Parvalbumin interneurons in human ventromedial prefrontal cortex: a comprehensive post-mortem study of myelination and perineuronal nets in neurotypical individuals and depressed suicides with and without a history of child abuse

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Abstract

Parvalbumin interneurons (PV+) are major regulators of excitatory/inhibitory cortical information processing. Their maturation is associated with the opening of developmental critical periods (CP), where neural circuits are more easily shaped by the external environment. Perineuronal net (PNN) maturation around PV+ cells is associated with the closure of these CP. Recent reports have revealed that cortical PV+ axons are myelinated, and that myelination also plays a role in ending CP. Exposure to child abuse (CA) during CP of development can have profound lasting structural and functional effects on brain organization which poses a significant risk for adult psychopathology. Our lab recently found that a history of CA is associated with increased PNNs in the ventromedial prefrontal cortex (vmPFC) and impaired myelination in the anterior cingulate cortex of adult depressed suicides (DS). Given that both axonal myelination and PNNs stabilize mature PV+ networks, we hypothesized that there is a relationship between PNNs and myelination around PV+ interneurons and that CA has a lasting impact on this relationship. Well-characterized post-mortem human vmPFC samples from adult male and female DS with or without a history of severe CA (DS-CA & DS) and matched healthy controls (CTRL) were acquired from the Douglas-Bell Canada Brain Bank (n=31). These samples were matched for age and post-mortem interval. Immunofluorescent (IF) labeling with three primary antibodies directed against PV, Myelin Basic Protein (MBP), and Neurofascin (axon initial segments), as well as with Wisteria Floribunda Lectin (WFL) to stain for PNNs was used to visualize proteins of interest. Multiarea timelapse z-stack image acquisitions were performed on a Laser Scanning Confocal Microscope using a 60x objective. Simple Neurite Tracer (SNT) plugin from Fiji was used to investigate the relationship between PV+ axon myelination and PNN coverage and split them into four categories (MBP+/PNN+, MBP+/PNN-, MBP-/PNN+, and MBP- /PNN-). We found that 81% of PV+ axons

were myelinated in CTRL samples, and that PV+ interneurons with a myelinated axon and a PNN were the most abundant category of PV+ cells in all three subject groups, suggesting a potential relationship between PNNs and myelination. Additionally, CA is associated with a three-fold increase in the number of unmyelinated PV+ cells with a PNN compared to CTRLs. PV+ cell body area of myelinated PV+ interneurons is significantly larger than unmyelinated PV+ axons in CTRLs and DS but not in DS-CA. Finally, PV+ interneurons with a myelinated axon and/or PNN have a significantly higher immunofluorescent intensity in DS-CA compared to CTRLs. These results are the first to quantify and characterize PV+ interneuron myelination and PNN coverage and their potential relationship as well as shed new light on possible long term maladaptive CA-associated changes in vmPFC networks.

Résumé

Les interneurones parvalbumine (PV+) sont d'importants régulateurs du traitement de l'information excitatrice/inhibitrice au sein du néocortex. Leur maturation est associée à l'ouverture de périodes critiques du développement (CP), période au cours de laquelle les circuits neuronaux sont plus facilement modelés par l'environnement externe. La maturation des filets périneuronaux (PNN) autour des cellules PV+ est associée à la fermeture de ces CP. De récentes études ont révélé que les axones PV+ corticaux sont myélinisés et que la myélinisation joue également un rôle dans la fermeture des CP. L'exposition à la maltraitance infantile (CA) au cours du développement peut avoir des effets structurels et fonctionnels profonds et durables sur l'organisation du cerveau, ce qui présente un risque important de psychopathologie tout au cours de la vie. Notre laboratoire a récemment découvert que des antécédents de maltraitance infantiles sont associés à une augmentation des PNN dans le cortex préfrontal ventromédian (vmPFC) et à une altération de la myélinisation au sein du cortex cingulaire antérieur chez les dépressifs suicidés (DS). Étant donné que la myélinisation axonale et les PNN stabilisent les réseaux PV+ matures, nous avons émis l'hypothèse qu'une relation existe entre les PNN et la myélinisation autour des interneurones PV+ et que la maltraitance infantile a un impact durable sur cette relation. Des échantillons post-mortem biens caractérisés de vmPFC humain provenant d'hommes et de femmes dépressifs suicidés avec ou sans antécédents de maltraitance infantile sévère (DS-CA & DS) et de témoins sains (CTRL) ont été obtenus de la Banque de cerveaux Douglas-Bell Canada (n=31). Ces échantillons ont été appariés en fonction de l'âge et du délai post-mortem. Le marquage par immunofluorescence (IF) réalisé avec trois anticorps primaires dirigés contre la PV, la protéine basique de la myéline (MBP) et la neurofascine (segments initiaux de l'axone), ainsi qu'avec la Wisteria Floribunda Lectin (WFL) pour colorer les PNN, a été utilisé pour visualiser les protéines d'intérêt. Des acquisitions

d'images *multiarea timelapse z-stack* ont été réalisées sur un microscope confocal en utilisant un objectif 60x. Le *plugin* Simple Neurite Tracer (SNT) de Fiji a été utilisé pour étudier la relation entre la myélinisation des axones PV+ et la couverture des PNN et les diviser en quatre catégories (MBP+/PNN+, MBP+/PNN-, MBP-/PNN+, et MBP- /PNN-). Nous avons constaté que 81 % des axones PV+ étaient myélinisés dans le groupe CTRL et que les interneurones PV+ présentant un axone myélinisé ainsi qu'un PNN représentaient la catégorie la plus abondante, ce qui suggère qu'il existe une relation entre ces deux attributs cellulaires. Fait à noter, la maltraitance infantile fut associée à la présence d'environ trois fois plus d'interneurones PV+ non myélinisées avec un PNN par rapport aux CTRL. La surface du corps cellulaire des interneurones PV+ myélinisés fut significativement plus grande que celle des interneurones PV+ non myélinisés dans les groupes CTRL et les DS, mais dans le groupe DS-CA. Finalement, les interneurones PV+ avec un axone myélinisé et/ou un PNN présentaient une intensité d'immunofluorescence PV+ significativement plus élevée dans le groupe DS-CA que dans le groupe CTRL. Cette étude est la première à quantifier et à caractériser la myélinisation des interneurones PV+ et leur couverture par un PNN dans le cerveau humain. Les résultats présentés dans ce mémoire jettent aussi un éclairage nouveau sur les changements inadaptés associés à la CA au sein du vmPFC.

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I would like to express my gratitude to my supervisor Dr. Naguib Mechawar, for his guidance and this amazing project idea. I would also like to thank my advisory committee members, Dr. Claire Dominique Walker and Dr. Kai-Florian Storch along with my mentor Mallar Chakravarty for their constructive feedback. The work presented here would not be possible without the generous donations of brain samples, so I would like to acknowledge these individuals and thank their families. Additionally, this research would be inconceivable without the help from the staff at the Douglas-Bell Canada Brain Bank and coordinators of the Molecular and Cellular Microscopy platform.

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To my family and friends, thank you for all your love and encouragement. Your motivation reminded me to be proud of my accomplishments and helped me become the person I am today. Finally, I am very grateful to my boyfriend and bestfriend, Dario. Your patience, love and comedic relief helped me remember to enjoy the process. So, thank you all from the bottom of my heart.

Contribution of Authors

Chapter I:

This section is a literature review that pertains to perineuronal nets and myelination surrounding PV+ interneurons along with the impact of depression and CA on these structures. The writing and research were conducted by the thesis author (ST) under the guidance of NM.

Chapter II:

This chapter contains unpublished original results from the manuscript "Parvalbumin interneurons in human ventromedial prefrontal cortex: a comprehensive post-mortem study of myelination and perineuronal nets in samples from depressed suicides with and without a history of CA and matched controls". ST and NM conceptualized the study. GT acquired and characterized the brain samples along with their phenotypic characterizations. ST, CB, DX, RK and MAD contributed to tissue dissections and immunofluorescent staining. ST and DX performed confocal imaging and cell body size and immunofluorescent intensity analysis. KP generated and analyzed the correlation between PV+ immunofluorescent intensity and PNN probability. ST executed all manual myelination tracing, PNN coverage counting and cortical layer analysis with Nissl staining. ST wrote the manuscript under the guidance of NM.

Chapter III:

This chapter contains a comprehensive scholarly discussion of all the findings along with a conclusion and summary mentioning how the objectives were reached while discussing the implications of the study. The writing and research were conducted by the thesis author under the guidance of NM.

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List of Abbreviations

ACC – Anterior cingulate cortex

AIS – Axon initial segment

BA – Broadmann area

CA – Child abuse

CECA – Childhood Experience of Care and Abuse

CP – Critical period

CSPG – Chondroitin sulphate proteoglycans

CTRL – Healthy control

DLPN – Deep layer pyramidal neurons

DS – Depressed suicides with no history of child abuse

DS-CA – Depressed suicides with a history of child abuse

ECM – Extracellular matrix

E/I – Excitatory/inhibitory

ELA – Early life adversity

HAS – Hyaluronan synthase

IF – Immunofluorescence

MBP- – Unmyelinated parvalbumin interneuron

MBP-/PNN- – Non myelinated parvalbumin interneuron without a perineuronal net

MBP-/PNN+ – Non myelinated parvalbumin interneuron surrounded by a perineuronal net

MBP+ – Myelinated parvalbumin interneuron

MBP+ – Myelinated parvalbumin interneuron

MBP+/PNN- – Myelinated parvalbumin interneuron without a perineuronal net

MBP+/PNN+ – Myelinated parvalbumin interneuron surrounded by a perineuronal net

MDD – Major depressive disorder

MMPs – Matrix metalloproteinases

mPFC – Medial prefrontal cortex

OPC – Oligodendrocyte precursor cells

PBS – Phosphate-buffered saline

PFC – Prefrontal cortex

PMI – Post-mortem interval

PNN- – Parvalbumin interneuron without a perineuronal net

PNN – Perineuronal net

PNN+ – Parvalbumin interneuron surrounded by a perineuronal net

PV+ – Parvalbumin positive

SDPS – Social defeat persistent stress

SNT – Simple Neurite Tracer

TNR – Tenascin-R

vmPFC – Ventromedial prefrontal cortex

WFL – Wisteria Floribunda Lectin

Chapter I: Introduction

Child abuse, Major depressive disorder and Suicide

Child abuse (CA) has substantial effects on brain development and poses a significant risk for adult psychopathology (Teicher et al., 2016). There are different types of maltreatment, namely sexual, physical, and emotional abuse along with neglect (World Health Organization, 2022). Large bodies of evidence show that CA alters the critical processes of brain maturation affecting sensory systems and network architecture (Baker et al., 2013). Many important brain systems such as the reward and anticipation, emotion regulation and threat detection circuitries are affected by CA (Teicher et al., 2016). In fact, individuals with a history of CA are twice more likely to develop Major Depressive Disorder (MDD) which affects 3.8% of the world population (Perez-Caballero et al., 2019; World Health Organization, 2017). MDD is characterized by various symptoms such as loss of interest in normally pleasurable activities (anhedonia), appetite/weight changes, trouble sleeping, difficulty concentrating/thinking and feeling useless (World Health Organization, 2017). Literature suggests that mood disorders such as MDD are associated with neural circuit disruptions in the limbic-cortical system, which regulates mood, emotions, and stress responsiveness (Ressler & Mayberg, 2007). More than 30% of individuals diagnosed with MDD suffer from ineffective antidepressant treatment (Kessler et al., 2003) making MDD a leading cause of disability that accounts for more loss of productivity than any other disorder globally (Perez-Caballero et al., 2019; World Health Organization, 2017). According to the World Health Organization, depression was considered a great contributor to suicide deaths in 2015, especially for people 15-29 years of age (Perez-Caballero et al., 2019). Individuals affected by depression are 20 times more at risk of dying by suicide when compared to the general population (Chesney et al., 2014). CA accounts for 67% of the population attributable risk of suicide attempts (Dube et al., 2003). Indeed, individuals

exposed to 6 or more traumatic events during their childhood can have up to 20 years taken off their adult life (Brown et al., 2009).

Overall, exposure to CA during critical periods of plasticity, when neural circuits are more easily shaped by external experiences, can have profound effects on the structural and functional organization of the brain (Cisneros-Franco et al., 2020). Despite years of research indicating a strong link between CA and psychopathology there is still much to be discovered at the cellular and molecular level. It is imperative to better understand the long-term impacts of CA on the human brain as it may lead to novel targets for treatment that can lessen the impact it has on an individual's life.

Perineuronal nets

During development, critical periods are important windows of plasticity where neural circuits are shaped more easily by the environment (Cisneros-Franco et al., 2020; Reh et al., 2020). This critical period of neuroplasticity coincides with synaptogenesis, synaptic refinement, myelination, and maturation of the nervous system (Gour et al., 2021; Wang & Fawcett, 2012). Perineuronal nets (PNNs) are important structures thought to be key regulators of plasticity (Bucher et al., 2021). PNNs are recruited during critical periods and halt this period of heightened plasticity (Carulli & Verhaagen, 2021). PNNs are lattice-like specialized extracellular matrix structures that condense around the soma, proximal dendrites and axon initial segments of neuronal subpopulations found in the cortex, amygdala, hippocampus, cerebellum and spinal cord (Lensjø et al., 2017; van 't Spijker & Kwok, 2017). PNNs are a meshwork supported by a hyaluronan backbone synthesized by hyaluronan synthase (HAS) expressed on the surface of neurons (Reichelt et al., 2019). This backbone provides a scaffold for the attachment of proteoglycans, link proteins and tenascin-R (TNR) (**Figure 1**) (Reichelt et al., 2019). These proteoglycans are from the lectican family

aggrecan, brevican, versican and neurocan (van 't Spijker & Kwok, 2017). The role for PNNs in stabilizing synaptic connectivity during development to regulate neuroplasticity and closing the critical period is well accepted in literature (Sorg et al., 2016). Evidence in support of this hypothesis demonstrates that chemically ablating PNNs with the bacterial enzyme chondroitinase ABC can restore plasticity resembling the critical period in the visual cortex and amygdala (Gogolla et al., 2009; Pizzorusso et al., 2002). Degradation of PNNs using this enzyme restored ocular dominance plasticity in the visual cortex and enabled the erasure of already acquired fear memories in adult rats (Gogolla et al., 2009; Pizzorusso et al., 2002). PNNs are generally found around parvalbumin (PV+) expressing interneurons throughout the cortex (Bucher et al., 2021; Tanti et al., 2022) and, more infrequently and with lesser intensity, around deep-layer pyramidal neurons (DLPNs).

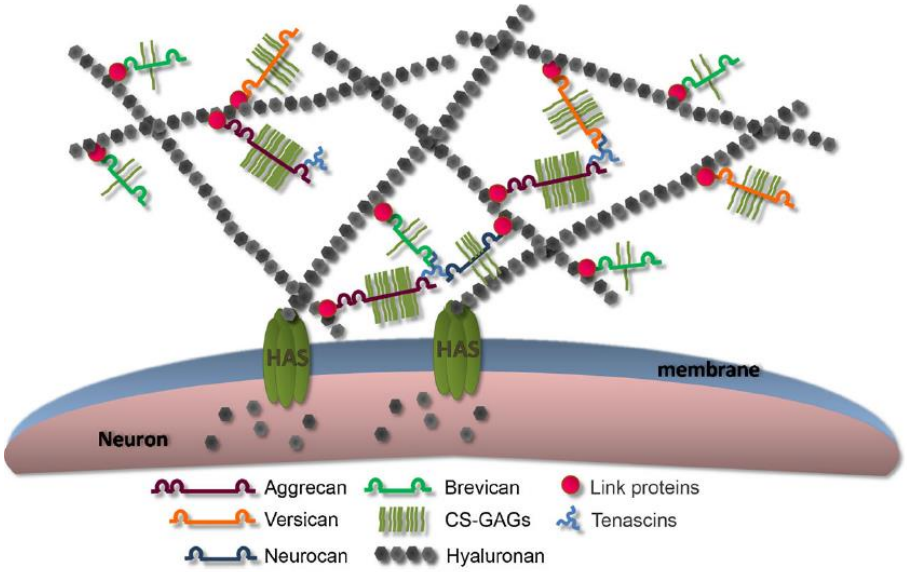

Figure 1: Molecular composition and structure of PNNs (Djerbal et al., 2017). PNNs are a meshwork of hyaluronan backbone synthesized by hyaluronan synthase which provides a scaffold for the attachement of CSPGs from the lectican family (aggrecan, versican, brevican and neurocan), link proteins and tenascin-R (TNR) (Djerbal et al., 2017).

Parvalbumin interneurons

PV+ interneurons are fast-spiking GABAergic interneurons that play a critical role in cortical inhibition (Nahar et al., 2021). PV+ interneurons exhibit two main subtypes, basket cell which have large-bodied cells and highly branching axonal arbors which synapse on cell somas and proximal dendrites or chandelier cell which are less common and have short rows of terminals which look like candlesticks and mostly synapse onto the axon initial segment (AIS) (DeFelipe et al., 1986, 1989; Tremblay et al., 2016). PV+ interneurons play a critical role in maintaining balance in the excitatory/inhibitory (E/I) system allowing for optimal information processing in the prefrontal cortex (PFC) (Ferguson & Gao, 2018). Disrupting this E/I balance can lead to several impairments in PFC dependent behaviors and has been found to lead to psychiatric disorders (Ferguson & Gao, 2018).

PV+ interneurons are important regulators of excitatory neuron activity by maintaining proper inhibitory control of pyramidal neurons like DLPNs through synapses on proximal dendrites or perisomatically (Baker et al., 2018; Milbocker et al., 2021). DLPNs are located in layers V and VI of the neocortex and have larger cells bodies compared to layer II/III pyramidal neurons (Baker et al., 2018). DLPNs are a major source of output for the neocortex by either sending their axons to make long-range projections to other brain regions and subcortical regions or sending short-range projections that act as feedback from higher cortical layers (Molnár & Cheung, 2006; Shepherd, 2013). Pyramidal neurons in the PFC make long-range projections to the amygdala, striatum and ipsilateral perirhinal cortex as well as to the contralateral striatum and cortex (Gabbott et al., 2005; Hirai et al., 2012). During the critical period of experience-dependent neuroplasticity, DLPNs of the PFC undergo many structural and functional changes which elicit long-term neural circuit changes (Bhattacherjee et al., 2019). The PFC has a protracted development that continues past

adolescence into adulthood (Anderson et al., 2010; Koss et al., 2014). Therefore, exposure to stressful experiences during this time of synaptic remodeling can have harmful effects that can persist into adulthood (Urban & Valentino, 2017).

Impact of early life adversity on cortical development

People in all stages of life adapt their behaviour to external environment, so adverse experiences during infancy like CA increase the risk for mental health disorders (Reh et al., 2020). Most of the evidence examining the impact of early life stress/adversity like CA on PNNs and PV+ interneurons during critical periods of development has come from studies conducted in rodents. A study in rats shows that social defeat-induced persistent stress (SDPS) induces depressive like state in rats, in which there was an increase in the expression of CSPGs and number of PNNs surrounding PV+ expressing interneurons in the hippocampus (Riga et al., 2017). More recently, another study demonstrated that maternal separation with early weaning mice to model early life adversity showed reduced intensity of PV+ as well as increased PNNs intensity around PV+ interneurons in the ventral hippocampus (Murthy et al., 2019). It is important to note that the intensity of a PNN is associated with maturation, so the brighter the PNN, the more mature it is (Sigal et al., 2019). Finally, a study using a scarcity/adversity model revealed an increased number of PNNs in the amygdala, but only in the right hemisphere of male rats (Guadagno et al., 2020).

Although evidence in humans is scarce, recent findings from our lab suggest that CA can indeed have long lasting impacts of cortical development and connectivity. Namely, our group found that post-mortem human brain samples of depressed suicides with a history of CA are associated with increased recruitment, morphological complexity, and maturation of PNNs around PV+ interneurons in the lower layers of the ventromedial prefrontal cortex (vmPFC) (Tanti et al., 2022). Previously, we had reported that a history of CA is associated with profound epigenetic and

transcriptomic alterations affecting anterior cingulate cortex (ACC) gray matter and hypomyelination in white matter (Lutz et al., 2017). Lastly, our lab also found an imbalance of oligodendrocyte cells at the cellular and transcriptional level along with a decreased expression of MBP expression, a major component of myelin in the vmPFC white matter of depressed suicides with a history of CA (Tanti et al., 2018). These findings highlight that myelination is regulated in early life by the social environment and that early life experiences, like CA, have an impact on myelination in the ACC and vmPFC.

Ventromedial prefrontal cortex

The vmPFC is located in the frontal lobe, at the bottom of the cerebral hemisphere, right behind our foreheads. This area, made up of Brodmann areas 11, 12, 24, 25 and 32, is essential for emotion regulation which is involved in coping with stress and reducing risk behavior (Martin & Delgado, 2011; Motzkin et al., 2015). Decision making processes are also supported by the vmPFC, since this area is considered to be one of the hubs for the Default Mode Network known to be activated during a day-dreaming or resting state (Lopez-Persem et al., 2019; Raichle, 2015). In addition, this region is important for cognitive and affective functions which are commonly disrupted in mental illness (Hiser & Koenigs, 2018). In fact, the vmPFC is involved in the development of depression due to functional and structural differences seen in both humans and rodents (Koenigs & Grafman, 2009). Imaging studies report that individuals with MDD have abnormally high levels of activity within the vmPFC (Greicius et al., 2007; Keedwell et al., 2009; Matthews et al., 2008; Mayberg et al., 2000; Mayberg et al., 2005). Moreover, many studies report that depressive symptoms were associated with not only lower global cortical thickness but cortical thinning in specific prefrontal cortex regions (Grieve et al., 2013; Mackin et al., 2013; Peng et al., 2015; Pink et al., 2017; Tu et al., 2012). Childhood maltreatment is also known to alter neurodevelopmental circuity in the

prefrontal cortex which is important for emotional and psychological brain development (McCrory et al., 2012; Teicher et al., 2016). Trauma experiences during childhood contribute to alteration seen in the fear inhibition circuitry composed of the amygdala, hippocampus and vmPFC (Jovanovic & Ressler, 2010). Connections between the amygdala and vmPFC are important for inhibiting and regulating the negative emotions and fear responses originating from the amygdala (Motzkin et al., 2015). Additionally, the communication between the vmPFC and hippocampus, a key brain region for memory and learning, also contributes to inhibition of fear memories, based on their context (Milad et al., 2007). All in all, the fronto-limbic network which includes the vmPFC has a leading role in emotion processing and cognitive functions that are affected due to depression and CA.

Parvalbumin interneuron myelination

Communication between neurons in different regions of the brain is achieved using white matter tracks also known as axonal projections wrapped in myelin (Bullock et al., 2022). Myelin was first thought to be still and fixed, however recent discoveries demonstrated that myelin changes occur with experience and learning new skills (de Faria et al., 2021). Mature oligodendrocytes are responsible for creating the myelin sheath in a constant fashion where increased neuronal activity results in increased myelination (Bradl & Lassmann, 2010). Improvements in myelin imaging brought to light that new myelin internodes appear, and existing myelin internodes elongate, retract and even disappear following experience and acquisition of new skills (de Faria et al., 2021). Several studies demonstrate that mice exposed to social isolation or sensory deprivation during the critical period of myelination display a decrease in number of myelinated axons, along with thickness or internode length in the medial prefrontal cortex (mPFC), somatosensory cortex, and optic nerve (Barrera et al., 2013; Liu et al., 2012; Makinodan et al., 2012; Osanai et al., 2018).

Also, early life adversity (ELA) in mice affects adolescent myelination leading to hypomyelination of white mater tracts in adult prefrontal cortex and hippocampus (Teissier et al., 2020).

Micheva and colleagues discovered that a large fraction of cortical myelin surrounds the axons of inhibitory neurons, specifically PV+ interneurons (Micheva et al., 2016). They found that 50% of all the myelin in layer II/III and 25% of all the myelin in layer IV covers the axons of PV+ interneurons (Micheva et al., 2016). As previously mentioned, cortical PV+ cells are fast-spiking interneurons and generate long trains of action potentials with very high frequencies (Canetta et al., 2022). Therefore, axonal myelination likely helps with the high energy demands, either by improving the energy efficiency of action potential propagation or by providing metabolic support (Micheva et al., 2016).

Myelinated axons and PNNs in cortical PV+ interneurons

There has been very little research on the possible relationship between PV+ interneuron myelination and PNNs (Browne et al., 2022). A study demonstrated that disrupting PV+ synapses onto oligodendrocyte precursor cells (OPC) resulted in abnormal myelination and axonal morphology of PV+ interneurons (Benamer et al., 2020). This revealed that PV+ interneuron axons are myelinated to ensure their morphological and functional integrity (Benamer et al., 2020). Our lab recently found that OPC express the canonical components of PNNs in human neocortex, suggesting that OPCs could be potential regulars of PNNs formation (Tanti et al., 2022). As previously mentioned, the formation and maturation PNNs mark the closure of the critical periods in development which coincides with synaptogenesis, synaptic refinement, and myelination (Gour et al., 2021; Wang & Fawcett, 2012). Therefore, we can speculate that OPC-PV+ interneuron communication during the critical windows of development may play a fundamental role in modeling cortical plasticity and the maturation of PNNs (Bucher et al., 2021). Thus, PV+ networks

can be physically and functionally stabilised by PNNs and axonal myelination (Bucher et al., 2021).

As previously mentioned, PV+ interneurons are important for maintaining the proper functioning of excitatory neurons like DLPNs. Therefore, both cell types are susceptible to harmful environmental experiences like CA during the critical periods of development. In fact, CpG and CAC methylation of DLPNs are globally dysregulated following CA in human post-mortem PFC and these differentially methylated regions altered downstream transcriptional programs involved in the regulation of neurodevelopment and synaptic signaling (Almeida, 2023). Also, human findings investigating around 80,000 nuclei from the dorsolateral prefrontal cortex of postmortem brain tissue of individuals with MDD found that DLPNs and immature oligodendrocyte precursor cells had the greatest dysregulation showing changes in gene expression (Nagy et al., 2020). In addition, experimental findings in neonatal mice found that repeated exposure to stress hampered dendritic arborization and impaired dendritic spine plasticity of layer V pyramidal neurons in the prelimbic cortex and dorsal agranular cingulate cortex of neonatal mice (Yang et al., 2015).

Rationale and objectives of the research

There is substantial evidence demonstrating that early life adversity has long lasting molecular and cellular impacts on the brain. Several studies investigate its pathological effects on PNNs and myelination, but few have examined their relationship in this context. Of those few, none were conducted in humans. This research was inspired by findings from our lab that a history of CA is associated with impaired myelination in the ACC and vmPFC white matter and increased recruitment of PNNs in the vmPFC (Lutz et al., 2017; Tanti et al., 2022; Tanti et al., 2018). We hypothesized that there is a relationship between PNNs and myelination around PV+ interneurons and that this is dysregulated in the vmPFC of depressed suicides with a history of CA (DS-CA)

compared to matched depressed suicides without a history of CA (DS) and healthy controls (CTRL). The main objective of this work was to quantify both myelination and PNNs around cortical PV+ interneurons in the human vmPFC as well as understand the potential relation between PNNs and myelination around these cells in CTRL, DS and DS-CA. As detailed in the next section, we investigated this relationship in the vmPFC, since it is important for emotional, cognitive, and affective functions which are commonly disrupted in mental illness (Hiser & Koenigs, 2018; Tanti et al., 2022). Our first aim was to determine the proportions of PV+ interneurons displaying a myelinated axon in the human vmPFC. In this aim, we quantified the number of PV+ interneurons displaying a myelinated (MBP+) versus non myelinated (MBP-) axon and examined the proportion of myelinated vs non-myelinated PV+ interneurons by cortical layer, specifically focusing on layers II-VI. We also examined cell body size of myelinated vs unmyelinated axons and compare PV+ interneuron myelination in CTRL, DS, and DS-CA samples. Our second aim was to determine the relationship between PNNs and PV+ interneuron axon myelination in human vmPFC. We began by quantifying the proportions of PV+ interneurons with (PNN+) and without (PNN-) a PNN and examined the relationship between PV+ myelinated axons and PNNs. We also compared the PV-immunofluorescence intensity of cells surrounded by a PNN versus those without and compared the relationship between PV+ axon myelination and PNNs in CTRL, DS, DS-CA vmPFC samples.

Chapter II: Body of the thesis

Parvalbumin interneurons in human ventromedial prefrontal cortex: a comprehensive post-mortem study of myelination and perineuronal nets in neurotypical individuals and depressed suicides with and without a history of child abuse

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Abstract

Cortical parvalbumin interneurons (PV+) are major regulators of excitatory/inhibitory information processing, and their maturation is associated with the opening of developmental critical periods (CP). Recent studies have revealed that cortical PV+ axons are myelinated, and that myelination along with perineuronal net (PNN) maturation around PV+ cells is associated with the closures of CP. Although PV+ interneurons are susceptible to early life stress, the relationship between myelination and PNNs around these cells remains unexplored. This study compared the fine features of PV+ interneurons in well-characterized human post-mortem ventromedial prefrontal cortex (vmPFC) samples (N=31) from depressed suicides with (DS-CA) or without (DS) a history of child abuse and matched controls. In healthy controls, 81% of all sampled PV+ interneurons displayed a myelinated axon, while 66% of these cells displayed a PNN as well as a myelinated axon, suggesting a relationship between both attributes. In samples from CA victims, a three-fold increase in the proportion of unmyelinated PV+ interneurons with a PNN was observed in pyramidal layers, along with greater PV-immunofluorescence intensity in myelinated PV+ cells with a PNN. This study, which is the first to provide normative data on myelination and PNNs around PV+ interneurons in human neocortex, sheds further light on the cellular and molecular consequences of early-life adversity on cortical PV+ interneurons.

Introduction

Cortical parvalbumin-expressing (PV+) interneurons play a critical role in maintaining balance in circuit excitation/inhibition, allowing for optimal information processing (1). Most of these cells are surrounded by perineuronal nets (PNN)s, which have been implicated in the closing of critical periods of developmental plasticity (2-4). Another distinctive feature displayed by PV+ interneurons is that a significant fraction of these cells extends a myelinated axon (5). Given that myelination has also been associated with neurodevelopmental plasticity, PV+ cells are uniquely positioned to lastingly shape the connectivity and properties of cortical circuits (6). In this context, the potential vulnerability of PV+ interneurons to early-life stress has recently become a focus of investigation in animals exposed to early-life stress and in postmortem human brain samples from individuals with a history of child abuse (CA) (7). The latter, defined as physical, sexual and/or psychological maltreatment can affect brain development and strongly predispose to psychopathologies and suicide (8). Indeed, individuals with a history of CA are twice as more likely to develop major depressive disorder (MDD), while CA accounts for 67% of the population attributable risk of suicide attempts (9-11). Overall, exposure to CA during critical periods of plasticity, when neural circuits are more easily shaped by external experiences, can exert profound effects on the structural and functional organization of the brain (12) and on the psychology of the individual. Although accumulating evidence indicates that early-life adversity has long-lasting cellular and impact on the brain, few studies have examined PV+ interneurons in this context, particularly in humans.

Our group reported that a history of severe CA is associated with altered oligodendrocyte function and hypomyelination in the anterior cingulate cortex of adult depressed suicides (13). More recently, we also observed a CA-associated increase in number and maturity of PNNs around PV+

interneurons in the ventromedial prefrontal cortex (vmPFC) (3). The current study was aimed at generating the first quantitative assessment of both myelination and PNNs around cortical PV+ interneurons in the human brain to examine whether there is a relationship between PNNs and myelination around these cells. Results were acquired in post-mortem vmPFC samples from individuals having died suddenly with no neurological nor psychiatric condition to generative normative data. The latter were compared to data acquired in parallel in matched samples from depressed suicides with and without a history of CA, in order to determine whether such relationship would be affected by a history of CA.

Materials and methods

Human brain samples

This project was approved by the Research Ethics Board of the Douglas Institute. Well-characterized post-mortem human vmPFC samples from males and females were provided by the Douglas-Bell Canada Brain Bank (https://douglasbrainbank.ca). These 31 samples were divided into three subject groups matched for age and post-mortem interval (PMI) (Table 1): healthy controls, who died suddenly by natural or accidental causes without any brain disorder nor history of CA (CTRL, n=11 (3F, 8M)) and depressed suicides with (DS-CA, n=8 (5F, 3M)) and without (DS, n=12 (3F, 9M) a history of CA. In collaboration with the Quebec Coroner's Office and with the participation of next of kin who consented to donating the brain of their loved one, standardized psychological autopsies were performed to retrieve phenotypic information, as described previously (14). Cases and controls were defined with the support of medical charts and Coroner records and diagnoses were assigned based on DSM-IV criteria. An adapted version of the Childhood Experience of Care and Abuse (CECA) interview assessed experiences of sexual and physical abuse, as well as neglect up to age 15 (15). Only cases with maximum severity ratings of

1 and 2 were included in the DS-CA group. Medication prescribed during the last three months of life as provided by interviews with next of kin are listed in Table 1. Some controls were prescribed or taking medication (e.g. benzodiazepines as sleeping aid) at time of death, but not for any Axis 1 disorder.

Tissue dissections

Cortical samples (Brodmann areas [BA]11/12) were dissected by expert brain bank staff from fresh-frozen 0.5cm-thick coronal sections, with the guidance of a human brain atlas (16). The samples were fixed overnight in 10% formalin and then transferred to a 30% sucrose solution until the tissue sunk. Then, samples were frozen in isopentane and cut on a cryostat into 40 µm-thick sections and these free-floating sections were stored at -20˚C in a cryoprotectant solution. This section thickness was selected because it allows for the visualization and analysis of entire PV+ interneuron cell bodies along with their emerging processes over a sufficient distance.

Immunostaining

Free-floating sections were rinsed in phosphate-buffered saline (PBS) and submerged in a blocking solution of PBS/0.5% Triton X/2% normal donkey serum for 1h at room temperature with constant agitation. Then, the sections were incubated for 48h at 4ºC in constant agitation with primary antibodies (chicken anti-Neurofascin (Invitrogen, PA5-47468, 1:500), rabbit anti-PV (Swant, PV27, 1:2500), mouse anti-myelin basic protein (MBP) (BioLegend, 808402, 1:2500), or lectin (biotinylated Wisteria Floribunda Lectin (WFL), Vector Laboratories, B-1355, 1:2500) diluted in the same blocking solution. Sections were then rinsed with PBS and incubated for 2h at room temperature with fluorophore conjugated secondary antibodies (Alexa-488 anti-chicken (Jackson ImmunoResearch, 703-545-155, 1:500) for neurofascin, Cy3 anti-rabbit (Jackson ImmunoResearch, 711-165-152, 1:500) for PV+, Alexa-680 anti-mouse (Jackson

ImmunoResearch, 715-625-150, 1:500) for MBP or Dylight-405-conjugated Streptavidin (Jackson ImmunoResearch, 016-470-084, 1:100) for the detection of PNN and diluted again in the same blocking solution. Sections were rinsed with PBS and mounted on Superfrost charged slides and coverslipped with Vectashield mounting medium (Vector Laboratories).

Confocal imaging

All images were acquired and analyzed in a blinded fashion, using slides coded by another investigator. Image acquisitions were performed on an Olympus FV1200 laser scanning confocal microscope with a motorized stage. For each subject, a multi-area timelapse of 63 stack tile images stitched together were taken twice, once with neurofascin-488, PV-Cy3 and MBP-680 to capture vmPFC layers II to VI using PV+ immunostaining as a reference, the second with WFL-405 and PV-Cy3 to capture the PV+ interneurons covered by a PNN. The same area was scanned twice since we were limited to 3 channels for the multi-area timelapse. Therefore, stacks were imaged with neurofascin, PV+ and MBP immunostaining and others with WFL and PV+ staining of the same within the section. Scanning twice with PV+ allowed to properly match the myelinated axon to the PV+ interneuron surrounded by a PNN by comparing both images side by side. DAPI was not used as a marker due to the four-probe limitation (neurofascin, WFL, PV+ and MBP) of this confocal system. Images were taken with a 60x objective (NA= 1.42) (1024x 1024 pixels) with a Z-spacing of 0.8µm and laser scanning speed of 2µs/pixel. This process was repeated several times for certain samples to obtain a minimum of 50 cells/subject in all three groups.

Manual myelination tracing and PNN coverage counting

Confocal z-stack images were transferred to FIJI (U.S. Department of Health and Human Services), where analysis and manual tracing was performed using the plugin Simple Neurite Tracer. Myelinated segments were traced manually in individual tile images, moving through the

z-stack, when neurofascin and PV+ were found to be co-localized while examining PV+ interneurons and their processes. Once this co-localization was observed, a segment of at least 75 µm was traced from the soma, along the axon. This minimal length is conservative given that myelination of PV+ axons begins on average 25-50 µm from the soma (17). PV+ interneurons for which neurofascin co-localization could not be visualized clearly were not considered further. Myelinated axons were marked as "MBP+" and non-myelinated axons as "MBP-", and PNN coverage of the cell was then assessed. Therefore, the PNN channel (WFL) was closed while tracing and opened to view PNN once the myelin tracing was completed. The PV+ interneurons surrounded by a PNN were marked "PNN+" and those without a PNN as "PNN-". Throughout the analysis, axon myelination and PNN ensheathment of PV+ interneurons were split into four categories: myelinated PV+ cells surrounded by a PNN (MBP+/PNN+), myelinated PV+ cells not surrounded by a PNN (MBP+/PNN-), non-myelinated PV+ cells surrounded by a PNN (MBP-/PNN+), and non-myelinated PV+ cells not surrounded by a PNN (MBP-/PNN-). A total of 575 PV+ interneurons were counted in the CTRL group, 649 PV+ interneurons in the DS group, and 423 PV+ interneurons in the DS-CA group.

Nissl staining and cortical layer analysis

Nissl staining was performed on sections previously stained by immunofluorescence (IF) to properly visualize cortical layers. Whole sections were imaged on an Olympus VS120 Slide Scanner at 20x to identify the region scanned at the confocal microscope. Coverslips were removed by leaving the slides in PBS overnight. Sections were dehydrated following a sequential immersion in ddH2O, 70% EtOH, 95% EtOH and 100% EtOH (1 min/solution) followed by 20 min in cresyl violet. Then, another dehydration step was performed with the same solutions for the same amount of time. The slides were left in xylene for 1 min before being cover slipped with Permount. Once

dried, the whole sections were imaged at 20x. Using QuPath, the immunofluorescence and cresyl violet slide images acquired with the slide scanner were overlayed. Using the transparency feature in QuPath on the immunofluorescence image, we were able to view the cresyl violet-stained Nissl bodies through the IF tissue and separate the layers, which were further defined with the aid of the Allen Brain atlas.

Cell body size and immunofluorescent intensity analysis

Sum slices projections were generated from the stacks to visualize PV+ cells at their largest diameter, using the freehand tool Fiji. The outline of the soma was manually traced ensuring to exclude dendritic and axonic protrusions. The area measurements, calculated within the plugin, were compared between myelinated and unmyelinated PV+ cells. They were also compared between the four PV+ interneuron categories defined above. PV+ interneurons that were seen overlapping or whose largest diameter was cut off by sectioning were excluded. PV+ cell immunofluorescent intensity was determined similarly. After tracing the cell body, PV-immunofluorescence intensity was determined by the mean gray value, i.e. the sum of the gray value of all the pixels in the selection divided by the number of pixels, and this selection was calculated with FIJI. Each image stack per subject was taken at different laser powers and voltage, however this was accounted for during the analysis by examining the meta-data for each image. To calculate the probability of PNN presence vs PV-immunofluorescence intensity, a linear model was built for PV-immunofluorescence intensity to control for age, PMI, antidepressant use and, laser power. Then, the residuals from that model were normalize to a range between 0 and 1. The normalized intensities were split into 4 categories (low PV, intermediate-low PV, intermediate-high PV, high PV) as described by Lupori and colleagues (18) and the probability of having a PNN

(number of PV+ cells with a PNN divided by total of PV cells) per intensity category was calculated.

Statistical analyses

Analyses were performed on Prism version 9 (GraphPad sofware) and R package ggplot2. Normality and lognormality tests of variances were assessed with D'Agostino & Pearson tests. ROUT tests were used to identify outliers. Myelination and PNN coverage of PV+ interneurons by layer were analyzed using a mixed-effects model using layer and group as fixed factors, followed by Tukey's honestly significant difference test. For all other statistical tests, one-way ANOVAs or Kruskal-Wallis tests and two-way ANOVAs followed by Tukey's honestly significant difference or Dunn's test were used. Two-sided linear regressions and Spearman correlations examined the relationship between dependant variables and covariates (age, PMI, antidepressants and laser power) (Supplementary information and Supplementary Table 1). Significance threshold was set at 0.05 and all data presented are mean ± SEM.

Results

Proportions of PV+ cells that are myelinated and surrounded by PNNs in CTRL vmPFC samples

In our experimental conditions, PV+ interneuron myelination and PNN coverage were reliably visualized in human vmPFC samples (Fig. 1A). In CTRL vmPFC samples, the great majority (81%) of the PV+ interneurons analyzed were found to extend a myelinated axon (Fig. 1B), while 59% were surrounded by a PNN (Fig. 1C). Of the myelinated PV+ cells, 66% also displayed a PNN (Fig. 1D). This proportion was more than twice as high than for unmyelinated PV+ interneurons, with only 28% of the latter presenting a PNN (Fig. 1E).

DS-CA vmPFC samples display a three-fold increase in unmyelinated PV+ interneurons with a PNN

The proportion of vmPFC PV+ interneurons that were myelinated was similar between groups (Fig. 1B; Kruskal-Wallis ANOVA: H(2) = 6.024, P = 0.0492, followed by Dunn's multiple comparison test). The proportions of PV+ cells with a PNN were also similar across groups (Fig. 1C; Ordinary one-way ANOVA: F(2, 28) = 0.7688, P = 0.4731, followed by Tukey's multiple comparisons test). The proportion of PV+ interneurons that were both myelinated and covered by a PNN (~63% in all groups) remained consistent across groups (Fig. 1D; Ordinary one-way ANOVA: F(2, 28) = 0.7629, P = 0.4758, followed by Tukey's multiple comparisons test). However, the proportion of unmyelinated PV+ interneurons with a PNN was robustly and significantly higher in the DS-CA compared to DS and CTRL groups (Fig. 1E). This greater proportion of unmyelinated PV+ interneurons with a PNN was additionally apparent when comparing the total PV+ interneuron proportions of each category per group (Fig. 1F). This translated into a significant three-fold greater number of unmyelinated PV+ interneurons with a PNN in DS-CA vmPFC compared to the other groups (Fig. 1G).

Significant CA-associated increase in unmyelinated PV+ interneurons with a PNN in vmPFC pyramidal layers

The distributions of PV+ cells by category and groups were examined across cortical layers II to VI, as outlined by Nissl staining (Fig. 2A). Again, total PNN coverage and myelination was only assessed for PV+ interneurons that could be traced and unambiguously classified as myelinated or unmyelinated (Fig. 2B). As expected, myelinated (Fig. 2B; group effect: F(2,28)=3.717, P = 0.0370; layer effect: F(2.417, 67.69)=58.58, P < 0.0001; layer x group: F(8, 112) = 0.8149, P = 0.5909, followed by Tukey's multiple comparison test) [(Layer III: ~26%, CTRL; 14%, DS; ~20%, DS-CA), (Layer V: ~31%, CTRL; ~37%, DS; ~30%, DS-CA)] and PNN-covered PV+

interneurons (Fig. 2C; group effect: F(2,28)=0.4314, P = 0.6539; layer effect: F(2.634, 73.10)=50.06, P < 0.0001; layer x group: F(8, 111) = 0.9242, P = 0.4995, followed by Tukey's multiple comparison test) [(Layer III: ~22%, CTRL; ~15%, DS; ~20%, DS-CA), (Layer V: ~21%, CTRL; ~22%, DS; ~21%, DS-CA)] were mostly observed in layers III and V for each group, and the significantly greater overall number of unmyelinated PV+ interneurons with a PNN in DS-CA vmPFC samples was found to occur more specifically in layers III and V (Fig. 2D).

The soma of MBP+ PV+ interneurons is generally larger than unmyelinated ones

When examining the relationship between PV+ cell size and myelination, it was found that cell area was significantly larger in myelinated compared to non-myelinated PV+ interneurons in samples from the CTRL and DS groups (Fig. 3A, B), but not in samples from the DS-CA group (Fig. 3C).

In DS-CA samples, PV-immunofluorescence is significantly more intense in interneurons with a myelinated axon and a PNN

Lastly, we investigated whether the presence of a PNN had any incidence on the intensity of PV-immunofluorescence. When combining all cells from the three subject groups, interneurons with high PV-immunofluorescence were found to be associated with a strong probability of being accompanied by a PNN (Fig. 4A). Moreover, the intensity of PV-immunofluorescence was significantly higher in DS-CA compared to CTRL samples in cells with a PNN (Fig. 4B) or that were myelinated (Fig. 4C). Finally, the combination of PNN and a myelinated axon was associated with significantly greater PV-immunofluorescence in DS-CA samples compared to either DS or CTRL samples (Fig. 4D).

Discussion

This study is the first to thoroughly analyze PV+ interneuron myelination in the human brain, and to examine a possible relationship between the presence of a PNN and axon myelination. To achieve these goals, we examined post-mortem vmPFC samples from healthy individuals as well as from DS, with or without a history of severe CA. Our main findings are that (1) there exists a potential relationship between the presence of a PNN and axon myelination around PV+ interneurons, given the greater prevalence of PNNs around PV+ interneurons with a myelinated axon; (2) CA is associated with a three-fold increase in the proportion of unmyelinated PV+ interneurons with a PNN in pyramidal layers; (3) PV+ interneurons with a myelinated axons are on average larger than unmyelinated PV+ cells, except in DS-CA samples; and (4) the presence of myelin and/or of a PNN is associated with a greater PV-immunofluorescence intensity in DS-CA vmPFC compared to CTRL samples.

Few studies have focused on PV+ interneuron myelination since Micheva and colleagues first reported this phenomenon in mice (5), and to our knowledge, only one investigation has been conducted in humans (17). The latter study indicated, based on 10 PV+ interneurons sampled in a resected sample of temporal cortex, that 100% of these cells were myelinated. Although regional differences cannot be excluded, our larger sample size of 575 PV+ interneurons revealed a lower proportion (about 81%) of vmPFC PV+ interneurons with a myelinated axon. This suggests at least two categories of PV+ fast-spiking interneurons in this cortical area, solely based on myelination, i.e. the speed of nerve impulse conduction. Even though only two morphological subtypes of PV+ interneurons are generally recognized, i.e. chandelier and basket cells, an increasing number of studies suggests that there are morphologically and electrophysiologically distinct subtypes of PV+ basket interneurons, even within the same cortical layer (6). We can speculate that the cell categories examined in the current study (MBP+/PNN+, MBP+/PNN-,

MBP-/PNN+, MBP-/PNN-) represent subgroups of PV+ basket cells. Although we could not provide morphological data in support of this speculation, the latter is based on the fact that PV+ chandelier cells make up less than 5% of all cortical interneurons, at least in mice (18). Moreover, there is no evidence showing that chandelier cells are covered by a PNN (19, 20) or more than a small proportion of these cells project a myelinated axon (5, 17).

This and previous reports in human neocortex have found that a majority of PV+ interneurons display a PNN, a finding consistent with rodent studies (2-4, 21). As in our recent study (Tanti et al., 2022) this proportion in CTRL and DS samples was around 60-65%. In DS-CA samples, however, it was only slightly (but non-significantly) higher than in the other groups, whereas in the Tanti et al. (2022) study, it was significantly higher. This discrepancy can likely be explained by differences in cell sampling strategies, as the one adopted in the current study might have prevented the generation of representative proportions within a lower pool of subjects. Indeed, for each cell to be considered for analysis, its axon had to be clearly visible and traceable for a certain distance, which allowed to determine whether it was myelinated or not. This main selection criterion had us exclude many cells, which is different than counting all PV+ interneurons (with or without a PNN) across the cortical thickness, as was done previously (Tanti et al., 2022). Unfortunately, the latter approach became impossible in the current study once the sections had been labeled, analyzed and then Nissl-stained. Despite these methodological limitations, we were able to measure a robust three-fold CA-associated increase in the number of unmyelinated PV+ interneurons with a PNN in layers III and V. This small (20%) fraction of the PV+ interneuron population, only about 4% of which displayed a PNN in CTRL and DS samples, may be particularly sensitive to early-life adversity. Given that the samples were from middle-aged individuals, if these changes are indeed due to CA, then they are long-lasting.

One of the main questions that stimulated this study was whether or not there is a relationship between axon myelination and PNN in human vmPFC PV+ interneurons. In all three groups, the most abundant category of PV+ cells was the MBP+/PNN+ population. Moreover, the least abundant category was the MBP-/PNN+ population. This could indicate that being myelinated predisposes to being PNN-embedded, or vice-versa, further suggesting that mature PV networks are physically and functionally stabilized by axonal myelination and being enwrapped by a PNN (2). Since both cortical myelination and PNN formation occur mainly during early childhood (22, 23) it is likely that both processes occur concomitantly. Interestingly, oligodendrocyte-lineage cells are implicated in myelination but also seem to participate, at least in human vmPFC, in PNN development and maintenance (3). Oligodendrocyte precursor cells (OPCs) in mouse neocortex, which are innervated by PV+ interneurons, have been shown to fine-tune PV+ axon myelination during the critical period following sensory experiences (24, 25). Previous post-mortem studies have suggested that CA has profound and lasting effects on oligodendrocyte-lineage cells, leading to impaired myelination in the ACC and vmPFC (13, 26).

PNN formation and maturation mark the closure of developmental critical periods of plasticity, and coincide with synaptogenesis, synaptic refinement, and myelination (22, 23). PNN maturation and myelination are thought to be molecular breaks that close critical periods, thus OPC-PV+ interneuron communication during this important developmental window may play a fundamental role in modeling cortical plasticity to maintain an excitatory/inhibitory balance in the vmPFC (2). Thus, with MBP+/PNN+ PV+ cells being the most abundant PV+ interneuron subtype, we can speculate that the relationship between PNNs and myelin plays a major role in shaping cortical connectivity. In addition, as discussed above, we can hypothesize that the MBP-/PNN+ category is more strongly affected by CA. Clearly, more work is needed to investigate PV+ interneuron

morphology and electrophysiology of these distinct categories of MBP+/- and PNN+/- interneurons to better understand their relationship and respective roles, and how they are each impacted by early-life adversity.

Similar to previous research that found that the probability of PV+ interneurons being myelinated increases with axonal diameter and cell soma size (27), we found that in the CTRL and DS groups, myelinated PV+ interneurons had a significantly larger cell soma area than their unmyelinated counterparts. This was not the case in the DS-CA group, however, in which myelinated and unmyelinated PV+ cells had on average the same cell soma area. This might constitute another feature linked to the apparent vulnerability displayed by PV+ interneurons to early-life adversity. Interestingly, a recent study conducted in various regions of the adult mouse brain revealed that as the intensity of PV-immunofluorescence increases, the probability of having a PNN increases as well (28). This was replicated here in human brain samples when combining PV+ cells from the three subject groups. When breaking down the data per group, however, PV+ interneurons that had a myelinated axon, a PNN, or both, displayed a greater PV-immunofluorescence intensity in the DS-CA compared to the CTRL group. Most studies investigating PV-immunofluorescence intensity in the PFC of stressed animals, whether looking at mRNA expression or immunostaining intensity have found mixed results (7). However, none of these studies took into consideration whether PV+ cells were myelinated or surrounded by a PNN. It has been proposed that two distinct PV+ interneurons might display two distinct network configurations: one more permissive towards plasticity, characterized by weak expression of PV+ and another that limits plasticity, characterized by a strong PV+ expression (29, 30). Following this interpretation, our results suggest that PV+ interneuron plasticity is limited in DS-CA compared to healthy CTRLs due to significantly higher PV-immunofluorescence. The balance of excitatory and inhibitory neural inputs is important for

the closing and opening of critical periods of neuroplasticity (31). Also, the co-development of PNN and myelination with PV+ expression is hypothesized to be an important step of development and maturation of the PV+ framework (2). Therefore, exposure to adversity during this sensitive time may have increased inhibitory neurotransmission from PV+ interneurons, speeding up their maturation (32). This, coupled with increased recruitment of PNNs as reported here and previously by our group, may affect the timing and synchronization associated with PV+ interneuron network maturity (3, 13, 30). This accelerated maturation could lead to reduced neuroplasticity of cortical circuits, causing maladaptive information processing persisting into adulthood (33, 34).

This study is not without limitations. One of the main ones is that it included a low number of female samples, which prevented an analysis of possible sex differences. Females have a higher prevalence of MDD, but suicide is more frequent in males (35, 36), resulting in more limited access to brain samples from female depressed suicides. Another limitation comes from the strict criteria adopted to determine unambiguously the presence or absence of PV+ interneuron myelination, which led to the exclusion of many PV+ interneurons from the analysis. Therefore, our results may not necessarily be fully representative of all vmPFC PV+ interneurons. Lastly, PNNs were labelled using WFL immunostaining, but these nets can also be identified using chondroitin sulphate proteoglycans (CSPGs) from the lectican family, including aggrecan, brevican, versican and neurocan (37). WFL staining may only label a fraction of the PNNs present in a brain section, since in both mouse and humans, aggrecan-only stained PNN have been observed (38-40).

In conclusion, this study provides the first normative data on myelination and PNNs around PV+ interneurons in human neocortex. Our results suggest a relationship between myelination and PNNs, with PV+ cells displaying both being the most abundant in the vmPFC. Moreover, we provide further data indicating that severe CA has a lasting influence on these cells, namely by

increasing the proportion of unmyelinated PV+ interneurons with a PNN, and potentially leading to maladaptive neuroplasticity of this cortical area. In sum, this work provides a greater insight into neuroplastic features of human PV+ interneurons and how CA may lead to lasting maladaptive changes in vmPFC networks, a consequence thought to increase an individual's vulnerability to psychopathologies and suicide.

Conflict of Interest

The authors declare there is no conflict of interest to declare.

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Table 1: Group information.

TABLE 1	CTRL	DS	DS-CA
N	11	12	8
Axis 1 diagnosis	0	MDD (10); DD-NOS (2)	MDD (6); DD-NOS (2)
Age (years) (*P* = 0.06)	52.9 ± 3.8	40.8 ± 5.2	49.2 ± 3.9
Sex (M/F)	8/3	9/3	3/5
PMI (h) (*P* = 0.89)	57.7 ± 4.3	51.9 ± 4.0	47.4 ± 1.5
Substance dependence	0	2	1
Medication in the last 3 months	Benzodiazepine (2); Therapeutic cannabis (1); SSRI (1); Stimulants (1); Antipsychotic (1)	Opiate; Antipsychotic (1); SSRI (4); SARI (1); Atypical antidepressant (1), Benzodiazepine (1); SNRI (1); Anticonvulsant (1)	Benzodiazepine (2); Sedative-hypnotics (1); antipsychotics (3); SSRI (4); SNRI (1)
Data represent mean ± SEM. P-values generated with one-way ANOVAs. *DD-NOS* depressive disorder not otherwise specified, *MDD* major depressive disorder, *PMI* post-mortem interval; *SSRI* selective serotonin reuptake inhibitor; *SNRI* selective norepinephrine reuptake inhibitor; *SARI* Serotonin antagonist and reuptake inhibitors			

Figures and figure legends

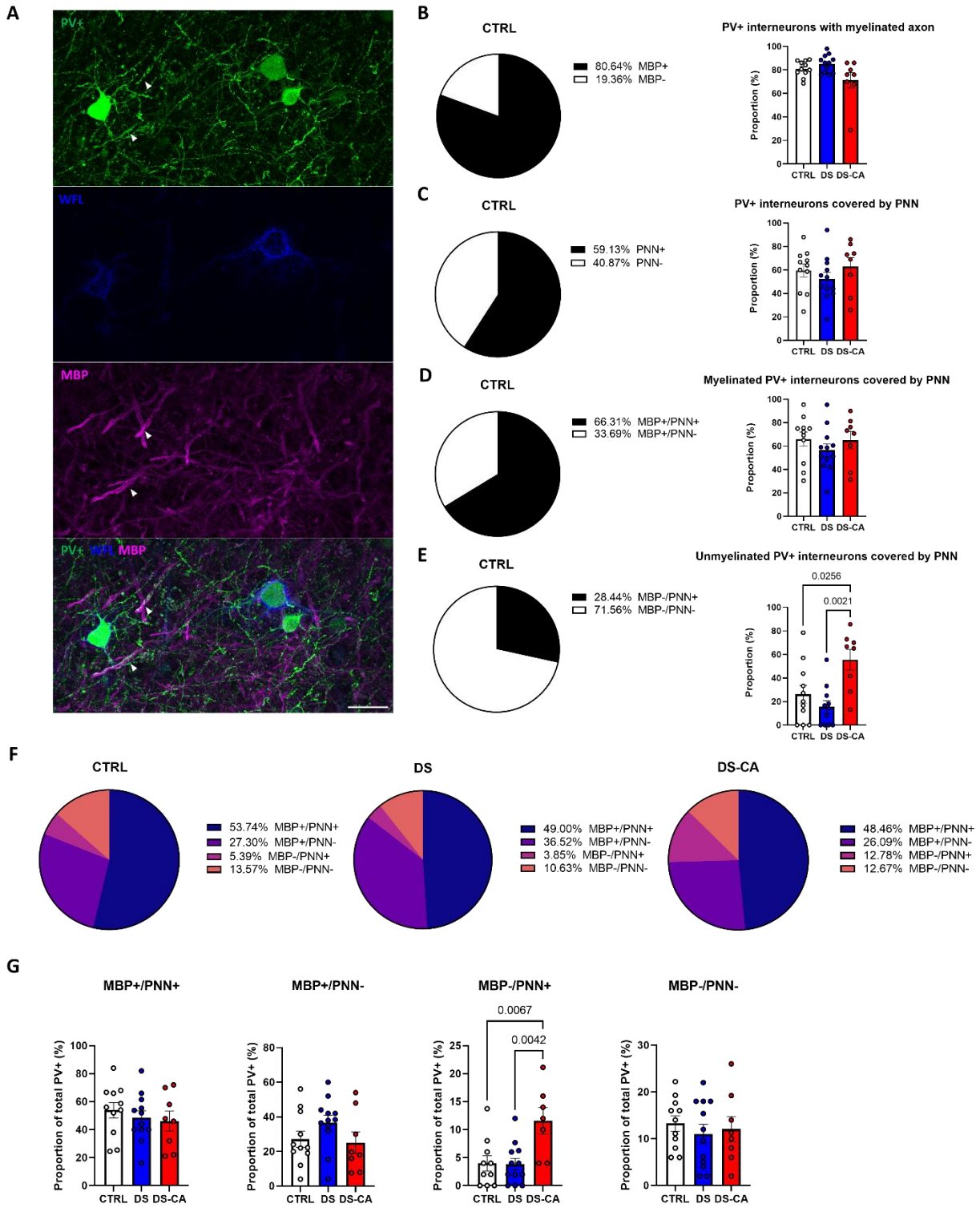

Figure 1: Proportions of PV+ interneurons that display a myelinated axon, a PNN, or both in vmPFC samples. (A) Representative high magnification images of three of the markers (PV+, WFL, MBP) used in this study, merged in the bottom panel. PV+ interneurons with and without a WFL-stained PNN are illustrated and myelinated PV+ axons labelled for MBP are highlighted by white arrows. Scale bar: 25µm (applies to all panels). (**B)** In CTRL samples, ~81% of PV+ interneurons have a myelinated axon. Group comparisons indicate similar proportions in DS and DS-CA samples. (**C)** In CTRL samples ~59% of PV+ interneurons are surrounded by a PNN, with DS and DS-CA samples displaying similar proportions. (**D)** In all three groups, the majority (~63%) of PV+ interneurons were found to be MBP+/PNN+ when looking at the proportion of myelinated PV+ interneurons surrounded by a PNN. **(E)** Significantly more MBP-/PNN+ were found in DS-CA compared to CTRL and DS samples while examining the proportion of unmyelinated PV+ interneurons surrounded by a PNN (Ordinary one-way ANOVA: $F(2, 27) = 7.474$, $P = 0.0026$, followed by Tukey's multiple comparisons test). **(F)** In all three groups, most PV+ interneurons are MBP+/PNN+ when investigating the proportion of all PV+ interneurons counted. **(G)** Proportion of PV+ interneurons in each category is not significantly different between groups in MBP+/PNN+ (Ordinary one-way ANOVA: $F(2, 28) = 0.4702$, $P = 0.6297$, followed by Tukey's multiple comparisons test), MBP+/PNN- (Ordinary one-way ANOVA: $F(2, 28) = 1.600$, $P = 0.2197$, followed by Tukey's multiple comparisons test) and MBP-/PNN- (Kruskal-Wallis ANOVA: $H(2) = 0.7213$, $P = 0.6972$, followed by Dunn's multiple comparison test), except in MBP-/PNN+ PV+ interneurons, which displayed a significant three-fold higher proportion in DS-CA samples compared to CTRL and DS samples (Ordinary one-way ANOVA: $F(2, 26) = 7.424$, $P = 0.0028$, followed by Tukey's multiple comparisons test). Data are presented as mean ± s.e.m.

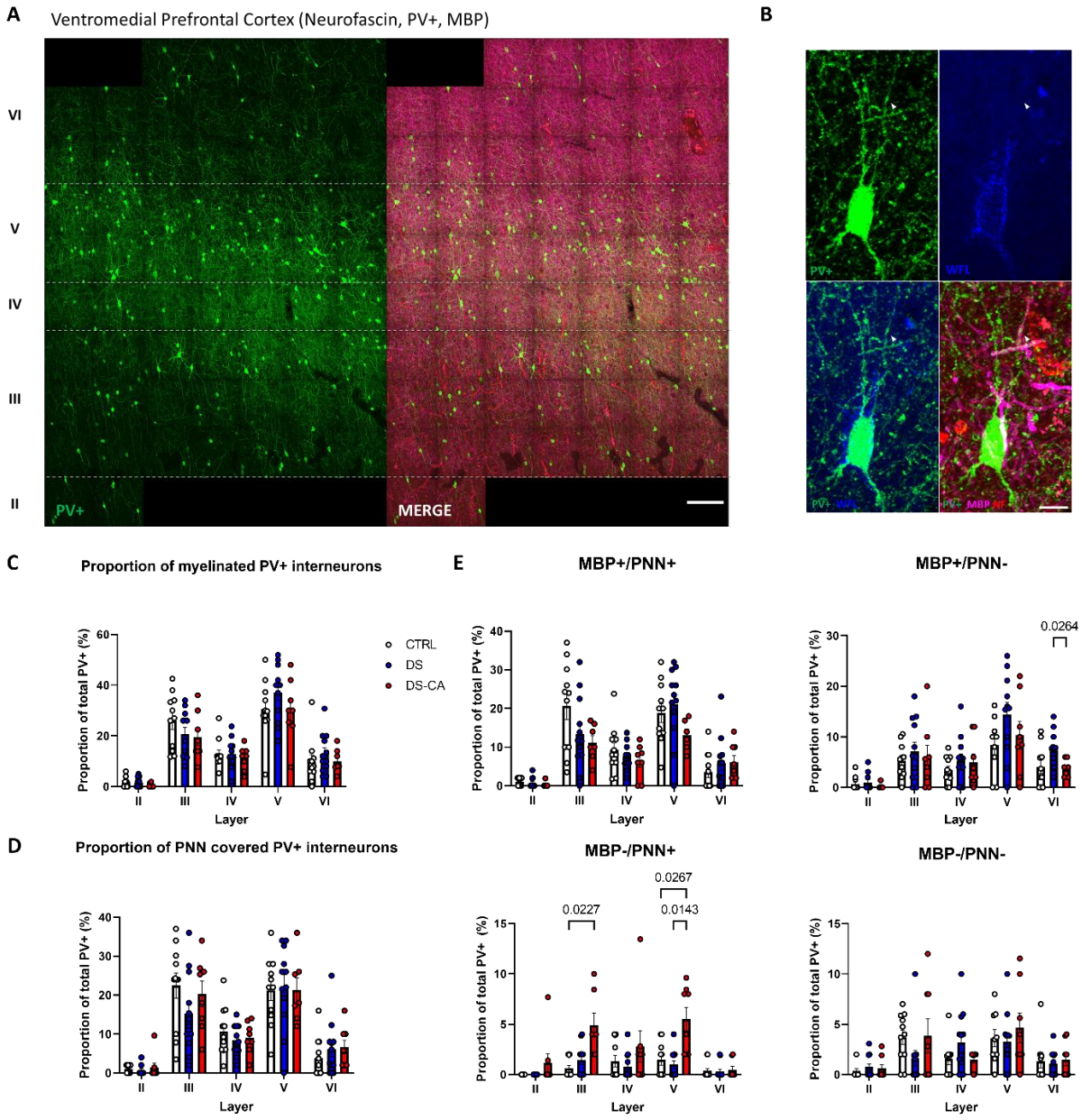

Figure 2: Significant CA-associated increase in unmyelinated PV+ interneurons with a PNN in vmPFC pyramidal layers. (A) Representative high magnification image of a stack of multi-area time-lapse confocal images illustrating the distribution of PV+ interneurons (green), Neurofascin-stained axons (red) and MBP-stained myelin (magenta) and their distribution throughout vmPFC pyramidal layers (60x magnification). Scale bar: 200µm (applies to both panels). **(B)** Representative high magnification images of a myelinated PV+ axon directly linked

to its PV+ cell body surrounded by a WFL-stained PNN (MBP+/PNN+). The myelinated axon labelled with MBP is highlighted with white arrows. Scale bar: 10μm (applies to all panels). **(C)** The majority of PV+ axons are myelinated in layers III and V, with similar proportions observed between groups. (**D)** The majority of PV+ interneurons are surrounded by a PNN in layers III and V, with no significant differences between groups. (**E)** The proportion of PV+ interneurons in each category through layers II to IV is not significantly different between groups for MBP+/PNN+ (group effect: $F(2,28)=2.045$, $P = 0.1483$; layer effect: $F(2.534, 69.04)=37.86$, $P < 0.0001$; layer x group: $F(8, 109) = 2.041$, $P = 0.0480$, followed by Tukey's multiple comparison test) and MBP-/PNN- PV+ interneurons (group effect: $F(2,28)=0.2748$, $P = 0.7617$; layer effect: $F(3.008, 81.23)=9.163$, $P < 0.0001$; layer x group: $F(8, 108) = 1.409$, $P = 0.2010$, followed by Tukey's multiple comparison test). The proportion of layer VI MBP+/PNN- cells was significantly higher in DS vs DS-CA samples (group effect: $F(2,28)=2.662$, $P = 0.0874$; layer effect: $F(2.722, 73.48)=22.42$, $P < 0.0001$; layer x group: $F(8, 108) = 0.7461$, $P = 0.6507$, followed by Tukey's multiple comparison test). The proportion of MBP-/PNN+ interneurons in layers III and V was found to be higher in DS-CA compared to CTRLs (group effect: $F(2,28)=8.510$, $P = 0.0013$; layer effect: $F(2.714, 73.27)=16.91$, $P < 0.0001$; layer x group: $F(8, 108) = 5.267$, $P < 0.0001$, followed by Tukey's multiple comparison test). Data are presented as mean ± s.e.m.

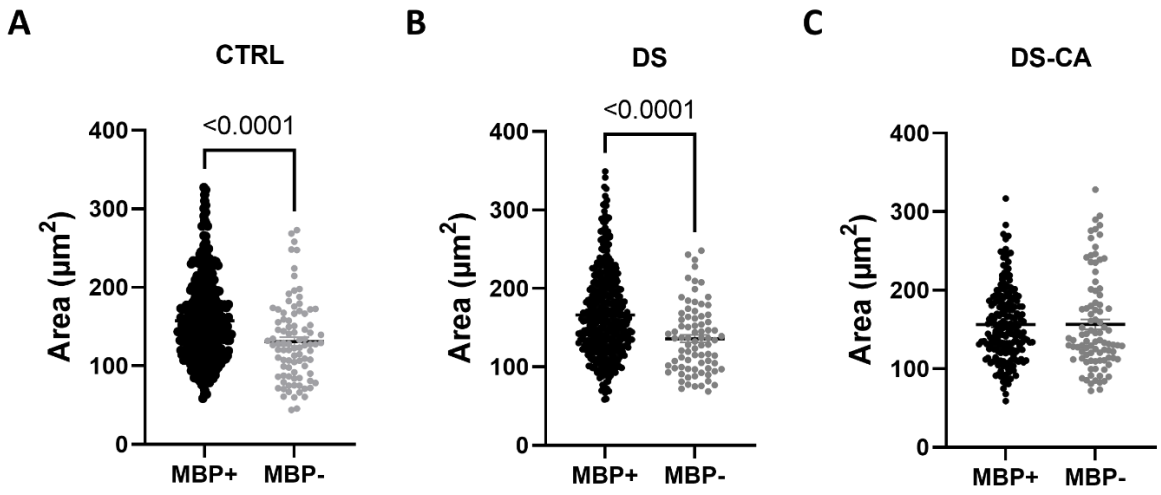

Figure 3: The cell body area of myelinated PV+ interneurons is significantly larger compared to unmyelinated PV+ interneurons in CTRL and DS but not in DS-CA vmPFC samples. (A) In CTRLs (*N*=11), myelinated PV+ interneurons have a significantly larger cell body area than unmyelinated PV+ interneurons (Mann-Whitney test: U = 10961, $P < 0.0001$). **(B)** Myelinated PV+ interneurons also have a significantly larger cell body area than unmyelinated PV+ interneurons in DS (*N*=12) (Mann-Whitney test: U = 8716, $P < 0.0001$). **(C)** In DS-CA (*N*=8), there are no significant differences in PV+ cell body area between myelinated and unmyelinated interneurons (Mann-Whitney test: U = 8924, $P = 0.3313$). Data are presented as mean ± s.e.m.

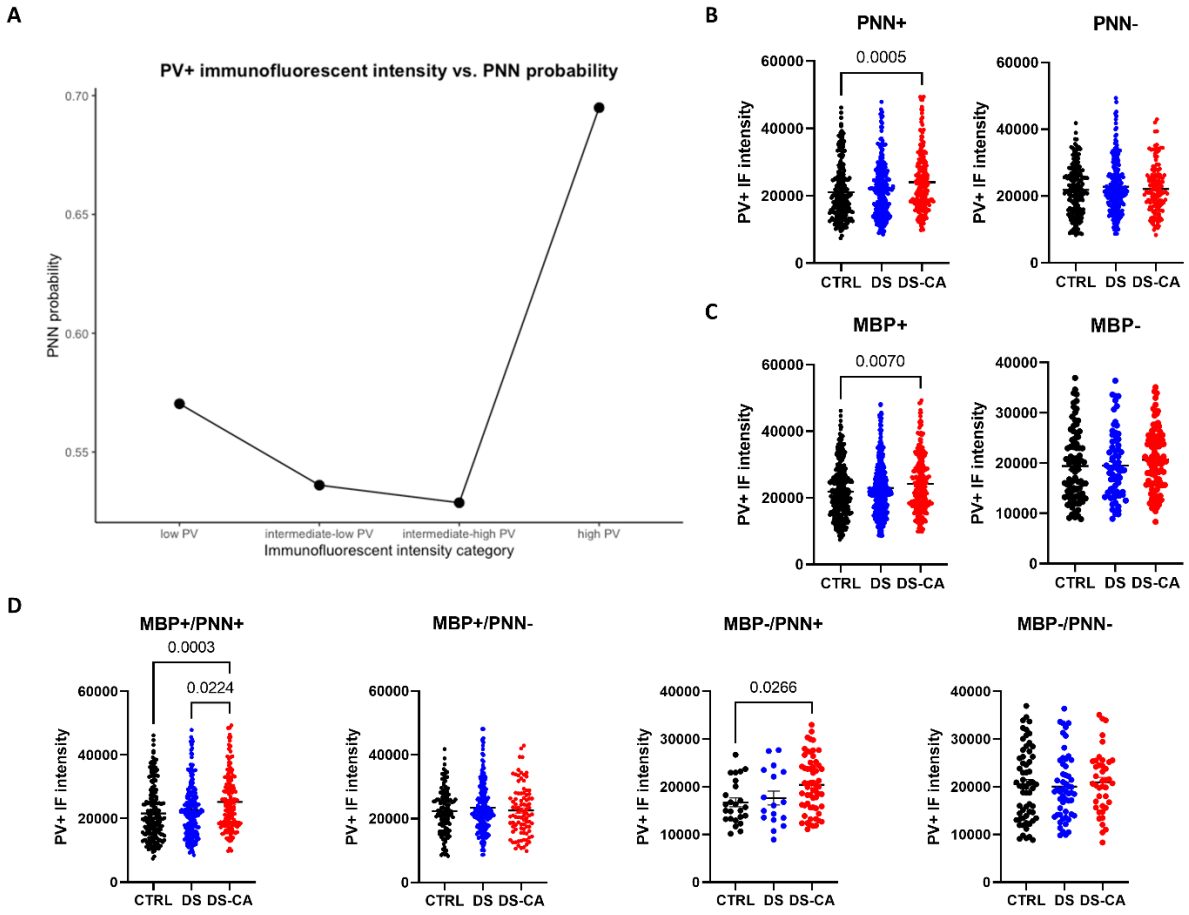

Figure 4: PV-immunofluorescence is significantly more intense in vmPFC interneurons with a myelinated axon and a PNN in DS-CA samples. **(A)** The probability for PV+ interneurons to be surrounded by a PNN is greater in the high PV-immunofluorescence intensity category than in the lower intensity categories. **(B)** The intensity of PV-immunofluorescence in cells with a PNN (PNN+) is significantly higher in DS-CA compared to CTRL samples (Kruskal-Wallis ANOVA: H(2) = 14.39, $P = 0.0007$, followed by Dunn's multiple comparison test). This is not the case for PV+ interneurons without a PNN (PNN-) (Kruskal-Wallis ANOVA: H(2) = 0.4362, $P = 0.8041$, followed by Dunn's multiple comparison test). **(C)** PV-immunofluorescence intensity of myelinated interneurons (MBP+) is significantly brighter in DS-CA and DS compared to CTRL samples (Kruskal-Wallis ANOVA: H(2) = 9.321, $P = 0.0095$, followed by Dunn's multiple

comparison test), however no significant difference was observed when looking at the immunofluorescence intensity of unmyelinated PV+ interneurons (MBP-) (Kruskal-Wallis ANOVA: H(2) =2.817, P = 0.2445, followed by Dunn's multiple comparison test). **(D)** PV-immunofluorescence was significantly more intense in DS-CA compared to CTRL samples for MBP+/PNN+ (Kruskal-Wallis ANOVA: H(2) = 15.41, P = 0.0005, followed by Dunn's multiple comparison test) and MBP-/PNN+ interneurons (Kruskal-Wallis ANOVA: H(2) = 7.970, P = 0.0186, followed by Dunn's multiple comparison test). No significant difference was found when comparing PV-immunofluorescence intensity between groups in the remaining categories: MBP+/PNN- (Kruskal-Wallis ANOVA: H(2) = 0.5752, P = 0.7501, followed by Dunn's multiple comparison test) and MBP-/PNN- (Kruskal-Wallis ANOVA: H(2) = 0.6758, P = 0.7133, followed by Dunn's multiple comparison test). Data are presented as mean ± s.e.m.

Supplementary Information

Percent of MBP+/PNN+ PV+ interneurons was correlated with age (Supplementary Table 1) but controlling for this factor did not change the outcome of group comparisons (ANCOVA with group as fixed factor with age as a covariate: group effect: F (2, 27) = 0.354, P = 0.705; age effect: P = 0.085).

Percent of MBP+/PNN- PV+ interneurons was inversely correlated with age (Supplementary Table 1) but controlling for this factor did not change the outcome of group comparisons (ANCOVA with group as fixed factor with age as a covariate: group effect: F (2, 27) = 0.678, P = 0.516; age effect: P = 0.049).

Supplementary Table 1: Correlation coefficients and P values for Spearman's rho non-parametric measure of association between co-variates and dependant variables.

Spearman's rho		MBP+/PNN+		MBP+/PNN-		MBP-/PNN+		MBP-/PNN-		Area	
		Percent	Intensity	Percent	Intensity	Percent	Intensity	Percent	Intensity	MBP+	MBP-
Age	Coef.	0.367	0.119	-0.427	-0.155	0.248	-0.151	-0.147	-0.045	0.137	-0.049
	Sig.	0.042	0.522	0.017	0.405	0.179	0.501	0.429	0.809	0.464	0.793
PMI	Coef.	0.117	0.19	-0.152	0.171	-0.047	-0.195	-0.047	-0.185	-0.143	-0.176
	Sig.	0.53	0.305	0.415	0.356	0.8	0.385	0.802	0.32	0.443	0.344
Antidepressants	Coef.	-0.174	0.136	-0.048	-0.191	0.118	0.212	0.135	0.139	-0.256	0.007
	Sig.	0.349	0.465	0.797	0.304	0.529	0.343	0.468	0.454	0.164	0.969
Laser Power	Coef.	-	-0.285	-	-0.115	-	0.275	-	-0.037	-	-
	Sig.	-	0.12	-	0.54	-	0.216	-	0.842	-	-

Chapter III: Discussion

Summary of research

This research is the first to have examined the proportions of cortical PV+ interneurons displaying a myelinated axon and PNN coverage in the human brain, and to explore how a history of CA and/or depression could impair these proportions. We found that in vmPFC samples from neurotypical individuals, 81% of PV+ interneurons display a myelinated axon, and that this proportion is similar in all groups examined. This is substantially different than what was previously found from the analysis of only 10 PV+ interneurons in resected temporal lobe (Stedehouder & Kushner, 2017). That study had concluded that 100% of cortical PV+ interneurons were myelinated in human cortex. Our results indicate a greater diversity of PV+ interneurons than previously thought. Interestingly, PV+ interneurons with a myelinated axon were found to display a significantly larger cell body area than unmyelinated PV+ interneurons in CTRL and DS but not in DS-CA vmPFC samples. This larger soma size is consistent with previous studies conducted in animal models (Stedehouder et al., 2019), and our result in DS-CA samples points to another potential sign of PV+ interneuron vulnerability to ELA.

Our investigation was also the first to examine the potential relationship between PV+ myelination and PNN presence in the human brain. The majority of PV+ interneurons were found to be myelinated and surrounded by a PNN (~63%) in all groups, indicating a strong association between the two. Moreover, our results revealed a significantly greater proportion of unmyelinated PV+ interneurons with a PNN in the vmPFC of DS-CA compared DS and CTRL, and this phenomenon was found to occur in pyramidal layers. Lastly, the intensity of PV-immunofluorescence in myelinated and/ or PNN embedded cells was significantly greater in DS-CA vmPFC compared to DS and CTRL samples.

Additional discussion arguments

It is a well-established fact that individuals with a history of CA are more susceptible to mental illnesses. A large number of studies report that this increased vulnerability is linked to a stress-activated accelerated maturation of the fronto-limbic network (Callaghan & Tottenham, 2016). Some of the earliest investigations in the amygdala report that ELA accelerated both behavioral and neural responses to stress which led to changes in amygdala volume across development (Moriceau et al., 2009; Tottenham et al., 2010; Whittle et al., 2013). The structural and functional development of the hippocampus and the mPFC, which regulate amygdala activity, are also accelerated by adversity. Studies in both the hippocampus and mPFC demonstrate smaller brain volumes in humans during childhood and adult-like emotion learning behaviors in rodents following ELA (Callaghan & Richardson, 2011; Cowan et al., 2013; Hanson et al., 2010; Hanson et al., 2015). A potential reasoning for why the interactions between these three regions look more adult-like following ELA may be due to a possible adaptive and protective mechanism put in place from the lack of parental care (Callaghan & Tottenham, 2016). This adaptation is most likely a survival mechanism able to alter developmental plasticity and establish lower threshold of stress responsiveness to meet certain emotional demands (Shonkoff et al., 2009). This can lead to long-term consequences that follow individuals into adulthood making them more susceptible to mental illnesses such as depression and lead to suicide.

The work conducted in our lab has shown that this adaptation has profound effects on neurodevelopment and neurocircuitry. With PNN formation and maturation known to mark the closure of critical periods in development, an increased recruitment, morphological complexity, and maturity of PNN was reported in the human vmPFC following a history of CA.(Tanti et al., 2022). As PNN mostly surrounds PV+ basket cells, these cells are mostly concentrated in layers

II-III and V and have axonal ramifications that project to both local and translaminar regions in the neocortex (Hendry et al., 1989; Packer & Yuste, 2011). In this project, we confirmed that myelinated PV+ interneurons with a PNN are also mainly found in layers III and V. Very few studies have addressed the proportion of myelinated PV+ interneurons by layer, and only in mice (Micheva et al., 2016; Stedehouder & Kushner, 2017). Fast-spiking PV+ basket cells play an important role in providing strong cortical inhibition to other PV+ interneurons and nearby excitatory pyramidal neurons with each layer V PV+ cell inhibiting more than around 1,000 pyramidal cells (Nahar et al., 2021; Naka & Adesnik, 2016; Packer & Yuste, 2011). Additionally, this study described that the PV-immunofluorescence intensity of myelinated PV+ interneurons and/or embedded in a PNN is significantly higher in depressed suicides with CA. Previous reports have highlighted that a high PV+ intensity is indicative of a PV+ network which limits synaptic plasticity (Carceller et al., 2020; Donato et al., 2013). Additionally, higher PV+ levels enhance the probability of PNN coverage further suggesting limited circuity of the PV+ network (Lupori et al., 2023). Myelination also plays an important role in halting critical periods of plasticity, and that disruptions in myelin formation can influence brain connectivity leading to various mood disorders (Hensch, 2005). Therefore, a greater number of unmyelinated PV+ interneurons surrounded by a PNN in DS-CA points to oligodendrocytes regulations and functions being affected by CA. In agreement, our lab has reported CA-associated impairments on oligodendrocyte lineage cells in the vmPFC and ACC white matter (Lutz et al., 2017; Tanti et al., 2018). Based on the findings that OPCs express the canonical components of PNNs, we can assume that OPC regulate both PNN formation and myelination, both processes which can be dysregulated by CA (Tanti et al., 2022). Thus, PNNs ability to stabilize synaptic plasticity during developmental critical periods may impair PV+ interneurons ability to regulate the excitatory/inhibitory balance which can lead to a

number of impairments in the PFC-dependant behaviors and psychiatric disorders (Ferguson & Gao, 2018; Wang & Fawcett, 2012)

PNN and myelin formation and maturation are regulated by various cues originating from both glia and neurons. Microglia, the resident immune cells of the brain, play an important role in responding to environmental stressors along with the regulation of phagocytosis for proper brain development and circuitry (Schramm & Waisman, 2022). In fact, microglia are believed to play two distinct roles in PNN and myelin regulation through the release of various molecules such as matrix metalloproteinases (MMPs) (Traiffort et al., 2020; Venturino et al., 2021). MMPs are endopeptidases able to degrade glycoproteins that make up the extracellular matrix (ECM) (Cabral-Pacheco et al., 2020). There are many different subtypes and classes of MMPs, but the most studied is MMP-9 as it plays a key role in myelination, synaptic pruning and ECM remodelling (Reinhard et al., 2015). Previous research demonstrates that when you knock out MMP-9 in mice, PNN densities surrounding PV+ interneurons increase in the auditory cortex (Wen et al., 2017) whereas there are transient reductions in the amount of MBP in the corpus collosum (Larsen et al., 2006). Microglia have recently gained interest for their role in regulating plasticity following early life stress through the modulation of myelin and the ECM, more specifically PNNs (Crapser et al., 2021; Rahimian et al., 2021; Reemst et al., 2022). Some unpublished data from our lab indicate a downregulation of multiple MMPs, including MMP-9 in isolated microglia and the vmPFC of human post-mortem depressed suicides with a history of CA compared to controls (data not shown). All in all, microglia along with other various PNN and myelin regulators could also be affected by CA causing us to see more unmyelinated PV+ interneurons surrounded by a PNN in DS-CA samples.

Future directions

Future studies could further investigate the relationship between axon myelination and PNNs while specifically looking at PV+ cell morphology, as these interneurons exhibit both basket and chandelier morphologies. Research on chandelier cells, which display different electrophysiological properties and synaptic patterns than basket cells, is more scarce as these interneurons are much less abundant (Tremblay et al., 2016). Another intriguing result which warrants further investigation has to do with the apparent particular sensitivity of unmyelinated PV+ interneurons to a history of CA. Lastly, the impact of a PNN on the transcriptome of PV+ interneurons is another interesting research avenue. Such work was recently initiated in the Mechawar lab, and vmPFC PV+ cells with and without a PNN are being isolated with laser-capture microdissection and compared between CTRL and DS-CA samples.

Conclusion

In conclusion, this research provides novel insight on the nature and diversity of PV+ interneurons in the human brain, and how these cells may be affected by a history of CA. Our findings suggest a potential relationship between myelination and PNNs with a majority of PV+ cells exhibiting both attributes in human vmPFC. These results improve our understanding of human cortical interneurons, their vulnerability to ELA, and their possible implication in depression and suicide.

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