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Nagaraj Swaminathan April 8th, 2021

The Structural and Molecular Changes associated with Altered Homeostatic Intrinsic Plasticity in the Axon Initial Segment and Dendrites of *Fmr1* KO Neurons

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Abstract

The Structural and Molecular Changes associated with Altered Homeostatic Intrinsic Plasticity in the Axon Initial Segment and Dendrites of *Fmr1* KO Neurons

By Nagaraj Swaminathan

Fragile-X Syndrome (FXS), often associated with autism and autism associated disorders, is characterized by intellectual disability, sensory hypersensitivity and sleep disorders. Several of the symptoms in FXS have been linked to increased neuronal excitability, a phenotype that has also been demonstrated in the FXS mouse model, the *Fmr1* knockout (KO). Though the cause for this cortical hyperexcitability remains a mystery, recent work from our lab is supporting the idea that one type of neuronal plasticity called Homeostatic Intrinsic Plasticity (HIP) is involved (Bülow et al., 2019, *Cell Reports*). HIP regulates the excitability of a neuron in response to activity perturbations and is thereby one of the fundamental mechanisms in maintaining normal neural activity levels during brain development. Studies conducted in our lab on cortical neurons from *Fmr1* KO mice have highlighted a dysregulation of HIP mechanisms and that these mechanisms were either exaggerated or absent in two subsets of excitatory neurons (Bülow et al., 2019, *Cell Reports*). To further expand on these results, we are now testing the hypothesis that altered HIP function is mediated by altered regulation of the Axon Initial Segment (AIS). The AIS is a specialized region at the start of the axon and is the site of action potential initiation due to high expression of voltage-gated sodium channels. In this region, both the length and the location of the AIS have previously been reported to be regulated by HIP, and a few recent studies have also demonstrated dysregulated AIS regulation in neurodegenerative and neurodevelopmental diseases. Additionally, loss of FMRP in FXS is also associated with

dysregulation of protein synthesis. Preliminary experiments measuring nascent protein synthesis using puromycin labelling and experiments analyzing translational regulation in the soma by measuring processing bodies(P bodies) in the soma revealed that *Fmr1* KO neurons display altered protein translational regulation. Therefore we tested whether changes to P bodies in the dendrites and AIS in response to TTX/APV treatment would mirror the phenotypic changes seen in the WT and KO neurons from the preliminary soma experiments.

Using immunocytochemical approaches, we find that the AIS is located farther away from the soma in the *Fmr1* KO at baseline when compared to WT counterparts. This is indicative of unique compensatory mechanisms engaged by the *Fmr1* KO neurons at baseline. We also found that *Fmr1* KO neurons do not exhibit any significant responses to treatment with gabazine. Immunocytochemical experiments conducted on the dendrites and AIS analyzing the P bodies revealed that there was no significant changes in the *Fmr1* KO or WT neurons at baseline or in response to treatment. This data did not align with our initial hypothesis and indicates that P bodies are potentially recruited to the soma following activity deprivation.

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Introduction:

Fragile X syndrome (FXS) is a debilitating disease that is characterized by severe intellectual disability (ID) caused by a mutation in the Fragile X Mental Retardation 1 (*FMR1*) gene (Bassell and Warren, 2008). The mutation causing FXS is also the leading cause of ID and inherited autism, accounting for their shared symptomology (Ciaccio et al., 2017). In a typical individual the 5' untranslated region of the *FMR1* gene contains around 5-49 CCG repeats. However, in people with FXS this repeat number is increased to above 200. This repeat expansion is associated with hypermethylation of the *FMR1* promoter, which leads to the silencing of the *FMR1* gene and thus loss of its protein product Fragile X Mental Retardation Protein (FMRP) (Bassell and Warren, 2008; Crawford et al., 2001). FMRP is found in most neurons and is best known as an RNA binding protein, where it regulates and often represses translation of various mRNAs (Bassell and Warren, 2008). FMRP regulates translation of both pre- and postsynaptic mRNA transcripts. For example, at the postsynaptic side, FMRP targets mRNAs encoding for NMDA receptor subunits (NR1, 2A, 2B and 3A), neuroligins 1-3, PSD-93 and PSD-95 (Darnell et al., 2011). Presynaptic targets for FMRP include transcripts involved with synaptic vesicle recycling and exocytosis (Deng et al., 2011). Therefore, *Fmr1* KO neurons often present with translational dysregulation due to the loss of FMRP.

One major phenotype observed in FXS patients and the FXS mouse model, the *Fmr1* knock out (KO), is neuronal hyperexcitability (Bassell and Warren 2008). This phenotype has been associated with various behavioral symptoms including sensory hypersensitivity, sleep disturbances and seizures, which are prominent in patients with FXS as well as autism (Contractor et al., 2015). Initial theories regarding this increased hyperexcitability attributed it to altered synaptic development and Hebbian plasticity, which involves modification of neuronal

synapses via long-term potentiation(LTP) and long-term depression(LTD) mechanisms in response to activity perturbation. A pivotal study found that *Fmr1* KO mice presented with exaggerated mGluR-dependent LTD, resulting from loss of FMRP mediated repression in protein translation (Bear et al., 2004). This exaggerated LTD response was a consequence of increased internalization of AMPA receptors. This theory prompted the use of mGluR5 inhibitors as a potential therapeutic option (Schraf et al., 2015). While there was compelling evidence for the therapeutic potential of mGluR5 inhibitors in the *Fmr1* KO mouse model, these results unfortunately were not observed in the human patient populations (Schraf et al., 2015).

Other theories hypothesized that cortical hyperexcitability arose from dysregulation of ion channel functioning in FXS. Ion channel regulation is especially important in determining intrinsic excitability of the cell as balance of inward and outward currents and is essential for normal action potential firing (Turrigiano et al., 2011). Studies show that ion channels such as Kv4.2, an A-type dendritic potassium channels that decreases back-propagation of action potentials in the neuron (Birnbaum et al., 2004, Chen et al., 2006), is regulated by FMRP dependent translational regulation of mRNA translation *in vivo* and *in vitro* (Gross et al., 2011). FMRP directly interacts with intrinsic membrane proteins as it is seen to modulate the gating of sodium-activated potassium channels Slack (Brown et al., 2010). FMRP also plays a role in regulating action potential(APs) duration and neurotransmitter release by interacting with the BKβ4 subunit of the calcium-activated potassium channel BK in excitatory hippocampal neurons (Deng et al., 2013, Deng et al., 2016). Therefore, the loss of FMRP alters both synaptic function and intrinsic excitability, both of which contribute to altered neuronal function and plasticity (Darnell et. al 2013).

However, it still remains unclear how hyperexcitability emerges in FXS, and if

hyperexcitability is primarily accounted for by dysfunction in synaptic or ion channel properties. The work of this Honor's Thesis will elaborate on recent studies from our lab identifying a novel mechanism contributing to the emergence of cortical hyperexcitability in FXS (Bülow et al 2019)

Many studies strongly support the idea that neuronal circuits possess mechanisms that allow them to maintain stable firing rates, and this is commonly known as homeostatic plasticity (Turrigiano et al., 2011). Homeostatic plasticity encompasses a set of mechanisms that are triggered during chronic activity perturbations to return firing activity to pre-perturbation levels. For example, treatment with the GABA-A receptor antagonist Bicuculine triggers an acute increase in firing activity, but after 24 hours, spiking activity returns to pre-perturbation levels despite the continued presence of the drug (Turrigiano et al., 1998). Thus, homeostatic plasticity mechanisms had been engaged to restore firing activity back to pre-perturbation levels.

Homeostatic plasticity is comprised of several distinct mechanisms. One of these homeostatic mechanisms is called synaptic scaling. Synaptic scaling is a cell autonomous process that involves the modulation of the firing rate of the neuron by altering the strength of the synapse (Turrigiano et al., 1998, Desai et al., 2002, Goel and Lee 2007). These modulations of firing rates are mediated by the synthesis and insertion of AMPA receptors on the postsynaptic neuron following activity perturbation, and can be measured as changes in the amplitude of the miniature excitatory postsynaptic potential (Turrigiano et al., 2011). In the context of FXS syndrome, Soden and Chen found that synaptic scaling is lost in activity deprived *Fmr1* KO hippocampal neurons when treated voltage-gated sodium channel blocker TTX and the NMDA receptor antagonist APV for 48 hours (Soden and Chen 2010). Thus, synaptic scaling in hippocampal neurons is dependent on FMRP expression. However, a recent study from our lab

revealed intact synaptic scaling cortical *Fmr1* KO neurons, suggesting that region and cell type may play role in the requirement for FMRP in scaling (Bülow et al., 2019). Further, it suggests that alterations in synaptic scaling do not contribute to cortical hyperexcitability in FXS.

Another type of homoeostatic mechanism is called homeostatic intrinsic plasticity (HIP). HIP maintains neuronal firing levels by adjusting the intrinsic membrane excitability of a neuron (Turrigiano et al., 1994, 1995, Desai et al., 1999). The intrinsic membrane excitability can be regulated via changing expression/function of various ion channels as well as regulating the axon initial segment (AIS), a specialized region in the axon that is the site of action potential initiation (Kuba et al., 2010, Grubb and Burrone 2010, Turrigiano et al.,, 1994, 1995, Desai et al., 1999). Although most work has focused on ion channel regulation in the soma and AIS, homeostatic regulation of ion channels on the dendritic membrane also plays an important role in adjusting excitability (Brager and Johnston 2014). Interestingly, There is a large overlap between the ion channels regulated by FMRP and HIP (Fig 1), such as *NaV 1.6, Kv1.2, Kv2.1, Kv7.2, N/L/T – Ca^{2+} Channels, NMDA receptors* (Contractor et al., 2015). This overlap motivated a previous study in our lab to test the novel hypothesis that loss of FMRP impairs HIP function. As I will elaborate on below, our lab found that HIP was impaired in cortical *Fmr1* KO neurons and this prompted us to test whether the AIS, a site enriched in ion channels and important for regulating neuronal excitability (Contractor et al., 2015), was altered in *Fmr1* KO neurons during HIP.

The AIS is a specialized region at the start of the axon and is the site of action potential initiation (Fig 2). This region is highly concentrated in voltage-gated sodium channels and also expresses voltage gated calcium and potassium channels (Fig 2)(Contractor et al., 2015). HIP regulates the expression of these channels in response to changes in neuronal activity (Desai et al., 1999). Seminal studies showed that chronically increasing or decreasing neuronal activity led

to compensatory changes in the AIS length or position (Grubb and Burrone 2010; Kuba et. al 2010). Regulation of the AIS position or length can have complex consequences on neuronal excitability. Increased length of the AIS is correlated with increased expression of voltage-gated sodium channels which should increase excitability, while movement of the AIS away from the soma will likely lead to increased current leak, reduced depolarizing drive and thus reduced excitability (Grubb and Burrone 2010; Kuba et. al 2010). Thus, HIP can regulate excitability through morphological changes of the AIS either by moving its location along the axon, or by increasing or decreasing its length based on the neuronal activity.

As already mentioned, FMRP and HIP regulate an overlapping set of ion channels. However, until recently, it was unknown if loss of FMRP impairs HIP function. A recent publication from our lab demonstrated abnormal HIP function in *Fmr1* KO primary cortical excitatory neurons (Fig 3) (Bülow et al., 2019). Our study utilized an activity deprivation paradigm with TTX/APV(TTX: voltage gated sodium channel antagonist, APV: NMDA receptor antagonist) and subsequently measured the number of spikes fired by the neurons in the absence of TTX/APV. As previously reported (Orlandi et al., 2011), we found that wild-type neurons increased the number of spikes they fired following treatment with TTX/APV, consistent with the idea that the neurons had compensated for the activity deprivation by increasing their intrinsic excitability. Interestingly, TTX/APV treated *Fmr1* KO neurons displayed an even greater compensation, measured as a larger number of action potential firing compared to TTX/APV treated wild-type cells. Our results suggested that HIP is exaggerated in *Fmr1* KO neurons, and more importantly, that abnormal HIP may drive the emergence of cortical hyperexcitability in FXS. These changes in spike rate are correlated with decreased action potential rise time, which could be a product of altered Na+ channel function (Bülow et al.,

2019). However, the mechanisms underlying altered HIP in *Fmr1* KO neurons remains unknown. Therefore, one of the primary goals of this project is to test the hypothesis that dysregulation of AIS position or length contributes to altered HIP in *Fmr1* KO neurons.

Previous studies have shown that the homeostatic regulation of the AIS is bidirectional (Kuba et al., 2010, Grubb and Burrone 2010). Previous research in our lab found that at baseline, the soma-AIS distance in the *Fmr1* KO neurons was significantly greater than their WT counterparts suggesting evidence of potentially novel compensatory mechanisms in the KO cells in response to the hyperexcitability phenotype (Fig 4A). Interestingly, TTX/APV treatment led to a unique relocation of the AIS closer to the soma in *Fmr1* KO neurons, such that *Fmr1* KO and WT control neurons no longer differed (Fig 4A). These results are interesting for two reasons: 1. At baseline, loss of FMRP alters the position of the AIS, either because loss of FMRP impairs AIS positioning or because *Fmr1* KO neurons attempt to compensate for a hyperexcitable environment during earlier developmental time points, or 2. TTX/APV treatment triggers a relocation of the AIS towards the soma, but only in *Fmr1* KO neurons. A shorter distance between the soma and the AIS correlates with increased excitability (Grubb and Burrone 2010), and these data support the idea that exaggerated HIP in *Fmr1* KO neurons may be mediated by closer proximity between the soma and AIS. (Bülow et al., 2019). Is AIS relocation specific to activity deprivation perturbation in the *Fmr1* KO or does AIS plasticity occur in response to all types of activity perturbations? Answering this question could further our understanding of the role of FMRP in AIS plasticity. Thus, one of the goals of this Honors Thesis Project is to test whether increasing neuronal activity, rather than reducing it, causes morphological changes to the AIS (Aim 1). If altered HIP function is bidirectional, this could suggest that *Fmr1* KO cortical neurons have a defect in the core machinery mediating HIP.

A secondary goal of this project was to investigate the molecular pathways underlying HIP. Previous work has shown that homeostatic plasticity is protein synthesis dependent (Sutton et al., 2004), and several candidate pathways have later been identified (Stellwagen and Malenka 2006, Aoto et al, 2008, Jakawich et al.,, 2010). Our lab has preliminary data to suggests that TTX/APV treatment leads to a significant downregulation of protein synthesis in wild-type but not *Fmr1* KO neurons (Fig 5). This downregulation occurred in both the soma, dendrites and AIS. Repressive granules, cytoplasmic translational repression elements, are important for regulating protein synthesis (Lai and Valdez-Sinon, 2020). Moreover, repressive granules have been reported to be dysregulated in FXS animal models (Valdez-Sinon et al., 2020, Aschrafi et al., 2005). This literature combined with our preliminary data, led us to hypothesize that altered HIP function in *Fmr1* KO neurons may be the result of dysregulated protein synthesis due to impaired repressive granule function.

Repressive granules or RNA granules are ubiquitous cytoplasmic organelles that provide functional compartmentalization of RNA within the cell (Tian et al., 2020). Given that they interact primarily with RNA, RNA granules have been primarily associated with post-transcriptional regulation. RNA granules can be classified in to three main types, namely: germ granules, stress granules, and processing bodies (P-bodies) (Tian et al., 2020). Germ granules are primarily associated with playing a role in early embryogenesis and directing germ cell fate in metazoans (Voronina and Seydoux 2010, Ephrussi et al., 1991) . Stress granules comprise of messenger ribonucleoprotein (mRNP) units which are formed in response to sudden stress such as hypoxia or heat shock. These accumulate translationally repressed mRNAs in response to stress and are quickly broken down after removal of the stressor (Wilbertz et al., 2019). These serve as intermediate translational repression elements as they either direct stored mRNA toward

processing bodies for degradation or back in to the cytoplasm once the stressor is removed (Lui et al., 2014). Lastly, Processing bodies (P-bodies), the group of repressive granules that this project will focus on, are cytoplasmic granules that serve as protein translation control elements in a wide variety of somatic cells as well as oocytes and neurons(Swetloff et al., 2009, Parker and Sheth 2007, Cougot et al., 2008) . Composed of a wide variety of miRNA machinery such as argonuate (Ago) proteins and mRNA de-capping enzymes Dcp1/Dcp2, these aggregates are commonly involved in translation repression through mRNA degradation processes or storage of mRNA (Parker and Sheth 2007, Cougot et al., 2008). Previous studies have identified P-body markers in the soma and dendrites of primary neurons (Cougot et al., 2008) and in axons of sensory neurons (Melemedijan et al.,, 2014). Furthermore, studies also highlight that proteins necessary for P body function, such as Ago1/2 and RCK, colocalize with FMRP. This colocalization of P-body proteins and FMRP is necessary P-body and/or FMRP-mediated repression of subsets of mRNA transcripts (Lee et al., 2010, Chu and Rana 2008). Studies have also highlighted that the location and functioning of these P bodies are regulated by synaptic activity (Cougot et al., 2008). Given the striking role that FMRP plays in these structures paired with the fact that their functioning is regulated by changes in neuronal activity, we hypothesized that P-bodies would be ideal targets to study in the context of homeostatic plasticity and FXS.

Our lab has exciting preliminary data demonstrating that P-bodies are upregulated in the soma of wild type neurons following activity deprivation with TTX/APV (Fig 6). An upregulation of P-bodies would suggest reduced protein synthesis, which is consistent with our data in Fig 6. Interestingly, *Fmr1* KO neurons had significantly increased levels of P-bodies in the soma at baseline, with only a slight upregulation following TTX/APV. This increase is consistent with the small decrease in protein synthesis that we observed at baseline. Although a

small increase in P-body numbers after TTX/APV in the *Fmr1* KO was observed, we hypothesize that this change may not be functionally significant. In summary, P body activity in the soma is upregulated in response to activity deprivation. These results led us to hypothesize that similar changes in P body expression also occur in dendrites and the AIS. Therefore, the final part of this project will focus on measuring the activity dependent regulation of P bodies in the AIS (Aim 2) as well as in the dendrites (Aim 3) of neurons. These results will contribute to our understanding of the protein synthesis dependent changes during HIP and will help elucidate the molecular changes that may be targeted to alleviate the pathology associated with FXS.

Aims:

The study described above can be summarized as *investigating the compartmentalized morphological and molecular mechanisms underlying homeostatic intrinsic plasticity in Fmr1 KO neurons in response to activity perturbation*. To narrow the scope of the experiment this can further be divided in to three aims.

Aim 1: To understand how the axon initial segment morphology changes in response to chronic activity increase with the GABA-A receptor antagonist Gabazine, and if this change is exaggerated in *Fmr1* KO neurons.

Aim 2: To investigate whether P-body number increases in the AIS to regulate protein synthesis in WT during TTX/APV treatment(chronic activity deprivation), but not in TTX/APV treated *Fmr1* KO neurons.

Aim 3: To investigate whether P-body number increases in the denndrites to regulate protein synthesis in WT during TTX/APV treatment(chronic activity deprivation), but not in TTX/APV treated *Fmr1* KO neurons.

Methods:

Mice:

FMR1^{HET} females were crossed with WT C57BL6 to generate pups of mixed genotypes(WT, *FMR1*^{HET}, *Fmr1* KO). For these experiments, *Fmr1* KO and WT pups were selected following verification of their genotype using PCR (postnatal days 0-2). The mice were housed in a 12 hr light/dark cycle and the animal protocol was approved by the Institutional Animal Care and Use Committees at Emory University.

Treatment:

We use primary cortical neuronal cultures from dissected cerebral cortices of WT and Fmr1 KO littermate pups (postnatal day 0-2). The culturing and dissection processes are similar to the methods described in Bülow et. al 2019. Pharmacological activity perturbation of the cells will be induced through Gabazine treatment (1 uM) (Aim 1) or TTX/APV treatment (1 uM/100 uM) (Aims 2, 3) wherein the drugs will be added to the glial conditioned growth media and introduced to the cells by complete media change on days in vitro (DIV) 10. Tetrodotoxin(TTX) functions by blocking the pore region of voltage gated sodium channels and this results in impaired generation of action potentials having an overall inhibitory effect on the activity of the neuron (Lorentz et al., 2016). (2R)-amino-5-phosphonovaleric acid (APV) is an NMDA receptor antagonist that has inhibitory effect on neuronal activity. Inhibition of NMDAR activity by APV has been linked to the rescue LTD function in Fmr1 KO neurons (Toft et al., 2016). It has also been linked to impaired fear contextualization in an *in vivo* model of dorsal hippocampal neurons in C57BL/6J mice (Steidl et al., 2000). Gabazine is a very effective $GABA_A$ receptor

antagonist that inhibits the receptor competitively in a high affinity state (Heaulme et al., 1986). This inhibition of $GABA_A$ receptors blocks inhibitory effects of GABA neurotransmission on these neurons resulting in an upregulation of excitatory input and depolarization. It is important to note that both TTX/APV and Gabazine treatment have proved to be effective agents of activity perturbations in *in vivo* and *in vitro models.* More specifically, electrophysiological studies measuring TTX/APV treatment's effect on in vitro cortical Fmr1 KO neurons has shown that this type of activity perturbation is effective in inducing activity deprivation in the neurons (Bülow et al. 2019). TTX/APV treatment experimental paradigms have also been used in in vitro studies of synaptic and homeostatic plasticity and are reflective of phenotypic changes seen in *in vivo* models (Soden and Chen, 2010, He and Bausch, 2013). Gabazine is an effective drug treatment when studying plasticity changes in cortical neurons as prior *in vitro* studies have used gabazine to study changes in homeostatic plasticity in rat visual cortex neurons (Roux et al. 2009). The control cultures will also undergo a media change without the added drugs (Bülow et. al 2019). This treatment is done two days (48 hours) prior to Immunocytochemistry (ICC).

Immunocytochemistry:

The cells are prepped for the ICC by fixing them with 4% PFA for 15 mins at room temperature. The cells will be washed with Tris-glycine buffer(20% Tris, 0.15% glycine) and then permeabilized with 0.2% Triton 1X PBS (2mL per well). Each coverslip will be blocked with 5% BSA solution and then stained with two or three different primary antibodies for 24 hours in a hydration chamber at 4 C. After 24 hours, the coverslips are washed with PBS, and then exposed to the secondary antibodies for 1 hour. The coverslips are then washed three times, mounted and prepped for imaging.

Primary antibodies:

For aim 1, the dendritic marker MAP 2 is paired with 2 cytoskeletal markers that colocalize with the AIS: Ankyrin G(AnkG) and pIkBa. Both of the AIS antibodies are used to allow for colocalization within the AIS and will verify that our stains are specific to the AIS. For aim 2, MAP2, AnkG, and a marker that localizes within P bodies called DCP1A will be used. Lastly, for aim 3 only MAP2 and DCP1A will be used.

Imaging and Morphology Analysis:

A Nikon confocal microscope will be utilized in the imaging acquisition. All aims will be done using a 60X objective. In experiment 1, only cells with colocalized expression of Ankyrin-G and PiKBA will be included. The images themselves will be Z-stacks (0.3 um steps), which after being obtained, will be deconvoluted and analyzed on the FIJI software.

AIS analysis: In aim 1, AISs will be analyzed as described previously (Grubb and Burrone, 2010; Evans et al., 2015). Specifically, fluorescence intensity values are averaged over a 3x3 pixel square, smoothened and normalized to the pixel with the maximal intensity. AIS start and end positions are the proximal and distal axonal positions where the normalized, smoothed profile declined to 0.33 (Fig 7). AIS length will be calculated as the axonal distance between start and end positions of the AIS, and AIS to soma distance is calculated from the proximal start position of the AIS to the most proximal part of the soma (outlined manually; see Fig. 7).

P-body analysis:

To test Aim 2, the same protocol employed in Aim 1 was used to define the start and the end position of the AIS. After this step, the AIS is outlined manually. This selection is then placed over a maximal intensity compressed 32-bit image of the DCP1A channel (Fig 8B) and another maximal intensity compressed DCP1A 8-bit image (Fig 8C) that has undergone automated thresholding (thresholding program = 'Triangle'). The two images are compared and the extent of thresholding in the 8-bit image is adjusted manually to match the visible P bodies seen in the 32-bit image within the outline. Once matched, the number of P bodies, area of the AIS and the thresholding values are automatically recorded and compared between conditions. To test cellular changes with regard to P bodies in the dendrite is tested by using the same analysis protocols as seen in 1B. The only change to this protocol was that specific dendritic ROIs 15um away from the soma are used to analyze the changes in the number of P bodies between the conditions.

Results:

Fmr1 KO neurons exhibit novel compensatory changes in soma-AIS distance at baseline:

At baseline, comparisons between the AIS length between the WT and the *Fmr1* KO neurons revealed that there was no significant changes between the two groups (Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p>0.05$) (Fig 9B). Additionally, these cell cultures revealed that Gabazine had no significant effect on AIS length following treatment in either the WT or the *Fmr1* KO neurons (Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p>0.05$) (Fig 9B). There was also no significant difference between the WT treatment group and the KO treatment group. These results were similar to the changes in AIS length that were observed in the TTX/APV experiments wherein no baseline or treatment effect differences were observed in the WT and *Fmr1* KO neurons following TTX/APV treatment (Fig 4B).

Comparing the soma-AIS distances between these groups revealed that at baseline *Fmr1* KO neurons present with a significantly greater soma- AIS distance compared to the WT counterparts (Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p<0.05$) (Fig 9A). These results are particularly interesting as the increase in soma-AIS distance at baseline is indicative of a novel compensatory mechanisms in response to hyperexcitability seen in the *Fmr1* KO neurons. Furthermore, these results also validate what was found in the preliminary TTX/APV experiments (Fig 4). Additionally, there were no treatment effects in either the WT or the *Fmr1* KO neurons following treatment with Gabazine. This was also the case when comparing the soma-AIS distance between the WT and KO treatment conditions(Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p>0.05$).

In summary results from the Gabazine experiments reveled that at baseline *Fmr1* KO neurons display altered AIS positioning compared to WT controls. This increase could be caused directly by the loss of FMRP, or alternatively, could represent a compensatory response to hyperexcitability at earlier developmental timepoints. Additionally, the results revealed that the abnormal HIP changes observed in the *Fmr1* KO neurons in response to TTX/APV treatment was not seen in the KO cells following Gabazine treatment. This suggests that activity deprivation(TTX/APV treatment) but not activity increase(Gabazine treatment) triggered unique homeostatic changes in the AIS of KO neurons. Therefore, we were unable to find evidence that supported our initial hypothesis that the abnormal HIP changes seen in the *Fmr1* KO neurons in response to TTX/APV treatment was bidirectional in nature. The results highlighted that activity deprivation but not activity increase in *Fmr1* KO neurons resulted in altered HIP changes in the AIS.

P-Bodies in the AIS were not upregulated as a consequence of activity deprivation:

Based on our preliminary experiments, we expected to see a greater number of P bodies in the AIS of WT neurons treated with TTX/APV when compared to the controls. In the KO TTX/APV condition, however, we hypothesized that the there are no changes in P-body number compared to controls, consistent with our preliminary data quantified in the soma. The results that we found in these experiments did not provide evidence for our hypothesis as there was no detectable increase in P bodies seen in the WT TTX/APV condition.

P-body quantification experiments conducted on *Fmr1* KO and WT neurons in response to TTX/APV treatment revealed results contrary to the preliminary experiments conducted in the

soma. At baseline, we found that there were no significant differences between the WT and the *Fmr1* KO neurons (Fig 10B). Furthermore we also found that there were no significant differences following TTX/APV treatment in either condition (Fig 10B) (Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p>0.05$).

P-Bodies in the dendrites were not upregulated as a consequence of activity deprivation.

In accordance with our preliminary results from the soma, we expected to see an increase in the number of P bodies in the dendrites of the WT in response to the activity perturbation with TTX/APV. In contrast, we did not expect to see a significant difference in number of P bodies between the KO conditions following treatment. The results from the experiments above shows that the data we found does not support this hypothesis and that there is no significant effect of TTX/APV treatment on the number of dendritic P bodies in either the WT or the KO condition.

In a set of experiments quantifying the number of P-bodies in the dendrites of *Fmr1* KO neurons following TTX/APV treatment we found results similar to the data obtained from the AIS experiments. At baseline, we found that there was no significant difference between the WT and KO neurons (Fig 11B) (Two-way ANOVA w multiple comparisons and Bonferroni corrections, $p>0.05$). Additionally, there were no treatment effects in either the WT or the KO neurons following TTX/APV treatment (Fig 11B).

In both the AIS and the dendritic P body experiments we observed variability between each individual experiment. This increased variability could confound the validity of the data and therefore motivates future studies that use a similar experimental paradigm with increased

replicates to better understand the trends in P body concentration following TTX/APV treatment in the Fmr1 KO and WT neurons.

Discussion:

In this study, one major goal was to test whether KO neurons lacking FMRP display abnormal AIS plasticity during chronic activity perturbations. Here, we further corroborated previous findings that *Fmr1* KO neurons display a novel compensatory mechanism at baseline in the regulation of soma-AIS distance. Furthermore, we also found evidence contrary to our preliminary hypothesis that we would detect a bidirectional effect following treatment with gabazine. We also examined the changes to molecular translational repression regulation in *Fmr1* KO neurons by analyzing changes to the number of P bodies in the AIS and dendrites following TTX/APV treatment. We hypothesized that we would detect a marked increase in the number of P bodies in the AIS and dendrites of the WT neurons but not the KO neurons following TTX/APV treatment. However, we found that there were no significant baseline differences or treatment effects in the WT and the KO neurons following treatment. These findings contradict our initial hypothesis in that the changes observed following gabazine treatment in the Fmr1 KO neurons were not bidirectional in nature and validated potential compensatory mechanisms in response to hyperexcitability at baseline in the Fmr1 KO neurons.

Preliminary experiments conducted to measure changes to AIS length and soma-AIS distance in response to TTX/APV treatment revealed that the WT cortical cells did not regulate the AIS length or position after treatment. In the experiments that we conducted using gabazine ($GABA_A$ receptor antagonist), we found that the WT neurons do not engage in AIS-related homeostatic plastic changes(length and location) in response to the perturbation. These findings

were especially interesting as studies in the past have shown that the location and length of the AIS are both regulated by HIP mechanisms following activity perturbation. For example, Grubb and Burrone showed that global depolarization of hippocampal neurons using 15 mM extracellular potassium resulted in significantly longer soma-AIS distances in these neurons (Grubb and Burrone 2010). Conversely, experiments conducted with auditory neurons from the chick found that the AIS length was significantly greater following activity deprivation (Kuba et al., 2010). One possible explanation for the lack of AIS plasticity in cortical WT neurons in our experiments may be due to unique cell-type specific responses to activity perturbation. Dopaminergic Cav3.2 receptors coupled in the AIS of inhibitory interneurons in the dorsal cochlear nucleus are inhibited via a β-arrestin dependent pathway involving protein kinase C in response to activity increase(Bender et al., 2010; Yang et al., 2016). Alternatively, Cav3.2 receptors in the hippocampal granule cells are found to be activated by muscarinic M1 receptor dependent pathway(Aznavour et al., 2005). This activation results in an increased influx of Ca^{2+} ions at the AIS consequently leading to a negative shift in the activation curve of Kv channels which in turn augments AP generation(Aznavour et al., 2005). To better understand that the lack of response to treatment seen in the WT cells, future electrophysiological studies could analyze ionotropic and metabotropic channel activity in the AIS following activity perturbation so that we can obtain a better picture of the biophysical changes that takes place in these neurons.

When comparing the responses of the *Fmr1* KO neurons to TTX/APV and Gabazine, we found that at baseline they had a significantly longer soma-AIS distance compared to their WT counterparts(WT = 8.81 μm, KO = 10.46 μm). This finding was especially remarkable as it possibly supports the hypothesis that the KO neurons engaged in a novel compensatory mechanism in response to the phenotype of hyperexcitability that we see in FXS.

The results observed in the *Fmr1* KO neurons at baseline may also be due to dysregulation of cytoskeletal components such as Ankyrin G that responsible for AIS structure and morphology. This is supported by studies conducted in drosophila d*Fmr1* KO neurons that highlighted a dysregulation of cytoskeletal remodeling mechanisms involved in synaptic remodeling that were regulated by dFmrp (Schnek et al., 2001). Studies have also revealed glycogen synthase kinase (GSK3) and β-catenin influence ankyrin-G and sodium channel levels in cultured cortical and hippocampal neurons both of which play a role in proper development of the AIS(Tapia et al., 2013). Loss of FMRP in *Fmr1* KO mice is associated with reduced inhibition of GSK-3 (Yuskaitis et al., 2010) indicating that the altered soma-AIS distance in the *Fmr1* KO neurons at baseline may be a product of altered Ankyrin-G synthesis and/or insertion rather than a compensatory response to treatment.

Intriguingly, we found that the KO neurons exhibit a significant reduction in soma-AIS distance following TTX/APV treatment similar to WT. In contrast, no significant change to soma-AIS distance was observed in the KO cells following Gabazine treatment. These results suggest that the baseline increase in soma-AIS distance of the *Fmr1* KO neurons decreases the excitability of these neurons. In the case of the TTX/APV experiments where the neuron is chronically activity deprived, it makes sense for the baseline compensation to be removed and the AIS moves closer to the soma in an attempt to increase neuronal excitability. However, in the case of the Gabazine experiments, the *Fmr1* KO neurons are exposed to chronic activity increase which in turn could prevent the neurons from removing their baseline compensatory adaptations to hyperexcitability and in turn engage in alternate homeostatic changes in response to activity perturbations. Therefore, the baseline compensation to hyperexcitability could not only be

attributed to a ceiling effect in the regulation of AIS location but also may help explain the lack of response in the *Fmr1* KO neurons following gabazine treatment.

Another goal of this Honors Thesis was to elucidate the molecular changes that contribute to the altered HIP phenotype observed in *Fmr1* KO neurons (Bülow et al.,2019). We wanted to analyze how translational repression elements called processing bodies (P-bodies) were altered in the dendrites and AIS of WT and *Fmr1* KO neurons in response to activity deprivation with TTX/APV. We made the novel discovery that the number of P-bodies are not regulated by neuronal activity in WT dendrites and the AIS contrary to what our lab previously reported in the soma of WT neurons. Moreover, we found no significant differences in the number of P bodies between the WT and the *Fmr1* KO cells at baseline in the dendrites and the AIS.

These results could be explained by the theory that the P bodies function in a highly localized manner. For example, it is possible that P bodies confer distinct functions in the soma and at the synapse. At the synapse, the P-bodies may be important for rapid release and capture of mRNAs important for synaptic plasticity, whereas P bodies in the soma are important for long term repression of mRNAs at the bulk level. This idea implies that TTX/APV treatment triggers formation of P-bodies in the soma rather than recruitment of P-bodies from the AIS or dendrite into the soma. This idea of localized P body function is further supported by previous studies reporting unique activity dependent changes in dendritic P-body localization. For example, one study reported that P-bodies were transported to more distal locations of the dendrite upon NMDA treatment (Cougot et al., 2008), supporting the idea of localized activity regulation of P-bodies and these results could account for some of the results in our study. Thus, a future study should investigate whether P-bodies are transported proximally in the dendrite and AIS after

TTX/APV treatment. We also found that when comparing the data between the experiments, there was a large amount of variability between individual experiments. In fact, we observed robust increases in the number of P-bodies in WT dendrites two out of four experiments. A third experiment measured a reduction in the number of P-bodies after TTX/APV and the fourth no difference with treatment. We consider two possible explanations for this variability: First, The high density of cells in the cultures made it challenging to obtain images individual neurons without overlap. While obvious instances of overlap were excluded from the data this overlap could have played a confounding role in the data. Secondly, due to time constraints due to COVID-19 only a small population of neurons were analyzed in the AIS experiments (n=10) which could further reduce the accuracy of the data as it may not be representative of the entire population. Future experiments, could look to use cultures with a lower density of neurons to allow for better visualization of individual cells as well as an increase in the population of neurons analyzed so as to gain a more accurate representation of the population.

Future Direction and Conclusion:

Overall, we found evidence that suggests novel compensatory mechanisms in response to hyperexcitability in the *Fmr1* KO neurons at baseline. Furthermore, the preliminary TTX/APV experiments show that there is a reduction in soma-AIS distance in the treatment conditions, which could be a mechanism leading to hyperexcitability following TTX/APV treatment. However, we did not observe any effect of Gabazine treatment, suggesting that activity deprivation but not activity increase triggers unique homeostatic mechanisms in the *Fmr1* KO neurons. In addition to perturbation specific effects on HIP, we also hypothesize that cell type could be important. Moreover, it is likely that specific ion channels or cytoskeletal proteins are regulated differently between WT and *Fmr1* KO neurons during HIP. Preliminary studies conducted in the soma measuring translational repression dysregulation revealed that P bodies were upregulated in the *Fmr1* KO neurons at baseline and were increased in both conditions following treatment with TTX/APV. However, our study was able to replicate these results in the AIS and the dendrites indicating that P body function and response may be localized depending on the type of activity perturbation.

To better understand our results and further test our hypothesis, additional experiments can be performed. The gabazine experiments revealed that abnormal HIP in the *Fmr1* KO may be mediated via alternate mechanisms of action in addition to just structural changes. Future experiments could investigate biophysical changes in the cell by conducting electrophysiological studies that monitor ionotropic and metabotropic receptor function following activity perturbation. Furthermore, to test if AIS location regulation plateau's after a certain level of activity perturbation, dose response curve experiments using increasing doses of Gabazine and TTX/APV should be conducted. It is also possible that the baseline compensatory changes

observed in *Fmr1* KO neurons in soma-AIS distance may be due to altered Ankyrin-G synthesis and/or insertion. To test this idea, future experiments should test Ankyrin G levels in *Fmr1* KO neurons following activity perturbation by conducting immunoblot experiments to quantify the amount of protein. Ankyrin-G insertion could be visualized using visual microscopy experiments that monitor activity perturbed neuron cultures with fluorescently tagged Ankyrin G. Lastly, with regard to our experiments measuring changes to P body concentration in the AIS and dendrites, the non-significant change may be a product of proximal movement of P bodies towards the soma in response to activity perturbation. While we did not see a decrease in the concentration of P bodies, it is possible that the synthesis of P bodies in this region is unimpaired**.** Future studies should test this idea by conducting live cell imaging studies that examine Dcp1a localization as a marker of P body movement following treatment with TTX/APV. Answers to these questions in the future paired with our findings in this study may help provide valuable information in identifying a clinical target that may help ameliorate symptoms of hyperexcitability in FXS in the future.

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Figures:

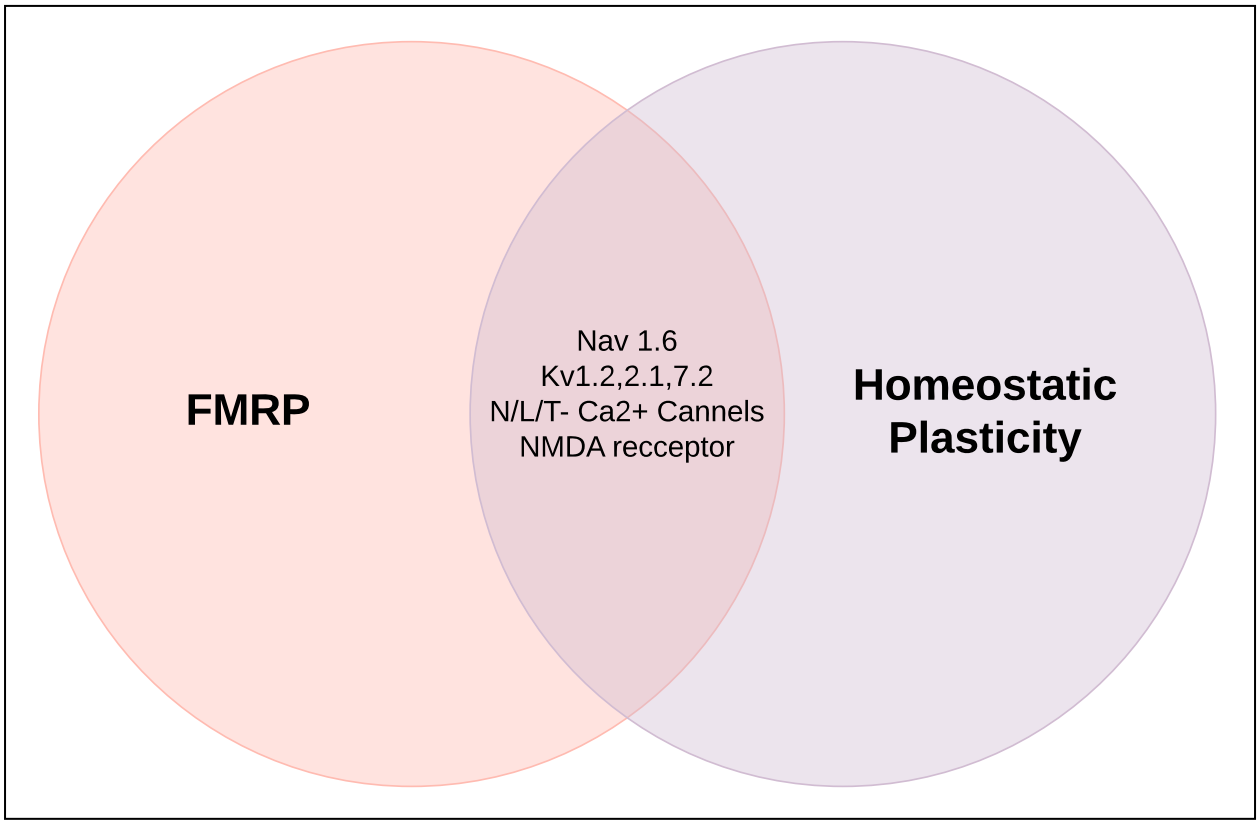


Fig 1. Ion channels involved in Homeostatic intrinsic plasticity(HIP) mechanisms are also regulated by FMRP. These channels play an important role in regulating neuron excitability. These ion channels are highly concentrated in the AIS motivating our study to examine morphological changes to the AIS in *Fmr1* KO neurons in response to activity perturbations.

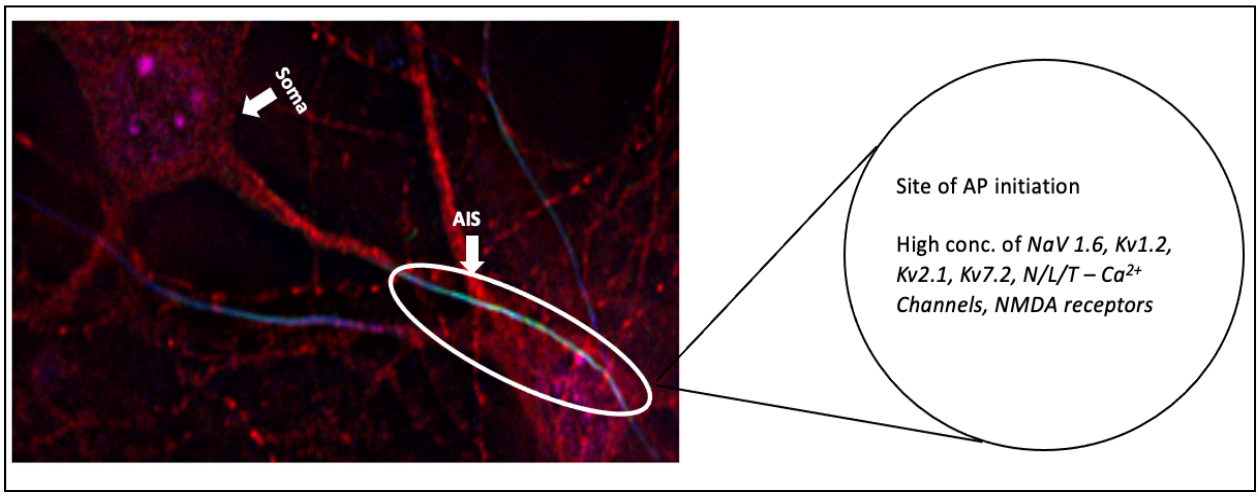


Fig 2. Fluorescent coimmunostaining of WT cortical neuron cultures using DAPI(nucleus), MAP2(dendrite and soma cytoskeleton), Ankyrin-G(AIS cytoskeleton), PiKBA(AIS cytoskeleton). The dendritic cytoskeletal marker MAP-2 was visualized in red using Cy5 coupled with an anti-guinea pig antibody. The AIS cytoskeletal markers Ankyrin-G was visualized in green using Cy3 with an anti-mouse antibody and PiKBA was visualized in blue using 488 with an anti-rabbit antibody.(*pictured on the left)* Characteristics of the axon initial segment.(*pictured on the right)*

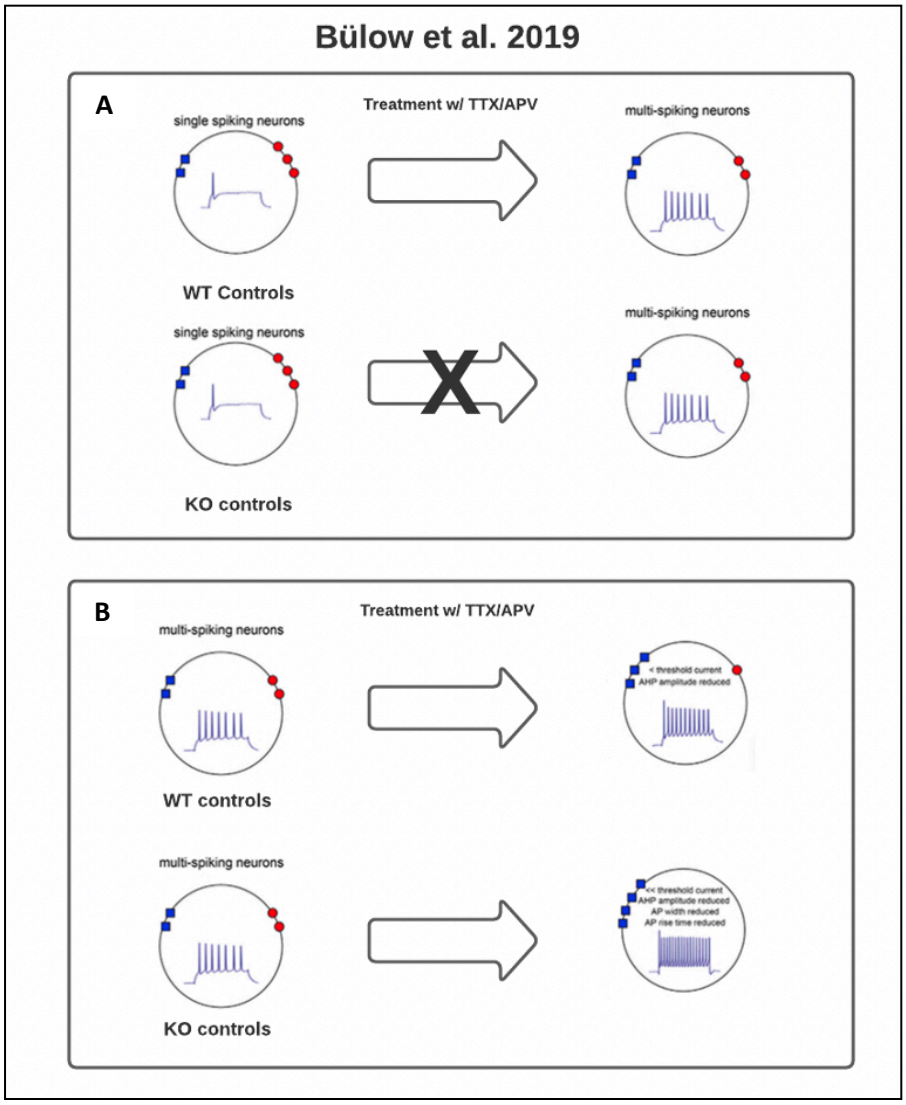
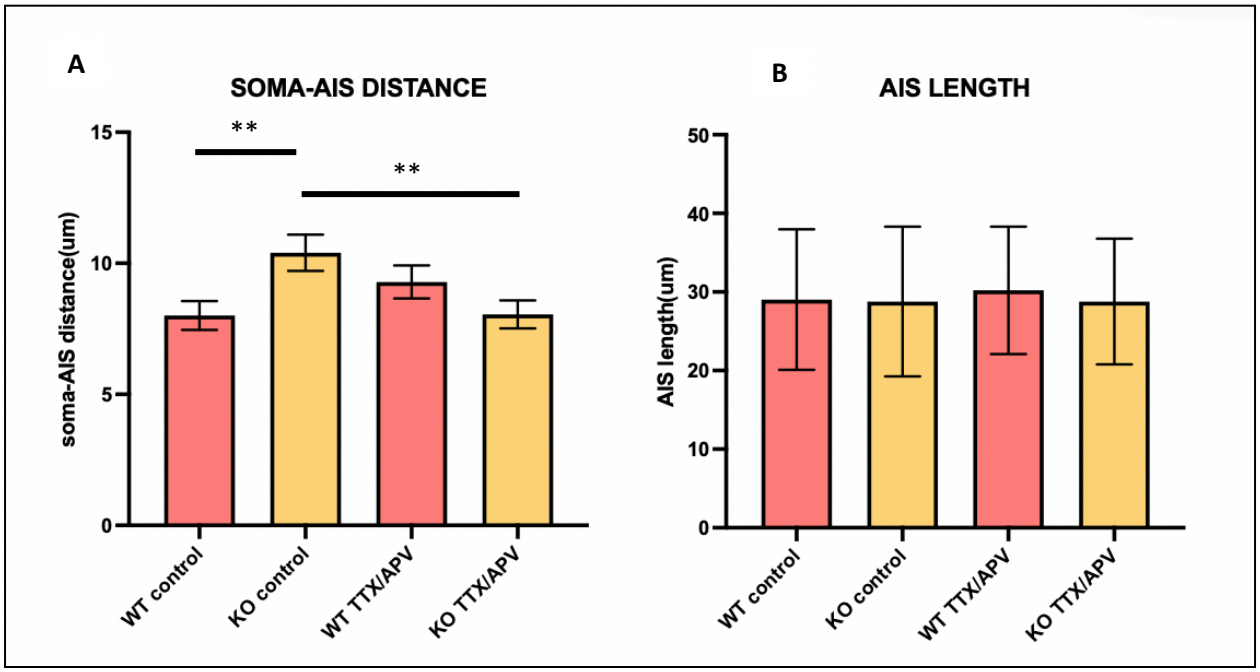


Fig 3. **Image from Bülow et al., 2019.** This paper showed that *Fmr1* KO neurons presented with abnormal HIP mechanisms in response to activity perturbation. (A) In response to TTX/APV treatment WT neurons were seen to convert from single spiking neurons to multi-spiking neurons. However *Fmr1* KO neurons did not exhibit this phenotypic change. This was not only evidence for a novel HIP mechanisms but also one that was not exhibited by *Fmr1* KO neurons. (B) Spiking activity and action potential parameters were measured between *Fmr1* KO neurons and their WT counter parts. *Fmr1* KO neurons presented with significantly greater increase in spiking frequency following treatment with TTX/APV when compared to the WT cells. Furthermore, *Fmr1* KO neurons displayed changes to action potential parameters.

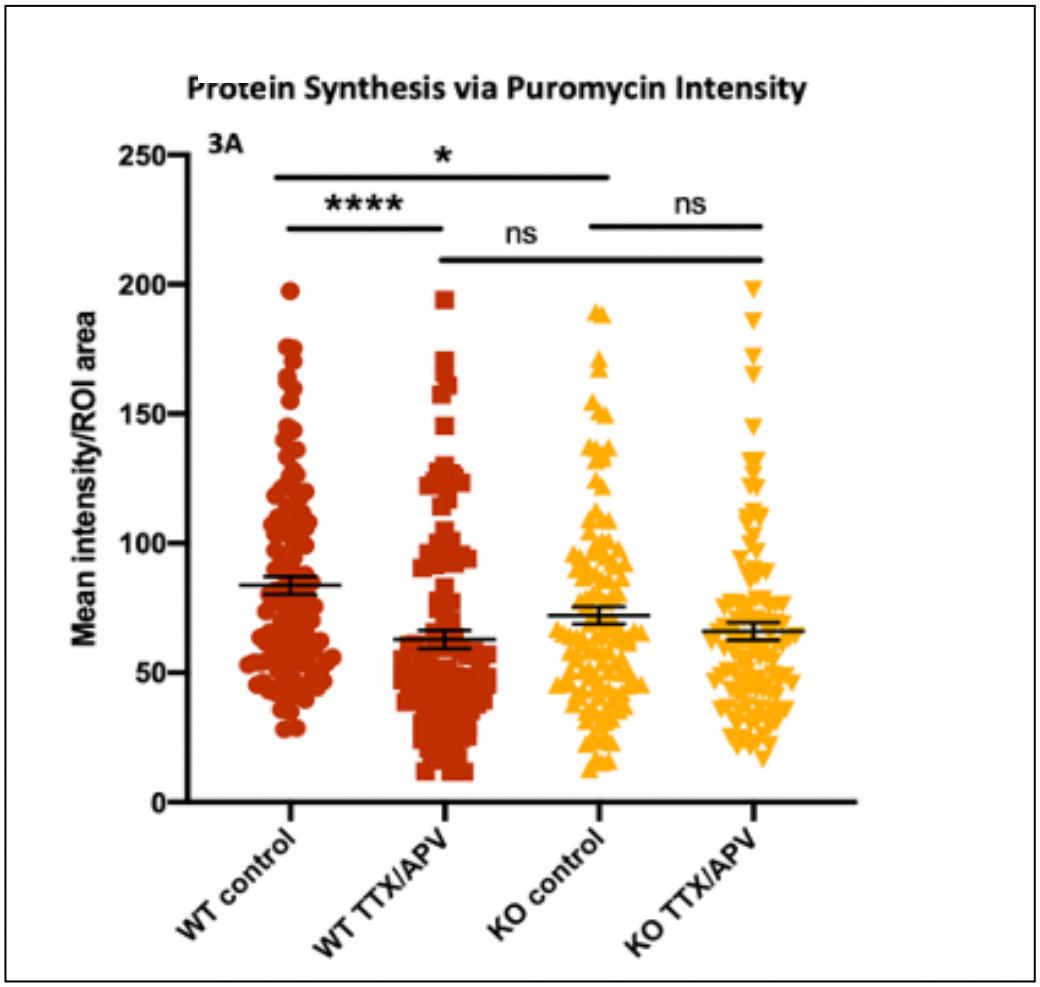


Data collected by Chayla Vasquez

Fig 4. Experiments conducted in WT and *Fmr1* KO neurons measuring morphological changes to soma-AIS distance and AIS length following treatment with TTX/APV.

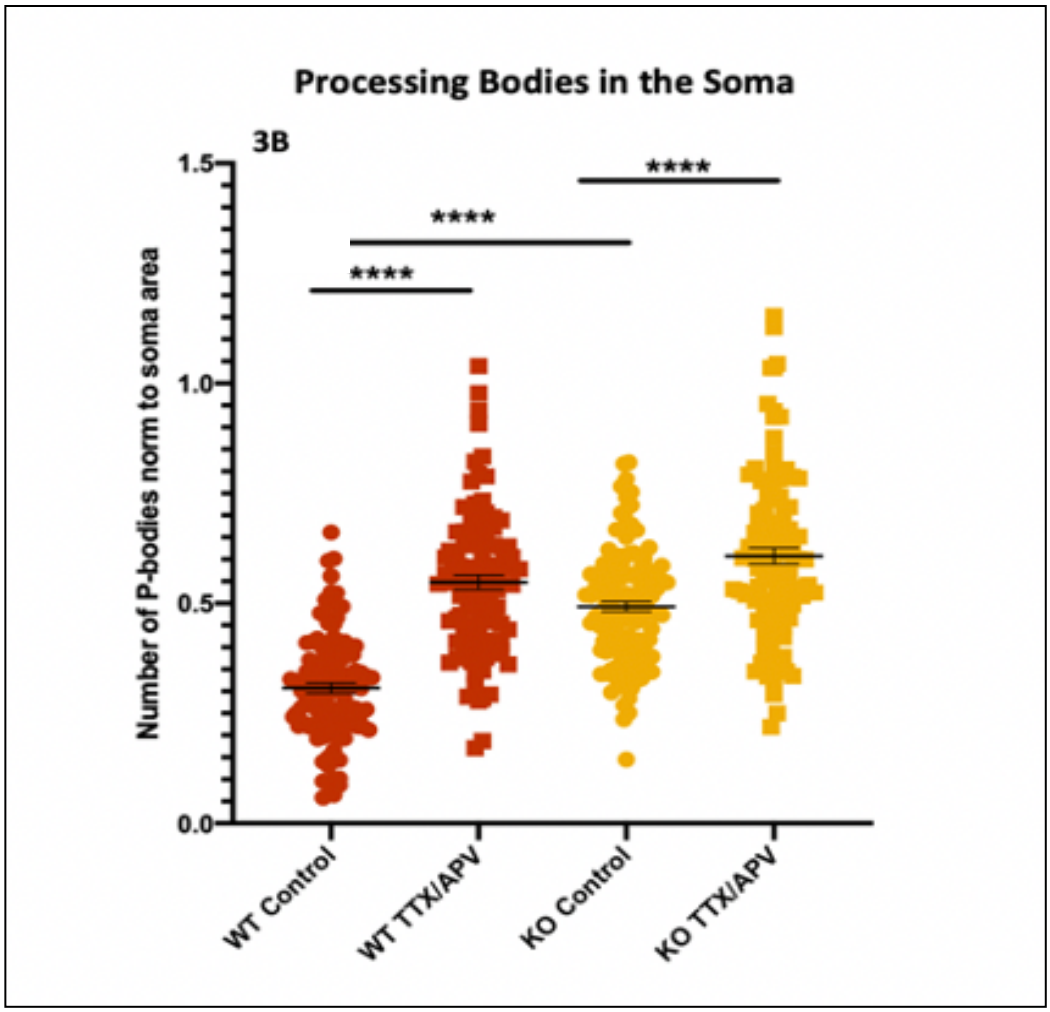
(A) Differences in soma-AIS distance between WT and *Fmr1* KO cortical neurons. *Fmr1* KO neurons at baseline present with significantly larger soma-AIS distance than WT counterparts, indicating potentially novel compensatory mechanisms. Additionally, the soma to AIS distance of the *Fmr1* KO neurons following treatment was significantly shorter(n = 40, * = $p < 0.05$, two-way ANOVA with multiple comparisons and Bonferroni corrections).

(B) Differences in AIS length between WT and *Fmr1* KO cortical neurons. AIS length was not significantly different between the two groups and did not exhibit any significant changes following treatment. (n = 40 , two-way ANOVA with multiple comparisons and Bonferroni corrections)



Data collected by Pernille Bülow

Fig 5. Highlights data from Puromycin labelling experiment to detect nascent protein synthesis in cortical neurons following TTX/APV activity perturbation. There was a significant lower levels of protein synthesis in the *Fmr1* KO neurons. WT neurons also presented with decreased protein synthesis following treatment with TTX/APV. (two-way ANOVA with multiple comparisons and Bonferroni corrections)



Data collected by Pernille Bülow

Fig 6. Preliminary experiment that examined the changes in the number of P bodies in the soma of WT and *Fmr1 KO* in response to TTX/APV treatment. At baseline, KO neurons had significantly greater number of P bodies compared to WT cells. Both WT and KO neurons exhibited a significantly increase in P bodies following TTX/APV treatment. (*= $p<0.05$, two-way ANOVA with multiple comparisons and Bonferroni corrections)

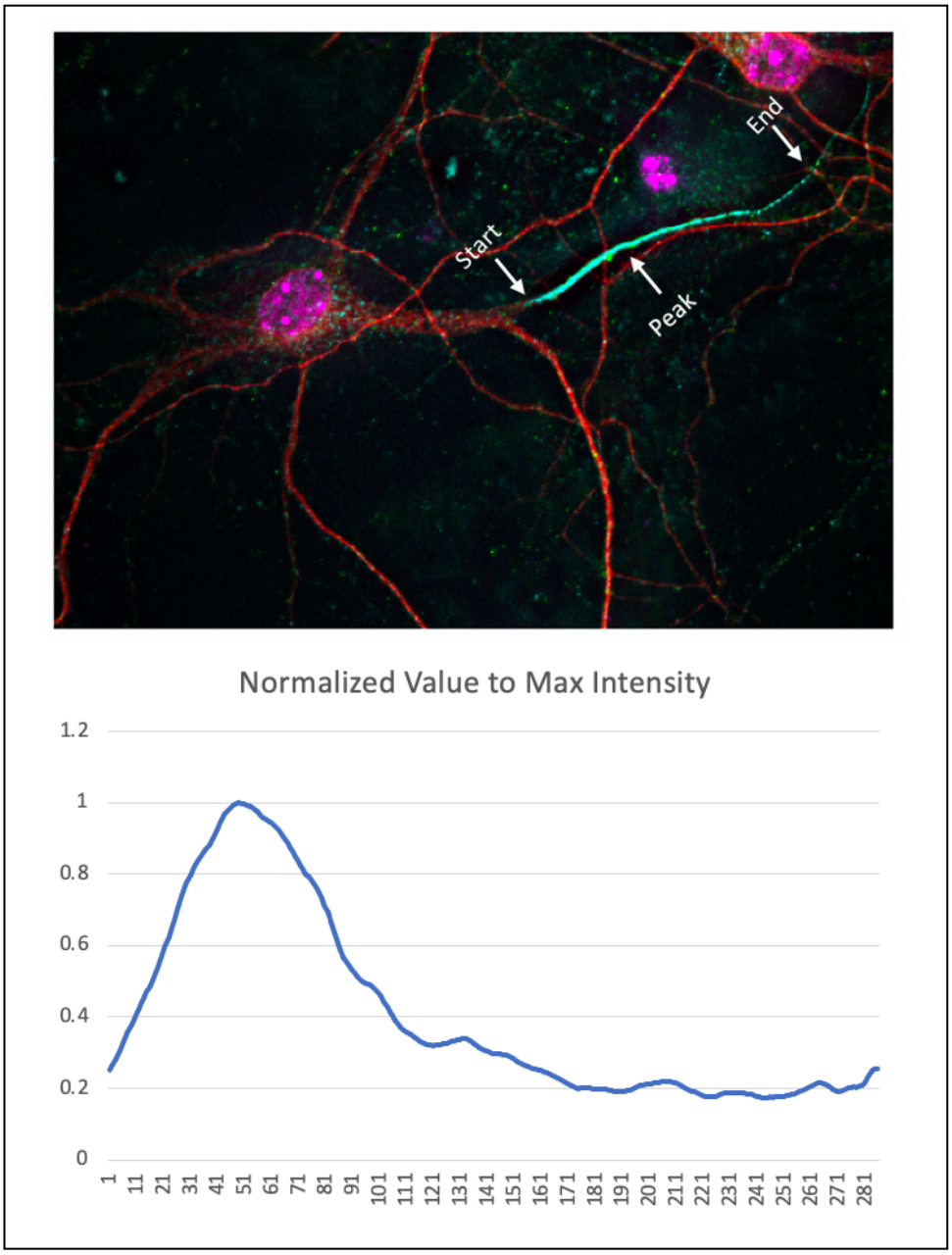


Fig 7. Image of typical neuron analyzed with start and end points(*top*). A graphical representation of fluorescent intensities along the AIS. The start and end of the AIS is defined as the pixel where it reaches 0.33 of its peak normalized intensity(*bottom)*.

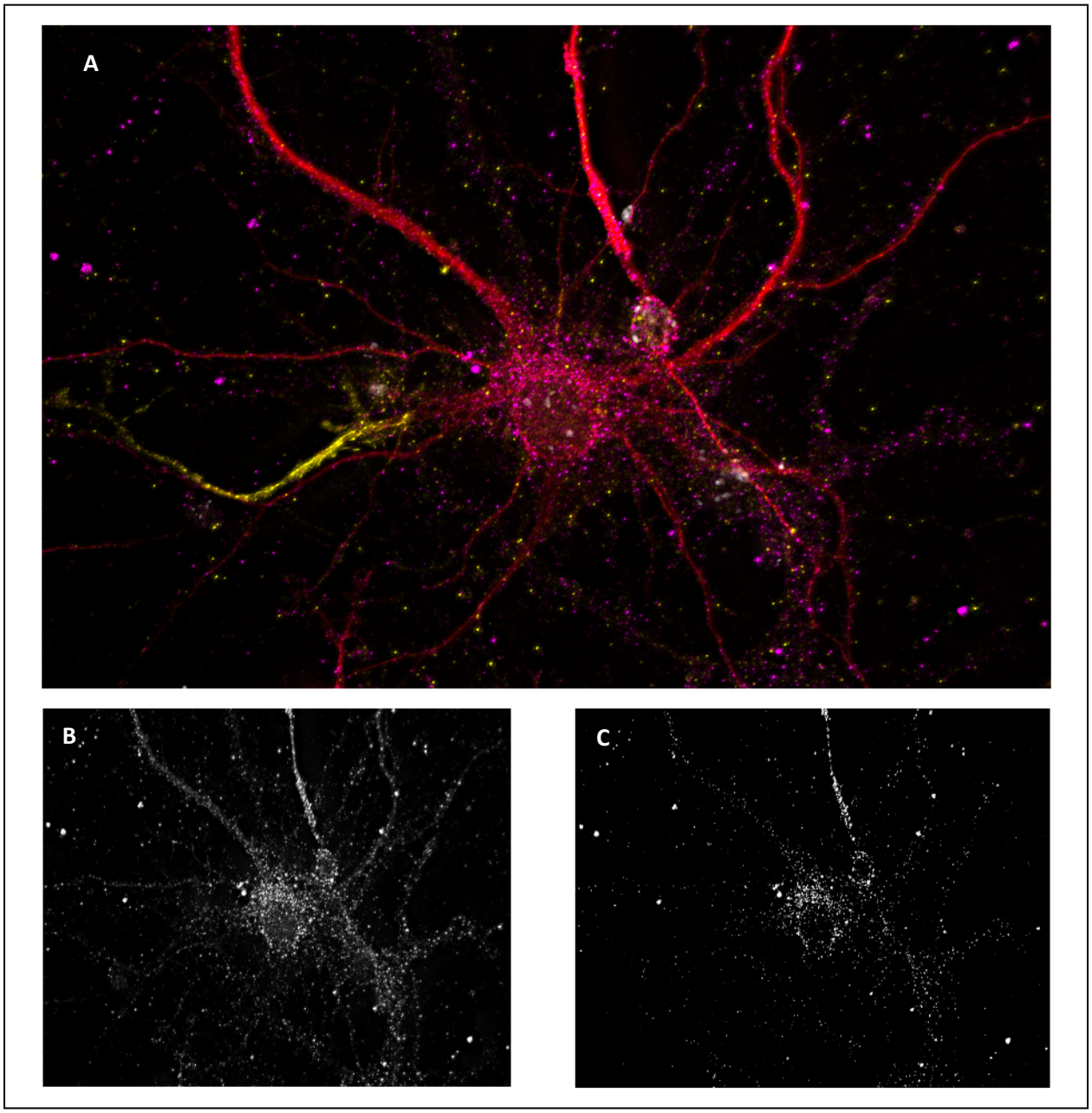


Fig 8. (A) Image of typical neuron analyzed to determine the number of P-bodies in the AIS. The dendrites and soma are visualized using MAP2(red), AIS is identified using cytoskeletal marker Ankyrin-G(yellow), and the P bodies are identified using Dcp1a marker(pink). (B) 32-bit image of the Dcp1a channel that is used to identify, confirm and count the Dcp1a positive P bodies. (C) 8-bit threshold image that was thresholded using "Triangle" software on FIJI. This channel is used to identify and count the number of Dcp1a positive puncta.

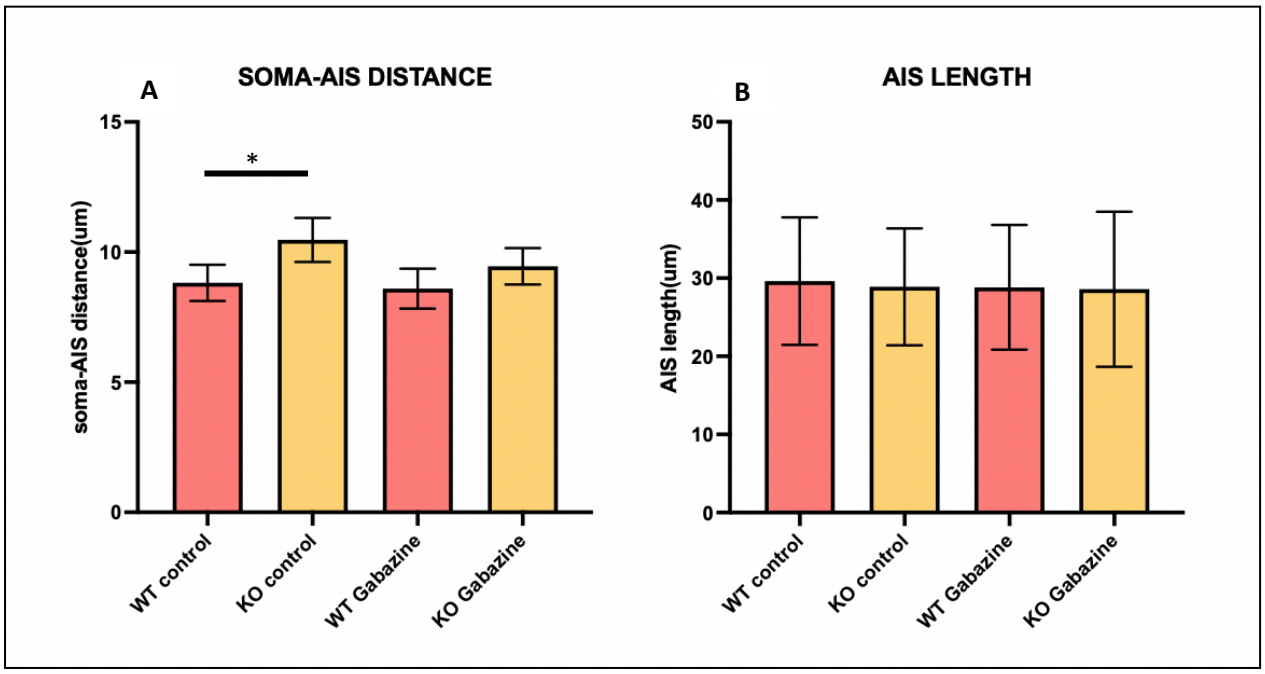


Fig 9. Experiments conducted in WT and *Fmr1* KO neurons measuring morphological changes to soma-AIS distance and AIS length following treatment with Gabazine.

(A) Differences in soma-AIS distance between WT and *Fmr1* KO cortical neurons. *Fmr1* KO neurons at baseline present with significantly larger soma-AIS distance than WT counterparts, indicating potentially novel compensatory mechanisms. Additionally, the soma to AIS distance of the *Fmr1* KO neurons following treatment was significantly shorter(n = 40, * = $p < 0.05$, two-way ANOVA with multiple comparisons and Bonferroni corrections).

(B) Differences in AIS length between WT and *Fmr1* KO cortical neurons. AIS length was not significantly different between the two groups and did not exhibit any significant changes following

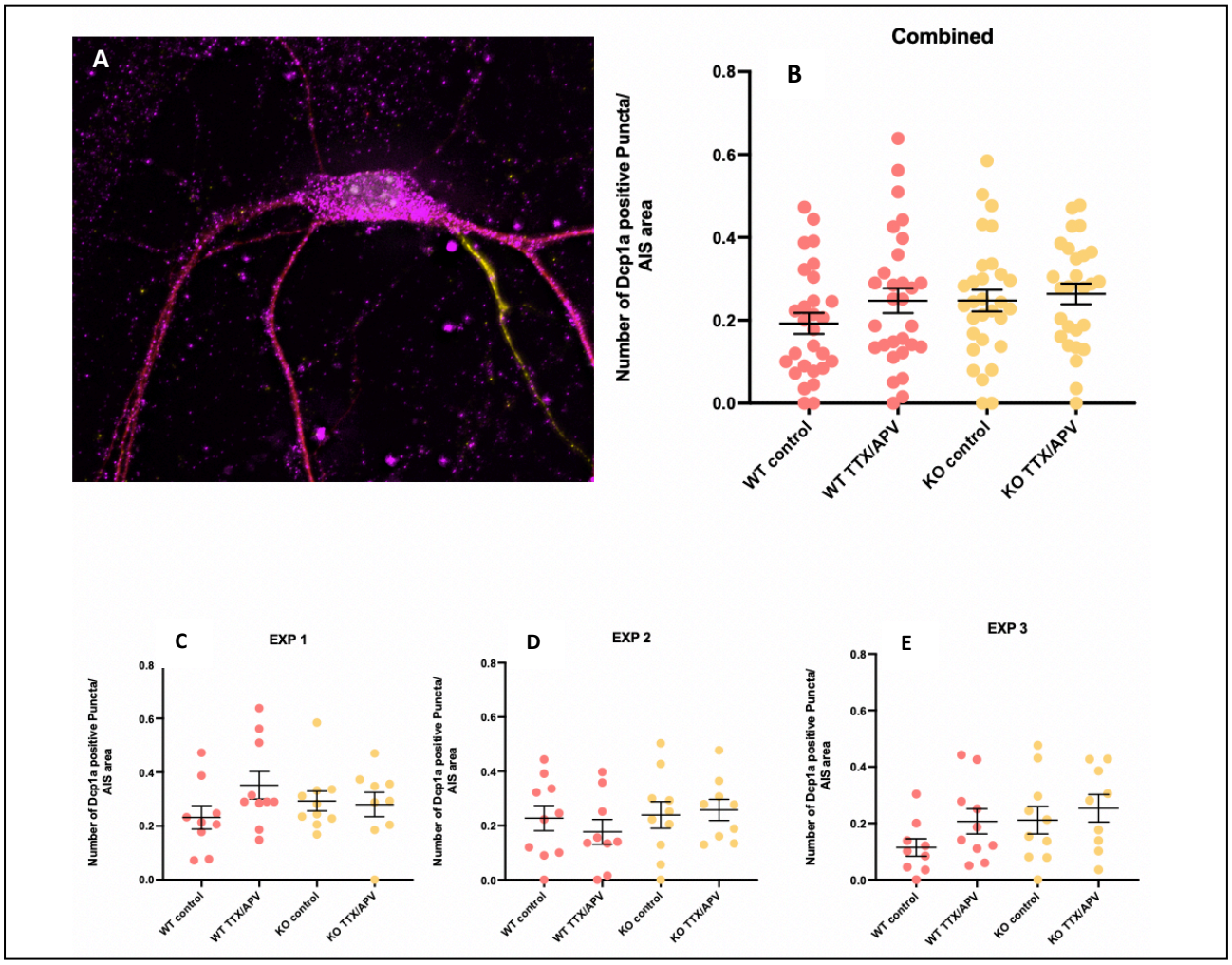


Fig 10. (A) Image of example neuron analyzed to determine the number of P bodies in the AIS following TTX/APV treatment. (B) highlights data testing translational repression in the AIS of cortical neurons. It represents a two way ANOVA with post-hoc Bonferroni to test differences in number of Processing Bodies after treatment with TTX/APV from all three experiments.(C-E) Represents data from each individual experiment that tested the differences in the number of P bodies between WT and KO neurons following TTX/APV activity perturbation. (n = 10 , two-way ANOVA with multiple comparisons and Bonferroni corrections)

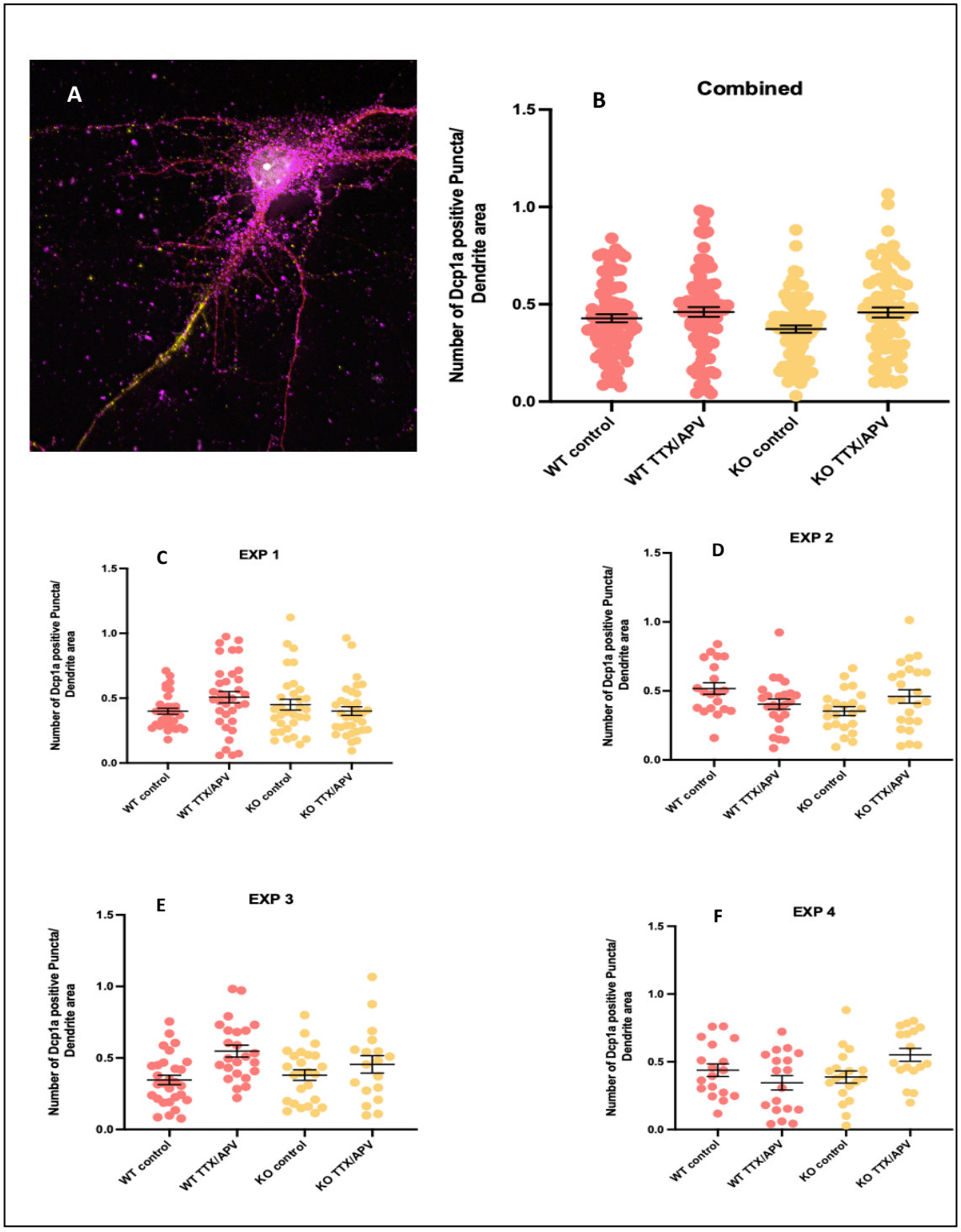


Fig 11. (A) Image of example neuron analyzed to determine the number of P bodies in the dendrites following TTX/APV treatment. (B) highlights data testing translational repression in the dendrites of cortical neurons. It represents a two way ANNOVA with post-hoc Bonferroni to test differences in number of Processing Bodies after treatment with TTX/APV from all four experiments.(C-F) Represents data from each individual experiment that tested the differences in the number of P bodies in the dendrites of WT and KO neurons following TTX/APV activity perturbation. (n = 30 , two-way ANOVA with multiple comparisons and Bonferroni corrections)