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Susana R. Louros[1,2]*, Sang S. Seo[1,2]*, Beatriz Maio[1,2], Cristina Martinez-Gonzalez[1,2], Miguel A. Gonzalez-Lozano[3], Melania Muscas[1,2], Nick C. Verity[1,2], Jimi C. Wills[4], Ka Wan Li[3], Matthew F. Nolan[1,2], Emily K. Osterweil[1,2,5]

* These authors contributed equally

Affiliations: [1] Centre for Discovery Brain Sciences, University of Edinburgh, UK
[2] Simons Initiative for the Developing Brain, University of Edinburgh, UK
[3] Department of Molecular and Cellular Neurobiology, Centre for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, Netherlands
[4] CRUK Edinburgh Centre, Institute of Genetics and Cancer, University of Edinburgh, United Kingdom
[5] Lead contact

Corresponding author: Emily K. Osterweil, University of Edinburgh,
Hugh Robson Building, George Square, Edinburgh, EH8 9XD, UK
Email: Emily.osterweil@ed.ac.uk

Summary:

In Fragile X Syndrome (FX), the leading monogenic cause of autism, excessive neuronal protein synthesis is a core pathophysiology, however an overall increase in protein expression isn't observed. Here, we tested whether excessive protein synthesis drives a compensatory rise in protein degradation that is protective for FX mouse model (*Fmr1*$^{-/y}$) neurons. Surprisingly, although we find a significant increase in protein degradation through ubiquitin proteasome system (UPS), this contributes to pathological changes. Normalizing proteasome activity with bortezomib corrects excessive hippocampal protein synthesis and hyperactivation of neurons in the inferior colliculus (IC) in response to auditory stimulation. Moreover, systemic administration of bortezomib significantly reduces the incidence and severity of audiogenic seizures (AGS) in the *Fmr1*$^{-/y}$ mouse, as does genetic reduction of proteasome specifically in the IC. Together these results identify excessive activation of the UPS pathway in *Fmr1*$^{-/y}$ neurons as a contributor to multiple phenotypes that can be targeted for therapeutic intervention.

Keywords: Fragile X, *FMR1*, UPS, proteasome, proteostasis, AGS, bortezomib, inferior colliculus

Introduction:

Fragile X Syndrome (FX) is a neurodevelopmental disorder affecting 1:4000 males and 1:8000 females, which arises from transcriptional silencing of the *FMR1* gene and subsequent loss of the RNA-binding protein Fragile X Messenger Ribonucleoprotein (FMRP) [1-4]. It is associated with a variety of symptoms including intellectual disability, hypersensitivity, ADHD, epilepsy, and it is the most commonly identified monogenic cause of autism [5,6]. Studies of *Fmr1* mutant animal models have identified alterations in plasticity and excitability in a number of different brain circuits [7-9], with several studies identifing excessive protein synthesis as a key contributor [9-14]. This has led to the development of therapies that normalize protein synthesis, including metabotropic glutamate receptor 5 ($mGlu_5$) antagonists and inhibitors of the extracellular regulated-kinase (ERK) and mammalian target of rapamycin (mTOR) signalling pathways [15-18].

FMRP localizes to polyribosome complexes and has a well-described role in the repression of translation [19-22]. There are over 1000 identified targets of FMRP including many that encode critical synaptic proteins, so a key question is whether the phenotypes in FX arise due to an excessive accumulation of proteins encoded by these targets [23-25]. While previous studies have shown that select targets are overexpressed in *Fmr1*$^{-/y}$ brain [26-28], recent evidence shows that the majority of FMRP target mRNAs are less abundant in the ribosome-bound population, and underexpressed in the synapse-enriched proteome of *Fmr1*$^{-/y}$ hippocampus [24,29-32]. Moreover, proteomics analyses of juvenile and adult *Fmr1*$^{-/y}$ brain have not identified a global change in protein accumulation to match the 15-20% increase in protein synthesis [32-35]. This suggests that the increased protein synthesis seen in *Fmr1*$^{-/y}$ brain does not result in a general accumulation of proteins at synapses.

One possibility for the lack of protein accumulation in *Fmr1*$^{-/y}$ neurons is that there is a compensatory increase in protein degradation in response to the elevation of protein synthesis, which keeps the steady-state proteome in balance. The major pathway responsible for targeted protein degradation is the ubiquitin proteasome system (UPS), which is comprised of a series of ubiquitin ligases that covalently attach ubiquitin molecules to proteins targeted for degradation, and the 26S proteasome complex that enzymatically digests these targets [36]. The UPS is also a critical regulator of cytoskeletal proteins that regulate dendritic spine

morphology, as well as postsynaptic receptors and presynaptic vesicle proteins [37-39]. As such it is essential for multiple synaptic and circuit functions including the long-term potentiation and depression (LTP/D) of synaptic strength and the maintenance of homeostatic plasticity [40,41].

Here, we show that activity of the proteasome is elevated in *Fmr1*$^{-/y}$ hippocampal neurons, with a concomitant increase in UPS components observed in neuron-specific Translating Ribosome Affinity Purification and RNA sequencing (TRAP-seq) datasets from *Fmr1*$^{-/y}$ mice. Proteomics analysis of hippocampal slices treated with the proteasome inhibitor MG132 reveals an increase in protein degradation. Pharmacological UPS inhibition normalizes excessive protein synthesis, showing that manipulation of protein degradation can beneficially impact the elevation in translation. Furthermore, systemic administration of the proteasome inhibitor bortezomib (BTZ) normalizes proteasome activity in neurons of the *Fmr1*$^{-/y}$ inferior colliculus (IC) and prevents the hyperactivation of these neurons in response to sound. Consequently, this treatment significantly reduces the incidence and severity of audiogenic seizures (AGS) in *Fmr1*$^{-/y}$ mice as does *Psmb5* shRNA expression in *Fmr1*$^{-/y}$ IC. Together these results define excessive protein degradation as a novel cellular pathology that can be targeted for therapeutic intervention in FX.

Results:

Proteasome activity and UPS expression are increased in *Fmr1*$^{-/y}$ neurons

To investigate UPS function in *Fmr1*$^{-/y}$ neurons, we designed a hippocampal slice assay to measure proteasome activity under the same conditions where excess protein synthesis and electrophysiological changes are observed [10]. Hippocampal slices were prepared from littermate *Fmr1*$^{-/y}$ and WT mice, and proteasome activity measured in slice lysates using a fluorescence assay that reports chymotrypsin-like activity with the fluorogenic substrate Suc-LLVY-AMC (**Fig. 1A**) [42]. Our results show a significant increase in proteasome activity in *Fmr1*$^{-/y}$ hippocampal slices versus WT (**Fig. 1B**). Incubating slices in the proteasome inhibitor MG132 for 1 hour results in a significant reduction in fluorescence in both genotypes, validating this assay as a faithful reflection of the basal proteasome activity in acute brain slices (**Fig. 1B**). Next, to confirm whether the increase in proteasome activity could be localized to synapses where many FX phenotypes are expressed, we performed the same proteasome activity assay on synaptoneurosome fractions isolated from *Fmr1*$^{-/y}$ versus WT hippocampal slices (**Fig. 1C**). Our results confirm elevated proteasome activity exists in *Fmr1*$^{-/y}$ synapses (**Fig. 1D**).

Our next question was whether the proteasome or other members of the UPS system were upregulated in *Fmr1*$^{-/y}$ neurons. The 26S proteasome is a macromolecular complex with two regulatory complexes (19S), and a cylindrical 20S core comprised of multiple subunits including three that confer catalytic activity (β1, β2 & β5) [43]. To investigate whether the proteasome was overexpressed we immunoblotted for the 20S complex, as well as the key 20S subunit Psmb5 (β5) and Rpt6 (encoded by *Psmc5*), an ATPase subunit of the 19S regulatory particle that has emerged as an important site for regulation of 26S proteasome function in neurons [39,44]. Our results show that these proteasome components are significantly overexpressed in *Fmr1*$^{-/y}$ hippocampus (**Fig. 1E, Fig. S1**). To determine whether these changes were localized to synapses, we repeated our immunoblotting on *Fmr1*$^{-/y}$ synaptoneurosome fractions. These results show the same overexpression of Psmb5 and Rpt6 proteasome subunits in *Fmr1*$^{-/y}$ synapses (**Fig. 1F, Fig. S1**).

We next asked whether the increase in proteasome expression could be seen specifically in neurons and also if this expanded to other UPS pathway members. To answer these questions, we investigated the expression of transcripts encoding UPS components in *Fmr1*$^{-/y}$ hippocampal neurons by analysing our recently published TRAP-seq dataset (**Fig. 1G-H, Fig. S1**) [32]. This dataset was generated using littermate WT and *Fmr1*$^{-/y}$ mice bred to be heterozygous for *Snap25*-EGFP/Rpl10a, resulting in a pan-neuronal expression of EGFP-tagged ribosomes. RNA-sequencing was performed on transcripts isolated from EGFP-tagged ribosomes in WT and *Fmr1*$^{-/y}$ animals, and DESeq2 analysis showed that 57 of the 640 transcripts upregulated at $p< 0.05$ in the *Fmr1*$^{-/y}$ fraction were UPS-related components [32] (**Fig. 1I**). Furthermore, a Gene Set Enrichment Analysis (GSEA) showed that 20 out of the 122 gene sets significantly upregulated in *Fmr1*$^{-/y}$ TRAP are related to proteasome and ubiquitin ligase function (padj< 0.1) (**Fig. 1J-L, Table S1**) [32]. A similar upregulation of UPS terms was seen in a CA1-specific TRAP-seq dataset from *Fmr1*$^{-/y}$ hippocampus published in [29]. Indeed, 13 of the 19 UPS-related gene sets significantly upregulated in the *Fmr1*$^{-/y}$ *Snap25*-TRAP dataset were also increased in the *Fmr1*$^{-/y}$ CA1-TRAP dataset (**Fig. S1**). These results indicate a general upregulation of the UPS pathway in *Fmr1*$^{-/y}$ neurons in the hippocampus.

Increased proteasome activity is downstream of excessive protein synthesis in *Fmr1*$^{-/y}$ hippocampus

A major function of the UPS is in co-translational quality control to prevent the creation of misfolded or otherwise malfunctional proteins [45-47]. We therefore wondered if the elevated proteasome activity we observed in *Fmr1*$^{-/y}$ neurons was related to the increased rate of protein

synthesis. To test this possibility, we examined the impact of multiple compounds that normalize translation in the *Fmr1*$^{-/y}$ hippocampus. Previous studies have shown that inhibition of $mGlu_5$ corrects hippocampal protein synthesis in the *Fmr1*$^{-/y}$ mouse (**Fig. 1M**) [10,16,48]. To investigate whether this treatment normalized proteasome activity, we incubated WT and *Fmr1*$^{-/y}$ slices in 10μM of the $mGlu_5$ negative allosteric modulator CTEP for 1 hour, a treatment previously shown to correct protein synthesis [16]. Our results reveal that inhibition of $mGlu_5$ corrects the elevated proteasome activity in the *Fmr1*$^{-/y}$ hippocampus (**Fig. 1N**). Next, we tested the statin drug lovastatin, which has been shown to normalize protein synthesis in FX animal models via reduction of Ras-ERK signalling [17,49]. Our results show that incubation of 50μM lovastatin, a dose that correct protein synthesis, is sufficient to normalize proteasome activity in the *Fmr1*$^{-/y}$ to WT levels (**Fig. 1O**).

Both CTEP and lovastatin have a selective impact on protein synthesis in the *Fmr1*$^{-/y}$ hippocampus due to a hypersensitivity to the $mGlu_5$-ERK pathway [10]. However, these compounds impact more than translation, and we therefore tested whether incubation with the protein synthesis inhibitor cycloheximide would similarly correct the elevated proteasome activity. Our results show that 60μM CHX reduces proteasome activity in both genotypes, however the effect is greater in the *Fmr1*$^{-/y}$ hippocampus (**Fig. 1P**). This results in a normalization of proteasome activity in *Fmr1*$^{-/y}$ hippocampus to WT levels by 60 min. Together, these results show that the elevation in proteasome activity is downstream of the excessive synthesis of new proteins in *Fmr1*$^{-/y}$ neurons.

Synaptic proteins are excessively degraded in *Fmr1*$^{-/y}$ neurons

A predicted consequence of an increased rate of degradation in the *Fmr1*$^{-/y}$ hippocampus, where protein synthesis is similarly increased, is that there should be an accumulation of proteins upon application of a proteasome inhibitor. To test this, we prepared hippocampal slices from WT and *Fmr1*$^{-/y}$ littermates and incubated in vehicle or 1μM of the proteasome inhibitor MG132 for 90 minutes (**Fig. 2A**). Although this timescale could lead to the identification of proteins with a relatively fast turnover, proteasome inhibition on this timescale has been shown to significantly alter the mGluR-LTD and epileptiform activity that are exaggerated in *Fmr1*$^{-/y}$ hippocampus [50,51]. Post-nuclear P2 fractions were prepared from treated hippocampal slices to enrich for changes at synapses, and these samples were then processed for label-free Mass Spectroscopy (MS) (**Figs. 2A, S2**). Synaptic enrichment was confirmed by the increased expression of PSD95 and exclusion of nuclear marker Histone H3 in these fractions (**Fig. S2**). Immunoblotting for K48 ubiquitin, which is specifically associated

with targets bound for proteasomal degradation, confirmed a significant build-up in MG132-treated slices (**Fig. S2**).

Similar to previous studies in adult *Fmr1*$^{-/y}$ brain, our differential expression analysis revealed few changes in WT versus *Fmr1*$^{-/y}$ vehicle-treated slices at p< 0.05 (**Fig. 2B, Table S2**) [33]. However, a comparison of MG132-treated slices reveals significantly more upregulated proteins between WT and *Fmr1*$^{-/y}$ (Veh: 26 proteins, MG132: 139 proteins). MG132 had little effect on protein accumulation in WT slices, most likely due to the short incubation time, resulting in only 35 proteins increased. In contrast, MG132 treatment in *Fmr1*$^{-/y}$ slices caused an upregulation of 177 proteins. Interestingly, 18 of the 35 proteins significantly upregulated in WT are also elevated in *Fmr1*$^{-/y}$, suggesting that the population excessively degrading in the *Fmr1*$^{-/y}$ is at least in part an amplification of the population undergoing basal turnover in WT (**Fig. 2C-D, Table S2**).

To further investigate the protein population accumulated with MG132 in *Fmr1*$^{-/y}$ hippocampus we performed pathway enrichment analyses. GSEA revealed a significant upregulation of gene sets involved in cytoskeleton regulation, translation, cell adhesion, and synaptic vesicle exocytosis suggesting proteins involved in multiple pathways are degrading faster in *Fmr1*$^{-/y}$ synapses (padj< 0.1) (**Fig. S2, Table S3**). A KEGG pathway analysis of the most significantly changed population identified several pathways involved in synaptic function (**Fig. 2E**). Interestingly, these pathways were identified by significant increases in α and ß subunits of Gi/o, a small heterotrimeric G-protein that is an essential mediator of adenylate cyclase signalling and cAMP production downstream of multiple synaptic receptors (**Fig. 2E, Fig. S2**) [52]. Immunoblotting on P2 fractions from additional sets of slices validated a significant increase in both Gnb1 and Gnai1 subunits in *Fmr1*$^{-/y}$ slices treated with MG132 (**Fig. 2F, Fig. S3**). Gnai1 expression is also constitutively reduced in vehicle-treated *Fmr1*$^{-/y}$ P2, consistent with accelerated degradation in the *Fmr1*$^{-/y}$ hippocampus. To determine whether these changes are restricted to the P2 fraction, which could suggest a change in trafficking to the synapse, we repeated our experiments and examined whole homogenates. Our results show the same increase in Gnai1 in *Fmr1*$^{-/y}$ homogenates with MG132 incubation indicating a cell-wide change in degradation (**Fig. 2G, Fig. S3**). As MG132 has additional activity on calpain cleavage and pathways involved in autophagy, we performed additional experiments with BTZ, a specific inhibitor of the β5-mediated catalytic activity of the proteasome [53,54]. These results show a similar increase in Gnai with BTZ treatment in *Fmr1*$^{-/y}$ slices (**Fig. 2H, Fig. S3**). Together, these results confirm an accelerated degradation of proteins, including Gi/o subunits, in *Fmr1*$^{-/y}$ hippocampal synapses.

Given our results showing that proteasome activity in the *Fmr1*$^{-/y}$ hippocampus is normalized with protein synthesis inhibitors, we wondered whether there was an overlap between the excessively-degrading and newly synthesized populations in *Fmr1*$^{-/y}$ neurons. To investigate this, we compared the proteins accumulated with MG132 in the *Fmr1*$^{-/y}$ synaptic proteome to the ribosome-bound mRNAs in our recently published neuron-specific TRAP-seq dataset [32]. Although this analysis does not allow for a direct comparison of translation and degradation in the same fraction, we reasoned that the neuron-specific information provided by TRAP-seq would be the most relevant to the changes seen in the synapse-enriched proteome. A population analysis shows that the group of 177 proteins upregulated with MG132 in *Fmr1*$^{-/y}$ is elevated in the *Fmr1*$^{-/y}$ TRAP-seq population (**Fig. 2I, Table S4**). This indicates that the population of proteins excessively degraded in *Fmr1*$^{-/y}$ hippocampus is overlapping with the over-synthesizing population in *Fmr1*$^{-/y}$ neurons. However, a GSEA comparison between *Fmr1*$^{-/y}$ MG132-accumulated and *Fmr1*$^{-/y}$ TRAP-seq populations reveals certain gene sets enriched in the degrading population are not similarly enriched in the translating population, suggesting an incomplete overlap (padj< 0.1) (**Fig. 2J**).

To further investigate the impact of degradation on the basal synaptic *Fmr1*$^{-/y}$ proteome, we asked whether the over-degrading population was expressed at WT levels. Analysis of the expression of the MG132-accumulated population in the basal *Fmr1*$^{-/y}$ synaptic proteome reveals a significant reduction (**Fig. 2K, Table S4**). This is reflected in the steady-state downregulation of most over-degrading synaptic KEGG pathways including “Glutamatergic synapse”, “Dopaminergic synapse”, and “Long-term depression” (**Fig. 2L**). Interestingly, “Ribosomes” is the only category significantly elevated at steady-state, which is due to an increased expression of ribosomal proteins (RPs). In contrast, G protein subunits are significantly downregulated as a group at steady-state (**Fig. 2M**). Together, our results suggest that excessive degradation is linked to excessive synthesis in the *Fmr1*$^{-/y}$ hippocampus, however an imbalance in the favour of degradation may result in an underrepresentation of regulatory proteins in the synaptic proteome.

Inhibiting UPS activity normalizes protein synthesis in *Fmr1*$^{-/y}$ hippocampus

Our proteomics experiments identified an overlap between TRAP-seq and MG132-accumulated proteomic datasets that points to a coupling between excessive synthesis and degradation phenotypes in the *Fmr1*$^{-/y}$ hippocampus. Previous studies have shown that proteasome inhibition reduces new protein synthesis in neurons [55-57]. We therefore wondered

whether UPS inhibitors could reverse excessive protein synthesis in *Fmr1*$^{-/y}$ neurons, which is thought to contribute to several pathological changes in plasticity and excitability.

To test whether UPS inhibition could normalize protein synthesis we used BTZ, as it is a specific inhibitor of the catalytic activity of the proteasome that is bioavailable and is currently used as a chemotherapeutic agent (**Fig. 3A**) [54]. Our goal was to lower proteasome activity to WT levels rather than completely inhibit activity, so that cytotoxic effects could be avoided. We therefore performed a dose-response experiment, which identified 3nM BTZ as a treatment that would have the maximum impact on proteasome activity in *Fmr1*$^{-/y}$ hippocampus without altering WT hippocampus (**Fig. 3B-C**). Using this concentration, we performed metabolic labelling on hippocampal slices prepared from littermate WT and *Fmr1*$^{-/y}$ mice. Our results show that 3nM BTZ significantly lowers protein synthesis in the *Fmr1*$^{-/y}$ hippocampus without altering levels in WT, indicating a correction of the excess protein synthesis phenotype in *Fmr1*$^{-/y}$ hippocampus (**Fig. 3D**).

Protein degradation by the UPS begins with the activation of ubiquitin by E1 ligases, which is subsequently conjugated to E2 ligases then E3 ligases that ultimately tag proteins for degradation [58]. To test whether inhibition of E1 ligase activity would have an impact on the *Fmr1*$^{-/y}$ hippocampus, we incubated *Fmr1*$^{-/y}$ and WT littermate slices in 50 μM PYR-41, previously shown to inhibit 95% of E1 activity by 15 min [59]. First, to confirm this treatment had an impact on UPS activity we measured proteasome activity in *Fmr1*$^{-/y}$ and WT hippocampus. Our results show that a 1 hour incubation with 50μM PYR-41 significantly reduces proteasome activity in *Fmr1*$^{-/y}$ slices (**Fig. 3E**). Next, we tested whether this treatment could similarly normalize protein synthesis. Metabolic labelling experiments show that PYR-41 corrects the excessive protein synthesis phenotype in *Fmr1*$^{-/y}$ hippocampus (**Fig. 3F**).

Given the relationship between the proteasome and protein synthesis phenotypes, we next asked whether raising proteasome activity would exacerbate the protein synthesis phenotype in *Fmr1*$^{-/y}$ hippocampus or whether this response would be occluded. To do this, we tested the impact of the drug IU1. This drug inhibits Usp14, a proteasome-associated deubiquitinating enzyme (DUB) that reduces proteasome activity by trimming ubiquitin chains from substrates before degradation. By inhibiting Usp14 activity, IU1 activates the proteasome [60,61]. To test whether raising proteasome activity using this compound changes protein synthesis, we performed metabolic labelling on WT and *Fmr1*$^{-/y}$ slices in 60μM IU1 or vehicle. These results show that IU1 causes a significant increase in protein synthesis in WT but not *Fmr1*$^{-/y}$ slices, indicating the saturation of protein synthesis occludes further increases in response to UPS activation (**Fig. 3G**). Together, our results show that proteasome activity

exists in a feedback loop with protein synthesis in the *Fmr1*$^{-/y}$ hippocampus, and that reducing UPS activity is a new approach to correcting excessive protein synthesis in FX.

Normalizing proteasome activity in *Fmr1*$^{-/y}$ IC neurons corrects hyperresponsiveness to sound

Our experiments showing that lowering UPS activity is beneficial for *Fmr1*$^{-/y}$ hippocampus led us to ask whether excessive proteasome activity was involved in other *Fmr1*$^{-/y}$ phenotypes. One of the most robust behavioural phenotypes in the *Fmr1*$^{-/y}$ mouse is an increased susceptibility to AGS [62-66], which models the hypersensitivity to sensory stimuli in FX patients [6,67-70]. Recent evidence indicates neurons of the IC in the expression of the AGS phenotype [71,72]. The IC is a subcortical structure that is a major relay for auditory information and processes multi-sensory information related to sound [73]. Recent investigations of the *Fmr1*$^{-/y}$ mouse reveal that exposure to sound results in greater activation of IC neurons as assessed by c-Fos immunostaining or by *in vivo* single-unit recordings [71]. Additionally, replacement of *Fmr1* in glutamatergic IC neurons, but not cortical neurons, is sufficient to block AGS in *Fmr1*$^{-/y}$ mice [72]. To investigate the role of the UPS in IC neurons, we measured proteasome activity in IC isolated from *Fmr1*$^{-/y}$ and WT littermates (**Fig. 4A**). Our results revealed a significant elevation of proteasome activity in the *Fmr1*$^{-/y}$ IC similar to what was observed in *Fmr1*$^{-/y}$ hippocampus (**Fig. 4B**). These results were similarly observed in *Fmr1*$^{-/y}$ mice reared on the FVB strain. Next, to investigate whether UPS pathway components were upregulated at IC synapses, we isolated P2 fractions from *Fmr1*$^{-/y}$ IC and performed label-free quantitative MS analysis. Differential expression analyses revealed 73 proteins significantly upregulated and 67 proteins significantly downregulated in *Fmr1*$^{-/y}$ versus WT IC ($p < 0.05$) (**Fig. 4C, Fig. S4, Table S5**). Similar to what was observed in the hippocampus, the upregulated population was enriched for UPS components including proteasome complex subunits and several ubiquitin ligase subunits. GSEA revealed that 6 of the 38 significantly upregulated categories are UPS-related, with "Proteasome complex" as the most upregulated term ($padj < 0.1$) (**Fig. 4D, Fig. S4, Table S6)**. These results show that UPS pathway members are upregulated in synaptically-enriched fractions of *Fmr1*$^{-/y}$ IC.

Previous mouse studies testing BTZ treatment for cancer show that i.p. injection leads to an accumulation in CNS tissue and a significant reduction in proteasome activity by 1 hour [74,75]. We therefore tested whether BTZ administration could normalize proteasome activity in *Fmr1*$^{-/y}$ IC. WT and *Fmr1*$^{-/y}$ littermates were injected with BTZ (5mg/kg) or vehicle, returned to the home cage for 1 hour, and IC tissue isolated and processed for proteasome activity (**Fig.**

4E). In order to ensure that any effects of BTZ would be seen under stimulated conditions, we isolated IC tissue from both naïve animals and those exposed to a 120dB stimulus that induces AGS. Our results show that BTZ significantly reduces proteasome activity in IC isolated from *Fmr1*$^{-/y}$ mice under both naïve and stimulated conditions (**Fig. 4F).** To confirm the impact of BTZ on proteasome activity *in vivo*, we measured the accumulation of proteins tagged with K48 ubiquitin that are readily degraded by the proteasome. Immunoblotting of IC tissue shows a significant accumulation of K48 ubiquitinated proteins in the *Fmr1*$^{-/y}$ IC at 1 hour post-injection with BTZ (**Fig. 4G, Fig. S4**). Together, these results confirm BTZ injection corrects excessive proteasome activity in *Fmr1*$^{-/y}$ IC.

Our next question was whether normalizing UPS activity could reduce the hyperactivation of *Fmr1*$^{-/y}$ IC neurons in response to auditory input. To test this, we performed c-Fos immunostaining to measure neuron activation in the IC of *Fmr1*$^{-/y}$ and WT mice exposed to an AGS stimulation. To isolate the effect of sound from subsequent seizure activity, we performed pilot experiments to determine a level of stimulation that would stop short of AGS expression (**Fig. S4**).

Animals were injected with BTZ or vehicle, and after 1 hour either exposed to 45 sec of AGS stimulus at 116dB or left in the home cage for an identical time period. After stimulation, mice were returned to the home cage for 45 min to allow for sufficient c-Fos expression in activated neurons (**Fig. 4H**). Immunostaining for c-Fos was performed on IC sections and the density of labelled cells quantified in z-stacks acquired with confocal imaging. Consistent with previous work, our results show that auditory stimulation results in an increased density of c-Fos positive (c-Fos+) neurons in *Fmr1*$^{-/y}$ versus WT IC (**Fig. 4I-J**). A stimulated increase was observed in both WT and *Fmr1*$^{-/y}$ IC, however this response was significantly greater in *Fmr1*$^{-/y}$ mice. Furthermore, BTZ treatment resulted in a striking correction of auditory-induced hyperactivation in *Fmr1*$^{-/y}$ IC (**Fig. 4I-J**). To confirm these results, c-Fos+ cell density was re-analysed using a separate analysis package, which revealed a similar significant increase in neuron activation in the *Fmr1*$^{-/y}$ IC that is blocked with BTZ treatment (**Fig. S4**). These results suggest that excessive UPS activity contributes to the hyperresponsiveness of *Fmr1*$^{-/y}$ IC neurons to sound.

Interestingly, studies of AGS in seizure-prone rats have indicated a regional difference in the activation of neurons in the IC central core (CIC), a major relay of sound information via the lemniscal pathway, and the external capsule (ECIC) and dorsal capsule (DCIC) regions that receive input from the CIC via the non-lemniscal pathway [73,76,77]. We therefore investigated changes in specific to these regions. Analysis of c-Fos+ neuron density reveals an

increased sound-driven activation of neurons in both lemniscal and non-lemniscal subregions the *Fmr1*$^{-/y}$ IC (**Fig. 4K**). Furthermore, BTZ prevents the hyperactivation in both IC subregions (**Fig. 4L**). These results show that sound exposure results in hyperactivation of neurons throughout the *Fmr1*$^{-/y}$ IC, and this is prevented by BTZ.

Proteasome inhibition with bortezomib prevents AGS in *Fmr1*$^{-/y}$ mice

The correction of neuronal activity in the *Fmr1*$^{-/y}$ IC with BTZ prompted us to investigate whether this treatment could correct the enhanced susceptibility to AGS. The AGS phenotype is one of the most reliable behavioural alterations observed in *Fmr1*$^{-/y}$ mice [78]. Multiple therapeutic strategies that alleviate AGS have gone on to be tested in clinical studies, suggesting this assay has predictive value [16,17,79,80]. To measure AGS we performed the same assay published in previous studies [29,81]. *Fmr1*$^{-/y}$ and WT littermates were injected with vehicle or 5mg/kg BTZ with the experimenter blind to both genotype and treatment. After 1 hour, animals were transferred to a test chamber where they were habituated for 1 min, then exposed to a 2-min sampling of a defined mixture of frequencies played at 120dB. Seizures were scored on a 1-3 scale of increasing severity: (1) wild running, (2) clonic seizure, and (3) tonic seizure (**Fig. 5A**, **Videos S1-S4**).

We first tested mice at a young age (P19-22) at which the AGS phenotype is most readily apparent. We used cohorts raised on a C57BL6/J x FVB hybrid background, which shows an increased susceptibility to AGS versus pure C57BL6/J [62]. Our results show the expected increase in both AGS incidence and severity in vehicle-treated *Fmr1*$^{-/y}$ mice versus vehicle-treated WT. Consistent with the elimination of hyperactivity in IC neurons, BTZ treatment caused a striking reduction in the incidence and severity of AGS in *Fmr1*$^{-/y}$ mice (**Fig. 5B**). Next, we repeated our experiments using *Fmr1*$^{-/y}$ and WT littermates raised on a pure FVB background [82]. Our results show a similar significant increase in AGS incidence and severity that is corrected with BTZ in *Fmr1*$^{-/y}$ mice (**Fig. 5C**). On the FVB background, *Fmr1*$^{-/y}$ mice exhibit the AGS phenotype into adulthood, and we therefore repeated our experiments on adult (>P60) *Fmr1*$^{-/y}$ and WT littermate mice. AGS testing reveals that there is a significant increase in AGS incidence and severity in *Fmr1*$^{-/y}$ adult mice that is eliminated with BTZ (**Fig. 5D**). These results show that normalizing proteasome activity is sufficient to correct AGS in *Fmr1*$^{-/y}$ mice raised on multiple background strains, and at different developmental ages.

Genetic reduction of proteasome activity in the IC prevents AGS in *Fmr1*$^{-/y}$ mice

Considering the pivotal role of IC in AGS and our novel observation that proteasome activity is elevated in this region, we wondered if specific reduction of proteasome activity in IC neurons would be sufficient to reduce AGS in *Fmr1*$^{-/y}$ mice. To test this, we reduced proteasome activity selectively in the IC through genetic reduction of *Psmb5* (**Fig. 6A**). The Psmb5 subunit is required for chymotrypsin-like enzymatic activity of the 26S proteasome, and previous studies have demonstrated that manipulation of this subunit alone is sufficient to change overall proteasome activity [83,84]. To reduce levels of *Psmb5* specifically in IC, we performed stereotaxic injections of AAV9 carrying *Psmb5* shRNA and allowed for 1 week of expression. *Fmr1*$^{-/y}$ and WT animals were injected with vector expressing either *Psmb5* shRNA or a scrambled shRNA sequence under the control of a U6 promoter, followed by an mCherry tag driven by a *hSYN1* promoter (**Fig. 6A**). Confocal imaging shows selective expression of virus in the IC, with good infectivity of neurons (**Fig. 6B**). A qPCR analysis of dissected IC confirms a significant reduction of *Psmb5* transcript in animals injected with AAV9-shRNA versus AAV9-scrambled after 1 week of expression (**Fig. 6C**). Immunoblotting reveals a similar significant reduction in Psmb5 protein level in shRNA-expressing *Fmr1*$^{-/y}$ IC (**Fig. S4**). Importantly, this reduction in *Psmb5* is sufficient to normalize the elevated proteasome activity seen in *Fmr1*$^{-/y}$ IC (**Fig. 6D**).

To test whether *Psmb5* knock-down in the IC was sufficient to correct AGS, *Fmr1*$^{-/y}$ and WT mice injected with either shRNA or scrambled control were tested in groups counterbalanced for genotype and treatment. As expected, our results show a significant increase in incidence and severity in *Fmr1*$^{-/y}$ versus WT scrambled-treated mice (**Fig. 6E**). Remarkably, *Psmb5* shRNA-treated *Fmr1*$^{-/y}$ mice showed a significant reduction in AGS incidence and severity (**Fig. 6E**, **Videos S5-S7)**. A significant increase in latency to first seizure also indicates a positive impact of *Psmb5* knock-down even in the mice expressing seizures (**Fig. 6F**). These results confirm that selective reduction of proteasome activity in IC neurons is sufficient to correct AGS in the *Fmr1*$^{-/y}$ mouse.

Discussion:

This study identifies excessive activity of the UPS as a novel cellular phenotype contributing to pathological changes in the *Fmr1*$^{-/y}$ brain. We find that proteasome activity is elevated in *Fmr1*$^{-/y}$ neurons, consistent with an upregulation of proteasome subunits and UPS pathway members seen by neuron-specific TRAP-seq. As a consequence, protein degradation is excessive in *Fmr1*$^{-/y}$ hippocampal synapses. Furthermore, the proteasome inhibitor BTZ and the upstream E1 ubiquitin ligase inhibitor PYR-41 normalize excessive protein synthesis in the

Fmr1$^{-/y}$ hippocampus, suggesting that excessive protein degradation is tightly linked to excessive protein synthesis. In addition to hippocampus, we show that UPS activity is elevated in the *Fmr1*$^{-/y}$ IC, which contributes to a hyperactivation of neurons in response to auditory stimulation. Consistent with these results, systemic administration of BTZ significantly reduces the incidence and severity of AGS in *Fmr1*$^{-/y}$ mice. The same reduction in seizures is seen with viral injection of *Psmb5* shRNA in *Fmr1*$^{-/y}$ IC, verifying the importance of UPS activity in this brain region for the expression of the AGS phenotype. Together, these results show that excessive activity of the UPS contributes to FX neuropathology, and this pathway can be targeted to correct multiple phenotypes in the *Fmr1*$^{-/y}$ mouse (**Fig. 6G**).

We were motivated to investigate protein breakdown based on an absence of protein accumulation despite an increase in protein synthesis in juvenile *Fmr1*$^{-/y}$ hippocampus. Our results show that not only is protein degradation excessive, but it is dynamically linked to the elevation in protein synthesis. Recent work implicates FMRP in ribosome stalling and shows that translation elongation is elevated in *Fmr1*$^{-/y}$ neurons [23,24,30,85,86]. Interestingly, the newly-synthesized protein population is targeted for degradation by the UPS at a greater rate versus existing proteins, which is mainly due to the high error rate that occurs during translation [45-47]. It is therefore possible that the elevated proteasome activity seen in *Fmr1*$^{-/y}$ neurons is a compensatory response to the proportional increase in the newly-synthesized fraction. This is consistent with our results showing that protein synthesis inhibitors have a greater impact on proteasome activity in the *Fmr1*$^{-/y}$ hippocampus (**Fig. 1N-P**), and that the excessively-degrading population is upregulated in the translating fraction of *Fmr1*$^{-/y}$ neurons (**Fig. 2I**).

If increased protein degradation compensates for the increase in mRNA translation, why would inhibition of UPS activity be beneficial? One possibility is that the reduction of proteasome activity normalizes protein synthesis, which ultimately resolves phenotypes that are driven by exaggerated translation (**Fig. 3D-F**). Indeed, a reduction in translation has been shown to be beneficial for myriad phenotypes in the *Fmr1*$^{-/y}$ mouse and in other FX models [13,15,87]. It is also possible that reducing proteasome activity prevents the aberrant breakdown of existing proteins that contribute to altered synaptic function (**Fig. 2K-M**). Interestingly, the over-degrading population is enriched for G-protein subunits including the alpha subunit of Gi/o. The activation of Gi/o is generally linked to an inhibition of adenylate cyclase activity and cAMP production [52]. It is therefore interesting that deficient cAMP production in neurons is seen in several FX models including mice, fly, and human [88-90], which has stimulated clinical trials for PDE4-D inhibitors that raise cAMP levels in FX patients [91]. It is interesting to speculate that the increased turnover in Gi/o may shift the proportion of active versus inactive

populations, altering cAMP production downstream of GPCRs. It is also interesting to note that decreased activity of Gi/o-linked GABA-BRs has been implicated in hyperexcitability in *Fmr1*$^{-/y}$ brain, and the GABA-BR positive allosteric modulator R-baclofen ameliorates many pathological phenotypes in the *Fmr1*$^{-/y}$ mouse [79,92,93]. Whether reduction of UPS activity beneficially impacts Gi/o-linked receptor functioning or signalling is an important next question for future studies.

We find that exposure to a stimulus that elicits AGS causes a hyperactivation of neurons in the *Fmr1*$^{-/y}$ IC, which is eliminated with BTZ (**Fig. 4**). Furthermore, selective reduction of proteasome activity in the IC through expression of *Psmb5* shRNA is sufficient to reduce AGS in the *Fmr1*$^{-/y}$ mouse (**Fig. 6**). These results are consistent with previous work indicating that the IC is a critical mediator of AGS in the *Fmr1*$^{-/y}$ mouse [66,71,72]. It has been shown that deletion of *Fmr1* from *vglut2*+ neurons is necessary to elicit AGS in otherwise WT mice, and that re-expression of *Fmr1* in *Ntsr1*+ neurons in the IC is sufficient to prevent AGS in otherwise *Fmr1*$^{-/y}$ mice [72]. This indicates that *Fmr1* loss in excitatory IC neurons is critical. However, it is not clear that these are the same neurons responsible for seizure generation. Indeed, evidence from somatosensory cortex indicates loss of *Fmr1* from excitatory neurons reduces feed-forward inhibition, ultimately resulting in an increase in firing [94]. Future work is therefore needed to identify which cell population is responsible for initiating seizure activity, and investigate how reduced UPS activity prevents this response.

Our results show that BTZ causes a striking reduction of hyperactivity in IC and blocks AGS in the *Fmr1*$^{-/y}$ mouse. Although the molecular mechanisms contributing to this rescue are not clear, there are many ways in which UPS activity contributes to neuronal excitability. The ubiquitination of GluA1 and GluA2 subunits contributes to the recycling of AMPA-Rs at excitatory synapses, and major scaffolding proteins that impact receptor function such as PSD-95, AKAP and Shank are targets for the UPS [38,41]. Multiple presynaptic proteins are also targeted for ubiquitination, and inhibition of the UPS alters the size of the recycling vesicle population at presynaptic terminals [95]. The expression of GABA-A receptors is also regulated by ubiquitination and degradation, which is enhanced in post-mortem brain tissue from ASD patients. Interestingly, inhibition of the ERAD pathway can reduce seizures in mice expressing GABA-A misfolding mutations simply by increasing surface expression [96,97]. Many ion channels that regulate neuronal excitability and are involved in epilepsy are also regulated by the UPS, including large-conductance potassium (BK) channels and voltage-independent Ca^{2+}-activated K^+ (SK2) channels, both of which are deficiently activated in *Fmr1*$^{-/y}$ neurons [98-100]. Moreover, studies of *Fmr1*$^{-/y}$ hippocampal neurons reveal a dysregulation in the expression of

the E3 ligase Nedd4-2, which modifies AMPA-R trafficking and targets several ion channels involved in excitability [101,102]. Future work determining the targets of enhanced UPS activity that underlie increased excitability in IC neurons will be important for determining the mechanistic cause of AGS in the *Fmr1*$^{-/y}$ mouse, and for identifying further therapeutic targets.

Our results provide important new information relevant to therapeutic strategies in FX. Original experiments identifying excessive protein synthesis downstream of $mGlu_5$ led to the development of multiple treatments that are being tested in clinical trials [103]. However, there is still no targeted treatment approved for FX, and novel strategies are urgently needed. In this study, we show that increased activity of the UPS is a pathological adaptation that accompanies excess protein synthesis in FX, and this can be targeted to correct multiple deleterious changes. This is particularly advantageous in light of the numerous UPS inhibitors that have been developed for the treatment of cancer and other disorders [54,58]. Although our study is focused on the question of FX, several mutations that alter UPS function have been linked to autism, intellectual disability (ID) and epilepsy [104-106]. It is therefore possible that our results will be relevant to other neurodevelopmental disorders.

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Author Contributions: EKO, SRL and SSS conceptualized the study and prepared the manuscript. MFN provided essential feedback on multiple aspects of the study, and was the major contributor to the conception, design and analyses of c-Fos+ imaging experiments and oversight of data acquisition by SRL and CMG. SRL designed, performed, and analysed hippocampal slice experiments and all biochemistry experiments, including the preparation of samples for proteomic analysis. SRL designed, performed and analysed AGS experiments, with assistance from MM and BM. SSS performed bioinformatics analyses of TRAP-seq datasets and proteomics datasets from hippocampus and IC. MAGL and KWL performed MS analysis of hippocampal samples. JCW performed MS analysis of IC samples. SSS and NCV performed viral injections, TRAP experiments, and qPCR with assistance from BM. SRL and BM performed c-Fos immunostaining and imaging experiments with assistance from CMG.

SRL and CMG performed c-Fos+ neuron quantification. All authors contributed to the review and editing of the manuscript, with major inputs from SRL, SSS and MFN.

Declaration of Interests: The authors declare no competing interests

Inclusion and Diversity: One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as living with a disability. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

Figure legends:

Figure 1. The UPS is elevated in *Fmr1$^{-/y}$* neurons. (**A**) Schematic of proteasome activity assay. (**B**) Proteasome activity is increased in *Fmr1$^{-/y}$* hippocampal slices and significantly reduced with 1µM MG132 for 1h (ANOVA treatment *p<0.0001; WT-veh vs. KO-veh *p=0.0108, KO-veh vs. KO-MG *p<0.0001, WT-veh vs WT-MG *p<0.0001; n=11). (**C**) Immunoblot shows enrichment of synaptophysin 1 and PSD95 in synaptoneurosome fractions. (**D**) Proteasome activity is increased in *Fmr1 $^{-/y}$* synaptoneurosomes (paired t-test p=0.0094, N=11). (**E**) Increased expression of proteasomal subunits Psmb5 and Rpt6 in *Fmr1$^{-/y}$* hippocampal slice homogenates (Psmb5: paired t-test *p=0.0270, N=8. 20S: paired t-test *p=0.033, N=9. Rpt6: paired t-test *p=0.0175, n=7). (**F**) Increased expression of Psmb5 and Rpt6 in *Fmr1$^{-/y}$* synaptoneurosomes (Psmb5: paired t-test *p=0.0225, n=7. Rpt6: paired t-test *p=0.0361, N=6). (**G**) Schematic representation of TRAP assay shows isolation of translating ribosomes (IP) from Input using anti-GFP coated beads. (**H**) IP shows enrichment of *Snap25* and depletion of *Gfap* versus Input fraction. (*Gfap*: paired t-test *p<0.0001, *Snap25*: *paired t-test p=0.006). (**I**) Differential expression analysis shows significant upregulation of many transcripts in *Fmr1$^{-/y}$* neuron-specific TRAP (red), including UPS related targets. (**J**) GSEA shows upregulation of several UPS terms in the *Fmr1$^{-/y}$* neuronal TRAP population. (**K**) Examples of UPS related GO terms show robust population changes of the transcripts involved in the term (Proteasome complex GSEA *padj=0.0008, Protein polyubiquitination GSEA *padj=0.0005). (**L**) Significantly upregulated targets include proteasome subunits (e.g., *Psmc6*, *Psmb5*, *Psmc2*), E3 ubiquitin ligases (e.g., *Arel1*, *Rnf114*, *Rnf180*), and related UPS pathway

members (e.g., *Yod1*, *Fbxo2*). (**M**) Excessive protein synthesis in *Fmr1*$^{-/y}$ hippocampus is corrected with inhibition of the mGlu$_5$-Ras-ERK1/2 pathway. (**N**) mGlu$_5$ negative allosteric modulator CTEP (10μM) significantly reduces proteasome activity in *Fmr1*$^{-/y}$ slices (ANOVA F(1,10)=8.71, treatment x genotype *p=0.0145, WT-veh versus KO-veh *p=0.0211, KO-veh versus KO-CTEP *p=0.0117, n=11). (**O**) Ras-ERK inhibitor lovastatin (50μM) significantly reduces proteasome activity in *Fmr1*$^{-/y}$ slices (ANOVA F(1,10)=17.93, treatment x genotype *p=0.0017, WT-veh versus KO-veh *p=0.0009, WT-veh vs WT-lova *p=0.0326, KO-veh versus KO-lova *p=0.0056, n=11). (**P**) Protein synthesis inhibitor cycloheximide (60μM) significantly reduces proteasome activity in *Fmr1*$^{-/y}$ slices (ANOVA treatment F(2,10)=13.08, *p=0.0002, genotype F(1,10)=14.72, *p=0.0033, treatment x genotype F(2,10)=3.85, *p=0.0386, WT-veh versus KO-veh *p=0.0005, WT-CHX-30' vs KO-CHX-30' *p=0.0046, WT-veh versus WT-CHX-30' *p=0.0399, WT-veh versus WT-CHX-60' *p=0.0005, KO-veh versus KO-CHX-30' *p=0.0054, KO-veh versus KO-CHX-60' *p<0.0001, n=11). N = number of littermate pairs. Data presented as mean ± SEM. All immunoblot samples loaded on the same gel but not side-by-side (see STAR methods). See also **Figure S1** and **Table S1**.

Figure 2. Protein degradation is excessive in *Fmr1*$^{-/y}$ hippocampal synapses. (**A**) Hippocampal slices from 5 *Fmr1*$^{-/y}$/WT littermate pairs were incubated in proteasome inhibitor MG132 for 90 min, and synapse-enriched P2 fractions isolated submitted for MS. (**B)** Volcano plot showing differential expression with significantly changed proteins ($p<0.05$) in red. The accumulation of proteins with MG132 in *Fmr1*$^{-/y}$ is significantly greater than WT (χ^2=100.1, *$p<2.2 \times 10^{-16}$) (**C-D)** MG132 treatment results in significant build-up of more proteins in *Fmr1*$^{-/y}$ vs WT (WT=35, *Fmr1*$^{-/y}$=177) (**E)** A cluster analysis of enriched KEGG pathways in the MG132-accumulated population in the *Fmr1*$^{-/y}$ (i.e., degrading population) shows 4 distinct clusters: Synaptic, Hippo/Thyroid signalling, PI3K-Akt signalling, and Ribosome. Red lines denote inter-cluster connections and black lines denote intra-cluster connections. Significantly changed genes that identify individual KEGG pathways are shown. (**F)** Immunoblotting confirms that Gnb1 is significantly accumulated in *Fmr1*$^{-/y}$ with MG132 treatment (ANOVA genotype x treatment F(1,6)=7.52, *p=0.0336; KO-veh vs. KO-MG *p=0.0242; n=7 littermate pairs), as is Gnai1 (ANOVA genotype F(1,6)=6.297, p=0.0459; WT-veh vs KO-veh *p=0.0174, KO-veh vs. KO-MG *p=0.0148; n=7). **(G)** Gnai1 accumulation with MG132 treatment is similarly seen in homogenate fractions from *Fmr1*$^{-/y}$ slices (ANOVA genotype x treatment F(1,5)=8.12, *p=0.0358; WT-veh vs KO-veh *p=0.0465, KO-veh vs. KO-MG

*p=0.0037; n=6). **(H)** Hippocampal slices were treated with 3nM BTZ and homogenates immunoblotted for Gnai1. Results show BTZ results in a significant accumulation of Gnai1 in *Fmr1*$^{-/y}$ slices, similar to what is seen with MG132 (ANOVA treatment F(1,8)=5.45, *p=0.0479, genotype F(1,8)=6.23, *p=0.0371; WT-veh vs KO-veh *p=0.0299, KO-veh vs. KO-BTZ *p=0.0163; n=9). (**I**) Expression of proteins in the degrading population were identified in neuron-specific TRAP-seq. T-statistic values (combined score) show expression of the degrading population is significantly elevated versus the mean distribution (z test z=3.6741, *p=0.00023). (**J**) A comparison of all GSEA categories significantly enriched in both the degrading population and the *Fmr1*$^{-/y}$ TRAP population shows a functional divergence in proteins over-translated and over-degraded (ribosomes), under-translated over-degraded (synaptic) and over-translated but not degrading (mitochondria). (**K**) Comparison of combined score shows a significant decrease of the degrading population from the mean distribution (z test, z=-3.375, *p=0.00075). **(L)** TRAP-seq (translation) changes and basal protein expression changes of the degrading population were compared. Most KEGG pathways significantly enriched in the degrading population are decreased in the basal population, with the exception of "Ribosome". (**M**) Density plot reveals different functional groups that are over-translated/overexpressed (ribosomal proteins), over-translated/underexpressed (G protein subunits) or under-translated and underexpressed (FMRP targets). G protein subunits show more accumulation upon MG132 compared to RPs. (Wilcoxon rank sum test G proteins vs total *p=0.0337, RPs vs total *p=0.3162). N = number of littermate pairs. Data presented as mean ± SEM. All immunoblot samples loaded on the same gel but not side-by-side (see STAR methods). See also **Figures S2-S3** and **Tables S2-S4**.

Figure 3. Inhibiting the UPS normalises protein synthesis in *Fmr1*$^{-/y}$ hippocampus. (**A**) Schematic of the UPS system showing which steps are inhibited by PYR-41, bortezomib (BTZ) and IU1. (**B**) Dose response to BTZ on WT hippocampal slices shows a significant decrease in proteasome activity for concentrations higher than 10nM (ANOVA F=60.97, *p<0.0001, WT-veh versus WT-BTZ-10 *p=0.0089, WT-veh versus WT-BTZ-100 *p<0.0001, n=7). (**C**) Bortezomib (3nM) significantly reduces proteasome activity in *Fmr1*$^{-/y}$ slices to WT levels (ANOVA treatment x genotype F(1,8)=17.32, *p=0.0032, WT-veh versus KO-veh *p=0.0031, KO-veh versus KO-BTZ *p=0.0007, n=9). (**D**) Bortezomib (3nM) normalizes protein synthesis in *Fmr1*$^{-/y}$ slices to WT levels (ANOVA treatment F(1,10)=6.15, *p=0.0325, genotype F(1,10)=8.39, *p=0.0159, treatment x genotype F(1,10)=11.66, *p=0.0066, WT-veh versus

KO-veh *p<0.0001, KO-veh versus KO-BTZ *p=0.0001, n=11). (**E**) Inhibiting E1 ligase with PYR-41 (50µM) restores proteasome activity in *Fmr1*$^{-/y}$ slices (ANOVA: genotype F(1,9)=11.39, *p=0.0082, WT-veh versus KO-veh *p=0.0034, KO-veh versus KO-PYR-41 *p=0.0043, n=10). (**F**) PYR-41 (50µM) significantly reduces protein synthesis rates in *Fmr1*$^{-/y}$ slices (ANOVA mixed effect analysis: treatment F(1,9)=5.54, *p=0.0430, WT-veh versus KO-veh *p=0.0204, KO-veh versus KO-PYR-41 *p=0.0190, n=10). (**G**) UPS activator IU1 (60µM) significantly increases protein synthesis in WT but not *Fmr1*$^{-/y}$ (ANOVA treatment F(1,7)=20.41, *p=0.0027, treatment x genotype F(1,7)=9.69, *p=0.0170, WT-veh versus KO-veh *p=0.0169, WT-veh versus WT-IU1 *p=0.0018, n=8). N = number of littermate pairs. Data presented as mean ± SEM.

Figure 4. Normalising proteasome activity in IC of *Fmr1*$^{-/y}$ mice with bortezomib reduces hyperexcitability in IC. (A) Inferior colliculi from WT and *Fmr1*$^{-/y}$ mice were processed for proteasome activity assay or MS analysis. **(B**) Proteasome activity is significantly increased in *Fmr1*$^{-/y}$ IC when reared on either FVB or C57BL6 background strains (FVB: paired t-test *p=0.0158. N=7; C57: paired t-test *p=0.0412. N=10 littermate pairs). **(C**) Volcano plot shows differentially expressed proteins in WT and *Fmr1*$^{-/y}$ IC with significantly changed proteins in red (p<0.05). Upregulated proteasome complex (dark red) and ubiquitin ligases (green) related proteins are highlighted. **(D**) GSEA of significantly enriched categories (padj<0.1) in the *Fmr1*$^{-/y}$ IC show upregulation of 6 UPS related categories. (**E**) WT and *Fmr1*$^{-/y}$ mice were injected with vehicle or BTZ (5mg/kg) and the inferior colliculi were dissected either 1h post injection (naïve) or after exposure to AGS stimulation (AGS) for proteasome activity and WB analysis. (**F**) Proteasome activity is reduced in IC by BTZ (3-ANOVA treatment F(1,12)=9.72, *p=0.0089; genotype x treatment F(1,12)=5.32, *p=0.0397. KO-HC-vs KO-HC-BTZ *p=0.0322, KO-AGS vs KO-AGS-BTZ *p=0.0119, n=7). (**G)** BTZ administration causes significant accumulation of K48 ubiquitinated proteins in *Fmr1*$^{-/y}$ IC (ANOVA treatment F(1,4)=21.84, *p=0.0095, genotype x treatment F(1,4)=14.21, *p=0.0196; KO-veh vs. KO-BTZ *p=0.0092; n=5). (**H**) WT and *Fmr1*$^{-/y}$ mice were injected with vehicle or BTZ (5mg/kg) and after 1h mice were either stimulated with AGS or kept in HC. After 45 min mice were perfused and brains processed for c-Fos immunostaining. Scale bars: left: 500µm, right: 100µm. (**I**) Representative sections from *Fmr1*$^{-/y}$ and WT brains immunostained for c-Fos in IC (scale bar 200µm). Full IC imaged for each condition is shown in **Fig. S4**. (**J**) c-Fos+ cell density is significantly increased by AGS stimulation in both genotypes, and this effect is

greater in the *Fmr1*$^{-/y}$. BTZ eliminates the increase in c-Fos+ cell density in the *Fmr1*$^{-/y}$ IC (3-ANOVA AGS F(1,3)=12.8, *p=0.0373; Genotype x treatment F(1,3)=20.8, *p=0.0197; AGS x treatment F (1,3)=15.4, *p=0.0293; WT-HC vs WT-AGS *p=0.03, WT-AGS vs KO-AGS *p=0.0105, KO-HC vs KO-AGS *p=0.0041, KO-AGS vs KO-BTZ-AGS *p=0.0334, n=4). (**K**) AGS stimulation increases c-Fos+ cell density in the lemniscal (CIC) and non-lemniscal (DCIC+ECIC) regions of the *Fmr1*$^{-/y}$ IC (3-ANOVA AGS F(1,3)=60.29, *p=0.0044, Genotype F(1,3)=27.88, *p=0.0132; CIC: KO-HC vs KO-AGS *p=0.0341, DCIC+ECIC: KO-HC vs KO-AGS *p=0.0246, WT-AGS vs KO-AGS *p=0.0461; n=4). (**L**) BTZ causes a significant reduction in AGS-induced c-Fos labelling in both lemniscal and non-lemniscal regions of the *Fmr1*$^{-/y}$ IC (3-ANOVA treatment F(1,3)=12.73, *p=0.0039, genotype x treatment F(1,3)=6.06, *p=0.030; CIC: WT-AGS vs KO-AGS *p=0.0438, KO-AGS vs KO-AGS-BTZ *p=0.009; DCIC+ECIC WT-AGS vs KO-AGS *p=0.0346, KO-AGS vs KO-AGS-BTZ *p=0.009; n=4). N = number of mice per group. Data presented as mean ± SEM. All immunoblot samples loaded on the same gel but not side-by-side (see STAR methods). Mouse silhouettes illustrated in panels A and E were sourced from https://scidraw.io and adapted from doi.org/10.5281/zenodo.3925903 and doi.org/10.5281/zenodo.3925901. See also **Figure S4** and **Tables S5-S6**.

Figure 5. Pharmacological inhibition of the proteasome prevents AGS in juvenile and adult *Fmr1*$^{-/y}$ mice. (**A**) *Fmr1*$^{-/y}$ and WT mice on the C57BL/6 x FVB (**B**) or FVB/N (**C,D**) backgrounds were injected with 5 mg/kg bortezomib (BTZ) or vehicle and tested for AGS after 1 h. Increasing severity was scored as wild running, clonic seizure and tonic seizure. (**B**) BTZ reduces the incidence of AGS in juvenile *Fmr1*$^{-/y}$ mice on the C57BL/6 x FVB background (Fisher's exact test WT vs KO-veh *p=0.0154, KO-veh vs KO-BTZ *p=0.0213, n=11-14). Comparison of AGS scores shows BTZ reduces seizure severity (Mann–Whitney WT vs KO-veh *p=0.0189, KO-veh vs KO-BTZ p=0.0040, n=11-14). (**C**) BTZ reduces the incidence of AGS in juvenile *Fmr1*$^{-/y}$ mice on the FVB/N background (Fisher's exact test WT-veh vs KO-veh *p<0.0001, KO-veh vs KO-BTZ *p<0.0001, n=11-12). Comparison of AGS scores shows BTZ reduces seizure severity (Mann–Whitney WT-veh vs KO-veh *p<0.0001, KO-veh vs KO-BTZ *p<0.0001, n=11-12). (**D**) BTZ reduces the incidence of AGS in adult *Fmr1*$^{-/y}$ mice on the FVB/N background (Fisher's exact test WT-veh vs KO-veh *p=0.0014, KO-veh vs KO-BTZ *p=0.0075. n=9-11). Comparison of AGS scores shows BTZ reduces seizure severity (Mann–Whitney WT-veh vs KO-veh *p=0.0026, KO-veh vs KO-BTZ *p=0.0094, n=9-11). N = number of mice per group. Data presented as mean ± SEM. See also **Videos S1-S4**.

Figure 6. Genetic reduction of proteasome activity in inferior colliculus prevents AGS in *Fmr1*$^{-/y}$ mice. (**A**) *Fmr1*$^{-/y}$ and WT mice were injected with AAV9-*Psmb5* shRNA-mCherry or AAV9-scramble-mCherry, tested for AGS after 6-8 days, and IC tissue analysed for *Psmb5* knock-down and proteasome activity. (**B**) Confocal imaging shows mCherry expression is specific for IC and is well expressed in neurons (identified by EGFP expression) (scale bars = 500μm and 50μm). (**C**) qPCR shows injection of AAV9-*Psmb5* shRNA significantly reduces *Psmb5* expression in *Fmr1*$^{-/y}$ IC. A significant increase in *Psmb5* expression in *Fmr1*$^{-/y}$ versus WT expressing control AAV9-scramble control is consistent with the basal increase seen in *Fmr1*$^{-/y}$ hippocampus (ANOVA *p<0.001; WT-scr vs KO-scr *p=0.0001, KO-scr vs KO-shRNA *p<0.0001; n=8-16) (**D**) Proteasome assays show injection of AAV9-*Psmb5* shRNA is sufficient to reduce proteasome activity in the *Fmr1*$^{-/y}$ IC (ANOVA F=14.82, *p=0.0002; WT-scr vs KO-scr *p=0.0123; KO-scr vs KO-shRNA *p<0.0001; n=6-8) (**E**) AGS assays of WT and *Fmr1*$^{-/y}$ injected animals shows that expression of AAV9-*Psmb5* shRNA in the IC is sufficient to significantly reduce the incidence of seizures in *Fmr1*$^{-/y}$ mice (Fisher's exact test WT-scr vs KO-scr *p=0.0062, KO-scr vs KO-shRNA *p=0.0032; n=8-16). A comparison of AGS scores shows that expression of AAV9-*Psmb5* shRNA in the IC is also sufficient to significantly reduce seizure severity (Mann–Whitney WT-scr vs KO-scr *p=0.0357, KO-scr vs KO-shRNA p=0.0193). (**F**) In the *Fmr1*$^{-/y}$ animals expressing AAV9-*Psmb5* that go on to have seizures, the average latency to the first seizure is longer than those expressing AAV9-scramble, indicating a reduced susceptibility to seizure generation (t-test KO-scr vs KO-shRNA *p=0.0226). (**G**) A model of excessive proteostasis in FX proposes that excess $mGlu_5$-ERK mediated protein synthesis causes a compensatory increase in protein degradation that contributes to pathological changes such as AGS. Importantly, the correction of neuropathology with bortezomib or *Psmb5* shRNA suggests that inhibition of the UPS is a novel therapeutic strategy for FX. N = number of mice per group. Data presented as mean ± SEM. Mouse head silhouette illustrated in panel A was sourced from https://scidraw.io and adapted from doi.org/10.5281/zenodo.3925903. See also **Videos S5-S7**.

STAR Methods:

Resource availability

Lead Contact

Further information and requests for resources, reagents or code should be directed to and will be fulfilled by the Lead Contact, Emily Osterweil (emily.osterweil@ed.ac.uk).

Materials Availability

This study did not generate new unique reagents.

Data and code availability

All the raw RNAseq and proteomics datasets have been deposited in GEO and PRIDE respectively with accession numbers listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

Experimental model

Fmr1$^{-/y}$ mice generated on a C57BL6/J (003025) or FVB/N (004624) background were obtained from Jackson Labs. *Fmr1*$^{-/y}$ and WT littermates on a C57BL6/J were bred using *Fmr1*$^{+/-}$ females and JAX C57BL/6J males. To generate WT and *Fmr1*$^{-/y}$ littermate pairs on the FVB/N background, the stock line 004624 was crossed with 004828 for one generation and inbred (*Fmr1*$^{+/-}$ females crossed with WT males) afterwards. *Snap25-EGFP/Rpl10a* mice were obtained from Jackson Labs and maintained on a C57BL/6J background. All experiments were carried out using male littermate mice and studied with the experimenter blind to genotype. Proteomics data generated from control WT/*Fmr1*$^{-/y}$ hippocampal slices in this study was used for a separate analysis published in [32] with accession number PXD031932. Mice were group housed (5 maximum) in non-environmentally enriched cages with unrestricted food and water access and a 12h light-dark cycle. Room temperature was maintained at 21 ± 2°C. Animal husbandry was carried out by University of Edinburgh technical staff. All procedures were in performed in accordance with ARRIVE guidelines and the regulations set by the University of Edinburgh and the UK Animals Act 1986.

Method details

Hippocampal Slice Preparation

Hippocampal slices were prepared from male P25-32 wildtype and *Fmr1*$^{-/y}$ littermates on a C57BL6/J background. All experiments were performed blind to genotype. Mice were anaesthetized with isoflurane and each hippocampus was rapidly dissected in ice-cold Artificial Cerebrospinal Fluid (ACSF) (124 mM NaCl, 3mM KCl, 1.25 mM NaH_2PO_4, 26 mM NaHCO3,

10 mM Dextrose, 1 mM MgCl2 and 2 mM CaCl2, (Sigma Aldrich) saturated with 95% O2 and 5% CO2). Dorsal slices of 500 µm thickness were prepared using a Stoelting Tissue Slicer and transferred into 32.5C ACSF saturated with 95% O_2 and 5% CO_2 within approximately 5 minutes, then left to recover for 4 hours.

Synaptoneurosome Preparation

Synaptoneurosomes were isolated from sets of 4 hippocampal slices prepared as above. Slices were homogenised in ice-cold homogenisation buffer (10 mM HEPES pH 7.4, 5 mM EDTA, 150 mM NaCl) in 2ml Dounce homogenisers. Homogenates were filtered through two 100 µm filters, followed by a 5 µm filter (both Millipore) and centrifuged at 10,000 x g for 10 minutes and supernatant was discarded.

Proteasome activity assay

Chymotrypsin-like activity of the proteasome was assayed by monitoring the production of 7-amino-4-methylcoumarin (AMC) from fluorogenic peptide Suc-LLVY-AMC (Enzo Lifesciences). Hippocampal slices or synaptoneurosomes were lysed in 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100. Inferior colliculi samples were homogenised in 0.32 M sucrose, 50 mM HEPES (pH 7.5), 2 mM ATP and centrifuged at 1000 x g for 10 minutes at 4^{o}C, and the supernatant (S1) re-centrifuged at 17,000 x g for 15 minutes at 4^{o}C. The proteasome activity was measured from the S2 fraction. Addition of 2 mM ATP to the lysate will improve the recovery of intact 26S proteasome and spun at 15,000 x *g* for 15 min. Samples (20-30 µg protein) were incubated with the fluorogenic substrate, 50 µM Suc-LLVY-AMC, in 250 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.001% SDS and 0.05% NP-40 buffer, in a final volume of 100 µl. The release of fluorescent AMC was measured at 37 °C using a microplate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, after 2 h. Specific activity was determined by subtracting the activity measured in the presence of 10 µM lactacystin (Enzo Lifesciences), a proteasome inhibitor. For analysis each sample was normalised to an AMC standard curve to determine concentration in µM, and values were then normalised to total protein amount based on results from BCA protein quantification kit (Biorad) to determine AMC(µM)/ug. Each value was normalised to the average fluorescence within the experiment.

Immunoblotting

Samples were boiled in Laemmli sample buffer, resolved on SDS polyacrylamide gels (10 or

12%), transferred to nitrocellulose membranes (BioRad), and stained for total protein (memcode staining kit, Pierce). Samples were loaded in a blinded fashion. Membranes were cut at appropriate molecular weights to allow immunoblotting of the same membrane for multiple proteins. Immunoblotting was performed with the following primary antibodies: 20S proteasomal subunits, Rpt6, PSMB5, PSD95, Synaptophysin 1, Gnai1, Gnb1, K48 ubiquitin. Following incubation in primary antibody overnight at 4C, immunoblots were incubated with appropriate HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Cell Signaling Technology (1:5,000-1:20,000)) diluted in 3% BSA in TBS-T, developed with Clarity Western ECL (BioRad) for 3 minutes, and exposed to film. For all immunoblotting experiments densitometry was performed on scanned blot films with Image Studio Lite while memcode stains (total protein) were quantified using Fiji. Data are expressed as protein signal divided by respective total protein value (memcode stain) and each signal was normalized to the average signal of all lanes in the same blot to account for blot-to-blot variance.

All samples were loaded blind to genotype and condition, and were thus randomized across the gel. This was done to prevent skewing that can occur when all samples of one condition are loaded towards the ends of the gel. For figures, different lanes from the same gel are shown next to one another with blank space to indicate they are not loaded side-by-side, and unprocessed blots are shown in supplementary figures.

RT-qPCR

RNA for each sample was converted into cDNA using Superscript VILO cDNA Synthesis Kit (Life Technologies) and RT-qPCR was performed using Quantitect SYBRgreen qPCR master mix (QIAGEN) according to the manufacturer's instructions. Samples were prepared in triplicate in 96-well reaction plates and run on a StepOne Plus (Life Technologies). Each target was normalized to *bAct* and *pib1* as the endogenous references. For primer sequences see key resources table.

P2 fractionation

Hippocampal slices or dissected inferior colliculi were prepared from WT and *Fmr1*$^{-/y}$ littermate pairs in an interleaved and blinded fashion. Tissue was homogenized in 50 mM HEPES, pH 7.4 plus 0.32 M sucrose supplemented with protease inhibitor cocktail (Roche). To isolate synapse-enriched fractions, homogenates were centrifuged at 1000 x g for 10 minutes at 4^{o}C, and the supernatant (S1) re-centrifuged at 17,000 x g for 15 minutes at 4^{o}C.

Mass Spectroscopy

Hippocampal slice:

Proteomics data generated from control WT/*Fmr1*$^{-/y}$ hippocampal slices in this study was used for a separate analysis published in [32] with accession number PXD031932. Hippocampal slices were prepared from littermate WT/*Fmr1*$^{-/y}$ mice and incubated with or without 1 µM MG132 for 90 min. P2 fractions were prepared and 20 µg of each sample was mixed with 75 µL 2% SDS, 1 mM Tris (2-carboxyethyl)phosphine and incubated at 55°C for 1 hour. Cysteines were blocked by adding 0.5 µL 200 mM methyl methanethiosulfonate and incubating 15 minutes at RT. After mixing with 200 µL 8 M Urea in Tris pH 8.8, samples were transferred to Microcon-30 filter tubes (Millipore) and centrifuged at 14,000 x *g* for 15 minutes at RT. Samples were washed 4X 200 µL 8 M urea, 4X 200 µL 50 mM ammonium bicarbonate. Proteins were digested with 0.7 µg Trypsin/Lys-C Mix (MS grade, Promega) in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides were recovered by centrifugation, pooled, washed with 50 mM ammonium bicarbonate, dried in a speed vac and stored at -20 °C until used.

Peptides were dissolved in 7 µL 2% acetonitrile, 0.1% formic acid solution containing iRT reference (Biognosys) and analysed by micro LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific). A 5 mm Pepmap 100 C18 column (300 µm i.d., 5 µm particle size, Dionex) and a 200 mm Alltima C18 column (100 µm i.d., 3 µm particle size) were used to trap and fractionate the peptides, respectively. Acetonitrile concentration in 0.1% formic acid was increased linearly from 5 to 18% in 88 minutes, to 25% at 98 minutes, 40% at 108 minutes and to 90% in 2 minutes, at a flow rate of 5 µL/minute. Peptides were electro-sprayed into the mass spectrometer with a micro-spray needle voltage of 5500 V. Each SWATH cycle consisted of a parent ion scan of 150 msec and 8 Da SWATH windows, with scan time of 80 msec, through 450-770 m/z mass range. The collision energy for each window was calculated for a 2+ ion centered upon the window (spread of 15 eV).

The data was analysed using Spectronaut with a spectral library previously generated from synaptosomal preparations by data-dependent acquisition [107]. The cross-run normalization based on total peak areas was enabled and the peptide abundances were exported for further processing using R language.

Inferior colliculus:

Inferior colliculi were dissected from juvenile WT and *Fmr1*$^{-/y}$ (P19-28) mice on the FVB/N background and snap frozen. P2 pellet fractions were isolated from 150 µg protein as described above, and lysed in 6M GnHCl, 200 mM tris HCl pH 8.5, 5 mM tris, 2-

carboxyethyl)phosphine, 10 nM chloroacetamide, probed sonicated for 10 seconds at 5 µM, and incubated at 95C for 5 minutes. Samples were diluted with water to 3 M GnHCl and digested with 0.5 µg Lysyl endopeptidase (WAKO) for 4 hours at 37C. The reactions were further diluted to 1M GnHCl with water and digest continued overnight with additional 0.3 µg trypsin (Thermo). Samples were acidified by addition of TFA and insoluble material removed by centrifugation. Liberated peptides were desalted on activated (methanol), equilibrated (0.1% TFA) C18 Stage tips [108]. Elution in 80% acetonitrile, 0.1% TFA was followed by vacuum concentration and reconstitution in 0.1% TFA. Peptides were separated on an Aurora nano packed emitter (IonOpticks, Australia) using a gradient from 4% to 25% acetonitrile in 120 minutes and to 37% in a further 10 minutes, delivered by an Ultimate 3000 (Thermo) with 0.5 % acetic acid throughout. Peptides were ionized and analyzed at 240K resolution on a Fusion Lumos (Thermo), scanning 350-1400. Advanced Peak Detection was employed, and data-dependent fragmentation spectra were acquired with Quadrupole isolation 0.7, HCD NCE 30%, and ion trap rapid scan. Primary data processing was performed with MaxQuant [109] version 1.6.17.0, searching mouse Uniprot reference proteome UP000000589 from release 2021_01-10-Feb-2021 [110].

Metabolic labelling

Hippocampal slices prepared and recovered as described above were incubated in ACSF containing either vehicle (0.002% DMSO in ddH_2O) or 3 nM Bortezomib (Seleckchem), 50 µM PYR-41, 60 µM IU1 or 60 µM cycloheximide for 50 min. Slices were then transferred to fresh ACSF containing 10 µCi/ml ^{35}S-Met/Cys (Perkin Elmer) with vehicle or drugs as listed above for another 10 min and snap frozen. Slices were homogenized in ice-cold buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, protease inhibitors and phosphatase inhibitors). To precipitate proteins, homogenates were incubated in trichloroacetic acid (TCA: 20% final) for 10 min on ice before being centrifuged at 16,000 rpm for 10 min. The pellet was washed in ice-cold ddH_2O and re-suspended in 1 N NaOH until dissolved, and the pH was re-adjusted to neutral using 0.33 N HCl. Triplicates of each sample were added to scintillation cocktail and read with a scintillation counter. Protein concentration of each sample was measured using BioRad DC (BioRad). Averaged triplicate counts per minute (CPM) values were divided by protein concentrations, resulting in CPM per µg protein. To control for daily variation in incorporation rate, the values obtained on each day were normalized to the ^{35}S-Met/Cys ACSF used for incubation, and the average incorporation of all slices analysed in that experiment.

c-Fos immunohistochemistry, imaging and quantification

Juvenile WT and *Fmr1*$^{-/y}$ (P21-22) mice on the FVB/N background were weighed and injected intraperitoneally (i.p.) with 5 mg/kg Bortezomib or vehicle (1% DMSO in PBS) and transferred to a quiet (< 70 dB ambient sound) room for 1 hr. Mice from the home cage group remained in this room while mice from the AGS group were transferred to a transparent plastic test chamber (39 cm x 23 cm) and, after 1 min of habituation, exposed to a stimulus of 116 dB (recorded sampling of a modified personal alarm, 2 × 50-W speakers (KRK Rokit RP5 G3 Active Studio Monitor) for 45 seconds, before any motor manifestations of wild running was observed. WT and *Fmr1*$^{-/y}$ mice were anaesthetized by a lethal dose of sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were post-fixed overnight in 4% PFA, then incubated overnight in 30% sucrose in PBS. Tissue was then placed in Optimum Cutting Temperature (OCT) embedding matrix and sliced sagittally in 50-µm thick sections using a freezing microtome. Free-floating sections containing the entire IC were collected and stored in PBS with Sodium Azide 0.05% (Sigma Aldrich) at 4°C until used. Sections were permeabilized and blocked with 0.3% Triton X-100, 5%NGS in PBS (PBST). Selected sagittal sections containing IC were incubated overnight in c-Fos (1:1000; abcam) in PBST at 4°C. Sections were then rinsed in PBS and incubated overnight in Alexa fluor 647 goat anti-rabbit (1:1000; Thermo Fisher). Finally, sections were rinsed in PBS and mounted on microscopy slides using FluorSave^{TM} (Millipore). Sections were imaged for fluorescence using a Zeiss LSM800 confocal. For each mouse, two-three sagittal sections around 50% of the rostrocaudal extent of the IC were imaged. Stacks of images (42 µm, 2 µm *z*-steps) containing the IC were acquired using a Zeiss Plain Apochromat 10X/0.45 objective.

For histological analysis, two authors were blinded to genotype and treatment. Author 1 quantified total c-Fos+ cell density in each IC section manually using Imaris 9.8.0. The same intensity threshold and cell soma size parameters were used for all images, and volumetric measurement of c-Fos+ nuclei was performed for each z-stack of IC images. Author 2 quantified c-Fos+ nuclei in IC subregions using Arivis Vision 4D software. Low-magnification images containing the entire brain in the sagittal plane were aligned to higher magnification stacked-images containing IC. A manually-delineated template atlas of IC was created in Arivis based on anatomical landmarks in the tissue corresponding to the level 0.96 mm lateral to Bregma for the acquired 42 µm z-stack images (The Mouse Brain in Stereotaxic Coordinates Third Edition, Fraklin and Paxinos; Figure 109). This template atlas was used for all animals.

Automated quantification of c-Fos+ nuclei in the entire z-stack of IC images was done using the same threshold, split sensitivity and cell soma size parameters for each image. These parameters were validated by manual quantification of n=100 cell nuclei before applying them to all analysed images.

Audiogenic seizures

Experiments were performed as previously described [29]. Naive male FVB/N or C57BL6 x FVB/N P21-70 mice were weighed and injected intraperitoneally (i.p.) with 5 mg/kg Bortezomib or vehicle (1% DMSO in PBS) and transferred to a quiet (< 60 dB ambient sound) room for 1 hr. For testing, animals were moved to a transparent test chamber (39 cm x 23 cm) equipped with speakers and a camera and allowed to habituate for 1 min. Audiogenic stimulation (recorded sampling of a modified personal alarm) was passed through an amplifier and 2 × 50-W speakers (KRK Rokit RP5 G3 Active Studio Monitor) to produce a stimulus of 120 dB for a maximum of 2 min. During each testing session mice from both genotype and treatment groups were included, with experimenter blind to genotype and treatment. A decibel meter was placed on a clear plexiglass sheet 28 cm above the arena, in order to monitor sound output at a fixed distance throughout the experiment. Incidence and severity of seizures was scored. AGS severity was calculated as follows: wild running (WR; pronounced, undirected running and thrashing), clonic seizure (violent spasms accompanied by loss of balance), or tonic seizure (postural rigidity in limbs). Any animal that reached tonic seizure was immediately humanely euthanized. Latency was measured as the number of seconds between onset of the AGS stimulus and appearance of the first seizure.

Viral injections

Bilateral injections of AAV9-U6-scramble-hsyn-mCherry or AA9-U6-mPSMB5 shRNA-hsyn-mCherry into the inferior colliculi were performed in mice age P21 – P35 using standard aseptic techniques (Vector Biolabs, shRNA sequence in the Key Resource table). Mice were anesthetized with isoflurane, shaved at the scalp and a rostro-caudal incision made to expose the skull. Body temperature was maintained throughout using a heating pad (37C). Inferior colliculus injection sites were identified using stereotaxic coordinates (relative to the lambda suture: y: -0.9mm ; x: ± 1.0 ; z: -1.75), and 500 nl of virus was delivered per hemisphere in a rate of 100 nl/min. Post-surgical injection of analgesic (Vetergesic) and 0.9% NaCl was delivered subcutaneously.

Stereotaxic coordinates were checked by performing injections in *Snap25*-EGFP/Rpl10a mice and immunostaining for mCherry (Abcam, ab 205402) to visualize viral spread. At 6-8 days post injection, mice were either perfused for immunostaining or tested for AGS as described, after which IC tissue was collected for downstream qPCR and proteasome activity assays. Imaging was performed on a Nikon A1R FLIM confocal microscope in collaboration with the IMPACT facility at the University of Edinburgh. Images were analyzed with ImageJ. All injections and analyses were performed blind to genotype and treatment.

Bioinformatics analysis

TRAP-Seq:

TRAP-seq data were generated in a recent study [32] and deposited under accession number GSE199328. 75 bp, Paired end reads were mapped to the *Mus musculus* primary assembly (Ensembl release v99) using STAR 2.7.1a. Reads that were uniquely aligned to annotated genes were counted with featureCounts function from subread 2.0.1. Differential expression analyses were performed with a paired design to account for matching littermate pairs using DESeq2 1.24.0 (Bioconductor) in R 3.6.1. Low count genes with average count of <5 across all samples were removed prior to DESeq2 analysis and $\log_2$ fold changes were moderated using lfcShrink function using type = Normal to account for high fold changes in low count genes. DESeq2 significance was set at $p < 0.05$ for inclusion in downstream analyses. GSEA was performed on TRAP-seq data with results included in our recently published work [32]. Significance was set at padj < 0.1. For GO term heatmaps, pairwise $\log_2$ fold change was calculated using normalised counts. GSEA was performed with moderated $\log_2$ fold change value using Piano 2.0.2 using all GO term information from biomaRt. GSEA was restricted to gene sets with >20 and <500 genes.

Hippocampus proteomics:

Peptide abundances from Spectronaut were normalised using variance stabilising normalisation using DEP 1.6.1 (Bioconductor). Detected proteins with where the values were missing for all samples in at least one condition were classified as missing not at random (MNAR) and all other missing values were classified as missing at random (MAR). MAR values were imputed using Bayesian PCA and MNAR values were imputed using quantile regression imputation of left censored data (QRILC). Duplicated protein IDs were removed and converted to mouse gene names using biomaRt (Bioconductor). After filtering for ambiguous gene names 2460 proteins were considered for downstream analysis. Differential

expression analysis was performed using Limma using empirical Bayesian normalisation and targets with a significance threshold of $p < 0.05$.

Functional enrichment analysis:

GO enrichment was performed using DAVID [111] with background correction for all detected proteins for each dataset and GO terms and KEGG pathways were tested. GSEA was performed with empirical bayes moderated t statistics value using Piano 2.0.2 using full GO terms obtained from biomaRt (Bioconductor).

KEGG pathway cluster analysis:

KEGG pathway information was obtained from the KEGG pathway database (https://www.genome.jp/kegg/pathway.html) and GO term information was obtained from biomaRt (2.40.5). Network of significant KEGG terms or GSEA GO terms was constructed by calculating the proportion of shared proteins from the total number of proteins in each term. Network of significant terms was clustered by weight using cluster_edge_betweenness function from igraph (1.2.6).

For comparison of KEGG terms in TRAP vs proteome, a z score for each GO term/ KEGG pathway was calculated by using circle_dat function from GOplot package (https://wencke.github.io/) which uses the formula below:

$$z\ score = Number\ of\ up\ regulated - Number\ of\ down\ regulated\ /\sqrt{Total}$$

For calculating z score of a GO/ KEGG pathway from other datasets, above formula was used with all proteins detected from the dataset with up/down regulation threshold of >/< 0 $\log_2$ fold change.

Comparison of translation and degradation in hippocampus:

T-statistic values of TRAP-seq targets identified as over-degrading (significantly upregulated with MG132 in *Fmr1*$^{-/y}$) were ranked with 0 indicating no change from the total TRAP-seq population. Population of proteins was compared to the average TRAP-seq population and significance determined by z-test.

Protein-protein interaction:

Protein-protein interaction dataset was obtained from STRING database (https://string-db.org/). All interactions with combined score >400 were used for network construction.

Network was constructed with igraph using the combined score as weight of the connections. Proteins were clustered using cluster_edge_betweenness function from igraph and different communities were colour coded.

Data Analysis and Quantification

All statistical analyses were performed using GraphPad Prism or R. For proteasome assays, protein synthesis assays, and immunoblot datasets, outliers (± 2 SD from the mean) were removed and significant effects determined using repeated-measures 2-way ANOVA followed by Benjamini and Hochberg FDR post-hoc tests (comparisons of 4 or more groups) or paired Student's t-test (2 groups). For c-Fos immunostaining experiments, groups were compared by 3-way ANOVA followed by FDR post-hoc. For viral injection experiments, data were analysed with ordinary 1-way ANOVA followed by FDR post-hoc. Significance for population comparisons was determined using Chi-squared test, and distributions were compared with z-test as indicated. AGS incidence scores were analysed using Fisher's Exact test and severity score distributions analysed by Mann-Whitney test.

Data Availability

The *Snap25* TRAP--seq data has been deposited in GEO with the dataset identifier GSE199328 (Seo *et al*, 2022) (**Fig. 1**). The mass spectrometry proteomics data from hippocampal P2 fractions have been deposited to the ProteomeXchange Consortium via the PRIDE, partner repository with the dataset identifier PXD031932 (Seo *et al*, 2022) (**Fig. 2**). The mass spectrometry proteomics data from inferior colliculus P2 fractions have been deposited to the ProteomeXchange Consortium via the PRIDE, partner repository with the dataset identifier PXD036296 (**Fig. 4**).

Table S1. Related to Figure 1. GSEA *Fmr1*$^{-/y}$ TRAP-seq. List of gene sets significantly changed in GSEA comparison of WT vs *Fmr1*$^{-/y}$ *Snap25* TRAP-seq (data originally presented in [32]).

Table S2. Related to Figure 2. Hippocampal P2 proteomics. Values for proteins identified by mass spectrometry of P2 fractions from WT and *Fmr1*$^{-/y}$ hippocampal slices treated with vehicle or MG132.

Table S3. Related to Figure 2. GSEA hippocampal P2 proteomics. List of gene sets significantly changed in GSEA comparison of proteins altered by vehicle versus MG132 treatment of WT and *Fmr1*$^{-/y}$ hippocampal slices.

Table S4. Related to Figure 2. Proteins accumulated with MG132 in *Fmr1*$^{-/y}$ hippocampal P2. Analysis of targets significantly accumulated with MG132 in *Fmr1*$^{-/y}$ hippocampal proteome in ribosome-bound hippocampal *Snap25*-TRAP fractions and in the steady-state hippocampal proteome.

Table S5. Related to Figure 4. Inferior colliculus proteomics. Comparison of proteins identified by mass spectrometry of P2 fractions of inferior colliculi isolated from WT and *Fmr1*$^{-/y}$ mice.

Table S6. Related to Figure 4. Inferior colliculus GSEA. List of gene sets significantly changed in GSEA comparison of proteins altered in WT and *Fmr1*$^{-/y}$ inferior colliculi.

Video S1. Related to Figure 5. KO veh AGS. Shows 1 min habituation followed by 2 min AGS stimulation of *Fmr1*$^{-/y}$ mouse injected with vehicle.

Video S2. Related to Figure 5. KO BTZ AGS. Shows 1 min habituation followed by 2 min AGS stimulation of *Fmr1*$^{-/y}$ mouse injected with 5 mg/kg BTZ.

Video S3. Related to Figure 5. WT veh AGS. Shows 1 min habituation followed by 2 min AGS stimulation of WT mouse injected with vehicle.

Video S4. Related to Figure 5. WT BTZ AGS. Shows 1 min habituation followed by 2 min AGS stimulation of WT mouse injected with 5 mg/kg BTZ.

Video S5. Related to Figure 6. WT AAV-scr AGS. Shows 1 min habituation followed by 2 min AGS stimulation of WT mouse expressing AAV9-scrambled in IC.

Video S6. Related to Figure 6. KO AAV-scr AGS. Shows 1 min habituation followed by 2 min AGS stimulation of *Fmr1*$^{-/y}$ mouse expressing AAV9-scrambled in IC.

Video S7. Related to Figure 6. KO AAV-shRNA AGS. Shows 1 min habituation followed by 2 min AGS stimulation of *Fmr1*$^{-/y}$ mouse expressing AAV9-*Psmb5* shRNA in IC.

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