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STEROID-DEPENDENT PLASTICITY IN THE SONG CONTROL SYSTEM : PERINEURONAL NETS AND HVC NEUROGENESIS

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ABSTRACT

The vocal control nucleus HVC in songbirds has emerged as a widespread model system to study adult brain plasticity in response to changes in the hormonal and social environment. I review here studies completed in my laboratory during the last decade that concern two aspects of this plasticity: changes in aggregations of extracellular matrix components surrounding the soma of inhibitory parvalbumin-positive neurons called perineuronal nets (PNN) and the production/incorporation of new neurons. Both features are modulated by the season, age, sex and endocrine status of the birds in correlation with changes in song structure and stability. Causal studies have also investigated the role of PNN and of new neurons in the control of song. Dissolving PNN with chondroitinase sulfate, a specific enzyme applied directly on HVC or depletion of new neurons by focalized X-ray irradiation both affected song structure but the amplitude of changes was limited and deserves further investigations.

1. Introduction

During the last 50 years, the song control system (SCS) of oscine birds has become a major topic of study in neurosciences (Marler and Slabbekoorn, 2004, Zeigler and Marler, 2008). This network of interconnected brain nuclei was initially discovered based on a series of retrograde tract-tracing studies designed to identify the brain structures that ultimately connect to the syrinx muscles in order to control song production (Nottebohm et al., 1976). Lesions experiments that followed during the next decade or two then identified the specific function of each node in this network in the control of song learning and song production (Brainard, 2008, Nottebohm, 1980, Wild, 1997, Wild, 2008).

The SCS is schematically composed of two main functional paths (Brenowitz et al., 1997b). A caudal path runs from HVC (initially High Vocal Center, now used as proper name: (Reiner et al., 2004)) to the robust nucleus of the arcopalium (RA) which then projects to the motoneurons of the

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12th nerve that directly innervate the synrigeal muscles (Schmidt and Wild, 2014, Wild, 1994, Wild, 2008). A more rostral pathway also connects HVC to RA but via projection from HVC to Area X of the basal ganglia, then to the medial part of the dorsolateral thalamic nucleus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) or in summary: HVC=>Area X=>DLM=>lMAN=>RA (Bottjer and Johnson, 1997). A multitude of studies clearly indicate that the caudal pathway is critical for the production of song while the rostral pathway is implicated in song learning during ontogeny and maintenance of song structure in adulthood (sumarized in (Jarvis, 2008)). Indeed songbirds learn their song during ontogeny. They first memorize the song of conspecifics (the father or other males living in the neighborhood, the sensory phase), then at a later time they start practicing vocalizations trying to match their vocal production to the template they have memorized (the sensorimotor phase) to finally reach a stage when they produce a mature song that will become fixed (crystalized) either for the entire life - in closed-ended learners such as zebra finches - or for the next reproductive season - in open-ended learners such as canaries or starlings –that will further change their song for one year to the next (Brainard and Doupe, 2002, Marler and Slabbekoorn, 2004).

Experimental work has identified a number of important features of the SCS (Brenowitz et al., 1997a) including its sexually differentiated nature (Ball et al., 2008, MacDougall-Shackleton and Ball, 1999, Nottebohm and Arnold, 1976) and its functional lateralization (Nottebohm, 1971, Nottebohm and Nottebohm, 1976, Suthers et al., 2004). Many nuclei of the SCS also express sex steroid receptors (Arnold et al., 1976, Gahr et al., 1993) and their function is clearly controlled by these hormones (Ball and Balthazart, 2010, Gahr, 2001, Schlinger, 1997). The expression of multiple genes in the SCS is indeed sensitive to androgens and/or estrogens (Choe et al., 2021, Ko et al., 2021, Voigt et al., 2004).

The SCS has thus become an easily tractable model for the study of all these topics. But above all, the SCS is known and studied for the extensive plasticity it displays throughout the life of the bird (Nottebohm, 1981). Indeed most avian species living in the temperate zones, including many songbirds, show pronounced annual cycles in their reproductive physiology and these endocrine changes have a much larger magnitude than observed in mammals. For example, the testes size varies 10 to a 100 fold between the two extremes while only one or two fold changes are usually detected in mammals, if any (Hurley et al., 2008, Nicholls et al., 1988, Nicholls et al., 1973, Niklowitz et al., 1994). This high degree of physiological plasticity in birds actually extends to many other domains. One could, for additional examples, cite the very large changes in body weight and fat storage occurring in relationship to migration (Majumdar et al., 2021, Wingfield et al., 1996) or the complete regression of the gonadotropin hormone-releasing hormone (GnRH) expression in hypothalamic neurons when birds become photorefractory (Dawson et al., 2001, Stevenson et al., 2012). Changes in blood concentrations of sex steroids observed across seasons in birds are similarly much larger than in mammals and it is therefore not surprising to see in parallel large changes in brain structure and function.

It was initially discovered that the volume of song control nuclei in canaries varies 2 or 3 fold during the annual cycle (Nottebohm, 1981). The search for the underlying mechanisms identified a

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host of cellular changes that take place between the breeding and the non-breeding seasons in the song nuclei of this species. These volumetric changes reflect increases in cell size, in cell spacing, in dendritic arborization and then, specifically in HVC, changes in neuron numbers associated with an active and seasonally changing neurogenesis (Brenowitz, 2008). The discovery of an active neurogenesis in the HVC of adult birds in the early 1980ies actually came as a big surprise (Burd and Nottebohm, 1985, Goldman and Nottebohm, 1983, Paton and Nottebohm, 1984). It was challenging a previous dogma according to which adult homoeothermic vertebrates are born with a complement of neurons and do not add new neurons at any point during their postnatal life (Gross, 2000). A large amount of research was therefore dedicated to this topic in the following decades. During the last 10 years we also paid attention to another form of plasticity that had been hypothesized to control the connectivity in the song control nuclei and in this way to regulate song crystallization during ontogeny, the perineuronal nets (PNN)(Celio et al., 1998).

2. Perineuronal nets

Perineuronal nets (PNN) are aggregations of extracellular matrix components that accumulate around specific neurons. PNN are namely composed of chondroitin sulfate proteoglycans, tenascin R, hyaluronic acid and binding proteins. They form a scaffold mainly around fast spiking GABAergic interneurons expressing parvalbumin (Deepa et al., 2006, Wang and Fawcett, 2012). It has been demonstrated that, in mammals, PNN play an important role in the closing of sensitive periods for sensory learning (Hensch, 2004, Hensch, 2005, Wang and Fawcett, 2012, Werker and Hensch, 2015). Indeed PNN limit synaptic plasticity in areas such as the somatosensory cortex (Nakamura et al., 2009) and the visual cortex where they develop in an experience-dependent manner following visual stimulation (Liu et al., 2013, Ye and Miao, 2013). In addition to these correlative studies relating PNN development to various functional outcomes, multiple experiments have shown that experimental degradation of PNN restores functional plasticity (e.g., (Galtrey and Fawcett, 2007, Karetko and Skangiel-Kramska, 2009, Pizzorusso et al., 2002, Wang and Fawcett, 2012). PNN around parvalbumin neurons of the CA1 part of the hippocampus also regulate the formation of very specific memories (Ramsaran et al., 2023).

When we initiated our work on this topic, one single study had analyzed PNN in songbirds. Teresa Nick and collaborators had demonstrated that, in zebra finches (*Taeniopygia guttata*) PNN are present in very high density in all telencephalic song control nuclei (HVC, RA, Area X and lMAN) and that their density increases between 30 days post-hatch (dph) and adulthood, i.e. during the period when birds learn, practice and crystallize their song (Balmer et al., 2009). In 65-day-old male birds that were approximately halfway through the sensorimotor phase of song learning, the density of PNN surrounding PV-positive neurons in HVC was also shown to correlate with various features of song such as the entropy variance or frequency variance, so that PNN development predicted to some extent the maturity of song or in other words its degree of crystallization. Quite importantly, this increase appeared to be experience-dependent as evidenced by the fact that males raised in isolation (without tutor) had when adults a much lower density of PNN in HVC (Balmer et al., 2009).

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2.1. ONTOGENY IN ZEBRA FINCHES

This study pointed to specific relationships between song and PNN development but did not allow to precisely identify when PNN density increases with respect to song development. To address this question, in a first set of experiments we collected brains from male and female zebra finches every 10 days from 10 dph until 60 dph and then in adult birds at 90 and 120 dph (Cornez et al., 2018). This study first confirmed the specific presence of high densities of PNN and of parvalbumin-positive neurons in the song control nuclei HVC, RA, Area X and LMAN (Fig. 1A-B) and the specific localization of PNN around the parvalbumin neurons (Fig. 1C-D). The experiments also demonstrated that PNN density increases progressively in the HVC and RA of males, but not of females. An increase in PNN density with age was also observed in the Area X of males but a similar increase was observed here in the corresponding region of the basal ganglia of females even if a clear delineation of Area X is not possible in females of this species (Cornez et al., 2018).

The higher density of PNN in the HVC and RA of males compared to females confirmed a sex difference that had been previously reported (Cornez et al., 2015, Meyer et al., 2014). This sex difference potentially relates to behavior since adult males sing a crystallized song while females never sing in this species (Arnold, 1975, Pröve, 1974). The increase of PNN density with age observed in males was relatively progressive and reached statistical significance at 60 dph in HVC but only at 90 dph in RA. It was therefore difficult to identify the aspects of song development that are correlated and are possibly modulated by PNN. Indeed zebra finches develop their song relatively rapidly and there is an overlap in time between the sensory (±10 to 60 dph) and sensorimotor (30 to 90 dph) phases of song learning before song crystallized around day 90 (Brainard and Doupe, 2002). A link with song crystallization was thus suggested but could not be confirmed.

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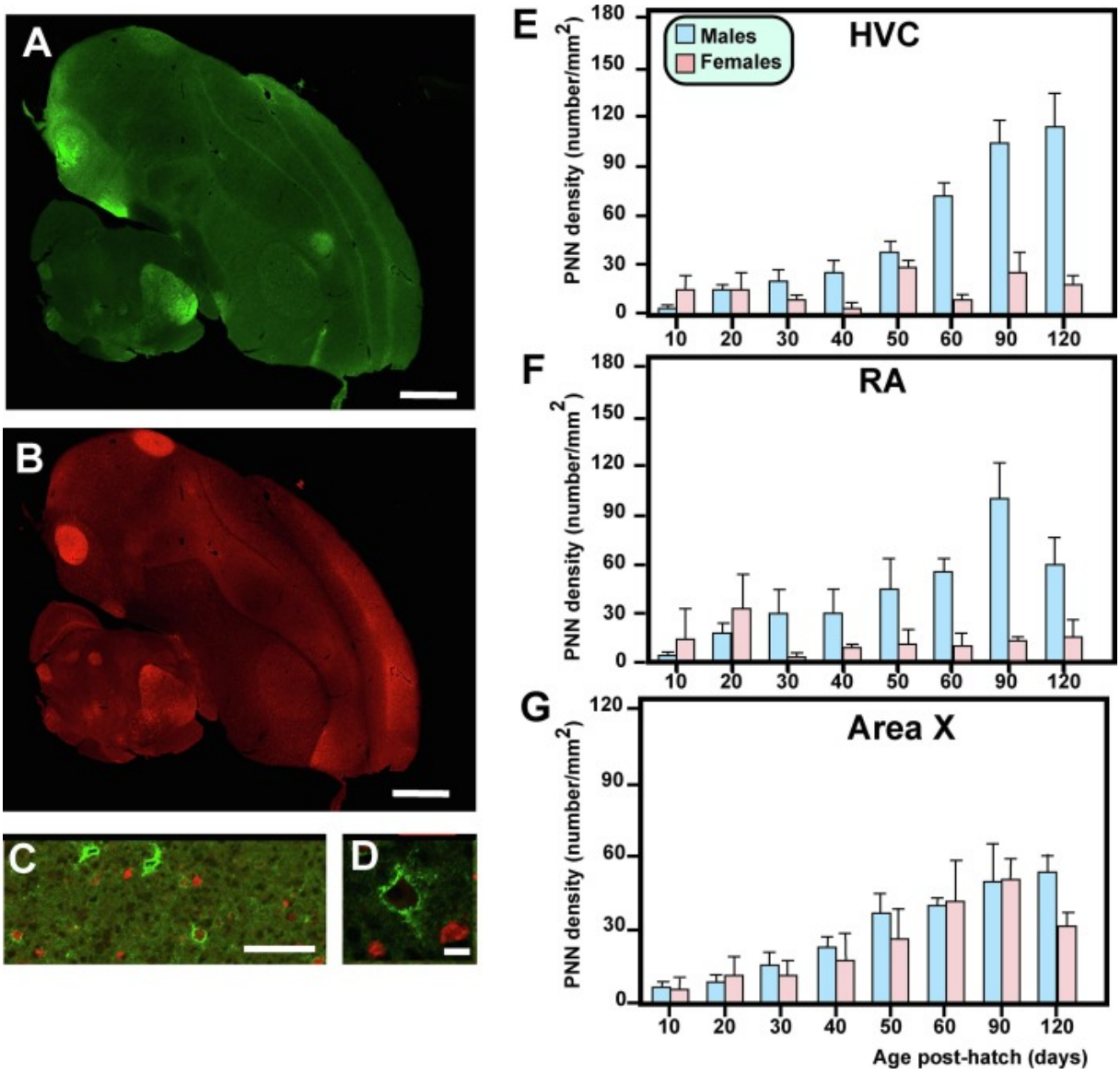
Figure 1. *Perineuronal nets and their development during ontogeny in male and female zebra finches.*



A-D. Photomicrographs of a sagittal section through the male zebra finch brain illustrating the distribution of perineuronal nets (PNN) stained by an antibody directed against chondroitin sulfate (A) and of parvalbumin-immunoreactive neurons (B). Both markers are co-expressed at high density in the three song control nuclei, HVC, RA and Area X. C, D. Medium high (C) and high (D) magnification of sections showing a few PNN (green) surrounding or not parvalbumin neurons (red). Magnification bar = 1 mm in A-B, 100 µM in C and 10 µM in D. E-G. Changes in PNN density (numbers /mm2) during ontogeny in three song control nuclei in male and female zebra finches. All data are means ± SEM of 3 to 7 data points per sex and age. Redrawn and rearranged from data in (Cornez et al., 2018, Cornez et al., 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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2.2. ONTOGENY IN CANARIES

To progress in the analysis of this question, a similar study was undertaken in canaries (Serinus canaria) who develop their song in a much slower manner between the spring-summer when they are born and the following spring. The density of PNN in song control nuclei was quantified in male canaries during their first year of life in 5 different groups of birds that correspond to specific stages in song development (Cornez et al., 2020b).

Canaries are normally born in the spring. Their sensory song learning starts around fledging time (±25–35 dph) and ends sometimes during the summer when adults of previous generations stop singing. Young birds are at that time between 50 and 100 days old, depending on their hatching date (Leitner et al., 2015). Sensorimotor learning then starts around 60 dph and extends until the first breeding season when birds are around one year old (Brainard and Doupe, 2002, Leitner et al., 2015). Increases of PNN expression occurring during the late spring/early summer would thus relate to sensory learning. If it occurred after the summer, it would consequently be associated with sensorimotor learning or with song crystallization if occurring late in the fall or winter.

Groups of newborn male canaries were obtained in the spring and kept indoors under a changing photoperiod that was modified each month to match the natural photoperiod at 52° north, the attitude of Belgium. They were continuously exposed to the vocalizations of 5 adult male tutors kept under the same photoperiod. Brains were collected in the late spring when birds were 55-day-old and only produced a rudimentary subsong (Group 1), in the summer when birds started singing a plastic song, practicing the song they had memorized earlier as a template (Group 2), in the autumn when plastic song had become more elaborate and it became possible to recognize distinct syllables (Group 3), in the winter when plastic song was progressively crystallizing in this final form (Group 4), and finally in the following spring when birds sang a fully crystallized adult song (Group 5; see Fig. 2A-D). Before brain collection, singing activity had been recorded during a 4 week-period in each group so that its degree of maturation could be analyzed (Cornez et al., 2020b).

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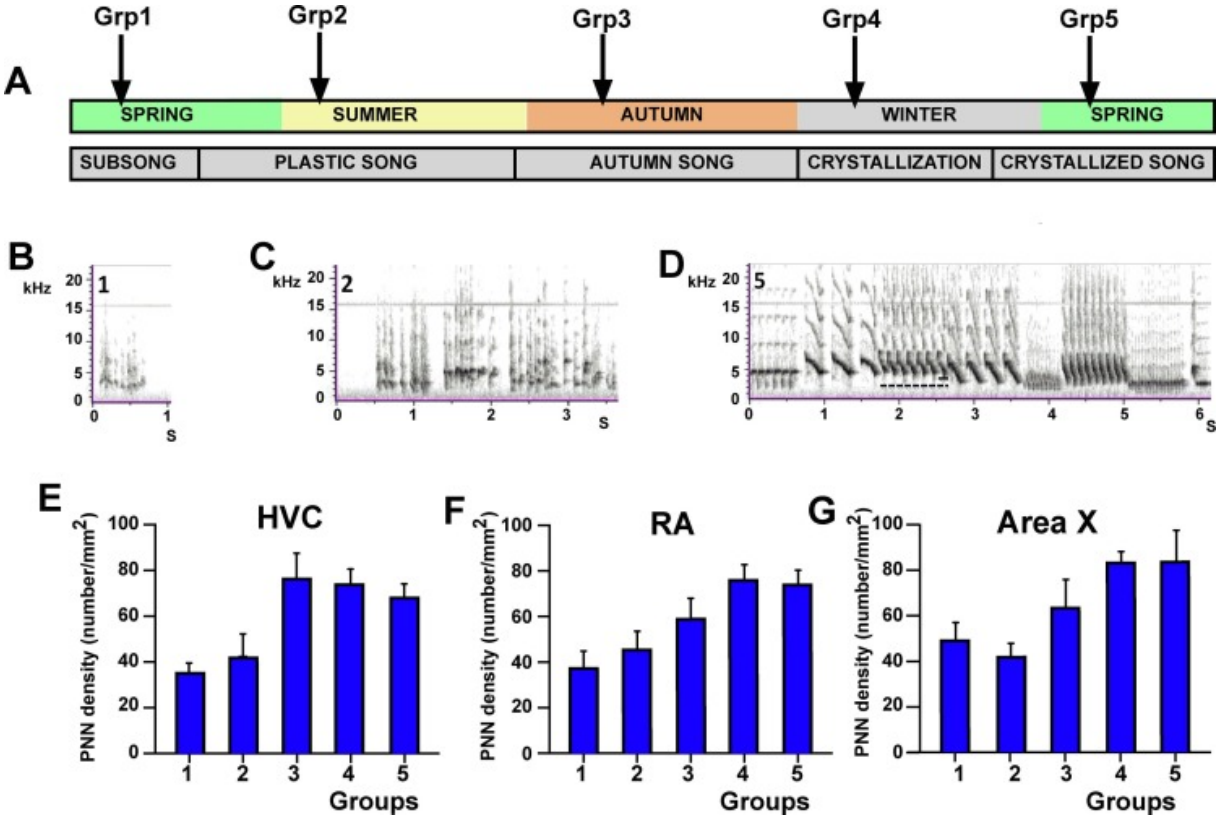
Figure 2. *Perineuronal nets and song development during ontogeny in male canaries.*



A. Schematic representation of song development in male canaries during their first year of life and distribution in time of the 5 experimental groups (Grp 1 to 5). B-D. Examples of spectrograms illustrating the 3 main stages of song development, subsong (B), plastic song (C) and crytallized song (D). E-G. Changes in PNN density (numbers /mm2) during ontogeny in three song control nuclei in male canaries. All data are means ± SEM of 7 to 10 data points per age. Redrawn and rearranged from data in (Cornez et al., 2020b).

Analysis of the recorded songs indicated that song learning had progressed in these birds raised in fully controlled conditions as predicted based on previous studies of wild birds. Birds in group 1 essentially did not sing yet whereas birds of group 2 sang more or less developed subsongs (example in Fig. 2B). Groups 3 and 4 sang progressively more complex plastic songs (Fig. 2C) that were approaching crystallization during the winter. Song in spring (group 5) was fully crystallized (Fig. 2D; See (Cornez et al., 2020b) for the detailed quantification of these songs).

In parallel, PNN density in the song control nuclei progressively increased: in HVC they reached their maximal density in group 3 (fall) whereas this maximum was only attained during the winter (group 4) in RA and Area X (Fig. 2E-G). Based on these data, it appears that the full development of PNN is correlated with the end of the plastic song period/beginning of its crystallization, i.e., the moment when song structure loses its plasticity and becomes relatively fixed, at least for the following reproductive season.

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2.3. SEASONAL CHANGES IN CANARIES

Canaries are indeed open-ended learners: they first learn their song during ontogeny and crystallize it before their first breeding season but then they are able to change it from one breeding season to the next, adding new and removing old syllables from their repertoire. To my knowledge, it is unknown at this point whether the syllables added during successive seasons are learned in adulthood, as clearly demonstrated in another songbird species the European starling (Sturnus vulgaris)(Bohner et al., 1990, Chaiken et al., 1994), or are just the expression of different parts of the repertoire that were learned during the first year of life.

It is well established that across successive years, the canary song alternates between periods of crystallized song (in spring) and more plastic song (during fall and winter) during which song structure can be modified (Leitner et al., 2001a, Leitner et al., 2001b). During their entire life canaries thus alternate between periods when they express a fully crystallized song and periods of plastic song during which they practice production of new song elements like they had done during their first fall and winter. To some extent they partly recapitulate an ontogenetic process during the annual cycle. If PNN play a role in song crystallization, it could then be expected that their density should fluctuate during the annual cycle and decrease in the fall when song becomes more plastic again.

We tested this idea in an additional set of experiments on male canaries of the Fife fancy breed (Cornez et al., 2020a). During all experiments the photoperiod of the aviaries was adjusted every month to match the natural photoperiod in Belgium (change from 8L:16D in December to 16.5L:7.5D in June). In a first study a group of 5 adult males (greater than 2 years old) arrived in the laboratory in May 2016 and were kept under this seasonally changing photoperiod until March 2019. During two years their song was periodically recorded and analyzed and blood samples were collected to assess plasma testosterone concentration by enzyme-immunoassay (EIA). As shown in Fig. 3A, the birds in these conditions displayed clear annual cycles of gonadal activity as reflected by the periodic changes in circulating testosterone concentrations that peaked in the summers of 2017 and 2018 (Cornez et al., 2020a). In parallel, the singing rate and various measures of song quality changed seasonally. For example song quality quantified on a 5 points scale ranging from 1 (subsong) to 5 (crystallized song) fluctuated seasonally reaching minimal values in the fall at scores of 2 to 4 that correspond to different degrees of plastic song (Fig. 3B)(Cornez et al., 2020a).

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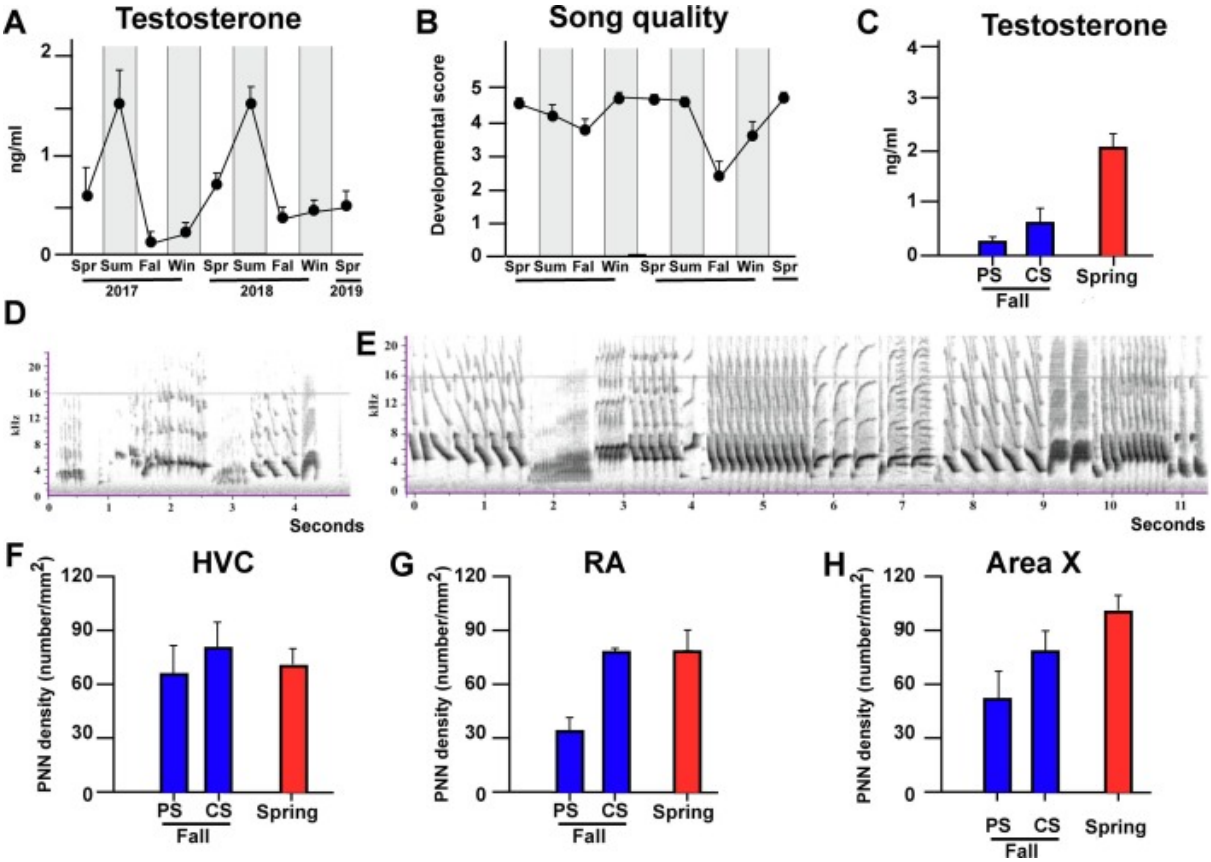
Figure 3. *Changes in song and perineuronal nets during the annual cycle in male canaries.*



A-B. Seasonal changes in plasma testosterone concentrations (A) and in song quality assessed on a qualitative scale from 1 to 5 (B) in a group of 5 males held under a photoperiod mimicking the natural photoperiod (Spr: spring, Sum: summer, Fal: fall, Win: winter). C. Plasma testosterone concentrations measured in the fall or spring in birds that were used to study PNN development (results in panels F-H). The fall birds were separated in two sub-groups depending of whether they sang a plastic (PS) or a crystallized (CS) song. D-E. Examples of spectrograms illustrating the plastic song produced in the fall (D) and the crystalized song (E) produced in the spring. F-H. Changes in PNN density (numbers /mm2) between the fall and spring in three song control nuclei in male canaries as defined in panel C. Redrawn and rearranged from data in (Cornez et al., 2020a).

Having confirmed the seasonal nature of gonadal activity and song production in canaries of the Fife fancy breed, we carried out two experiments aimed at comparing song quality and PNN development in birds collected when song is supposed to be plastic and when song is fully crystallized. The first of these experiments compared brains of birds collected on December 13 (Fall), February 7 (Winter) or March 28 (Spring) (Cornez et al., 2020a). Song had been recorded from these birds during the month before brain collection. As anticipated testosterone concentration and testes weight was low in the fall, intermediate in the winter and high in the spring birds, thus confirming the anticipated seasonal changes. However the volumes of HVC and RA were already at nearly maximal levels in the fall samples and there was little variation in singing activity or song quality between these three groups of birds. The fall samples had apparently been collected too late in the season and song had thus already progressed toward crystallization. Accordingly we did not detect a significant difference in the density of PNN in HVC nor RA between these three groups of birds even if values were numerically lower in the fall birds.

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In the second experiment, we compared samples in the fall and spring but collected the fall birds at an earlier date (September 27) in the hope that their song would still be plastic and not yet crystallized (Cornez et al., 2020a). Spring samples were collected on March 27 and as in the previous experiment, song was recorded before brain collection. When these recordings were analyzed, it was discovered that one half of the fall birds (N = 5) were singing a plastic song but somewhat unexpectedly the other half (N = 5) had already engaged in the song crystallization process. These two sub-groups of subjects were therefore analyzed separately and compared to the spring birds.

This distinction was reflected at the brain level. The volumes of HVC, RA and Area X were significantly different across the three groups and post-hoc analyses indicated that spring volumes were significantly larger than volumes in the fall-plastic song group. The fall birds that had crystallized their song had intermediate volumes of the song control nuclei that were not significantly different from volumes in the two other groups. The total numbers of PNN in these nuclei and their density (number/mm2) showed a similar pattern (Fig. 3F-H): values were lower in the fall birds singing a plastic song than in those singling a crystallized song and in the spring birds. These differences were significant for RA and Area X but not for HVC (see (Cornez et al., 2020a) for detail of the statistical results).

2.4. EFFECTS OF TESTOSTERONE

Interestingly, the increase in PNN density in the SCN occurred both during the ontogeny and the seasonal studies at a time when testosterone plasma concentration begins to increase. Therefore we wondered whether, this increase could possibly be caused at least in part by the action of testosterone in addition to possible internal timing mechanisms. It is indeed established that at least during ontogeny, a treatment with exogenous testosterone induces a premature crystallization of song (Marler et al., 1988).

To test this idea, a group of castrated male canaries was treated with one 10 mm-long Silastic™ capsule filled with crystalline testosterone while another group of castrated males received an empty Silastic™ implant as a control. Singing activity was then recorded every day in the morning for 2 h starting immediately after lights on. As anticipated, control birds never sang except for one bird singing at a very low rate. Treatment with testosterone induced an active singing activity beginning for the earliest birds on day 3 after implantation, and all of them were singing after one week. Song quality of the birds increased over time and their variability as measured by the entropy decreased suggesting that this song was progressively crystallizing (Cornez et al., 2020c).

Brains were collected after 24 days of treatment and their analysis revealed that the volume of the three song control nuclei (HVC, RA and Area X) had been significantly increased by testosterone as compared to control birds. The density of PNN in the three song control nuclei did not differ significantly between the two groups of birds but since the volume of the nuclei had increased, the total number of PNN was significantly increased in the three nuclei.

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A similar experiment was carried out in females who had been exposed to a short photoperiod so that their gonadal activity was basal. This experiment additionally assessed the rate at which brain changes develop after exposure to the steroid. Four groups of females were treated with exogenous testosterone (again one 10 mm Silastic™ implant filled with testosterone) while four other groups received empty implants. Brains were collected in one control and one testosterone-treated group after 1, 2, 9 or 21 days of treatment (Cornez et al., 2020c).

Overall the volume of the three SCN was increased by testosterone but the effects only became significant after 21 days. As observed in males, no overall change in PNN density was observed in these three nuclei even if a numerical increase was present on day 21. Once again, since the volume of the nuclei had markedly increased, the total number of PNN was significantly changed and post-hoc tests confirmed that the increase was present on day 21 only.

Together these two experiments indicated that testosterone action is at least part of the causes that induce an increase in PNN expression in the SCN in parallel with song crystallization. The brain changes appear to develop relatively slowly (3 weeks at least) which is consistent with the fact that song control nuclei volumes also takes quite a bit of time to develop, as does the increase in singing activity and subsequently in song stability.

2.5. COMPARISONS BETWEEN SPECIES

Given that the expression of PNN in SCN seems to relate (and possibly cause) to song crystallization, we were curious to investigate PNN expression in the species that is best known for its song plasticity in adulthood, the European starling (Sturnus vulgaris). This species is indeed one of the most extreme examples of open-ended learners. Males in this species can learn new songs at any stage of their reproductive cycle throughout their adult life (Bohner et al., 1990, Chaiken et al., 1994).

PNN expression was quantified in the brains of three groups of male starlings that had been collected when in physiological states representing the three extremes of the annual cycle: when photosensitive as they are in the winter, photostimulated as seen in the spring after exposure to a few weeks of long days and finally photorefractory which takes place in the summer after a prolonged exposure to long photoperiod. In this last condition, gonads are completely regressed and this is the only period of the year when birds completely stop singing (Dawson et al., 2001, Riters et al., 2000). Quite interestingly PNN density was very low in starlings and did not vary seasonally in these birds (Cornez et al., 2017). Given that HVC and RA volumes were significantly larger in photostimulated than in photosensitive birds, we also calculated the total numbers of PNN in these nuclei but even these total numbers did not change significantly as a function of the physiological condition.

Because the three species investigated so far differ markedly in the plasticity of their adult song (zebra finches: closed-ended learners; canaries and starlings: open-ended learners that learn (starling) or possibly do not learn (canary) new syllables in adulthood; see before),(Brainard and Doupe, 2002, Brenowitz, 2008, Chaiken et al., 1994), we finally wondered whether PNN density

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would vary accordingly (Cornez et al., 2017). Quite interestingly the measures of PNN density in these three different songbird species differed markedly in HVC, RA and Area X. Zebra finches who have a completely fixed adult song displayed by far he highest PNN density while starlings showed PNN densities that were approximately 10 times slower. Canaries were intermediate (Cornez et al., 2017).

The measures that were compared concern in each species males that were in reproductive condition and thus presumably presented the largest PNN densities that can be observed in the species. It cannot be excluded that the absolute maximum for each species was not identified but given the magnitude of the species differences observed, it is unlikely that this would change the main conclusion that PNN density across species correlates with adult song plasticity. One obvious weakness of this conclusion is of course that is it based on only three species that differ by their adult song plasticity but also by many other physiological characteristics. This conclusion should therefore be tested further by comparing a larger number of species distributed along the gradient of song plasticity ranging from full closed-ended to full open-ended learners (Brenowitz, 1997, Brenowitz, 2008).

2.6. EXPERIMENTAL ANALYSIS OF PNN: DEGRADATION BY CHONDROITINASE ABC

All these data were thus suggesting that PNN relate to and probably cause song crystallization in songbirds. In a last set of three experiments, we attempted to demonstrate this link in a direct causal manner with the help of a technique initially developed for songbirds in the laboratory of Teresa Nick based on the dissolution of PNN by local application of chondroitinase ABC, ChABC (Best et al., 2011). ChABC is an enzyme that is capable of disorganizing and dissolving the PNN by degrading chondroitin sulfate, one of the key components. In mammals, it was demonstrated in multiple experiments that local injection of ChABC in relevant brain sites restores behavioral flexibility in a variety of animal models (See introduction). For example, treatment with ChABC promotes recovery from early monocular deprivation of the visual system in adult rats (Pizzorusso et al., 2002). Removal of PNN in the secondary visual system also disrupts the recall of visual fear memory ((Thompson et al., 2018); for review see: (Celio et al., 1998, Karetko and Skangiel-Kramska, 2009, Miao et al., 2014, Werker and Hensch, 2015)).

In our experiments a window was opened in the skull on both sides just above HVC and a small piece of foam containing ChABC was applied directly on the brain (Cornez et al., 2021). The enzyme can in this way diffuse through HVC and dissolve PNN in the nucleus. Preliminary experiments clearly demonstrated that if the enzyme was applied on one side of the brain only, the density of PNN on this side was drastically decreased (6 to 10 fold decrease) after one or three days by comparison with the untreated side that was kept as a control (the foam was only soaked with saline). In another group of birds, ChABC was applied on both sides of the brain and PNN in HVC were quantified after one month of treatment. There was in this case an average 50 % decrease by comparison to control birds with some individual variation so that the difference was at the edge of significance ($p = 0.059$) (Cornez et al., 2021). Together these data suggest that the enzyme

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extensively degrades the PNN for a while but that after a few weeks a progressive recovery is observed. The bilateral application of ChABC directly on HVC was thus used in three separate experiments (Cornez et al., 2021).

The first of these experiments was started in November with 16 adult males who were singing at low rate a relatively plastic song. They all received a Silastic™ implant filled with testosterone to increase singing activity and hopefully crystallize the song. Half of them were bilaterally treated with ChABC while the others received the control saline treatment. Song was recorded for 4 weeks during which birds were exposed to the playback of tutor songs. The tapes were obtained from the University of Maryland (laboratory of Gregory F. Ball) and contained songs that our birds born in Belgium could never have heard so that any new song element that would be incorporated could easily be detected. Quantitative analysis of multiple song features quantified with a specialized MATLAB routine (Dos Santos et al., 2022, Shevchouk et al., 2018) detected no major difference with control birds following ChABC treatment. Manual analysis of the song repertoire also detected no difference before and 2 weeks after treatment between ChABC and control birds. A small decrease in repertoire size was observed on week 4 in the ChABC birds who had dropped a few syllables by comparison with the pretest recordings. Very few syllables from the tutor tapes (0 to 2 depending on the subject) had also been incorporated and none of these effects was significantly different from what was observed in control birds.

For the second experiment we reasoned that the testosterone treatment during the previous experiment might have rapidly crystalized the song thus preventing any major effect of the PNN degradation. Therefore we now initiated the ChABC or control treatment in early July in males that had been kept under the external photoperiod in Belgium. At that time plasma testosterone was very low in all birds but one, indicating they were probably photorefractory. These concentrations remained low until the end of September. Birds were recorded during the next four weeks during which they were also exposed to the song of tutors as done in the previous experiment. From that point, they were then left undisturbed until September. They were then recorded during four weeks and since their singing activity was still very low, they received a testosterone Silastic™ implant in early October and their song was recorded again during the next four weeks until their brain was collected.

Unfortunately for unidentified reasons (probably blood-sucking parasites that had invaded the animal facility at that time), many birds died following surgeries reducing sample size to 7 ChABC and 2 control males. Subsequent analyses had therefore very little power and controls can only be used as a qualitative reference point. It can however be said that no significant change in 12 song traits took place during the 4 weeks following ChABC application. No significant change in song repertoire also took place during this period. The two controls however added more syllables from the tutor tapes (3 each) than the ChABC-treated birds did (1 or 2) but this difference must be taken with great caution given the small sample size.

In September most males were essentially silent. Testosterone treatment induced a proficient singing activity, but again no quantitative difference could be detected in the 12 traits that were

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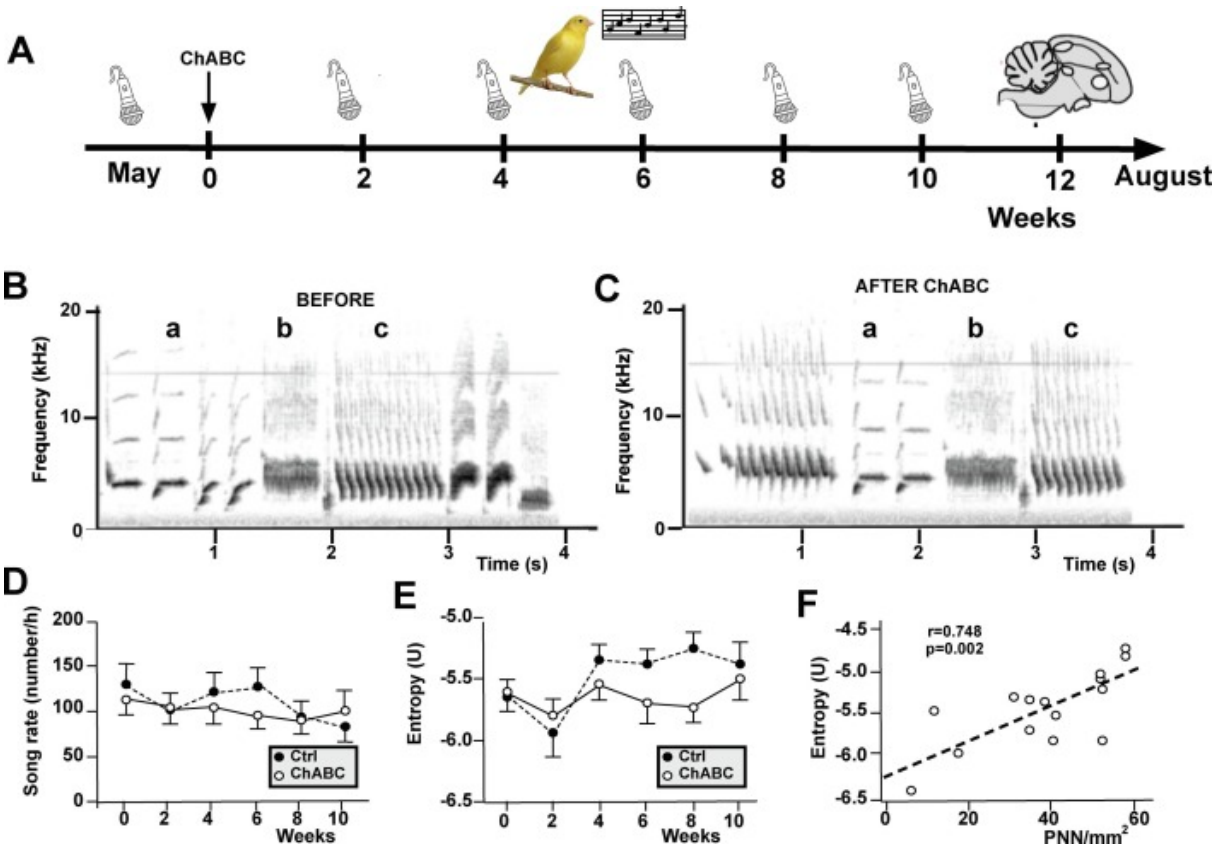
quantified. Density of PNN was similar in both groups of birds at that time, 4 months after ChABC application, suggesting that PNN had been fully reconstructed during this period.

The third and final experiment performed on this topic was initiated during the breeding season when birds were singing a stable song at high rate, in order to be as close as possible to the natural conditions. One group of birds was treated with ChABC and another one with the saline control procedure and their singing activity was then recorded every two weeks for 10 weeks. Final sample sizes were here 8 ChABC and 7 controls (Fig. 4A). No additional testosterone was provided and on week 12, when brains were collected, PNN density had apparently recovered in the ChABC birds to the control level.

Figure 4. *PNN degradation by direct application of chondroitinase ABC (ChABC) over HVC results in minor changes in song structure.*



A. Schematic representation of the experimental protocol of experiment 3 during which song was recorded and analyzed during 12 weeks after the ChABC application. B-C Representative spectrograms of a song sample in one bird before and after application of ChABC. Some syllables labeled here a-c are still clearly recognizable after treatment. D-E. Quantitative analysis revealed that song rate was not affected by the treatment (D) but song entropy was increased reflecting increased overall variability (E). F. In the group of 15 birds used in this experiment, song entropy was negatively correlated with the density of PNN in HVC. Redrawn and rearranged from data in (Cornez et al., 2021) and unpublished data in Panel F.

Once again, few changes in singing activity were observed: syllables were still clearly recognizable after the treatment (Fig. 4B-C) and the singing rate was not affected (Fig. 4D). There was however a significant interaction of time by treatment affecting the song Wiener entropy: measures of entropy that were nearly identical before treatment were significantly more negative in the ChABC

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than in the control group (Fig. 4E). Entropy was computed by the MATLAB routine for the entire song and not for individual syllables as done for example in studies of zebra finch (Tchernichovski et al., 2021, Tchernichovski et al., 2000). The measure of entropy considered here is the logarithm in base e of the Wiener entropy and thus ranges for 0 to minus infinity. This measure integrates multiple dimensions of the song including its duration, the number of syllables included, the repertoire and the bandwidth. A more negative value thus reflects a less structured (more variable) song as observed in castrated males compared to testosterone-treated ones. It can thus be inferred that ChABC treated birds produced more variable, presumably less crystallized, songs than their controls.

Finally, although there was no longer a difference in the densities of PNN observed between the ChABC and control birds 12 weeks after the treatment, we computed the correlation between the entropy of songs in the 15 males included in this experiment and the density of PNN in their HVC. As seen in Fig. 4F, there was a significantly negative correlation between these 2 variables indicating that birds with a lower density of PNN in HVC have a more plastic song ($r = 0.748$). This correlation was also positive when the two groups of birds were considered separately, but it was only significant in the 8 ChABC males ($r = 0.800$, $p = 0.017$), not in the 7 controls ($r = 0.608$, $p = 0.146$).

2.7. CONCLUSIONS, LIMITATIONS OF THESE RESULTS AND FUTURE DIRECTIONS OF PNN RESEARCH

Taken together, all these experiments indicate that the development of PNN in SCN correlates with the status of song development. PNN are clearly associated with the last phases of learning, probably the final crystallization, even if the resolution in time of most experiments carried out so far does not permit to make sure of the specific phase of song development implicated. This correlation is observed in multiple dimensions. It is observed in the context of sex differences in zebra finches (males who develop a crystallized song have large densities of PNN while females who do not sing do not; (Cornez et al., 2015, Meyer et al., 2014)), during ontogeny (PNN appear at the end of song development; (Balmer et al., 2009, Cornez et al., 2020b, Cornez et al., 2018)), across seasons (PNN increase when song crystallizes in the fall/winter before the next breeding season; (Cornez et al., 2020a)) and across species (a closed-ended learner species has more PNN than the open-ended learners; (Cornez et al., 2017)). Changes in PNN seem, in addition, to be controlled by the circulating concentrations of testosterone (Cornez et al., 2020c).

The attempts to demonstrate the causal meaning of these correlations was however only partly successful (Cornez et al., 2021). Dissolution of the PNN in HVC by local application of ChABC had limited effects on song structure. Nevertheless limited changes in song repertoire were detected and the third experiment demonstrated an increased song plasticity after PNN removal as well as a negative correlation between PNN density and song stability. These experiments thus suggest that PNN are not necessary for song production (song rate was never affected) but that they probably have more specific effects on song structure.

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Multiple reasons could clearly explain this relative failure to reliably affect song after PNN removal. It could first be assumed that the three experiments that were performed did not have sufficient power but this would then mean that changes to be expected have a limited effect size. We could also have failed to analyze the relevant features of song structure but this also seems unlikely since both the song repertoire and a wide list of quantitative parameters were considered and quantified by the MATLAB routine.

The most likely reasons relate in our opinion to the timing of the experiments or to the limited degradation of PNN both in terms of duration and anatomical localization. Based on the ontogeny and seasonal experiments, it appears that PNN probably play a role at specific times in the bird's life. To affect song structure in a reliable manner, the experimental degradation of PNN must therefore take place at a very specific stage of song development. Even if the three experiments we performed had a very different timing aimed at probing different stages of song development, it remains possible that the key target was missed. In addition, ChABC as applied here only affected PNN in HVC and probably did so for only a limited period of time; a major recovery had already taken place one month after application of the enzyme. PNN are expressed and vary in a major way in the three song control nuclei, HVC, RA and Area X. It can thus be speculated that to affect song structure in a more important manner, PNN should be depleted in all three nuclei, possibly over a longer period (see (Cornez et al., 2021) for more detail). The available results indeed indicate that after local application of ChABC in HVC, PNN are markedly depleted one or three days later but they have partly recovered after one month and completely recovered after four months (Cornez et al., 2021). The exact duration of the depletion is unknown but it can be speculated that this depletion might not be long enough to affect song structure in a major way.

There are thus multiple tracks that could be followed to solve these questions and I hope that the present review will stimulate other researchers to continue this promising investigation avenue. Studies in mammals continue to accumulate and demonstrate a substantial role of PNN on brain plasticity in relation to multiple functional outputs. The development and stability of song in oscines is an outstanding model to pursue these questions. My laboratory being now closed, the topic is, as far as I know, essentially open.

3. DCX neurogenesis and X-Ray irradiation

When seasonal changes in volume of song control nuclei were discovered (Nottebohm, 1981), the search for underlying mechanisms relatively quickly identified the presence of a very active neurogenesis that leads to the replacement of a large number of HVC neurons projecting to RA in adult birds, up to 1.5 % of them each day (Alvarez-Buylla et al., 1988, Goldman, 1998, Goldman and Nottebohm, 1983). This was a real revolution in the neurosciences since it had been assumed so far that homeotherms (birds and mammals) are unable to replace neurons in adulthood (Gross, 2000). This initiated a host of research in both birds and mammals (Kemperman, 2011), but songbirds and canaries in particular remained a favorite model for this research because neurogenesis is at least one order of magnitude (often more) more active in HVC of this species than in the hippocampus or

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olfactory pathway of mammals where it has also been identified (Brenowitz and Larson, 2015, Kirn et al., 1994, Kirn and Nottebohm, 1993). In addition, songbirds such as canaries display much larger seasonal changes in gonadal activity than mammals and, as a result, brain plasticity and in particular neurogenesis changes markedly between the breeding and non-breeding seasons thus offering an outstanding tool to study the control mechanisms (Dawson et al., 2001).

3.1. EXOGENOUS VERSUS ENDOGENOUS MARKERS OF NEUROGENESIS

Historically, various tools have been used to study and quantify neurogenesis. These studies initially relied on exogenous markers, first tritiated thymidine that had to be revealed by in situ autoradiography, a very tedious and technically challenging procedure, and later bromodeoxyuridine (BrdU) that can more easily be visualized by immunocytochemistry (Balthazart and Ball, 2014b). These two markers are incorporated into DNA when a cell is preparing its division. This essentially takes place at the level of the ventricle wall (Fig. 5A-B) and the new neurons then migrate into the brain parenchyma to occupy their final position (Alvarez-Buylla and Nottebohm, 1988). If this division was the last one in the genesis of a neuron, the label of the cell will remain available for detection for extensive periods of time. It the cells however continue to divide, the markers will progressively be diluted and become undetectable after several cycles of division.

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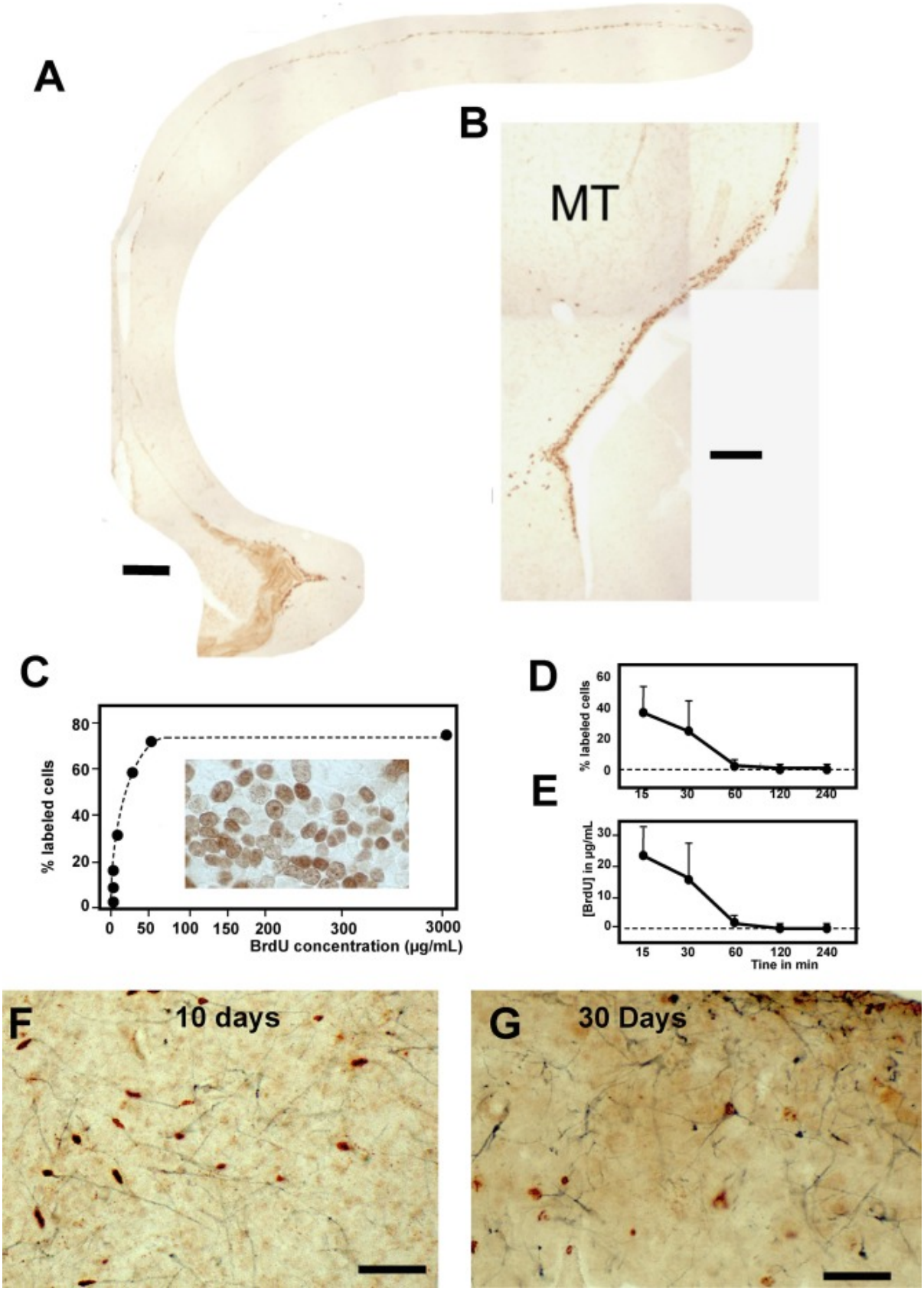
Figure 5. *New neurons labeling in the canary HVC by exogenous BrdU or the endogenous marker DCX.*



A-B. Photomicrographs of coronal sections through the brain of a male canary that had been previously injected with

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BrdU. Panel A is a photomontage showing the entire length of the lateral ventricle at a level just rostral to HVC. It shows the dense spot of neurogenesis at the ventral tip of the ventricle. Panel B presents a higher magnification of the lateral ventricle more clearly showing the BrdU labeled cellsin a male treated with testosterone (MT). C-E. Determination of the duration during which BrdU injected in a canary remains accessible for new neuron labeling. Panel C also shows a photomicrograph of a HEK cell culture labeled by BrdU. See text for additional explanations. F-G. Photomicrographs of sections through HVC in canaries that had been injected with BrdU 10 days (F) or 30 days (G) before. Sections were double-labeled by immunocytochemistry for BrdU (brown) and DCX (blue). At 10 days most of the cells doubled labeled by BrdU and DCX have a fusiform morphology but at 30 days some are already multipolar. Magnification bar is 250 µM in A, 150 µM in B, and 25 µM in E-F. Redrawn and arranged from (Barker et al., 2014) (A-B), (Barker et al., 2013) (C-E) and unpublished material related to (Balthazart et al., 2008) (E-F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These techniques have in common a limitation: the marker has to be injected to experimental subjects, which can be a problem to study wild species. Moreover BrdU remains present in the blood and available for incorporation into DNA for a limited period only (approximately 2 h in mammals, (Boswald et al., 1990, Miller and Nowakowski, 1988, Nowakowski and Rakic, 1974, Packard et al., 1973)). Birds however have a higher body temperature and metabolism and it was not know until recently how these mammalian data concerning BrdU availability can be extrapolated to canaries. We quantified this aspect with the help of an indirect bioassay based on the labeling of HEK293T cells in culture (Barker et al., 2013).

Adult canaries were injected with a single dose of BrdU (100 mg/kg) and blood was subsequently collected 15, 30, 60, 120 and 240 min later. HEK cells were cultured on glass coverslips and incubated at 37 °C during 4 h in a culture medium to which we added 10 µL of a BrdU solution to bring the final BrdU concentration to a range between 0 and 3 mg/mL. Parallel cultures were similarly incubated in medium to which we added 10 µL of the blood serum collected from the injected canaries. After 4 h, cells were fixed with paraformaldehyde and stained for BrdU by immunocytochemistry. The percentage of labeled cells was then counted on standardized photomicrographs of these slides.

As shown in Fig. 5C, incubation with increasing concentrations of BrdU resulted in the labeling of an increasing proportion of HEK cells reaching a plateau at a concentration between 50 and 100 µg/mL. At lower concentrations, the percentage of labeled cells was linearly correlated with the BrdU concentration. In slides incubated in parallel with the serum of BrdU-injected canaries, a substantial number of cells were labeled if serum had been collected 15 min after the BrdU injection but the percentage of labeled cells already decreased markedly when blood was collected at the 30 min time point and this percentage approached zero at the 60 min time point (Fig. 5D). Reporting this percentage on the calibration curve (Fig. 5C) then allowed determining the evolution of BrdU concentration in the blood of injected canaries (Fig. 5E).

As suspected based on mammalian data, availability of BrdU for labeling new neurons is very short in canaries. It must also be considered that this duration might be changing with the physiological condition or sex of the subject and this would then induce a bias in the relative quantification of neurogenesis.

The study of neurogenesis via an exogenous marker (BrdU or tritiated thymidine) thus provides a snapshot of the degree of DNA replication and neurogenesis at a very specific time point but this

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approach can easily miss a change in the rate of neurogenesis that would not (no longer) be present when the marker was injected.

More recently, a number of endogenous markers have been introduced as research tools to quantify neurogenesis. Markers are available that label all stages of a neuron's life ranging from the initial mitotic stages (Ki67, PCNA, pHH3, nestin) to the later postmitotic condition (NeuN, Calbindin, …). The obvious advantage of these markers is that they provide an integrated view of neurogenesis over a longer period of time, variable according to the marker used. They also do not require injections of an exogenous compound, which facilitates studies of wild species. One limitation relates however to their specificity that might not be as high for a given stage of neurogenesis as sometimes required and needs to be validated (see for review in birds: (Balthazart and Ball, 2014b)).

Of particular interest, doublecortin (DCX) labels young neurons from a few days after they final mitotic division for a limited period ranging from a couple of weeks to about one month ((Brown et al., 2003, Couillard-Despres et al., 2005), see for review: (Balthazart and Ball, 2014b)). DCX which can be visualized by immunocytochemistry is a protein associated with microtubules and as such a part of the intracellular machinery that mediates the migration of young neurons (Bai et al., 2003, Jin et al., 2004, Moores et al., 2004). It has been and is still used as a marker of young neurons in a huge number of studies of the mammalian brain (Bonfanti and Charvet, 2021, Brown et al., 2003, Encinas et al., 2013, Francis et al., 1999, Rao and Shetty, 2004).

Until relatively recently, the presence of the DCX mRNA and of the corresponding protein had been identified in the embryonic and early post-natal brain of two avian species, the chicken and the zebra finch, but few studies had considered DCX in the adult brain and it was though that the density of the protein was markedly decreased in parallel with the decrease in neurogenesis that is observed as subjects become older (Couillard-Despres et al., 2005, des Portes et al., 1998). In an initial descriptive neuroanatomical study, we demonstrated that neurons immunoreactive for DCX (DCX-ir) are still present in large numbers throughout the telencephalon of adult canaries with the exception of the arcopallium, i.e., in brain regions that were previously shown to incorporate new neurons in adulthood (Boseret et al., 2007). In particular, high densities of DCX-ir neurons are present in HVC and in most birds allow a delineation of the nucleus by contrast with the lower densities of positive cells in the surrounding nidopallium.

As described in mammals, DCX is present in two types of neurons that correspond to successive phases in the development of these cells. Very young DCX-ir neurons that are still migrating from the ventricle wall where they were born to their final destination in the brain display a characteristic fusiform uni- or bi-polar shape (Fig. 5F). Older DCX-ir neurons that have reached their destination and begun their final differentiation have a multipolar shape (Fig. 5G) and will soon after loose their DCX expression. Both types of neurons are present in HVC (Balthazart et al., 2008, Boseret et al., 2007).

DCX expression in young neurons seems to last about one month according to some studies in mammals although this might vary depending on the brain area or species considered (Bonfanti and Peretto, 2011, Zhao et al., 2008). We confirmed during one experiment in canaries the notion

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that young neurons are indeed temporarily labeled by DCX and that the two morphologies of these cells correspond to very young (fusiform) or slightly older (multipolar) neurons (Balthazart et al., 2008).

Males canaries received twice daily injections of BrdU (50 mg/kg) during 5 consecutive days. Brains were then collected 10 or 30 days later and labeled for both BrdU and DCX. As anticipated at 10 days BrdU-positive nuclei were mostly located in fusiform DCX-ir neurons whereas in brains collected at 30 days fusiform BrdU-DCX double-labeled neurons had decreased in number to the benefit of double-labeled multipolar cells. Cells counts in a standardized square area (200 × 200 µM) at the 10-day time point found 27.2 ± 10.8 fusiform but only 1.5 ± 0.6 round BrdU+-DCX+ neurons whereas at 30 days these numbers were respectively 5.7 ± 3.1 and 10.5 ± 4.2 fusiform and multipolar cells (Balthazart et al., 2008).

Overall more that 70 % of the BrdU+ cells also expressed DCX at both time points (73.4 ± 7.6 and 75.7 ± 13.2 at 10 and 30 days respectively) and conversely more than 75 % of DCX+ neurons contained BrdU in their nucleus at 10 days (72.1 ± 10.2 fusiform and 5.0 ± 2.5 mutipolar) but this figure dropped to 55 % at 30 days (30.6 ± 13.5 fusiform and 24.17 ± 8.4 multipolar). This demonstrates that DCX-ir neurons are essentially young neurons that replicated their DNA at the time of BrdU injections. One could wonder why these numbers never reach 100 %. This is probably explained by the fact that even with the multiple injections schedule used here, BrdU was only available for incorporation during brief periods of time so that DCX neurons still could be born before or after these brief periods. In addition, some of the BrdU+ cells had a probably non-neuronal nature (glial, endothelial).. Overall, these data are thus supporting the notion that fusiform DCX-ir cells are young neurons and they later mature into round multipolar DCX cells before they stop expressing this protein (see (Balthazart et al., 2008) and (Balthazart and Ball, 2014b) for more discussion).

It must however be recognized that a few cells located in brain regions that are not known for experiencing adult neurogenesis also display a weak DCX immunoreactivity (Boseret et al., 2007). This probably reflects minor rearrangements of neuronal architecture that are also known for involving the activity of microtubules. These cells (neurons?) are however present in small numbers and their location, morphology and low level of immunoreactivity allows to distinguish them relatively easily.

Some doubts have been raised about he usefulness and accuracy of this marker in the study of neurogenesis (Vellema et al., 2010) and most of these arguments have been considered and largely refuted (Balthazart and Ball, 2014a). This endogenous marker has indeed been widely used to study neurogenesis in both mammals (Couillard-Despres et al., 2005, Francis et al., 1999, Gleeson et al., 1999, Rao and Shetty, 2004) and birds (Balthazart et al., 2008, Diez et al., 2021, LaDage et al., 2010, Melleu et al., 2013) during the last decades. Searching the keyword doublecortin in Pubmed indeed retrieves more than 3600 references and combining it with bird or avian still finds more than 50 publications (on May 2023). This avian research also comes from a variety of laboratories around the world thus indicating the adoption of DCX as a valuable tool to assess neurogenesis in the avian brain.

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In my laboratory and in the laboratory of Gregory Ball who has been a collaborator for almost 40 years, multiple experiments have used DCX to analyze neurogenesis in HVC and its control by exogenous testosterone (Balthazart et al., 2008, Shevchouk et al., 2017) or its androgenic and estrogenic metabolites (Yamamura et al., 2011) or by changes in photoperiod and in the photoperiodic condition of the birds (Alward et al., 2014, Balthazart et al., 2008). This marker was also used to assess sex differences in HVC neurogenesis (Dos Santos et al., 2022). Finally, in a suite of experiments we showed that implantation of testosterone in the preoptic area increases the singing motivation in male and female canaries, and the increased singing activity was shown to enhance neurogenesis in HVC as assessed by quantification of DCX-ir neurons (Alward et al., 2013, Alward et al., 2016, Vandries et al., 2019). Because there is no direct mono-synaptic connection between the preoptic area and HVC and the steroid implanted in the preoptic area could not diffuse to HVC, these data support the idea that neurogenesis can, at least in part, be controlled by the singing activity thus supporting earlier work using different experimental protocols (Alvarez-Borda and Nottebohm, 2002, Li et al., 2000, Rasika et al., 1999, Rasika et al., 1994).

3.2. BRDU AND DCX PROVIDE DIFFERENT BUT COMPLEMENTARY QUANTIFICATIONS OF NEUROGENESIS

In one experiment we also specifically compared the measures of neurogenesis obtained with the use of the exogenous marker BrdU and the endogenous marker DCX. This experiment was carried out on castrated males in which circulating testosterone concentrations were clamped to a high level typical of reproductively active birds. The effects of the social situation were then assessed by comparing neurogenesis in males kept in isolation (M) and males kept with another male (M−M) or with a female treated with exogenous estradiol to make her sexually receptive (M−F). Neurogenesis was also quantified in these females used as stimulus (F). These different situations had been shown to modulate the singing activity of the birds and the volume of their HVC (Boseret et al., 2006). Birds were injected with BrdU (50 mg/kg, 5 times, 2 h apart on the same day). Song rate was periodically quantified and brains were collected 21 days later. Coronal sections were then double-stained for BrdU and DCX (Shevchouk et al., 2017). An alternate set of section were Nissl-stained and used to measure HVC volume.

As previously shown (Boseret et al., 2006), males kept with a female sang less than males housed with a male, and both group sang less than males kept alone (Fig. 6B). HVC volume was larger in males than in females and HVC differences in males followed the same pattern as singing activity (M > M−M > M−F) although these differences did not reach statistical significance because sample size was too small (Fig. 6C).

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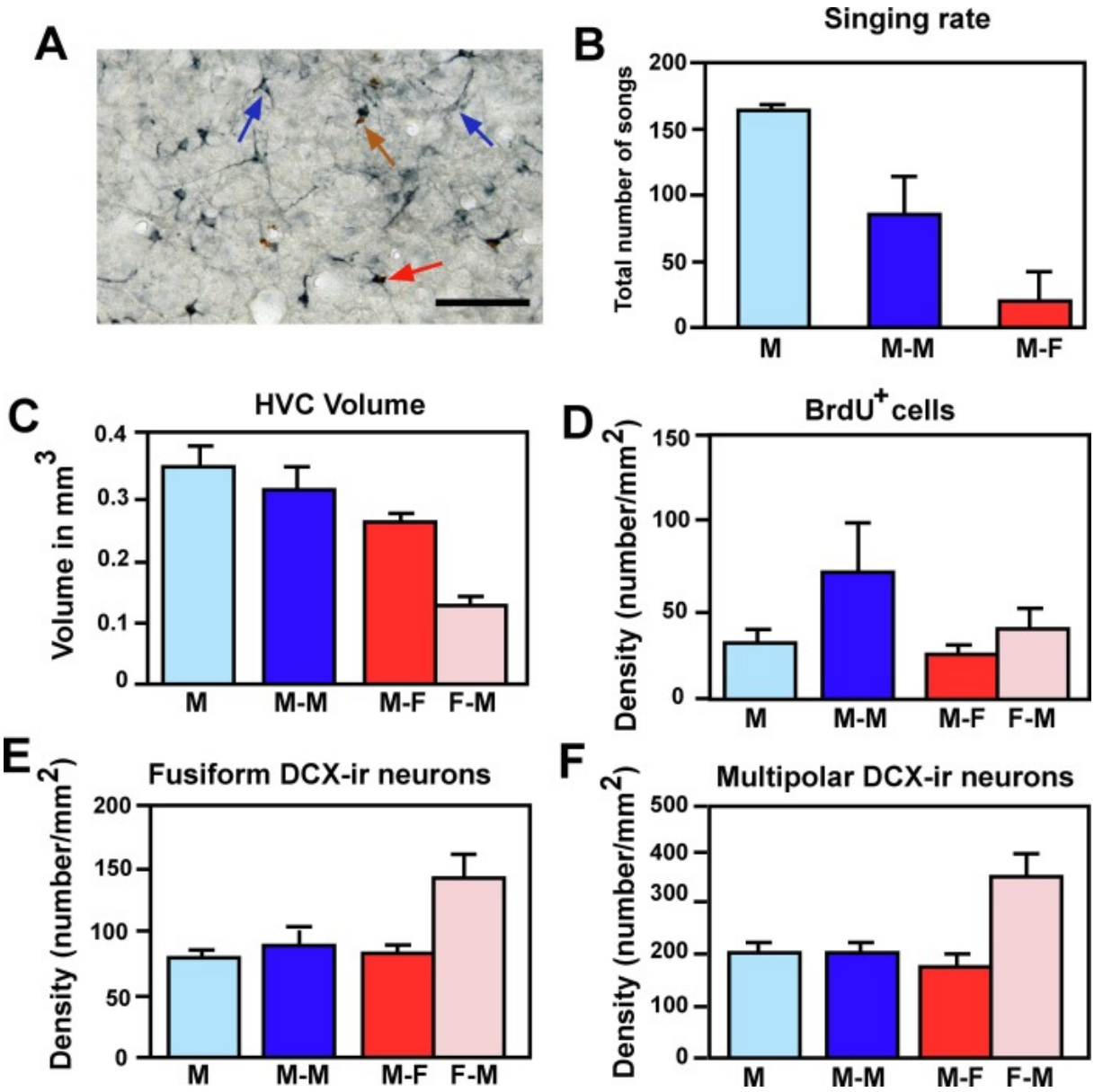
Figure 6. *Effects of social conditions on neurogenesis in HVC as assessed by the exogenous marker BrdU and the endogenous marker DCX.*



Males were housed alone (M) or with a male (M−M) or with a female (M−F). Neurogenesis was quantified after 21 days of exposure to these conditions and injection of BrdU, in these 3 groups and in the stimulus females (F). A. Photomicrograph of a section through HVC double labeled for BrdU (brown) and DCX (dark blue). Blue arrows point to DCX neurons not labeled by BrdU, the brown arrow points to a cell labeled by BrdU only and the red arrow points to a double-labeled neuron. Magnification bar = 50 µM. B. Singing rate in the 3 groups of males kept in different housing conditions. C. Volume of HVC in these 4 groups of birds. D. Density of BrdU + cells. E-F. Density of fusiform (C) and multipolar (D) DCX-ir neurons. Redrawn form data in (Shevchouk et al., 2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The average density of BrdU+ cells was larger in the M−M group than in the others although differences were not significant. The overall densities of DCX-ir neurons were much higher than for BrdU and in addition followed a completely different pattern with the highest densities observed for both the fusiform and multipolar neurons in the female group. This difference between males

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and females was statistically significant. Note however that this higher density in females was associated with a smaller HVC volume so that the total number of DCX-ir neurons in the female HVC was lower than in males, and this difference was significant for the fusiform cells. Densities of both types of DCX neurons were similar in the three groups of males but since HVC volumes were slightly different, the total numbers of fusiform DCX-ir neurons computed for the entire HVC was significantly smaller in the M−F and the two other male groups.

These data clearly highlight the fact that BrdU and DCX provide a different view of the dynamics of neurogenesis. DCX integrates the production of new neurons over a long period (around one month) whereas BrdU only labels cells for a limited period (around one hour for each injection). In addition BrdU labels all cell types, not only neurons, and an additional marker such as NeuN must be used to obtain specific neuronal counts.

More generally data indicate that social stimuli, independently of steroid action (clamped at a high concentrations by the Silastic™ implant), modulate neurogenesis in HVC and they do so is an anatomically specific manner since DCX-ir cell densities were usually not affected in the adjacent parts of the nidopallium. DCX revealed that total neurogenesis was decreased in the M−F groups as compared with the two other male groups but this effect only developed progressively toward the end of the experimental period: it was therefore not captured by BrdU injected at the beginning of the experiment and there was not enough time for the effect to affect multipolar DCX neurons. This experiment highlights the value of using multiple markers to fully quantify the dynamic of neurogenesis.

3.3. CAUSAL ANALYSIS OF NEUROGENESIS BASED ON FOCAL IRRADIATION WITH X-RAYS

Since its discovery in the early 1980ies, neurogenesis in the songbird brain has been the topic of a multitude of studies. This research has identified the site of production of new neurons and their fate from the division of the undifferentiated neural progenitors to the mature neurons incorporated into functional circuits (reviewed in (Balthazart and Ball, 2016, Brenowitz and Larson, 2015, Goldman, 1998, Nottebohm, 1985, Nottebohm, 2008). It was namely shown that new neurons originate at the level of walls of the lateral ventricles adjacent or ventral to HVC and that following their last division the new neurons take about a week to reach HVC (Alvarez-Buylla et al., 1988). The peak of new neurons density is reached around 15 days later and then a large fraction of them will die so that only a fraction will survive at one month or later (Kirn et al., 1991).

This research has also discovered multiple factors that control the production of new neurons and has correlated changes in the neurogenesis rate with changes in song production and structure. It was shown that neurogenesis in HVC varies across the annual cycle in temperate zone birds such as canaries and these changes correlate with the annual changes in singing behavior (Alvarez-Buylla and Nottebohm, 1988, Nottebohm et al., 1987). Male canaries sing a crystallized stable song during the breeding season, they stop singing during the mold period in the summer and then in the fall they start singing a modified repertoire in a trial and error manner, the so-called plastic

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song which will progressively become more stable as winter progresses to reach a new crystalized song in the next spring (Brainard and Doupe, 2002, Marler, 1987, Marler, 1997). The most prominent control of these seasonal changes is obviously exerted by testosterone which increases the rate of incorporation and long-term survival of new HVC neurons (Nottebohm et al., 1994, Rasika et al., 1994) with a subsidiary positive effect on the rate of neuroblast divisions leading to immature new cells with a neuronal fate (Barker et al., 2014). Modulatory influences of photoperiod and of the social environment, both unrelated to testosterone, have also been identified (reviewed in (Balthazart and Ball, 2016).

The period of plastic song in the fall corresponds to an increase in the incorporation of new neurons and the two phenomena have long been thought to be causally related (Alvarez-Buylla et al., 1988, Kirn et al., 1994) but this was never formally demonstrated. More generally, the specific function of the new neurons in HVC resulting in changes in HVC overall volume are poorly understood. The initial work in Nottebohm's lab suggested that larger HVC volumes are related to increased song repertoire by analogy with the size of a library relating to the amount of information it contains (Nottebohm, 1981). However this view was later challenged by a number of negative results (Bernard et al., 1996, Brenowitz et al., 1995, Brenowitz et al., 1991, Leitner and Catchpole, 2004) and it was proposed that the volume of HVC (and possibly the rate of neurogenesis) rather relates to the amount of singing performed by a bird by analogy with muscles developing with exercise (reviewed in (Ball et al., 2002)).

Multiple correlations between HVC volume or HVC neurogenesis and singing activity or song quality have thus been described but no study almost has been able to establish a direct causal link between the new neurons and changes in singing behavior. This situation is largely related to the fact that it is very difficult to modify HVC neurogenesis in a direct and specific manner.

The causal analysis of neurogenesis in mammals is often based on the injection of antimitotic drugs such as arabinofuranosyl cytidine (AraC) or methylazoxy-methanol acetate (MAM) to block cell divisions. Most of these compounds have however a number of side effects and if their action cannot be limited to the specific site where new neurons are produced, they are likely to have major undesired effects obscuring the outcome of the study, especially if it concerns behavior. Avian studies using these compounds are rare. One study based on systemic MAM injections demonstrated however a link between neuronal recruitment in the hippocampus and spatial learning in black-capped chickadee, Poecile atricapillus (Hall et al., 2014). It was also shown that inhibition of cell divisions in the hypothalamic region by AraC infusion at the surface of the brain delays behavioral recovery after a localized hypothalamic lesion in ring doves, Streptopelia risoria (Chen and Cheng, 2007). However, to our knowledge, no paper has reported a use of antimitotic drugs to analyze the role of adult neurogenesis in HVC probably because the drug would have to be applied in the lateral ventricles in order to affect most of the ventricle wall and non-specific effects would likely occur.

Alternatively, viral methods have been developed to specifically ablate neural progenitors and immature dentate granule cells in the mouse hippocampus (e.g., (Johnston et al., 2021)) but the

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use of these techniques in avian system remains technically difficult. As a consequence, the specific role of new neurons recruited during adulthood in HVC remains elusive to this date.

Focal irradiation with gamma or X-rays has also been used to study neurogenesis in mammals (Achanta et al., 2012, Ford et al., 2011, Lazarini et al., 2009, Manda et al., 2009, Wojtowicz, 2006). Neural progenitors are more sensitive than mature neurons to the effects of ionizing radiations. These radiations break DNA and as a consequence disrupt protein synthesis and other biochemical pathways. Immature rapidly dividing cells are therefore likely to be affected more profoundly. By exposing proliferating precursors to a limited dose of radiation (range 10 to 20 Gray), it is thus possible to selectively kill them without (too much) collateral damage (see (Wojtowicz, 2006) for a detailed discussion of this specificity aspect). In addition, this irradiation can easily be focalized to the zone of interest with the use of a small animal irradiator and scanner (Small Animal Radiation Therapy with advanced precision, SmART; Precision X-Ray North Branford, CT, USA). This further increases the specificity of effects.

We recently used this approach in two separate experiments to study HVC neurogenesis in canaries (Chiver et al., 2023). A first methodological experiment demonstrated the usefulness of the method by quantitatively comparing neurogenesis in the two brain hemispheres of males that had been irradiated on one side (23 Gy split in three converging beams) while the other side was kept as a control. Birds received a Silastic™ implant filled with testosterone to optimize the rate of neurogenesis and were injected with BrdU on the same day (5 injections at 50 mg/kg two hours apart). Brains were collected two weeks later at a time point that corresponds to the peak of arrival of new neurons in HVC. Coronal sections were then stained by double-label immunocytochemistry for BrdU and DCX. Both markers provided converging evidence demonstrating that focal irradiation had decreased neurogenesis in the irradiated brain hemisphere. The density of BrdU+ cells was significantly decreased in the irradiated side and a similar nearly significant trend was observed for DCX-ir neurons. This effect was not fully significant because there was actually no decrease at all of the multipolar DCX-ir neurons and these neurons were presumably born before the irradiation. In contrast, fusiform DCX-ir neurons and especially those double labeled by BrdU were decreased by irradiation.

This experiment was thus consistent with several of the previous conclusions namely that fusiform DCX-ir neurons are younger than multipolar DCX-ir neurons: the former only were affected and only them were double-labeled by BrdU; we also did not find multipolar DCX-ir cells that contained BrdU. In addition, DCX-ir neurons were more numerous than BrdU+ cells confirming that DCX labels neurons for a longer period than BrdU.

Based on these encouraging results, a second experiment investigated the functional consequences of a bilateral irradiation of the neurogenic zone adjacent to HVC on the development of testosterone-induced singing in adult females. Experimental birds were thus bilaterally irradiated (23 Grays again) by two parallel focal beams focused on HVC and its adjacent ventricle (Fig. 7A-B) while a group of females were kept as control and only submitted to the same anesthesia. On the same day, all birds also received a Silastic ™ implant filed with testosterone and on the next day they received five injections two hours apart of BrdU (50 mg/kg). Song was then

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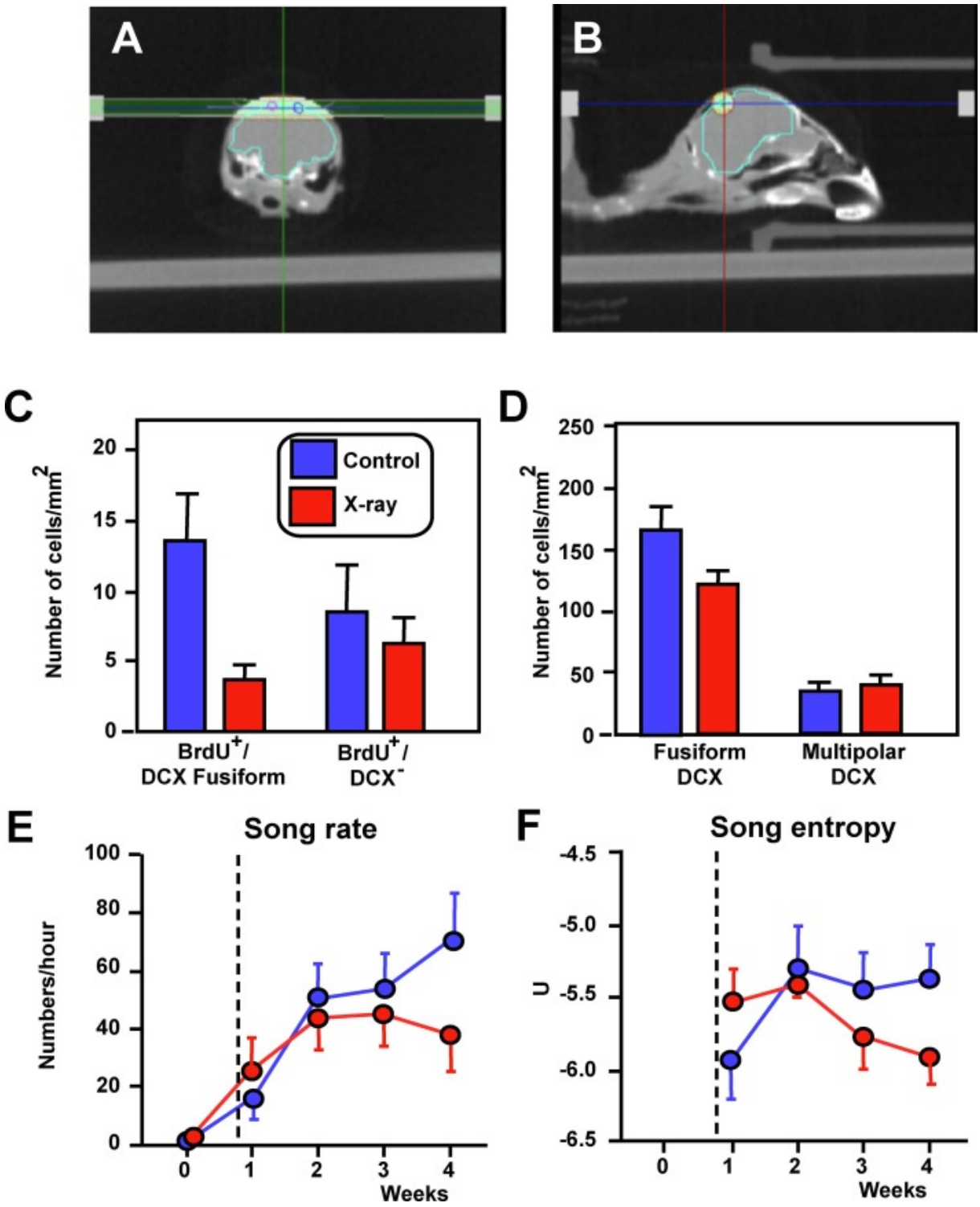
recorded and quantified during 4 weeks before brains were collected and stained for BrdU and DCX.

Figure 7. *Focal irradiation depletes neurogenesis in HVC and affects song structure.*



A-B. Selected coronal (A) and sagittal (B) views of the irradiator computer screen illustrating the bilateral X-Ray beams focused on the ventricle dorsal to the 2 HVCs. C. Density of BrdU+ cells that were or were not double labeled with DCX. D. Total densities of fusiform or multipolar DCX-ir cells. E-F. Changes in time of song rate and song entropy in the irradiated

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and control groups. In panels C-F, control data are in blue and data from X-rayed birds are in red. Redrawn from data in (Chiver et al., 2023). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As shown in the previous experiment, irradiation clearly decreased the density of fusiform DCX-ir neurons whereas multipolar ones were again not affected (Fig. 7D) despite the longer duration between irradiation and brain collection (4 versus 2 weeks). This might potentially suggest that DCX-ir neurons retain a fusiform morphology for longer than initially expected and this topic should be further investigated. These data are also consistent with the idea that effects of irradiation were limited to the progenitor cells and did not affect more mature neurons. The new neuron depletion was even more prominent when assessed by the density of BrdU+ cells, in particular young neurons that were also marked by DCX with a fusiform morphology (Fig. 7C). There was however also a decrease in BrdU+ cells not labeled by DCX that represented non-neuronal (glial or endothelial) cells. Once again no multipolar DCX-ir neuron was labeled by BrdU thus confirming the relatively long duration needed for a young neuron to begin differentiating in its target location and acquiring a multipolar morphology.

The female singing activity was very low at the beginning of the experiment but rapidly increased following implantation with testosterone-filed capsules. This progressive increase in song rate is illustrated in Fig. 7E. It was observed both in irradiated and in control females but the singing activity of the irradiated females reached a plateau after two weeks so that a significant interaction between treatment and time effects was present. Many features of the songs produced by irradiated birds were not affected by comparison with the songs of controls (e.g., their duration was similar throughout) but a few important differences were noted. In particular there was a progressive decrease in the entropy values of the whole songs following irradiation (Fig. 7F). As in previoulsy discussed experiments, the Wiener entropy was measured here for the entire songs and integrates multiple dimensions of songs including their duration, number of syllables per song, syllable repertoire, sequence and frequency bandwidth. The more negative values in irradiated birds thus indicate a decrease in song stability but further research will be needed to identify more precisely which aspects of song are affected by the depletion of new neurons in HVC.

It is important to highlight that the irradiation resulted in very specific changes in song structure without affecting many other aspects. This procedure thus did not simply lesion HVC. It had more specific effects that presumably relate to the decrease in neurogenesis. This notion is supported by two observations. First, the observed change in entropy only developed progressively and was thus not resulting from a direct effect of the X-rays but was rather a consequence of the progressive depletion in new neurons. Second, analysis of individual differences indicated that entropy values on week 4 were correlated with the density of fusiform DCX-ir-BrdU+ neurons in HVC: more negative entropy values were measured in birds with the lowest densities of these neurons.

3.4. CONCLUSIONS CONCERNING NEUROGENESIS

Together these studies document new technical possibilities in the study of HVC neurogenesis. Combining an exogenous (BrdU) and an endogenous (DCX) marker of new neurons leads to a more

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complete description of the process and also provides some information on the time course of the process. Analysis of the evolution in time of new neurons with BrdU requires multiple injections and multiple brain collection times to get information on the numbers of new neurons at different stages of their life and obtain measures of the rate of production, migration, differentiation, and death. In addition if the researcher wants to study the effects on neurogenesis of an experimental manipulation that takes place at a specific time (e.g. a treatment with a steroid), he/she needs to study multiple injection dates before and at various intervals after the manipulation to study the effects in time. The study of such a question thus requires development of a bi-dimensional protocol of injections and brain collection times that ends up to be time consuming, financially expensive and costly in terms of experimental subjects. This approach has therefore rarely if ever been implemented.

The simultaneous use of DCX and BrdU provides in part this type of temporal information since these markers will provide an estimate of the numbers of neurons that were born (entered in their last division) at the time of BrdU injection(s) and an integrated view of all very young (fusiform) and slightly older (multipolar) neurons. All this can be done with the same subjects by just double-labeling sections for both markers. This approach is therefore highly recommendable.

On the other hand, our recent study based on focalized X-ray irradiation represents a clear proof of concept indicating that this technique is able to affect in an apparently specific manner the number of new neurons migrating and incorporating into HVC. Additional work should obviously be carried out to demonstrate the absence of non-specific effects and then identify more precisely the functional consequences of the new neurons depletion. This technique does not suffer from the non-specific and potentially systemic effects of the injection of an anti-mitotic drug and it is easily transferable from one animal model to another contrary to the viral approaches that always require specific adaptation of the viral serotype to the target species.

Manipulations of HVC neurogenesis by focal X-ray irradiation thus appears to be a very promising technical approach to modify this process in an anatomically specific manner and therefore produce causal analyses of its role in song production. It offers a great alternative to chemical or viral methods to assess the role of neurogenesis in this experimental model.

4. Conclusions

This selective review on plasticity in the songbird brain was focused on two main topic that were investigated in my laboratory in the last 10 to 15 years: the perineuronal nets surrounding mostly inhibitory parvalbumin-positive neurons in three telencephalic control nuclei and neurogenesis in HVC. As illustrated, despite the fact that neurogenesis in HVC was discovered about 40 years ago, multiple questions remain concerning the nature of the process, its external controls, the underlying cellular mechanisms and above all the function of the new neurons in the control of song. New questions are raised by the discovery of PNN: what exactly controls their development, how do they affect neuronal physiology at the cellular/molecular level and most importantly what

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are the consequences for song learning and stability. This review also demonstrates that focal irradiation can be an efficient tool to study in a directly causal manner the role of new HVC neurons in the control of singing behavior. Research on songbird brain plasticity has been very active over the past decades. It is hoped that this will continue to be the case and that studies of this outstanding model will lead to fundamental discoveries that could have an impact in the field of neuroscience in general.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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