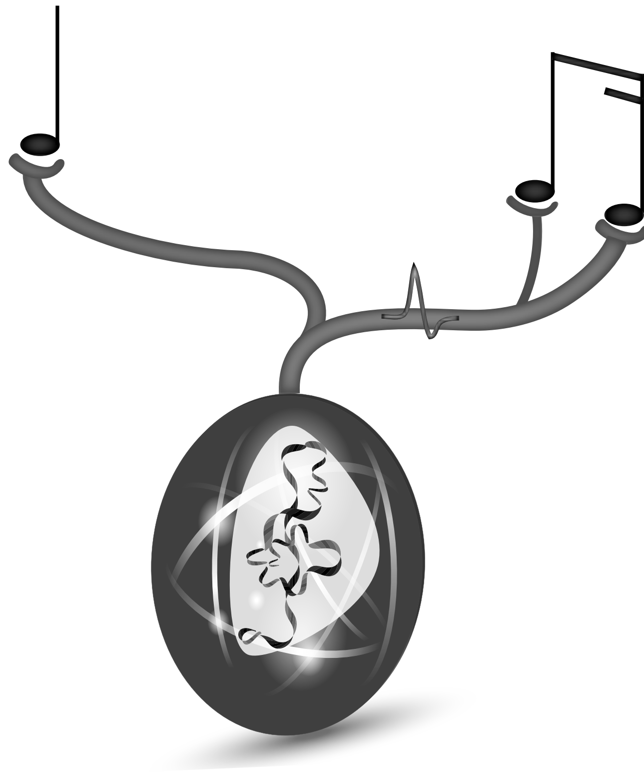
When new neurons
become functional:
On the importance of activity and connectivity of new neurons in the adult zebra finch



Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
Kirill Igorevich Tokarev
from Rubezhnoye, Ukraine

2009

Diese Arbeit wurde von Juni 2006 bis November 2009 am Max-Planck-Institut für Molekulare Genetik und der AG Verhaltensbiologie der Freien Universität Berlin unter der Leitung von Prof. Constance Scharff angefertigt.

1. Gutachter: Prof. Constance Scharff, Ph.D.

2. Gutachter: Prof. Dr. Hans-Joachim Pflüger

Die Dissertation wurde eingereicht am 21. Dezember 2009.

Tag der Disputation ist der 25. Februar 2010.

Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe angefertigt zu haben und alle Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben. Des weiteren versichere ich, dass die vorliegende Arbeit nie Gegenstand eines früheren Promotionsverfahrens war.

Die dem Verfahren zugrunde liegende Promotionsordnung ist mir bekannt.

21. Dezember 2009

____

Unterschrift

Acknowledgements

The wonderful three and a half years that I have spent for my PhD thesis at the Animals Behavior Group, first at the Max-Planck-Institute for Molecular Genetics and then at the Free University Berlin, have resulted in the work presented in this dissertation. This would not be possible without the many outstanding people who have provided invaluable support and mentorship during this time. It is to them that my sincere gratitude goes to.

First, I would like to thank my advisor Professor Constance Scharff for providing a rather exceptional scientific setting in the lab, which allowed me on the one hand to move freely in my research path towards this thesis, and on the other, to receive a state-of-the-art expertise in the field. She has been much more than merely a scientific advisor to me.

I am grateful to my many colleagues for providing a stimulating and fun environment in which to work grow. It was a pleasure to learn many necessary techniques from Arpenik Nshdejan, Sophie Scotto-Lomassese and Judith Rautenkranz in our lab, as well as Arndt Pechstein, Kira Gromova and Michael Krauß in the neighboring Laboratory of Membrane Biochemistry & Molecular Cell Biology. On the other hand, I was happy to pass my knowledge to Gala A. Claßen, Philip Rebensburg and Arjen Boender, all of whom happened to be also extremely helpful! I am deeply thankful to Marina Hoffmann for her incredibly efficient administrative work, and to Janett and David Birkenfeld for their attentive care of our birds. Anjelik Capar, Christopher K. Thompson, Sebastian Haesler, Silke Kipper, Silke Heucke, Henrike Hultsch, Sandra Wohlgemuth, Sarah Kiefer, Kim Mortega, Steffen Schulz, Micha Weiss, Iris Adam, Conny Bartsch, Jörg Böhner, and Mariam Honarmand made the lab so diverse in both scientific and social meanings. Some of the lab-mates have become good friends also outside of the lab, among them I would like to express my gratitude to Christina Roeske, Jörg Schreiber, Arpenik Nshdejan, Sophie Scotto-Lomassese, Christelle Rochefort, Jana Petri, and especially Ezequiel Mendoza together with his family.

Many thanks also go to Dr Christopher K. Thompson for his rational advice and comments on my manuscript, and to Professor Hans-Joachim Pflüger for being the co-supervisor of my thesis. Mariam Honarmand deserves a special thank for the help with translation of the summary into German.

Neither can I help thanking the supervisors of my Diploma thesis – Anna Tiunova and Konstantin Anokhin, who introduced me to the exciting world of the neurobiology of vocal learning.

Of course this work would not be possible without funding from Boehringer Ingelheim Fonds. BIF sponsored my PhD study during three years, for which I am highly indebted to them, but on top of that I would like to acknowledge their unparalleled 'family' attitude towards scholarship fellows. I am also grateful to Professor Constance Scharff for funding additionally the first month of my work and one month after the end of my scholarship.

I would also like thank my capoeira trainer, Professor Zorro, and break-dance teacher, Arndt Pechstein, for keeping me fit and active, which I hope have supported my neurogenesis at high levels.

Last, but certainly not the least, I am endlessly grateful to my family and friends. Through my childhood and study career, my parents have always encouraged me to follow my dreams. And I would like to dedicated my thesis to my babushka who passed away when I started writing it, but whose love and zeal for knowledge I would like to keep in me for the rest of my life. Special thanks go to Natasha, Jonathan, Matteo, Grigory, EJ, Brendan and Matjaž for their multidimensional backing during my PhD. I cannot find enough words to thank my dear Pantelako, for understanding and love, for arranging the most comfortable and inspiring place for writing this thesis, for so needed support during last months, and for calling me 'little doctor'...

'They have a sacred bird called the phoenix which I myself have never seen, except in pictures. Indeed it is a great rarity only coming there once in 500 years, when the old phoenix dies. They tell a story of what this bird does, which does not seem to me to be credible' (Herodotus, during his travels in Egypt in the 500´s B.C.)

Abbreviations 9

ABSTRACT 10

Zusammenfassung (German) 11

I INTRODUCTION 12

1.1 Adult neurogenesis in the avian and mammalian brain 12

1.1.1 Mechanisms and functions of adult neurogenesis 13

Neural stem cells and their niche 15

Molecular regulation of adult neurogenesis 15

Synaptic integration of adult-born neurons 20

Survival, activation, and function of adult-born neurons 22

Summary of specific properties of adult-born neurons 24

1.1.2 BrdU as a cell birth marker 25

1.2 Song control system in the songbird brain 27

Song system in the concept of functional systems 30

1.3 Immediate-early genes 33

1.4 Aim of this thesis 38

II EXPERIENCE-DEPENDENT TRANSCRIPTIONAL ACTIVATION OF ADULT-BORN NEURONS IN THE SONG SYSTEM 40

1 Introduction 40

2 Materials & Methods 42

2.1 Animals and schedule of experiments 42

2.2 BrdU injections 43

2.3 Retrograde labeling of projection neurons in the song nuclei 43

2.4 Tissue preparation and immunohistochemistry 44

2.5 Image analysis and quantification 45

2.6 Statistical analyses 46

3 Results 47

3.1 IEG induction in telencephalic nuclei of the song system during singing 47
3.2 Activation of adult-born neurons in the song system during vocal production..47
3.2.1 Activation of new neurons in HVC 47
3.2.2 Activation of new neurons in Area X 50
3.2.3 LMAN - new neurogenic region of the song system? 52
3.2.4 New neurons in HVC express IEGs irrespective of the type of singing..52
3.3 Age-dependent recruitment of new neurons into vocal production circuits 54
3.4 New HVC neurons are preferentially activated during singing..........59
4 Discussion 60
4.1 Relation to the data on maturation of adult-born neurons.................60
4.2 Are young HVC-RA and mature HVC-X neurons sensitive to the social context of vocalization? 63
4.3 Is activity of new HVC neurons related to vocal plasticity? 66
4.4 Mapping of IEG expression is an effective approach to the function of adult neurogenesis...70
III PHARMACOLOGICAL INHIBITION OF ADULT NEUROGENESIS IN THE ZEBRA FINCH? 72
1 Introduction 72
2 Materials & Methods 75
2.1 Animals and schedule of treatment 75
2.2 Injections of TMZ...76
2.3 BrdU injections...76
2.4 Immunohistochemistry...76
2.5 Quantification of newly-generated cells in the ventricular zone adjacent to HVC..77
3 Results 78
4 Discussion 79

IV A ROLE FOR FoxP1/FoxP2 IN ADULT NEUROGENESIS? 81
1 Introduction 81
2 Materials & Methods 85
2.1 Animals..85
2.2 BrdU injections..85
2.3 Retrograde labeling of projection neurons in HVC.............................85
2.4 Tissue preparation and immunocytochemical labeling of HVC interneurons...86
2.5 Immunohistochemistry...86
2.6 Image analysis 87
3 Results 88
3.1 FoxP1 expression in adult-born HVC cells..88
3.2 FoxP1 expression is not restricted to the replaceable type of HVC neurons 88
3.3 FoxP1 is not expressed in parvalbumin-positive HVC interneurons .88
3.4 Expression of nNOS, target of FoxP2/1, in the VZ, including one-day old cells 90
3.5 Absent or low expression of nNOS in more mature and in FoxP2+ cells 90
4 Discussion..92
4.1 FoxP1 and development of projection neurons 92
4.2 NO and neurogenesis 94
V CONCLUDING REMARKS and SUMMARY OF the FINDINGS 97
VI Bibliography 100

Abbreviations

Area X – Area X of the medial striatum

BrdU – 5-bromo-2´-deoxyuridine

BDNF – brain-derived neurotrophic factor

CMM – caudomedial mesopallium

CTB – subunit B of cholera toxin

DLM – dorsolateral medial nucleus of the thalamus

DMP – nucleus dorsomedialis posterior of the thalamus

GABA – γ-Aminobutyric acid

HVC – nucleus HVC of the nidopallium, formerly, the high vocal center

HVC-RA – HVC neurons projecting to the robust nucleus of the arcopallium, RA

HVC-X – HVC neurons projecting to Area X of the medial striatum

IEG – immediate-early gene

LMAN – lateral magnocellular nucleus of the anterior nidopallium

LoC – locus coeruleus of the midbrain

LTP – long-term potentiation

MMAN – medial magnocellular nucleus of the anterior nidopallium

NCM – caudomedial nidopallium

NIf – interfacial nucleus of the nidopallium

NMDA – *N*-methyl-D-aspartate

NMDAR – *N*-methyl-D-aspartate (NMDA) receptor

nNOS – neuronal nitric oxide synthase

NO - nitric oxide

NSC – neural stem cell

nXIIts – the tracheosyringeal portion of the twelfth cranial nerve

POM – preoptic nucleus of the midbrain

RA – robust nucleus of the arcopallium

TMZ – temozolomide

VTA – ventral tegmental area

VZ – ventricular zone

ABSTRACT

New neurons continuously replace dying ones in adult HVC, a forebrain pallial region that is important for song production in adult zebra finches. Despite the ongoing replacement of HVC neurons, zebra finch song remains relatively stable with no evident learning of new syllables. New neurons project to their target (nucleus RA) and respond to auditory stimulation (in anesthetized animals) which suggests that they are functionally integrated into the premotor circuit controlling song production, but this has not been demonstrated directly.

We used immediate-early gene (IEG) expression and retrograde tracer injections to track the functional recruitment of new neurons labeled by cell-birth-dating marker 5-bromo-2′-deoxyuridine (BrdU) into premotor circuits involved in singing in freely behaving zebra finches. We found that in HVC adult-born neurons at least 3 weeks old express IEGs during singing, as well as in other vocal control areas – Area X and LMAN. This is also the first case to our knowledge when adult-born neurons in LMAN have been documented. Surprisingly, in HVC, IEG expression in new neurons is probably not dependent upon their connectivity to the target region RA, suggesting that these neurons may participate in processing of premotor information through local microcircuitry within HVC before their axons reach RA. However, already at 3 weeks, the population of adult-generated neurons that had been connected to RA by the time of singing were as likely to be activated as the pre-existing HVC-RA projection neurons. Nevertheless, among adult-born HVC cells, the portion of neurons that expressed IEGs and were connected to RA increased with their age from 3 to 8 weeks. Interestingly, we show that social context of singing has an impact on IEG expression in new HVC neurons: At the age of 3 weeks they were more likely to be activated during undirected singing than during directed singing.

To analyze requirement of adult neurogenesis for singing in zebra finches, we injected temozolomide (TMZ), the active component of a brain tumor drug, but did not detect any effect on the rates of neurogenesis due to extremely high variability of this parameter in both treated and control conditions.

In addition, comparison of expression patterns suggests that FoxP2, a gene linked to human speech and language impairment, and its closest homolog, FoxP1 may interfere with adult neurogenesis by down-regulation of NO pathway, and FoxP1 may be a lineage marker for projection neurons in HVC.

Summing up, our results demonstrate that adult-born HVC neurons are activated during vocal production in an age-dependent manner and may contribute specifically to vocal plasticity (minor changes of acoustic parameters of song elements).

Zusammenfassung (German)

Im adulten HVC, einem pallialen Teil des Vorderhirns, welcher essentiell für die Gesangsproduktion adulter Zebrafinken ist, werden kontinuierlich sterbende, alte Neuronen durch neue ersetzt. Trotz dieses ständigen Austauschs von Neuronen in HVC, bleibt der Gesang von Zebrafinken relativ stabil und es werden augenscheinlich keine neuen Silben gelernt. Neue Neuronen projizieren nicht nur zu ihrer Zielregion (Nucleus RA) sondern reagieren auch auf auditorische Stimulation (anästhesierte Tiere). Dies lässt vermuten, dass sie funktionell in die prämotorischen Schaltkreise, die die Gesangsproduktion kontrollieren, integriert sind. Dies wurde bislang nicht experimentell belegt.

Wir haben die Expression von „immediate-early" Genen (IEG) und die retrograde Injektion von Tracern genutzt, um die funktionelle Rekrutierung von neuen, 5-Brom-2´-Desoxyuridin (BrdU) markierten Neuronen in die prämotorischen Schaltkreise singender Zebrafinken zu verfolgen. Wir konnten zeigen, dass in neuen, mind. drei Wochen alten, Neuronen die Expression von IEGs durch Singen ausgelöst wurde; dies war der Fall in HVC und auch anderen vokalen Kontrollarealen, AreaX und LMAN. Nach unserem Wissensstand ist dies auch die erste Studie, die adulte Neurogenese in LMAN detektiert. Überraschenderweise scheint die Expression von IEGs in neu geborenen HVC Neuronen nicht von deren Projektion zu ihrer Zielregion RA abzuhängen. Dies könnte ein Hinweis auf die Beteiligung dieser Neuronen bei der Prozessierung von Prämotorinformationen durch die lokalen Mikroschaltkreise innerhalb HVCs sein, zu einem Zeitpunkt bevor die Axone ihr Projektionsziel RA erreichen. Bereits nach drei Wochen ist die Wahrscheinlichkeit einer durch Singen erfolgreichen Aktivierung für die Population der neu geborenen projizierenden Neuronen und die bereits existierenden HVC-RA Projektionsneuronen gleich groß. Nichtsdestotrotz, stieg innerhalb der neu geborenen Neuronen der Anteil an IEG exprimierenden und RA-projizierenden Neuronen mit dem Alter; im Zeitraum von drei bis acht Wochen.Interessanterweise finden wir einen Einfluss des sozialen Gesangskontextes auf die Expression von IEGs in den neu geborenen HVC-Neuronen: Drei Wochen alte Neuronen wurden mit einer größeren Wahrscheinlichkeit durch ungerichteten Gesang, als durch gerichteten Gesang aktiviert.

Um die Notwendigkeit adulter Neurogenese für Gesang in Zebrafinken zu untersuchen, haben wir Temozolomid (TMZ), die aktive Komponente eines Medikaments zur Behandlung on Hirntumoren, appliziert. Wir konnten aufgrund der hohen Variabilität der Neurogeneserate keinen Effekt zwischen Behandlungs- und Kontrollgruppe feststellen.

Eine Vergleich von Expressionsmustern deutet darauf hin, dass FoxP1 durch Herunterregulation des NO-Signaltransduktionsweges Einfluss auf die Neurogeneserate nehmen könnten. FoxP1 ist das nächste homologe Gen zu FoxP2, welches mit einer menschlichen Sprachstörung in Verbindung gebracht wird. Daneben zeigen wir dass FoxP1 ein Abstammungsmarker für Projektionsneuronen in HVC sein könnte.

Zusammenfassend zeigen unsere Resultate, dass adult geborene HVC-Neurone durch vokaler Produktion altersabhängig aktiviert werden und spezifisch zur vokalen Plastizität beitragen können (minimale Änderungen akustischer Parameter von Gesangselementen).

I INTRODUCTION

1.1 Adult neurogenesis in the avian and mammalian brain

Although it is assumed that the majority of neuron types may persist our entire lifespan since being formed during gestation (Rakic, 2002), in at least two areas of the human brain, hippocampus and olfactory bulb, new neurons appear even during adulthood (Eriksson et al., 1998; reviewed in Rakic, 2002, and Gould, 2007). In songbirds, brain areas incorporating adult-born neurons include vocal control nuclei of the song system HVC[1] and Area X (Goldman & Nottebohm, 1983; Alvarez-Buylla & Nottebohm, 1988), and higher auditory area NCM (Barkan et al., 2007). Adult neurogenesis represents a process of adult neural stem cell proliferation, their fate specification and differentiation, maturation, migration and incorporation into the existing neural circuitry in the mature nervous system (Ming & Song, 2005).

Since the discovery of adult neurogenesis in mammals (Altman & Das, 1965; Altman, 1969) and birds (Goldman & Nottebohm, 1983; Alvarez-Buylla & Nottebohm, 1988), one of the essential issues in neurobiology has been whether or not the new neurons are functionally important, because it appears that, in «higher» vertebrates, adult neurogenesis may be just a vestigial process, as with evolutionary progression in vertebrates fewer and fewer brain areas incorporate new neurons and the rate of such incorporation decreases (reviewed in Chapouton et al., 2007). Nevertheless, although some contradictory findings (Shors et al., 2002; Imayoshi et al., 2008; Hernandez-Rabaza et al., 2009) and difficulty interpreting many results lend credence to skepticism on the role and sometimes even existence of adult-born neurons in some brain areas (Rakic, 2002), a growing body of evidence suggests that adult neurogenesis is involved (Magavi et al., 2005; Ramirez-Amaya et al., 2006; Kee et al., 2007; Trouche et al., 2009) and even necessary (Shors et al., 2001, 2002; Bruel-Jungerman et al., 2005; Madsen et al., 2003; Rola et al., 2004; Dupret et al., 2007; Imayoshi et al., 2008; Hernandez-Rabaza et al., 2009, Garthe et al., 2009) for some behaviors.

It remains unclear why few areas in the adult brain are neurogenic (Rakic, 2002; Gould, 2007) and particular types of neurons are renewed (Scotto-Lomassese et al., 2007; Nottebohm, 2002), whereas the major mechanism of plasticity in the brain is through

---

[1] See the list of abbreviations.

remodeling of dendrites and synaptic connections (reviewed in Rose, 1991; Lledo et al., 2006; Bruel-Jungerman et al., 2007; Feldman, 2009). Why was adult neurogenesis maintained in specific brain regions during the course of vertebrate evolution? One hypothesis is that given their unique physiological properties, developing adult-born neurons transiently serve as mediators for experience-driven plasticity and selectively integrate as special units within the adult circuitry where they contribute to specific brain functions (Ge et al., 2008).

1.1.1 *Mechanisms and functions of adult neurogenesis*

Some of the mechanisms of development and integration into functional networks have been elucidated for adult-generated neuron types in songbirds (reviewed in Nottebohm, 2004) and rodents (Zhao et al., 2008; Ma et al., 2009b). Development of adult-generated neurons is perhaps most comprehensively described for the rodent hippocampus, as summarized on the scheme on **Fig. 1**.

Despite many similarities between early postnatal and adult neurogenesis (as those found by Espósito et al., 2005, in mice), major differences have been identified as well. Most strikingly, adult neurogenesis is dynamically regulated by stimuli that modulate the activity of the existing neuronal circuitry, as discussed in the following paragraphs. Adult neurogenesis also exhibits a more prolonged time course of neuronal maturation. For example, dendritic development of adult-born dentate granule cells lasts more than four weeks and formation and maturation of synaptic spines over eight weeks, while the same process occurs largely within two weeks during early postnatal dentate development (Ge et al., 2006).

Aided by new methodologies for birth-dating and tracking new neurons in the adult brain, recent studies have elucidated the basic process of adult neurogenesis (Alvarez-Buylla & Lim, 2004; Ming & Song, 2005; Lledo et al., 2006; Zhao et al., 2008). In particular, studies using 5-bromo-2′-deoxyuridine (BrdU) cell birth-dating[2] and immunohistochemistry of cell type specific markers have identified a series of intermediate developmental stages during adult neurogenesis (Kempermann et al., 2004). In addition, electrophysiology and imaging studies of new neurons labeled with retroviruses and in transgenic reporter mice have characterized the maturation and integration of new neurons in the adult brain (Duan et al., 2008). Furthermore, genetic manipulation of adult neural

[2] For the introduction to BrdU-based method of cell birth-dating, see section 1.1.2 of this chapter.

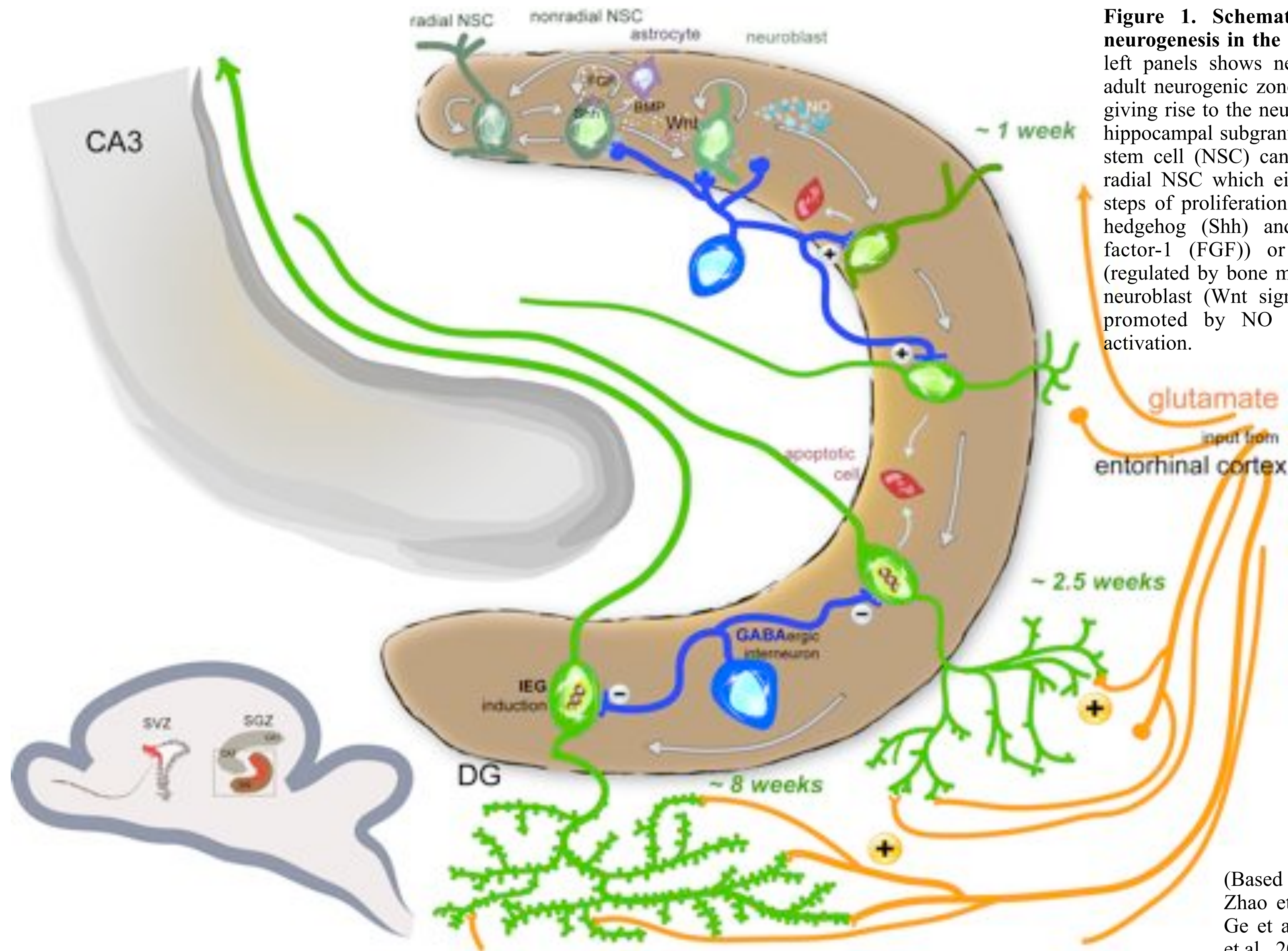


Figure 1. Schematic representation of adult neurogenesis in the rodent dentate gyrus. Lower-left panels shows neuroanatomical localization of adult neurogenic zones, subventricular zone (SVZ), giving rise to the neurons of the olfactory bulb, and hippocampal subgranular zone (SGZ). Radial neural stem cell (NSC) can give rise to pluripotent non-radial NSC which either may go through multiple steps of proliferation (influenced by intrinsic sonic hedgehog (Shh) and extrinsic fibroblast growth factor-1 (FGF)) or differentiate into astrocyte (regulated by bone morphogenic protein (BMP)) or neuroblast (Wnt signaling). Neuronal fate is also promoted by NO signaling and tonic GABA activation.

On 2nd-3rd weeks, new neurons start receiving phasic (dendritic and perisomatic) GABA input, which changes its effect on membrane potential from depolarization to hyperpolarization.

Transition to inhibition by GABA coincides with receiving of glutamatergic excitatory input, which in turn can induce immediate-early gene (IEG) expression. This period is characterized by competitive survival of new neurons followed by their enhanced long-term potentiation. And further arborization and growth of synaptic spines continues.

(Based on schemes and data from Zhao et al., 2008; Duan et al., 2008; Ge et al., 2008; Jagasia 2006; Cheng et al., 2003).

stem cells and their local environment, or ‘neurogenic niche’, is starting to reveal key molecular mechanisms underlying adult neurogenesis and its activity-dependent regulation (reviewed by Alvarez-Buylla & Lim, 2004; Ge et al., 2008; Ma et al., 2009b,2009a).

Neural stem cells and their niche

Neuronal progenitor cells destined to pallium and striatum in adult birds and olfactory bulb in mammals undergo their last division on the walls of the lateral ventricle (ventricular zone (VZ) in adult birds and subventricular zone (SVZ) in adult mammals), and those residing in subgranular zone (SGZ) cells of the dentate gyrus in the hippocampal formation in mammals generate neurons destined to the dentate gyrus itself (for review see Chapouton et al., 2007).

It was first shown in songbirds that many migrating cells are tightly apposed to the radial processes of the radial glial cells, and it was hypothesized that radial glia are the precursors of neurons (Alvarez-Buylla & Nottebohm, 1988; reviewed in Alvarez-Buylla et al., 2002). Radial glia astrocytes are also considered to play the central role in adult hippocampal neurogenesis in rodents both being a neural stem cell (NSC) and providing a ‘niche’ for developing and migrating neurons (reviewed in Alvarez-Buylla et al., 2002; Alvarez-Buylla & Lim, 2004; Ma et al., 2005). In contrast to developmental neurogenesis, however, neurons incorporating into the adult olfactory bulb originate from non-radial astrocytes (Doetsch et al., 1999). Newly-born neurons are connected to adjacent radial glia with gap junctions, and expression of connexin (structural protein of gap junctions) by radial glia-like cells is required for neurogenesis in the adult dentate gyrus (Kunze et al., 2009). In addition to providing structural support to developing and migrating adult-born neurons, astrocytes are known to express secreted and membrane-associated molecules, including cytokines, growth factors, and neurotransmitters, in response to physiological and pathological stimuli (Schipke & Kettenmann, 2004; Schipke & Kettenmann, 2004). Astrocytes are particularly well suited to integrate local environmental signals because of the unique syncytium structure formed via gap junctions between them, through which intercellular signaling might propagate (Schipke & Kettenmann, 2004).

Molecular regulation of adult neurogenesis

During the last couple of decades some of the key regulators of adult neurogenesis have been identified, both of intrinsic and extrinsic origin.

Intrinsic hedgehog signaling[3] is activated in quiescent adult NSCs in the adult rodent SVZ and SGZ (Ahn & Joyner, 2005) and appears to be required for both establishment and maintenance of proper NSC pools (Balordi & Fishell, 2007; Han et al., 2008). In addition, expression of leukemia inhibitory factor (LIF) promotes the self-renewal of adult NSC and prevents their differentiation, leading to an expansion of the NSC pool in the adult SGZ (Bauer & Patterson, 2006). Little is known about the transcriptional regulation of this process. Adult mice lacking either the E-box containing transcription factor E2F1, or the basic helix–loop helix transcription factor NPAS3, have reduced proliferation in the postnatal dentate gyrus (Cooper-Kuhn et al., 2002). The orphan nuclear receptor TLX (tailless homolog) is also expressed in the postnatal hippocampus, where it appears to maintain neurogenic proliferation by repressing glial fate (Shi et al., 2004). Genetic knock-down and correlative evidences suggest that, like in the embryonic telencephalon, members of the winged-helix/forkhead family of transcription factors may be important regulators of adult neurogenesis: FoxG1 is expressed in adult-generated neurons in the murine dentate gyrus and is required for their formation (Shen et al., 2006); FoxP2 is expressed in newborn neurons in the striatal nucleus Area X of songbirds (Rochefort et al., 2007).

Astroglia derived Wnt signaling has been shown to be a key extrinsic pathway to promote neurogenesis of adult NSCs, as manipulation of this signaling *in vivo* by over-expressing Wnt3 or a Wnt inhibitor in the dentate gyrus leads to enhanced or diminished adult neurogenesis, respectively (Lie et al., 2005). Autonomous Wnt signaling is a conserved feature of the neurogenic niche that preserves the delicate balance between NSC maintenance and differentiation (Jagasia et al., 2006; Wexler et al., 2009). By contrast, signaling from the bone morphogenic protein (BMP) family instructs adult NSCs to follow glial fate (Hsieh et al., 2004).

Furthermore, a number of cell adhesion molecules (e.g. b1-integrin, PSA-NCAM, and tenascin-R) and extracellular cues (e.g. GABA, neuregulins, and slits) regulate the stability, motility, or directionality of neuronal migration (Ming & Song, 2005; Zhao et al., 2008). Some of the intrinsic regulators in neuronal migration have been identified as well. For

---

[3] Hedgehog family of intercellular signaling proteins have become recognized as key mediators of many fundamental processes in embryonic development. Their activities are central to the growth, patterning, and morphogenesis of many different regions within the body plans of vertebrates and insects, and probably other invertebrates. In some contexts, Hedgehog signals act as morphogens in the dose-dependent induction of distinct cell fates, in others as mitogens regulating cell proliferation or as inducing factors controlling the form of a developing organ (reviewed by Ingham & McMahon, 2001)

instance, knockdown of Disrupted-in-Schizophrenia 1 (DISC1) in newborn granule cells leads to overextended migration and aberrant positioning in the outer granule cell layer and molecular layer in the dentate gyrus (Duan et al., 2007).

One hallmark of adult neurogenesis is its regulation by the activity of the existing neuronal circuitry. GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, for mature neurons in the adult brain and also serve as extrinsic regulators for immature adult-born neurons. These neurotransmitters activate neurons not only locally within synaptic clefts (phasic activation), but also at a distance after diffusion out of synapses (tonic activation) (Farrant & Nusser, 2005). GABA-A receptors (GABAARs) are chloride (Cl^-) permeable channels and the polarity of GABA action depends on the Cl^- gradient across the membrane, which in turn is determined by the developmentally regulated expression of Cl^- transporters (Tozuka et al., 2005; Ge et al., 2006). Adult hippocampal neural progenitors and immature neurons express functional ionotropic GABAARs (Tozuka et al., 2005; Ge et al., 2006). Newborn granule cells exhibit a gradual decrease in the expression of NKCC1, a Cl^- importer, with a concurrent increase in the expression of KCC2, a Cl^- exporter, during their maturation process. As a consequence, GABA initially depolarizes immature new neurons because of their high Cl^- content and such depolarization is gradually converted to hyperpolarization over a period of 2–3 weeks (Ge et al., 2006).

Unusual (compared to mature neurons) regulation of the transmembrane flow of positively charged ions also contributes to the high excitability of adult-born neurons: The early expression of voltage-gated Na^+ and K^+ channels together with the delayed expression of K^+ inward rectifier (Kir) allow high excitability of 3-4 week old dentate gyrus neurons despite weak glutamatergic inputs from perforant path[4] stimulation (Mongiat et al., 2009).

Glutamate has long been implicated in regulating adult hippocampus neurogenesis through *N*-methyl-D-aspartate (NMDA) receptors (NMDARs)[5]. Direct injection of NMDA down-regulates cell proliferation in the adult rat dentate gyrus (Cameron et al., 1995), while induction of long-term potentiation (LTP) at the glutamatergic perforant path

[4] Perforant pathway connects entorhinal cortex to the hippocampal formation.

[5] NMDA receptors are heteromultimeric ion channels composed of the NR1 subunit and one or more of the NR2A, -B, -C, or -D subunits (Cull-Candy et al., 2001), but only NR2A and NR2B are typical for the pallial regions in mammals (Standaert et al., 1994), and birds (Wada et al., 2004).

to dentate granule cells promotes the proliferation of adult neural progenitors and survival of newborn neurons in an NMDAR-dependent fashion (Chun et al., 2006). These results suggest that glutamate has both cell autonomous effects in immature neurons and extrinsic impacts through modulation of existing neuronal circuits. Immunohistochemistry studies suggest that two subunits of NMDAR (NR1 and NR2B) are expressed at early stages during adult neurogenesis (Nacher et al., 2007). Interestingly, direct NMDAR signaling has been shown to regulate newborn neurons during two critical periods (Ge et al., 2008). In the first phase, NMDAR activation of new neurons promotes their competitive survival, whereas genetic deletion of NR1 in proliferating adult neural progenitors reduces the survival of their neuronal progeny between 2 and 3 weeks after their birth (Tashiro et al., 2006). Interestingly, such a critical period is coincident with a transition from excitatory to inhibitory GABA signaling. Whether GABA cooperates with glutamate signaling in regulating the survival of new neurons during this critical period remains to be determined. In the second phase, NR2B-dependent activation of NMDARs is required for the enhanced synaptic plasticity of glutamatergic inputs to new neurons during the 4–6 weeks after their birth, potentially serving as a substrate for learning from new experience (Ge et al., 2007; Kee et al., 2007). Parallel to such a direct impact of glutamate on newborn neurons, NMDAR activation in mature neurons is known to lead to a gene expression program that produces diffusible factors that profoundly up-regulate adult neurogenesis (Ma et al., 2009b).

It is well established that retinoic acid plays a role in developmental neurogenesis (reviewed by Maden, 2007), and there is growing evidence from rodents that it is also involved in the control of adult neurogenesis. First, specific pattern of retinoid signaling occurs at sites of adult neurogenesis, the olfactory bulb and hippocampus (Thompson Haskell et al., 2002). Second, in mice fed on retinoid-deficient diet, differentiation and survival of new hippocampal neurons is impaired (Jacobs et al., 2006). Finally, in cultured slices, retinoic acid promotes proliferation of neuroblasts from the murine SVZ (Wang et al., 2005b).

Another neuron-derived regulator which acts on proliferation and differentiation of NSCs is nitric oxide (NO), a gaseous paracrine messenger synthesized in the brain by specific neurons expressing the neuronal isoform of NO synthase (nNOS). This molecule has been demonstrated to down-regulate proliferation of NSCs in adult but not postnatal

mice (Romero-Grimaldi et al., 2008). NO does not simply inhibit cell proliferation, however; it also stimulates NSCs to switch from a state of proliferation to neuronal differentiation (Cheng et al., 2003). The effects of nNOS-derived NO on neurogenesis in the SGZ are probably mediated by NMDAR activity, cAMP second messenger cascade and cyclic AMP response element binding protein (CREB) (Zhu et al., 2006); the latter two are involved in all stages of adult neurogenesis from proliferation to survival (Nakagawa et al., 2002). Furthermore, it is possible that there is a feedback mechanism between nNOS and NMDARs, given that nNOS is a signaling enzyme of these receptors (Aarts et al., 2002).

Finally, adult neurogenesis is influenced by neurotrophins, such as brain-derived neurotrophic factor (BDNF), as shown in numerous studies using canaries (for example, Li et al., 2000; for review see Nottebohm, 2004) and mice (Lee & Son, 2009). BDNF signaling stimulates NO-driven switch from proliferation to neuronal differentiation of neuronal progenitor cells (Cheng et al., 2003). BDNF itself is regulated by neuronal depolarization (Shieh et al., 1998; Tao et al., 1998) and affects dendritic and axonal morphology (McAllister et al., 1996; Horch et al., 1999), synaptogenesis (Causing et al., 1997) and synaptic efficacy (Lohof et al., 1993; Kang & Schuman, 1995; Patterson et al., 1996). Among other trophic factors, the vascular endothelial growth factor (VEGF) and insulin-like growth factors (IGFs) are known to influence adult neurogenesis positively (for review see Lee & Son, 2009).

A recent study by Ma et al., 2009a, showed that neuronal circuit activity in the adult dentate gyrus regulates expression of paracrine neurogenic niche factors from mature neurons through region-specific DNA demethylation by a product of the immediate-early gene (see chapter I.1.3) called *Gadd45b*. These paracrine regulators include BDNF and yet another growth factor FGF-1 (fibroblast growth factor–1), which exhibits robust mitogenic activity on neural progenitor proliferation in vitro. As a result, *Gadd45b* expression in mature neurons driven by firing up-regulates proliferation of NSCs and arborization of immature neurons in adult hippocampus (Ma et al., 2009a).

Thus, GABA, glutamate, NO, immediate early genes like *Gadd45b* and neurotrophic factors provide a link between neuronal activity on the one hand and proliferation/ differentiation of neural progenitors as well as their further integration into existing neural circuitry on the other.

Synaptic integration of adult-born neurons

A quarter of a century ago, ultrastructural analysis with electron microscopy by Burd & Nottebohm, 1985, revealed that adult-born HVC neurons receive three morphologically distinct types of synaptic input. Even today, however, the neurochemical nature of those synapses is unclear.

Over the past few years engineered oncoretrovirus (van Praag et al., 2002) and transgenic reporter mice (Overstreet et al., 2004) have greatly facilitated functional analysis of new neurons in vivo. Studies using these tools have revealed a stereotypic process in establishing connections by newborn granule cells in the adult hippocampus, from initial tonic activation by ambient GABA, to dendritic GABAergic synaptic inputs, followed by dendritic glutamatergic inputs and finally perisomatic GABAergic inputs (Espósito et al., 2005; Tozuka et al., 2005; Ge et al., 2006; **Fig. 1**). Apparently synaptogenesis and arborization of developing adult-born neurons are heavily influenced by behavioral activity (Ambrogini et al., 2009). After a prolonged maturation phase, adult-born neurons exhibit electrical and synaptic properties that are indistinguishable from those of neighboring mature granule cells when examined by electrophysiology at the single-cell level (van Praag et al., 2002; Laplagne et al., 2006; Ge et al., 2007). Alternative approaches such as imaging of dendritic spines (Zhao et al., 2006) and analysis of immediate early gene expression (Jessberger & Kempermann, 2003) in adult-born granule cells suggest a similar prolonged time course of synaptic integration and maturation of adult-born neurons.

Analysis of axonal and dendritic development of new neurons using retrovirus-mediated labeling has revealed similar patterns of targeting as their neighboring mature neurons (Ming & Song, 2005). For example, newborn dentate granule cells rapidly extend their axons through the hilus region to reach the CA3 region within two weeks after birth, while their dendrites reach the molecular layer within one week and continue to elaborate for at least four weeks (Ge et al., 2006, 2007). The environmental cues that guide axons and dendrites of newborn neurons in the adult brain have not yet been identified. A number of molecular players have been shown to regulate the tempo of dendritic development, though. For example, during adult hippocampal neurogenesis GABA-induced depolarization (Ge et al., 2006) and Notch signaling (Breunig et al., 2007) promote

dendritic growth, whereas DISC1 limits dendritic initiation and outgrowth of new neurons (Duan et al., 2007).

Interestingly, neural progenitors and immature neurons are tonically activated by ambient GABA before receiving any functional synaptic inputs during adult SVZ and SGZ neurogenesis (Jessberger et al., 2005). For new dentate granule cells, formation of dendritic GABAergic synaptic inputs (approximately one week after birth) initiates before formation of glutamatergic synaptic inputs (approximately two weeks after birth), and perisomatic GABAergic inputs appear to form last (Espósito et al., 2005). During the initial stage of neuronal maturation, GABA depolarizes newborn neurons, due to the high chloride content which promotes formation of GABAergic and glutamatergic synaptic inputs to new neurons in vivo (Ge et al., 2006).

Adult-generated neurons in birds probably also have delayed synaptogenesis. New neurons in HVC form synapses mediating stimulus-evoked and spontaneous action potentials during the second-third week *in vitro* (Goldman & Nedergaard, 1992); and *in vivo* they were reported to obtain synaptic input from approximately 3 weeks (Burd & Nottebohm, 1985).

Very little is known about synaptic outputs of newborn neurons in the adult brain. It remains to be determined whether new neurons innervate the same targets as their neighboring mature neurons. While axons of adult-born granule cells have been shown to extend into the CA3 subfield of the hippocampus (Markakis & Gage, 1999; Zhao et al., 2006), little is known about properties of their synaptic outputs due to technical hurdles. Electron microscopy reconstruction of the mossy fiber synaptic boutons of retroviral-labelled adult-born neurons suggests that their synaptic outputs exhibit a similar time course of synaptogenesis and maturation to their synaptic inputs (Ge et al., 2008).

Thanks to the possibility of using retrograde tracers to track projections within the song system of songbirds, it was established that adult-born neurons of HVC project to the same area as existing neurons of that type (Scotto-Lomassese et al., 2007). It is estimated that this connectivity develops gradually in the population of newly incorporated HVC neurons: 15-18 days old neurons only start reaching their target area (at this survival time, only about 1% of them were backfilled with retrograde tracer injected into their target region); this number increases to 24% by 22-25 days and to 43% by 31 days (Kirn et al.,

1999; a similar percentage of connected one month old neurons in HVC was observed by Scotto-Lomassese et al., 2007).

Survival, activation, and function of adult-born neurons

Like during early development, new neurons in the adult brain are produced in excess, and as a result, about two-thirds of the young neurons migrating to HVC in songbirds and nearly half of the newly generated neurons in the adult rodent brain die (Alvarez-Buylla & Kirn, 1997; Ming & Song, 2005; Alonso et al., 2006; Tashiro et al., 2006). But unlike during embryonic development, this life-or-death decision for adult-born neurons is probably based on behavior-driven neuronal activity in the neurogenic areas. It is assumed that the survival of individual neurons is competitive, so that only new neurons mediating information-relevant activity are selectively preserved and integrated into the circuitry (Ma et al., 2009b).

Interestingly, neuronal death itself may trigger adult neurogenesis. In HVC, production of new neurons and death of older ones are parallel and interrelated processes and neurogenesis is not merely a process of production of new neurons but a process of neuronal replacement. Direct evidence that neuronal replacement takes place came from a study on canaries in which all HVC neurons of a particular projection type (HVC-RA) present in April were labeled with a vital retrograde marker; 40–50% of these cells were lost and replaced by newly incorporated ones by October (Kirn & Nottebohm, 1993). Experimentally induced targeted death of these replaceable HVC neurons results in increased production of neurons of this type (Scharff et al., 2000), and recently it has been shown that experimental decrease of seasonal apoptosis causes reduction of neurogenesis in HVC (Thompson & Brenowitz, 2009). Furthermore, even brain areas that do not replace neurons in normal conditions (striatum and cortex in mammals, including humans) may receive new neurons after stroke (Parent, 2003; Jin et al., 2006).

But what kind of behavioral activities are interrelated with adult neurogenesis? One of the first hypotheses was that new neurons are involved in acquisition of new memories, and studies in birds were the first to provide evidence for a positive correlation between adult neurogenesis and learning. The production of new HVC neurons in canaries positively correlates with hormonal and seasonal differences in vocal plasticity – acquisition of new syllables (Goldman & Nottebohm, 1983; Kirn et al., 1994). Likewise, in the hippocampal region of black-capped chickadees, a seasonal fluctuation in adult

neurogenesis is positively related to engaging in spatial learning behaviors, namely seed storage and retrieval (Barnea & Nottebohm, 1994). Similar correlations were found also between spatial learning and survival of newborn neurons (Gould et al., 1999) as well as cell proliferation (Döbrössy et al., 2003) in the adult dentate gyrus of rodents. Remarkably, spatial learning in a water maze was also linked to a decrease in the number of newborn neurons in the adult dentate gyrus, and this decline was the strongest in the rats which showed the best memory performances (Döbrössy et al., 2003). A study by Dupret et al., 2007, explained this phenomenon showing that spatial learning indeed increased cell proliferation in SGZ and resulted in higher survival rates of the newly incorporated neurons of about 2 weeks of age but also induced apoptosis of less mature newly incorporated neurons of 5-8 days of age. This supports the idea that cell proliferation, apoptosis and survival of new neurons in the adult brain are interrelated processes regulated by behavioral activity.

However, although positive correlations between the number of new neurons and learning performance imply a relationship between neurogenesis and learning, it is not necessarily a causal one. Moreover, increased/more variable sensory input and/or motor activity are capable of up-regulating adult neurogenesis as well. For example, compared with littermates housed in standard cages, significantly more new neurons were found in the dentate gyrus of mice exposed to an enriched environment (Kempermann et al., 1997). More specifically, long term exposure to an odor-enriched environment increased the number of surviving new 3-week-old neurons in the olfactory bulb (Rochefort & Lledo, 2005). Surprisingly, van Praag et al., 1999, showed that mere voluntary exercise (running), without other components of an enriched environment, was sufficient to enhance neurogenesis. On the other hand, negative emotional activity, including stress and depression, has been shown to be a potent inhibitor of adult hippocampal neurogenesis (Sahay & Hen, 2007).

Numerous studies addressed whether adult neurogenesis is necessary for learning in the tasks dependent on the neurogenic regions by selective (to different extent) ablation of neurogenesis but have not produced an unambiguous body of evidence (see review by Leuner et al., 2006, and discussion in II.4.4 and III.1).

Another approach addressing the functional importance of adult-generated neurons is to assess their activation during certain behaviors. Functional activation of new neurons was

first demonstrated 25 years ago in a landmark study where 1 month after adult canaries were injected with [^{3}H]-thymidine to label new cells, four randomly recorded neurons (out of a total of 74 neurons) within HVC exhibited electrical responses to auditory stimulation and were positive for [^{3}H]-thymidine labeling (Paton & Nottebohm, 1984). However, functionality of such activation is not certain, because the birds were anesthetized, and the replaceable type of HVC neurons does not fire during hearing of the song in awake birds (Rauske et al., 2003; Prather et al., 2008). Thus, the circumstances under which these new neurons would become active in the awake birds are still unknown

Other studies used expression of immediate-early genes (IEGs) as markers of neuronal activity and plasticity and showed that new neurons in the adult rodent dentate gyrus from the age of 2 weeks can be activated by several hippocampus-dependent activities (Jessberger & Kempermann, 2003), and at the age of 4-6 weeks they are even preferentially activated in spatial learning compared to existing neurons (Kee et al., 2007); in the olfactory bulb such preferential activation of new neurons by olfactory stimuli starts even earlier - at the age of 2-3 weeks (Magavi et al., 2005).

Using retrovirus-mediated birth-dating and labeling in combination with electrophysiology analysis, Ge et al., 2007, identified a crucial period when new granule cells (neurons of the dentate gyrus) exhibit NR2B-dependent enhanced LTP with a reduced induction threshold and increased amplitude. This effect is present when new adult-born neurons are 4–6 weeks old and thus these findings may provide a physiological mechanism for experience-dependent preferential recruitment of new neurons into memory circuits as suggested by Kee et al., 2007.

Although by 4 weeks of age, new neurons in the dentate gyrus begin to display the features typical of mature granule cells such as axosomatic, axodendritic and axospinous input, further modification takes place (Espósito et al., 2005; Zhao et al., 2006; Ge et al., 2006, 2007): Newborn dentate granule cells become physiologically and morphologically indistinguishable from the pre-existing population only by about 4 months (van Praag et al., 2002; Laplagne et al., 2006).

Summary of specific properties of adult-born neurons

Adult-born hippocampal neurons of rodents exhibit a number of unique properties that are distinct from the existing mature neurons transiently during their integration and maturation process (also see the scheme on **Fig. 1**), including (i) depolarization by GABA

(<3 weeks old) (Ge et al., 2007), (ii) enhanced excitability and low LTP induction threshold (~2–4 weeks old) (Schmidt-Hieber et al., 2004; Ge et al., 2007), (iii) larger LTP amplitude (~4–6 weeks old) (Ge et al., 2007), and (iv) delayed formation of perisomatic GABAergic innervations (>4 weeks old) (Espósito et al., 2005). In addition, there are also two critical periods when adult-born neurons are subjected to activity-dependent selective integration into the existing neuronal circuitry, including (i) NMDAR-dependent competitive survival among cohorts of new neurons (~2–3 weeks old) (Ma et al., 2009b), and (ii) NR2B-dependent enhanced synaptic plasticity of new neurons (~4–6 weeks old) (Ge et al., 2008). Further studies should elucidate whether adult-born neurons in other systems, like rodent olfactory bulb and avian hippocampus and the song system, also posses these unique features that can allow making specific contributions into behavioral activities.

1.1.2 BrdU as a cell birth marker

Since the 1950's, tritiated ([^{3}H]) thymidine autoradiography has been used to study cell proliferation in situ and to determine the time of origin, migration, lineage and fate of neuronal cells in the developing central nervous system (for example, Sidman et al., 1959). It also provided the first lines of evidence of neurogenesis in the adult mammalian brain (Altman & Das, 1965; Altman, 1969).

5-bromo-2´-deoxyuridine (BrdU) labeling was developed as an alternative approach for determining the proliferative index of tumors (Hoshino et al., 1989). BrdU is a thymidine analog and incorporates DNA of dividing cells during the S-phase of the cell cycle, and *in vitro* studies suggest that BrdU is transported by the same nucleoside transporter as thymidine (Lynch et al., 1977). It is estimated that after systemic injection BrdU is available for incorporation in the adult brain for approximately a 2-h period (Hayes & Nowakowski, 2000).

Since BrdU can be revealed by immunohistochemical staining procedure using a monoclonal antibody (Gratzner, 1982), this method has generally replaced [^{3}H]-thymidine autoradiography and is now the most common tool to track newly generated neurons, despite some restrictions and certain level of ambiguity (reviewed in Taupin, 2007). The main concern is that BrdU is not a marker of the S-phase of the cell cycle per se but a marker of DNA synthesis, which occurs not only during cell proliferation but is also involved in DNA repair, abortive cell cycle reentry and gene duplication. However,

standard protocols used in studies of neurogenesis in the adult brain by BrdU immunohistochemistry are not sensitive enough to label cell undergoing DNA repair, because DNA repair *in vivo* occurs normally through a mechanism that replaces 1–2 nucleotides at each site, as opposed to cell division where the entire genome is replicated (Taupin, 2007). Neither cell cycle reentry is a common mechanism of neurodegeneration, since most dying neurons do not pass the G1/S-phase checkpoint to resume DNA synthesis (Bauer & Patterson, 2005) unless in specific conditions of combined hypoxia and ischemia (Kuan et al., 2004).

Another concern is that BrdU is a toxic substance. The integration of halogenated thymine analogs into the DNA alters its stability, increasing the risk of sister-chromatid exchanges, mutations, DNA double-strand breaks, and lengthens the cell cycle of cells that incorporate it (Bannigan & Langman, 1979; Saffhill & Ockey, 1985; Morris, 1991, Morris et al., 1992). Therefore, BrdU is likely to have toxic effects on newly generated neuronal cells in the adult brain, particularly when BrdU is administered orally or multiple doses are administered (Sekerkova et al., 2004). In the CNS, high doses of BrdU 60–600 mg/kg (body weight, i.p.) trigger neuronal cell death during embryonic and neonatal development (Bannigan, 1985; Nagao et al., 1998). Nevertheless, lower doses of BrdU, e.g. 50 mg/kg of body weigh (single injection), have no apparent toxic effect on development of the cortical ventricular zone (Miller & Nowakowski, 1988; Takahashi et al., 1995). To study adult neurogenesis in rodents and non-human primates, most investigators use 50–100 mg of BrdU per kg of body weight (reviewed in Taupin, 2007). BrdU 50 mg/kg (body weight, i.p.) for up to 12 days, as well as single doses as high as 300 mg/kg have no physiological side effects, like weight loss or behavioral changes in adult rats, and no apparent toxic effect on dividing cells in the DG (Cameron & McKay, 2001; Cooper-Kuhn & Kuhn, 2002).

Thus, under standard conditions and with commonly used protocols, BrdU labeling is a reliable method of cell birth dating during adult neurogenesis.

1.2 **Song control system in the songbird brain**

Like humans, songbirds have specialized vocal areas in the forebrain, which sets them apart from species of birds and mammals that do not exhibit vocal learning. In the latter, vocalizations are controlled on the level of the midbrain (Jarvis, 2004). Similar to humans, songbirds acquire species-specific vocalizations by means of imitative learning during a sensitive (critical) period in which they develop their innate vocal abilities to match vocalizations of a tutor, typically a parent (Marler, 1970; Doupe & Kuhl, 1999). Brain regions of songbirds involved in this process comprise the so-called song system (Nottebohm et al., 1976, 1982).

Functionally, the song system may be divided into three pathways: song production, learning and song perception (see the scheme on **Fig. 2**), which has been revealed in canaries, zebra finches and some other songbirds in numerous studies utilizing lesions, electrophysiological activity recordings, immediate-early genes expression etc. These three pathways are interconnected in HVC (formerly, higher vocal center, currently, abbreviation used as a proper name, Reiner et al., 2004) in the dorsal nidopallium (Reiner et al., 2004). HVC microcircuitry is remarkably complex, consisting of numerous extrinsic inputs as well as local connections (Mooney & Prather, 2005). HVC contains three morphologically and physiologically distinct types of neurons - interneurons and two types of projection neurons with distinct targets (Dutar et al., 1998).

The majority of HVC neurons project to RA (robust nucleus of arcopallium), which in turn controls vocal-respiratory nuclei of the brainstem (Wild, 1993; Wild et al., 2009). Thus, HVC and RA form the telencephalic part of the posterior motor pathway that is necessary for song production (Nottebohm et al., 1976, 1982; Aronov et al., 2008). Induction of immediate-early gene expression in these nuclei was observed as a result of the motor act of singing (Kimpo & Doupe, 1997; Jarvis & Nottebohm, 1997). HVC encodes the temporal sequence in which vocal sounds are generated (Yu & Margoliash, 1996; Hahnloser et al., 2002). HVC-RA projecting neurons generate single bursts of spikes (sparse code) at precisely defined times within the song motif (Hahnloser et al., 2002), and RA transforms this sparse firing to continuous signals that contain a wide range of time scales reflecting the motif, syllable, and subsyllable acoustic structure. These signals ultimately control syringeal and respiratory muscles (Leonardo & Fee, 2005).

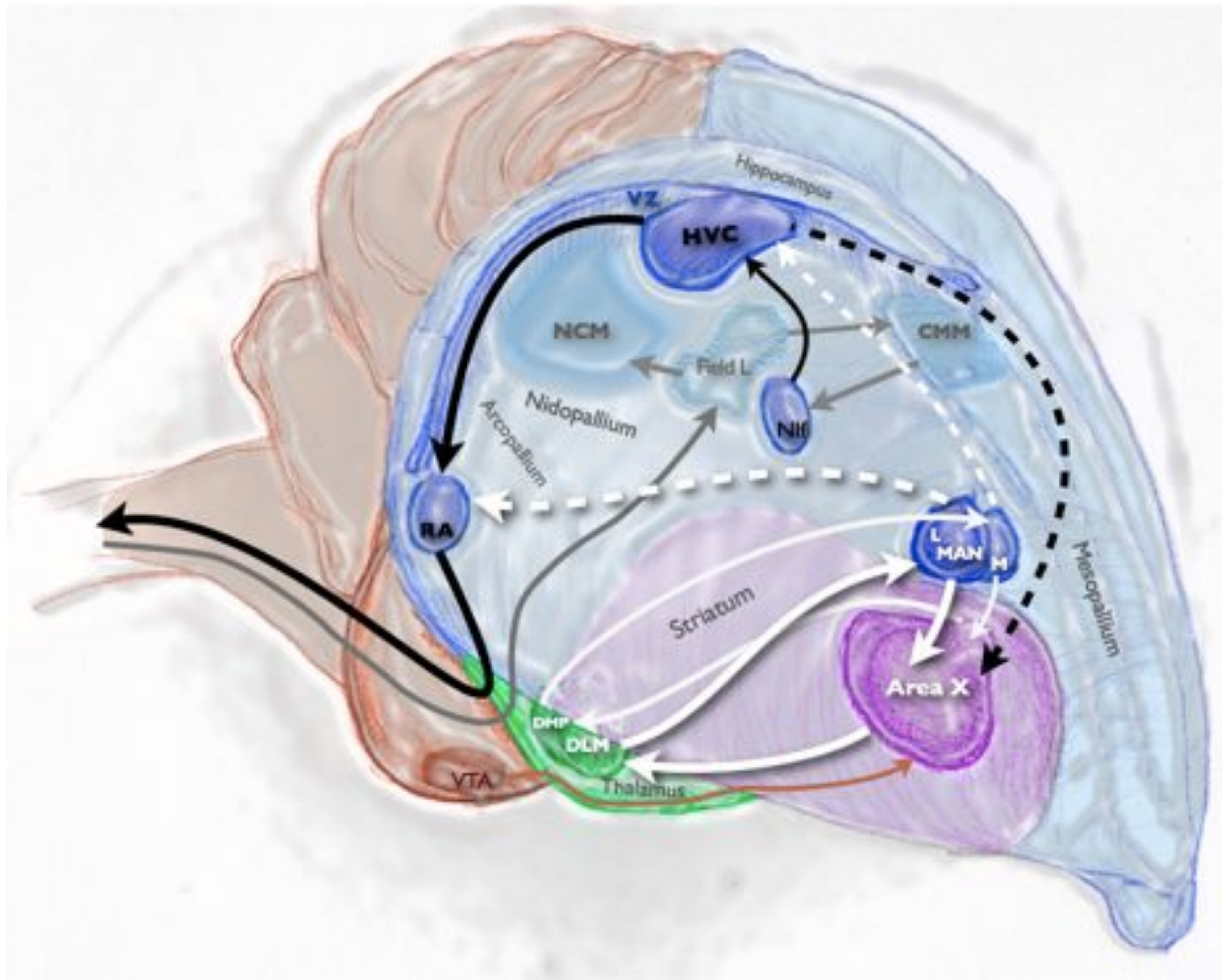


Figure 2. A simplified semi-3D view of the song system in the zebra finch brain. The structures of the song system are organized into three pathways: posterior (connections shown in black), anterior (white; and connections between these two, dashed), and auditory (gray). The posterior pathway is necessary for normal song production in adulthood, as it processes premotor stimulation and directly projects to the brainstem nuclei (not shown) innervating the vocal organ (syrinx) and respiratory muscles involved in singing. Anterior forebrain pathway (AFP) is necessary for vocal learning, as it is involved in recognition of bird's own song, and for some aspects of singing after song crystallization, e.g. related to social context, such as enhanced stereotypy during singing directed to another individual. AFP represents a basal ganglia-thalamo-'cortical' loop and receives input from midbrain areas such as VTA, which may convey context-related stimulation. Shown auditory areas are responsible for perception of species-specific vocalizations and storage of tutor song template. Such auditory information may reach the posterior pathway through NIf. Dorso-caudal part of the ventricular zone (VZ) is shown as well.

Another projection type of HVC neurons targets Area X (Dutar et al., 1998) and thus starts the so-called anterior forebrain pathway (AFP). AFP includes Area X in medial striatum, which is a homolog of mammalian basal ganglia (Person et al., 2008; Jarvis et al., 2005), LMAN (lateral magnocellular nucleus of the anterior nidopallium, which may be homologous to the cortex (Jarvis et al., 2005) and DLM (dorsal lateral nucleus of the medial thalamus), and it forms the basal ganglia-thalamo-'cortical' loop and indirectly connects HVC to RA (Luo et al., 2001; **Fig. 2**). The AFP is essential for the sensorimotor phase of song learning (Bottjer et al., 1984; Scharff & Nottebohm, 1991; Aronov et al., 2008). In adult zebra finches, when the song is stereotyped, activity of AFP becomes rather context dependent (Jarvis et al., 1998; Hessler & Doupe, 1999b) and may add variability to the song (Scharff & Nottebohm, 1991; Williams & Mehta, 1999; Aronov et al., 2008). AFP receives motor stimulation from HVC via HVC-X projecting neurons (Hessler & Doupe, 1999a, 1999b; Troyer & Doupe, 2000), but some neurons of Area X and LMAN are also selectively activated in anesthetized and awake birds by hearing bird's own (Hessler & Doupe, 1999a; Troyer & Doupe, 2000) or tutor's song (Solis & Doupe, 1999; Solis et al., 2000).

For perception of song, the caudal medial nidopallium (NCM) and caudal medial mesopallium (CMM) serve as higher auditory areas, specialized for perception of conspecific song (Mello et al., 1992; Jin & Clayton, 1997; Stripling et al., 1997) and storage of memorized tutor song (Bolhuis et al., 2000; London & Clayton, 2008). In NCM/CMM, enhancement of immediate-early gene expression induced by a song presentation was more robust if this song was similar to the tutor song to which the bird had been exposed during sensitive period (Bolhuis et al., 2000; Terpstra et al., 2004), and ERK signaling, a molecular pathways regulating immediate-early gene expression in this area during tutor exposure, is required for accurate tutor song copying (London & Clayton, 2008).

Finally, afferents from at least one of the HVC input areas, the interfacial nucleus (NIf) (Nottebohm et al., 1982), convey auditory information also to HVC (Cardin & Schmidt, 2004)[6], which underlies the selective responses to the bird's own song that are characteristic of HVC-X neurons (Prather et al., 2008).

---

[6] A recent study (Roy & Mooney, 2009) has shown that lesions of NIf spared selective auditory responses of HVC, so there should be also another source of auditory input to HVC such as CMM.

Song system in the concept of functional systems

One of the striking features of the brain systems underlying a particular behavior is distributed neuroanatomical localization of their elements, which was summarized by P.K. Anokhin in the concept of functional systems, saying that "the most diverse 'anatomical systems' can participate and integrate in a functional system on the basis of their synchronous activation during performance of one or another function of the organism" (Anokhin, 1937 (in Russian); Anokhin, 1974). This pioneering concept featuring feedback loops was developed in 1930's as an alternative to the predominant at those times concept of reflexes and has been used till nowadays in cybernetics and artificial intelligence studies (Red'ko et al., 2004). Other models using networks and feedback loops have been applied to describe long-term memory formation (Butz et al., 2009), speech production (Moore, 2007), and learning to use brain-machine interface (Jimenez et al., 2009).

Vocal learning in songbirds has not been described within the concept of functional systems, but the song control system providing production of species-specific vocalization comprises a well defined functional system. Indeed, the song system includes brain areas that are different in their topography, development and evolutionary origin but unified by the common function – production of species-specific vocalization. Connections and distinct roles of these structures fit well in the scheme of the functional system proposed by P.K. Anokhin (**Fig. 3**). During the sensorimotor phase of vocal learning in young songbirds the song of an adult tutor (typically father) serves as a "stimulus afferentation" (Anokhin, 1974) and is probably processed by higher auditory areas in nidopallium (NCM) and mesopallium (CMM) (Mello et al., 1992; Jin & Clayton, 1997), where it is stored as a template (Bolhuis et al., 2000; Terpstra et al., 2004; London & Clayton, 2008)."Contextual afferentaion" (Anokhin, 1974) during learning includes live contact with the tutor, as fledglings memorize the song from alive tutor better than from loudspeakers, given that they prefer the song of a tutor with whom they have had social contacts (Houx & Ten Cate, 1999; Tchernichovski & Nottebohm, 1998). In adulthood, presence of another individual (usually female) may be "contextual afferentation", as they would sing differently directed to this individual than in solo context (Woolley & Doupe, 2008). "Motivation" for singing, at least its sexual component, is probably mediated through the medial preoptic nucleus (POM) and ventral tegmental area (VTA) (Heimovics & Riters, 2005). NIf and HVC in

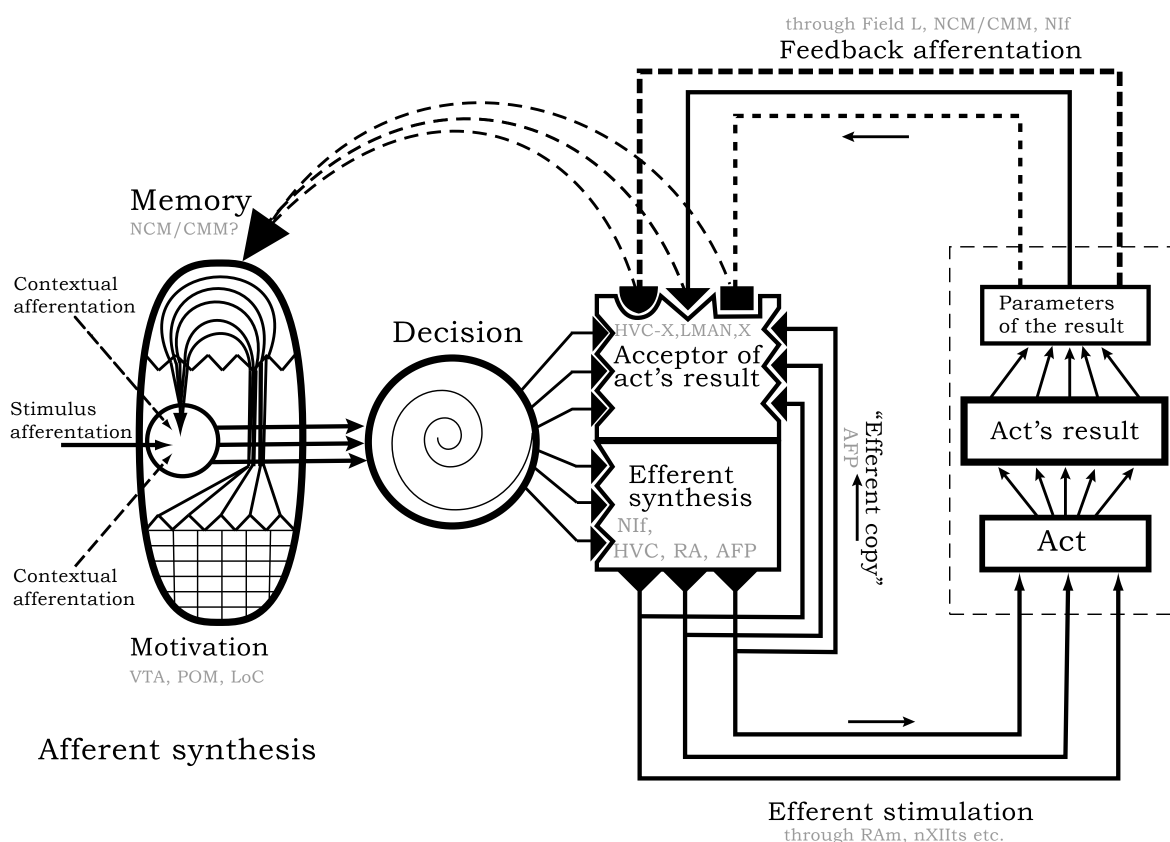


Figure 3. A scheme of the functional system by P.K. Anokhin *(translated from Russian)* and neural correlates in the song system of zebra finches. Operation of the functional system includes: 1) preparation for decision making (afferent synthesis), 2) decision making (selection of an act), 3) forecast of the act's result (generation of acceptor of act's result), 4) backward afferentation (comparison between the actual result and forecast). Neural correlates for the functional system of singing are given in gray. See the text for details.

nidopallium are probably involved in the decision to start singing ("decision making"), as they are the first nuclei of the song system showing premotor electrophysiological activity correlating with the sequencing of syllables (Yu & Margoliash, 1996; Hahnloser et al., 2002; Hahnloser & Fee, 2007). Further on, RA in arcopallium receiving direct efferent input from HVC-RA neurons joins HVC at the stage of "efferent synthesis" (Anokhin, 1974) - premotor activity correlated with different parameters of syllables (Yu & Margoliash, 1996; Hahnloser et al., 2002; Leonardo & Fee, 2005), Simultaneously, an "efference copy" is sent from HVC-X neurons by AFP to RA – through Area X in striatum, DLM in thalamus and LMAN in nidopallium (Hessler & Doupe, 1999a; Troyer & Doupe, 2000), and probably also back to HVC – through the medial part of Area X and MMAN (Kubikova et al., 2007). One of the functions of this efferent copy is perhaps to add information from "contextual afferentation" to the "efferent synthesis", as neural activity in Area X and LMAN is different during directed or undirected singing (Jarvis et al., 1998;

Hessler & Doupe, 1999b; Kao et al., 2005). After "efferent synthesis", the efferent stimuli go to the brainstem nuclei controlling vocal (syrinx) and respiratory muscles (Wild, 1993; Wild et al., 2009) resulting in the motor "act" of singing. Acoustic features of this vocalization ("parameters of the result") are detected by HVC-X neurons (Prather et al., 2008), and specialized LMAN (and to a less extent Area X) neurons that are tuned to bird's own song ("feedback afferentation"; Hessler & Doupe, 1999a; Troyer & Doupe, 2000). Experiments with lesions showed that Area X and LMAN are necessary for the bird's ability to distinguish its own song from those of other conspecifics (Scharff et al., 1998); also, song deterioration caused by deafening (i.e. lack of auditory feedback) requires intact AFP (Solis et al., 2000). This makes such neurons primary candidates for comparison of bird's own song with the "efference copy" in these nuclei (Hessler & Doupe, 1999a; Troyer & Doupe, 2000) and with memorized tutor's song (Solis & Doupe, 1999; Solis et al., 2000), the template of which may be stored in NCM/CMM (Bolhuis et al., 2000; Terpstra et al., 2004). Thus, the AFP, and particularly LMAN, form the "acceptor of act's result", which provides evaluation of matching between expected (stored song template) and actual results (assessment of "feedback afferentation") in order to reach similarity between them by means of correction of efferent stimulation (Solis et al., 2000).

1.3 **Immediate-early genes**

Immediate-early genes (IEGs) are commonly used to visualize neuronal activity. They are the first group of genes to be expressed in neurons following specific extracellular signals. IEGs are operationally defined as those RNAs expressed in the presence of protein synthesis inhibitors, and thereby do not require *de novo* protein synthesis for RNA transcription (Guzowski et al., 2005).

It is generally considered that in the intact mature brain only neurons express IEGs. It should be noted that the only known example of IEG expression in glia is a basal expression of c-Fos by astrocytes in the suprachiasmatic nucleus that increases following perturbation of the circadian rhythm (Bennett & Schwartz, 1994; see review by Herdegen & Leah, 1998).

IEG expression in neurons of resting animals is extremely low and can be due either to genetic programs operating continually and regulated from within the cells (constitutive expression) or to ongoing, physiologically normal input from synapses or neuroactive hormones (basal expression) (Herdegen & Leah, 1998). Expression of IEGs is rapidly and dramatically increased, however, following patterned neural activity associated with the induction of synaptic plasticity (Cole et al., 1989) and neural activity associated with attentive brain states (Guzowski et al., 1999). The molecular cascade of induction and function of IEGs is schematically depicted in **Fig. 4**; some of the IEGs known to be induced in HVC by singing (Wada et al., 2006) are shown on the scheme, but the molecular mechanisms of their activation have not been studied directly in this paradigm.

Amino acids, like glutamate, excite neurons electrophysiologically by acting at AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), kainate, and NMDA receptors, but induce IEG expression principally via the NMDA receptors (Herdegen & Leah, 1998; see **Fig. 4**). IEG induction also follows activation of α_1 and β adrenoreceptors, as well as inhibition of auto-inhibitory α_2 receptors by yohimbine (Bing et al., 1991; Gubits et al., 1989). Stimulation by dopamine agonists in rodent striatum produces IEG expression as well as by glutamate agonists (Berretta et al., 1992), and this expression is probably induced by stimulation of either D1 or D2 receptors in two different populations of striatal projection neurons (summarized in Wirtshafter & Asin, 1995). Input through serotonergic receptors was also shown to modulate expression of IEGs in several brain regions (Humblot et al., 1998; Pei et al., 2000). Other modes of IEG induction include Ca^{++} influx,

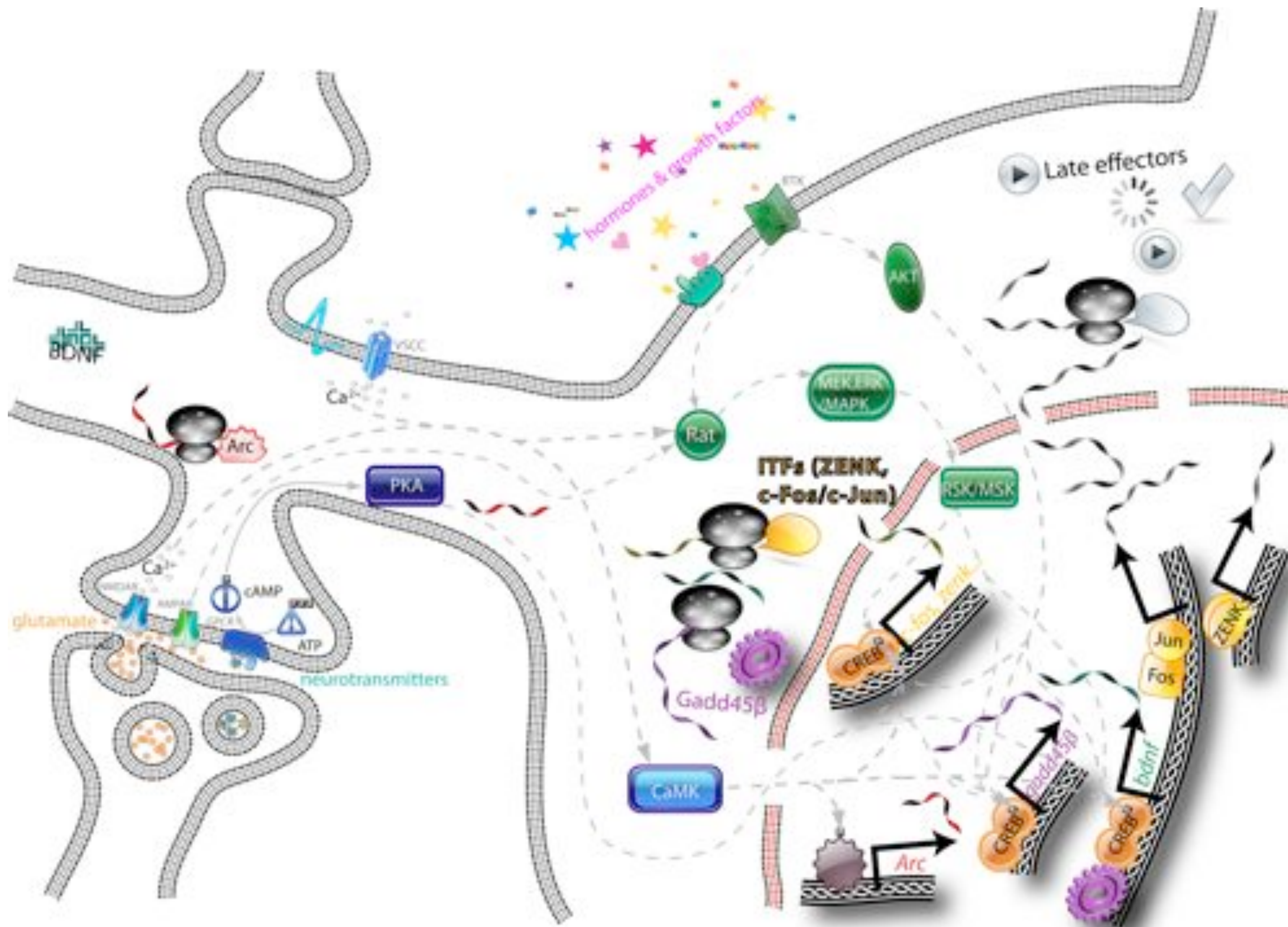


Figure 4. A simplified scheme of the molecular cascade of immediate-early gene (IEG) activation and function in a hypothetical HVC neuron. Activation of IEGs involves multiple steps of phosphorylation (and sometimes as well as dephosphorylation, not shown). The molecular cascade starts from transmembrane receptors, which may be activated by action potential (voltage-sensitive calcium channels, VSCC), glutamate (NMDA and AMPA receptors) and other neurotransmitters (G protein-coupled receptors, GPCR), or from hormones and growth factors (receptor tyrosine kinase, RTK). They activate phosphotransferases (kinases), such as protein kinase A (PKA), Ca2+/calmodulin-dependent protein kinase (CaMK), mitogen-activated protein kinase (MAPK), extracellular signal-regulated protein kinases (ERK), mitogen- and stress-activated protein kinase (MSK) etc. For many IEGs the final complex activating their translation includes phosphorylated CREB. IEGs encode: 1) effectors, such as Arc, involved in synaptic vesicle trafficking, and BDNF, regulating dendrite outgrowth amongst other functions; 2) inducible transcription factors (ITFs), such as c-Fos and ZENK, which regulate expression of late effectors involved in neuronal plasticity. Some IEGs can also regulate expression of other IEGs, e.g. Gadd45β demethylates the gene of BDNF, which is essential for its translation. Based on schemes and data from Alberini (2009), Greer & Greenberg (2008), Clayton (2000), Ma et al. (2009), Velho & Mello (2008). IEGs induced in HVC during singing (Wada et al., 2006) are shown.

some hormones and neurotrophins (reviewed in Herdegen & Leah, 1998; Curran & Morgan, 1987; Clayton, 2000; Greer & Greenberg, 2008; see **Fig. 4**).

IEGs encode a diverse range of proteins, including regulatory transcription factors, structural and scaffolding proteins, signal transduction proteins, growth factors, adhesion molecules, proteases, enzymes (Lanahan & Worley, 1998; Clayton, 2000; Flavell & Greenberg, 2008) and DNA-demethylating proteins (Ma et al., 2009a). The IEGs most commonly used for detection of behaviorally relevant neuronal activation are *Arc* (activity-regulated cytoskeletal-associated gene, aka *Arg3.1*), *c-fos* and *zenk* (aka *zif268*, *egr1*, *ngfi-a* and *krox24*). The former encodes an "effector" protein Arc (activity-regulated cytoskeletal-associated protein) involved in growth of the F-actin cytoskeleton that is thought to underlie stable changes in spine morphology (reviewed in Bramham, 2008). The latter two encode inducible transcription factors c-Fos and ZENK which may indirectly influence cellular physiology by regulating expression of specific “downstream” genes (Herdegen & Leah, 1998; O'Donovan et al., 1999; Clayton, 2000; see **Fig. 4**). The Fos proteins heterodimerize with Jun proteins binding with "leucine zippers"; such dimers form a particular group of inducible transcription factors because only they bind to the AP-1 (activator protein 1) DNA sequence with a high affinity, and the binding of other regulators to this DNA sequence is strictly dependent on forming dimers with them. They can also dimerize with CREB (cyclic AMP response element binding protein) and bind to CRE (cAMP response element) sites and rapidly effect transcription (Herdegen & Leah, 1998; Flavell & Greenberg, 2008; Alberini, 2009). ZENK belongs to the EGR (early growth response) family of transcription factors, a defining feature of which is a highly conserved DNA-binding domain composed of three zinc-finger motifs; together, these fingers recognize a nine-base-pair segment of DNA, with each finger spanning three nucleotides (O'Donovan et al., 1999; Alberini, 2009). ZENK is a bifunctional regulatory protein because the N-terminus is a potent activator of transcription whilst the amino acid segment 281–314 displays repressive effects (Molnar et al., 1994).

Importantly, IEGs are expressed only a limited time after induction which allows one to associate their expression with certain changes in behavior. For example singing induces rapid expression of at least 33 genes in the song system of zebra finches (Wada et al., 2006). A single short stimulus typically initiates a rapid expression of IEG mRNAs that peaks at 15 to 30 min and is gone by 60 min, and an expression of IEG proteins that peaks

at 1h and disappears by 3 to 4h (Herdegen & Leah, 1998; Clayton, 2000). These fast kinetics can also occur when the stimulation is continuous (Stripling et al., 1997). The mRNA and protein expression times can vary amongst individual IEGs and different types of neurons. For example, methamphetamine induces an expression of c-Fos in the sensory cortex and striatum that peaks at 1h and is gone by 3h, whereas ZENK expression in the cortex plateaus for 1 to 2h and disappears only after 6h, and in the striatum it peaks at 1h and returns to basal levels at 6h (Wang et al., 1995). Rapid down-regulation of *c-fos* and *zenk* transcription occurs when the Fos and ZENK proteins cis- or trans-repress (e.g. Fos can repress *zenk*) their own promoter, but there are also other ways they are regulated (Herdegen & Leah, 1998; O'Donovan et al., 1999). IEGs are among the most rapidly degraded of all proteins, suggesting breakdown by the calpain and ubiquitin systems operating through lysosomes and proteasomes (Herdegen & Leah, 1998).

It is worth noting that not all stimulation resulting in firing induces IEGs expression in neurons. For example, in adult zebra finches, hearing a conspecific song triggers ZENK expression in NCM/CMM (Mello et al., 1992), but it is abolished by repetitions of the song, whereas the neurons continue to fire at high rate even after 180 repetitions (Stripling et al., 1997). However, it is possible to re-induce ZENK response after habituation by a novel conspecific song (Mello et al., 1995), or if the repeated song is presented in a novel context (Kruse et al., 2004).

Given both similar and different mechanisms of induction and regulation of different IEGs, it is not surprising that they can be induced simultaneously or differentially by the same stimuli in different brain systems. For instance, Arc shows constitutive expression in adult-born murine hippocampal neurons from 1 to 28 days after their birth but not LTP-induced expression unlike in mature neurons, whereas ZENK needs LTP stimulation for its induction and this takes an effect not earlier than two weeks after birth of these neurons (Kuipers et al., 2009). In juvenile zebra finches, c-Fos induction in NCM after exposure to conspecific song was observed only in females, while ZENK was seen only in males (Bailey & Wade, 2003). But in the study of Velho et al., 2005, on adult zebra finches, in a similar context of song auditory stimulation (and singing), expression of all mentioned above IEGs (c-Fos, ZENK and Arc) co-localized at the cellular level.

Functional gene targeting and knockdown studies have shown that IEG expression plays a crucial role in stabilizing recent changes in synaptic efficacy, and is important for the

molecular processes underlying memory consolidation (reviewed in Guzowski, 2002). It is assumed that c-Fos is one of the essential factors on the initial phase of memory consolidation (Anokhin et al., 1991; Anokhin & Rose, 1991), being a transcription factor for many later genes, products of which may function in morphological or synaptic remodeling of neurons on later phases of memory formation (Patel & Stewart, 1988; Rose & Stewart, 1999; Clayton, 2000). A recent study on songbirds indicates that ZENK and c-Fos regulate transcription of synapsins (Velho & Mello, 2008), which are phosphoproteins associated with the synaptic vesicle membrane and thought to modulate different aspects of synaptic transmission (Greengard et al., 1993; Hilfiker et al., 1999; Gitler et al., 2004). Using administration of antisense oligonucleotides that caused amnesic effect, it was demonstrated that c-Fos expression is necessary for long-term memory formation in chicks (Mileusnic et al., 1996) and rodents (Lamprecht & Dudai, 1996; Morrow et al., 1999). It was also shown that a molecular pathway regulating ZENK expression (extracellular signal–regulated kinase (ERK), according to Cheng & Clayton, 2004) is required for accurate tutor song memorization in songbirds (London & Clayton, 2008). A recent study by Ma et al., 2009a, suggests that a gene called *Gadd45b* is expressed like an IEG in mature hippocampal neurons and up-regulates proliferation of NSCs and arborization of immature adult-born neurons by demethylating genes of paracrine neurotrophic factors. Therefore, IEGs may regulate not only synaptic plasticity, but also plasticity provided by incorporation of new neurons in an activity-dependent manner.

Thus, expression of immediate-early genes can serve as a molecular marker pointing to the neural circuits with behaviorally relevant activation and plasticity (**Fig. 4**; reviewed by Rose, 1991; Clayton, 1997, 2000; Guzowski et al., 2005; Poirier et al., 2008; Alberini, 2009). IEGs have been used extensively to map brain areas in the songbird brain that are activated during song perception (Mello et al., 1992), learning (Whitney et al., 2000) and production (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Jarvis et al., 1998), as well as during stereotyped movements (Feenders et al., 2008).

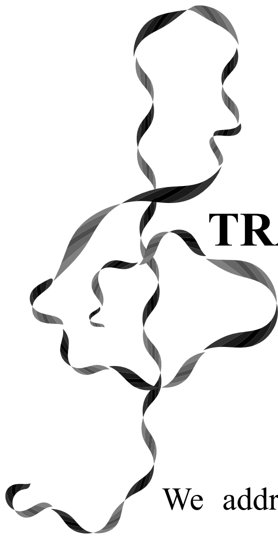
1.4 **Aim of this thesis**

The work presented in this thesis aims at dissecting the functional significance of neurogenesis in the song system of adult zebra finches.

First we addressed functional activation of adult generated neurons in the song system during singing (chapter II). We found that from the age of 3 weeks newly incorporated neurons in HVC and Area X of adult zebra finches expressed activity-dependent IEGs (c-Fos and ZENK) during singing. Surprisingly, in HVC such an activation of new neurons probably is not dependent on their connectivity to the target structure (RA), suggesting that these neurons may participate in processing of premotor information through local microcircuitry within HVC before their axons reach RA. Already at 3 weeks, the population of adult-generated neurons that had been connected to RA by the time of singing did not differ from the pre-existing HVC-RA neurons in the level of IEG induction. Nevertheless, overall, the portion of new neurons that expressed IEGs and were connected to RA increased with their age from 3 to 8 weeks. We addressed whether new HVC neurons are specifically related to vocal plasticity in adult zebra finches by comparing their activation during directed and undirected singing. Although undirected singing involves much higher level of variability (Sossinka & Böhner, 1980; Woolley & Doupe, 2008), we found that new neurons were activated during both types of singing. Nevertheless, the portion of 3-week-old HVC neurons expressing c-Fos was significantly higher among undirected singers than in directed ones. Thus, new neurons in HVC not only replace dying ones of the same type (Kirn et al., 1994; Alvarez-Buylla & Kirn, 1997; Scharff et al., 2000), but, as our data suggest, they may also make specific contributions to the plasticity of vocal production.

I also tried to address the requirement of adult generated HVC neurons for singing (chapter III) by blocking neurogenesis in adult zebra finches with temozolomide (TMZ, active compound of Temodal®). Temozolomide recently has been shown to selectively reduce neurogenesis in adult mice by up to 90% without noticeable side effects (Garthe et al., 2009). Unfortunately I did not detect any effect of TMZ treatment on the rate of cell proliferation in the VZ adjacent to HVC due to extremely high individual variability of this parameter in the adult zebra finches of both treated and control groups.

Finally, I analyzed potential roles in adult neurogenesis for FoxP2, a gene linked to human speech and language impairment (Fisher & Scharff, 2009), and its closest homolog, FoxP1 (chapter IV). We did not find FoxP1 labeling in the VZ of adult zebra finches but confirmed findings of Rochefort et al., 2007, that FoxP2 is expressed in the striatal VZ of songbirds. Nevertheless, FoxP1 is highly expressed in HVC and is evident in one-week old cells. We then addressed if FoxP1 was exclusive to the replaceable type of neurons in HVC (HVC-RA projection neurons), but discovered that it was expressed in both types of HVC projection neurons, but not in parvalbumin-positive interneurons. In combination with previous findings in the striatum of rodents (Tamura et al., 2004), this is consistent with a role of FoxP1 in the development of projection neurons. In addition, comparison of expression patterns suggests that FoxP2 and FoxP1 may relate to adult neurogenesis by down-regulation of the NO pathway.



II EXPERIENCE-DEPENDENT TRANSCRIPTIONAL ACTIVATION OF ADULT-BORN NEURONS IN THE SONG SYSTEM

1 Introduction

We addressed functional activation of adult-born neurons by analysis of IEG expression in the song system of adult zebra finches after singing. IEGs have been used extensively to map brain areas in the songbird brain that are activated during perception (Mello et al., 1992), learning (Whitney et al., 2000) and production (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Jarvis et al., 1998) of vocalizations (for a simplified scheme of the song system see **Fig. 2** and chapter I.1.2). We labeled adult-generated cells with the proliferation marker BrdU (see chapter I.1.1.2; Nowakowski et al., 1989; Taupin, 2007). Only two types of neurons from the areas associated with song production have been documented to be born and incorporated during adulthood: HVC-RA projection neurons (Scotto-Lomassese et al., 2007) and interneurons in Area X, a nucleus of the basal ganglia (Rochefort et al., 2007). HVC-RA projection neurons belong to the posterior, or motor, pathway of the song system. Two to three weeks after birth, new neurons form synaptic connections within HVC (Goldman & Nedergaard, 1992; Burd & Nottebohm, 1985), and send axons to their target area, RA (Kirn et al., 1999). However, it is not known, whether these new neurons are activated during vocal production. We detected IEG expression immuhistochemically and used retrograde tracers to reveal functional integration of new neurons in premotor circuits of the song system. To identify neurons processing vocal production stimulation, after song recordings, we analyzed expression of two IEGs, c-Fos and ZENK, which are associated with neuronal activity and plasticity (see chapter I.1.3 and **Fig. 4**; reviewed by Rose, 1991; Clayton, 1997; Guzowski et al., 2005; Poirier et al., 2008; Alberini, 2009). Expression of c-Fos and ZENK in HVC is linked to the motor act of singing (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997). To track HVC neurons that send their axons to RA, we injected a retrograde tracer (subunit B of cholera toxin, CTB) into RA four days before perfusion, enough time for it to accumulate in the soma of HVC neurons projecting to the site of injection (RA). We then assayed IEG expression in

retrogradely labeled neurons and unlabeled neurons 1, 3, 5 and 8 weeks after their birth in singing or quiet control conditions.

2 Materials & Methods

2.1 Animals and schedule of experiments

Thirty six male zebra finches (*Taeniopygia guttata*) were chosen from our breeding colony at the Free University Berlin, where they were maintained at constant room temperature and 12:12 hr light/dark cycle. All of them were the age of four months or older, the age when their adult song is formed and the song system is mature (Immelman, 1969; Funabiki & Funabiki, 2009). When divided into groups for analysis of neurons of particular age (1, 3, 5 and 8 weeks, see **Fig. 5** for design of the experiments), the birds were distributed evenly according to their ages, because it was shown that age of zebra finches correlates with the level of neurogenesis (Pytte et al., 2007). For song recordings the birds were kept in song recording boxes with a 3 day adaptation period.

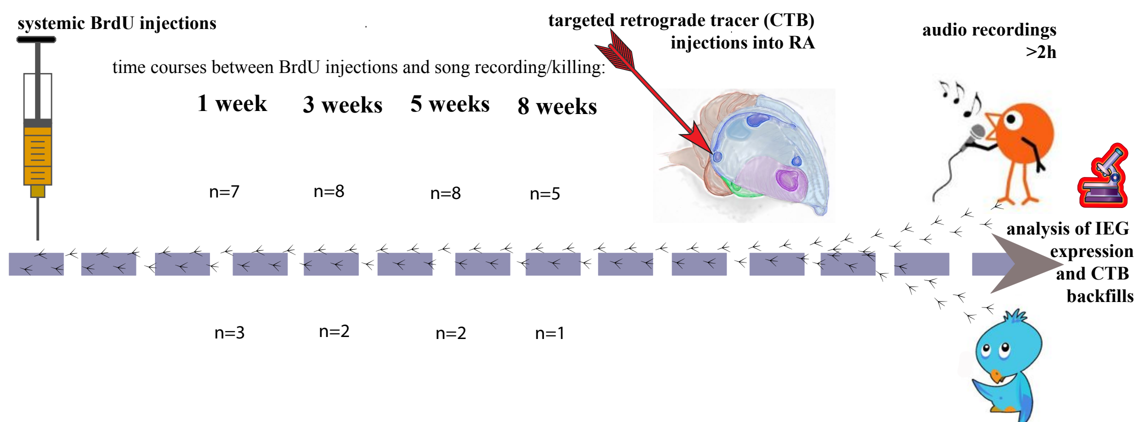


Figure 5. Scheme of the experimental procedures for analysis of singing-driven IEG expression in adult-born neurons. BrdU was injected intramuscularly 3 times per day during first 4 days of the experiment. And 4 days before the end of experiment, a retrograde tracer (CTB) was unilaterally injected into RA with stereotaxic apparatus. One, 3, 5 or 8 weeks after the last BrdU injections the birds' singing behavior was analyzed and recorded for at least 2h before killing. Afterwards, we analyzed connectivity of BrdU+ neurons to RA (judging by CTB backfill) and singing-induced IEG expression.

The experiment with the song group birds (n=28) started on the fourth day immediately after the beginning of the light period. Half of the birds were presented with a female to evoke directed singing, and another half sang spontaneously (undirected singing). The

evoke directed singing, and another half sang spontaneously (undirected singing). The birds' behavior was registered by the experimenter, and their singing was automatically recorded (Sound Analysis Pro 2 software, as described in Tchernichovski et al., 2000; http://forum.sci.ccny.cuny.edu/Members/ofer/sound-analysis-pro) to assure that during the last 90 min before they would be sacrificed, they had sung at least 10 song bouts (succession of unseparated song motifs) to induce sufficient IEG expression (Kruse et al., 2000). After this criterion was reached the birds received anesthesia overdose and then were perfused with PFA (see below, section 2.4).

Eight zebra finches of the quiet control group were maintained and recorded in quiet conditions (closed sound-proof recording chambers overnight) for the last 8 hrs before killing. Audio recording did not detect any singing, and therefore minimal activity was expected. After this period the birds received anesthesia overdose and then were perfused with PFA (see below, section 2.4).

2.2 BrdU injections

BrdU was injected three times daily (Sigma; 50 mg per kg of body weight, dissolved in 0.007 N NaOH solution with 0.9% NaCl) into the pectoral muscle (i.m.) during four successive days 1, 3, 5, or 8 weeks prior to song recordings. This design assessed singing-induced IEG expression in new neurons at different stages of their incorporation into the song system (**Fig. 5**).

2.3 Retrograde labeling of projection neurons in the song nuclei

Four days prior to the song recording and subsequent perfusion, the birds were unilaterally injected into RA with retrograde neuronal tracers using a stereotaxic apparatus (MyNeurolab, St Louis, USA) to label projection neurons in HVC connected to RA. In addition, we injected contralateral Area X to check for possible differences in IEG expression in two populations of HVC projection neurons. The injections were performed unilaterally to reduce the risk to affect singing abilities.

The birds received painkiller Rimadyl® (active is carprofen, dose 25 mg per kg body weight) intramuscularly half hour before the surgery and during it were anesthetized by constant inhalation of isoflurane (Baxter). Isoflurane evaporation was mixed with oxygen at the level of 1-2% and was delivered to the beak through a pipe system at 1l/min flow

rate; they were given another painkiller (Meloxidyl; active is meloxicam, dose 0.1 mg per kg body weight) with food once per day for three consecutive days to eliminate possible discomfort caused by pain sensation after surgery. Importantly, isoflurane anesthesia was shown to have no effect on the rates of neurogenesis in rats (Tung et al., 2008). Stereotaxic coordinates used were initially determined using coordinates derived from the canary atlas of Stokes et al., 1974, and subsequently confirmed empirically. After the bird showed no pain reflexes and the breathing was stable, its head was placed in a stereotaxic head holder at an angle of 45°, and the skin was opened with sharp scissors. The bifurcation point of the midsagittal sinus that lies just anterior to the rostral tip of the cerebellum served as stereotaxic point 0.0 both for the anteroposterior and mediolateral axes. To target RA we used the following coordinates: 1.5 and 1.8 mm posterior, ±2.4 mm lateral, 1.8 and 2 mm below the dura and an injection angle tilted 9° relative to the vertical plane (to avoid passing through HVC, for it lies above RA). To target Area X we penetrated the brain perpendicular to the surface of the brain (90° vertical injection angle relative to the horizontal plane) and used the following coordinates: 3.8 and 4 mm anterior, ±1.5 mm lateral, 4 and 4.2 mm deep. A small opening in the skull above the expected site of injection was made with a curette (delicate bone scraper, FST 100075-16) and pulled off with sharp forceps (Dumont). Then, by slow injection using a hydraulic micromanipulator (Narishige, Japan) approximately 200nL of retrograde tracer solution was delivered through the injection needles with a plunger that had been constructed from pulled capillary tubes (Drummond® Wiretrol), tips of which had been cut to 20–50µm inner diameter. We used cholera toxin subunit B conjugated with deep-red fluorescent dye Alexa fluor 647 (CTB Alexa 647; 0.2% diluted in 0.1M phosphate buffer saline, Molecular Probe, Karlsruhe, Germany). After the injections the piece of the skull from the opening was returned back and the skin was closed with collodion glue. The animals resumed normal activity 5-10 min following surgery and were returned to their home cages.

2.4 Tissue preparation and immunohistochemistry

Each subject was deeply anesthetized and perfused transcardially with saline (NaCl 0.9%) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS; pH 7.4). The brains were then excised and kept overnight in PFA and subsequently overnight in 30% sucrose at 4˚C. Sagittal sections of 40µm were serially cut using a

vibrating microtome (VT1000S, Leica), collected in PBS and stored in cryoprotective solution (25% ethylene glycol, 25% glycerin, and 0.05 mol/L phosphate buffer) at -20˚C.

Six sections containing HVC and/or Area X (at least four containing HVC and two containing Area X, but some contained both) were selected for immunohistochemical analyses. All immunohistochemical reactions were carried out on free floating sections under continuous gentle agitation. Before incubation with primary antibodies the sections were washed in PBS and incubated in 2N HCl at 37˚C for DNA denaturation to reveal incorporated BrdU for the antibodies (rat anti-BrdU; OBT0030, Oxford Biotechnology). Expression of ZENK was detected with rabbit anti-Egr-1 (Egr-1 (C-19):sc-189; Santa Cruz), c-Fos – rabbit anti-Fos (c-Fos, NB100-1875; Novus). Primary antibody incubations were carried out for 24h in PBS/0.3% TritonX100 containing 3% normal goat serum (NGS) at room temperature. Secondary antibody and streptavidin conjugate incubations lasted for 2h in PBS/0.3% TritonX100 at room temperature. Negative controls involved the omission of one or more of the primary antibodies to check for cross-reactivity. To determine the phenotype of neurons, double immunolabelling and detection by retrograde tracers were performed. Four sections with HVC and two sections with Area X from each hemisphere were reacted with combination of antibodies for BrdU (1:200) and ZENK (1:500) or c-Fos (1:1000), which were subsequently revealed with Alexa 488-conjugated donkey anti-rabbit IgG (1:200; A21206, Molecular Probes) and Alexa 568-labelled goat anti-rat IgG (1:200; A11077; Molecular Probes). Afterwards the sections were rinsed and coverslipped with fluorescent mounting medium (Fluoromount G, SouthernBiotech, Alabama, USA).

2.5 Image analysis and quantification

Immunofluorescent sections were analyzed using epifluorescent and confocal microscopes (Zeiss Axiovert 200M inverted microscope and Perkin Elmer Ultraview Spinning disk confocal microscope) equipped with filters for blue, green, red and deep-red fluorescence and coupled to CCD and EMCCD (Electron Multiplying Charge Coupled Device) cameras, respectfully.

To asses cell densities, the immunopositive cells were counted on digitized images from the epifluorescent microscope with a 20x objective using the cell count tool of Image J64 software. IEG+ and CTB+ cells were counted on 145x145μm^2 frame on every second

section analyzed. BrdU+ cells were counted on all stained sections with boundaries of the analyzed structures as the counting frame boundaries. Area X and HVC were defined according to the stereotaxic canary brain atlas (Stokes et al., 1974). HVC was also delimited by the presence of neurons backfilled by the tracers. The structures analyzed in the current work and their localization within the brain are schematically depicted on the **Fig. 2**.

To determine colocalization of BrdU signal with IEG expression and CTB backfill, we examined BrdU+ cells under 40, 63 and 100 magnifications at up to 20 focal planes under epifluorescent and confocal microscopes (**Fig. 8,10,12-13,16**); when colocalization was difficult to determine, 3D images with z-stack and deconvolution were taken with Slidebook Digital Microscopy software (Intelligent Imaging Innovations). This allowed us to calculate the percentage of new neurons (labelled by BrdU) expressing c-Fos/ZENK in relation to all new cells of each structure; in HVC we also examined if BrdU+ neurons were also colocalized with CTB signal, e.g. connected to RA.

2.6 Statistical analyses

The differences in numbers and fractions of immunoreactive cells between singing and quiet control groups were analyzed using t-tests, and between singing birds from different neuron age groups with one-way ANOVA followed by LSD post-hoc test. For assessment of the inter-hemispheric differences within each group, paired-samples t-test was applied (SPSS 16.0 software).

3 Results

3.1 ***IEG induction in telencephalic nuclei of the song system during singing***

Changes in the expression of IEGs such as c-Fos and ZENK are correlated with neuronal firing, and therefore they can be used as neuronal activity markers in the brain (see <u>chapter I.1.3</u>; reviewed by Rose, 1991; Clayton, 1997; Guzowski et al., 2005; Poirier et al., 2008; Alberini, 2009). In preliminary studies, we first established that c-Fos and ZENK expression in HVC is limited to neurons (**Fig. 6**). In our experiments, these genes were induced in all singing birds in HVC (**Fig. 7**) and RA, and in the case of undirected singers also in LMAN and Area X, consistent with previous studies (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Jarvis et al., 1998).

Thanks to the use of the retrograde backfilling, we could also assess expression of IEGs in two populations of HVC projection neurons. ZENK was reported to be activated in both Area X and RA-projecting HVC neurons (Jarvis et al., 1998), whereas c-Fos was found only in the latter type (Kimpo & Doupe, 1997). We found, however, that during undirected singing Area X projecting HVC neurons expressed c-Fos as well (n=2, **Fig. 8A**). Absence of c-Fos induction in this type of HVC neuron in a previous study (Kimpo & Doupe, 1997) was probably due to the fact that all birds used sang directed songs; we also did not find c-Fos expression in these neurons in directed singers (n=2, **Fig. 8B**). This was not consistent through all of the birds (n=1), perhaps because they might perform undirected singing even though in presence of females.

3.2 ***Activation of adult-born neurons in the song system during vocal production***

3.2.1 *Activation of new neurons in HVC*

We next examined whether adult-generated neurons in HVC and Area X are activated during singing. Zebra finches were initially treated with BrdU and their singing activity was then analyzed 1, 3, 5 or 8 weeks later. Zebra finches in each group underwent the same experience before being sacrificed, which therefore ruled out the possibility of nonspecific effects of the experimental procedures (for example, stress and arousal) to influence group differences. Zebra finches were killed following 90 min of the recording in which they had sung more than 10 song bouts, and ZENK/c-Fos and BrdU expression were quantified

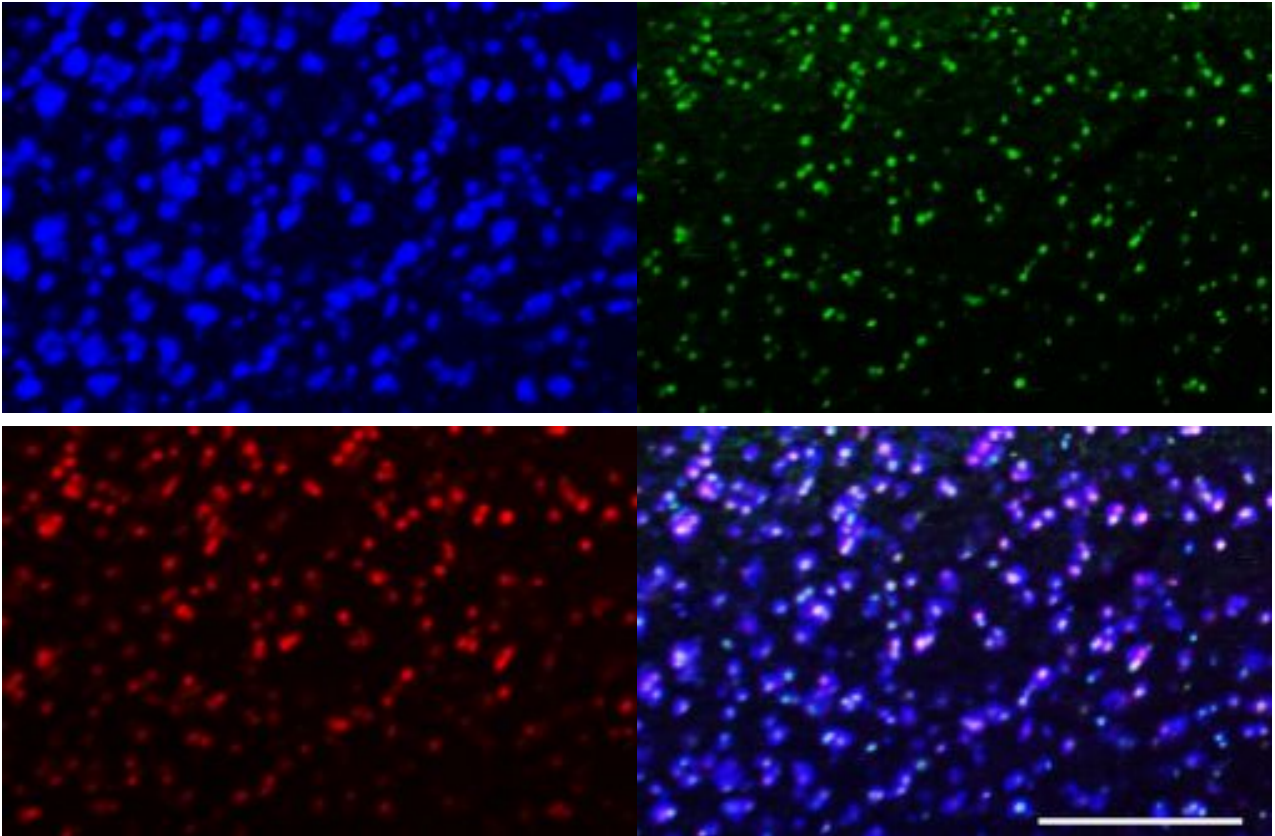


Figure 6. Expression of c-Fos and ZENK is limited to neurons in HVC during singing. Representative images of an area of HVC with NeuN+ cells (neurons, blue), ZENK (green) and c-Fos (red) expressing cells, merged on the lower right image. Of 184 ZENK+ and 177 c-Fos+ cells, all were NeuN+. On average about 60±8.5% (Mean ± SE) of NeuN+ cells expressed c-Fos, and 39±11% ZENK. Scale bar = 100 µm.

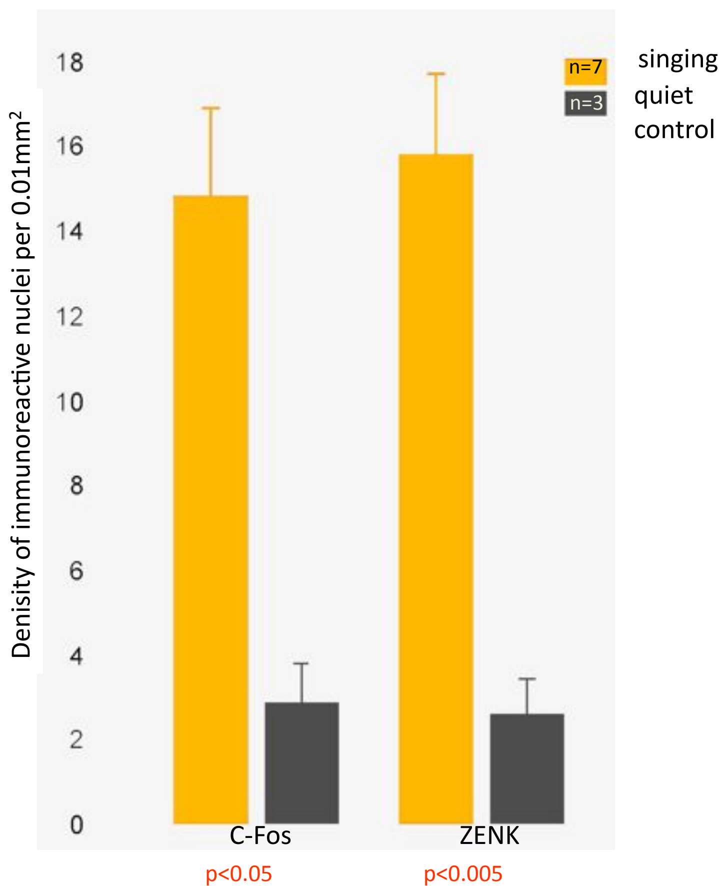
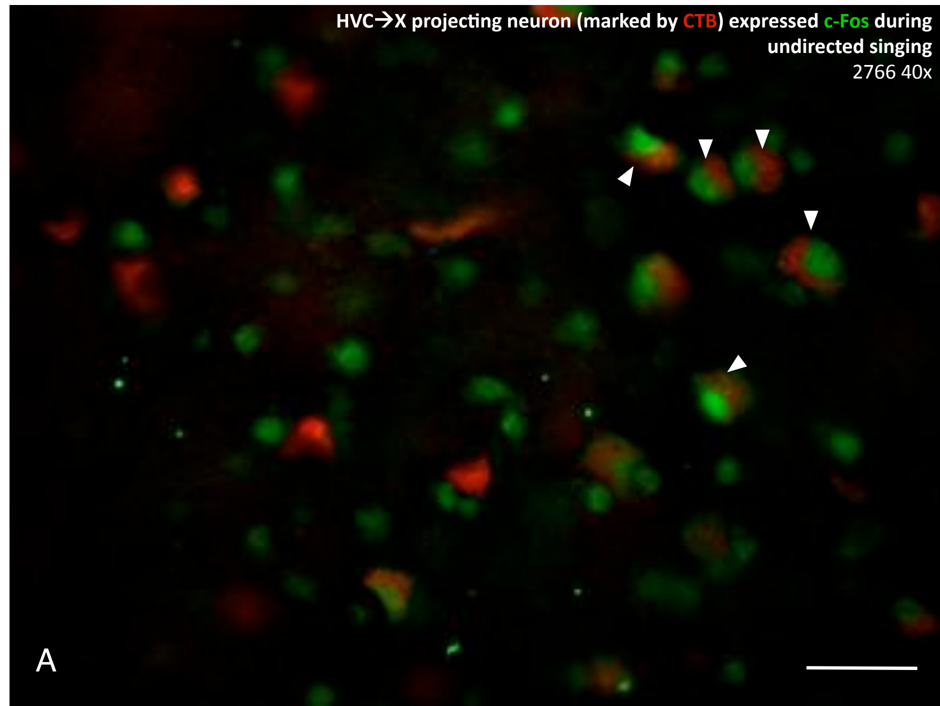
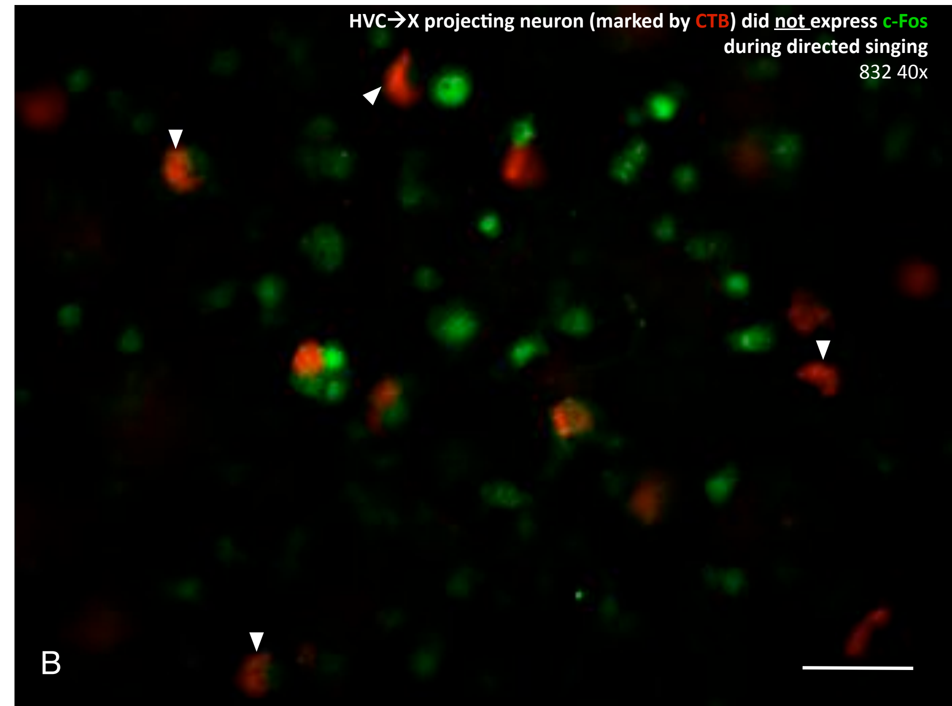
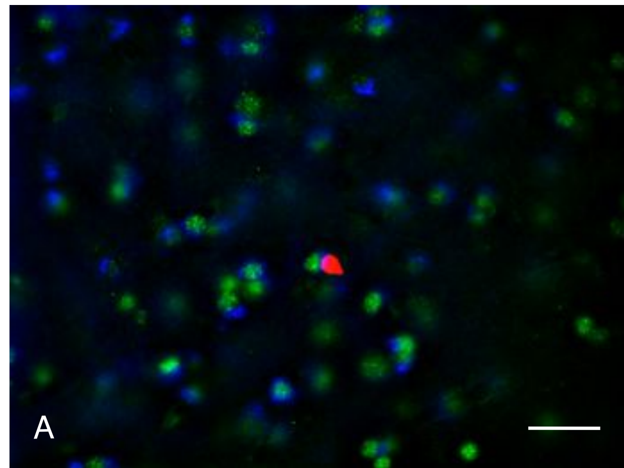


Figure 7. Expression of c-Fos and ZENK in HVC is upregulated during singing. Density of c-Fos+ and ZENK+ neurons was significantly higher in the singing group comparing to the quiet control ($p<0.05$; t-tests).







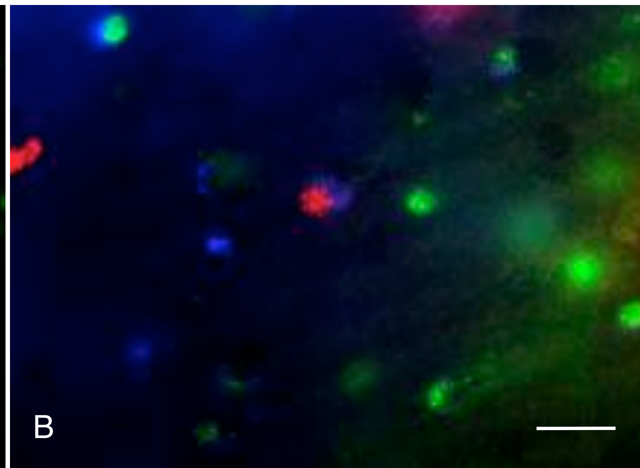


Figure 8. Expression of c-Fos in Area X projection neurons of HVC during singing. Representative images of an area of HVC with c-Fos+ and CTB+ cells. C-Fos was expressed in Area X projection neurons during undirected singing (A), but we failed to find consistent expression of c-Fos in this type of neurons during directed singing (B). Scale bar = 25 µm.

Figure 9. One week old cells of HVC labelled by BrdU did not express ZENK (or c-Fos, not shown) during singing. (***A***) An example of BrdU+ cell one week after BrdU injections which did not contain ZENK although surrounded by ZENK+ neurons and neighboring one of them. Scale bar = 50 µm. (***B***) An example of BrdU+ cell one week after BrdU injections which did not contain ZENK although already connected to RA (contained retrograde tracer CTB). Scale bar = 25 µm.

using immunohistochemical techniques. We predicted that if newly generated BrdU-labeled neurons are incorporated into circuits of the song system involved in song production at the time of experiment, then there should be IEG expression in BrdU+ cells following singing. After the probe test, we identified many c-Fos+, ZENK+ and BrdU+ cells in the HVC and Area X (**Fig. 6,8-10,12,16**).

Across all groups, density of ZENK or c-Fos expressing cells in HVC was similar, 275±20 and 210±20 immunoreactive cells per 0.1mm^2 respectively (P>0.05; ANOVA) (**data not shown**, but see **Table 1**), consistent with the idea that activity in these neurons is a constant attribute of song production (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Jarvis et al., 1998). BrdU+ cells in the HVC and Area X were also identified in all groups of zebra finches. There was a substantial difference, however, between the groups in the extent to which these cell populations overlapped. Most strikingly, the overlap between the IEG+ and BrdU+ populations depended on the age of newborn HVC cells. One week old neurons did not express either of the studied IEGs (out of 718 analyzed BrdU+ HVC cells, none expressed c-Fos or ZENK; for representative images see **Fig. 9**). But singing-driven IEG expression appeared in BrdU+ cells 3, 5 and 8 weeks after BrdU injections (for representative images see **Fig. 10**). As the sacrifice times following BrdU treatment increased, the number of c-Fos+BrdU+ cells increased (P<0.005, ANOVA; **Fig. 11**), and at 8 weeks it was significantly higher than at 3 and 5 (P<0.05, LSD test), suggesting that as adult-generated HVC cells mature, they are increasingly likely to be incorporated into song system circuits supporting song production. Similar dependency was also found for ZENK+BrdU+ neurons (P<0.005, ANOVA; **Fig. 11**), although without such a sharp increase by 8 weeks as with c-Fos (**Fig. 11**).

3.2.2 *Activation of new neurons in Area X*

Likewise, at the age of 1 week none of BrdU+ cells in Area X expressed c-Fos or ZENK, but from 3 weeks on, adult-born Area X neurons express IEGs (**Fig. 12**). However, we could not asses whether there was also an age dependent increase in the portion of new neurons expressing IEGs in Area X due to insufficient number of birds with IEG expression in this brain region, because only half of the birds in each group sang undirected song.

Figure 10. Expression of **IEGs in 8-week old HVC-RA projection neurons during singing.** (***A***) 8-week old neuron (marked by BrdU and CTB) expressed ZENK during singing as well as other HVC neurons. (***B***) 8-week old neuron (marked by BrdU and CTB) expressed c-Fos during singing as well as other HVC neurons. Quasi-confocal images, 63x. (***C***) Expression of IEGs in adult-born neurons during singing was confirmed by confocal microscopy. A representative confocal image showing colocalization of ZENK expression with BrdU signal and CTB backfill. Scale bar = 16 µm.

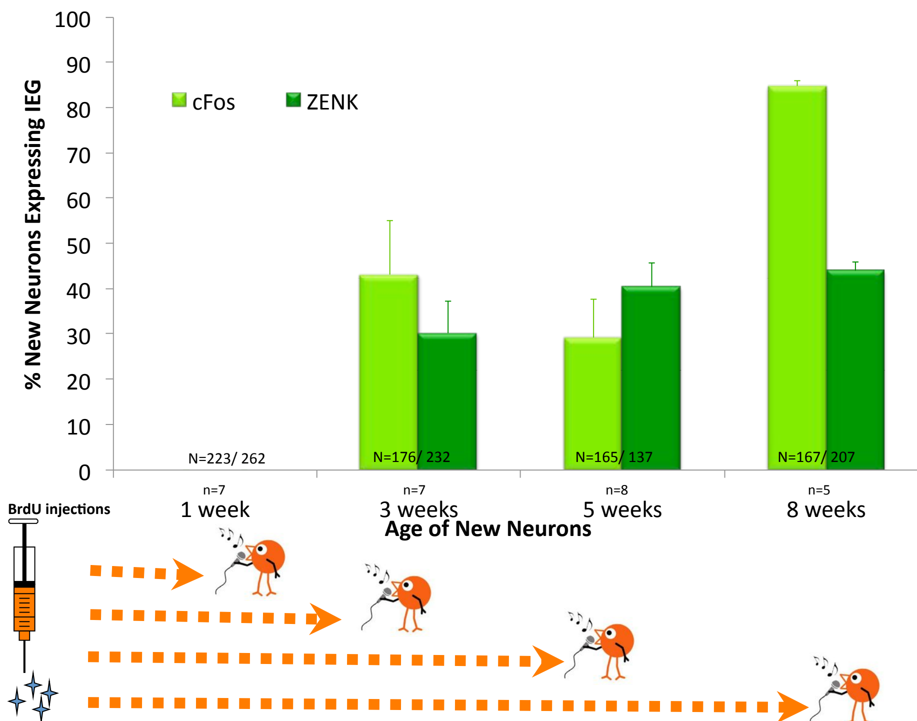


Figure 11. Age-dependent activation of adult-born HVC neurons in singing birds. We found a significant effect of age on probability of IEG expression by adult-born neurons in singing birds ($P<0.05$; ANOVA); and post-hoc test revealed that the percentage of adult-born neurons expressing c-Fos was significantly higher in the birds analyzed 8 weeks after BrdU injections comparing to earlier ages ($P<0.05$; LSD), whereas ZENK was expressed in similar portions of BrdU+ neurons from 3 to 8 weeks after BrdU injections. Also note decreasing variability (error bars) in the levels of IEG expression. N = # of cells analyzed, n = # of animals.

3.2.3 *LMAN - new neurogenic region of the song system?*

Surprisingly, we also discovered adult-born cells in yet another nucleus of the song system - LMAN, which has not been reported before, and they also expressed IEGs during singing (**Fig. 13**). It has been believed that with the exception of Area X and HVC, none of the other song control nuclei receive new neurons in adulthood (Nottebohm, 1993; Alvarez-Buylla & Kirn, 1997). Thanks to the CTB backfill from RA injection we could see the boundaries of LMAN, as its neurons project to RA; this boundary outlined the area of increased ZENK/c-Fos expression in undirected singers. So we could clearly detect that BrdU+ cells were indeed within LMAN limits. However, we did not see CTB+BrdU+ neurons, perhaps because topographically LMAN lies farther from RA than HVC and it may take longer time to establish connectivity between new LMAN neurons and RA, but further analysis is necessary. Nevertheless, it is unlikely that those were glial nuclei, because Kirn et al., 1999, reported that diameter of nuclei of neurons of 2 weeks of age or more is as much as twice bigger than those of glia, and in our work, BrdU+ LMAN nuclei appear even bigger. Also, IEG induction in glia in the intact adult brain to our knowledge has been reported only once in a completely different system (Bennett & Schwartz, 1994) despite the vast number of studies on patterns of IEG expression; c-Fos and ZENK expression was also limited to neurons in HVC after singing (**Fig. 6**).

3.2.4 *New neurons in HVC express IEGs irrespective of the type of singing*

Given that half of the birds sang spontaneously (undirected) and another half directed to a female, we could assess whether new neurons in HVC are activated during both types of singing. Densities of c-Fos+ and ZENK+ nuclei in HVC did not differ between directed and undirected singers ($p>0.05$, t-tests). We also found ZENK+/c-Fos+BrdU+ cells in birds that sang under both conditions. In contrast to c-Fos expression in the existing population of HVC cells, the difference in percentage of activated new neurons between directed and undirected singers reached significant level at the age of 3 weeks (33±19%, n=4, and 51±17%, n=3, respectively; $p<0.05$, t-test) and was marginally significant in 5-week old neurons ($p=0.06$, t-test). Proportions of new neurons expressing c-Fos at the age of 8 weeks and ZENK at all analyzed ages did not differ after directed and undirected singing ($p>0.05$, t-tests).

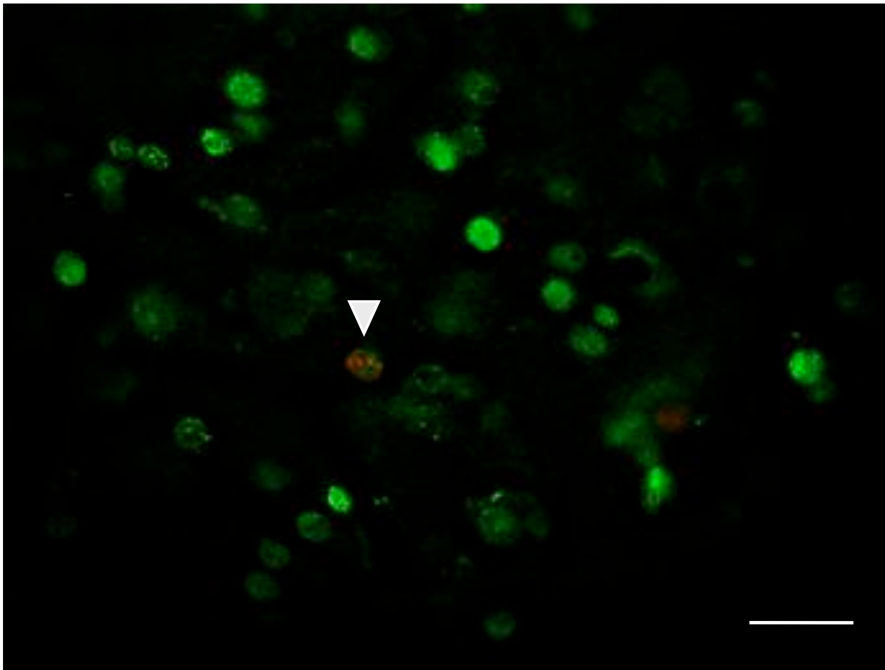
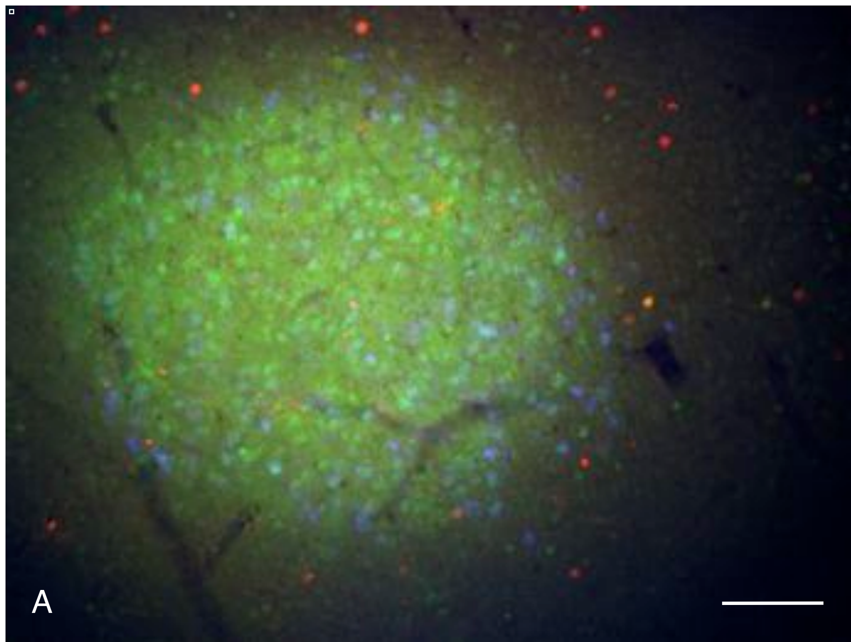


Figure 12. Expression of IEGs in adult-born Area X neurons during singing. A representative image of ZENK+BrdU+ neuron in Area X three weeks after BrdU injections. Scale bar = 25 µm.



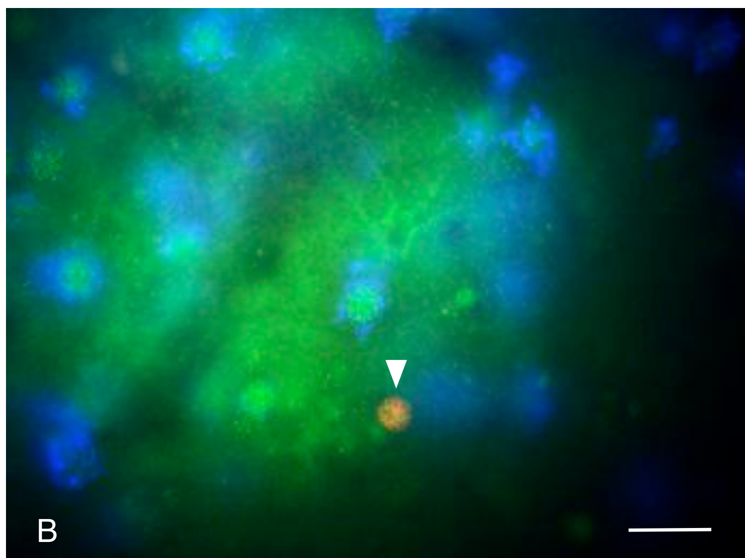


Figure 13. LMAN probably also incorporates functional new neurons during adulthood. (*A*) Representative image of an area of LMAN with 8 week old BrdU+ cells; LMAN is outlined by the backfill of CTB from RA injection and high level of ZENK expression. Scale bar = 100 µm. (*B*) High magnification (40x) quasiconfocal image of such an 8 week old cell within LMAN that expressed ZENK during singing, although not backfilled by CTB. Scale bar = 16 µm.

3.3 *Age-dependent recruitment of new neurons into vocal production circuits*

We next studied whether adult-generated HVC cells are incorporated into premotor circuits in the posterior pathway of the song system by the time they become active (express IEGs). For this we injected the retrograde tracer CTB into RA unilaterally, that allowed us to examine which neurons of the ipsilateral HVC were connected to their projection site, RA. Similar to the work of Kirn et al., 1999, we found that with age new neurons became increasingly connected to RA, although our results showed slightly higher rates of incorporation (**Fig. 14**) and capacity for this axonal connection already from one week of age (**Fig. 9B**), not just from two weeks (Kirn et al., 1999).

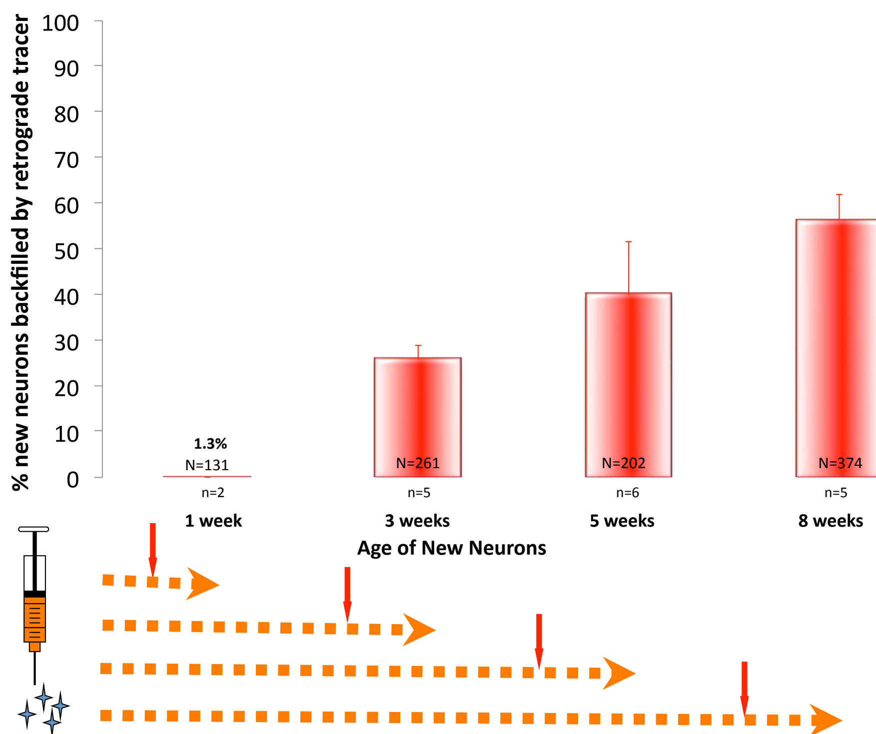


Figure 14. Age-dependent increase in connectivity of HVC-RA projection neurons. The graph shows distribution of portions of new HVC neurons that were backfilled by retrograde tracer (CTB) across different age groups. Although their axons can reach RA as early as one week after birth, only during next weeks substantial portion of them becomes connected and increases with age significantly ($P<0.05$, ANOVA; LSD). N = # of cells analyzed, n = # of animals.

We predicted that if BrdU-labeled neurons were functionally incorporated into motor circuits of the song system at the time of the experiment, then they should contain CTB and express IEGs after singing. And indeed we found many of CTB+ cells that expressed c-Fos or ZENK after singing and were labeled by BrdU injected at least 3 weeks before; and among 8-week old neurons, portions of c-Fos+CTB+BrdU+ and ZENK+CTB+BrdU+ cells were significantly higher than at earlier ages ($P<0.05$, LSD test; **Fig. 15,** ***top***), although the density of CTB+ cells was similar across the groups ($P>0.05$, ANOVA). Our

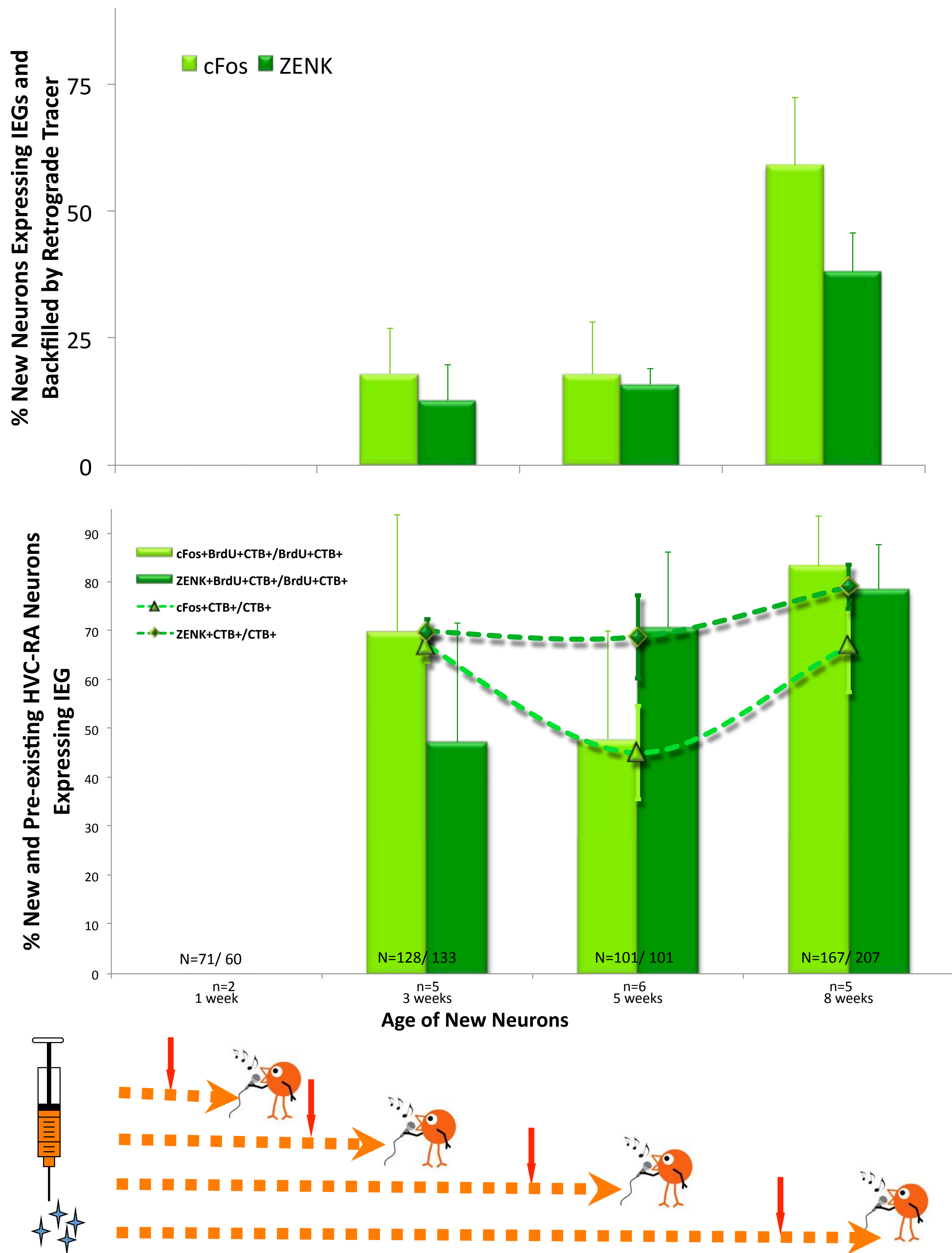
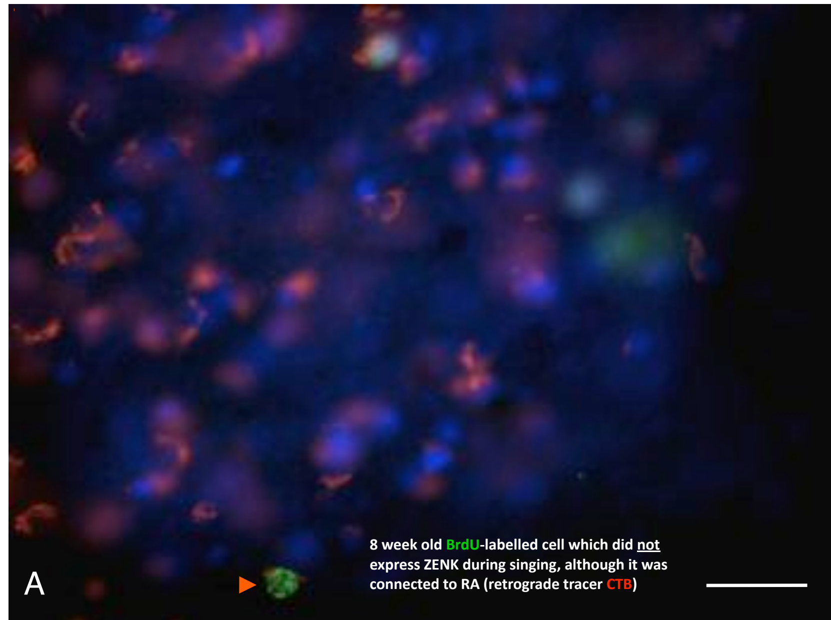


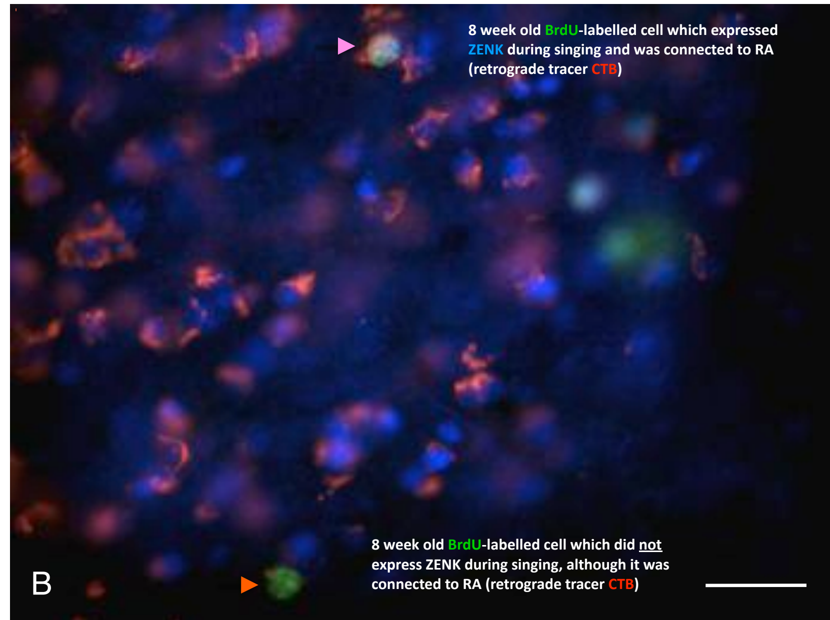
Figure 15. Age-dependent integration of adult-born HVC neurons into premotor circuits of the song system. ***Upper graph*****:** We found significant effect of age on probability of IEG expression during singing in adult-born HVC neurons and their connectivity to RA ($P<0.05$; ANOVA); and post-hoc test revealed that percentage of adult-born neurons connected to RA and expressing IEGs was significantly higher when the singing recordings and subsequent brain analysis were taken 8 weeks after BrdU injections comparing to earlier ages ($P<0.05$; LSD). ***Lower graph*****:** Analysis of backfilled HVC-RA neurons revealed that the percentage of activated new neurons that had been connected to RA was as high as among pre-existing population of HVC-RA neurons starting from 3 weeks, however earlier ages are characterized by higher variability (error bars). N = # of cells analyzed, n = # of animals.

results show, however, that HVC-RA projection neurons can become connected to their target neurons as early as 7 days after their birth (**Fig. 9B**), but even then none of 718 analyzed BrdU+ cells in the 1-week group expressed IEGs. Yet it should be noted that in the 3-, 5- and 8-week groups there were CTB backfilled BrdU+ neurons that did not express IEGs during singing (**Fig. 16A, 17**). And, surprisingly, we found that up to a third of new neurons did express c-Fos or ZENK after singing, although they were not backfilled (**Fig. 16B,17**). Such cells were found in all groups, and their amount did not correlate with the total number of CTB backfilled neurons in HVC of each bird (for example, even a bird with the highest density of CTB+ neurons had 33% of c-Fos+BrdU+ 8-week neurons that were not backfilled), so it is unlikely that all IEG+BrdU+CTB- cells could be a consequence of incomplete retrograde tracer labeling. This suggests that singing-driven activation of new HVC neurons does not depend on synapsing onto RA.

Nevertheless, when the level of IEG activation is assessed only in the backfilled BrdU+ neurons (IEG+CTB+BrdU+/CTB+BrdU+) and compared with that of all existing HVC-RA neurons (IEG+CTB+/CTB+), starting from the age of 3 weeks, there was no statistical difference between portions of activated neurons ($P>0.05$, ANOVA followed by post-hoc LSD test; **Fig. 15, *bottom***). Therefore, once connected, new HVC neurons are not distinguishable from the existing cells of their type by the level of IEG activation during singing already by 3 weeks after their birth.

Altogether, these data support the idea that as adult-generated HVC cells mature, they are increasingly likely to be integrated into the song system circuits and involved in song production.





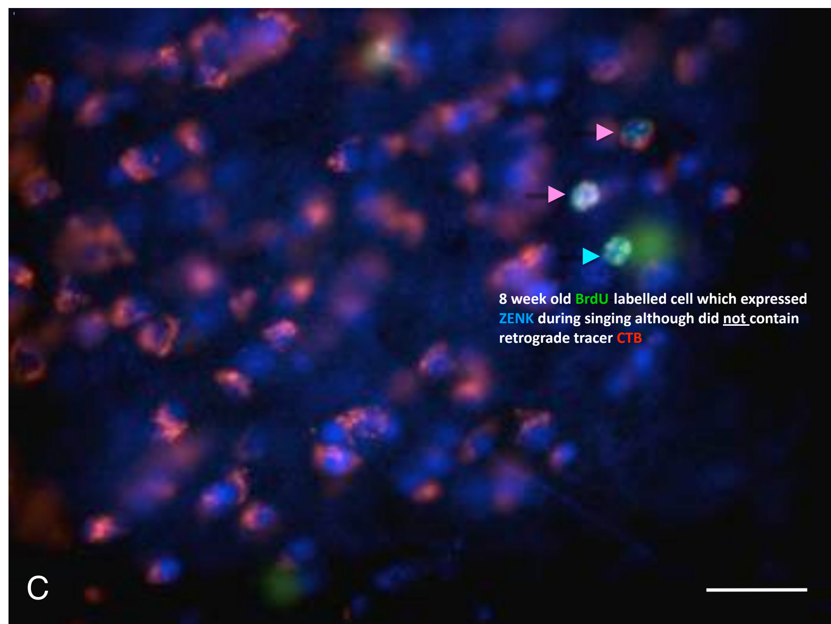


Figure 16. Singing-driven activation of new HVC neurons and their connectivity with the target area are not causally related. Representative quasi-confocal images form z-stack series (A-C) of ZENK expression in 8-week old BrdU+ neurons showing that in the same HVC area in close vicinity there were adult-born neurons connected to RA (CTB backfilled) but not expressing ZENK during singing (***A***, red arrow), such neurons that also did express ZENK (***B***, pink arrow), and also non-backfilled cells that expressed ZENK (***C***, blue arrow) during singing. Scale bar = 25 µm.

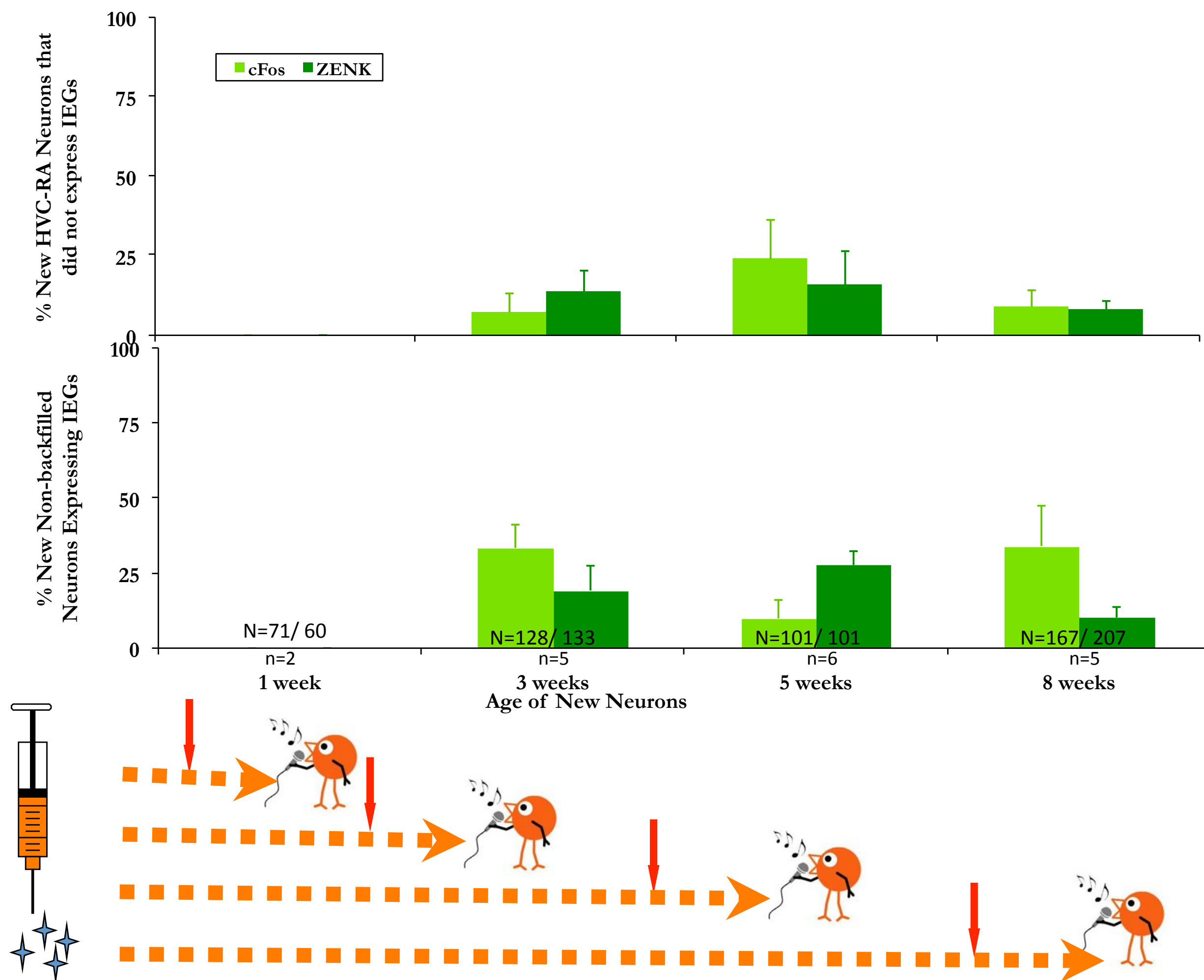


Figure 17. Age distribution of portions of new neurons that were backfilled by retrograde tracer but did not express IEGs during singing and neurons that were not backfilled but expressed IEGs. ***Upper graph*****:** Relatively low percentages of new neurons that were connected to RA but did not express IEGs during singing were found at all analyzed ages. Their amount was not age-dependent (P>0.5; ANOVA). ***Lower graph*****:** Also, among the new neurons of 3-8 weeks of age, significant fractions were found to express IEGs during singing despite lack of connectivity to RA judging by CTB backfills. The percentage of such cells was not age-dependent (P>0.5; ANOVA).

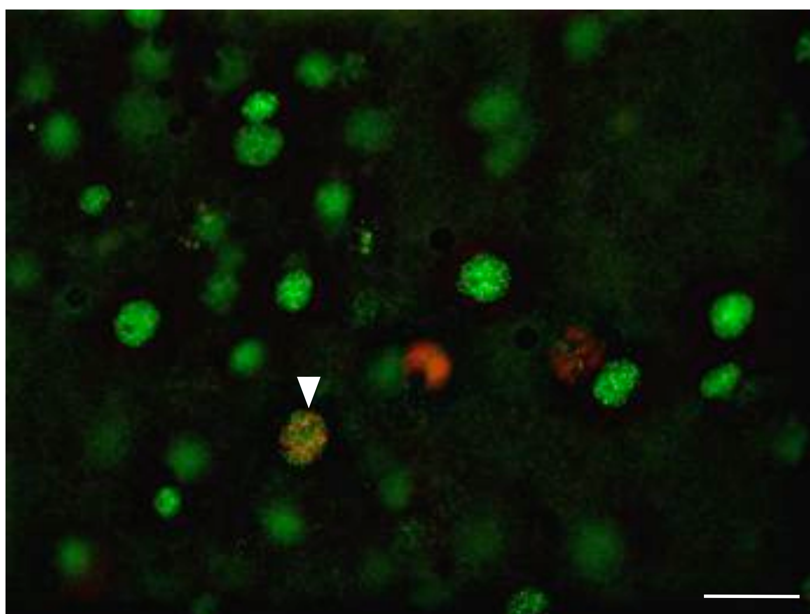


Figure 18. Adult-born IEG+ neurons do not necessarily neighbor other IEG+ neurons. Representative high magnification (63x) multi-plane quasi-confocal image of an area of HVC with 5 week old BrdU+ (red) cells and ZENK expression (green); the arrowhead points to the BrdU+ neuron that expressed ZENK during singing and was not surrounded by other ZENK+ neurons. Scale bar = 16 μm.

3.4 *New neurons are preferentially activated during singing*

Another notable feature of IEG induction in adult-born HVC neurons was that inter-animal variability decreased with the age of neurons: in the 8-week group the percentage of BrdU+ cells expressing IEGs was very similar across birds and its standard deviation was very low (2.7 for c-Fos and 4.3 for ZENK) comparing to 3- and 5-week groups (31.6 and 23.9 for c-Fos, and 18.7 and 15 for ZENK, respectively; **Table 1** and **Fig. 11**). Thus at the age of 8 weeks adult-born neurons are activated during singing at equally high level across individuals, despite the fact that in each group the birds originated from different parents and their age varied from 120 days to 3 years, and most strikingly in spite of larger variability of general density of IEG+ neurons in their HVC (**Table 1**).

Thus our data show specific qualities of IEG expression in adult generated HVC neurons that are dependent on their age.

Table 1. Descriptive statistics of densities of IEG+ neurons and portions of IEG+ neurons among adult-born cells of different age groups in HVC.

	age of neurons, weeks	N	Mean	Std. Deviation	Min	Max
density, ZENK+ per .02mm^2	3	4	46	15	27	64
	5	5	53	17	39	83
	8	5	64	9	54	77
density, c-Fos+ per .02mm^2	3	5	45	8	34	54
	5	5	35	7	24	45
	8	5	51	18	24	68
% ZENK+BrdU+/ BrdU+	3	7	30.2	18.7	0	52.6
	5	8	35.4	19.9	0	56.3
	8	5	44	4.3	36.4	46.8
% cFos+BrdU+/ BrdU+	3	7	43.2	31.6	0	92.3
	5	7	29.3	23.9	0	59.6
	8	5	84.9	2.7	83.3	88.9

4 Discussion

4.1 *Relation to the data on maturation of adult-born neurons*

Our experiments reveal that as adult-generated cells in HVC mature, they are increasingly likely to be recruited into premotor circuits involved in singing. This age-dependent recruitment tracks the morphological and physiological maturation of new neurons according to the data obtained on mammalian hippocampus (reviewed by Ge et al., 2008; Duan et al., 2008; Ma et al., 2009b) and from avian brain explants containing HVC *in vitro* (Goldman & Nedergaard, 1992). After migrating into the HVC from the adjacent ventricular zone (Scott & Lois, 2007), immature neurons in HVC may receive GABA-mediated excitatory synaptic and tonic inputs similarly to the adult-generated neurons in rodent hippocampus (Ge et al., 2008), because about 10% of HVC neurons are GABA-expressing interneurons (Scotto-Lomassese et al., 2007). It would be interesting to examine whether activity-dependent maturation, integration, and survival of new neurons also recruits GABA-mediated regulation in HVC. Adult-generated neurons in HVC form synapses supporting stimulus-evoked and spontaneous action potentials from the second to third week *in vitro* (Goldman & Nedergaard, 1992); and *in vivo* they were reported to obtain three morphologically distinct types of synaptic input from approximately 3 weeks on (Burd & Nottebohm, 1985) and extend their axons into RA (Kirn et al., 1999, and our data, **Fig. 14**). This is consistent with the data on new neurons in the dentate gyrus, where major glutamatergic synaptic activation does not occur until new neurons are 2-4 weeks old, when spines begin to form (reviewed in Duan et al., 2008). Our data indicate that by 3 weeks of age new neurons in the song system obtain glutamatergic input through NMDARs, as IEG expression is induced principally via these receptors (Herdegen & Leah, 1998), and we observed singing-driven IEG expression in new neurons starting from this age. Unlike in the studies of Magavi et al., 2005, in the murine olfactory bulb and Kee et al., 2007, on the dentate gyrus, IEGs were expressed in similar portions of newly incorporated and pre-existing HVC-RA neurons from 3 weeks on. Nevertheless, when inter-animal differences are considered, it appears that at the age of 8 weeks newly incorporated HVC neurons are activated at equally-high levels across different birds in contrast to individually variable IEG induction in pre-existing HVC neurons. This indicates at preferential activation of adult-born HVC neurons of certain age (8 weeks). Perhaps

newly incorporated neurons in HVC have a lower threshold of activation comparing to more mature ones, as was shown for the granule cells in the dentate gyrus and olfactory bulb (Schmidt-Hieber et al., 2004; Mongiat et al., 2009; Nissant et al., 2009).

It has been shown that new neurons in HVC can be electrically active in anesthetized birds in response to an auditory stimulus (Paton & Nottebohm, 1984). However HVC is assumed to play a pivotal role in song production as a motor act (Nottebohm et al., 1976; Yu & Margoliash, 1996; Jarvis & Nottebohm, 1997; Hahnloser et al., 2002), and RA-projecting neurons of HVC (the replaceable type) do not fire during hearing of the song in awake birds, probably due to inhibition provided by local GABAergic interneurons (Rauske et al., 2003; Prather et al., 2008). Due to very limited numbers of recorded neurons in the latter two studies, it is highly probable that the relatively rare newly incorporated HVC-RA neurons were not included in the analysis. Given that for adult-generated neurons in the dentate gyrus GABA provides excitatory (depolarizing) input during the first two weeks unlike for mature neurons (see corresponding section in I.1.1.1; Ge et al., 2008), such young new neurons in HVC could actually fire in response to auditory stimulation also in awake birds but without subsequent IEG induction, although in fact GABAergic stimulation induces c-Fos expression in immature hippocampal neurons *in vitro* (Berninger et al., 1995). This also seems unlikely for adult-born neurons of one month of age that were used in the study of Paton & Nottebohm, 1984, however, as they already express IEGs and hence probably receive normal excitatory glutamatergic input through NMDARs (Herdegen & Leah, 1998).

Notably, expression of IEGs reveals that functional activation of new neurons may appear irrespectively of whether they are connected to their target neurons or not. At all studied neuronal ages starting from 3 weeks, there were non-backfilled neurons that expressed c-Fos or ZENK (**Fig. 16-17**), and as the percentage of backfilled new neurons did not correlate with the success of backfilling, measured by the density of CTB+ cells, this probably reflects individual differences in connection of those new neurons to their targets, even though we cannot rule out that some 'HVC-RA' neurons never connect to RA. Thus, behavior-driven activation of new HVC neurons does not necessarily require established synaptic output to the RA target, although HVC-RA neurons provide effector stimulation. HVC-RA projection neurons, like other types of HVC neurons, extend axonal processes within HVC as well, affording the means for local synaptic processing (Katz &

Gurney, 1981; Mooney, 2000; Mooney & Prather, 2005). It is likely that newly incorporated neurons can participate in this processing even without axons reaching RA. Consistent with the idea of prolonged maturation of adult born neurons (Ge et al., 2006), even by 8 months 20-25% of newborn neurons in HVC of adult canaries are not retrogradely filled, suggesting that they do not have established axonal connections with RA (Kirn et al., 1991), perhaps indicating that maintenance of newly incorporated neurons without efferent outputs outside HVC for such a long time serves some specific function for local synaptic processing in HVC. Nonetheless, once connected, already at 3 weeks after birth new HVC neurons are not distinguishable from the pre-existing cells of their type by the level of IEG activation during singing (**Fig. 15**), whereas it was relatively low among non-backfilled BrdU+ cells (**Fig. 17**).

It is important to know how information about "normal" singing is preserved within a chain of neurons many of which are continually being replaced. It was suggested that critical information is stored in long-lived neurons, such as HVC-X ones, or motor-related information might be transferred from older cells to newer cells within the RA-projecting population and these cells have a way of ‘‘tutoring’’ new RA-projecting cells through gap junctions (Alvarez-Buylla & Kirn, 1997). This idea is supported by ultrastructural studies that showed that HVC cells are often found in clusters and within clusters they make direct soma-soma contact (Burd & Nottebohm, 1985); and gap junctions have been localized to HVC soma membranes (Gahr & Garcia-Segura, 1996). Gap junctions have been shown to serve as channels for the transfer of trophic molecules (reviewed by Guthrie & Gilula, 1989) and apoptotic signals (Lin et al., 1998). In accord with Kirn et al., 1999, and Scotto-Lomassese et al. (unpublished data), we observed that adult-born HVC neurons are sometimes packed tightly together with other neurons, forming cell clusters. But gap junctions are unlikely to be the principal mechanism of how older neurons may influence the maturation of new neurons, because we also found single adult-born HVC neurons that were connected to RA and expressed IEGs but were not surrounded by other backfilled or IEG+ cells (see multiple planes image at **Fig. 18,** and also **Fig. 10**). Local dendritic and axonal connections within HVC microcircuits may be more important for activity-dependent maturation of newly incorporated neurons.

Another way pre-existing HVC neurons may influence adult-born ones could be through paracrine neurotrophic factors. For example, the gene for insulin-like growth

factor II (IGF-II) is expressed by HVC-X neurons and the IGF-II protein is accumulated by the RA-projecting neurons, that raises the possibility that within HVC, IGF-II acts as a paracrine signal between non-replaceable Area X-projecting neurons and replaceable RA-projecting neurons, a mode of action that is compatible with the involvement of IGF-II in the replacement of neurons (Holzenberger et al., 1997). HVC-X neurons also produce retinoic acid, a substance known to have trophic activity (Denisenko-Nehrbass et al., 2000). But BDNF expression is enhanced mostly in HVC-RA neurons and, importantly, is singing-dependent and is correlated with elevated level of survival of adult-born neurons in HVC (Li et al., 2000). It is plausible that this singing-driven BDNF expression is mediated through recently discovered DNA demethylation of its gene by a product of an IEG *Gadd45b* (aka *Gadd45β*) (Ma et al., 2009a, see chapter I.1.1.1), because *Gadd45b* is also induced in HVC by singing (Wada et al., 2006). Furthermore, since we found that adult-born neurons express ZENK and c-Fos during singing, it is likely that they can also express *Gadd45b*, and consequently BDNF, starting from 3 weeks. And as BDNF affects dendritic and axonal morphology (McAllister et al., 1996; Horch et al., 1999), synaptogenesis (Causing et al., 1997) and synaptic efficacy (Lohof et al., 1993; Kang & Schuman, 1995; Patterson et al., 1996), it could provide a mechanism for competitive survival of newly incorporated HVC neurons that are activated during singing, similarly to competitive survival proposed for new neurons in the adult dentate gyrus (Ma et al., 2009b). Yet another target of *Gadd45b* discovered by Ma et al., 2009a, FGF-1, could up-regulate proliferation of NSCs in the VZ adjacent to HVC as well.

4.2 *Are young HVC-RA and mature HVC-X neurons sensitive to the social context of vocalization?*

Male zebra finches, like many other songbirds, perform singing in two contexts: directed to another individual, whether as a part of a courtship "dance" or as a territorial defense, or undirected, either alone or not orienting toward any other bird in particular, that is thought to be used for practice or advertisement (Sossinka & Böhner, 1980; Catchpole & Slater, 1995; Dunn & Zann, 1996). Even in adult zebra finches, who sing relatively stable song after sexual maturation (Immelman, 1969; Funabiki & Funabiki, 2009), undirected singing is significantly more variable (Woolley & Doupe, 2008), suggesting that when the bird is not focused on sending communicative signals to another individual some kind of

motor practicing can take place. Consistent with that, transcriptional activity as of IEGs (Jarvis et al., 1998) and electrophysiological firing (Hessler & Doupe, 1999b) are induced in AFP, which is known to instigate plasticity in singing according to lesion studies (Williams & Mehta, 1999; Aronov et al., 2008).

Also, these studies reported that during directed singing c-Fos is expressed only in HVC-RA projecting neurons (Kimpo & Doupe, 1997) whereas ZENK is expressed in both types of projection neurons in HVC during directed and undirected singing (Jarvis et al., 1998). The present data argue that during undirected singing c-Fos is also induced in HVC-X neurons (**Fig. 8A**) and is expressed in significantly more relatively young HVC-RA neurons during directed singing.

What are the potential functions of this transcriptional activation at the level of -Fos and how are they related to the specific qualities of HVC-X and young HVC-RA neurons? As noted above and in previous studies (Jarvis et al., 1998), relatively variable undirected singing may be a kind of “rehearsal” for relatively stable directed singing. Then perhaps during such a practice the bird is keener on hearing himself, and some premotor neurons could undergo singing-driven plastic changes provided by c-Fos expression but under control of the stimulation from hearing bird's own song (BOS). As was suggested in the model of the song system as a "functional system" (see chapter I.1.2), such comparison of "feedback afferentation" (hearing BOS) with the "efference copy" in HVC-X neurons and AFP might be used for correction of efferent stimulation, and to fix the changes, expression of IEGs such as c-Fos could be necessary in some HVC-X neurons. Intriguingly, findings of Lovell et al., 2008, indicate that the serotonin receptor subunit 5HT1F is enriched in this type of HVC neurons, and serotonin is involved in "the evaluation of future rewards" (for review see Schweighofer et al., 2007; Doya, 2008), so while singing alone, a male zebra finch could anticipate attractiveness of certain acoustic characteristics of his song on females as a delayed reward. Consistent with that, out of three types of HVC neurons, only HVC-X projecting neurons seem to have significant firing activity in response to hearing BOS in awake state (Rauske et al., 2003; Prather et al., 2008), but to our knowledge a case of differential regulation of ZENK and c-Fos by serotonin input has not been reported so far. However, studies on anesthetized birds indicate that all HVC neuron types receive high excitatory BOS-selective stimulation form NIf and differences in their response depend on local microcircuitry involving inhibition by GABA (Rosen & Mooney, 2006). Then, as was

shown for one-month old HVC neurons in anesthetized birds (Paton & Nottebohm, 1984), 3-week old HVC neurons in awake birds could also be responsive to auditory stimulation if GABA still has depolarizing effect on them similar to young hippocampal granule cells (Ge et al., 2008). Expression of c-Fos in these neurons is probably still related to the efferent motor stimulation (Kimpo & Doupe, 1997), but unlike in mature neurons it could be facilitated by GABA depolarizing activity (Berninger et al., 1995). Then, in the concept of selective survival of immature neurons (Ma et al., 2009b), enhanced c-Fos expression may drive plasticity in young HVC-RA neurons whose efferent bursting activity correlate with stimulation from the "feedback afferentation". Further studies are necessary to verify that immature HVC-RA neurons can be responsive to BOS in freely behaving animals and whether it depends on the context of singing, however.

Another hypothesis is that HVC could receive direct input about the social context from motivation related midbrain areas such as the locus coeruleus (LoC) (Mello et al., 1998; Hara et al., 2007) or from VTA and AFP through MMAN (Kubikova et al., 2007). Intriguingly, LoC may provide noradrenergic innervation of HVC (Mello et al., 1998; Hara et al., 2007), and there are certain differences between the responses of ZENK and c-Fos gene expression to the input through adrenergic receptors: For example, administration of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine, a selective noradrenergic neurotoxin, suppresses the basal and light-induced expression of c-Fos in the visual cortex (Yamada et al., 1999) but induces it in the hippocampus in adult rats (Sanders et al., 2008) in contrast to no effect on ZENK expression in the same animals. The noradrenergic system was also shown to be necessary for down-regulation of ZENK during directed singing in Area X (Castelino & Ball, 2005).

In addition, different IEG responses on social context afferentation may be mediated through NMDARs containing different subunits. Sexual maturation in zebra finches, accompanied by crystallization of the song, is characterized by a developmental shift from NR2B to NR2A expression in HVC and other song nuclei (Scott et al., 2004). However, adult-born HVC-RA neurons could recapitulate this process during their maturation, as immunohistochemistry studies show that NR2B subunit (along with NR1, of course) is expressed at early stages of adult hippocampal neurogenesis (Nacher et al., 2007). Also, it is possible that the shift from NR2B to NR2A is more characteristic for HVC-RA neurons, although HVC-X could preserve NR2B expression (but since HVC-RA is the most

numerous neuronal type in HVC (Scotto-Lomassese, unpublished results), this tendency might be taken for the whole HVC). Then stimulation through NR2A-containing NMDARs in both projection neuron types of HVC could induce major ZENK and c-Fos expression related to the motor act of singing, while solo-context stimulation via NR2B-containing NMDARs in HVC-X and young adult-born HVC-RA neurons could evoke additional expression of c-Fos related to specific aspects of undirected singing, such as its higher plasticity. Kubikova et al., 2007, showed that the input from the medial part of AFP (through MMAN), which may convey social context information, influences levels of ZENK expression in HVC, but it was impossible to determine whether it is related to the social context per se, because ZENK expression correlated with the amount of singing and undirected singers sang more than directed singers. But then perhaps stimulation from MMAN through NR2B-containing NMDARs could also regulate c-Fos expression in HVC but in more distinct manner, as found in our experiments. Consistent with this, a study with specific NMDAR inhibitors indicates that NR2B-containing NMDARs are mainly involved in mediating haloperidol-induced c-Fos expression in the medial, or "limbic", striatum, and NR2A-containing NMDARs, in the lateral, or "motor", striatum in rats (Lee & Rajakumar, 2003).

Thus, although c-Fos expression in HVC is driven by singing as a motor act (Kimpo & Doupe, 1997), in HVC-X and young HVC-RA neurons it may be dependent upon hearing bird's own song or presence of another individual. Current knowledge about differences between ZENK and c-Fos and in synaptic inputs to different types of HVC neurons is not sufficient to determine why in the same neuronal population ZENK is expressed during two modes of singing and c-Fos preferentially during undirected song. Nevertheless, we have proposed two hypotheses with possible mechanisms of this finding, as described above.

4.3 *Is activity of new HVC neurons related to vocal plasticity?*

The incorporation of new neurons in the dentate gyrus of hippocampus and olfactory bulb in mice and rats has been extensively studied and is well accepted to have a function in learning and memory (reviewed in Leuner et al., 2006; Zhao et al., 2008). Although numerous attempts to show the requirement of new neurons for hippocampus-dependent spatial learning could not provide unambiguous evidence (Leuner et al., 2006), some

detailed studies succeeded to show that new neurons contribute only to specific aspects of spatial learning (Garthe et al., 2009) or are preferentially activated during particular periods during their maturation (Magavi et al., 2005; Kee et al., 2007).

Using immunohistochemical approaches to visualize new neurons in freely behaving animals, we provide the first evidence that adult-born neurons are activated during vocal production, and that these neurons are effectively integrated into the functional system of singing in an age-dependent manner. To identify BrdU-labeled HVC cells processing premotor activity, we analyzed expression of two immediate-early genes, c-Fos and ZENK, following singing. The expression of both c-Fos and ZENK was limited to neurons at least in HVC, and these genes were also induced in RA, LMAN and Area X of the song system. Significant recruitment of new neurons occurred at least from 3 weeks on after their birth.

The findings of the present work provide evidence that not only do new neurons arrive to the vocal nuclei HVC, Area X and LMAN, but that they become functionally integrated into neuronal networks supporting vocal production. But is there a specific function for these neurons? Do new neurons in the song system also make a unique contribution to the motor control of singing?

Previous studies used similar immunohistochemical approaches to show that adult-generated granule cells in rodent hippocampus can be activated by a variety of stimulation, including water maze training (Jessberger & Kempermann, 2003), spatial exploration (Ramirez-Amaya et al., 2006) and spatial memory (Kee et al., 2007; Trouche et al., 2009); and similarly, also new neurons in the murine olfactory bulb were shown to be activated by olfactory stimulation (Magavi et al., 2005). Although those behavioral procedures involved motor activity, the main focus in all those studies was on whether new neurons respond to environmental stimulation (Magavi et al., 2005; Ramirez-Amaya et al., 2006) or get involved in memory formation (Kee et al., 2007; Trouche et al., 2009). It was also shown that new neurons in HVC fire during auditory stimulation in anesthetized canaries (Paton & Nottebohm, 1984), but normally, in awake birds, this type of neurons does not respond to hearing song (Rauske et al., 2003; Prather et al., 2008). Also, a role in learning was proposed for neurogenesis in the song system (Nottebohm, 2004), because seasonal peaks of incorporation of new neurons in HVC of wild canaries coincide with periods when they acquire new song syllables (Kirn et al., 1994). Given that zebra finches are so called ‘closed’ vocal learners and do not acquire new song syllables in adulthood (Immelman,

1969; Funabiki & Funabiki, 2009), new neurons are probably not involved in vocal learning in the strict sense.

To explain this contradiction, another idea was suggested. New neurons may simply allow more plasticity in neurogenic brain regions (Scott et al., 2000; Pytte et al., 2007), and consistent with this, neurogenesis persists through adulthood also in the zebra finch, but as neurogenesis in HVC decreases with age, so does the variability of acoustic features of their songs (Pytte et al., 2007). Given that this also means that the song becomes more and more stable with age, it is possible that even in adult zebra finches vocal plasticity includes so called "action-based" learning when the songbird selects particular song types out of its "over-productive" repertoire, which may involve "inventions" and "improvisations", for further maintenance (for review see Marler, 1997). Furthermore, a recent study has shown that adult zebra finches amend the fine structure of their song syllables according to the acoustic environment i.e. songs of their cage mates but then can be retuned again when re-exposed to the same tutor they had in youth (Funabiki & Funabiki, 2008), which means that even after sexual maturation zebra finches rely upon the songs of other adult males as a basis for their vocal plasticity, though apparently to a substantially lesser extent than in developmental vocal learning during sensitive period.

Similar to the study of Kirn et al., 1999, we found that new HVC neurons were incorporated into the posterior pathway of the song system, as many of them were backfilled by retrograde tracer injected into RA. Thanks to combination of retrograde tracing with analysis of IEG expression we could also assess functional recruitment of these neurons into premotor circuits in the posterior pathway. Consistent with an enduring role for this pathway in singing as a motor act (Nottebohm et al., 1976; Yu & Margoliash, 1996; Jarvis & Nottebohm, 1997; Hahnloser et al., 2002), our experiments reveal that as adult-generated cells in HVC mature, they are increasingly likely to be recruited into premotor circuits involved in singing. Importantly, our findings also show that new neurons may contribute to the processing of premotor information even before they are connected to RA (**Fig. 16-17**), probably through local connections within HVC.

Although in zebra finches directed singing is stereotyped in contrast to the more variable undirected singing (Sossinka & Böhner, 1980; Woolley & Doupe, 2008), we found that new neurons in HVC were activated also when the birds directed song to other individuals (females), despite the above proposed role of new neurons in plasticity of

singing in adult zebra finches. Apparently undirected singing may involve more newly incorporated neurons than directed singing, and intriguingly such a difference appeared at the earliest age when we detected c-Fos expression in adult-born neurons (3 weeks), but with maturation of new neurons directed and undirected singing seem to induce c-Fos in similar portions of adult-born HVC neurons. In Area X and LMAN, expression of IEGs in new neurons, like in the pre-existing neuronal population, was only induced during undirected singing.

Bearing in mind the hypothesis of activity-dependent competitive survival of new neurons in the rodent hippocampus (Ma et al., 2009b; see also chapter I.1.1.1), one could also imagine that adult-born neurons in HVC undergo selective survival depending on their activation during singing. Within the concept of functional systems (see chapter I.1.2), HVC-RA neurons is a crucial part of the "efferent stimulation" providing the act of singing which in turn is assessed by the "acceptor of the act's result" (e.g. HVC-X neurons and parts of the AFP); then probably newly incorporated HVC-RA neurons whose activation during undirected singing produces a better match to the stored song template could obtain growth factors or other stimulants providing their competitive survival (or alternatively, the mismatching neurons could be eliminated). Thus, with the maturation of new neurons, only the ones 'approved' by the acceptor of the act's result would survive, which could explain why older HVC-RA neurons are activated in directed and undirected singing with similar likelihood. And on other hand, aging birds would accumulate more and more HVC-RA neurons producing a song matching to the stored model, which would explain stabilization of the adult song in zebra finches continuing after their sexual maturation (Pytte et al., 2007).

Since expression of c-Fos and ZENK in HVC, Area X and LMAN is a well known attribute of singing as a motor act and is independent of the acoustic feedback of singing (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Jarvis et al., 1998), and apparently there is no vocal learning in zebra finches in adulthood (Immelman, 1969; Funabiki & Funabiki, 2009), our data suggest that adult-born neurons in these song control nuclei are involved in the motor act of singing. To our knowledge this is the first evidence of activation of adult-born neurons in vocal production, and, although we do not know the exact mechanisms, most probably they contribute to the brain plasticity related to the variability of vocalizations.

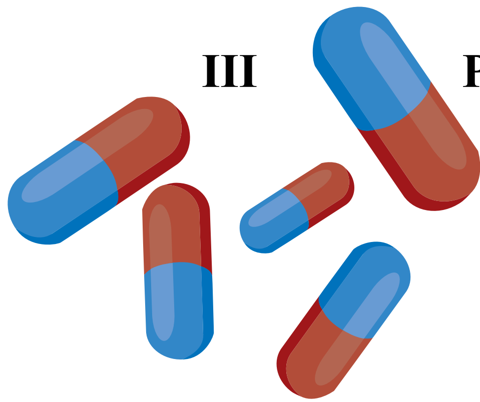
4.4 ***Mapping of IEG expression is an effective approach to the function of adult neurogenesis***

Historically, the first approach to determine the function of adult neurogenesis was an observation of rates of neuronal proliferation and survival in animals in specific behavioral states, particularly learning (see review by Leuner et al., 2006). This approach has been productive in finding correlations (mostly positive) between adult neurogenesis and numerous behavioral activities, including seasonal vocal plasticity in canaries (Kirn et al., 1994). However, correlative evidences cannot contribute to our understanding of causal relations between these processes, although it gives a hint on how adult neurogenesis might contribute to behavioral activity.

Numerous studies have used irradiation, antimitotic agents, or genetic tools to suppress NSC proliferation in order to assess requirement of adult neurogenesis in learning and memory, as discussed in the following chapter III.1. But results across these studies are contradictory. Positive ones are summarized in chapter III.1, but there are probably an equal number of studies that have failed to demonstrate that newly generated cells are involved in hippocampal-dependent learning (Leuner et al., 2006), for example, treatment with antimitotic drug MAM, which significantly reduced the population of new cells, or their depletion by ionizing irradiation did not result in a deficit in spatial learning in the Morris water maze task (Shors et al., 2001, 2002; Madsen et al., 2003; Snyder et al., 2005). This can be explained by the possibility that for some tasks only a limited number of cells may be needed to sustain performance (Shors et al., 2002; Dupret et al., 2005). Moreover, even in the case of complete ablation of neurogenesis, learning may be supported by the remaining mature neurons, and the absence of an effect of inhibition of neurogenesis does not necessarily mean that new neurons do not participate in learning.

The correlative approach has already been used in songbirds to demonstrate a possible relation between the formation of adult-born HVC neurons and the seasonal acquisition of new syllables by canaries (Kirn et al., 1994) or maintenance of vocal plasticity in bengalese and zebra finches (Scott et al., 2000; Pytte et al., 2007). We aimed to establish a pharmacological method of depletion of neurogenesis in the adult zebra finches but failed to show an effect of the tested drug (TMZ) on cell proliferation in the VZ, as discussed in the chapter III.4. But other studies circumvented the issues with ablation of neurogenesis and uncertainties of correlative interpretation by using immunohistochemical approaches

to visualize the activation of new neurons associated with IEG expression in odor discrimination and spatial learning in intact mice, and they identified the ages at which new neurons in the olfactory bulb and hippocampus are preferentially activated in olfactory and spatial memory circuits (Jessberger & Kempermann, 2003; Magavi et al., 2005; Kee et al., 2007). The immunohistochemical analysis of IEG expression, which is an attribute of neuronal activity and plasticity (see chapter I.1.3; reviewed by Rose, 1991; Clayton, 1997; Guzowski et al., 2005; Poirier et al., 2008; Alberini, 2009), proved to be a good method of revealing behavior-driven activation of new neurons also in our work. Such activation during singing was evident only in the new neurons of three weeks or more and increased with age. Thus, these methods allow us to show involvement of new neurons in a particular behavior (singing) and also relate it to the data on maturation of adult-born neurons. It does not, however, answer the question whether new neurons in the song system are required for normal song production. If we are to inhibit neurogenesis in future studies, we will have to maintain the suppression for several weeks, as it seems that new neurons are involved in singing only from about 3 weeks and at 8 weeks may contribute specifically to the song production, expressing c-Fos with higher probability than existing HVC neurons.



III PHARMACOLOGICAL INHIBITION OF ADULT NEUROGENESIS IN THE ZEBRA FINCH?

1 Introduction

The present data revealed that new neurons in the song system of zebra finches are activated during song production, as discussed in the previous chapter II. Based on correlative evidence, neurogenesis in HVC in adult canaries has been linked to vocal learning (Kirn et al., 1994; Nottebohm, 2002) and in adult bengalese and zebra finches to vocal plasticity (Scott et al., 2000; Pytte et al., 2007). Perhaps the only way to obtain definitive evidence for a requirement of new neurons in vocal behavior is by demonstrating deficits in such learning/plasticity following selective depletion of new neurons, as has been done in the studies addressing requirement of adult neurogenesis for the the function of hippocampus and olfactory bulb in rodents, as discussed below. Designing such experiments has been difficult for two major reasons (reviewed by Leuner et al., 2006). The first hurdle is to selectively deplete new neurons without affecting other aspects of brain function. Imayoshi et al., 2008, recently used genetic approaches that specifically and efficiently labeled NSCs and ablated newly formed neurons in the adult brain of mice, but such genetic tools are not (yet) available in birds. Almost all published studies have used either antimitotic drugs or irradiation to decrease adult neurogenesis, but both of these methods can induce nonspecific effects on performance or brain function raising the possibility of false positive results. For example, an artificial decrease of adult neurogenesis in HVC was achieved after disrupting the critical retinoic acid pathway by disulfiram, but it was not possible to dissect whether this was not an effect of neurotoxicity of the treatment (Roeske, 2009). Second, the timing and duration of neuron depletion may be a critical factor, as new neurons may be required for learning/plasticity only during a discrete period after their formation. False negative results could occur if neuron depletion is insufficient in length or the interval between depletion and training is inappropriate.

The antimitotic agent methylazomethanol acetate (MAM) has been used to block adult neurogenesis in rats (Shors et al., 2001, 2002). A substantial reduction in the number of adult born cells resulting from MAM treatment over a 2-week period was associated with

an impaired ability to acquire the trace eyeblink conditioning task. Similar treatment in a separate group of animals was not associated with deficits during training on delay conditioning, using parameters that do not depend on the hippocampus. When the population of new neurons was allowed to replenish itself, the ability to acquire trace memories was restored (Shors et al., 2001). Although such findings suggest a causal relationship between neurogenesis and learning, it is impossible to rule out the possibility that MAM's effects are associated with its side effects. First, being an anti-mitotic drug, it can affect not only NSCs but any other dividing cells including glia which may influence brain functions. And although known negative effects of high doses of MAM on general health were avoided in studies showing learning impairments (Shors et al., 2001, 2002; Bruel-Jungerman et al., 2005) by using lower but still efficient doses (Shors et al., 2001; Dupret et al., 2005), it is possible that undetectable yet detrimental effects of the drug on health or performance could contribute to the deficits in learning.

To circumvent some of the problems associated with systemic administration of cytostatic agents such as MAM, some studies have used localized irradiation to reduce the cell production in the dentate gyrus (reviewed by Wojtowicz, 2006). For example, the performance of irradiated rats was impaired on a hippocampal-dependent place recognition task, but not on an object recognition task, which is not dependent on the hippocampus (Madsen et al., 2003; Rola et al., 2004). Irradiation has the advantage over antimitotic agents in that the population of new cells is usually depleted completely rather than just reduced and it seems that neural progenitors are much more sensitive to it than glia (Wojtowicz, 2006), so possible conclusions regarding the involvement of adult-generated neurons in learning become more convincing. However, like the antimitotic agents, there are some possible side effects of using irradiation to block adult neurogenesis, which could inadvertently affect performance. Most notably, irradiation can induce inflammatory responses, which can impact aspects of behavior and physiology that may in turn impact performance during learning tasks (Monje et al., 2002; Rola et al., 2004). Thus, the extent to which cognitive deficits following irradiation are attributable to a loss of newly born cells in the dentate gyrus remains unknown.

Our attention was drawn to temozolomide (TMZ, active compound of Temodal®), a drug used to treat brain tumors in humans (EMEA, 2005; Rhee et al., 2009) without detectable toxic effects when applied as a single agent (Schiff et al., 2009). Recently it has

been shown to selectively reduce neurogenesis in adult mice by up to 90% and without side effects on general health and activity and even production of microglia during one month treatment and afterwards (Garthe et al., 2009).

Hence, TMZ appeared to us as a perfect candidate to try to inhibit neurogenesis in songbirds and check the consequent effects on vocalizations. First we had to establish whether it has an effect in zebra finches, however. We used BrdU-labeling of recently divided cells in the VZ adjacent to HVC but failed to find differences between treated and non-treated groups of animals due to very high individual differences in the rates of cell proliferation.

2 Materials & Methods

2.1 Animals and schedule of treatment

Twenty two mature (>100 days) male zebra finches (*Taeniopygia guttata*) were chosen from our breeding colony at the Free University Berlin, where they were maintained at constant room temperature and 12:12 hr light/dark cycle. When divided into groups, the birds were distributed evenly according to their ages, because it was shown that age of zebra finches correlates with the level of neurogenesis (Pytte et al., 2007); we also took care not to allocate brothers only in one experimental group, as the origin of zebra finches influences rates of adult neurogenesis (Hurley et al., 2008).

The birds underwent series of injections of TMZ or saline (control group) in one or two cycles followed by of BrdU injections one day before killing (one cycle is one week: 3 days of single TMZ/saline injections and 4 days of recovery, see the scheme on **Fig. 19**) (Rhee et al., 2009).

The day after BrdU injection the birds received anesthesia overdose and then were perfused with PFA (see below).

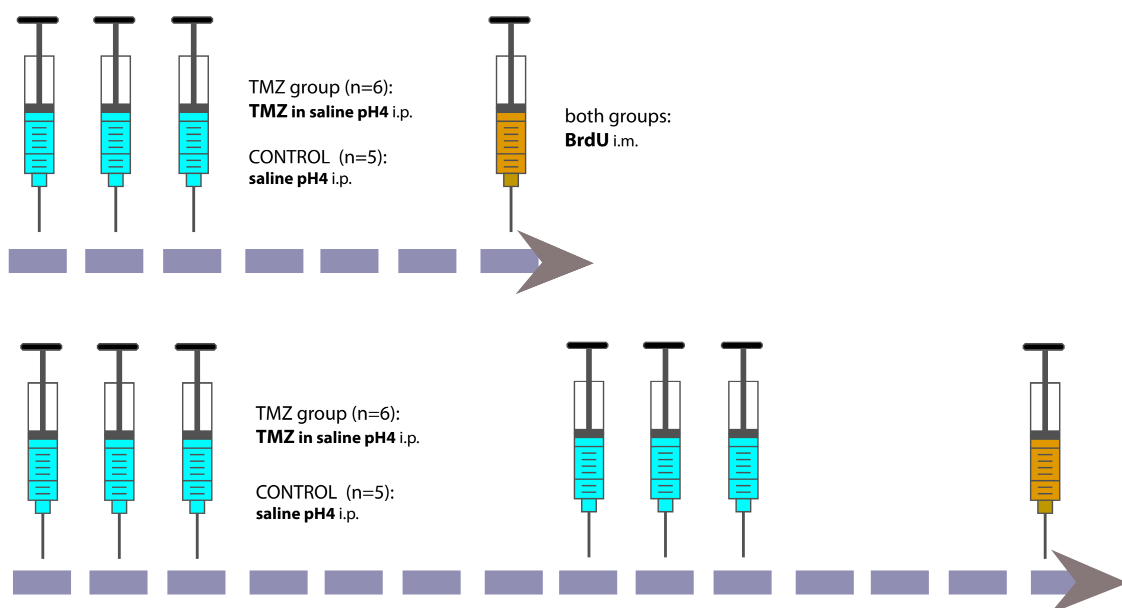


Figure 19. Schedule of TMZ and BrdU injections. The birds of TMZ group underwent either one or two cycles of temozolomide (TMZ) treatment, each consisted of 3 daily intraperitoneal (i.p.) injections followed by 4 recovery days. Control groups underwent similar procedures with sham injections of saline pH4. BrdU was injected intramuscularly (i.m.) 5 times every 2h on the last day of the experiment.

2.2 Injections of TMZ

Temozolomide (TMZ, Temodal®, SP Europe) is a lipid-soluble methylating agent that readily crosses the blood–brain barrier (Patel et al., 2003). It was found to be effective in suppressing adult neurogenesis in mice at a dose of 25 mg/kg body weight (Garthe et al., 2009). Following this study, we injected the zebra finches with TMZ at 25 mg/kg body weight (i.p., 2,5 mg/ml in 0.9% NaCl, pH4) once per day, whereas the control group received sham injections of saline pH4 only. This regimen was given on the first three days of a week for either one or two weeks to resemble paradigms used for glioma treatment in humans (**Fig. 19**).

Fresh TMZ solution was prepared on the day of injection from two commercial pills of Temodal® (SP Europe). The content (powder) of two 5 mg capsules was put into 15 ml Falcon tube and dissolved in 4ml 0.9%NaCl and about 100µl 1N HCl to achieve pH4, which is necessary to prevent deterioration of TMZ (Garthe A., personal communication). After that the solution was placed for 10 min on a shaker and left for sedimentation. The supernatant was filtered with sterile 0.22µm filter and aliquoted by 1ml in Eppendorf tubes. Animals were injected with 100µl of this solution per 10g of body weight.

2.3 BrdU injections

We labeled newly divided cells with a DNA synthesis marker BrdU (Sigma). One day before killing, each bird received 5 BrdU injections (every 2h). BrdU was administered into the pectoral muscle (50mg/kg of body weight per injection, dissolved in 0.007 N NaOH solution with 0.9% NaCl). This design allowed us to assess numbers of the newborn one day old cells in the VZ.

2.4 Immunohistochemistry

Each subject was deeply anesthetized and perfused transcardially with saline (NaCl 0.9%) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS; pH 7.4). The brains were then excised and kept overnight in PFA and subsequently overnight in 30% sucrose at 4˚C. Sagittal sections of 40µm were serially cut using a vibrating microtome (VT1000S, Leica), collected in PBS and stored in cryoprotective solution (25% ethylene glycol, 25% glycerin, and 0.05 mol/L phosphate buffer) at -20˚C.

Four sections with HVC from each hemisphere were selected for immunohistochemical analyses. All immunohistochemical reactions were carried out on free floating sections under continuous agitation. Before incubation with primary antibodies the sections were washed in PBS and incubated in 2N HCl at 37˚C for DNA denaturation to reveal incorporated BrdU for the antibodies (rat anti-BrdU, OBT0030, Oxford Biotechnology; diluted 1:200). Primary antibody incubations were carried out for 24h in PBS/0.3% TritonX100 containing 3% normal goat serum (NGS) at room temperature. Incubation with secondary antibody (Alexa 568-labelled goat anti-rat IgG , Molecular Probe; 1:200) lasted for 2h in PBS/0.3% TritonX100 at room temperature. Negative controls involved omission of the primary antibody to check for unspecific staining. Afterwards the sections were rinsed and cover-slipped with fluorescent mounting medium (Fluoromount G, SouthernBiotech, Alabama, USA).

2.5 Quantification of newly-generated cells in the ventricular zone adjacent to HVC

To estimate rates of neurogenesis we counted BrdU+ cells in the VZ adjacent to HVC. According to the study of Scott & Lois, 2007, HVC-RA projecting neurons originate from this part of the VZ, and this is the only type of neurons undergoing replacement in adult zebra finches (Scotto-Lomassese et al., 2007). The counting was done at 20x magnification, as newly-generated one day old cells are all aligned to the VZ and it is easy to distinguish as separate cells. In rare cases of extremely dense and small neurogenic areas, higher magnifications were used. The numbers of BrdU+ cells were divided by the length of the VZ adjacent to HVC, and thus we obtained average densities of one day old cells per mm of the VZ.

Statistical differences between groups were assessed with one-way ANOVA followed by LSD post-hoc test (SPSS 16.0 software).

3 Results

All BrdU+ nuclei in the vicinity of HVC were aligned to the walls of the ventricle, consistent with previous data that newborn cells do not yet migrate perpendicularly away from the VZ at the age of one day (Barami et al., 1995).

Our data showed very high individual variability of rates of neurogenesis within each group (**Table 2**), and no effect of TMZ treatment on the densities of newborn cells was found (P>0.5, ANOVA; **Fig. 20**).

Neither did we observe any effect of TMZ (or saline) injections on general health and behavior of the birds; body weight remained the same over the time of the experiment (P>0.5, ANOVA; **data not shown**).

Table 2. Descriptive statistics of densities of one day old cells per mm of the VZ above HVC in adult zebra finches treated with TMZ or saline. Note the large differences between minimal and maximal values in each group.

Treatment	N	Mean	Std. Deviation	Std. Error	Min	Max
saline, 1 cycle	5	13.4460	6.24558	2.79311	6.38	20.67
TMZ, 1 cycle	6	10.4483	9.25257	3.77735	2.88	24.88
saline, 2 cycles	5	19.6260	13.81668	6.17901	6.75	41.75
TMZ, 2 cycles	6	15.6633	13.38106	5.46280	4.75	41.63
Total	22	14.6377	10.88047	2.31972	2.88	41.75

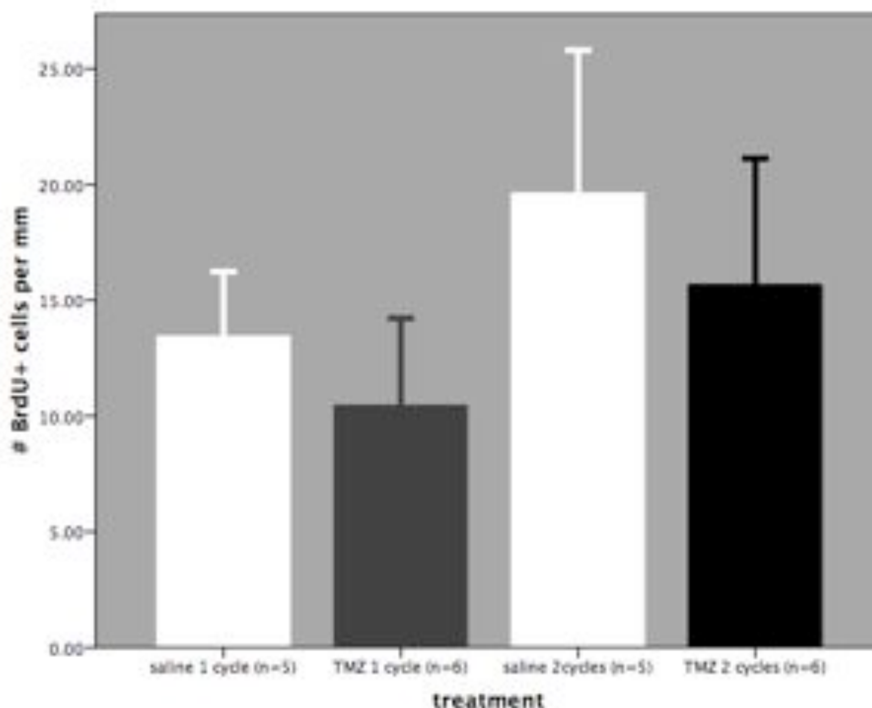


Figure 20. No detected effect of TMZ on cell proliferation in the VZ above HVC. Analysis of density of one-day-old cells in the VZ adjacent to HVC revealed no statistical difference between zebra finches treated with TMZ and saline; neither during one, nor two cycles (P>0.5, ANOVA).

4 **Discussion**

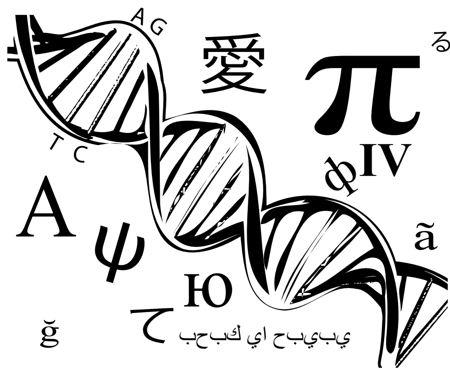
Although we followed the protocol of TMZ preparation and administration used by Garthe et al., 2009[7], we did not find any effect of TMZ on the densities of newborn cells in the VZ adjacent to HVC in adult zebra finches either after one or two cycles of treatment. Since TMZ has not been tested on birds before (to our knowledge), we do not know whether they may have stronger resistance to TMZ comparing to mammals, as *in vitro* avian cells demonstrate higher resistance to DNA damage than mammalian ones (Ogburn et al., 1998) and DNA repair modulates the vulnerability of the brain to alkylating agents (Kisby et al., 2009).

However, given high variability in the rates of neurogenesis in zebra finches observed in our experiments, it is likely that we would need a much larger and more genetically uniform sample of birds, more comparable to that used in the study of Garthe et al., 2009, on the single-strained mice of similar age (6-8 weeks). The age of zebra finches correlates with levels of adult neurogenesis in HVC (Pytte et al., 2007), but unfortunately at the time of experiments we could not find a large enough group of male zebra finches of the same age in our colony. Nevertheless, in our experiments the birds of different ages were distributed evenly across groups, that is each group contained similar numbers of relatively young birds (around 130 days), "middle-aged" (about 1 year) and older (3 years) birds, and thus the age impact could not contribute to the lack of difference between differently treated birds. Moreover, even among the birds of the same age groups, the density of BrdU + cells varied dramatically; for example, in the group injected with saline for 2 cycles, one bird of 130 days had on average about 7 BrdU+ cells/mm, and another, nearly 42! It will be interesting to find out, whether differences in vocal plasticity found by Pytte et al., 2007, in zebra finches of different ages are as evident among zebra finches of the same age but having different rates of neurogenesis.

Hurley et al., 2008, also observed high variability in adult neurogenesis in zebra finches and found that nest of origin determines its levels. Unfortunately, it seems nearly impossible to find at the same time enough brothers even for two representative enough groups of differently treated zebra finches to show the effects on the level of neurogenesis unless they are drastic. Perhaps, future studies on songbirds should use focal radiation, since it can block neurogenesis completely (Wojtowicz, 2006) and to the benefit of the

[7] With correction according to personal communication with the author, A. Garthe.

method, HVC is located very close to the surface of the brain. Meanwhile, we addressed the question of functionality of adult-born neurons in songbirds by analyzing their activation during singing (see the previous chapter II).



A ROLE FOR FOXP1/FOXP2 IN ADULT NEUROGENESIS?

1 Introduction

The Fox gene family encodes a large group of transcription factors, which share a common DNA-binding domain called the forkhead motif (or winged helix). The forkhead domain was defined in 1990 by the homology between the DNA-binding domain of hepatocyte nuclear factor-3 and the Drosophila forkhead gene, suggesting the existence of a novel family of transcription factors that is conserved between Drosophila and mammals (Lai et al., 1990; Weigel & Jackle, 1990). Members of this family have been shown to play critical roles in a wide range of normal developmental events, including the control of cellular differentiation and proliferation, pattern formation, and signal transduction (Lai et al., 1993; reviewed by Kaufmann & Knochel, 1996). For example, the Forkhead box M1 (FoxM1) gene is essential for G1/S transition, as it regulates transcription of cell cycle genes critical for progression into S-phase and mitosis (Wang et al., 2005a). Mutations of Fox genes have been implicated in a range of human developmental disorders (for review see Carlsson & Mahlapuu, 2002), including a severe speech and language impairment caused by FoxP2 mutations (Lai et al., 2001; reviewed in Fisher & Scharff, 2009). An animal model for vocal impairment similar to that seen in humans was established in the zebra finch by producing a lentiviral FoxP2 knock-down in Area X during song learning (Haesler et al., 2007). Despite significant advances in accumulating the knowledge about FoxP2 functions (for review see Fisher & Scharff, 2009), its exact cellular mechanisms are far from clear. One open question is whether FoxP2 and its closest homolog, FoxP1, are involved in adult neurogenesis, as they are expressed in two songbird brain areas that incorporate neurons during adulthood, Area X and HVC (Haesler et al., 2004; Rochefort et al., 2007).

Members of the winged-helix/forkhead family of transcription factors are involved in the development of the central nervous system, especially in the development of the telencephalon. One of them, FoxA2, is specifically expressed in adult dopamine neurons and their precursors in the medial floor plate and is required to generate dopamine neurons during fetal development and from embryonic stem cells (Kittappa et al., 2007). FOXO transcription factors are closely related to the FoxA genes and have a central role in

neuronal survival (Brunet et al., 2001). Most notably, FoxG1 (formerly BF-1), is localized in the progenitor cells of the telencephalic neuroepithelium, and Foxg1 null mutant mice have dramatically reduced cerebral hemispheres (Tao & Lai, 1992; Xuan et al., 1995). This reduction in the size of the cerebral hemispheres is thought to be a result of a premature differentiation of telencephalic neuroepithelial cells, leading to a decrease in the progenitor cell population of the ventricular zone (Xuan et al., 1995). These studies suggest that FoxG1 is a key regulator in the development of the cerebral cortex through its regulation of progenitor cell proliferation and differentiation. Similarly, Shen et al., 2006, have demonstrated that FoxG1 also regulates neurogenesis in the adult hippocampus. Finally, correlative data suggests that yet another member, FoxP2, may be involved in neurogenesis in zebra finches during vocal learning as it is expressed in the striatal VZ and newly generated interneurons of the Area X (Rochefort et al., 2007).

In addition to a forkhead domain, the members of the FoxP subfamily are characterized by a zinc finger domain and a leucine zipper motif (Shu et al., 2001), which allow them to homo- or heterodimerize (Li et al., 2004), similar to such transcription factors as ZENK and c-Fos which also have zinc fingers or leucine zippers, respectively, as discussed in the chapter I.1.3. But unlike ZENK and c-Fos, FoxP2 usually *represses* transcription of other genes in the brain (Vernes et al., 2007; Spiteri et al., 2007). Although *FoxP* genes seem to encode constitutive transcription factors and are not listed as IEGs, some evidence suggests that FoxP2 is related to induced neuronal plasticity. Miller et al., 2008, showed that the social context of singing (presence a female) causes rapid down-regulation of FoxP2 in Area X of adult zebra finches. There are issues with this result, however, because the western-blot levels of FoxP2 were normalized against GAPDH protein control. The study did not determine whether GAPDH expression in Area X is indeed unaltered by song and in fact, its levels in HVC-X neurons are reduced by >6 times 90 min after singing (Lombardino et al., 2006; for review see Fisher & Scharff, 2009).

Nevertheless, constitutive expression of FoxP2 may be indeed involved in neuronal plasticity. For example, on performing electrophysiological examinations of key neural Foxp2-expression sites known to be important for motor-skill learning, Groszer et al., 2008, found abnormalities in synaptic plasticity – the way in which synapse sensitivity is modulated in response to prior stimulation – in FoxP2 mutants. And at the periods when juvenile zebra finches learn to sing or adult canaries remodel their songs, FoxP2 levels are

transiently elevated in Area X (Haesler et al., 2004) and thus correlate with phases of vocal plasticity. But importantly, such an elevation was observed in the birds that had not sung in the hours before analysis and thus is not caused by singing itself.

A search for FoxP2 target genes suggests that it can play a role in both neuronal development and in neuronal activity/plasticity (direct function). For instance, FoxP2 binds to CNTNAP2, a gene encoding 'contactin associated protein-like 2', a member of the neurexin superfamily. Neurexins are neuronal transmembrane proteins involved in cell adhesion and function primarily as polymorphic synaptic receptors, whereas contactin-associated proteins have been implicated in neuron–glia interactions (Inda et al., 2006). Two ChIP/chip studies combining FOXP2–chromatin-immunoprecipitation (ChIP) to screening of microarrays ('chip) representing 5000 human promoters uncovered several hundred potential target genes. Among these were many involved in signal transduction, neurite outgrowth and axon guidance, neurotransmission and synaptic plasticity (Vernes et al., 2007; Spiteri et al., 2007). With such a broad spectrum of regulatory activity, FoxP2 could also be involved in adult neurogenesis. Intriguingly, FoxP2 expression in the tectum, mid-hindbrain boundary and hindbrain during development in zebrafish is driven by Lef1, a member of a family of transcription factors activated by Wnt signaling (Bonkowsky et al., 2008), which in turn is also a key regulator during adult hippocampal neurogenesis (Lie et al., 2005).

If FoxP2 is to have a role in adult neurogenesis, it is more likely to have one in the striatum of songbirds than in neurogenic areas of mammals. FoxP2 is expressed in the avian striatal VZ (Rochefort et al., 2007), but not in the germinal zones of mammals (Rochefort et al., 2007; Ferland et al., 2003). FoxP2 is not expressed in the adult dentate gyrus, but it is expressed in the olfactory bulb, another mammalian brain area incorporating new neurons in adulthood (Ferland et al., 2003). Thus, FoxP2 in songbirds could act on newly born striatal neurons at the earliest stages after their formation while they are in the VZ (Rochefort et al., 2007), while the expression and developmental data from rodents favor the view that FoxP2 and its closest homolog FoxP1 are expressed only in postmigratory neurons, since their expression begins when most cells should have already reached their postmigratory position (Ferland et al., 2003). Is the difference in FoxP2 expression between songbirds and rodents also evident for its sibling FoxP1? We addressed this question by immunocytochemical analysis of FoxP1 expression in HVC,

another area of the song system which incorporates neurons during adulthood (Goldman & Nottebohm, 1983; Alvarez-Buylla & Nottebohm, 1988; our data in the chapter II.3.2.1), which lacks FoxP2 expression but strongly expresses FoxP1 (Haesler et al., 2004; Teramitsu et al., 2004).

And what are the potential pathways through which these transcription factors could regulate neurogenesis in adulthood? One of the target genes of both FoxP2 and FoxP1 is *nNOS* (aka *NOS1*, Spiteri et al., 2007). As discussed in the chapter I.1.1.1, NO production stimulates NSCs to switch from proliferation to neuronal differentiation (Cheng et al., 2003). We explored whether the sites of nNOS and FoxP2/1 expression are coincident in the adult songbird brain.

2 **Materials & Methods**

2.1 Animals

The data discussed in this chapter were obtained from eight adult male zebra finches (*Taeniopygia guttata*) from our breeding colonies at the Free University and the Max Planck Institute for Molecular Genetics, Berlin, where they were maintained at constant room temperature and 12:12 hr light/dark cycle.

2.2 BrdU injections

Newly born cells were labeled with the DNA synthesis marker BrdU (Sigma). BrdU was administered into the pectoral muscle (50mg/kg of body weight per injection, dissolved in 0.007 N NaOH solution with 0.9% NaCl). Each bird received 5 BrdU injections in one day (one every 2h) and was subsequently killed either 1 or 7 days later. This design allowed us to assess expression of nNOS, FoxP2 or FoxP1 in the newborn cells in the striatal and pallial VZ or at the time when they start incorporating into HVC. The sections of the brains with three-week-old BrdU+ cells for additional nNOS staining were obtained from the birds used in previous experiments, see chapter II.2.2 for the protocol of BrdU injections.

2.3 Retrograde labeling of projection neurons in HVC

To label two types of projection neurons in HVC, we bilaterally injected retrograde neuronal tracers into RA and Area X using a stereotaxic apparatus (MyNeurolab, St Louis, USA) four days prior to killing. We used the same procedure as described in the chapter II. 2.3, except that the injections were carried out bilaterally with different tracers for RA and Area X. Cholera toxin subunit B conjugated with deep-red fluorescent dye Alexa fluor 647 (CTB Alexa 647; 0.2% diluted in 0.1M phosphate buffer saline, Molecular Probe, Karlsruhe, Germany) was injected into RA, and green latex microspheres (Lumafluor RetroBeads™ IX) into Area X. As the animals resumed normal activity 5-10 min following surgery, they were returned to their home cages.

2.4 Tissue preparation and immunocytochemical labeling of HVC interneurons

Each subject was deeply anesthetized and perfused transcardially with saline (NaCl 0.9%) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS; pH 7.4). The brains were then excised and kept overnight in PFA and subsequently overnight in PBS at 4˚C. Sagittal (for HVC) or frontal (for striatal VZ and Area X) sections of 40µm were serially cut using a vibrating microtome (VT1000S, Leica), collected in PBS and stored at 4˚C until processed for immunohistochemical analysis within next days.

Calcium-binding proteins are used to define interneurons in HVC (Wild et al., 2005; Scotto-Lomassese et al., 2007). To label HVC interneurons we used antibodies to calcium-binding protein parvalbumin (mouse anti-PV, 1:2000; Swant, Switzerland), which according to the data from Wild et al., 2005, is expressed in about three-quarters of HVC interneurons.

2.5 Immunohistochemistry

Two sections from each hemisphere containing HVC or Area X were selected for immunohistochemical analyses. All immunohistochemical reactions were carried out on free floating sections under continuous agitation. Before incubation with primary antibodies the sections were washed in PBS and, if BrdU identification were necessary, incubated in 2N HCl at 37˚C for DNA denaturation to reveal incorporated BrdU for the antibodies (rat anti-BrdU; OBT0030, Oxford Biotechnology). Expression of FoxP1 was detected with rabbit anti-FoxP1 (1474), courtesy of the Morrisey Lab, University of Pennsylvania, (diluted 1:1000), FoxP2 – goat anti-FoxP2 (ab1307, Abcam; 1:1000), nNOS – rabbit anti-nNOS (61-7000, Zymed; 1:200), neuronal marker Hu[8] – mouse anti-HuC/D (Chemicon; 1:200). Primary antibody incubations were carried out for 24h in PBS/0.3% TritonX100 containing 3% normal goat serum (NGS) at room temperature. They were subsequently revealed with Alexa 568-labelled goat anti-rat IgG (A11077, Molecular Probes; 1:200), Alexa 488-conjugated donkey anti-rabbit IgG (A21206, Molecular Probes; 1:200), Alexa 405-conjugated donkey anti-mouse or anti-goat IgG (Molecular Probes; 1:200). Secondary antibody incubations lasted for 2h in PBS/0.3% TritonX100 at room

[8] Hu is a family of neuronal RNA-binding proteins which are used as pan-neuronal markers and are expressed in newly formed neurons just several hours after the last division (Barami et al., 1995).

temperature. Negative controls involved the omission of one or more of the primary antibodies to check for cross-reactivity. To determine the phenotype of neurons, parvalbumin immunolabeling and detection by retrograde tracers were performed (see above, IV.2.3 and IV.2.4). Afterwards, the sections were rinsed and coverslipped with fluorescent mounting medium (Fluoromount G, SouthernBiotech, Alabama, USA).

2.6 Image analysis

Immunofluorescent sections were analyzed using epifluorescent and confocal microscopes (Zeiss Axiovert 200M and Zeiss LSM Meta 510, respectively) equipped with filters for blue, green, red and deep-red fluorescence and coupled to CCD (Charge Coupled Device) cameras.

Cell counts were performed on digitized images using Image J64 software. Area X and HVC were defined according to the stereotaxic canary brain atlas (Stokes et al., 1974). HVC was also delimited by the presence of neurons backfilled by the retrograde tracers. The structures analyzed in the current work and their localization within the brain are schematically depicted on the **Fig. 2**.

To determine colocalization of BrdU signal with nNOS or FoxP1 expression, or the latter with CTB backfill, we examined BrdU+ and FoxP1+ cells under 40, 63 and 100 magnifications at up to 20 focal planes at epifluorescent and confocal microscopes (**Fig. 22**). When colocalization was difficult to determine, 3D images with z-stack and deconvolution were taken with Slidebook Digital Microscopy software (Intelligent Imaging Innovations).

3 **Results**

3.1 ***FoxP1 expression in adult-born HVC cells***

FoxP2 and FoxP1 are not expressed in the germinal zones of rodents (Rochefort et al., 2007; Ferland et al., 2003), but FoxP2 expression is present in the VZ of zebra finches (Rochefort et al., 2007). We addressed whether the pattern of FoxP1 expression also shows such differences between these species. However, after immunostaining of brain sections of adult zebra finches with FoxP1 antibodies we could not find typical nuclear FoxP1 labeling in the VZ (**Fig. 21**), although FoxP2 was indeed expressed in the striatal VZ (**Fig. 23B**).

Nevertheless, FoxP1 is expressed in 1 week old neurons (**Fig. 21**). Although we did not perform counterstaining with neuronal markers on those sections, we assume that these newborn FoxP+ cells are neurons, because previously it has been shown that FoxP1 expression in the developing and adult rodent brain is restricted to neurons based on nuclear morphology as well as colabeling with a neuronal marker NeuN (Ferland et al., 2003), similarly to exclusively neuronal expression of FoxP2 also in the avian brain, as assessed by colocalization with another neuronal marker, Hu (Haesler et al., 2004).

3.2 ***FoxP1 expression is not restricted to the replaceable type of HVC neurons***

After we found out that FoxP1 is expressed by adult generated neurons in HVC, we examined whether this expression is exclusive to the replaceable type of neurons, HVC-RA projection neurons. Although indeed many HVC-RA neurons expressed FoxP1, so did non-replaceable HVC-X neurons (**Fig. 22B,C**). This rejects the possibility that FoxP1 could specifically regulate adult neurogenesis in HVC but on the other hand suggests a possibility for more general role in regulation of development/function of these projection neurons.

3.3 ***FoxP1 is not expressed in parvalbumin-positive HVC interneurons***

Furthermore, to determine whether FoxP1 is specifically expressed in the projection neurons of HVC, we analyzed PV+ neurons which according to the data from Wild et al., 2005, represent about three-quarters of HVC interneurons. Out of 235 PV+ HVC neurons

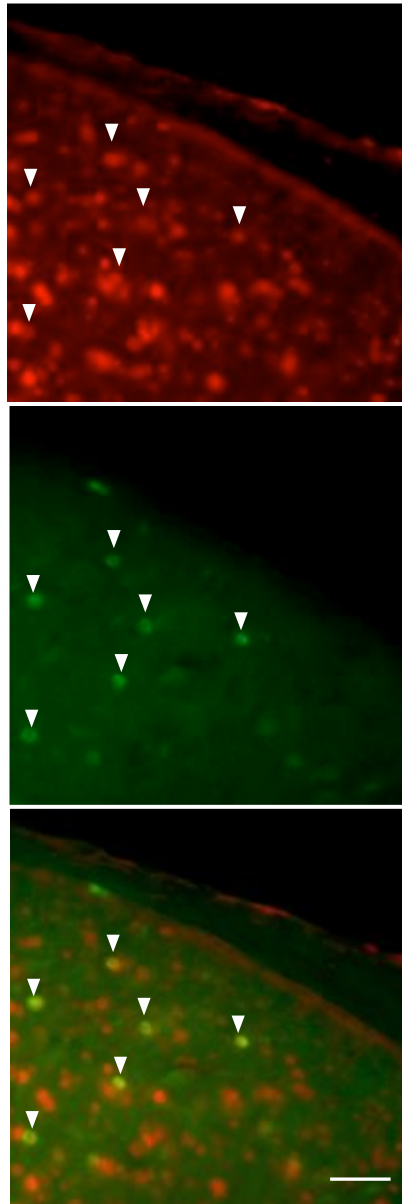
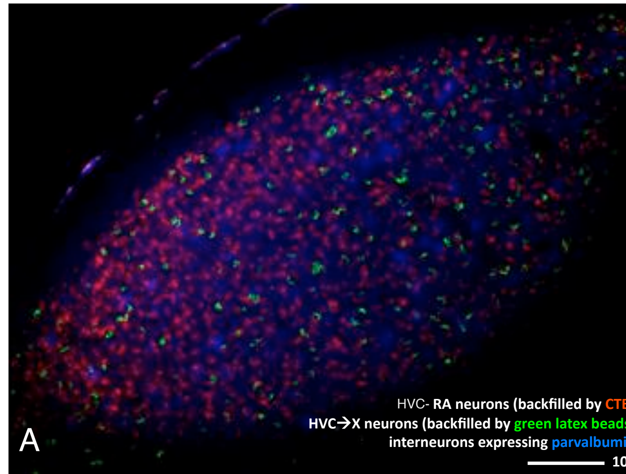
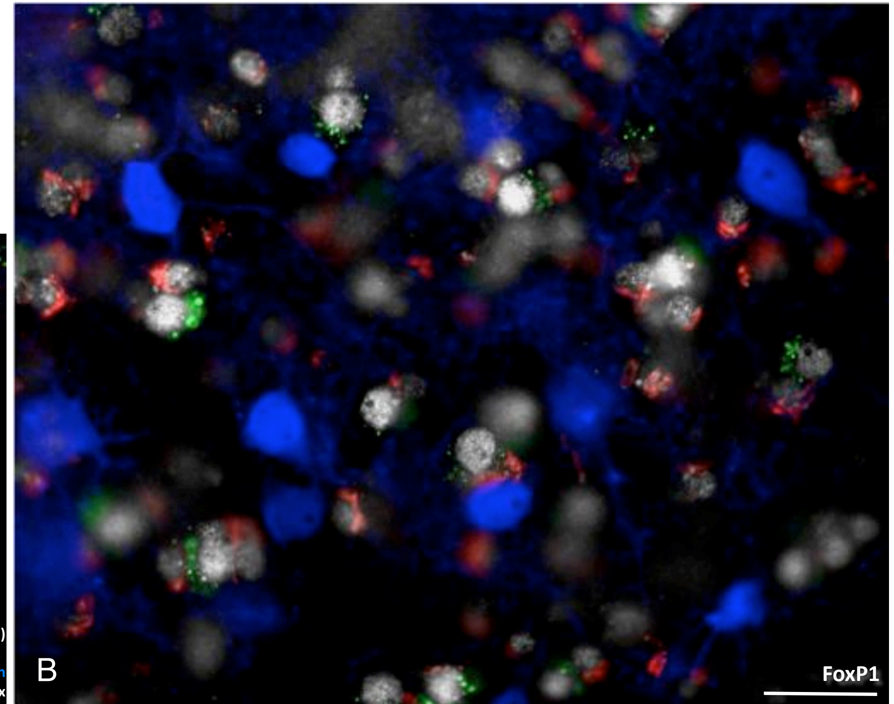


Figure 21 (left panel)**. FoxP1 is expressed in adult-born neurons in HVC.** Representative images of an area of HVC with FoxP1 expression (red) and 7 days old cells labeled by BrdU (green), merged in the right one. Cell nuclei with colocalized BrdU signal and FoxP1 expression are indicated by arrowheads. Note that nuclear FoxP1 expression is absent in the VZ above HVC. Scale bar = 50 µm.





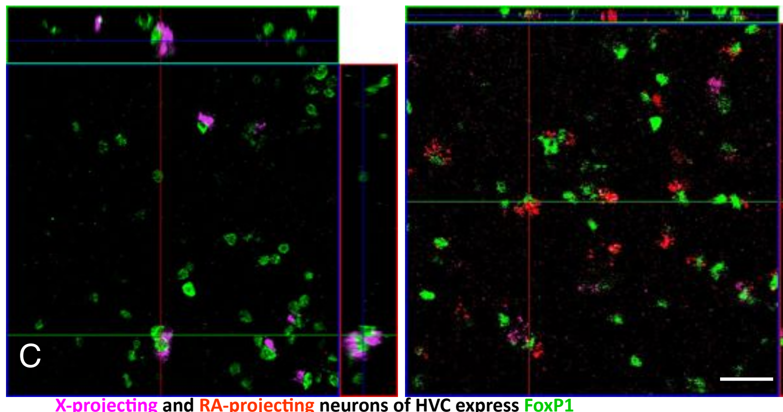


Figure 22. FoxP1 is expressed in projection neurons but not in parvalbumin expressing interneurons in HVC. ***(A)*** Three populations of neurons in HVC were labeled immunocytochemically (interneurons expressing parvalbumin) or with retrograde tracers injected into Area X and RA (HVC-X and HVC-RA projection neurons, respectively). Scale bar = 100 µm. ***(B)*** Representative image of an area of HVC with PV + interneurons and FoxP1 expression (white) in HVC-X (green) and HVC-RA (red) neurons. Scale bar = 16 µm. ***(C)*** Confocal images of FoxP1 staining (green) colocalized with the backfill of retrograde tracers injected into Area X and RA. Scale bar = 30 µm.

analyzed in 3 birds, none expressed FoxP1 (**Fig. 22B**). This suggests that indeed FoxP1 expression may be exclusive to the projection neurons in HVC. However, not all backfilled, i.e. projecting, neurons expressed FoxP1 (not quantified).

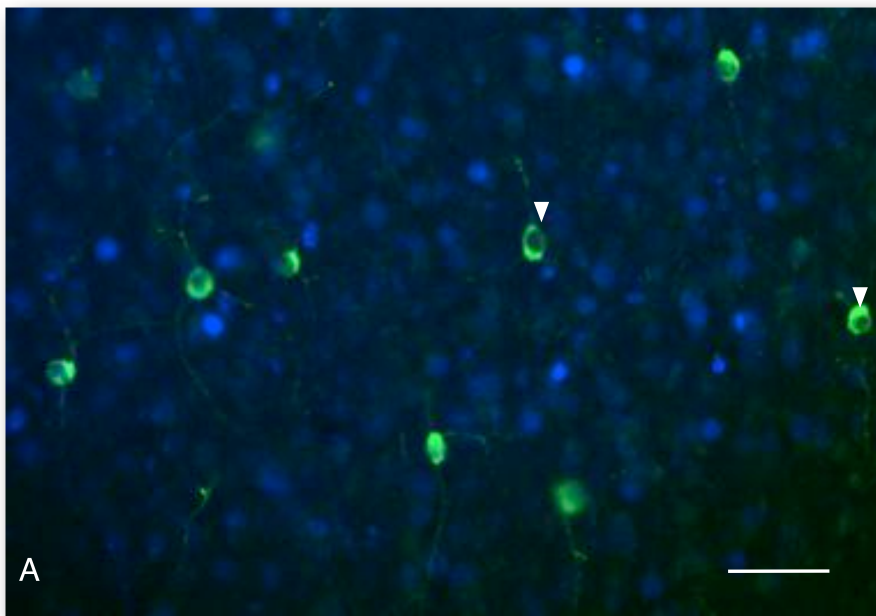
3.4 *Expression of nNOS, target of FoxP2/1, in the VZ, including one-day old cells*

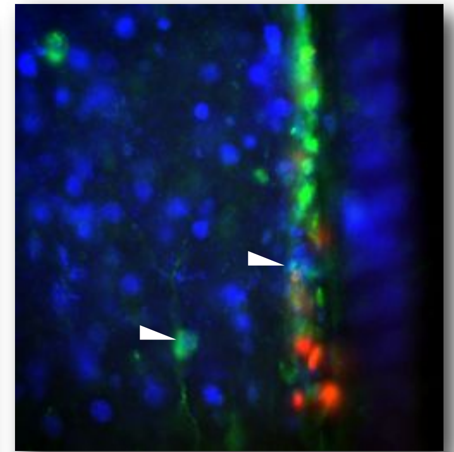
We proposed that FoxP2 and FoxP1 might regulate adult neurogenesis in Area X and HVC of zebra finches through a molecular pathway involving NO. We found that in the adult zebra finches nNOS is highly expressed in the VZ adjacent to Area X (**Fig. 23B,D**) and HVC (**Fig. 23C**), including one-day old neurons as revealed by colocalized BrdU and Hu staining (**Fig. 23D**).

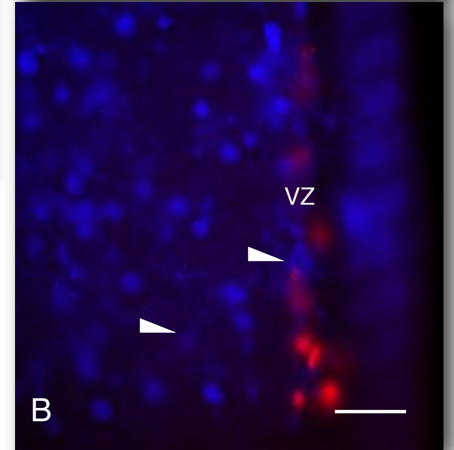
3.5 *Absent or low expression of nNOS in more mature and in FoxP2+ cells*

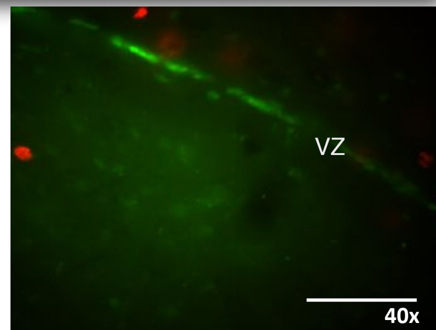
Though we observed high expression of nNOS in the VZ, it apparently diminishes and becomes more specific with maturation of neurons. In HVC and Area X, we found relatively few neurons expressing nNOS (**Fig. 23A**). These neurons were apparently mature as we could see their arborization with nNOS staining (**Fig. 23A**). We did not find 3 week old cells expressing nNOS, but we assessed this only in one animal, so given relatively scarce expression of nNOS in mature neurons, it is plausible that nNOS is expressed in adult-born neurons during maturation but under specific conditions, not as almost universally as in the VZ.

Interestingly, both in the VZ and in mature neurons, we could find nNOS expression only in the cells with no or very low FoxP2 expression, and vice versa, FoxP2+ nuclei did not colocalize with nNOS expression (**Fig. 23A,B**).









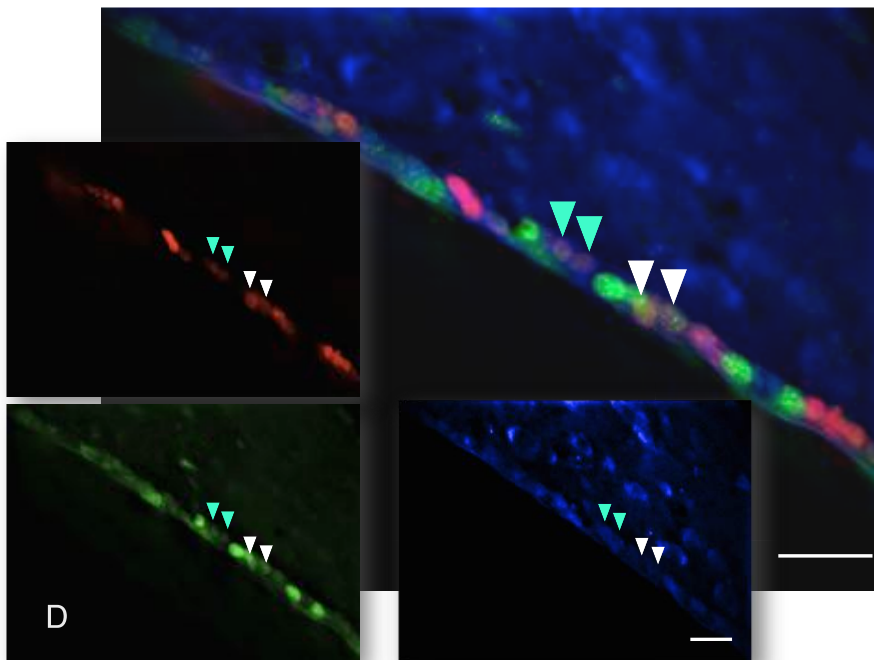


Figure 23. Expression of nNOS in the VZ and newborn cells in the adult zebra finches. ***(A)*** FoxP2+ neurons in Area X did not express nNOS (target of FoxP2), and vice versa, nNOS+ neurons lacked FoxP2 labeling in their nuclei (arrow heads point to two nNOS+ neurons with their nuclei in focus). Scale bar = 50 µm. ***(B)*** In the striatal VZ and neighboring part of striatum, however, some nNOS+ cells colocalized with low signal of FoxP2 labeling (arrow heads). Overall, nNOS is strongly expressed in the VZ. Scale bar = 50 µm. ***(C)*** nNOS was also expressed in the VZ above HVC; we did not find 3 week old HVC cells (BrdU+) expressing nNOS, though only one animal was checked. Scale bar = 200 µm. ***(D)*** However, in the striatal VZ, nNOS is expressed in 1 day old cells (BrdU+, arrowheads), including those expressing neuronal marker Hu (light blue arrowheads). Scale bar = 25 µm.

4 **Discussion**

4.1 ***FoxP1 and development of projection neurons***

Unlike in mammals, FoxP2 in juvenile zebra finches was found to be expressed in the striatal VZ (Rochefort et al., 2007), and it was suggested that FoxP2 could regulate some steps of neurogenesis in the birds specifically (Rochefort et al., 2007). In the present work we examined whether FoxP1 could also be linked to adult neurogenesis.

We also observed expression of FoxP2 in the striatal VZ of adult zebra finches (**Fig. 23B,D**), but FoxP1 expression was absent in both striatal and pallial parts of the VZ (**Fig. 21**). This finding implies that FoxP1 cannot be a regulator of early stages (proliferation and differentiation) of adult neurogenesis in songbirds, but perhaps FoxP1 could be involved in maturation of newly generated neurons? Indeed, we found that adult-born one week old neurons expressed FoxP1 (**Fig. 21**).

Only one type of neuron in HVC is generated during adulthood – HVC-RA projection neurons (Scotto-Lomassese et al., 2007). We sought to determine if FoxP1 expression is unique to this class. We found that FoxP1 was highly expressed also in non-replaceable HVC-X projection neurons, suggesting that FoxP1 is not involved in maturation of newborn neurons specifically. Since FoxP2 is expressed in medium spiny neurons of the striatal Area X in zebra finches, which undergo neurogenesis (Rochefort et al., 2007), and in the striatum of mice (Haesler et al., 2004; Teramitsu et al., 2004), where adult neurogenesis under normal conditions has not been reported so far, it is plausible that FoxP2 is primarily a lineage marker e.g. for spiny neurons as suggested by Rochefort et al., 2007. Then could FoxP1 be a lineage marker for the premotor projection neurons of HVC? In support of this idea we found that FoxP1 was not expressed in PV+ interneurons (**Fig. 22B**), suggesting that out of three neuronal types of HVC (Dutar et al., 1998) only projection neurons express FoxP1, similar to FoxP1 expression in the striatum of mice where it is also exclusive to the projection neurons (Tamura et al., 2004). This is also consistent with the crucial role FoxP1 plays in the development and establishing of correct axonal connectivity in spinal motor neurons (Dasen et al., 2008; Rousso et al., 2008).

What are the possible mechanisms of how FoxP1 could orchestrate axonal connectivity in the two types of projection neurons of HVC which have different anatomical targets? FoxP1 was found to be an indispensable Hox accessory factor during the programming of

spinal motor neuron motor neuron diversity and connectivity (Dasen et al., 2008): specification during the transcriptional programming of motor neuron columnar and pool[9] identity alternates between Hox proteins and FoxP1. Initially, differences between Hox6, 9, and 10 paralog identity set the level of FoxP1 expression in motor neurons. Once induced, however, FoxP1 is the primary determinant of columnar specificity in a dose-dependent manner. But for the program of motor pool allocation, the onus of specificity in target gene activation reverts to Hox proteins and acts in the context of uniformly high-level FoxP1 expression (Dasen et al., 2008). Unlike spinal motor neurons, premotor projection neurons of HVC have different developmental origin, as they arise from different loci in the VZ (Scott & Lois, 2007). Perhaps they express different "master" genes à la Hox which could either determine different levels of FoxP1, leading to a differential transcriptional activation/suppression of their target genes, or set a specific profile of gene available for FoxP1 binding, and thus two distinct types of projection neurons could use the same FoxP1-dependent machinery to build up axonal connections to their different targets depending on available intrinsic and extrinsic signals.

One mechanism through which FoxP1 could mediate its function in axonal connectivity is through regulation of cell surface glycoproteins such as neuropilins (Npn) and cadherins[10]. Neuropilin was identified as the receptor for semaphorin (Sema) (Kolodkin et al., 1997) and another family of axon guidance molecules called plexins (Ohta et al., 1992); it mediates the attractive or repulsive signals of semaphorins into the growth cone via the cytoplasmic domain of plexins (Tamagnone et al., 1999; Price et al., 2002). Among them, Npn-1 and Plexin-A4 are strongly expressed in vocal control areas of songbirds and parrots, whereas their ligand Sema3A, on the contrary, outside of the vocal nuclei (Lovell et al., 2008; Matsunaga & Okanoya, 2009b), suggesting a potential role of Sema/Npn/Plexin signaling in vocal circuit formation (Lovell et al., 2008; Matsunaga & Okanoya, 2009a); some cadherins are also specifically expressed in the song system during development and adulthood (Lovell et al., 2008; Matsunaga & Okanoya, 2008). Intriguingly, Dasen et al., 2008, have shown that FoxP1 is necessary for the expression of

---

[9] Motor neurons first organize into longitudinal columns that extend along the rostrocaudal axis of the embryo to facilitate the matching of motor neurons with their synaptic targets, for example trunk vs limb muscles. Once this columnar organization has been established, they subdivide into even smaller groups, termed motor pools, which innervate the individual muscles within each target region (reviewed by Jessell, 2000).

[10] Cadherins are cell surface glycoproteins involved in cell–cell interaction through homophilic binding (Takeichi, 2007).

two cadherins, Cad-8 and Cad-20, and of Sema3E. Also, given that FoxPs are mostly known as transcriptional repressors (Vernes et al., 2007; Spiteri et al., 2007), it is possible that FoxP1 expression could down-regulate some cadherins as well: For instance, in birds, expression of N-cadherin is expressed in the neuronal progeny of NSCs and is down-regulated upon their emigration from the VZ 4 to 7 days after differentiation (Barami et al., 1995), and according to our data, FoxP1 is absent in the VZ but appears in 7-day-old cells in HVC (**Fig. 21**). However, it is not known whether this cadherin is a target of FoxP1.

Finally, expression of the retinoic acid producing enzyme (retinaldehyde dehydrogenase-2, RALDH2) in specific subsets of spinal motor neurons is dependent on convergent FoxP1 and Hox activity (Dasen et al., 2008). Retinoic acid is known to have trophic activity (Briscoe & Novitch, 2008) and plays a well established role in developmental (reviewed by Maden, 2007) and adult neurogenesis, as noted in the introduction chapter I.1.1.1. Moreover, after pharmacological inhibition of retinoic acid synthesis in HVC by disulfiram, rates of new neuron incorporation and/or survival decreased, however, it was not clarified whether the effect was not caused by neurotoxicity of the treatment (Roeske, 2009). In HVC, retinoic acid is specifically produced by X-projecting neurons (Denisenko-Nehrbass et al., 2000). So far, *RALDH2*, is the only gene that is known to be exclusively expressed only in one of the two types of HVC projection neurons, and thus it could be an example of a morphogenic gene induced by some unknown factor, which is unique to HVC-X neurons, but subsequently controlled by FoxP1, which is expressed in both types of projection neurons (**Fig. 22B,C**), as proposed above.

4.2 ***NO and neurogenesis***

Among the known targets of FoxP2 and FoxP1 (Vernes et al., 2007; Spiteri et al., 2007), nNOS[11] perhaps has the best documented role in neurogenesis (see introduction chapter I. 1.1.1). We observed high expression of nNOS in the VZ of adult zebra finches (**Fig. 23B,C,D**) in accord with the crucial role of NO on initial stages of neurogenesis when it stimulates NSCs to switch from proliferation to neuronal differentiation (Cheng et al., 2003).

[11] Probably nNOS is also a target of another FoxP member expressed in the CNS, FoxP4, as it is found in FoxP4+ neurons in FoxP1 knockdown mice (Dasen et al., 2008).

Using Ingenuity pathway-analysis software to analyze the 34 core central nervous system (CNS) targets of FoxP2, Spiteri et al., 2007, identified its several possible functions, including branching of dendrites through regulation of nNOS. In support of the idea that FoxP2 might regulate nNOS expression in the adult brain, we found that FoxP2+ cells in the VZ and striatum lacked nNOS labeling, and contrariwise, nNOS+ cells did not express FoxP2, or did so at a level hardly detectable with our immunohistochemical method (**Fig. 23A,B**). This observation is consistent with the findings of Spiteri et al., 2007, showing that FoxP2 overexpression leads to a significant decrease of nNOS expression. In addition to the role in the arborization of neurons, we suggest that FoxP2 may also regulate differentiation of NSCs in the VZ of birds through its target nNOS.

Since the work of Cheng et al., 2003, indicates an existence of a positive loop between BDNF expression and NO synthesis (see chapter I.1.1.1), it is likely that BDNF production and down-regulation of FoxP2/1 and following up-regulation of nNOS are parallel processes, though we know nothing about causal relations between them. BDNF can be regulated by neuronal depolarization (Shieh et al., 1998; Tao et al., 1998), and its expression in zebra finches correlates with singing activity (Li et al., 2000); effects of nNOS-derived NO on neurogenesis in the murine SGZ are probably also mediated by NMDAR activity (Zhu et al., 2006). Thus BDNF and NO could serve as paracrine messengers controlling the proliferation and differentiation of NSCs in the VZ (Cheng et al., 2003) and thus providing the link between functional activity of mature neurons and neurogenesis. If FoxP2 is fast-regulated by the context of singing as proposed by Miller et al., 2008, we could expect a causal relationship between neuronal activity and down-regulation of FoxP2/up-regulation of nNOS, which in turn is coincident with BDNF expression. However *in situ* hybridization and immunohistochemical analyses suggest that levels of FoxP2 and FoxP1 expression in the song system of songbirds can also vary depending on developmental state independent of recent singing activity (Haesler et al., 2004; Rochefort et al., 2007). Thus, these transcriptional factors appear as plausible regulators that allow certain developmental levels of neuronal plasticity, which is independently induced by neuronal activity. We have only begun to unveil possible molecular pathways through which FoxPs could regulate such processes as differentiation and maturation of newly generated neurons and plasticity of mature ones. Apparently, one of the key FoxP-dependent pathways includes nNOS expression, as was shown in

developing spinal motor neurons (Dasen et al., 2008). Our data indicate that FoxPs could also control NO production from nNOS in the song system of adult zebra finches.

In summary, although it does not appear that FoxP1 and FoxP2 have specific functions in adult neurogenesis, our findings, in combination with the previous research on these genes, suggest that they may regulate neuronal differentiation and plasticity by controlling their target genes such as nNOS.

V CONCLUDING REMARKS AND SUMMARY OF THE FINDINGS

In conclusion, we provide the first evidence that adult-born neurons in the song system of zebra finches are activated during singing, and we have elucidated some stages of this functional maturation of newly-generated neurons (see summarizing scheme at **Fig. 24**). First, our data demonstrate that the rates of cell proliferation in the neural germinal zone adjacent to HVC are highly variable in adult zebra finches with up to 6-fold differences among the birds of the same age. Further studies should reveal if this individual proliferative variability is reflected in the rates of incorporation of new neurons in HVC of the birds of the same age and whether it correlates with the degree of vocal plasticity, as was seen in zebra finches of different ages by Pytte et al., 2007. The results of immunohistochemical analyses indicate that in both striatal and pallial VZ of adult zebra finches, neuronal progenitors could be under the control of the NO signaling pathway, which is known to induce the switch from proliferation to differentiation of neural progenitors (Cheng et al., 2003). Expression of the enzyme responsible for NO synthesis, nNOS, in Area X and HVC and adjacent VZs may be down-regulated by FoxP2 and FoxP1. By the end of the first week after birth, newly formed neurons do not express plasticity-related IEGs such as c-Fos and ZENK. But in HVC, they express FoxP1 and already start sending axons reaching their target area (RA). FoxP1 could be involved in control of axonal connectivity of newborn neurons as it seems to be specific to projection neuron types of HVC. By the third week, new neurons in HVC, Area X and LMAN express ZENK and c-Fos during singing, providing the first evidence of functional activation of adult-born neurons during vocal production; to our knowledge, this is also the first example of incorporation of new neurons in LMAN during adulthood. At this young age, new neurons in HVC are more likely to express c-Fos during undirected singing than during directed singing. We have hypothesized that unlike mature HVC-RA projecting neurons, immature ones could be similar to HVC-X neurons in their responsiveness to bird's own song or receive afferentation related to social context; this could be mediated through unique qualities of young neurons, such as depolarization by GABA, reported for immature adult-born neurons in rodent hippocampus (Ge et al., 2006), or, as suggested by us, due to recapitulation of developmental shift in expression of two different subunits of

NMDA receptors. First, it should be established that indeed newly incorporated HVC-RA neurons respond to auditory stimulation in awake birds or fire differently depending on social context. Similar to the study of Kirn et al., 1999, we found that new HVC neurons become increasingly connected to RA with age. But, surprisingly, activation of new HVC neurons does not depend on innervation of their target, RA, suggesting that newly incorporated neurons may participate in processing of premotor stimulation through local microcircuitry of HVC before their axons reach RA. Once connected to RA, however, the population of 3 to 8 weeks old adult-born neurons was not distinguishable by the level of IEG induction during singing from pre-existing neurons of this type, although the portion of new neurons that express IEGs during singing and are connected to RA relatively to all new neurons increases significantly from 3 to 8 weeks. Albeit we do not know the exact mechanisms, probably they contribute to the brain plasticity related to variability of vocalizations.

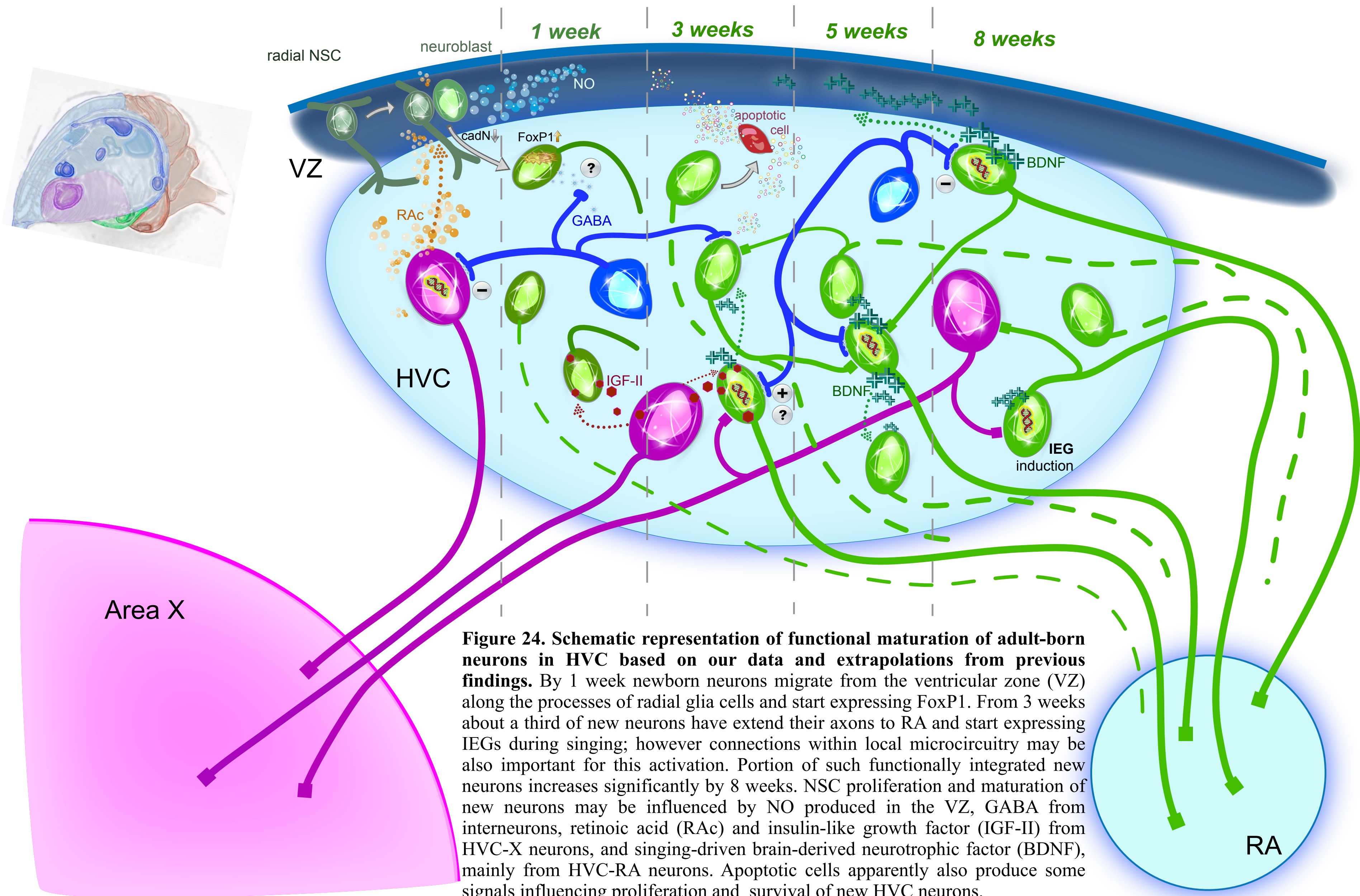


Figure 24. Schematic representation of functional maturation of adult-born neurons in HVC based on our data and extrapolations from previous findings. By 1 week newborn neurons migrate from the ventricular zone (VZ) along the processes of radial glia cells and start expressing FoxP1. From 3 weeks about a third of new neurons have extend their axons to RA and start expressing IEGs during singing; however connections within local microcircuitry may be also important for this activation. Portion of such functionally integrated new neurons increases significantly by 8 weeks. NSC proliferation and maturation of new neurons may be influenced by NO produced in the VZ, GABA from interneurons, retinoic acid (RAc) and insulin-like growth factor (IGF-II) from HVC-X neurons, and singing-driven brain-derived neurotrophic factor (BDNF), mainly from HVC-RA neurons. Apoptotic cells apparently also produce some signals influencing proliferation and survival of new HVC neurons.

VI BIBLIOGRAPHY

Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. Science 298(5594): 846-50.

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437(7060): 894-7.

Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89(1): 121-45.

Alonso M, Viollet C, Gabellec MM, Meas-Yedid V, Olivo-Marin JC, Lledo PM (2006) Olfactory discrimination learning increases the survival of adult-born neurons in the olfactory bulb. J Neurosci 26(41): 10508-13.

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. 3. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. J Comp Neurol 136(3): 269-93.

Altman J, Das GD (1965) Post-natal origin of microneurones in the rat brain. Nature 207 (5000): 953-6.

Alvarez-Buylla A, Kirn JR (1997) Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. J Neurobiol 33(5): 585-601.

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41(5): 683-6.

Alvarez-Buylla A, Nottebohm F (1988) Migration of young neurons in adult avian brain. Nature 335(6188): 353-4.

Alvarez-Buylla A, Seri B, Doetsch F (2002) Identification of neural stem cells in the adult vertebrate brain. Brain Res Bull 57(6): 751-8.

Ambrogini P, Cuppini R, Lattanzi D, Ciuffoli S, Frontini A, Fanelli M (2009) Synaptogenesis in adult-generated hippocampal granule cells is affected by behavioral experiences. Hippocampus.

Anokhin KV, Mileusnic R, Shamakina IY, Rose SP (1991) Effects of early experience on c-fos gene expression in the chick forebrain. Brain Res 544(1): 101-7.

Anokhin KV, Rose SP (1991) Learning-induced Increase of Immediate Early Gene Messenger RNA in the Chick Forebrain. Eur J Neurosci 3(2): 162-167.

Anokhin PK (1937) New data on the problem of interactions between center and periphery in the neural activity. Archive of Biol. Sci 12: 290-338.

Anokhin PK (1974) Biology and Neurophysiology of the Conditioned Reflex and Its Role in Adaptive Behavior. Oxford.

Aronov D, Andalman AS, Fee MS (2008) A specialized forebrain circuit for vocal babbling in the juvenile songbird. Science 320(5876): 630-4.

Bailey DJ, Wade J (2003) Differential expression of the immediate early genes FOS and ZENK following auditory stimulation in the juvenile male and female zebra finch. Brain Res Mol Brain Res 116(1-2): 147-54.

Balordi F, Fishell G (2007) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. J Neurosci 27 (22): 5936-47.

Bannigan J, Langman J (1979) The cellular effect of 5-bromodeoxyuridine on the mammalian embryo. J Embryol Exp Morphol 50: 123-35.

Bannigan JG (1985) The effects of 5-bromodeoxyuridine on fusion of the cranial neural folds in the mouse embryo. Teratology 32(2): 229-39.

Barami K, Iversen K, Furneaux H, Goldman SA (1995) Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. J Neurobiol 28(1): 82-101.

Barkan S, Ayali A, Nottebohm F, Barnea A (2007) Neuronal recruitment in adult zebra finch brain during a reproductive cycle. Dev Neurobiol 67(6): 687-701.

Barnea A, Nottebohm F (1994) Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. Proc Natl Acad Sci U S A 91(23): 11217-21.

Bauer S, Patterson PH (2005) The cell cycle-apoptosis connection revisited in the adult brain. J Cell Biol 171(4): 641-50.

Bauer S, Patterson PH (2006) Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. J Neurosci 26(46): 12089-99.

Bennett MR, Schwartz WJ (1994) Are glia among the cells that express immunoreactive c-Fos in the suprachiasmatic nucleus? Neuroreport 5(14): 1737-40.

Berninger B, Marty S, Zafra F, da Penha Berzaghi M, Thoenen H, Lindholm D (1995) GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro. Development 121(8): 2327-35.

Berretta S, Robertson HA, Graybiel AM (1992) Dopamine and glutamate agonists stimulate neuron-specific expression of Fos-like protein in the striatum. J Neurophysiol 68(3): 767-77.

Bing GY, Filer D, Miller JC, Stone EA (1991) Noradrenergic activation of immediate early genes in rat cerebral cortex. Brain Res Mol Brain Res 11(1): 43-6.

Bolhuis JJ, Zijlstra GG, den Boer-Visser AM, Van Der Zee EA (2000) Localized neuronal activation in the zebra finch brain is related to the strength of song learning. Proc Natl Acad Sci U S A 97(5): 2282-5.

Bonkowsky JL, Wang X, Fujimoto E, Lee JE, Chien CB, Dorsky RI (2008) Domain-specific regulation of foxP2 CNS expression by lef1. BMC Dev Biol 8: 103.

Bottjer SW, Miesner EA, Arnold AP (1984) Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science 224(4651): 901-3.

Bramham CR (2008) Local protein synthesis, actin dynamics, and LTP consolidation. Curr Opin Neurobiol 18(5): 524-31.

Breunig JJ, Silbereis J, Vaccarino FM, Sestan N, Rakic P (2007) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proc Natl Acad Sci U S A 104(51): 20558-63.

Briscoe J, Novitch BG (2008) Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. Philos Trans R Soc Lond B Biol Sci 363 (1489): 57-70.

Bruel-Jungerman E, Davis S, Laroche S (2007) Brain plasticity mechanisms and memory: a party of four. Neuroscientist 13(5): 492-505.

Bruel-Jungerman E, Laroche S, Rampon C (2005) New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. Eur J Neurosci 21(2): 513-21.

Burd GD, Nottebohm F (1985) Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol 240(2): 143-52.

Butz M, Wörgötter F, van Ooyen A (2009) Activity-dependent structural plasticity. Brain Res Rev 60(2): 287-305.

Cameron HA, McEwen BS, Gould E (1995) Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci 15(6): 4687-92.

Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435(4): 406-17.

Cardin JA, Schmidt MF (2004) Auditory responses in multiple sensorimotor song system nuclei are co-modulated by behavioral state. J Neurophysiol 91(5): 2148-63.

Carlsson P, Mahlapuu M (2002) Forkhead transcription factors: key players in development and metabolism. Dev Biol 250(1): 1-23.

Castelino CB, Ball GF (2005) A role for norepinephrine in the regulation of context-dependent ZENK expression in male zebra finches (Taeniopygia guttata). Eur J Neurosci 21(7): 1962-72.

Catchpole CK, Slater PJB (1995) Bird Song: Biological Themes and Variations. Cambridge, UK, Cambridge Univ. Press.

Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, Kuchel G, Miller FD (1997) Synaptic innervation density is regulated by neuron-derived BDNF. Neuron 18 (2): 257-67.

Chapouton P, Jagasia R, Bally-Cuif L (2007) Adult neurogenesis in non-mammalian vertebrates. Bioessays 29(8): 745-57.

Cheng A, Wang S, Cai J, Rao MS, Mattson MP (2003) Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. Dev Biol 258(2): 319-33.

Cheng HY, Clayton DF (2004) Activation and habituation of extracellular signal-regulated kinase phosphorylation in zebra finch auditory forebrain during song presentation. J Neurosci 24(34): 7503-13.

Chun SK, Sun W, Park JJ, Jung MW (2006) Enhanced proliferation of progenitor cells following long-term potentiation induction in the rat dentate gyrus. Neurobiol Learn Mem 86(3): 322-9.

Clayton DF (1997) Role of gene regulation in song circuit development and song learning. J Neurobiol 33(5): 549-71.

Clayton DF (2000) The genomic action potential. Neurobiol Learn Mem 74(3): 185-216.

Cole AJ, Saffen DW, Baraban JM, Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature 340(6233): 474-6.

Cooper-Kuhn CM, Kuhn HG (2002) Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res 134(1-2): 13-21.

Cooper-Kuhn CM, Vroemen M, Brown J, Ye H, Thompson MA, Winkler J, Kuhn HG (2002) Impaired adult neurogenesis in mice lacking the transcription factor E2F1. Mol Cell Neurosci 21(2): 312-23.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11(3): 327-35.

Curran T, Morgan JI (1987) Memories of fos. Bioessays 7(6): 255-8.

Dasen JS, De Camilli A, Wang B, Tucker PW, Jessell TM (2008) Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell 134 (2): 304-16.

Denisenko-Nehrbass NI, Jarvis E, Scharff C, Nottebohm F, Mello CV (2000) Site-specific retinoic acid production in the brain of adult songbirds. Neuron 27(2): 359-70.

Döbrössy MD, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2003) Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Mol Psychiatry 8(12): 974-82.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97(6): 703-16.

Doupe AJ, Kuhl PK (1999) Birdsong and human speech: common themes and mechanisms. Annu Rev Neurosci 22: 567-631.

Doya K (2008) Modulators of decision making. Nat Neurosci 11(4): 410-6.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007) Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 130(6): 1146-58.

Duan X, Kang E, Liu CY, Ming GL, Song H (2008) Development of neural stem cell in the adult brain. Curr Opin Neurobiol 18(1): 108-15.

Dunn AM, Zann RA (1996) Undirected song in wild zebra finch flocks: Contexts and effects of mate removal. Ethology 102(529-539).

Dupret D, Fabre A, Döbrössy MD, Panatier A, Rodriguez JJ, Lamarque S, Lemaire V, Oliet SH, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS Biol 5(8): e214.

Dupret D, Montaron MF, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2005) Methylazoxymethanol acetate does not fully block cell genesis in the young and aged dentate gyrus. Eur J Neurosci 22(3): 778-83.

Dutar P, Vu HM, Perkel DJ (1998) Multiple cell types distinguished by physiological, pharmacological, and anatomic properties in nucleus HVc of the adult zebra finch. J Neurophysiol 80(4): 1828-38.

EMEA (2005) European public assessment report: Temodal. H/C. 0229.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4(11): 1313-7.

Espósito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci 25(44): 10074-86.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6(3): 215-29.

Feenders G, Liedvogel M, Rivas M, Zapka M, Horita H, Hara E, Wada K, Mouritsen H, Jarvis ED (2008) Molecular mapping of movement-associated areas in the avian brain: a motor theory for vocal learning origin. PLoS One 3(3): e1768.

Feldman DE (2009) Synaptic mechanisms for plasticity in neocortex. Annu Rev Neurosci 32: 33-55.

Ferland RJ, Cherry TJ, Preware PO, Morrisey EE, Walsh CA (2003) Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. J Comp Neurol 460(2): 266-79.

Fisher SE, Scharff C (2009) FOXP2 as a molecular window into speech and language. Trends Genet 25(4): 166-77.
Flavell SW, Greenberg ME (2008) Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu Rev Neurosci 31: 563-90.
Funabiki Y, Funabiki K (2008) Song retuning with tutor model by adult zebra finches. Dev Neurobiol 68(5): 645-55.
Funabiki Y, Funabiki K (2009) Factors limiting song acquisition in adult zebra finches. Dev Neurobiol 69(11): 752-9.
Gahr M, Garcia-Segura LM (1996) Testosterone-dependent increase of gap-junctions in HVC neurons of adult female canaries. Brain Res 712(1): 69-73.
Garthe A, Behr J, Kempermann G (2009) Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One 4(5): e5464.
Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439(7076): 589-93.
Ge S, Sailor KA, Ming GL, Song H (2008) Synaptic integration and plasticity of new neurons in the adult hippocampus. J Physiol 586(16): 3759-65.
Ge S, Yang CH, Hsu KS, Ming GL, Song H (2007) A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 54(4): 559-66.
Gitler D, Takagishi Y, Feng J, Ren Y, Rodriguiz RM, Wetsel WC, Greengard P, Augustine GJ (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. J Neurosci 24(50): 11368-80.
Goldman SA, Nedergaard M (1992) Newly generated neurons of the adult songbird brain become functionally active in long-term culture. Brain Res Dev Brain Res 68(2): 217-23.
Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A 80(8): 2390-4.
Gould E (2007) How widespread is adult neurogenesis in mammals? Nat Rev Neurosci 8 (6): 481-8.
Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2(3): 260-5.
Gratzner HG (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 218(4571): 474-5.
Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259(5096): 780-5.
Greer PL, Greenberg ME (2008) From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron 59(6): 846-60.
Groszer M, Keays DA, Deacon RM, de Bono JP, Prasad-Mulcare S, Gaub S, Baum MG, French CA, Nicod J, Coventry JA, Enard W, Fray M, Brown SD, Nolan PM, Pääbo S, Channon KM, Costa RM, Eilers J, Ehret G, Rawlins JN, Fisher SE (2008) Impaired synaptic plasticity and motor learning in mice with a point mutation implicated in human speech deficits. Curr Biol 18(5): 354-62.
Gubits RM, Smith TM, Fairhurst JL, Yu H (1989) Adrenergic receptors mediate changes in c-fos mRNA levels in brain. Brain Res Mol Brain Res 6(1): 39-45.
Guthrie SC, Gilula NB (1989) Gap junctional communication and development. Trends Neurosci 12(1): 12-6.

Guzowski JF (2002) Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. Hippocampus 12(1): 86-104.

Guzowski JF, McNaughton BL, Barnes CA, Worley PF (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci 2(12): 1120-4.

Guzowski JF, Timlin JA, Roysam B, McNaughton BL, Worley PF, Barnes CA (2005) Mapping behaviorally relevant neural circuits with immediate-early gene expression. Curr Opin Neurobiol 15(5): 599-606.

Haesler S, Rochefort C, Georgi B, Licznerski P, Osten P, Scharff C (2007) Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. PLoS Biol 5(12): e321.

Haesler S, Wada K, Nshdejan A, Morrisey EE, Lints T, Jarvis ED, Scharff C (2004) FoxP2 expression in avian vocal learners and non-learners. J Neurosci 24(13): 3164-75.

Hahnloser RH, Fee MS (2007) Sleep-related spike bursts in HVC are driven by the nucleus interface of the nidopallium. J Neurophysiol 97(1): 423-35.

Hahnloser RH, Kozhevnikov AA, Fee MS (2002) An ultra-sparse code underlies the generation of neural sequences in a songbird. Nature 419(6902): 65-70.

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11(3): 277-84.

Hara E, Kubikova L, Hessler NA, Jarvis ED (2007) Role of the midbrain dopaminergic system in modulation of vocal brain activation by social context. Eur J Neurosci 25(11): 3406-16.

Hayes NL, Nowakowski RS (2000) Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. Dev Neurosci 22(1-2): 44-55.

Heimovics SA, Riters LV (2005) Immediate early gene activity in song control nuclei and brain areas regulating motivation relates positively to singing behavior during, but not outside of, a breeding context. J Neurobiol 65(3): 207-24.

Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28(3): 370-490.

Hernandez-Rabaza V, Llorens-Martin M, Velazquez-Sanchez C, Ferragud A, Arcusa A, Gumus HG, Gomez-Pinedo U, Perez-Villalba A, Rosello J, Trejo JL, Barcia JA, Canales JJ (2009) Inhibition of adult hippocampal neurogenesis disrupts contextual learning but spares spatial working memory, long-term conditional rule retention and spatial reversal. Neuroscience 159(1): 59-68.

Hessler NA, Doupe AJ (1999a) Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. J Neurosci 19(23): 10461-81.

Hessler NA, Doupe AJ (1999b) Social context modulates singing-related neural activity in the songbird forebrain. Nat Neurosci 2(3): 209-11.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354(1381): 269-79.

Holzenberger M, Jarvis ED, Chong C, Grossman M, Nottebohm F, Scharff C (1997) Selective expression of insulin-like growth factor II in the songbird brain. J Neurosci 17 (18): 6974-87.

Horch HW, Kruttgen A, Portbury SD, Katz LC (1999) Destabilization of cortical dendrites and spines by BDNF. Neuron 23(2): 353-64.

Hoshino T, Nagashima T, Cho KG, Davis RL, Donegan J, Slusarz M, Wilson CB (1989) Variability in the proliferative potential of human gliomas. J Neurooncol 7(2): 137-43.

Houx AB, Ten Cate C (1999) Song learning from playback in zebra finches: is there an effect of operant contingency? Anim Behav 57(4): 837-845.

Hsieh J, Aimone JB, Kaspar BK, Kuwabara T, Nakashima K, Gage FH (2004) IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J Cell Biol 164(1): 111-22.

Humblot N, Thiriet N, Gobaille S, Aunis D, Zwiller J (1998) The serotonergic system modulates the cocaine-induced expression of the immediate early genes egr-1 and c-fos in rat brain. Ann N Y Acad Sci 844: 7-20.

Hurley P, Pytte C, Kirn JR (2008) Nest of origin predicts adult neuron addition rates in the vocal control system of the zebra finch. Brain Behav Evol 71(4): 263-70.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11(10): 1153-61.

Immelman K (1969). Song development in the zebra finch and other estrilid finches. Bird vocalizations. RA Hinde. New York, Cambridge University Press: 61–74.

Inda MC, DeFelipe J, Munoz A (2006) Voltage-gated ion channels in the axon initial segment of human cortical pyramidal cells and their relationship with chandelier cells. Proc Natl Acad Sci U S A 103(8): 2920-5.

Ingham PW, McMahon AP (2001) Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15(23): 3059-87.

Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH, Evans RM (2006) Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proc Natl Acad Sci U S A 103(10): 3902-7.

Jagasia R, Song H, Gage FH, Lie DC (2006) New regulators in adult neurogenesis and their potential role for repair. Trends Mol Med 12(9): 400-5.

Jarvis ED (2004) Learned birdsong and the neurobiology of human language. Ann N Y Acad Sci 1016: 749-77.

Jarvis ED, Güntürkün O, Bruce L, Csillag A, Karten H, Kuenzel W, Medina L, Paxinos G, Perkel DJ, Shimizu T, Striedter G, Wild JM, Ball GF, Dugas-Ford J, Durand SE, Hough GE, Husband S, Kubikova L, Lee DW, Mello CV, Powers A, Siang C, Smulders TV, Wada K, White SA, Yamamoto K, Yu J, Reiner A, Butler AB (2005) Avian brains and a new understanding of vertebrate brain evolution. Nat Rev Neurosci 6(2): 151-9.

Jarvis ED, Nottebohm F (1997) Motor-driven gene expression. Proc Natl Acad Sci U S A 94(8): 4097-102.

Jarvis ED, Scharff C, Grossman MR, Ramos JA, Nottebohm F (1998) For whom the bird sings: context-dependent gene expression. Neuron 21(4): 775-88.

Jessberger S, Kempermann G (2003) Adult-born hippocampal neurons mature into activity-dependent responsiveness. Eur J Neurosci 18(10): 2707-12.

Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp Neurol 196(2): 342-51.

Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1(1): 20-9.

Jimenez J, Heliot R, Carmena JM (2009) Learning to use a brain-machine interface: Model, simulation and analysis. Conf Proc IEEE Eng Med Biol Soc 1: 4551-4.

Jin H, Clayton DF (1997) Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. Neuron 19(5): 1049-59.

Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA (2006) Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A 103(35): 13198-202.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267(5204): 1658-62.

Kao MH, Doupe AJ, Brainard MS (2005) Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song. Nature 433(7026): 638-43.

Katz LC, Gurney ME (1981) Auditory responses in the zebra finch's motor system for song. Brain Res 221(1): 192-7.

Kaufmann E, Knochel W (1996) Five years on the wings of fork head. Mech Dev 57(1): 3-20.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10(3): 355-62.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27(8): 447-52.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386(6624): 493-5.

Kimpo RR, Doupe AJ (1997) FOS is induced by singing in distinct neuronal populations in a motor network. Neuron 18(2): 315-25.

Kirn JR, Alvarez-Buylla A, Nottebohm F (1991) Production and survival of projection neurons in a forebrain vocal center of adult male canaries. J Neurosci 11(6): 1756-62.

Kirn JR, Fishman Y, Sasportas K, Alvarez-Buylla A, Nottebohm F (1999) Fate of new neurons in adult canary high vocal center during the first 30 days after their formation. J Comp Neurol 411(3): 487-94.

Kirn JR, Nottebohm F (1993) Direct evidence for loss and replacement of projection neurons in adult canary brain. J Neurosci 13(4): 1654-63.

Kirn JR, O'Loughlin B, Kasparian S, Nottebohm F (1994) Cell death and neuronal recruitment in the high vocal center of adult male canaries are temporally related to changes in song. Proc Natl Acad Sci U S A 91(17): 7844-8.

Kisby GE, Olivas A, Park T, Churchwell M, Doerge D, Samson LD, Gerson SL, Turker MS (2009) DNA repair modulates the vulnerability of the developing brain to alkylating agents. DNA Repair (Amst) 8(3): 400-12.

Kittappa R, Chang WW, Awatramani RB, McKay RD (2007) The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. PLoS Biol 5(12): e325.

Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD (1997) Neuropilin is a semaphorin III receptor. Cell 90(4): 753-62.

Kruse AA, Stripling R, Clayton DF (2000) Minimal experience required for immediate-early gene induction in zebra finch neostriatum. Neurobiol Learn Mem 74(3): 179-84.
Kruse AA, Stripling R, Clayton DF (2004) Context-specific habituation of the zenk gene response to song in adult zebra finches. Neurobiol Learn Mem 82(2): 99-108.
Kuan CY, Schloemer AJ, Lu A, Burns KA, Weng WL, Williams MT, Strauss KI, Vorhees CV, Flavell RA, Davis RJ, Sharp FR, Rakic P (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci 24 (47): 10763-72.
Kubikova L, Turner EA, Jarvis ED (2007) The pallial basal ganglia pathway modulates the behaviorally driven gene expression of the motor pathway. Eur J Neurosci 25(7): 2145-60.
Kuipers SD, Tiron A, Soule J, Messaoudi E, Trentani A, Bramham CR (2009) Selective survival and maturation of adult-born dentate granule cells expressing the immediate early gene Arc/Arg3.1. PLoS One 4(3): e4885.
Kunze A, Congreso MR, Hartmann C, Wallraff-Beck A, Huttmann K, Bedner P, Requardt R, Seifert G, Redecker C, Willecke K, Hofmann A, Pfeifer A, Theis M, Steinhauser C (2009) Connexin expression by radial glia-like cells is required for neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A 106(27): 11336-41.
Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP (2001) A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 413(6855): 519-23.
Lai E, Clark KL, Burley SK, Darnell JE, Jr. (1993) Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biologic function. Proc Natl Acad Sci U S A 90(22): 10421-3.
Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE, Jr. (1990) HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev 4(8): 1427-36.
Lamprecht R, Dudai Y (1996) Transient expression of c-Fos in rat amygdala during training is required for encoding conditioned taste aversion memory. Learn Mem 3(1): 31-41.
Lanahan A, Worley P (1998) Immediate-early genes and synaptic function. Neurobiol Learn Mem 70(1-2): 37-43.
Laplagne DA, Esposito MS, Piatti VC, Morgenstern NA, Zhao C, van Praag H, Gage FH, Schinder AF (2006) Functional convergence of neurons generated in the developing and adult hippocampus. PLoS Biol 4(12): e409.
Lee E, Son H (2009) Adult hippocampal neurogenesis and related neurotrophic factors. BMB Rep 42(5): 239-44.
Lee J, Rajakumar N (2003) Role of NR2B-containing N-methyl-D-aspartate receptors in haloperidol-induced c-Fos expression in the striatum and nucleus accumbens. Neuroscience 122(3): 739-45.
Leonardo A, Fee MS (2005) Ensemble coding of vocal control in birdsong. J Neurosci 25 (3): 652-61.
Leuner B, Gould E, Shors TJ (2006) Is there a link between adult neurogenesis and learning? Hippocampus 16(3): 216-24.
Li S, Weidenfeld J, Morrisey EE (2004) Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Mol Cell Biol 24(2): 809-22.

Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F (2000) A relationship between behavior, neurotrophin expression, and new neuron survival. Proc Natl Acad Sci U S A 97(15): 8584-9.

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437(7063): 1370-5.

Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M (1998) Gap-junction-mediated propagation and amplification of cell injury. Nat Neurosci 1(6): 494-500.

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7(3): 179-93.

Lohof AM, Ip NY, Poo MM (1993) Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363(6427): 350-3.

Lombardino AJ, Hertel M, Li XC, Haripal B, Martin-Harris L, Pariser E, Nottebohm F (2006) Expression profiling of intermingled long-range projection neurons harvested by laser capture microdissection. J Neurosci Methods 157(2): 195-207.

London SE, Clayton DF (2008) Functional identification of sensory mechanisms required for developmental song learning. Nat Neurosci 11(5): 579-86.

Lovell PV, Clayton DF, Replogle KL, Mello CV (2008) Birdsong "transcriptomics": neurochemical specializations of the oscine song system. PLoS One 3(10): e3440.

Luo M, Ding L, Perkel DJ (2001) An avian basal ganglia pathway essential for vocal learning forms a closed topographic loop. J Neurosci 21(17): 6836-45.

Lynch TP, Cass CE, Paterson AR (1977) Defective transport of thymidine by cultured cells resistant to 5-bromodeoxyuridine. J Supramol Struct 6(3): 363-74.

Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H (2009a) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323(5917): 1074-7.

Ma DK, Kim WR, Ming GL, Song H (2009b) Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. Ann N Y Acad Sci 1170: 664-73.

Ma DK, Ming GL, Song H (2005) Glial influences on neural stem cell development: cellular niches for adult neurogenesis. Curr Opin Neurobiol 15(5): 514-20.

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8(10): 755-65.

Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G (2003) Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience 119(3): 635-42.

Magavi SS, Mitchell BD, Szentirmai O, Carter BS, Macklis JD (2005) Adult-born and preexisting olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses in vivo. J Neurosci 25(46): 10729-39.

Markakis EA, Gage FH (1999) Adult-generated neurons in the dentate gyrus send axonal projections to field CA3 and are surrounded by synaptic vesicles. J Comp Neurol 406 (4): 449-60.

Marler P (1970) Birdsong and speech development: could there be parallels. Am. Sci. 58: 669-73.

Marler P (1997) Three models of song learning: evidence from behavior. J Neurobiol 33 (5): 501-16.

Matsunaga E, Okanoya K (2008) Expression analysis of cadherins in the songbird brain: relationship to vocal system development. J Comp Neurol 508(2): 329-42.

Matsunaga E, Okanoya K (2009a) Evolution and diversity in avian vocal system: an Evo-Devo model from the morphological and behavioral perspectives. Dev Growth Differ 51 (3): 355-67.

Matsunaga E, Okanoya K (2009b) Vocal control area-related expression of neuropilin-1, plexin-A4, and the ligand semaphorin-3A has implications for the evolution of the avian vocal system. Dev Growth Differ 51(1): 45-54.

McAllister AK, Katz LC, Lo DC (1996) Neurotrophin regulation of cortical dendritic growth requires activity. Neuron 17(6): 1057-64.

Mello C, Nottebohm F, Clayton D (1995) Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. J Neurosci 15(10): 6919-25.

Mello CV, Pinaud R, Ribeiro S (1998) Noradrenergic system of the zebra finch brain: immunocytochemical study of dopamine-beta-hydroxylase. J Comp Neurol 400(2): 207-28.

Mello CV, Vicario DS, Clayton DF (1992) Song presentation induces gene expression in the songbird forebrain. Proc Natl Acad Sci U S A 89(15): 6818-22.

Mileusnic R, Anokhin K, Rose SP (1996) Antisense oligodeoxynucleotides to c-fos are amnestic for passive avoidance in the chick. Neuroreport 7(7): 1269-72.

Miller JE, Spiteri E, Condro MC, Dosumu-Johnson RT, Geschwind DH, White SA (2008) Birdsong decreases protein levels of FoxP2, a molecule required for human speech. J Neurophysiol 100(4): 2015-25.

Miller MW, Nowakowski RS (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res 457(1): 44-52.

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28: 223-50.

Molnar G, Crozat A, Pardee AB (1994) The immediate-early gene Egr-1 regulates the activity of the thymidine kinase promoter at the G0-to-G1 transition of the cell cycle. Mol Cell Biol 14(8): 5242-8.

Mongiat LA, Esposito MS, Lombardi G, Schinder AF (2009) Reliable activation of immature neurons in the adult hippocampus. PLoS One 4(4): e5320.

Monje ML, Mizumatsu S, Fike JR, Palmer TD (2002) Irradiation induces neural precursor-cell dysfunction. Nat Med 8(9): 955-62.

Mooney R (2000) Different subthreshold mechanisms underlie song selectivity in identified HVc neurons of the zebra finch. J Neurosci 20(14): 5420-36.

Mooney R, Prather JF (2005) The HVC microcircuit: the synaptic basis for interactions between song motor and vocal plasticity pathways. J Neurosci 25(8): 1952-64.

Moore RK (2007) Spoken language processing: Piecing together the puzzle. Speech Communication 49(5): 418-435

Morris SM (1991) The genetic toxicology of 5-bromodeoxyuridine in mammalian cells. Mutat Res 258(2): 161-88.

Morris SM, Domon OE, McGarrity LJ, Kodell RL, Casciano DA (1992) Effect of bromodeoxyuridine on the proliferation and growth of ethyl methanesulfonate-exposed P3 cells: relationship to the induction of sister-chromatid exchanges. Cell Biol Toxicol 8 (1): 75-87.

Morrow BA, Elsworth JD, Inglis FM, Roth RH (1999) An antisense oligonucleotide reverses the footshock-induced expression of fos in the rat medial prefrontal cortex and the subsequent expression of conditioned fear-induced immobility. J Neurosci 19(13): 5666-73.

Nacher J, Varea E, Miguel Blasco-Ibanez J, Gomez-Climent MA, Castillo-Gomez E, Crespo C, Martinez-Guijarro FJ, McEwen BS (2007) N-methyl-d-aspartate receptor expression during adult neurogenesis in the rat dentate gyrus. Neuroscience 144(3): 855-64.

Nagao T, Kuwagata M, Saito Y (1998) Effects of prenatal exposure to 5-fluoro-2'-deoxyuridine on developing central nervous system and reproductive function in male offspring of mice. Teratog Carcinog Mutagen 18(2): 73-92.

Nakagawa S, Kim JE, Lee R, Malberg JE, Chen J, Steffen C, Zhang YJ, Nestler EJ, Duman RS (2002) Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. J Neurosci 22(9): 3673-82.

Nissant A, Bardy C, Katagiri H, Murray K, Lledo PM (2009) Adult neurogenesis promotes synaptic plasticity in the olfactory bulb. Nat Neurosci 12(6): 728-30.

Nottebohm F (1993) The search for neural mechanisms that define the sensitive period for song learning in birds. Neth. J. Zool. 43(193-234).

Nottebohm F (2002) Why are some neurons replaced in adult brain? J Neurosci 22(3): 624-8.

Nottebohm F (2004) The road we travelled: discovery, choreography, and significance of brain replaceable neurons. Ann N Y Acad Sci 1016: 628-58.

Nottebohm F, Kelley DB, Paton JA (1982) Connections of vocal control nuclei in the canary telencephalon. J Comp Neurol 207(4): 344-57.

Nottebohm F, Stokes TM, Leonard CM (1976) Central control of song in the canary, Serinus canarius. J Comp Neurol 165(4): 457-86.

Nowakowski RS, Lewin SB, Miller MW (1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol 18(3): 311-8.

O'Donovan KJ, Tourtellotte WG, Millbrandt J, Baraban JM (1999) The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. Trends Neurosci 22(4): 167-73.

Ogburn CE, Austad SN, Holmes DJ, Kiklevich JV, Gollahon K, Rabinovitch PS, Martin GM (1998) Cultured renal epithelial cells from birds and mice: enhanced resistance of avian cells to oxidative stress and DNA damage. J Gerontol A Biol Sci Med Sci 53(4): B287-92.

Ohta K, Takagi S, Asou H, Fujisawa H (1992) Involvement of neuronal cell surface molecule B2 in the formation of retinal plexiform layers. Neuron 9(1): 151-61.

Overstreet LS, Hentges ST, Bumaschny VF, de Souza FS, Smart JL, Santangelo AM, Low MJ, Westbrook GL, Rubinstein M (2004) A transgenic marker for newly born granule cells in dentate gyrus. J Neurosci 24(13): 3251-9.

Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. Neuroscientist 9(4): 261-72.

Patel M, McCully C, Godwin K, Balis FM (2003) Plasma and cerebrospinal fluid pharmacokinetics of intravenous temozolomide in non-human primates. J Neurooncol 61(3): 203-7.

Patel SN, Stewart MG (1988) Changes in the number and structure of dendritic spines 25 hours after passive avoidance training in the domestic chick, Gallus domesticus. Brain Res 449(1-2): 34-46.

Paton JA, Nottebohm FN (1984) Neurons generated in the adult brain are recruited into functional circuits. Science 225(4666): 1046-8.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16(6): 1137-45.

Pei Q, Lewis L, Sprakes ME, Jones EJ, Grahame-Smith DG, Zetterström TS (2000) Serotonergic regulation of mRNA expression of Arc, an immediate early gene selectively localized at neuronal dendrites. Neuropharmacology 39(3): 463-70.

Person AL, Gale SD, Farries MA, Perkel DJ (2008) Organization of the songbird basal ganglia, including area X. J Comp Neurol 508(5): 840-66.

Poirier R, Cheval H, Mailhes C, Garel S, Charnay P, Davis S, Laroche S (2008) Distinct functions of egr gene family members in cognitive processes. Front Neurosci 2(1): 47-55.

Prather JF, Peters S, Nowicki S, Mooney R (2008) Precise auditory-vocal mirroring in neurons for learned vocal communication. Nature 451(7176): 305-10.

Price SR, De Marco Garcia NV, Ranscht B, Jessell TM (2002) Regulation of motor neuron pool sorting by differential expression of type II cadherins. Cell 109(2): 205-16.

Pytte CL, Gerson M, Miller J, Kirn JR (2007) Increasing stereotypy in adult zebra finch song correlates with a declining rate of adult neurogenesis. Dev Neurobiol 67(13): 1699-720.

Rakic P (2002) Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci 3(1): 65-71.

Ramirez-Amaya V, Marrone DF, Gage FH, Worley PF, Barnes CA (2006) Integration of new neurons into functional neural networks. J Neurosci 26(47): 12237-41.

Rauske PL, Shea SD, Margoliash D (2003) State and neuronal class-dependent reconfiguration in the avian song system. J Neurophysiol 89(3): 1688-701.

Red'ko VG, Prokhorov DV, Burtsev MS (2004) Theory of functional systems, adaptive critics and neural networks. IEEE International Joint Conference on Neural Networks. (3) 1787-1792.

Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M, Ball GF, Durand S, Gunturkun O, Lee DW, Mello CV, Powers A, White SA, Hough G, Kubikova L, Smulders TV, Wada K, Dugas-Ford J, Husband S, Yamamoto K, Yu J, Siang C, Jarvis ED (2004) Revised nomenclature for avian telencephalon and some related brainstem nuclei. J Comp Neurol 473(3): 377-414.

Rhee DJ, Kong DS, Kim WS, Park KB, Lee JI, Suh YL, Song SY, Kim ST, Lim DH, Park K, Kim JH, Nam DH (2009) Efficacy of temozolomide as adjuvant chemotherapy after postsurgical radiotherapy alone for glioblastomas. Clin Neurol Neurosurg 111(9): 748-51.

Rochefort C, He X, Scotto-Lomassese S, Scharff C (2007) Recruitment of FoxP2-expressing neurons to area X varies during song development. Dev Neurobiol 67(6): 809-17.

Rochefort C, Lledo PM (2005) Short-term survival of newborn neurons in the adult olfactory bulb after exposure to a complex odor environment. Eur J Neurosci 22(11): 2863-70.

Roeske CC (2009) The role of retinoid signaling in the song control system of the zebra finch. PhD. Berlin, Free University Berlin.

Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, Fike JR (2004) Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol 188(2): 316-30.

Romero-Grimaldi C, Moreno-López B, Estrada C (2008) Age-dependent effect of nitric oxide on subventricular zone and olfactory bulb neural precursor proliferation. J Comp Neurol 506(2): 339-46.

Rose SP (1991) How chicks make memories: the cellular cascade from c-fos to dendritic remodelling. Trends Neurosci 14(9): 390-7.

Rose SP, Stewart MG (1999) Cellular correlates of stages of memory formation in the chick following passive avoidance training. Behav Brain Res 98(2): 237-43.

Rosen MJ, Mooney R (2006) Synaptic interactions underlying song-selectivity in the avian nucleus HVC revealed by dual intracellular recordings. J Neurophysiol 95(2): 1158-75.

Rousso DL, Gaber ZB, Wellik D, Morrisey EE, Novitch BG (2008) Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. Neuron 59(2): 226-40.

Roy A, Mooney R (2009) Song decrystallization in adult zebra finches does not require the song nucleus NIf. J Neurophysiol 102(2): 979-91.

Saffhill R, Ockey CH (1985) Strand breaks arising from the repair of the 5-bromodeoxyuridine-substituted template and methyl methanesulphonate-induced lesions can explain the formation of sister chromatid exchanges. Chromosoma 92(3): 218-24.

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10 (9): 1110-5.

Sanders JD, Happe HK, Bylund DB, Murrin LC (2008) Differential effects of neonatal norepinephrine lesions on immediate early gene expression in developing and adult rat brain. Neuroscience 157(4): 821-32.

Scharff C, Kirn JR, Grossman M, Macklis JD, Nottebohm F (2000) Targeted neuronal death affects neuronal replacement and vocal behavior in adult songbirds. Neuron 25(2): 481-92.

Scharff C, Nottebohm F (1991) A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J Neurosci 11(9): 2896-913.

Scharff C, Nottebohm F, Cynx J (1998) Conspecific and heterospecific song discrimination in male zebra finches with lesions in the anterior forebrain pathway. J Neurobiol 36(1): 81-90.

Schiff D, Wen PY, van den Bent MJ (2009) Neurological adverse effects caused by cytotoxic and targeted therapies. Nat Rev Clin Oncol 6(10): 596-603.

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429(6988): 184-7.

Schweighofer N, Tanaka SC, Doya K (2007) Serotonin and the evaluation of future rewards: theory, experiments, and possible neural mechanisms. Ann N Y Acad Sci 1104: 289-300.

Scott BB, Lois C (2007) Developmental origin and identity of song system neurons born during vocal learning in songbirds. J Comp Neurol 502(2): 202-14.

Scott LL, Nordeen EJ, Nordeen KW (2000) The relationship between rates of HVc neuron addition and vocal plasticity in adult songbirds. J Neurobiol 43(1): 79-88.

Scott LL, Singh TD, Nordeen EJ, Nordeen KW (2004) Developmental patterns of NMDAR expression within the song system do not recur during adult vocal plasticity in zebra finches. J Neurobiol 58(4): 442-54.

Scotto-Lomassese S, Rochefort C, Nshdejan A, Scharff C (2007) HVC interneurons are not renewed in adult male zebra finches. Eur J Neurosci 25(6): 1663-8.

Sekerkova G, Ilijic E, Mugnaini E (2004) Bromodeoxyuridine administered during neurogenesis of the projection neurons causes cerebellar defects in rat. J Comp Neurol 470(3): 221-39.

Shen L, Nam HS, Song P, Moore H, Anderson SA (2006) FoxG1 haploinsufficiency results in impaired neurogenesis in the postnatal hippocampus and contextual memory deficits. Hippocampus 16(10): 875-90.

Shi Y, Chichung Lie D, Taupin P, Nakashima K, Ray J, Yu RT, Gage FH, Evans RM (2004) Expression and function of orphan nuclear receptor TLX in adult neural stem cells. Nature 427(6969): 78-83.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20(4): 727-40.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410(6826): 372-6.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12 (5): 578-84.

Shu W, Yang H, Zhang L, Lu MM, Morrisey EE (2001) Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors. J Biol Chem 276(29): 27488-97.

Sidman RL, Miale IL, Feder N (1959) Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. Exp Neurol 1: 322-33.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. Neuroscience 130(4): 843-52.

Solis MM, Brainard MS, Hessler NA, Doupe AJ (2000) Song selectivity and sensorimotor signals in vocal learning and production. Proc Natl Acad Sci U S A 97(22): 11836-42.

Solis MM, Doupe AJ (1999) Contributions of tutor and bird's own song experience to neural selectivity in the songbird anterior forebrain. J Neurosci 19(11): 4559-84.

Sossinka R, Böhner J (1980) Song types in the zebra finch (Poephila guttata castanotis). Z.Tierpsychol. 53: 123-132.

Spiteri E, Konopka G, Coppola G, Bomar J, Oldham M, Ou J, Vernes SC, Fisher SE, Ren B, Geschwind DH (2007) Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am J Hum Genet 81(6): 1144-57.

Standaert DG, Testa CM, Young AB, Penney JB, Jr. (1994) Organization of N-methyl-D-aspartate glutamate receptor gene expression in the basal ganglia of the rat. J Comp Neurol 343(1): 1-16.

Stokes TM, Leonard CM, Nottebohm F (1974) The telencephalon, diencephalon, and mesencephalon of the canary, Serinus canaria, in stereotaxic coordinates. J Comp Neurol 156(3): 337-74.
Stripling R, Volman SF, Clayton DF (1997) Response modulation in the zebra finch neostriatum: relationship to nuclear gene regulation. J Neurosci 17(10): 3883-93.
Takahashi T, Nowakowski RS, Caviness VS, Jr. (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15(9): 6046-57.
Takeichi M (2007) The cadherin superfamily in neuronal connections and interactions. Nat Rev Neurosci 8(1): 11-20.
Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM (1999) Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99(1): 71-80.
Tamura S, Morikawa Y, Iwanishi H, Hisaoka T, Senba E (2004) Foxp1 gene expression in projection neurons of the mouse striatum. Neuroscience 124(2): 261-7.
Tao W, Lai E (1992) Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron 8(5): 957-66.
Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20(4): 709-26.
Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH (2006) NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. Nature 442(7105): 929-33.
Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53(1): 198-214.
Tchernichovski O, Nottebohm F (1998) Social inhibition of song imitation among sibling male zebra finches. Proc Natl Acad Sci U S A 95(15): 8951-6.
Tchernichovski O, Nottebohm F, Ho CE, Pesaran B, Mitra PP (2000) A procedure for an automated measurement of song similarity. Anim Behav 59(6): 1167-1176.
Teramitsu I, Kudo LC, London SE, Geschwind DH, White SA (2004) Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. J Neurosci 24(13): 3152-63.
Terpstra NJ, Bolhuis JJ, den Boer-Visser AM (2004) An analysis of the neural representation of birdsong memory. J Neurosci 24(21): 4971-7.
Thompson CK, Brenowitz EA (2009) Neurogenesis in an adult avian song nucleus is reduced by decreasing caspase-mediated apoptosis. J Neurosci 29(14): 4586-91.
Thompson Haskell G, Maynard TM, Shatzmiller RA, Lamantia AS (2002) Retinoic acid signaling at sites of plasticity in the mature central nervous system. J Comp Neurol 452 (3): 228-41.
Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47(6): 803-15.
Trouche S, Bontempi B, Roullet P, Rampon C (2009) Recruitment of adult-generated neurons into functional hippocampal networks contributes to updating and strengthening of spatial memory. Proc Natl Acad Sci U S A 106(14): 5919-24.
Troyer TW, Doupe AJ (2000) An associational model of birdsong sensorimotor learning I. Efference copy and the learning of song syllables. J Neurophysiol 84(3): 1204-23.

Tung A, Herrera S, Fornal CA, Jacobs BL (2008) The effect of prolonged anesthesia with isoflurane, propofol, dexmedetomidine, or ketamine on neural cell proliferation in the adult rat. Anesth Analg 106(6): 1772-7.

van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2(3): 266-70.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415(6875): 1030-4.

Velho TA, Mello CV (2008) Synapsins are late activity-induced genes regulated by birdsong. J Neurosci 28(46): 11871-82.

Velho TA, Pinaud R, Rodrigues PV, Mello CV (2005) Co-induction of activity-dependent genes in songbirds. Eur J Neurosci 22(7): 1667-78.

Vernes SC, Spiteri E, Nicod J, Groszer M, Taylor JM, Davies KE, Geschwind DH, Fisher SE (2007) High-throughput analysis of promoter occupancy reveals direct neural targets of FOXP2, a gene mutated in speech and language disorders. Am J Hum Genet 81(6): 1232-50.

Wada K, Howard JT, McConnell P, Whitney O, Lints T, Rivas MV, Horita H, Patterson MA, White SA, Scharff C, Haesler S, Zhao S, Sakaguchi H, Hagiwara M, Shiraki T, Hirozane-Kishikawa T, Skene P, Hayashizaki Y, Carninci P, Jarvis ED (2006) A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. Proc Natl Acad Sci U S A 103(41): 15212-7.

Wada K, Sakaguchi H, Jarvis ED, Hagiwara M (2004) Differential expression of glutamate receptors in avian neural pathways for learned vocalization. J Comp Neurol 476(1): 44-64.

Wang IC, Chen YJ, Hughes D, Petrovic V, Major ML, Park HJ, Tan Y, Ackerson T, Costa RH (2005a) Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol Cell Biol 25(24): 10875-94.

Wang JQ, Smith AJ, McGinty JF (1995) A single injection of amphetamine or methamphetamine induces dynamic alterations in c-fos, zif/268 and preprodynorphin messenger RNA expression in rat forebrain. Neuroscience 68(1): 83-95.

Wang TW, Zhang H, Parent JM (2005b) Retinoic acid regulates postnatal neurogenesis in the murine subventricular zone-olfactory bulb pathway. Development 132(12): 2721-32.

Weigel D, Jackle H (1990) The fork head domain: a novel DNA binding motif of eukaryotic transcription factors? Cell 63(3): 455-6.

Wexler EM, Paucer A, Kornblum HI, Plamer TD, Geschwind DH (2009) Endogenous Wnt signaling maintains neural progenitor cell potency. Stem Cells 27(5): 1130-41.

Whitney O, Soderstrom K, Johnson F (2000) Post-transcriptional regulation of zenk expression associated with zebra finch vocal development. Brain Res Mol Brain Res 80 (2): 279-90.

Wild JM (1993) Descending projections of the songbird nucleus robustus archistriatalis. J Comp Neurol 338(2): 225-41.

Wild JM, Kubke MF, Mooney R (2009) Avian nucleus retroambigualis: cell types and projections to other respiratory-vocal nuclei in the brain of the zebra finch (Taeniopygia guttata). J Comp Neurol 512(6): 768-83.

Wild JM, Williams MN, Howie GJ, Mooney R (2005) Calcium-binding proteins define interneurons in HVC of the zebra finch (Taeniopygia guttata). J Comp Neurol 483(1): 76-90.

Williams H, Mehta N (1999) Changes in adult zebra finch song require a forebrain nucleus that is not necessary for song production. J Neurobiol 39(1): 14-28.

Wirtshafter D, Asin KE (1995) Dopamine antagonists induce fos-like-immunoreactivity in the substantia nigra and entopeduncular nucleus of the rat. Brain Res 670(2): 205-14.

Wojtowicz JM (2006) Irradiation as an experimental tool in studies of adult neurogenesis. Hippocampus 16(3): 261-6.

Woolley SC, Doupe AJ (2008) Social context-induced song variation affects female behavior and gene expression. PLoS Biol 6(3): e62.

Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E (1995) Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron 14(6): 1141-52.

Yamada Y, Hada Y, Imamura K, Mataga N, Watanabe Y, Yamamoto M (1999) Differential expression of immediate-early genes, c-fos and zif268, in the visual cortex of young rats: effects of a noradrenergic neurotoxin on their expression. Neuroscience 92(2): 473-84.

Yu AC, Margoliash D (1996) Temporal hierarchical control of singing in birds. Science 273(5283): 1871-5.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132(4): 645-60.

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26(1): 3-11.

Zhu XJ, Hua Y, Jiang J, Zhou QG, Luo CX, Han X, Lu YM, Zhu DY (2006) Neuronal nitric oxide synthase-derived nitric oxide inhibits neurogenesis in the adult dentate gyrus by down-regulating cyclic AMP response element binding protein phosphorylation. Neuroscience 141(2): 827-36.