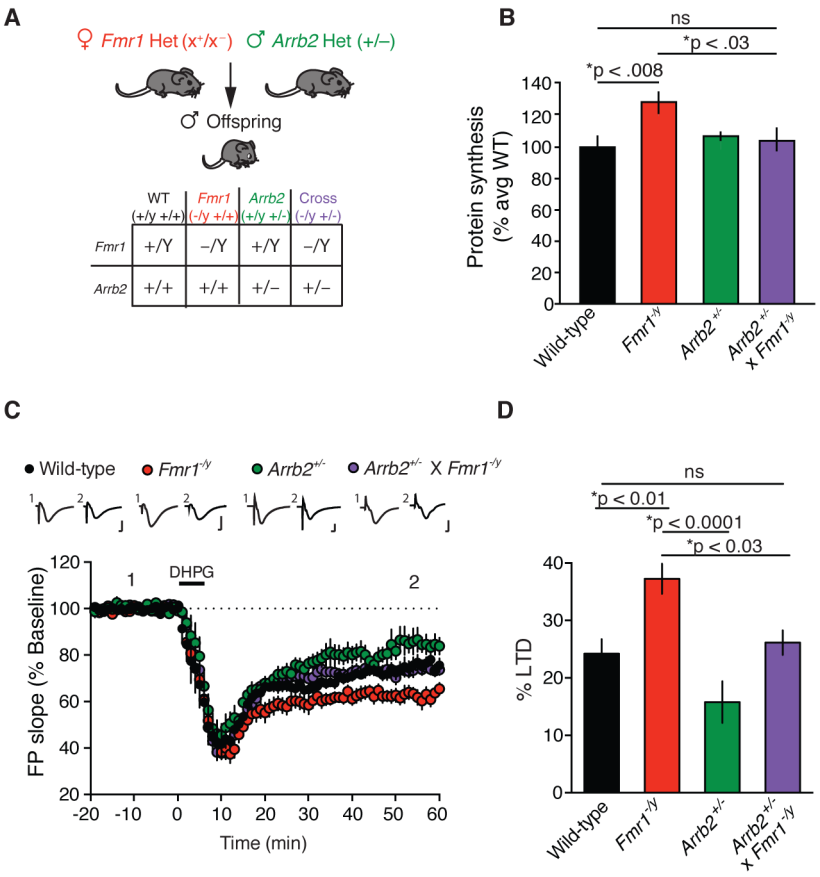

Figure 2. Genetic reduction of β-arrestin2 in *Fmr1*$^{-/y}$ mice corrects exaggerated protein synthesis and mGlu-LTD

(**A**) Genetic rescue strategy. (**B**) Basal protein synthesis is significantly increased in slices from *Fmr1*$^{-/y}$ mice compared with WT slices (two-tailed *t* test, t = 3.0689, *p = 0.0078, n = 9 animals each genotype). Basal protein synthesis is comparable in slices from *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice and WT mice (two-tailed *t* test, t = 0.4821, p = 0.6363, n = 9 animals each genotype). Basal protein synthesis is significantly increased in slices from *Fmr1*$^{-/y}$ mice compared with *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ slices (two-tailed *t* test, t = 2.5243, *p = 0.0225, n = 9 animals each genotype). Mean ± SEM ^{35}S incorporation (%CMP/μg): WT = 2.9183 ± 0.1988; *Fmr1*$^{-/y}$ = 3.7697 ± 0.1934; *Arrb2*$^{+/-}$ = 3.135 ± 0.0747; *Fmr1*$^{-/y}$ x *Arrb2*$^{+/-}$ = 3.0563 ± 0.2060. (**C**) The magnitude of DHPG-induced LTD in slices from *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice is significantly different from *Fmr1*$^{-/y}$ slices and is indistinguishable from WT slices (One-way ANOVA, *p = 0.0001, F = 8.715, with Bonferroni multiple comparisons: *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ vs. *Fmr1*$^{-/y}$, *p < 0.03, t = 2.971, *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ vs. WT, p = 0.999, t = 0.5741, *Arrb2*$^{+/-}$ vs. *Fmr1*$^{-/y}$, *p < 0.0001, t = 5.033, WT n = 15 animals, *Fmr1*$^{-/y}$ n = 10 animals, *Arrb2*$^{+/-}$ n = 9 animals, *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ n = 16 animals). Representative FP traces (average of 10 sweeps) were taken at times indicated by numerals. Scales bars equal 0.5 mV, 2ms. (**D**) Summary of LTD data. Bar graphs, percentage decrease from baseline in FP slope.

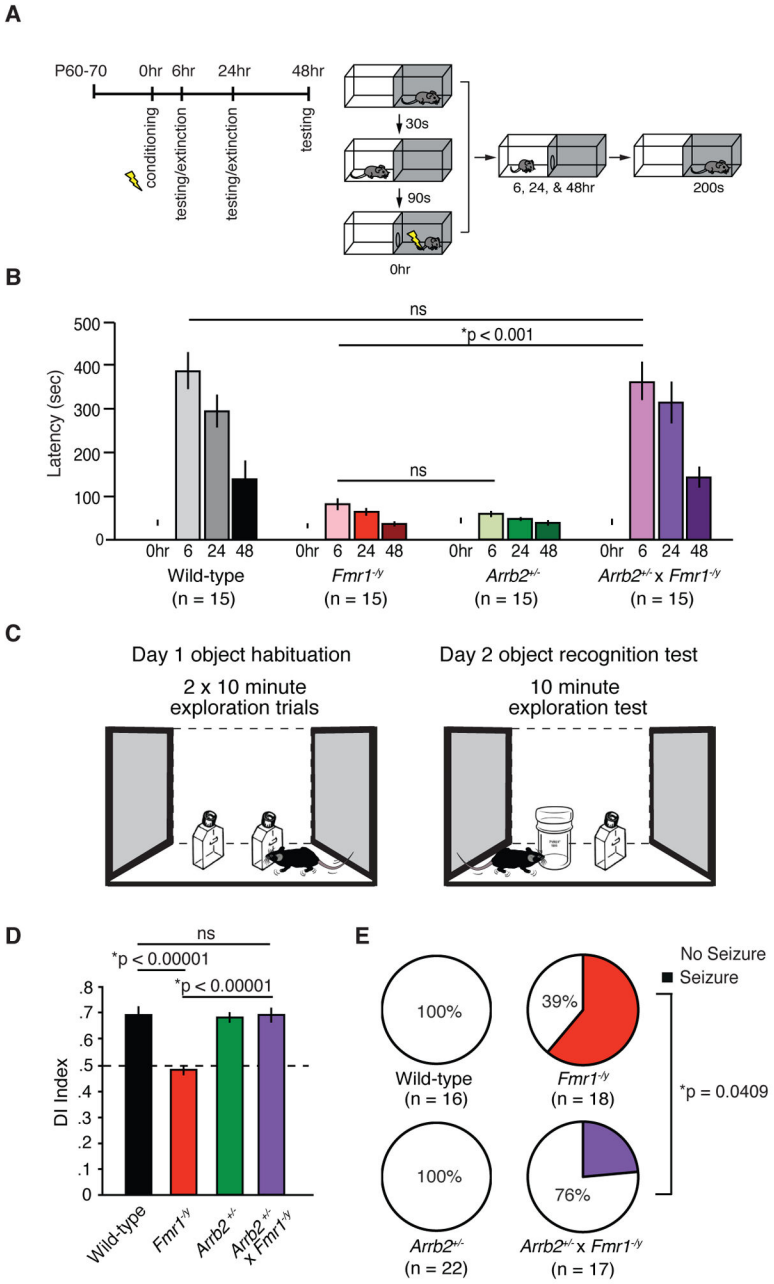

Figure 3. Genetic reduction of β-arrestin2 in *Fmr1*$^{-/y}$ mice corrects behavioral and cognitive deficits

(**A**) Experimental design of inhibitory avoidance learning task. (**B**) *Fmr1*$^{-/y}$ mice and *Arrb2*$^{+/-}$ mice show impaired acquisition of inhibitory avoidance learning compared to WT mice (two-way ANOVA, *p < 0.001 for each comparison, wild-type vs. *Fmr1*$^{-/y}$ (F = 12.760); WT vs. *Arrb2*$^{+/-}$ (F = 12.525). *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice show comparable acquisition and extinction of inhibitory avoidance to WT mice (two-way ANOVA, F = 0.145, p = 0.933). There is a statistically significant interaction between genotype and time point across groups (repeated measures two-way ANOVA, F = 12.425, *p = < 0.001). (**C**) Experimental design of familiar object recognition task. (**D**) *Fmr1*$^{-/y}$ mice show impaired

novelty detection on experimental test day 2 when presented with a familiar and novel object compared to WT (two-tailed *t* test, t = 7.1445, *p < 0.00001, n = 10 animals each genotype). In comparison, *Arrb2*$^{+/-}$ x *Fmr1*$^{-/y}$ demonstrate a discrimination index that is not significantly different from wild-type mice (two-tailed *t* test, t = 0.0511, p = 0.9598, n = 10 animals each genotype). (**E**) *Fmr1*$^{-/y}$ mice exhibit increased susceptibility to audiogenic seizure activity compared to WT (two-tailed Fisher's exact test, *p = 0.0001, n = 16, 18 animals) and *Arrb2*$^{+/-}$ mice (*p = 0.0001, n = 18, 22 animals). Genetic reduction of *Arrb2* in *Fmr1*$^{-/y}$ mice significantly reduces the incidence of seizure activity (*p = 0.0409, n = 18, 17).

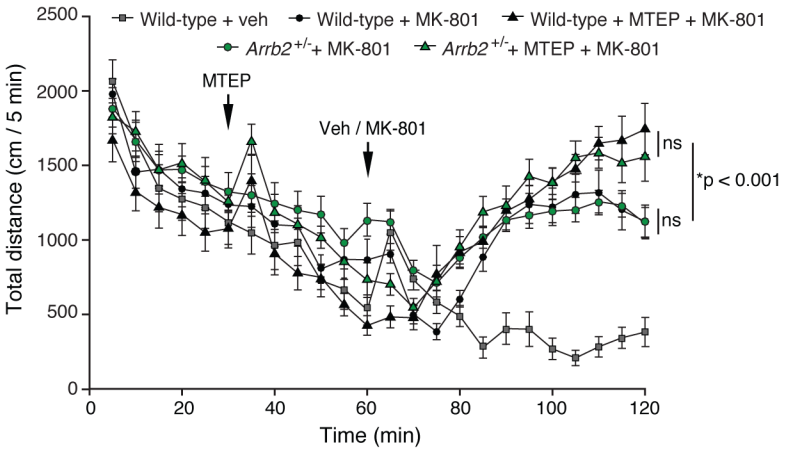

Figure 4. Genetic reduction of β-arrestin2 does not potentiate the psychotomimetic effects of MK801

WT and *Arrb2*$^{+/-}$ mice injected intraperitoneally with the NMDAR antagonist MK801 (0.3 mg/kg) show comparable hyperlocomotion 60 minutes post-treatment compared to vehicle (N = 10 mice per group). Data points represent distance travelled in cm over 5 minute bins, averaged as pooled animals per treatment group. Pre-treatment with MTEP (10 mg/kg, i.p.) potentiates hyperlocomotion in both WT and *Arrb2*$^{+/-}$ mice (N = 9 mice per group). Two-way ANOVA for genotype: F = 0.468, p = 0.499, and for treatment: F = 13.597, *p < 0.001; no significant interaction between genotype and treatment: F = 0.352, p = 0.557. Two-tailed *t* test, WT + MK801 vs. WT + MTEP + MK801: t = 2.9358, *p = 0.0092. Two-tailed *t* test, *Arrb2*$^{+/-}$ + MK801 vs. *Arrb2*$^{+/-}$ + MTEP + MK801: t = 2.2603, *p = 0.0372.