Modeling the effects of age, experience and perineuronal net expression on parvalbumin neurons in sensorimotor circuitry

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Abstract

Parvalbumin (PV) expressing interneurons and the perineuronal nets (PNNs) that preferentially surround them are present in various brain regions throughout development and play an important role in neural plasticity. Parvalbumin is associated with increased plasticity while PNNs are thought to restrict the plasticity of the neurons they surround. For example, the increased expression of PNNs around PV interneurons in sensory areas is thought to consolidate mechanisms of sensory processing, thus limiting plasticity. Given their effects on neural function and behavioural plasticity, it is important to understand factors that shape the characteristics of PV neurons. Here, I summarize a series of experiments aimed to reveal how age, experience, and PNN expression influence the amount of PV that is expressed in PV neurons. I investigate PV neuron characteristics primarily in songbirds (namely zebra finches), which are an excellent model for this investigation because PV neurons are abundant within sensory and sensorimotor circuits for birdsong and implicated in vocal learning. To this end, we analyzed PV intensity (a proxy for PV abundance) in PV neurons with or without PNNs in sensory and sensorimotor brain structures in young and older finches and in finches with or without tutor experience. Investigations into PV abundance in rodents were also conducted to complement findings in songbirds. Our data demonstrate that regardless of age, tutor experience, and species, PV intensity is greater in PV neurons surrounded by PNNs than in PV neurons not surrounded by PNNs in most sensory and sensorimotor brain areas examined. Furthermore, degrading the PNNs via chondroitinase ABC (ChABC) results in decreased PV intensity in zebra finches, suggesting a causal role of PNNs to PV intensity and highlighting additional similarities to rodents. Together, these findings underscore the relationship between PV intensity and PNNs as well as the translational impact of such examinations in songbirds.

Résumé

Les interneurones exprimant la parvalbumine (PV) et les réseaux périneuronaux (PNN) qui les entourent en particulier sont présents dans diverses régions du cerveau au cours du développement et jouent un rôle important dans la plasticité neuronale. La parvalbumine est associée à une plasticité accrue, tandis que les PNN limiteraient la plasticité des neurones qu'ils entourent. Par exemple, l'expression accrue des PNN autour des interneurones PV dans les zones sensorielles est supposée consolider les mécanismes de traitement sensoriel, limitant ainsi la plasticité. Compte tenu de leurs effets sur la fonction neuronale et la plasticité comportementale, il est important de comprendre les facteurs qui façonnent les caractéristiques des neurones PV. Je résume ici une série d'expériences visant à révéler comment l'âge, l'expérience et l'expression des PNN influencent la quantité de PV exprimée dans les neurones PV. J'étudie les caractéristiques des neurones PV principalement chez les oiseaux chanteurs (notamment les diamants mandarins), qui constituent un excellent modèle pour cette étude car les neurones PV sont abondants dans les circuits sensoriels et sensori-moteurs du chant des oiseaux et sont impliqués dans l'apprentissage vocal. À cette fin, nous avons analysé l'intensité des PV (une approximation de l'abondance des PV) dans les neurones PV avec ou sans PNN dans les structures cérébrales sensorielles et sensorimotrices chez les diamants mandarins jeunes et âgés,avec ou sans expérience de tuteur. Des études sur l'abondance des PV chez les rongeurs ont également été menées pour compléter les résultats obtenus chez les oiseaux chanteurs. Nos données démontrent que, indépendamment de l'âge, de l'expérience du tuteur et de l'espèce, l'intensité de la PV est plus grande dans les neurones PV entourés de PNN que dans les neurones PV non entourés de PNN dans la plupart des zones cérébrales sensorielles et sensorimotrices examinées. En outre, la dégradation des PNN par la chondroïtinase ABC (ChABC) entraîne une diminution de l'intensité des PV chez les

diamants mandarins, ce qui suggère un rôle causal des PNN dans l'intensité des PV et souligne d'autres similitudes avec les rongeurs. Ensemble, ces résultats soulignent la relation entre l'intensité de la PV et les PNN ainsi qu’un potentiel translationnel chez les oiseaux chanteurs.

Acknowledgements

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Contribution of authors

The images of rat tissue analyzed in this thesis were provided by the lab of Dr. Etienne de Villers-Sidani. The images of PV neurons throughout development were provided by Dr. Jacques Balthazart. Xinghaoyun Wan and Daria Storch helped with various aspects of image acquisition and quantification. Surgeries were done by Anca Vochin and Xinghaoyun Wan. Statistics were run by Dr. Jon T. Sakata and me. I collected, quantified, and analyzed all other data presented in this thesis. Dr. Jon T. Sakata designed the experiments and provided guidance and assistance with data analysis and thesis revisions.

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List of Abbreviations

AFP	Anterior forebrain pathway
BCAN	Brevican
ChABC	Chondroitinase ABC
DLM	Dorsolateral division of the medial thalamus
dph	Days post-hatch
GPe	External globus pallidus
LMAN	Lateral magnocellular nucleus of the anterior nidopallium
NCM	Caudomedial nidopallium
nXIIts	Tracheosyringeal nucleus of the XII cranial nerve
Otx2	Orthodenticle homeobox 2
PNN	Perineuronal net
PV	Parvalbumin
RA	Robust nucleus of the arcopallium
TBS	Tris-buffered saline

Chapter 1: Introduction

1.1 Critical periods and song learning in songbirds

Over development, there are multiple periods of heightened plasticity called critical periods. These phases of enhanced plasticity allow for growth and learning of a variety of behaviours, as seen in examples such as imprinting, ocular dominance plasticity, and speech and language acquisition (Crair et al., 1998; Lenneberg, 1967; Lorenz, 1935; Wiesel & Hubel, 1963). Understanding the neural mechanisms regulating plasticity throughout the lifespan has been an important pursuit.

In songbirds, vocal communication signals (i.e., songs) are learned during the critical period. The development of these vocalizations has many similarities with human speech acquisition, such as reliance on auditory feedback and the neural circuits involved in learning and production (Bolhuis & Gahr, 2006; Brainard & Doupe, 2002; Doupe & Kuhl, 1999; Fee & Scharff, 2010; Sakata & Yazaki-Sugiyama, 2020). Thus, songbirds are an excellent model for human speech development. Zebra finches (*Taeniopygia guttata*) are often used for such research, and in this species, only the males sing and their critical period for song learning and production ends at around 90 days post-hatch (dph) (Brainard & Doupe, 2002; Cornez et al., 2015), at which point they reach the end of their sensorimotor critical period and their song becomes crystallized, meaning that it becomes highly stereotyped and much less dependent on auditory feedback. This unique feature of song crystallization makes zebra finches closed-ended learners and allows for comparison between the animals in a plastic and non-plastic state (i.e., juveniles vs adults).

Songbirds have a discrete neural network that involves two major pathways, the anterior forebrain pathway (AFP) and the vocal motor pathway. The vocal motor pathway is a feed-

forward circuit that includes HVC (acronym used as the proper name, the songbird analogue to the premotor cortex) and the robust nucleus of the arcopallium (RA, the songbird analogue to the laryngeal motor cortex). Neurons in HVC project to neurons in RA, which in turn project further downstream to the tracheosyringeal nucleus of the XII cranial nerve (nXIIts) to eventually allow for vocal production. The AFP similarly starts with HVC and leads to projections to RA via Area X (the vocal portion of the basal ganglia), the dorsolateral division of the medial thalamus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN, a frontal cortical nucleus). The four mentioned telencephalic song control nuclei (HVC, RA, LMAN, Area X) are crucial for song learning, production, and maintenance into adulthood (Ali et al., 2013; Aronov & Fee, 2012; Bottjer et al., 1984; Kao et al., 2005; Roberts & Mooney, 2013; Sakata & Yazaki-Sugiyama, 2020; Scharff & Nottebohm, 1989).

1.2 Parvalbumin neurons, perineuronal nets, and neural plasticity

Parvalbumin (PV) is a calcium-binding protein expressed in GABAergic fast-spiking interneurons that is essential for neural plasticity across a variety of brain systems and species (reviewed in Hensch, 2004, 2005). In mice, the emergence of PV neurons in the visual cortex coincides with the start of the critical period (del Rio et al., 1994), and in the hippocampus, levels of PV increase during the critical period for memory development (Miranda et al., 2022). Manipulations that cause a decrease in PV expression also result in a return to a plastic state in the mouse visual system (Hou et al., 2017). In rats, chemogenetic silencing of PV neurons is able to reactivate plasticity in the auditory cortex (Cisneros-Franco & Villers-Sidani, 2019). In other systems, it has been shown that PV is more abundant in the orofacial primary motor cortex of hominids when compared to macaques, which do not learn vocalizations (Sherwood et al., 2004). This difference between vocal learning and non-learning primates suggests that PV neurons are

integral for the ability to learn vocalizations. A role of PV in vocal learning is suggested because PV levels are significantly higher in the vocal motor pathway of vocal learners than of vocal non-learners (Hara et al., 2012).

While PV neurons are implicated in promoting plasticity, other neurobiological factors can shape the influence of PV neurons. Many PV-expressing neurons are surrounded by perineuronal nets (PNNs), and the emergence of PNNs are tied to a decrease in neural plasticity in multiple animal models, such as rats and zebra finches (Balmer et al., 2009; Cornez et al., 2015; Pizzorusso et al., 2002). In the primary visual cortex, the formation of PNNs (in particular around PV neurons) coincides with the end of the critical period (Hensch, 2005; Pizzorusso et al., 2002). In the deep cerebellar nucleus of mice, PNNs naturally decrease during learning (i.e. plastic state) and are restored when the memory is consolidated (i.e. non-plastic state) (Carulli et al., 2020). Further study in this region showed that degrading the PNNs via chondroitinase ABC (ChABC) improves cerebellum-dependent learning (memory acquisition) (Carulli et al., 2020). Similarly, PNNs in both the hippocampus and anterior cingulate cortex are necessary for memory consolidation and recall (Shi et al., 2019). While PNN expression has been more associated with the closing of critical periods, it has also been shown that PNNs, or more specifically chondroitin sulfate, are necessary for the onset of critical periods as well (Hou et al., 2017).

Given the close relationship of PV and PNNs, it is important to investigate how they might modulate each other. PNNs may inhibit synaptogenesis by creating a physical barrier that prevents new synaptic contacts on PV neurons (Carulli et al., 2010; Dityatev et al., 2007; Karetko & Skangiel-Kramska, 2009). Since PV neurons also receive lateral inhibition from other PV neurons (Hu et al., 2014), removal of PNNs could lead to a decrease in PV expression. Indeed we see that PNNs affect the activity of PV-positive neurons (Härtig et al., 1999). For

example, enzymatic degradation of PNNs using ChABC decreases PV intensity (a proxy for PV abundance) in the hippocampus (Yamada et al., 2015), and decreasing chondroitin sulfate (a component of PNNs) in the visual system via knockout of a catalyzing synthesis enzyme resulted in decreased PV intensity as well as number of PV cells (Hou et al., 2017). PV neuron activity can also affect PNN expression: inhibition of PV neurons in the mouse visual cortex has been shown to induce PNN regression (Devienne et al., 2021), and manipulations of PV neuron activity in the primary auditory cortex of adult rats decrease PNN expression (Cisneros-Franco & Villers-Sidani, 2019). One possible factor in the relationship between PV and PNNs is orthodenticle homeobox 2 (Otx2 homeoprotein), which binds to PNNs (Beurdeley et al., 2012). In the mouse visual cortex, ChABC reduces Otx2 in PV cells, causing PV to also decrease (Beurdeley et al., 2012). Given that other studies have shown that ChABC degrades PNNs and thus decreases PV abundance (Yamada et al., 2015), this suggests that Otx2 could be the connecting link in the mechanism of how PNNs regulate PV expression.

In the zebra finch song system, it has been shown that the percent of neurons surrounded by PNNs increases from juveniles in their critical periods to adults with crystallized songs (Balmer et al., 2009). Since zebra finches are closed-ended learners, meaning that the adult birds do not have a plastic song system, this finding indicates that the presence of PNNs increases with loss of plasticity. In contrast, European starlings are able to modify their songs in adulthood (i.e., open-ended learners), meaning they retain more plasticity into adulthood as compared to zebra finches. These starlings express fewer PNNs in the neural circuits for vocal learning and performance when compared to zebra finches (Cornez et al., 2017). These data suggest that PNNs are related to song crystallization and loss of plasticity.

1.3 Rationale behind studies in thesis

Much of the work on PV and PNNs has been conducted in the sensory system and in mammals, but little is known about the relationship between PV neurons and PNNs, and PV intensity in particular, in sensorimotor systems or in other species. However, sensorimotor structures are replete with PV neurons and PNNs not only in mammals but also in birds. Revealing the relationship between PV neurons and PNNs in sensorimotor structures in songbirds can broaden our understanding of the connection between PV neurons and PNNs and find common principles of brain function and organization. Additionally, given that song learning in zebra finches has many parallels to the acquisition of human speech (Brainard & Doupe, 2013; Chen et al., 2016; Doupe & Kuhl, 1999; Lipkind et al., 2013; Prather et al., 2017), further investigation of the relationship of PV abundance and PNNs in the song system would be valuable for understanding neural plasticity in humans.

In this thesis, I investigate the relationship between the intensity of PV within PV neurons and PNN expression and how this relationship is modulated by factors including age and sensory experience (i.e., tutoring), as well as by manipulations of PNNs. In addition, I pursue studies in rodents to complement the findings in songbirds. Given the similarities in the patterns of PV and PNNs in mammals and birds in existing literature, I hypothesized that our results would be congruent across species. Specifically, I hypothesized that there would be a positive correlation between PV intensity and presence of PNNs and that this would hold true following the patterns expected with other variables (e.g., increased PNNs with age). These data provide insight into PV and PNNs and allow for further research on the plasticity of the sensorimotor system.

Chapter 2: Methods

2.1 Animals

Eighteen normally-reared (i.e., with mother and father) zebra finches (seven juvenile males 50-70 days post-hatch (dph, mean ± SEM: 62 ± 2.4 days), eleven adult males 1-3.5 years post-hatch (19 ± 3.9 months)) were raised and housed in our colony ("tutored" birds). Five male zebra finches (juveniles 50-70 dph, 59 ± 2.1 days) were raised without an adult male (e.g., just mother) from 5-7 dph during their critical periods in sound-attenuating chambers (TRA Acoustics, Ontario, Canada) until they could survive independently, at which point they were housed individually in their own sound-attenuating chambers. These birds constitute our "untutored" birds, meaning that they were not exposed to song during or after their critical periods. All birds were housed on a 14:10 light-dark cycle with food and water *ad libitum*. 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.

2.2 Tissue collection

Subjects were anesthetized with isoflurane vapour and transcardially perfused with heparinized saline (100 IU/100mL) 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 solution for cryoprotection. Brains were cut on a freezing microtome (Leica Biosystems, Wetzlar, Germany) in 40 µm sagittal sections and collected in 1X Tris-buffered saline (TBS).

2.3 Immunocytochemistry (ICC)

Previous literature has shown that there are no significant hemispherical differences of PV distribution in HVC nor caudomedial nidopallium (NCM; an auditory area) (Pagliaro et al., 2020). Thus, one set of tissue from one hemisphere of each bird was processed for both PNN and PV. Free-floating sections were washed for 5 min three times in 1X TBS then blocked for 30 min in TBS + 5.0 donkey serum + 0.1% Triton-X. Then the tissue was incubated overnight at 4°C in a mouse monoclonal anti-chondroitin sulfate (C8035; Sigma-Aldrich; 1:500) and a rabbit polyclonal anti-PV (ab11427; abcam; 1:2000). Afterward, the sections were washed for 5 min three times in TBS followed by a 2 h incubation at room temperature in donkey anti-mouse secondary conjugated to Alexa Fluor 488 (10 µl/ml; ThermoFisher) and donkey anti-rabbit conjugated to Alexa Fluor 594 (5 µl/ml; ThermoFisher) in TBS + 0.1% Triton-X. The tissue was then washed for 5 min three times in TBS and transferred to TBS before mounting. Sections were coverslipped with Prolong Gold Antifade (Life Technologies, 2491361).

2.4 Image analysis

Images of HVC, RA, LMAN, Area X, and Field L were taken with a Zeiss Axio Imager.A2 microscope with a 40x objective for PNN (488nm) and PV (594nm) expression using the ZEN Imaging software (Carl Zeiss).

PV cell counts were done in Fiji. The images were converted to grayscale (16-bit) and the cells were manually counted by a single experimenter across three to five images per song nucleus per bird. The experimenter was blind to the experimental condition of the birds. After identifying the PV cells, PV cell intensity was measured using Fiji; for this, the mean gray values of all cells identified were measured in a 30x30 pixel square centered in the cell. Background measurements

were done in a similar fashion, measuring the mean gray value of a 30x30 pixel square in three randomly chosen locations across the image that did not contain cells. An average of the background measurements was subtracted from the PV cell intensities of the corresponding image to acquire normalized PV intensities, hereafter referred to simply as PV intensity.

PNN counts were done in Fiji independent of PV quantification. Images were converted to grayscale (16-bit), and neurons surrounded by PNNs were manually counted by a single experimenter across three to five images per song nucleus per bird. 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. Again, the experimenter was blind to the condition for image quantification.

Images of additional subjects were acquired from Cornez et al. (2018) of song nuclei at time points 20, 40, 60, 90, and 120 dph. These images were immuno-labeled with antibodies against PV (C8035, Sigma Aldrich; 1:1000) and PNN (C8035, Sigma Aldrich; 1:500). We analysed these images using the same method, covering 3-8 sections per bird from 4-7 birds per age group.

Images of PV neurons and PNNs within the auditory cortex of rats were acquired from Cisneros-Franco et al. (2018). These images were immuno-labeled with antibodies against PV (#P-3088; Sigma-Aldrich; 1:10,000) and PNN (fluorescein wisteria floribunda lectin #FL-1351; Vector Laboratories; 1:200) and were analysed using the same method, covering 7-21 sections per rat from 9 rats.

2.5 Statistical analysis

We used mixed effects models to analyze the effect of PNNs, species, brain region, age, tutoring, and ChABC on PV intensity. Bird ID and Section ID nested in Bird ID were used as random

variables. These random variables were included because multiple PV neurons were measured per section and multiple sections were imaged per animal. Post-hoc tests (Tukey's HSD) were computed for cases where $p<0.05$. All statistics were computed using JMP 13.0 (SAS, Cary, NC), with $\alpha=0.05$ for all analyses.

2.6 Surgery

To experimentally assess how PNNs contribute to PV abundance in sensorimotor structures, we examined how degradation of PNNs in the sensorimotor structure HVC affects the intensity of PV in PV neurons. For surgery, adult birds (n=6, 25 ± 7.3 months) were anesthetized with an intramuscular injection of ketamine (0.03 mg/g i.m.) and midazolam (0.0015 mg/g i.m.) followed by vapourized 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 bilateral craniotomy, chondroitinase ABC (ChABC; C3667; Sigma-Aldrich; 100 U/mL, in 0.1% BSA in PBS) was injected into HVC using stereotaxic coordinates (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). ChABC was injected into HVC using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific, Broomall, PA) assembled with a glass pipette. The injection was performed at the rate of 10 nL/s with the amount of 50 or 100 nL in each cycle, 1-3 cycles in each side of HVC, and the glass pipette was left *in situ* for around 2 min before retraction. Birds were perfused 1-7 days following surgery. After tissue collection, both hemispheres of each bird were processed for both PNN and PV following the same procedure detailed above.

Chapter 3: Results

3.1 Effects of species on PV intensity with relation to PNNs in the primary auditory cortex

We first examined the degree to which the relationship between PV intensity and PNNs was similar in the primary auditory cortex of songbirds and rodents. (Only the auditory cortex was analyzed across these species because of clear homology between these areas (Woolley & Woolley, 2020) and because of lack of a "song system" in rats.) Field L is homologous to the primary auditory cortex of mammals, and PV neurons in Field L demonstrated some variation in PV intensity depending on whether they were surrounded by PNNs or not. For example, in the image in Figure 1A, there are four PV neurons surrounded by a PNN (PV+PNN; white arrows) and two PV neurons not surrounded by a PNN (PV-PNN; orange arrow). PV appears more abundant in the four PV+PNN neurons compared to the two PV-PNN neurons. Across all PV neurons measured in adult male zebra finches (n=6 birds), PV intensity was significantly higher in PV neurons surrounded by PNNs ($F_{1,169.7}$=9.6, p=0.0023) (Fig. 1B).

Just as in male zebra finches, the abundance of PV within PV neurons in the auditory cortex of male rats seems to vary depending on whether the PV neuron was ensheathed in a PNN or not. In Fig. 1C, PV+PNN neurons (white arrows) are more intense with PV than the PV-PNN neuron (orange arrow). Across all measured PV neurons in male rats (n=9 rats), PV intensity was significantly higher in cells surrounded by PNNs ($F_{1,388.2}$=10.5, p=0.0013) (Fig. 1D).

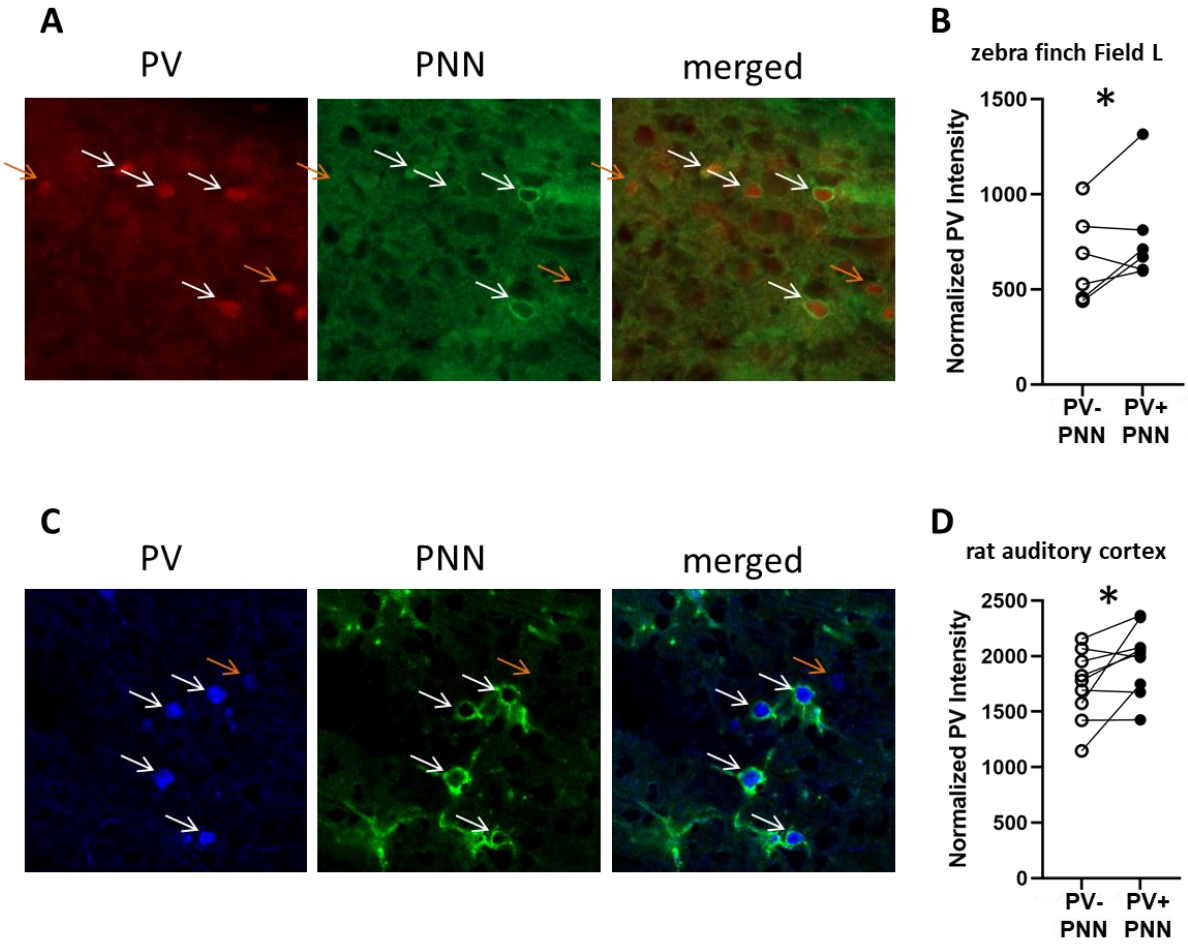

Figure 1. PV intensity of PV-PNN and PV+PNN neurons in zebra finch Field L and rat auditory cortex. *Representative data from a single section in (A) zebra finch and (C) rat. PV intensity of PV-PNN (white circles) and PV+PNN neurons (black circles) in (B) Field L of adult zebra finches (n=6, 13-45 months post-hatch, 25.7 ± 6.5 months) and (D) auditory cortex of adult rats.*

3.2 Effects of age, sensory (tutoring) experience, and PNNs on PV intensity in sensorimotor structures

Parvalbumin neurons and PNNs are abundant within brain areas for song learning and production: namely HVC, RA, LMAN, and Area X (Balmer et al., 2009; Cornez et al., 2015, 2017, 2018; Gogola et al., 2019; Martin Wild et al., 2001; Olson et al., 2015; Wild et al., 2005). As neurons in these areas are affected by age and tutoring experience (Ikeda et al., 2020; reviewed in Sakata & Yazaki-Sugiyama, 2020), we investigated the extent to which age and

tutoring experience affected PV abundance within PV neurons and modulated the relationship between PV intensity and PNN ensheathment. We specifically investigated PV intensity as a function on PNNs in untutored juveniles, tutored juveniles, and tutored adults (see Methods). This allowed us to analyze the effect of age in tutored birds and the effect of tutoring in juvenile birds in conjunction with the effect of PNN ensheathment on PV intensity.

In HVC, there were main effects of group ($F_{2,13.9}$=5.3, p=0.0197) and the presence of PNNs ($F_{1,304.4}$=55.1, p<0.0001) on PV intensity. There was no significant effect of age in tutored birds (p=0.0744) or effect of tutoring in juveniles (p=0.1460; fig. 2A). However, PV intensity was significantly higher in untutored juveniles compared to tutored adults (p=0.0062; fig. 2A). With regard to the effect of PNNs, PV intensity was significantly higher in PV+PNN neurons than in PV-PNN neurons (fig. 3).

In RA, there was a main effect of PNNs on PV intensity ($F_{1,408.9}$=72.4, p<0.0001), with PV intensity being higher in PV+PNN neurons than in PV-PNN neurons (figs. 2B and 3). There was no significant difference in PV intensity across groups.

In LMAN, there was a significant interaction between group and presence of PNNs on PV intensity ($F_{2,503.7}$=7.9, p=0.0004; fig. 2C). Post-hoc contrasts indicate a significant effect of PNNs in tutored juveniles (p<0.0001) and untutored juveniles (p<0.0001) but not in tutored adults (p=0.1748; fig. 2C). There was also a main effect of PNNs ($F_{1,504.5}$=48.9, p<0.0001; fig. 3), with PV intensity being overall higher in PV+PNN neurons.

In Area X, there was a main effect of PNNs on PV intensity ($F_{1,402.5}$=11.4, p=0.0008, fig. 2D and 3), with PV intensity being lower in PV+PNN neurons than in PV-PNN neurons.

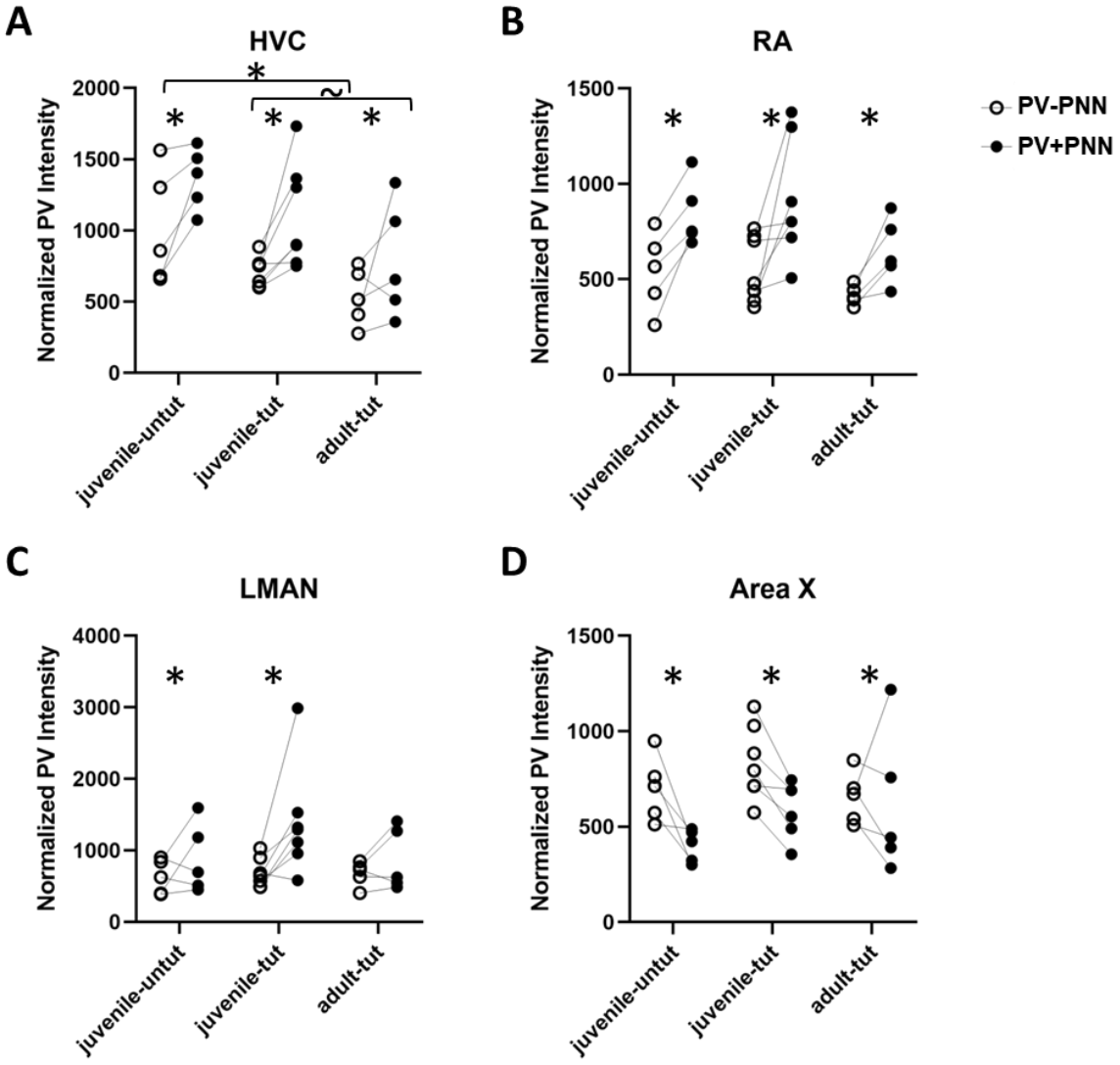

Figure 2. PV intensity of PV-PNN and PV+PNN neurons in untutored juvenile, tutored juvenile, and tutored adult zebra finch song nuclei. *PV intensity of PV-PNN (white circles) and PV+PNN neurons (black circles)) in (A) HVC, (B) RA, (C) LMAN, and (D) Area X of juvenile and adult zebra finches (n=12, 50-70 dph, 60.9 ± 1.6 days and n=5, 10-15 months post-hatch, 11.7 ± 1.0 months, respectively) that were tutored ("tut") or untutored ("untut"). "*" denotes p<0.05, "~" denotes p<0.10.*

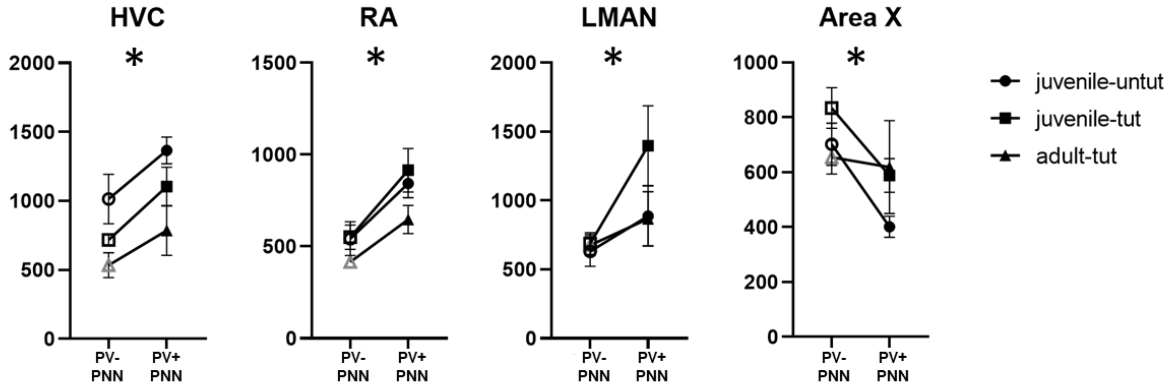

Figure 3. Summary of PV intensity of PV-PNN and PV+PNN neurons in untutored juvenile, tutored juvenile, and tutored adult zebra finch song nuclei. *Summary of PV intensities of PV-PNN (white shapes) and PV+PNN neurons (black shapes) in song nuclei of juvenile and adult zebra finches (n=12, 50-70 dph, 60.9 ± 1.6 days and n=5, 10-15 months post-hatch, 11.7 ± 1.0 months, respectively) that were tutored ("tut") or untutored ("untut"). Plotted are mean±SEM for each group. These plots are useful to visualize the effect of PNNs on PV intensity, regardless of age and tutoring experience.*

3.3 Effects of age and PNNs throughout development on PV intensity in sensorimotor structures

The previous analysis highlights differences in PV intensity between juvenile and adult zebra finches, which were, on average, nine months apart. Many changes to neural circuitry and behavior occur during development; consequently, we collected and analyzed images at different developmental time points (i.e., 20, 40, 60, 90, and 120 dph) from a previously published study (Cornez et al., 2018). Due to the lack of data for 20 dph, that age was not included in further analyses. We did not find a significant effect of age on PV intensity in any song nucleus, though there was a trend for a decrease in PV intensity with age in the cortical-like areas, particularly RA ($F_{3,16.57}$=2.1, p<0.15) (Fig. 4). Nevertheless, there was a significant effect of PNNs in RA and LMAN, with PV+PNN neurons having higher PV intensity ($F_{1,501.1}$=4.6, p=0.0316; $F_{1,592.1}$=16.1, p<0.0001), and similar trend in HVC ($F_{1,776.2}$=3.6, p=0.0568; Figure 5). There was a marginally significant difference in PV intensity between PV+PNN and PV-PNN neurons in Area X, with

PV+PNN neurons having lower PV intensity ($F_{1,368.3}$=2.9, p=0.0869) (Fig. 5). The regional variation in the relationship between PV intensity and PNN expression are consistent with that of our own dataset (Figures 2 & 3).

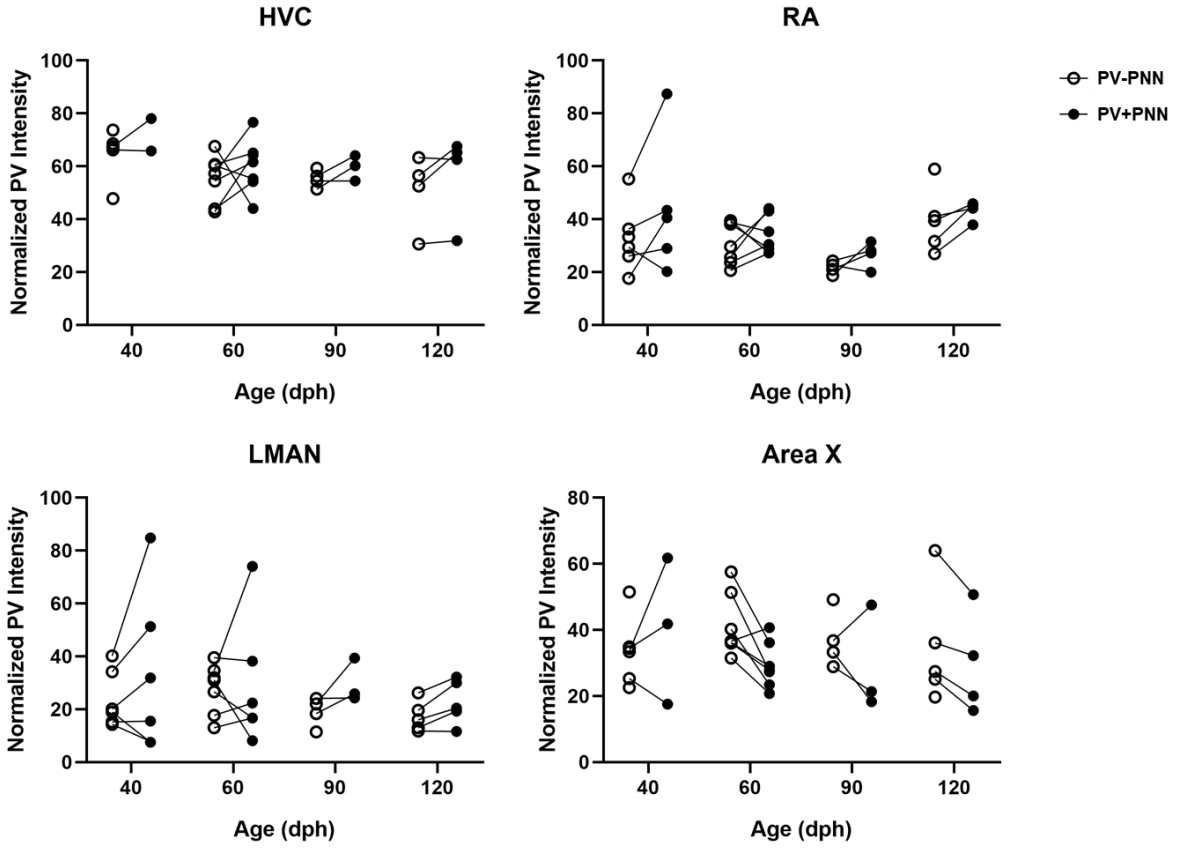

Figure 4. PV intensity of PV-PNN and PV+PNN neurons in zebra finch song nuclei at various points throughout development. *PV intensity of PV-PNN (white circles) and PV+PNN neurons (black circles) of zebra finches at 40, 60, 90, and 120 dph (n=6, 7, 4, and 5, respectively) in (A) HVC, (B) RA, (C) LMAN, and (D) Area X.*

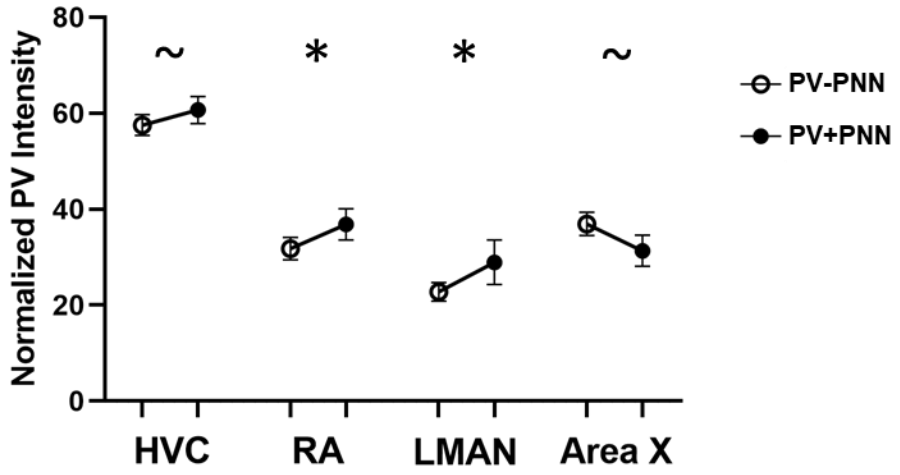

Figure 5. Mean PV intensity of PV-PNN and PV+PNN neurons in zebra finch song nuclei across all time points. *Mean PV intensity of PV-PNN (white circles) and PV+PNN neurons (black circles) of zebra finches across all time points (n=22) in HVC, RA, LMAN, and Area X. "*" denotes p<0.05, "~" denotes p<0.10.*

3.4 Effects of ChABC infusions on PV intensity in the pre-motor nucleus HVC

The preceding analyses suggest the possibility that PNNs could augment PV expression in the PV neurons they ensheath or that PNNs could differentially surround PV neurons that express more PV. To test the former hypothesis, we investigated how degrading PNNs (by infusing ChABC) affected PV expression within PV neurons in HVC. Based on the results described above, we predicted that degradation of PNN should decrease the intensity of PV expression within PV neurons.

In many instances, our ChABC infusions were slightly off target such that PNNs were not removed throughout the entirety of HVC (Fig. 6). However, we took advantage of this to test the effect of PNN degradation. Specifically, these partial PNN degradations allowed us to quantify the intensity of PV within PV neurons in areas of HVC with PNN degradation (PV+ChABC neurons in dorsal HVC; Fig. 6) as well as within PV neurons in parts of HVC without PNN

degradation (PV-PNN and PV+PNN neurons in ventral HVC; Fig. 6). PV intensity was significantly different among these three types of PV neurons ($F_{2,520}$=27.3, p<0.0001). Further, in parts of HVC without PNN degradation, we could measure PV intensity in PV-PNN and PV+PNN neurons. We confirmed that, in parts of HVC without PNN degradation, PV+PNN neurons demonstrated more intense PV staining than PV-PNN neurons (p<0.0001; Fig. 7). Moreover, compared to PV neurons in parts of HVC without PNN degradation, the intensity of PV+ChABC neurons was significantly lower than that of PV+PNN neurons (p<0.0001) and not different from that of PV-PNN neurons (p=0.7944). These data suggest that degradation of PNNs caused a decrease in PV intensity.

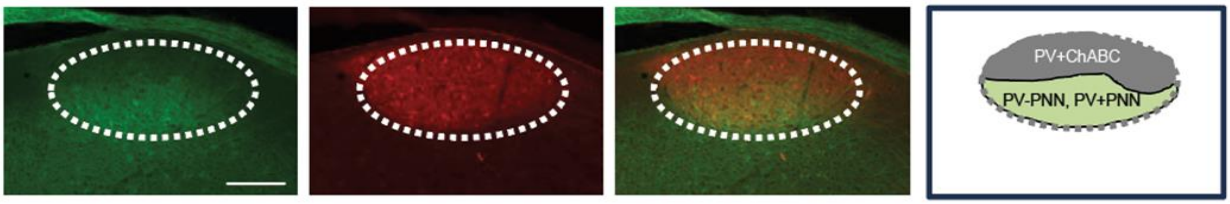

Figure 6. Representative image of HVC after partial ChABC infusion. *Representative image of HVC after partial ChABC infusion with PNNs shown in green and PV shown in red where PNNs were only partially degraded (in this example, the dorsal portion of HVC was degraded (i.e., PV+ChABC) while the ventral portion is intact (i.e., PV-PNN, PV+PNN)).*

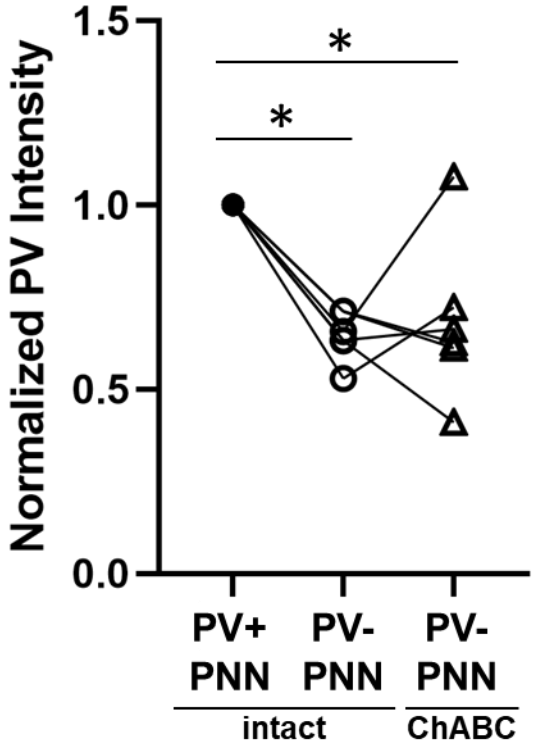

Figure 7. PV intensity of PV+PNN, PV-PNN, and PV+ChABC neurons in zebra finch HVC. *PV intensities of PV+PNN (black circles), PV-PNN (white circles), and PV+ChABC neurons (white triangles) of zebra finches (n=6, 4-49 months post-hatch, 25.2 ± 7.3 months) in HVC. All data were normalized to the mean PV intensity of PV+PNN neurons for each respective bird (since all three categories of PV neurons are observed in birds with partial PNN degradation within HVC). "*" denotes p<0.05.*

Chapter 4: Discussion

Many studies have investigated PV and PNN expression over development (Balmer et al., 2009; Cornez et al., 2018) or PV intensity in areas involved in sensory processing or cognition (Cisneros-Franco & Villers-Sidani, 2019; Hou et al., 2017), but there is a lack of such research on PV intensity in sensorimotor systems. Thus, we analyzed the effect of PNNs on PV intensity in the song system of birds of various ages and with different sensory experiences. In Field L (the homologue to the primary auditory cortex in mammals), we found that PV+PNN neurons had significantly higher PV intensity (a proxy for PV abundance) than PV-PNN neurons. Similarly, in our complementary investigation into the primary auditory cortex of rats, we also found that PV+PNN neurons had significantly higher PV intensity than PV-PNN neurons. In the song nuclei, we found the same result in HVC, RA, and LMAN, while in Area X we found the opposite effect, namely that PV-PNN neurons had significantly higher PV intensity than PV+PNN neurons. We did not find an effect of experience (i.e., tutoring) or age on PV intensity, though there was a trend in HVC and RA for PV intensity to decrease with age. Furthermore, manipulation of PNNs via ChABC infusions showed that removal of PNNs significantly decreased PV intensity.

The regional variation in PV intensity in PV+PNN neurons and PV-PNN neurons suggest that PNNs might function differently in the cortex and basal ganglia. In previous literature, PNNs have been suggested to promote neural activity. The PV-positive interneurons in HVC, many of which are surrounded by PNNs, are known to have high rates of spontaneous firing (Wild et al., 2005). This is likely due to the fact that PNNs act as an insulator that reduces membrane capacitance of fast-spiking interneurons, allowing them to fire at much higher frequencies (Tewari et al., 2018). In mouse somatosensory cortex, tumor-induced PNN removal resulted in

increased cell capacitance and thus decreased excitability (Tewari et al., 2018). Similarly, degradation of PNNs in the visual cortex or deep cerebellar nucleus lowers the spontaneous activity of neurons (Carulli et al., 2020; Lensjø et al., 2017). In contrast, other literature shows that in mouse hippocampal cultures, ChABC decreases the firing threshold and afterhyperpolarization of fast-spiking neurons (Dityatev et al., 2007), and the removal of PNNs increases interneuron excitability (Dityatev et al., 2007). Though the relationship of PV intensity and PNNs in the basal ganglia is less studied, the regional variation in other areas shows that PNNs could act differently in the cortical song nuclei compared to the basal ganglia.

The difference in PV intensity of PV+PNN cells and PV-PNN cells could also be due to the neurons themselves. In the mouse hippocampus, cells with high PV intensity were usually PV+PNN neurons while cells with low PV intensity tended to be PV-PNN neurons (Yamada et al., 2015). Furthermore, the former lacked somatostatin and neuropeptide Y while the latter expressed them (Yamada et al., 2015). In songbirds, studies have also shown differences in neurons that express PV (Braun et al., 1985; Martin Wild et al., 2001; Wild et al., 2005; Zengin-Toktas & Woolley, 2017). For example, RA projection neurons were moderately PV-positive while the putative interneurons were intensely PV-stained (Martin Wild et al., 2001). Similarly, in HVC, the projection neurons did not have detectable calcium-binding protein immunoreactivity, while interneurons did (Wild et al., 2005). Given that our data shows that PV+PNN neurons have higher PV intensity, these findings suggest that PNNs preferentially surround interneurons in RA and HVC. In Area X, it has been shown that there are two cell types of PV neurons, one larger and weakly stained and one smaller and intensely stained (Braun et al., 1985). Other studies in Area X have shown that the larger PV neurons were putative external globus pallidus (GPe) neurons while the smaller PV neurons were putative fast-spiking

interneurons (Carrillo & Doupe, 2004; Reiner et al., 2004; Zengin-Toktas & Woolley, 2017). Given that our data shows that PV+PNN neurons have significantly lower PV intensity in Area X, these findings suggest that PNNs preferentially surround the larger, putative GPe PV neurons in this area. Additionally, PV appears differently in various regions of the auditory midbrain; at times the cells show punctate staining while at others they lack such staining but still show dark neuropil, fibers, and somata and processes (Logerot et al., 2011). These studies suggest that there is regional variation in PV expression and neurons.

Given the association of PV and PNNs to plasticity, we further investigated the changes in PV intensity over development. Our initial dataset, which included juveniles in their critical periods (50-70 dph) and adults well past their critical periods (10-15 months post-hatch), showed that there was a trend for juveniles to have higher PV intensity in some song nuclei compared to adults. Similarly, our second dataset, which included birds at 40, 60, 90, and 120 dph, showed that there was a trend for PV intensity to decrease with age in some song nuclei, though there was variation in the data. Previous literature has shown that PV expression increases over development (Olson et al., 2015) from juveniles at 20 dph to adults over 90 dph. While these findings are in contrast to our data, it could be due to a difference in methodology and our small sample size. Given that our data had inconsistencies, further research would be needed to parse out the details of changes in PV intensity over development.

Previous literature has shown that PV intensity is affected by experience. Specifically, in HVC, PV intensity was significantly lower in birds that were isolated from song tutors than those that were not (Balmer et al., 2009). Though we did not see an effect of tutoring on PV intensity in our data, this could be due to a difference in methodology and could warrant further investigation.

Manipulation of PNNs via ChABC infusions in HVC showed similar results as in mammals. Degradation of PNNs decreases PV intensity in the rodent hippocampus (Yamada et al., 2015) and visual cortex (Beurdeley et al., 2012; Hou et al., 2017; Rowlands et al., 2018). Our data similarly shows decreased PV intensity in HVC after degradation of PNNs via ChABC. Given that PNNs, and their removal, affect the activity of the cells they surround (Carulli et al., 2020; Dityatev et al., 2007; Lensjø et al., 2017; Tewari et al., 2018), these findings suggest that PNNs could similarly modulate the cells in song circuitry of songbirds. While the precise mechanism for how such modulation occurs is not known, possible factors are Otx2 and brevican (BCAN). In mice, it has been shown that PNNs facilitate the movement of Otx2 into PV-expressing interneurons (Beurdeley et al., 2012) and decreasing the Otx2 results in a decrease in PV intensity (Spatazza et al., 2013; Sugiyama et al., 2008). As for BCAN, a component of PNNs, it affects the synaptic inputs of PV interneurons via potassium channels and AMPA receptors (Favuzzi et al., 2017). The PV+BCAN cells also show higher PV intensity than PV-BCAN cells (Favuzzi et al., 2017). Given that our data similarly show that PV+PNN cells have higher PV intensity than PV-PNN cells and that our rodent and songbird data show the same results, these findings suggest that Otx2 and BCAN could be acting in a similar fashion in songbirds as they do in mammals.

This thesis aimed to investigate the relationship between PV and PNNs, specifically with regards to how presence of PNNs affects PV intensity. The results demonstrate that in most sensory and sensorimotor brain areas examined, PV intensity is greater in PV neurons surrounded by PNNs than in PV neurons not surrounded by PNNs. These findings show the translational impact of the findings between mammals and songbirds and further bolster songbirds as a way to study sensorimotor systems. These data open the doors for further research into the mechanisms and

model of PV and PNNs and neuroplasticity and allow for songbird findings to benefit research into human speech and illness.

References

Ali, F., Otchy, T. M., Pehlevan, C., Fantana, A. L., Burak, Y., & Ölveczky, B. P. (2013). The basal ganglia is necessary for learning spectral, but not temporal features of birdsong. *Neuron*, *80*(2), 494–506. https://doi.org/10.1016/j.neuron.2013.07.049

Aronov, D., & Fee, M. S. (2012). Natural Changes in Brain Temperature Underlie Variations in Song Tempo during a Mating Behavior. *PLOS ONE*, *7*(10), e47856. https://doi.org/10.1371/journal.pone.0047856

Balmer, T. S., Carels, V. M., Frisch, J. L., & Nick, T. A. (2009). Modulation of perineuronal nets and parvalbumin with developmental song learning. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *29*(41), 12878–12885. https://doi.org/10.1523/JNEUROSCI.2974-09.2009

Beurdeley, M., Spatazza, J., Lee, H. H. C., Sugiyama, S., Bernard, C., Nardo, A. A. D., Hensch, T. K., & Prochiantz, A. (2012). Otx2 Binding to Perineuronal Nets Persistently Regulates Plasticity in the Mature Visual Cortex. *Journal of Neuroscience*, *32*(27), 9429–9437. https://doi.org/10.1523/JNEUROSCI.0394-12.2012

Bolhuis, J. J., & Gahr, M. (2006). Neural mechanisms of birdsong memory. *Nature Reviews. Neuroscience*, *7*(5), 347–357. https://doi.org/10.1038/nrn1904

Bottjer, S. W., Miesner, E. A., & Arnold, A. P. (1984). Forebrain Lesions Disrupt Development But Not Maintenance of Song in Passerine Birds. *Science*, *224*(4651), 901–903. https://doi.org/10.1126/science.6719123

Brainard, M. S., & Doupe, A. J. (2002). What songbirds teach us about learning. *Nature*, *417*(6886), 351–358. https://doi.org/10.1038/417351a

Brainard, M. S., & Doupe, A. J. (2013). Translating birdsong: Songbirds as a model for basic and applied medical research. *Annual Review of Neuroscience*, *36*, 489–517. https://doi.org/10.1146/annurev-neuro-060909-152826

Braun, K., Scheich, H., Schachner, M., & Heizmann, C. W. (1985). Distribution of parvalbumin, cytochrome oxidase activity and 14C-2-deoxyglucose uptake in the brain of the zebra finch. *Cell and Tissue Research*, *240*(1), 101–115. https://doi.org/10.1007/BF00217563

Carrillo, G. D., & Doupe, A. J. (2004). Is the songbird Area X striatal, pallidal, or both? An anatomical study. *Journal of Comparative Neurology*, *473*(3), 415–437. https://doi.org/10.1002/cne.20099

Carulli, D., Broersen, R., de Winter, F., Muir, E. M., Mešković, M., de Waal, M., de Vries, S., Boele, H.-J., Canto, C. B., De Zeeuw, C. I., & Verhaagen, J. (2020). Cerebellar plasticity and associative memories are controlled by perineuronal nets. *Proceedings of the National Academy*

of Sciences of the United States of America, *117*(12), 6855–6865. https://doi.org/10.1073/pnas.1916163117

Carulli, D., Pizzorusso, T., Kwok, J. C. F., Putignano, E., Poli, A., Forostyak, S., Andrews, M. R., Deepa, S. S., Glant, T. T., & Fawcett, J. W. (2010). Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. *Brain*, *133*(8), 2331–2347. https://doi.org/10.1093/brain/awq145

Chen, Y., Matheson, L. E., & Sakata, J. T. (2016). Mechanisms underlying the social enhancement of vocal learning in songbirds. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(24), 6641–6646. https://doi.org/10.1073/pnas.1522306113

Cisneros-Franco, J. M., & Villers-Sidani, É. de. (2019). Reactivation of critical period plasticity in adult auditory cortex through chemogenetic silencing of parvalbumin-positive interneurons. *Proceedings of the National Academy of Sciences*, *116*(52), 26329–26331. https://doi.org/10.1073/pnas.1913227117

Cornez, G., Haar, S. M. ter, Cornil, C. A., & Balthazart, J. (2015). Anatomically Discrete Sex Differences in Neuroplasticity in Zebra Finches as Reflected by Perineuronal Nets. *PLOS ONE*, *10*(4), e0123199. https://doi.org/10.1371/journal.pone.0123199

Cornez, G., Jonckers, E., Ter Haar, S. M., Van der Linden, A., Cornil, C. A., & Balthazart, J. (2018). Timing of perineuronal net development in the zebra finch song control system correlates with developmental song learning. *Proceedings. Biological Sciences*, *285*(1883), 20180849. https://doi.org/10.1098/rspb.2018.0849

Cornez, G., Madison, F. N., Van der Linden, A., Cornil, C., Yoder, K. M., Ball, G. F., & Balthazart, J. (2017). Perineuronal nets and vocal plasticity in songbirds: A proposed mechanism to explain the difference between closed-ended and open-ended learning. *Developmental Neurobiology*, *77*(8), 975–994. https://doi.org/10.1002/dneu.22485

Crair, M. C., Gillespie, D. C., & Stryker, M. P. (1998). The Role of Visual Experience in the Development of Columns in Cat Visual Cortex. *Science (New York, N.Y.)*, *279*(5350), 566–570.

del Rio, J., de Lecea, L., Ferrer, I., & Soriano, E. (1994). The development of parvalbumin-immunoreactivity in the neocortex of the mouse. *Developmental Brain Research*, *81*(2), 247–259. https://doi.org/10.1016/0165-3806(94)90311-5

Devienne, G., Picaud, S., Cohen, I., Piquet, J., Tricoire, L., Testa, D., Nardo, A. A. D., Rossier, J., Cauli, B., & Lambolez, B. (2021). Regulation of Perineuronal Nets in the Adult Cortex by the Activity of the Cortical Network. *Journal of Neuroscience*, *41*(27), 5779–5790. https://doi.org/10.1523/JNEUROSCI.0434-21.2021

Dityatev, A., Brückner, G., Dityateva, G., Grosche, J., Kleene, R., & Schachner, M. (2007). Activity-dependent formation and functions of chondroitin sulfate-rich extracellular matrix of perineuronal nets. *Developmental Neurobiology*, *67*(5), 570–588. https://doi.org/10.1002/dneu.20361

Doupe, A. J., & Kuhl, P. K. (1999). Birdsong and human speech: Common themes and mechanisms. *Annual Review of Neuroscience*, *22*, 567–631. https://doi.org/10.1146/annurev.neuro.22.1.567

Favuzzi, E., Marques-Smith, A., Deogracias, R., Winterflood, C. M., Sánchez-Aguilera, A., Mantoan, L., Maeso, P., Fernandes, C., Ewers, H., & Rico, B. (2017). Activity-Dependent Gating of Parvalbumin Interneuron Function by the Perineuronal Net Protein Brevican. *Neuron*, *95*(3), 639-655.e10. https://doi.org/10.1016/j.neuron.2017.06.028

Fee, M. S., & Scharff, C. (2010). The Songbird as a Model for the Generation and Learning of Complex Sequential Behaviors. *ILAR Journal*, *51*(4), 362–377. https://doi.org/10.1093/ilar.51.4.362

Gogola, J. V., Gores, E. O., & London, S. E. (2019). Inhibitory cell populations depend on age, sex, and prior experience across a neural network for Critical Period learning. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-56293-2

Hara, E., Rivas, M. V., Ward, J. M., Okanoya, K., & Jarvis, E. D. (2012). Convergent differential regulation of parvalbumin in the brains of vocal learners. *PloS One*, *7*(1), e29457. https://doi.org/10.1371/journal.pone.0029457

Härtig, W., Derouiche, A., Welt, K., Brauer, K., Grosche, J., Mäder, M., Reichenbach, A., & Brückner, G. (1999). Cortical neurons immunoreactive for the potassium channel Kv3.1b subunit are predominantly surrounded by perineuronal nets presumed as a buffering system for cations. *Brain Research*, *842*(1), 15–29. https://doi.org/10.1016/s0006-8993(99)01784-9

Hensch, T. K. (2004). Critical period regulation. *Annual Review of Neuroscience*, *27*, 549–579. https://doi.org/10.1146/annurev.neuro.27.070203.144327

Hensch, T. K. (2005). Critical period plasticity in local cortical circuits. *Nature Reviews. Neuroscience*, *6*(11), 877–888. https://doi.org/10.1038/nrn1787

Hou, X., Yoshioka, N., Tsukano, H., Sakai, A., Miyata, S., Watanabe, Y., Yanagawa, Y., Sakimura, K., Takeuchi, K., Kitagawa, H., Hensch, T. K., Shibuki, K., Igarashi, M., & Sugiyama, S. (2017). Chondroitin Sulfate Is Required for Onset and Offset of Critical Period Plasticity in Visual Cortex. *Scientific Reports*, *7*, 12646. https://doi.org/10.1038/s41598-017-04007-x

Hu, H., Gan, J., & Jonas, P. (2014). Fast-spiking, parvalbumin+ GABAergic interneurons: From cellular design to microcircuit function. *Science*, *345*(6196), 1255263. https://doi.org/10.1126/science.1255263

Ikeda, M. Z., Trusel, M., & Roberts, T. F. (2020). Memory circuits for vocal imitation. *Current Opinion in Neurobiology*, *60*, 37–46. https://doi.org/10.1016/j.conb.2019.11.002

Kao, M. H., Doupe, A. J., & Brainard, M. S. (2005). Contributions of an avian basal ganglia–forebrain circuit to real-time modulation of song. *Nature*, *433*(7026), Article 7026. https://doi.org/10.1038/nature03127

Karetko, M., & Skangiel-Kramska, J. (2009). Diverse functions of perineuronal nets. *Acta Neurobiologiae Experimentalis*, *69*(4), 564–577.

Lenneberg EH (1967) Biological foundations of language. New York: Wiley.

Lensjø, K. K., Lepperød, M. E., Dick, G., Hafting, T., & Fyhn, M. (2017). Removal of Perineuronal Nets Unlocks Juvenile Plasticity Through Network Mechanisms of Decreased Inhibition and Increased Gamma Activity. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *37*(5), 1269–1283. https://doi.org/10.1523/JNEUROSCI.2504-16.2016

Lipkind, D., Marcus, G. F., Bemis, D. K., Sasahara, K., Jacoby, N., Takahasi, M., Suzuki, K., Feher, O., Ravbar, P., Okanoya, K., & Tchernichovski, O. (2013). Stepwise acquisition of vocal combinatorial capacity in songbirds and human infants. *Nature*, *498*(7452), 104–108. https://doi.org/10.1038/nature12173

Logerot, P., Krützfeldt, N. O. E., Wild, J. M., & Kubke, M. F. (2011). Subdivisions of the Auditory Midbrain (N. Mesencephalicus Lateralis, pars dorsalis) in Zebra Finches Using Calcium-Binding Protein Immunocytochemistry. *PLOS ONE*, *6*(6), e20686. https://doi.org/10.1371/journal.pone.0020686

Lorenz, K. (1935). Der Kumpan in der Umwelt des Vogels. *Journal für Ornithologie*, *83*(2), 137–213. https://doi.org/10.1007/BF01905355

Martin Wild, J., Williams, M. N., & Suthers, R. A. (2001). Parvalbumin-positive projection neurons characterise the vocal premotor pathway in male, but not female, zebra finches. *Brain Research*, *917*(2), 235–252. https://doi.org/10.1016/S0006-8993(01)02938-9

Miranda, J. M., Cruz, E., Bessières, B., & Alberini, C. M. (2022). Hippocampal parvalbumin interneurons play a critical role in memory development. *Cell Reports*, *41*(7), 111643. https://doi.org/10.1016/j.celrep.2022.111643

Olson, C. R., Hodges, L. K., & Mello, C. V. (2015). Dynamic gene expression in the song system of zebra finches during the song learning period. *Developmental Neurobiology*, *75*(12), 1315–1338. https://doi.org/10.1002/dneu.22286

Pagliaro, A. H., Arya, P., Sharbaf, Y., & Gobes, S. M. H. (2020). Hemispheric asymmetry of calbindin-positive neurons is associated with successful song imitation. *Brain Research*, *1732*, 146679. https://doi.org/10.1016/j.brainres.2020.146679

Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J. W., & Maffei, L. (2002). Reactivation of ocular dominance plasticity in the adult visual cortex. *Science (New York, N.Y.)*, *298*(5596), 1248–1251. https://doi.org/10.1126/science.1072699

Prather, J. F., Okanoya, K., & Bolhuis, J. J. (2017). Brains for birds and babies: Neural parallels between birdsong and speech acquisition. *Neuroscience and Biobehavioral Reviews*, *81*(Pt B), 225–237. https://doi.org/10.1016/j.neubiorev.2016.12.035

Reiner, A., Laverghetta, A. V., Meade, C. A., Cuthbertson, S. L., & Bottjer, S. W. (2004). An immunohistochemical and pathway tracing study of the striatopallidal organization of area X in the male zebra finch. *Journal of Comparative Neurology*, *469*(2), 239–261. https://doi.org/10.1002/cne.11012

Roberts, T. F., & Mooney, R. (2013). Motor circuits help encode auditory memories of vocal models used to guide vocal learning. *Hearing Research*, *303*, 48–57. https://doi.org/10.1016/j.heares.2013.01.009

Rowlands, D., Lensjø, K. K., Dinh, T., Yang, S., Andrews, M. R., Hafting, T., Fyhn, M., Fawcett, J. W., & Dick, G. (2018). Aggrecan Directs Extracellular Matrix-Mediated Neuronal Plasticity. *Journal of Neuroscience*, *38*(47), 10102–10113. https://doi.org/10.1523/JNEUROSCI.1122-18.2018

Sakata, J. T., & Yazaki-Sugiyama, Y. (2020). Neural Circuits Underlying Vocal Learning in Songbirds. In J. T. Sakata, S. C. Woolley, R. R. Fay, & A. N. Popper (Eds.), *The Neuroethology of Birdsong* (pp. 29–63). Springer International Publishing. https://doi.org/10.1007/978-3-030-34683-6_2

Scharff, C., & Nottebohm, F. (1989). Lesions in area X affect song in juvenile but not adult male zebra finches. *Soc. Neurosci. Abstr.*, *15*.

Sherwood, C. C., Holloway, R. L., Erwin, J. M., & Hof, P. R. (2004). Cortical Orofacial Motor Representation in Old World Monkeys, Great Apes, and Humans. *Brain, Behavior and Evolution*, *63*(2), 82–106. https://doi.org/10.1159/000075673

Shi, W., Wei, X., Wang, X., Du, S., Liu, W., Song, J., & Wang, Y. (2019). Perineuronal nets protect long-term memory by limiting activity-dependent inhibition from parvalbumin

interneurons. *Proceedings of the National Academy of Sciences*, *116*(52), 27063–27073. https://doi.org/10.1073/pnas.1902680116

Spatazza, J., Lee, H. H. C., Di Nardo, A. A., Tibaldi, L., Joliot, A., Hensch, T. K., & Prochiantz, A. (2013). Choroid-Plexus-Derived Otx2 Homeoprotein Constrains Adult Cortical Plasticity. *Cell Reports*, *3*(6), 1815–1823. https://doi.org/10.1016/j.celrep.2013.05.014

Sugiyama, S., Nardo, A. A. D., Aizawa, S., Matsuo, I., Volovitch, M., Prochiantz, A., & Hensch, T. K. (2008). Experience-Dependent Transfer of Otx2 Homeoprotein into the Visual Cortex Activates Postnatal Plasticity. *Cell*, *134*(3), 508–520. https://doi.org/10.1016/j.cell.2008.05.054

Tewari, B. P., Chaunsali, L., Campbell, S. L., Patel, D. C., Goode, A. E., & Sontheimer, H. (2018). Perineuronal nets decrease membrane capacitance of peritumoral fast spiking interneurons in a model of epilepsy. *Nature Communications*, *9*, 4724. https://doi.org/10.1038/s41467-018-07113-0

Wiesel, T. N., & Hubel, D. H. (1963). Effects of visual deprivation on morphology and physiology of cells in the cat's lateral geniculate body. *Journal of Neurophysiology*, *26*(6), 978–993. https://doi.org/10.1152/jn.1963.26.6.978

Wild, J. M., Williams, M. N., Howie, G. J., & Mooney, R. (2005). Calcium-binding proteins define interneurons in HVC of the zebra finch (Taeniopygia guttata). *Journal of Comparative Neurology*, *483*(1), 76–90. https://doi.org/10.1002/cne.20403

Woolley, S. C., & Woolley, S. M. N. (2020). Integrating Form and Function in the Songbird Auditory Forebrain. In J. T. Sakata, S. C. Woolley, R. R. Fay, & A. N. Popper (Eds.), *The Neuroethology of Birdsong* (pp. 127–155). Springer International Publishing. https://doi.org/10.1007/978-3-030-34683-6_5

Yamada, J., Ohgomori, T., & Jinno, S. (2015). Perineuronal nets affect parvalbumin expression in GABAergic neurons of the mouse hippocampus. *The European Journal of Neuroscience*, *41*(3), 368–378. https://doi.org/10.1111/ejn.12792

Zengin-Toktas, Y., & Woolley, S. C. (2017). Singing modulates parvalbumin interneurons throughout songbird forebrain vocal control circuitry. *PLOS ONE*, *12*(2), e0172944. https://doi.org/10.1371/journal.pone.0172944