Timing of perineuronal net development in the zebra finch song control system correlates with developmental song learning

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ABSTRACT

The appearance of perineuronal nets (PNN) represents one of the mechanisms that contribute to the closing of sensitive periods for neural plasticity. This relationship has mostly been studied in the ocular dominance model in rodents. Previous studies also indicated that PNN might control neural plasticity in the song control system (SCS) of songbirds. To further elucidate this relationship, we quantified PNN expression and their localization around parvalbumin interneurons at key time-points during ontogeny in both male and female zebra finches and correlated these data with the well-described development of song in this species. We also extended these analyses to the auditory system. The development of PNN during ontogeny correlated with song crystallization although the timing of PNN appearance in the four main telencephalic song control nuclei slightly varied between nuclei in agreement with the established role these nuclei play during song learning. Our data also indicate that very few PNN develop in the secondary auditory forebrain areas even in adult birds, which may allow constant adaptation to a changing acoustic environment by allowing synaptic reorganization during adulthood.

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1. Introduction

Learning-related neuroplasticity is expressed at its highest level during ontogeny and supports progressive shaping of the adult brain. Extensive neurogenesis and synaptic organization take place during this plastic developmental period and are shaped by the environment. These neurobiological processes are fundamental for the normal development of brain and behavior both in humans and animals. At the end of development, neural plasticity and new learning decrease drastically but the underlying mechanisms involved remain poorly understood.

It has been suggested that the appearance of perineuronal nets (PNN) is one of the mechanisms that contribute to the closing of sensitive periods for neural plasticity and associated learning[1]. In mammals, an increased PNN expression in the somatosensory visual cortex correlates with the end of the sensitive period for visual[2–7] and other aspects of learning[8–10]. These PNN are aggregates of chondroitin sulfate proteoglycan chains (CSPGs) associated with hyaluronic acid and tenascin R that form a scaffold generally surrounding GABAergic interneurons expressing the calcium-binding protein parvalbumin (PV)[2,11]. They develop in an experience-dependent manner at the end of the sensitive period for visual learning in the visual cortex[6] and constitute a physical barrier precluding new synaptic contacts[2]. On another hand, the development of PV interneurons is usually marking the onset of sensitive periods for somatosensory learning, while formation of PNN around them marks the end of such sensitive periods [2,3].

The song of oscines (songbirds) is learned during development by imitation of a conspecific tutor, a rare feature only observed in a few groups of mammals (humans, some bats, whales and elephants) and birds (songbirds, some hummingbirds and parrots)[12]. Vocal learning in songbirds is associated with sensitive periods for sensory learning (song memorization) and sensorimotor learning (vocal practice) that finally lead to the crystallization of the mature adult song [13,14]. The sensorimotor stages of song learning (subsong, plastic song and crystallized song) correspond to human speech learning progressing from babies' babbling to grammatically correct language[15–17].

The neural network underlying these processes is analogous if not homologous. In songbirds, the interconnected brain nuclei involved in song learning and production is called the song control system. It comprises two main pathways. The vocal motor pathway, which connects HVC (previously High Vocal Center, now used as a proper name) directly to the premotor nucleus RA (robustus nucleus of the arcopallium), is similar to the connection between Broca's area and the laryngeal cortex in humans. HVC and RA are also indirectly connected through Area X from the basal ganglia and LMAN (lateral magnocellular nucleus of

the anterior nidopallium) that form the anterior forebrain pathway (AFP) which corresponds to the corticostriatal motor loop in humans[18–20]. These functional and anatomical similarities make songbirds a unique model to study brain mechanisms underlying vocal learning.

In zebra finches (*Taeniopygia guttata*), sensory learning (template memorization) takes place between 25 and 65 days post-hatching (dph)[12,13,16]. Sensorimotor learning starts around 30dph in zebra finches and thus overlaps with sensory learning. At that time, the song is still variable but it will progressively match the memorized template through a feedback process[15] to reach around 90 dph a fully mature crystallized song that will remain stable for the rest of each individual's life.

Between hatching and 90 dph the song control nuclei HVC, RA and Area X undergo major morphological changes including global increases in volume and neuronal size, development of long distance connections and synaptic reorganization[21,22,23]. LMAN, which directly projects to RA and modulates song variability, increases in volume until 20dph and then regresses later on[23]. Sex differences also appear during song learning so that HVC and RA become much bigger in males than females (Area X is essentially not visible in females)[23,24]. The connections in the anterior forebrain pathway are established before song production begins but will be refined during song learning[21]. For example, the connection from LMAN to RA undergoes synaptic pruning that leads to a topographic reorganization[25] that is probably essential for the progressive transition from a variable to a crystallized song when the HVC to RA connection is fully established.

It was previously shown that adult male zebra finches have significantly more PNN especially around PV neurons in HVC and RA than 33 day-old juveniles and this measure in HVC was positively correlated with song stereotypy. Isolation from tutor song during development decreased numbers of PNN around PV neurons in HVC[26]. Adult males who sing proficiently also have more PNN especially around PV neurons in HVC and RA than females who do not sing[27,28]. Moreover adult male zebra finches who are closed-ended learners have a higher PNN density in most song control nuclei compared to other songbird species able to modify their song during adulthood[29]. Together these correlations suggest a causal relationship between high PNN expression in the song control system and decreased neural and song plasticity. Nevertheless the specific time-course of PNN development across developmental song learning is not known. To elucidate this question, we quantified here PNN expression and their localization around PV interneurons throughout ontogeny (every 10 days from 10 to 60 dph then at 90 and 120 dph) in 4 song control nuclei and 4 auditory nuclei in both male and female zebra finches and correlated these data with the well-described development of song

and of song control nuclei in this species. The results allow us to draw clear links between PNN in specific brain regions and different aspects of song learning. Specifically, we show that PNN expression increases markedly in song control nuclei at the end of the sensory phase of vocal learning (±50-60 dph) although maximal levels are reached only later during the sensory-motor phase in a nucleus-specific manner. Sex differences in PNN expression also appear in parallel in HVC and RA but surprisingly not in area X or LMAN. These data suggest specific roles of PNN expressed in different song control nuclei in song learning or production.

2. Material and methods

(a) Subjects & tissue collection

46 male and 32 female zebra finches (*Taeniopygia guttata*) were raised in a common indoor aviary containing nesting material, along with their parents and a large group of conspecifics on a 13L:11D dark/light cycle with food and water *ad libitum*. Brains were collected from 8 developmental ages (10, 20, 30, 40, 50, 60, 90 and 120 dph; 3-6 females and 4-7 males/group; see figures to determine the actual number of individual data points in each case) after perfusion under anesthesia (0.5 ml Nembutal™ at 0.6 mg/ml) with phosphate buffer saline (PBS) followed by paraformaldehyde (4% in PBS). After 24 hours postfixation and cryoprotection in 30% sucrose in PBS, brains were cut on a cryostat in 30 μm coronal sections that were collected in 6 series of 4 wells.

(b) Immunohistochemistry

One series from each bird was stained by double-label immunohistochemistry for parvalbumin and perineuronal nets as previously described[26,28,29]. Free floating sections were incubated in a mouse monoclonal anti-chondroitin sulfate (1:500) specific for the glycosaminoglycan portion of the CSPGs that are main components of the PNN and a polyclonal rabbit anti-parvalbumin (1:1000) and visualized using using Alexa-coupled secondary antibodies (see SI for details).

One series generally contained at least 4 sections within each Region Of Interest (ROI). However, this was not the case for younger birds and some females. For those birds a second full series was consequently stained to ensure appropriate sampling of all ROIs in most subjects. Even with this second series of sections, song control nuclei could not be detected in all subjects at 10 dph, resulting in a smaller number of data point at this age. Sections from the first series were stained in 3 batches each containing individuals from all age groups to prevent

any possible batch effect. Sections for the second series were stained in one batch. No statistical differences between the 2 staining series could be detected and analysis of data including or excluding series 2 led to similar statistical results. Only global analyses including all subjects and sections will thus be presented.

(c) PV & PNN quantifications

Staining was bilaterally quantified in the 4 main song control nuclei (HVC, RA, Area X and LMAN) and in 4 auditory nuclei: CMM (caudomedial mesopallium), NCM (caudomedial nidopallium), Field L complex that are directly or indirectly connected to HVC[30,31] and in NCC (caudocentral nidopallium), a multisensory integrative forebrain area processing acoustic signals and their use in mate choice[32]. Detection of the ROIs was based on the bright PV and/or PNN staining background of these areas except for NCM & NCC which were located based on the zebra finch atlas[33]. NCM is a large telencephalic region extending rostro-caudally from the caudal end of Field L to the caudal edge of the medial telencephalon. NCC is similarly located but lateral to NCM. In this area, pictures were always taken dorsal to the arcopallium at the level of RA, between the caudal end of HVC and the rostral end of RA.

Photomicrographs were acquired bilaterally, with a Leica fluorescence microscope with a 40X objective and fixed settings, in 3-4 sections equally spaced in the rostro-caudal axis for each ROI. The numbers of PV-positive cells (PV), of cells surrounded by PNN (PNN) and of PV-positive cells surrounded by PNN were counted in all photomicrographs with Image J software (https://imagej.nih/ij) as previously described[34]

For each ROI, PV and PNN were quantified as density/mm^2 and as total number/nuclei. Additionally we calculated the % PV surrounded by PNN (%PVwithPNN) and the % PNN surrounding PV (%PNNwithPV) (see SI for details). Pictures were also acquired at 5X magnification (within 1 or 2 full series) for sections containing HVC, RA or Area X to quantify their volume as previously described[34].

All counting and measurements were done blind to the age and sex of the subjects, except for Area X that does not exist in females in which counts were obtained in an equivalent area located at the same neuroanatomical coordinates.

(d) Statistics

All data were analyzed independently for each ROI using a 2-Way ANOVA with sex (n=2) and age (n=8) as independent factors. Significant interactions or age effects were further analyzed using Tukey post hoc tests. Significance level was set at $p < 0.05$. Some statistical tendencies at

189   *p*<0.10 are also shown when important for data interpretation. Results of all ANOVAs (F and p)

190   are reported in SI Tables 1 and 2.

191

192   **3. Results**

193

194   *(a) Song control nuclei volume*

195   The volume of HVC and RA increased significantly with age and was different between sexes.

196   Post hoc analysis of the significant sex by age interaction confirmed that the increase appeared

197   in males only, starting at 40 dph in HVC and 50 dph in RA, with the sex difference appearing at

198   the same age (see Fig.1a-b). Similarly, the volume of Area X increased with age in males starting

199   at 50 dph (see Fig. 1c). The maximum volume was reached at 40 dph in HVC and at 50 dph in

200   RA and Area X, (see Fig. 1a-b-c), as previously reported[23].

201

202   Insert figure 1 about here

203

204   *(b) PNN increases around PV cells in the vocal motor pathway after the end of sensory learning*

205   In HVC, the density of PNN+ cells increased progressively with age and this increase was

206   significant from 60 dph until adulthood (90-120dph) in males but not in females (see Fig.2a).

207   Similarly, the total number of PNN in the nucleus increased significantly from 60 dph in males

208   only and the plateau was reached at 90 dph (see Fig. 2e). In both measures, a significant sex

209   difference was present from 60 till 120 dph (see Fig. 2a and 2e). In RA, a similar pattern of

210   progressive PNN increase (density and number/nuclei) was detected in males but the

211   significant sex difference appeared at 90 dph only (see Fig.2b and f).

212   In parallel, we observed a significant drop of the density of PV+ cells in HVC and RA after

213   10 dph (see Fig. 3a-b). However, the total number of PV+ cells in the entire nucleus was not

214   affected by age in HVC (see Fig. 3e) and it was significantly increased from 50 dph on in the RA

215   of males only (see Fig. 3f). In HVC, whereas females had overall a higher density of PV+ cells,

216   males had a higher number of PV in the entire nucleus (see Fig. 3a-e). This opposite sex

217   difference relates to the large sex difference in the volume of this nucleus appearing around 40

218   dph. In RA, no sex difference was detected in the density of PV+ cells but males had also more

219   PV than females in the entire nucleus (see Fig. 3b-f).

220   Interestingly the percentage of PV cells surrounded by PNN increased following a

221   pattern very similar to the increase of PNN density and number/nuclei in both HVC and RA. In

222   HVC, this percentage was significantly increased from 60 until 120 dph (see SI Fig. 1a), while in

223 RA, the increase was significant from 60 until 90 dph but no longer at 120dph (see SI Fig. 1b).
224 This suggests that, in the vocal motor pathway of males, a large amount of PNN starts
225 developing specifically around PV+ interneurons when the sensory learning period ends and
226 these PNN continue to increase until the end of the sensorimotor period (90 dph) when PNN
227 density, PNN/nuclei and the percentage of PV+ neurons with PNN reach a plateau.

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229   Insert figure 2 about here

230

231 *(c) PNN development in the anterior forebrain pathway*
232 In Area X and LMAN the density of PNN increased with age in both males and females starting
233 at 60 dph in Area X (see Fig. 2c) and at 50 dph in LMAN (see Fig. 2d). Interestingly, no sex
234 difference was detected in Area X or LMAN despite the fact that Area X is not visible in females
235 and counts were made in an anatomically equivalent area. It should also be noted that the
236 average density of PNN in LMAN of males already reached a level similar to 50-60 dph at 30-40
237 dph (see Fig. 2d), which was not the case for females. This is probably why the statistical
238 significance of the age effect (in the absence of a significant interaction) appeared only at 50
239 dph when both males and females reached the same level of PNN density. In Area X, the total
240 number of PNN also increased with age in males but the effect only became significant at 90
241 dph only (see Fig. 2g). Total numbers of PNN could not be calculated for the female Area X nor
242 for LMAN in both sexes since because boundaries of these ROI could not be delineated.

243   As seen in HVC and RA, the density of PV+ cells dropped significantly after 10 dph in
244 Area X and LMAN (see Fig. 3c-d). Interestingly, whereas the PV density remained stable at later
245 ages in HVC, RA and Area X, it increased significantly in LMAN at 90 dph and even further at
246 120 dph. The total number of PV+ cells in the Area X of males followed a different pattern with
247 a main effect of age essentially due to the 50 dph males that had more PV than 10 dph males
248 (see Fig. 3g). The percentage of PV surrounded by PNN in Area X also increased with age, but
249 the effect became significant only at 90 dph (see SI Fig. 1c) i.e., when the total number of PNN
250 increased. In LMAN a progressive increase in this measure was present but the percentage was
251 significantly different only between the 60 and 120 dph birds versus the 20 dph birds (a
252 tendency was nevertheless observed at 90 dph: p=0.0506).
253   As in HVC and RA, the PNN density, total PNN/nuclei and percentage of PV neurons
254 surrounded with PNN in the anterior forebrain pathway were thus increasing with age but in
255 both males and females instead of males only, and the PNN development seemed to occur
256 earlier in LMAN (especially in males). Nevertheless, as in the vocal motor pathway, PNN

257　development starts near the end of the sensitive period for sensory learning and takes place

258　mostly during the sensorimotor period for song learning.

259

260　*(d) The percentage of PNN located around PV+ interneurons varies between sexes*

261　In HVC and RA, the percentage of PNN surrounding PV was not affected by age but differed

262　between sexes. Overall the majority of PNN were located around PV cells and this localization

263　was more prominent in males than in females (HVC: 79% vs. 61%; RA: 85% vs. 49%)(see SI Fig.

264　2a-b). In contrast, in Area X, a higher proportion of PNN were surrounding PV in females (72%)

265　than in males (50%) (see SI Fig. 2c). In LMAN there was no sex difference affecting this

266　percentage but it was lower at earlier ages (10-20 dph) and significantly higher at 120dph

267　(73%) compared to 20 dph birds (see SI Fig. 2d). Taken together, these data indicate that PNN

268　stabilize the connectivity of different cell types (PV-positive and others) depending on the sex

269　and the nucleus considered but this association with different cell types does not change with

270　age except in LMAN.

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274　*(e) PNN and PV expression in auditory nuclei during development*

275　Overall, the density of PNN in the auditory nuclei CMM, NCM and NCC was very low compared

276　to densities observed in the four main song control nuclei. In the majority of birds, the density

277　was smaller than 20 PNN/mm^2 and the mean of each group rarely exceed 10 PNN/mm^2 (see SI

278　Fig. 3b-c-d). This density was ten times higher in HVC and RA and five times higher in Area X

279　and LMAN in adult males. The PNN density in these three nuclei did not change as a function of

280　age or sex (see SI Fig. 3b-c-d) nor did the percentage of PV+ cells surrounded by PNN (see SI

281　Fig. 1f-g-h). PNN in CMM, NCM and NCC are thus unlikely involved in the song crystallization

282　process. In contrast, a high density of PNN was found in Field L and it significantly increased

283　with age (see SI Fig. 3a for detail of post-hoc tests comparing ages). As observed in Area X and

284　LMAN, this increase occurred in both males and females.

285　　　　The densities of PV+ cells in CMM, NCM and NCC were comparable to the densities

286　observed in song control nuclei and they were significantly affected by the sex of the subjects.

287　Females had a slightly higher PV density in CMM and NCM (see SI Fig. 4b-c, only in older ages in

288　the latter case, there was a significant interaction of sex by age) whereas males had slightly

289　more PV in NCC (see SI Fig. 4d). This density of PV cells also increased with age in NCM (120

290　dph had more PV/mm^2 than 10-50 dph birds; SI Fig. 4c) and CMM in which 10 dph had fewer

PV/mm^2 than all the other age groups (see SI Fig. 4b). In NCM, the interaction showed that this age effect was partly due to 120 dph females who had a significantly higher PV density than-20-60 dph males (statistics not shown in the graph). The low density of PNN in these nuclei was reflected in the percentage of PV surrounded by PNN that was lower than 3% in most groups and was not affected by age or sex (see SI Fig. 1f-g-h).

The density of PV+ cells was also affected by age in Field L essentially because it was much higher at 10 dph than in all older age groups (see SI Fig. 4a). Note however that in the brain of 10 dph birds Field L was not clearly discernable as it is in older birds. We therefore quantified photomicrographs taken at the same location within the telencephalon but corresponding results should be taken cautiously. Counts at 10 dph could relate to another adjacent area. Even ignoring the 10 dph data, the percentage of PV neurons with PNN increased with age in parallel to the PNN density reaching maximal levels around 50 dph (see SI Fig. 1e for detail of post-hoc tests) in the absence of any sex difference.

4. Discussion

This study demonstrates that in the four main song control nuclei PNN and their localization around PV interneurons generally start increasing around 50-60 dph, an age that corresponds to the end of the sensory phase of vocal learning when the tutor song has been memorized. The maximum PNN expression is reached later at an age that varies between nuclei. A sex difference in PNN expression and/or colocalization with PV also develops starting at 60 dph in HVC and RA that are involved in song production, but not in Area X and LMAN that play a role in song learning and variability.

A similar pattern of changes with age was observed in the auditory Field L but increases were observed earlier (first increase at 30 dph and plateau at 50 dph) and were not associated with a sex difference. Surprisingly, however, in the other telencephalic auditory areas, few PNN were detected and their density did not change as a function of age suggesting that these nuclei possibly retain their plasticity in adulthood.

(a) PNN increase in the motor pathway corresponds to the end of the sensorimotor sensitive period and song crystallization

In HVC and RA, the largest increase in PNN expression started at the end of the sensitive period for sensory learning (60 dph) and reached a plateau at the end of the sensorimotor learning period (90 dph). This temporal pattern is consistent with a role of PNN in song crystallization. As the majority of PNN are located around GABAergic inhibitory interneurons expressing PV[35]

and PNN are known to increase fast spiking activity of these neurons[36,37], song crystallization could result from an increased inhibition within HVC, resulting in a decreased synaptic plasticity[2]. A higher neural inhibition in HVC indeed correlates with song stability in adult zebra finches[38]. In RA the density of PNN and the percentage of PV neurons with PNN followed a similar pattern of increase although absolute numbers were smaller and the peak observed at 90 dph was followed by a small (non-significant) decrease at 120 dph. The PNN density and the percentage of PV neurons with PNN were thus maximal at 90 dph in both HVC and RA suggesting a clear link between PNN in these nuclei and song crystallization that is completed at 90 dph..

In both HVC and RA, males had a higher proportion of PNN around PV-interneurons (more than 80%) than females (60% in HVC, 50% in RA). In the female HVC and RA, PNN thus develop around different neuronal types or around interneurons expressing different calcium-binding proteins such as calretinin or calbindin. It is nevertheless likely that PNN are expressed mainly around GABAergic interneurons as shown in mammals[37,39]. Together with the higher density of PNN in males, this suggests a differential role of PNN in the vocal motor pathway of the two sexes in relation with the fact that females do not sing and only produce calls[12].

(b) PNN increase in the AFP corresponds to the progressive decrease of song variability during the sensorimotor period

In agreement with previously published studies on adult zebra finches[27,28], no sex difference in the PNN density could be detected in Area X and LMAN throughout development. This is particularly surprising for Area X since this nucleus is not visible in Nissl-stained sections of females and quantification was made here in the corresponding part of the basal ganglia. In males, Area X was discernible based on a higher background staining for PV and a higher density of PNN compared to the rest of the basal ganglia. These differences were not clearly visible in females although PV background density tended to be higher in the lateral part of the basal ganglia (but no shape reminiscent of Area X was detectable) and PNN density tended to be higher in the female "Area X" than in the rest of the basal ganglia. It should be noted that an anterograde and retrograde tract-tracing study published in poster form demonstrated that HVC in female zebra finches projects to a clearly defined cluster of cells in the basal ganglia (Shaughnessy D.W., Flores D., Moore L., Bertram R., Wu W., Hyson R.L. & Johnson F. 2016. Brain connectivity not to blame for a sex difference in singing behavior of zebra finches. Abstract 629.04/AAA23, Society for Neuroscience, San Diego CA). A functional Area X might thus exist in females also.

PNN density increased significantly starting at 60 dph in Area X and 50 dph in LMAN but these changes were similar in both sexes. Here again, the beginning of this rise corresponds to the end of the sensory learning period (60 dph) but it is not followed by any major changes later in development contrary to what is observed in the motor pathway. However, the AFP has never been associated with song memorization. Moreover the <u>total</u> number of PNN in Area X continued to increase after 60 dph, in relation to the global increase in volume of the nucleus, to reach a plateau at 90 dph at end of the sensorimotor period. The development of PNN in the AFP is consequently more likely related to the sensorimotor than to the sensory learning stage.

Knowing that a) LMAN plays a key role in vocal learning at early stages of sensorimotor learning[21,40], b) song variability[13] is highest between 30 and 60 dph[41], c) LMAN volume decreases between 20 and 50 dph in male zebra finches and no change occurs later[23] and d) LMAN lesions between 55 and 65 dph are less effective in disturbing song production than lesions performed between 35 and 50 dph[42], it is interesting that this is the nucleus where the increase in PNN density occurred at the earliest age (around 50 dph or maybe even earlier in males). A large (though not significant, possibly due to the large number of comparisons) increase of the average PNN density (4x) and of the percentage of PV neurons with PNN (5.5x) takes place between 20 and 30dph in the LMAN of males specifically. These measures remain relatively constant afterwards, whereas they only increase after 50 dph in females to reach male levels. This early increase of PNN in LMAN of males was not observed in Area X.

This specific timing of PNN development in LMAN and Area X suggests a delay between the synaptic maturation of these two nuclei in males only. Area X sends inhibitory projections into DLM which in turn sends excitatory projections to LMAN[13]. LMAN is known to induce song variability through its connectivity with RA[43]. LMAN activity could thus be stabilized early (30 dph) by the development of PNN in males to control the firing of the LMAN-RA projection neurons during a period of high song variability (30-50 dph). The subsequent development of PNN around PV interneurons in Area X would then progressively inhibit the excitatory action of DLM on LMAN, in turn decreasing its activity and thus song variability when the song becomes completely stereotyped. This scenario is consistent with the fact that, in males, the percentage of PNN neurons around PV in Area X (50%) and LMAN (56%) is much lower than in HVC (79%) and RA (85%) indicating that in the AFP a large portion of PNN potentially develop around neurons that are not expressing PV and are eventually projection neurons. Furthermore, PV positive cells can also be projection neurons[44].

(c) Role of PNN in the auditory nuclei

We observed an early increase of PNN density and of the percentage of PV neurons with PNN in Field L between 10 and 30 dph. This increased expression of PNN in Field L occurs during the early steps of song memorization. Field L, as the mammalian primary auditory cortex, relays the auditory information directly to HVC but also to CMM and NCM[31]. This brain region is however processing all kinds of auditory stimuli besides species-specific vocalizations and it is difficult to imagine that plasticity in this area should decrease with age. The absence of sex differences in Field L is however in agreement with the fact that both males and females need to be able to discriminate male songs.

In the other auditory nuclei, the density of PNN was much lower (PNN density peak in males: CMM: 16.6/mm^2, NCM: 7.2/mm^2, NCC: 6.8/mm^2) as previously observed in adult male starlings[34] and adult male and female canaries (unpublished data from our lab). Moreover, the density of PNN was not affected by age or sex in these regions. Either synaptic plasticity in these auditory forebrain areas is controlled by other mechanisms or it is surprisingly retained in adulthood. CMM and NCM are thought to be involved in song perception and decoding[31] and NCM could, in addition together with the vocal pathway, contain (in part) the memory of the tutor song template[46]. Neurons in both Field L, CMM and NCM respond selectively to the species-specific songs during development[47-50] but CMM and NCM neurons respond in a non-predictable manner and this response continuously evolves with the acoustic environment even in adulthood which is not the case for Field L[51-54]. The relative absence of PNN in the NCM and CMM may consequently allow constant adaptation of adult zebra finches to a changing acoustic environment by providing a larger possibility of synaptic reorganization.

(e) Conclusions

We demonstrate here sex-specific and age-specific changes in the expression of PNN and of PV interneurons in the song control nuclei and auditory areas of developing zebra finches that display temporal relationships with the sensory and sensorimotor phases of song development. The differences observed between nuclei suggest that each nucleus has its own timing of connectivity stabilization (sensitive period?) during which it plays one key role in the vocal learning process. These correlations raise a number of important questions. One type of question concern the role of PNN in song learning. Are they slowing down or inhibiting the learning process? Are they implicated in the crystallization of song? Are different nuclei differentially affected as a function of their role in song acquisition and production as suggested by differences in the temporal patterns of expression? The other type of questions relates to the mechanisms controlling PNN formation. Do they develop directly in response to physiological,

in particular hormonal, changes that take place as the bird ages or are they the consequence of changes in the vocal behavior that would feedback on the underlying brain structures?

We previously showed that the density of PNN is lower in open-ended learner species that are still able to modify their song in adulthood (canaries, starlings) than in closed-ended learner species such as the zebra finch that crystallize a song when reaching adulthood and will no longer change it during the rest of their life. This suggests that PNN development might relate to song crystallization and the developmental patterns identified here are consistent with this view. The functional significance of these correlations should now be tested by causal manipulations, in particular experiments that would experimentally dissolve the PNN in specific brain regions and assess whether this would result in a regain of behavioral plasticity as previously shown in a number of experimental models in mammals. An initial attempt published only as a poster obtained mixed results: application of chondroitinase ABC dissolved the PNN in HVC but no clear modification of song structure was detected in males kept in acoustic isolation. However, in males maintained with a female after PNN dissolution, song became more plastic: song duration decreased, some syllables were lost while others were added (Best B.J., Day N.F., Larson G.T., Carels V.M. & Nicks T.A. 2011. Vocal effects of perineuronal net destruction in adult zebra finch. 303.18/XX51, Society for Neuroscience, Washington DC). We also observed some alterations of song in male canaries following application of chondroitinase ABC over HVC (Cornez G., Cornil C.A. & Balthazart J, unpublished data). Future work will have to better characterize these changes in vocal production and learning in order to assign a specific role to PNN in each song control nuclei. The age- and nuclei-specific changes described here will clearly help formulating specific hypotheses and testing them.

Ethics. All experimental procedures were in agreement with the Belgian laws on animal experimentation and had been approved by the local Animal Care Committee (Commission d'Ethique de l'Utilisation des Animaux d'Expérience de l'Université de Liège; Protocol number 1396).

Data accessibility.

All data raw data are available for review at datadryad.org (doi:10.5061/dryad.515n9k9)

474

Figure legends

476

477   Figure 1: HVC (a), RA (b) and Area X (c) volumes of males (blue) and females (red) across ages

478   10-120 dph. Results of the ANOVAs are summarized in the insets. If a significant sex by age

479   interaction was detected, significant sex differences within age groups as determined by Tukey

480   post hoc tests are shown as follows: *p<0.05, **p<0.01, ***p<0.001, (*)p<0.10. Letters indicate

481   significant (p<0.05) age differences within males only as follows: a=different from 10-40 dph,

482   b= different from10-30 dph, c= different from 10-20 dph, d= different from 10 dph. Bars

483   represent means ± SEM but all individual values are also plotted.

484

485   Figure 2: PNN density (left; a-d) and number/nuclei (right; e-g) in the song control system of

486   males (blue) and females (red) across ages 10-120 dph. Statistical results of the ANOVA are

487   summarized in the insets. If a significant sex by age interaction was detected, significant sex

488   differences within age groups as determined by Tukey post hoc tests are shown as follows:

489   *p<0.05, **p<0.01, ***p<0.001, (*)p<0.10. Letters indicate significant (p<0.05) age differences

490   within males only (when interaction is significant) or among all birds (when a main effect of

491   age without interaction is found) as follows: a= different from 10-60 dph, b= different from 10-

492   50 dph, c= different from 10-40 dph, d= different from 10-30 dph, e= different from 10 dph, f=

493   different from 20 dph (f). Panel h shows representative photomicrographs of PV+PNN double

494   staining in HVC of an adult male (upper left), a 20 dph males (down left), an adult female (down

495   right) and higher magnification of a PV cell surrounded by PNN in the adult male. Bars

496   represent means ± SEM but all individual values are also plotted.

497

498   Figure 3: PV density (left; a-d) and number/nuclei (right; e-g) in the song control system of

499   males (blue) and females (red) across ages 10-120 dph. Statistical results of the ANOVA are

500   summarized in the insets. If a significant sex by age interaction was detected, significant sex

501   differences within age groups as determined by Tukey post hoc tests are shown as follows:

502   *p<0.05, **p<0.01, ***p<0.001, (*)p<0.10. Letters indicate significant (p<0.05) age differences

503   within males only (when interaction is significant) or among all birds (when a main effect of

504   age without interaction is found) as follows: a= different from 20-120 dph, b= different from

505   20-90 dph, c= different from 20-60 dph, d= different from 50-120 dph, e= different from 10

506   dph, f= different from 20 dph, g= different from 90 dph. Panel h shows representative

507   photomicrographs of PV+PNN double staining in RA of an adult male (upper left), a 20 dph

508   males (down left), an adult female (down right) and higher magnification of a PV cell

509   surrounded by PNN in the adult male. Bars represent means ± SEM but all individual values are

510   also plotted.

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