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FoxP2 in songbirds

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Humans with mutations in the transcription factor FOXP2 display a severe speech disorder. Songbirds are a powerful model system to study FoxP2. Like humans, songbirds communicate *via* vocalizations that are imitatively learned during critical periods and this learning is influenced by social factors and relies on functionally lateralized neural circuits. During the past five years significant progress has been made moving from a descriptive to a more mechanistic understanding of how FoxP2 functions in songbirds. Current evidence from molecular and electrophysiological studies indicates that FoxP2 is important for shaping synaptic plasticity of specific neuron populations. One future goal will be to identify the transcriptional regulation orchestrated by FoxP2 and its associated molecular network that brings about these physiological effects. This will be key to further unravel how FoxP2 influences synaptic function and thereby contributes to auditory guided vocal motor behavior in the songbird model.

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Introduction

Forkhead box proteins (Fox) belong to a large family of winged helix transcription factors that bind to regulatory regions of target genes and have essential functions in development and disease [1]. Two members of the *FOXP* family, *FOXP1* and *FOXP2*,[1] have received particular attention for their role in speech and language. Patients carrying FOXP2 mutations causing haploinsufficiency have difficulty mastering complex sequences of mouth movements underlying speech (developmental verbal dyspraxia, DVD), and have impaired expressive and receptive language, whereas other aspects of cognition and development are relatively spared [2,3,74]. *FOXP1* mutations are also associated with language skills [1,4].

Because song learning in birds and speech learning in humans bear many parallels, songbirds emerged as a powerful model system to study the function of *FoxP2*, its associated molecular network and its relation to vocal learning. Like language, song of many birds is learned by imitation of adult conspecifics. Learned song is subserved by a discrete and anatomically well-characterized neural circuit. This circuit consists of two pathways: the descending motor pathway and the anterior-forebrain pathway, which contains the pallial song nucleus LMAN and the striatal song nucleus Area X and resembles the mammalian cortico-basal ganglia-thalamo-cortical loop [5]. These forebrain structures are exclusive to avian vocal learners and absent in birds that do not learn their vocalizations by imitation ('non-learners').

Modifying the expression of targeted genes in specific brain regions and measuring the effects on singing behavior and neural activity *in vivo* has recently become possible [6,7••]. These methodological advances increase the power and sophistication with which we can address how genes affect the function and refinement of complex neural circuits underlying vocal learning. Here we review recent literature from the songbird field that starts to elucidate the role of *FoxP2* and its associated signaling network for the development and function of neural circuits mediating vocal production learning.

FoxP2 expression pattern in songbirds: similarities and differences with other vertebrates

The expression pattern of *FoxP2* in birds is overall very similar to that of rodents and other vertebrates, including humans. *FoxP2* expression is prominent in the striatum, dorsal thalamus and the olivar-cerebellar system [8,9,10•,11–13]. *FoxP1* partially overlaps *FoxP2* expression, for example in the striatum, but co-expression is not obligatory, for example cerebellar Purkinje cells express *FoxP2* but not *FoxP1* [9,11].

In the pallium of different rodent species expression of Foxp2 is always present in cortical layer VI [14–16] but varies in layer V. Whether these differences correlate with

[1] Following nomenclature proposed by Kaestner and colleagues (Kaestner KH, Knochel W, Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 14;2000:142–6). Upper case (FOXP2) and lower case (FoxP2) refer to human and non-human transcription factors, respectively. Italicized letters (*FOXP2 and FoxP2*) refer to the genes.

different vocal behaviors in the studied species is not known yet [12]. In different bird species there is no evidence so far that song complexity is related to qualitative or quantitative variations in *FoxP2* expression in the song system [17]. For instance, Bengalese finches have a larger repertoire of song elements and sing much more variable sequences than zebra finches, but neural expression patterns of *FoxP2* (and *FoxP1*) in the two species are very similar on the gross morphological level [10•].

The pallium of different bird species with the exception of the budgerigar, expresses little *FoxP2* [9,10•]. However, the mesopallium of budgerigars expresses more *FoxP2* than the mesopallium of finches. Interestingly, the song nucleus MO that is embedded in the mesopallium expresses conspicuously less *FoxP2* than the surrounding tissue [9]. How cortical layers in mammals relate to the different pallial regions in birds continues to be an area of controversy (for a recent summary see editorial [18]).

In gymnotiform fish *FoxP2* is expressed in the dorsocentral pallium (DC) supporting the proposed homology between DC and layers V and VI of the mammalian isocortex [19]. The authors propose a comparative approach across gymnotiform fish to relate *apteronotid FoxP2* expression patterns to communication behavior.

Because of the known relevance of corticostriatal circuitry for auditory guided vocal communication in songbirds and in humans [20], most attempts to elucidate the cellular functions of *FoxP2* have centered on the *FoxP2* expressing striatal spiny neurons (SN) [7••,9,21–23] and the circuitry they are embedded in.

Cues to *FoxP2* function from expression differences

Foxp2 plays a role during early patterning of the mouse nervous system. It regulates embryonic cortical neurogenesis [24] and promotes the differentiation of medium spiny neurons derived from the lateral ganglionic eminence without affecting cell proliferation or survival [25]. In birds, the presence of *FoxP2* expression in the embryonic ventricular zone giving rise to SN is consistent with a similar role [9,21].

FoxP2 expression increases during the period of development when song learning occurs and is lower in adult birds in Area X, which is important for song learning [9,26]. Because the neuron density in Area X does not vary significantly during this time, parsimony suggests that FoxP2 expression in individual neurons changes over the course of development [21]. Using a statistically unbiased analysis Thompson *et al.* [27•] found that FoxP2-expressing neurons fall into two different classes of FoxP2-immunoreactivity (IR): weakly stained and intensely stained. Interestingly, only the intensely stained ones decline with age whereas the density of the weakly stained neurons remains stable. Furthermore, adult generated neurons are more likely to strongly express FoxP2, suggesting that intensely IR neurons may represent a 'younger' fraction of FoxP2 IR cells. Like the decline in intensely stained cells, neural recruitment to Area X also decreases during development [21]. The relation between *FoxP2* and the integration and differentiation of newly generated neurons into Area X needs further investigation. Studies in mouse and chicken support the role of *FoxP2* in neurogenesis [24,28]. It is possible that adult neurogenesis also occurs in the human striatum [29] as suggested by accumulation of the thymidine analogue IdU and ^{14}C. A possible caveat is that IdU and ^{14}C are not only incorporated into DNA during cell division but also during DNA repair or DNA methylation, respectively. Interestingly, in contrast to BDNF-treated mice [30] and intact birds [21], the putative human adult-born striatal neurons are not of the FoxP2 expressing medium spiny neuron type, but interneurons [29].

In the light of gender differences in language acquisition, it is interesting that the amount of FOXP2 expression in the brains of young boys and girls and in juvenile rats is sexually dimorphic [16]. However, in songbird species where both females and males sing, *FoxP2* is expressed in Area X to similar degrees [9].

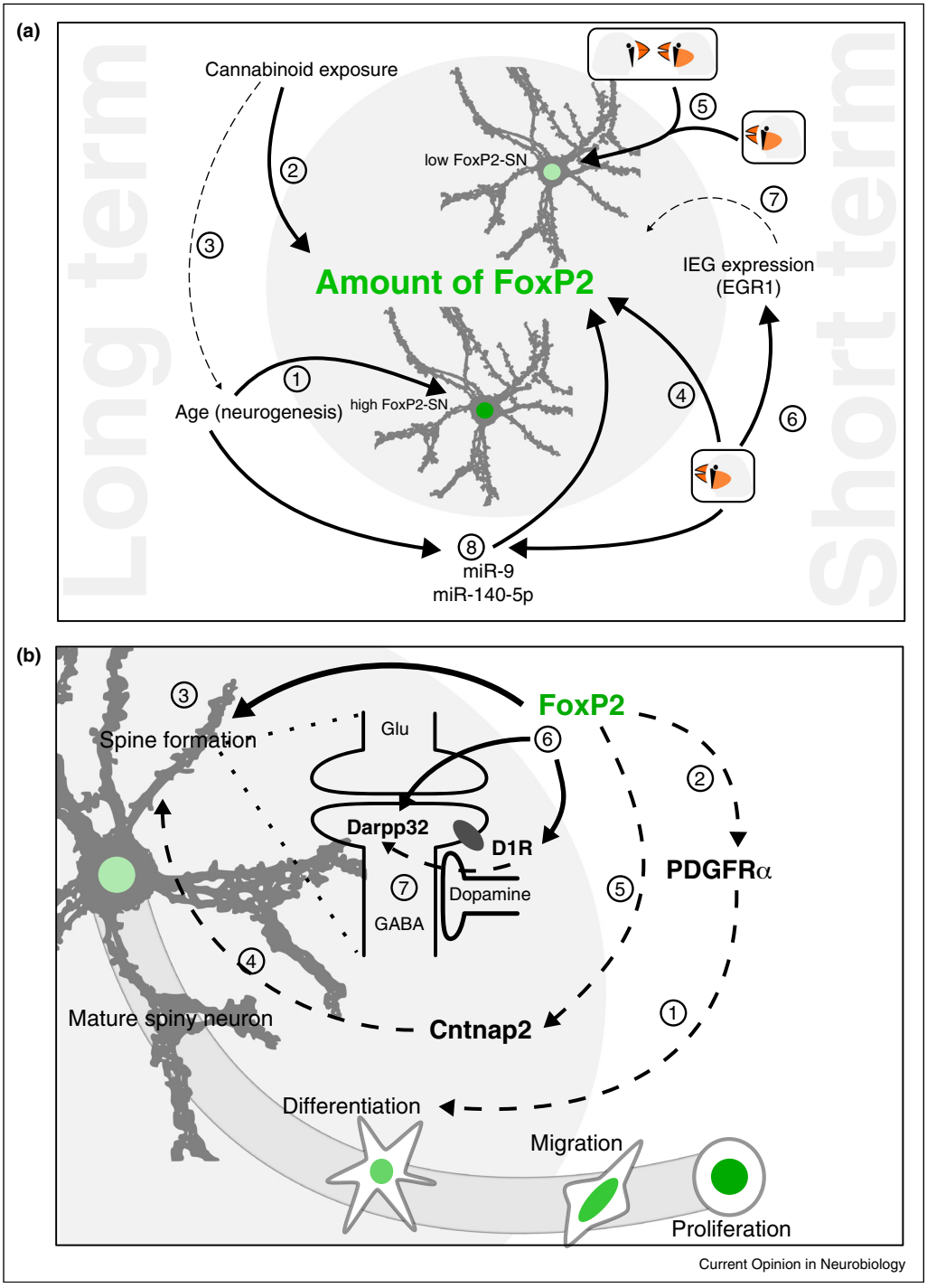
In summary, the time, place and amount of expression during embryogenesis and postnatal neural differentiation of songbirds is consistent with experimental evidence in mice that *Foxp2* partakes in shaping neural differentiation. In addition, there is increasing evidence that at later life stages *FoxP2* functions also at a shorter timescale affecting neural transmission and behavior.

Behavioral modulation of the *FoxP2* expression

Zebra finches sing in different social contexts. During courtship, males direct their song towards females ('directed song') but they also sing song that is not directed at conspecifics or while they are alone ('undirected song'). Female-directed song differs acoustically from undirected song [31] as does the accompanying neural activity [32] and immediate early gene expression, such as *EGR1* [33,34]. Many studies implicate dopamine acting on Area X neurons in the regulation of these neural and behavioral differences associated with singing in the two different social contexts [35–43].

FoxP2 mRNA expression also varies with social context. It decreases during undirected but not during directed singing [26,44]. The expression of *FoxP2* mRNA correlates negatively with the amount of undirected singing, both in zebra finches [26,45••] and in Bengalese finches

Figure 1



(A) Upstream of *FoxP2*: multiple factors influence the expression level of *FoxP2* in Area X (symbolized by the grey circle). These changes can either manifest on a long term scale or be shorter termed. FoxP2 is expressed in the spiny neurons (SN) of Area X and can occur in two rather distinct 'states': SNs expressing high levels of FoxP2 (high FoxP2-SN, bright green) or low levels of FoxP2 (low FoxP2-SN, light green). The high FoxP2-SNs have a higher proportion of adult generated neurons than the low FoxP2-SNs and often show an elongated nucleus indicating that they are indeed adult born new neurons (1; Thompson *et al.*, 2013). The density of high FoxP2-SNs declines with age as does the neuronal recruitment to Area X (1; Thompson *et al.*, 2013; Rochefort *et al.*, 2007). Cannabinoid exposure in late postnatal development leads to a sustained elevation of FoxP2 levels (2; Soderstrom *et al.*, 2010). Cannabinoid exposure also increases adult neurogenesis in the mouse hippocampus (3; Jiang *et al.*, 2005). On an hour time scale, FoxP2 mRNA and protein levels are regulated by singing. Singing undirected song (symbolized by a pictogram of a male singing alone) for 2 hours leads to a downregulation of *FoxP2*-mRNA (4; Teramitsu *et al.*, 2006) whereas both, undirected and directed song downregulates FoxP2 protein in the low FoxP2-SNs (5; Miller *et al.*, 2008; Thompson *et al.*, 2013). Undirected singing also triggers immediate early gene expression, peaking 1 hour after the

[10•]. However, this correlation does not hold in deaf zebra finches [26], indicating that *FoxP2* expression in Area X is modulated by motor activity and sensory activity. Interestingly, in contrast to the mRNA, FoxP2 protein expression is downregulated after singing in both social contexts [27•,46] raising the possibility of differential posttranscriptional regulation. This dissociation between mRNA and protein levels may be related to sensory input [47].

Upstream of *FoxP2*

Which factors regulate *FoxP2* expression in Area X? (Figure 1A). The major *FoxP2* transcript in the zebra finch brain is 6.5 kb long, with only a small part coding for the 710 aa long protein [9], providing a substrate for complex posttranscriptional regulation. A recent paper implicates miRNAs in this process. Given the relationship between singing and *FoxP2* expression, and between singing and miRNAs expression [48,49], Shi *et al.* [50••] hypothesized that miRNAs regulate *Foxp2* expression in songbirds and identified *miR-9* and *miR-140-5p* as potential regulators. The authors characterized the *FoxP2* 3′-UTR and identified miRNA binding sites using bioinformatic sequence analysis combined with PCR-based cDNA amplification. The specificity of those sequences for miRNA binding was confirmed with luciferase reporter assays. Furthermore, over-expressed *miR-9* and *miR-140-5p* reduced levels of *Foxp2* mRNA and protein in SH-SY5Y cells. The authors also quantified the expression levels of both miRNAs and of *FoxP2* mRNAs in Area X tissue punches by QPCR and found that *FoxP2* mRNA levels were elevated at 45 days of age when the miRNA expression was reduced, complementing the *in vitro* data. After undirected singing the expression of the two miRNAs was inversely related to *FoxP2* expression. Consistent with an evolutionary conserved regulatory role of miRNAs on *FoxP2* expression, mi*R-9* and mi*R-132* can repress ectopically expressed *FoxP2* in embryonic mouse cortex [51].

Because undirected singing is associated with upregulation of the immediate early gene *EGR1* and downregulation of *FoxP2*, direct interactions are a possibility. In fact, the 5′ flanking region of human and songbird *FoxP2* contains predicted binding sites for EGR1 [48,52]. However, a significant downregulation of *FoxP2* is only observed after very vigorous and sustained singing [45••] (and personal observations). In canaries the amount of *EGR1* expression in Area X does not predict the amount of FoxP2 expression [9]. Together these facts suggest that *EGR1* represses *FoxP2* slowly or inefficiently. However, one has to keep in mind that the effect of singing on *FoxP2* so far has always been measured in Area X as a unit. If neural activity regulates the level of *FoxP2* expression through IEGs in some spiny neurons but not others, the true extent of regulation might only become apparent by analyzing the relationship in single cells.

In addition to miRNAs, cannabinoids impact FoxP2 expression in zebra finches. Systemic administration of the cannabinoid agonist WIN55212-2 in juvenile birds increases the numbers of IR FoxP2 neurons in Area X [53]. Cannabinoid exposure during song learning leads to a reduction of song element imitation and increases song sequence variability [54,55]. The activation of type 1 cannabinoid receptors in Area X reduces synaptic strength onto FoxP2—expressing spiny neurons through reduced presynaptic release probability [56].

Hormones may also be involved in regulation of *FoxP2* expression. In canaries, hormones mediate seasonally changing singing behavior and song plasticity [57] coincident with changing *FoxP2* expression levels. *FoxP2* levels are highest in Area X during the months of the year when testosterone levels are low and canaries sing variable, non-stereotyped song [9]. It would thus be interesting to further pursue the potential molecular relationship between hormones and *FoxP2*.

Together, the above observations provide hints about some factors, predicted or verified, that contribute to *FoxP2* regulation in Area X, and in turn, song learning. Additionally, studies in cell lines, as well as in developing fish, mice and humans, suggest that regulators of FoxP2 can in turn be targets of FoxP2. For instance, in zebra fish *lef1*, a member of the Lef/Tcf family of transcription factors activated by Wnt signaling binds to enhancers in the *foxP2* genomic locus, regulating region-specific

(Figure 1 Legend continued) start of singing (6; Jarvis *et al.*, 1998). The human and songbird genome both contain EGR1-binding sites in the promoter region of *FoxP2* opening the opportunity for a singing driven downregulation of FoxP2 mediated by IEGs (7; Bruce and Margolis, 2002). *MiR 9* and *miR-140-5p* are able to bind and downregulate *FoxP2* expression. Both miRNAs are expressed in Area X in an inverse pattern to that of *FoxP2* (lower in juveniles compared to adults) and are upregulated after undirected singing in adult males (8; Shi *et al.*, 2013). This together with the EGR1 expression might explain the downregulation of the *FoxP2* mRNA only in undirected song. The upstream factors influencing the protein expression in directed song are still elusive. **(B)** Downstream of *FoxP2*: In developing mice, Foxp2 promotes the differentiation of medium spiny neurons derived from the lateral ganglionic eminence *via* PDGFRalpha (1; Chiu *et al.*, 2014), a direct target of FOXP2 in a human cell line (2; Konopka *et al.*, 2009). Proliferation and migration do not seem to be affected. In mature SNs FoxP2 knockdown leads to a reduction of spines (3; Schulz *et al.*, 2013). In mice, spine formation is promoted by Cntnap2 (4; Anderson *et al.*, 2012), a FOXP2 target gene (5; Vernes *et al.*, 2008). In zebra finches *FoxP2* is involved in the integration of dopaminergic and glutamatergic signaling at the corticostriatal synapse in Area X. In mammalian medium spiny neurons D1R signals *via* Darpp32 (7; Surmeier *et al.*, 2007). In Area X *FoxP2* knockdown leads to a severe decrease in DARPP32 expression as well as a slighter decrease in D1R expression (6; Murugan *et al.*, 2013). Direct experimental evidence in songbirds is symbolized by black bold arrows, whereas potential links derived from other species or brain regions are symbolized by dashed arrows.

expression [58]. Components of the Wnt signaling pathway also emerge as FOXP2 targets, identified by ChIP experiments [59,60]. Likewise, miRNAs cannot only regulate FoxP2 but also be downstream targets regulated by Foxp2 [22]. Thus, FoxP2 abundance in neurons, particularly during development, may be subject to tight control *via* feedback mechanisms.

Downstream of *FoxP2*

Studies using chromatin immunoprecipitation (ChIP) followed by microarray analysis revealed about 2000 potential targets of FOXP2 [22,59,60,74]. While both mice and songbirds depend on intact FoxP2 function in their striatal circuits for learned fine motor skills [6,7••,61] it is not clear whether the evolution of auditory-guided vocal motor learning in songbirds went hand in hand with a diversification of FoxP2 target genes. If so, this could result in the elaboration of slightly different circuitry or different neural function during song learning and singing behavior, or both.

So far, there are no microarray or deep sequencing data published that directly address the global transcriptional regulation by FoxP2 in songbirds (Figure 1B). However a recent publication provides indirect evidence. A differential expression study in zebra finches used weighted gene coexpression network analysis on microarray data to identify groups of genes co-regulated during singing [45••]. Among the more than 2000 genes regulated by singing in Area X many were known to be targets of human *FOXP2*, identified using various methods and tissues [22,59,60,62,63]. For example of 175 targets found in human fetal basal ganglia [59] 56 were also in the singing-regulated network, many of them downregulated by singing. Functional annotation of the singing regulated genes led the authors to postulate a role for *FoxP2* in singing-related NMDAR-mediated synaptic plasticity and cytoskeletal rearrangement, as well as MAPKK and tyrosine phosphatase signaling.

Another target identified by ChIP on human neuronal like cells is contactin-associated protein-like 2 (*CNTNAP2*), a member of the neurexin superfamily that has been linked to speech pathologies and autism [64]. The expression pattern of the *CNTNAP2* mRNA [65] inversely mirrors the developmental modulation of the *FoxP2* expression in Area X of zebra finches [9]. In contrast to the mRNA levels, more CNTNAP2 protein is detected in Area X than in the surrounding striatopallidum [66•]. Cntnap2 has been linked to synaptic remodeling in rodents [67] but the functional relevance in songbirds awaits experimental manipulation.

Recently, two components of the dopamine signaling pathway, the D1R and DARPP32 have also been shown to be affected by experimental manipulation of *FoxP2* expression in Area X of adult male zebra finches [7••]. *Darpp32* had already been found in the target screen by Vernes *et al.* [22]. These findings, and functional studies (see below) suggest a link between FoxP2 and dopaminergic signaling which is additionally supported by several studies in mice [74].

Effect of *FoxP2* on vocal learning and neural processing

Ultimately, gene function studies require targeted genetic manipulations, but in contrast to mice, germline transgenesis in songbirds is still inefficient [68,69]. However, lentivirally mediated RNAi-based knockdown of *FoxP2* has been successfully used in Area X in juvenile and adult zebra finches [6,7••]. *FoxP2* knockdown in Area X of juvenile zebra finches results in incomplete and inaccurate song imitation and leads to greater variability in song delivery in adults [6,7••]. Knockdown of *FoxP2* in adult Area X prevents the social context dependent 'switch' of the singing mode. Variability of the fundamental frequency of song elements is usually lower in directed song than in undirected song. After knockdown this is no longer the case. The behavioral read-out is mirrored by neural activity in the pallial song nucleus LMAN, two synapses downstream of Area X. In control birds, the mean firing rate in LMAN is usually higher in undirected song than in directed song, as is the number of bursts (*e.g.* interspike intervals of less than 5 ms). After *FoxP2* knockdown in Area X, burstiness and mean firing rate during directed song remain as high as during undirected song. Interestingly a recent study reveals that the difference in AFP firing between directed and undirected song initially emerges in Area X [70••]. In addition, after knockdown the signal propagation from the pallial nucleus HVC through Area X to LMAN is accelerated by 3 ms. Importantly, dopamine 1 receptor agonists and antagonists modulate this signal propagation speed in control birds, but not in knockdown birds. Whether these manipulations affect pre-motor spike timing in RA was not tested *in vivo*. However, in a slice preparation of unmanipulated birds; differences in the arrival times on the same order as those observed after *FoxP2* knockdown in Area X can significantly affect RA spike timing variability. This could be the mechanism by which millisecond changes in the anterior forebrain signal contribute to song variability. Further investigation is necessary to test whether FoxP2 dysfunction leads to a cell-type-specific reshaping of cortico-striatal connectivity and function. Similarly to the observed excessive bursting after *FoxP2* knockdown in the pallial nucleus LMAN, dopamine alterations in the striatum of mammals can rapidly affect correlated activity in cortical areas ([71]; see also [72]). Based on the latter findings Woolley *et al.* [70••] proposed a model how the influence of dopamine on the correlated activity of SNs in Area X could lead to social context dependent modulation of firing in pallidal output neurons. Taken together this leads to the question if *FoxP2* likewise affects the correlated activity between

SNs. Another potential link to *FoxP2* pathomechanisms in relation to synaptic function is its involvement in spine formation. Knockdown of *FoxP2* in spiny neurons of zebra finches reduces spine density *in vivo*. This effect is even more pronounced when neurons receive the knockdown before differentiation, that is as neuroblasts in the ventricular zone, where adult neurogenesis takes place [73]. The exact chain of events leading from reduced *FoxP2* levels in spiny neurons to altered neural and behavioral function remains to be uncovered (Figure 1B).

Conclusion

In this review we focused on songbirds as a model to address how the expression of *FoxP2* affects neural circuits underlying vocal plasticity.

Data point towards a dichotomous role of *FoxP2*: On the one hand it is clearly involved in the formation of the circuitry employed for sensory guided motor learning by influencing neuronal differentiation, in particular dendritic outgrowth and spine formation. On the other hand *FoxP2* is needed for the proper function of these circuits: Vocal learning in juveniles and social context dependent 'switching' of singing style in adults. Whether *FoxP2* subserves these two aspects *via* the same or different mechanism needs to be followed up further.

Future research should aim at solving the following open questions:

- Target genes and signaling cascades: Microarray, ChIP or Next Generation Sequencing studies to identify which genes are regulated by FoxP2 during song learning and maintenance are needed. Among those genes, which are the most relevant ones in juvenile and adult animals? Such studies promise to shed light on the specific contribution of particular molecular modules, *e.g.* the Wnt signaling pathway to vocal communication.
- Structure-function relationships: *FoxP2* is involved in spine-formation, dendritic branching and outgrowth in SNs. Whether and how this impacts intrinsic and synaptic properties of these neurons remains largely elusive and thus important to investigate.
- Signal propagation and processing: *FoxP2* knockdown in Area X of adults changes signal propagation speed on the one hand and neural output patterns on the other hand. Future research should address whether this is also the case in juveniles and whether the changes in signaling speed and output patterns are the consequence of similar or distinct pathomechanisms.
- Area X microcircuits: The question how *FoxP2* affects the micro circuitry of Area X merits more attention. Sophisticated molecular tools in combination with classical tracing techniques promise insights whether and how *FoxP2* alters the formation and function of the circuits within Area X.

Conflict of interest

Nothing declared.

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