Role of FoxP2 during functional recruitment of post-hatch-generated medium spiny neurons in a brain region relevant for vocal learning in male zebra finches

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Table of contents

Abstract

Adult neurogenesis is a process in which new neurons are generated in neurogenic niches and become recruited into distinct regions of the mature brain. In the adult songbird brain, new neurons are incorporated into areas that facilitate learning, production and maintenance of song. The striatal song nucleus Area X constantly receives new medium spiny neurons (MSNs) throughout adulthood, but it was not known if they are functionally integrated into the preexisting circuitry. To address this question, I applied Bromodeoxyuridine (BrdU) and lentiviral vector-mediated labelling of progenitor cells and examined the maturation, connectivity and singing elicited activation and of their progeny in Area X after different survival periods. Six weeks after their birth, the majority of new neurons expressed a marker for mature MSNs, show pre- and postsynaptic connections and expressed dopamine receptors, indicative of dopaminergic innervation. The expression of the immediate early gene EGR-1 (early growth response protein 1) was used to assess if and at what age new neurons were activated by singing. Already three weeks after their labelling, a small fraction of new MSNs expressed EGR-1 after singing and this fraction increased with progressing maturation. Measuring MSN densities in zebra finches up to seven years of age provided insights into the dynamics of striatal adult neurogenesis and revealed that it is a process of constant new neuron addition.

New MSNs that are recruited into Area X express the forkhead box protein P2 (FoxP2). This transcription factor has important functions in mammalian brain development and mutations in FOXP2 cause speech and language impairments in humans. In zebra finches, correct FoxP2 expression levels in Area X are crucial for successful song learning and for song modulation between different social contexts. FoxP2 levels in Area X are high during the phase of song learning but generally low in adults and are downregulated by singing. MSNs in Area X exhibit different FoxP2 expression levels. Since FoxP2 downregulation after singing only occurs in MSNs with low FoxP2 levels ($FoxP2^{low}$) and not in MSNs with high FoxP2 levels ($FoxP2^{high}$), I postulated that the latter were recently recruited and need to become $FoxP2^{low}$ MSNs before they would be activated by singing. This hypothesis was tested by measuring FoxP2 protein levels and EGR-1 expression in individual new MSNs of singing and non-singing birds at different time points after BrdU birth dating. Interestingly, $FoxP2^{high}$ and $FoxP2^{low}$ MSNs were equally activated during singing, indicating that this is a process independent of FoxP2 levels. Further, I identified that one third of new MSNs expressed FoxP2 at high levels during early stages of their maturation. However, the majority of matured MSNs expressed FoxP2 at low levels, indicating an age-related decrease of FoxP2 levels in a subset

of newly recruited MSNs. Because Foxp2 was shown to enhance neuronal outgrowth and differentiation, I analyzed the dendrite morphology and the density of dendritic spines of FoxP2^{high} and FoxP2^{low} new MSNs that were virally labelled and expressed the green fluorescent protein. FoxP2^{high} new MSNs had more complex dendrites and a higher density of the mature mushroom spines than FoxP2^{low} new MSNs and thus probably received more pallial inputs during a narrow timeframe of their maturation. Comparing my results to what is known about MSNs of the direct and indirect pathway of the basal ganglia of rodents, I hypothesize that early differences in FoxP2 levels and concomitant diverging new MSNs morphology might indicate the existence of distinct MSN subtypes in Area X of zebra finches.

Altogether, the presented data illustrate that new MSNs recruited into Area X of adult zebra finches are functional and might play a role for the maintenance of song. Within the first six weeks after their birth new MSNs exhibited dynamic FoxP2 expression levels which are liked to their dendritic arborization and spine density, thus broadening FoxP2 function by an implication in striatal adult neurogenesis.

General Introduction

A brief history of adult neurogenesis

As many fundamental scientific discoveries, the first detection of newly generated neurons in adult brains was a coincidence. In the early sixties, Joseph Altman studied glia proliferation after injury and observed many newly generated cells far away from the lesion site (Altman, 1962b). He suspected that new neurons were generated in the adult brain (Altman, 1962a) and systematically injected rats and cats with tritiated thymidine at different ages (Altman, 1963). In follow-up studies he described postnatal neurogenesis in different species and brain regions, including the hippocampus and the olfactory bulb (Altman and Das, 1965a; b; 1967; Altman, 1969). Despite publishing his reports on postnatal neurogenesis in respected journals (Altman and Das, 1965b; 1967; Altman, 1969) strong criticism by prominent scientists of the time who maintained that neurogenesis was limited to pre-natal development (Rakic, 1974) caused these findings to be largely forgotten for two decades (Altman, 2011). The claims that *"adult centers, the nerve paths are something fixed, ended, and immutable"* and *"everything may die, nothing may be regenerated"* postulated by Ramón y Cajal in 1913 (Ramón y Cajal, 1913) kept being the dogma for another 20 years.

In 1983, Fernando Nottebohm was wondering about the seasonal volume changes in nuclei of the canary brain. Might fluctuations of neurons account for the volume differences? Using similar techniques as Altman had used, Nottebohm and his PhD student Steve Goldman found unequivocal evidence that neurons were born in the adult songbird brain (Goldman and Nottebohm, 1983). Tour-de-force follow up paper demonstrated that these new neurons were incorporated into functioning neural networks by showing that they responded physiologically to sound and made synapses with neighboring neurons (Paton and Nottebohm, 1984; Burd and Nottebohm, 1985). This "rediscovery" of neurogenesis in the adult telencephalon opened doors for many further investigations of adult neurogenesis in songbirds as well as in mammals (Doetsch and Scharff, 2001; Gould, 2007; Barnea and Pravosudov, 2011; Kempermann et al., 2015).

Songbirds as a model to study adult neurogenesis

Neural plasticity, the brains ability to adapt constantly to changing conditions, can be observed on many levels ranging from single molecules, strengthening or weakening of synapses to new connections within neuronal circuits (Citri and Malenka, 2008; Ho et al., 2011; Frisen, 2016). The addition of new neurons into preexisting functional circuits is another intriguing way to

generate plasticity, because neurons as single units can be variably tuned to current demands (Toda and Gage, 2018). Songbirds offer an excellent model to study neurogenesis in the adult brain because many new neurons are recruited into brain areas that are exclusively associated with one particular behavior: singing. By studying adult neurogenesis in birds, it is possible to relate new neuron addition to a measurable behavioral output and thus interpret its role for vocal learning and song production.

Vocal learning and song production

Vocal communication is a common trait in the animal kingdom, but vocal production learning (i.e. the imitation of an acoustically perceived sound) has been demonstrated only in eight animal groups: three groups of birds (hummingbirds, parrots and songbirds) and five groups of mammals (bats, cetaceans, elephants, humans and pinnipeds) (Janik and Slater, 1997; 2000). In the majority of the more than 4500 songbird species both males and females sing, which also seems to have been the ancestral state (Odom et al., 2014). However, in other species, including the Australian zebra finch, and also in most of those living in the temperate zone, only the males sing. The time and duration when songbirds learn to produce the acoustic elements of their song also varies considerably. Open-ended learners like the European starling incorporate new song elements throughout their lives, whereas closed learners like the zebra finch only sing the elements that they learned during a sensitive period early in their lives (Brainard and Doupe, 2002; London, 2017). During a sensorimotor phase, juvenile zebra finches hear and store the tutor's song while they simultaneously produce a subsong. At around 90 days post hatch the song has crystallized and usually resembles the tutor's song to a large extent. In comparison to many other songbirds, adult zebra finch song is quite stereotyped, with little variation between song renditions. It consists of multiple elements that form a motif. Introductory notes followed by multiple motifs form a song bout. Zebra finches sing in two different social contexts; they either direct their song towards a conspecific, in most cases during courtship (directed song), or they sing without directing their song towards a conspecific (undirected song, Sossinka and Böhner, 1980). Directed song is more stereotyped and faster than undirected song and often accompanied by a courtship dance (Sossinka and Böhner, 1980; Cooper and Goller, 2006; Ullrich et al., 2016). Despite subtle differences in song features, directed and undirected song elicit different neuronal activity, gene expression and neurotransmitter release (Jarvis et al., 1998; Leblois et al., 2010; Woolley et al., 2014).

The song system

The neural substrate underlying hearing, producing and learning song consists of several song nuclei in three interconnected pathways (Fig. 1). The motor pathway controls song production and connects the pallial ("cortical" like) nucleus HVC (proper name) to the robust nucleus of the archistriatum (RA). RA in turn connects to motor neurons in the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which innervates the vocal organ, called syrinx (Nottebohm et al, 1976). The auditory pathway processes auditory information entering the brain via the ears, ascending through a vertebrate-canonical series of nuclei and regions and also connects indirectly towards HVC and other pallial nuclei (Vates et al., 1996; Mandelblat-Cerf et al., 2014; Murphy et al., 2017). The anterior forebrain pathway (AFP) enables song learning, song maintenance and social context dependent modulation of song (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Murugan et al., 2013; Kubikova et al., 2014; Woolley and Kao, 2015; Kojima et al., 2018; Xiao et al., 2020). It forms a pallial-basal ganglia-thalamo-pallial feedback loop and connects HVC and lateral magnocellular nucleus of the anterior nidopallium (LMAN) to RA via striatal Area X and the medial dorsolateral nucleus of the anterior thalamus (DLM). Area X receives dopaminergic innervation from the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc, Lewis et al., 1981; Bottjer, 1993; Gale et al., 2008).

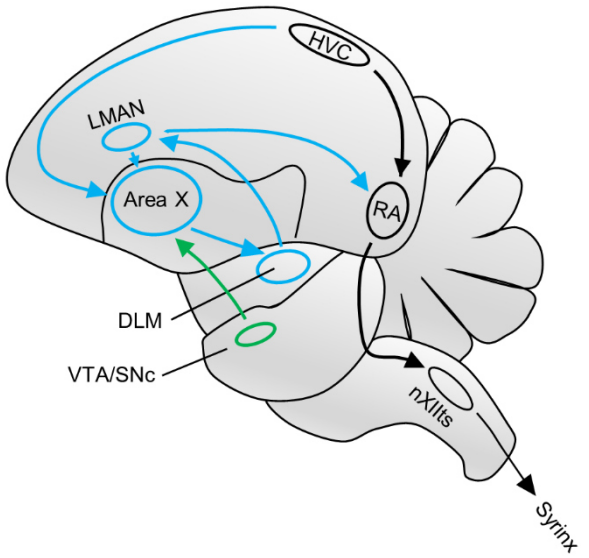

Figure 1. The song system
The song motor pathway (shown in black) controls the vocal organ (syrinx) via HVC >RA >nXIIts. The anterior forebrain pathway (AFP, shown in blue) forms a pallial-basal ganglia-thalamic-pallial loop, connecting HVC and RA via Area X>DLM>LMAN. Area X receives dopaminergic innervation from VTA and SNc (shown in green).

Adult neurogenesis in nuclei of the song system

In the adult songbird brain, new neurons are generated, migrate into the telencephalon and incorporate into existing circuits (Alvarez-Buylla and Nottebohm, 1988). Proliferation hot spots at the wall of the lateral ventricle give rise to new neurons that migrate along radial glia cells into the parenchyma (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988b; 1990). In the song system, pallial HVC, the caudomedial nidopallium (NCM, part of the auditory pathway) and striatal Area X receive new neurons (Alvarez-Buylla and Nottebohm, 1988; Nordeen and Nordeen, 1988a, Fig. 2). First, I will expand on adult neurogenesis in HVC, then on adult neurogenesis Area X. HVC contains two types of projection neurons: HVC_{RA} neurons are part motor pathway and project to RA whereas HVC_X neurons send their axons to Area X (Nottebohm et al., 1976). Only HVC_{RA} neurons are generated postnatally and in adulthood, in contrast to HVC_X neurons, that are mainly generated *in ovo* (Alvarez-Buylla et al., 1988a; Nordeen and Nordeen, 1988b; Scotto-Lomassese et al., 2007). The specific ablation of either HVC_{RA} or HVC_X neurons leads to an increased recruitment of only HVC_{RA} neurons, indicating that cell death of HVC_X neurons does not induce their recruitment (Scharff et al., 2000). New HVC neurons can be detected as early as one week after their birth and their connection to RA is robustly established between 22 and 31 days after their birth (Burek et al., 1994; Kirn et al., 1999; Tokarev et al., 2015). They respond to auditory stimuli (Paton and Nottebohm, 1984) and robustly express immediate early genes after singing as early as three weeks after they were born, indicating that they are firing during singing (Tokarev et al., 2015). New neuron recruitment in songbirds can either occur as a process of addition or replacement. In the seasonally breeding canary, new neurons in HVC replace older ones that have died (Kirn and Nottebohm, 1993). In the zebra finch, new HVC neurons are added to the existing circuitry, resulting in a doubling of neuron density over time (Walton et al., 2012).

Age, experience during early development, social environment and behavior impact on new neuron recruitment and/or survival in HVC. As zebra finches age, the rate of new neuron addition declines in HVC but not in other parts of the song system (Wang et al., 2002; Pytte et al., 2007). Since singing enhances new neuron survival in HVC of canaries (Li et al., 2000; Alvarez-Borda and Nottebohm, 2002), it might be possible that decreased singing rates in aged zebra finches cause a decline of adult neurogenesis in HVC. However, in Pytte et al. (2007), there was no difference in motifs per song bout between young and old zebra finches but a detailed analysis of singing rates across different ages might reveal a relationship between age-dependent singing rates and the recruitment of new neurons into HVC.

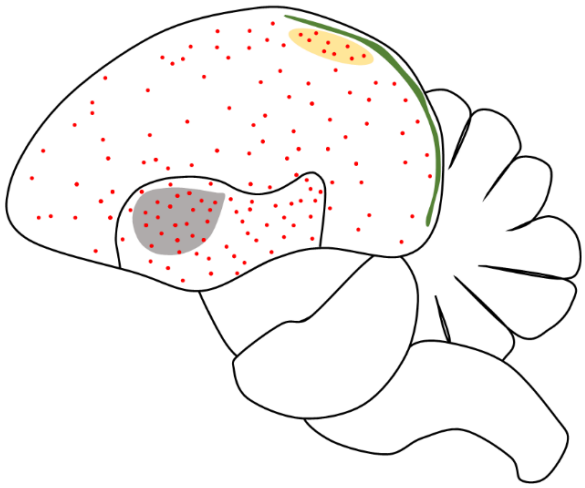

Figure 2. Adult neurogenesis in the songbird brain
New neurons (represented by red dots) are generated in the ventricular zone (shown in green) and migrate towards many regions of the telencephalon, including song system nuclei HVC (shown in yellow) and Area X (shown in gray).

Besides age, social environment influences adult neurogenesis in HVC. New neuron number in HVC co-varies among related and even unrelated adult male zebra finches when they shared the same nest (Hurley et al., 2008) and individuals that were held in a large mixed sex group had more new neurons in HVC and Area X compared to zebra fiches that were held in pairs or in isolation (Lipkind et al., 2002). On a cellular level, postsynaptic neural activity in RA was shown to be crucial for the survival of new HVC neurons (Larson et al., 2013). Further, singing induced brain-derived neurotrophic factor (BDNF) expression enhances the survival of new neurons in HVC of canaries (Li et al., 2000) and its interaction with testosterone influences proliferation and new neuron survival especially in seasonally breeding songbirds (Brenowitz, 2014). HVC receives input from the auditory pathway (Vates et al., 1996) and new neurons in HVC respond to sound early during their maturation (Paton and Nottebohm, 1984). Auditory deprivation caused by bilateral deafening of adult zebra finches decreases the total number of new neurons in HVC indicating that auditory input is necessary for their survival (Wang et al., 1999). Other studies found a positive or no effect of deafening on HVC neurogenesis in adults (Hurley et al., 2008; Pytte et al., 2012), whereby social context and different composition of nest mates may account for the differences between the studies. The quality of song structure and rates of adult neurogenesis in HVC are connected; the magnitude of song deterioration after paralysis of syringeal muscles and the rate of song recovery correlate positively with the number of new neurons incorporated into HVC (Pytte et al., 2011). If song quality affects rates of new neurons in HVC or vice versa awaits further investigation.

The following paragraph will summarize the knowledge on adult neurogenesis in Area X, of which much less is known compared to adult neurogenesis in HVC. Area X is formed from the Islet1+ ventral striatal domain between 8 and 10 days after hatching and increases in volume until 40 days after hatching (Nixdorf-Bergweiler, 1996; Garcia-Calero and Scharff, 2013). Islet1 is a marker for the lateral ganglionic eminence where medium spiny neurons originate during brain development in mammals (Stenman et al., 2003). Newly generated neurons in Area X are also MSNs, which is the most abundant neuron type in the striatum of mammals and birds (Freund et al., 1984; Farries and Perkel, 2000; Rochefort et al., 2007; Scott and Lois, 2007). New MSNs that migrate into Area X originate at the ventricular zone (VZ) at the wall of the lateral ventricle adjacent to the striatum (Alvarez-Buylla et al., 1990; Scott and Lois, 2007). MSNs are characterized by a medium sized cell soma, spiny dendrites and distinct electrophysiological and transcriptional profiles (Surmeier et al., 2007; Cepeda et al., 2008; Gokce et al., 2016; Stanley et al., 2019).

MSNs in Area X receive glutamatergic (excitatory) innervation from HVC and LMAN and dopaminergic innervation from VTA/SNc. Both glutamatergic and dopaminergic projections converge onto dendritic spines of the same MSN (Bouyer et al., 1984; Kornfeld et al., 2020). MSNs are GABAergic (inhibitory), they show sparse firing during singing and their function is feed-forward inhibition of pallial signaling (Goldberg and Fee, 2010) . They do not project out of Area X but innervate pallidal-like neurons (PNs) that project to thalamic DLM (Farries and Perkel, 2002; Kornfeld et al., 2020). Two types PNs can be distinguished in Area X; direct PNs that innervate DLM and indirect PNs that only innervate direct PNs (Farries et al., 2005; Goldberg et al., 2010; Xiao et al., 2020, Figure 3). Based on electrophysiological recordings, PNs have been proposed to resemble the mammalian external and internal segments of the globus pallidus (Goldberg et al., 2010), that are innervated by different populations of MSNs (Calabresi et al., 2014, Fig. 3). Gene expression profiles of single PNs in Area X, however, contradict this hypothesis and show that PNs appear more similar to arkypallidal cells of the external globus pallidus (Xiao et al., 2020) that do not project forward to neurons of the subthalamic nucleus but project back to the striatum (Mallet et al., 2012; Abdi et al., 2015). The existence of different MSN subtypes that exclusively innervate direct or indirect PNs in songbirds has been proposed but was not yet proven by electrophysiological recordings (Gale and Perkel, 2010; Pidoux et al., 2015). A recent study that analyzed the Area X transcriptome distinguishes even five different MSNs clusters (Xiao et al., 2020). Differential gene expression analysis delineated MSNs clusters that expressed classical markers of MSNs

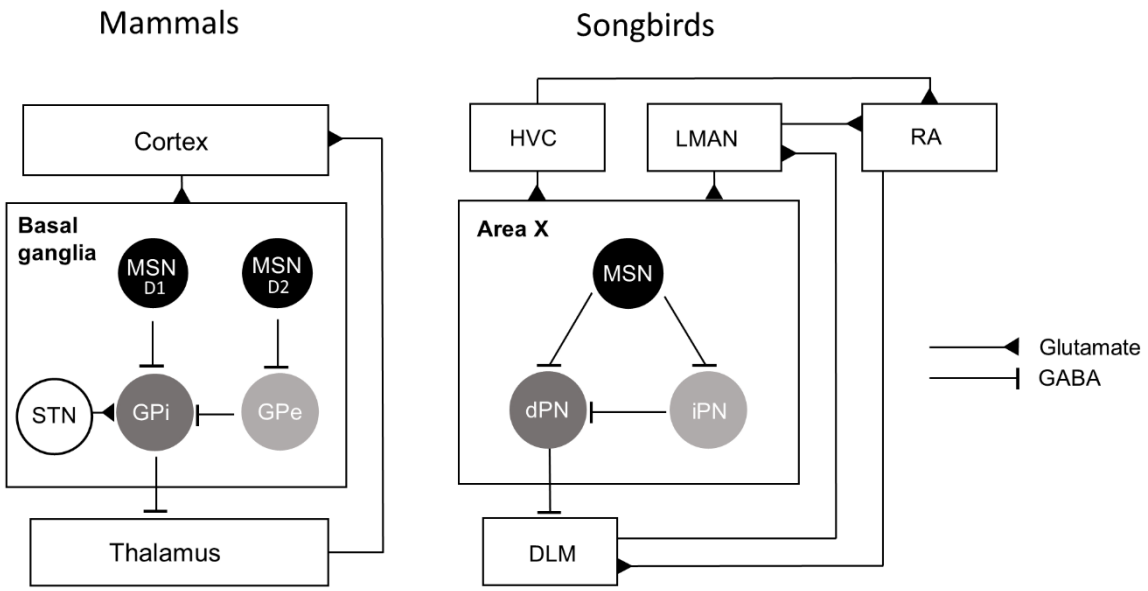

Figure 3. Basal ganglia microcircuitry in mammals and songbirds
In the mammalian striatum, dopamine receptor type 1 (D1) expressing MSNs are part of the direct pathway and directly innervate the internal globus pallidus (GPi), which inhibits the thalamus. Dopamine receptor type 2 (D2) expressing MSNs are part of the indirect pathway and project to the external GP (GPe). In the songbird striatum, neurons in Area X receive glutamatergic innervations from HVC and LMAN. MSNs in Area X inhibit direct and indirect pallidal-like neurons (dPN, iPN). Only dPNs project to the thalamic nucleus DLM that connects to RA via LMAN. RA directly innervates the AFP via DLM. Graphic adapted from Pidoux et al. (2015) and Kosubek-Langer et al. (2017).

that innervate the direct and indirect pathway of the mammalian basal ganglia (Xiao et al., 2020, Fig. 3). In rodents, direct pathway MSNs express the dopamine receptor type 1 (Drd1) and the forkhead box protein P2 (Foxp2[1]), a transcription factor implicated in striatal function (Enard, 2011, and see next section). MSNs of the indirect pathway express the dopamine receptor type 2 (Drd2) but no or only little Foxp2 (Vernes et al., 2011; van Rhijn et al., 2018; Stanley et al., 2019). In zebra finch Area X, FoxP2 expression is less segregated between MSN cluster; about 60% and 20% of the MSNs that correspond to the direct or indirect pathway express FoxP2, respectively (Xiao et al., 2020).

New MSNs that migrate into Area X also express FoxP2 (Rochefort et al., 2007). FoxP2 downregulation in the SVZ decreased new MSN spine density in Area X and led to a small but statistically not significant effect on the rate of recruited new MSNs during song development in juveniles (Schulz et al., 2010). In mammals, Foxp2 is implicated in embryonic development of the cortex and the striatum (Tsui et al., 2013; Chiu et al., 2014; Kast et al., 2019), but which role FoxP2 plays for the process of adult neurogenesis in songbirds is not known.

[1] *FOXP2* refers to the human gene, *Foxp2* refers to the mouse gene and *FoxP2* refers to all other species. FOXP2, Foxp2 and FoxP2 correspond to the protein product (Kaestner et al., 2000).

One striking difference between the process of adult neurogenesis in HVC and Area X is that in the latter the rate of recruited neurons does not decrease with age (Pytte et al., 2007), which is why I suspected that is has an ongoing function for song maintenance. To find out whether new neurons in Area X are functionally integrated into the circuitry and behaviorally relevant, in **Publication A** of this dissertation, I addressed the maturation course of new MSNs, their participation in singing related activity and the question if their integration into Area X is a process of replacement or addition.

FOXP2 – a transcription factor implicated in speech and language

Mutations of the transcription factor FOXP2 cause a severe speech and language impairment in humans, called childhood apraxia of speech (CAS, Lai et al., 2001; MacDermot et al., 2005; Morgan et al., 2016). Besides a mildly impaired language perception, affected individuals mostly have difficulties performing fine orofacial movements that underlie speech production (Vargha-Khadem et al., 1998). Functional imaging revealed that the brains of affected individuals show structural and functional differences in cortical, cerebellar and basal ganglia regions (Vargha-Khadem et al., 1998; Watkins et al., 2002; Liégeois et al., 2003; Liégeois et al., 2016). Additional to its role in speech and language impairments, *FOXP2* variants are associated with attention deficit/ hyperactivity disorder and *FOXP2* is a risk gene in autism spectrum disorders (Demontis et al., 2019; Satterstrom et al., 2020). Considering the underlying mechanism, both brain development and later brain function may be involved (Ehninger et al., 2008). Consistent with the former, many of FOXP2's target genes are linked to neurodevelopmental disorders (Mukamel et al., 2011). Deciphering Foxp2 expression pattern during brain development and adulthood is crucial to understand which cell types and neuronal circuits are affected by Foxp2 variants or mutations. Foxp2 expression patterns in cortex, cerebellum, thalamus and striatum are strongly conserved across reptiles, birds and mammals (Ferland et al., 2003; Takahashi et al., 2003; Haesler et al., 2004; Takahashi et al., 2008; Campbell et al., 2009; Rodenas-Cuadrado et al., 2018). In the cortices of mice, Foxp2 is enriched in corticothalamic projection neurons but not in corticocortical projection neurons of layer 6 (Kast et al., 2019). In cerebellar cortex, Foxp2 is expressed in Purkinje cells, which send the main motor coordination output signals to deep cerebellar nuclei. In the striatum, Foxp2 is expressed in MSNs of the striosome compartment but not in the ones of the matrix (Takahashi et al., 2003; Takahashi et al., 2008; Chen et al., 2016). In the striosome, Foxp2 is enriched in dopamine receptor 1 (Drd1) expressing MSNs that innervate the direct pathway of the cortico-striatal-thalamic motor circuit (Vernes et al., 2011; van Rhijn et al., 2018, Fig. 3). Only a small fraction of dopamine receptor 2 expressing (Drd2) MSNs of the indirect pathway express Foxp2 (van Rhijn et al., 2018; Stanley et al., 2019).

Given that the learned song in songbirds and speech in humans exhibit molecular, neural and behavioral similarities (Fee and Scharff, 2010; Brainard and Doupe, 2013), it is interesting that FoxP2 expression is low in pallial ("cortical") regions of songbirds and high in striatum and thalamus (Haesler et al., 2004). In Area X of the avian striatum, FoxP2 expression levels are developmentally and behaviorally regulated. This is consistent with an important conserved role for FOXP2/FoxP2 function in the basal ganglia of humans and songbirds (Bolhuis et al.,

2010). In fact, FoxP2 levels are upregulated in zebra finches during the phase of song learning and decrease with age (Haesler et al., 2004). In addition to the developmental regulation, singing activity itself downregulates FoxP2 expression in juveniles and adults (Teramitsu and White, 2006; Miller et al., 2008; Teramitsu et al., 2010). Curiously, FoxP2 expression intensities vary in individual MSNs; some neurons show very intense others quite weak immunostaining for FoxP2 (Thompson et al., 2013). The proportion of weakly stained neurons increases with age and decreases with undirected singing. In contrast, newly generated MSNs in Area X are more likely to be intensely stained for FoxP2 21 days after they were born in the ventricular zone (Thompson et al., 2013). Altogether this indicates that FoxP2 levels in Area X depend on the age of the animal, on the age of individual neurons and on behavioral processes that regulate its expression.

Aberrant Foxp2 protein levels impair learning and striatal signaling in mammals and songbirds

To elucidate FOXP2 function and the mechanisms underlying the speech impairment CAS, transgenic mouse models were generated that either lack one or both Foxp2 alleles or carried Foxp2 mutations similar to those found in CAS patients. These mutations include an arginine to histidine substitution within the Foxp2 DNA binding domain (R553H, Lai et al., 2001) or a nonsense mutation which leads to a truncated protein product that lacks the DNA binding domain (S321X, MacDermot et al., 2005). Heterozygous *Foxp2* mutations (*R552H* and *S321X*) cause deficits in motor behavior and motor learning (Groszer et al., 2008; French et al., 2012; Kurt et al., 2012; van Rhijn et al., 2018). Interestingly, mice lacking *Foxp2* exclusively in Purkinje cells show the largest motor deficits compared with *Foxp2* KO mice that lack *Foxp2* only in cortical neurons or striatal MSNs (French et al., 2019). On a neurophysiological level, the *Foxp2-R552H* mutation specifically influence properties of the corticostriatal synapse and results in altered glutamate receptor ratios and impaired long-term depression (LTD) in striatal MSNs (Groszer et al., 2008; French et al., 2012; van Rhijn et al., 2018). In contrast to Foxp2 mutant mice, mice carrying humanized Foxp2 alleles ($Foxp2^{hum/hum}$) show a stronger LTD in striatal MSNs (Enard et al., 2009; Reimers-Kipping et al., 2011; Schreiweis et al., 2014).

In zebra finches, the reduction of FoxP2 protein expression via RNA interference (FoxP2 knock down, KD) or FoxP2 overexpression in striatal Area X impairs song learning in juveniles (Haesler et al., 2007; Heston and White, 2015). Decreased or elevated FoxP2 expression in adults abolishes social context dependent changes in song variability and causes syllable repetition (Murugan et al., 2013; Day et al., 2019; Xiao et al., 2020). Further, FoxP2

KD in adult birds decreases glutamate receptor ratios, the expression of Drd1 and of DARPP-32, which is an integrator protein of glutamatergic and dopaminergic signaling in MSNs (Murugan et al., 2013; Adam et al., 2016). Taken together, these studies highlight the importance of a tight regulation of FoxP2 expression levels and behaviorally driven regulation for its correct on-line function during song learning and song production.

Foxp2 expression promotes developmental neurogenesis in mice

Many lines of evidence indicate that Foxp2 is involved in the formation of the nervous system during development. The first study that addressed the role of Foxp transcription factors in the context of neurogenesis found that orchestrated expression of Foxp2 and Foxp4 facilitate the delamination of neuronal progenitor cells from the ventricular zone of the spinal cord during development in mice and chicken (Rousso et al., 2012).

There are controversial reports about Foxp2s' role in development of the cortex. In one study, mouse cortices were electroporated in utero at stage E13/14 with a plasmid containing a short hairpin that decreased Foxp2 protein levels. This Foxp2 KD perturbed the generation of neurons from precursors, more specifically the transition from radial precursors to intermediate progenitors (Tsui et al., 2013). Other studies using genetic ablation of cortical *Foxp2* did not find abnormal cortical patterning or connectivity of corticothalamic projection neurons in layer 6 (Co et al., 2019; Kast et al., 2019; Medvedeva et al., 2019). One explanation for the discrepancy of the studies might be the lower level of Foxp2 reduction when using short hairpin mediated KD compared to homozygous genetic ablation (Kast et al., 2019). Interestingly, cortical Foxp2 ablation decreases Drd1 expression in corticothalamic and corticocortical projection neurons in layer 6 and increases the generation of immature interneurons (Co et al., 2019). Experiments using cell cultures of neuronal progenitors from the medial ganglionic eminence (MGE) have also shown that normal Foxp2 expression is necessary for the generation of cortical interneurons during development (Chiu et al., 2014).

During brain development, Foxp2 expressing MSNs of the striatum originate in the lateral ganglionic eminence (LGE). Here, the neurons of the striosome compartment (Foxp2+) develop earlier than the neurons of the matrix (Foxp2-) (van der Kooy and Fishell, 1987). Foxp2 is differentially expressed in the LGE of developing mice and this is associated with progenitor morphology during their migration. Low expression levels in the subventricular zone (SVZ, a proliferative zone containing progenitor cells) of the LGE are associated with multipolar morphology of progenitors. As progenitors migrate towards their final location, the mantle zone, their Foxp2 levels increase and are associated with bipolar morphology (Garcia-Calero et

al., 2016). Further, Foxp2 expression promotes differentiation of neuronal progenitors from the LGE to mature, DARPP-32 positive MSNs during development (Chiu et al., 2014).

Foxp2 enhances neuronal outgrowth

The role of Foxp2 in neuronal outgrowth was elucidated by the analysis of its target genes and from Foxp2 KD, mutation or overexpression both *in vitro* and *in vivo*. First, analysis of Foxp2/FOXP2 target genes links it to neuronal outgrowth and synaptic plasticity (Spiteri et al., 2007; Vernes et al., 2007; Vernes et al., 2011). Second, in mouse and human neuronal cell lines, Foxp2/FOXP2 drives neuronal differentiation, affects gene expression and promotes neuronal outgrowth (Vernes et al., 2011; Devanna et al., 2014). Primary neurons harvested from ganglionoic eminences of *Foxp2-R552H* mutated mice show decreased neurite outgrowth compared to wild type neurons (Vernes et al., 2011). Short hairpin mediated Foxp2 KD in progenitors from the LGE reduced their differentiation into DARPP-32 positive MSNs (Chiu et al., 2014). Third, Foxp2 overexpression in the cortical and striatal SVZ promotes bipolar morphology of migrating neurons and increased their neurite length *in vivo* (Garcia-Calero et al., 2016). In juvenile zebra finches, lentiviral mediated FoxP2 KD in striatal progenitors in the VZ decreased spine density of new Area X MSNs (Schulz et al., 2010). In mouse cerebellar Purkinje cells, Foxp2 KD decreases their dendritic length and dendritic branching (Usui et al., 2017). Other evidence for Foxp2's role in neuronal outgrowth stems from $Foxp2^{hum/hum}$ mice. Their MSNs and other neuron types of the cortico-striatal circuitry possess longer dendrites compared to the same neurons in wild type mice (Enard et al., 2009; Reimers-Kipping et al., 2011). Concerning MSNs, this could be due to a greater proportion of MSNs with high Foxp2 expression found in the striatum of $Foxp2^{hum/hum}$ mice (Schreiweis et al., 2019). In summary, it has been shown with varying experimental approaches that Foxp2 is crucial for neuronal outgrowth in different species.

Thesis outline

As one part of my thesis I studied basic principles of adult neurogenesis in Area X. **Publication A** addresses how new medium spiny neurons are incorporated into the existing Area X circuit, how they mature and become active during singing. To label newly generated neurons, adult male zebra finches were injected with Bromdesoxyuridin (BrdU). This synthetic nucleoside is an analogue of thymidine and becomes incorporated into newly synthesized DNA during cell proliferation. It can be immunohistochemically detected in fixed tissue and the nuclear staining indicates that cells have been recently dividing. Because I wanted to visualize

not only the nucleus but entire new neurons, I applied a second approach and virally labelled cells in the ventricular zone (VZ). Therefore, a lentiviral vector carrying the green fluorescent protein reporter gene (GFP) was injected into the VZ adjacent to the lateral ventricle, where new neurons destinated for Area X emerge (Alvarez-Buylla et al., 1990; Scott and Lois, 2007). After sufficient survival time, GFP expressing new MSNs were analyzed in Area X. BrdU and virally mediated labelling techniques were applied in the experiments underlying the datasets presented in both publications. The results of **Publication A** show that new medium spiny neurons in Area X show the same properties as older, resident MSNs. Once incorporated, they are robustly active during singing behavior. Further, new neurons are constantly added to Area X, leading to a more than doubling of the neuronal density in aged zebra finches.

The second part of this thesis especially addresses FoxP2's role in the process of adult neurogenesis in Area X. Previous studies indicated that Foxp2 is implicated in both neurogenesis and neural outgrowth. It was known that new neurons in Area X expressed FoxP2 but why this might be so was unknown. In **Publication B**, I virally labelled progenitor cells and later analyzed the decedents of these cells once they differentiated into new MSNs in Area X. The results of Publication B show that new MSNs in Area X express dynamic FoxP2 levels in an age dependent manner, which influence their morphology at specific timepoints during their maturation.

Publication A

Maturation, Behavioral Activation, and Connectivity of Adult-Born Medium Spiny Neurons in a Striatal Song Nucleus

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ORIGINAL RESEARCH
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Maturation, Behavioral Activation, and Connectivity of Adult-Born Medium Spiny Neurons in a Striatal Song Nucleus

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Neurogenesis continues in the adult songbird brain. Many telencephalic song control regions incorporate new neurons into their existing circuits in adulthood. One song nucleus that receives many new neurons is Area X. Because this striatal region is crucial for song learning and song maintenance the recruitment of new neurons into Area X could influence these processes. As an entry point into addressing this possibility, we investigated the maturation and connectivity within the song circuit and behavioral activation of newly generated Area X neurons. Using BrdU birth dating and virally mediated GFP expression we followed adult-generated neurons from their place of birth in the ventricle to their place of incorporation into Area X. We show that newborn neurons receive glutamatergic input from pallial/cortical song nuclei. Additionally, backfills revealed that the new neurons connect to pallidal-like projection neurons that innervate the thalamus. Using *in situ* hybridization, we found that new neurons express the mRNA for D1- and D2-type dopamine receptors. Employing DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) and EGR-1 (early growth response protein 1) as markers for neural maturation and activation, we established that at 42 days after labeling approximately 80% of new neurons were mature medium spiny neurons (MSNs) and could be activated by singing behavior. Finally, we compared the MSN density in Area X of birds up to seven years of age and found a significant increase with age, indicating that new neurons are constantly added to the nucleus. In summary, we provide evidence that newborn MSNs in Area X constantly functionally integrate into the circuit and are thus likely to play a role in the maintenance and regulation of adult song.

Keywords: adult neurogenesis, songbird, basal ganglia, Area X, EGR-1, DARPP-32, dopamine

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INTRODUCTION

Adult neurogenesis is an enigmatic trait. Only some neurons continue to be generated in adulthood whereas the majority are born during development and persist throughout the animal's life. Why these differences exist is still not known but much progress has been made elucidating the mechanism and function of adult neurogenesis during the past decades (Song et al., 2016). Neurons born in adulthood originate in regions adjacent to the ventricles that also give rise to neurons during development. From these neurogenic niches, neural precursors delaminate and then migrate through the dense parenchyma, incorporate into functional circuits and influence behavior (Paredes et al., 2016).

Considerable differences exist with respect to the extent of adult neurogenesis in different species. As a rule of thumb, adult-born new neurons are recruited to many brain regions in vertebrates like teleost fish, amphibians, and reptiles, whereas in birds the extent is still widespread but more restricted to the forebrain (Kaslin et al., 2008). In mammals, there are even fewer regions that continue to recruit new neurons in adulthood, principally the dentate gyrus (DG) of the hippocampal formation (Kempermann et al., 2015) and the olfactory bulb (Lim and Alvarez-Buylla, 2016). Interestingly, in rats, rabbits, monkeys and humans but not in mice, adult-generated neurons have also been observed in the striatum (Bedard et al., 2002; Dayer et al., 2005; Tonchev et al., 2005; Luzzati et al., 2006; Ernst et al., 2014). In these cases, the newly generated neurons belong primarily to the class of GABAergic interneurons, which constitute less than 5% of the striatal neurons (Tepper et al., 2010). The most abundant striatal cell type are medium spiny projection neurons (MSNs) (Gerfen and Wilson, 1996). In adult rodents, generation of MSNs has only been reported in response to experimentally induced stroke, ischemia, or lesions (Arvidsson et al., 2002; Tattersfield et al., 2004; Hou et al., 2008). In contrast, in songbirds adult MSNs keep immigrating in substantial numbers into the striatum under natural conditions (Alvarez-Buylla et al., 1990). Striatal newborn neurons originate from the progenitor containing subpallial region in the lateral ventricle that expresses the transcription factors ISL-1/2, NKX2.1, and DLX but not TBR1 (Scott and Lois, 2007). Of particular interest is the recruitment of MSNs into Area X (Nordeen and Nordeen, 1988; Rochefort et al., 2007; Scott and Lois, 2007) a region unique to songbirds relevant for song plasticity in juveniles and adults (Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Jarvis et al., 1998; Hessler and Doupe, 1999; Woolley et al., 2014). In songbirds, new neurons destined for Area X migrate between 1,000 and 2,000 μm to their final destination.

The dynamics of neural recruitment are best understood in the DG and the olfactory bulb. In the former, new neurons are added, whereas in the latter, they replace older neurons that undergo apoptosis (Crespo et al., 1986; Imayoshi et al., 2008). In both cases, the time it takes for new neurons to incorporate into preexisting circuits is similar (Deshpande et al., 2013). In songbirds, the dynamics of neural recruitment have only been studied in the pallial/cortical song control region HVC (proper name, **Figure 1A**), where glutamatergic projection neurons undergo neurogenesis (Kirn et al., 1999; Scott and Lois, 2007; Tokarev et al., 2016).

To gain insight into the integration of GABAergic MSNs into existing circuits, we studied their differentiation, connectivity and activation by singing in Area X. To do so we traced new neurons by injections of green fluorescent protein (GFP)-expressing lentivirus into the lateral wall of the lateral ventricle and with systemic injections of the cell birth marker 5-bromo-2′-deoxyuridine (BrdU). We also injected retrograde tracer into one of the target regions of Area X, and used immuno- and *in situ*-histochemistry to characterize the new neurons. We report that adult born MSNs receive glutamatergic and dopaminergic input, connect to pallidal-like projection neurons and are activated during singing like older, resident MSNs.

Because new HVC neurons seem to replace older ones in canaries (Kirn and Nottebohm, 1993), whereas in zebra finches constant neuronal addition was observed (Walton et al., 2012) we also addressed the issue of replacement vs. addition. We quantified neuron numbers in adult zebra finches of varying age and found that the density of MSNs in Area X increased with age, supporting the idea of neuron addition rather than replacement. Overall, our results suggest that Area X receives a constant addition of functional new GABAergic MSNs.

MATERIALS AND METHODS

Animals

Adult male zebra finches (*Taeniopygia guttata*) were bred and housed at the Department of Animal Behavior at Freie Universität Berlin. The colony was kept under a 12:12 h light:dark-cycle and food and water were available *ad libitum*. All procedures were reviewed and approved by the veterinary department of the Freie Universität Berlin and by the ethics committee of the Regional Office for Health and Social Affairs Berlin (LAGeSo). The permit numbers are G0116/13 and G0296/15. In total, we used 53 adult male zebra finches. For the expression analysis of the early growth response protein 1 (EGR-1) and the dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) in newborn cells we used 29 birds (age 462 ± 158 days, mean ± standard deviation, SD). Dopamine (DA) receptor expression was studied in 5 birds (age 172 days ± 13 days, mean ± SD). Five birds received lentiviral injections (age 367 days ± 109 days, mean ± SD). Density measures in Area X were performed in 14 birds (age ranging from 372 to 2,526 days).

BrdU Injections

Birds for EGR-1 and DA receptor analysis received BrdU (50 μg/g) via intramuscular injections in the mornings for 5 consecutive days. Birds were assigned to three groups with different survival times after BrdU injection (21, 31, and 42 days). We choose the first survival time to be 21 days, because BrdU+ neurons in Area X were previously shown to express immediate early genes after singing at that time (Tokarev et al., 2016).

Song Monitoring

For subsequent EGR-1 analysis, birds were kept in sound attenuated chambers for three nights and were perfused in the morning of the 4th day 1.5 h after the lights went on. Vocalizations were continuously monitored via Sound Analysis Pro (Tchernichovski et al., 2000). During those 1.5 h birds had to sing at least 150 motifs to be included in the subsequent analysis of EGR-1 expression.

Birds that received lentiviral injections and retrograde tracer were isolated in sound attenuated chambers for one night before sacrifice. Birds were kept from singing by the experimenter sitting nearby for 1.5 h after lights went on in the morning and then killed. This was necessary because we used some of the brain sections in another experiment to be reported elsewhere.

Birds used for DA receptor analysis were decapitated without previous song monitoring and their brains were quickly dissected

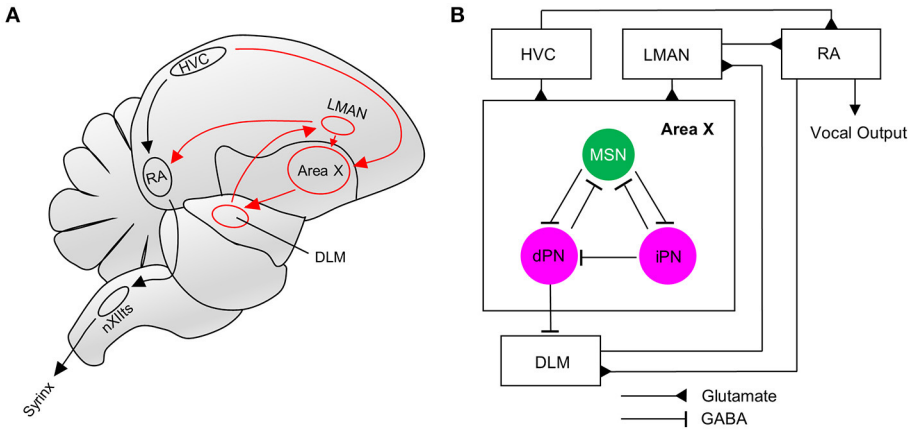

FIGURE 1 | The song system and connectivity within Area X. **(A)** The song motor pathway (shown in black) controls the vocal organ (syrinx) via HVC->RA->nXIIts. The anterior forebrain pathway (AFP, shown in red) forms a cortico-basal ganglia-thalamic-cortical loop, connecting HVC and RA via Area X->DLM->LMAN. **(B)** Neurons in Area X receive glutamatergic innervations from HVC and LMAN. MSNs in Area X inhibit direct and indirect pallidal-like neurons (dPN, iPN). Both types can project to MSNs, but only dPNs project to the thalamic nucleus DLM that connects to RA via LMAN (Farries et al., 2005; Goldberg et al., 2010). RA directly innervates the AFP via DLM. RA, Robust nucleus of the arcopallium; LMAN, Lateral magnocellular nucleus of the anterior nidopallium; XII, Nucleus; NXIIts, tracheosyringeal part; DLM, Dorsal lateral nucleus of the medial thalamus.

1.5 h after the lights went on. All birds were killed by isoflurane overdose.

Lentiviral Vector Injection and Backfill

To label progenitors in the lateral wall of the ventricle, the lentiviral expression vector pFUGW (Lois et al., 2002) containing a GFP reporter gene was stereotactically injected into the ventricular zone under isofluorane anesthesia. Birds were fixed in a stereotaxic head holder, with the beak in a 45° angle from the vertical axis. In each hemisphere, we injected four sites with approximately 200 µl of viral construct using the following coordinates relative to the bifurcation of the midsagittal sinus: anterior-posterior 3.8–4.1, medial-lateral −1.3/+1.3, dorsal-ventral −5.0, injection angle AP 10°. To label pallidal-like projection neurons, we injected approximately 600 µl tetramethylrhodamine coupled with biotin (BDA, 3,000 MW, Molecular Probes) into DLM 4–5 days before sacrifice at day 42. We used the following coordinates: anterior-posterior 1.2, medial-lateral −1.3/+1.3, dorsal-ventral −4.5. After surgeries birds were transferred to their home cages. To confirm that the virus infected proliferating cells, some birds were injected with BrdU (50 µg/g) on the day of surgery.

Immunohistochemistry and Image Analysis

For immunohistochemistry birds were overdosed with isoflurane and then perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. After dissection, brains were post-fixed for one night, washed for another night in PBS and cut sagitally or coronally into 50 µm sections using a vibrating microtome (VT1000S, Leica). For BrdU antigen retrieval, sections were incubated in 2 N HCl for 30 min at 37°C and neutralized with borate buffer. All other immunostainings were performed according to standard protocols. The following antibodies were used; primary: anti EGR-1 (rabbit, Santa Cruz sc-189), anti DARPP-32 (mouse, kindly provided by H.C. Hemmings, Jr., Weill Cornell Medical College, New York), anti DARPP-32 (rabbit, abcam ab40801), anti BrdU (rat, Bio-Rad MCA2060), anti VGLUT2 (mouse, abcam ab79157), anti GFP (rabbit, abcam ab290). Fluorescent Secondary: anti-rabbit-Alexa-Fluor-568 (life technologies, A10042), anti-mouse-Alexa-Flour-568 (Life technologies, A10037), anti-rat-Alexa-Fluor-488 (Life Technologies, A21208), anti-rabbit-Alexa-Fluor-488 (Life Technologies, A21206). Biotinylated dextran signal was amplified using Streptavidin-Alexa-Fluor-568 (Life Technologies, S11226). Sections were counterstained with 4′,6-Diamidin-2-phenylindol (DAPI, Serva). Z-Stacks were obtained with a SP8 confocal microscope (Leica) and processed using the Fiji software package (Schindelin et al., 2012). Colors of images were adjusted ("false-colored") to improve visibility, particularly for readers with red-green blindness. Axons were traced using the Simple Neurite Tracer plugin in Fiji (Schindelin et al., 2012), starting at the soma and using the smooth axonal morphology (in contrast to spiny dendrites) as a criterion. MSN density was analyzed in 40 µm sagittal sections containing Area X. For each bird, we analyzed two to four different sections of both hemispheres. Within those we counted the number of labeled neurons in at least eight stacks, each with the measures 100 × 100 × 8 µm and used the average of those to calculate density. We counted all nuclei (DAPI+) and all DARPP-32+ cells using the cell counter plugin in the Fiji software package (Schindelin et al., 2012).

In situ Hybridization

Hemispheres of birds used for *in situ* hybridization were separately frozen in Tissue-Tek O.T.C. Compound medium (Sakura) and stored at −80°C. Hemispheres were cut in 12 µm sagittal sections using a cryostat (Cryo-Star HM 560

Cryostat, MICROM). Sections were fixed with 4% PFA for 10 min and then acetylated with 0.25% acetic anhydride in triethanolamine for 10 min. Sections were rinsed in 2x in saline sodium citrate (SSC) buffer, dehydrated (75% EtOH, 95% EtOH, and 100% EtOH, each for 2 min) and air dried. Sections were prehybridized for 1 h at 60°C in a hybridization mix consisting of 50% deionized formamide, 5x SSC (pH 4.5), 2% blocking reagent (Roche, 11096176001) in 1x maleic acid buffer, 2% sodium dodecyl sulfate, yeast tRNA (Invitrogen, 0.25 mg/ml), and heparin (Polysciences, 0.1 mg/ml). Sections were hybridized overnight with 1% digoxigenin or fluorescin labeled RNA probe in hybridization mix at 60°C in a mineral oil bath. The next day, slides were rinsed twice with chloroform followed by 2x SSC and 1x SSC. A series of post-hybridization washes followed: 30 min in 1x SSC containing 50% formamide at hybridization temperature (60°C). Then, sections were washed once in 2x SSC and twice in 0.2x SSC 20 min each at hybridization temperature. After the post-hybridization washing steps, sections were washed twice in 1x MABT (pH 7.5), consisting of 100 mM maleic acid, 150 mM NaCl and 0.1% Tween-20. Afterwards, sections were incubated in 1x Roti-ImmunoBlock (Carl Roth) in 1x MABT for 30 min, then with either alkaline phosphatase (AP)-conjugated sheep anti-DIG antibody (Roche) or AP-conjugated sheep anti-fluorescein antibody (Roche), that were diluted 1:200 in 1x Roti-ImmunoBlock in 1x MABT. Slices were incubated overnight at 4°C in a humidity chamber. After antibody incubation, slides were washed with 1x MABT 4 times for 5 min and equilibrated in alkaline phosphatase buffer NTMT, consisting of 100 mM NaCl, 100 mM Tris hydrochloride pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween-20 for 10 min. AP-labeled probes were detected colorimetrically via the nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate substrate system (NBT/BCIP; Roche). NBT (final concentration: 337.5 µg/ml) and BCIP (final concentration: 175 µg/ml) were diluted in NTMT and slices were covered with this solution. Slices were incubated for 6–8 h, then fresh NBT/BCIP solution was added and sections were incubated overnight. The reaction was stopped by 10 min of incubation in a stop solution consisting of 10 mM Tris hydrochloride pH 8.0 and 1 mM EDTA. Afterwards, slides were washed three times with 1x PBS for 5 min. Sections were further used for immunohistochemical BrdU detection (see Immunohistochemistry) and examined with a Zeiss Axiovert 200 fluorescent microscope.

Analysis and Statistics

Data were analyzed with the data analysis software R (R Development Core Team, 2013) and GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA). Data for EGR-1, DARPP-32 and DA receptor expression passed the D'Agostino's K^2 test for normal distribution and were then evaluated with an analysis of variance (ANOVA) followed by a *post hoc* Tukey's Honestly Significant Difference test (HSD). To test the correlation between DARPP-32 density and age, we performed a linear regression analysis. Significance level was $p < 0.05$ for all tests.

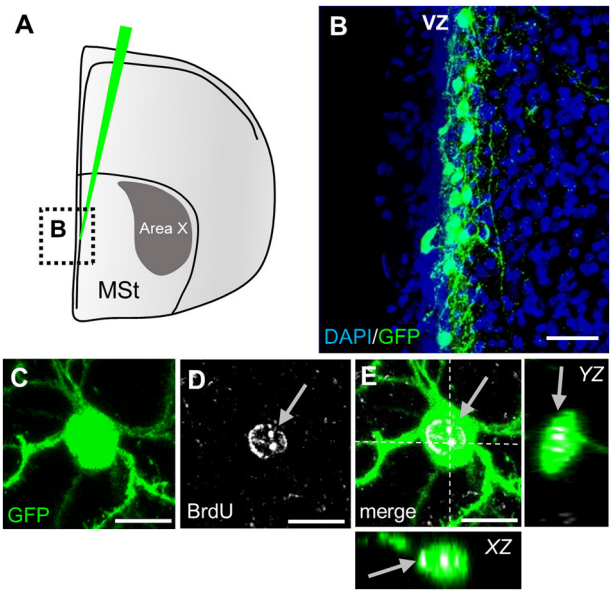

FIGURE 2 | Labeling of striatal progenitors. **(A)** Lentiviral vector injections were surgically targeted at the wall of the lateral ventricle adjacent to the medial striatum (MSt). The area outlined is depicted in B. **(B)** Many cells in the ventricular zone (VZ) were infected, as shown by virally mediated GFP expression in a coronal section. The ventricle is on the left side of the image. **(C–E)** GFP+ neuron in the striatum recently divided and incorporated BrdU (arrows). Dashed lines **(E)** indicate the planes used to generate orthogonal views of the Z-stack (*YZ, XZ*). Scale bars: 25 µm **(B)**, 10 µm **(C–E)**.

RESULTS

Newborn MSNs Receive Glutamatergic Input and Connect to Pallidal Output Neurons

To investigate whether and when newborn neurons in Area X are integrated into existing circuits, we used a lentivirally mediated approach to label progenitor cells in the striatal ventricular zone of adult male zebra finches (**Figures 2A,B**). By 31 days post injection (dpi), newly generated neurons in Area X exhibited the typical MSN morphology with relatively small nuclei (5–9 µm) and spiny dendrites. Co-labeling with BrdU confirmed that GFP+ cells in Area X recently divided and originated from the progenitor pool (**Figures 2C–E**).

Newly generated granule neurons in the adult murine DG first receive long-range cortical inputs at 3 weeks of age, whereas granule cells in the olfactory bulb connect already at 2 weeks of age to presynaptic cortical neurons (Deshpande et al., 2013). We wanted to know if and when newborn MSNs in Area X receive glutamatergic inputs from afferent cortical song nuclei. Using VGLUT2 (vesicular glutamate transporter 2) (**Figure 3A**) as a marker we found glutamatergic synapses at spines of newly generated MSNs at 31 dpi (**Figures 3B–E**). These glutamatergic innervations are likely to originate from the pallial song nuclei HVC and LMAN (**Figure 1**). We also noticed spines without VGLUT2 immunoreactivity (**Figures 3B,F–H**).

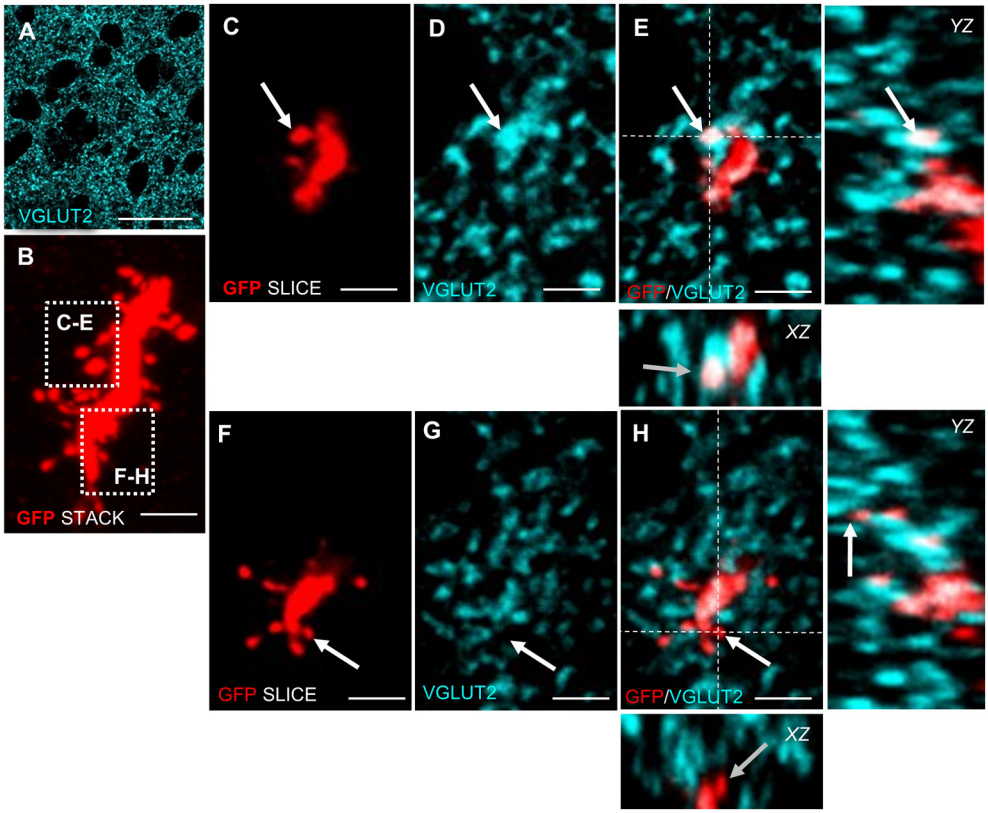

FIGURE 3 | Adult generated MSNs in Area X receive glutamatergic input. **(A)** VGLUT2 is expressed in a punctate pattern in the neuropil, corresponding to presynaptic glutamatergic terminals in Area X. **(B)** High-resolution scan of an adult generated MSN dendrite (GFP+, red). The Z-scan was collapsed. The focus planes of spines in dashed boxes are shown in **C–H**. **(C–E)** Arrow points to a dendritic spine of an adult generated MSN that colocalized with VGLUT2. **(F–H)** Arrow points to a spine of new MSN that did not colocalize with VGLUT2. Dashed lines **(E,H)** indicate the planes used to generate orthogonal views of the Z-stack (*YZ, XZ*). Scale bars: 2.5 μm **(B–H)**, 25 μm **(A)**.

After confirming glutamatergic input onto new MSNs, we tested if they contribute to signal transmission via pallidal-like output neurons. In the adult HVC, newborn projection neurons were found to be connected to their target nucleus at 3 weeks of age (Tokarev et al., 2016). We therefore predicted that newborn MSNs connected to their target cells in a similar way. Additional to GFP-labeling of progenitors in the VZ, we retrogradely labeled one class of pallidal-like neurons that project directly from Area X to the thalamic nucleus DLM (**Figures 1**, **4A,B**; Goldberg et al., 2013).This neuron type is considered to be homologous to primate internal pallidal neurons (Goldberg and Fee, 2010). Retrogradely labeled neurons had big somata and smooth, aspiny dendrites; consistent with this cell type (Reiner et al., 2004; **Figure 4C**). We found connections from newborn MSNs to pallidal-like neurons at 31 dpi and 42 dpi. We observed connections between axons and axonal boutons of new MSNs and dendrites of pallidal-like neurons; in that case, axons often wrapped around pallidal-like neuronal dendrites (**Figures 4G–J**). Additionally, their axons were often found in close apposition to the somata of pallidal-like neurons (**Figures 4D–F**). We specifically searched for backfilled pallidal-like neurons with new MSNs (GFP+) nearby. At 31 dpi, we observed that in a fraction of 0.73 of pallidal-like neurons, new MSN axons contacted their dendrites. In a fraction of 0.27 of pallidal-like neurons, both their somata and dendrites received contacts by new MSNs axons (in total 22 pallidal-like neurons, 2 animals). At 42 dpi, we found that in a fraction of 0.69 of pallidal-like neurons, new MSN axons contacted their dendrites. In a fraction of 0.31 of pallidal-like neurons, both their somata and dendrites received contacts by new MSNs axons (in total 26 pallidal-like neurons, 2 animals).

Newborn MSNs Receive Dopaminergic Innervation

Besides glutamatergic input from the song nuclei HVC and LMAN, MSNs in Area X also receive dopaminergic innervations from the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc), (Lewis et al., 1981; Bottjer, 1993; Gale et al., 2008). DA signaling via D1 receptors modulates social context dependent song variability; DA concentration in Area X is higher during female directed courtship song than when birds sing by themselves (Sasaki et al., 2006; Leblois et al., 2010). DA signaling can either be activating or inhibiting, depending on the receptor it is binding to Gerfen and Surmeier (2011). DA

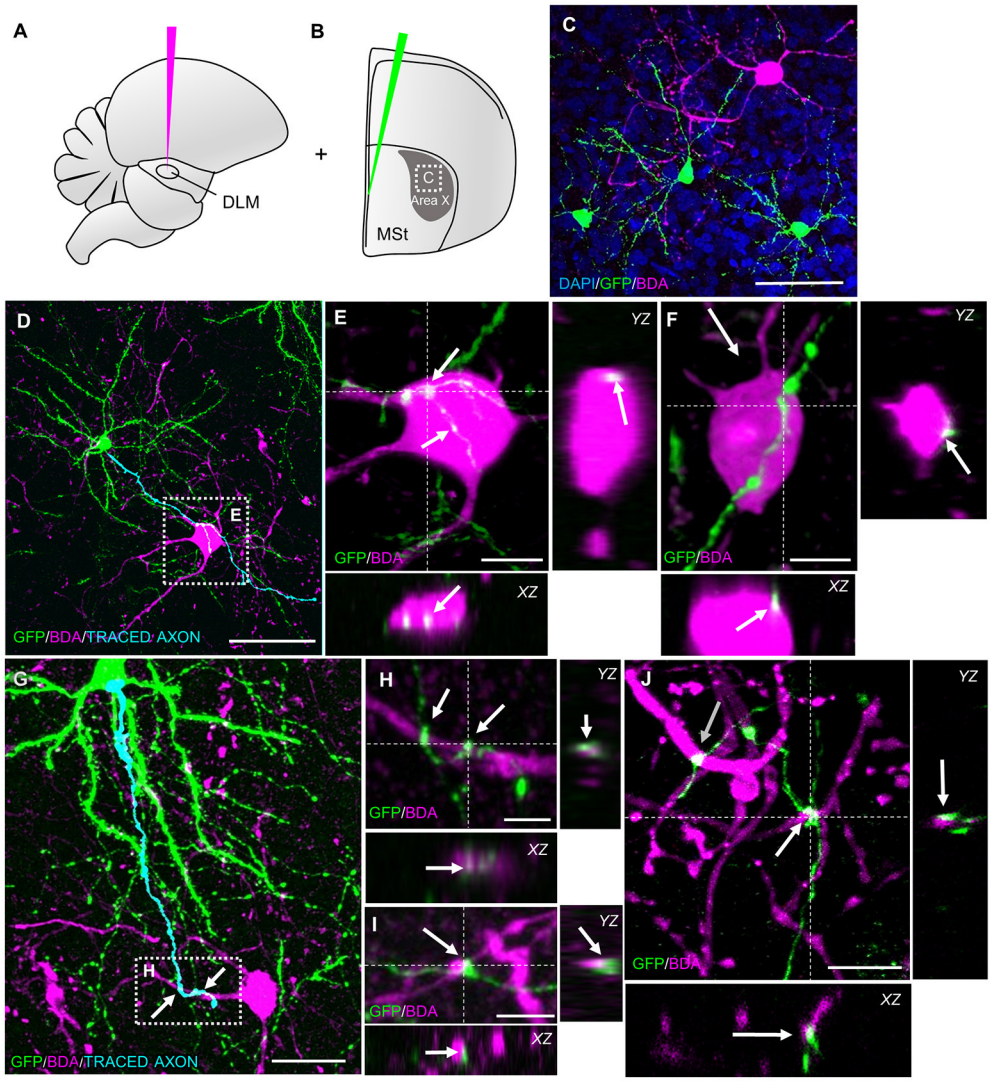

FIGURE 4 | New MSNs have axosomatic and axodendritic contacts to pallidal-like projection neurons in Area X. **(A)** Pallidal-like projection neurons in Area X were labeled via retrograde tracing. BDA was injected into thalamic nucleus DLM, the target of pallidal-like projection neurons in Area X. **(B)** Additionally, progenitors were labeled in the VZ via lentivirally mediated GFP expression. The area outlined is depicted in **C**. **(C)** Newborn MSNs (GFP+) and pallidal-like neurons were both present in sections of Area X. **(D)** The axon of a newborn MSN passed the soma of a pallidal-like projection neuron (BDA). The pallidal-like neuron in the dashed box is magnified in **E**,**F**. **(E,F)** Axosomatic contacts (arrows) of new MSN on pallidal-like neuron somata. **(G)** The axon a newborn MSN wrapped around dendrites of a pallidal-like neuron. The area in the dashed box is magnified in **H**. **(H–J)** Axodendritic contacts (arrows) of newborn MSN onto pallidal-like neurons. Dashed lines **(E,F,H–J)** indicate the planes used to generate orthogonal views of the Z-stack (*YZ, XZ*). Scale bars: 5 μm **(H,I)**, 10 μm **(E,F,J)**, 25 μm **(G)**, 50 μm **(C,D)**.

binding to D1-like receptors rises the resting potential and hence increases the chance of an action potential, whereas DA binding to D2-like receptors has the opposite effect. Neurons in the avian striatum express four types of dopamine receptors. Different from mice, up to 50% of MSNs in songbirds express both D1 and D2 receptor types (Kubikova et al., 2010). To test if newborn neurons differ from older, resident neurons in Area X in their expression of DA receptors, we combined *in situ* hybridization to detect DA receptor mRNA with BrdU labeling (**Figures 5A–G**). Because the majority of new neurons were mature at 42 days after BrdU labeling (**Figure 6L**), we decided to analyze DA receptor expression at that point. We found that a fraction of 0.89 ± 0.03 of new neurons expressed D1A, 0.94 ± 0.02 D1B, and 0.69 ± 0.03 D2 receptor mRNA (**Figure 5H**).

These results did not differ statistically from DA receptor mRNA expression values we found in non-BrdU labeled cells (0.9 ± 0.08 D1A, 0.95 ± 0.05 D1B, and 0.66 ± 0.08 D2). The averages of single-labeled D1A and D2 cells added up to more than 1, indicating that at least a fraction of 0.58 of BrdU+ cells co-expressed both receptor types. The averages of single-labeled D1B and D2 indicate that at least a fraction of 0.63 of BrdU+ cells co-expressed D1B and D2 receptors.

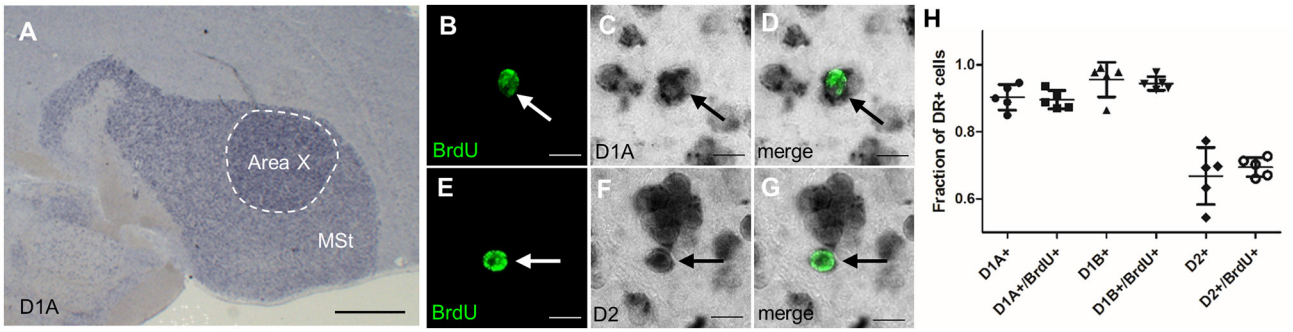

FIGURE 5 | Newborn MSNs do not differ from mature MSNs in their DA receptor expression. **(A)** Dopamine receptors were highly expressed in the MSt and Area X shown here for D1A in a non-fluorescent *in situ* hybridization (blue precipitate). **(B–D)** New MSNs (BrdU+, arrow, fluorescent green label) expressed dopamine receptor D1A (dark precipitate). **(E–G)** New MSNs (BrdU+, arrow) expressed DA receptor D2. **(H)** There was no significant difference in the expression of dopamine receptor types between older neurons (BrdU-) and 42-day-old neurons (BrdU+). One data point represents one animal. Shown are mean and SD. Scale bars: 10 μm **(B–F)**, 500 μm **(A)**.

Age Dependent Activation of Newborn MSNs during Singing Behavior

Having confirmed that newborn MSNs receive both glutamatergic and dopaminergic input and are connected to output neurons, we tested if they participate in signal transduction during singing. We used the immediate early gene EGR-1 as an indicator for neuronal activity (Knapska and Kaczmarek, 2004) in Area X and quantified its expression after singing in new neurons at different survival times (**Figure 6A**). Undirected singing resulted in elevated EGR-1 expression in Area X (**Figure 6B**), as expected (Jarvis et al., 1998; Mello and Ribeiro, 1998).

The fraction of singing-activated, newborn neurons in Area X (BrdU+/EGR-1+, **Figures 6C–F**) cells increased from 0.18 ± 0.17 at 21 dpi to 0.72 ±0.07 at 42 dpi ($F = 13.05$, $p = 0.00038$, **Figure 6K**). There was no significant difference in activation of new neurons between 21 and 31 dpi ($F = 13.05$, $p = 0.149$), but between 31 and 42 dpi ($F = 13.05$, $p = 0.019$, **Figure 6K**). Additionally, we evaluated the maturation course and quantified the expression of the MSN marker DARPP-32 in newborn neurons (BrdU+/DARPP-32+, **Figures 6G–J**). DARPP-32 expression significantly increased from 0.0075 ± 0.015 at 21 dpi to 0.44 ± 0.09 at 31 dpi ($F = 180.8$, $p = 6.3 \times 10^{-6}$) to 0.9 ±0.01 at 42 dpi ($F = 180.8$, $p = 8.3 \times 10^{-6}$, **Figure 6L**).

Age Dependent MSN Density in Area X

When studying adult neurogenesis, it is always of concern whether newly-generated neurons are added continuously to an existing circuit or if they replace older neurons. Both strategies can occur in the same organism: newly generated granule cells in the mouse DG are added to the existing cell pool, whereas in the olfactory bulb new granule cells replace old neurons (Crespo et al., 1986; Imayoshi et al., 2008).

In the canary song control nucleus HVC, newly generated projection neurons are replaced seasonally, while in the zebra finch HVC, new neurons are continuously added to the existing circuit (Walton et al., 2012). To investigate which strategy applies in Area X of zebra finches, we quantified the density of MSNs in zebra finches at different ages. MSN density in Area X increased significantly between 1 and 6 years of age (linear regression, $R^2 = 0.679$ $p = 0.0003$, **Figure 7G**). MSN packing density increased from 78 × 10^4 cells/mm^3 in Area X of a 1-year-old zebra finch (**Figures 7A–C**) to 163 × 10^4 cells/mm^3 in as 6-year-old zebra finch (**Figures 7D–F**). Assuming an Area X size of 1.532 mm^3 (Nixdorf-Bergweiler, 1996) the total number of MSNs in Area X more than doubled from 1.2 to 2.5 million within 5 years. The fraction of MSNs out of all DAPI+ cells also increased significantly with MSN density (linear regression, $R^2 = 0.34$ $p = 0.0286$, **Figure 7H**).

DISCUSSION

In the present study, we investigated key features of adult-generated MSN that integrate into the avian striatal song nucleus Area X. Area X receives long-range cortical glutamatergic innervations from premotor nuclei HVC and LMAN (Bottjer and Johnson, 1997). We tested whether newborn MSNs in Area X receive this input by searching for glutamatergic presynaptic terminals on GFP-labeled newborn neurons after their migration from the ventricular zone. We found those contacts as early as 31 dpi. This time frame of being contacted by long-range excitatory input is similar to that reported for newborn hippocampal granule cells in mice (Deshpande et al., 2013), even though the migration distance of new MSNs from the VZ to Area X is considerably longer. This suggests that glutamatergic innervation of adult-born neurons is more a question of absolute age than a question of time of arrival at their final destination. We did not find presynaptic terminals on all dendritic spines, perhaps because those were in the process of being contacted or eliminated (Ramiro-Cortes et al., 2014).

Besides glutamate, dopaminergic innervation from VTA and SNc is the second main input to Area X (Lewis et al., 1981; Bottjer, 1993; Gale et al., 2008). By combining BrdU birth

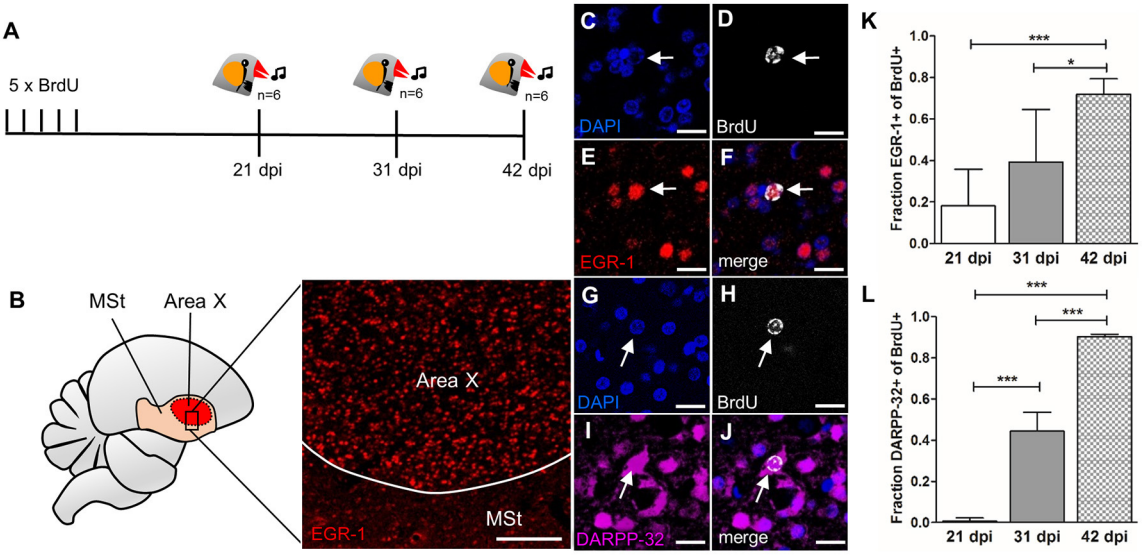

FIGURE 6 | Newborn MSNs are activated during singing in an age-dependent manner. **(A)** Experimental design: Adult male zebra finches ($n = 6$ per age group) received BrdU injections and were sacrificed at 21, 31, or 42 dpi after singing. **(B)** Region specific EGR-1 expression in Area X but not in the surrounding striatum after singing. **(C–F)** Newborn neuron (31 dpi, arrow) expressed EGR-1 after singing. **(G–J)** Newborn neuron (31 dpi arrow) expressed DARPP-32. **(K)** The fraction of new cells (BrdU+) that were activated after singing (EGR-1+) increased significantly between 21 and 42 dpi and between 31 and 42 dpi (shown are mean and SD). **(L)** The fraction of new cells (BrdU+) that express DARPP-32 increased significantly from 21 to 42 dpi (shown are mean and SD). $^*P \leq 0.05$; $^{***}P \leq 0.001$. Scale bars: 10 μm **(C–J)**, 100 μm **(B)**.

dating with *in situ* hybridization for DA receptors we established that 6-weeks old MSNs in Area X expressed mRNA for D1- and D2-type receptors in the same fractions as older, resident neurons. This suggests that newborn Area X neurons participate in dopaminergic signaling in the same way as older neurons do. It would be interesting to test if a time-dependent dopamine receptor expression in new neurons was crucial for specific stages of neurogenesis. For example, dopaminergic innervation via D3 receptors stimulates the very early process of progenitor proliferation in mammals and birds (Coronas et al., 2004; Lukacova et al., 2016) and in new murine granular cells, D1-type receptor expression is found earlier than D2-type receptor expression (Mu et al., 2011).

Having established the inputs onto new MSNs we were interested in their connection to pallidal-like projection neurons inside Area X. Direct pallidal-like neurons project to thalamic nucleus DLM and exhibit different firing patterns than indirect pallidal-like neurons (Goldberg and Fee, 2010; Woolley et al., 2014). We observed terminal boutons of newborn MSNs in close proximity to somata and dendrites of direct pallidal-like neurons. This suggests that newborn Area X neurons participate in signal transduction via the pallidal-like projection neurons. Future studies might address whether the innervation and connectivity to output neurons occurs even earlier than by 31 days after generation in the VZ, the time point we chose.

Given that newborn MSN have the morphological hallmarks to receive and transmit signals within Area X, we tested whether they are active during production of undirected song, which is known to induce EGR-1 protein expression (Jarvis et al., 1998; Mello and Ribeiro, 1998). We found that 20% of 21 day old MSN expressed EGR-1 after singing, but DARPP-32 was not detected in any MSN at that age. By 42 days of age, the majority of newborn MSNs expressed both proteins, raising the possibility that new MSNs may have to be physiologically active to trigger their further maturation. This is consistent with the fact that in mammals EGR-1 acts as a transcriptional activator of DARPP-32 (Keilani et al., 2012). One interpretation of our data is that singing-driven EGR-1 triggers maturation of newborn MSNs. This idea is supported indirectly; in mammals, the brain-derived neurotrophic factor (BDNF) enhances EGR-1 binding to the *Darpp-32* gene (Keilani et al., 2012). In canaries, BDNF levels are positively correlated with singing and enhance the survival of newly recruited HVC neurons (Rasika et al., 1999; Li et al., 2000). Similar mechanisms were shown in rodents; voluntary running exercise increases BDNF levels (Kobilo et al., 2011) and individual running activity positively correlates with rates of neurogenesis in the DG (Kodali et al., 2016). If overall individual singing activity influenced neuronal maturation via a BDNF/EGR-1/DARPP-32 pathway, it could explain the high variance in the fraction of activated new MSN during the early maturation phase (31 dpi) in contrast to the later maturation phase (42 dpi). New neurons that survived by then might have reached a stable state, whereas others that were not reliably EGR-1 activated by behavior were eliminated, similar to mechanisms found in the DG of mice (Veyrac et al., 2013).

Are new neurons in Area X added to existing circuits as a replacement of neurons that have died or are they added to the existing cell pool? In the songbird HVC both strategies exist: in canary HVC, seasonal fluctuations in projection neuron death and the recruitment of new neurons are correlated and

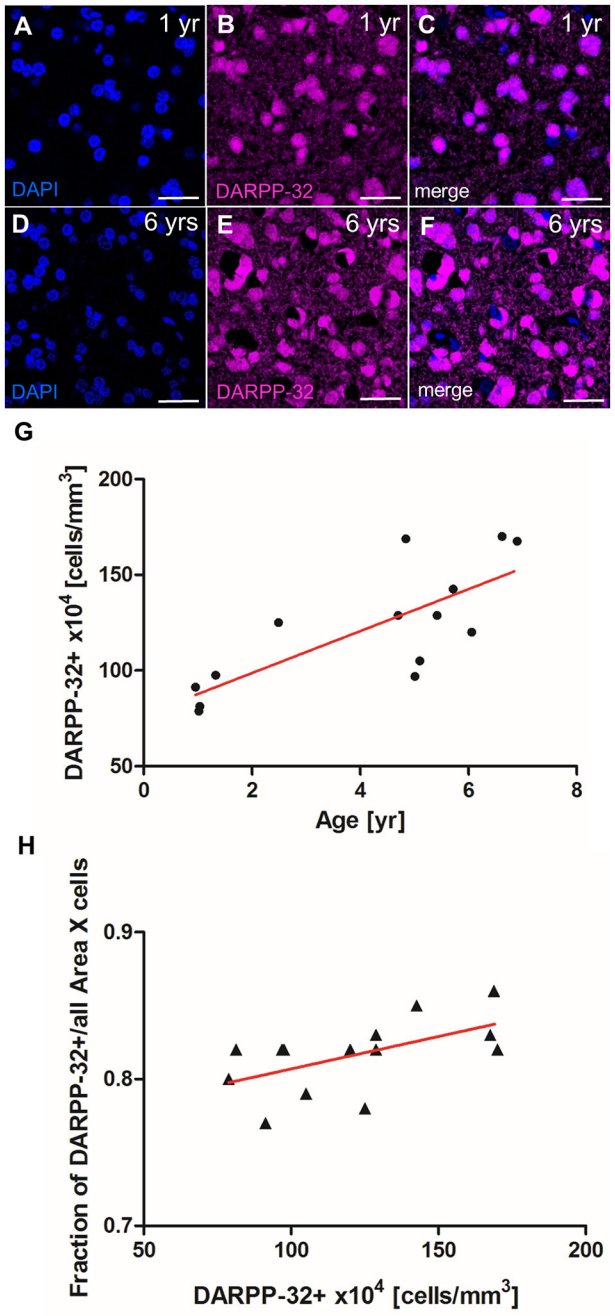

FIGURE 7 | MSN density in Area X increases with age. **(A–C)** MSNs (DARPP-32+) in Area X of a 1-year-old zebra finch male. **(D–F)** MSN (DARPP-32+) in Area X of a 6-year-old zebra finch male. **(G)** The density of MSNs (Darpp-32+) increased significantly with age. **(H)** The fraction of MSNs (DARPP-32+) of all DAPI+ nuclei increased with MSN density. In **(G,H)** one data point represents one animal (mean of both hemispheres). Scale bars: 20 μm **(A–F)**.

the peaks of neural recruitment coincide with the incorporation of new song elements. Together these data are consistent with a replacement strategy (Kirn et al., 1994). In the zebra finch HVC, new projection neurons are added constantly to HVC, resulting in an increasing density within the nucleus (Walton et al., 2012). Correlative evidence suggests that the age-dependent decline of new neuron addition in HVC is associated with increasing song stereotypy (Pytte et al., 2007). Together, these data are best explained by an addition strategy. In the present dataset, we show that the density of DARPP-32 positive MSNs in Area X increased significantly with age, implying that new MSNs were constantly added to the circuit. This does not exclude the possibility that some new neurons replaced apoptotic cells. In fact, experimentally induced apoptosis correlates with replacement by new neurons in zebra finch HVC (Scharff et al., 2000). Further, we found that the fraction of cells that were DARPP-32+ relative to all Area X cells also increased with age. Since the DARPP-32 neurons constitute the majority of cells that undergo adult neurogenesis, this finding emphasizes that increased cell density in Area X is a consequence of continued recruitment of newly born MSN during the course of aging.

Our findings suggest that, once matured, newborn MSNs fulfill the same function as older, resident MSNs, at least concerning the features we analyzed. MSNs function via feed forward inhibition, e.g. sparsely spiking MSNs inhibit tonically active pallidal-like projection neurons. Their high frequency bursts can evoke spiking of DLM neurons via inhibitory rebound (Person and Perkel, 2005, 2007; Kojima and Doupe, 2009). This process in modulated by dopaminergic signals from VTA/SNc. Dopaminergic neurons in VTA/SNc encode performance errors in singing zebra finches (Gadagkar et al., 2016).

We end on some speculations how constant addition of new neurons might affect the AFP and in turn the motor pathway. Constant MSN addition in face of an unchanged number of pallidal-like neurons would be expected to cause stronger inhibitory MSN action on pallidal-like neurons. In turn, DLM would experience fewer inhibitory rebound spikes, causing lower activation of LMAN neurons. Ultimately this would result in reduced excitation of motor nucleus RA by the AFP. If this hypothesis holds true, signaling through the AFP would diminish, as birds get older. In adult birds, the AFP mediates differences in song variability (Hessler and Doupe, 1999; Woolley et al., 2014). Song variability, including deterioration, can be induced experimentally by distorting auditory feedback via deafening or tracheosyringeal nerve cut (Williams and McKibben, 1992; Hough and Volman, 2002; Nordeen and Nordeen, 2010). The AFP seems to mediate this degradation process, since lesions of the AFP output nucleus LMAN prevent song deterioration after auditory feedback distortion (Brainard and Doupe, 2000). Interestingly, song deterioration after deafening is less severe in old birds compared to young birds, and song becomes more stereotyped with age, consistent with our hypothesis (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001; Pytte et al., 2007, 2012). This scenario does not exclude the possibility that new MSNs initially might undergo a narrow plastic phase, during which they can be tuned and possibly counteract song drift. In summary, we demonstrate that within a month after their generation newly generated MSNs in Area X of adult zebra finches are connected to other song nuclei and participate in neuronal firing during song production. The net increase of Area X neurons with age

might provide a mechanism to achieve the equilibrium between plasticity and stereotypy needed to sustain adult song behavior.

ETHICS STATEMENT

This study was carried out in accordance with the governmental law (TierSchG). The protocol was approved by the LAGeSo, Berlin.

AUTHOR CONTRIBUTIONS

JK and CS planned experiments, JK and LS conducted experiments, JK and CS wrote the manuscript.

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Publication B

Dynamic FoxP2 levels in male zebra finches are linked to morphology of adult-born Area X medium spiny neurons

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Dynamic FoxP2 levels in male zebra finches are linked to morphology of adult-born Area X medium spiny neurons

Jennifer Kosubek-Langer* & Constance Scharff

The transcription factor FOXP2 is crucial for the formation and function of cortico-striatal circuits. *FOXP2* mutations are associated with specific speech and language impairments. In songbirds, experimentally altered FoxP2 expression levels in the striatal song nucleus Area X impair vocal learning and song production. Overall FoxP2 protein levels in Area X are low in adult zebra finches and decrease further with singing. However, some Area X medium spiny neurons (MSNs) express FoxP2 at high levels (FoxP2^{high} MSNs) and singing does not change this. Because Area X receives many new neurons throughout adulthood, we hypothesized that the FoxP2^{high} MSNs are newly recruited neurons, not yet integrated into the local Area X circuitry and thus not active during singing. Contrary to our expectation, FoxP2 protein levels did not predict whether new MSNs were active during singing, assayed via immediate early gene expression. However, new FoxP2^{high} MSNs had more complex dendrites, higher spine density and more mushroom spines than new FoxP2^{low} MSNs. In addition, FoxP2 expression levels correlated positively with nucleus size of new MSNs. Together, our data suggest that dynamic FoxP2 levels in new MSNs shape their morphology during maturation and their incorporation into a neural circuit that enables the maintenance and social modulation of adult birdsong.

The forkhead box P2 transcription factor (FOXP2) is linked to speech and language disorders. Heterozygous *FOXP2* mutations in humans affect both the coordination of fine orofacial movements and language perception[1–3]. Because songbirds – like humans – need to learn most of their communicative vocalizations, they offer a unique model to study the role of FoxP2 (for nomenclature FOXP2/FoxP2 see Methods) for vocal learning and for the maintenance of learned vocalizations as adults[4]. Studying the relationship between FoxP2 and vocal learning in songbirds may inform the neurogenetic mechanism underlying the speech deficits in patients carrying FOXP2 mutations for the following reasons. The *FoxP2* protein coding sequence is highly conserved between humans and songbirds as are the brain expression patterns, notably in the cerebellum and striatum[5–7]. Moreover, genetic manipulations of FoxP2 expression levels in the striatal song nucleus Area X during the critical phase of song learning lead to inaccurate and incomplete imitation of the tutor's song and more variable vocal production[3,8–10]. This phenotype bears similarities to the specific speech deficits called developmental verbal dyspraxia, DVD (or childhood apraxia of speech), that patients carrying *FOXP2* mutations suffer from. The core-phenotype of DVD consists of altered precision, consistency and sequencing of movements underlying speech in the absence of neuromuscular deficits[11]. In addition, altered FoxP2 levels in adult Area X affect the dopaminergic modulation of corticostriatal signaling important to song variability and affect song maintenance[12,13], stressing the fact that tight regulation of FoxP2 expression is a prerequisite for correct neural transmission in differentiated neural circuits. Additional effects of Foxp2 and its disruption on the embryonic development and the function of neural circuits have been described in mice[14–23]. Further evidence for the biological relevance of tight regulation of FoxP2 expression levels comes from the following studies. FoxP2 expression levels in Area X transiently increase during song learning but are lower in adults[7,24]. Singing decreases overall FoxP2 levels in Area X but not in the surrounding striatum and the degree of FoxP2 down regulation correlates with the amount of produced song[25–27]. This relationship is missing in deafened birds, pointing to an important role of auditory feedback for singing-driven FoxP2 down regulation[27]. How does singing affect FoxP2 expression at the cellular level? Medium spiny neurons

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(MSNs), the most abundant cell type in the avian striatum, predominantly express FoxP2 at low levels (FoxP2^{low}) while a subset expresses FoxP2 at very high levels (FoxP2^{high}). Both subtypes are not equally affected by singing; the density of FoxP2^{high} MSNs is not measurably different after singing, contrary to the decreasing density of FoxP2^{low} MSNs[24]. The authors hypothesized that the difference might be due to the neuronal age. Adult Area X constantly receives new MSNs that originate at the ventricular zone[28–33]. FoxP2^{high} MSNs colocalize more frequently with a marker for new neurons than FoxP2^{low} MSNs[24]. Recently we showed that new MSNs mature and participate in singing activity – as measured by immediate early gene activation – within a timeframe of six weeks[34]. Whether FoxP2 influences not only the function but also the integration of new neurons into existing circuits is still an open question. Based on the results of Thompson *et al*. (ref. [21]) we hypothesized that FoxP2^{high} MSNs are newly recruited into Area X and need to become FoxP2^{low} MSNs before they can participate in singing. To test this, we labelled neuronal progenitors in adult zebra finches. At different time points after these cells had migrated into Area X, we quantified their expression levels of FoxP2 and whether they also expressed the immediate early gene expression EGR-1 after singing.

In rodents, Foxp2 expression is associated with neurite outgrowth, neuronal morphology and synapse formation in cortico-striatal circuits[18,19,35–37]. Foxp2 expression levels vary in striatal MNSs and these differences may be relevant for the morphology of striatal MSNs. Dopamine receptor 1 (D1) expressing MSNs[38] express Foxp2 at higher levels than dopamine receptor 2 (D2) expressing MSNs[23,35]. These differences in Foxp2 levels may be linked to anatomical differences between D1 and D2 MSNs[35,39]. Furthermore, in mice carrying humanized Foxp2 alleles (Foxp2$^{hum/hum}$ mice), Foxp2^{high} MSNs are more numerous in the dorsal striatum and their MSNs have longer dendrites than wildtype mice[36,37]. Based on the latter results we hypothesized that FoxP2 levels of new MSNs in adult songbirds correlate with their neural morphology. To test this, we virally labelled neural progenitors and analyzed their FoxP2 expression, dendrite complexity and spine density after migration into Area X.

Results

Dynamic FoxP2 levels in new MSNs in Area X. To assess FoxP2 protein levels in individual newborn neurons we labelled progenitor cells with Bromodeoxyuridine (BrdU) and detected BrdU+/FoxP2+ cells after 21, 31 and 42 days post BrdU injection (dpi) in Area X of adult male zebra finches (Fig. 1a,b). We found that FoxP2 expression in Area X was very variable, with some neurons expressing FoxP2 at particularly high levels and some at low levels (Fig. 1c). At 21 dpi and at 31 dpi, the mean pixel intensities of all BrdU+/FoxP2+ cells formed a bimodal distribution, whereas at 42 dpi the distribution was unimodal and shifted to low FoxP2 expression (Fig. 1d). We classified all neurons that had expression intensities within the top 30% of the measured pixel intensity distribution as FoxP2^{high} neurons. Neurons within the bottom 30% of the measured pixel intensity distribution were considered as FoxP2^{low}. Because we were interested in the two extremes of the expression levels in this study, we did not analyze the new neurons with intermediate FoxP2 expression levels further (29.3% ± 9.4, SD, see Methods). At 21 dpi and at 31 dpi 36.17% ± 6.02 (SEM) and 34.91% ± 2.53 (SEM) percent of all BrdU+ cells were FoxP2^{high} neurons, respectively. At 42 dpi the percentage of FoxP2^{high} cells had significantly decreased to 12.95% ± 2.87 (SEM) on average ($p = 0.0077$, Kruskal-Wallis test, Fig. 1f,g). The percentage of FoxP2^{low} neurons increased significantly from 21 dpi (34.59 ± 5.86, SEM) and 31 dpi (34.06 ± 3.35, SEM) to 42 dpi (59.19 ± 4.02, SEM, $p = 0.013$, ANOVA, Fig. 1e,g). We also noticed that BrdU+ cells varied in their nucleus size. Quantification revealed that the distribution of the nucleus size was shifted towards bigger nuclei at 31 dpi (data not shown). BrdU+/FoxP2+ cells at 31 dpi had significantly bigger nuclei (7.37 µm ± 0.94 (SD) than BrdU+/FoxP2+ cell at 21 dpi (6.88 µm ± 0.9 (SD), $p = 0.00025$, chi-square = 75.358, df = 2) or 42 dpi (6.74 µm ± 0.59 (SD), $p = 2 \times 10^{-6}$, chi-square = 75.358, df = 2, data not shown). Interestingly there was a significant positive relationship with a low effect size between nucleus size and FoxP2 expression levels in all three experimental groups (21 dpi: $r^2 = 0.086$, $p = 0.017$, 31 dpi: $r^2 = 0.083$, $p = 5.2 \times 10\text{-}7$, 42 dpi = $r^2 = 0.039$, $p = 0.0012$, Fig. 1h).

Singing induced activity of new MSNs is independent of FoxP2 levels. In adult zebra finches FoxP2 expression levels in Area X are behaviourally regulated. Undirected singing leads to downregulation of FoxP2 mRNA and protein[24–26]. Undirected singing is also associated with expression of the immediate early gene *EGR-1* in Area X, which is therefore often used as a molecular readout of the neuronal activity associated with undirected song[40–44]. We hypothesized that FoxP2 levels in new FoxP2^{high} neurons needed to be downregulated before activation by singing could occur, resulting in EGR-1 expression. Consequently, we did not expect to find BrdU+/FoxP2^{high}/EGR-1+ MSNs in Area X. To test this, we analyzed BrdU+/EGR-1 ± /FoxP2+ cells in birds that had sung before sacrifice after 21 dpi, 31 dpi and 42 dpi.

Contrary to our hypothesis we found BrdU+/FoxP2^{high}/EGR-1+ cells in Area X (Fig. 2a) in all groups, with large differences between birds. At 31 dpi, on average 41.45% ± 15.43 (SEM) of new neurons expressed FoxP2^{high} and were also activated by singing (BrdU+/FoxP2^{high}/EGR-1+) and 40.28% ± 13.34 (SEM) of the new neurons that expressed FoxP2^{high} were not activated by singing (BrdU+/FoxP2^{high}/EGR-1-) (Fig. 2b). At 42 dpi, the more birds had sung the fewer FoxP2^{high} new neurons were found, resulting in a significant negative relationship between the number of BrdU+/FoxP2^{+}/EGR-1+ neurons and the number of motifs sung before sacrifice ($r^2 = 0.753$, $p = 0.025$, Fig. 2c) which was not the case at 21 dpi ($r^2 = 0.168$, $p = 0.493$, data not shown) nor at 31 dpi ($r^2 = 0.164$, $p = 0.425$, data not shown).

FoxP2 levels in new MSNs influence dendritic arborization and spine density. Because of the relationship of FoxP2 levels and nucleus size we wanted to further characterize the morphology of new neurons expressing different FoxP2 levels. We used a lentiviral approach to express Green Fluorescent Protein (GFP) in progenitor cells at their place of birth in the lateral wall of the lateral ventricle (Fig. 3a). After 31 and 42 dpi we found labeled neurons (GFP+) in Area X and the surrounding striatum (Fig. 3b). GFP+ neurons exhibited the typical morphology of medium spiny neurons with small somata and spiny dendrites (Fig. 3c) and expressed

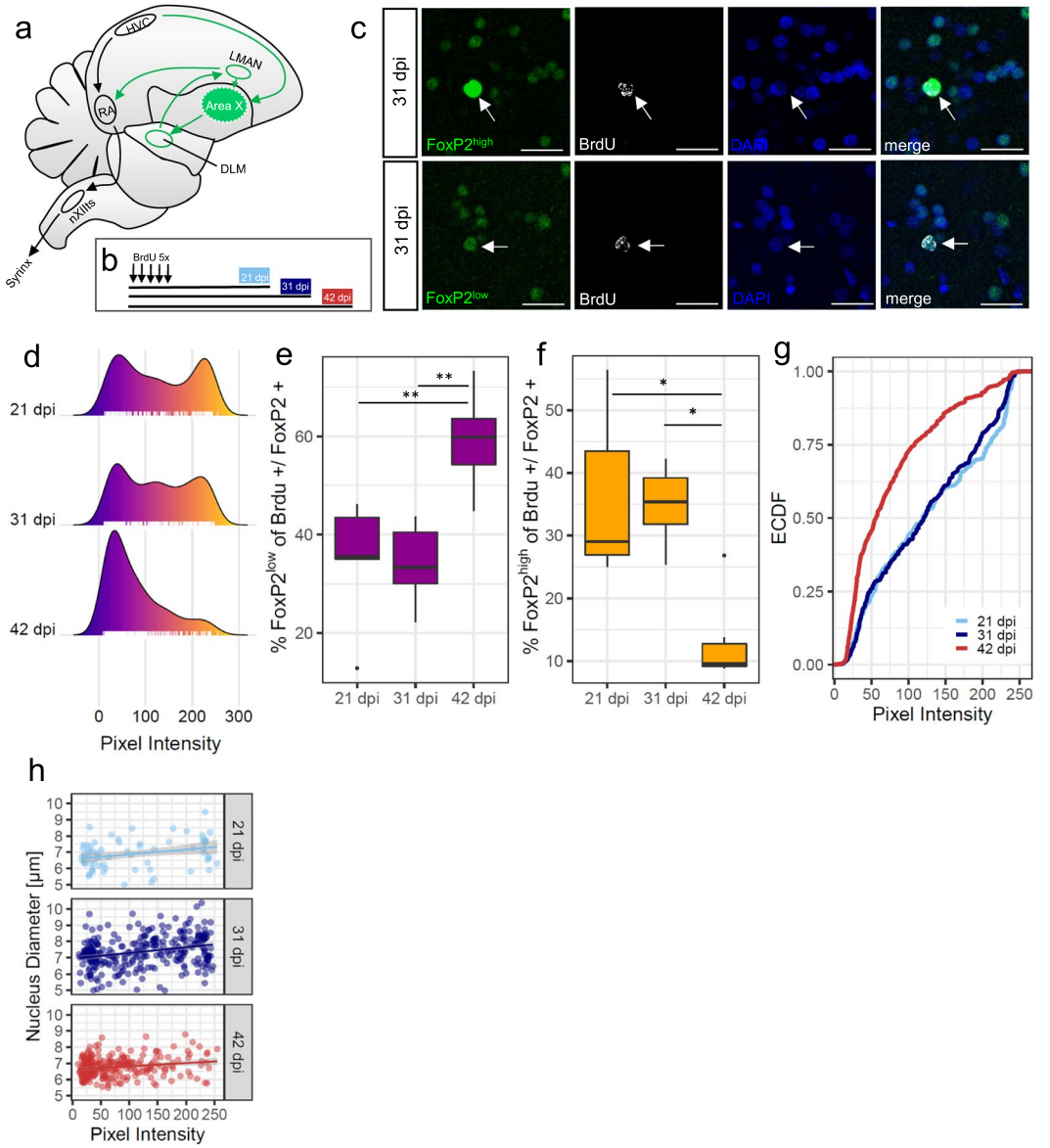

Figure 1. Dynamic FoxP2 expression levels in new MSNs. (**a**) The song motor pathway (main nuclei outlined in black) controls the vocal organ (syrinx). The anterior forebrain pathway (shown in green) forms a cortico (HVCm, lMAN) -basal ganglia (Area X) –thalamo (DLM) –cortical (RA) loop. (**b**) Experimental schedule: adult male zebra finches received BrdU on five consecutive days and were sacrificed 21, 31 or 42 days after injections (dpi). (**c**) Examples of FoxP2^{high} (top row, immunoreactivity shown in green) and FoxP2^{low} (bottom row) new (BrdU+ immunoreactivity shown in white) MSNs in Area X at 31 dpi. Nuclear expression of FoxP2 and BrdU coincides with DAPI label in blue. (**d**) Density plots of FoxP2 pixel intensities of individual new MSNs in Area X at different time points after BrdU injections. The color scheme indicates increasing pixel intensity from low (blue) to high (yellow) intensity. Ticks at the bottom of each plot represent individual MSNs. (**e**) Percentage of new FoxP2^{low} MSNs significantly increases from 21/31 dpi to 42 dpi. (**f**) Percentage of new FoxP2^{high} MSNs significantly decreases from 21/31 dpi to 42 dpi. (**g**) Empirical cumulative distribution function (ECDF) of FoxP2 pixel intensities of individual new MSNs in Area X. FoxP2 pixel intensities are similar at 21 and 31 dpi and are lower at 42 dpi. (**h**) FoxP2 pixel intensities of new MSNs positively correlate with nucleus diameter. Each dot represents one new MSNs. Sample size (d-h): 733 MSNs of 17 zebra finches. *$p \leq 0.05$, **$p \leq 0.01$. Scale bar: 20 µm (**g**). RA, Robust nucleus of the arcopallium; LMAN, Lateral magnocellular nucleus of the anterior nidopallium; NXIIts, tracheosyringeal part of the hypoglossal nucleus; DLM, Dorsal lateral nucleus of the medial thalamus.

FoxP2 (Fig. 3d,e). First, we traced FoxP2^{high} and FoxP2^{low} new neurons at 31 dpi (Fig. 4a) and found that FoxP2^{high} neurons had more primary dendrites ($p = 0.021$, Mann Whitney test, Fig. 4b), a higher total branch length ($p = 0.003$, Mann Whitney test, Fig. 4e) and thicker dendrites than FoxP2^{low} neurons ($p = 0.003$, t-test, Fig. 4f). Second, we analyzed the extent of dendritic arborization of GFP+/FoxP2+ neurons using a Sholl analysis (see

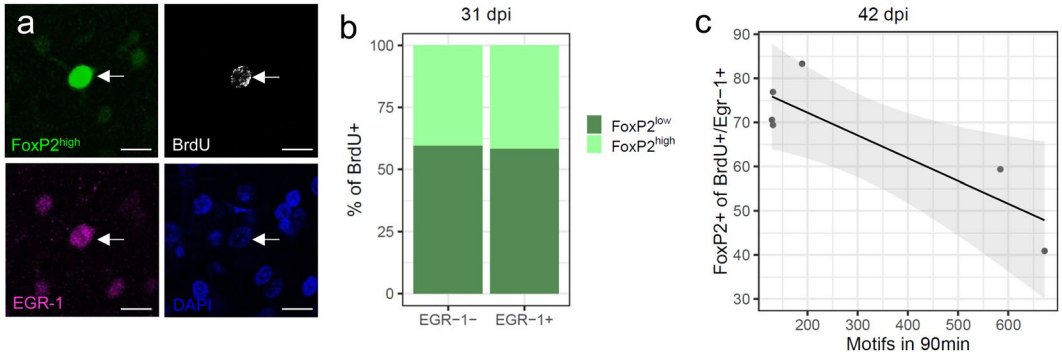

Figure 2. Singing-induced EGR-1 activation of new MSNs is independent of FoxP2 levels. (**a**) The white arrow in all 4 panels points to a new (BrdU+ immunoreactivity, white) MSN that expresses FoxP2^{high} (immunoreactivity, green) and also EGR-1 (immunoreactivity, purple) after undirected singing. The blue DAPI staining shows other cells that are not new, but express FoxP2 at low levels, some of which also express EGR-1. Scale bar: 10 µm. (**b**) At 31 dpi new neurons can either be activated by undirected singing (EGR-1+, right column) or not (EGR-1-, left column). In both cases, the new MSN can either express FoxP2^{low} or FoxP2^{high}. (**c**) At 42 dpi the number of FoxP2/BrdU+/EGR-1+ neurons negatively correlate with the number of motifs sung during the 90 min before sacrifice. Sample size (**b**): 108 MSNs of 6 zebra finches. Sample size (**c**): 156 MSNs of 6 zebra finches.

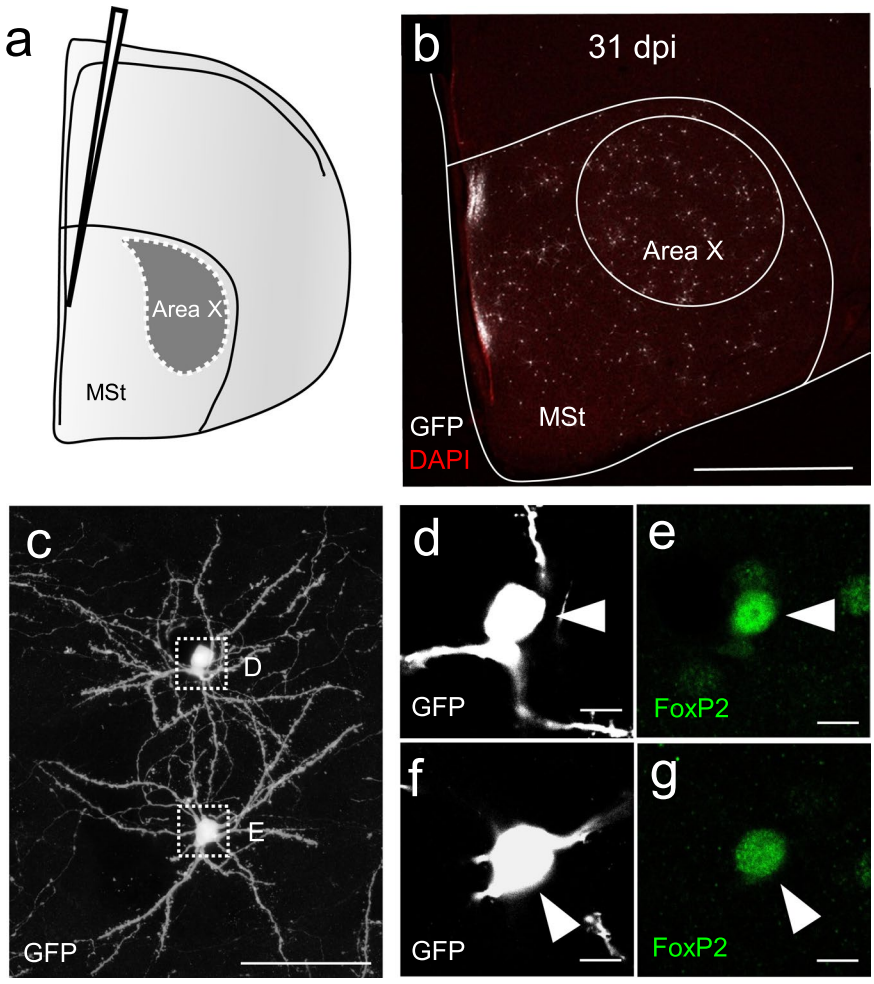

Figure 3. New MSNs that were GFP-labeled as progenitors at the ventricular zone and migrated to Area X express FoxP2. (**a**) Injections with a lentivirus into the ventricular zone resulted in GFP expression in the neural progenitors of MSN. (**b**) 31 dpi after viral injections, many GFP-labelled neurons can be observed in Area X and the surrounding striatum. (**c**) New GFP-expressing MSNs in Area X. Somata in the dashed boxes are magnified in (**d**) and (**e**). (**d**,**e**) Virally labelled MSNs (GFP immunoreactivity, white) express FoxP2 (immunoreactivity, green). Scale bars: 10 µm (d, e), 50 µm (**c**), 1 mm (**b**).

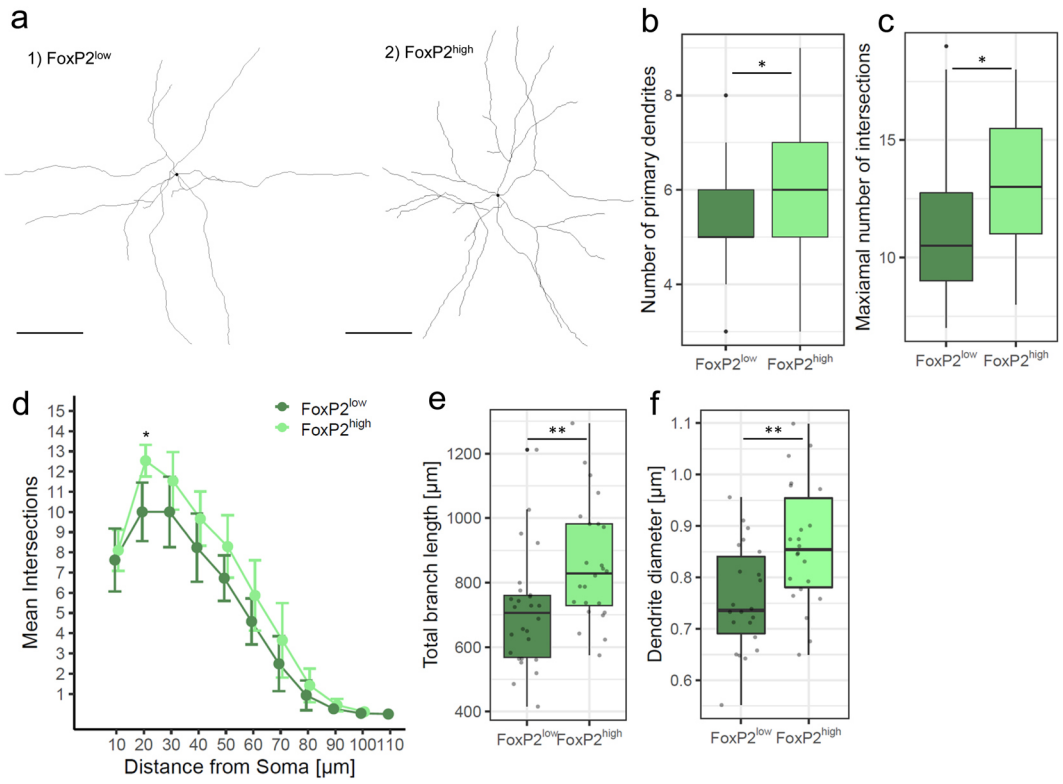

Figure 4. FoxP2 expression levels are linked to distinct morphologies of new MSNs at 31 dpi. (**a**) Examples of dendrite tracings of new FoxP2^{low} and FoxP2^{high} MSNs. The black dot marks the center of the soma. (**b**) Number of primary dendrites is significantly increased in FoxP2^{high} compared to FoxP2^{low} new MSN. (**c**) Maximal number of intersections between dendrites and Sholl circles is significantly higher in FoxP2^{high} than in FoxP2^{low} new MSN. (**d**) Sholl analysis revealed that dendrites of new FoxP2^{high} MSNs had more complex arborizations as indicated by more intersections at 20 µm from the soma than new FoxP2^{low} MSNs. Shown are mean ± SEM. Data points of FoxP2^{high} neurons were slightly shifted to the right for better visibility. (**e**) FoxP2^{high} new MSNs have a significantly higher total branch length than FoxP2^{low} new MSNs. (**f**) At 31 dpi new FoxP2^{high} MSNs have thicker dendrites than new FoxP2^{low} MSNs. Sample size (a-f): 52 MSNs of 4 zebra finches. Scale bars: 25 µm. *p ≤ 0.05; **p ≤ 0.01.

Methods). At 31 dpi FoxP2^{high} neurons had more intersections at 20 µm distance from the soma ($p = 0.024$, paired t-test, Fig. 4a,d) and a higher number of maximal intersections ($p = 0.019$, t-test, Fig. 4c) than FoxP2^{low} neurons, reflecting more extensive dendritic arborizations in FoxP2^{high} than FoxP2^{low} neurons. Second, Third, we used a semi-automated quantification approach to assess the number of dendritic spines in GFP+/FoxP2+ neurons at 31 dpi. FoxP2^{high} neurons in Area X had more dendritic spines than FoxP2^{low} neurons ($p = 0.034$, paired t-test, Fig. 5a–c). Overall, FoxP2^{high} neurons had more mushroom spines than FoxP2^{low} neurons ($p = 0.0186$, Mann Whitney test, Fig. 5d). There was no difference in the number of stubby spines ($p = 0.819$, Mann Whitney test) or thin spines ($p = 0.409$, Mann Whitney test) between FoxP2^{high} and FoxP2^{low} neurons (Fig. 5d). Because of the difference in mushroom spine number, we assumed that FoxP2 expression levels might influence mushroom spine head size, too. However, quantification revealed no difference in mushroom spine head size between FoxP2^{high} and FoxP2^{low} neurons ($p = 0.317$, Mann Whitney test, Fig. 5e). In a last step we compared spine densities between new MSNs at 31 dpi and 42 dpi. Since at 42 dpi only few new neurons were FoxP2^{high} we included only FoxP2^{low} new neurons in this analysis (Fig. 5f). At 42 dpi, the spine density of FoxP2^{low} new neurons was higher than in FoxP2^{low} neurons at 31 dpi ($p = 2.517 \times 10^{-4}$, t-test, Fig. 5f). This elevated spine density was largely due to an increase in thin spines ($p = 8.422 \times 10^{-4}$, Mann Whitney test) and not mushroom spines ($p = 0.39$, t-test) or stubby spines ($p = 0.119$, Mann Whitney test).

Discussion

In the present study we investigated the dynamics of FoxP2 expression in adult-born MSNs in the striatal song nucleus Area X of adult male zebra finches. We show that the new MSN strongly expressed FoxP2 at their arrival in Area X from the ventricular zone (VZ) where they were born 21 days prior. During this stage and at intermediate maturation stages (31 days) one third of new MSNs expressed FoxP2 at high levels. At the late maturation stage (42 days) most new MSNs expressed FoxP2 at low levels (Fig. 6). Together with our previous data we conclude that reaching low FoxP2 levels is a sign that adult-born born MSN in Area X have reached maturity by 6 weeks after their generation in the VZ[34].

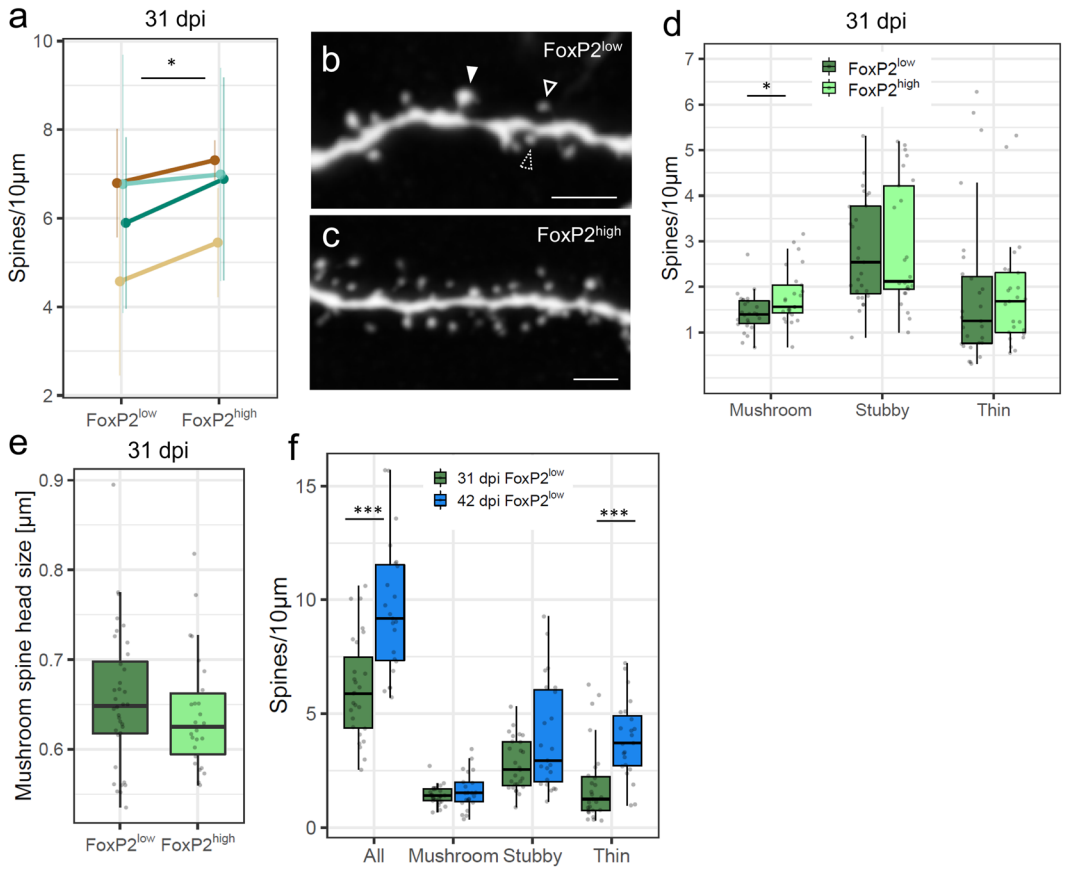

Figure 5. FoxP2 levels are associated with dendritic spine density of new MSNs. (**a**) New FoxP2^{high} MSNs had significantly more dendritic spines than new FoxP2^{low} MSNs at 31 dpi (shown are mean ± SEM). Lines connect data from the same animal. (**b**) Confocal 12 µm projection showing an example of FoxP2^{low} new MSN dendrite with mushroom (filled arrow), stubby (dashed arrow) and thin spines (unfilled arrow). (**c**) Example of FoxP2^{high} new MSNs dendrite. (**d**) New FoxP2^{high} MSNs have more mushroom spines than FoxP2^{low} new MSN at 31 dpi. (**e**) Mushroom spine head size is not different between MSNs with different FoxP2 levels. (**f**) New FoxP2^{low} MSNs at 42 dpi show overall more dendritic spines and more thin spines than new FoxP2^{low} MSNs at 31 dpi. Sample size (**a**,**d**,**e**,**f**): 52 MSNs of 4 zebra finches. Sample size (**f**): 23 MSNs of 3 zebra finches. *$p \leq 0.05$; ***$p \leq 0.001$. Scale bars: 5 µm (**b**,**c**).

Since our previous work demonstrated that new MSNs participate in singing-associated neural activity in Area X[34] here we asked if this was linked to FoxP2 levels. After singing FoxP2 mRNA and protein levels are lower in Area X tissue[25–27] than when birds were silent whereas the expression of the immediate early gene EGR-1 increases linearly the more birds sing undirected song[40,41]. Analyzing expression levels in individual neurons revealed that only FoxP2^{low} MSNs, but not Foxp2^{high} MSNs seemed to be subject to singing induced FoxP2 downregulation[24]. We therefore hypothesized that FoxP2^{high} MSNs were not yet connected into the circuit and therefore not regulated by singing. Our data contradict this hypothesis. We show that the induction of the immediate early gene EGR-1 in MSNs after singing was equally likely in FoxP2^{low} and FoxP2^{high} MSNs, suggesting that both were functionally incorporated into the song circuit.

Previous work showed that the degree of FoxP2 downregulation correlates with the quantity of produced song and depends on auditory feedback, which is relayed to Area X via the cortical song nucleus HVC[27,45]. In our study, the relationship between FoxP2 downregulation and song quantity was present in new MSNs at 42 dpi but not earlier and we therefore suggest that new MSNs start to receive auditory feedback signals between 31 and 42 dpi.

What might cause the age-dependent FoxP2 downregulation in new MSNs? One possibility is that intrinsic mechanisms, depending more on cell age than on extracellular inputs, downregulate FoxP2 during maturation. Another possibility is that EGR-1 gradually decreases FoxP2 levels during every singing event. This latter scenario is consistent with the findings that the *FoxP2* promoter contains EGR-1 binding sites[4,46] and that EGR-1 expression is crucial for functional integration of new neurons in the adult rodent hippocampus[47].

We also tested if varying FoxP2 levels affect neuronal morphology in adult male zebra finches and analyzed dendrite complexity and spine density of virally labelled new FoxP2^{low} and FoxP2^{high} MSNs. We now show differences between the morphology of adult generated MSNs that express FoxP2 at high or low levels; high FoxP2 levels were associated with greater dendrite complexity and higher dendritic spine density in comparison to neurons with low FoxP2 levels (Fig. 6). Concerning spine density, our results are consistent with previous studies

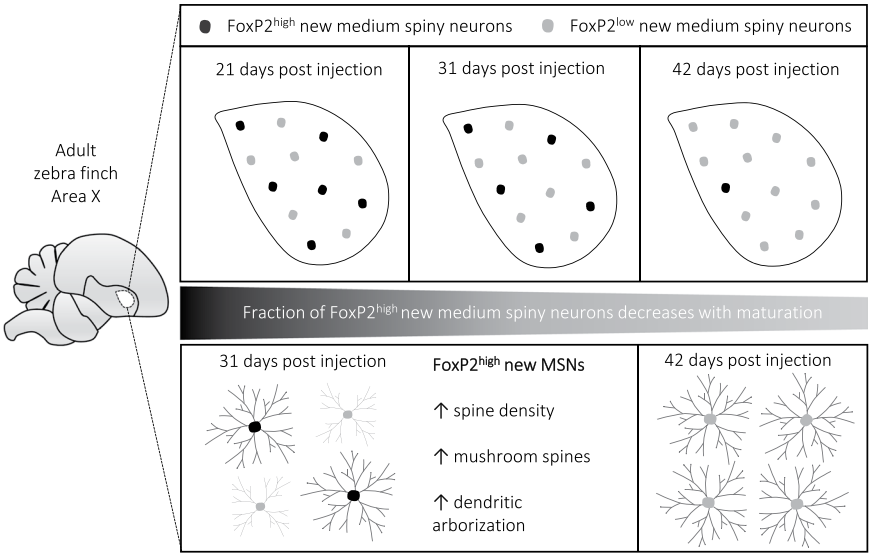

Figure 6. Graphical summary of the main results. The fraction of adult generated FoxP2^{high} MSNs from zebra finch Area X decreases with maturation. FoxP2^{high} new MSNs show higher spine densities, more mushroom spines and a more complex dendritic arborization than FoxP2^{low} new MSNs.

in juvenile zebra finches, since experimental FoxP2 knockdown decreased overall spine densities of new Area X MSNs[48]. Moreover, our results are in line with findings in mice that link Foxp2 to neuronal outgrowth and spine density in striatal neurons and their progenitors[18,19,35–37]. Additionally, we find similarities between mice and birds on the level of spine types. We show that FoxP2^{low} new MSNs 31 dpi have fewer mushroom spines than FoxP2^{high} new MSNs. In mice, striatal spiny neurons of heterozygous Foxp2 knockdown mice show specifically a decrease of mushroom and branched spines whereas stubby and thin spines are not affected[19]. In birds and mammals, dendritic spines of striatal MSNs receive both glutamatergic dopaminergic input from cortical/pallial regions and the midbrain, respectively. In the case of new MSNs in Area X, we hypothesize that high FoxP2 expression levels during their maturation might increase their capacity for receiving both inputs.

What mechanisms might account for morphological differences between FoxP2^{low} and FoxP2^{high} MSNs? They may originate from differential target gene activation in FoxP2^{low} and FoxP2^{high} MSNs. FoxP2 has hundreds of downstream target genes of which many are part of networks associated with neurite development[35,49–52]. One specific candidate is the myocyte enhancer factor 2C (Mef2c), a negative regulator of synaptogenesis[19]. Foxp2 specifically promotes corticostriatal synaptogenesis via the repression of Mef2c. Whether a similar mechanism shapes the integration of new MSNs into the avian corticostriatal network remains to be elucidated by future studies. In zebra finch Area X, two direct FoxP2 targets are associated with neuronal outgrowth; the very-low-density-lipoprotein receptor (VLDLR) and the contactin-associated protein-like 2 (CNTNAP2). Their expression correlates positively with FoxP2 in juveniles and in singing adults[53,54]. Thus, in new Area X neurons, VLDLR and CNTNAP2 would be expected to be highly activated during singing in FoxP2^{high} MSN but not in FoxP2^{low} MSNs and may thus generate the diverging MSNs morphology we found.

We would like to propose some speculations regarding possible functions of two MSNs subpopulations that differ in FoxP2 expression levels. We found that these populations differed in nucleus size, dendritic complexity and spine density during an early time period of their integration into Area X. We do not know if the observed morphological differences persist long-term because of a lack of markers that could distinguish former FoxP2^{high} MSNs from former FoxP2^{low} MSNs in later maturation phases. If these two subpopulations persist long-term, they might resemble striato-nigral and striato-pallidal MSNs of the direct and indirect pathway in mammals. These MSNs subtypes are morphologically and neurochemically different. Direct pathway MSNs express the dopamine receptor D1 and their dendrites are more complex than indirect pathway MSNs that express the dopamine receptor D2[39,55]. High Foxp2 levels in D1 MSNs and low Foxp2 in D2 MSNs have been proposed to be linked to this anatomical dichotomy[35]. The avian direct and indirect pathway through the basal ganglia however is not characterized by different MSN projections but rather by direct and indirect pallidal-like output neurons that project from Area X to the thalamus[56]. To date, it is not known if MSN subtypes exclusively synapse on either direct or indirect pallidal-like neurons[57]. Contrary to mammalian MSNs, more than half of the Area X MSNs express multiple dopamine receptors[58] so that they cannot be used as markers for indirect versus direct pathway neurons. Investigating potential avian MSNs subtypes and the developmental role FoxP2 plays in those will be of interest for future studies.

What might be the function of new MSNs in Area X and how is it affected by FoxP2 expression levels? Our previous study showed that once matured, new MSNs have similar characteristics as older, resident MSNs and are active during singing[34]. General MSNs function is feed forward inhibition within a cortico-striatal-thalamo-cortical loop during singing[59]. We hypothesize that new MSNs in adult zebra finches are entrained to produce a correct firing pattern in a plastic phase during their maturation and thus may counteract

song drift, as has been suggested before[33,60]. This process might be in influenced by varying FoxP2 expression levels and the resulting morphological differences between putative subpopulations of new MSNs. FoxP2^{high} new neurons with a higher dendrite complexity and more dendritic spines might be more receptive to tuning than FoxP2^{low} new neurons. For further interpretations of our findings it will be crucial to gain additional knowledge on the microcircuitry of Area X and on the role neurons play for its function.

In summary, FoxP2 expression levels vary in adult-born MSN at different maturation times after they have been recruited to Area X. We show that the different FoxP2 expression levels correlate with neuronal morphology and spine density. Varying FoxP2 expression levels during a specific time window might permit different target gene activation important for correct incorporation and function of new MSNs in Area X.

Methods

FoxP2 nomenclature. We follow the nomenclature proposed by[61], *FOXP* refers to the human gene, *Foxp* refers to the mouse gene and *FoxP* refers to all other species. FOXP, Foxp2 and FoxP2 correspond to the protein product.

Animals. 42 adult male zebra finches (*Taeniopygia guttata*, age >120 days) were used in the present study, bred and housed at the Department of Animal Behaviour at Freie Universität Berlin. The colony was kept under a 12:12 h light:dark-cycle with food and water ad libitum. All experiments were reviewed and approved by the veterinary department of the Freie Universität Berlin and by the ethics committee of the Regional Office for Health and Social Affairs Berlin and were performed in accordance with relevant guidelines and regulations. The permit numbers are G0116/13 and G0296/15.

Experiments. We conduced three experiments. In the first, we analyzed FoxP2 expression levels of new neurons (BrdU+, see below) in Area X at three time points, e.g. at 21 days (5 birds, 166 neurons), 31 days (6 birds, 295 neurons) or 42 days (6 birds, 272 neurons) after BrdU injections (dpi). In the second experiment we analyzed FoxP2 expression levels and the expression of the early growth response protein 1 (EGR-1) at 21 dpi (6 birds, 127 neurons), 31 dpi (6 birds, 108 neurons) and at 42 dpi (6 birds 156 neurons). In the third experiment we analyzed FoxP2 expression levels, dendrite morphology and spine density of new neurons that were labelled via lentiviral infection. In total we analyzed 52 neurons of 4 zebra finches (13 ± 3 neurons/bird, mean $\pm$ SD) at 31 dpi and 23 neurons of 3 zebra finches at 42 dpi (7.6 ± 0.5 neurons/bird, mean $\pm$ SD).

BrdU injections. For the analysis of FoxP2 levels and EGR-1 expression in newborn neurons 35 birds received BrdU (50 µg/g) via intramuscular injections in the mornings for 5 consecutive days. Birds were assigned to three groups with different survival times (21, 31, and 42 days after BrdU injection, dpi).

Song monitoring. For FoxP2 expression level analysis after BrdU treatment or lentiviral injections, 17 birds were isolated in sound attenuated chambers for one night before sacrifice. In the following morning, birds were kept from singing by the experimenter sitting nearby for 1.5 h after lights went on. For EGR-1 expression analysis in new neurons after singing, 18 birds were kept in sound attenuated chambers for three nights and were perfused in the morning of the 4th day 1.5 h after the lights went on. During those 1.5 h birds had to sing at least 150 motifs to be included in the subsequent analysis of EGR-1 expression. Vocalizations were continuously monitored via Sound Analysis Pro[62].

Lentiviral Vector injection. To label progenitor cells at the lateral wall of the ventricle, the lentiviral expression vector pFUGW[63] containing a GFP reporter gene was generated as described in Lois *et al.*[63] and stereotactically injected into the ventricular zone of 7 birds under isoflurane anesthesia. Titers ranged from 2×10^6 and 3×10^7 viral particles/µl. Birds were fixed in a stereotaxic head holder, with the beak in a 45° angle from the vertical axis. In each hemisphere, approximately 200 µl of virus containing solution were injected into four sites. Following coordinates relative to the bifurcation of the midsagittal sinus were used: anterior-posterior 3.8–4.1, medial-latera −1.3/+1.3, dorsal-ventral −5.0, injection angle: 10° lateral.

Immunohistochemistry. For immunohistochemical staining birds were overdosed with isoflurane and immediately perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected, post-fixed in 4% PFA for one night and washed for another night in PBS. Brains were cut sagitally or frontally into 50 µm sections using a vibrating microtome (VT1000S, Leica). BrdU antigen retrieval required incubation in 2N HCl for 30 min at 37 °C and neutralization with borate buffer. GFP signal was enhanced via antibody staining. All immunostainings were performed according to standard protocols. The following primary antibodies were used: anti FoxP2 (goat, Abcam ab1307, dilution:1:1000), anti EGR-1 (rabbit, Santa Cruz sc-189, dilution:1:600), anti BrdU (rat, Bio-Rad MCA2060, dilution: 1:200), anti GFP (rabbit, Abcam ab290, dilution:1:1000). Fluorescent secondary antibodies were the following: anti-rat-Alexa-Fluor-488 (Life Technologies, A21208, dilution: 1:200), anti-rabbit-Alexa-Fluor-568 (Life Technologies, A10042, dilution: 1:200), anti-goat-Alaxa-Fluor-647 (Life Technologies, A21447, dilution: 1:200). To visualize nuclei, all sections were counterstained with 4′, 6-Diamidin-2-phenylindol (DAPI, Serva).

Confocal imaging and image processing and quantification. Z-Stacks of BrdU+ or GFP+ cells in Area X were obtained with a SP8 confocal microscope (Leica). For FoxP2 scanning, all microscope settings were kept constant. Scans of BrdU+ nuclei were performed using a 63x lens (digital zoom 2.0), an image size of 1024×1024 pixels and a z-stack size of 1 µm. Whole neurons (GFP+) were imaged using a 63x lens, an image size of 2042×2042 pixel and a z-stack size of 1 µm. Acquired images were processed using the Fiji software package[64]. Only neurons with spiny long dendrites were included in the analysis. The Rolling Ball Background Subtraction

plugin was used to subtract background. We measured the mean pixel intensity of nuclear FoxP2 expression, by positioning a circle of 4 µm in diameter (12.56 µm^2) in the center of the BrdU+ nucleus. In total we analyzed the intensity of the FoxP2 expression dependent fluorescence of 166 BrdU+ cells at 21 dpi (n = 5), 295 BrdU+ cells at 31 dpi (n = 6) and 272 BrdU+ cells at 42 dpi (n = 6). FoxP2^{high} were defined as cells that fell into the top 30% of measured mean pixel intensities in one animal (i.e. if the highest mean pixel intensity in one animal was 240 we counted all BrdU+ cells that had a FoxP2 mean pixel intensity between 168 and 240 as FoxP2^{high} neurons). We decided on the 30% value because it covered the FoxP2^{high} expressing cells in the bimodal distribution of all FoxP2 intensities. We defined the neurons that fell into the bottom 30% of measured mean pixel intensities as FoxP2^{low}. As for the FoxP2^{high} cutoff, the bottom 30% contained the low-intensity peak of the bimodal density distribution. Because we particularly wanted to address the effect of high and low FoxP2 expression levels on neuronal properties, neurons with intermediate FoxP2 expression levels were not considered for further analysis. The Simple Neurite Tracer plugin (Fiji) was used to trace individual neurons and we analyzed their total branch length and number of primary dendrites. The traces were then used by the Sholl analysis plugin in Fiji[65]. We measured intersections of dendrites with concentric circles that were placed every 10 µm starting from the center of the soma. The maximal number of intersections per neuron was extracted from the Sholl analysis dataset. For dendritic spine analysis images were deconvolved using the Tikhonov-Miller algorithm in the DeconvolutionLab plugin in Fiji[66]. Prior to deconvolution an individual point spread function was generated for each image using the Born and Wolf 3D optical model in the PSF Generator plugin in Fiji[67]. Semiautomated dendritic spine counts were performed using the software NeuronStudio[68] that uses a spine classification algorithm. For spine classification, the default settings were used to classify spines as mushroom, stubby or thin spines: a head-to-neck ratio threshold of 1.1 µm, a height-to-width ratio threshold of 2.5 µm and a minimum mushroom head size of 0.35 µm. A spine is considered mushroom if the head-to-neck ratio is above the threshold and its head is larger than 0.35 µm. A spine is considered stubby if its head-to-neck ratio and its heights-to-width ratio are below threshold. In all other cases spines were classified as thin. On average, we analyzed spines densities on secondary dendrites along the length of 118 µm ± 1.92 (mean, SEM) per neuron. In total, we analyzed spines of 52 individual neurons of 4 animals in experimental group 31 dpi, and 23 neurons of 3 animals at 42 dpi. Additionally, we measured the dendrite diameter of 44 new neurons in 4 animals (8–12 neurons per animal) using the line measuring tool in Fiji[64]. We took 5 measures on each of 3 secondary dendrites per neuron (in total 15 measures per neuron). The experimenter was blind to FoxP2 levels of individual neurons during the whole quantification process, because cells were selected for quantification based on their BrdU+ fluorescence or their EGR-1 fluorescence and FoxP2 fluorescence in a different channel was quantified last. The datasets generated and analysed during the current study are available from the corresponding author on request.

Statistics. The software R was used to analyze data[69]. Significance level was $p < 0.05$ for all tests. Plots were generated using the ggplot package in R[70]. For the analysis of FoxP2^{high} neurons we used a Kruskal-Wallis test followed by a Dunn's test for pairwise comparison. For the analysis of FoxP2^{low} neurons we used ANOVA followed by a Bonferroni's multiple comparison test. The relationship of (a) FoxP2 levels and nuclear diameter as well as (b) FoxP2+ neurons and singing were determined using a linear regression analysis. Dendritic spine data (all spines) and Sholl data were analyzed using the paired Student's t-test. Data from the spine type analysis, mushroom head size, total dendrite length, number of primary dendrites and dendrite thickness were analyzed using the Mann Whitney test. Number of maximal intersections was analyzed using the Student's t-test. Choice of test was based on previous analysis for normality using the Shapiro-Wilk-test and variance analysis using the F-test or Levene's test.

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General Discussion

Songbirds offer a model for studying functional adult striatal neurogenesis and its role for the maintenance of a learned behavior

In this thesis, I present evidence for functional adult neurogenesis in the striatal song nucleus Area X, which is implicated in song learning as well as in the long-term maintenance of song (Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Ali et al., 2013; Kubikova et al., 2014; Kojima et al., 2018). In Publication A, I applied BrdU birth dating of new neurons and immuno-histochemically analyzed their maturation course and singing induced activity in Area X after varying survival times. Further, I virally labelled progenitor cells in the neurogenic niche adjacent to the lateral ventricle and imaged the descending new medium spiny neurons (MSNs) in Area X. To elucidate the connectivity of new MSNs, I retrogradely traced their postsynaptic target cells, labelled presynaptic contacts from pallial nuclei and detected indicators of dopaminergic signaling. The results presented in **Publication A** provide evidence that new MSNs in Area X mature within a time frame of six weeks and are robustly active during singing. Their connectivity within the local circuitry resembles the one of older, resident MSNs. By analyzing MSNs densities in zebra finches up to seven years of age, I found that adult neurogenesis is a process of constant addition, most likely increasing inhibitory signaling within Area X. Altogether, the data presented in Publication A filled several knowledge gaps about the processes of striatal adult neurogenesis and illustrate that adult born neurons in Area X fulfill many essential prerequisites for a functional role in adult song maintenance.

Because adult neurogenesis in HVC has been intensively studied I will outline similarities and differences between adult neurogenesis in HVC and Area X. One essential similarity is that in both nuclei only one cell type is subject to adult neurogenesis; in HVC only HVC_{RA} but not HVC_X projection neurons and in Area X only MSNs but not pallidal-like neurons are recruited (Alvarez-Buylla et al., 1988a; Nordeen and Nordeen, 1988b; Rochefort et al., 2007; Scotto-Lomassese et al., 2007). This indicates that the parts of the ventricular zone (VZ) that give rise to pallial and striatal cells are either determined to produce a specific neuron type like in the mammalian subventricular zone (Kelsch et al., 2007; Merkle et al., 2007; Merkle et al., 2014) or that local cues in HVC and Area X only allow differentiation into one neuron type. Since HVC_{RA} neurons are mainly born post hatch, while most of HVC_X neurons are generated *in ovo* (Alvarez-Buylla et al., 1988a), it is conceivable that adult neurogenesis represents a prolongation of juvenile neurogenesis. The fact that adult neurogenesis in HVC decreases with age supports this notion (Wang et al., 2002; Wilbrecht et al., 2002; Pytte et al.,

2007). Striatal neurons are born during early embryonic development and after hatching. Compared to other telencephalic regions, the striatum receives the highest number of new neurons after hatching (Alvarez-Buylla et al., 1994) and Area X forms about 8-10 days post hatch (Garcia-Calero and Scharff, 2013). Assuming that adult neurogenesis in Area X is an extension of juvenile neurogenesis, I would expect that its rate decreased with age, like in HVC. Interestingly, this is not the case and rates of Area X neurogenesis remain constant in adults (Pytte et al., 2007).

Another similarity is that both new neuron types connect to their targets. New HCV_{RA} neurons connect to their target RA, e.g. are retrogradely labelled, by 21 days after they originated in the pallial VZ (Kirn et al., 1999; Tokarev et al., 2015). **Publication A** shows that new MSNs in Area X locally contacted pallidal-like neurons as early as 31 days after their generation in the striatal ventricular zone. New MSNs migrate at least 1000 µm from the VZ to Area X whereas new HVC neurons only migrate approximately less than 100 µm. Taking the longer migration time of new MSNs from the VZ to Area X into account it is plausible that the time between birth dating and connection to target neurons is very similar in new HVC neurons and new MSNs.

Both new HVC and Area X neurons are activated by singing, as shown by the induction of immediate early gene transcription (Tokarev et al., 2015; Kosubek-Langer et al., 2017). In **Publication A**, I analyzed the percentage of new MSNs that expressed the immediate early gene (IEG) EGR-1 after undirected singing . A methodologically similar study investigated IEG expression in new HVC_{RA} neurons after directed or undirected singing (Tokarev et al., 2015). Comparing only the fraction of neurons that were new (BrdU+) and active during singing (IEG+), it becomes clear that their proportion increased significantly with age. After 6 or 8 weeks, between 70-80% of new neurons were activated by singing, both in Area X and HVC respectively (Kosubek-Langer et al., 2017; Tokarev et al., 2015). In HVC, a stable fraction of new neurons was IEG+ without being retrogradely filled from RA, e.g. are probably not connected (Tokarev et al., 2015). In new MSNs it is not clear if singing related activity depends on coincident postsynaptic connections. Furthermore, the variability of how many new neurons are active during singing decreases with survival time in both nuclei, i.e. the older the new neurons become the more robust is their activation by singing across animals. EGR-1 expression is crucial for the survival of new granule neurons in the murine dentate gyrus of the hippocampal formation (Veyrac et al., 2013). Further, EGR-1 induces the expression of DARPP-32, a marker for mature MSNs, in primary striatal neurons (Keilani et al., 2012). Taken

together, singing-induced IEG expression most likely promotes both the maturation and survival in new HVC and Area X neurons.

Another feature common between adult neurogenesis in HVC and Area X is that it is a process of addition rather than of replacement. In both song nuclei the density of the added neuron type increases with age and their total neuron number doubles within 11 or 5 years in HVC and Area X, respectively (Walton et al., 2012; Kosubek-Langer et al., 2017). In contrast, in seasonal breeding songbirds like canaries, new HVC neurons replace neurons that have died after the preceding breeding season (Kirn et al., 1994; Thompson and Brenowitz, 2009). When neurons are constantly added, as is the case in zebra finches, the anterior forebrain pathway faces two challenges. First, all new MSNs have to be innervated by an unchanging number of HVC_X and LMAN neurons. Second, the pallidal-like neurons receive increasing inhibitory input from an increasing number of MSNs. The same would be true for neurons in VTA/SNc that sent dopaminergic innervation to Area X. Further experiments need to elucidate the concrete timeline of all innervations on new MSNs and how pre- and postsynaptic neurons adapt to increasing neuron numbers in Area X.

Many questions about the process of adult neurogenesis remain unanswered. What are the cues that stop migration of new neurons and how do they become entrained to their specific firing pattern, especially during singing? Adult-generated granule cells (GCs) in the mammalian hippocampus receive input from mature GCs during a very restricted early time window of their maturation. As their inputs from the entorhinal cortex become stronger, the local inputs from mature GCs decrease (Vivar et al., 2012). If a similar scenario applied to new MSNs in Area X they would be first entrained by older MSNs, before they would receive pallial inputs from HVC and LMAN. Some evidence supports the hypothesis that new neurons are entrained by local mature neurons the songbird brain. First, HVC neurons are often found in clusters with direct soma-soma contact and new neurons can be part of these clusters (Burd and Nottebohm, 1985). Second, new post migratory HVC neurons are found in closer proximity than still migratory neurons (Scott et al., 2012). The notion that mature and new neurons form clusters opens the possibility that they might use gap junctions for electrical coupling (Gahr and Garcia-Segura, 1996; Alvarez-Buylla and Kirn, 1997). If neuronal clusters indeed are involved in the maturation of new HVC and Area X neurons awaits further studies. In Area X, dopaminergic signaling from VTA encodes performance errors (i.e. the matching of prediction and actual song performance, Gadagkar et al., 2016; Kearney et al., 2019) and may be another mechanism involved in the entrainment of new MSNs. Since new MSNs express Drd1A, Drd1B

and Drd2 dopamine receptors (Kosubek-Langer et al., 2017), their entrainment by VTA dopaminergic signals is likely.

In the following section, the possible function of adult neurogenesis in Area X and HVC will be discussed. Two lines of evidence suggest that in zebra finches new HVC_{RA} neurons are involved in adult song stereotypy (Pytte, 2016). First, specific ablation of HVC_{RA} neurons impairs song structure and the addition of new HVC_{RA} neurons is associated with the recovery of the stereotyped song (Scharff et al., 2000). Second, the recovery of distorted song after paralysis of syringeal muscles or deafening correlates with the numbers of new neurons in HVC (Pytte et al., 2011; Pytte et al., 2012). In Area X, I propose that adult neurogenesis also serves to keep adult song from drifting and that it has two temporally segregated effects, an immediate effect and a long-term effect. New neurons might provide a tool to counterbalance slight changes in song features originating from physiological changes. Adult generated granule cells in the dentate gyrus of mice undergo a sensitive phase between 1 and 1.5 months of their maturation in which they exhibit enhanced synaptic plasticity that may contribute to behavioral driven plasticity (Ge et al., 2007). Accordingly, each new MSN might have a sensitive time window during maturation, in which it can be entrained to the song template, correct subtle song changes and ultimately prevent song drift. **Publication A** shows that very young and probably not fully matured new MSNs were active during singing and singing was shown to enhance new neuron survival (Li et al., 2000). Therefore, it is conceivable that singing plays a dual role for new neurons: (a) triggering the production of neurotrophins that secure their survival and (b) entrainment to firing patterns during singing. Whether singing is relevant during a sensitive period or if it affects the (electro-) physiological properties of new neuron maturation and whether gap junctions between young and old MSNs are involved in this process awaits further studies.

A long-term consequence of adult striatal neurogenesis would be song stabilization by progressive “silencing” of the anterior forebrain pathway (AFP). In this scenario, the addition of new MSNs would increase inhibition onto pallidal-like neurons within Area X and ultimately lead to fewer excitatory outputs to the motor pathway. Two lines of evidence support this hypothesis. First, specific loss of MSNs in an avian model for Huntington’s Disease leads to decreased inhibition on pallidal-like neurons in Area X and abnormal firing in downstream LMAN (Tanaka et al., 2016), indicating that the total number of MSNs in Area X does matter for proper signaling in downstream nuclei of the AFP. Second, studies that tested the effect of lacking auditory feedback on song production provide evidence that feedback from AFP to the motor pathway decreases with age. Song deteriorates after the ablation of auditory feedback by

deafening, but this process can be prevented by lesions within the AFP (Brainard and Doupe, 2000; Kojima et al., 2013). In old adults, deafening has only moderate effects on song compared to young adults, indicating that the AFP of old birds is less sensitive to the ablation of auditory feedback, probably to a large extent because of memory consolidation (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). But constant addition of new inhibitory neurons within Area X might serve as an additive factor that decreases AFP feedback onto the motor pathway with age. Increased feed forward inhibition is also one proposed function of new granule neurons within the hippocampal dentate gyrus-CA3 circuit (Miller and Sahay, 2019). If the prediction about the immediate (variability) and long-term (stability) effect of adult neurogenesis in Area X holds true, can be tested in further experiments.

Beyond its putative role in maintaining birdsong, why is studying striatal adult neurogenesis in birds of interest? First, striatal MSNs are implicated in motor related neurodegenerative disorders like Huntington's and Parkinson's disease (Reiner et al., 2011; Nelson and Kreitzer, 2014). Two studies already addressed the effect of the mutated huntingtin protein on avian basal ganglia function, either in transgenic zebra finches that express the human mutant huntingtin protein (mHTT, Liu et al., 2015) or by expressing the mutated huntingtin gene fragment in Area X of adult zebra finches using local virus injections (Tanaka et al., 2016). These studies found aberrant song development in juveniles, sequence instability in adults and loss of MSNs in Area X concomitant with decreased inhibition of pallidal-like neurons. In mHTT transgenic zebra finches the song degradation subsided and stabilized after 9-15 months of age and this was proposed to be linked with the replacement of dead MSNs with new MSNs (Liu et al., 2015). Since Area X recovers extensively within six months after neurotoxic lesions and incorporate new, functional MSNs (Kubikova et al., 2014; Lukacova et al., 2017), recovery from MSNs loss in mHTT birds seems possible. The second reason why studying striatal adult neurogenesis in songbirds is interesting is that the natural generation of new striatal MSNs does not occur in adult mammals. It can be experimentally induced by stroke, ischemia or lesions, but the survival rates are low (Arvidsson et al., 2002; Parent et al., 2002; Tattersfield et al., 2004; Yamashita et al., 2006). Only GABAergic interneurons are generated under natural conditions and after brain damage in the striatum of adult rats, rabbits, monkeys and humans (Bédard et al., 2002; Tonchev et al., 2003; Dayer et al., 2005; Tonchev et al., 2005; Luzzati et al., 2006; Yang et al., 2008; Liu et al., 2009; Wei et al., 2011; Ernst et al., 2014). In rodents, two other prominent neurogenic niches persist during adulthood; the subventricular zone that gives rise to granule interneurons that incorporate into the olfactory bulb (Lledo and Valley, 2016) and the subgranular zone of the dentate gyrus (DG) of the

hippocampal formation (Altman and Das, 1965a; Kempermann et al., 2015). In the latter, new granule cells (GCs) are generated from radial glia cells and incorporate into the granule cell layer of the DG, where they become fully mature GCs within 4-8 weeks (Esposito et al., 2005). They are embedded in the hippocampal-entorhinal formation that plays a major role in the formation of episodic memories. New GC function in adult rodents is implicated in context discrimination, memory consolidation, forgetting, pattern separation and feed-forward inhibition in DG-CA3 (Miller and Sahay, 2019). Their recruitment and survival are positively influenced by exercise and environmental enrichment (Vivar et al., 2013; Kempermann, 2019). If neurogenesis in the hippocampus persists in the adult human brain is currently highly debated, with differences in human tissue fixation protocols being the main contentious issue (Boldrini et al., 2018; Cipriani et al., 2018; Kempermann et al., 2018; Paredes et al., 2018; Sorrells et al., 2018; Moreno-Jimenez et al., 2019; Tobin et al., 2019). Taken together, songbirds provide a unique model for studying the natural generation of a neuron type that is highly relevant for movement, learning and cognition and that is not renewed in mammals.

FoxP2 function is extended by an implication in adult neurogenesis within a pallial-basal ganglia-thalamo-pallial circuit

How do levels of a transcription factor in individual neurons influence their morphology and function? With my thesis work I tried to answer that question by studying the protein levels of FoxP2 in new MSNs in Area X. The motivation for this study came from a report that MSNs in Area X expressed FoxP2 at either high and low levels ($FoxP2^{high}$ or $FoxP2^{low}$ MSNs) and that the distribution of expression intensities was age dependent with $FoxP2^{high}$ neurons decreasing as zebra finches aged (Thompson et al., 2013). From the observation that singing only decreases the density of $FoxP2^{low}$ and not $FoxP2^{high}$ neurons (Thompson et al., 2013), I hypothesized that FoxP2 levels in young neurons need to decrease before they participate in singing activity. **Publication B** of this thesis indeed illustrates that FoxP2 expression levels decrease with age; 30-40 % of new MSNs in Area X expressed FoxP2 at high levels during early maturation stages (21 and 31 days after BrdU labelling) and this fraction of $FoxP2^{high}$ MSNs decreased with maturation (Kosubek-Langer and Scharff, 2020). At a later maturation stage (42 days after BrdU labelling) only approximately 10% of new MSNs were $FoxP2^{high}$. Contrary to my hypothesis, new MSNs participated in singing activity (measured by EGR-1 expression) independent of their FoxP2 expression level. **In Publication B**, I measured the FoxP2 expression levels of individual GFP expressing new MSNs and analyzed their dendrite morphology and spine density. Interestingly, FoxP2 expression levels in individual new MSNs

correlated with both. FoxP2^{high} new MSNs had more elaborate dendrites and a higher density of dendritic spines than FoxP2^{low} new MSNs. In detail, the mature mushroom spines were more numerous in FoxP2^{high} than in FoxP2^{low} new MSNs, indicating that FoxP2^{high} new MSNs have built stronger connections with their presynaptic inputs. MSNs dendrites form synaptic contacts with axons from HVC and LMAN (Kornfeld et al., 2020). The former guides the temporal features of song (Long and Fee, 2008), whereas the latter generates variability during singing (Kao et al., 2005; Ölveczky et al., 2005; Kao and Brainard, 2006; Kao et al., 2008; Ölveczky et al., 2011). The majority of HVC axons synapse onto MSN dendritic spines whereas LMAN axons preferentially terminate on MSN dendritic shafts (Kornfeld et al., 2020). Moreover, HVC synapses onto MSN spines are larger than those of LMAN (Kornfeld et al., 2020). Given that FoxP2^{high} new neurons had more of the large mushroom spines than FoxP2^{low} new neurons, I speculate that they receive more inputs form HVC and become tightly entrained to song timing. If FoxP2^{low} new neurons, on the other hand, receive more LMAN synapses on dendritic shafts and thus a higher proportion of variable inputs awaits further investigation. Nevertheless, the differences in dendritic spine densities in FoxP2^{high} and FoxP2^{low} new MSNs during a narrow time window of their maturation might reflect differential innervation by upstream song nuclei with opposing functions.

The results presented in **Publication B** are consistent with previous studies that linked Foxp2/FoxP2 expression with neuronal outgrowth and spine density (Enard et al., 2009; Schulz et al., 2010; Reimers-Kipping et al., 2011; Vernes et al., 2011). Many studies describe the role of Foxp2 during developmental neurogenesis (Rousso et al., 2012; Tsui et al., 2013; Chiu et al., 2014; Co et al., 2019; Kast et al., 2019), but **Publication B** is the first report that links FoxP2 expression to the process of adult neurogenesis. This expands FoxP2 function by neuronal outgrowth of newly recruited striatal MSNs.

Varying Foxp2 levels have been previously detected. On a cellular level, Foxp2 expression is enriched in Drd1 MSNs of the direct pathway, whereas only few Drd2 MSNs of the indirect pathway express Foxp2 (Vernes et al., 2011; van Rhijn et al., 2018). These immunohistochemical results are supported by single cell RNA sequencing of the adult murine striatum showing that Foxp2 in only enriched in Drd1 MSNs (Stanley et al., 2019). But Foxp2 expression levels are also compartmentalized; Foxp2^{high} MSNs are more numerous in the striosome than in the matrix compartment (Takahashi et al., 2003; Takahashi et al., 2008; Chen et al., 2016; Schreiweis et al., 2019). The striosome forms a three-dimensional labyrinth-like structure that permeates the matrix and comprises 10-15% of the striatum. Striosome and matrix are neurochemically different but both harbor MSNs of the direct and indirect pathway

(Brimblecombe and Cragg, 2017). Dopamine levels are higher in the matrix than in the striosomes (Salinas et al., 2016). In genetically engineered mice that carry humanized Foxp2 alleles ($Foxp2^{hum/hum}$) the density of $Foxp2^{high}$ MSNs increased in the striosome of the dorsal striatum in comparison to wildtype littermates. In the ventral striatum, however, the density of $Foxp2^{high}$ MSNs increased in the matrix, not in the striosome. It is not clear which (developmental) processes lead to the increase in $Foxp2^{high}$ MSNs, nor in which MSNs subtype Foxp2 expression is elevated in $Foxp2^{hum/hum}$ mice. Further, it needs to be determined how increased Foxp2 levels relate to the increased dendrite length found in striatal MSNs and other neurons of the striatal-basal ganglia circuit of $Foxp2^{hum/hum}$ mice (Enard et al., 2009; Reimers-Kipping et al., 2011).

In **Publication B**, I present data that FoxP2 expression levels distinguish fractions of new MSNs in Area X during a short, specific time window of their maturation. Older, mature MSNs cannot be distinguished by their FoxP2 expression levels. I argue that these early differences in FoxP2 expression and concomitant morphological features may imprint young, new MSNs and give rise to different MSNs subtypes, as is the case in mice. In mice, Drd1 direct and Drd2 indirect pathway striatal MSNs (see Fig. 3 of the Introduction) cannot only be segregated by the expression of dopamine receptors but by many other characteristics. Dopamine modulates the response of MSNs to cortical inputs; Drd1s increase the intrinsic excitability of MSNs, whereas Drd2s decrease the intrinsic excitability of MSNs (Surmeier et al., 2014). Direct pathway activation promotes movement, while indirect pathway activation suppresses motor behaviors (Kravitz et al., 2010). Further, direct and indirect MSNs differ in electrophysiological properties (Cepeda et al., 2008; Day et al., 2008), morphology (Gertler et al., 2008), synaptic plasticity (Kreitzer and Malenka, 2007; Shen et al., 2008), cortical inputs (Wall et al., 2013) and brain-wide responses (Lee et al., 2016).

There is nothing known about the connectivity or electrophysiology of MSNs subtypes in birds, although their existence has been presumed (Gale and Perkel, 2010; Pidoux et al., 2015). But gene expression data provides hints for the existence of MSNs subtypes. Single nucleus RNA sequencing identified five MSN cluster, of which two resembled the gene expression profile of direct and indirect MSNs of the murine striatum (Xiao et al., 2020). However, mRNA and protein abundance do not always correlate (Vogel and Marcotte, 2012) and RNA profiles reflect one snapshot of a dynamic system, hence RNA profiling alone is insufficient to determine neuronal subtypes. Corresponding to the murine basal ganglia, avian indirect MSNs would exclusively innervate indirect pallidal-like neurons that inhibit the direct pallidal-projection neurons. Following evidence underpin the hypothesis that early FoxP2

expression levels may indicate MSN subtype specification. First, mouse Drd1 direct MSNs and corresponding direct MSNs in zebra finches both express FoxP2 at high levels whereas Drd2 indirect MSNs in mammals and birds express only low levels of Foxp2 (Vernes et al., 2011; van Rhijn et al., 2018; Xiao et al., 2020). Second, both FoxP2^{high} new MSNs in zebra finches and Drd1 MSNs in mice had (a) more intersections in the Sholl analysis, which indicates a higher dendritic complexity (b) a higher cumulative dendritic length, and (c) more primary dendrites compared to FoxP2^{low} new MSNs and Drd2 MSNs, respectively (Gertler et al., 2008; Kosubek-Langer and Scharff, 2020). To test if this hypothesis holds true it requires suitable markers, tracings and electrophysiological recordings. Unfortunately, a single avian MSNs often expresses multiple dopamine receptors (Kubikova et al., 2010; Kosubek-Langer et al., 2017; Xiao et al., 2020) and therefore MSNs in songbirds cannot be distinguished by their dopamine receptors, which is possible in rodent MSNs. It is however possible that a combination of several marker proteins will reveal MSN subtypes also in birds. Another challenge in the detection of MSN subtypes is the fact that their targets, the direct and indirect pallidal-like projection neurons, lie within Area X and this complicates their detection via retrograde tracings. But since MSN subtypes may have different roles during song learning as well as in the modulation of variability during directed and undirected singing, their detection and how FoxP2 expression is involved in their subtype specification is of particular interest.

In summary, proper amounts of Foxp2 protein are crucial for a wide array of neurobiological processes including brain development and motor learning in mammals (French et al., 2007; Fujita et al., 2008; Groszer et al., 2008; Enard et al., 2009; Reimers-Kipping et al., 2011; French et al., 2012; Kurt et al., 2012; Tsui et al., 2013; Chiu et al., 2014; Chen et al., 2016; French et al., 2019; Kast et al., 2019). In zebra finches, the tight regulation of FoxP2 protein levels is particularly important for its on-line function during song learning in juveniles and for context-dependent song variability in adults (Haesler et al., 2004; Haesler et al., 2007; Murugan et al., 2013; Heston and White, 2015; Day et al., 2019). **In Publication B**, I present the first evidence that FoxP2 protein levels are as well relevant during the process of striatal adult neurogenesis (Kosubek-Langer and Scharff, 2020), which broadens FoxP2 function in adult songbirds.

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List of Publications

Publication A (S. 20)

Kosubek-Langer, J., Schulze, L., and Scharff, C. (2017). Maturation, Behavioral Activation, and Connectivity of Adult-Born Medium Spiny Neurons in a Striatal Song Nucleus. *Frontiers in Neuroscience*
https://doi.org/10.3389/fnins.2017.00323

Publication B (S. 34)

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Zusammenfassung

Adulte Neurogenese ist ein Prozess, bei dem neue Nervenzellen in einer neurogenen Nische gebildet werden und in bestimmte Regionen des adulten Gehirns einwandern. Im Gehirn von erwachsenen Singvögeln werden neue Nervenzellen in Regionen integriert, die das Lernen, die Produktion und die Erhaltung des Gesangs steuern. Neue *medium spiny neurons* (dornenbesetzte Nervenzellen von mittlerer Größe, folgend MSNs) wandern kontinuierlich in den striatalen Basalgangliennukleus Area X ein, es war jedoch nicht bekannt, ob sie funktionell in die vorhandenen Schaltkreise integriert werden. Um diese Frage zu beantworten, habe ich Vorläuferzellen mit dem chemischen Basenanalogon Bromdeoxyuridin oder durch eine lentiviral vermittelte Expression eines fluoreszierenden Proteins markiert. Neue Nervenzellen in Area X wurden nach unterschiedlichen Zeitspannen auf ihre Reifung, Vernetzung und neuronale Aktivität während des Gesangs hin untersucht. Bereits sechs Wochen nach ihrem Entstehen sind die meisten neuen Nervenzellen in Area X ausgereift, weisen prä- und postsynaptische Verbindungen auf und exprimieren Dopaminrezeptoren, die auf dopaminerge Innervation hinweisen. Um zu beantworten ob und ab welchem Alter neue Nervenzellen durch Singen aktiviert werden, wurde die Expression des Gens EGR-1 (*early growth response 1*) genutzt, weil es schon wenige Minuten nach Beginn der neuronalen Aktivität transkribiert wird. Bereits drei Wochen nach ihrer Entstehung exprimiert ein kleiner Teil der neuen Nervenzellen EGR-1 als Folge deren Aktivierung durch Singen und mit fortschreitender Reifung nimmt der Anteil der durch Singen aktivierten neuen Nervenzellen zu. Um die Dynamik der striatalen adulten Neurogenese zu verstehen, wurde die Dichte von MSNs in Zebrafinken bis zu einem Alter von sieben Jahren untersucht. Die Ergebnisse deuten darauf hin, dass es sich bei der striatalen adulten Neurogenese um einen Prozess handelt, bei dem neuen Nervenzellen dem Nervengeflecht konstant hinzugefügt werden.

MSNs die in Area X einwandern exprimieren das Forkhead-Box-Protein P2 (FoxP2). Dieser Transkriptionsfaktor hat wichtige Funktionen bei der Entwicklung des Gehirns von Säugetieren und dessen Mutation verursacht beim Menschen eine Sprachstörung (Verbale Entwicklungsdyspraxie). Bei Zebrafinken sind korrekte Expressionsniveaus von FoxP2 in Area X entscheidend für erfolgreiches Gesangslernen und für die Gesangsmodulation in verschiedenen sozialen Kontexten. FoxP2 Expression in Area X ist während der Phase des Gesangslernens hoch, jedoch niedrig bei adulten Tieren und während des Gesangs wird die FoxP2 Expression sogar herunterreguliert. MSNs in Area X weisen unterschiedliche FoxP2 Expressionsniveaus auf. Die Herunterregulierung von FoxP2 nach dem Singen tritt nur in

MSNs mit niedrigem FoxP2 Expressionsniveau und nicht in MSNs mit hohem FoxP2 Expressionsniveau auf. Daher postulierte ich, dass MSNs mit hohem FoxP2 Expressionsniveau erst kürzlich in Area X eingewandert sind und deren FoxP2 Expression erst verringert werden muss, bevor sie durch Singen aktiviert werden können. Diese Hypothese wurde getestet, indem das FoxP2 Expressionsniveau und die EGR-1-Expression in einzelnen neuen MSNs von singenden und nicht singenden Zebrafinken zu verschiedenen Zeitpunkten nach ihrer Entstehung bestimmt wurde. Interessanterweise war die Aktivierung der neuen MSNs unabhängig von ihrem FoxP2 Expressionsniveaus. Ein weiteres Ergebnis war, dass ein Drittel der neuen MSNs in frühen Reifungsstadien FoxP2 in hohen Mengen exprimierte. Die Mehrheit der gereiften MSNs exprimierte FoxP2 jedoch in geringen Mengen, was auf eine altersbedingte Abnahme der FoxP2 Proteinmengen in einer Fraktion der neuen MSNs hinweist. Da gezeigt wurde, dass Foxp2 das Wachstum und die Differenzierung von Neuronen fördert, habe ich die Morphologie der Dendriten und die Dichte der Dornenfortsätze von neuen MSNs mit hohem oder geringem FoxP2 Expressionsniveau analysiert. Im Vergleich hatten neue MSNs mit hohem FoxP2 Expressionsniveau komplexere Dendriten und eine höhere Dichte an pilzförmigen Dornenfortsätzen. Dies deutet darauf hin, dass sie während eines kurzen Zeitraums ihrer Reifung mehr Verknüpfungen mit vorgeschalteten Gesangsnuklei ausbildeten. Ein Verglich der hier präsentieren Ergebnisse mit den MSNs der direkten und indirekten Verbindungen der Basalganglien von Nagetieren, lässt schlussfolgern, dass unterschiedliche FoxP2 Expressionsniveaus und die damit einhergehende dichotome Morphologie neuer MSNs auf die Existenz unterschiedlicher Subtypen ebendieser in Area X von Zebrafinken hindeuten. Zusammenfassend zeigen die präsentierten Daten, dass die neuen MSNs, die in Area X von erwachsenen Zebrafinken rekrutiert werden, funktionsfähig sind und für die Erhaltung des Gesangs eine wichtige Rolle spielen könnten. Innerhalb der ersten sechs Wochen nach ihrer Bildung weisen neue MSNs dynamische FoxP2 Expressionsniveaus auf. Letztere korrelieren positiv mit der Komplexität der dendritischen Verzweigungen und der Dichte der Dornenfortsätze von neuen MSNs, wodurch die Funktion von FoxP2 um eine Rolle in der striatalen adulten Neurogenese erweitert wurde.

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Eidesstattliche Erklärung

Hiermit versichere ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben. Bei der Verfassung der Dissertation wurden keine anderen als die im Text angegebenen Quellen und Hilfsmittel verwendet. Ein Promotionsverfahren wurde zu keinem früheren Zeitpunkt an einer anderen Hochschule oder bei einem anderen Fachbereich beantragt.

Berlin, den 16.04.2020

Jennifer Kosubek-Langer