A Developmental Timeline of Parvalbumin-Expressing Neuron Addition to the Zebra Finch HVC During the Critical Period

by

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Abstract

The cortical pre-motor nucleus HVC plays a key role in regulating song learning, production, and maintenance in oscine birds. The HVC is located at the center of both major pathways involved in song learning and is believed to work as the implicit timekeeper of the song system. During the critical period, a time early in development during which a bird must be exposed to its species-specific song if it is to learn to sing, the HVC experiences high rates of neurogenesis that decrease considerably once the critical period has ended around 60 to 90 days post-hatch, although the nucleus continues to recruit new neurons throughout life. If a bird is isolated during the critical period so that it has no exposure to a male tutor, high rates of neuron addition to the HVC are prolonged and the bird can learn to copy another male’s song later into adulthood, although isolates generally produce a more variable song regardless of eventual tutor exposure.

To date, investigations into the nature of neurogenesis to the HVC have focused on the addition of excitatory projection neurons that connect to RA, another nucleus involved in vocal production. However, it has been shown that inhibitory interneurons are also added to the HVC, at least during the critical period. Since parvalbumin-expressing interneurons are associated with plasticity in other sensory systems and have even been shown to induce ocular dominance plasticity in mice
after the critical period has ended, we wondered what role parvalbumin-positive interneuron addition may play in regulating plasticity of the song system. To investigate this question, we quantified the rates of parvalbumin-expressing neuron addition to the zebra finch HVC during and directly after the critical period and analyzed these trends in relation to vocal learning.

Neurons were birth-dated using thymidine analog injections at 20, 40, 60, or 120 days post-hatch in male zebra finches raised with male tutors. Birds were perfused at least 60 days after injection, and immunohistochemical techniques were used to quantify new parvalbumin-positive neurons added at these different time points. Before perfusion, birdsong was recorded, and computer-driven similarity scores between a bird’s song and its tutor’s were used to analyze the degree to which a bird had effectively copied its tutor. In addition, rates of parvalbumin-positive neurogenesis were investigated in a population of birds isolated from male tutors.

In normally raised birds, rates of parvalbumin-positive neuron addition reflected what is known about neurogenesis to the HVC during the critical period, with a peak at 40 days post-hatch and a gradual decline thereafter. Rates of parvalbumin-positive neuron addition to the HVC at 60 days post-hatch positively correlated with tutor song copying. The addition of neurons expressing parvalbumin occurred at a higher rate in isolates than in birds reared with a tutor, and by 180 days post-hatch, isolated birds displayed significantly more parvalbumin-positive neurons throughout the HVC than birds reared with a tutor. These data suggest that parvalbumin-expressing neurons are at least one factor mediating plasticity in the zebra finch vocal learning system.
Introduction

Songbirds and Humans

Songbirds provide a useful animal model for studying human vocal development because songbirds, like humans, are vocal learners. Songbirds must form auditory memories of adult songs, engage in vocal motor practice, and receive auditory feedback in order to perfect and maintain their songs. They also have an early critical period during which they must be exposed to a tutor’s song if they are ever to learn to sing. A similar critical period has been observed in human vocal learning. Lastly, discrete brain regions have been shown to play key roles in vocal learning and production in both songbirds and humans, making songbirds a convenient model for understanding neural correlates of behavior (Doupe and Kuhl 1999; Kuhl 2003).

Zebra-finch Song Structure and Learning Process

Zebra finch songs have a specific structure. They usually consist of a few introductory notes, followed by 4-7 repeated strings of stereotyped syllables, called “motifs”. Motifs are often repeated several times in succession, referred to as a “bout” (Kirn 2010).

Only male zebra finches sing. A zebra finch learns its song from one or more tutors, other males of the same species. The bird forms an auditory memory of these adult songs beginning at 20-25 days post-hatch and starts to imitate them from days 30-90. It is believed that the tutor’s song becomes a template that the bird attempts to match using vocal-motor practice and auditory feedback during the sensorimotor stage (Eales 1985). At first, the bird produces a sub-song, an elementary song that
doesn’t much resemble the finished product. With time, the bird will begin to produce more recognizable notes or syllables, but these will change greatly from bout to bout, a period known as the plastic song stage. Finally, at song crystallization, the song is highly stereotyped from one rendition to the next and will only become more refined with age, as long as the bird continues to receive auditory feedback. After crystallization, a zebra finch will not learn song from other conspecifics (Zevin, Seidenberg, and Bottjer 2004; Eales 1985).

*Critical Periods*

The zebra finch is a critical period species, meaning that zebra finches require exposure to song during a specific timeframe early in development if they are to copy it, and most song learning is completed by the time the bird reaches sexual maturity, around 90-120 days after hatching. For this reason, the zebra finch is referred to as a “close-ended” or “age-limited” species (Kirn 2010). There is some evidence that vocal motor practice must occur during a specific critical period as well; temporarily disrupting vocal muscles during the late adolescent stage when adult song starts to emerge disturbs the development of song permanently, but similar disruptions do not have this effect at any other period during the bird’s life (Pytte and Suthers 2000).

While zebra finches raised around male tutors will develop a stereotyped song during days 65-90, isolating a zebra finch during development can delay the closing of the critical period. Zebra finches raised in visual or auditory isolation from adult males can still alter their songs to match that of a male tutor after 80 days post-hatch,
in young adulthood. Generally, however, the songs of isolates are more variable throughout life than birds raised with a male tutor (Wilbrecht et al. 2006).

**HVC and neurogenesis**

While the exact neural mechanisms of critical period plasticity are not known, neurogenesis, or the birth of new neurons, may contribute. In vertebrates, new neurons are added to certain regions of the brain through adulthood (Kaplan and Hinds 1977; Goldman and Nottebohm 1983). Songbirds are unique, however, in that new neurons are added throughout the entire telencephalon and usually replace neurons that have died (Alvarez-Buylla and Kirn 1997). However, new evidence suggests that, at least in zebra finches, neurons added to the HVC do not replace ones that have died but just integrate into the existing population and grow more densely packed with age (Walton, Pariser, and Nottebohm 2012). All neurons of the telencephalon arise from cells lining the walls of the lateral ventricles (Alvarez-Buylla et al. 1998; Alvarez-Buylla and Nottebohm 1988; Goldman and Nottebohm 1983).

The high vocal center, or HVC (acronym used as proper name), experiences neurogenesis at a high rate during the early critical period, but significantly fewer new neurons are added after 65 days post-hatch, when song becomes more crystallized and less learning occurs (Wilbrecht, Crionas, and Nottebohm 2002). HVC neurogenesis continues to taper off into adulthood with increasing song stereotypy (Wilbrecht and Kirn 2004). However, when male birds are isolated during the critical period so that the end of the period is delayed, the HVC receives significantly more new neurons after day 60 than birds reared normally. By 180 days post-hatch, isolated
birds do not appear to have any higher new-neuron recruitment to the HVC than birds housed with a tutor, but they do have higher neuron numbers in the HVC generally, which suggests that isolated birds may retain neurons more than socially-reared birds. In addition, birds with higher rates of neurogenesis to the HVC display higher song variability (Wilbrecht et al. 2006).

In the seasonally breeding canary, neurogenesis to the HVC occurs year-round but occurs at the highest rate when new song learning is taking place (Alvarez-Buylla, Kirn, and Nottebohm 1990; Kirn et al. 1994). Between spring, when singing rates are highest and song stereotypy is at its peak, and fall, when song is most variable, nearly 30-50% of the canary HVC neuron population projecting to RA (robust nucleus of the arcopallium), another nucleus involved in song learning, is lost and replaced (Kirn and Nottebohm 1993), raising the possibility that neurogenesis promotes new song learning. However, it is also possible that neurogenesis reflects song instability regardless of learning as song progresses toward stereotypy (Kirn 2010); in western song sparrows, which learn their songs as juveniles but experience periods of song variability throughout life, neurogenesis to the HVC is highest during these periods of song instability (Tramontin and Brenowitz 1999). It has been suggested that, when neurons are added to the HVC, they facilitate motor plasticity and their survival depends on their usefulness in producing the target song. In such a model, new cells would receive feedback signals when they are first active to indicate their participation in a more or less correct version of song production, and this would determine their survival (Wilbrecht and Kirn 2004).
The HVC plays a critical role in both song learning and song production. About 60% of HVC neurons project to the RA, which connects directly and indirectly to regions of the brain stem innervating respiratory and vocal motor neurons (Alvarez-Buylla, Theelen, and Nottebohm 1988; Kirn, Alvarez-Buylla, and Nottebohm 1991; Kirn et al. 1999). This is referred to as the “motor pathway”, and lesions at any point in this pathway have been shown to cause significant disruption to vocal production at any point in life (Margoliash 1997). HVC also begins a second major pathway, referred to as the anterior forebrain pathway (AFP), which is analogous to the basal ganglia-cortico-thalamic circuitry in mammals (Bottjer 2004). A collection of HVC cells distinct from those connecting to RA project to a striatal region called Area X, which projects to the dorsal lateral nucleus of the medial thalamus (DLM). DLM projects to the lateral magnocellular nucleus of the anterior nidopallium, or LMAN, the neurons of which have bifurcating axons that synapse on neurons in Area X and RA (Vates and Nottebohm 1995). Lesions in this pathway do not impact fully crystallized song but greatly disrupt song learning (Bottjer, Miesner, and Arnold 1984; Kao, Doupe, and Brainard 2005; K. W. Nordeen and Nordeen 1993; Scharff and Nottebohm 1991; Sohrabji, Nordeen, and Nordeen 1990; Williams and Mehta 1999).

Although it is intuitive to divide these two pathways into a production pathway and a learning pathway, there is evidence that both pathways play a role in song learning and maintenance throughout life. Neurons in both pathways respond to sound stimuli, and some neurons in both pathways respond most strongly to playbacks of bird’s own song (BOS). In RA, BOS selectivity is dependent on HVC
input (Doupe and Konishi 1991; Vicario and Yohay 1993). Many neurons that respond selectively to BOS also exhibit pre-motor activity (Mooney 2000; Rosen and Mooney 2003). Finally, regions of both pathways receive new neurons throughout life, indicating a continuing role in song maintenance (Kirn 2010).

It is believed that another brain region, the caudomedial nidopallium (NCM), is responsible for storing the tutor song template. The cells of NCM respond selectively to tutor song and not to BOS, and the strength of cellular response to tutor song reflects the extent to which tutor song has been copied (Phan, Pytte, and Vicario 2006). This has been supported by studies showing that disruption of immediate early gene (IEG) expression in NCM during the sensory stage prevents song copying without impacting performance on auditory tasks (London and Clayton 2008). It is possible that NCM and the surrounding structures store the song template and relay this information to the HVC and subsequent AFP in order to guide auditory vocal-motor practice (Bolhuis and Gahr 2006; Phan, Pytte, and Vicario 2006).

Changes in the balance of RA input from HVC and LMAN appear to drive song development. Before 45 days post-hatch, lesioning HVC neurons projecting to RA has little impact on song structure, while inactivating LMAN during this time abolishes sub-song (Aronov, Andalman, and Fee 2008). This is unexpected, as inactivating HVC also inactivates HVC neurons projecting to Area X, the beginning of the AFP, of which LMAN is a part. However, after 45 days post-hatch, eradicating HVC has a much more substantial impact on song development while lesioning LMAN has a more modest effect (Aronov, Andalman, and Fee 2008).
These changes are reflected in neurophysiological developments in the song system. In the HVC, most neurons projecting to Area X are formed before hatching (Alvarez-Buylla and Kirn 1997; Alvarez-Buylla, Theelen, and Nottebohm 1988), while most RA-projecting neurons are produced exclusively after hatching (Alvarez-Buylla, Theelen, and Nottebohm 1988; Alvarez-Buylla, Kirn, and Nottebohm 1990; K. W. Nordeen and Nordeen 1988). Neurons projecting to RA begin to form connections 30-35 days post-hatch, around the time the bird begins to practice (Konishi and Akutagawa 1985; Mooney and Rao 1994), while most of the neurons of the AFP, including HVC neurons projecting to Area X and LMAN neurons, have formed connections by day 20 (Johnson and Bottjer 1992; Mooney 1992; Sohrabji, Nordeen, and Nordeen 1993; Mooney and Rao 1994). HVC and RA both grow substantially in volume between 12 and 53 days post-hatch; while HVC receives a great number of new neurons during this time, RA receives few, indicating that there is a significant increase in synapses on RA from HVC as song learning progresses. This has been supported by quantitative analysis. In contrast, LMAN shrinks significantly with age, and RA synapses originating in LMAN are decreased by about 80% during the same time interval (Herrmann and Arnold 1991).

Thus, it appears that LMAN inputs to RA dominate when song is in early development and HVC inputs are more prevalent as song is crystallized. This has been supported by recent studies showing that lesioning HVC in adult zebra finches can contribute to song variability which can be blocked by LMAN lesions (Thompson and Johnson 2007), indicating that LMAN may play a role in allowing or inducing song plasticity. Similarly, lesioning LMAN can prevent changes to song that usually
occur when a bird is deafened or after tracheosyringeal nerve transection (Brainard and Doupe 2000; Williams and Mehta 1999).

HVC and LMAN projections to RA are glutamatergic. HVC projections to RA consist of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazoleproprianate (AMPA) receptors, while LMAN to RA synapses consist mostly of NMDA receptors (Mooney 1992; Mooney and Konishi 1991; Stark and Perkel 1999), which are implicated in Hebbian plasticity in many systems (Tsien 2000). As song develops, the number and distribution of these receptors decreases, as does the duration of receptor currents, which may occur progressively as synapses are strengthened in correlation with more precise song output. NMDA receptor activation is necessary for song learning, as it has been shown that applying an NMDA receptor antagonist during exposure to tutor song disrupts song learning (Aamodt, Nordeen, and Nordeen 1996).

HVC neurons projecting to RA fire once at most during every vocalization. This firing is time-locked with a motif, occurs every time the motif is sung, and reliably drives activity in RA in both awake singing birds and birds exposed to song during sleep. This strongly supports that the HVC-RA connection is a site for learning plasticity, as it can act as a “timekeeper” for premotor and motor neurons downstream (Hahnloser, Kozhevnikov, and Fee 2002). It has been suggested that the HVC acts as an implicit metronome, as stimulating HVC slices in vitro produces rhythmic postsynaptic potentials and local field potentials with similar timing to syllable production (Solis and Perkel 2005). However, analysis of songs produced by the same bird over time indicates that syllables and gaps do not vary on the same time scale,
indicating that HVC activity in vivo may be entrained to maintain different tempos for different aspects of song production rather than one consistent rhythm (Glaze and Troyer 2006). Interestingly, cooling the HVC has been shown to slow down a bird’s song significantly without changing other aspects of the acoustic structure, while manipulating the temperature of RA does not impact song production, providing further evidence that the HVC is the timekeeper of the song learning system (Long and Fee 2008).

Different HVC neuron types interconnect within the nucleus, which most likely drives its pattern-forming activity. HVC-RA neurons excite inhibitory interneurons, which subsequently inhibit HVC-X neurons in the AFP. Many interneurons in the HVC innervate both types of projection neurons, and many excitatory and inhibitory neurons within the HVC form mutual connections, all of which support its role in integrating and consolidating sensory input and motor output (Mooney and Prather 2005).

HVC volume has been found to co-vary with song complexity across species, genera, and families of songbirds (Airey and DeVoogd 2000; Devoogd 2004), although these studies did not measure whether variations in volume correlated with variations in neuron size, number, or density. In zebra finches, neuron number in the HVC appears to correlate with the accuracy with which a bird has copied its tutor (Ward, Nordeen, and Nordeen 1998). Studies have also indicated that HVC and RA volume show a strong positive correlation with amount of singing in several songbird species, including the canary (Ball et al. 2004; Brenowitz 2004).
Figure 1: The songbird song-learning system. HVC connects both directly, via what is referred to as the “motor pathway” (solid line), and indirectly, via the “anterior forebrain pathway” (dotted line) to the Robust Nucleus of the Arcopallium (RA). RA projects to regions of the brain stem that innervate respiratory and motor neurons (Krn 2010).
Role of inhibitory interneurons in learning and plasticity

Inhibitory interneurons, in particular parvalbumin-expressing neurons, have been found to play a key role in critical period plasticity in sensory systems, most commonly the visual system. Parvalbumin (PV) is a calcium-binding protein found in fast-spiking GABAergic interneurons (Celio 1986; Braun et al. 1988; Wild et al. 2005). In mice, knocking out the gene for enzymes that produce GABA eliminates the plasticity that allows cells to switch responsiveness toward a dominant eye when one eye is blinded (Hensch et al. 1998). Transplanting immature inhibitory neurons into the ocular dominance column reinstates plasticity after the critical period. Interestingly, one study in mice showed a peak in plasticity 33-35 days following transplantation of cells from the medial ganglionic eminence, which is the age when ocular dominance plasticity is naturally at its highest, indicating that the maturity of these inhibitory neurons plays a large role in inducing plasticity (Southwell et al. 2010). Further study found that implanting either primarily PV-positive or somatostatin-positive interneurons was sufficient to induce ocular dominance plasticity, but MGE transplants depleted of both PV and somatostatin did not induce plasticity (Tang et al. 2014). However, it appears that in the zebra finch vocal system, inhibition is related to song-learning progress and not age. Two-photon targeted voltage-clamp recordings revealed no age-related change in the strength or timing of firing in inhibitory neurons connected to premotor neurons in the HVC during tutor song playback. However, birds that exhibited better overall copying of the tutor song revealed significantly higher charge, amplitude, frequency, and accuracy of inhibitory events between bouts. In general, tutor song-evoked premotor activity was seen in projection
neurons in the HVC in juvenile birds and not in adults, which appeared to be a result of PV-positive interneuron maturation. Applying a GABA antagonist reinstated this premotor activity. However, this was observed in all adults regardless of how well they had learned their songs, indicating that there is at least one other neural factor contributing to this pattern besides the learning-related strengthening of inhibitory currents within HVC (Vallentin et al. 2016).

Preliminary studies indicate that a homeoprotein, Otx2, stimulates the maturation of PV-expressing interneurons, and thus the opening of the critical period, in the mammalian visual system. Otx2 is a transcription factor involved in embryonic head development but is isolated to the visual cortex after birth. Otx2 expression in PV-positive neurons in the visual cortex increases with PV-expression until the critical period peak at 28-30 days and persists into adulthood. The influx of non-cell-autonomous Otx2 into the visual cortex depends on visual input, as presence of the protein in dark-reared mice is significantly reduced. Furthermore, delivering the protein into one hemisphere of the visual cortex in animals raised in darkness significantly increases the number of PV-expressing neurons and perineuronal nets in comparison to the non-treated hemisphere (Sugiyama et al. 2008). Although no studies of Otx2 in songbirds have yet been published, its close ties to PV may make the protein an apt subject of future studies regarding song-system neurogenesis and critical period plasticity.

During the critical period for ocular dominance plasticity, the primary visual cortex exhibits prolonged cellular bursting, high PV expression, changes in excitatory-inhibitory balance, and the presence of extracellular matrix perineuronal
nets (PNNs, discussed in next section) (Hockfield and McKay 1983; Sur 1988). Prolonged bursting is also seen in the HVC during the critical period, and the shortening of bursts in HVC correlates with maturation of song (Crandall et al. 2007). In addition, fast-spiking interneurons in the HVC are active in juveniles and not in adults before, during, and after singing (Crandall et al., 2006). Prolonged interneuron bursts after singing in juveniles may represent a window for sensory feedback to be received after motor output, which is reduced with decreasing plasticity and increasing stereotypy (Troyer and Doupe 2000; Crandall et al. 2007).

PV-expressing interneurons are added to the zebra finch HVC during song learning, as well as at least one other type of GABAergic interneuron (Scott and Lois 2007). Interneurons in the HVC have been found to express parvalbumin, calbindin, calretinin, or any combination of the three (Wild et al. 2005). It has been thought that no new inhibitory interneurons are added to the zebra finch HVC once it reaches adulthood (Scotto-Lomassese et al. 2007; Walton, Pariser, and Nottebohm 2012).

PV-expressing interneurons in the HVC may work to suppress premotor activity for learned syllables of a song. When birds are exposed to tutor song, interneurons fire reliably and produce inhibitory currents on premotor neurons precisely when they hear syllables that they have already learned well, and it has been suggested that these interneuron circuits protect learned information from incoming sensory input (Vallentin et al. 2016).

A recent model of song learning has suggested that inhibitory interneurons work as a “stabilizing critic” in the song system, orchestrating song learning by limiting the windows of time within which neurons can fire. In this model, certain
inhibitory neurons in the HVC work as “disinhibitory neurons”, projecting to other inhibitory neurons that synapse on excitatory premotor and motor neurons in other nuclei. As a bird’s song begins to match its stored template, the disinhibitory neurons fire with increased synchronicity, perhaps through the mechanism of gap-junction coupling (Saraga, Ng, and Skinner 2006), reinforcing the coinciding motor output (Nick 2014). In this model, HVC receives input from an afferent region that compares sensory feedback to tutor song (Nick 2014). Although the author does not name possible nuclei, NCM could play a role in this process (see previous section).

**Perineuronal nets**

In mammalian cortical systems, the formation of perineuronal nets (PNNs) marks the closing of a critical period (Hensch 2005). Perineuronal nets are lattice-like structures of proteins, chondroitin sulphate proteoglycans (CSPGs), that form in the extracellular matrix surrounding mostly parvalbumin-positive GABAergic neurons, although they are occasionally seen surrounding pyramidal neurons as well. They are believed to work as a buffer for cations in the extracellular space, allowing the interneurons to fire in rapid succession (Härtig et al. 1999). In doing so, they may preserve inhibitory interneurons (Morawski et al. 2004). It has also been proposed that PNNs contribute to closing periods of plasticity by structurally limiting the growth of new synapses (Nick 2014). When a molecule that contributes to normal PNN formation is altered, the strength of inhibition by interneurons projecting to pyramidal neurons in the hippocampus is weakened, excitatory neuron firing increases, and long-term potentiation is decreased (Saghatelyan et al. 2001). Disruption of PNN’s can
also reinstate ocular dominance plasticity after the critical period in the visual cortex (Pizzorusso et al. 2002).

In adult zebra finches, PNNs were seen surrounding PV-positive interneurons in all song nuclei investigated (HVC, Area X, LMAN, and RA). HVC showed the highest percentage of PV-positive neurons surrounded by PNNs; about 80% of PNNs in HVC surrounded PV-positive interneurons (Balmer et al. 2009). In all four nuclei, the presence of PNNs and the percentage of PV-positive neurons surrounded by PNNs co-varied with the bird’s age, although the total number of Area X neurons surrounded by PNNs surprisingly appeared to decrease. Higher percentages of PV-expressing interneurons surrounded by PNNs in HVC correlated with song stereotypy. In addition, zebra finches that were isolated during the critical period so that the closure of the critical period was delayed had significantly fewer PV-positive neurons at 90 days than birds reared with a tutor, but there was no significant difference in the number of neurons surrounded by PNNs in total, meaning that the number of PV-negative neurons surrounded by PNNs was higher in isolated birds. Interestingly, in this study, staining for PV and PNNs in isolates was less efficient, yielding a lower signal-to-noise ratio in isolates than in birds reared with a tutor. In the most general sense, this indicates that isolation has some sort of impact on these critical period indicators (Balmer et al. 2009).

Hypothesis and Predictions

Neurogenesis to the HVC has been a great focus of birdsong research, but most of these studies have focused specifically on RA-projecting neurons. The
present research was motivated by a desire to understand whether PV-expressing interneuron addition also plays a role in critical period dynamics. While it is already known that the maturation of inhibitory interneurons in the HVC is correlated with tutor song copying (Vallentin et al. 2016) and that PV-positive neurons are added to the HVC during the critical period (Scott and Lois 2007), a timeline of this interneuron addition throughout the critical period would aid in understanding its relationship with song-learning, a process that is already well documented.

We predicted that PV-expressing interneuron addition would be higher during the critical period than in adulthood, both because neurogenesis to the HVC in general is higher during periods of learning and because PV-positive interneurons play such a vital role in mediating critical period plasticity in other sensory systems (Hensch 2005; Alvarez-Buylla 1990; Kirn et al. 1994; Kirn 2010; Wilbrecht et al. 2006; Tang et al. 2014). We also predicted that the relative level of PV-positive neuron addition would predict the extent to which tutor song had been copied. In addition, we expected to see a prolonged period of PV-positive neuron addition to the HVC in birds raised in social isolation.
Methods

Subjects: All experiments were done in accordance with the Wesleyan University Institutional Animal Care and Use Committee and NIH guidelines. Only male zebra finches were used in the study. Birds in normally-reared group (with a male tutor) were raised in single-family cages until approximately ninety days after hatching, then moved to single-sex group cages. These birds were injected with BrdU to label cells born at 20, 40, or 60 days post-hatch (dph) or in adulthood (125-166 dph). Isolated birds were not exposed to male tutors at all from birth. Isolated zebra finches were divided into two cohorts to label new neurons, around 60 dph or in adulthood (120 dph). All birds had access to food and water ad libitum. The colony room was maintained in a 14:10 light: dark cycle with a controlled temperature of approximately 22º C. One week before perfusion, birds were moved to soundproof recording chambers for song recordings. Conditions in recording chambers were similar to those of colony room.

BrdU Injections: Each zebra finch received an intramuscular injection of bromodeoxyuridine (BrdU; 100mg/kg body weight, dissolved in 0.1M Tris-buffered saline, pH: 7.6; Roche Diagnostics, North America) twice daily, for four consecutive days. All birds in the 20, 40 and 60 dph cohorts, including isolates in the 60 dph cohort, were allowed to survive until 120 dph. The birds in the adult cohorts were allowed to survive for 60 days after the BrdU injections.
**Song Recording and Analysis:** All birds were housed individually in soundproof chambers and recorded continuously for a week before perfusion. The recordings were carried out using a recording interface (PreSonus FireStudio Project; PreSonus Audio Electronics Inc., Baton Rouge, LA) connected to Sound Analysis Pro Software (Ofer Tchernichovski, Hunter College, City University of New York). Song similarity analysis between each bird and its tutor was carried out using Sound Analysis Pro. Birds who did not produce songs were eliminated from this aspect of study.

**Histology:** At ages described above, the birds were removed from their home cages and anesthetized with methoxyflurane (Metofane; Mallinckrodt Inc., Mindelgn, IL). The birds were then transcardially perfused with 20 mL of 0.1M phosphate buffer (PB; pH: 7.4) followed by 50 mL of 4% paraformaldehyde (pH: 7.4). The brain was post-fixed in 4% paraformaldehyde at room temperature for one hour, rinsed in 1x phosphate-buffered-saline (PBS; pH: 7.4), dehydrated in increasing concentrations of ethanol (50%, 70%, 95% and 100%) and embedded in polyethylene glycol (PEG; Polysciences, Inc., Warrington, PA). Six-micron parasagittal sections were cut using a rotary microtome and every eighth section containing HVC was mounted on a positively charged microscope slide (AmFrost Ultra+; American Laboratory Supply LLC, Bradenton, Fl). The slides were stored at -20º C.

**Immunohistochemistry:** The slides were brought to room temperature and incubated in 1% H₂O₂ in 0.1M PB for ten minutes, followed by a 0.1M PB wash for
five minutes. The slides were then exposed to 0.01M citrate buffer (pH: 5.6-6) at 10ºC using a vegetable steamer, washed in 0.1M PB for five minutes at 37ºC, incubated in 2.5% pepsin in 0.1M PB at 37ºC, and washed in 0.1M PB for five minutes at room temperature. The slides were blocked for thirty minutes at room temperature using 10% normal donkey serum (Sigma-Aldrich Co. LLC., St. Louis, MO) in 1x PBS containing 0.3% Triton-X (PBST, EMD Millipore, Billerica, MA), followed by overnight exposure to sheep anti-BrdU (1:100; Capralogics Inc., Hardwick, MA), rabbit anti-parvalbumin (1:1000; Swant, Switzerland) and mouse anti-Hu (1:40; Life Technologies, Grand Island, NY) at room temperature. After three five-minute PBST washes, the slides were incubated with biotinylated donkey anti-sheep (1:200; EMD Millipore, Billerica, MA), HRP-conjugated donkey anti-rabbit (1:1000; Life Technologies, Grand Island, NY) and donkey anti-mouse conjugated with Alexa 647 (1:1000; Life Technologies, Grand Island, NY) for two hours at room temperature, followed by three five-minute PBST washes. Then the slides were developed using a Tyramide Signal Amplification (TSA) kit (1:50; Perkin Elmer, Waltham, MA) for ten minutes at room temperature, followed by three five-minute PBST washes. The slides were incubated with Streptavidin conjugated with Alexa 488 (1:800 in PBST; Life Technologies, Grand Island, NY) for two hours at room temperature, followed by three five-minute PBST washes, and coverslipped using Aqua-Poly Mount (Polysciences, Inc., Warrington, PA).
**Data Collection:** Data collection was carried out without knowledge of bird identity. Labeled cells of HVC were imaged using confocal microscopy (Zeiss LSM 510; Zeiss USA). A Z-stack of every section was taken with a 25x objective lens using three color channels. Individual images were stitched together using the merge function in Adobe Photoshop. Boundaries of HVC were drawn where larger Hu-labeled neurons were surrounded by smaller Hu-labeled neurons in line-like formation. Hu-, PV-, and BrdU-labeled neurons were quantified manually. At least five full sections throughout the HVC were quantified and averaged for each bird.

**Statistics:** Statistical analysis was carried out using JMP® software (Version 11, SAS Institute Inc., Cary, NC, 1989-2007). Data was compared using one-way and two-way ANOVA, and Student’s t-Test was used for pairwise comparisons. Significance was denoted at $p < 0.05$. 
Figure 2: Experiment Timeline. Birds raised with a tutor were injected with BrdU to label neurons born at 20, 40, 60, or roughly 120 dph. Birds raised in isolation from a male tutor were injected with BrdU at 60 or 120 dph. Birds injected before adulthood were perfused at 120 dph, while birds injected in adulthood were perfused 60 days later, near 180 dph. Green bars indicate rates of high neuron addition to the HVC associated with the critical period for song learning. When birds are isolated from tutor song exposure, high rates of neuron addition persist past the normal drop-off point (Wilbrecht et al. 2006).
Results

Figure 3: Immunohistochemical Identification of Cells in HVC. A: Hu, a neuronal marker; B: Parvalbumin; C: BrdU, cell birthdate marker; D: Merged image of all three channels. White arrow points to a triple-labeled cell, indicating a new PV-expressing neuron born at the time of BrdU injection.
Figure 4: Sample Triple-Labeled Neuron. BrdU (green) is restricted to the nucleus of a cell, while Hu (blue) and PV (red) are seen in the cytoplasm.
Timeline for Parvalbumin-Expressing Neuron Addition to HVC

A peak in neurogenesis of neurons surviving into adulthood in the HVC, although not statistically significant, was seen in neurons formed at 40 dph (neuron addition: \( F = 1.8517, p = 0.1962 \)). This number was lower for neurons formed at 60 dph and decreased further for neurons formed at 120 dph (Figure 5). Similarly, the number of new neurons expressing PV that survived into adulthood was highest in neurons formed at 40 dph, although again not significantly, and slightly more new parvalbumin-expressing neurons were added at 120 dph than at 60 (\( F = 2.6217, p = 0.1031 \)) (Figure 6). One-way ANOVA indicated that the ratio of parvalbumin-expressing neurons to all new neurons varied significantly with age (\( F = 3.8976, p = 0.0403 \)) (Figure 7). Student’s t-Test revealed that the percentage of neurons added that expressed PV was significantly higher at 40 dph and 120 dph than at 60 dph (40 dph vs 60 dph: \( t = 2.63, p = 0.0236 \); 120 dph vs 60 dph: \( t = 2.98, p = 0.0125 \)).
Figure 5: In birds reared with a tutor, an insignificant peak in the rate of neurogenesis to the HVC of neurons surviving into adulthood is seen at 40 dph and continues to drop into adulthood \((F = 1.8517, p = 0.1962)\). Numbers above error bars represent \(n\)-numbers for each cohort.
Figure 6: In birds reared with a tutor, an insignificant peak in the rate of neurogenesis to the HVC of PV-expressing neurons is seen at 40 dph. The rate is lower at 60 dph and increases slightly in adulthood (F= 2.6217, p = 0.1031).
Figure 7: The percent of PV+ new neurons added was greatest at 120 dph. The ratios of new neurons added that were PV+ were significantly greater at 40 and 120 dph than at 60 dph. (40 dph vs 60 dph: t = 2.63, p = 0.0236 ; 120 dph vs 60 dph: t = 2.98, p = 0.0125).
The Relation Between PV-Positive Neuron Addition and Tutor Song Copying

The rate of PV-expressing neuron addition at 60 dph positively correlated with amount of tutor song copying, as measured with similarity ratings between a bird’s song and its tutor’s ($R^2 = 0.57397$) (Figure 8). There was no correlation between rate of PV-positive neuron addition at 20 dph and tutor song copying. This could not be measured for birds injected with BrdU at 40 dph because there were not enough birds in the cohort that produced song.
Figure 8: The rate of PV-expressing neuron addition to the HVC at 60 dph positively correlates with the amount a bird had copied its tutor’s song ($R^2 = 0.57397$).
Effects of Social Isolation on Neuron Addition to HVC

Isolated birds exhibited a higher rate than controls of neurogenesis of neurons that formed at 60 and 120 dph and lived for at least 60 days, although this difference was not significant (Two-Way ANOVA, 60 dph vs. 120 dph; $F = 0.7460, p = 0.5451$) (Figure 9). A similar insignificant trend was seen in new PV-expressing neurons ($F = 1.6831, p = 0.2233$) (Figure 10). The ratio of new neurons that expressed PV to all new neurons added to the HVC was also higher in isolates than in socially reared birds ($F = 4.8193, p = 0.0199$) (Figure 11). Student’s t-Test revealed that the amount of new neurons expressing PV was significantly higher at 120 days than at 60 (120 dph vs. 60 dph: $t= 2.86, p = 0.0144$). Specifically, the ratio of new parvalbumin-expressing neurons to all new neurons added at 120 dph in normally raised birds was significantly higher than at 60 dph (120 N vs. 60 N: $t = 2.26, p = 0.0433$). The total number of neurons expressing PV in the HVC at the time of perfusion (120 dph for birds injected with BrdU at 60 dph, 180 dph for birds injected at 120 dph) was also higher in isolated birds ($F = 12.8577, p = 0.0005$) (Figures 12 and 13). Two-way ANOVA yielded an effect of age and an interaction between social isolation and age on the number of PV-positive neurons at perfusion. The number of PV-expressing neurons was higher in birds injected at 120 dph than at 60 dph (Student’s t-Test, 120 vs. 60: $t = 3.41, p = 0.0052$). Specifically, the number of PV-expressing neurons in the HVCs of isolates at 180 dph was significantly higher than in any other group (120 I vs. 60 I: $t=4.91, p = 0.0004$; 120 I vs. 60 N: $t=5.64, p= 0.0001$; 120 I vs. 120 N: $t = 5.12, p= 0.0003$).
**Figure 9:** Birds isolated from male tutors during the critical period exhibited slightly higher rates of neuron addition to the HVC at 60 and 120 dph. This was not significant (60 dph and 120 dph; $F = 0.7460, p = 0.5451$).
Figure 10: Isolated birds exhibited slightly higher rates of PV-expressing neuron addition to the HVC at 60 and 120 dph. This was not significant ($F = 1.6831, p = 0.2233$).
Figure 11: A greater percent of neurons added to the HVC are PV-positive at 120 dph than at 60 dph ($F = 4.8193, p = 0.0199$) (120 dph vs 60 dph: $t = 2.86, p = 0.0144$). The percent of new neurons that expressed PV is higher in isolates than birds reared with a tutor, although this is not significant.
Figure 12: The total number of neurons in the HVC expressing PV at the time of perfusion (120 dph for birds injected with BrdU at 60 dph, 180 dph for birds injected at 120 dph) is higher in isolated birds ($F = 12.8577, p = 0.0005$). Specifically, the number of PV-expressing neurons in isolates at 180 dph is significantly higher than in isolates at 120 dph or in normally raised birds at either time point (120 I vs. 60 I: $t = 4.91, p = 0.0004$; 120 I vs. 60 N: $t = 5.64, p = 0.0001$; 120 I vs. 120 N: $t = 5.12, p = 0.0003$).
Figure 13: More cells express PV in adult isolates than in adults reared with a tutor. Images 1A and 1B are from two different adult males raised with a tutor, perfused at 180 dph, and images 2A and 2B are from two different isolated males perfused at 180 dph. White dotted line indicates HVC boundary.
Discussion

Parvalbumin-expressing interneurons have gained attention as a potential factor mediating critical period plasticity. While it has previously been shown that high rates of neurogenesis to the HVC are characteristic of the critical period for song learning in zebra finches and that isolating a bird during the critical period can prolong this neurogenesis, no study to date has investigated the nature of PV-expressing interneuron addition specifically during this time (Wilbrecht, Crionas, and Nottebohm 2002; Wilbrecht and Kirn 2004; Wilbrecht et al. 2006). This study was driven by a desire to understand how PV-positive interneuron addition during the critical period may mediate plasticity, and how presence of PV-positive neurons in the HVC may impact a bird’s ability to effectively copy its tutor’s song.

We observed a pattern of neurogenesis to the HVC during the critical period that is consistent with previous studies. The most neurons added to the HVC that survived until adulthood, out of the four time points that we looked at, were born at 40 dph, and this number continued to decrease from 60 to 120 dph, the natural end of the critical period (Wilbrecht, Crionas, and Nottebohm 2002). This trend was not significant, although this may be due to the small sample size used in this study. The rate of new parvalbumin-expressing neuron addition was also highest, although not significantly, at 40 dph, which is during the critical period as we predicted. It is known that PV-positive interneurons take about 30 days to mature in mice (Southwell et al. 2010). If this time frame is similar in birds, then interneurons added at 40 dph would be mature around 70 days and those added at 60 dph would be mature at 90 days of age, when song is stereotyped (Wilbrecht and Kirn 2004). This is consistent
with a recent study that suggested that inhibitory interneurons selectively inhibit excitatory neurons in the HVC coding for song syllables that are well learned (Vallentin et al. 2016); it would make sense for the HVC to recruit inhibitory interneurons within a time frame such that they reach maturity as song reaches stereotypy.

This explanation does not account for our finding that a comparable percent of all new neurons added to the HVC following BrdU injections at 120 days and 40 days expressed PV, which was not expected. While it has previously been shown that the HVC receives fewer neurons after the critical period has ended, as our data supports, it appears that a greater percentage of the neurons that are added in adulthood are PV-positive. It has been previously thought that inhibitory interneurons are not added to the HVC in adulthood (Scotto-Lomassese et al. 2007; Walton, Pariser, and Nottebohm 2012). However, both of these studies detected new neurons added to the HVC in adulthood that were not backfilled from RA or Area X and were not immunopositive for any of the three inhibitory interneuron markers (parvalbumin, calretinin, or calbindin). It is possible that our immunostaining technique for PV, using TSA as a signal amplifier, allowed us to identify PV-positive neurons that previous studies did not reveal. Our results also reflect a different timeline from either of these previous studies. Scotto-Lomassese et al., injected BrdU at 120 days and perfused one month later, allowing only half of our survival time, and it is possible that the interneurons were not given adequate time to mature and fully express calcium-binding protein markers (Scotto-Lomassese et al. 2007). Walton et al., on the other hand, injected BrdU around 90 days and investigated interneuron survival four
years later (Walton, Pariser, and Nottebohm 2012). This leaves room for the possibility that the interneurons that we identified as being added after the critical period could have survived at least 60 days but died somewhere between 180 days and four years post-hatch.

It should be noted that the patterns of PV-positive neuron addition seen here are similar to patterns recently seen in NCM (Kirn Lab, unpublished data). In the NCM, rates of neuron addition and parvalbumin-expressing neuron addition were highest for cells formed on day 40 and dropped at day 60 with further decreases into adulthood. Because both of these nuclei have been shown to play a critical role in tutor song copying (Roberts et al. 2012), it is possible that this pattern of PV-positive interneuron addition may relate to the storage of a song template. While NCM responds selectively to tutor song and not to BOS, HVC contains neurons that respond to both. Since zebra finches begin vocal motor practice around day 45, at the start of the sensorimotor stage, it is possible that the peak in parvalbumin-expressing interneuron addition around this time in these two regions may mediate the relationship between tutor song template storage and sensory-motor feedback. Similar to Vallentin et al.’s suggestion that interneurons “protect” learned song, perhaps these interneurons also work to facilitate the accurate relay of information from neurons encoding for the tutor song template (Vallentin et al. 2016).

Our results also suggest that higher rates of parvalbumin-expressing neuron addition at 60 days may correlate with similarity to tutor song, another one of our hypotheses. Keeping in mind that the neurons we identified as being added at different time points are only those that survived until 120 dph, this trend most likely
reflects the number of inhibitory neurons added that were incorporated into functional circuits and not the transient total number of PV-positive neuron addition at different times, which is likely to be much higher. One may ask whether birds with more inhibitory interneurons form more precise song circuits, or whether precise song circuits recruit more PV-positive neurons. Further investigation might involve observing synapse formation of PV-positive interneurons as they are incorporated into the existing population of HVC cells, perhaps using electrophysiological techniques.

Our data from birds isolated during the critical period indicate that, not only is neuron addition to the HVC extended past the critical period, but PV-positive interneuron addition is extended as well, supporting our predictions. This is consistent with the wealth of data indicating that inhibitory interneurons are an important marker of plasticity and can even reinstate plasticity after the normal closing of critical periods in sensory systems (Tang et al. 2014). Perhaps the HVC continues to recruit new interneurons at a higher rate in isolates in order to sustain the possibility of forming tutor song-related memories. Balmer et al. previously showed a marked decrease in PV expression in the HVCs of isolated birds as compared to birds raised with a tutor at 90 days of age (Balmer et al. 2009). Interestingly, while we showed no significant difference in PV expression between isolates and socially reared birds at 120 dph, isolated birds exhibited a significantly greater number of PV-positive neurons in the HVC by day 180. This is consistent with our finding that, out of all age groups studied, adult isolates appeared to recruit the highest percentage of PV-positive new neurons in relation to all new neurons added to the HVC.
A previous study showed that, when birds undergo visual or auditory isolation during the critical period, a greater number of neurons are added to the HVC after the critical period until 150 dph, at which point the rate of neurogenesis is comparable to that of socially reared birds (Wilbrecht et al. 2006). However, at 180 days, the HVCs of isolates contained about 40% more neurons than at 150 days, an increase which was not evident before this time point. This indicates that, before 150 days, there may be a higher rate of cell turnover, and after a certain time point near 150 dph there is an increase in cell survival (Wilbrecht et al. 2006). Our data support such a trend; perhaps a greater number of PV-positive neurons added to the HVC in isolates is preserved after a certain time point before 180 days, leading to an elevated level of PV expression as observed here. It is fairly undisputed that RA-projecting neurons are added to the HVC at a high rate during the critical period and continue to be added throughout life to a lessening degree (Walton, Pariser, and Nottebohm 2012; Wang et al. 2002; Kirn 2010). If a greater number of neurons are retained in isolates after a certain point in adulthood, it is possible that many of these are RA-projecting neurons, but this also offers potential insight into the high rates of parvalbumin-expressing neuron addition in adulthood seen here; perhaps inhibitory neurons are simply needed in HVC to maintain an excitatory-inhibitory balance. Excitatory neurons projecting to Area X and RA are densely interconnected with inhibitory interneurons in HVC (Mooney and Prather 2005); therefore, co-varying rates of adult parvalbumin-expressing interneuron addition may accompany excitatory RA-projecting neuron addition throughout life.
There is an alternative explanation to this data: perhaps the PV-positive neurons so prevalent in our adult isolates were not all inhibitory interneurons. In our confocal images of the adult isolate HVC, a majority of the neurons appeared to express PV at first glance, and this was consistent across all three birds in the cohort. This is reflected in the numerical data; about half of the neurons in the HVCs of isolated birds at 180 dph appeared PV-positive. In the average socially reared zebra finch, about 60% of neurons project to RA, and another segment of the population projects to Area X. Therefore, if we assume that all of the neurons counted as PV-positive are inhibitory interneurons, then isolation must have a drastic impact on the cellular makeup of HVC. However, Wild et al. observed that some RA-projecting cells appear to express PV to a faint degree (Wild et al. 2005). Perhaps, then, it is possible that lack of exposure to birdsong during the critical period impacts protein expression in HVC neurons later in life, causing more projection neurons to express PV. One study observed a potential impact of isolation on PV and PNN expression in the HVC, claiming that the signal to noise ratio in isolates was markedly lower despite applying the same immunohistochemical processes to all images (Balmer et al. 2009). While our results do not necessarily reflect the same trend, it cannot be ruled out that isolation may affect the expression of PV or any other neuronal indicator in HVC cells of varying types. It is not known what impact an increased expression of PV would have in excitatory projection neurons, although it is known that isolated birds produce aberrant song, as our results also indicate. A further study may investigate this question by repeating the experiment described here, using the
same age divisions and immunohistochemical techniques, but also backfilling RA and Area X projecting neurons in the HVC to determine whether there is any co-labeling.

What exactly is the role of inhibitory interneurons in regulating plasticity? Two useful models have been suggested. One involves the time-locked regulation of LTP and LTD by coordinating pre- and postsynaptic inputs; because PV-positive interneurons are fast spiking, quick inhibitory inputs on the soma of projection neurons may serve the purpose of suppressing back-propagation of action potentials that are not useful to the production of the ideal song, preventing the potentiation of excess circuits. This is referred to as the “instructive model”. The second, which may be called a “permissive model”, involves the ability of a large population of interneurons to process synchronous inputs through gap junction coupling. When they receive strong coincident input, they may release factors that stimulate dendrite or axon growth on the cells near them. In this model, interneurons act as a sort of mediator between competing circuits responding to input and selectively advance the most useful circuits (Hensch 2005).

It is critically important to keep in mind, however, that these models are based almost exclusively on what is known of the visual cortex, a sensory system. A sensorimotor system such as the songbird vocal learning pathway is likely to require different mechanisms for plasticity. Therefore, it is useful to examine other critical period indicators in the context of a brain consistently receiving new neurons to understand how these nuclei may moderate sensory inputs and motor outputs throughout life.
Perineuronal nets, for instance, seem to play an integral role in critical period mechanisms of the visual system. In the mouse visual cortex, PNN’s preferentially surround PV-positive interneurons, and destroying PNNs can reinstate plasticity after the critical period has closed (Pizzorusso et al. 2002). However, Balmer et al. showed that, when a bird is isolated so that the critical period is delayed, there is no significant change in the amount of PNN’s in the HVC at age 90, but, as they exhibit fewer PV-positive neurons, significantly less PNNs surround PV-positive interneurons specifically (Balmer et al. 2009). These results provide some interesting potential interpretations of our data. On one hand, if PNNs do work to regulate the environment for PV-positive interneurons, thus increasing their rate of survival (Morawski et al. 2004; Cabungcal et al. 2013), it is possible that the lack of PNNs surrounding PV-positive interneurons in isolates leads to an increased turnover of these neurons, which would provide some justification for the increased rates of PV-positive neuron addition seen in adult isolates. Alternatively, if isolation does upregulate the expression of a PV-encoding gene in projection neurons, as previously discussed, then this could also account for the greater amount of PNNs surrounding projection neurons in isolates. It is also possible that PNNs just happen to form in the extracellular matrix regardless of experience and surround more projection neurons in isolates because there are more of them to surround; however, evidence from the visual system suggests that PNN formation and PV-cell maturation are linked and both rely on sensory input. What remains unclear, however, is whether auditory information alone registers as “experience” for the HVC or whether this is song-specific; a future experiment may investigate PV expression and PNN formation in
deafened birds and birds that undergo different types of isolation. Although PNNs may not play as crucial a role in critical period modulation as they do in the mammalian visual system, as evidenced by some data suggesting that destroying PNNs in the song system does not reinstate plasticity, it does result in some song variability (Balmer, unpublished observations). Therefore, the distinct presence of PNNs in many song nuclei and their close association with PV-expressing interneurons make them a site of interest for future plasticity research.
Conclusion

The HVC has a central location in the song system of oscine birds, making it a prime site for integrating sensory information and vocal-motor output. It contains cells that respond to a bird’s own song as well as that of its tutor, it connects directly to the motor pathway for vocal production, and it appears to keep rhythm throughout it all. In zebra finches raised with a tutor, song is usually crystallized around day 90, after which point the HVC receives a relatively low number of new, mostly RA projecting neurons throughout life. The data presented here suggest a model in which PV-positive interneurons are added to the HVC during the critical period, particularly during the sensorimotor stage, to mediate the relationship between song template and BOS and to optimize vocal production circuits. If this is successfully done, PNNs may surround these interneurons, solidifying the circuits and the song patterns they encode. Any PV-positive interneuron addition after this point may occur to maintain the excitatory-inhibitory balance previously established. However, in birds isolated during the critical period, PV-positive interneurons added during development do not have a template signal to guide them, and this may lead to an increased rate of PV-positive cells addition late into adulthood. There is also a possibility presented here, although highly speculative, that isolation may impact the expression of PV in RA-projecting neurons. Future experiments may include investigating the various signals, such as the Otx2 homeoprotein, that may encode for sensory experience in the HVC and how these impact the development and connectivity of PV-positive interneurons.
References


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