CDKL5 deficiency results in atypical subregion-specific expression of perineuronal nets in the mouse primary visual cortex

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Cyclin-dependent kinase-like 5 (CDKL5) deficiency disorder is an X-linked neurodevelopmental disorder, where children exhibit motor, speech, cognitive and visual deficits during development as well as early onset of seizures and infantile spasms. CDKL5 disorder results from de novo mutations in CDKL5, which phosphorylates proteins including MECP2, an epigenetic regulator of critical period plasticity. How mutations in CDKL5 gene result in these neurological deficits is unknown. Using a male CDKL5-null mouse model, we systematically characterize the expression of perineuronal nets, which are extracellular matrix structures surrounding parvalbumin+ GABAergic neurons in the primary visual cortex. Perineuronal nets (PNNs) are considered to be barriers to synaptic plasticity, particularly at the end of critical period plasticity in binocular zone. In CDKL5-null male visual cortex, here we report an increase in high-intensity PNN expression in the binocular zone, a decrease in overall PNN expression in the monocular zone, and no differences in PNN expression in rostral V1. These results suggest that at the closure of the visual critical period plasticity, subregion-specific variations in PNN expression lead to functional differences in synaptic plasticity and ultimately, changes in visual function in male CDKL5-null mice. Additionally, the systematic characterization of subregion-specific expression patterns of perineuronal nets lay the groundwork for further studies to probe the roles for perineuronal nets beyond the binocular zone of the mouse primary visual cortex.

Introduction:

Cortical circuits are shaped by experience during sensitive time windows of early postnatal life. In the developing binocular zone of the visual cortex, these critical periods are crucial for experience-dependent formation of synaptic connectivity, and wiring of neuronal pathways that underlie visual function. The postnatal maturation of cortical interneurons, specifically parvalbumin+ (PV) GABAergic neurons, is critical for controlling the timing of critical period plasticity and visual function (Hensch, 2005; Hensch et al., 1998; Huang et al., 1999). This postnatal experience-dependent maturation of PV neurons is concomitant with the expression of specialized extracellular matrix structures called perineuronal nets (PNNs) on PV neurons (Begum and Sng, 2017; Gundelfinger et al., 2010; Hartig et al., 1992; Hou et al., 2017; Kosaka and Heizmann, 1989; Krishnan et al., 2015; Nakagawa et al., 1986; Orlando et al., 2012; Sigal et al., 2019; Ueno et al., 2018; Ye and Miao, 2013). PNNs are mainly composed of lecticans, chondroitin sulfate proteoglycans, hyaluronan glycosaminoglycan chains, link proteins and tenascin-R (Bignami et al., 1992; Carulli et al., 2007; Kwok et al., 2010; Miyata and Kitagawa, 2017). Mature PNNs are thought to restrict plasticity, as their removal in adult visual cortex restores ocular dominance plasticity (Beurdeley et al., 2012; Hou et al., 2017; Pizzorusso et al., 2002, 2006). Disruptions to the timing of critical period plasticity via PV neurons, or alterations in GABA transmission and connectivity change PNN expression, affect cortical plasticity and are associated with neurodevelopmental disorders such as Rett syndrome, CDKL5 deficiency disorder and Fragile X syndrome (Carstens et al., 2021; Krishnan et al., 2015; Krishnan et al., 2017; Lau et al., 2020a; Lau et al., 2020b; Patrizi et al., 2020; Pizzo et al., 2016; Reinhard et al., 2019; Wen et al., 2018).

CDKL5 deficiency disorder is a neurodevelopmental disorder characterized by seizures, intellectual disability, motor and social impairment. Patients with CDKL5 deficiency disorder often exhibit cerebral visual impairment (Brock et al., 2021; Kind and Bird, 2021; Mari et al., 2005; Quintiliani et al., 2021). In null and heterozygous rodent models for CDKL5 deficiency disorder, persistent reduction in response

Author Contributions:
KK supervised the project, designed experiments and analyzed data. BAE and ME perfused animals, performed sectioning, immunostained and mapped sections, and manually counted PNNs. BAE imaged sections. ME and BYBL performed data analysis and data visualizations. BYBL trained BAE and ME in technical aspects of the project. BAE, ME, BYBL and KK wrote and edited the manuscript.

amplitude, reduced visual acuity and defective contrast function are observed by measuring visually evoked potentials (Mazziotti et al., 2017). Interestingly, these deficits in visually evoked responses are observed at the peak of visual critical period plasticity (~P28) and in adults (~P60-80), but not before (~P25). These results correlate with increased expression of PNNs in the binocular zone at ~P35 (Pizzo et al., 2016). Together, these results suggest that the timing of critical period plasticity and proper wiring of visual cortical circuits are affected in rodent models for CDKL5 deficiency disorder.

Though the role of cortical PNNs were established by pivotal studies in the mouse primary visual cortex (wV1), all of these studies have only focused on the binocular zone (V1B). However, PNNs are widely expressed in other sub-regions of the wV1 (Figure 1). Their formation, maintenance and function in synaptic plasticity and/or overall function in the wV1 are unclear. Thus, we performed systematic characterization of PNN expression, using wisteria floribunda agglutinin, over the entire mouse visual cortex at the peak of critical period plasticity in both male wild-type (WT) and CDKL5-null mouse model (null) of CDKL5 deficiency disorder. We found subregion-, rostra-caudal axis-specific changes in PNN expression in CDKL5-null mouse model, compared to age-matched WT controls. Additionally, in both WT and null brains, PNN expression was similar across both hemispheres, showing a lack of hemisphere laterality in the early adolescent visual cortex. These results are in contrast to our previous work showing hemisphere laterality in PNN expression in the adult primary somatosensory cortex (Lau et al., 2020b), suggesting regional- and age-specific changes in PNN expression. Together, these results set the stage for studying the role of PNNs in diverse cortical areas over different ages.

Materials and Methods:

Animals

Young male mice (P15, P30, n=5 mice per genotype, per age used in this study) were maintained on a 12-hour light-dark cycle. Lights turned on at 07:00 AM and the mice received food ad libitum. Genotypes used were CDKL5 hemizygous (B6.129 (FVB) - Cdkl5$^{tm1.1Joez}$/J) (null) and wild type (C57Bl/6) (Wang et al., 2012). All breeders were obtained from Jax Laboratory. Procedures were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Protocols were approved by the University of Tennessee in Knoxville's Institutional Animal Care and Use Committee.

Immunohistochemistry

On P15 and P30, male null and wild type mice were perfused with 1X phosphate buffer solution (PBS) followed by 4% paraformaldehyde dissolved in PBS. Brains were extracted and additionally fixed overnight at 4°C. Prior to sectioning, brains were treated with 30% sucrose in PBS overnight at room temperature. Coronal brain sections at 70µm were collected using a freezing microtome. Immunohistochemistry was performed as previously described (Krishnan et al., 2015). Briefly, free-floating brain sections were blocked in 10% normal goat serum (NGS) and 0.5% Triton-X in PBS for 3 hours. Then, sections were incubated overnight with biotin conjugated Lectin from Wisteria Floribunda (L1516, Sigma-Aldrich) (1:500) in a 5% NGS and 0.25% Triton-X in PBS. Some P15 brain sections were also co-labeled with BCL11B/CTIP2 (PA5-83952, ThermoFisher Scientific) (1:1,000). The next day, sections were incubated for four hours with appropriate secondary antibody (1:1000) in a 5% NGS and 0.25% Triton-X in PBS. In the final step, sections were stained with the marker DAPI (1:1000) for five minutes, and mounted on slides in Fluoromount-G.

Image Acquisition and Analysis

For PNN analysis, 10X single-plane PNN images of the entire visual cortex from each brain section were acquired on a motorized stage epifluorescent microscope (Keyence BZ-X710; Keyence Corp.) and stitched using Keyence BZ-X Analyzer. Imaging settings were established based on null (Pizzo et al., 2016) having the most PNN expression compared to the wild type, and standardized across cohorts. After image acquisition, analysis was performed in ImageJ. Rostal V1 (V1), binocular zone (V1B) and monocular zone (V1M) of each image were identified and outlined by overlaying corresponding atlas maps (Paxinos and Franklin, 2013) with matching identifiable landmarks such as the hippocampus. We counted PNNs as previously described (Lau et al., 2020b). Briefly, to count high-intensity mature PNNs, the "contrast" setting from ImageJ was maximized to filter out weaker signals. The remaining signals were manually quantified as a "mature" PNN if it retained 80% of its original shape (before contrast adjustment) and had proximal processes before contrast adjustment. All PNNs were manually quantified from the 'original' image (no contrast nor brightness adjustment), which included high-intensity PNNs as well. These PNNs are complete in shape with 100% complete borders and proximal processes. All statistical analysis and graphs were generated using GraphPad Prism.

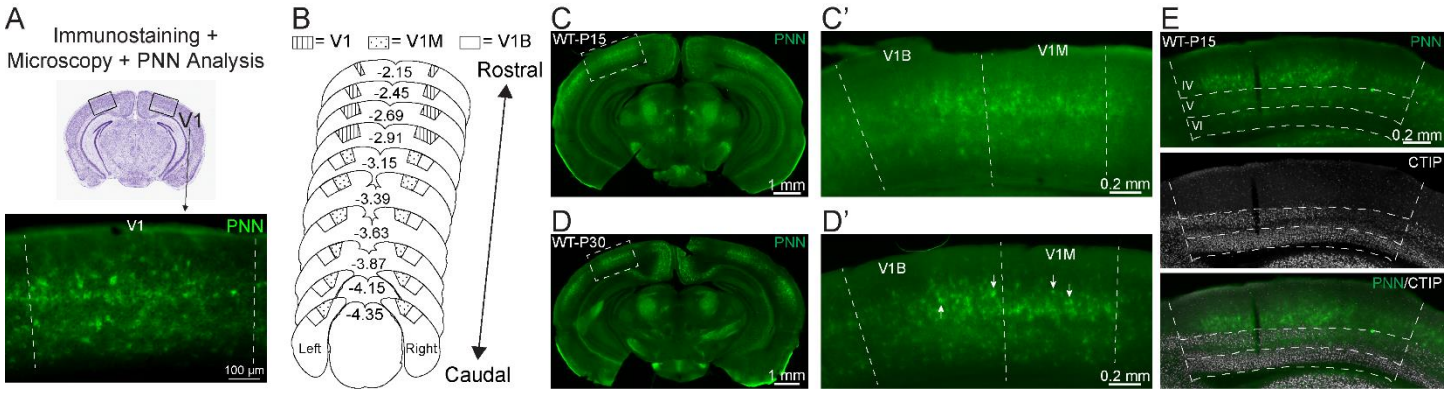

Figure 1: Expression of PNNs in subregions of the whole visual cortex (wV1) during WT critical period. (A) Schema representing mouse brain section (top), and immunostaining with WFA to identify PNNs (bottom). **(B)** Schema of coronal mouse brain sections, depicting rostral to caudal subregions of wV1 analyzed for this study. **(C -D)** Representative epifluorescent images of PNN expression at P15 (C) and P30 (D) of WT. Boxed regions highlight the binocular (V1B) and monocular (V1M) zones and are magnified in C' (P15) and D' (P30). High-intensity PNNs, used for quantification in this study, are marked with arrows. **(E)** Representative epifluorescent images of P15 WT showing PNN expression (green) in layer IV, with minimal co-labeling with layers V and VI cells, as identified by CTIP2 expression (grey).

Results:

We performed a systematic characterization of PNN expression across the subregions of the visual cortex by collecting all coronal brain sections covering the entire visual cortex (wV1), rather than the standard approach of analyzing representative sample sections (Fig. 1A). We analyzed 10–15 coronal brain sections (at 70 µm each, in both left and right hemispheres) per brain. The wV1 subregions are composed of rostral V1 (V1), monocular zon (V1M) and binocular zone (V1B). They are found at Bregma coordinates -2.15 mm (rostral) to -4.35 mm (caudal), which encompass ~2.2 mm of the mouse brain (Figure 1B). PNN expression, as assessed by wisteria floribunda agglutinin (WFA) staining, is weak and immature in male wild type mice (WT) early visual cortex development, around P15 (Figure 1C, 1C'). These PNNs surround soma and proximal dendrites of parvalbumin+ GABAergic neurons, and are found predominantly in layer IV, with minimal expression in layer V. Figure 1E). Early identification of deeper cortical layers was performed with co-immunostaining for CTIP2, which labels pyramidal neurons in those layers. By P30, more mature and higher intensity PNNs (Figure 1D, 1D') appear in both V1M and V1B.

As there were too few clear PNN structures at P15 in the different subregions, we focused our further analysis around P30, towards the end of mouse critical period. Qualitatively, in WT, V1M and V1B PNNs were more distinct than rostral V1 (Figure 2A, B), which suggest "immaturity" of PNNs at this time point (Krishnan et al., 2015). By manual quantification of all PNNs (immature and mature PNNs, see Methods for detail) in the visual cortical subregions, we found that V1B had a significantly higher density, in comparison to V1 and V1M (Figure 2C). However, high-intensity PNN (mature PNNs only) density did not differ between the subregions (Figure 2D). The percentage of high-intensity PNNs to all PNNs did not vary significantly between subregions (Figure 2E), suggesting that though there are more PNNs in V1B, a higher proportion of them are not high-intensity PNNs. Together, these results suggest expression and maturation of PNNs is subregion-dependent in the mouse visual cortex during the end of critical period.

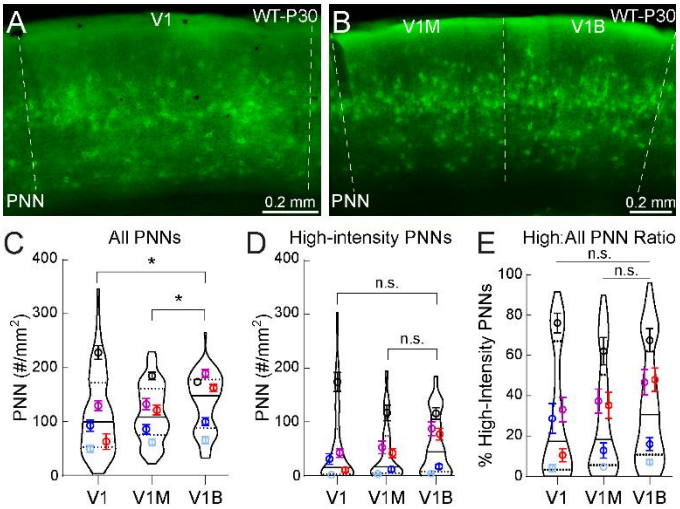

Figure 2: Binocular zone of V1 exhibits higher PNN density than other subregions at P30 WT. (A-B) Representative epifluorescent images of PNN expression in rostral V1 (V1, A), monocular (V1M) and binocular (V1B) zones (B). **(C)** V1B exhibited significantly higher all-PNN density, compared to V1M and V1. **(D)** No significant differences were observed in high-intensity PNNs between all 3 subregions. **(E)** Statistical analysis of high-intensity to All PNN ratio revealed no significant differences between subregions. For C-E, V1 (64 images), V1M (76 images) and V1B (76 images). *Kruskal-Wallis followed by Dunn's test*, *$p < 0.05$, *n.s. = no significance*. Super plots show median (solid line), 25th and 75th quartiles (dash lines) with maximum and minimum, width of violins represents frequency of data points in each region. Each circle + lines represent mean ± s.e.m. for each color-coded cohort.

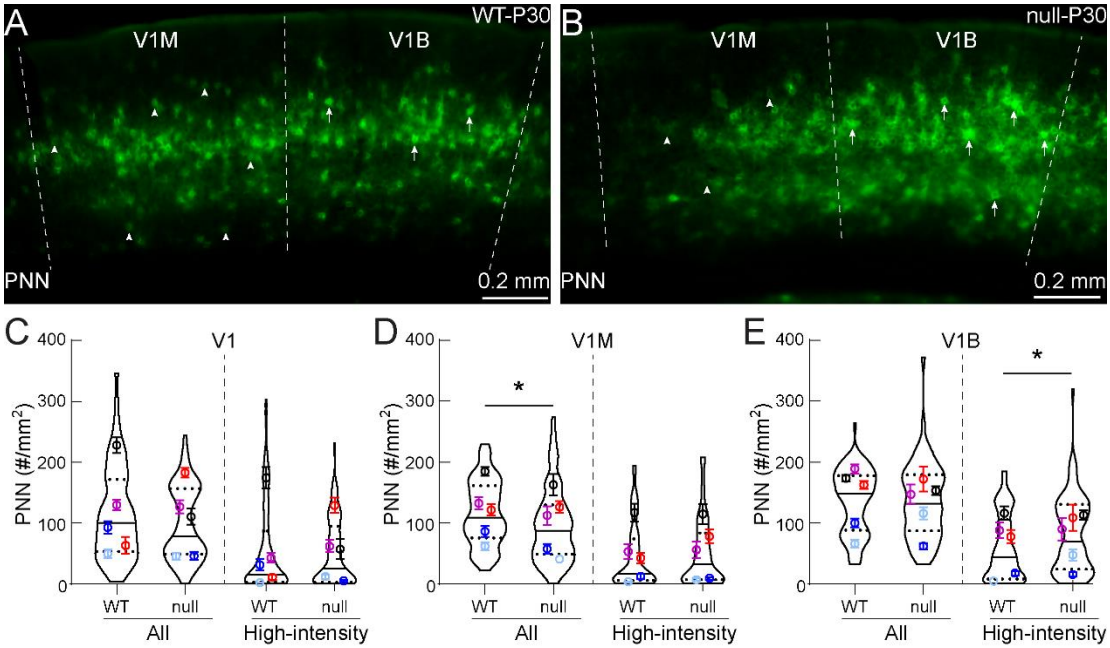

Figure 3: Subregion-specific changes in PNN expression in male CKLD5-null mice. (A-B) Representative epifluorescent images of PNN expression at P30 in V1M and V1B of WT (A) and CDKL5-null (null) (B) show fewer PNNs in V1M, and higher intensity in V1B. Arrowheads and arrows indicate lower intensity and high-intensity PNNs, respectively. **(C)** In rostral V1 (V1), PNN densities were not significantly different between WT and null (WT: n = 64 images; null: n = 62 images; *Mann-Whitney test*, $p = 0.059$). **(D)** However, in V1M, null showed significantly decreased density of all PNNs, with no change in high-intensity PNNs. **(E)** In V1B, null did not show significant change in all PNN density, but expressed significantly more high-intensity PNNs. For D and E, WT: n = 76 images; null: n = 72 images; *Mann-Whitney test, *p < 0.05*. For C-E, super plots: violin plots show median (solid line), 25th and 75th quartiles (dash lines) with maximum and minimum, width of violins represents frequency of data points in each region. Circles + lines represent mean ± s.e.m. for each color-coded cohort.

Next, we determined if the male mouse model for CDKL5 disorder, which was previously shown to have aberrant PNN expression (Pizzo et al, 2016), also displayed subregion-specific changes. Qualitatively, compared to the WT, expression of PNNs in null appeared to be fewer in V1M and with brighter intensity in V1B (Figure 3A, B). With manual quantification, we found that in null, all PNN density was significantly decreased in V1M, with no significant changes in high-intensity PNNs, compared to WT (Figure 3D). In V1B, high-intensity PNN density was significantly higher in null than WT, with no significant differences in all PNN density (Figure 3E). However, we did not observe significant differences in PNN density between WT and null in rostral V1 (Figure 3C). Together, these results suggest that subtle yet significant subregion-specific differences exist in PNN density within the CDKL5-null mouse visual cortex.

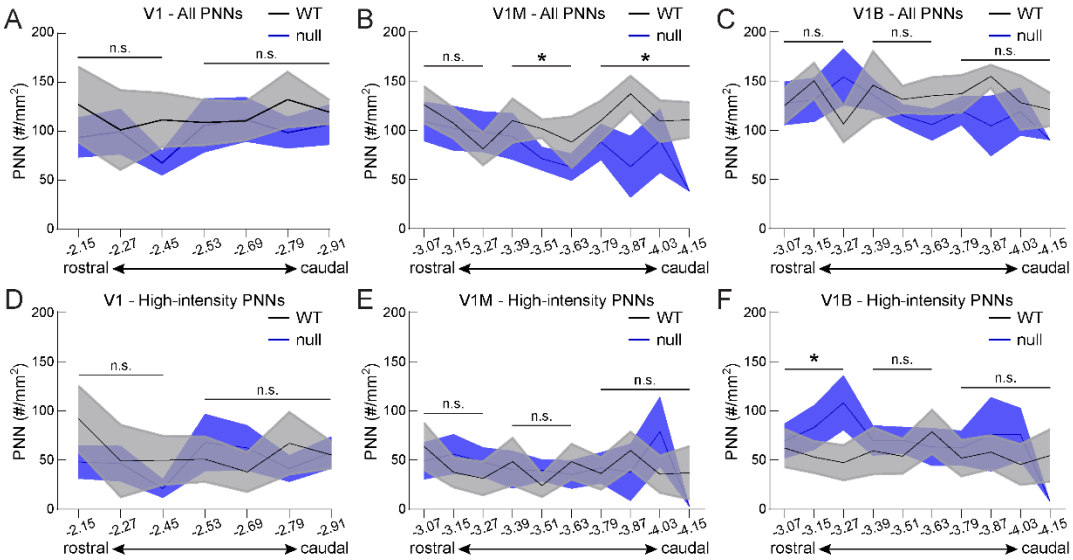

Figure 4: P30 CDKL5-null males exhibit altered PNN expression in specific subregions of visual cortex. (A-F) Distribution of all PNN density (A-C) and high-intensity PNN density (D-F) in WT (grey) and null (blue) in rostral V1 (A, D), V1M (B, E), and V1B (C, F) across rostral-caudal axis. Null exhibited a significant reduction in all PNN density in more caudal regions of V1M (B) and a significant increase in high-intensity PNN density in rostral V1B (F). N = 1-13 images per Bregma coordinate. Solid lines + shades represent mean ± s.e.m. *Mann-Whitney test* within each subregion between WT and null: **p < 0.05, n.s. = no significance.*

Previously, we reported lateral-medial changes in high-intensity PNN density in the primary somatosensory cortex of ~12 week old adult female mice (Lau et al., 2020b). To determine if such anatomical axes differences existed in ~4 week old (P30) male wV1, we plotted individual

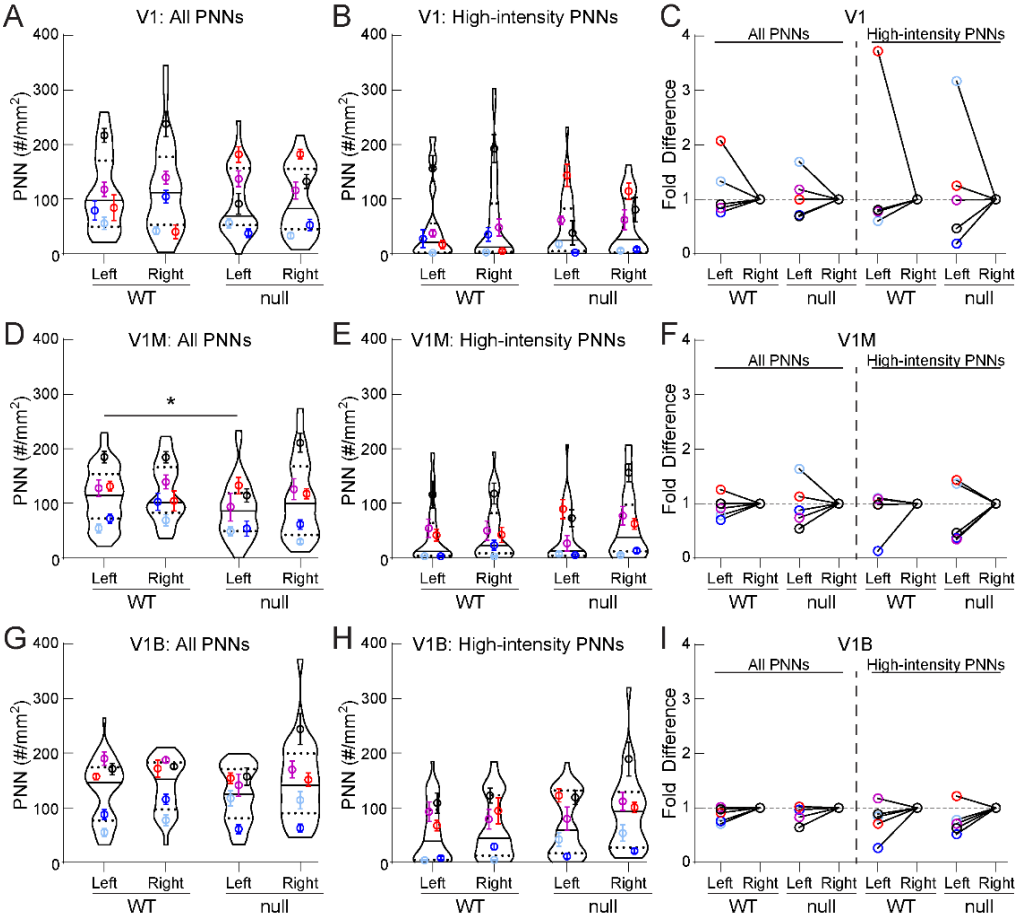

Figure 5: Individual brains exhibit similar hemispheric PNN density in all V1 subregions. On average and within genotype, WT and null displayed no asymmetry in all PNN expression **(A, D, G)** or in high-intensity PNNs **(B, E, H)** between hemispheres of rostral V1 (A, B), V1M (D, E) and V1B (G, H). Overall, there were no hemisphere-specific changes between WT and null visual cortex, except for V1M, where null showed a significant reduction in all PNN density in the left hemisphere (D). **(C, F, I)** Individual brains displayed natural variations in asymmetry in both all PNN and high intensity PNN densities in rostral V1 (C), and minimally in V1M (F) and V1B (I). For each brain, PNN density from the left hemisphere was normalized to its right hemisphere. (A-B, D-E, G-H) Super plots: violin plots showing median (solid line), 25th and 75th quartiles (dash lines) with maximum and minimum, width of violins represents frequency of data points in each region. Each circle + lines represent mean ± s.e.m. for each color-coded cohort. N = 31-42 images. *Kruskal-Wallis followed by Dunn's test*, $*p < 0.05$.

PNN density per section in the rostral-caudal axis. In contrast to the adult primary somatosensory cortical PNN density distribution, we did not observe significant differences in all or high-intensity PNN density within genotypes along the rostral-caudal axis (Figure 4). Comparing WT to null, we did observe significant decreases in all PNN density in the more caudal areas of null V1M (Figure 4B) and increases in high-intensity PNN density in the rostral areas of null V1B (Figure 4F). We did not observe significant differences in all or high-intensity PNN density between WT and null rostral V1 (V1) along the rostral-caudal axis (Figure 4A, D). Together, these results point to specific disruptions in a subregion- and axis-specific manner in the null visual cortex.

Previously, we reported lateralized, subregion-specific expression of high-intensity PNNs in the 12 weeks old primary somatosensory cortex (Lau et al., 2020b). Thus, we investigated if lateralized PNN expression can be observed in early postnatal development in the wV1. Interestingly, we did not observe significant lateralization in either all or high-intensity PNN expression in wV1 subregions (Figure 5). However, we did note individual brains with lateralized high-intensity PNNs in the rostral V1 (Figure 5C). Together, these results suggest that lateralization of PNNs has not been established at this early stage in the visual cortex and is not dysregulated in the male CDKL5-null visual cortex.

Discussion:

PNNs are widely expressed in the rodent brain. Particularly, the different sensory cortical regions display dense PNNs surrounding the soma of neurons. Though their relative functions in individual brain regions and cell types may vary (Balmer, 2016; Carstens et al., 2016; Dauth et al., 2016; Devienne et al., 2021; Krishnan et al., 2017; Tewari et al., 2018), overall PNNs regulate the function of inhibitory networks during development and adulthood. As PNNs are predominantly found on PV+ GABAergic neurons in the cortex, they are intimately associated with and contribute to PV+ network function including gamma oscillations, and ultimately behavior. Thus, determining the function of PNNs in different regions and biological contexts requires a thorough characterization of their expression across the whole brain. Towards this bigger goal, we have focused our efforts on the primary visual cortex during the well-accepted critical period time period.

Most of the knowledge of PNN expression and function in the mouse V1 is based on the binocular zone. The known electrophysiological

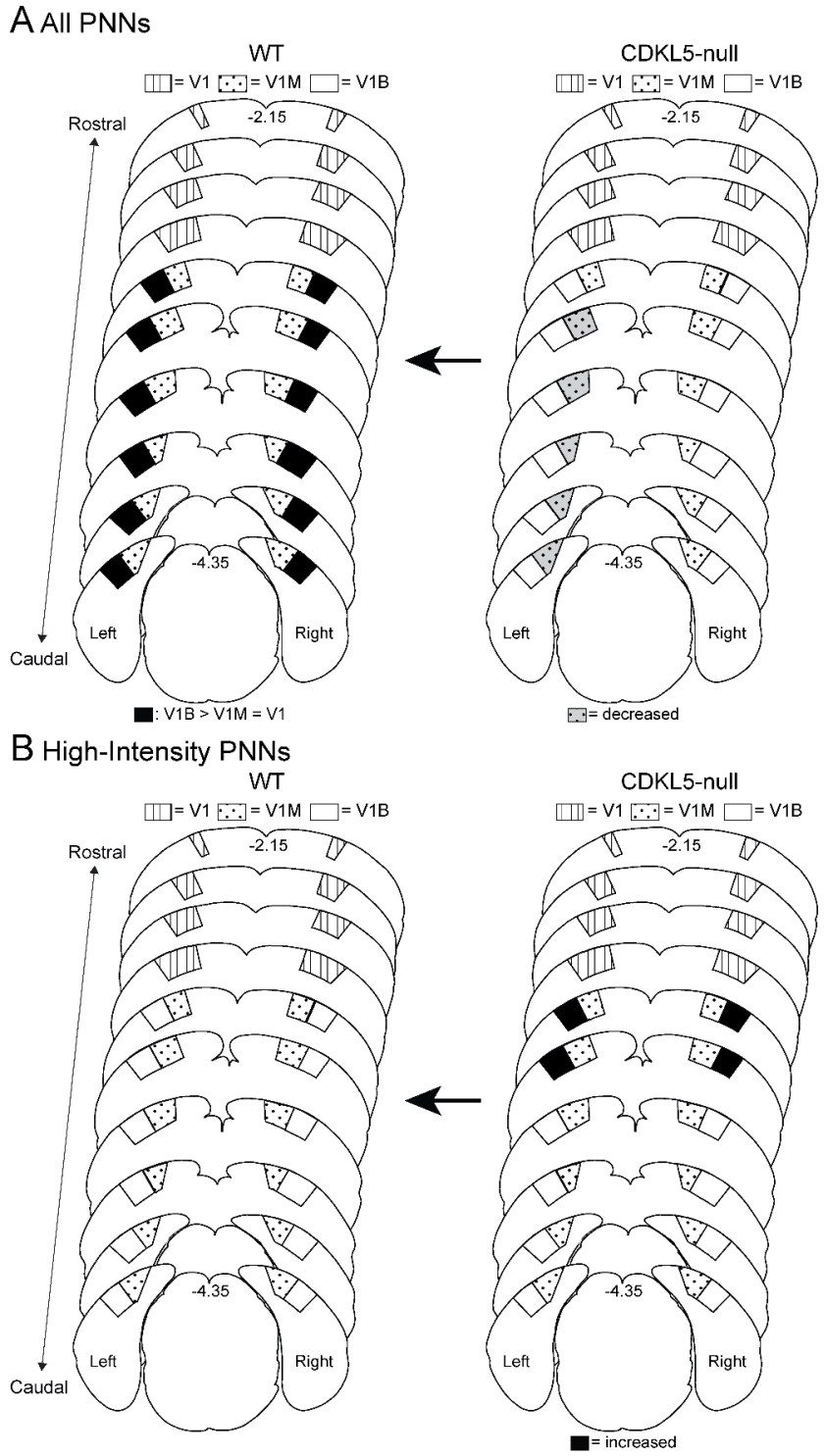

Figure 6: Summary diagram - Perineuronal net populations in the primary visual cortex vary by monocular, binocular and rostral subregions. Overall, WT and CDKL5-null male brains express more overall PNNs in the binocular zone relative to rostral V1 and monocular zone within genotype **(A: left, only WT is shown)**, while expressing similar level of high-intensity PNNs throughout the whole V1 **(B: left, only WT is shown)**. Compared to WT (**arrow in A, B**), CDKL5-null have lesser overall PNN expression in the left hemisphere of the monocular zone **(A: right, grey)**, more high-intensity PNN expression in both hemispheres of the binocular zone **(B: right, black)**, and no difference in rostral V1 (A, B). These results suggest that at the closure of the visual critical period plasticity, subregion-specific variations in PNN expression could lead to functional differences in synaptic plasticity and ultimately, changes in visual function in male CDKL5-null mice. The systematic characterization of PNNs in the rostral-caudal axis lay the groundwork to probe the roles for PNNs beyond the binocular zone of the mouse primary visual cortex.

properties and roles of the binocular zone in ocular dominance plasticity, binocular matching and depth perception have allowed for elucidating the importance of PNNs in brain function. Here, we present the first systematic characterization of PNN expression with subregion-, rostrocaudal axis-, and hemisphere/laterality-specificity in the whole primary visual cortex at the peak of critical period (Figure 6). We report that the absolute density of all PNN expression in the wild-type monocular V1 and rostral V1 is substantial. The functional relevance of this expression in these sub-regions, with a similar timeline of maturation as V1B, is unclear. Typically, neurons of monocular V1 are thought to encode for receptive field properties including orientation and direction selectivity. However, the role of neuronal activity and/or visual experience in initial formation and maturation of these properties is controversial (Chapman and Stryker, 1993; Hagihara et al., 2015; Ko et al., 2013; Miller et al., 1999). Furthermore, the role of inhibitory neurons in the establishment and maintenance of receptive field properties in monocular V1 remains to be determined. Emergence of multi-unit recordings and wide-field imaging techniques with natural scenery stimuli in different species could begin to shed light on the role of V1M and ultimately, on PV interneurons and PNNs. Furthermore, results from the comparative analysis with CDKL5-null V1 suggest that PNN expression is malleable in all subregions of V1, and is impacted by CDKL5.

It was reported previously that the total density of PNNs is increased in CDKL5 mutant mice at the closure of the critical period (Pizzo et al., 2016). Our findings are consistent with their results, and add to the idea that excitation/inhibition imbalance in CDKL5-null V1 is likely to contribute to the visual impairments associated with CDKL5 deficient pathology. Further electrophysiological studies are necessary to determine how a decrease in all PNN expression in V1M, an increase in high-intensity PNN expression in V1B, and no differences in PNN expression in rostral V1 (Figures 3, 6) ultimately contribute to the differentially evoked activity, *in vivo* neural activity and/or synchrony in CDKL5-null

wV1. These subregional differences are particularly important in the context of the rostrocaudal position, with intermediate-caudal V1M showing fewer all PNNs, while more V1B showing increase high-intensity PNNs (Figure 4, 6). Such positional information points to nuanced and likely compensatory synaptic plasticity mechanisms in the CDKL5-null brain. As PV neuronal activity influences PNN maintenance, it is likely that PV network activity and/or gamma oscillations are disrupted in a subregion-specific manner in the CDKL5-null whole V1 (Bartos et al., 2002; Devienne et al., 2021). CDKL5 deficiency disorder was once classified as atypical Rett syndrome, due to the similarities in clinical phenotypes, and due to the commonalities in molecular pathways between CDKL5 and MECP2. CDKL5 is one of the kinases that phosphorylates MECP2, the epigenetic regulator mutated in Rett syndrome. Based on PNN and visually evoked activity comparisons, the male null model for CDKL5 deficiency seems milder, compared to the MECP2 null male model during visual cortical critical period, suggesting distinct cellular phenotypes in similar clinical presentations of neurodevelopmental disorders.

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