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Impaired cognitive function after perineuronal net degradation in the medial prefrontal cortex

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Abstract

Perineuronal nets (PNNs) are highly organized components of the extracellular matrix that surround a subset of mature neurons in the central nervous system. These structures play a critical role in regulating neuronal plasticity, particularly during neurodevelopment. Consistent with this role, their presence is associated with functional and structural stability of the neurons they ensheath. A loss of PNNs in the prefrontal cortex has been suggested to contribute to cognitive impairment in disorders such as schizophrenia. However, the direct consequences of PNN loss in medial prefrontal cortex (mPFC) on cognition has not been demonstrated. Here, we examined behavior after disruption of PNNs in mPFC of Long-Evans rats following injection of the enzyme Chondroitinase ABC (ChABC). Our data show that ChABC-treated animals were impaired on tests of object oddity perception. Performance in the cross-modal object recognition task was not significantly different for ChABC-treated rats, although ChABC-treated rats were not able to perform above chance levels whereas control rats were. ChABC treated animals were not significantly different from controls on tests of prepulse inhibition, set-shifting, reversal learning, or tactile and visual object recognition memory. Posthumous immunohistochemistry confirmed significantly reduced PNNs in mPFC due to ChABC treatment. Moreover, PNN density in the mPFC predicted performance on the oddity task, where higher PNN density was associated with better performance. These findings suggest that PNN loss within the mPFC impairs some aspects of object oddity perception and recognition and that PNNs contribute to cognitive function in young adulthood.

48 **Significance Statement**

49 Perineuronal nets (PNNs) are organized components of the extracellular matrix that surround
50 mature central nervous system neurons and are critical for the regulation of neuronal plasticity. A
51 loss of PNNs has been observed in schizophrenia and other central nervous system diseases but
52 the exact functional contribution of these structures or the consequences of their loss are not well
53 understood. Here, we show that targeted degradation of PNNs within the medial prefrontal
54 cortex disrupts performance of some tests of object oddity perception and recognition memory.
55 These findings suggest that PNNs and their loss in CNS diseases may contribute directly to the
56 presentation of cognitive dysfunction.

Introduction

Perineuronal nets (PNNs) are highly organized components of the extracellular matrix that surround the cell body, proximal dendrites, and initial axon segment of mature central nervous system (CNS) neurons (Hockfield and McKay, 1983; Wang and Fawcett, 2012). These structures play a critical role in the regulation of neuronal plasticity in the CNS (Pizzorusso et al., 2002; Sorg et al., 2016). PNNs act as a physical barrier to structural changes in the neurons and also stabilize the functional properties of these neurons. Consistent with this, PNNs are sparse early in development when plasticity is generally at its highest and increase throughout the postnatal lifespan, particularly following critical periods of plasticity (Mauney et al., 2013; Pizzorusso et al., 2002). Within these periods, cortical tissue undergoes dramatic structural reorganization of neural connectivity in response to the appropriate stimulus (Hensch, 2005). These changes are followed by a period of synaptic pruning, and then stabilization of the network long-term. In line with a role in regulating plasticity, PNN expression increases at the closure of these critical periods and degradation of PNNs can re-open these windows of heightened plasticity in adulthood (Lensjø et al., 2017; Pizzorusso et al., 2002).

Several recent studies suggest that PNNs are reduced in the post-mortem tissue of patients suffering from CNS disorders such as schizophrenia, epilepsy, and Alzheimer's disease (Baig et al., 2005; Berretta et al., 2015; Bitanihirwe and Woo, 2014; McRae and Porter, 2012; Okamoto et al., 1994; Pollock et al., 2014). In schizophrenia, post-mortem analyses of the prefrontal cortex, amygdala, and superior temporal cortex suggest reduced PNN density (Enwright et al., 2016; Mauney et al., 2013; Pantazopoulos et al., 2010). This finding has been replicated in animal models of the disease and coincides with the development of cognitive impairment (Paylor et al., 2016; Steullet et al., 2017). Post-mortem analysis of Alzheimer's

patients has also revealed deficits in PNNs in the frontal lobe (Baig et al., 2005; Brückner et al., 1999; Morawski et al., 2010). Moreover, PNNs protect against Alzheimer's pathology and their loss may render neurons particularly vulnerable to the disease pathology (Okamoto et al., 1994). PNN loss and the degradation of extracellular matrix components have also been implicated in epileptogenesis and the maintenance of seizures in epilepsy (McRae and Porter, 2012; Pollock et al., 2014). While this observational evidence is a compelling indicator that PNNs are involved in CNS disorders, our current understanding of their functional significance is limited. Studies that show coincidental PNN loss and behavioral disturbances are intriguing, but do not necessarily implicate the loss of PNNs as sufficient for causing cognitive dysfunction.

We have previously observed a reduction of PNNs in medial prefrontal cortex (mPFC) of the offspring of rats exposed to polyI:C during pregnancy (Paylor et al., 2016). As an extension of these findings, the present study examined cognitive function after targeted reduction of PNNs in the mPFC of rats using Chondroitinase ABC (ChABC). ChABC catalyzes the breakdown to glycosaminoglycan subunits of chondroitin sulfate proteoglycans (CSPGs), which are the primary component of PNNs (Brückner et al., 1998; Crespo et al., 2007). This treatment has been used extensively to degrade CSPGs in PNNs and the surrounding interstitial matrix (Fawcett, 2015). After injection, we assessed cognitive function using tasks where performance is impaired in the offspring of rats subjected to polyI:C during pregnancy, including altered object oddity preference, recognition memory, sensorimotor gating, and cognitive flexibility (set-shifting and reversal learning; Ballendine et al., 2015; Bissonette et al., 2013; Kamiński et al., 2017; Latif-Hernandez et al., 2016; Lins et al., 2018; Yang et al., 2014). We found that ChABC treatment reduced overall extracellular matrix staining within the mPFC as well as a reduced density of PNNs. These cellular changes were associated with impaired performance on an object oddity

task, and performance at chance levels in a task measuring cross-modal object recognition. Interestingly, linear regression showed that PNN density predicted performance on the oddity task. Conversely, PNN digestion did not affect performance on measures of prepulse inhibition, set-shifting, reversal learning, or tactile and visual object recognition memory. Thus, our findings support a nuanced effect of degrading mPFC PNNs on cognitive functions related to schizophrenia.

Methods

Subjects

Adult male Long Evans rats (n = 80; 300-350 g; Charles River Laboratories, Kingston, NY, USA) were used for all experiments. After their arrival, animals were pair housed in ventilated plastic cages and left undisturbed for 1 week with food and water *ad libitum* (Purina Rat Chow). A 12:12-h lighting cycle was used with lights on at 7:00am. Animals were given environmental enrichment in their home cage in the form of a plastic tube throughout the experiment. Following acclimatization, animals used for operant conditioning were maintained at 90% of free feeding weight and singly housed to ensure the appropriate amount of food was consumed by each rat in the home cage after behavioral testing. All animal procedures were performed in accordance with the [Author University] animal care committee's regulations.

Behavioral Measures.

All rats were handled for at least 5 min/day for 3 days before behavioral testing. They were also habituated to transport in an elevator from the vivarium to the testing rooms. Rats were randomly assigned to one of two groups for behavioral testing. Group 1 had ChABC or PEN infused into mPFC prior to testing two weeks later on prepulse inhibition (PPI), the cross-modal object

recognition (CMOR) battery, and the oddity task. Group 2 was food restricted and then trained to press levers for food reward in the operant conditioning chambers. After passing set-shifting (SS) Train (see below), ChABC or PEN was infused into mPFC. Two weeks later, the rats were retrained on the SS Train (3-4 days) and then tested on visual cue discrimination, set-shifting, and reversal learning.

Prepulse Inhibition (PPI): PPI measures the percent attenuation of motor response to a startling tone when that tone is preceded by a brief prepulse. Two SR-LAB startle boxes (San Diego Instruments, San Diego, CA, USA) were used. Each session had a constant background noise (70 dB) and began with 5 min of acclimatization, followed by 6 pulse-alone trials (120 dB, 40 ms). Pulse-alone (6 trials), prepulse alone (18), prepulse + pulse (72), and no stimulus (6) trials were then presented in a pseudorandom order, followed by 6 additional pulse-alone trials. Prepulse + pulse trials began with a 20 ms prepulse of 3, 6, or 12 dB above background (70 dB). Prepulse–pulse intervals (time between the onset of the prepulse and the 120 dB pulse) were short (30 ms) or long (50, 80, or 140 ms). The inter-trial interval varied randomly from 3 to 14 s (Howland et al., 2012; Lins et al., 2017).

Cross-Modal Object Recognition (CMOR) Battery: This task uses spontaneous exploratory behavior to assess visual memory, tactile memory, and visual-tactile sensory integration (Ballendine et al., 2015; Winters and Reid, 2010). The testing apparatus was a Y-shaped maze with 1 start arm and 2 object arms (10 × 27 cm) made of white corrugated plastic. A white plastic guillotine-style door separated the start arm from the object arms, and Velcro at the distal end of the object arms fixed objects in place. A removable, clear Plexiglas barrier could be inserted in front of the objects. A tripod positioned above the apparatus held a video camera that recorded the task activity. Rats were habituated to the apparatus twice for 10 min. Lighting alternated

148 during habituation between white light (used during visual phases) and red light (used during
149 tactile phases) for 5 min each with the order counterbalanced, and the clear barriers were in place
150 for one day of habituation and removed for the other with order counterbalanced between all rats.
151 Test days consisted of a 3 min sample phase with two identical copies of an object attached with
152 Velcro to the maze, a 60 min delay, and then a 2 min test phase with a third copy of the original
153 object and a novel object placed in the maze. Rats began each phase in the start arm; the
154 guillotine door was opened and closed once the rat entered the object arms. This task consisted
155 of 3 distinct tests performed on 3 separate days: tactile memory (day 1), visual memory (day 2)
156 and cross-modal memory (day 3). Red light illuminated the tactile phases allowing the rats'
157 behavior to be recorded while preventing the rats' visual assessment of the objects and the
158 removal of the clear barriers allowed for tactile exploration. White light was used during visual
159 phases, but clear Plexiglas barriers in front of the objects prevented tactile exploration. CMOR
160 had a tactile sample phase (red light, no barriers) and a visual test phase (white light, clear
161 barriers). Recognition memory was defined as significantly greater exploration of the novel
162 object than the familiar object. Video recordings of behavior were manually scored by
163 investigators blind to the treatment status of the rats and identity of the objects. Novel object
164 preference was reported as a discrimination ratio (time exploring novel object – time exploring
165 familiar object)/(total time exploring both objects) of the first minute of the test phase.

166 Oddity Discrimination: The oddity discrimination test measures object perception using
167 presentation of 3 copies of one object and a fourth distinct or 'odd' object (Bartko et al., 2007).
168 The testing apparatus was a square arena (60 x 60 x 60 cm) constructed of white corrugated
169 plastic with Velcro in each of the 4 corners. Following two days of habituation to the arena (10
170 min sessions), the test day was conducted. On test day, 3 identical objects and one different or

'odd' object made of glazed ceramic (a round 'owl' statue, 9.5 cm in diameter x 8 cm tall) or plastic (a square Lego statue, 5.5 cm (w) x 7 cm (h)) were fixed to the Velcro and the rats' activity were recorded for 5 min using a video camera mounted to the ceiling. The odd object and its location was counterbalanced among the rats in both treatment groups. Object exploration times were hand scored by an investigator blind to the treatment status of the rats. Object examination was counted when a rat's face was oriented toward the object at a maximum distance of 2 cm. Odd object preference was reported as a percentage of the total time exploring the odd object. Note that 25% is chance performance in this task (Lins et al., 2018).

Operant Set-Shifting Task (OSST): Eight operant conditioning chambers (MedAssociates Systems, St. Albans, VT, USA) in sound-attenuating cubicles were used. The chambers contained two retractable levers and two stimulus lights positioned on either side of a food port used to deliver food rewards (Dustless Precision Pellets, 45 mg, Rodent Purified Diet; BioServ, Frenchtown, NJ). A 100 mA house light illuminated the chamber. Sessions began with levers retracted and the chamber in darkness (inter-trial state), with the exception of lever training days in which the trial began with levers exposed to allow for baiting with ground reward pellets. Rats were tested once each day. *Lever training*. Rats were trained to press the levers as described previously and immediately after reaching criterion, side preference was determined (Floresco et al., 2008; Thai et al., 2013; Zhang et al., 2012). *Visual-cue discrimination*. Rats were trained to press the lever indicated by a stimulus light illuminated above it. Trials (every 20 s) began with an illumination of one stimulus light, followed 3 s later by the house light and insertion of both levers. A correct press of the lever underneath the illuminated stimulus light caused retraction of both levers and the delivery of a reward pellet. The house light remained illuminated for an additional 4 s before the chamber returned to the inter-trial state. An incorrect press returned the

chamber to the inter-trial state (all lights off) with no reward. Failure to press a lever within 10 s of their initial insertion was scored as an omission and the immediate return of the chamber to the inter-trial state. *Strategy set-shift (shift to response discrimination).* The visual-cue rule from the previous stage was reinforced with 20 trials where the rat was required to press the lever below the illuminated stimulus light. Subsequently, rats were required to change their response from the visual cue to a spatial cue (the lever opposite to their side preference, regardless of whether the stimulus light was illuminated) to receive a reward pellet. *Reversal learning*. Rats were required to press the lever opposite to the one rewarded during set-shifting. Criterion was 10 consecutive correct responses for each testing day and errors for each testing day were coded as described previously (Floresco et al., 2008; Thai et al., 2013; Zhang et al. 2012). Rats were tested for a minimum of 30 trials per day and a maximum of 150 trials per day. If a second day of testing was required, trials per criterion were calculated as the sum of the trials completed on all testing days for a given discrimination.

mPFC Infusions of ChABC or Penicillinase (PEN)

Prior to and during the procedure, rats were anesthetized with the inhalant anesthetic isoflurane (Janssen, Toronto, ON). Pre-operatively, all rats were administered a 0.5 mg/kg subcutaneous dose of the analgesic Anafen (Merial Canada Inc, QC). After animals were positioned in the stereotaxic apparatus, the scalp was cut and retracted to expose the skull. Holes were drilled above mPFC and injectors made from 35Ga silica tubing (WPI, Sarasota, FL) glued to PE-50 tubing were inserted bilaterally to the following coordinates: anteroposterior (AP) +3.0 mm; lateral (L) 0.7 mm; dorsoventral (DV) 4.4 mm relative to bregma. Either ChABC (100 units/ml) or PEN (100 units/ml) was infused (0.1 ul/min) for 2 min at DV coordinates -4.4 mm, -4.2 mm, and -3.9 mm (total infusion volume 0.6 ul/side). Injectors were left in place for an additional 6

min to allow for diffusion of the solution away from the last infusion site. Injectors were then slowly removed, the holes filled with bone wax, and wound was closed with stitches.

Tissue Collection

Following behavioral testing, rats were deeply anesthetized with isoflurane and transcardially perfused with PBS followed by 4% paraformaldehyde using infusion pumps. After perfusion, brains were extracted and stored in 4% paraformaldehyde at 4°C. One-day later, brains were transferred to 30% sucrose for several days and then frozen in isopentane and optimal cutting temperature (OCT) gel. Frozen brains were sectioned at 25 µm on a cryostat. For cFos staining, animals (PEN = 8, ChABC = 8) were time-perfused 100 minutes after assessment on the oddity object task.

Immunohistochemistry

Slides were warmed to room temperature for 20 min and then given three washes in 1X PBS for 10 min each. After which slides were incubated for 1 hour with 10% Protein Block, Serum-Free (Dako, Missisauga, ON) in 1X PBS. Slides were then incubated overnight at room temperature with a primary antibody in a solution of 1% Protein Block, 1% Bovine Serum Albumin, and 99.9% 1X PBS with 0.1% Triton X-100. Primary antibodies were as follows: Mouse anti-Chondroitin-4-Sulfate (C4S; 1:400; Millipore, Etobicoke, ON), Wisteria Floribunda Agglutinin (WFA; 1:1000; Vector Labs, Philadelphia, PA), mouse anti-Parvalbumin (1:1000; Swant, Switzerland), rabbit anti-Parvalbumin (1:1000; Swant, Switzerland); rabbit anti-IBA1 (1:200; Dako, Mississauga, ON); mouse anti-GFAP (1:200; Sigma-Aldrich, Oakville, ON); c-Fos (1:400; Cell Signaling, Whitby, ON); mouse anti-GAD67 (1:400; Millipore, Etobicoke, ON); anti-Gephryin (1:500; ThermoFisher; Rockford, IL). After overnight incubation, slides were

washed three times, twice in 1X PBS with 1% tween-20 and once in 1X PBS. Slides were then incubated for 1h with secondary antibodies in antibody solution (as above). Secondary antibodies were as follows: Streptavidin 647 (1:200; Invitrogen, Burling, ON), Donkey anti-Mouse Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR), Donkey anti-Rabbit Alexa Fluor 647 (1:200; Molecular Probes, Eugene, OR), and Donkey anti-Mouse 647 (1:200, Molecular Probes, Eugene, OR). After 1 hour incubation slides were washed again three times and mounted with DAPI (4',6-diamidino-2-phenylindole) in vectashield mounting medium (Vector Labs, Philadelphia, PA).

Microscopy

Images were acquired using a Leica DMI6000B Microscope with LAS AF computer software. The mPFC was identified using The Rat Brain in Stereotaxic Coordinates and selected based on landmarks in the DAPI nuclear staining pattern (Paxinos and Watson, 2007). The mPFC was identified between +2.76mm and +3.24mm anterior to Bregma with the imaging window aligned to the midline and extending through cortical layers 1-6. All imaging was captured at 10X magnification with a total of 6 images taken bilaterally in adjacent sections (~250µm apart). Images from the primary somatosensory jaw (S1J) area were also taken from within the same slices (directly lateral) as images of the mPFC, as a control region outside of the targeted injection area. A constant gain, exposure, and light intensity was used across all animals. Gephyrin and Neuronal Nuclei (NeuN) confocal imaging was conducted on a LEICA SP5 Confocal microscope. For each animal, four 2x2 tile scans were conducted at 25X magnification over the mPFC.

Image analysis

Analysis was completed on unmodified images by an observer blind to the experimental condition of the tissue analyzed. Cell counts for DAPI+, IBA+, PV+, c-Fos+ cells and Gephyrin+ puncta were performed using the Image-based Tool for Counting Nuclei (Centre for Bio-image Informatics, UC Santa Barbara, CA, USA) plugin for NIH ImageJ software. PNNs were counted manually using ImageJ Cell Counter function. For cell specific Gephryin+ puncta, 4 cells were selected per image from each quadrant (total number of cells analyzed = 229). For PV+ immunofluorescence and GAD67 colocalization, an overlay for all PV+ cells was generated using the ImageJ Analyze Particles function and mean brightness values taken from both PV+ and GAD67+ channels within cell marked areas. A second analysis for PV+ and c-Fos+ cell density and colocalization was conducted using a custom automated detection script in Python (Python Software Foundation. Python Language Reference, version 2.7. Available at http://www.python.org). For all images a standard rectangular area was drawn over the region of interest, spanning cortical layers 1-6, within which cells were identified and measurement parameters kept constant. For each stain measurements of mean brightness within the area were also taken. Quantification of densities are expressed as a 100x100 micron square (10000μm^2).

Statistical Analyses

All data are presented as mean ± SEM. Statistical analyses were conducted in PRISM Software (Prism Software, Irvine, CA) and significance was set at $p < 0.05$. For experiments in Figure 1-3 and 6-7, unpaired students t-tests were used to compare PEN to ChABC. Simple linear regressions were used to examine the predictive value of behavioural performance on PNN densities. For Figure 4, a two-way ANOVA of Treatment Group and Prepulse Intensity was conducted to probe deficits in prepulse inhibition. In Figure 5, in addition to unpaired students t-tests, we utilized one-sample t-tests against chance performance to probe animals performance

on object recognition. One sample t-tests to chance performance are frequently used in behavioral neuroscience to determine whether performance of a given group differs significantly from chance (Gervais et al., 2016; Jacklin et al., 2016; Lins et al., 2018).

Results

Perineuronal Nets & Interstitial Matrix. To confirm the degradation of CSPGs and PNNs after treatment with ChABC, we stained with Chondroitin-4-Sulfate (C-4-S), a marker for cleaved components of CSPGs, and Wisteria Floribunda Agglutinin (WFA), a marker for the CSPGs that preferentially labels PNNs (PEN = 40, ChABC = 40). Treatment with ChABC did not alter total cellular density (Figure 1. E) in the mPFC ($t(77)=0.37$, $p = 0.72$). Staining intensity for C-4-S was significantly greater in ChABC treated animals than controls (Figure 1. F; $t(76)=12.56$, $p < 0.0001$). ChABC treatment induced a significant reduction in WFA staining intensity (Figure 1. G; $t(77)=4.83$, $p < 0.0001$) and a reduction in PNN density within the mPFC (Figure 2. E; $t(77)=6.403$, $p < 0.0001$). As a control to demonstrate selective digestion of PNNs at the site of injection, we assessed the same measures in the S1J, lateral from the mPFC, from within the same tissue slices. Within the S1J, total cellular density was not altered by ChABC treatment (Figure 1. E; $t(76)=1.327$, $p = 0.19$). C4S staining intensity (Figure 1. F; $t(76)=0.07$, $p = 0.94$) and WFA staining intensity (Figure 1. G; $t(76)=1.03$, $p = 0.30$) within the S1J were also unaffected by ChABC treatment. We also visually inspected slides anterior of the mPFC, including the frontal association cortex and regions of the orbitofrontal cortex and found no signs of elevated C4S or reduced WFA staining intensity. Similarly, there was no overt C4S or WFA alterations posterior in regions such as the hippocampus (data not shown).

Parvalbumin-expressing (PV+) Interneurons. PNNs most frequently surround PV+ inhibitory interneurons (Härtig et al., 1992). To assess whether changes in PNNs were paralleled by cellular

loss of these inhibitory interneurons, immunostaining for an antibody specific to PV+ was performed (PEN = 40, ChABC = 40). Despite the close association between PNNs and PV+ inhibitory interneurons, the total density of PV+ cells was unchanged (Figure 2. F; $t(77)=0.74$, $p = 0.46$). However, the percentage of PV+ cells surrounded by a PNN was significantly reduced in ChABC treated animals (Figure 2. G; $t(77)=2.71$, $p < 0.01$).

GAD67 Expression. To assess whether ChABC affected the integrity of PV+ cells, immunostaining for GAD67+, a critical GABA synthesis enzyme present in PV+ cells, was performed along with PV+ staining (PEN = 16, ChABC = 16). Across all images there was no difference between PEN and ChABC groups in terms of the number of cells analyzed ($t(29)=1.28$, $p = 0.21$). PV+ fluorescence within PV+ cells did not differ between groups (Figure 3. F; $t(29)=1.17$, $p = 0.25$). Similarly, ChABC treatment did not result in an overall change in GAD67+ fluorescence from within PV+ cells (Figure 3. G; $t(29)=0.99$, $p = 0.33$).

Gephryin+ Puncta. To further examine the cellular consequences of ChABC treatment, we assessed Gephryin, a major scaffolding protein at inhibitory synapses, to determine whether PNN loss resulted in changes in inhibitory connectivity (PEN = 8, ChABC = 8).Within the mPFC, the total number of Gephryin+ puncta was not affected by ChABC treatment ($t(14)=1.30$, $p = 0.22$). Next, we assessed Gephryin+ puncta colocalized with NeuN, a marker for neuronal cells. A total of 229 cells were analyzed (avg = 14.31 per animal) and measured cell size did not differ between PEN or ChABC animals ($t(14)=0.27$, $p = 0.82$). The number of Gephyrin+ puncta colocalized with NeuN did not differ between groups (Figure 3. H; $t(14)=0.67$, $p = 0.51$)

Immune Cell Labeling. To assess the degree of reactive inflammation to the injection of ChABC or PEN, immunostaining for IBA1+ microglia and GFAP+ astrocytes was performed (PEN = 16, ChABC = 16). Intensity of IBA1+ immunofluorescence was not altered by ChABC, (Figure 4.

C; $t(30)=0.50$, $p = 0.61$) but IBA1+ microglia cell density was significantly increased in treated animals (Figure 4. D; $t(30)=2.31$, $p < 0.05$). Treatment with ChABC did not significantly alter GFAP+ immunoreactivity (Figure 4. E; $t(30)=0.28$, $p = 0.79$).

Prepulse Inhibition. To assess whether PNN degradation resulted in deficits in sensorimotor gating, rats were tested on a (PPI) task using the presentation of acoustic stimuli. Rats showed a robust startle response to presentation of 120-db tones in all treatment groups (PEN = 25, ChABC = 24). We observed a main effect of pulse block ($F(2,141) = 56.65$, $p < 0.0001$) indicating habituation of the startle response over the testing session. ChABC treatment resulted in a marginally increased startle response but this effect was not significant ($F(1,141) = 3.20$, $p = 0.08$). Rats in both treatment groups displayed greater PPI for trials with louder prepulses (Figure 4. B). A main effect of prepulse intensity ($F(2,141) = 35.44$, $p < 0.0001$) confirmed this observation (Figure 4. B). There was no main effect of treatment with ChABC on prepulse inhibition ($F(1,141) = 0.01$, $p = 0.93$) and no interaction between prepulse intensity and treatment ($F(2,141) = 0.25$, $p = 0.78$). Linear regression was used to investigate the relationship between PNN density and prepulse inhibition for 12 dB prepulses but no significant relationship was detected ($R^2 < 0.01$, $p = 0.91$)

CMOR. To assess whether PNN degradation affected recognition memory we assessed rats on a CMOR task (PEN = 20, ChABC = 23). Both groups showed similar levels of total object exploration during the sample phases of all three tests (tactile: PEN=43.02±2.44 s, ChABC=47.57±3.12 s; visual: PEN=7.92±0.68 s, ChABC=7.92±0.50 s; cross-modal: PEN=46.14±3.69 s, ChABC=42.74±3.12 s; statistics not shown). In the tactile object recognition testing phase, both groups had similar total exploration time of the objects (Figure 6. B; $t(46)=1.31$, $p = 0.26$) and discrimination ratio for the novel object was not affected by treatment

($t(46)=0.32$, $p = 0.75$). One sample t-tests revealed that rats in both groups displayed a preference for the novel object significantly greater than expected by chance (PEN $t(23)=6.80$, $p<0.001$; ChABC $t(23)=8.59$, $p<0.001$). In the visual object recognition testing phase, both groups had similar total exploration time of the objects (Figure 6. C; ($t(46)=0.21$, $p = 0.83$) and discrimination ratio for the novel object was not affected by treatment ($t(46)=0.19$, $p = 0.85$). Rats in both groups displayed a preference for the novel object significantly greater than expected by chance (one sample t-tests, PEN $t(23)=1.97$, $p=0.03$; ChABC $t(23)=2.35$, $p = 0.01$). In the CMOR testing phase, both groups had similar total exploration time of the objects ($t(41)=1.54$, $p = 0.87$). When comparing the discrimination ratio for the novel object, rats treated with ChABC were not significantly different than control rats (Figure 6. D; $t(41)=0.86$, $p = 0.39$). However, a comparison against chance showed that PEN rats performed significantly better than to be expected if rats had no recollection of the objects (one sample t-test: $t(19) = 2.80$, $p = 0.01$) whereas rats treated with ChABC did not perform significantly better than chance ($t(22) = 1.39$, $p = 0.09$). Linear regression did not reveal significant relationships between PNN density and performance on visual, tactile, or cross modal object recognition (Figure 6. E-G; visual: $R^2 = 0.02$, $p = 0.37$; tactile: $R^2 = 0.01$, $p = 0.62$; CMOR: $R^2 = 0.02$, $p = 0.44$).

Oddity Task. As a second assessment of recognition memory function, rats (PEN = 8, ChABC = 8) were tested on an oddity task to determine if ChABC treatment impaired the ability to perceive and maintain representations of odd stimuli in their environment. There was no difference between total time exploring the objects for PEN or ChABC groups ($t(14)=0.04$, $p = 0.96$). When the percentage of exploration for the odd object was evaluated, ChABC-treated rats spent significantly less time inspecting the odd object compared to the duplicate objects than PEN-treated rats (Figure 7. B; $t(14)=2.55$, $p < 0.05$). Linear regression analysis identified a

significant relationship between PNN density and the odd object preference in both groups (Figure 6. C; $R^2 = 0.36$, $p < 0.05$).

Rats evaluated on the oddity task were perfused 100 minutes after completion of the test to permit analysis of c-Fos immunoreactivity as a marker of neuronal activity in the mPFC (PEN = 8, ChABC = 8). Treatment with ChABC did not significantly alter the total number of c-Fos+ cells (Figure 8. F; $t(14)=0.33$, $p = 0.75$) nor was there a change in the intensity of c-Fos+ immunofluorescence in the cell soma (Figure 8. H, $t(14)=0.56$, $p = 0.59$). However, a comparison of the number of PV+ cells that co-localized with c-Fos+ immunoreactivity in ChABC animals relative to controls approached statistical significance (Figure 8. G, $t(14)=2.10$, $p = 0.054$).

Set-Shifting & Reversal Learning. To determine if animals treated with ChABC had deficits in cognitive flexibility and learning, rats were assessed in set-shifting and reversal learning paradigms. Rats in both groups (PEN, n=15; ChABC, n=16) had similar trials to reach criterion for the set-shifting task ($t(29)=0.16$, $p = 0.87$) and a similar number of total errors ($t(29)=0.16$, $p = 0.87$). Comparison of perseverative errors only revealed no significant differences between treatment groups ($t(29)=0.51$, $p = 0.61$) nor did they differ statistically in regressive errors ($t(29)=0.83$, $p = 0.42$). A simple linear regression was utilized to determine the relationship between PNN density and total errors committed in the set-shifting task but no relationship was found ($R^2 = 0.03$, $p = 0.34$)

With regards to reversal learning, both PEN (n=16) and ChABC (n=16) rats required a similar number of trials to reach criterion ($t(29)=0.34$, $p = 0.74$) and committed a similar number of total errors ($t(29)=0.04$, $p = 0.97$). Errors committed by the two groups also did not differ when subdivided into perseverative errors ($t(29)=0.57$, $p = 0.57$) or regressive errors ($t(29)=1.22$, $p =$

399 0.23). A simple linear regression was utilized to determine the relationship between PNN density
400 and total errors committed. There was a weak negative relationship between PNN density and
401 total errors, but this effect was not significant ($R^2 = 0.10$, $p = 0.08$).

402 **Discussion**

403 Here, targeted delivery of ChABC was used to degrade CSPGs and PNNs in the mPFC of
404 adult rats. Immunohistochemistry confirmed that ChABC treatment elevated staining for C-4-S
405 stubs, the cleaved disaccharide components of PNNs, and decreased WFA staining, a marker for
406 CSPGs in the extracellular matrix. The density of PNNs was significantly decreased in mPFC by
407 ChABC treatment. There was no change in the density of PV+ inhibitory interneurons, but the
408 number of PV+ cells surrounded by a PNN was reduced. Furthermore, PV+ cells also had no
409 change in the fluorescence of PV+ protein, c-Fos+, gephyrin or GAD67. ChABC treatment
410 significantly increased the density of IBA1+ microglia within the mPFC. Notably, PNN loss in
411 the mPFC was accompanied by behavioral impairments in an oddity task and in CMOR, whereas
412 prepulse inhibition, set-shifting, and reversal learning were unaffected.

413 *Perineuronal Nets & Cognitive Function*

414 The battery of tasks used in the present study was developed from previous research
415 conducted to assess behavioral effects in the offspring of rats subjected to treatment with polyI:C
416 during pregnancy. As the offspring of polyI:C-treated dams display altered behavior in these
417 tasks (Howland et al. 2012; Zhang et al. 2012; Ballendine et al. 2015; Lins et al. 2018) and have
418 reduced PNNs in mPFC (Paylor et al. 2016), we reasoned it would be valuable to assess behavior
419 in the same tasks following ChABC infusions in young adulthood. In general, behavior of the
420 PEN-treated rats was similar to that previously reported for these tasks (Ballendine et al. 2015;

421 Marks et al. 2016; Lins et al. 2018); thus, we are confident in our testing protocols for these
422 groups of rats. ChABC did not significantly affect PPI or alter the startle response. Although the
423 mPFC is involved in the modulation of PPI in rats, an array of other brain areas are also involved
424 (Swerdlow et al., 2001). Therefore, it is likely that the relatively subtle manipulation of mPFC
425 PNNs we performed was insufficient to disturb the global activity of this circuit. Previously,
426 deficits in frontal-dependent object recognition tasks, including object-in-place and CMOR, were
427 observed in the male offspring of polyI:C treated dams (Howland et al. 2012; Ballendine et al.
428 2015). Other tasks, such as object recognition or the tactile and visual variants of the CMOR
429 battery, were unaffected (Howland et al. 2012; Ballendine et al. 2015). Lesions of the
430 orbitofrontal, but not mPFC, cortex impair performance of the CMOR task (Reid et al., 2014).
431 As a result, it was somewhat unexpected that injections of ChABC into mPFC impaired
432 performance of CMOR. Reconciling the effect of mPFC ChABC injections on CMOR with the
433 lack of effect on the operant conditioning-based discrimination, set-shifting, and reversal
434 learning task battery is also difficult. In particular, temporary inactivation of the mPFC impairs
435 the set-shifting aspect of the task (Floresco et al. 2008). Thus, given the relatively subtle nature
436 of the observed impairment of CMOR following mPFC ChABC injection, replication in future
437 studies is important. The circuitry involved in the object oddity task is incompletely
438 characterized, although no study to our knowledge has directly implicated the mPFC in this task.
439 Previous work has shown the involvement of lateral cortical regions including perirhinal cortex
440 in object oddity tasks (Bartko et al. 2007). As mPFC interactions with the perirhinal cortex are
441 necessary for some object memory tasks (Hannesson, 2004), it is possible that interactions
442 between these areas are also involved in the oddity task. However, this speculation will need to
443 be tested directly.

These data contribute to a growing body of literature that suggests PNNs play an important role in cognitive function. PNN loss is associated with behavioral changes in several brain disorders (Pantazopoulos and Berretta, 2016), but relatively few studies have directly examined the effect of targeted PNN degradation on cognition. PNN degradation in the mPFC was recently shown to decrease the frequency of inhibitory currents onto mPFC pyramidal cells and impair cocaine-induced conditioned-place preference memory (Slaker et al., 2015). Consistent with our findings, PNN degradation was not associated with elevated network activity as indicated by the density of c-Fos+ cells, but the number of c-Fos+ cells ensheathed by a PNN was decreased. These findings differ from the trend towards elevated c-Fos+ expression in PV+ inhibitory interneurons observed in our data. Elevated c-Fos in PV+ neurons is consistent, however, with recent data showing ChABC treatment in the anterior cingulate cortex increased the fast rhythmic activity of GABAergic interneurons (Steullet et al., 2014). Interestingly, PNN degradation by genetic knockout of the PNN component Cartilage-link-1 protein or with ChABC treatment into the perirhinal cortex enhanced object recognition (Romberg et al., 2013). Similarly, genetic depletion of Tenascin-R, a PNN component, improved performance in reversal learning and working memory paradigms (Morellini et al., 2010). In contrast, genetic knockout of Tenascin-C produced deficits in hippocampal-dependent contextual memory (Strekalova, 2002). These discrepancies may be explained by differences in the method and location of PNN manipulation, the memory task studied, and the time course of degradation and behavioral assessment. Memory impairment due to PNN disruption using ChABC depends on the timing of treatment in relation to memory formation. For example, removal of PNNs within the basolateral amygdala impairs conditioned fear memories but only if given prior to fear conditioning and extinction (Gogolla et al., 2009). Conversely, removal of PNNs within the basolateral amygdala

467 impairs drug-associated memories, but only if given after memory formation but prior to
468 extinction (Xue et al., 2014). Slaker et al. (2015) found that that WFA intensity after ChABC
469 injection into the mPFC was reduced 3, 9, and 13 days following treatment but not at 30 days
470 (Slaker et al., 2015), whereas PNN density was only significantly reduced 3 days post-injection
471 and returned to control levels by 9 days. Conversely, our data shows that PNN density and WFA
472 labelling intensity is still significantly reduced ~ 25 days post-injection. These differences might
473 be explained by animal strain differences (Sprague Dawley vs Long-Evans rats in our study) or
474 injection volume (0.6 µl total volume vs. 0.6 µl/side in our study) as ChABC concentration used
475 were similar (0.09 units/µl vs 0.1 units/µl in our study).

476 *Functional Consequences of PNN Degradation*

477 The effects of PNN degradation on neuronal structure and function are still poorly
478 understood but can be considered in light of known PNN functions, including: (1) the regulation
479 of GABAergic transmission, (2) restriction of neural plasticity, and (3) protection from oxidative
480 stress and other environmental factors. PNNs are most frequently associated with PV+ fast-
481 spiking GABAergic inhibitory interneurons. PV+ cells typically express the potassium channel
482 KV3.1b, which is thought to give rise to their rapidly repolarizing action potentials. PNNs are
483 thought to support these highly metabolically active neurons by acting as a buffers of excess
484 cation changes in the local extracellular space (Härtig et al., 1999). The loss of PNNs has also
485 been suggested to disrupt ion homeostasis and contribute to changes in functional activity of host
486 neurons (e.g., hyperexcitability; Brückner et al., 1993). PNNs are important regulators of
487 receptor function and localization on interneurons. During periods of elevated activity, synaptic
488 glutamate AMPA receptors become desensitized and are exchanged for naïve receptors from the
489 extrasynaptic pool (Heine et al., 2008). PNNs restrict this process, allowing for desensitization of

490 synapses (Frischknecht et al., 2009). Degradation of PNNs might contribute to the hyper-
491 excitability in neuronal cells that previously hosted PNNs. This is consistent with previous
492 findings that ChABC treatment increases the firing rate of inhibitory interneurons (Dityatev et
493 al., 2007). Our c-Fos immunolabeling did not conclusively identify increased immediate early
494 gene activity in PV+ cells in ChABC-treated rats following the oddity task, but a comparison of
495 the number of PV+ cells expressing c-Fos (relative to controls) approached statistical
496 significance ($p = 0.054$).

497 PNNs also play a critical role in the regulation of neural plasticity, as evidenced by their
498 role regulating critical periods of heightened plasticity during development (Sorg et al., 2016;
499 Takesian and Hensch, 2013). Notably, PV upregulation denotes the onset of critical periods and
500 the appearance of PNNs expression indicates the closure of critical periods (Hensch, 2005;
501 McRae et al., 2007; del Rio et al., 1994; Takesian and Hensch, 2013). In maturity, the
502 degradation of PNNs can re-open critical periods of elevated structural and functional plasticity
503 (Gogolla et al., 2009; Pizzorusso et al., 2002). Moreover, genetic knockouts that disrupt PNNs
504 (e.g. Cartilage-link protein 1) can permanently delay the closure of the critical period and
505 maintain a juvenile state of elevated plasticity well into adulthood (Carulli et al., 2010). Outside
506 of critical periods, PNNs maintain similar plasticity-restricting properties. The degradation of
507 PNNs with microinjections of ChABC enhances spine dynamics in hippocampal pyramidal cells
508 (Orlando et al., 2012). Similarly, injections of ChABC into the visual cortex of adult mice can
509 enhance spine dynamics and contribute to long term functional synaptic plasticity (Pizzorusso et
510 al., 2006; de Vivo et al., 2013). While digestion of PNNs in mPFC in our study was associated
511 with varying degrees of impairment on cognitive tasks, we did not evaluate markers of

512 neuroplasticity and it remains to be determined if CSPG digestion induced aberrant
513 neuroplasticity that contributed to these deficits.

514 Finally, PNNs may be protective against oxidative stress and other pathological processes
515 in CNS disease (Morawski et al., 2004; Suttkus et al., 2016). Fast-spiking PV+ interneurons are
516 highly susceptible to oxidative stress and their association with PNNs is protective in immature
517 and mature PV cells (Cabungcal et al., 2013) (Suttkus et al., 2012). While it has not been directly
518 demonstrated that PNN degradation in otherwise healthy animals results in oxidative stress
519 injury, their loss may render neurons more susceptible to insult or disease. A recent study
520 analyzed numerous genetic and environmental animal models of schizophrenia and identified
521 oxidative stress in PV+ interneurons as a common feature in 12 of 14 models evaluated (Steullet
522 et al., 2017). PNN loss was also present in 12 out of 14 of those models. While we did not detect
523 overt loss of PV+ interneurons, increased oxidative stress in PV+ cells after PNN digestion could
524 contribute to altered cognitive performance.

525 *PNNs in CNS Disease*

526 Our findings contribute to a growing body of literature that implicates PNNs and their
527 loss in the symptomatology of CNS disorders such as schizophrenia, epilepsy, and Alzheimer's
528 (Baig et al., 2005; Berretta et al., 2015; Bitanihirwe and Woo, 2014; McRae and Porter, 2012;
529 Okamoto et al., 1994; Pantazopoulos and Berretta, 2016; Pollock et al., 2014; Winship et al.,
530 2018). Decreased PNN density in the prefrontal cortex, superior temporal cortex, and amygdala
531 has been reported in post-mortem tissue from patients diagnosed with schizophrenia (Enwright et
532 al., 2016; Mauney et al., 2013; Pantazopoulos et al., 2010). The loss of PNNs in the mPFC has
533 also been recapitulated in animal models of schizophrenia (Paylor et al., 2016; Steullet et al.,
534 2017). Our finding that PNN loss can disrupt performance on the CMOR task are of particular

535 importance in this context, as polyI:C affected animals present with a CMOR deficit (Ballendine
536 et al., 2015). In schizophrenia, disturbances to the inhibitory system have been reported,
537 including loss of PV+ expression and GAD67, the GABA synthesis enzyme (Enwright et al.,
538 2016; Glausier et al., 2014; Kimoto et al., 2014; Volk et al., 2000). CSPG digestions with
539 ChABC did not induce significant changes in PV+ or GAD67+ fluorescence within PV+ cells.
540 ChABC digestion induces a transient loss of CSPGs and PNNs, and it may be that altered PV
541 and GAD67 expression in schizophrenia may results from chronic absence of PNNs around PV+
542 cells. Conversely, PNN decreases in schizophrenia may be the result of long term, developmental
543 dysregulation of PV+ cells which also disrupts the healthy expression of PV and GAD67.
544 Similarly, we did not detect significant changes in the density of Gephyrin+ puncta, which can
545 be used to identify the presynaptic terminals of inhibitory synapses in the CNS. This suggests
546 that our ChABC injections did not grossly modify the number of inhibitory synapses. However,
547 our measurements are only sensitive to a net gain or loss of inhibitory synaptic contacts, and not
548 changes to the turnover rate. Previous studies using in vivo imaging have shown that ChABC can
549 destabilize dendritic spines and increase their motility while not affecting the net number, length,
550 or volume (de Vivo et al., 2013).

551 *Conclusion*

552 Our findings demonstrate that ChABC degrades PNNs and the interstitial matrix of the
553 extracellular matrix in the mPFC. The loss of PNNs was associated with impairment in oddity
554 object identification and object recognition memory. These findings contribute to growing body
555 of literature suggesting that PNNs play an important role in healthy cognitive function and may
556 have relevance for brain disorders (e.g., schizophrenia) where the pathology includes a loss of
557 PNNs. While the mechanisms by which PNNs are reduced in these diseases is not well

understood, interventions that target the loss of PNNs or stimulate their development could reduce cognitive impairment in neurodevelopmental or neurodegenerative diseases.

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Figures

Figure 1. ChABC treatment increases C4S staining for cleaved CSPG stubs and decreases WFA expression of the extracellular matrix. Representative images of DAPI (A), C4S (B), WFA (C), and merges images (D). Within the mPFC, PEN-treated and ChABC-treated animals had no difference in total cellular density (E). PEN animals had minimal expression of C4S for cleaved CSPG stubs but after ChABC treatment this significantly increased (F). There was also a significant reduction in WFA expression in ChABC treated animals (G). Similar analysis of the S1 (middle panels) of the same tissue slices from PEN-treated and ChABC-treated animals revealed no differences in C4S or WFA consistent with the localized injection and degradation we observed. Higher magnification images (left) images are 100x100 um ($10000um^2$) insets taken from white-lined boxes (D, left). Scale bar (white) is 100 microns. PEN, n=40; ChABC, n=40. * = $p < 0.05$

Figure 2. ChABC treatment reduced PNN density but did not affect PV+ interneurons. Representative images of DAPI (A), WFA (B), PV+ (C), and merges images (D). An examination of PNN density (E) showed that ChABC-treated animals had a significant reduction in PNNs. The density of PV+ interneurons was unchanged after PNN degradation (F). Higher magnification images (middle right) from the mPFC of PEN and ChABC showed that significantly less PV+ cells were surrounded by a PNN in ChABC treated animals (G). Higher magnification images are 100x100 um (10000um^2) insets taken from white-lined boxes (D, left). Scale bar (white) is 100 microns. PEN, n=40; ChABC, n=40. * = $p < 0.05$

Figure 3. To evaluate the effect of ChABC treatment on PV+ cells (A), we examined PV+ and GAD67+ (B), cell fluorescence (merged in C). Additionally, we examined the number of Gephryin+ puncta on neuronal cells labelled with NeuN (D,E; NeuN+ cell = green, colocalized Gephyrin+ puncta = black, puncta not colocalized with NeuN = red). ChABC treatment did not result in any change in PV+ fluorescence within PV+ cells (F). Similarly, GAD67+ expression in PV+ was not affected by ChABC. The number of Gephyrin+ puncta colocalized with NeuN+ cells was also unaffected by ChABC treatment. Images are 100x100 um (10000um^2) in size. PEN, n=16; ChABC, n=16. * = $p < 0.05$.

Figure 4. ChABC treatment increased microglial density but did not result in a robust immune response over PEN-treated control animals. Representative images are shown for DAPI (A), IBA1 (B), GFAP (C), and merged images (D). ChABC treatment did not result in overall changes in IBA1 staining intensity (C), but did cause a small but significant increase in IBA1+ cell density (D). Similar to IBA1, ChABC injection did not result in overt changes in GFAP staining intensity for astrocytes (E). Higher magnification images (middle right) are 100x100 um ($10000um^2$) insets taken from white-lined boxes (D, left). Scale bar (white) is 100 microns. * = $p < 0.05$ Scale bar (white) is 100 microns. PEN, n=16; ChABC, n=16. * = $p < 0.05$.

Figure 5. PNN degradation did not affect PPI. (A) Graphic representation of the behavioral assay. (B) Rats showed greater PPI for trials with increasingly loud prepulses. However, ChABC treatment did not affect PPI at any prepulse intensity. PEN, n=25; ChABC, n=24.

Figure 6. PNN degradation resulted impaired cross-modal recognition memory. (A) Graphic illustration of the behavioral assay. To emphasize the tactile modality (top) in object recognition, the lights are turned off during the task to limit rat's ability to gather visual information about the object. In the visual phase (middle), the lights are on but the glass pane is positioned between the rat and the object, preventing them from gathering tactical information about the object. In the cross-modal phase (lower), animals are trained in one modality (e.g. tactile) and tested in the other (e.g. visual) to challenge integration across sensory modalities. ChABC treatment did not result in any changes in performance in tactile (B) or visual OR (C) and after ChABC treatment, animals still performed significantly better than chance. In the cross-modal OR (D) phase, animals treated with ChABC were not able to perform at better than chance levels whereas PEN treated animals were. Linear regression were conducted to determine the predictive value of animals performance on the task of their PNN density, but no relationship was observed for the tactile (E), visual (F), or cross-modal (G) components of the task. PEN, n=20; ChABC, n=23. * = $p < 0.05$.

Figure 7. PNN degradation impaired performance on the oddity task and performance was predictive of PNN density. (A) Graphic illustration of the oddity task. Animals are presented with 4 objects, 3 of which are common and 1 of which is odd. (B) Animals treated with ChABC had a significant impairment in % exploration for the odd object compared to PEN animals. (C) Linear regression showed that animal's PNN density, irrespective of treatment group, was predictive of performance on the oddity task. PEN, n=8; ChABC, n=8. * = $p < 0.05$.

Figure 8. To evaluate the effect of behavioural testing on cellular activity, we time-perfused (100-minutes) a subset (n=16) of animals after the oddity object experiment and examined c-Fos+ expression, a marker of heightened neuronal activity. Representative images for PV+ cells (A), c-Fos (B), and merged images (C). ChABC treatment did not result in a change in the total number of c-Fos+ cells within the mPFC, (F) it did however result in an slight increase in the number c-Fos+ colocalized with PV+, but this effect did not reach statistical significance (G). ChABC treatment did not affect c-Fos+ fluorescence within PV+ cells (H). Images are 100x100 um (10000um^2). PEN, n=8; ChABC, n=8. * = $p < 0.05$.