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Report

# Lovastatin Corrects Excess Protein Synthesis and Prevents Epileptogenesis in a Mouse Model of Fragile X Syndrome

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## SUMMARY

**Many neuropsychiatric symptoms of fragile X syndrome (FXS) are believed to be a consequence of altered regulation of protein synthesis at synapses. We discovered that lovastatin, a drug that is widely prescribed for the treatment of high cholesterol, can correct excess hippocampal protein synthesis in the mouse model of FXS and can prevent one of the robust functional consequences of increased protein synthesis in FXS, epileptogenesis. These data suggest that lovastatin is potentially disease modifying and could be a viable prophylactic treatment for epileptogenesis in FXS.**

## INTRODUCTION

Seizures and EEG abnormalities were noted in some of the earliest studies of autism (Kanner, 1943; Tuchman et al., 2010), and it is now appreciated that approximately 30% of the ASD population have epilepsy. The etiologic heterogeneity of autism and epilepsy have made it difficult to understand the molecular mechanisms linking the two disorders. One approach is to focus on syndromic disorders that have a known genetic etiology and valid animal models. In the current study, we have used a mouse model of fragile X syndrome (FXS), a single-gene developmental disorder characterized by increased incidence of both autism and epilepsy (Berry-Kravis, 2002; Musumeci et al., 1999). The *Fmr1*$^{-/y}$ (knockout [KO]) mouse exhibits robust epilepsy phenotypes, both in vitro, as measured by ictal-like discharges in hippocampal slices and neocortical hyperexcitability, and in vivo, as measured by increased susceptibility to audiogenic seizures (AGS) (Yan et al., 2004).

The *FMR1* gene encodes the translational repressor fragile X mental retardation protein (FMRP). Pathological changes observed in FXS are believed to stem in part from an elevation of basal protein synthesis downstream of an extracellular signal-regulated kinase (ERK1/2) signaling pathway (Bhakar et al., 2012). ERK1/2 is a key member of the larger MAP kinase (MAPK) signaling pathway, which is involved in regulation of multiple biochemical processes including the initiation of cap-dependent translation. At the head of this intracellular cascade lies the small GTPase Ras. The Ras/MAPK pathway is a major regulator of cell growth, and thus a strong inhibitor of Ras-ERK1/2 would have deleterious consequences on the developing brain. However, a previous study reported that lovastatin, an HMG-CoA reductase inhibitor in widespread use for the treatment of hypercholesterolemia in both children and adults, could correct cognitive deficits caused by excess Ras activity in the mouse model of neurofibromatosis type 1 (Li et al., 2005). Lovastatin can achieve a mild reduction in Ras-ERK1/2 activation by interfering with the recruitment of Ras to the membrane, a process that is required to transition from the inactive guanosine diphosphate (GDP)-bound form to the active guanosine triphosphate (GTP)-bound form (Kloog et al., 1999; Schafer et al., 1989). The interaction of Ras with the membrane requires the posttranslational addition of a farnesyl group to the C terminus and lovastatin inhibits Ras farnesylation by targeting the upstream mevalonate pathway (Figure 1A) (Li et al., 2005; Mendola and Backer, 1990; Schafer et al., 1989). We therefore wondered whether lovastatin could prevent pathological changes in FXS that lie downstream of excessive ERK-mediated protein synthesis and contribute to epileptogenesis. Unless otherwise stated, we tested lovastatin in male KO and wild-type (WT) mice in the C57BL/6 background.

## RESULTS

### Lovastatin Normalizes Excessive Protein Synthesis in the *Fmr1* KO

We showed previously that partial inhibition of ERK1/2 was sufficient to restore normal levels of protein synthesis in the *Fmr1* KO hippocampus (Osterweil et al., 2010). To determine whether reduction of Ras signaling has the same effect, we used farnesyl thiosalicylic acid (FTS), which dislodges Ras from the membrane (Figure 1A) (Weisz et al., 1999). Hippocampal slices were prepared from juvenile *Fmr1* KO and WT mice and preincubated in 25, 50 μM FTS or vehicle for 30 min. Protein synthesis was measured for another 30 min via incorporation of a $^{35}$S-labeled methionine/cysteine ($^{35}$S-Met/Cys) mix. We found that 50 μM FTS reduces protein synthesis in the *Fmr1* KO down to WT levels

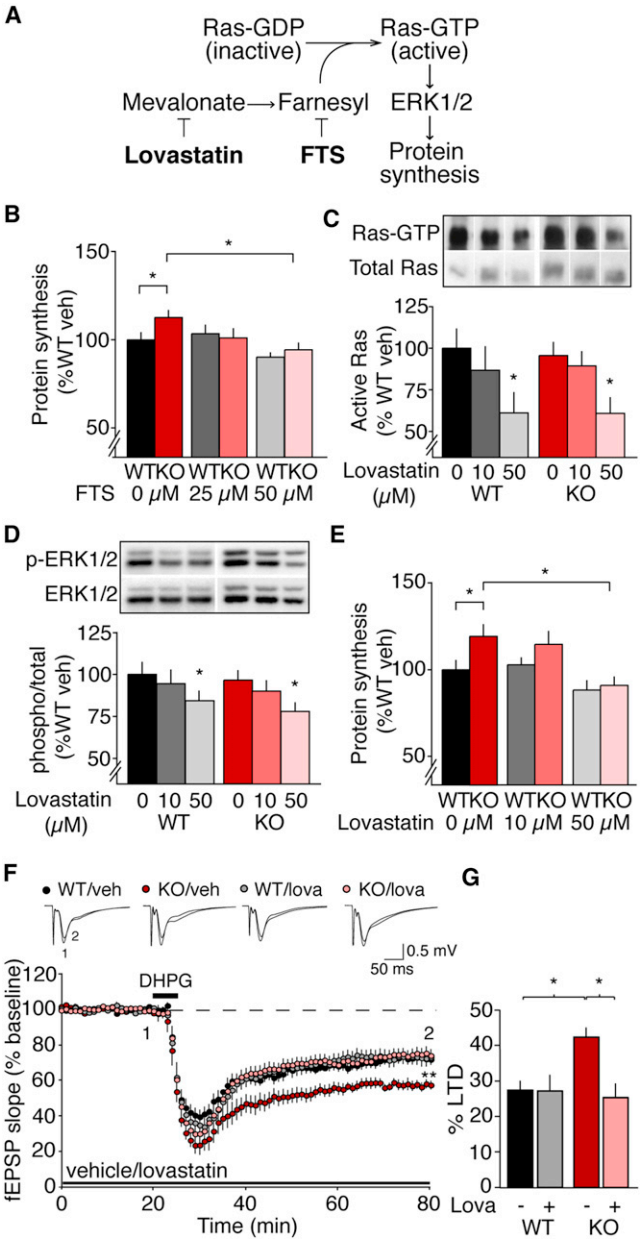


**Figure 1. Lovastatin Inhibits Ras-ERK1/2 Signaling, Normalizes Excessive Protein Synthesis, and Corrects Exaggerated mGluR-LTD in the *Fmr1* KO Hippocampus**

(A) Model of the mechanism by which lovastatin and FTS reduce Ras-ERK1/2 activation and normalize protein synthesis in the *Fmr1* KO. (B) Application of FTS corrects excessive protein synthesis in the *Fmr1* KO (WT: veh 100% ± 4%, 25 μM 104% ± 5%; 50 μM 90% ± 2%; KO: veh 113% ± 4%, 25 μM 101% ± 5%; 50 μM 94% ± 4%; ANOVA treatment *p = 0.0017; WT versus KO veh *p = 0.041, KO veh versus 50 μM *p = 0.00002; n = 15). A small but significant reduction of protein synthesis is also observed with 50 μM FTS in WT (*p = 0.0316). (C) Lovastatin inhibits Ras activation in hippocampal slices (WT: veh 100% ± 11%, 10 μM 87% ± 14%; 50 μM 61% ± 12%; KO: veh 96% ± 8%, 10 μM 89% ± 8%; 50 μM 61% ± 9%; ANOVA treatment *p = 0.0266; WT *p = 0.039, KO *p = 0.015; n = 6). (D) Lovastatin downregulates ERK1/2 (WT: veh 100% ± 7%, 10 μM 94% ± 8%; 50 μM 84% ± 5%; KO: veh 97% ± 5%, 10 μM 90% ± 6%; 50 μM 78% ± 5%; ANOVA treatment *p = 0.0037; WT *p = 0.035, KO *p = 0.008; n = 15). (E) Lovastatin normalizes protein synthesis in *Fmr1* KO hippocampal slices (WT: veh 100% ± 5%, 10 μM 103% ± 4%; 50 μM 88% ± 5%; KO: veh 119% ± 6%, 10 μM 115% ± 7%; 50 μM 91% ± 4%; ANOVA genotype x treatment *p = 0.0332; WT versus KO veh *p = 0.019, KO veh versus 50 μM *p = 0.011; n = 11). (F) LTD was induced with 50 μM R,S-DHPG and extracellular recordings were performed in area CA1. In the presence of vehicle, greater LTD is observed in the *Fmr1* KO versus WT (WT veh 72.5% ± 2.5%, KO veh 57.5% ± 2.5%, *p = 0.005, n = 9–10). Fifty micromolar lovastatin significantly reduces LTD magnitude in the *Fmr1* KO to WT levels (WT lova 72.7% ± 4.4%, KO lova 74.5% ± 3.4%; KO veh versus lova *p < 0.001, n = 11–13) but has no significant effect on LTD in the WT (WT veh versus lova p = 0.869). Field potential traces are averages of all experiments and were taken at times indicated by numerals; scale bars represent 0.5 mV, 5 ms. (G) Lovastatin significantly reduces LTD magnitude in the *Fmr1* KO to WT levels (ANOVA genotype × treatment *p = 0.021). LTD magnitude was assessed by a comparison of the averaged last 5 min pre-DHPG and the last 5 min of recordings (minutes 55–60 post-DHPG). n = animals. Error bars represent SEM.

(WT versus KO veh *p = 0.041, KO veh versus 50 μM FTS *p = 0.00002) (Figure 1B).

We next asked whether lovastatin could inhibit Ras in our slices as has been shown in other systems. Hippocampal slices were prepared from WT and *Fmr1* KO, then incubated with 10–50 μM lovastatin or vehicle. GTP-bound Ras was isolated using a glutathione S-transferase (GST) pull-down assay and compared to total Ras. Our results show that 50 μM lovastatin significantly reduces the amount of active Ras in both WT and *Fmr1* KO hippocampal slices (WT *p = 0.039, KO *p = 0.015) (Figure 1C).

Western blot analysis revealed that 50 μM lovastatin also significantly reduces ERK1/2 activation in both WT and *Fmr1* KO slices (WT *p = 0.035, KO *p = 0.008) (Figure 1D). To examine the consequence of this modest reduction in ERK1/2 activity on hippocampal protein synthesis, we performed metabolic labeling on WT and *Fmr1* KO slices in the presence of 10–50 μM lovastatin. We found that inhibition of the Ras-ERK1/2 pathway by 50 μM lovastatin is sufficient to restore WT levels of protein synthesis in hippocampal slices from the *Fmr1* KO (WT versus KO veh *p = 0.019, KO veh versus 50 μM *p = 0.011) (Figure 1E). Consistent with previous findings, there was no difference in basal levels of Ras and ERK1/2 in the *Fmr1* KO hippocampus, suggesting that the excessive protein synthesis in the *Fmr1* KO is due to a hypersensitivity to, not hyperactivation of, the Ras-ERK1/2 signaling pathway (Osterweil et al., 2010). To see whether the effects of FTS and lovastatin on protein synthesis might be due to inhibition of the homologous GTPase Rheb instead of Ras (Makovski et al., 2012), we measured the phosphorylation (activation) of p70S6 kinase (p70S6K) and S6 ribosomal protein (see Supplemental Information available online). We failed to detect changes in these downstream markers of Rheb activity (Figures S1 and S2), suggesting that inhibition of Rheb is not likely to be the mechanism of action for FTS or lovastatin in our preparation.

An electrophysiological signature of altered hippocampal protein synthesis in the *Fmr1* KO mouse is exaggerated long-term synaptic depression (LTD) induced by activation of metabotropic glutamate receptor 5 (mGluR5) (Huber et al., 2002). We therefore examined the effect of lovastatin on LTD induced by the mGluR5 agonist 3,5-dihydroxyphenylglycine (DHPG) in

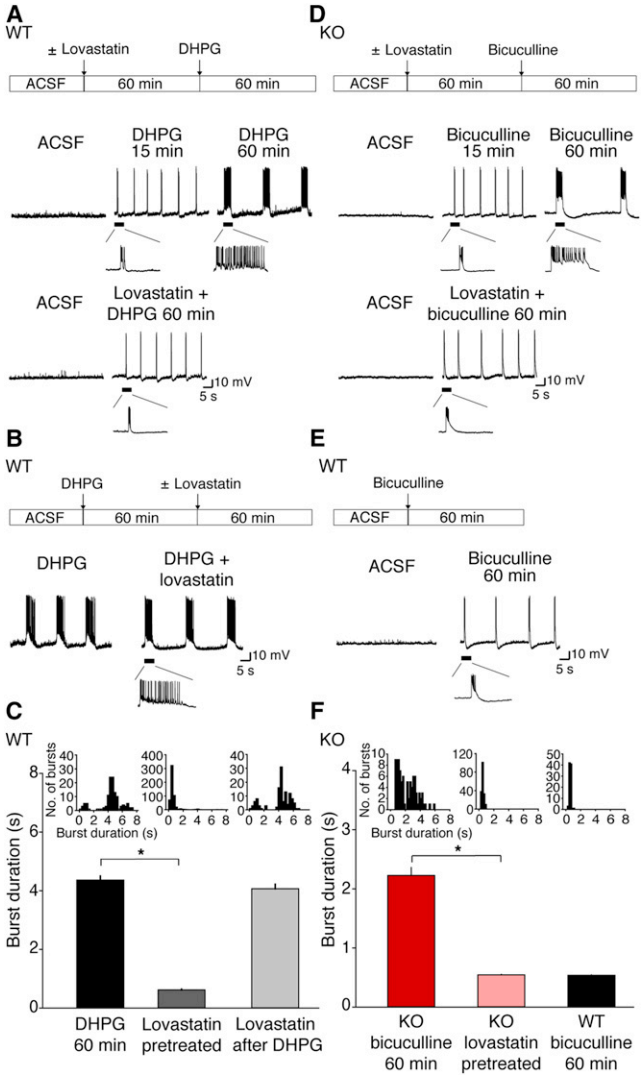


**Figure 2. Lovastatin Blocks mGluR-Mediated Epileptiform Bursting in the *Fmr1* KO Hippocampus**

Intracellular recordings were performed on CA3 pyramidal neurons in WT and *Fmr1* KO hippocampal slices. (A) In WT slices, 50 μM R,S-DHPG leads to bursting from CA3 neurons, which progresses to epileptiform discharges by 60 min. This epileptiform activity is prevented by a 60 min pretreatment with 50 μM lovastatin. (B) Addition of lovastatin after 60 min post-DHPG did not affect the duration of epileptiform discharges. (C) Mean epileptiform burst durations from WT slices under the following conditions: at 60 min post-DHPG (4.359 ± 0.14 s; n = 125 discharges, six slices from six animals), lovastatin-pretreated (0.618 ± 0.03 s; n = 545 discharges, 12 slices from seven animals), and lovastatin after DHPG (4.066 ± 0.15 s; n = 124 discharges, five slices from five animals). Slices pretreated with lovastatin showed significant reduction in burst duration compared to vehicle-pretreated slices (*p = 0.0000218), while there was no significant difference in burst duration between DHPG 60 min and lovastatin after DHPG (p = 0.072). (D) In *Fmr1* KO slices, synaptic mGluR activation by spontaneous activity in bicuculline induces prolonged epileptiform discharges, which are blocked with 60 min pretreatment with 50 μM lovastatin. (E) In contrast to *Fmr1* KO slices, bicuculline fails to induce prolonged epileptiform discharges in WT slices. (F) Plot of mean epileptiform burst durations from *Fmr1* KO slices 60 min after bicuculline ±50 μM lovastatin and WT slices 60 min after bicuculline. *Fmr1* KO slices pretreated with lovastatin showed significant reduction in burst duration (0.528 ± 0.01 s; n = 182 discharges; ten slices from five animals) compared to vehicle-pretreated slices (2.227 ± 0.13 s; n = 90 discharges; eight slices from six animals; *p = 0.000022), while there was no significant difference in burst duration between lovastatin-pretreated *Fmr1* KO slices and bicuculline-treated WT slices (0.537 ± 0.01 s; n = 88 discharges; five slices from five animals; p = 0.995). Insets indicate summary frequency histograms of synchronized discharges in each experimental condition. Error bars represent SEM.

hippocampal area CA1 of WT and *Fmr1* KO mice. Slices were incubated in vehicle or 50 μM lovastatin, extracellular field potentials were recorded in area CA1 in response to Schaffer collateral stimulation, and mGluR-LTD was induced with a 5 min bath application of 50 μM DHPG with the experimenter blind to genotype and treatment. We found that lovastatin corrects the exaggerated mGluR-LTD observed in the *Fmr1* KO to WT levels (WT veh versus KO veh *p = 0.005, KO veh versus lova *p < 0.001) but had no significant effect on mGluR-LTD in the WT (WT veh versus lova p = 0.869) (Figures 1F and 1G). These electrophysiological findings corroborate the biochemical results and support the conclusion that lovastatin can correct excessive protein synthesis in the *Fmr1* KO.

## Lovastatin Prevents mGluR-Induced Epileptogenesis in Hippocampal Slices

Previous studies in slices of hippocampal area CA3 have shown that a lasting consequence of mGluR5- and ERK-mediated protein synthesis is the generation of epileptiform activity (Merlin et al., 1998; Zhao et al., 2004). We therefore investigated the effect of lovastatin in this model of epileptogenesis. Intracellular recordings were made from CA3 pyramidal cells in WT hippocampal slices ±1 hr preincubation with 50 μM lovastatin. Single-cell behavior and network discharge behavior can be differentiated in these intracellular recordings in that the occurrence, rhythm, and duration of network discharges are not affected by depolarizing or hyperpolarizing the recorded cell (see Taylor et al., 1995). In control slices, addition of DHPG first elicited short (~500 ms) synchronized discharges, which gradually extended to reach an average duration of 4.359 ± 0.14 s at 60 min (Figure 2A). Preincubation with 50 μM lovastatin significantly reduced the average duration of synchronized discharges to 0.618 ± 0.03 s at 60 min (*p = 0.0000218; Figures 2A and 2C). These results suggest that lovastatin is sufficient to block the induction of mGluR-mediated epileptiform activity in hippocampal slices.

The epileptiform discharges observed after sustained DHPG application persist after the agonist is removed. This maintenance phase is not affected by inhibition of protein synthesis or ERK1/2, suggesting that translation plays a specific role in epileptogenesis (Zhao et al., 2004). Similarly, exposure of slices to 50 μM lovastatin 60 min after DHPG had no effect on average burst duration (average duration 4.066 ± 0.15 s; p = 0.072; Figures 2B and 2C). These results are consistent with the action of lovastatin as an inhibitor of ERK-mediated protein synthesis (Merlin et al., 1998).

In the *Fmr1* KO, spontaneous activity is sufficient to elicit epileptiform discharges in hippocampal CA3, suggesting a hypersensitive response to mGluR5-ERK1/2 activation (Chuang et al., 2005). Thus, unlike WT slices, which require DHPG to

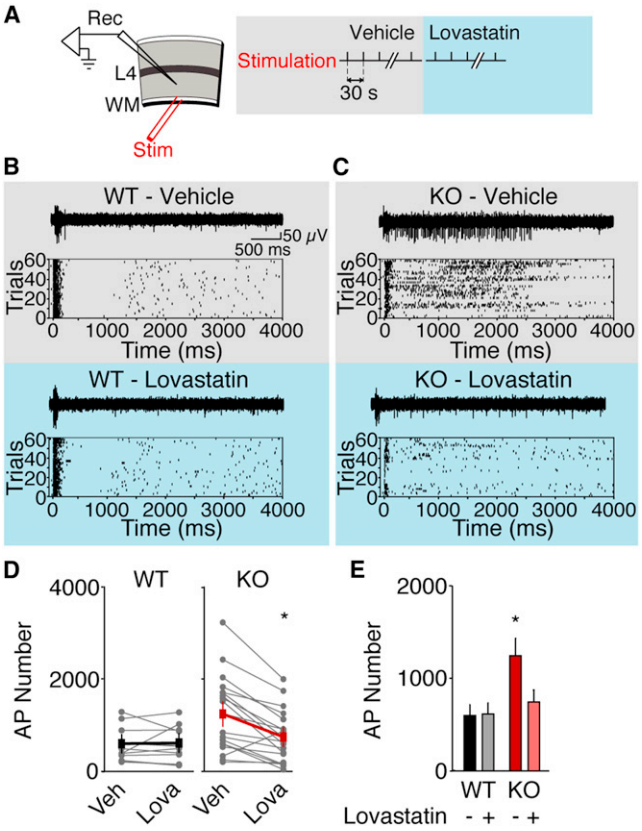


**Figure 3. Lovastatin Reduces Excitability in the *Fmr1* KO Visual Cortex**

(A) Extracellular recordings were performed in layer 5 of visual cortical slices. Trains of action potentials (APs) were evoked with 60 trials of white matter stimulation. Responses were collected in ACSF plus vehicle, then ACSF plus 50 μM lovastatin. (B and C) Representative traces and raster plots of recordings from WT (B) and *Fmr1* KO (C). Prolonged firing in the *Fmr1* KO is corrected with 50 μM lovastatin application. (D) Lovastatin significantly reduces firing in *Fmr1* KO but not WT visual cortical slices. (E) The mean number of action potentials is significantly higher in *Fmr1* KO slices versus WT slices, and 50 μM lovastatin restores normal firing to the *Fmr1* KO slices (WT veh 100% ± 20%, WT lova 103% ± 20%, KO veh 203% ± 44%, KO lova 113% ± 29%; ANOVA genotype x treatment *p = 0.0022; WT versus KO veh *p = 0.0258, KO veh versus lova *p = 0.0001, WT veh versus lova p = 0.8412; WT n = 10 slices from five animals, KO n = 19 slices from nine animals). Error bars represent SEM.

induce ictal discharges, epileptiform activity can be induced in *Fmr1* KO slices simply by applying the GABA-A receptor antagonist bicuculline to increase recurrent network activity (Bianchi et al., 2009). Intracellular recordings were therefore performed on bicuculline-treated *Fmr1* KO slices ±1 hr preincubation of 50 μM lovastatin. In untreated KO slices, bicuculline elicited short (<1 s) synchronized discharges that increased progressively in duration over the course of 60 min (average duration 2.227 ± 0.13 s at 60 min). This progression was prevented by preincubation in lovastatin (Figures 2D and 2F). The average burst duration of *Fmr1* KO slices in lovastatin (0.528 ± 0.01 s at 60 min) was not significantly different from discharges elicited by bicuculline in the WT (0.537 ± 0.01 s; p = 0.995; Figures 2E and 2F). These results show that acute application of lovastatin blocks epileptogenesis in the *Fmr1* KO hippocampus.

## Lovastatin Dampens Hyperexcitability in the *Fmr1* KO Visual Cortex

Increased excitability has been observed in the neocortex of the *Fmr1* KO. This phenotype is caused by specific changes in excitatory synaptic transmission and neuronal membrane excitability and can be rescued by inhibition or reduced expression of mGluR5 (Hays et al., 2011). To investigate whether lovastatin could similarly reduce cortical hyperexcitability, we prepared acute slices from visual cortex and measured persistent activity in layer 5 pyramidal neurons. Spiking activity was evoked with electrical stimulation of the underlying white matter every 30 s for a total number of 60 trials in artificial cerebrospinal fluid (ACSF) plus vehicle, followed by 60 trials in ACSF plus 50 μM lovastatin (Figure 3A). We found that the barrage of action potentials evoked by stimulation of white matter is significantly longer in the *Fmr1* KO (Figures 3B and 3C) but is reduced to WT levels by the application of 50 μM lovastatin (Figures 3B and 3C). Analysis of the total number of action potentials evoked revealed a significant dampening effect of lovastatin in *Fmr1* KO but not WT slices (WT versus KO veh *p = 0.0258, KO veh versus lova *p = 0.0001; Figures 3D and 3E). These results suggest that lovastatin corrects the hyperexcitability in the *Fmr1* KO visual cortex.

## Lovastatin Corrects AGS in the *Fmr1* KO

We next tested whether lovastatin could ameliorate AGS observed in the *Fmr1* KO in vivo. *Fmr1* KO and WT mice were injected intraperitoneally (i.p.) with 30 mg/kg lovastatin and, after 1 hr, exposed to a 130 dB stimulus for 2 min. Four stages of increasing AGS severity were scored: wild running, clonic seizure, tonic seizure, or death (Osterweil et al., 2010; Yan et al., 2004). Vehicle-treated *Fmr1* KO mice exhibited a 74% incidence of AGS, and no seizures were observed in vehicle-treated WT mice (WT versus KO *p = 0.0002). Acute injection of lovastatin reduced the incidence of AGS to 28% in the *Fmr1* KO (KO veh versus lov *p = 0.009) and significantly lessened the severity (Figure 4A). A higher dose of 100 mg/kg lovastatin showed a similar reduction in AGS incidence (KO veh versus lov 85% to 23%; *p = 0.005) and severity (KO veh versus lov *p = 0.015).

To determine whether the effect of lovastatin on AGS susceptibility is independent of the genetic background, we performed another set of experiments using *Fmr1* KO mice in the seizure prone FVB strain. Consistent with previous studies, we observed an 85% incidence of AGS in vehicle-treated *Fmr1* KO FVB mice (Yan et al., 2004), which was significantly higher than the 11% incidence observed in vehicle-treated WT mice (*p = 0.002). Interestingly, *Fmr1* KO FVB mice injected with 30 mg/kg lovastatin still exhibit a 64% incidence of AGS incidence, which is not significantly different from the vehicle-treated group (p = 0.357). The severity of AGS is similarly unaffected by this treatment (p = 0.862) (Figure 4B).

Previous work has shown that higher doses of mGluR5 inhibitors are required to reduce AGS in *Fmr1* KO mice bred on the FVB/NJ background versus the C57BL/6J × FVB/NJ

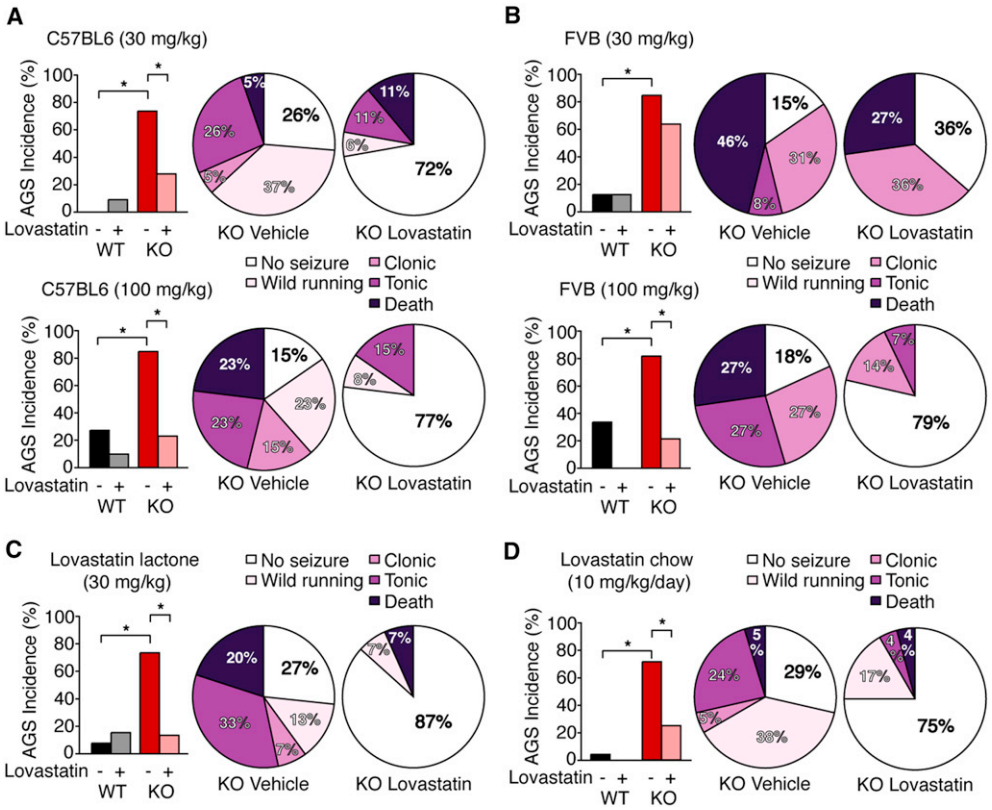


**Figure 4. Lovastatin Significantly Reduces AGS Incidence and Severity in the *Fmr1* KO**

*Fmr1* KO and WT mice on the C57BL/6 (A,C, and D) or FVB (B) backgrounds were treated as indicated, tested for AGS, and scored for wild running, clonic seizure, tonic seizure, and death. (A) Injection of 30 mg/kg lovastatin acid significantly reduces AGS incidence in the *Fmr1* KO on the C57BL/6 background (*p = 0.009; n = 18–19). Pie charts show a significant shift in the severity distribution of *Fmr1* KO mice treated with vehicle versus 30 mg/kg lovastatin (WT versus KO veh *p = 0.002, WT versus KO lov p = 0.999; KO veh versus lov *p = 0.041). Injection of a higher 100 mg/kg dose of lovastatin also significantly reduces AGS incidence (*p = 0.005; n = 13) and attenuates AGS severity (KO veh versus lov *p = 0.015; WT versus KO veh. *p = 0.04; WT versus KO lov p = 0.999) in *Fmr1* KO mice on the C57BL/6 background. (B) In *Fmr1* KO mice bred on the FVB background, injection of 30 mg/kg lovastatin does not significantly reduce AGS incidence (KO veh 85%, KO lov 64%, p = 0.357) or severity (KO veh versus lov p = 0.862); however, a higher dose of 100 mg/kg lovastatin significantly reduces both AGS incidence (KO veh 82%, KO lov 21%, *p = 0.005; n = 11–14) and severity (KO veh versus lov *p = 0.022; WT versus KO veh *p = 0.016; WT versus KO lov p = 0.999). (C) Injection of 30 mg/kg lovastatin in the lactone form significantly reduces AGS incidence (*p = 0.009; n = 15) and severity (KO veh versus lov *p = 0.009; WT versus KO veh *p = 0.005; WT versus KO lov p = 0.999) in *Fmr1* KO mice. (D) Feeding of 0.01% lovastatin chow for 48 hr significantly reduces AGS incidence (KO con 71%, KO lov 25%, *p = 0.003; n = 21–24) and severity (KO veh versus lov *p = 0.016; WT versus KO veh *p = 0.0001; WT versus KO lov p = 0.461) in *Fmr1* KO mice. n = animals.

hybrid background (Yan et al., 2005). In addition, a difference in lovastatin metabolism has been observed between different mouse strains (Gegg et al., 2005). Based on this literature, we repeated our experiments using a higher dose of lovastatin. The results show that an increased dose of 100 mg/kg lovastatin significantly lowers AGS incidence (KO veh 82%, KO lov 21%, *p = 0.005) and reduces AGS severity (KO veh versus lov *p = 0.022) in FVB *Fmr1* KO mice (Figure 4B). Thus, although the effective dose range differs, lovastatin is effective in correcting AGS in *Fmr1* KO mice bred on multiple background strains.

For human use, lovastatin is administered as a prodrug in its lactone form, which is metabolized into an active conformation (lovastatin acid) (Lambert et al., 1996). We therefore wished to confirm that lovastatin lactone could also reduce AGS incidence and severity in the *Fmr1* KO. Consistent with our previous results, we found that lovastatin lactone (30 mg/kg) significantly reduces AGS incidence from 73% to 20% (*p = 0.009) and reduces AGS severity (KO veh versus lov *p = 0.009) in the *Fmr1* KO (Figure 4C).

We also wanted to verify that oral administration could similarly correct AGS in the *Fmr1* KO. To address this question, we fed WT and *Fmr1* KO mice standard rodent chow supplemented with 0.01% lovastatin, which corresponds to a dose of 10 mg/kg/day (Yamada et al., 2000). After a 48 hr exposure to either lovastatin chow or control chow with the same formulation, mice were tested for AGS. Our results show that this oral administration of lovastatin significantly reduces AGS incidence (KO con 71%, KO lov 25%, *p = 0.003) and severity (KO veh versus lov *p = 0.016) in the *Fmr1* KO mouse (Figure 4D). These results suggest that oral administration of pharmaceutical grade lovastatin is effective in correcting AGS susceptibility in the *Fmr1* KO.

## DISCUSSION

Based on the insights that an ERK1/2 signaling pathway lies upstream of the excessive hippocampal protein synthesis in the *Fmr1* KO (Osterweil et al., 2010) and that lovastatin can, among other actions, inhibit Ras-ERK1/2 signaling in hippocampal neurons (Li et al., 2005), we wondered whether lovastatin could correct core biochemical and electrophysiological consequences of the loss of the translational repressor FMRP in FXS. We thought this was a particularly exciting prospect because lovastatin is widely prescribed, has a known safety profile, and is approved for use in children (to treat hypercholesterolemia). Our results show that lovastatin can indeed correct excessive protein synthesis and prevent the emergence of epileptiform activity in the *Fmr1* KO hippocampus in vitro and can protect the *Fmr1* KO mice from AGS in vivo.

It has been suggested that seizures in children with developmental disorders such as FXS could worsen the progression and severity of other symptoms including autism (Berry-Kravis, 2002; Tuchman et al., 2010). Once manifest, seizures can be controlled with conventional anticonvulsant medications, but these drugs themselves have side effects that can exacerbate other symptoms, such as cognitive impairment. Further, prodromal changes may be as deleterious for brain development as full-blown seizures. Therefore, preventing epileptogenesis is an important goal.

Statins have been studied previously in rodent models of epilepsy in which seizures are initiated or kindled in vivo by administration of drugs or by direct electrical stimulation of the temporal lobes. Some studies have suggested that statins can lessen seizure activity (Lee et al., 2008; Ramirez et al., 2011; Xie et al., 2011), whereas others have reported no effect (van Vliet et al., 2011) or an exacerbation of seizures (Serbanescu et al., 2004). Beneficial effects have typically been attributed to reduced brain inflammation associated with seizures. In contrast to previous work, we have used a genetically engineered mouse model of FXS in which seizure phenotypes are not caused by artificial induction of inflammatory responses and neurodegeneration. Our findings point to an entirely different mechanism of action, by which lovastatin corrects multiple *Fmr1* KO phenotypes including, but not limited to, in vivo seizure activity by downregulating Ras-ERK1/2 and protein synthesis.

It should be noted, however, that the connection between seizure phenotypes in the mouse model and childhood epilepsy in humans with FXS remains to be firmly established. For example, AGS may be largely a reflex event driven by activation of brainstem nuclei with limited relevance to cortical electroencephalographic seizures (Raisinghani and Faingold, 2003). Arguing against this point of view is the observation that audio stimulation rapidly elicits (within 2 min) generalized tonic-clonic seizures in most *Fmr1* KO mice, showing that hyperexcitability extends beyond brainstem neurons. Nevertheless, epileptogenesis in fragile X patients is manifest as the appearance of spontaneous seizures, which are not observed in the mouse model. The issue of whether the pathogenesis of epilepsy is shared by mice and humans lacking FMRP could be addressed if the effects of a prophylactic treatment were compared in the two species. Our findings that lovastatin, already approved for use in humans, can prevent epileptogenesis and hyperexcitability in the *Fmr1* KO suggest that carefully controlled human clinical trials are warranted.

As a negative regulator of the mevalonate pathway, lovastatin can impact the production of multiple compounds involved in intracellular signaling, including ubiquinone, dolichol, and isoprenoids (Goldstein and Brown, 1990). Therefore, we cannot rule out the possibility that some beneficial effects of lovastatin could be due to actions other than the reduction in Ras-ERK1/2 signaling, including a reduction in the farnesylation of other target proteins. However, like lovastatin, inhibitors of Ras or ERK1/2 are sufficient to normalize protein synthesis, and downregulation of the ERK1/2 pathway also blocks hippocampal epileptogenesis and eliminates AGS in the *Fmr1* KO (Chuang et al., 2005; Osterweil et al., 2010). Thus, although the precise molecular mechanisms by which lovastatin confers benefit remain to be determined, the weight of the evidence suggests that the effects are due to a reduction in Ras-ERK1/2 signaling.

This study was focused on the seizure phenotype because it has both construct (genotypic) and face (phenotypic) validity with the human disorder (with the caveats raised above). However, previous work has suggested that multiple fragile X symptoms can arise from the same core pathophysiology (Bear et al., 2004; Bhakar et al., 2012). The finding that lovastatin normalizes mGluR-LTD in the *Fmr1* KO hippocampus to WT levels (Figure 1F) is consistent with this idea. This result is intriguing, however, as protein synthesis inhibitors cannot correct the exaggerated mGluR-LTD in the *Fmr1* KO (reviewed in Bhakar et al., 2012). The implication is that lovastatin is exerting additional beneficial effects on the *Fmr1* KO beyond the reduction of protein synthesis. What these effects are remains to be determined; however, it should be noted that the selective reduction of mGluR-LTD in the *Fmr1* KO has been observed in a previous study using lithium (Choi et al., 2011).

It will be of interest to assess in future studies the effect of lovastatin treatment on the full spectrum of fragile X phenotypes. Indeed, the findings that lovastatin can selectively quiet cortical hyperexcitability in the *Fmr1* KO (Figure 3) as well as exaggerated LTD suggest it could potentially improve sensory and cognitive functions. Moreover, there is growing appreciation that disruptions in Ras signaling (Krab et al., 2008) and synaptic protein synthesis (Kelleher and Bear, 2008) may lie at the core of many autisms of unknown etiology, suggesting that lovastatin could have therapeutic utility in other ASDs as well as in other symptom domains.

## EXPERIMENTAL PROCEDURES

### Mice

*Fmr1* KO (Jackson Laboratory) and WT littermates, bred on the C57BL/6 or FVB background strains, were group housed and maintained in a 12:12 hr light:dark cycle. All animals were treated in accordance with NIH, MIT, and SUNY guidelines.

### Metabolic Labeling

Experiments were performed on male WT and *Fmr1* KO mice (postnatal days 25–32 [P25–P32]), blind to genotype, as described previously (Osterweil et al., 2010). Details appear in Supplemental Experimental Prodecures.

### Immunoblotting

Immunoblotting was performed blind to genotype and treatment, as described previously (Osterweil et al., 2010), using primary antibodies to Ras (Pierce), p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p70S6K (Thr389), p70S6K, p-S6 (Ser240/244), and S6 (Cell Signaling Technology) and HRP-conjugated secondary antibodies (GE Healthcare).

### Ras Activation Assay

Slices (four to six per animal) were prepared exactly as for metabolic labeling, and Ras-GTP was isolated using the Active Ras Pull-Down and Detection kit (Pierce) according to manufacturer's instructions.

### Hippocampal LTD

Experiments were performed blind to genotype, as described previously (Huber et al., 2002). Lovastatin (50 μM) was dissolved in vehicle comprising 0.05% DMSO in ACSF. Slices were exposed to drug or vehicle from 30 min prior to application of DHPG until the end of the experiment. Details appear in Supplemental Experimental Procedures.

### Hippocampal CA3 Recordings

Epileptiform activity was elicited using DHPG or bicuculline and intracellular CA3 recordings were performed as previously described (Chuang et al., 2005). Details appear in Supplemental Experimental Procedures.

### Cortical Slice Electrophysiology

Slices of visual cortex were prepared from P16–P21 WT or *Fmr1* KO animals, blind to genotype, as described previously (Philpot et al., 2001). Submerged slices were maintained at 30°C in a modified ACSF that closely mimics physiological CSF (124 mM NaCl, 3.5 mM KCl, 1.25 mM $NaH_2PO_4$, 26 vNa$HCO_3$, 10 mM dextrose, 0.8 mM $MgCl_2$, 1 mM $CaCl_2$, saturated with 95% $O_2$ and 5% $CO_2$) and promotes spiking in response to electrical stimulation. Stimulation electrodes (clustered bipolar tungsten, FHC) were positioned in white matter and extracellular recordings were performed in layer 5 using glass recording electrodes (~1 MΩ). Baseline responses were collected every 30 s, using a stimulus intensity of 35–80 μA, 0.2 ms duration. Responses were collected in ACSF plus vehicle, then ACSF + 50 μM lovastatin. Extracellular recordings made using Axopatch 200B (Axon Instruments) were amplified 1,000 times, filtered between 0.3 and 3 Hz, and digitized at 25 kHz. Further details are provided in Supplemental Experimental Procedures.

### AGS

Experiments were performed using male WT and *Fmr1* KO littermates (P17–P25), blind to genotype, as described previously (Osterweil et al., 2010). For acute exposure experiments, mice were injected i.p. with drug or vehicle and returned to their home cages for 1 hr. For oral administration experiments, mice were weaned onto standard rodent chow (Bio-serv) formulated with 100 mg/kg IC lovastatin (40 mg tablets, Mylan) or identical chow containing no lovastatin and allowed to feed ad libitum for 48 hr. Immediately prior to testing, mice were transferred to a quiet (<60 dB ambient sound) room for 1 hr, then transferred to a transparent plastic test chamber. After 1 min of habituation, mice were exposed to a 130 dB stimulus (recorded sampling of a modified personal alarm, Radioshack model 49-1010) for 2 min, and the incidence and severity of AGS was scored. Further details are provided in Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.01.034.

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