Published in final edited form as:

Neurobiol Dis. 2012 March ; 45(3): 1145–1152. doi:10.1016/j.nbd.2011.12.037.

Lithium reverses increased rates of cerebral protein synthesis in a mouse model of fragile X syndrome

Zhong-Hua Liu, Tianjian Huang, and Carolyn Beebe Smith

Section on Neuroadaptation and Protein Metabolism, National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, U.S.A.

Abstract

Individuals with fragile X syndrome (FXS), an inherited form of cognitive disability, have a wide range of symptoms including hyperactivity, autistic behavior, seizures and learning deficits. FXS is caused by silencing of *FMR1* and the consequent absence of fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that associates with polyribosomes and negatively regulates translation. In a previous study of a mouse model of FXS (*Fmr1* knockout (KO)) we demonstrated that *in vivo* rates of cerebral protein synthesis (rCPS) were elevated in selective brain regions suggesting that the absence of FMRP in FXS may result in dysregulation of cerebral protein synthesis. Lithium, a drug used clinically to treat bipolar disorder, has been used to improve mood dysregulation in individuals with FXS. We reported previously that in the *Fmr1* KO mouse chronic dietary lithium treatment reversed or ameliorated both behavioral and morphological abnormalities. Herein we report that chronic dietary lithium treatment reversed the increased rCPS in *Fmr1* KO mice with little effect on wild type mice. We also report our results of analyses of key signaling molecules involved in regulation of mRNA translation. Our analyses indicate that neither effects on the PI3K/Akt nor the MAPK/ERK 1/2 pathway fully account for the effects of lithium treatment on rCPS. Collectively our findings and those from other laboratories on the efficacy of lithium treatment in animal models support further studies in patients with FXS.

Keywords

Protein synthesis; fragile X syndrome; lithium; *Fmr1*; mouse; brain; PI3K/Akt; MAPK/ERK1/2; hippocampus

Corresponding author: Carolyn Beebe Smith Section on Neuroadaptation and Protein Metabolism Bldg. 10, 2D54, 10 Center Drive National Institutes of Health Bethesda, MD 20892-1298, U.S.A. 01-301-402-3120 (telephone) 01-301-480-1668 (FAX) beebe@mail.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Classification: Biological Sciences, Neurobiology

Introduction

Individuals with fragile X syndrome (FXS), an inherited form of intellectual disability, show a broad spectrum of morphologic, cognitive, behavioral, neurological, and psychological features (Chonchaiya et al., 2009). FXS is caused by the silencing of the *FMR1* gene and the consequent absence of its protein product, fragile X mental retardation protein (FMRP) (Brown, 2002). FMRP is a RNA-binding protein that associates with polyribosomes and negatively regulates translation of certain mRNAs (Penagarikano et al., 2007). It is thought that absence of FMRP results in a dysregulation of protein synthesis and that it is this dysregulation that has such profound consequences for development and function of the nervous system. Protein synthesis in the nervous system is required for normal nervous system development and maintenance. It is also essential for implementation of long lasting changes in synaptic strength such as occur in long term potentiation (LTP) and long term depression (LTD). Some forms of both LTD and LTP are impaired in *Fmr1* knockout (KO) mice (Huber et al., 2004; Larson et al., 2005; Li et al., 2002; Suvrathan et al., 2010; Wilson and Cox, 2007; Zhao et al., 2005), and it has been proposed that a dysregulation of protein synthesis may underlie these impairments.

In accord with the idea that a dysregulation of protein synthesis may be at the heart of the defect in FXS, we have shown that adult male *Fmr1* KO mice have elevated regional rates of cerebral protein synthesis (rCPS) compared with wild type (WT) littermates (Qin et al., 2005). We measured rCPS *in vivo* with a quantitative autoradiographic method (Smith et al., 1988). Effects were regionally selective occurring mainly in hippocampus, thalamus and hypothalamus. Other investigators have monitored the incorporation of ³⁵S-methionine/cysteine into new protein *in vitro*. Their findings also indicate that incorporation is higher in hippocampal slices from *Fmr1* KO mice (Dölen et al, 2007; Osterweil et al, 2010).

Treatment for FXS is largely symptom-based and multidisciplinary. Current treatments include special education, behavioral interventions, physical therapy and symptom-directed pharmacotherapy. There are several classes of pharmacotherapeutic agents currently under investigation in FXS that are aimed at the biochemical processes thought to underlie the disease. Among them are mGluR antagonists, antibiotics, and GABA agonists (Levenga et al., 2010). Lithium, an effective mood stabilizer for the treatment of bipolar disorder, has been used successfully in individuals with FXS to stabilize mood dysregulation (Al-Semaan et al., 1999). Results of a pilot add-on trial demonstrated that lithium may improve behavior, adaptive skills, and verbal memory in patients with FXS (Berry-Kravis et al., 2008). We and other groups have demonstrated that chronic dietary lithium treatment ameliorates many of the behavioral deficits seen in *Fmr1* KO mice (Liu et al., 2011; Mines et al., 2010; Yuskaitis et al., 2010). Moreover, lithium treatment partially normalized the immature dendritic spine morphology in medial prefrontal cortex (Liu et al., 2011). Lithium has also been shown to modify abnormal behavior and morphology in a *Drosophila* model of FXS (McBride et al., 2005). Taken together results from these studies suggest that lithium could provide significant therapeutic benefits in FXS.

How lithium treatment may effect these therapeutic outcomes is not understood. In the current study, we measure rCPS in WT and *Fmr1* KO mice to investigate whether lithium

may act through an effect on the control of translation. Our results indicate that, in addition to its effects on behavior and morphology, lithium also normalizes the elevated rCPS in *Fmr1* KO mice. We also present in this paper effects of lithium treatment on some of the steps in signaling pathways that control cap-dependent translation.

Materials and methods

Animals

Male WT and *Fmr1* KO mice (n=79), generated by FVB/NJ-*fmr1*^{tm1Cgr} breeding pairs (heterozygous females and homozygous males), were used. The generation of *Fmr1* KO mice and their genotyping by PCR amplification of tail DNA were described previously (Qin et al., 2005). All mice were group housed in a central facility and maintained under controlled conditions of normal humidity and temperature with standard alternating 12-hr periods of light and darkness. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Four groups of mice were studied: 1) WT fed control diet (WT-C) (n=23); 2) KO fed control diet (KO-C) (n=22); 3) WT fed lithium-supplemented diet (WT-Li) (n=17); 4) KO fed lithium-supplemented diet (KO-Li) (n=17). The base diet was NIH-31 and the lithium-supplemented diet was NIH-31 to which 0.3% (w/w) lithium carbonate had been added (Harlan Teklad, Madison, WI). Feeding commenced at weaning (3 weeks of age) and was provided *ad libitum* until mice were studied at 12 weeks of age. Mice fed lithium-supplemented chow were given drinking water with 1.5% (w/v) sodium chloride to counteract potential toxicity of lithium. Mice fed control chow were provided tap water. As we reported previously (Liu et al, 2011), whole blood lithium concentrations were 0.38 ± 0.03 (n = 4) and 0.38 ± 0.02 mEq/L (n = 4) in WT and *Fmr1* KO mice, respectively. No toxicity of the lithium treatment was observed. This concentration is equivalent to a plasma lithium level that is at the low end of the therapeutic range of plasma lithium for treatment of bipolar disorder in human subjects (0.4-1.2 mEq/L).

2.2. Determination of rCPS

Mice, under light isoflurane anesthesia, were prepared for studies of rCPS by insertion of polyethylene catheters (PE-10) into a femoral artery and vein. Mice recovered from the surgery overnight and food and water were available *ad libitum*. Body temperature was maintained by warming the bottom of the cage with hand warmers (Grabber, Grand Rapids, MI). Mice were permitted to move freely throughout the recovery and experimental periods. To monitor the physiological state of each mouse, mean arterial blood pressure, hematocrit and arterial plasma glucose concentrations were measured at 0 and 45 min during the rCPS experiment.

The experimental period was initiated by an intravenous pulse injection of 100 μ Ci/kg of L-[1-¹⁴C]leucine (specific activity, 60 mCi/mmol, Moravsek Biochemicals and Radiochemicals, Brea, CA). Timed arterial samples were collected during the following 60 min for determination of the time courses of plasma concentrations of leucine and [¹⁴C]leucine.

Labeled and unlabeled leucine concentrations in the acid-soluble fractions of arterial plasma were assayed by liquid scintillation counting and amino acid analysis, respectively. At the end of the experimental interval, brains were removed and frozen, and serial sections, 20 μm in thickness, were prepared for quantitative autoradiography by means of a Leica cryostat (Leica Microsystems, Inc, Buffalo Grove, IL). Sections were mounted on gelatin-coated slides, fixed and washed in 10% formalin, and exposed to Ektascan B/RA film (Eastman Kodak, Rochester, NY) along with calibrated [^{14}C]methylmethacrylate standards as described previously (Qin et al., 2005). Autoradiograms were digitized (MCID Analysis, Interfocus Imaging Ltd, Linton, Cambridge, UK), the concentration of ^{14}C in each region of interest was determined, and rCPS was calculated by means of the operational equation of the method (Smith et al, 1988). The value of lambda in the equation was 0.603 (Qin et al, 2005). Brain regions were identified by reference to a mouse brain atlas (Paxinos and Franklin, 2001).

2.3. Western blotting

At 12 weeks of age, 32 mice ($n = 8$ per group) were decapitated and brains were removed quickly and placed on ice. Hippocampi were dissected immediately, and snap frozen in dry ice. To prepare lysates, frozen tissues were thawed on ice and homogenized in 5% (w/v) ice-cold T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) with 1% Halt protease inhibitor (Thermo Scientific) and 1% phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged ($12,000 \times g$, 4°C , 15 min) and supernatant fractions were collected. Protein concentrations were determined by BCA protein assay kit (Thermo Scientific). Protein samples were subjected to electrophoresis on NuPAGE 10% Bis–Tris or 3–8% Tris-Acetate gels (Invitrogen Life Technologies, Grand Island, NY), and transferred electrophoretically to nitrocellulose membranes. Membranes were incubated with phospho-Akt (Ser 473) monoclonal antibody (1:4,000), Akt antibody (1:5,000), phospho-mTOR (Ser2448) antibody (1:1,000), mTOR antibody (1:2,000), phospho-p70 S6K1 (Thr389) antibody (1:500), p70 S6K1 (Thr389) antibody (1:1000), phospho-p44/42 ERK1/2 (Thr202/Tyr204) antibody (1:2000), p44/42 ERK1/2 antibody (1:5000), β -tubulin monoclonal antibody (1:8,000). β -tubulin was used as a loading control. All antibodies were obtained from Cell Signaling Technology, Inc (Danvers, MA). Immunoblots were developed by Western Breeze Chemiluminescent kit (Invitrogen). Signals of protein bands were quantified by densitometry with the MCID image processing system.

2.4. Immunohistochemistry

Mice (5/group, WT-C & KO-C; 2/group, WT-Li & KO-Li) were deeply anesthetized and perfused transcardially with normal saline containing 2% sodium nitrite followed by 4% paraformaldehyde containing 2.5% acrolein. Brains were removed and immersed in 30% sucrose at 4°C . Cryostat sections, 30 μm in thickness, were prepared. Floating sections were incubated with p-mTOR (Ser 2448) rabbit monoclonal antibody (Cell signaling) (1:3,000) for 1 h at room temperature followed by 48 h at 4°C , and then secondary biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) (1:600). Sections were stained with nickel-3,3'-diaminobenzidine chromogen (Sigma-Aldrich, St. Louis, MO) (Hoffman et al, 2008).

2.5. Statistical analysis

The rCPS data were analyzed by 3-way repeated measures analysis of variance (RM ANOVA) with genotype (WT, KO) and treatment (untreated, lithium-treated) as between-subjects factors and brain region as a within-subjects factor. The Western blotting data were analyzed by 2-way ANOVA with genotype and treatment as factors. *Post hoc* pair-wise comparisons were further conducted with Bonferroni *t*-tests. The significance level was set at $p = 0.05$. We used the SPSS program (IBM, Armonk, NY) for statistical computations.

3. Results

3.1. Cerebral protein synthesis

We studied WT and *Fmr1* KO mice under two conditions, control with normal rodent chow and experimental with lithium-supplemented chow. Physiological variables were measured at the time of determination of rCPS. The four groups were well matched with regard to age, body weight, and physiological status (Table 1). Arterial plasma leucine concentration was affected by the lithium treatment; leucine concentrations were lower in the lithium-treated mice regardless of genotype ($F_{(1, 29)} = 30.51, p < 0.001$).

We determined rCPS in 27 regions of brain (Fig. 1&2). In general, rCPS values were higher in KO-C mice compared with WT-C mice (3-20%). With lithium treatment rCPS values were decreased in both genotypes, but the effects were consistent and significant only in *Fmr1* KO mice. Data were analyzed by means of 3-way RM ANOVA with genotype, treatment and region as factors and repeated measures on region. The 3-way interaction was statistically significant ($F_{(4.8, 139.6)} = 4.50, p < 0.01$). *Post hoc* pair-wise comparisons revealed that compared with WT-C mice, rCPS values were higher ($p = 0.05$) in KO-C mice in 6 out of 27 regions including basolateral amygdala, paraventricular nucleus, supraoptic nucleus, arcuate nucleus, CA1 pyramidal cell layer of hippocampus and granule cell layer of dentate gyrus (Fig.1&2). Increases ranged from 11-20% in these regions. In posterior parietal cortex rCPS was 9% higher in KO-C compared with WT-C ($p = 0.055$). Values of rCPS in other regions analyzed were 3-10% higher in KO-C than WT-C mice, but these changes were not statistically significant. In *Fmr1* KO mice lithium treatment resulted in statistically significant 9-20% decreases in rCPS in 23 of the 27 regions analyzed. In the other four regions decreases approached statistical significance ($0.05 < p < 0.10$). In WT mice, decreases (1-11%) in rCPS after lithium treatment were smaller than in *Fmr1* KO mice and none reached or approached statistical significance. These data indicate that chronic lithium treatment may have very minor effects on rCPS in WT mice, but lithium reverses increased rCPS in *Fmr1* KO mice.

3.2. Akt/mTOR and MAPK/ERK1/2 signaling

It has been proposed that the therapeutic actions of lithium likely involve alterations in signaling pathways and gene expression (Chiu and Chuang, 2010). One of the principal targets of lithium may be the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. We examined the phosphorylation state of Akt in hippocampus of control and lithium-treated mice and found that lithium decreased phospho-Akt (p-Akt) (Ser 473) in both WT (20% decrease, $p=0.02$) and *Fmr1* KO (31% decrease, $p<0.001$) mice and had no

effects on total Akt (Fig. 3A&B). In untreated mice, average p-Akt was 16% higher in *Fmr1* KO mice compared with WT ($p < 0.01$), whereas in lithium-treated mice there was no difference between the two genotypes. Neither the interaction between genotype and treatment ($F_{(1, 28)} = 2.81$, $p = 0.105$) nor the main effect of genotype ($F_{(1, 28)} = 3.189$, $p = 0.08$) reached statistical significance. The main effect of treatment was statistically significant ($F_{(1, 28)} = 35.0$, $p < 0.001$), indicating that chronic lithium decreased the level of p-Akt regardless of genotype. With respect to the level of total Akt, neither the interaction between genotype and treatment ($F_{(1, 28)} = 0.06$, *NS*) nor the main effect of treatment ($F_{(1, 28)} = 1.28$, *NS*) was statistically significant. The main effect of genotype was statistically significant ($F_{(1, 28)} = 7.969$, $p < 0.01$), but the effect was very small (4% higher in KO compared with WT).

We also examined the phosphorylation state of mammalian target of rapamycin (mTOR) in hippocampus from treated and untreated WT and *Fmr1* KO mice (Fig. 3C&D). The signaling kinase, mTOR, is downstream from PI3K/Akt, and is a pivotal regulator of protein synthesis. mTOR has been shown to participate in control of cell growth and proliferation and is thought to be involved in synaptic plasticity (Jaworski and Sheng, 2006). In untreated mice, the average level of phospho-mTOR (p-mTOR) (Ser2448) was higher in *Fmr1* KO mice by 11% ($p = 0.05$). Treatment with lithium lowered p-mTOR by 11% in *Fmr1* KO mice ($p = 0.03$) and increased it by 9% in WT (*NS*). For p-mTOR the interaction between genotype and treatment was statistically significant ($F_{(1, 28)} = 8.29$, $p < 0.01$) indicating a difference in the response to lithium treatment between the two genotypes. Total mTOR levels did not differ significantly among groups (Interaction: $F_{(1, 28)} = 0.06$, *NS*; genotype: $F_{(1, 28)} = 0.02$, *NS*; treatment: $F_{(1, 28)} = 0.29$, *NS*).

We further examined the hippocampus for changes in p-mTOR by means of immunohistochemistry in a parallel series of mice (Fig. 4). Expression of p-mTOR in WT-C was highest in the *fasciola cinereum* and in the polymorph layer of the dentate gyrus. Staining was also intense in layers just above and below the pyramidal cell layer of CA1. The heavier band of staining is the innermost portion of the *stratum oriens*, the locus of the basal dendrites of the pyramidal cells. The layer of staining below the pyramidal cell layer is the innermost portion of *stratum radiatum* and the locus of the portions of apical dendrites closest to the soma. There is also discernable staining in the granule cell layer of the dentate gyrus and at the hippocampal fissure. Comparison of the staining pattern in KO-C with WT-C suggests that staining in the layers above and below the pyramidal cells and in *stratum radiatum* is higher in the KO-C mice. Following lithium treatment this enhanced staining in the KO mice appears to be abated.

One of the downstream targets of mTOR is a ribosomal protein p70 S6 kinase (p70 S6K1). We found no differences in the relative levels of either the total or phosphorylated forms of p70 S6K1 in hippocampal extracts between WT and *Fmr1* KO mice with or without chronic lithium treatment (Fig. 3E&F).

We also considered the possibility that lithium may act through effects on the mitogen-activated protein kinase (MAPK)/ extracellular-regulated kinase (ERK1/2) pathway. We analyzed the effects of lithium treatment on phosphorylated (Thr202/Tyr204) and total

ERK1/2 in hippocampal homogenates. Similar to our results on p70 S6K1 we found no statistically significant effects, although there was a tendency for p-ERK1/2 levels to be higher in untreated *Fmr1* KO mice (Fig. 5).

4. Discussion

The central finding of our study is that chronic treatment with lithium reversed the increased rCPS found in *Fmr1* KO mice and had little, if any, effect on rCPS in WT mice. Increased rCPS in the untreated *Fmr1* KO mice were found primarily in the limbic system and hypothalamus, whereas the effects of lithium occurred throughout the brain. We extended our studies to examine in hippocampus the effects of lithium treatment on some of the signaling pathways that influence translation. Our results indicate that neither effects on the PI3K/Akt nor the ERK 1/2 pathway can fully account for the effects on rCPS under basal conditions.

The protein absent in *Fmr1* KO mice is FMRP, an RNA-binding protein that negatively regulates translation of specific mRNAs (Lagerbauer et al., 2001; Li et al, 2001; Zhang et al., 2001). We posit that a dysregulation of general translation is the primary defect of FXS that underlies the wide range of signs and symptoms of the disease. Protein synthesis is an integral part of the plasticity response in nervous tissue. As such, its tight regulation is vital for this critical brain function. We have shown previously that adult *Fmr1* KO mice have elevated rCPS in selective regions of the brain including the hippocampus, parts of the hypothalamus and thalamus, amygdala, and frontal and parietal cortex (Qin et al., 2005). We used the *in vivo* autoradiographic leucine method (Smith et al., 1988) to quantify protein synthesis rates in our studies. This methodology made it possible to measure steady state rates of ongoing protein synthesis in conscious functioning mice. We took care not to disturb the metabolic state of the animals. During the study we administered a tracer amount of a radiolabeled naturally occurring amino acid, and we sampled arterial blood to monitor the clearance of the tracer and the concentrations of amino acids in the arterial plasma while the animals were awake and freely moving. Since we did nothing to perturb the metabolic state by our procedures, measurements should reflect ongoing processes in the intact nervous system. Our measurements are average rates of leucine incorporation into all tissue proteins; they do not target specific proteins. The spatial resolution of the autoradiographic method is 50 μm (Smith, 1983). This permits us to distinguish specific nuclei and cell layers, including dendritic-rich and cell body layers. We cannot, however distinguish specific cell types within a layer.

After chronic dietary lithium treatment, the increased rCPS in *Fmr1* KO mice was reversed. Because the effects on protein synthesis are so close to the genetic abnormality, we think that this may underlie the amelioration of lithium on behavioral deficits and immature dendritic spine morphology. We and others have previously discovered that the same lithium treatment strategy leads to multiple beneficial effects on behavioral phenotypes in *Fmr1* KO mice such as hyperactivity, low generalized anxiety, impaired social interaction, impaired performance on the passive avoidance test (Liu et al., 2011; Mines et al., 2010; Yuskaitis et al., 2010) and increased incidence of audiogenic seizures (Min et al., 2009). We have also

found that lithium corrected the abnormally increased spine length and density in medial frontal cortex of fragile X mice (Liu et al., 2011).

In the literature there are reports of some indirect measures that suggest a negative effect of lithium on mRNA translation in brain (Berg, 1968; De Bernardi et al., 1969; Wolcott, 1981; Wolcott, 1982; Kuznetsov, 1989). Studies employing microarray analysis of rodent brain showed that 20-50 transcripts were significantly down-regulated after chronic lithium treatment; none of the transcripts was up-regulated (Bosetti et al., 2002a; Chetcuti et al., 2008). The concentration of a key regulator of translation, eIF-2Be, was decreased in rat brain extracts by 29% following chronic treatment with lithium (Bosetti et al., 2002b). In our study, negative effects of lithium treatment on rCPS were clear in *Fmr1* KO mice.

The process of protein synthesis is primarily regulated by translation factors that transiently associate with ribosomes. Numerous components involved in the initiation and elongation stages of translation are regulated by mTOR, a protein kinase that phosphorylates 4E-BP (eIF4E-binding proteins) and the ribosomal protein p70 S6 kinase 1 (p70 S6K1). The PI3K/Akt signaling pathway is an important upstream regulator of mTOR. At the synapse, this pathway is coupled to membrane receptors. Results of recent studies indicate that PI3K/Akt signaling may be dysregulated in FXS. Basal PI3K activity in cortical synaptoneurosomes from developing *Fmr1* KO mice was found to be excessive likely due to increased synthesis and synaptic localization of its catalytic subunit, p110 β , both of which are normally regulated by FMRP (Gross et al., 2010). In hippocampal slices from *Fmr1* KO mice, mTOR signaling was also found to be elevated possibly due to an increase in PI3K enhancer (PIKE) (Sharma et al., 2010), which is also normally regulated by FMRP. It has been suggested that these effects lead to the exaggerated protein synthesis in the *Fmr1* KO mice.

In the present study, we looked at the phosphorylation status of some components of the PI3K/Akt pathway to try to understand the effects of lithium treatment. Herein we investigated the expression of total and phosphorylated forms of Akt, mTOR, and p70 S6K1 in homogenates of hippocampus. Relative levels of p-Akt ($p=0.01$) and p-mTOR ($p=0.05$) were elevated in untreated *Fmr1* KO mice, but not in the downstream component, p-p70 S6K1. Our results in the untreated mice agree with other measurements of p-Akt and p-mTOR in lysates of freshly dissected hippocampus (Osterweil et al., 2010; Sharma et al., 2010), although the percent changes in our study were less than previously reported. Our result on p-p70 S6K1 contrasts with that of a previous report (Sharma et al., 2010) in which p-S6K was increased by 32% in *Fmr1* KO mice, but in the previous study a 60 kDa band was analyzed whereas we analyzed a 70 kDa band (Shima et al., 1998; Ruvinsky and Meyuhas, 2006). It is also possible that quantitative differences between our results and those of others are due to differences in the ages of animals studied. In both of the other studies, mice were 28-42 days of age (Osterweil et al., 2010; Sharma et al., 2010), whereas in our study mice were 80-90 days of age. Another factor that may play a role is the effect of postmortem ischemia which may vary with preparation time and conditions.

Chronic lithium treatment resulted in substantial decreases in p-Akt in both genotypes, but effects of lithium on p-mTOR and p-p70 S6K1 were slight and were only seen in *Fmr1* KO

mice. The effects of lithium on GSK-3 β activity may play a role in the diminished effects further down the PI3K/Akt pathway. In our previous study, we showed that whole brain extracts from adult *Fmr1* KO mice had a decreased (~30%) level of the phosphorylated form of glycogen synthase kinase-3 β (Ser9) (GSK-3 β) indicating an increased activity compared to WT; this was reversed by lithium treatment (Liu et al., 2011). Decreases in both Akt and GSK-3 β activities may have opposing effects on mTOR via their opposite effects on tuberous sclerosis protein 2 (TSC2). GSK-3 β activates TSC2 and consequently further suppresses mTOR, whereas Akt inhibits TSC2 thus decreasing TSC2 suppression of mTOR. Assuming that the effects of lithium on GSK-3 β activity that we found in whole brain homogenates (Liu et al, 2011) also occur in the hippocampus, these opposing effects on TSC2 may explain the diminished effects of the lack of FMRP and of lithium treatment on p-mTOR and p-p70 S6K1.

We also considered the possibility that effects of lithium on translation may occur through the MAPK/ERK1/2 pathway. Our analysis of the relative level of the phosphorylated form of ERK1/2 in the hippocampus under basal conditions indicates no difference between WT and *Fmr1* KO mice in agreement with others (Osterweil et al, 2010; Gross et al, 2010). Moreover, we found no effect of lithium treatment.

Collectively our results clearly indicate that lithium treatment ameliorates increased protein synthesis in *Fmr1* KO mice but they do not support an effect of lithium treatment on protein synthesis by means of either the PI3K/Akt or the MAPK/ERK1/2 pathway. The mechanisms underlying the up-regulation of protein synthesis and the effects of lithium treatment in fragile X are likely more complicated than originally thought. Multiple pathways may be involved. These will be the focus of future studies.

These findings are of interest in light of a model proposed for the bimodal mechanism of action of lithium (Jope, 1999). Lithium, a drug used primarily in the treatment of bipolar disorder, has been shown to attenuate both hyperactivity (Jope, 1999) and hypoactivity (Lerer et al., 1980; Smith, 1988). Lithium elevates basal adenylyl cyclase activity, but it decreases receptor-coupled stimulation of adenylyl cyclase (Jope, 1999). In mouse brain slices, lithium acutely inhibits and chronically upregulates and stabilizes glutamate uptake (Dixon and Hokin, 1999). In animal studies lithium has been shown to activate Akt (Chalecka-Franaszek and Chuang, 1998) and to downregulate Akt (Erdal et al., 2005; Nemoto et al., 2008). In the bimodal model of lithium action, lithium reduces the low and high extremes, resulting in a stabilization of fluctuations in activity (Jope, 1999).

Our data add credence to the idea that dysregulated protein synthesis in the nervous system is central to FXS. We have confirmed our original finding that mice lacking FMRP have elevated *in vivo* rCPS (Qin et al., 2005). We further show that chronic lithium treatment of these mice reverses the excessive translation, but we do not find evidence that this effect occurs through PI3K/Akt or MAPK/ERK1/2 signaling. Moreover, chronic lithium treatment ameliorates the behavioral and morphological characteristics of the *Fmr1* KO mouse (Liu et al., 2011). These results in the mouse model coupled with the results from other laboratories studying the mouse model (Min et al., 2009), the *Drosophila* model (McBride et al., 2005),

and human subjects (Berry-Kravis et al., 2008) make a strong case for instituting a placebo-controlled trial of lithium in subjects with FXS.

Acknowledgements

We thank Zengyan Xia for overseeing the breeding colony and Tom Burlin for analyzing plasma samples for amino acid concentrations. We also thank Dr. De-Maw Chuang for helpful discussions at the outset of these studies. The research was supported by the Intramural Research Program of the National Institute of Mental Health, National Institutes of Health.

Abbreviations Footnote

FXS	fragile X syndrome
FMRP	fragile X mental retardation protein
rCPS	regional rate of cerebral protein synthesis
WT	wild type
KO	knock out
Akt	protein kinase B
mTOR	mammalian target of rapamycin
PI3K	phosphatidylinositol 3-kinase
ERK	extracellular-signal-regulated kinase
p70 S6K	p70 S6 kinase
PIKE	PI3K enhancer
4E-BP1	eIF4E-binding protein

References

- Al-Semaan Y, Malla AK, Lazosky AI. Schizoaffective disorder in a fragile-X carrier. *Aust N Z J Psychiatry*. 1999; 33:436–40. [PubMed: 10442803]
- Berg WE. Effect of lithium on the rate of protein synthesis in the sea urchin embryo. *Exp Cell Res*. 1968; 50:133–9. [PubMed: 5650856]
- Berry-Kravis E, Sumis A, Hervey C, Nelson M, Porges SW, Weng N, Weiler IJ, Greenough WT. Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr*. 2008; 29:293–302. [PubMed: 18698192]
- Bosetti F, Seemann R, Bell JM, Zahorchak R, Friedman E, Rapoport SI, Manickam P. Analysis of gene expression with cDNA microarrays in rat brain after 7 and 42 days of oral lithium administration. *Brain Res Bull*. 2002a; 57:205–9. [PubMed: 11849827]
- Bosetti F, Seemann R, Rapoport SI. Chronic lithium chloride administration to rats decreases brain protein level of epsilon (epsilon) subunit of eukaryotic initiation factor-2B. *Neurosci Lett*. 2002b; 327:71–3. [PubMed: 12098503]
- Brown, WT. The molecular biology of the fragile X mutation.. In: Hagerman, RJ.; Hageman, PJ., editors. *Fragile X Syndrome: Diagnosis, Treatment and Research*. The Johns Hopkins University Press; 2002. p. 110-135.

- Chalecka-Franaszek E, Chuang DM. Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. *Proc Natl Acad Sci USA*. 1999; 96:8745–50. [PubMed: 10411946]
- Chetcuti A, Adams LJ, Mitchell PB, Schofield PR. Microarray gene expression profiling of mouse brain mRNA in a model of lithium treatment. *Psychiatr Genet*. 2008; 18:64–72. [PubMed: 18349697]
- Chiu CT, Chuang DM. Molecular actions and therapeutic potential of lithium in preclinical and clinical studies of CNS disorders. *Pharmacol Ther*. 2010; 128:281–304. [PubMed: 20705090]
- Chonchaiya W, Schneider A, Hagerman RJ. Fragile X: a family of disorders. *Adv Pediatr*. 2009; 56:165–86. [PubMed: 19968948]
- De Bernardi F, Leonardi Cigada M, Maci R, Ranzi S. On protein synthesis during the development of lithium-treated embryos. *Experientia*. 1969; 25:211–3. [PubMed: 5786115]
- Dixon JF, Hokin LE. Lithium acutely inhibits and chronically up-regulates and stabilizes glutamate uptake by presynaptic nerve endings in mouse cerebral cortex. *Proc Natl Acad Sci USA*. 1998; 95:8363–8. [PubMed: 9653192]
- Dölen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, Chattarji S, Bear MF. Correction of fragile X syndrome in mice. *Neuron*. 2007; 56:955–62. [PubMed: 18093519]
- Erdal E, Ozturk N, Cagatay T, Eksioğlu-Demiralp E, Ozturk M. Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells. *Int J Cancer*. 2005; 115:903–10. [PubMed: 15723355]
- Gross C, Nakamoto M, Yao X, Chan C-B, Yim SY, Ye K, Warren ST, Bassell GJ. Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome. *J Neurosci*. 2010; 30:10624–10638. [PubMed: 20702695]
- Hoffman GE, Le WW, Sita LV. The importance of titrating antibodies for immunocytochemical methods. *Curr Protoc Neurosci*. 2008 Chapter 2: Unit 2.12.
- Huber KM, Gallagher SM, Warren ST, Bear MF. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci USA*. 2004; 99:7746–50. [PubMed: 12032354]
- Jaworski J, Sheng M. The growing role of mTOR in neuronal development and plasticity. *Mol Neurobiol*. 2006; 34:205–19. [PubMed: 17308353]
- Jope RS. A bimodal model of the mechanism of action of lithium. *Mol Psychiatry*. 1999; 4:21–5. [PubMed: 10089004]
- Kuznetsov DA. Lithium salts adopted for psychopharmacological use as the modulators of protein synthesis in the brain: analytical review. *Int J Neurosci*. 1989; 45:1–6. [PubMed: 2654043]
- Lagerbauer B, Ostareck D, Keidel EM, Ostareck-Lederer A, Fischer U. Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum Mol Genet*. 2001; 10:329–38. [PubMed: 11157796]
- Larson J, Jessen RE, Kim D, Fine A-KS, Hoffmann J. Age-dependent and selective impairment of long-term potentiation in the anterior piriform cortex of mice lacking the fragile X mental retardation protein. *J Neurosci*. 2005; 25:9460–9. [PubMed: 16221856]
- Lerer B, Ebstein RP, Felix A, Belmaker RH. Lithium amelioration of reserpine-induced hypoactivity in rats. *Int Pharmacopsychiatry*. 1980; 15:338–43. [PubMed: 7298265]
- Levenga J, de Vrij FM, Oostra BA, Willemsen R. Potential therapeutic interventions for fragile X syndrome. *Trends Mol Med*. 2010; 16:516–27. [PubMed: 20864408]
- Li J, Pelletier MR, Velazquez J-LP, Carlen PL. Reduced cortical synaptic plasticity and GluR1 expression associated with fragile X mental retardation protein deficiency. *Mol cell neurosci*. 2002; 19:138–51. [PubMed: 11860268]
- Li Z, Zhang Y, Ku L, Wilkinson KD, Warren ST, Feng Y. The fragile X mental retardation protein inhibits translation via interacting with mRNA. *Nucleic Acids Res*. 2001; 29:2276–83. [PubMed: 11376146]
- Liu ZH, Chuang DM, Smith CB. Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome. *Int J Neuropsychopharmacol*. 2011; 14:618–30. [PubMed: 20497624]
- McBride SM, Choi CH, Wang Y, Liebelt D, Braunstein E, Ferreira D, Sehgal A, Siwicki KK, Dockendorff TC, Nguyen HT, McDonald TV, Jongens TA. Pharmacological rescue of synaptic

plasticity, courtship behavior, and mushroom body defects in a *Drosophila* model of fragile X syndrome. *Neuron*. 2005; 45:753–64. [PubMed: 15748850]

Min WW, Yuskaitis CJ, Yan Q, Sikorski C, Chen S, Jope RS, Bauchwitz RP. Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential. *Neuropharmacology*. 2009; 56:463–72. [PubMed: 18952114]

Mines MA, Yuskaitis CJ, King MK, Beurel E, Jope RS. GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism. *PLoS One*. 2010; 5:e9706. [PubMed: 20300527]

Nemoto T, Kanai T, Yanagita T, Satoh S, Maruta T, Yoshikawa N, Kobayashi H, Wada A. Regulation of Akt mRNA and protein levels by glycogen synthase kinase-3 β in adrenal chromaffin cells: effects of LiCl and SB216763. *Eur J Pharmacol*. 2008; 586:82–9. [PubMed: 18395711]

Osterweil EK, Krueger DD, Reinhold K, Bear MF. Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *J Neurosci*. 2010; 30:15616–27. [PubMed: 21084617]

Paxinos, G.; Franklin, KBJ. The mouse brain in stereotaxic coordinates. Academic Press; New York: 2001.

Penagarikano O, Mulle JG, Warren ST. The pathophysiology of fragile x syndrome. *Annu Rev Genomics Hum Genet*. 2007; 8:109–29. [PubMed: 17477822]

Qin M, Kang J, Burlin TV, Jiang C, Smith CB. Postadolescent changes in regional cerebral protein synthesis: an in vivo study in the FMR1 null mouse. *J Neurosci*. 2005; 25:5087–95. [PubMed: 15901791]

Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *TIBS*. 2006; 31:342–348. [PubMed: 16679021]

Sharma A, Hoeffler CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, Zukin RS. Dysregulation of mTOR signaling in fragile X syndrome. *J Neurosci*. 2010; 30:694–702. [PubMed: 20071534]

Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the p70^{s6k}/p85^{s6k} gene reveals a small mouse phenotype and a new functional S6 kinase. *The EMBO J*. 1998; 17:6649–6659. [PubMed: 9822608]

Smith, CB. Localization of activity-associated changes in metabolism of the central nervous system with the deoxyglucose method: prospects for cellular resolution.. In: Barker, JL.; McKelvey, JF., editors. *Methods in Cellular Neurobiology*. Vol. 1. John Wiley & Sons; New York: 1983. p. 269-317.

Smith CB, Deibler GE, Eng N, Schmidt K, Sokoloff L. Measurement of local cerebral protein synthesis in vivo: influence of recycling of amino acids derived from protein degradation. *Proc Natl Acad Sci USA*. 1988; 85:9341–5. [PubMed: 3057507]

Smith DF. Lithium attenuates clonidine-induced hypoactivity: further studies in inbred mouse strains. *Psychopharmacology*. 1988; 94:428–30. [PubMed: 3128821]

Suvrathan A, Hoeffler CA, Wong H, Klann E, Chattarji S. Characterization and reversal of synaptic defects in the amygdala in a mouse model of fragile X syndrome. *Proc Natl Acad Sci USA*. 2010; 107:11591–6. [PubMed: 20534533]

Wilson BM, Cox CL. Absence of metabotropic glutamate receptor-mediated plasticity in the neocortex of fragile X mice. *Proc Natl Acad Sci USA*. 2007; 104:2454–9. [PubMed: 17287348]

Wolcott DL. Effect of potassium and lithium ions on protein synthesis in the sea urchin embryo. *Exp Cell Res*. 1981; 132:464–8. [PubMed: 7215455]

Wolcott DL. Does protein synthesis decline in lithium-treated sea urchin embryos because RNA synthesis is inhibited? *Exp Cell Res*. 1982; 137:427–31. [PubMed: 7056296]

Ye K, Hurt KJ, Wu FY, Fang M, Luo HR, Hong JJ, Blackshaw S, Ferris CD, Snyder SH. Pike: A nuclear gtpase that enhances PI3kinase activity and is regulated by protein 4.1N. *Cell*. 2000; 103:919–30. [PubMed: 11136977]

Yuskaitis CJ, Mines MA, King MK, Sweatt JD, Miller CA, Jope RS. Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome. *Biochem Pharmacol*. 2010; 79:632–46. [PubMed: 19799873]

- Zhang YQ, Bailey AM, Matthies HJ, Renden RB, Smith MA, Speese SD, Rubin GM, Broadie K. *Drosophila fragile X*-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell*. 2001; 107:591–603. [PubMed: 11733059]
- Zhao MG, Toyoda H, Ko SW, Ding HK, Wu LJ, Zhuo M. Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J Neurosci*. 2005; 25:7385–92. [PubMed: 16093389]

Research Highlights

- Chronic lithium reverses increased cerebral protein synthesis in *Fmr1* KO mice
- Chronic lithium has little effect on cerebral protein synthesis in WT mice
- PI3K/Akt pathway signaling does not fully account for effects on hippocampal rCPS
- MAPK/ERK 1/2 pathway signaling does not fully account for effects on hippocampal rCPS

Fig. 1.

Effects of chronic lithium treatment on rCPS in WT and *Fmr1* KO mice. Bars represent the means $\pm$ SEM for WT-C (n=10), KO-C (n=9), WT-Li (n=7) and KO-Li (n=7) mice.

Abbreviations are as follows: FrA, frontal association cortex; mPPr, medial prefrontal cortex (infralimbic and prelimbic); Cg, anterior cingulate cortex; M1, primary motor cortex; S1, somatosensory cortex; PPtA, posterior parietal cortex; VHi, ventral hippocampus; CA1 pyr, CA1 sector of the pyramidal cell layer of ventral hippocampus; Rad, stratum radiatum of ventral hippocampus; GrDG, granule cell layer of the dentate gyrus of ventral hippocampus; BLA, basal lateral amygdala; CeA, central amygdaloid nucleus; DTh, dorsal thalamus; VTh, ventral thalamus; LHb, lateral habenula; MHb, medial habenula; LH, lateral hypothalamic area; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus, Arc, arcuate nucleus; SuMM, Supramammillary nucleus; S, septal nucleus; CPu, caudate putamen; DR, dorsal raphe; MR, median raphe; Ce, cerebellum; CC, corpus callosum. Data

were analyzed by means of 3-way RM ANOVA, followed by pair-wise comparisons with Bonferroni t-tests. The 3-way interaction (genotype x treatment x region) was statistically significant ($F_{(4,8,139.6)}=4.50$, $p<0.01$).

Post-hoc comparisons:

Statistically significantly different from WT-C, *, 0.01 P 0.05; **, 0.001 P 0.01; #, P 0.06 Statistically significantly different from KO-C, †, 0.01 P 0.05; ††, 0.001 P 0.01

Fig. 2.

Digitized autoradiographic images color-coded for rCPS at the level of dorsal hippocampus (A-D) and at the level of the anterior hypothalamus (E-H). Representative images at the level of dorsal hippocampus from each experimental group are as follows: (A) WT-C, (B) KO-C, (C) WT-Li, (D) KO-Li. Upper color bar and the scale bar in A (2 mm) apply to all four images (A-D). Representative images from each experimental group at the level of anterior hypothalamus are as follows: (E) WT-C, (F) KO-C, (G) WT-Li, (H) KO-Li. Lower color bar and the scalebar in E (1 mm) apply to all four images (E-H). The paraventricular nuclei (PVN) are indicated in E.

Fig. 3.

Western blot analysis and representative Western blots of (A) p-Akt (ser473), (B) total Akt, (C) p-mTOR (ser2448), (D) total mTOR levels (E) p-p70 S6K1 (Thr389), and F p70 S6K1 in hippocampal lysates. Optical densities were normalized to β -tubulin and values were expressed as percent of WT-C. A. p-Akt, both the genotype $\times$ treatment interaction ($F_{(1, 28)} = 2.81$, $p = 0.11$) and the main effect of genotype ($F_{(1, 28)} = 3.19$, $p = 0.09$) approached statistical significance, and the main effect of treatment was statistically significant ($F_{(1, 28)} = 35.00$, $p < 0.001$). Because the interaction only approached statistical significance pairwise

comparisons are not strictly valid. B. Total Akt, neither the genotype x treatment interaction ($F_{(1, 28)} = 0.06$, NS) nor the main effect of treatment ($F_{(1, 28)} = 1.28$, NS) was statistically significant, but the main effect of genotype ($F_{(1, 28)} = 7.97$, $p < 0.01$) was. C. p-mTOR, the genotype x treatment interaction ($F_{(1, 28)} = 8.29$, $p < 0.01$) was statistically significant. D. Total mTOR, the genotype x treatment interaction ($F_{(1, 28)} = 0.06$, NS) was not statistically significant, neither was the main effect of treatment ($F_{(1, 28)} = 0.29$, NS) nor genotype ($F_{(1, 28)} = 0.02$, NS). E. p-p70 S6K1, the genotype x treatment interaction ($F_{(1, 28)} = 1.79$, NS) was not statistically significant, neither was the main effect of treatment ($F_{(1, 28)} = 0.57$, NS) nor genotype ($F_{(1, 28)} = 0.66$, NS). F. Total p70 S6K1, the genotype x treatment interaction ($F_{(1, 28)} = 0.41$, NS) was not statistically significant, neither was the main effect of treatment ($F_{(1, 28)} = 0.91$, NS) nor genotype ($F_{(1, 28)} = 0.20$, NS). Results of *post-hoc* Bonferroni *t*-tests are shown on the figure; *, $p \leq 0.05$; **, $p \leq 0.001$.

Fig. 4.

Immunohistochemical localization of p-mTOR (Ser 2448) in the dorsal hippocampus from (A & B) WT-C, (C & D) WT-Li, (E & F) KO-C, and (G & H) KO-Li mice. Scale bars in A and B are 250 and 25 μm , respectively. Boxes in the low power images (A, C, E, & G) indicate the area from which the high power images were obtained (B, D, F, & H). Staining is particularly intense in *fasciola cinereum* (visible in C & D), CA2, and in the polymorph layer of the dentate gyrus. Staining is also noteworthy in layers just above and below the pyramidal cell layer of CA1. The upper band of staining is the innermost portion of the *stratum oriens*, the locus of the basal dendrites of the pyramidal cells. The layer of staining below the pyramidal cell layer is the innermost portion of *stratum radiatum* and the locus of the portions of apical dendrites closest to the soma. In the higher power images (B, D, F, & H) there is clear activity in the cytoplasm of the pyramidal cells and in the pyramidal cell dendrites. There is also discernable staining in the granule cell layer of the dentate gyrus and at the hippocampal fissure. High p-mTOR activity in pyramidal cell dendrites (particularly proximal segments) (F) appears to be normalized in lithium-treated mice (H).

Fig. 5.

Western blot analysis and representative Western blots of (A) phospho-p44/42 ERK1/2 (Thr202/Tyr204), (B) total p44/42 ERK1/2 in hippocampal lysates. Optical densities were normalized to β-tubulin and values were expressed as percent of WT-C. A. p-p44/42 ERK1/2 (Thr202/Tyr204), the genotype x treatment interaction ($F_{(1, 28)} = 0.56$, NS) was not statistically significant, neither was the main effect of treatment ($F_{(1, 28)} = 0.35$, NS) nor genotype ($F_{(1, 28)} = 0.80$, NS). B. Total p44/42 ERK1/2, the genotype x treatment interaction ($F_{(1, 28)} = 0.13$, NS) was not statistically significant, neither was the main effect of treatment ($F_{(1, 28)} = 2.30$, $p=0.14$) nor genotype ($F_{(1, 28)} = 1.83$, NS).

Table 1

Physiological variables

	WT-C (10)	KO-C (9)	WT-Li (7)	KO-Li (7)
Age (days)	87.4 ± 0.9	87.0 ± 0.9	87.0 ± 1.0	87.1 ± 0.9
Body weight (g)	28.0 ± 0.5	29.4 ± 0.7	28.5 ± 0.9	27.7 ± 0.7
Hematocrit (%), 0 min	43.3 ± 0.4	42.4 ± 0.5	42.1 ± 0.7	42.2 ± 0.8
45 min	39.0 ± 0.4	38.6 ± 0.5	38.5 ± 0.6	38.4 ± 0.9
Arterial plasma glucose concentration (mg/dl), 0 min	9.10 ± 0.25	7.94 ± 0.38	8.29 ± 0.64	8.57 ± 0.72
45 min	9.12 ± 0.18	8.33 ± 0.48	8.73 ± 0.71	8.69 ± 0.42
Mean arterial blood pressure (mmHg), 0 min	116.6 ± 2.4	112.0 ± 1.6	126.5 ± 3.4	125.0 ± 2.3
45 min	113.8 ± 3.8	107.3 ± 2.6	117.7 ± 4.4	115.2 ± 2.1
Arterial plasma leucine concentration (nmol/ml)	135.5 ± 4.2	142.7 ± 4.5	116.7 ± 7.5	104.8 ± 4.1

Values were means ± SEM for the number of mice indicated in parentheses. Arterial plasma leucine concentrations are the means for each animal of samples drawn over the 60 min experimental interval weighted by the integrated [¹⁴C]leucine activity during the sampling interval. There were no statistically significant interactions between genotype and treatment (2-way ANOVA) for any of the variables measured. The main effect of treatment, but not genotype, was statistically significant for arterial plasma leucine concentration ($P < 0.001$).