Glial dysregulation in human brain in Fragile X-related disorders

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Abstract: While large trinucleotide repeat expansions at the *FMR1* locus cause Fragile X Syndrome (FXS), smaller "premutations" are associated with the late-onset condition Fragile X-associated tremor/ataxia syndrome (FXTAS), which shows very different clinical and pathological features, with no clear molecular explanation for these marked differences. One prevailing theory posits that the premutation uniquely causes neurotoxic increases in FMR1 mRNA (i.e., 4-8-fold increases), but evidence to support this hypothesis is largely derived from analysis of peripheral blood. We applied single-nucleus RNA-sequencing to post-mortem frontal cortex and cerebellum from 9 individuals with Fragile X mutations as well as age and sex matched controls (n=6) to assess cell-type specific molecular neuropathology. We found robust reduction of FMR1 mRNA in FXS as expected, with modest but significant upregulation (~1.3 fold) of FMR1 in glial clusters associated with premutation expansions. In premutation cases we identified alterations in glia number in cortex and cerebellum. Differential expression analysis demonstrated altered cortical oligodendrocyte development, while gene ontology analysis revealed alterations in neuroregulatory roles of glia, such as glial modulation of neurotransmission and synaptic structure. We identified significant enrichment of known FMR1 protein target genes in differentially expressed gene lists in FXS as well as the premutation, suggesting FMR1 protein target pathways may represent a shared source of dysfunction in both conditions despite opposite FMR1 mRNA changes. These findings challenge existing dogma regarding FXTAS and implicate glial dysregulation as a critical facet of premutation pathophysiology, representing novel therapeutic targets directly derived from the human condition.

Introduction: *FMR1* related disorders contribute to neurologic dysfunction across the lifespan (Leehey, 2009; Hagerman et al., 2017). Large trinucleotide (CGG) expansion (i.e., full mutations, FM) in the 5' UTR of the *FMR1* gene are associated with the neurodevelopmental disorder Fragile X Syndrome (FXS) while smaller, premutations (PM) are associated with Fragile X-associated tremor and ataxia syndrome (FXTAS), a late onset condition characterized by executive functioning decline and progressive cerebellar ataxia, presenting in 40-70% of PM carriers (Fu et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991; Jacquemont et al., 2003; Grigsby et al., 2016). In the latter, neuropathological and imaging studies have identified intranuclear neuronal and astrocytic inclusions, prominent white matter abnormalities including myelin pallor and spongiosis, and characteristic T2 white matter hyperintensities on MRI (Jacquemont et al., 2003; Tassone et al., 2004; Cohen et al., 2006; Greco et al., 2006; Schwartz et al., 2021). In contrast, in Fragile X syndrome, an early-onset neurodevelopmental disorder characterized by intellectual disability, autistic symptoms and characteristic facial features (Martin & Bell, 1943; Turner et al., 1975), only subtle functional changes in white matter in humans have been identified on imaging (Hallahan et al., 2011; Sandoval et al., 2018; Swanson et al., 2018). The molecular correlates of these white matter findings in both conditions are unknown.

The FM is associated with hypermethylation and transcriptional silencing of the *FMR1* locus, and absence of FMR1 protein (FRMP), while the PM has been reported to be paradoxically associated with increases in FMR1 mRNA, particularly in blood, with only variable reductions in FMRP levels (Fu et al., 1991; Oberle et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991; Tassone et al., 1999; Tassone, Hagerman, Chamberlain,

et al., 2000; Tassone, Hagerman, Taylor, et al., 2000; Kenneson et al., 2001). Although individuals with the PM may present with alterations in typical neurodevelopment, FXS patients generally do not present with features of FXTAS. These divergent clinical and molecular phenotypes have led to the hypothesis that the clinical symptomatology associated with FXTAS is related to a neurotoxic effect of increased levels of FMR1 mRNA in the nervous system. This argument is bolstered by findings of a 4-8 fold increase of FMR1 mRNA in peripheral blood cells of individuals with the PM (Tassone, Hagerman, Taylor, et al., 2000). However, prior bulk studies of human post-mortem brain tissue from individuals with the PM have revealed more modest ~0.9-1.5 fold changes in FMR1 mRNA (Tassone et al., 2004; Pretto et al., 2014). Prior studies in post-mortem human brain in both FXS and FXTAS have focused on bulk cellular analysis, which does not resolve cell-type specific molecular alterations. Whereas it is possible that the cellular heterogeneity of the human CNS may mask toxic levels of FMR1 mRNA, other hypotheses, including an inappropriate DNA damage response, mitochondrial stress, and polyglycine-containing peptide accumulation, have been put forth as alternative hypotheses to explain the pathophysiology of FXTAS (Garcia-Arocena & Hagerman, 2010; Sellier et al., 2017; Schwartz et al., 2021). It is also possible that reduced FMRP contributes to PM pathology in a developmentally distinct manner from the total loss that occurs in FXS. Finally, while studies of the impact of *FMR1* disruption have been focused on post-mitotic neurons, there is increasing evidence implicating important roles for *FMR1* in a diversity of cellular subtypes at multiple points in nervous system development, including in glia (Giampetruzzi et al., 2013; Martínez Cerdeño et al., 2018; Doll et al., 2020; Doll et al., 2021; Raj et al., 2021).

Despite these gaps in knowledge, there has been no cell-type specific analysis of transcriptional changes related to Fragile X in human brain to date.

To understand the molecular and cellular perturbations associated with Fragile X expansion in human brain across the lifespan with an unbiased approach, we applied single nuclei RNA-sequencing (snRNA-seq) to post-mortem frontal cortex and cerebellar hemisphere of individuals with *FMR1* PMs and FXS. We identified changes in *FMR1* expression, cellular proportion, and global gene expression that challenge current assumptions about molecular mechanisms underlying FXTAS pathogenesis, and specifically implicate glial dysregulation as critical in Fragile X molecular neuropathology.

Results: Prior to tissue processing we reviewed available medical records to ensure that clinical and neuropathological data were consistent with genetic diagnoses

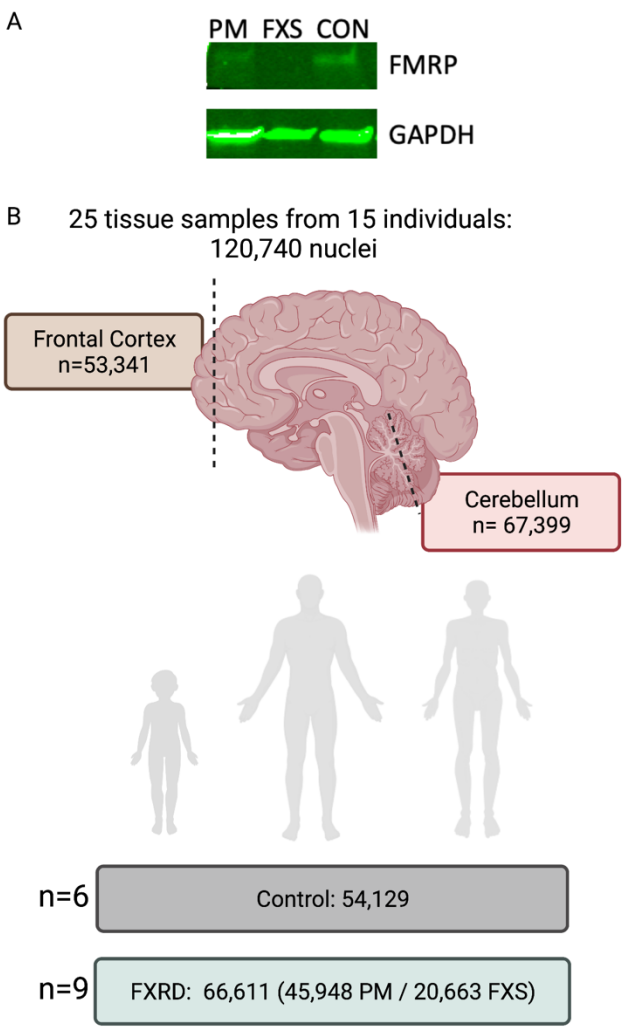

Figure 1: **Tissue validation and sample size overview.** A. Representative western blot of frontal cortex protein lysate demonstrating absent FMRP in FXS and variably reduced FMRP in PM case. B. Summary of sample size and final filtered nuclei number per condition and region, representing samples from across the lifespan in all conditions. FXRD: Fragile X related disorders.

(Table 1, Methods). All cases here have been previously presented in prior published work (Lohith et al., 2013; Esanov et al., 2016; Tran et al., 2019). Although we focus on the PM, we also included two known cases of FXS, to assess whether well-known effects on *FMR1* expression were present in our dataset. One case of FXS due to a deletion of *FMR1* was included given the known shared molecular consequences of *FMR*1 deletion and trinucleotide expansion (Gedeon et al., 1992). Neither FXS case had neuropathological abnormalities noted, consistent with expectations. The majority of PM cases had either clinical and/or neuropathological evidence of FXTAS. We identified one case that in the past was mistakenly categorized as FXS, but whose clinical records and genetic testing revealed it to be a PM (see Table 1). We validated functional effects of Fragile X disruption with western blotting of FMRP directly on frontal cortex tissue (Figure 1A) and obtained expected results: absent FMRP in FXS in both the FM and gene deletion, and variably reduced FMRP in PM cases.

Following unsupervised clustering of single nuclei, and filtering, we obtained over 120,000 high quality nuclei for further analysis across samples, including nuclei from 6 age- and sex-matched controls (Figure 1B, Table 2). We applied known cell type-specific markers to assess the specificity and accuracy of unsupervised clustering (Fig 2). For both prefrontal cortex and cerebellar hemisphere we identified specific classification of cellular subtypes for both neurons and glia. To further validate our annotation, we also identified layer-specific excitatory neurons and inhibitory neuron subclusters in the frontal cortex which were consistent with broader categorizations and prior annotations (Figure 2 figure supplement 1) (Lake et al., 2016; Hodge et al., 2019;

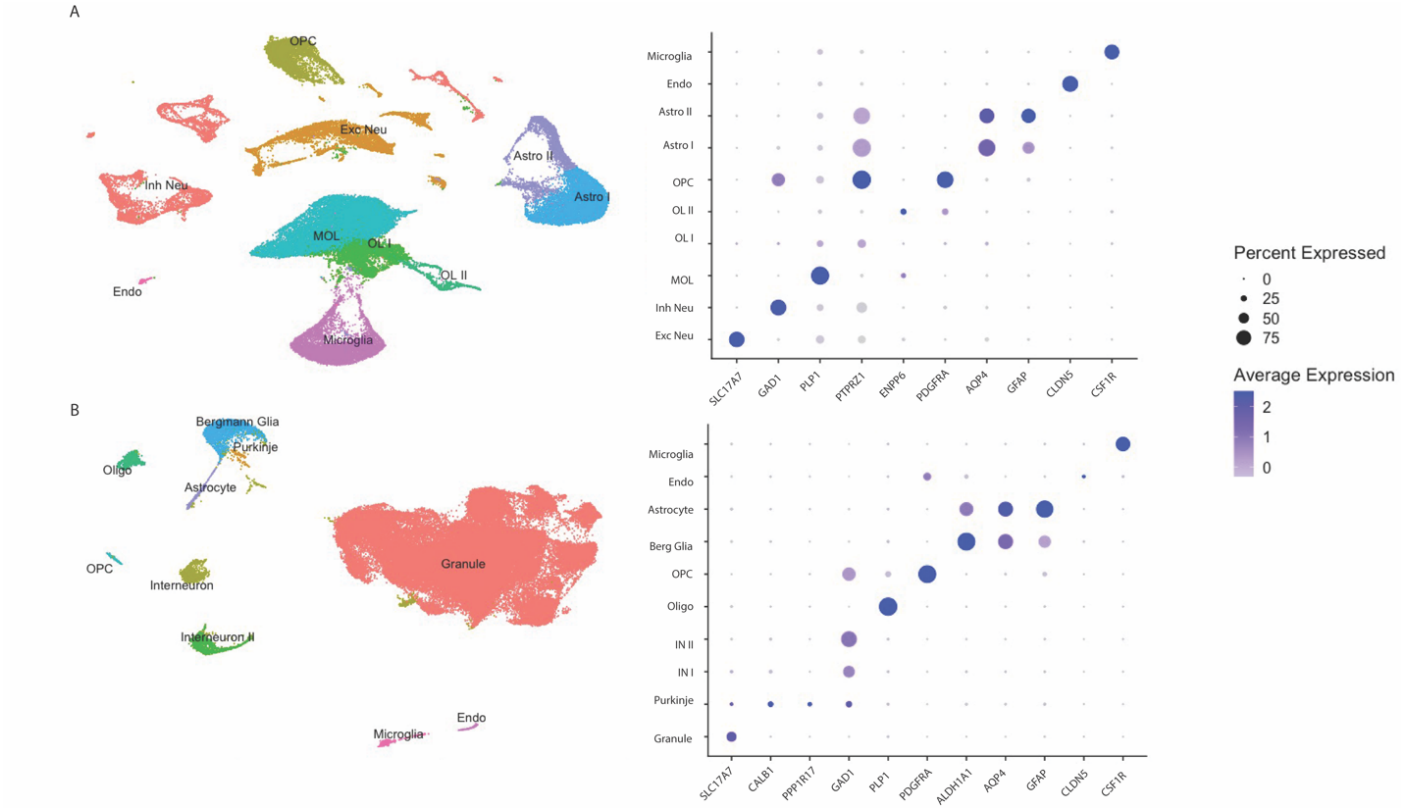

Figure 2: **snRNA-seq of frontal cortex and cerebellum.** Unsupervised clustering and visualization in Seurat visualized with UMAP (left) as well dot plot of cell type specific gene expression (right) in frontal cortex (A) and cerebellum (B) reflects accurate and specific cell type classification. Sample size as in Figure 1. Abbreviations: Astro: astrocyte, endo: endothelial, OPC: oligodendrocyte progenitor, OL: oligodendrocyte lineage, MOL: mature cortical oligodendrocyte, Inh: inhibitory, Exc: excitatory, Berg glia: Bergmann glia, Oligo: mature cerebellar oligodendrocyte, IN: cerebellar interneuron.

Velmeshev et al., 2019; Langseth et al., 2021) We chose to group excitatory subtypes, and group inhibitory subtypes respectively, to maximize power for downstream analysis.

There were distinctions between cerebellum and cortex in overall glial cell composition. In frontal cortex we identified several distinct clusters appearing to reflect different states of oligodendrocyte development, including PDGFRA + oligodendrocyte progenitor cells (OPCs), two intermediate clusters (OLI-ENPP6+ and OLII-TFC7L2+), and a mature myelinating oligodendrocyte (MOL) cluster. We compared the

transcriptional profile of OLI and OLII to oligodendrocyte lineage clusters identified in mouse (Marques et al., 2016), and found that OLI gene expression resembled mouse committed oligodendrocyte progenitors (COPs) and OLII resembled immature, newly formed, non-myelinating oligodendrocytes. On the other hand, in the cerebellum, although granule cells accounted for the majority of nuclei captured, as expected, we also identified a cerebellar specific Bergmann glia cluster. OLI and OLII clusters were not identified in cerebellar samples.

Comparison of average FMR1 mRNA across all nuclei in frontal cortex and cerebellum revealed regional differences in relative expression between neurons and glia. In the frontal cortex, expression of *FMR1* was higher in excitatory and inhibitory neurons compared to glia, as expected (Figure 2 figure supplement 2, Figure 3). However, in the cerebellum, *FMR1* expression was expressed in more non-neuronal subtypes at baseline. We used an independent harmonized single cell transcriptomic resource to confirm these findings and identified similar region-specific patterns of expression with higher relative glial to neuron FMR1 mRNA expression in cerebellum. (Figure 2 figure supplement 3) (Song et al., 2021).

Analysis of individual cluster *FMR1* expression revealed cell type-specific effects of Fragile X status on *FMR1* transcription. In both cases of Fragile X syndrome, we identified equal and total abrogation of *FMR1* expression, as expected (Figure 2 figure supplement 4). Although our sample of FXS cases is small, this provides important proof of principle that expected transcriptional signatures are present within the snRNA-seq data. Indeed, despite the smaller sample size and nuclei number, reduced *FMR1*

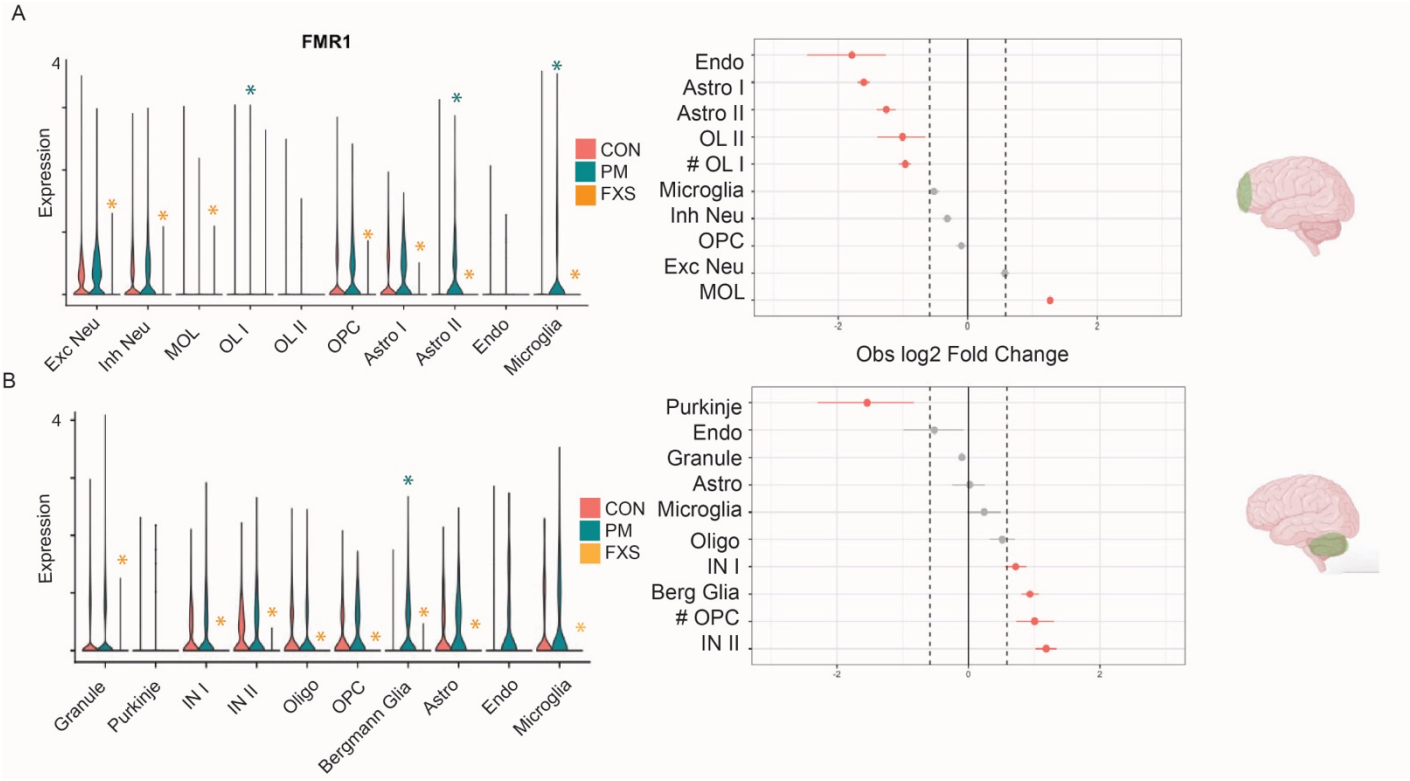

Figure 3: **FMR1 mRNA dysregulation in FXS and PM cases, and cluster proportion analysis for PM cases.** Frontal cortex (**A**) and cerebellar (**B**) changes in FMR1 expression in FXS and PM (left) and cellular proportion alterations in PM cases vs control (right) A. Violin plots demonstrate reduced FMR1 mRNA expression in multiple clusters in FXS, while FMR1 mRNA is variably increased in PM clusters, primarily in glia. Cluster abbreviations as in Fig. 2. Orange *: reduced FMR1 p-value in FXS vs CON padj< .05, teal *: increased FMR1 in PM vs. CON padj < .05. Right panel shows permutation plot for PM cluster proportions demonstrating glial cell number alterations in cortex and cerebellum. Red: FDR < .05 and abs(log2FC) > 0.58. # indicates significance of this cluster is not robust to outlier sample removal.

expression was robust among different clusters in FXS in both neuronal and glial subpopulations across the brain, consistent with the large effect size of this genetic driver (Figure 2 figure supplement 4, Figure 3). The effect of the PM on FMR1 expression, despite the larger sample size, was more modest and demonstrated cluster-specific heterogeneity. In PM cases, the only clusters that demonstrated significantly increased FMR1 mRNA expression in either the frontal cortex or cerebellum were non-neuronal, such as cerebellar Bergmann glia and cortical microglia

(Figure 3). Although the absence of significant FMR1 upregulation in a small number of clusters in PM cases may be due to inadequate power, most clusters included more than enough nuclei (including frontal cortex excitatory and inhibitory neurons, as well as cerebellar granule cells and interneurons) to rule this explanation out (see Methods). Thus, in general, the lack of significant upregulation of *FMR1* mRNA in neuronal subclusters in the PM cases is not due to a lack of power. Rather, it suggests that overall, the increase in FMR1 expression in brain caused by the PM is far more modest than the 4-8 fold increase observed in blood, and shows a preferential impact on glia, in the regions assessed here.

In the cerebellum, we identified changes in nuclei number in PM cases that recapitulated past neuropathological studies, specifically previous work demonstrating cerebellar Purkinje cell loss and Bergmann cell gliosis in individuals with the PM (Hagerman, 2013). Consistent with this, we identified fewer Purkinje cell nuclei, and greater Bergmann cell nuclei, in the cerebellum of PM carriers versus age matched controls (Figure 3). This orthogonal validation of prior findings reinforces the utility of our molecular approach to identify bonafide biological phenomenon.

We also identified changes in glial cell number in the frontal cortex (primarily BA10) in association with the PM. PM cases demonstrated fewer than expected astrocytes and endothelial cells, with an increase in mature oligodendrocytes compared to controls, observations surprisingly consistent across samples and not reflecting the presence of outliers (Figure 3, Methods). We assessed BA22 in one PM sample and identified MOL

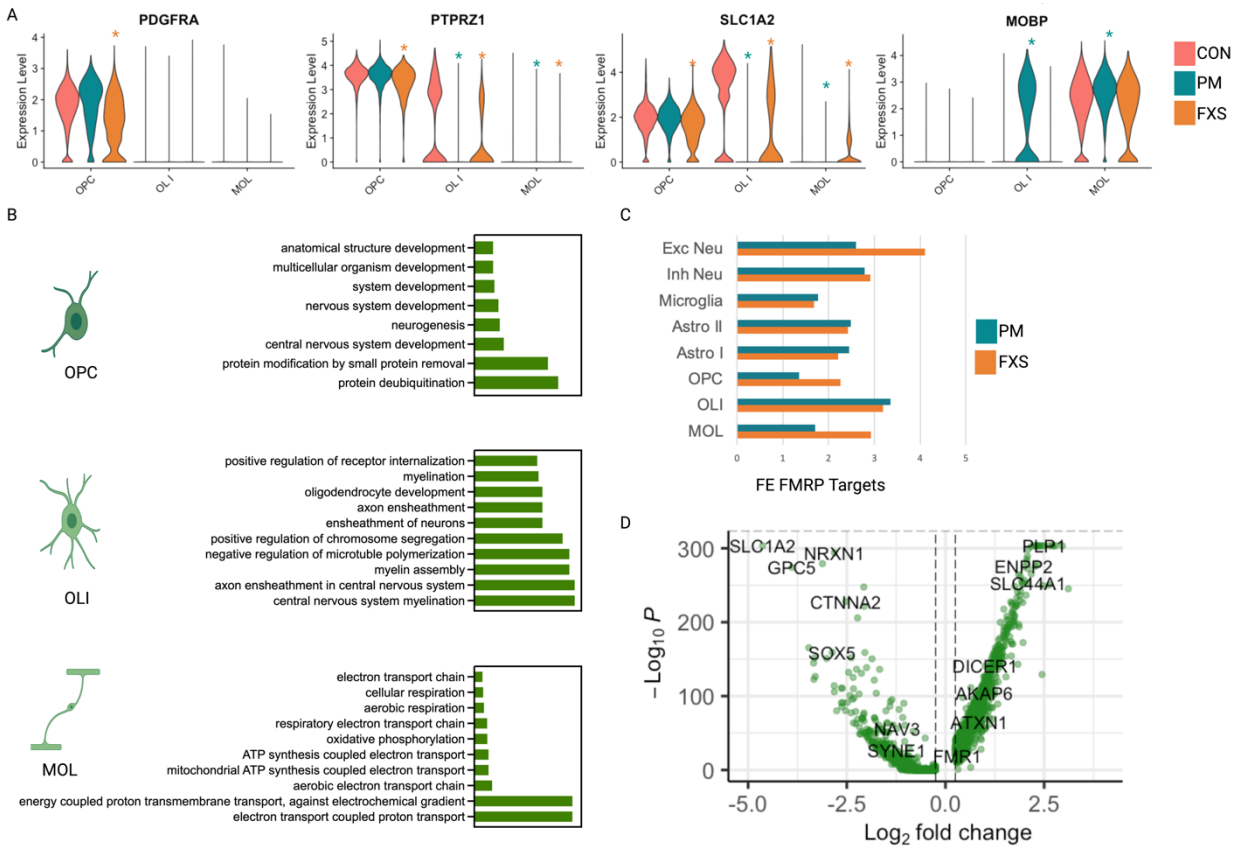

Figure 4: **Global glial transcriptional dysregulation in frontal cortex.** A. Violin plots demonstrating alteration in oligodendrocyte development in PM and FXS. Orange * indicates FXS vs. CON padj < .05, teal * indicates PM vs CON padj < .05. B. GO analysis of upregulated differentially expressed genes in oligodendrocyte clusters in frontal cortex demonstrate increased metabolic stress in MOLs and upregulation of myelination in OLI. C. Significant fold enrichment (FE) of FMRP target genes across clusters and conditions, all comparisons padj <.05 except for OPC in PM cases which was non-significant. D. Volcano plot of differentially regulated genes in PM cases in OLI demonstrate 3:1 increased: decreased DEGs, genes of potential interest noted.

levels and astrocyte levels to be similar to control, suggesting these findings may reflect a sub-cortical specific effect. Because changes in glial number have been observed in normal aging (Peters et al., 1991; Peters & Sethares, 2004; Soreq et al., 2017; Salas et al., 2020; Xu et al., 2020) we assessed the impact of age on these clusters and did not identify a significant association with age for these clusters in control individuals (Figure 3 figure supplement 1). We wondered whether age associated changes in neuronal

composition however might mask more subtle effects of the PM on neuronal clusters, given that age-associated changes in interneuron density have also been observed (Hua et al., 2008; Stanley et al., 2012; Rozycka & Liguz-Lecznar, 2017) In this case, we identified a significant age-related decline in inhibitory neuron/total neuron composition in frontal cortex as previously reported (Majdi et al., 2007), although there was no detectable effect of Fragile X PM status on this decline (Figure 3 figure supplement 2). Thus, we identified unexpected novel alterations in glial number in frontal cortex in PM cases.

Although limited by small sample size, we also assessed cluster proportions in FXS and found that alterations in cellular composition was markedly distinct from PM cases. For example, frontal cortex astrocyte and endothelial cell depletion were not identified in FXS. (Figure 3 figure supplement 3). In cerebral cortex in FXS there was an increase in OPC proportion; this finding has previously been observed in animal models (Doll et al., 2021) although to our knowledge not previously described in humans. Thus, FXS and PM cases are associated with distinct alterations in the brain's cellular architecture.

In frontal cortex, we identified alterations in glial cell transcriptional regulation indicating widespread perturbations in PM pathology (Figure 4). We focused on the frontal cortex given the broader and more equitable distribution of cell types, which allows for more direct comparisons between clusters. In PM cases, we identified a marked trend towards increased expression of myelination markers and reduced immature markers in the intermediate committed oligodendrocyte progenitor OLI cluster (Figure 4A). The increase in mature markers in an intermediate progenitor offers a

possible explanation for the increased number of MOLs in PM frontal cortex despite no change in the OPC pool number. Indeed, committed oligodendrocyte progenitors are known to serve as a reservoir for rapidly generating myelinating mature oligodendrocytes (Lee et al., 2012; Lecca et al., 2020). The OLI nuclei cluster was not the only cluster with alterations in critical glial developmental gene expression in PM cases. For example, within PM OPCs, upregulation of CSPG4 (i.e., NG2) (Ampofo et al., 2017) was observed in frontal cortex (Figure 4 supplemental figure 1). Intriguingly, FXS clusters also demonstrated dysregulation of several of glial markers, but with distinct directionality and magnitude of changes, and preferential impact on OPCs. There are thus cortical/cerebellar and condition specific patterns to glial transcriptional dysregulation in Fragile X related disorders.

Gene ontology analysis (see Methods, Figure 4) suggested that individual gene expression changes represented broader disturbances in biological functioning. Interestingly, in PM cases, frontal cortex MOLs demonstrated unique evidence of increased metabolic stress, consistent with neuropathological findings of oligodendrocyte dysfunction. The OLI cluster in PM cases was the only group in either condition or region to demonstrate myelination upregulation (Figure 4B), demonstrating that the gene expression changes described above were specific to this cell cluster. This is of particular interest given that several myelination related genes are known FMRP targets (Wang et al., 2004; Darnell et al., 2011; Giampetruzzi et al., 2013) - yet, these were not ubiquitously impacted in all oligodendrocyte lineage clusters. Downregulated categories in PM cases implicated dysfunction of glial regulatory roles. For example, there was evidence of downregulation of synaptic organization and

activity, decreased neuronal cell adhesion, and downregulation of glutamatergic signaling in a variety of oligodendrocyte lineage clusters (Figure 4 figure supplement 2). Inspection of biological processes disturbed in PM cases in astrocytes and microglia similarly revealed downregulation of several neuronal regulatory roles as well as evidence of inflammation (Figure 4 figure supplement 3). There was also evidence of neuronal dysfunction: neurons in PM cases demonstrated evidence of metabolic stress and downregulation of critical neuronal function and structure, such as postsynaptic organization and dendritic spine morphogenesis (Figure 4 figure supplement 4). Inspection of differentially regulated genes in FXS revealed widespread evidence of increased metabolic and energetic stress, across both neurons and glia with known FMRP targets present among differentially regulated genes (Fig 4 figure supplements 5-8).

Given that we observed the presence of FMRP targets in both neuronal and glial differentially expressed gene lists in both FXS and PM cases, we investigated whether this reflected statistically significant enrichment (Figure 4C). We had hypothesized that there would be significant enrichment of FMRP target genes in FXS but not PM cases, and greater enrichment in neuronal clusters vs non-neuronal clusters. To our surprise, we identified significant enrichment for FMRP targets among differentially expressed genes across many cell clusters in both FXS and PM cases. Excitatory neurons demonstrated the highest fold enrichment of known FMRP targets in FXS as expected, but significant enrichment was observed in non-neuronal clusters in FXS as well. Additionally, there was significant enrichment of FMRP target genes in differentially expressed genes across most clusters in the PM cases, in many cases very similar to

FXS, suggesting a role for FMRP dysfunction in PM pathogenesis. The highest enrichment for FMRP targets among PM clusters was not neuronal but rather the OL1 cluster. Interestingly, this cluster exhibited global transcriptional dysregulation similar to FMRP loss in FXS in excitatory neurons including ~ 3:1 increased:decreased significant differentially expressed genes (Fig 4D, Figure 4, figure supplement 9), reflecting a de-repressed state. However, known FMRP targets were not restricted to the upregulated list in this cluster (Figure 4D), potentially highlighting dual roles of FMRP as transcriptional repressor and in mRNA stabilization (Hale et al., 2021).

Discussion:

We present the first cell type specific analysis of gene expression of Fragile X related disorders in human brain. We identified changes in FMR1 mRNA expression, cellular proportion, and cell-type specific gene expression that sheds light on molecular perturbations associated with *FMR1* and specifically highlights an important role for glial molecular dysregulation in PM pathology.

Our data suggest that FMR1 mRNA expression in PM cases, at least in the brain regions analyzed here, is more modestly affected than has been observed in peripheral blood cells and furthermore that it preferentially effects glial cells more than neurons. Given the robust elimination of FMR1 mRNA expression observed here in association with FXS, regardless of the genetic driver (trinucleotide expansion vs gene deletion), nuclei cluster, or brain region, we are confident in the validity of our approach. In the PM cases, we identified modest upregulation of *FMR1* expression, with glial subclusters in both cerebellum and cortex demonstrating the most marked increases. Rather than

296 extreme neurotoxic increases in FMR1 mRNA, our findings suggest a more modest,
297 ~1.3 fold, increase in *FMR1* transcript levels, paralleling past studies of brain
298 homogenate (Tassone et al., 2004; Pretto et al., 2014). Although we cannot rule out that
299 neural cells expressing toxic levels of FMR1 transcript are selectively vulnerable and
300 preferentially lost with time, our cellular proportion analysis (see below) does not
301 support this interpretation, as one would expect clusters that are disproportionately lost
302 to have relatively higher increases in *FMR1* mRNA. We also include in our analyses one
303 21 year-old PM case, whose data is very similar to the other aged PM cases, which
304 further argues against age-related loss. Finally, changes in FMR1 expression were
305 comparable between clusters known to be vulnerable to PM associated intranuclear
306 inclusions (neurons, astrocytes) and those known to be spared (oligodendrocytes),
307 arguing against inclusion presence as being a confounding factor in FMR1 mRNA
308 measurement. Although our work challenges the causal role of extremely elevated
309 FMR1 mRNA in human brain, it is possible that more modest increases of CGG
310 containing FMR1 RNA still lead to cellular dysfunction, through previously posited
311 mechanisms including trinucleotide repeat toxicity. Thus, our work provides an
312 important foundation to understanding FMR1 mRNA levels that are relevant to
313 neurological pathophysiology in animal and human model systems and broadens the
314 scope of cellular subtypes that warrant further investigation within this context.

315 ***Cellular proportion***

316 Alterations in cell number in PM cases in both cerebellum and cortex also
317 implicated glial dysregulation. Our finding of reduced Purkinje cells and Bergmann cell

318 increases in the cerebellum in PM cases parallels well-described neuropathological
319 findings (Greco et al., 2006), and reinforces the validity of our approach, and suggests
320 that loss of Purkinje cells may contribute to FXTAS signs and symptoms. In PM cases,
321 we also identified a proportional decrease in endothelial cells and astrocytes, with
322 increases in MOLs in frontal cortex, a finding not explained by age or the presence of
323 outliers. The relationship of basal FMR1 mRNA expression, change in FMR1
324 expression, and cellular proportion was not straightforward, arguing against a simplistic
325 relationship between cellular proportion and FMR1 toxicity. For example, glial cells in
326 the frontal cortex that demonstrated modest differential expression of FMR1 also
327 demonstrated the most marked changes in cellular proportion. This may be related to
328 earlier developmental time points that are impacted, cellular extrinsic effects on survival
329 and proliferation, or both. Given the findings of global brain atrophy, white matter
330 abnormalities, and the reported decline in executive functioning deficits reported in
331 FXTAS (Brunberg et al., 2002; Greco et al., 2006; Brega et al., 2008; Grigsby et al.,
332 2008), these changes in cellular proportion warrant further exploration of the role of glia
333 in FXTAS associated cognitive decline and in other cortical areas.

334 The findings of changes in endothelial cell proportion are also interesting considering
335 the recent description of microangiopathy in FXTAS neuropathology (Salcedo-Arellano
336 et al., 2021). In fact, MRI findings in FXTAS of T2 hyperintensity have some similarities
337 to microvascular ischemia (Leehey, 2009), also suggesting cerebrovascular dysfunction
338 as an important facet of FXTAS. Past work has demonstrated that disruption of the
339 blood brain barrier can lead to increased OPC NG2 expression (Rhodes et al., 2006),
340 observed in our data in the frontal cortex in PM cases only. These findings highlight the

need for additional work to interrogate the developmental mechanisms and regional specificity of *FMR1* effects on the endothelium on a more comprehensive level.

We identified global transcriptional alterations associated with PM status that support the now well-established principle that glia play central roles in neurodevelopment and disease (Teismann et al., 2003; Croisier & Graeber, 2006; Lee et al., 2012; Liu et al., 2015). For example, OPCs are known to form synaptic like structures and respond to neuronal activity (Bergles & Richardson, 2015) and glia more generally are critical in neuronal development, axonal integrity and behavior (Nave, 2010; Fernandes et al., 2017; Nagai et al., 2021). Differential gene regulation in PM glia frequently identified perturbations in glial-neuronal signaling, maintenance of synaptic structure and function, and altered neurotransmission in multiple glial lineage clusters in addition to evidence of energetic stress specifically in MOLs. Indeed, white matter abnormalities in Fragile X related disorders more broadly, may reflect subtle disruption of glial regulatory roles in neuronal homeostasis. Determination of whether these glial abnormalities contribute causally to clinical symptomatology or represent a secondary response to neuronal dysfunction, will require further work in human model systems. Support for the former is corroborated by the fact that we identified significant enrichment of FRMP target genes in differentially expressed genes in both FXS and PM glial clusters. Although the magnitude of FMRP target enrichment was largest in excitatory neurons in FXS, there were surprising similarities in the magnitude of enrichment between FXS and PM. Although our work can not address causality, this suggests that shared loss of FMRP contributes to some degree to both disorders in a variety of cell types. There was also evidence of cell-type specific transcriptional

364 alterations. For example, the OLI cluster, uniquely demonstrated significant changes in
365 expression in myelin related genes (known FMRP targets) in PM cases, suggesting that
366 cellular states even within a single glial lineage may be differentially vulnerable to
367 Fragile X disruption in a context dependent manner. Thus, our findings in PM post-
368 mortem brain, in light of known neuropathological and imaging abnormalities, support
369 the interpretation of FXTAS as a disorder defined by glial dysfunction.

370 Our small sample size of Fragile X syndrome increases chances that changes in
371 cellular proportion or transcriptional dysregulation may be due to stochastic artifacts.
372 However, replication of past findings including reduced FMR1 expression (Pieretti et al.,
373 1991; Bhattacharyya & Zhao, 2016), increased OPC proportion (Doll et al., 2021), and
374 widespread evidence of metabolic stress (Donnard et al., 2020; Kang et al., 2021),
375 corroborate known molecular neuropathology of the disorder (Licznerski et al., 2020).
376 Thus, our findings highlight the need for more comprehensive study of Fragile X in
377 human tissue directly in a variety of different cell types.

378 In conclusion, we provide compelling evidence from human brain regarding cell type-
379 specific molecular neuropathology that helps contextualize the clinical heterogeneity
380 associated with genetic variation at the *FMR1* locus in neurodevelopment and
381 neurodegeneration and specifically implicates glial dysregulation in PM pathology.

382 **Materials and Methods**

383 ***Samples***

Post-mortem tissue was obtained from either the University of Maryland Neurobiobank and for one control case, from Autism Brain Net. All tissue was from deceased individuals and as such is not considered human subjects research. Fragile X mutation status/repeat size was determined through direct review of de-identified clinical records, and cross-referenced with prior published validation of the same cases (Table 1). Most of the PM cases had clinical symptomatology or neuropathological evidence of FXTAS (Table 1). Samples were matched for age, sex, and PMI but no cut-offs were utilized to exclude any cases (see Appendix Figure 1 for further details.)

Western Blotting

~25-50 mg frozen frontal cortex was homogenized in RIPA buffer + protease inhibitors and centrifuged, total protein content was then quantified. Laemelli sample buffer was added to the protein supernantant and boiled for 5 minutes. Equal amounts of protein (10 ug) were loaded onto precast SDS-Page gels with molecular weight ladders. Samples were transferred to membranes, blocked with Licor block (Lincoln, NE), and incubated in primary antibody overnight diluted in block at four degrees. Following four washes in tris buffered saline + tween (TBS+T), blots were incubated with LI-COR secondary fluorescent antibodies in the dark at room temperature for one hour. After further washing including a final wash of TBS, the blots were scanned on a LI-COR Odyssey imager and images analyzed with Image-J software. The following primary antibodies and dilutions were used: GAPDH (Cell Signaling # 2118S) 1:15,000; FMRP (Cell Signaling 4317S) 1:1,000.

406 **Isolation of post-mortem nuclei**

407 Frozen tissue (~ 25 mg) from either section 1 frontal cortex or section 5
408 cerebellar hemisphere (see table of demographics) was dissected at -20 and subjected
409 to dounce homogenization followed by sucrose gradient centrifugation as previously
410 described. Nuclei were filtered and incubated for 5 minutes in 1:1000 Hoechst
411 (Invitrogen H3569, Waltham MA). 10,000 Hoechst + nuclei from the suspension were
412 then sorted directly into 10x Genomics RT buffer (Pleasanton, CA) on a chilled plate
413 holder to remove doublets, debris, and dying nuclei on a FACS Aria (BD Biosciences,
414 Franklin Lakes, NJ) with low pressure nozzle (Appendix Figure 2). Following sorting,
415 reverse transcriptase enzyme was added on ice, and nuclei were immediately
416 processed for encapsulation in the 10x Chromium controller. cDNA and libraries were
417 prepared according to the 10x documentation protocol for 3' gene expression. Libraries
418 were prepared in matched batches and represent over 16 individual days of sample
419 preparation and 4 separate sequencing runs.

420 ***Sequencing and Quality Control***

421 Following pooling of samples, pools were run on a Novaseq 6000 (Illumina, San
422 Diego, CA) to obtain high coverage and saturation, and demultiplexed with bcl2fastq.
423 CellRanger Count was utilized to generate count matrices with introns included, given
424 intronic information is known to be informative for nuclear preparations. To remove
425 remaining ambient RNA and debris, and obtain a final high quality nuclei set, filtering
426 metrics were applied to nuclei in Seurat including: # UMIs> 500, # Genes > 250,
427 log10GenesPerUMI (complexity measure) > 0.8, and mitoRatio < 0.1. Datasets were

processed with SCTransform and integrated, and potential sources of variation were assessed with principal component analysis. Unsupervised clustering was performed with different resolutions followed by application of known cell type markers. For cerebellar Purkinjee and endothelial cells, which represented a small percentage of the total nuclei sample, cell type markers (CALB1; CLDN5) were used to manually select cell clusters using the SelectCells feature in Seurat.

Analysis

Prism and R studio were used for analysis of demographic and single nuclei data, respectively, using statistical tests as indicated in each figure. Computationally intensive work was conducted on the Harvard Computing Cluster, O2. For analysis of demographic data, one PM case that was extremely aged is listed as 89+ to ensure sample de-identification and sample points were removed from any graphs presented here to ensure de-identification. For cluster proportion analysis, scProportion package (Miller et al., 2021) was used to perform a permutation test to calculate a p-value for each cluster, with a confidence interval returned via bootstrapping. (https://github.com/rpolicastro/scProportionTest). Although this approach is particularly amenable to small sample sizes, the outcome could still be impacted by samples with outlier cluster proportions. To assess the robustness of the data to the presence of outliers, cluster proportions from each individual sample were compared, and Grubb's test used to identify the presence of outliers that may have contributed to significant results in scProportion. Samples which contributed to the presence of outliers were then systemically removed from the dataset and scProportion rerun. We

identified only rare cases of outlier clusters that impacted significance of findings: One young control sample had a high proportion of the committed progenitor OL I. One PM sample demonstrated higher than expected OPC number. Other outliers did not impact significance of findings in PM cases and include: the FXS case with the gene deletion demonstrated an unusually high presence of the newly-formed, non-myelinating immature OLII cluster, and a different control sample had a higher proportion of endothelial cells. The plots presented represent the entire dataset with no data removed. Instead, cluster changes that were not robust to the removal of outlier samples are indicated on the graph with a # sign.

Differential expression analysis was done with the FindMarkers functionality in Seurat using default settings of Wilcoxon rank sum test, FDR < .05, log fold change > 0.25 for all cell clusters. A priori we calculated that 400 cells/condition cluster are required to detect 80% of differentially expressed genes with a false discovery rate of 5%. Thus, our analyses are adequately powered for the majority of cell cluster comparisons. We chose to omit downsampling to preserve power. Results were compared to a subset downsampled dataset for select clusters with larger nuclei number, and results were found to be similar both in the pattern of differentially regulated genes as well as the specific genes present in the data set (data not shown). Additionally, no association between cluster cell number and number of differentially expressed genes were identified. To generate a set of putative FMRP target genes in humans, the list from (Darnell et al., 2011) was transformed from mouse to human gene symbols resulting in a gene list of 745 predicted targets that were functionally validated in animal models. A hypergeometric test was performed for each cluster and condition,

comparing the observed # FMRP target genes in differentially expressed lists vs predicted using a total gene set defined as 20,000. Gene ontology analysis was conducted with the web interface of Panther (http://geneontology.org) with default settings for statistical significance testing, including Fisher's exact test and FDR < .05.

Data and materials availability

All sequencing data and code are available from dbGaP (accession number pending).

ID	Sample*	Regions	Age	Race	Sex	PMI	RIN	Notes	Prior Published Genetic & Functional Validation (for cases)
1793	CON	FC, CBL	11	Black	M	19			
5408	CON	FC	6	Black	M	16	7.6		
5497	CON	FC, CBL	68	White	M	13	8		
5541	CON	FC, CBL	84	White	M	12	8.3		
5657	CON	FC, CBL	82	White	M	22	8.4		
AN10723	CON	CBL	60		M	24			
4555	FXPM (67)	FC, CBL	80	White	M	12		clinical diagnosis of Parkinson's	CGG repeat-primed PCR (Esanov 2016)
4664	FXPM (100)	FC, CBL	71	White	M	3		progressive neurological decline, history of hydrocephalus	# CGG repeat-primed PCR (D'Gama 2015, Esanov 2016)
4751	FXPM (88)	FC, CBL	21	White	M	5		perinatal ischemic event, seizures	CGG repeat-primed PCR (Esanov 2016)
5006	FXPM (150)	FC, CBL	85	White	M	5	7.1	FXTAS neuropathology	# CGG repeat-primed PCR (D'Gama 2015, Esanov 2016)
5212	FXPM	CBL	80	White	M	12		FXTAS neuropathology, progressive neurolgocial decline	CGG repeat-primed PCR (Esanov 2016), western blotting (Tran 2019)
5529	FXPM (58)	FC	89+**	White	M	16	7.1	FXTAS neuropathology	western blotting (Tran 2019)
5746***	FXPM (116)	CBL, BA22	80	White	M	22		progressive neurolgical decline, white matter abnormalities on MRI including MCP sign	western blotting (Tran 2019)
4806	FXS	FC, CBL	9	White	M	22	4.3	FXS, gene deletion, no neuropathological findings	
5319	FXS (450+)	FC, CBL	71	White	M	17	6.6	FXS, full mutation, no neuropathological changes	CGG repeat-primed PCR (Esanov 2016)
* (approximate repeat size if applicable)									
** age de-identified									
*** misclassified as FXS in Tran 2019 despite only partially reduced FMRP									
# in addition to premtuation, mosaic full mutation identified in small percent of cells, records consistent with premutation associated pathology and clinical presentation									

Table 1: Demographic information for post-mortem samples used. Repeat size if applicable ascertained from clinical records and prior published work. FC: frontal cortex, CBL: cerebellum

Cluster	CON	FXPM	FXS	Total
Cerebellum				
Granule	25067	24237	9831	
Oligo	366	542	130	
Bergmann Glia	643	1276	334	
Interneuron	497	848	155	
Interneuron II	395	930	97	
Microglia	219	268	85	
Astrocyte	263	276	104	
OPC	157	327	79	
Endothelial	87	63	20	
Purkinje	59	21	23	
Cerebellar Total	27753	28788	10858	67399
Cortex				
Inh Neu	3916	2049	1081	
Exc Neu	2776	2689	868	
OPC	2411	1467	1591	
OL I	3173	1058	522	
OL II	250	81	630	
MOL	4381	6890	1767	
Astro I	4264	914	1577	
Astro II	1719	468	614	
Microglia	3348	1518	1092	
Endo	138	26	63	
Cortex Total	26376	17160	9805	53341

Table 2: Filtered nuclei number by brain region and cluster status.

Appendix (Supplemental Methods)

We found no association between: PMI & RIN, age and PMI, and age and RIN, as expected (Appendix Figure 1). There was no difference in average RIN, PMI, or age between PM and controls. We did observe a reduction in RIN in the FXS samples as compared to controls but not PM cases.

Nuclear staining and sorting was conducted to select against dying cells, debris, and doublets (Appendix Figure 2).

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References:

Ampofo, E., Schmitt, B. M., Menger, M. D., & Laschke, M. W. (2017). The regulatory mechanisms of NG2/CSPG4 expression. *Cell Mol Biol Lett*, *22*, 4. https://doi.org/10.1186/s11658-017-0035-3

Bergles, D. E., & Richardson, W. D. (2015). Oligodendrocyte Development and Plasticity. *Cold Spring Harb Perspect Biol*, *8*(2), a020453. https://doi.org/10.1101/cshperspect.a020453

Bhattacharyya, A., & Zhao, X. (2016). Human pluripotent stem cell models of Fragile X syndrome. *Mol Cell Neurosci*, *73*, 43-51. https://doi.org/10.1016/j.mcn.2015.11.011

Brega, A. G., Goodrich, G., Bennett, R. E., Hessl, D., Engle, K., Leehey, M. A., Bounds, L. S., Paulich, M. J., Hagerman, R. J., Hagerman, P. J., Cogswell, J. B., Tassone, F., Reynolds, A., Kooken, R., Kenny, M., & Grigsby, J. (2008). The primary cognitive deficit among males with fragile X-associated tremor/ataxia syndrome (FXTAS) is a dysexecutive syndrome. *J Clin Exp Neuropsychol*, *30*(8), 853-869. https://doi.org/10.1080/13803390701819044

Brunberg, J. A., Jacquemont, S., Hagerman, R. J., Berry-Kravis, E. M., Grigsby, J., Leehey, M. A., Tassone, F., Brown, W. T., Greco, C. M., & Hagerman, P. J. (2002). Fragile X premutation carriers: characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. *AJNR Am J Neuroradiol*, *23*(10), 1757-1766. https://www.ncbi.nlm.nih.gov/pubmed/12427636

Cohen, S., Masyn, K., Adams, J., Hessl, D., Rivera, S., Tassone, F., Brunberg, J., DeCarli, C., Zhang, L., Cogswell, J., Loesch, D., Leehey, M., Grigsby, J., Hagerman, P. J., & Hagerman, R. (2006). Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. *Neurology*, *67*(8), 1426-1431. https://doi.org/10.1212/01.wnl.0000239837.57475.3a

Croisier, E., & Graeber, M. B. (2006). Glial degeneration and reactive gliosis in alpha-synucleinopathies: the emerging concept of primary gliodegeneration. *Acta Neuropathol*, *112*(5), 517-530. https://doi.org/10.1007/s00401-006-0119-z

Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y., Mele, A., Fraser, C. E., Stone, E. F., Chen, C., Fak, J. J., Chi, S. W., Licatalosi, D. D., Richter, J. D., & Darnell, R. B. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell*, *146*(2), 247-261. https://doi.org/10.1016/j.cell.2011.06.013

Doll, C. A., Scott, K., & Appel, B. (2021). Fmrp regulates oligodendrocyte lineage cell specification and differentiation. In *Glia*.

Doll, C. A., Yergert, K. M., & Appel, B. H. (2020). The RNA binding protein fragile X mental retardation protein promotes myelin sheath growth. In *Glia*.

Donnard, E., Shu, H., & Garber, M. (2020). Single cell transcriptomics reveals dysregulated cellular and molecular networks in a fragile x syndrome model. In *bioRxiv*.

Esanov, R., Andrade, N. S., Bennison, S., Wahlestedt, C., & Zeier, Z. (2016). The FMR1 promoter is selectively hydroxymethylated in primary neurons of fragile X syndrome patients. In *Human Molecular Genetics*.

579 Fernandes, V. M., Chen, Z., Rossi, A. M., Zipfel, J., & Desplan, C. (2017). Glia relay
580 differentiation cues to coordinate neuronal development in Drosophila. *Science*,
581 *357*(6354), 886-891. https://doi.org/10.1126/science.aan3174
582 Fu, Y. H., Kuhl, D. P. A., Pizzuti, A., Pieretti, M., Sutcliffe, J. S., Richards, S., Verkert, A.
583 J. M. H., Holden, J. J. A., Fenwick, R. G., Warren, S. T., Oostra, B. A., Nelson, D.
584 L., & Caskey, C. T. (1991). Variation of the CGG repeat at the fragile X site
585 results in genetic instability: Resolution of the Sherman paradox. In *Cell* (Vol. 67,
586 pp. 1047-1058): Cell Press.
587 Garcia-Arocena, D., & Hagerman, P. J. (2010). Advances in understanding the
588 molecular basis of FXTAS. *Hum Mol Genet*, *19*(R1), R83-89.
589 https://doi.org/10.1093/hmg/ddq166
590 Gedeon, A. K., Baker, E., Robinson, H., Partington, M. W., Gross, B., Manca, A., Korn,
591 B., Poustka, A., Yu, S., Sutherland, G. R., & et al. (1992). Fragile X syndrome
592 without CCG amplification has an FMR1 deletion. *Nat Genet*, *1*(5), 341-344.
593 https://doi.org/10.1038/ng0892-341
594 Giampetruzzi, A., Carson, J. H., & Barbarese, E. (2013). FMRP and myelin protein
595 expression in oligodendrocytes. *Mol Cell Neurosci*, *56*, 333-341.
596 https://doi.org/10.1016/j.mcn.2013.07.009
597 Greco, C. M., Berman, R. F., Martin, R. M., Tassone, F., Schwartz, P. H., Chang, A.,
598 Trapp, B. D., Iwahashi, C., Brunberg, J., Grigsby, J., Hessl, D., Becker, E. J.,
599 Papazian, J., Leehey, M. A., Hagerman, R. J., & Hagerman, P. J. (2006).
600 Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). In
601 *Brain*.
602 Grigsby, J., Brega, A. G., Bennett, R. E., Bourgeois, J. A., Seritan, A. L., Goodrich, G.
603 K., & Hagerman, R. J. (2016). Clinically significant psychiatric symptoms among
604 male carriers of the fragile X premutation, with and without FXTAS, and the
605 mediating influence of executive functioning. In *The Clinical Neuropsychologist*
606 (Vol. 30).
607 Grigsby, J., Brega, A. G., Engle, K., Leehey, M. A., Hagerman, R. J., Tassone, F.,
608 Hessl, D., Hagerman, P. J., Cogswell, J. B., Bennett, R. E., Cook, K., Hall, D. A.,
609 Bounds, L. S., Paulich, M. J., & Reynolds, A. (2008). Cognitive profile of fragile X
610 premutation carriers with and without fragile X-associated tremor/ataxia
611 syndrome. *Neuropsychology*, *22*(1), 48-60. https://doi.org/10.1037/0894-
612 4105.22.1.48
613 Hagerman, P. (2013). Fragile X-associated tremor/ataxia syndrome (FXTAS): pathology
614 and mechanisms. *Acta Neuropathol*, *126*(1), 1-19.
615 https://doi.org/10.1007/s00401-013-1138-1
616 Hagerman, R. J., Berry-Kravis, E., Hazlett, H. C., Bailey, D. B., Moine, H., Kooy, R. F.,
617 Tassone, F., Gantois, I., Sonenberg, N., Mandel, J. L., & Hagerman, P. J. (2017).
618 Fragile X syndrome. In *Nature Reviews Disease Primers 2017 3:1* (Vol. 3, pp. 1-
619 19): Nature Publishing Group.
620 Hale, C. R., Sawicka, K., Mora, K., Fak, J. J., Kang, J. J., Cutrim, P., Cialowicz, K.,
621 Carroll, T. S., & Darnell, R. B. (2021). FMRP regulates mRNAs encoding distinct
622 functions in the cell body and dendrites of CA1 pyramidal neurons. *Elife*, *10*.
623 https://doi.org/10.7554/eLife.71892

624 Hallahan, B. P., Craig, M. C., Toal, F., Daly, E. M., Moore, C. J., Ambikapathy, A.,
625 Robertson, D., Murphy, K. C., & Murphy, D. G. (2011). In vivo brain anatomy of
626 adult males with Fragile X syndrome: an MRI study. *Neuroimage*, *54*(1), 16-24.
627 https://doi.org/10.1016/j.neuroimage.2010.08.015
628 Hodge, R. D., Bakken, T. E., Miller, J. A., Smith, K. A., Barkan, E. R., Graybuck, L. T.,
629 Close, J. L., Long, B., Johansen, N., Penn, O., Yao, Z., Eggermont, J., Hollt, T.,
630 Levi, B. P., Shehata, S. I., Aevermann, B., Beller, A., Bertagnolli, D., Brouner, K.,
631 . . . Lein, E. S. (2019). Conserved cell types with divergent features in human
632 versus mouse cortex. *Nature*, *573*(7772), 61-68. https://doi.org/10.1038/s41586-
633 019-1506-7
634 Hua, T., Kao, C., Sun, Q., Li, X., & Zhou, Y. (2008). Decreased proportion of GABA
635 neurons accompanies age-related degradation of neuronal function in cat striate
636 cortex. *Brain Res Bull*, *75*(1), 119-125.
637 https://doi.org/10.1016/j.brainresbull.2007.08.001
638 Jacquemont, S., Hagerman, R. J., Leehey, M., Grigsby, J., Zhang, L., Brunberg, J. A.,
639 Greco, C., Des Portes, V., Jardini, T., Levine, R., Berry-Kravis, E., Brown, W. T.,
640 Schaeffer, S., Kissel, J., Tassone, F., & Hagerman, P. J. (2003). Fragile X
641 premutation tremor/ataxia syndrome: Molecular, clinical, and neuroimaging
642 correlates. In *American Journal of Human Genetics*.
643 Kang, Y., Zhou, Y., Li, Y., Han, Y., Xu, J., Niu, W., Li, Z., Liu, S., Feng, H., Huang, W.,
644 Duan, R., Xu, T., Raj, N., Zhang, F., Dou, J., Xu, C., Wu, H., Bassell, G. J.,
645 Warren, S. T., . . . Wen, Z. (2021). A human forebrain organoid model of fragile X
646 syndrome exhibits altered neurogenesis and highlights new treatment strategies.
647 *Nat Neurosci*, *24*(10), 1377-1391. https://doi.org/10.1038/s41593-021-00913-6
648 Kenneson, A., Zhang, F., Hagedorn, C. H., & Warren, S. T. (2001). Reduced FMRP and
649 increased FMR1 transcription is proportionally associated with CGG repeat
650 number in intermediate-length and premutation carriers. In *Human Molecular*
651 *Genetics*.
652 Lake, B. B., Ai, R., Kaeser, G. E., Salathia, N. S., Yung, Y. C., Liu, R., Wildberg, A.,
653 Gao, D., Fung, H. L., Chen, S., Vijayaraghavan, R., Wong, J., Chen, A., Sheng,
654 X., Kaper, F., Shen, R., Ronaghi, M., Fan, J. B., Wang, W., . . . Zhang, K. (2016).
655 Neuronal subtypes and diversity revealed by single-nucleus RNA sequencing of
656 the human brain. *Science*, *352*(6293), 1586-1590.
657 https://doi.org/10.1126/science.aaf1204
658 Langseth, C. M., Gyllborg, D., Miller, J. A., Close, J. L., Long, B., Lein, E. S., Hilscher,
659 M. M., & Nilsson, M. (2021). Comprehensive in situ mapping of human cortical
660 transcriptomic cell types. *Commun Biol*, *4*(1), 998.
661 https://doi.org/10.1038/s42003-021-02517-z
662 Lecca, D., Raffaele, S., Abbracchio, M. P., & Fumagalli, M. (2020). Regulation and
663 signaling of the GPR17 receptor in oligodendroglial cells. *Glia*, *68*(10), 1957-
664 1967. https://doi.org/10.1002/glia.23807
665 Lee, Y., Morrison, B. M., Li, Y., Lengacher, S., Farah, M. H., Hoffman, P. N., Liu, Y.,
666 Tsingalia, A., Jin, L., Zhang, P. W., Pellerin, L., Magistretti, P. J., & Rothstein, J.
667 D. (2012). Oligodendroglia metabolically support axons and contribute to
668 neurodegeneration. *Nature*, *487*(7408), 443-448.
669 https://doi.org/10.1038/nature11314

Leehey, M. A. (2009). Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS): Clinical Phenotype, Diagnosis and Treatment. In *Journal of Investigative Medicine* (Vol. 57, pp. 830-836).

Licznerski, P., Park, H. A., Rolyan, H., Chen, R., Mnatsakanyan, N., Miranda, P., Graham, M., Wu, J., Cruz-Reyes, N., Mehta, N., Sohail, S., Salcedo, J., Song, E., Effman, C., Effman, S., Brandao, L., Xu, G. N., Braker, A., Gribkoff, V. K., . . . Jonas, E. A. (2020). ATP Synthase c-Subunit Leak Causes Aberrant Cellular Metabolism in Fragile X Syndrome. *Cell*, *182*(5), 1170-1185 e1179. https://doi.org/10.1016/j.cell.2020.07.008

Liu, L., Zhang, K., Sandoval, H., Yamamoto, S., Jaiswal, M., Sanz, E., Li, Z., Hui, J., Graham, B. H., Quintana, A., & Bellen, H. J. (2015). Glial lipid droplets and ROS induced by mitochondrial defects promote neurodegeneration. *Cell*, *160*(1-2), 177-190. https://doi.org/10.1016/j.cell.2014.12.019

Lohith, T. G., Osterweil, E. K., Fujita, M., Jenko, K. J., Bear, M. F., & Innis, R. B. (2013). Is metabotropic glutamate receptor 5 upregulated in prefrontal cortex in fragile X syndrome? *Mol Autism*, *4*(1), 15. https://doi.org/10.1186/2040-2392-4-15

Majdi, M., Ribeiro-da-Silva, A., & Cuello, A. C. (2007). Cognitive impairment and transmitter-specific pre- and postsynaptic changes in the rat cerebral cortex during ageing. *Eur J Neurosci*, *26*(12), 3583-3596. https://doi.org/10.1111/j.1460-9568.2007.05966.x

Marques, S., Zeisel, A., Codeluppi, S., van Bruggen, D., Mendanha Falcao, A., Xiao, L., Li, H., Haring, M., Hochgerner, H., Romanov, R. A., Gyllborg, D., Munoz Manchado, A., La Manno, G., Lonnerberg, P., Floriddia, E. M., Rezayee, F., Ernfors, P., Arenas, E., Hjerling-Leffler, J., . . . Castelo-Branco, G. (2016). Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science*, *352*(6291), 1326-1329. https://doi.org/10.1126/science.aaf6463

Martin, J. P., & Bell, J. (1943). A Pedigree of Mental Defect Showing Sex-Linkage. *J Neurol Psychiatry*, *6*(3-4), 154-157. https://doi.org/10.1136/jnnp.6.3-4.154

Martínez Cerdeño, V., Hong, T., Amina, S., Lechpammer, M., Ariza, J., Tassone, F., Noctor, S. C., Hagerman, P., & Hagerman, R. (2018). Microglial cell activation and senescence are characteristic of the pathology FXTAS. In *Movement Disorders*.

Miller, S. A., Policastro, R. A., Sriramkumar, S., Lai, T., Huntington, T. D., Ladaika, C. A., Kim, D., Hao, C., Zentner, G. E., & O'Hagan, H. M. (2021). LSD1 and Aberrant DNA Methylation Mediate Persistence of Enteroendocrine Progenitors That Support BRAF-Mutant Colorectal Cancer. In *Cancer Research* (Vol. 81, pp. 3791-3805): American Association for Cancer Research.

Nagai, J., Yu, X., Papouin, T., Cheong, E., Freeman, M. R., Monk, K. R., Hastings, M. H., Haydon, P. G., Rowitch, D., Shaham, S., & Khakh, B. S. (2021). Behaviorally consequential astrocytic regulation of neural circuits. *Neuron*, *109*(4), 576-596. https://doi.org/10.1016/j.neuron.2020.12.008

Nave, K. A. (2010). Myelination and support of axonal integrity by glia. *Nature*, *468*(7321), 244-252. https://doi.org/10.1038/nature09614

Oberle, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boue, J., Bertheas, M., & Mandel, J. (1991). Instability of a 550-base pair DNA segment

and abnormal methylation in fragile X syndrome. In *Science* (Vol. 252, pp. 1097-1102).

Peters, A., Josephson, K., & Vincent, S. L. (1991). Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex. *Anat Rec*, *229*(3), 384-398. https://doi.org/10.1002/ar.1092290311

Peters, A., & Sethares, C. (2004). Oligodendrocytes, their progenitors and other neuroglial cells in the aging primate cerebral cortex. *Cereb Cortex*, *14*(9), 995-1007. https://doi.org/10.1093/cercor/bhh060

Pieretti, M., Zhang, F., Fu, Y.-H., Warren, S. T., Oostra, B. A., Caskey, C. T., & Nelson, D. L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. In *Cell* (Vol. 66, pp. 817-822).

Pretto, D. I., Kumar, M., Cao, Z., Cunningham, C. L., Durbin-Johnson, B., Qi, L., Berman, R., Noctor, S. C., Hagerman, R. J., Pessah, I. N., & Tassone, F. (2014). Reduced EAAT1 and mGluR5 expression in the cerebellum of FMR1 premutation carriers with FXTAS. In *Neurobiology of aging* (Vol. 35, pp. 1189): NIH Public Access.

Raj, N., McEachin, Z. T., Harousseau, W., Zhou, Y., Zhang, F., Merritt-Garza, M. E., Taliaferro, J. M., Kalinowska, M., Marro, S. G., Hales, C. M., Berry-Kravis, E., Wolf-Ochoa, M. W., Martinez-Cerdeño, V., Wernig, M., Chen, L., Klann, E., Warren, S. T., Jin, P., Wen, Z., & Bassell, G. J. (2021). Cell-type-specific profiling of human cellular models of fragile X syndrome reveal PI3K-dependent defects in translation and neurogenesis. In *Cell Reports* (Vol. 35, pp. 108991): Cell Press.

Rhodes, K. E., Raivich, G., & Fawcett, J. W. (2006). The injury response of oligodendrocyte precursor cells is induced by platelets, macrophages and inflammation-associated cytokines. *Neuroscience*, *140*(1), 87-100. https://doi.org/10.1016/j.neuroscience.2006.01.055

Rozycka, A., & Liguz-Lecznar, M. (2017). The space where aging acts: focus on the GABAergic synapse. *Aging Cell*, *16*(4), 634-643. https://doi.org/10.1111/acel.12605

Salas, I. H., Burgado, J., & Allen, N. J. (2020). Glia: victims or villains of the aging brain? *Neurobiol Dis*, *143*, 105008. https://doi.org/10.1016/j.nbd.2020.105008

Salcedo-Arellano, M. J., Wang, J. Y., McLennan, Y. A., Doan, M., Cabal-Herrera, A. M., Jimenez, S., Wolf-Ochoa, M. W., Sanchez, D., Juarez, P., Tassone, F., Durbin-Johnson, B., Hagerman, R. J., & Martínez-Cerdeño, V. (2021). Cerebral Microbleeds in Fragile X-Associated Tremor/Ataxia Syndrome. In *Movement disorders : official journal of the Movement Disorder Society* (Vol. 36, pp. 1935-1943): Mov Disord.

Sandoval, G. M., Shim, S., Hong, D. S., Garrett, A. S., Quintin, E. M., Marzelli, M. J., Patnaik, S., Lightbody, A. A., & Reiss, A. L. (2018). Neuroanatomical abnormalities in fragile X syndrome during the adolescent and young adult years. *J Psychiatr Res*, *107*, 138-144. https://doi.org/10.1016/j.jpsychires.2018.10.014

Schwartz, J. L., Jones, K. L., & Yeo, G. W. (2021). Repeat RNA expansion disorders of the nervous system: post-transcriptional mechanisms and therapeutic strategies. *Crit Rev Biochem Mol Biol*, *56*(1), 31-53. https://doi.org/10.1080/10409238.2020.1841726

760 Sellier, C., Buijsen, R. A. M., He, F., Natla, S., Jung, L., Tropel, P., Gaucherot, A.,
761 Jacobs, H., Meziane, H., Vincent, A., Champy, M. F., Sorg, T., Pavlovic, G.,
762 Wattenhofer-Donze, M., Birling, M. C., Oulad-Abdelghani, M., Eberling, P.,
763 Ruffenach, F., Joint, M., . . . Charlet-Berguerand, N. (2017). Translation of
764 Expanded CGG Repeats into FMRpolyG Is Pathogenic and May Contribute to
765 Fragile X Tremor Ataxia Syndrome. In *Neuron*.
766 Song, L., Pan, S., Zhang, Z., Jia, L., Chen, W. H., & Zhao, X. M. (2021). STAB: a
767 spatio-temporal cell atlas of the human brain. *Nucleic Acids Res*, *49*(D1), D1029-
768 D1037. https://doi.org/10.1093/nar/gkaa762
769 Soreq, L., Consortium, U. K. B. E., North American Brain Expression, C., Rose, J.,
770 Soreq, E., Hardy, J., Trabzuni, D., Cookson, M. R., Smith, C., Ryten, M., Patani,
771 R., & Ule, J. (2017). Major Shifts in Glial Regional Identity Are a Transcriptional
772 Hallmark of Human Brain Aging. *Cell Rep*, *18*(2), 557-570.
773 https://doi.org/10.1016/j.celrep.2016.12.011
774 Stanley, E. M., Fadel, J. R., & Mott, D. D. (2012). Interneuron loss reduces dendritic
775 inhibition and GABA release in hippocampus of aged rats. *Neurobiol Aging*,
776 *33*(2), 431 e431-413. https://doi.org/10.1016/j.neurobiolaging.2010.12.014
777 Swanson, M. R., Wolff, J. J., Shen, M. D., Styner, M., Estes, A., Gerig, G., McKinstry, R.
778 C., Botteron, K. N., Piven, J., Hazlett, H. C., & Infant Brain Imaging Study, N.
779 (2018). Development of White Matter Circuitry in Infants With Fragile X
780 Syndrome. *JAMA Psychiatry*, *75*(5), 505-513.
781 https://doi.org/10.1001/jamapsychiatry.2018.0180
782 Tassone, F., Hagerman, R. J., Chamberlain, W. D., & Hagerman, P. J. (2000).
783 Transcription of the FMR1 gene in individuals with fragile X syndrome. In
784 *American Journal of Medical Genetics - Seminars in Medical Genetics*.
785 Tassone, F., Hagerman, R. J., Garcia-Arocena, D., Khandjian, E. W., Greco, C. M., &
786 Hagerman, P. J. (2004). Intranuclear inclusions in neural cells with premutation
787 alleles in fragile X associated tremor/ataxia syndrome. In *Journal of Medical*
788 *Genetics* (Vol. 41, pp. e43-e43): BMJ Publishing Group Ltd.
789 Tassone, F., Hagerman, R. J., Iklé, D. N., Dyer, P. N., Lampe, M., Willemsen, R.,
790 Oostra, B. A., & Taylor, A. K. (1999). FMRP expression as a potential prognostic
791 indicator in fragile X syndrome. In *American Journal of Medical Genetics*.
792 Tassone, F., Hagerman, R. J., Taylor, A. K., Gane, L. W., Godfrey, T. E., & Hagerman,
793 P. J. (2000). Elevated Levels of FMR1 mRNA in Carrier Males: A New
794 Mechanism of Involvement in the Fragile-X Syndrome. In *The American Journal*
795 *of Human Genetics* (Vol. 66, pp. 6-15): Am J Hum Genet.
796 Teismann, P., Tieu, K., Cohen, O., Choi, D. K., Wu, D. C., Marks, D., Vila, M., Jackson-
797 Lewis, V., & Przedborski, S. (2003). Pathogenic role of glial cells in Parkinson's
798 disease. *Mov Disord*, *18*(2), 121-129. https://doi.org/10.1002/mds.10332
799 Tran, S. S., Jun, H. I., Bahn, J. H., Azghadi, A., Ramaswami, G., Van Nostrand, E. L.,
800 Nguyen, T. B., Hsiao, Y. E., Lee, C., Pratt, G. A., Martinez-Cerdeno, V.,
801 Hagerman, R. J., Yeo, G. W., Geschwind, D. H., & Xiao, X. (2019). Widespread
802 RNA editing dysregulation in brains from autistic individuals. *Nat Neurosci*, *22*(1),
803 25-36. https://doi.org/10.1038/s41593-018-0287-x

804 Turner, G., Eastman, C., Casey, J., McLeay, A., Procopis, P., & Turner, B. (1975). X-
805 linked mental retardation associated with macro-orchidism. *J Med Genet*, *12*(4),
806 367-371. https://doi.org/10.1136/jmg.12.4.367
807 Velmeshev, D., Schirmer, L., Jung, D., Haeussler, M., Perez, Y., Mayer, S., Bhaduri, A.,
808 Goyal, N., Rowitch, D. H., & Kriegstein, A. R. (2019). Single-cell genomics
809 identifies cell type-specific molecular changes in autism. *Science*, *364*(6441),
810 685-689. https://doi.org/10.1126/science.aav8130
811 Verkerk, A. J. M. H., Pieretti, M., Sutcliffe, J. S., Fu, Y.-H., Kuhl, D. P. A., Pizzuti, A.,
812 Reiner, O., Richards, S., Victoria, M. F., Zhang, F., Eussen, B. E., van Ommen,
813 G.-J. B., Blonden, L. A. J., Riggins, G. J., Chastain, J. L., Kunst, C. B., Galjaard,
814 H., Thomas Caskey, C., Nelson, D. L., . . . Warren, S. T. (1991). Identification of
815 a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster
816 region exhibiting length variation in fragile X syndrome. In *Cell* (Vol. 65).
817 Wang, H., Ku, L., Osterhout, D. J., Li, W., Ahmadian, A., Liang, Z., & Feng, Y. (2004).
818 Developmentally-programmed FMRP expression in oligodendrocytes: a potential
819 role of FMRP in regulating translation in oligodendroglia progenitors. *Hum Mol*
820 *Genet*, *13*(1), 79-89. https://doi.org/10.1093/hmg/ddh009
821 Xu, Z. X., Kim, G. H., Tan, J. W., Riso, A. E., Sun, Y., Xu, E. Y., Liao, G. Y., Xu, H., Lee,
822 S. H., Do, N. Y., Lee, C. H., Clipperton-Allen, A. E., Kwon, S., Page, D. T., Lee,
823 K. J., & Xu, B. (2020). Elevated protein synthesis in microglia causes autism-like
824 synaptic and behavioral aberrations. *Nat Commun*, *11*(1), 1797.
825 https://doi.org/10.1038/s41467-020-15530-3
826

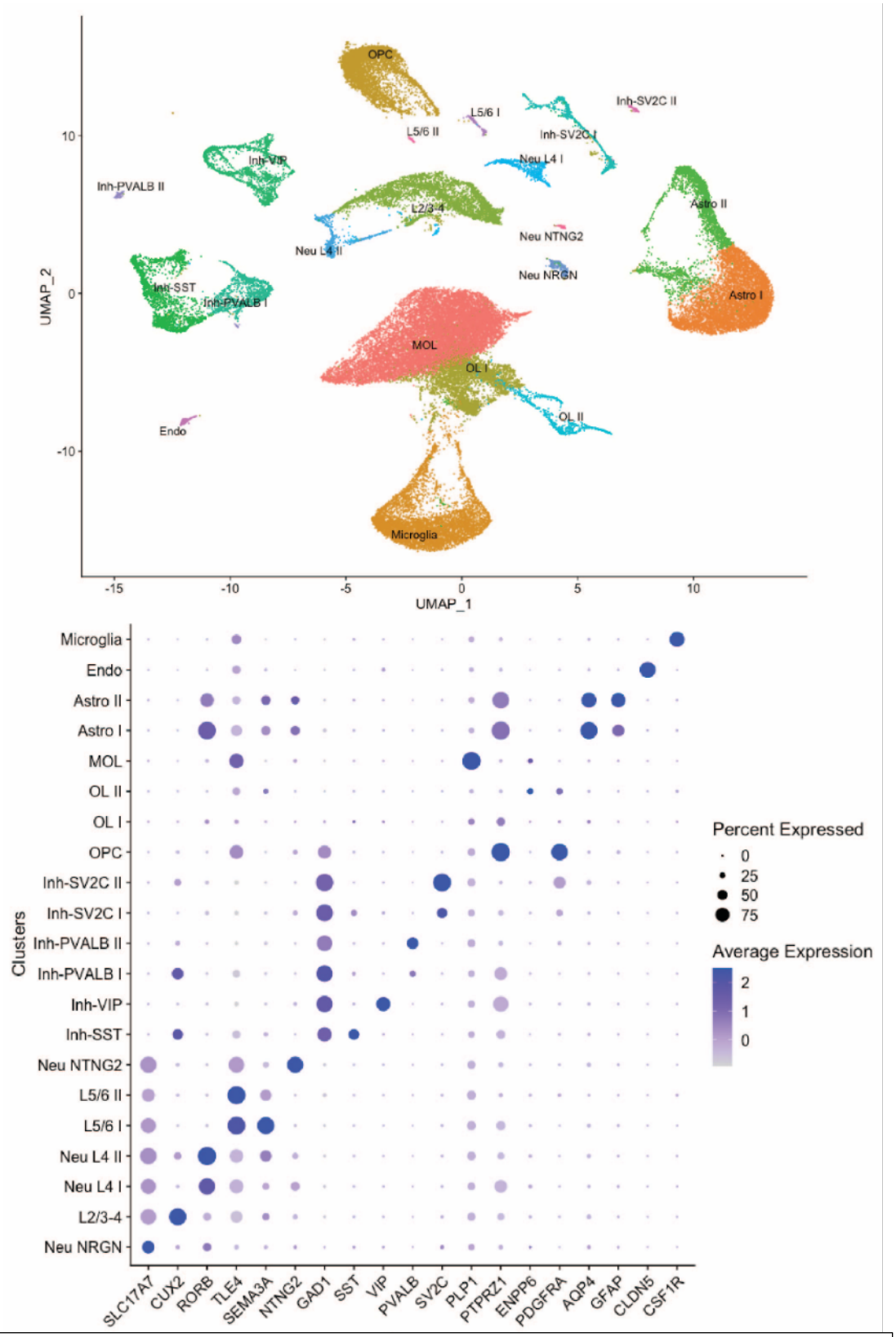


Figure 2 figure supplement 1: High resolution clustering of frontal cortex corroborates more general classification. Nuclei clustering of frontal cortex reveals accurate assignment of inhibitory neuronal subclusters and layer specific markers. Abbreviations as per Figure 2. Top panel is integrated UMAP, bottom is dot plot of cell type and layer specific marker expression by cluster.

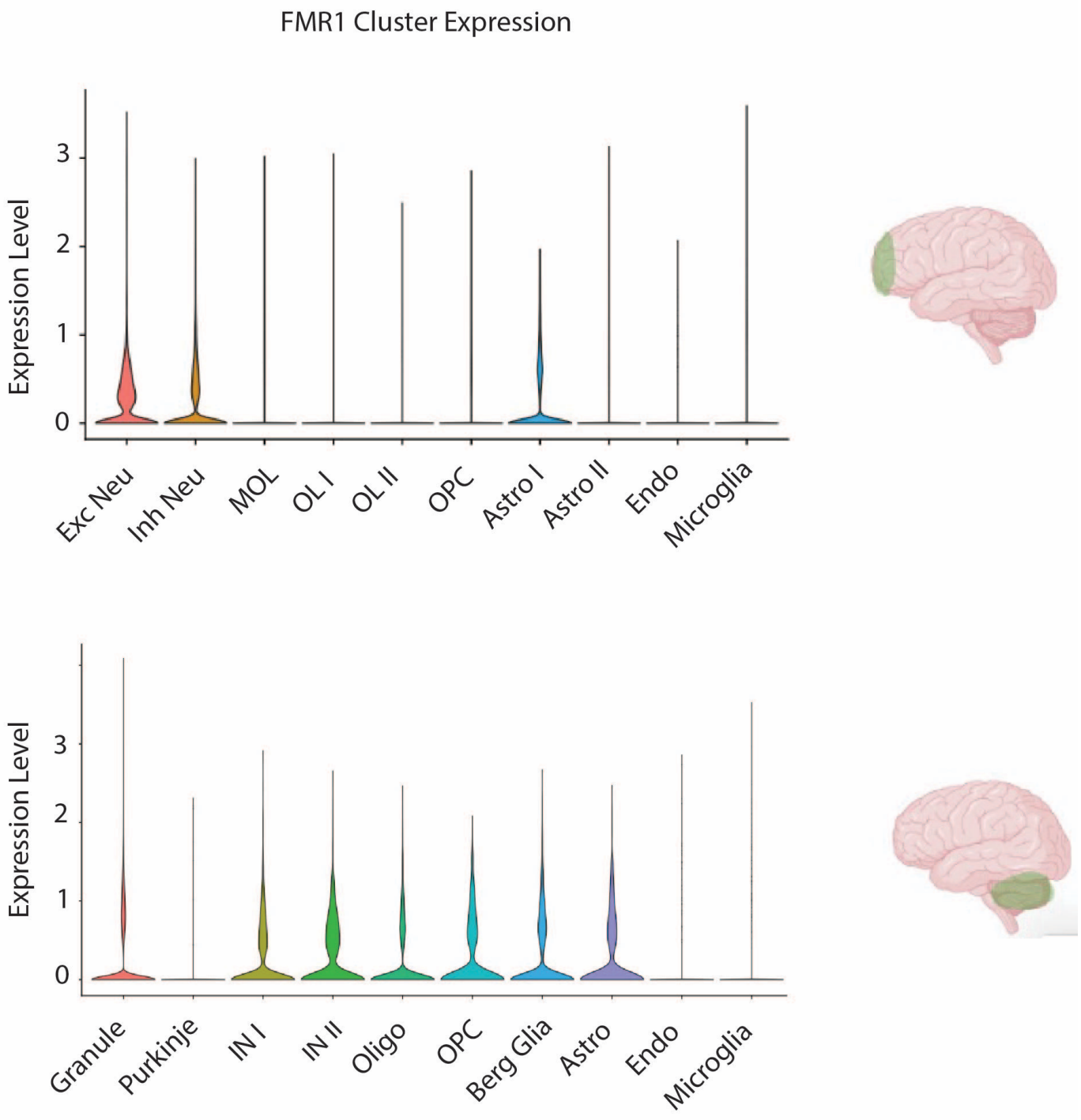


Figure 2 figure supplement 2: Average FMR1 expression by cluster and region. Abbreviations as per Figure 2.

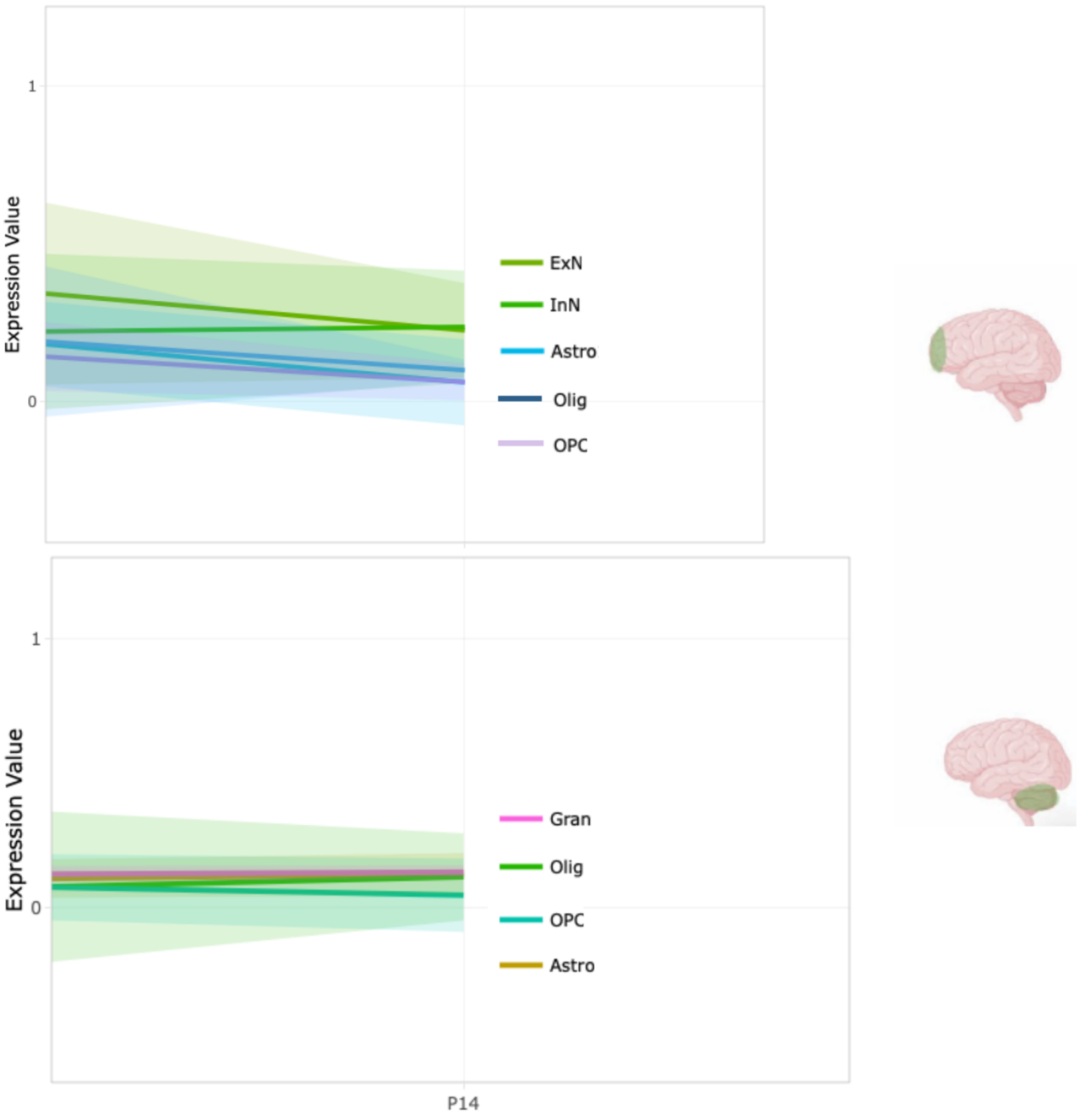


Figure 2 figure supplement 3: FMR1 expression in human frontal cortex (top) and cerebellum (bottom) in independent scRNA dataset replicates higher cerebellar glial expression P14: ages 40-59. Generated from stab.comp-sysbio.org on 1/8/21. Additional cell types hidden for clarity of presentation.

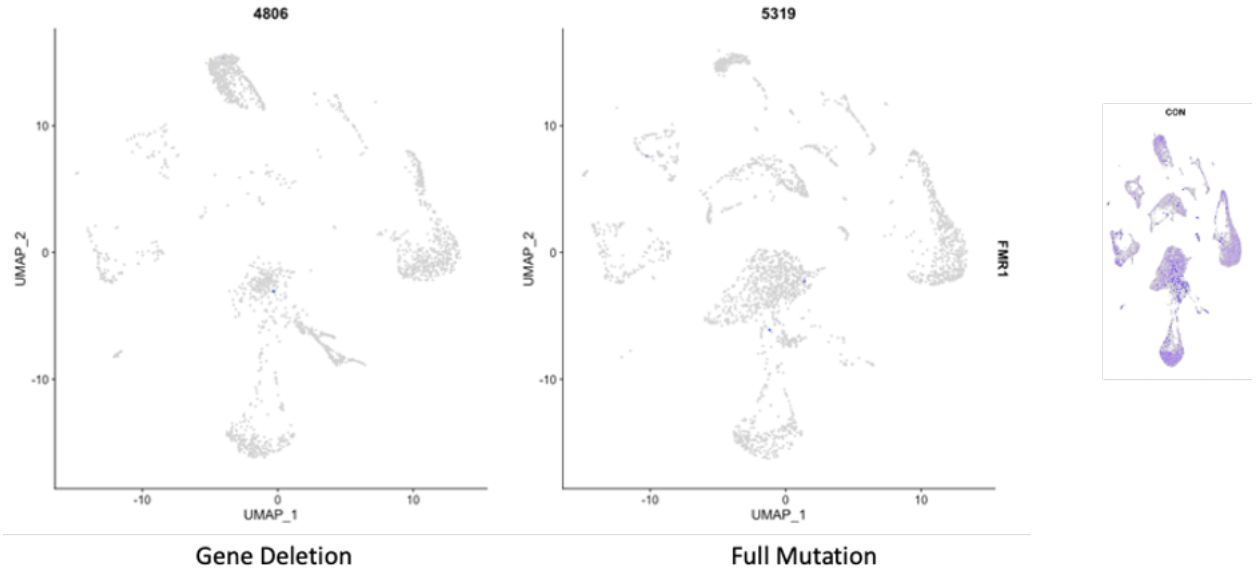


Figure 2 figure supplement 4: *FMR1* expression is eliminated in all cell clusters in Fragile X syndrome, regardless of genetic mechanism. Expected expression (purple) presented in right panel demonstrates average control expression. Sort cell feature in Seurat applied to visualize loss of expression. Frontal cortex UMAP is presented, results were identical in cerebellum.

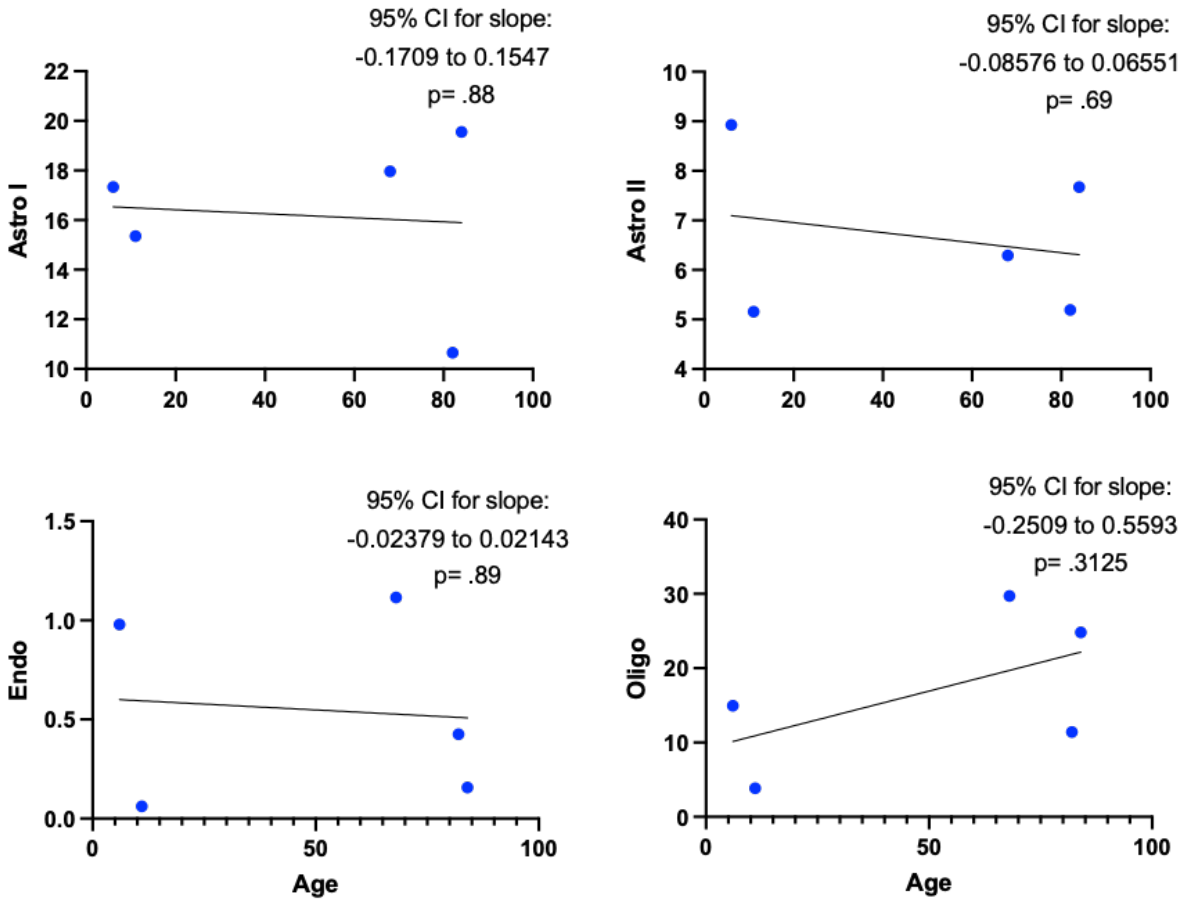


Figure 3 figure supplement 1: Linear regression for cluster proportion and age revealed no significant association with age for glial subclusters. P-value on graph for non-zero slope. Astro- astrocyte, Endo- endothelial, Oligo- mature oligodendrocyte (MOL).

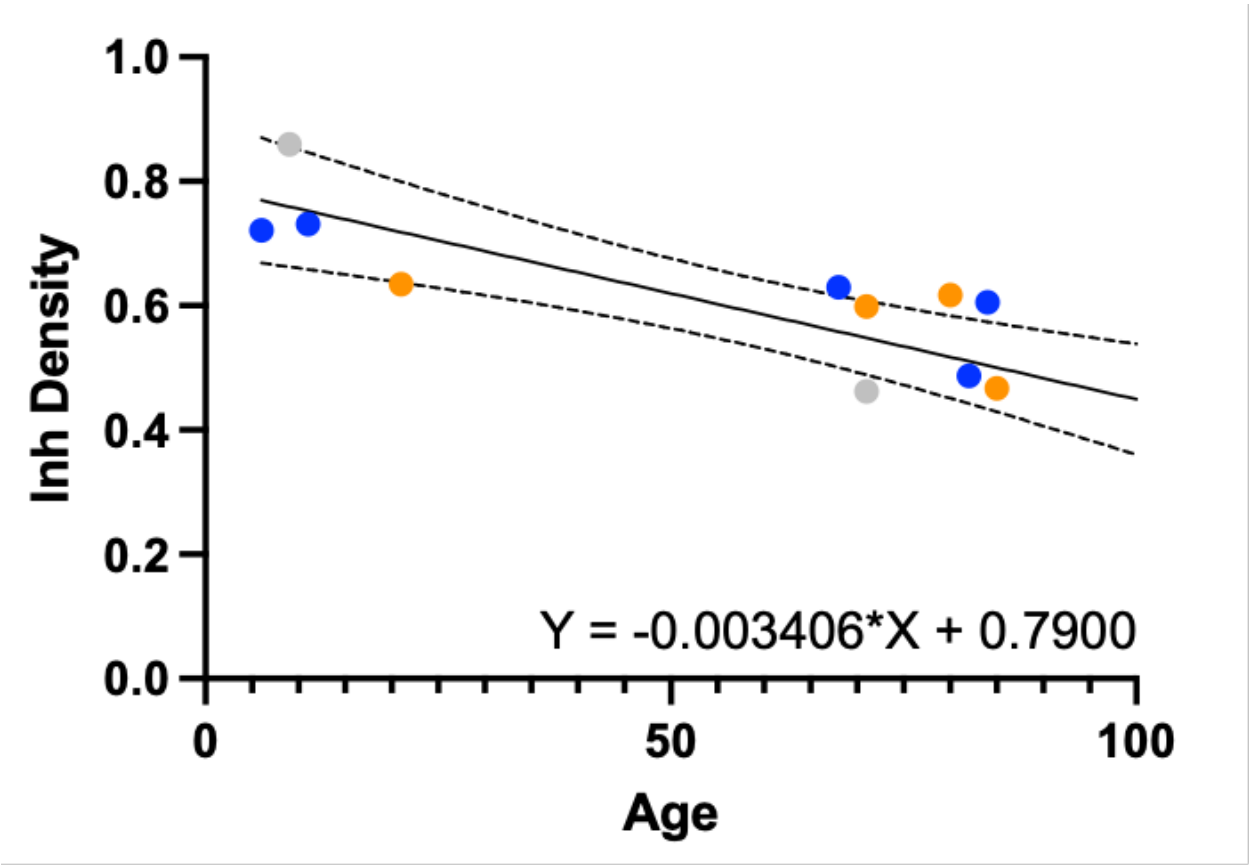


Figure 3 figure supplement 2: Linear regression for inhibitory/total neuron density in frontal cortex reveals significant age related decline that is not related to Fragile X status. Grey- FXS, blue- control, orange- PM. p-value of non-zero slope < .001.

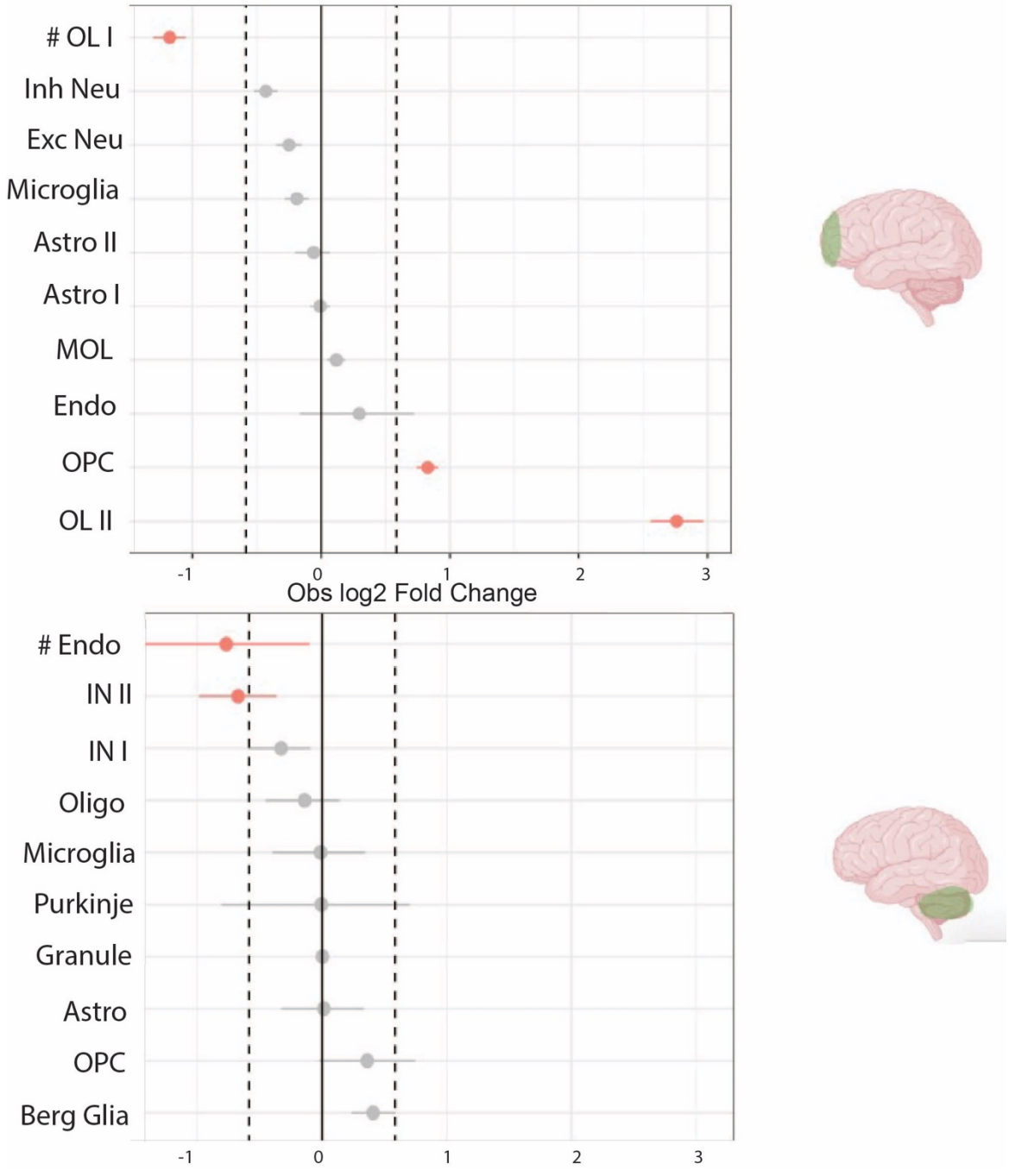


Figure 3 figure supplement 3: Alterations in cellular composition in Fragile X syndrome. Permutation plot resulting from scProportion() with red indicating FDR < .05 and abs(log2FC) > 0.58. # indicates significance of this cluster is not robust to outlier sample removal.

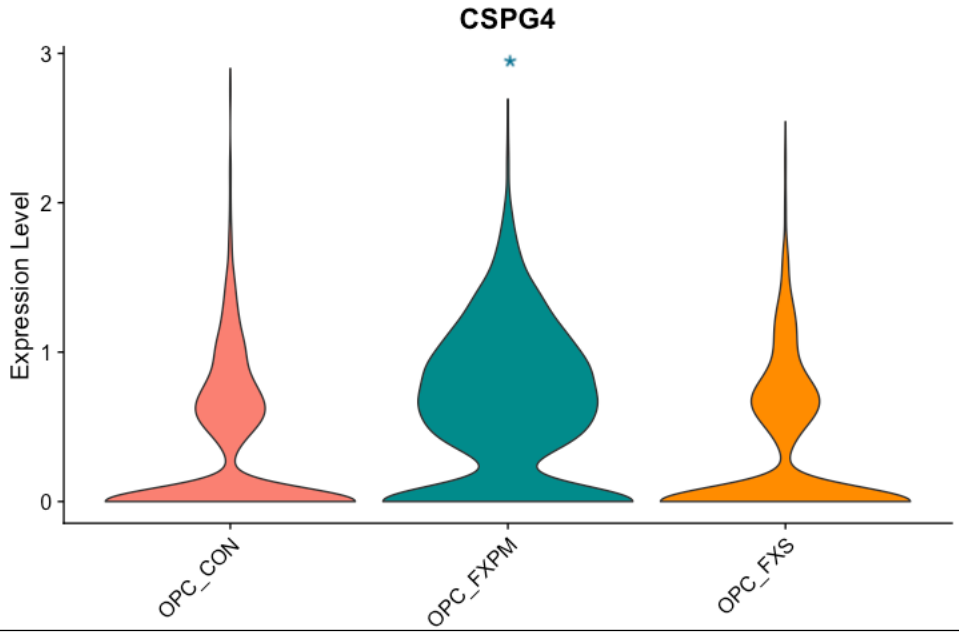


Figure 4 figures supplement 1: **Increased CSPG4 (NG2) expression in OPCs in PM cases in frontal cortex.** * indicates comparison vs control, padj <.05.

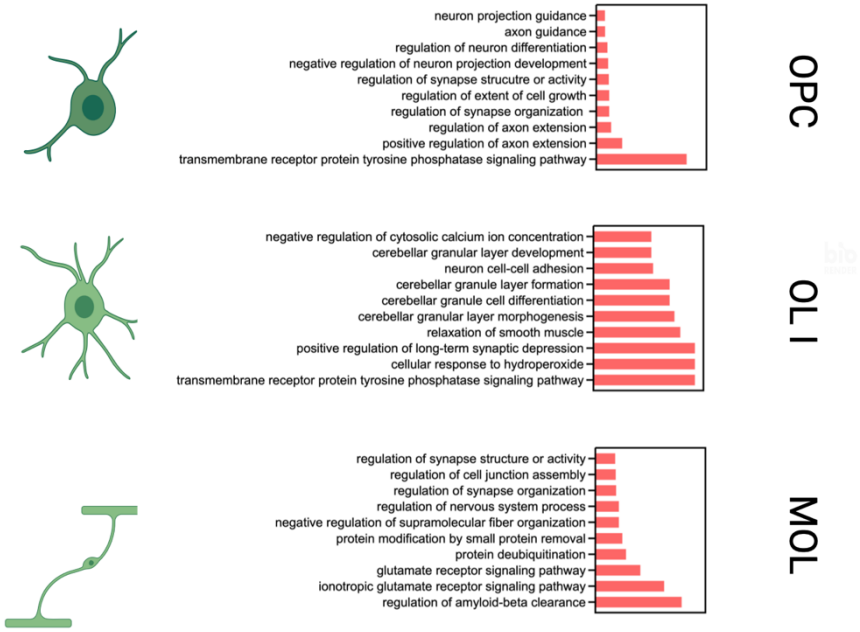


Figure 4 figure supplement 2: GO analysis of downregulated genes in oligodendrocyte clusters in PM cases. Analysis reveals disturbances in synaptic and neuronal organization and regulation.

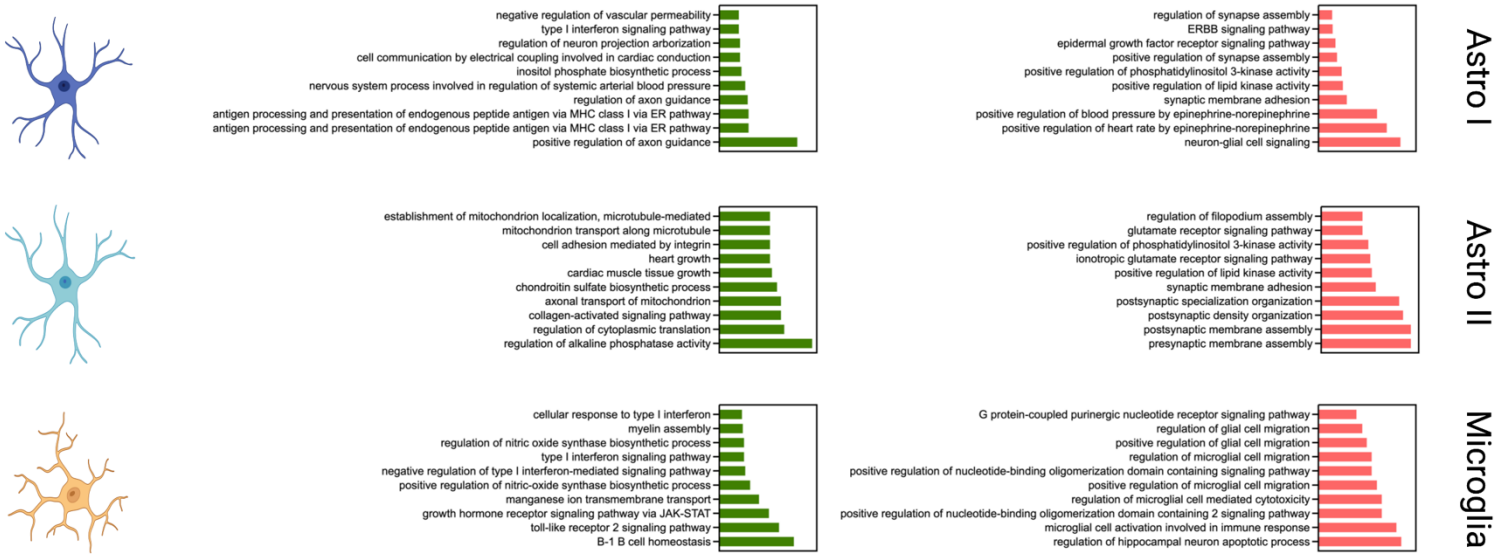


Figure 4 figure supplement 3: **GO analysis of upregulated (green) and downregulated (red) genes in astrocyte and microglia clusters in PM cases.** Analysis reveal upregulation of inflammation as well as disturbances in synaptic and neuronal organization and regulation.

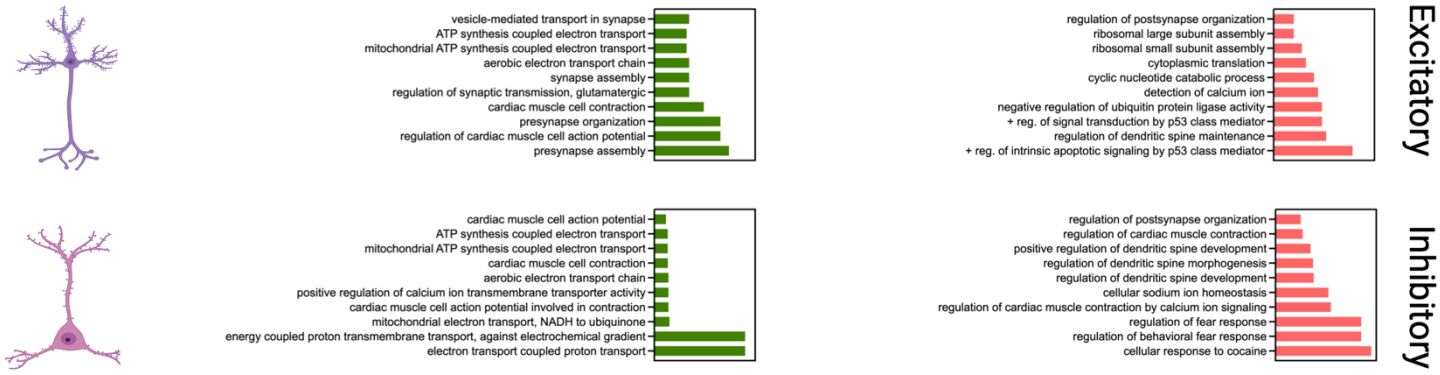


Figure 4 figure supplement 4: **GO analysis of upregulated (green) and downregulated (red) genes in neuronal clusters in PM cases.** There is evidence of metabolic stress and downregulation of synaptic and dendritic organization and development.

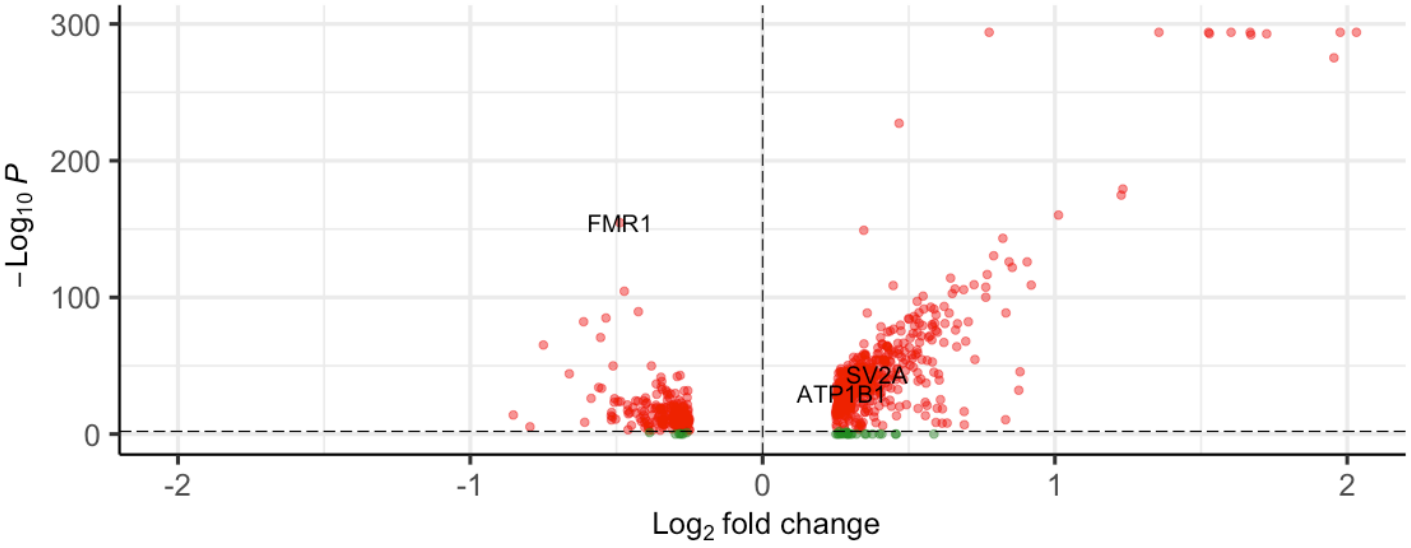


Figure 4 figure supplement 5: **Volcano plot of differentially expressed genes in FXS in frontal cortex excitatory neurons**. Note expected loss of FMR1 and deregulation of select FMRP target genes (ATP1B1, SV2A).

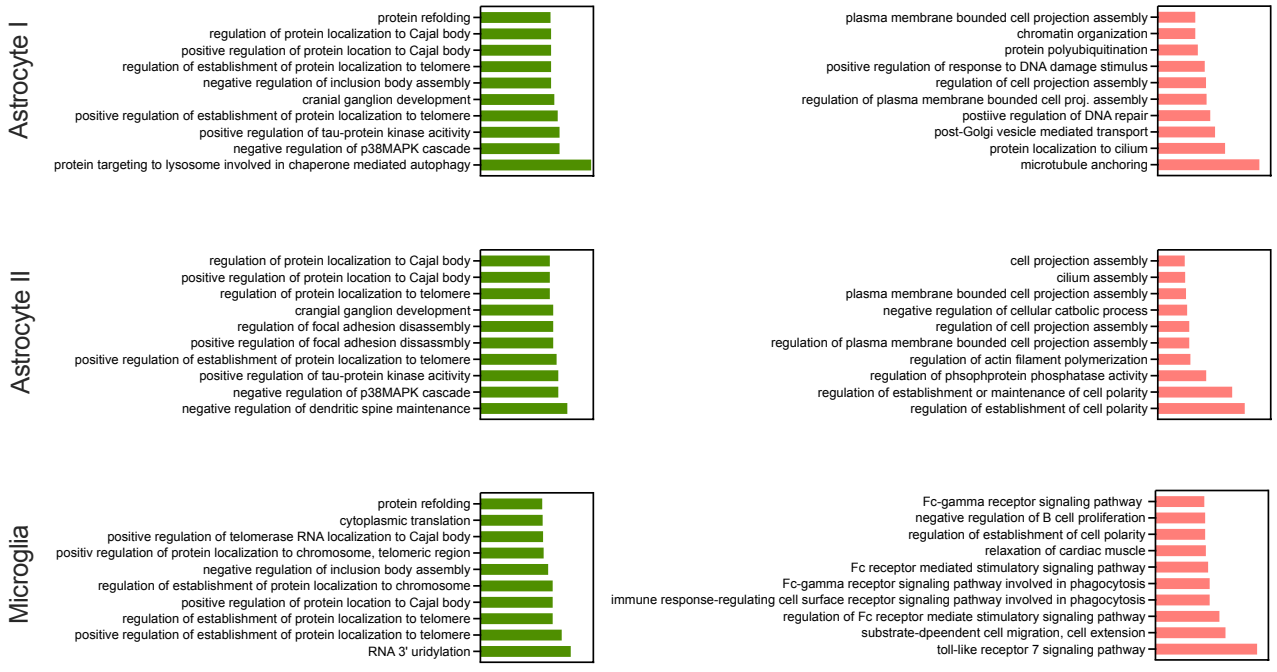


Figure 4 figure supplement 6: **GO analysis of upregulated (green) and downregulated (red) differentially regulated genes in astrocytes and microglia in FXS**.

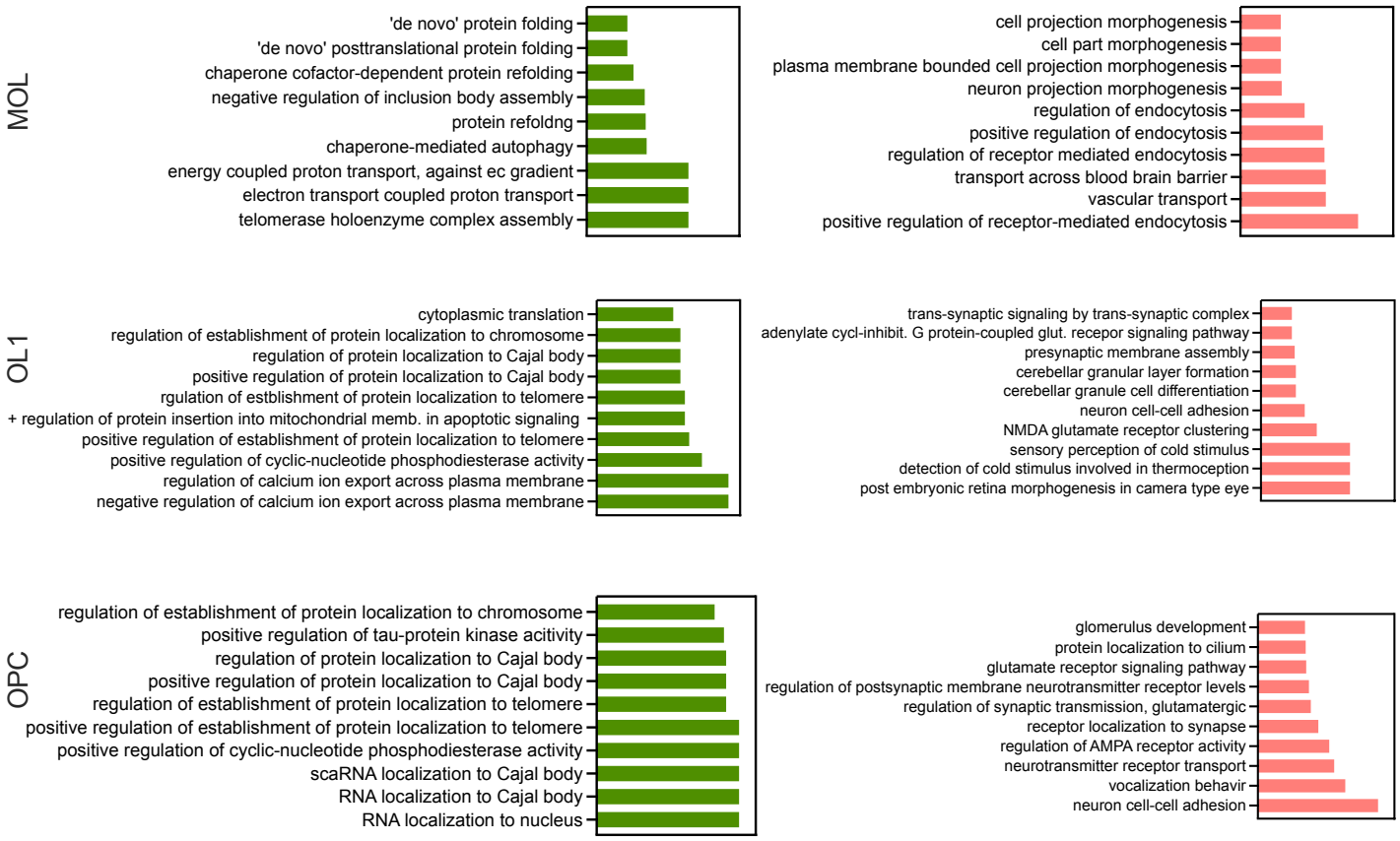


Figure 4 figure supplement 7: **GO analysis of upregulated (green) and downregulated (red) differentially regulated genes in oligodendrocyte lineage in FXS.**

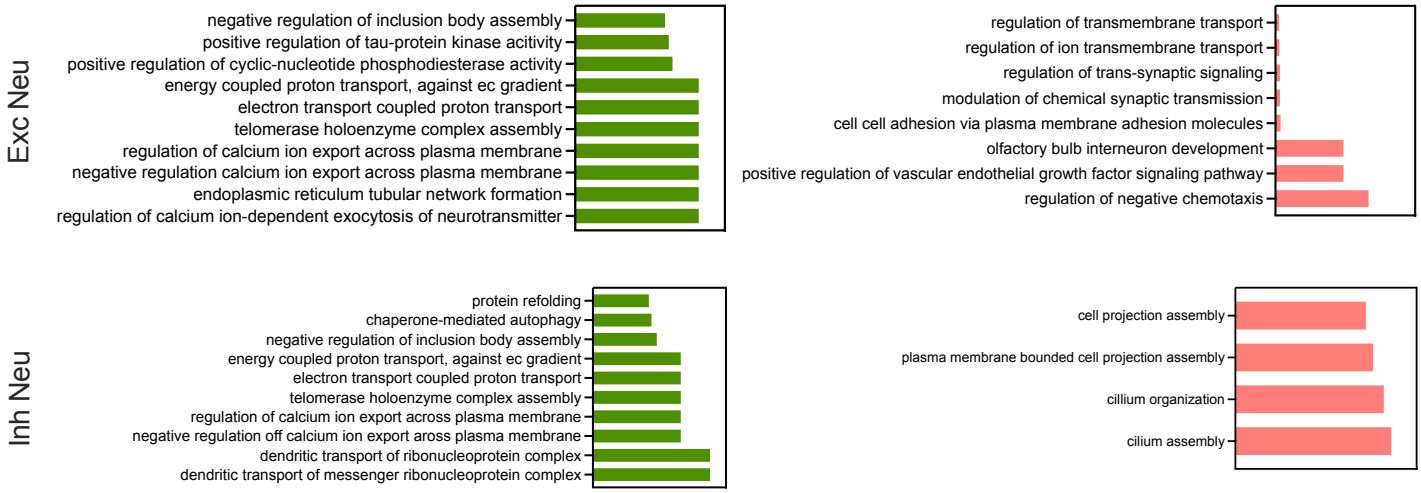


Figure 4 figure supplement 8: **GO analysis of upregulated (green) and downregulated (red) differentially regulated genes in neurons in FXS.**

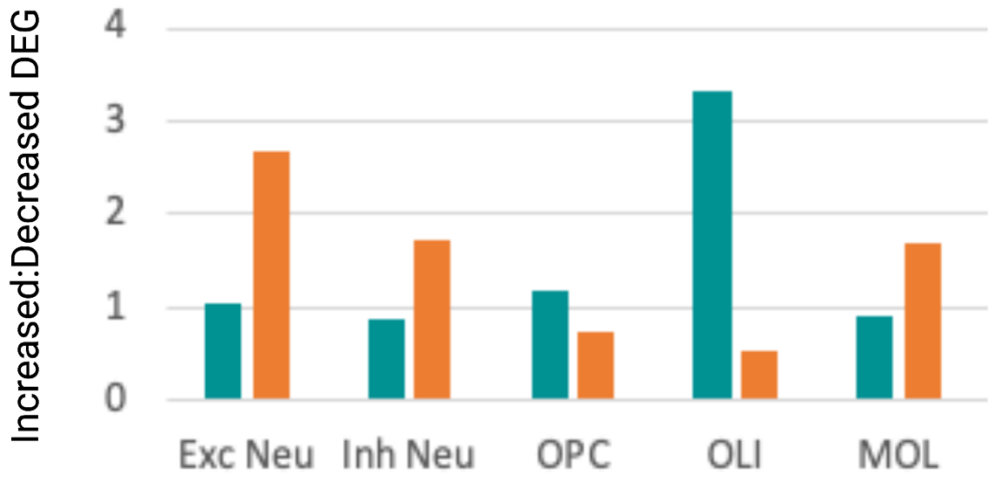
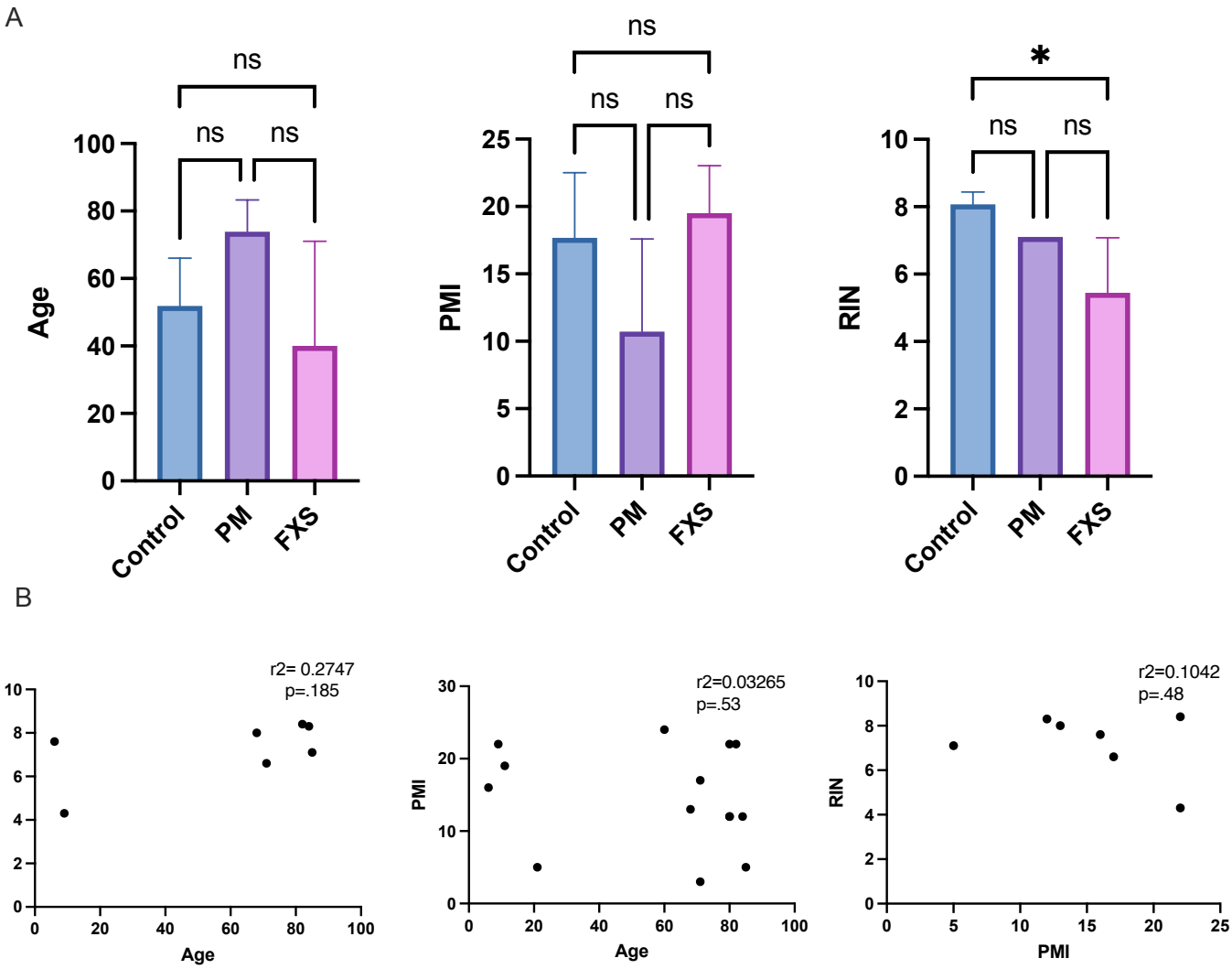
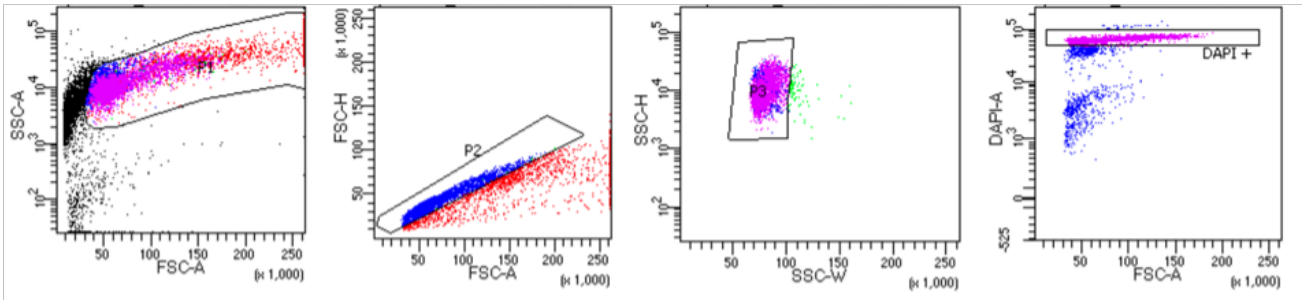


Figure 4 figure supplement 9: **Comparison of ratio of increased:decreased differentially expressed genes** in select clusters in PM cases (teal) and FXS (orange) reveal distinct patterns of global transcriptional dysregulation.



Appendix Fig 1: A. Age, PMI, and RIN by condition, ns= non-significant, one-way ANOVA, Tukey's multiple comparison test, * p < .05. B. No significant association between RIN and age, PMI and age, and RIN and PMI.



Appendix Fig 2: Representative flow cytometry results for nuclear selection- ~ 5-10% parent population is selected.