Title

Dysregulation of astrocyte-secreted pleiotrophin contributes to neuronal structural and functional phenotypes in Down Syndrome

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Summary

Neuronal dendrite structure and synapse formation is tightly regulated during development to promote proper connectivity. Astrocyte-secreted proteins act as guidance and pro-synaptogenic factors, and astrocyte protein secretion is dysregulated in neurodevelopmental disorders with aberrant circuitry. Here we identify down-regulation of the astrocyte-secreted molecule pleiotrophin as a major contributor to neuronal phenotypes in the Ts65Dn mouse model of Down Syndrome. Through histological characterizations of Ts65Dn mutant and pleiotrophin knockout mice, we find overlapping phenotypes in neuronal dendrites, spines and intracortical synapses. By targeting pleiotrophin overexpression to astrocytes in Ts65Dn mutant mice *in vivo*, we show that pleiotrophin can rescue and/or partially restore these phenotypes, and we further correlate structural changes to improved cognitive function. Our findings identify pleiotrophin as a molecule that can be targeted in Down Syndrome to promote proper circuit connectivity, even at later stages of development after typical periods of circuit refinement have closed.

Keywords

Astrocyte, dendritic spines, synapse, pleiotrophin, dendrite, neuron, Down Syndrome, neurodevelopmental disorder, trisomy 21, visual cortex

Introduction

Down Syndrome (DS) is the most common form of genetic intellectual disability, caused by trisomy of human chromosome 21 (HSA21) and affecting 1 in 700 live births[1]. Individuals with DS have neurodevelopmental changes that result in hyperactivity, as well as altered information processing and memory impediments[2]. Histological studies have identified changes to neuronal morphology during development in DS which may be linked to these functional impairments. For example, Layer 5 pyramidal neurons in the visual cortex have shorter and less complex dendrites than those from neurotypical individuals[3]. Additionally, decreased spine density is reported in pyramidal neurons of the motor cortex, visual cortex and hippocampus[4-6]. These classical studies were performed decades ago, but the field is still in search of therapies that can improve neuronal structure, synaptic circuitry and cognitive function.

It is now well known that the morphology of neurons and density of synapses is not necessarily regulated in a cell-autonomous manner. Rather, astrocytes, an abundant type of glial cell in the brain, play essential roles in regulating neuronal synapse abundance and activity during development[7]. One key mechanism by which astrocytes modulate synapses is through the secretion of proteins that guide various stages of synaptic development, including synapse formation and maturation[8-10]. With respect to DS, there is accumulating evidence for astrocyte involvement. One study demonstrated that wildtype (WT) neurons, cultured *in vitro* with DS astrocytes or with DS astrocyte conditioned media (ACM), acquired DS phenotypes such as reduced spine density and retention of immature filopodia spines. Thrombospondin1 (Tsp1) was identified as an astrocyte-secreted protein that was down-regulated in DS astrocytes and addition of Tsp1 to the media could partially recover these phenotypes[11]. However, the incomplete rescue with Tsp1 suggests there are additional factors that need to be identified. In support of this, a comprehensive mass spectrometry screen performed on the ACM of cultured primary mouse cortical astrocytes isolated from the Ts65Dn mouse model of DS identified nearly 800 differentially secreted proteins in mutant (Mut) astrocytes[12]. This study revealed that astrocyte protein secretion in DS is highly dysregulated, suggesting that altered secretion of a variety of proteins from astrocytes may have detrimental effects on neuronal circuit formation in DS.

In the present study we used the mass spectrometry screen performed by Caldwell and colleagues (2022) to identify the heparin-binding secreted growth factor, Pleiotrophin (Ptn), as a molecule with significantly decreased secretion from DS astrocytes. Ptn was a prime candidate due to previous descriptions of its role in dendrite regeneration after cortical prick injury[13]. Another study demonstrated that *Ptn* knockdown from neural stem cells in the dentate gyrus decreased dendrite length and complexity as well as spine density in newborn neurons[14]. The known roles of Ptn in regulating neuronal morphology and synaptic connectivity in the contexts of brain injury and circuit integration of immature neurons creates a strong link between its down-regulation in DS and the observed neurodevelopmental phenotypes.

Here we use the visual cortex as a model system to study the role of Ptn in DS, chosen due to its well-characterized circuitry and previously described phenotypes of shorter and less complex dendrites in pyramidal neurons in DS[3,6]. *Ptn* is highly enriched in cortical astrocytes[15] and its expression is developmentally regulated to peak during a robust period of synaptogenesis in the visual cortex[16]. We show that Ptn has important roles in neurotypical development, and that genetic loss of *Ptn* in a mouse model results in neuronal phenotypes overlapping with those observed in DS. Finally, we demonstrate that viral-mediated targeting of Ptn overexpression specifically to astrocytes in Ts65Dn mice can ameliorate many aspects of the aberrant circuit formation *in vivo*, as well as improve cognitive function in two separate memory tasks. Altogether, the results of this study pinpoint Ptn as a modulator of dendrite morphology and a

pro-synaptogenic factor during development. We provide evidence that dysregulation of Ptn has functional impact in DS, which may be translatable to other neurodevelopmental disorders with similar circuit aberrations.

Results

Ptn expression is developmentally regulated and is decreased in Ts65Dn astrocytes *in vivo*

The study by Caldwell and colleagues (2022) provides a resource of differentially expressed genes and secreted proteins in cultured Ts65Dn Mut astrocytes, isolated at postnatal day (P)7. Ptn was identified from this dataset as showing robust decreases at both the mRNA and secreted protein levels (Figure S1A-B; RNA-Seq: 173.8 ± 8.4 FPKM in WT, 114.9 ± 16.7 in Ts65Dn; Mass Spectrometry: 0.50 ± 0.13 NSAF in WT, 0.088 ± 0.034 NSAF in Ts65Dn ACM). In neurotypical development *Ptn* has widespread expression throughout the brain, and prominent expression in upper cortical layers (Figure S1C-C'). To ask how *Ptn* expression correlates with important periods of synaptic development, we utilized a previously published astrocyte RiboTag RNA-sequencing dataset of the visual cortex[16] to determine the temporal profile of *Ptn* expression. In the visual cortex dendrite outgrowth is prominent from birth[17], synapse formation is high from P7[18,19], synaptic maturation begins ~P14[20] and circuit stabilization is achieved by ~P120 (Figure 1A). We found that peak *Ptn* expression correlates with periods of synaptogenesis and synaptic maturation (Figure 1B).

We validated the expression profile of *Ptn* using single molecule fluorescent in situ hybridization (smFISH) in Aldh1L1-EGFP mice at P7, P14 and P30, where astrocytes are labeled with EGFP (Figure 1C-E). The upper cortical layers were chosen for quantification based on a previous report that *Ptn* is an upper layer astrocyte marker[16]. We found that *Ptn* has highest expression at P14 (Layer 1: 30.9 ± 3.8 a.u.; Layer 2/3: 40.2 ± 4.4 a.u.) and significantly decreases by P30 (Layer 1: 18.8 ± 2.1 a.u.; Layer 2/3: 21.1 ± 3.2 a.u.; Figure 1F-G). Next, we assessed whether *Ptn* expression is reduced in DS astrocytes *in vivo*. *Ptn* expression was quantified within *Slc1a3*+ astrocytes from Ts65Dn WT and Mut mice at P14 (Figure 1H-I; 1H'-I'). Ts65Dn Mut astrocytes have a significant reduction in *Ptn* in Layer 1 and Layer 2/3 astrocytes (Layer 1: 0.86 ± 0.043-fold, Layer 2/3: 0.90 ± 0.034-fold; Figure 1J-K). Altogether these data demonstrate that *Ptn* expression is developmentally regulated to coincide with peak synapse formation and maturation in the visual cortex, and that *Ptn* is decreased in Ts65Dn Mut astrocytes, suggesting it may contribute to neuronal and synaptic phenotypes in DS.

Astrocyte-secreted pleiotrophin promotes dendrite outgrowth and branching

Given that Layer 5 pyramidal neurons have outgrowth and branching impairments in humans with DS[3,6] and that pyramidal neuron dendrites project to upper layers where *Ptn* is highly expressed[16], we aimed to test if Ptn can induce dendrite outgrowth in pyramidal cells *in vitro.* This was approached by culturing cortical astrocytes from WT or a previously characterized Ptn KO mouse model[21], validated using smFISH that Ptn KO mice do not have detectable *Ptn* (Figure S2A). We utilized a cell culture model where WT cortical neurons are grown in minimal media (without growth factors added), ACM from WT astrocytes, ACM from Ptn KO astrocytes, or Ptn KO ACM + recombinant Ptn (rescue ACM; Figure 2A). We added recombinant Ptn at 0.025 ug/well after determining a physiological range of Ptn secretion from WT cultured astrocytes (Figure S2B). Dendrites were labeled with Map2, combined with Ctip2 (Figure 2B-2E) to selectively identify Layer 5 pyramidal neurons[22]. As previously reported, neurons cultured with WT ACM showed enhanced dendrite outgrowth compared to minimal media (average of 155.6 ± 9.1 um in MM, 225.7 ± 19.7 um in WT ACM). However, neurons cultured with Ptn KO ACM had significantly stunted dendrite outgrowth (115.8 ± 10.1 um). When neurons were exposed to Ptn KO ACM + recombinant Ptn protein, the total dendrite length was recovered (180.1 ± 13.2 um; Figure 2E; Figure S2C). We also used Sholl analysis to determine if there were significant differences in branching patterns between the conditions as a measure of complexity. The number of Sholl intersections was graphed as a function of distance from the cell body (Figure

2G) and the area under the curves was quantified (Figure 2H). We observed that WT ACM induced more complex branching of dendrites (area of 121.7 ± 7.8 in MM, 181.3 ± 16.2 in WT ACM), whereas Ptn KO ACM impaired dendrite branching (90.4 ± 8.1) and Ptn KO ACM + recombinant Ptn protein was able to recover this phenotype (144.1 ± 11.0). These experiments highlight the role of astrocyte-secreted Ptn in regulating neuronal dendrite outgrowth and branching, providing further rationale that decreased levels of Ptn in DS may play a causal role in aberrant neural circuit formation.

Ptn KO mice phenocopy Ts65Dn Mut mice in dendrite outgrowth, dendrite branching and spine density phenotypes *in vivo*

Knowing that Ptn can induce dendrite outgrowth and branching *in vitro*, we next assessed whether Ptn KO mice have *in vivo* phenotypes similar to those reported in humans with DS, and whether these phenotypes are also present in Ts65Dn mice. We crossed the Ptn KO and Ts65Dn lines to a Thy1-YFP reporter line that sparsely labels Layer 5 pyramidal neurons[23]. Total dendrite length, dendritic complexity and spine density were analyzed at P30 and P120 to represent developmental and mature timepoints, respectively. We found that Layer 5 pyramidal neurons of Ts65Dn Mut mice have significantly shorter total dendrite length at P30 (average of 718.9 ± 108.4 um in WT, 432.5 ± 53.6 um in Mut, Figure A-C) and at P120 (644.7 ± 35.3 um in WT, 519.5 ± 42.4 um in Mut; Figure 3C), reflecting a developmental impairment that does not recover. Sholl analysis showed decreased dendritic complexity at both ages when area under the curve was quantified (P30: area of 537.4 ± 75.7 in WT, 305.1 ± 46.9 in Mut; P120: 454.2 ± 31.2 in WT, 333.7 ± 34.2 in Mut; Figure 3D). This validates that Ts65Dn Mut mice accurately reflect the human phenotypes observed in DS. We next performed the same quantifications in the Ptn x Thy1-YFP line. We observed that Ptn KO mice have shorter total dendrite length at P30 (average of 635.7 ± 81.2 um in WT, 389.0 ± 57.8 µm in KO; Figure 3E-G), but interestingly the dendrite length recovers by P120 (506.5 ± 74.5 um in WT, 535.1 ± 75.5 um in KO; Figure 3G). Similarly, Sholl analysis revealed decreased dendritic complexity in KO mice at P30 (area of 506.7 ± 61.4 um in WT, 310.8 ± 48.2 µm in KO) but the phenotype was recovered by P120 (367.0 ± 46.5 in WT, 391.7 ± 47.7 in KO; Figure 3H). Distributions for individual dendrite lengths and Sholl graphs are shown in the associated supplemental figure (Figure S3A-D).

As spine number and morphology are altered in DS, we quantified spine density, focusing on dendrites that had their first primary branching point in upper layers (most commonly Layer 2/3). Ts65Dn Mut mice had a significantly decreased spine density at P120 (average of 1.44 ± 0.054 spines/um in WT, 1.17 ± 0.038 spines/um in Mut; Figure 3I-J and M), but no statistically significant difference at P30 (average of 1.17 ± 0.068 spines/um in WT, 0.98 ± 0.083 spines/um in Mut; Figure 3M). Ptn KO mice also had reduced spine density at P120 (average of 1.67 ± 0.070 spines/um in WT, 1.30 ± 0.047 spines/um in KO; Figure 3K-L and N) but no phenotype was observed at P30 (average of 1.46 ± 0.096 spines/um in WT, 1.31 ± 0.066 spines/um in KO; Figure 3N). Data from individual dendrites are shown in the associated supplemental figure (Figure S3E and G). Spine morphology was also assessed using previously published criteria for immature filopodia, thin, and long thin spines as well as more mature stubby and mushroom morphologies[24,25]. A previous *in vitro* report demonstrated that mouse WT neurons cultured with DS astrocytes or ACM from DS astrocytes had a higher proportion of immature filopodia spines[11]. We observed an increased percentage of filopodia spines at P120 in Ts65Dn Mut mice (average of 11.5 ± 1.0% of total spines in WT, 20.3 ± 2.1% of total spines in Mut (Figure S3F), with Ptn KO mice showing the same trend (Figure S3H). The parallel phenotypes observed in Ptn KO and Ts65Dn Mut mice suggest that Ptn contributes to dendrite morphology as well as spine formation and/or maturation during neurotypical development, and further that decreased Ptn in Ts65Dn Mut astrocytes may contribute to neuronal phenotypes.

Overlapping synaptic phenotypes are observed in Ptn KO and Ts65Dn Mut mice

Ptn expression peaks during a robust period of synapse formation and maturation (Figure 1), so we hypothesized it may be involved in regulating excitatory synapse number. We assessed whether Ts65Dn Mut and Ptn KO mice have altered numbers of excitatory synapses compared to their WT counterparts. We used antibodies against the vesicular glutamate transporter 1 (Vglut1) and the GluA2 AMPA receptor subunit to label pre- and post-synaptic puncta of intracortical synapses, imaged in Layer 2/3. Ts65Dn Mut mice have a significant decrease in colocalized puncta (Figure 4A) at P30 (0.89 ± 0.028-fold compared to WT; Figure 4B) and this phenotype remains at P120 (0.82 ± 0.062-fold compared to WT; Figure 4C-D). Similarly, we found significantly decreased colocalized puncta (Figure 4E) at P30 in Ptn KO mice (0.83 ± 0.049-fold compared to WT), driven by a decrease in GluA2 puncta (0.92 ± 0.026-fold compared to WT) with no effect on Vglut1 puncta (0.97 ± 0.022-fold compared to WT; Figure 4F). These impairments remained at the P120 timepoint (Colocalized: 0.83 ± 0.043-fold, GluA2: 0.94 ± 0.019-fold, Vglut1: 0.96 ± 0.026-fold; Figure 4G-H).

We also examined thalamocortical synapses using Vglut2 with GluA2 colocalization in Layer 1. Interestingly, there were clear distinctions between the Ts65Dn Mut and Ptn KO mice in these analyses. Ts65Dn Mut mice did not show any deficits in GluA2, Vglut2, or colocalized puncta (GluA2: 1.022 ± 0.014-fold, Vglut2: 1.015 ± 0.035-fold, Colocalized: 1.024 ± 0.029-fold; Figure S4A) in Layer 1 at P30 (Figure S4B) or at P120 (GluA2: 1.068 ± 0.050-fold, VGlut2: 0.96 ± 0.055-fold, Colocalized: 0.99 ± 0.074-fold; Figure S4C-D). On the other hand, at P30 Ptn KO mice had decreased GluA2 (0.91 ± 0.025-fold), decreased Vglut2 (0.92 ± 0.021-fold) and decreased colocalized puncta (0.87 ± 0.046-fold; Figure 4SE-F). These differences remained at P120 (GluA2: 0.91 ± 0.028-fold, Vglut2: 0.90 ± 0.041-fold, Colocalized: 0.82 ± 0.044-fold; Figure S4G-H). The collective evidence demonstrates that both Ts65Dn Mut and Ptn KO mice have impaired intracortical synapse number in Layer 2/3, but only Ptn KO mice have impaired thalamocortical synapses. This implies additional roles of Ptn in thalamocortical synapse targeting that may be compensated for in Ts65Dn Mut mice.

Astrocyte-targeted pleiotrophin overexpression rescues dendrite and spine phenotypes in Ts65Dn Mut mice

The overlapping phenotypes observed in Ts65Dn Mut and Ptn KO mice provided a rationale to investigate if targeting overexpression of Ptn to Ts65Dn Mut astrocytes can rescue or ameliorate the neuronal phenotypes. A viral-mediated strategy was designed to overexpress Ptn, or the control spaghetti monster fluorescent protein (smFP)[26], using the minimal GfaABC1d (Gfap) promoter (Figure 5A). The PHP.eB serotype of adeno-associated virus was used to achieve brain-wide targeting (Figure 5SA-B), which allowed for assessment of complex behaviors that involve multiple brain regions. We injected virus at P60, after the dendrite and synapse phenotypes are detected at P30, allowing us to evaluate if Ptn can rescue circuit aberrations rather than just prevent the phenotypes from developing. Four subject groups of the Ts65Dn x Thy1-YFP mice were utilized: WT mice with smFP control (WT smFP), WT mice with Ptn overexpression (WT Ptn), Ts65Dn Mut mice with smFP control (Ts65Dn Mut smFP) and Ts65Dn Mut mice with Ptn overexpression (Ts65Dn Mut Ptn). YFP+ mice were used for dendrite and spine quantifications and YFP- mice were used for synaptic puncta quantifications. Behavioral analysis was initiated 7 weeks post-injection and tissue was collected 1 week later at 8 weeks post-injection (Figure 5B). Quantification of virus penetrance and specificity was performed in the visual cortex. Both the Gfap-Ptn and Gfap-smFP viruses (HA labeling, cyan) targeted ~60% of Sox9+ (magenta) cortical astrocytes (smFP: 58.2 ± 6.9%, Ptn: 62.9 ± 2.0%; Figure 5C-D). Both viruses were highly specific for astrocytes and transduction of NeuN+ neurons (magenta)

was negligible (smFP: 0.3 ± 0.3% of NeuN+ cells, Ptn: 0.3 ± 0.07% of NeuN+ cells; Figure 5E-F).

We analyzed total dendrite length, dendritic complexity and spine density, with representative filament tracings of dendrites depicted in (Figure 6A-D). Quantification showed that Ts65Dn Mut smFP dendrites are truncated compared to WT smFP (average of 789.2 ± 51.3 in WT smFP, 591.0 ± 55.5 um in Mut smFP) and overexpression of Ptn fully recovered the phenotype (818.3 ± 44.2 um). Overexpression of Ptn in WT mice showed a nonsignificant trend towards reduced dendrite length (647.6 ± 21.2 um; Figure 6E) that reached significance when analyzing the combined dendrites from all biological replicates (Figure S6A-B). Sholl analysis revealed that Ptn overexpression was also able to rescue the dendritic complexity phenotype in Ts65Dn Mut mice. The Ts65Dn Mut smFP dendrites had a decreased area under the curve (average area of 573.4 ± 36.4 in WT smFP, 391.4 ± 42.3 in Mut smFP) and Ts65Dn Mut Ptn dendrites did not differ from WT smFP (area of 552.6 ± 14.9). In this measure WT Ptn dendrites did not significantly differ from WT smFP (area of 479.7 ± 19.7; Figure 6F-G). Ts65Dn Mut smFP dendrites had reduced spine density compared to WT smFP (average of 1.66 ± 0.032 spines/um in WT smFP, 1.34 ± 0.091 spines/um in Mut smFP). Ts65Dn Mut Ptn dendrites had a full recovery of the spine density phenotype (1.69 ± 0.094 spines/um). WT Ptn dendrite spine density was not significantly altered from WT smFP (1.50 ± 0.077; Figure 6H-L; S6C). We observed an increased percentage of filopodia in Ts65Dn Mut smFP mice (average of 9.9 ± 0.8% in WT smFP, 15.0 ± 1.4% in Mut smFP), which was not impacted by Ptn overexpression (Mut Ptn 14.2 ± 1.5%). Interestingly, WT Ptn dendrites also had increased filopodia (14.60 ± 1.0%; Figure S6D). Altogether these findings reveal that increasing levels of astrocyte-derived Ptn can rescue neuronal dendrite length/complexity and spine density in DS.

Astrocyte-targeted pleiotrophin overexpression improves excitatory synapse phenotypes in Ts65Dn Mut mice

To ask if Ptn overexpression could improve the deficits in intracortical synapse formation we observed in Ts65Dn Mut mice, we performed immunohistochemical analysis of pre- and postsynaptic markers Vglut1 and GluA2 in WT smFP, WT Ptn, Ts65Dn Mut smFP and Ts65Dn Mut Ptn mice (Figure 7A-D). Ts65Dn Mut smFP mice had decreased colocalized puncta (Figure 7A-D) compared to WT smFP (0.66 ± 0.065-fold), while the Ts65Dn Mut Ptn mice showed no significant change compared to WT smFP (0.93 ± 0.092-fold), indicating an improvement of the synaptic phenotype with Ptn overexpression. WT Ptn mice also did not significantly deviate from WT smFP (0.86 ± 0.085-fold; Figure 7G). Vglut1 and GluA2 puncta were quantified individually, showing Ts65Dn Mut smFP mice have a significant decrease in Vglut1 puncta, while Ts65Dn Mut Ptn mice do not significantly deviate from WT smFP (Vglut1: 0.80 ± 0.049-fold in Mut smFP, 1.01 ± 0.092-fold in Mut Ptn, 0.96 ± 0.060-fold in WT Ptn; Figure 7E). There are no significant differences in GluA2 puncta in any group (GluA2: 0.84 ± 0.063-fold in Mut smFP, 1.030 ± 0.076-fold in Mut Ptn, 0.94 ± 0.060-fold in WT Ptn; Figure 7F). These results highlight the ability of Ptn to promote synapse formation in Ts65Dn Mut mice, even at more mature timepoints after developmental synaptogenesis has largely ceased.

Astrocyte-targeted pleiotrophin overexpression improves some aspects of cognitive function

Histological evaluation of the effects of Ptn overexpression in Ts65Dn Mut astrocytes demonstrated that Ptn can rescue dendrite outgrowth/branching and spine density phenotypes, as well as improve impairments in intracortical excitatory synapses. We next asked if the effects of Ptn overexpression can improve performance in behavioral assays. Previous work demonstrated that Ts65Dn Mut mice are hyperactive in the open field and have memory impairments in the spontaneous alternation Y maze and fear conditioning assays[27]. We tested

mice in these assays 7 weeks post-injection (Figure 8A). Locomotor activity in the open field was assessed over 10 minutes (Figure 8B). We detected a main genotype effect of hyperactivity in Ts65Dn Mut mice in the 2nd 5-minute block, but not the 1st 5-minute block. The observed hyperactivity was not rescued with Ptn (Figure 8C-D). We next assessed working memory using the spontaneous alternation Y maze. We found that Ts65Dn Mut smFP mice perform significantly fewer correct alternations than WT smFP (average of 47.0 ± 1.8% in Mut smFP, 56.9 ± 2.9% in WT smFP) and the Ts65Dn Mut Ptn mice displayed a ~4% recovery of this phenotype (51.0 ± 2.2%). WT Ptn mice performed similarly to WT smFP (57.0 ± 3.0%; Figure 8E). The Ts65Dn Mut mice, regardless of treatment, were hyperactive in this test and completed more arm entries than WT smFP mice (WT smFP: average of 45.5 ± 2.4 entries, WT Ptn: 49.7 ± 1.9, Mut smFP: 65.7 ± 4.0, Mut Ptn: 63.1 ± 3.2; Figure 8F).

We used fear conditioning to test associative learning, as well as contextual and cued memory. We did not observe differences in learning, as each group significantly increased freezing behavior after the 3rd shock compared to baseline (Figure 8G). Twenty-four hours later contextual and cued memory were assessed. For contextual memory, we did not detect any genotype difference in Ts65Dn Mut mice compared to WT smFP (WT smFP: 25.9 ± 4.1%, Mut smFP: 21.3 ± 2.9%, Mut Ptn: 26.1 ± 2.9%; Figure 8H). For cued memory, Ts65Dn Mut smFP mice were significantly impaired compared to WT smFP (average of 14.0 ± 2.67% in Mut smFP, 26.0 ± 3.5% in WT smFP). Ts65Dn Mut Ptn mice were not significantly different from WT smFP in freezing behavior (17.0 ± 3.1%) and WT Ptn mice performed similarly to WT smFP (25.5 ± 3.5%; Figure 8I). Behaviors for each group were also assessed as z-scores deviating from WT smFP, with three main findings remaining consistent. First, Ts65Dn Mut smFP mice have significantly impaired working memory in the Y maze, but Ts65Dn Mut Ptn mice do not significantly deviate from WT smFP. Second, Ts65Dn Mut mice, regardless of treatment, are hyperactive in the number of arm entries in the Y maze. Third, Ts65Dn Mut smFP mice have significantly impaired cued fear memory while Ts65Dn Mut Ptn are not significantly changed from WT smFP. In addition, when assessed by z-score, WT Ptn mice exhibit significantly reduced freezing in the contextual memory paradigm (Figure 8J). These findings demonstrate that Ptn treatment in the Mut context can modestly improve function on memory tasks but does not affect hyperactivity. Additionally, aberrantly high levels of Ptn in the WT context can dampen contextual memory.

Discussion

We find that astrocyte-derived pleiotrophin is decreased in the Ts65Dn mouse model of DS, and through characterization of Ptn KO mice we identify important neurodevelopmental phenotypes that are mirrored in Ts65Dn Mut mice. The use of systemic injections of AAV PHP.eB serotype allowed for localized analysis of neuronal morphology and synapses in a well-characterized region, as well as evaluation of circuit level cognitive function that involves multiple brain regions. We demonstrate that targeting Ptn overexpression specifically to astrocytes in Ts65Dn rescues neuronal dendrite length/complexity and spine density phenotypes, as well as partially improves the observed deficits in excitatory synapse number. These histological findings correlate with functional improvements in memory tasks that are known to be perturbed in DS. Importantly, these improvements are seen when delivering Ptn to Ts65Dn mice after phenotypes are already established, showing reversibility. The results of this study highlight the importance of astrocyte regulation of neuronal structure/morphology and neuronal synapses in the context of neurodevelopmental disorders, and provide evidence that manipulating levels of pro-synaptogenic astrocyte-secreted molecules may be an effective therapeutic strategy even after the classical neurodevelopmental windows for synapse formation have closed.

Roles of astrocyte-derived Ptn in the context of DS and other neurodevelopmental disorders

Multiple studies on Ptn demonstrate its regulatory roles in axon and dendrite outgrowth and maintenance of spine density[13,14,28]. Stunted dendrite outgrowth/branching and decreased spine density are well-characterized phenotypes in the cortex and hippocampus of DS brains. However, a link between Ptn and DS had not been previously reported and is a major finding of the present study. Importantly, we identified Ptn as a candidate molecule through mining of our recently published dataset describing transcriptomic and proteomic changes in astrocytes from several neurodevelopmental disorders[12]. Ptn protein was found at significantly decreased levels in the ACM from Rett Syndrome and DS astrocytes and there was also a strong trend towards reduction in Fragile X Syndrome astrocytes, implying that Ptn reduction may be a commonality in the neural circuit impairments observed across multiple neurodevelopmental disorders.

The data in this study support the concept that astrocyte-secreted proteins can be used as therapeutic tools in DS, and to our knowledge this is the first in vivo demonstration of this concept. Previous in vitro work demonstrated that WT neurons cultured with DS astrocytes develop spine density and synaptic deficits[11,29,30]. The present study demonstrates that in vivo astrocytes can be manipulated to correct circuit aberrations in a murine model. Another group showed in vivo recovery of spine density with interventions to deplete microglia and reduce inflammation using anti-inflammatory drug treatment. These interventions were performed in the Dp(16) mouse model at P16 and P20, which roughly correlates to childhood to early pubescent years (~3-15 years) in a human[31,32]. Our intervention at P60 is more equivalent to young adulthood, demonstrating that circuit impairments can be reversed. The findings of this study highlight the therapeutic potential of targeting astrocyte-secreted proteins to promote proper circuit formation and that this approach can prove effective even outside of the typical developmental windows.

Roles of Ptn in neurotypical development

In the present study we found remarkable overlap in the neurodevelopmental phenotypes observed in Ts65Dn Mut and Ptn KO mice but there were also notable differences. Both mouse models had decreased levels of intracortical excitatory synapses in the upper cortical layers. However, Ptn KO mice also had distinct synaptic phenotypes from Ts65Dn Mut mice, showing reduced levels of Vglut2 in Layer 1 which correspond to the thalamocortical axon projections[33].

Given the demonstrated roles of Ptn in neurite outgrowth, we hypothesize that Ptn may act as a guidance factor for the long-range thalamocortical projections during early cortical development. These Vglut2 deficits were present at P30 and P120, implying that this is not merely a developmental delay in circuit formation and rather could be related to impaired navigation to the target innervation zone during development. It will be interesting in future studies to investigate if Ptn KO mice have any deficits in visual acuity.

Secondly, the total dendrite length and complexity phenotypes in Ts65Dn mice are present at both the P30 and P120 timepoints, but Ptn KO mice recover from this phenotype by P120. These findings suggest that in the case of genetic loss of *Ptn*, the phenotype may be related to a developmental delay, or there may be other local compensatory factors that are able to recover dendrite outgrowth. For example, midkine is a closely related paralogue of Ptn that has overlapping, but not identical, functions[34]. The Layer 5 pyramidal neurons that were stunted in their length and complexity have dendrites that grow towards the pial surface, and again, a functional role of Ptn as a guidance factor may come into play during the development of cortical circuitry. A genetic loss of *Ptn* could be compensated for by midkine in the Ptn KO model, whereas an incomplete loss of Ptn in Ts65Dn Mut mice may not trigger the same compensatory mechanisms.

Importance of maintaining homeostatic levels of astrocyte-derived factors

Many astrocyte-derived factors have been shown to promote synapse formation and maturation. However, most of these studies were performed with KO mouse models showing that loss of the factor of interest is harmful to the circuitry[10,25,35-37]. There is currently a knowledge gap regarding circuit outcomes when astrocyte-derived factors are expressed at levels higher than what is physiological. In this study we find that overexpression of Ptn in the WT context impairs total dendrite length. We also observed decreased freezing with Ptn overexpression in WT mice in the contextual memory assay. Work from the hippocampus has shown that Ptn is an injury-induced factor. *Ptn* mRNA is decreased transiently following forebrain ischemic insult or excitotoxic injury from kainic acid injection, but four days post-injury *Ptn* is up-regulated[38]. The transient decrease in *Ptn* expression after excitotoxic injury suggests it may be down-regulated in response to high levels of activity but up-regulated when tissue repair begins and formation of new synapses is prioritized. In a non-diseased context, up-regulation of Ptn may have harmful effects on synaptic transmission, but in a diseased context where synapses are not maintained at homeostatic levels then Ptn overexpression may be beneficial, as we have demonstrated in the context of DS. These are important considerations when evaluating astrocyte-secreted proteins as potential therapeutics during safety studies in clinical trials that assess healthy subjects.

In conclusion we present evidence that Ptn is an astrocyte-secreted protein that regulates neuronal dendrite morphology and synaptic circuitry during neurotypical development. We demonstrate that Ptn is down-regulated in DS astrocytes from a commonly used mouse model and use viral-mediated overexpression to fully rescue dendrite and spine phenotypes, partially restore excitatory intracortical synapse phenotypes and improve behavioral outcomes. This work opens investigative avenues for astrocyte-based therapeutics that may be relevant to multiple neurodevelopmental disorders.

Supplemental Information

Supplemental information includes 6 figures.

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Author Contributions

ANB and NJA conceived the project, designed experiments, analyzed data, and wrote the manuscript. ANB, QNA and MM performed experiments and analyzed data.

Declaration of interests

The authors declare no competing interests.

Figure Legends

Figure 1. Developmental expression of astrocyte *Ptn* mirrors the timeframe for formation and maturation of cortical synapses and is reduced in Ts65Dn Mut astrocytes.

(A) Timeline of developmental events in visual cortex. (B) Astrocyte RiboTag RNA-Sequencing data from visual cortex shows peak *Ptn* expression in astrocytes corresponding to timeframe for synapse formation and maturation. Data from Farhy-Tselnicker et al., 2021. (C-E) Representative images of *Ptn* mRNA (magenta) in Aldh1L1-EGFP+ astrocytes (cyan) at P7, P14 and P30. Scale bars = 10 um. (F-G) Quantification of *Ptn* area (a.u.) in astrocytes across development in Layer 1 and Layer 2/3. Statistics by one-way ANOVA with Tukey's. (H-I) Example images of *Ptn* (magenta) in *Slc1a3*+ astrocytes (cyan) at P14 in Layer 2/3 of WT and Ts65Dn Mut mice. Scale bars = 10 um. (H'-I') Zoomed images of *Ptn* within WT and Ts65Dn Mut astrocytes. Scale bars = 5 um. (J-K) Quantification of average *Ptn* area within Ts65Dn Mut astrocytes normalized to WT in Layer 1 and Layer 2/3. Statistics by one-sample t-test. N = 5 mice/timepoint in C-G and N = 5 mice/group in H-K.

Figure 2. Genetic loss of Ptn from astrocytes impairs dendrite outgrowth and dendritic complexity *in vitro*.

(A) Schematic demonstrating the *in vitro* experimental design to determine if astrocyte-derived Ptn modulates dendrite outgrowth and branching in WT cortical neurons. (B) Example images of each culture condition (MM: top left; WT: top right; Ptn KO: bottom left; Ptn KO + recombinant Ptn: bottom right) showing pyramidal neurons labeled by Ctip2 (magenta; arrowheads) and their dendrites labeled by Map2 (cyan). Scale bars = 20 um. (C) Quantification of average total dendrite length for each condition shows that WT ACM induces dendrite outgrowth, whereas Ptn KO ACM fails to induce outgrowth above minimal media. Addition of recombinant Ptn (0.025 ug) to Ptn KO ACM restores dendrite outgrowth. Statistics by one-way ANOVA with Tukey's. (D) Sholl curves of each culture condition representing the number of dendrite branches at 10 um increments from the cell body. (E) Quantification of area under the curve for Sholl graphs. WT ACM induces dendrite branching whereas Ptn KO ACM impairs branching and addition of Ptn protein to KO ACM restores branching. Statistics by one-way ANOVA with Tukey's. N = 4 independent experiments.

Figure 3. Ptn KO and Ts65Dn Mut mice display parallel dendrite and spine phenotypes

(A-B) Example images of Ts65Dn WT and Mut Layer 5 pyramidal neurons at P30. Scale bars = 50 um. (C) Data showing total dendrite length is reduced in Ts65Dn Mut mice at P30 and P120. (D) Quantification of area under the curve from Sholl analysis shows that Ts65Dn Mut mice have less complex branching patterns than WT mice at both ages. (E-F) Example images of dendrites in Ptn WT and KO mice at P30. Scale bars = 50 um. (G) Total dendrite length is reduced in Ptn KO mice at P30 but recovers at P120. (H) Quantification of area under the curve for Sholl analysis shows that Ptn KO mice have reduced dendritic complexity at P30 that recovers by P120. (I-L) Example images of dendritic spines in Ts65Dn WT and Mut mice (I-J) and Ptn WT and KO mice (K-L) at P120, imaged in Layers 1-3. Scale bars = 2 um. (M-N) Quantification of average spine density shows Ts65Dn Mut mice have reduced spine density at P120 (M) and Ptn KO mice have reduced spine density at P120 (N). N = 6 mice/group. All statistics by unpaired t-test except P120 Ts65Dn AUC (D) and P30 Ts65Dn spine density (M) with Mann-Whitney.

Figure 4. Ts65Dn Mut and Ptn KO mice have similar phenotypes in intracortical excitatory synapses

(A) Example images of Vglut1 (magenta) and GluA2 (cyan) labeling pre- and postsynaptic puncta, respectively, and their colocalization (white arrows) in the merged images at P30 in Ts65Dn WT and Mut mice, in Layer 2/3 of visual cortex. (B) Quantification at P30 shows a significant decrease in colocalized puncta. (C) Example images as in (A) at P120 in Ts65Dn WT and Mut mice. (D) Quantification shows Ts65Dn Mut mice have decreased colocalized puncta at P120. (E) Example images of Vglut1 (magenta) and GluA2 (cyan) and their colocalization (white arrows) at P30 in Ptn WT and KO mice. (F) Ptn KO mice have a significant decrease in post-synaptic GluA2 puncta and colocalized puncta at P30. (G) Representative images as in (E) at P120 in Ptn WT and KO mice. (H) Quantification at P120 shows the decreased GluA2 and colocalized puncta phenotypes remain at this age. Scale bars for all images = 2 um. N = 6-8 mice/group. Statistics by one-sample t-test for all except P120 Ts65Dn Colocalized Puncta and P120 Ptn KO GluA2 puncta assessed by one-sample Wilcoxon Signed-Rank test.

Figure 5. Viral overexpression strategy efficiently and specifically targets astrocytes.

(A) Cartoon schematic of targeting viruses. The *GfaABC1d* promoter was used to target astrocytes to either overexpress the control smFP containing 10 HA tags (bottom) or Ptn with an HA tag (top). (B) Timeline of experimental design for viral injection, behavior and collection for histology. (C) Example images of virus labeling with anti-HA (cyan) in Sox9+ (magenta) astrocytes. (D) Quantification of the percentage of Sox9+ astrocytes expressing HA for each virus. (E) Representative images showing that virus (anti-HA, cyan) does not co-label with NeuN (magenta). (F) Quantification of virus expression in neurons shows <0.5% of total neurons have off-target labeling. Scale bars = 50 um. N = 4 mice/group. Statistics by unpaired t-test.

Figure 6. Astrocyte-derived Ptn is a sufficient factor to rescue dendrite and spine phenotypes in Ts65Dn Mut neurons.

(A-D). Example filament tracings of dendrites at P120 for each experimental group: WT smFP, WT Ptn, Ts65Dn Mut smFP, Ts65Dn Mut Ptn. The cell bodies of the pyramidal neurons reside in Layer 5 (L5) and the dendrites grow towards Layer 1 (L1). Scale bars = 100 um. (E.) Total dendrite length is shorter in Ts65Dn Mut smFP compared to WT smFP, and Ts65Dn Mut Ptn dendrites are rescued. N = 6 mice/group. Statistics by two-way ANOVA with Tukey's test. Genotype: p = 0.7636; Treatment: p = 0.3532; Interaction: p = 0.0006. (F) Sholl curves quantifying dendrite branching points at 50 um increments from the cell body. (G) Area under the curve is decreased in the Ts65Dn Mut smFP group and Ts65Dn Mut Ptn dendrites recover from the reduced branching phenotype. Statistics by two-way ANOVA with Tukey's. Genotype: p = 0.0889; Treatment: p = 0.2815; Interaction: p = 0.0005. (H-K) Example images of dendritic spines for WT smFP (H), WT Ptn (I), Ts65Dn Mut smFP (J) and Ts65Dn Mut Ptn (K) groups. Scale bars = 2 um. (L) Spine density is decreased in the Ts65Dn Mut smFP group and Ts65Dn Mut Ptn dendrites recover from the phenotype. N = 8 mice/group. Statistics by two-way ANOVA with Tukey's. Genotype: p = 0.3753; Treatment: p = 0.2279; Interaction: p = 0.0027.

Figure 7. Synaptic phenotypes are improved with Ptn overexpression.

(A-D) Example images of Vglut1 (magenta), GluA2 (cyan) and colocalized puncta (white arrows) in Layer 2/3 for WT smFP (A), WT Ptn (B), Ts65Dn Mut smFP (C) and Ts65Dn Mut Ptn (D). Scale bars = 2 um. (E) Vglut1 puncta are decreased in the Ts65Dn Mut smFP group compared to WT smFP. WT Ptn and Ts65Dn Mut Ptn groups have similar Vglut1 levels as WT smFP. (F) GluA2 puncta levels are similar between all groups. (G) Colocalized puncta are decreased in the Ts65Dn Mut smFP group compared to WT smFP. WT Ptn and Ts65Dn Mut Ptn have similar levels of colocalized puncta as compared to WT smFP. All groups normalized to average of WT smFP group. Statistics by two-way ANOVA with Dunnett's. For Vglut1: Genotype: p = 0.179; Treatment: p = 0.1431; Interaction: p = 0.0398. For GluA2: Genotype: p = 0.5986; Treatment: p

= 0.3386; Interaction: p = 0.0790. For Colocalized puncta: Genotype: p = 0.1746; Treatment: p = 0.5103; Interaction: p = 0.0382. N = 8 mice/group.

Figure 8. Ptn treatment results in mild improvement on memory tasks in Ts65Dn Mut mice

(A) Cartoon schematic of behavioral testing schedule. (B) The distance traveled in the open field across a 10-minute duration in 1-minute blocks is shown for each group. (C-D) Quantification of total distance during the 1st 5-minute block (C) and 2nd 5-minute block (D) shows a main genotype effect of hyperactivity in Ts65Dn Mut mice in the 2nd 5-minute block. Statistics by Kruskal-Wallis with Dunn's (C; p = 0.9296) and two-way ANOVA with Dunnett's (D; Genotype: p = 0.0100; Treatment = 0.8414; Interaction: p = 0.5368). (E) Ts65Dn Mut smFP mice have a decreased percentage of correct alternations in the spontaneous Y maze test. WT Ptn and Ts65Dn Mut Ptn performed at similar levels to WT smFP. Statistics by two-way ANOVA with Dunnett's. Genotype: p = 0.0033; Treatment: p = 0.4207; Interaction: p = 0.4647. (F) Ts65Dn Mut mice regardless of treatment entered more arms during the test duration than their WT counterparts. Statistics by two-way ANOVA with Dunnett's. Genotype: p < 0.0001; Treatment: p = 0.7771; Interaction: p = 0.2457. (G) In fear conditioning mice from each group learned to associate the tone with the foot shock as assessed by the percentage of time freezing after the 3rd shock compared to baseline freezing within a group. Statistics by multiple Mann-Whitney tests with Benjamini, Krieger and Yekutieli correction. (H) We did not observe statistically significant differences in post-hoc comparisons between groups in the percentage of time freezing during the contextual memory paradigm, but the main ANOVA effect is significant. Statistics by Kruskal-Wallis with Dunn's. p = 0.0504. (I) Ts65Dn Mut smFP mice exhibited significantly reduced freezing behavior compared to WT smFP in the cued memory paradigm. WT Ptn performed similarly to WT smFP. Ts65Dn Mut Ptn were not significantly different than WT smFP. Statistics by Kruskal-Wallis with Dunn's. p = 0.0196. (J) A summary of z-scores for each behavioral test is presented as the deviation of each group from WT smFP. Blue shading signifies a decrease in average z-score and red shading signifies an increase in average z-score. The numeric value of the z-score for each comparison is listed in each box. *** p ≤ 0.001; ** p ≤ 0.01, * p ≤ 0.05. Statistics by two-way ANOVA with Dunnett's (open field and spontaneous Y maze) or Kruskal-Wallis with Dunn's (fear conditioning). For all tests, N = 18-24 mice/group.

STAR Methods

Resource Availability

Lead contact

Requests for additional information and sharing of resources and reagents should be directed to the lead contact, Nicola J. Allen (nallen@salk.edu).

Materials availability

The GfaABC1d-smFP-HA and GfaABC1d-Ptn-HA AAV PHP.eB viral constructs were generated for this study. Original plasmids are available upon request.

Experimental model and subject details

Mice

All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Salk Institute for Biological Studies. Mice were housed in the Salk Institute Animal Resources Department on a light cycle of 12 hours light:12 hours dark with access to food and water *ad libitum*. Mice of both sexes were used for all experiments between the ages of postnatal day (P)0 and P120. For experiments using developmental timepoints, littermates were used. For P120 experiments within-colony controls were used.

Aldh1L1-EGFP mice

Tg(Aldh1l1-EGFP)OFC789Gsat/Mmucd (Stock 011015-UCD) were used for analysis of developmental *Ptn* mRNA expression in neurotypical development (in situ data).

Ptn KO mice

Ptn WT and KO mice were used for *in vitro* neuron and astrocyte cell culture experiments. Homozygous WT and homozygous KO crosses were used to obtain larger numbers of pups of each genotype for *in vitro* assays. For propagation and maintenance of the Ptn line, heterozygous crosses were used. WT and KO progeny from the heterozygous crosses were used for the synaptic characterization experiments. Mice were a kind gift from T.F. Vogt at Princeton University and were originally described in[21].

Ptn x Thy1-YFP mice

The Ptn line was crossed to the B6.Cg-Tg(Thy1-YFP)HJrs/J line (Jax Stock 003782) to sparsely label Layer 5 pyramidal neurons[23]. The line was maintained as heterozygous for YFP. The YFP+ progeny that were WT for Ptn or KO for Ptn were used for characterization of dendrite length, dendritic complexity and spine density.

Ts65Dn mice

B6EiC3Sn.BLiA-Ts(1716)65Dn/DnJ mice were originally described in[39] and obtained from Jax (Stock 005252). These mice were used for synaptic characterizations. The line was propagated and maintained by crossing female mutants to wildtype males on the same background due to male sterility.

Ts65Dn x Thy1-YFP mice

The Ts65Dn line was crossed to the B6.Cg-Tg(Thy1-YFP)HJrs/J line (Jax Stock 003782) to obtain YFP-labeled Layer 5 pyramidal neurons. The line was maintained as heterozygous for YFP. Due to the Thy1-YFP mice being on a different background than the Ts65Dn mice, only the F1 progeny were used for experiments to avoid extensive backcrossing. Thy1-YFP breeders were imported from Jax as needed. The euploid and trisomic YFP+ progeny were used for characterization of dendrite length, dendritic complexity and spine density experiments, as well as the associated rescue experiments. The euploid and trisomic YFP- progeny were used for the synaptic rescue experiments.

Cell cultures

Primary astrocyte and neuron cultures were obtained using the Miltenyi MACS isolation systems for astrocytes and neurons described in detail below. P6-7 pups (4 pups/experiment) were used

for primary astrocyte isolation. P0-1 pups (2 pups/experiment) were used for primary neuron isolation.

Primary astrocyte cultures

The cortices were dissected and meninges removed in ice-cold DPBS. The cortices were cut into ~1mm^3 pieces using a razor blade to allow for optimal enzymatic digestion. Papain digestion was performed according to the kit instructions (Miltenyi MACS Neural Tissue Dissociation Kit 130-092-628). Digestion was performed using the gentleMACS Octo Dissociator with Heaters (Miltenyi 130-096-427) using the 37C_NTDK_1 protocol provided by Miltenyi with a total time of 22 minutes. Cells were centrifuged at 4C 300 g for 10 minutes and then resuspended in AstroMACS buffer and passed through a 70-um basket filter to remove clumps. Astrocytes were subsequently isolated using the Miltenyi MACS Anti-ACSA-2 Microbead kit (Miltenyi 130-097-678) according to the manufacturer's instructions, with slight modifications to deplete myelin debris and microglial cells prior to positive astrocyte selection. Myelin was depleted using a 10-minute incubation at 4C with anti-myelin beads (Miltenyi 130-104-257). The MS magnetic separator column was then used to deplete the myelin from the cell suspension via magnetic separation. The flow-through was collected and centrifuged at 4C 300 g to remove excess beads. Microglia depletion was performed using anti-CD11b beads (Miltenyi 130-093-634) for an incubation period of 10 minutes at 4C. The MS magnetic separator column removed the microglia from the cell suspension via magnetic separation. The flow-through was collected and centrifuged again at 4C 300 g to remove excess beads. The supernatant was collected and subsequent positive selection for astrocytes was performed. First a 10-minute incubation with FcR blocking beads was used to minimize any nonspecific binding. ACSA2-conjugated beads were then used to positively select for astrocytes during a 15-minute incubation period at 4C (FcR and ACSA2 beads in kit, Miltenyi 130-097-678). The MS column was used to magnetize the ACSA2-beads and collect the astrocytes. The magnetized astrocytes were plunged into a new tube with AstroMACS buffer and centrifuged again at 4C 300 g to remove excess beads. Cells were counted using Trypan Blue solution (Gibco 15250-061) and plated in 6-well plates (Falcon 353046) coated with poly-D-lysine (Sigma P64047) at a density of 40,000-60,000 cells per well. Astrocytes were grown to confluency over 7 days *in vitro* (DIV) in astrocyte growth medium containing: 50% DMEM (Thermo Fisher Scientific 11960044), 50% Neurobasal (Thermo Fisher Scientific 21103049), Penicillin-Streptomycin (LifeTech 15140-122), Glutamax (LifeTech 35050-061), sodium pyruvate (LifeTech 11360-070), N-acetyl-L-cysteine (Sigma A8199), SATO (containing transferrin (Sigma T-1147), BSA (Sigma A-4161), progesterone (Sigma P6149), putrescine (Sigma P5780) and sodium selenite (Sigma S9133)), and heparin binding EGF like growth factor (HbEGF,5 ng/mL; R&D systems 259-HE/CF). Astrocyte cultures were maintained in a humidified incubator at 37C/10% CO2.

Collection of astrocyte conditioned media (ACM)

The growth media was removed from the 6-well plates after 7DIV and the wells were washed three times with warm Dulbecco's Phosphate Buffered Saline (DPBS; HyClone SH30264.01). Astrocytes were then cultured in minimal low protein conditioning media containing: 50% DMEM, 50% Neurobasal, penicillin-streptomycin, glutamax, sodium pyruvate, and HbEGF. The culture was maintained in a humidified incubator at 37C/10% CO2 for 4DIV. The conditioned media was concentrated using Vivaspin-6 centrifugal concentrators with a molecular weight cut-off of 10 kDa (Sartorius VS0602). Protein concentration in the ACM was assessed using a Qubit 4 fluorometer (Invitrogen Q33238) and the Qubit protein assay kit (Invitrogen Q33211). The ACM for neuronal dendrite outgrowth assays was stored at 4C for no longer than 1 week before use. The ACM used for the neuronal dendrite outgrowth assays was produced from either WT astrocytes or Ptn KO astrocytes.

Primary neuron cultures

The cortices were dissected and meninges removed in ice-cold DPBS as described above. Papain digestion was performed the same as for primary astrocyte isolation. Cells were

centrifuged at 4C 300 g for 10 minutes and then resuspended in cold DPBS and passed through a 70-um basket filter to remove clumps. Neurons were subsequently isolated by biotin-conjugated magnetic bead depletion of non-neuronal cells using the Miltenyi Neuron Isolation Kit according to the manufacturer instructions (Miltenyi 130-115-389). Depletion with the non-neuronal antibody cocktail was performed for 5 minutes at 4C followed by centrifugation at 300 g (4C) for 10 minutes. Anti-biotin beads were then used to remove the non-neuronal cells during a 10-minute incubation at 4C. The cell suspension was applied to the Miltenyi LS column and the flow-through (now a neuron-enriched cell suspension) was collected. Trypan Blue was used to count cells and the neurons were plated on glass coverslips (12 mm diameter, Carolina Biological Supply 633029) coated with poly-D-lysine and laminin (R&D 3400-010-01) in 24-well plates (Falcon 353047) at a density of 40,000-60,000 cells per well. Neurons were cultured in either minimal media (MM), MM + WT ACM, MM + Ptn KO ACM or Ptn KO ACM + 0.25 ug recombinant Ptn ("Rescue ACM"; see below). The MM contained 50% DMEM, 50% Neurobasal, Penicillin-Streptomycin, Glutamax, sodium pyruvate, N-acetyl-L-cysteine, insulin (Sigma I6634), triiodo-thyronine (Sigma T6397), SATO, B27 (containing L-carnitine hydrochloride (Sigma C0283), ethanolamine (Sigma E9508), D(+)-galactose (Sigma G0625), putrescine (Sigma P5780), sodium selenite (Sigma S9133), corticosterone (Sigma C2505), linoleic acid (Sigma L1012), lipoic acid (Sigma T1395), progesterone (Sigma P8783), retinyl acetate (Sigma R7882), retinol (Sigma 95144), D,L-alpha tocopherol acetate (Sigma T3001), bovine albumin (Sigma A4161), catalase (Sigma C40), glutathione (Sigma G6013), insulin (Sigma I6634), superoxide dismutase (Sigma S5395), transferrin (Sigma T5391), triiodol-l-thyronine (Sigma T6397)), and forskolin (Sigma F6886). Cortical neuron cultures were maintained in a humidified incubator at 37C/10% CO2.

Method Details

Treatment of neuronal cultures for dendrite outgrowth assays

Primary neurons isolated from WT mice were cultured for 4DIV in either MM, WT ACM, Ptn KO ACM or Ptn KO ACM + 0.025 ug recombinant human Ptn (Rescue ACM, R&D Systems 252-LP-050). 1.5 ug/well of ACM was added to MM for the conditions receiving ACM treatment. The neurons were treated twice with ACM, at 0DIV and again at 2DIV. At 4DIV the neurons were collected for analysis (see methods details for immunocytochemistry below).

Dendrite outgrowth assay/immunocytochemistry

The cover slips with neurons were washed three times with warm DPBS followed by 20 minutes fixation with room temperature 4% paraformaldehyde (PFA, Electron Microscopy Sciences 50980487) diluted in PBS. Coverslips were washed three times with room temperature DPBS and subsequently blocked/permeabilized in a solution of 50% goat serum (Life Technologies 16210072)/50% antibody buffer (containing 150 mM sodium chloride (Sigma S5886); 50 mM Trizma base (Sigma T6066), 100 mM L-lysine (Sigma L5501), 1% bovine albumin (Sigma A2153), pH 7.4) and 0.5% Triton X-100 (Sigma T9284). The blocking step was performed for 30 minutes. Coverslips were then washed once in PBS and incubated overnight at 4C with antibodies against Map2 (1:2500, chicken, EnCor Biotechnologies CPCA-Map2) and Ctip2 (1:500, rat, Abcam ab18465). The next day coverslips were washed three times in PBS and incubated for two hours with appropriate secondary antibodies (goat anti-chicken 488, 1:500, Thermo Fisher Scientific A-11039) and goat anti-rat 555, 1:500, Thermo Fisher Scientific A-21434) in a solution of antibody buffer with 10% goat serum. Coverslips were washed three times in room temperature PBS and then stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Thermo Fisher Scientific 62247) for 5 minutes. Excess DAPI was washed off with one PBS wash and then coverslips were mounted onto glass slides using Fluoromount G (Southern Biotech 0100-01). Imaging was performed with a 20x objective on a Zeiss LSM 700 confocal microscope. Z-stacks were collected with 1.0 um steps and a total of 5 steps. The maximum

intensity projection images were used to assess total dendrite length and complexity. For each condition a total of 3 coverslips (technical replicates) were imaged per experiment. Ten images were acquired for each coverslip for a total of 30 images per condition in each experiment. Each Ctip2+ cell in the image was quantified if its entirety of dendrite labeling was captured in the image. Quantification of total dendrite length and Sholl analysis for dendritic complexity was performed in Imaris v9.2.0 (Oxford Instruments) using the filament tracing tool. Sholl analysis was performed with a resolution of 10 um. Four independent experiments were performed and analysis of dendrite length was conducted on both the average of cells within an experiment (Figure 2) and on a per cell basis (Figure S2) with the same results.

Western blotting

Western blotting was used to determine the physiological range of Ptn secretion from astrocytes. For determination of the physiological range of Ptn secretion from cultured astrocytes, four independent culture experiments were used. The ACM total protein concentration was assessed using the Qubit Protein Assay kit and ACM was loaded into the wells at inputs of 3 ug, 6 ug and 9 ug. Recombinant human Ptn protein was added into the wells at: 0.1 ug, 0.2 ug and 0.3 ug. The ACM and recombinant protein samples were heated in reducing loading dye (Thermo 39000) at 55C for 45 minutes. The samples were separated on 4-12% Bis-Tris gradient gels (Invitrogen NW04122BOX) at 80V for 1.5 hours in MOPS SDS buffer (Invitrogen B0001.02). Proteins were transferred to PVDF membrane (Millipore IPFL00010) at 100V for 1 hour in an ice bucket using Tris-Glycine buffer (Thermo Fisher Scientific 28363) with 20% methanol (Fisher A412-4). The membrane was blocked in blocking buffer containing 0.1% Casein (BioRad 1610782) in Tris Buffered Saline (TBS, BioRad 105300272) for one hour at room temperature on a shaker. The membrane was incubated overnight at 4C in blocking buffer with anti-Ptn antibody (1:500, goat, R&D Systems AF-252-PB). The next day the membrane was washed three times for ten minutes each in TBS with 0.1% Tween-20 (Sigma P9416) followed by secondary antibody (diluted 1:20,000 in blocking buffer + 0.1% Tween, donkey anti-goat 680, Invitrogen A21084) incubation for one hours at room temperature on a shaker. The membrane was washed three times for ten minutes each in TBS and the bands were visualized using the Odyssey Clx Infrared Imager (Li-Cor) and the band intensity was analyzed using Image Studio software (Li-Cor). The band intensities for WT ACM inputs were compared as ratios to the known input of recombinant Ptn to get an estimate of the fraction of Ptn protein of total protein in the ACM. These experiments yielded a range of 0.01-0.048 ug of Ptn in 1.5 ug of WT ACM. Based on this range, the final value of 0.025 ug Ptn was chosen for the dendrite outgrowth experiments described above. Molecular weights were visualized using either Precision Plus All Blue Protein standard loading dye (BioRad 1610373.

Cloning of plasmids for viral vectors

Untagged mouse Ptn cDNA clone in a PCMV6-Kan/Neo vector was obtained from oriGene (Accession No. NM_008973, Origene MC204839). The pCAG_smFP-HA vector was obtained from Addgene (#59759). Ptn and smFP-HA were separately inserted into pZac2.1 GfaABC1d-tdTomato vector obtained from Addgene (#44332). The In-Fusion HD cloning kit (Takara 638909) was used according to the manufacturer's instructions. The GfaABC1d-tdTom vector was linearized at the NheI and XbaI (New England Biolabs R3131S, R0145S) restriction sites for three hours at 37C and the linearized vector was purified from an agarose gel and cleaned using Nucleospin columns (Macherey-Nagel 740609.50). The Ptn cDNA was PCR amplified and an HA tag added to the C-terminus using the following primer sequences with 15-16 bp overlapping sequences to the linearized vector:

Forward (5' to 3'): ctcactataggctagcatgtcgtcccagcaatatca

Reverse (5' to 3'): caggagaagatgctggattatccgtatgatgttccggattatgcatagtctagagtcga

The PCR product was subsequently gel purified and cleaned with the Nucleospin columns. The In-Fusion HD cloning enzyme premix was used to insert the Ptn-HA sequence into the vector.

The smFP-HA was cloned into the GfaABC1d-tdTom vector using the same procedure. The primer sequences used for smFP are as follows:

Forward (5' to 3'): ctcactataggctagcatgtacccttatgatgtgcccgattatgct

Reverse (5' to 3'): gtacgatgtcccggactacgcttaatctagagtcgacccgg

Stellar competent E. Coli cells (Takara 636763) were then transformed with the plasmids via heat-shock according to the manufacturer's instructions. Bacteria were screened using carbenicillin resistance (100 ug/mL; Teknova C2130). The colonies were expanded in an overnight LB culture. The DNA was purified using an endotoxin-free plasmid MaxiPrep kit according to the manufacturer's instructions (Qiagen 12362).

Viral vector generation

Plasmids were generated into viral constructs at the Salk Institute Viral Vector (GT3) core facility. Viruses were packaged as AAV PHP.eB serotype to allow for systemic retroorbital delivery in order to obtain brain-wide transduction of astrocytes.

Retroorbital virus injections

Mice at P60 were anesthetized with 2-3% isofluorane in oxygen by constant flow through a nose cone. Virus was injected via retroorbital administration at a dose of 1.0E12 viral genome copies/mL. The virus was diluted in DPBS just prior to injection. Mice were allowed to recover from anesthesia and then returned to the housing room.

Tissue collection and preparation for histological experiments

Mice were deeply anesthetized with intraperitoneal injection of a mixture of 100 mg/kg Ketamine (Victor Medical Company 1699053) and 20 mg/kg Xylazine (Victor Medical Company 1264078) and transcardially perfused. For fixed tissue collection, mice were perfused first with room temperature PBS followed by 4% PFA. The brains were rapidly dissected and post-fixed overnight in 4% PFA (Electron Microscopy Sciences 19208) at 4C. After 24 hours the brains were moved to 30% sucrose (Invitrogen 1503-022) for 24-48 hours. The brains were then embedded in TFM (Electron Microscopy Sciences 72593) and frozen in a dry ice/ethanol slurry and stored at -80C until use. For fresh frozen tissue collection, mice were perfused with room temperature PBS and the brain was rapidly dissected and embedded in OCT (TissueTek 4583). The brains were frozen in a dry ice/ethanol slurry and stored at -80C until use as described above.

Single molecule fluorescent in situ hybridization (smFISH)

smFISH was used to characterize the developmental pattern of *Ptn* expression in astrocytes, confirm loss of *Ptn* in Ptn KO mice and validate the reduction of Ptn mRNA in Ts65Dn astrocytes. FISH was performed with the RNAScope Fluorescent Multiplex Detection kit (ACD 320851) according to the manufacturer's instructions for fixed and fresh frozen experiments (protocols ACD 320535-TN and ACD 320513-USM, respectively).

<u>Characterization of developmental Ptn expression in astrocytes</u>

Sagittal sections of 16 um were obtained using a cryostat (Hacker Industries OTF5000). Sections were obtained from Aldh1L1-EGFP mice at P7, P14 and P30. An epitope retrieval step was performed at 95C for 20 minutes using the ACD epitope retrieval buffer. Slides were then washed once in PBS and dehydrated in 100% ethanol before beginning protease treatment. Protease III was used for P7 samples and Protease IV was used for P14 and P30 samples. All protease treatment was performed for 30 minutes at 40C in the hybridization oven. *Mus musculus Ptn* probe (ACD 486381) was hybridized for 2 hours at 40C. Amplification steps were

performed according to the manufacturer's instructions without deviation. *Ptn* probe was conjugated to the 550-channel. After completion of the probe labeling, immunohistochemical labeling was performed to amplify the transgenic GFP signal in astrocytes. Briefly, slides were blocked and permeabilized in blocking buffer containing 10% goat serum in PBS with 0.2% Triton for 1 hour at room temperature. Slides were then incubated overnight at 4C with anti-GFP antibody (1:500, chicken, Novus Biological NB100-1614) diluted in an antibody dilution buffer containing 5% goat serum in PBS with 0.2% Triton. The next day slides were washed in PBS three times for 5, 10 and 15 minutes. Secondary antibody was applied (1:500, goat anti-chicken 488) for 2 hours at room temperature in antibody dilution buffer. Slides were washed three times for five, ten and fifteen minutes. DAPI was applied (1:1000) for five minutes. Slides were washed once in PBS and then coverslips (24 x 60 mm and 1.5 thickness, Fisher 12541037) were mounted with Fluoromount G. Five independent experiments were performed. Imaging was performed using a 40x objective on a Zeiss LSM 700 confocal microscope. Laser and gain intensities were maintained as constant for each image. Tiled z-stacks were obtained from the visual cortex with 0.4 um z-steps and a total of 12 steps, focusing on the upper layers due to description of *Ptn* as an upper layer astrocyte marker [16]. Three slices were imaged for each mouse. Layer 1 and Layer 2/3 were segmented out as separate images in FIJI using the DAPI channel. Images were quantified using a custom macro in FIJI that masks the astrocyte cell bodies and measures the thresholded mRNA signal in each cell as previously described [37]. The threshold was maintained as constant for each image within an experiment. Example images in Figure 1 show the maximum intensity projection for each timepoint. Overview images in Figure S1 were collected using a 20x objective. A z-step size of 3.5 um was used with a total of 9 steps. Tiled images of the entire cortex were collected and the image shown is a maximum intensity projection.

Confirmation of Ptn KO

Coronal sections of 16 um were obtained from WT and *Ptn* KO mice at P14. Epitope retrieval, FISH and immunohistochemical labeling for GFP was performed on fixed tissue in the same manner as described above. Images were obtained on the LSM 700 confocal microscope using a 40x objective. Tiled z-stacks were collected from the visual cortex with 1 um z-steps and a total of 7 steps. Constant laser and gain settings were used for WT and KO pairs. Images shown are maximum intensity projections.

Validation of reduced *Ptn* expression in Ts65Dn Mut astrocytes

FISH was used to validate that *Ptn* expression is reduced *in vivo* in Ts65Dn Mut astrocytes because the candidate molecule was identified based on *in vitro* studies. Sagittal sections of 16 um were collected from Ts65Dn mice at P14. Dual-probe FISH was performed on fresh frozen tissue for these experiments, as recommended by the manufacturer. Slides were fixed in 4% PFA at 4C for 15 minutes and then sequentially dehydrated in 50, 70 and 100% ethanol. Protease IV treatment was applied for 8 minutes at room temperature. Hybridization was performed as described above with *Ptn* probe and *Mus musculus Slc1a3* probe (ACD 430781-C2) to label astrocytes. Amplification steps were performed as described above and not deviating from the manufacturer's instructions. *Ptn* was conjugated to the 550-channel and *Slc1a3* was conjugated to the 488-channel. Five independent experiments were performed. Imaging was performed using a 20x objective on a Zeiss LSM 700 confocal microscope. Laser and gain intensities were maintained as constant for each image. Three slices were imaged for each mouse. Tiled z-stacks were obtained from the visual cortex with 0.4 um z-steps and a total of 12 steps, again focusing on the upper layers. Layer 1 and Layer 2/3 were segmented in FIJI as described above. The same custom macro was utilized for quantification as described above, but masking on *Slc1a3*+ signal. Example images in Figure 1 show the maximum intensity projection for each timepoint. The fresh frozen protocol yielded more variability between independent experiments so the *Ptn* signal in Ts65Dn Mut astrocytes was normalized to the WT values within each experiment.

Validation of viral constructs

The penetrance and specificity of the GfaABC1d-Ptn-HA and GfaABC1d-smFP-HA viruses were quantified. Viruses were injected and expressed for two months before collection of the brains. Coronal sections of 16 um were obtained from mice injected with each virus. TFM was removed with a PBS wash. Slices were blocked and permeabilized in blocking buffer (10% goat serum in PBS with 0.2% Triton) for one hour at room temperature. Overnight staining with antibody dilution solution (5% goat serum in PBS with 0.2% Triton) was performed at 4C. Antibodies for the astrocyte marker Sox9 (1:1000, rabbit, Abcam ab185966) and the neuronal marker NeuN (1:500, rabbit pre-conjugated to 647, Abcam ab190565) were used in separate tissue sections and each co-labeled with anti-HA antibody (1:500, rat, Sigma 11867423001). The next day slices were washed three times in PBS with 0.2% Triton for 5, 10 and 15 minutes. Secondary antibody labeling was performed in 5% goat serum in PBS with 0.2% Triton for two hours at room temperature. Appropriate secondary antibodies were used (1:500, goat anti-rabbit 488, Thermo Fisher Scientific A11034; 1:500, goat anti-rat 555, Thermo Fisher Scientific A21434). Washes in PBS with 0.2% Triton were performed three times for 5, 10 and 15 minutes. DAPI was applied (1:1000) for 5 minutes followed by a PBS wash. Coverslips were mounted with Fluoromount G. Quantification of viral penetrance and specificity was performed in four mice for each virus. Images were obtained on an LSM 700 confocal microscope using a 20x objective. Tiled z-stacks in the visual cortex were acquired with 2.0 um z-step increments and a total of 8 z-steps. Three slices/mouse were imaged. The images were stitched and maximum intensity projections were created in Zen software. The images were then imported into FIJI for cell counting analysis using the cell counter plug-in. The number of Sox9+ and NeuN+ cells were counted in each image to obtain the total number of astrocytes and neurons, respectively. Then the number of HA+/Sox9+ and HA+/NeuN+ cells were counted. The viral penetrance was quantified as the percentage of Sox9+ cells that were also HA+ for each virus. The off-target expression in neurons was quantified as the percentage of NeuN+ cells that were also HA+. The hippocampus and lateral amygdala were also imaged to show viral transduction in these brain regions. Images shown in Figure 5 and the associated supplemental figure are maximum intensity projections.

***Ex vivo* dendrite length, dendritic complexity, spine density and spine morphology analyses**

Fixed tissue coronal slices of 50 um thickness were obtained from the Ptn x Thy1-YFP and Ts65Dn x Thy1-YFP lines. These preparations were used for the characterizations of total dendrite length, dendritic complexity, spine density and spine morphology of Layer 5 pyramidal neurons described in Figure 3/Figure S3, as well as the rescue experiments described in Figure 6/Figure S6. TFM was removed with a PBS wash and coverslips were mounted using Fluoromount G. Triangular pieces of parafilm were cut and placed at the northwest and southeast corners of the slides to prevent the coverslip from crushing the thick tissue and obscuring spine morphology. All subsequent analyses were performed blinded to genotype and treatment.

<u>Imaging and analysis of total dendrite length and complexity</u>

Imaging was performed on an LSM 700 confocal microscope. Six independent experiments were performed for Ptn WT vs. KO, Ts65Dn WT vs. Mut and the 4-group rescue experiments (WT smFP, WT Ptn, Ts65Dn Mut smFP and Ts65Dn Mut Ptn). Laser settings and gain were maintained constant within each experiment. Tiled z-stacks of the visual cortex were obtained to include at least 5 labeled cells per image. Images were obtained using a 20x objective. The z-steps were obtained at 0.5 um increments with a total of 60 steps. Three slices/per mouse were imaged for each experiment. Zen Blue 3.3 was used to stitch the images and the stitched z-stack images were then imported into Imaris where the filament tracing tool was used to manually trace individual dendrites. Dendrites were traced if they were completely contained in

the z-stack and did not appear to be cut through the z-dimension. Imaris outputs total dendrite length as the sum of all individual processes for a given filament (dendrite). Sholl analysis was performed using 50 um increments from the cell body. Imaris outputs the number of branches intersecting with the Sholl circles as a function of distance from the cell body. Quantifications for total dendrite length and Sholl analysis were performed on both the average of all dendrites analyzed/mouse as well as the combined dendrites for each dataset (all dendrites analyzed from 6 mice combined) with the same results. Images shown in Figure 3 are maximum intensity projections.

Imaging and analysis of spine density and morphology

Imaging of dendritic spines was performed on a Zeiss LSM 880 confocal microscope with Airyscan imaging modality. Six independent experiments were performed for Ptn WT vs. KO, Ts65Dn WT vs. Mut and eight independent experiments were performed for the 4-group rescue experiments (WT smFP, WT Ptn, Ts65Dn Mut smFP and Ts65Dn Mut Ptn). Laser settings and gain were maintained constant within each experiment. Images were obtained using a 63x objective with 2.0 zoom. The z-steps were obtained at 0.17 um increments and the number of steps for each image was determined on an individual dendrite basis to encompass the entire dendrite. Dendrites were chosen for imaging based on the following criteria: 1.) the cell body resided in Layer 5 and the dendrite reached to the upper layers (Layers 1-3), 2.) the primary branch point on the dendrite resided in the upper layers, and 3.) the dendrite in the image could be resolved from surrounding dendrite branches when focusing through the z-stack. For each individual mouse, five separate dendrites were imaged that were sampled from at least two separate tissue sections. The images were Airyscan processed in Zen and exported as TIFF files. These images were imported into NeuronStudio for spine density and morphology analysis. Spine heads were labeled within NeuronStudio and the spine head width and neck length measurements were exported. These measurements were used to assign spine morphology based on the classifications described previously [24,25]. Quantification of spine density (number of spines/um) was performed on the average of analyzed dendrites/mouse and the combined total dendrites analyzed/dataset yielding the same results, with the exception that spine density in P30 Ts65Dn Mut mice was significantly reduced when combining all dendrites but not at the average of the biological replicate level (Figure 4 and Figure S4). Spine morphology quantifications were performed on the total of all dendrites analyzed per mouse and presented as the percentage of total spines for each morphological category. Images presented in Figure 3 and Figure 6 are maximum intensity projections.

Immunohistochemical synaptic staining, imaging and analysis

Fixed tissue coronal slices of 16 um thickness were obtained from the Ptn, Ts65Dn, Ptn x Thy1-YFP and Ts65Dn x Thy1-YFP lines. These preparations were used for the characterizations of synaptic puncta described in Figure 4/Figure S4 (Ptn and Ts65Dn lines), as well as the rescue experiments described in Figure 7 (YFP- mice from the Ptn x Thy1-YFP and Ts65Dn x Thy1-YFP lines). TFM was removed with a PBS wash. Slices were blocked and permeabilized in blocking buffer (10% goat serum in PBS with 0.2% Triton) for one hour at room temperature. Primary antibodies were diluted in antibody dilution solution (5% goat serum in PBS with 0.2% Triton) and stained overnight in a humidified chamber at 4C. The antibodies used for pre-synaptic puncta were Vglut1 (1:2000, guinea pig, Millipore AB5905) and Vglut2 (1:2000, guinea pig, Millipore AB2251-I). Post-synaptic puncta were labeled with GluA2 (1:400, rabbit, AB1768-I). The next day the slides were washed in PBS with 0.2% Triton three times for 5, 10 and 15 minutes. Appropriate secondary antibodies were used in antibody dilution solution. Secondary antibodies were applied for two hours at room temperature followed by three washes of 5, 10 and 15 minutes in PBS with 0.2% Triton. DAPI was applied (1:1000) for five minutes followed by a PBS wash. Coverslips were mounted with Fluoromount G. For the characterization experiments (Figure 4 and Figure S4) GluA2 was labeled on the 488-channel (1:500, goat anti-

rabbit 488) and Vglut1/2 were labeled on the 594-channel (1:500, goat anti-guinea pig 594, Thermo Fisher Scientific A11076). For the rescue experiments, Vglut1 was labeled on the 488-channel (1:500, goat anti-guinea pig 488, Thermo Fisher Scientific A11073) and GluA2 was labeled on the 647-channel (1:500, goat anti-rabbit 647, Thermo Fisher Scientific A21245). In these stainings, anti-HA antibody (1:500, rat) was added to confirm viral transduction within each mouse and HA was labeled on the 555-channel (1:500, goat anti-rat). Experiments in each dataset were repeated over 6-8 independent experiments. Images were obtained using a 63x objective with 1.2 zoom. The laser settings and gain were maintained constant within each experiment. Z-stacks of 0.4 um increments were obtained separately in Layer 1 and Layer 2/3 with a total of 10 z-steps per image. Three slices/per mouse were imaged for each experiment. Quantification of colocalized puncta was performed as previously described using the Imaris spots tool and the colocalization algorithm [10,25,37]. Spot sizes were set to 0.4 um for Vglut1 and GluA2 puncta and 0.5 um for Vglut2 puncta with a colocalization distance of 0.7 um. Variability between individual staining experiments necessitated the data for Ptn KO or Ts65Dn Mut mice to be normalized to the WT within each experiment. Images shown in Figure 4 and Figure 7 are single planes.

Behavioral analysis

Behavioral testing was performed in the Salk Institute's Behavioral Testing Core (BTC). Behavioral tests were assayed in the following order with 1-2 days rest in between each test: open field, spontaneous alternation Y maze and fear conditioning. All tests were performed in the morning between the hours of ZT1 to ZT5.5. Brain tissue was collected immediately after fear conditioning. All behavioral tests were performed blinded to genotype.

Open Field

Mice were habituated to the testing room for thirty minutes prior to initiation of the test. The room was lit by indirect lighting of ~30 lux. Activity Monitor software (Med Associates) was used to assess the locomotor activity over a ten-minute period. Mice were placed into the open field chamber (43.2 x 43.2 x 30.5 cm; Med Associates Inc. ENV-515S-A) facing the outer plexiglass wall. Motion data was collected with Activity Monitor v5.92 software (Med Associates Inc.) and analyzed. At the conclusion of the test, mice were returned to their home cage and placed back into the housing facility. Analysis was performed using Activity Monitor v6.02 (Med Associates Inc.). Data was analyzed as 5-minute blocks across the ten-minute test duration. The total distance traveled between the 4 groups tested (WT smFP, WT Ptn, Ts65Dn Mut smFP and Ts65Dn Mut Ptn) was assessed.

Spontaneous Alternation Y Maze

Mice were habituated to the testing room for thirty minutes prior to testing. The room was lit by indirect lighting of ~30 lux. The mouse was placed at the distal end of the starting arm (Arm A) and allowed to explore freely in any of the three arms (Arm A, Arm B, Arm C) over a testing period of eight minutes. The dimensions of arms were 17.0 cm x 6.5 cm x 23.0 cm. After the completion of the testing period the mouse was returned to its home cage. ANY-maze v7.2 software (San Diego Instruments) was used for motion tracking and automated analysis. The percentage of correct alternations was quantified as well as the total number of arm entries during the test duration. A correct alternation is defined as a triad of three entries without a repeat entry into a previous arm in the triad.

Fear Conditioning

Mice were habituated to the testing room for one hour prior to testing. The room was lit by indirect lighting of ~30 lux. The fear conditioning protocol used in this study is a two-day protocol. On the first day, mice were placed into the conditioning chamber (24.0 x 30.0 x 21.0 cm, Med Associates Inc. VFC-008) for a 3-minute habituation period before being exposed to a series of foot shocks that are paired to an acoustic tone. The tone (90 dB/5000 Hz) is presented prior to each foot shock. The tones were presented at 180, 300 and 420 seconds for 30-second

durations. The foot shocks were delivered directly after termination of the tone at 0.5 mA for two seconds. The mice learn to associate the foot shock with the tone and typically exhibit a freezing behavior. The mice were kept outside of the testing room in an anteroom to avoid pre-exposure to the acoustic tone. On the second day, the mice were tested in two separate paradigms. The first paradigm was used to test the contextual memory of the chamber. The mice were placed into the same chamber configuration as on the first day during the training period. The test duration was three minutes. In this paradigm there was no foot shock or tone and the mice exhibited freezing behavior simply in response to the contextual memory of the chamber. After the contextual memory paradigm, the mice were tested in a novel context and with the auditory cue and distinct visual, tactile and olfactory cues from the previous chamber. This paradigm tests the memory association of the auditory cue with the foot shock. The test duration was three minutes. The tone was presented three times at 20, 70 and 120 seconds for a duration of 30 seconds. Video Freeze software v.2.07.0.106 (Med Associates) was used for data analysis. Freezing was defined as a minimum motion threshold of 18 over 15 frames (0.5 seconds). The freezing behavior was quantified as the percentage of total time freezing within the test periods for the same context and novel context paradigms. For the learned association paradigm on the first day, the freezing behavior was quantified as the percentage of time freezing within defined periods (pre-shock, after first shock to second shock, second shock to third shock, and remainder of time after third shock).

Quantification and statistical analysis

Statistical analysis and data visualization was performed in Graphpad Prism 10. All tests were two-tailed. Data was tested for normality using either Shapiro-Wilk or Kolmogorov-Smirnov tests. For normally distributed data, T-tests (or one-sample t-tests) were used to compare two groups, one-way ANOVAs were used to compare three groups and two-way ANOVAs were used to compare multiple groups and treatments. If data was not normally distributed, pairwise comparisons were performed using Mann Whitney Rank Sum test (or one-sample Wilcoxon Signed Rank sum) and multiple comparisons were performed using Kruskal-Wallis on ranks. All violin plots represent median (solid line) with upper and lower quartiles denoted by dashed lines. All bar graphs and line graphs represent mean ± SEM. Figures were generated using Adobe Illustrator 2022 and Biorender.

Additional resources

The data that support the findings of this study are available from the lead contact upon reasonable request.

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Figure 1

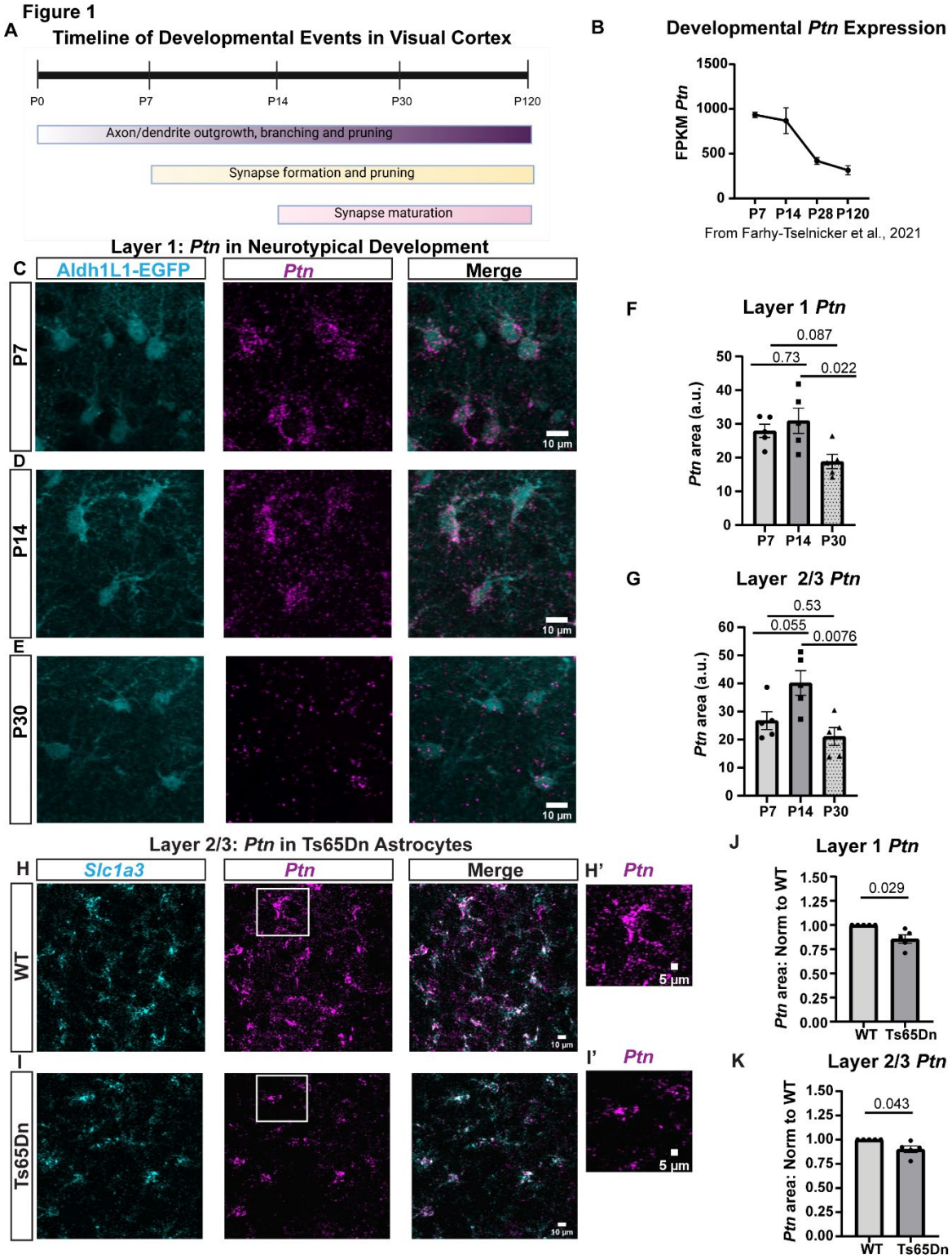

A
Timeline of Developmental Events in Visual Cortex
P0
P7
P14
P30
P120
Axon/dendrite outgrowth, branching and pruning
Synapse formation and pruning
Synapse maturation
B
Developmental Ptn Expression
FPKM Ptn
1500
1000
500
0
P7 P14 P28 P120
From Farhy-Tselnicker et al., 2021
Layer 1: Ptn in Neurotypical Development
C
Aldh1L1-EGFP
Ptn
Merge
P7
D
P14
E
P30
10 µm
F
Layer 1 Ptn
0.087
0.73
0.022
Ptn area (a.u.)
P7 P14 P30
G
Layer 2/3 Ptn
0.53
0.055
0.0076
Ptn area (a.u.)
P7 P14 P30
Layer 2/3: Ptn in Ts65Dn Astrocytes
H
Slc1a3
Ptn
Merge
WT
H'
Ptn
5 µm
I
Ts65Dn
I'
Ptn
J
Layer 1 Ptn
0.029
Ptn area: Norm to WT
WT Ts65Dn
K
Layer 2/3 Ptn
0.043
Ptn area: Norm to WT
WT Ts65Dn

Figure 2

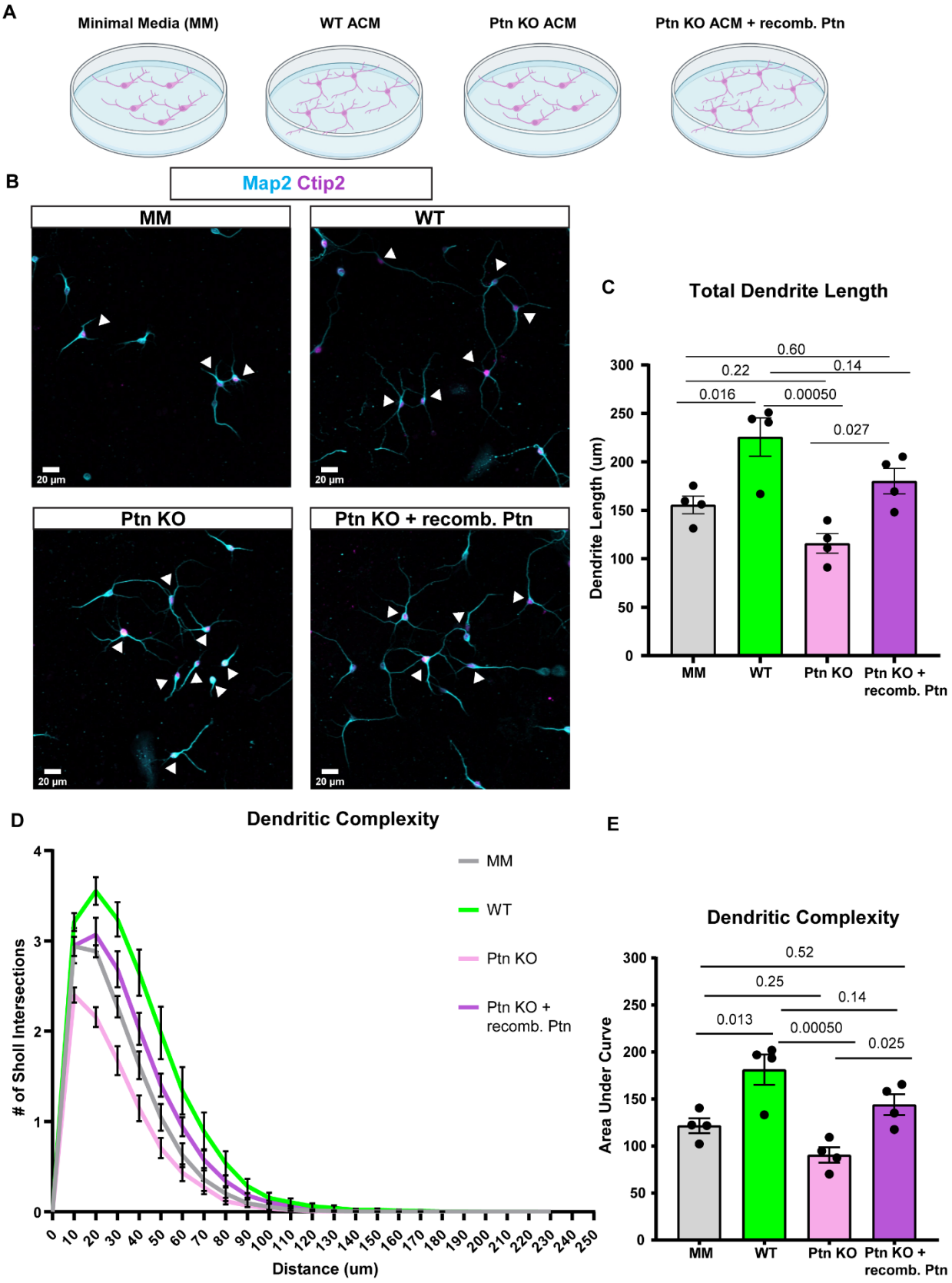

A
Minimal Media (MM)
WT ACM
Ptn KO ACM
Ptn KO ACM + recomb. Ptn
B
Map2 Ctip2
MM
WT
Ptn KO
Ptn KO + recomb. Ptn
20 µm
C
Total Dendrite Length
Dendrite Length (um)
0.60
0.22
0.14
0.016
0.00050
0.027
MM
WT
Ptn KO
Ptn KO + recomb. Ptn
D
Dendritic Complexity
# of Sholl Intersections
Distance (um)
MM
WT
Ptn KO
Ptn KO + recomb. Ptn
E
Dendritic Complexity
Area Under Curve
0.52
0.25
0.14
0.013
0.00050
0.025
MM
WT
Ptn KO
Ptn KO + recomb. Ptn

Figure 3

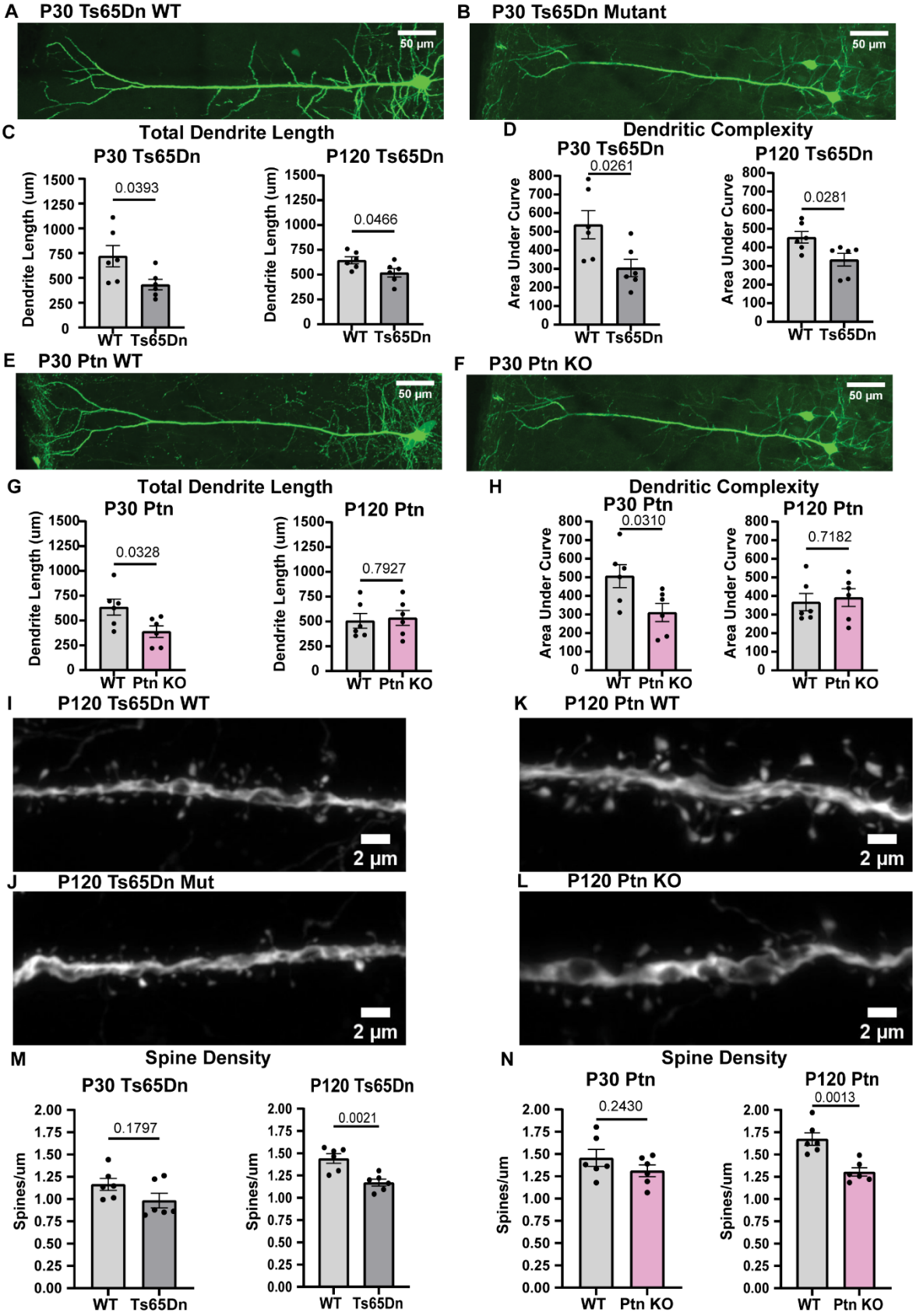

A P30 Ts65Dn WT
50 µm
B P30 Ts65Dn Mutant
50 µm
C Total Dendrite Length
P30 Ts65Dn
P120 Ts65Dn
Dendrite Length (um)
0.0393
0.0466
WT Ts65Dn
D Dendritic Complexity
P30 Ts65Dn
P120 Ts65Dn
Area Under Curve
0.0261
0.0281
WT Ts65Dn
E P30 Ptn WT
50 µm
F P30 Ptn KO
50 µm
G Total Dendrite Length
P30 Ptn
P120 Ptn
Dendrite Length (um)
0.0328
0.7927
WT Ptn KO
H Dendritic Complexity
P30 Ptn
P120 Ptn
Area Under Curve
0.0310
0.7182
WT Ptn KO
I P120 Ts65Dn WT
2 µm
K P120 Ptn WT
2 µm
J P120 Ts65Dn Mut
2 µm
L P120 Ptn KO
2 µm
M Spine Density
P30 Ts65Dn
P120 Ts65Dn
Spines/um
0.1797
0.0021
WT Ts65Dn
N Spine Density
P30 Ptn
P120 Ptn
Spines/um
0.2430
0.0013
WT Ptn KO

Figure 4

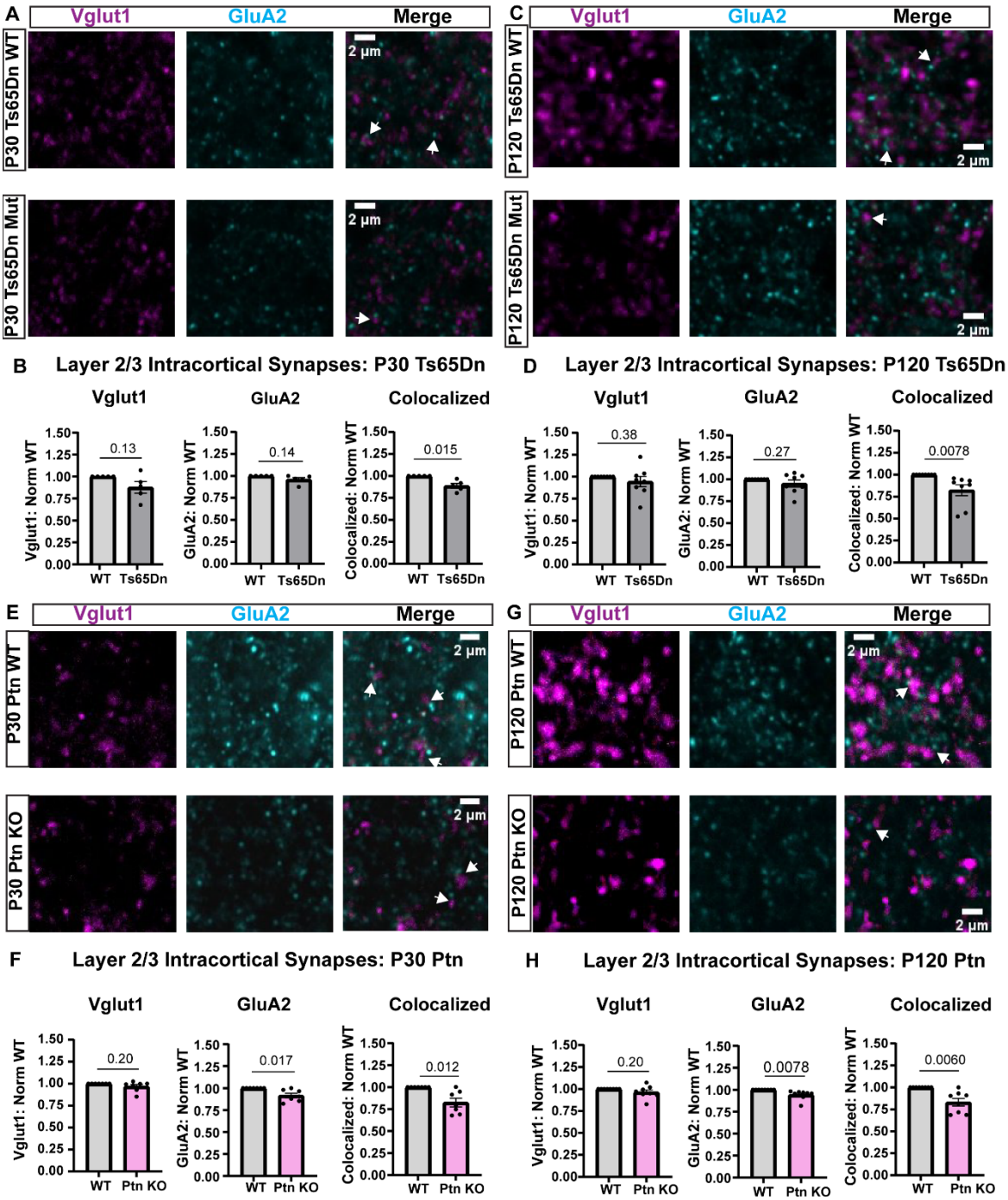

A
Vglut1
GluA2
Merge
P30 Ts65Dn WT
P30 Ts65Dn Mut
2 µm
C
P120 Ts65Dn WT
P120 Ts65Dn Mut
B
Layer 2/3 Intracortical Synapses: P30 Ts65Dn
Vglut1
GluA2
Colocalized
Vglut1: Norm WT
GluA2: Norm WT
Colocalized: Norm WT
0.13
0.14
0.015
WT
Ts65Dn
D
Layer 2/3 Intracortical Synapses: P120 Ts65Dn
0.38
0.27
0.0078
E
P30 Ptn WT
P30 Ptn KO
G
P120 Ptn WT
P120 Ptn KO
F
Layer 2/3 Intracortical Synapses: P30 Ptn
0.20
0.017
0.012
Ptn KO
H
Layer 2/3 Intracortical Synapses: P120 Ptn
0.20
0.0078
0.0060

Figure 5

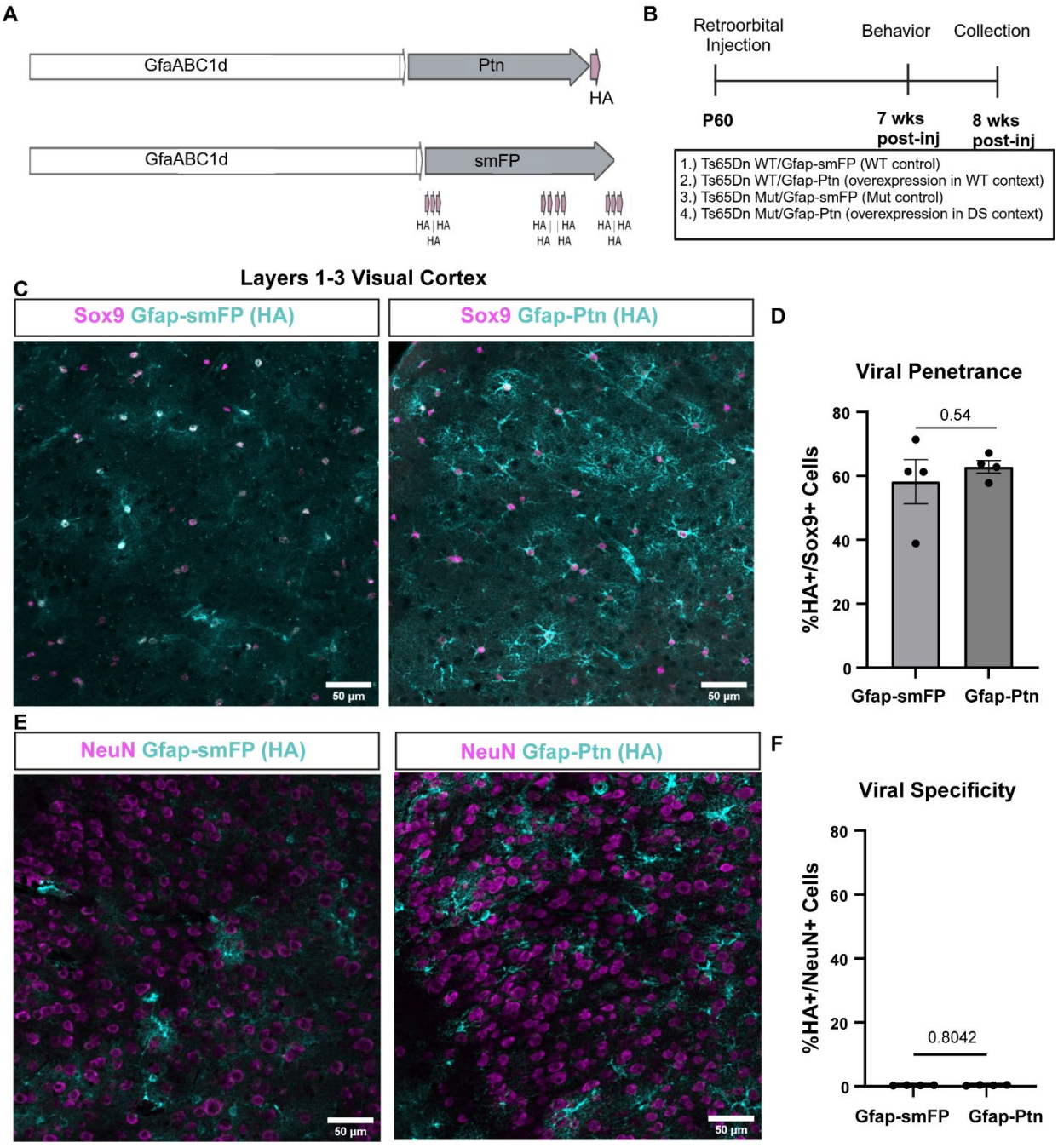

A
GfaABC1d
Ptn
HA
GfaABC1d
smFP
HA | HA
HA
HA | | HA
HA HA
HA | HA
HA
B
Retroorbital Injection
Behavior
Collection
P60
7 wks post-inj
8 wks post-inj
1.) Ts65Dn WT/Gfap-smFP (WT control)
2.) Ts65Dn WT/Gfap-Ptn (overexpression in WT context)
3.) Ts65Dn Mut/Gfap-smFP (Mut control)
4.) Ts65Dn Mut/Gfap-Ptn (overexpression in DS context)
C
Layers 1-3 Visual Cortex
Sox9 Gfap-smFP (HA)
Sox9 Gfap-Ptn (HA)
50 µm
50 µm
D
Viral Penetrance
%HA+/Sox9+ Cells
0.54
80
60
40
20
0
Gfap-smFP
Gfap-Ptn
E
NeuN Gfap-smFP (HA)
NeuN Gfap-Ptn (HA)
50 µm
50 µm
F
Viral Specificity
%HA+/NeuN+ Cells
0.8042
80
60
40
20
0
Gfap-smFP
Gfap-Ptn

Figure 6

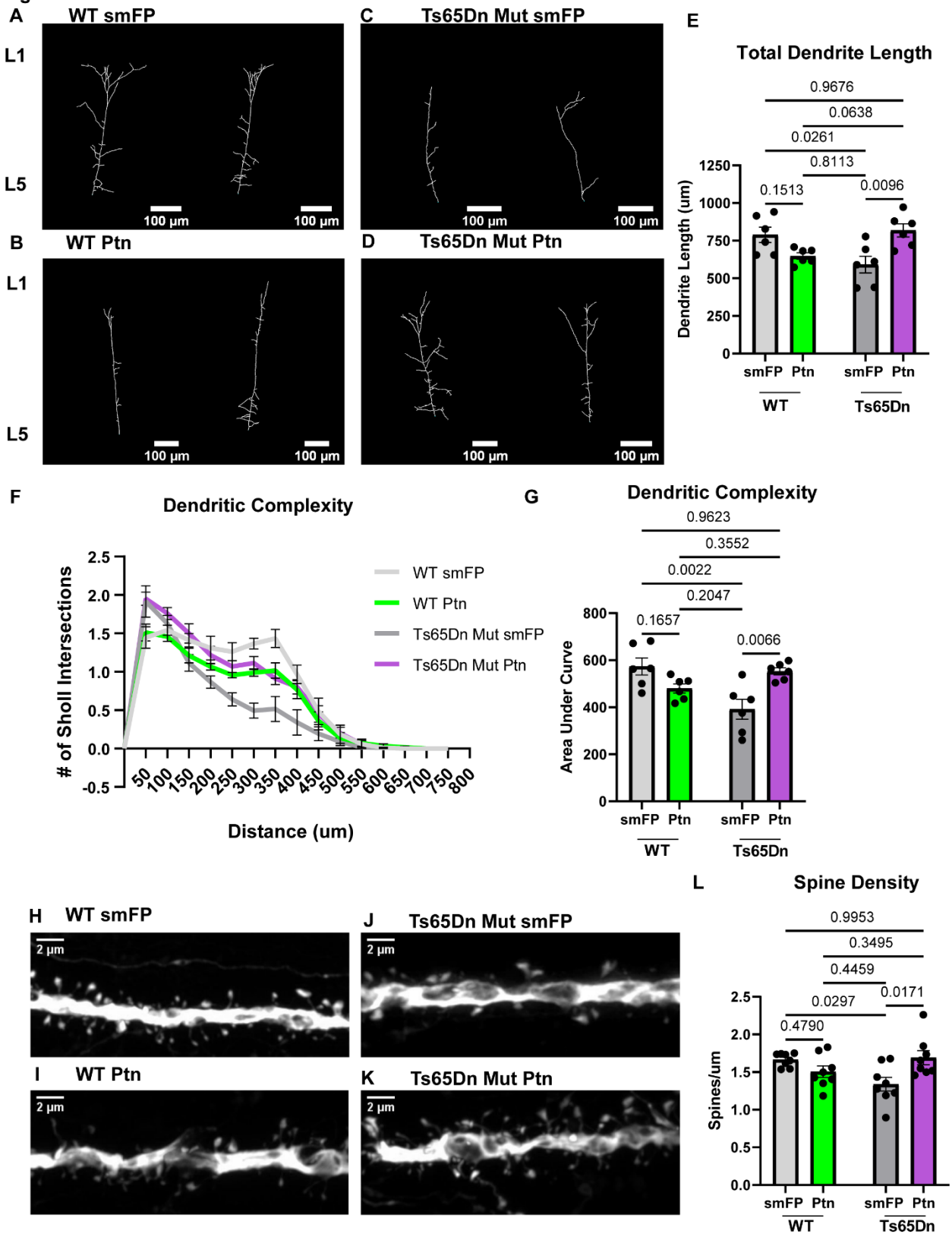

A
WT smFP
C
Ts65Dn Mut smFP
L1
L5
100 µm
B
WT Ptn
D
Ts65Dn Mut Ptn
E
Total Dendrite Length
Dendrite Length (um)
0.9676
0.0638
0.0261
0.8113
0.1513
0.0096
smFP
Ptn
WT
Ts65Dn
F
Dendritic Complexity
# of Sholl Intersections
Distance (um)
WT smFP
WT Ptn
Ts65Dn Mut smFP
Ts65Dn Mut Ptn
G
Dendritic Complexity
Area Under Curve
0.9623
0.3552
0.0022
0.2047
0.1657
0.0066
H
WT smFP
J
Ts65Dn Mut smFP
I
WT Ptn
K
Ts65Dn Mut Ptn
2 µm
L
Spine Density
Spines/um
0.9953
0.3495
0.4459
0.0297
0.0171
0.4790

Figure 7

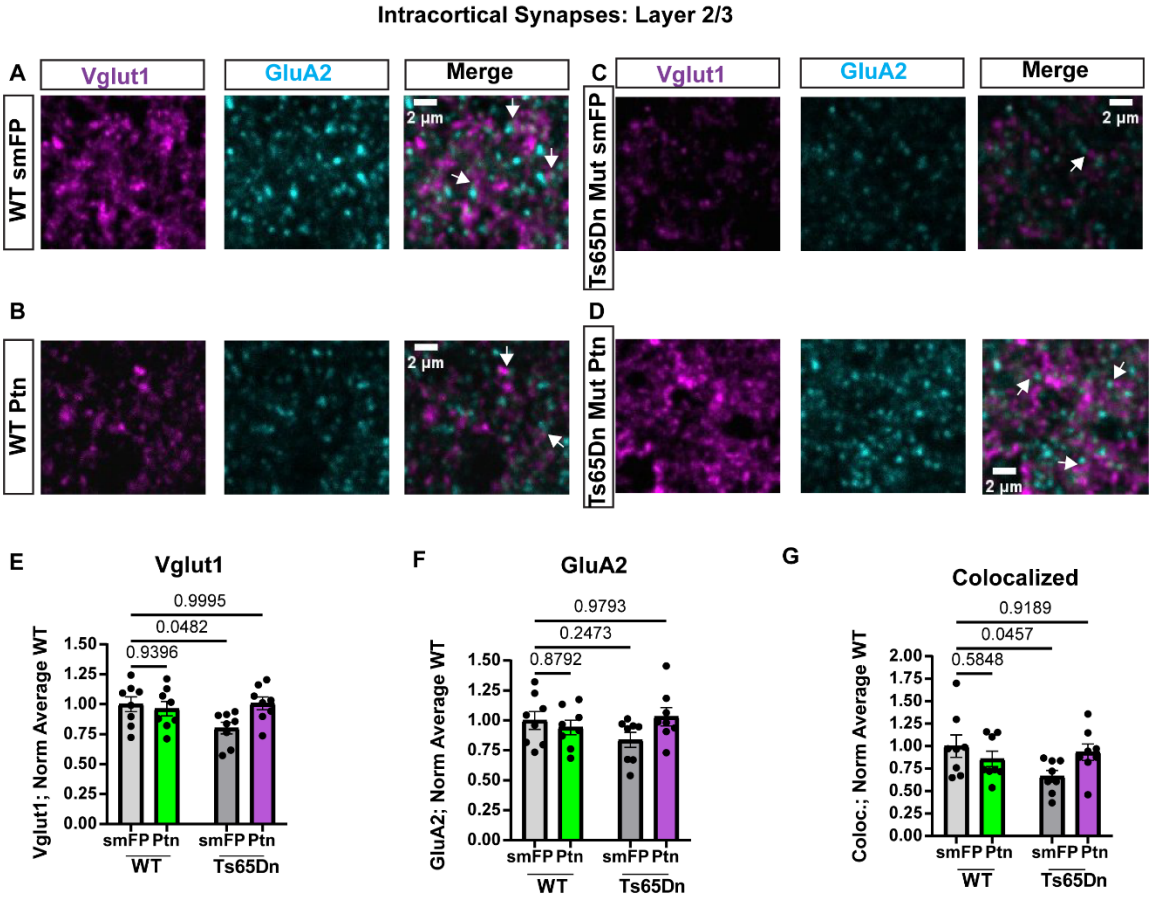
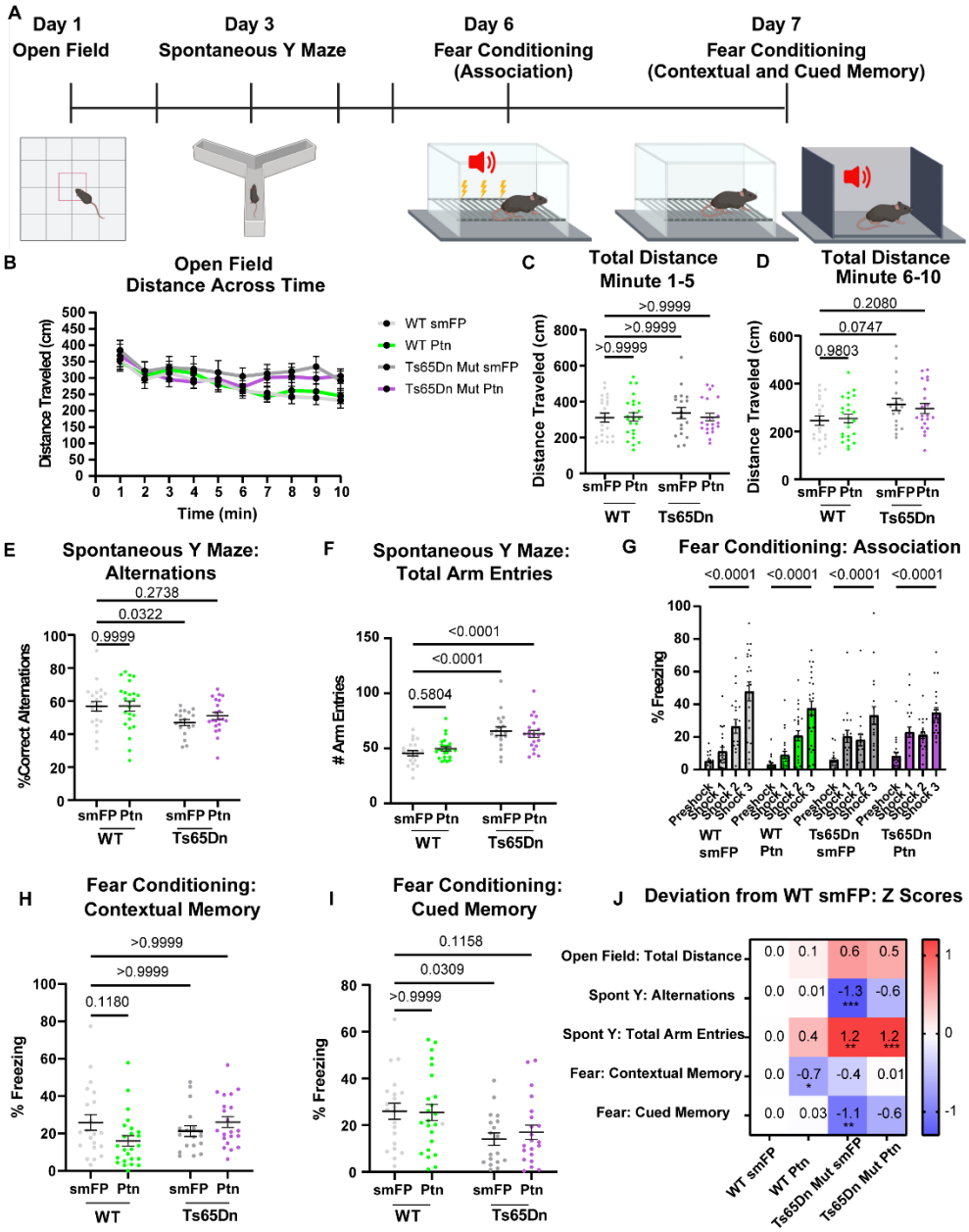

Intracortical Synapses: Layer 2/3
A
Vglut1
GluA2
Merge
WT smFP
2 µm
B
WT Ptn
C
Ts65Dn Mut smFP
D
Ts65Dn Mut Ptn
E
Vglut1
Vglut1; Norm Average WT
0.9995
0.0482
0.9396
smFP Ptn
WT
Ts65Dn
F
GluA2
GluA2; Norm Average WT
0.9793
0.2473
0.8792
G
Colocalized
Coloc.; Norm Average WT
0.9189
0.0457
0.5848

Figure 8


A
Day 1
Open Field
Day 3
Spontaneous Y Maze
Day 6
Fear Conditioning
(Association)
Day 7
Fear Conditioning
(Contextual and Cued Memory)
B
Open Field
Distance Across Time
Distance Traveled (cm)
Time (min)
WT smFP
WT Ptn
Ts65Dn Mut smFP
Ts65Dn Mut Ptn
C
Total Distance
Minute 1-5
>0.9999
>0.9999
>0.9999
D
Total Distance
Minute 6-10
0.2080
0.0747
0.9803
smFP Ptn smFP Ptn
WT Ts65Dn
E
Spontaneous Y Maze:
Alternations
%Correct Alternations
0.2738
0.0322
0.9999
F
Spontaneous Y Maze:
Total Arm Entries
Arm Entries
<0.0001
<0.0001
0.5804
G
Fear Conditioning: Association
% Freezing
<0.0001 <0.0001 <0.0001 <0.0001
Preshock
Shock 1
Shock 2
Shock 3
WT smFP
WT Ptn
Ts65Dn smFP
Ts65Dn Ptn
H
Fear Conditioning:
Contextual Memory
% Freezing
>0.9999
>0.9999
0.1180
I
Fear Conditioning:
Cued Memory
% Freezing
0.1158
0.0309
>0.9999
J
Deviation from WT smFP: Z Scores
Open Field: Total Distance
Spont Y: Alternations
Spont Y: Total Arm Entries
Fear: Contextual Memory
Fear: Cued Memory
0.0 0.1 0.6 0.5
0.0 0.01 -1.3 *** -0.6
0.0 0.4 1.2 ** 1.2 ***
0.0 -0.7 * -0.4 0.01
0.0 0.03 -1.1 ** -0.6
WT smFP
WT Ptn
Ts65Dn Mut smFP
Ts65Dn Mut Ptn
1
0
-1