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Cross-species conservation in the regulation of parvalbumin by perineuronal nets

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Parvalbumin (PV) neurons play an integral role in regulating neural dynamics and plasticity. Therefore, understanding the factors that regulate PV expression is important for revealing modulators of brain function. While the contribution of PV neurons to neural processes has been studied in mammals, relatively little is known about PV function in non-mammalian species, and discerning similarities in the regulation of PV across species can provide insight into evolutionary conservation in the role of PV neurons. Here we investigated factors that affect the abundance of PV in PV neurons in sensory and motor circuits of songbirds and rodents. In particular, we examined the degree to which perineuronal nets (PNNs), extracellular matrices that preferentially surround PV neurons, modulate PV abundance as well as how the relationship between PV and PNN expression differs across brain areas and species and changes over development. We generally found that cortical PV neurons that are surrounded by PNNs (PV+PNN neurons) are more enriched with PV than PV neurons without PNNs (PV-PNN neurons) across both rodents and songbirds. Interestingly, the relationship between PV and PNN expression in the vocal portion of the basal ganglia of songbirds (Area X) differed from that in other areas, with PV+PNN neurons having lower PV expression compared to PV-PNN neurons. These relationships remained consistent across development in vocal motor circuits of the songbird brain. Finally, we discovered a causal contribution of PNNs to PV expression in songbirds because degradation of PNNs led to a diminution of PV expression in PV neurons. These findings reveal a conserved relationship between PV and PNN expression in sensory and motor cortices and across songbirds and rodents and suggest that PV neurons could modulate plasticity and neural dynamics in similar ways across songbirds and rodents.

KEYWORDS

songbird, zebra finch, auditory cortex, motor cortex, basal ganglia, globus pallidus, striatum, development

Introduction

Parvalbumin (PV) neurons play a central role in regulating neural plasticity and in shaping neural dynamics. For example, the emergence of PV neurons in the visual cortex of rodents coincides with the onset of the critical period for visual plasticity (del Río et al., 1994; Sugiyama et al., 2008; Takesian and Hensch, 2013; Cisneros-Franco et al., 2020), and manipulations of PV

expression affect plasticity and the timing of critical periods for sensory and cognitive systems (Murray et al., 2015; Wöhr et al., 2015; Hou et al., 2017; Xia et al., 2017; Cisneros-Franco and de Villers-Sidani, 2019; Deng et al., 2019; Miranda et al., 2022). Furthermore, manipulations of PV neuron activity affects neural dynamics across various brain systems (e.g., Spiro et al., 1999; Cardin et al., 2009; Sohal et al., 2009; Pyka et al., 2011; Albéri et al., 2013; Liu et al., 2013; Hu et al., 2014; Xue et al., 2014; Wöhr et al., 2015; Xia et al., 2017; Carceller et al., 2020), and PV neuron dysfunction can lead to cognitive and behavioral dysfunctions (Cardin et al., 2009; Sohal et al., 2009; Hu et al., 2014; Wöhr et al., 2015; Enwright et al., 2016; Favuzzi et al., 2017; Xiang et al., 2022). Therefore, it is important to reveal the factors that influence PV expression.

Developmental experiences and the expression of various molecular and cellular markers are known to affect the abundance of PV within PV neurons (i.e., PV intensity). For example, as PV neurons in the visual cortex mature and accumulate more of the orthodenticle homeobox protein Otx2, PV neurons become more enriched with PV, and manipulations that revert PV neurons to an immature and plastic state (e.g., inhibition of Otx2 expression) decrease PV intensity (Sugiyama et al., 2008; Beurdeley et al., 2012; Spatazza et al., 2013). Further, PV neurons are preferentially surrounded by perineuronal nets (PNN), and PNN ensheathment affects not only the excitability and plasticity of PV neurons but also the intensity of PV expression within PV neurons (Hensch et al., 1998; Pizzorusso et al., 2002; Dityatev et al., 2007; Carulli et al., 2010; Beurdeley et al., 2012; Wang and Fawcett, 2012; Takesian and Hensch, 2013; Yamada et al., 2015; Hou et al., 2017; Rowlands et al., 2018; Gogola et al., 2019; Shi et al., 2019; Carceller et al., 2020; Carulli et al., 2020). Changes in PV intensity are informative because PV intensity can serve as a proxy for the excitability, activity, and state of PV neurons (e.g., Kinney et al., 2006; Favuzzi et al., 2017).

To date, most of our understanding of factors that affect PV expression stems from studies in mammals, in particular rodents. Given that important forms of plasticity shape the behavior and cognition of many non-mammalian species (e.g., critical periods for vocal learning and individual recognition in birds: Brainard and Doupe, 2002, 2013; Horn, 2004; Gobes et al., 2019; Sakata and Woolley, 2020), it is important to assess the role of PV neurons in shaping these forms of plasticity (e.g., Hara et al., 2012; Sakata and Woolley, 2022). Previous research indicates that brain areas important for vocal learning and performance in songbirds are replete with PV neurons (Braun et al., 1985, 1991; Wild et al., 2001, 2005; Balmer et al., 2009; Cornez et al., 2015, 2017, 2018, 2020a,b; Olson et al., 2015), and it has been proposed that PV interneurons provide strategic inhibition to coordinate the motor outputs necessary for singing (Spiro et al., 1999). Brain areas active during the performance of non-vocal communicative displays (e.g., drumming in woodpeckers) also express a high number of PV neurons (Schuppe et al., 2022). Collectively, these data motivate research into the factors that affect PV expression in brain circuits in non-mammalian vertebrates.

Here we analyzed the effects of PNN ensheathment on the intensity of PV expression within PV neurons in auditory and motor areas of songbirds and rodents, as well as how the effect of PNN ensheathment on PV intensity could vary across development in songbirds. We focused our attention in songbirds on the song system - a collection of interconnected forebrain areas that regulate vocal performance and learning and that consist of the forebrain areas HVC

(acronym used as a proper noun), the robust nucleus of the arcopallium (RA), the lateral magnocellular nucleus of the anterior nidopallium (LMAN), and Area X (the vocal portion of the avian basal ganglia) - as well as the primary auditory cortex (Field L). We measured PV expression in the motor and auditory cortices, dorsolateral striatum (DLS), and external nucleus of the globus pallidus (GPe) in rodents because many brain areas within the song and auditory systems of songbirds are analogous to these areas (Leblois and Perkel, 2020; Sakata and Yazaki-Sugiyama, 2020; Woolley and Woolley, 2020; Colquitt et al., 2021). Finally, we assessed the causal contribution of PNNs to PV intensity in songbirds by analyzing how degradation of PNNs in HVC affects the intensity of PV within PV neurons.

Materials and methods

Animals

Thirty-one normally reared (i.e., with mother and father) zebra finches [$n = 7$ juvenile males: 50–70 days post-hatch (dph), $n = 24$ adult males: 0.3–3.5 years old] were raised in our colony. Six adults were used for an initial investigation of the relationship between PNN ensheathment and PV intensity; 12 individuals were used in an analysis of age on PV intensity ($n = 7$ juveniles and $n = 5$ adults); and 13 adults were used to analyze the effects of PNN degradation on PV intensity. All birds were housed on a 14:10 light–dark cycle with food and water provided *ad libitum*. In addition, we analyzed the effect of PNN ensheathment on PV intensity in $n = 7$ adult male mice (22–27 months) that were hemizygous or wildtype from strain B6.Cg-Tg (Pcp2-cre)3555Jdhu/J; stock number: 010536 (Jackson Laboratory). Hemizygous mice express Cre under the L7 promoter, which should not alter brain development or function. All procedures were approved by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Surgical procedures

To experimentally assess how PNNs contribute to PV abundance in sensorimotor structures, we examined how degradation of PNNs in the sensorimotor structure HVC affected the intensity of PV in PV neurons. We selected HVC for this experiment because of the consistent relationship between PV intensity and PNN ensheathment (see Results) and because HVC is necessary for song learning and production (reviewed in Murphy et al., 2020; Sakata and Yazaki-Sugiyama, 2020). For surgery, birds were anesthetized with intramuscular injections of ketamine (0.03 mg/g) and midazolam (0.0015 mg/g) followed by vaporized isoflurane (0.2–3.0% in oxygen) to maintain a deep state of anesthesia throughout the surgical procedure. Birds were placed in a stereotaxic device, with their beaks stabilized at a 45° angle. Following a craniotomy, chondroitinase ABC (ChABC; Sigma C3667; 100 U/mL, in 0.1% BSA in PBS) or penicillinase (PEN; Sigma P038; 100 U/mL) was bilaterally injected into HVC (0.8 mm rostral from the caudal edge of the bifurcation of the midsagittal sinus, 1.8 mm lateral from the midline, and 0.5 mm in depth) using a Nanoject III Programmable Nanoliter Injector

(Drummond Scientific, Broomall, PA) assembled with a glass pipette. ChABC is an enzyme that degrades chondroitin sulfate proteoglycans, a major structural component of PNNs, whereas PEN is often used as a control enzyme for experiments involving ChABC because it does not degrade PNNs (e.g., Pizzorusso et al., 2002; Yamada et al., 2015; Carceller et al., 2020). Drugs were infused at 10 nL/s with 50 or 100 nL per cycle and 1–3 cycles for each hemisphere, and the glass pipette was left in place for ~2 min before retraction. Brains of ChABC- or PEN-treated birds were collected 6–7 days after surgery.

Tissue collection

Zebra finches were deeply anesthetized with isoflurane vapor and transcardially perfused with heparinized saline (100 IU/100 mL) followed by 150 mL of 4% paraformaldehyde (PFA; pH 7.4). Brains were left to postfix overnight at 4°C then moved to 30% sucrose PBS solution for cryoprotection. Brains were cut on a freezing microtome (Leica Biosystems, Wetzlar, Germany) in 40 µm sagittal sections and collected in Tris-buffered saline (TBS) with sodium azide.

Mouse brains were collected in 4% PFA. Briefly, mice were deeply anaesthetized using 2,2,2-tribromoethanol (Avertin) injected intraperitoneally. PBS (0.1 M, pH 7.4) with heparin salt (5.6 µg/mL) was perfused transcardially, followed by 30 mL ice-cold 4% PFA in Phosphate Buffer (PB, pH 7.4). Brains were extracted and allowed to postfix in 4% PFA at 4°C for a further 24 h before being transferred for long-term storage at 4°C in PBS with 0.5% sodium azide. Brains were moved to a 30% sucrose PBS solution for cryoprotection before cutting. Coronal sections (40 µm) were cut on a freezing microtome and collected in PBS with sodium azide.

Immunohistochemistry (IHC)

One set of brain sections from one hemisphere of each bird was processed for both PNN and PV expression. Free-floating sections were washed 3X for 5 min in 1X TBS and then blocked for 30 min in TBS + 5% donkey serum + 0.1% Triton-X. Then the tissue was incubated overnight at 4°C in a mouse monoclonal anti-chondroitin sulfate (ACS; C8035; Sigma-Aldrich; 1:500) and a rabbit polyclonal anti-PV (ab11427; Abcam; 1:2000). Thereafter, sections were washed 3X for 5 min in TBS followed by a 2-h incubation at room temperature with secondary antibodies [donkey anti-mouse Alexa Fluor 488: 1:100 (ThermoFisher); donkey anti-rabbit Alexa Fluor 594: 1:200 (ThermoFisher)] in TBS + 0.1% Triton-X. The tissue was then washed 3X for 5 min in TBS and transferred to TBS before mounting. Sections were coverslipped with Prolong Gold Antifade (Life Technologies, P36930).

Rat and mouse brains were processed in a similar manner as finch brains, albeit with different primary compounds (Cisneros-Franco et al., 2018). Briefly, rat brains were immunolabeled with an antibody against PV (#P-3088; Sigma-Aldrich; 1:10,000), and PNN were stained with fluorescein *Wisteria floribunda* lectin (WFL; #FL-1351; Vector Laboratories; 1:200). For mouse sections, PV neurons were stained using same PV antibody used in finches (ab11427; 1:500), while PNNs

were stained using *Wisteria floribunda* lectin (Sigma L1516; 1:1000). Secondary antibodies for this staining included donkey anti-rabbit Alexa Fluor 594 (1:200) and Streptavidin conjugated to Alexa Fluor 488 (1:500; S11223; Life Technologies).

Different methods were used to visualize PNNs in zebra finches and rodents because *Wisteria floribunda* lectin (WFL; aka WFA), the most widely used tool to visualize PNNs in mammals, does not work in zebra finches (A.S. Wang, Y. Yazaki-Sugiyajma, & J.T. Sakata, unpublished data). However, both WFL and ACS bind to the glycosaminoglycan portion of chondroitin sulfate (CS) proteoglycans of PNNs, so we anticipate that the antibodies target similar types of PNNs. Similarly, just as ChABC injections reduce WFL staining in rodents, ChABC injections reduce ACS labeling in songbirds (Balmer et al., 2009; Cornez et al., 2021; this paper).

Imaging and analysis

We analyzed images of PV neurons and PNNs acquired in our lab as well as images obtained from previously published studies (Cisneros-Franco et al., 2018; Cornez et al., 2018). Images of HVC, RA, LMAN, Area X, and Field L in juvenile and adult male zebra finches from our lab were taken with a Zeiss Axio Imager.A2 microscope at 40X using the ZEN Imaging software (Carl Zeiss). These areas in the songbird brain are readily identifiable following staining for PV and PNNs (e.g., Balmer et al., 2009; Cornez et al., 2015; see also zebrafinchatlas.org).

Brain areas in the mouse brain were identified based on previous studies as well as the Allen Brain atlas. In the mouse brain, images of the motor cortex were taken around bregma -1.1 to -1.6 mm; for the external nucleus of the globus pallidus (GPe) around bregma -1.0 to -1.5 mm; for the dorsolateral striatum (DLS) around 0.8 to 1.3 mm; and for the primary auditory cortex around -2.0 to -3.3 mm. 40X images were taken for mouse motor and auditory cortices, and 20X images were taken for the DLS and GPe in mouse brains. 20X images were taken of the DLS because of its low density of PV neurons (a lower magnification enabled us to count more PV neurons), and we took images at the same magnification and exposure times for the GPe to enable comparisons of PV intensities between these parts of the basal ganglia (see Discussion). We imaged cortical layers with the highest density of PV neurons and PNNs (Alpár et al., 2006; Fader et al., 2016; Lupori et al., 2023); for the auditory cortex, PV neurons and PNNs in layer IV were imaged and quantified, and for the motor cortex, PV neurons and PNNs in layers II/III and V were separately imaged and quantified (Supplementary Figure 1). No layers are found in Field L or in brain areas surrounding the song circuitry in songbirds, and PV neurons and PNN expression were analyzed in parts of the brain areas that were representative of the brain area.

Exposure times were kept constant for all images within a given brain area and immunocytochemical batch. Exposure times were optimized for each brain area and were generally different for different brain areas; this allowed for maximal sensitivity to detect effects of age and PNNs on PV intensity. An exception was the mouse basal ganglia, wherein exposure times were the same for GPe and DLS neurons, allowing for quantification of regional variation in PV intensity. This was particularly useful to draw parallels to Area X, which consists of

pallidal and striatal neurons (Carrillo and Doupe, 2004; Reiner et al., 2004; reviewed in Leblois and Perkel, 2020).

In addition to images acquired in our lab, images of PV neurons and PNNs within the auditory cortex of adult male Long-Evans rats ($n=9$; age: 2–25 months) were acquired from Cisneros-Franco et al. (2018) (bregma: -3.7 to -6.2 mm), and images of PV neurons and PNNs across various developmental ages in male zebra finches were acquired from Cornez et al. (2018). For the latter, we obtained and analyzed images of PV neurons and PNNs in HVC, RA, LMAN, and Area X of male zebra finches that were 40, 60, 90, and 120 dph (see Cornez et al., 2018 for details of methods).

Quantification of PV neurons and PNNs was conducted in Fiji, with each fluorophore imaged independently. The images were converted to grayscale (16-bit), and cells were manually counted by a single experimenter across 3–8 images per brain area per individual. The experimenter was blind to the experimental condition (e.g., age) of the animals. After identifying the PV neurons, PV intensity was measured; for this, the mean gray value of each individual cell identified was measured in a 30×30 pixel square placed in the center of the cell body. Background measurements were done in a similar fashion, measuring the mean gray value of a 30×30 pixel square in three randomly chosen locations across the image that did not contain cells. The average background intensity was computed per section and this value was subtracted from the raw PV intensities. From here on, “PV intensity” reflects this background-subtracted value and is reported in arbitrary units (a.u.).

Identification of neurons surrounded by PNNs was conducted independent of PV quantification. Images were converted to grayscale (16-bit), and neurons surrounded by PNNs were manually identified by a single experimenter across 3–8 images per brain area per animal. Neurons were identified as being surrounded by a PNN if the PNN surrounded $>75\%$ of the circumference of the cell body. After identifying neurons surrounded by PNNs, the experimenter compared the PV and PNN images and determined whether the PV neuron was or was not surrounded by PNNs (PV+PNN or PV-PNN, respectively). This allowed us to classify each PV intensity into a PV+PNN or PV-PNN category. Again, the experimenter was blind to experimental condition.

Statistical analyses

We used mixed-effects models to analyze how various parameters affected PV intensity. The specific independent variables included in the model depended on the experimental analysis but PNN ensheathment (PV+PNN vs. PV-PNN) was always an independent variable. When birds of different ages were analyzed, a full-factorial model with PNN ensheathment and age (juvenile vs. adult, days post-hatching) was used to analyze variation in PV intensity. For all models, animalID and sectionID nested within animalID were included as random effects because multiple PV neurons were measured per brain section and multiple sections were measured per animal. In situations in which multiple batches of IHCs were conducted (e.g., analysis of mouse sections as well as data from Cornez et al., 2018), batch was included as a random effect. In cases where significant interactions between PNN ensheathment and other variables were found, planned post-hoc contrasts were performed (see Results). JMP v16 (SAS, Cary, NC) were used for all analyses, with $\alpha=0.05$ throughout.

Results

Modulation of PV intensity by PNNs in the primary auditory cortex of adult songbirds and rodents

In both rodents and songbirds, PV neurons are abundant in the primary auditory cortex, and many of these PV neurons are surrounded by PNNs. Field L is homologous to the primary auditory cortex of mammals (Woolley and Woolley, 2020), and PV neurons in Field L demonstrated variation in PV intensity depending on whether they were surrounded by PNNs or not. For example, in Figure 1A, there are two PV neurons surrounded by a PNN (PV+PNN; white arrows) and three PV neurons not surrounded by a PNN (PV-PNN; orange arrows), and PV appears more abundant in the two PV+PNN neurons compared to the three PV-PNN neurons. This variation was consistently observed, and across all PV neurons in Field L ($n=6$ birds), PV intensity was significantly higher for PV+PNN neurons ($F_{1,169.7}=9.6$, $p=0.0023$; Figure 1B).

PNNs are abundant within layer IV of the auditory cortex (Fader et al., 2016; Lupori et al., 2023), and just as in male zebra finches, the abundance of PV within PV neurons in the auditory cortex of male rats and mice seems to vary depending on PNN ensheathment. In Figure 1C, PV+PNN neurons (white arrows) in a male rat are more intense with PV than the PV-PNN neuron (orange arrow). Overall, PV intensity was significantly higher for PV+PNN neurons than for PV-PNN neurons in rats ($n=9$ rats; $F_{1,388.2}=10.5$, $p=0.0013$; Figure 1D) and mice ($n=7$ mice; $F_{1,315}=13.8$, $p=0.0002$; Figure 1E).

Modulation of PV intensity by PNNs in motor circuitry of adult songbirds and rodents

The song circuit consists of four forebrain areas – HVC, RA, LMAN, and Area X – that are critical for song learning and performance and are replete with PV neurons and PNNs (Figures 2A,B; Supplementary Figure S2). HVC and RA have been proposed to be analogous, respectively, to premotor and motor cortices, whereas Area X is a basal ganglia structure with both striatal and pallidal components (reviewed in Doupe et al., 2005; Leblois and Perkel, 2020). The intensity of PV was significantly higher in PV+PNN neurons than in PV-PNN neurons in HVC ($n=6$ adults; $F_{1,140.8}=6.5$, $p=0.0118$) and LMAN ($F_{1,172.9}=15.5$, $p=0.0001$; Figure 2B). On the other hand, there were no significant differences in PV intensity between PV+PNN and PV-PNN neurons in RA or Area X ($p>0.15$ for each).

Similar relationships between PV intensity and PNN ensheathment were observed in the motor cortex and basal ganglia of mice ($n=7$ male mice; Figure 3). In both layer II/III and V of the motor cortex, there was a significant difference in PV intensity between PV+PNN and PV-PNN neurons, with PV+PNN neurons being more enriched with PV than PV-PNN neurons (layer II/III: $F_{1,208.9}=21.0$, $p<0.0001$; layer V: $F_{1,203.4}=14.9$, $p=0.0002$; Figures 3A,B). PV intensities were also significantly higher in PV+PNN neurons than in PV-PNN neurons in the external nucleus of the globus pallidus (GPe; $F_{1,646}=6.4$, $p=0.0114$; Figure 3C). PV intensities were not significantly different between PV+PNN and PV-PNN neurons in the

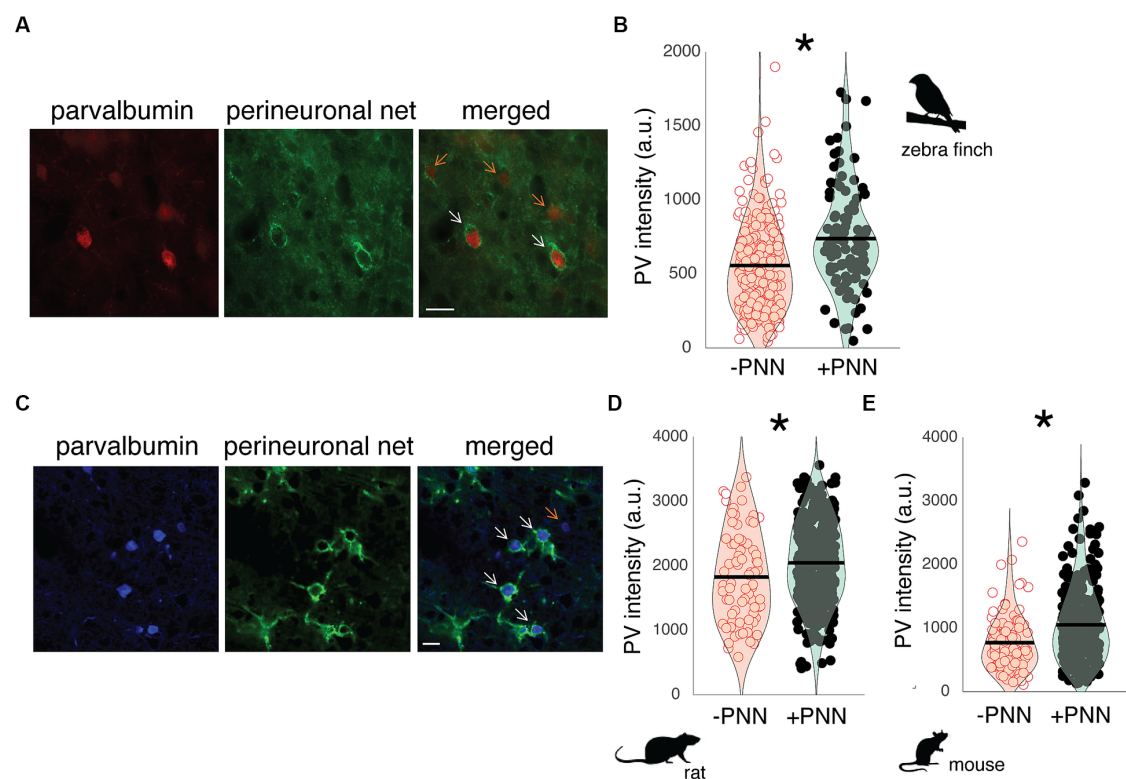


FIGURE 1

PV intensity of PV neurons and PNNs in the primary auditory cortices of zebra finches, rats, and mice. (A) Representative image of PV neurons (red) and PNNs (green) in Field L of adult zebra finches. White arrows point to PV neurons with PNNs (PV+PNN) while orange arrows point to PV neurons without PNNs (PV-PNN). Scale bar = 20 μm ; (B) PV intensity [in arbitrary units (a.u.)] is significantly higher for PV+PNN (filled circles) neurons than for PV-PNN neurons (empty circles) in Field L of adult zebra finches ($n = 175$ neurons in 6 finches; 13–45 months). (C) Representative image of PV neurons (blue) and PNNs (green) in the primary auditory cortex of adult rats. White arrows point to PV neurons with PNNs (PV+PNN) while the orange arrow points to a PV neuron without PNNs (PV-PNN). Scale bar = 20 μm . PV intensity is significantly higher for PV+PNN (filled circles) neurons than for PV-PNN neurons (empty circles) in layer IV of the primary auditory cortices of (D) adult rats ($n = 401$ neurons in 9 rats; 5–25 months) and (E) mice ($n = 341$ neurons in 7 mice; 22–26 months). Each symbol in (B), (D), and (E) represents the PV intensity of a neuron. A violin plot summarizing the distribution is plotted on top of the raw data, with the black line representing the mean of the distribution. ** denotes $p < 0.05$ (mixed-effects model).

dorsolateral striatum (DLS; $F_{1,209.8} = 2.6$, $p = 0.1076$; Figure 3D). This lack of statistical difference seems to be caused by a single high value for PV-PNN neurons, and removal of this point leads to the observation of higher PV intensity for PV+PNN neurons in the DLS ($F_{1,209.5} = 5.2$, $p = 0.0240$).

Variation in PV intensity in motor circuitry across development in songbirds

Neurons in song control circuitry change in various ways over development, and we investigated the extent to which PV intensity and the relationship between PV intensity and PNN expression changed across development. We first analyzed differences in PV expression between juvenile (~2 months old; $n = 7$) and adult zebra finches (10–15 months old; $n = 5$; Figure 4). Overall, PV intensities were significantly higher in PV+PNN neurons than in PV-PNN neurons in HVC ($F_{1,201.3} = 37.4$, $p < 0.0001$), RA ($F_{1,260.1} = 49.1$, $p < 0.0001$), and LMAN ($F_{1,338.5} = 34.0$, $p < 0.0001$; Figures 4A–C). However, we found the opposite pattern in Area X, with PV intensities being significantly lower for PV+PNN neurons than for PV-PNN neurons ($F_{1,286.1} = 5.7$, $p = 0.0172$; Figure 4D). While there were no

significant differences between juveniles and adults, there was a trend for PV intensities to be lower in adults than in juveniles in HVC ($F_{1,10.0} = 4.4$, $p = 0.0619$). Interestingly, there was a significant interaction between age and PNN expression in LMAN ($F_{1,338.5} = 20.2$, $p < 0.0001$) and a trend for an interaction in RA ($F_{1,260.1} = 3.2$, $p = 0.0759$). In LMAN, PV intensities were significantly higher for PV+PNN neurons compared to PV-PNN neurons in juveniles but not in adults, and PV intensities decreased with age for PV+PNN neurons but not for PV-PNN neurons (planned contrasts, $p < 0.01$). Similar patterns were observed for RA.

The previous analysis highlights differences in PV intensity between juvenile and adult zebra finches that were, on average, nine months apart. Many changes to neural circuitry and behavior occur during the first few months of development; consequently, we analyzed images at different developmental time points (i.e., 40, 60, 90, and 120 dph) from a previously published study (Corney et al., 2018; Figures 5A–D). There was no significant change in overall PV intensity across development for HVC, LMAN, or Area X but there was a trend for PV intensity to decrease over development up to 90 dph for RA ($F_{3,19.1} = 2.8$, $p = 0.0657$; Figure 5B). Over development, PV+PNN neurons were significantly more enriched with PV than PV-PNN neurons in LMAN ($F_{1,592.1} = 16.1$, $p < 0.0001$; Figure 5C), with a similar

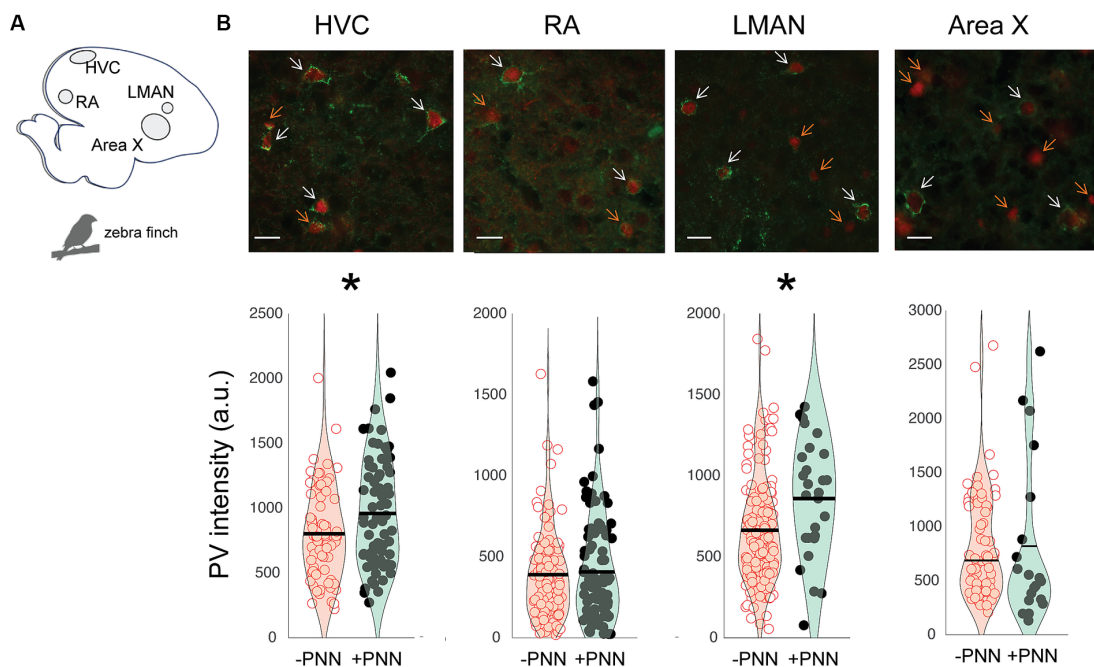


FIGURE 2

Regional variation in the effect of PNN ensheathment on PV intensity in the song system. **(A)** Sagittal representation of the zebra finch brain (left=posterior, right=anterior). **(B)** Representative images of PV neurons (red) and PNNs (green) in HVC, RA, LMAN, and Area X, with white arrows pointing to PV+PNN neurons and orange arrows pointing to PV-PNN neurons. Below the images are PV intensities [in arbitrary units (a.u.)] of PV+PNN (filled circles) and PV-PNN neurons (empty circles) in HVC ($n = 145$ neurons), RA ($n = 188$ neurons), LMAN ($n = 182$ neurons), and Area X ($n = 84$ neurons) of adult zebra finches ($n = 6$ birds). Different exposure times were used for different brain nuclei; therefore, differences in PV intensity across brain regions should not be taken to reflect differences in PV intensity. Each symbol represents the PV intensity of a neuron. A violin plot summarizing the distribution is plotted on top of the raw data, with the black line representing the mean of the distribution. "*" denotes $p < 0.05$ (mixed-effects model).

trend for HVC ($F_{1,785.9} = 3.6$, $p = 0.0586$; Figure 5A). Additionally, PV+PNN neurons tended to be less enriched with PV than PV-PNN neurons in Area X ($F_{1,368.3} = 2.9$, $p = 0.0869$; Figure 5D).

Causal contribution of PNNs to PV intensity in adult songbirds

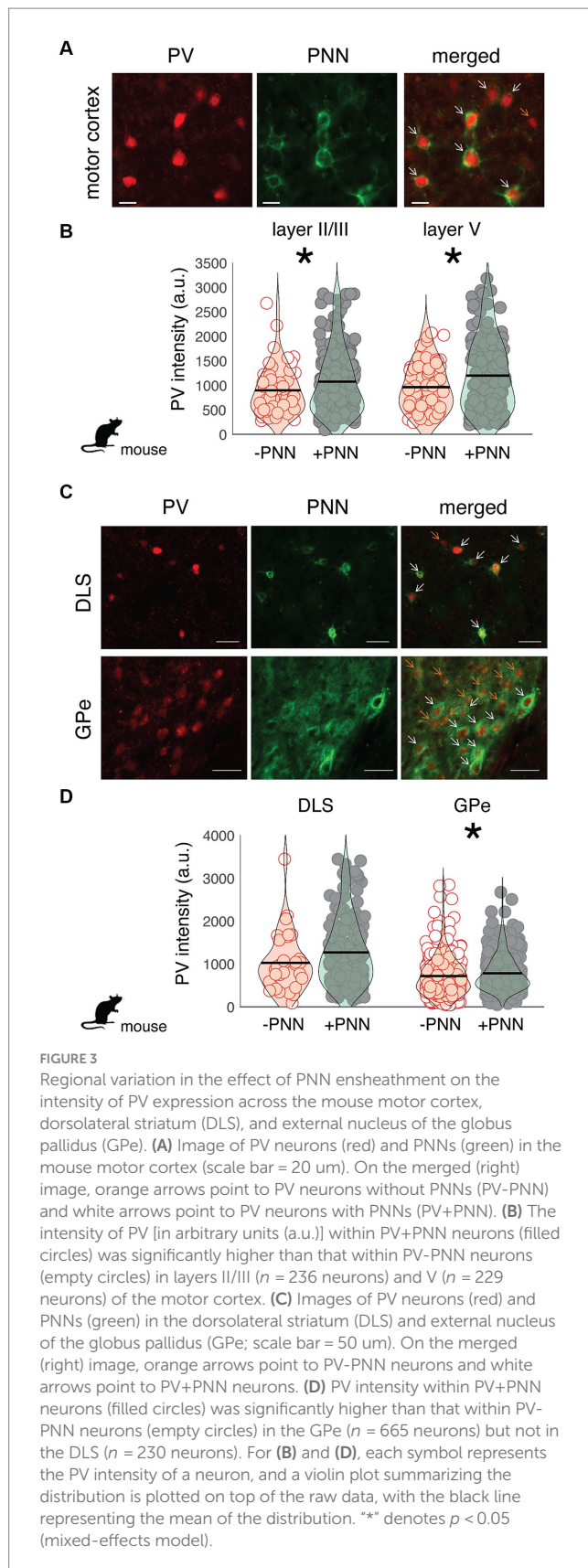
The preceding analyses suggest that PNNs could augment PV expression in the PV neurons they surround or that PNNs could differentially surround PV neurons that express more PV. To test the former hypothesis, we infused the HVCs of individual birds with either chondroitinase ABC (ChABC; $n = 7$ birds) or a control enzyme penicillinase (PEN; $n = 6$ birds) and investigated how degrading PNNs (via ChABC) affected PV expression within PV neurons in HVC. We hypothesized that PV neurons in parts of HVC with degraded PNNs will express less PV than PV+PNN neurons but will display the same degree of PV as PV-PNN neurons.

Similar to mammals, PNNs in HVC were degraded following ChABC infusions but remained intact following PEN infusions (Figures 6A–C). In many instances, ChABC infusions failed to remove PNNs throughout all of HVC (Figures 6A,D); we took advantage of this and analyzed PV intensities within PV+PNN and PV-PNN neurons in the non-affected part of HVC as well as PV intensities of PV neurons in the affected portion (PV+ChABC neurons).

Consistent with the preceding analyses, PV+PNN neurons in the non-affected portion of HVC in ChABC-treated birds were more

enriched with PV than PV-PNN neurons in the same region ($F_{1,97.6} = 3.8$, $p = 0.0543$). This same trend was observed between PV+PNN and PV-PNN neurons in the HVC of PEN-treated birds ($F_{1,151.7} = 3.6$, $p = 0.0584$). Not surprisingly, when the PV intensities of PV+PNN and PV-PNN neurons of birds treated with ChABC or PEN were analyzed together, there was a significant effect of PNN ensheathment (PV+PNN > PV-PNN; $F_{1,248.6} = 6.6$, $p = 0.0109$). Importantly, for this analysis of parts of HVC without PNN degradation, there was no significant effect of group (ChABC vs. PEN) or significant interaction between group and PNN ensheathment ($p > 0.8$ for each), indicating that the effect of PNN ensheathment was consistent across groups.

We next compared PV intensities of PV+ChABC neurons to PV+PNN and PV-PNN neurons in ChABC- and PEN-treated birds. In ChABC-treated birds, there was no statistically significant difference in PV intensity among PV+PNN, PV-PNN, and PV+ChABC neurons ($p > 0.15$), but PV+PNN neurons displayed on average the most intense PV staining. When comparing PV intensities of PV+ChABC neurons in ChABC-treated birds and PV+PNN and PV-PNN neurons in PEN-treated birds, no significant difference was observed ($p > 0.10$), though PV+PNN neurons were the most enriched with PV among the three. Because these two analyses of the effects of ChABC could be underpowered (due to small sample sizes), we analyzed the PV intensities of PV+ChABC neurons, PV+PNN, and PV-PNN neurons in ChABC- and PEN-treated birds simultaneously. When analyzed together, there was a significant difference between PV+ChABC, PV+PNN, and PV-PNN neurons ($F_{2,352.8} = 3.8$, $p = 0.0241$; Figure 6E), with PV+PNN neurons being more enriched with PV than



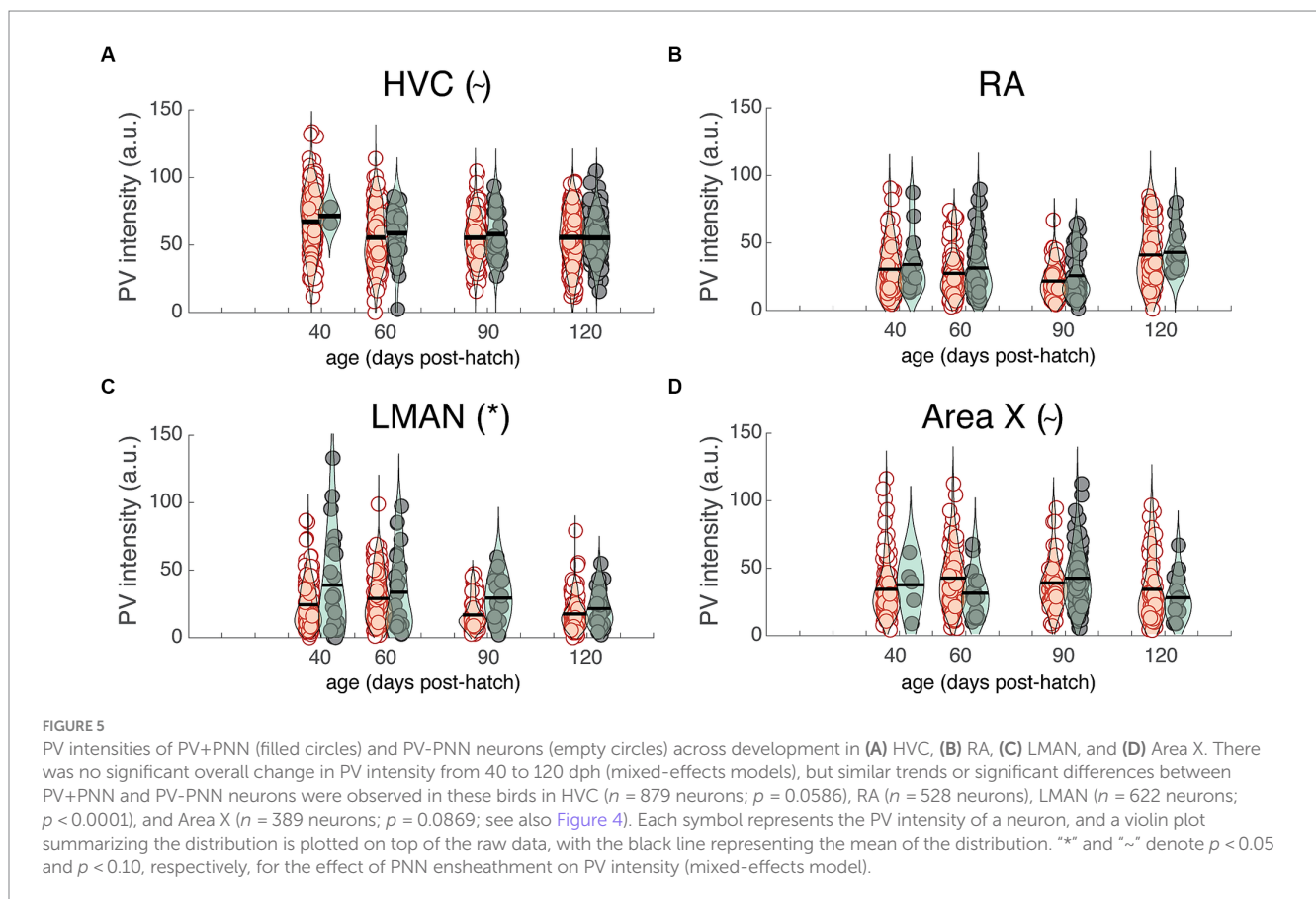
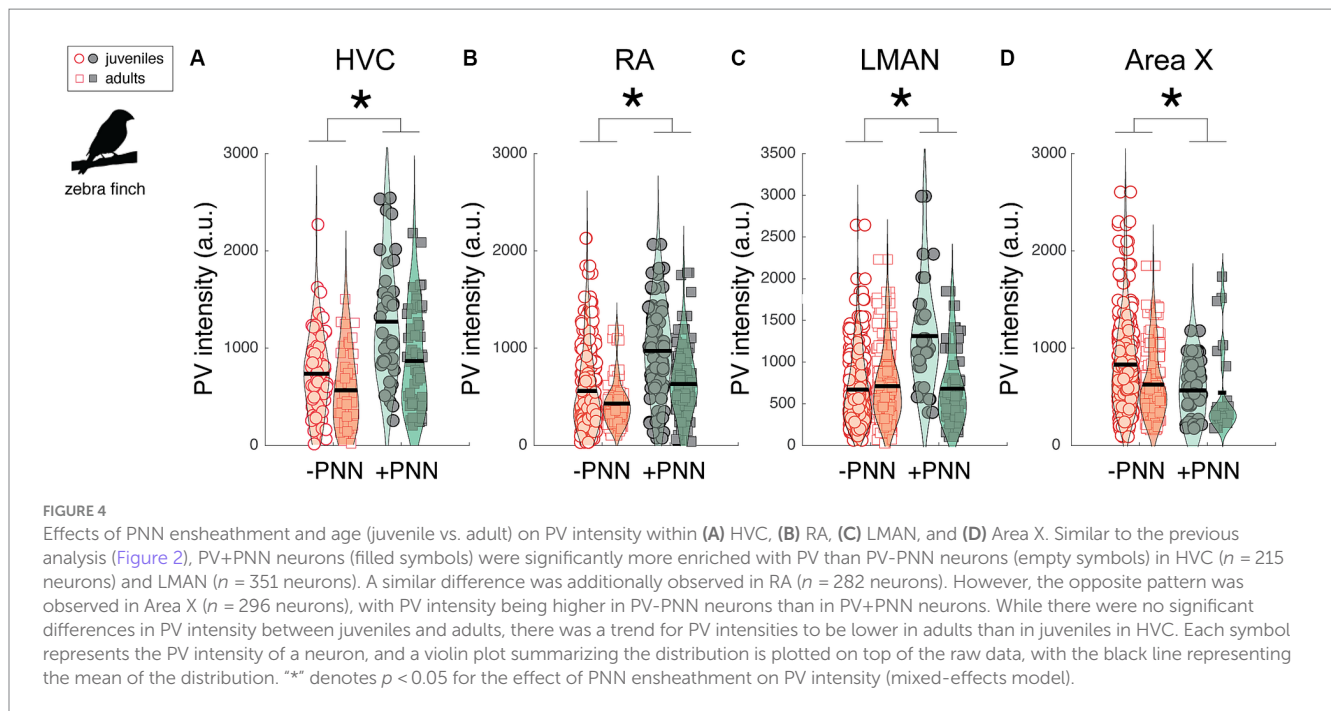
PV+ChABC and PV-PNN neurons (planned contrasts; $p < 0.02$ for each) and with PV+ChABC neurons expressing indistinguishable levels of PV as PV-PNN neurons ($p > 0.60$).

Discussion

Despite the prevalence of parvalbumin (PV) neurons across vertebrate taxa, little is known about the regulation of PV in non-mammalian species. Additionally, little is known about the regulation of PV in motor circuitry in either mammals or non-mammalian species. We investigated the degree to which PV expression was modulated by perineuronal nets (PNNs), extracellular matrices that affect plasticity and functional properties of neurons they surround, in the auditory cortex, motor cortex, and basal ganglia of songbirds and rodents. We specifically analyzed differences in PV intensity between PV neurons surrounded or not surrounded by PNNs within each species, and then discerned common patterns of differences across species. We also investigated the causal contribution of PNNs to PV intensity in the songbird brain.

We discovered similarities and differences in the relationship between PV and PNN expression between songbirds and rodents and between motor systems and other circuitry. PV intensity was higher for PV+PNN neurons in the auditory cortices of mice, rats, and songbirds, in the motor cortex of mice, and in the cortical-like areas HVC, RA, and LMAN of songbirds. Despite different methods to visualize PNNs in zebra finches compared to rodents (see Methods), this pattern was consistent across auditory and motor cortices in songbirds and rodents. Our data additionally highlight similarities in the relationship between PV and PNN expression in auditory and motor circuitry and other brain areas including the visual, prefrontal, and dorsolateral cortices and hippocampus in rodents (Yamada and Jinno, 2013; Yamada et al., 2015; Enwright et al., 2016; Favuzzi et al., 2017; Hou et al., 2017; Rowlands et al., 2018; Slaker et al., 2018; Carceller et al., 2020; Lupori et al., 2023). We observed some species variation in the relationship between PNN expression and PV intensity: whereas differences between PV+PNN and PV-PNN neurons in the DLS and GPe of mice were similar to those observed in motor and auditory cortices (PV+PNN > PV-PNN), PV expression was lower for PV+PNN neurons than for PV-PNN neurons in the basal ganglia nucleus Area X of songbirds. Although Area X consists of striatal- and pallidal-like neurons (Leblois and Perkel, 2020), Area X itself is not considered directly homologous to DLS and GPe; therefore, differences between Area X, DLS, and GPe might not be unexpected.

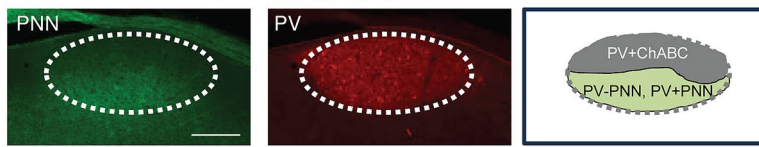
This regional variation in the relationship between PNN and PV expression in the songbird brain supports findings from a recent, comprehensive study of PNN and PV expression in the rodent brain (Lupori et al., 2023) and suggests that PNNs differentially affect neural activity across cortical and basal ganglia circuitry in songbirds. Regional variation in the effects of PNNs on neural activity have been previously documented; for example, decreases in PNN expression in the mouse somatosensory cortex, visual cortex, and deep cerebellar nucleus are associated with decreased neural activity (Lensjø et al., 2017; Tewari et al., 2018; Carulli et al., 2020). In contrast, enzymatic removal of PNNs in mouse hippocampal cultures decreases the amount of depolarizing current required to generate action potentials and reduces after-hyperpolarizations, thereby increasing firing rates (Dityatev et al., 2007). Given the regional variation in the relationship between PV and PNN expression in the song system, our data suggest that PNNs could differentially modulate neural activity and dynamics in HVC, RA, and LMAN compared to Area X. Differences in the relationship



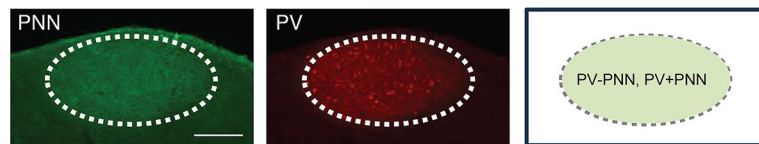
between PV ensheathment and PV intensity across brain areas in the song system could also be linked to regional variation in neuronal types (e.g., Pfenning et al., 2014; Colquitt et al., 2021).

Differential PV expression depending on PNN ensheathment suggests that PV+PNN and PV-PNN neurons could represent different neuronal subtypes and could differentially regulate neural

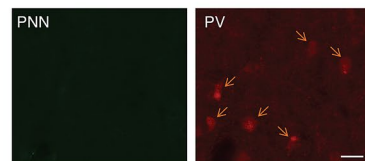
A Chondroitinase (ChABC) into HVC



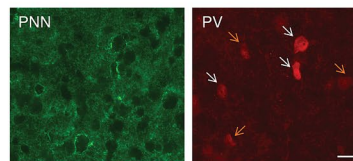
B Penicillinase (PEN) into HVC



C



D



E

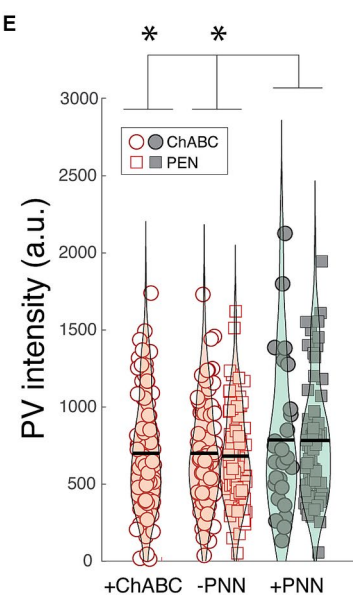


FIGURE 6

Effects of PNN degradation in HVC on PV intensities. **(A)** Infusions of ChABC degraded PNNs in HVC. In many instances, the infusions did not cover all of HVC, leaving some portion of HVC with intact PNNs. In this example, PNNs (green) are degraded in the dorsal portion of HVC whereas PNNs remain intact in the ventral portion. The intensity of PV (red) within PV neurons in the affected region (PV+ChABC neurons) and within PV+PNN and PV-PNN neurons in the unaffected region of HVC were analyzed. Scale bar = 200 μ m. **(B)** Image of intact PNNs and PV neurons in the HVC of a bird infused with PEN (i.e., infusions of PEN did not degrade PNNs). Scale bar = 200 μ m. **(C)** High magnification image of PNN degradation (left) and PV neurons (right) following a ChABC infusion into HVC. **(D)** High magnification image of intact PNNs (left) and PV neurons from a portion of the HVC without PNN degradation from the same bird as in **(C)**. For both **(C)** and **(D)**, white arrows point to PV+PNN neurons and orange arrows point to PV-PNN neurons, and scale bar = 20 μ m. **(E)** PV+ChABC neurons were comparable in PV intensity to PV-PNN neurons and less intensely stained with PV than PV+PNN neurons in ChABC-treated birds ($n = 212$ neurons) and PEN-treated birds ($n = 154$ neurons). Each symbol represents the PV intensity of a neuron, and a violin plot summarizing the distribution is plotted on top of the raw data, with the black line representing the mean of the distribution. “*” denotes $p < 0.05$ (mixed-effects model).

dynamics. In the rodent hippocampus, PV neurons with lower PV expression are less likely to be surrounded by PNNs and express somatostatin and neuropeptide Y, whereas PV neurons that express high levels of PV are more likely to be surrounded by PNNs and do not express somatostatin and neuropeptide Y (Yamada et al., 2015). Previous studies in songbirds document that distinct cell types within song circuitry of songbirds differ in PV intensity (Braun et al., 1985; Wild et al., 2001, 2005; Zengin-Toktas and Woolley, 2017). For example, interneurons in RA are enriched with PV whereas RA projection neurons only moderately express PV (Wild et al., 2001). Given that PV neurons in RA that are surrounded by PNNs are more enriched with PV than PV neurons not surrounded by PNNs, this suggests that PNNs might differentially surround RA interneurons. Similarly, a previous study identified at least two types of PV neurons in Area X (Braun et al., 1985): a population of large cells (~16 μ m in diameter) that were relatively weakly stained with PV and a population of small cells (~10–13 μ m in diameter) that were more intensely stained with PV. Because a number of our analyses suggest that PV+PNN neurons in Area X are less enriched with PV than PV-PNN neurons in Area X, it is possible that large, weakly stained PV neurons in Area X are differentially surrounded by PNNs compared with small, intensely stained PV neurons. Further, larger PV neurons in Area X are putatively analogous to mammalian GPe neurons whereas smaller PV neurons are more analogous to mammalian striatal fast-spiking interneurons (Carrillo and Doupe, 2004;

Reiner et al., 2004; Woolley, 2016; Zengin-Toktas and Woolley, 2017; Kumar et al., 2020; reviewed in Leblois and Perkel, 2020). Our data support this contention because PV neurons in the GPe are less intensely stained with PV than PV neurons in the DLS (Figure 3).

The effect of PNN degradation on PV intensity in the songbird nucleus HVC resembles the effect of PNN degradation in various parts of the mammalian brain. For example, just as experimental reductions of PNNs decrease in PV intensity in PV neurons in the hippocampus and visual cortex (Beurdeley et al., 2012; Yamada et al., 2015; Hou et al., 2017; Rowlands et al., 2018), degradation of PNNs decreased the intensity of PV expression within PV neurons in HVC. Given that PNN degradation also modulates neural activity and oscillations of various types of neurons in mammals, including PV neurons (Dityatev et al., 2007; Balmer, 2016; Carceller et al., 2020), we propose that degradation of PNNs in the song circuitry of songbirds could also modulate neural activity and dynamics and, consequently, vocal performance (e.g., canary: Cornez et al., 2021).

How PNNs modulate PV intensity in songbirds remain unknown. In mammals, PV expression is influenced by Otx2 or brevican. Specifically, PNNs allow for the transfer of Otx2 from the extracellular environment into PV neurons, which subsequently increases PV intensity (Sugiyama et al., 2008; Spatzza et al., 2013). Brevican, a protein commonly expressed in PNNs, affects the localization of potassium channels and AMPA receptors in PV neurons in rodents

(Favuzzi et al., 2017), and PV neurons surrounded by brevicin are innervated by more excitatory inputs than PV neurons not surrounded by brevicin (Favuzzi et al., 2017). Therefore, depending on the degree to which the composition of PNNs vary between mammals and birds, Otx2 and brevicin could contribute to elevated PV expression within PV neurons in areas like HVC, RA, LMAN, and Field L.

Taken together, these findings expand the similarities in neural processes and function across songbirds and mammals. These data further support the utility of songbirds as model organisms for the study of sensory and sensorimotor circuitry, especially as they relate to vocal learning and performance. This research also motivates subsequent studies into differences between PV+PNN and PV-PNN neurons in sensory and motor circuitry in mammals and songbirds as well as the contribution of PNNs in motor circuitry to motor performance and plasticity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the McGill University Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AW: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. XW: Data curation, Investigation, Visualization, Writing – review & editing. D-SS: Data curation, Investigation, Visualization, Writing – review & editing. VL: Data curation, Investigation, Visualization, Writing – review & editing. GC: Data curation, Writing – review & editing, Investigation, Visualization. JB: Data curation, Writing – review & editing, Funding acquisition. JC-F: Data curation, Investigation, Writing – review & editing. EV-S: Conceptualization, Data curation, Funding acquisition, Investigation, Writing – review & editing. JTS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fncir.2023.1297643/full#supplementary-material>

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