Singing is silver, hearing is gold: impacts of local FoxP1 knockdowns on auditory perception and gene expression in female zebra finches

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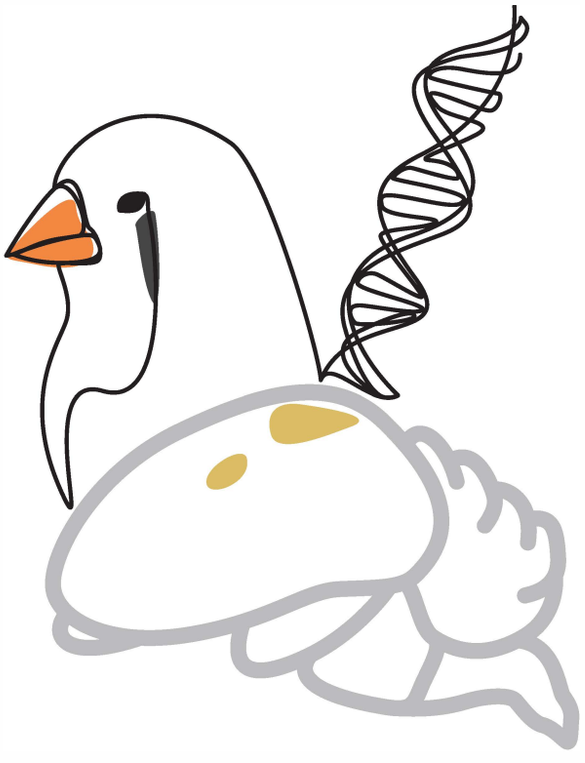
Chapter 1

General Introduction



Chapter 1: General Introduction

Human spoken language, speech, is acquired by vocal learning without the need for specific training (Friederici, 2011). In rare cases, impairments in the development of speech and language can be linked to disruptions of individual genes. While this allowed to begin the deciphering of molecular processes underlying speech and language, the functional roles of the relevant genes are difficult to examine in humans (Fisher *et al.*, 2003; Vernes and Fisher, 2009; Graham and Fisher, 2013; Szalontai and Csiszar, 2013; Deriziotis and Fisher, 2017).

However, a number of candidate genes has been identified during extensive research over the last decades. This thesis focuses on one gene of particular interest, forkhead box transcription factor 1 (*FOXP1*) which is a member of the p subfamily of forkhead box transcription factors (Shu *et al.*, 2001). FOXPs[1] have been implicated in human speech and language (Takahashi *et al.*, 2009; Co *et al.*, 2020a) and are highly homologous across vertebrates (Hannenhalli and Kaestner, 2009; Golson and Kaestner, 2017). Their contributions to brain development have been thoroughly investigated following the discovery that rare heterozygous disruptions of the human *FOXP2* gene are associated with childhood apraxia of speech (CAS) and further language impairments (Lai *et al.*, 2001; Morgan *et al.*, 2017). Next to *FOXP2*, mutations of two other genes of the *FOXP* subfamily, *FOXP1* (Pariani *et al.*, 2009) and *FOXP4* (Snijders Blok *et al.*, 2021) have been implicated in human neurodevelopmental disorders that include speech- and language-related disruptions. Heterozygous mutations of *FOXP1* result in a syndrome involving intellectual disability and/or autism spectrum disorder, often accompanied by speech and language deficits (Sollis *et al.*, 2016; Siper *et al.*, 2017), while those affecting *FOXP4* lead to a less severe and more variable phenotype with speech and language delays, growth defects, and congenital abnormalities (Snijders Blok *et al.*, 2021). *FOXP3*, the last member of the FOXP subfamily has not been implicated in cognitive or language related disorders in humans or vocal production in animals. Instead, *FOXP3* is related to immunological processes and specifically T regulatory cell functions (Hori *et al.*, 2003; Marson *et al.*, 2007; Colamatteo *et al.*, 2020) and thus lies outside of this thesis' scope. The observed

[1]Note the different spellings of FOXP depending on the context. FOXP refers to the human version of the protein, or the general subclass of transcription factors. *FOXP* refers to the human version of the gene, or the gene subfamily in general. Foxp refers to the mouse protein, and *Foxp* to the mouse gene, respectively. FoxP relates to songbird proteins, while *FoxP* describes songbird genes.

impacts of *FOXP1, 2* and *4* disruptions provide an important entry to examine the molecular underpinnings of speech and language, and more broadly the neurogenetic pathways involved in vocal learning (Vernes and Fisher, 2009; Deriziotis and Fisher, 2013; Oller *et al.*, 2013). Animal models can provide a potential window into the neurogenomic basis of speech and language, as they allow experimental insights into functions of genes for circuitries and their relevance for certain behaviours. To date, the implication of FOXP2 for vocalisations is demonstrated best as experimental genetic manipulations of orthologues of *FOXP2* have been shown to affect vocal behaviours in mice and songbirds (Shu *et al.*, 2005; Haesler *et al.*, 2007). One area of special interest concerns the potential contribution of FOXP transcription factors to auditory-guided vocal learning, a crucial element for acquisition of human speech. Suitable animal models are rare due to the limited occurrence of vocal learning among animal taxa. Vocalisations of both pups and adult mice do not obligatorily rely on experience as they are not impaired by a lack of auditory feedback or by deafness (Hammerschmidt *et al.*, 2012; Mahrt *et al.*, 2013). However, when auditory instruction is available, mice possess limited vocal learning abilities expressed by vocal flexibility based on experience (Arriaga *et al.*, 2012; Arriaga and Jarvis, 2013; Lattenkamp and Vernes, 2018; Martins and Boeckx, 2020). Extensive vocal learning occurs in songbirds (Nottebohm *et al.*, 1990; Braaten *et al.*, 2006), seals and cetaceans (Janik and Slater, 1997; Petkov and Jarvis, 2012) and certain species of bats (Knörnschild, 2014; Vernes, 2017). Due to limited options for experimental studies and practical and ethical considerations in seals and cetaceans, songbirds have emerged as tractable models for studying vocal learning. They learn their vocalisations, particularly their song, from adult tutors (Nottebohm *et al.*, 1990; Doupe and Kuhl, 1999). Despite considerable neuroanatomical differences, the pallial, striatal and pallidal brain regions of songbirds involved in song learning and its perception are functionally and transcriptionally similar to humans and mice (Pfenning *et al.*, 2014; Colquitt *et al.*, 2021). For example, Area X in the songbird striatum which is essential for song learning shows convergent gene expression compared to areas of the human striatum which are activated during speech. The robust nucleus of the arcopallium (RA) in songbirds shows transcriptional similarities to human laryngeal motorcortical areas which are also active during speech production (Reiner *et al.*, 2004; Jarvis *et al.*, 2013; Pfenning *et al.*, 2014).

Even when behavioural changes are thoroughly studied in animals with experimentally altered expression or functionality of FoxPs and in humans with aetiological *FOXP* mutations, it can be difficult to identify the underlying mechanisms. Altered vocalisations after *FOXP* manipulations could result from impaired sensory or motor learning (or both). An animal could memorise a song and form a song template but might subsequently fail to reproduce the model correctly. Conversely, impairments during sensory or cognitive processing and memorisation of perceived auditory stimuli could lead to impaired sensory memories. If these subsequently form the template for developing a vocal motor program, song of impaired birds will show little resemblance with the initial model which was poorly memorised. Probably due to the problem that impairments of adult vocalisations do not allow to discern either process, these two possibilities have rarely been investigated separately. As reviewed in further detail below, the expression of the different *FOXPs* is highly localised across different, functionally specialised areas of the songbird vocal system.

This thesis aims to increase the understanding whether some of the disturbances in vocal development related to *FOXPs*, and *FoxP1* in particular, are caused by impaired auditory learning. The pronounced sex differences in song learning in zebra finches (*Taeniopygia guttata*) allow an experimental approach where auditory learning and vocal production learning can be studied separately. Male and female zebra finches both memorise songs heard early in life, but only adult males produce learnt song. Studying song memorisation learning in female zebra finches in combination with neuromolecular approaches such as transcriptome sequencing should be applicable to answer whether *FoxP1* expression in certain key brain regions impacts auditory processing and learning. More broadly, these studies may help increase understanding of how *FOXP* genes contribute to vocal behaviours. This first Chapter reviews the prior knowledge about *FOXPs* and how auditory perception could be affected by these transcription factors, with a focus on *FOXP2* (which has been studied most extensively) and the less dominant but mounting evidence for a functional involvement of *FOXP1* in the development of vocal communication (which is the primary topic of this thesis). In addition, brief overviews of the three subsequent Chapters are given, which describe the various experiments that were conducted during this thesis project.

Molecular functions of FOXPs

Like other transcription factors, Forkhead box (FOX) proteins do not control physiological functions directly. Instead, they bind DNA and regulate the transcription of genes in proximity of the binding motif (Fisher *et al.*, 2003; Wang *et al.*, 2003; Stroud *et al.*, 2006). Thus they affect diverse developmental processes and disruptions of *FOX* genes are implicated in many diseases (Tuteja and Kaestner, 2007a, 2007b). Most vertebrates express four different FOX proteins of the p-subfamily: from *FOXP1* to *4* (Hannenhalli and Kaestner, 2009; Viscardi *et al.*, 2017), while only one *FOXP* gene is described in invertebrates (Mazet *et al.*, 2003; Lawton *et al.*, 2014) which shows highest homology to vertebrate *FOXP1* (Santos *et al.*, 2011). Functional domains of *FOXP* genes are highly conserved among each other and across different phyla (Hannenhalli and Kaestner, 2009). In FOXP2, for example, only two amino acids changed since the split between the chimpanzee and human lineages. It has been hypothesised that *FOXP2* underwent accelerated evolution in hominids (Enard *et al.*, 2002; Zhang *et al.*, 2002) even though it has not been subjected to more recent selection in humans (Atkinson *et al.*, 2018). An accelerated evolutionary change of *FoxP2* has also been observed in different bat species in comparison to other mammals (Li *et al.*, 2007) even though regulatory elements that further control *FoxP2* expression and additional genes and genetic regions contributing to bat vocalisations require further investigation (Vernes and Wilkinson, 2020).

All FOXP transcription factors are similarly structured (Figure 1) and contain a conserved DNA binding motif called Forkhead box or FOX, as well as a zinc-finger domain and a leucine-rich-zipper region which both enable protein-protein interactions (Wang *et al.*, 2003; Li *et al.*, 2004). With the exception of FOXP3, FOXPs contain a glutamine-rich region with polyglutamine repeats in FOXP1 and FOXP2. Nuclear localisation signals are shared among all family members (Mizutani *et al.*, 2007; Vernes *et al.*, 2007a). While earlier studies assumed a direct repressing function of FOXPs due to a transcriptional repressor domain (Myatt and Lam, 2007; Grundmann *et al.*, 2013), more recent results show both activating and repressing effects on gene transcription by FOXPs (Sin *et al.*, 2014; Araujo *et al.*, 2015, 2017; Li *et al.*, 2015).

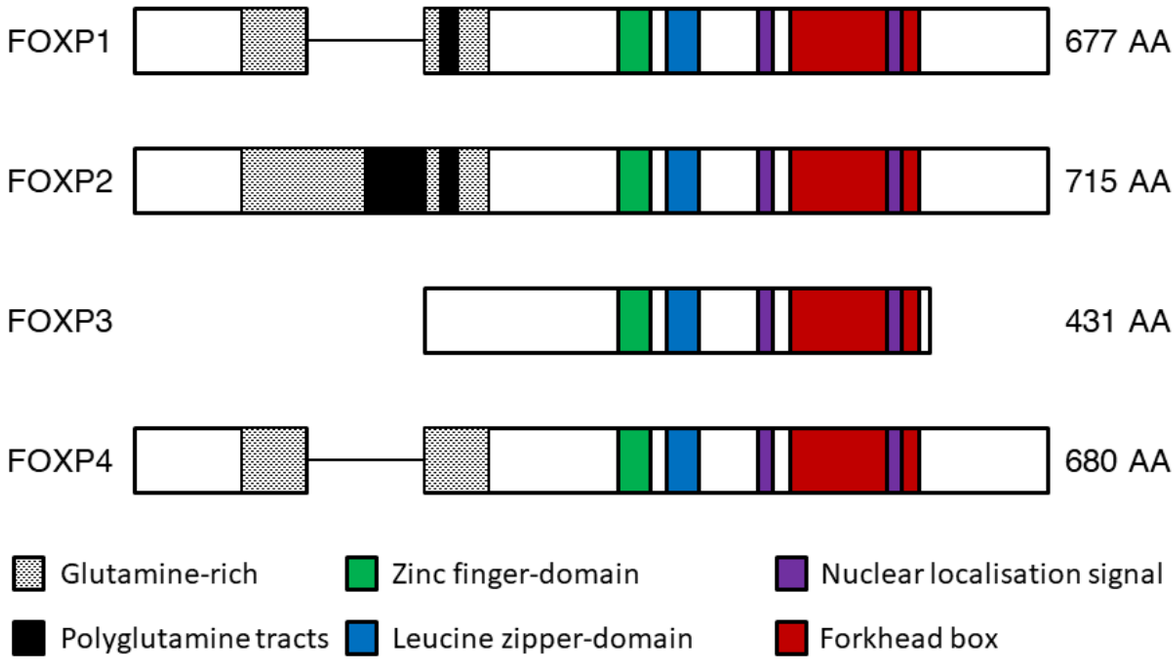


Figure 1: *Homologous structures of human main isoforms of FoxP transcription factors. All known FoxP members contain highly similar zinc-finger and leucine-zipper domains followed by nuclear localisation signals prior to and embedded in the shared forkhead box domain. Glutamine-rich regions have been described for all FOXPs but FOXP3. FOXP1 and FOXP2 have either one or two repetitive polyglutamine tracts within their glutamine-rich regions. Sizes of FoxP transcription factor proteins vary; in humans the main isoforms of the different orthologues consist of 431 to 715 amino acids (AA).*

DNA binding of FOXPs can occur either by individual proteins or complexes of multiple copies of similar (homodimers) or different (heterodimers) FOXP proteins (Sin *et al.*, 2014; Mendoza *et al.*, 2015). This flexibility further increases the diversity of processes that they can be involved in, depending on the composition of a dimer, and suggests an overlap of FOXP-regulated transcripts i.e. the existence of shared downstream targets that can be jointly regulated by different FOXPs. Due to the contribution of multiple FOXPs as binding partners during transcriptional regulation, altered gene expression of either partner could have overlapping effects on downstream targets. This overlap of transcriptional targets could result in comparable phenotypic effects if one of multiple FOXP binding partners is impaired or reduced in its expression.

Broadened phenotypic effects might also emerge based on the multiple dimer constructs an impaired binding partner contributes to.

Expression Patterns of *FOXP1* and *2* are comparable between humans, rodents, bats, and songbirds

Identifying and characterizing genes which are regulated by FOXP proteins can help to elucidate their potential functions. Further insights can also be gained by assessing spatial and temporal expression patterns of these transcription factors during development and in adult tissues of an organism. *FOXPs* are expressed in a range of different cell-types in multiple organs in vertebrates (Shu *et al.*, 2001; Lu *et al.*, 2002; Tamura *et al.*, 2003; Wang *et al.*, 2004; Zhao *et al.*, 2015) including in neuronal subpopulations of the brain (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015; Fong *et al.*, 2018). In particular, the expression patterns and potential roles of *FOXP1* and *FOXP2* in different brain structures and their putative links to neuronal development, vocal behaviours and learning have been studied extensively in vertebrates, and especially in songbirds and rodents (Takahashi *et al.*, 2009; Scharff and Petri, 2011; Deriziotis and Fisher, 2013, 2017; Co *et al.*, 2020a). Although also involved in development of the brain (among other organs), few studies of *FOXP4* have been published (Lu *et al.*, 2002; Norton *et al.*, 2019; Snijders Blok *et al.*, 2021). *FOXP3* is primarily a regulator of the immune system with little relevance for neural tissues (Rudensky, 2011; Deng *et al.*, 2020). Hence, *FOXP1* and *FOXP2* will be the focus of this Chapter.

During human embryonic development, *FOXP1* and *FOXP2* are also both highly expressed in the striatum, thalamus and the cerebellum during the first 24 weeks post conception (Lai *et al.*, 2003; Teramitsu *et al.*, 2004), *FOXP2* is also expressed in the alar plate of the cerebellum during Carnegie-state 23 prior to birth and the cerebellar piriform layer during later developmental stages at the time of birth (Lai *et al.*, 2003). *FOXP1* shows elevated expression levels in the primary somatosensory cortex of fetal and newborn brains up to one year of age (Teramitsu *et al.*, 2004). In adult humans, *FOXP1* is mostly expressed in upper layers of the neocortex whereas *FOXP2* shows highest expression levels in lower layers (Hisaoka *et al.*, 2010). *FOXP2* is highly expressed in parietal and temporal regions including cortical brain regions associated to auditory perception and language comprehension (Saygin *et al.*, 2003; Miller *et al.*, 2014). Low but distinctively elevated expression levels in comparison to the

surrounding tissue of *FOXP2* have been reported for the globus pallidus (Ferland *et al.*, 2003; Lai *et al.*, 2003; Teramitsu *et al.*, 2004). In the hippocampus, the amygdala and the primary motor cortex of adult humans, expression of *FOXP1* and *FOXP2* remains stable up to 40 years of age (Miller *et al.*, 2014; Li *et al.*, 2018; Co *et al.*, 2020a).

In rodents, *Foxp1* is expressed in the motor region of the spinal cord during embryonic development of mice (Shu *et al.*, 2001). *Foxp1* in mice drives development of stem-cells into motor neurons (Adams *et al.*, 2015) and further determines subtype identity and affects columnar fate dose-dependently and is involved in axon guidance (Dasen *et al.*, 2008; Rousso *et al.*, 2008). *Foxp1* expression is also documented in developing medium spiny neurons in the striatum of rats and mice (Ferland *et al.*, 2003; Delli Carri *et al.*, 2013). Further, *Foxp1* is widely expressed in excitatory projection neurons in the cortex, hippocampus and thalamic nuclei (Tamura *et al.*, 2004). In mice, *Foxp2* is expressed in Purkinje cells and deep cerebellar nuclei of the cerebellum (Shu *et al.*, 2001; Hisaoka *et al.*, 2010) and cortical layers V and VI, and thalamic as well as subthalamic nuclei (Ferland *et al.*, 2003; Van Rhijn and Vernes, 2015). Albeit in different layers, *Foxp1* and *Foxp2* are both expressed in the auditory cortices of developing and adult mice, and the neopallial cortices and ventral interneurons in the spinal cords of adults, as well as in mouse and rat striatum (Takahashi *et al.*, 2003; Fong *et al.*, 2018). Analyses of *FOXP1* and *2* expression patterns in mice and humans show notable overlaps at comparable developmental stages, suggesting high levels of evolutionary conservation in this regard (Ferland *et al.*, 2003; Lai *et al.*, 2003).

Unlike mice or non-human primates, some bat species are vocal learners (Knörnschild *et al.*, 2010; Knörnschild, 2014). In two vocal learning bat species, FoxP1 is absent in the auditory thalamus but shows high expression in the amygdala. It is also abundant across cortical layers II to VI, the striatum and the hippocampus while *FoxP2* is highly expressed in the bat auditory thalamus but absent in the amygdala. Further, *FoxP2* is present in cortico-striatal and cortico-cerebellar circuits. Except for contrasting expression in *FoxP2* in the hippocampus and a lack of *FoxP2* in the cortex of one species, expression of *FoxP1* and *FoxP2* largely overlaps with reports on human and rodent expression patterns (Rodenas-Cuadrado *et al.*, 2018). It has been hypothesised that *FoxP2* also plays a role for bat echolocation or social calls as well as in the sensorimotor integration of these behaviours (Li *et al.*, 2007).

The brains of songbirds consist of nuclei instead of layered cortices (Reiner *et al.*, 2004; Jarvis *et al.*, 2005), and the expression of *FoxP1* and *FoxP2* is spread over distinct regions (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015). Regions which are thought to be homologous to mammalian brain areas related to vocal production show congruent expression of *FoxP1* and *FoxP2* in birds (Teramitsu *et al.*, 2004; Pfenning *et al.*, 2014). In zebra finches, distinct expression of either one or multiple *FoxPs* can be seen in several different brain nuclei. *FoxP1* is most prominently expressed in HVC, RA, the mesopallium and the striatum, while most *FoxP2* expression is seen in the striatum. *FoxP1* expression in HVC, RA, the mesopallium and the striatum is stable during the first 100 days of developing zebra finches while *FoxP2* expression is increased in Area X, during the sensitive phase for song learning but not in adults (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015). In zebra finch embryos, *FoxP2* is expressed in developing nuclei of the striatum as well as the pallium that are relevant for song learning and production (Haesler *et al.*, 2004). Expression patterns of *FoxP1* and *FoxP2* in vocal learning birds seem to be conserved as similar patterns have been observed in various songbirds, such as the zebra finch, canary (*Serinus canaria*), Bengalese finch (*Lonchura striata*) and the budgerigar (*Melopsittacus undulatus*), a parrot species (Haesler *et al.*, 2004; Chen *et al.*, 2013; Hara *et al.*, 2015).

When comparing mammals and birds it becomes apparent that mammals show localised expression of *FoxP1* and *FoxP2* in complementary layers in the cortex. Upper layers express more *FoxP1* while deeper layers express more *FoxP2*. Notably, in the songbird brain, which is organised in individual nuclei rather than layers, *FoxP1* is expressed in more dorsal areas, while *FoxP2* expression is elevated in more ventral nuclei. Even though no distinct cellular layers of a cortex-like structure exist in songbirds where only four transcriptionally similar pallial subdivisions are suggested (Gedman *et al.*, 2021), cortex-like structures have been reported in pigeons (Stacho *et al.*, 2020) which would align upper cortical layers with regions of high *FoxP1* expression and lower cortical layers with regions of high *FoxP2* expression.

Moreover, expression of *FoxP1* and *FoxP2* in the basal ganglia seems to be conserved across vertebrates. Thalamic regions of songbirds and mammals also show comparable expression levels of both transcription factors even though compared to FoxP1, FoxP2 shows a more distributed pattern throughout the thalamus (Haesler *et*

al., 2004; Teramitsu *et al.*, 2004; Takahashi *et al.*, 2009; Mendoza *et al.*, 2015; Co *et al.*, 2020a).

Similar expression patterns of *FOXP1* and *FOXP2* across vocal learning humans, songbirds and bats (Teramitsu *et al.*, 2004; Rodenas-Cuadrado *et al.*, 2018; Co *et al.*, 2020a) but also other species that do not learn their vocalisations such as mice, doves or crocodiles (Haesler *et al.*, 2004) indicate that the presence of these transcription factors in brain areas related to vocal production or perception does not necessarily result in vocal learning capabilities. Yet in vocal learning species, FOXP1 and FOXP2 both play an important role for the imitation and perception of complex vocalisations.

FOXP1 **and** ***FOXP2*** **influence vocal production, vocal learning and complex behaviours**

To allow a broad comparison of the consequences of disrupting or manipulating FOXP1 or FOXP2 and their orthologues in other species, Table 1 summarises phenotypes in humans carrying aetiological variants, knockout and knockin experiments in mice, and knockdown experiments in birds. Depending on the nature of the underlying change, altered functionality of FOXP1 and FOXP2 can have a range of effects. In humans, these include general effects such as developmental delay or other congenital abnormalities but also cognitive impairments such as intellectual disabilities or memory deficits. However, all reported cases of humans with a disruptive mutation in *FOXP1* include intellectual disabilities, speech and language delays. Some cases also include traits associated to autism spectrum disorders (Table 1, Figure 2a). *FOXP2* disruptions in humans result in delayed onset of speech, articulatory impairments and dyspraxia. Nonetheless, perceptual or memory related deficits e.g. impaired language comprehension are also widely documented (Table 1, Figure 2a,c). After developmental delay, both impaired vocal production and impaired perception and comprehension are reported the most often in human case studies on *FOXP1* (Figure 2a) or *FOXP2* (Figure 2c) mutations included in this overview.

As genetic manipulations in animal models were in part informed by findings from the associated human disorders, impaired vocal production was often (but not always) a primary focus of that work, contributing to the discrepancy between the number of studies referred to in Table 1 and Figure 2 describing impaired vocalisations and those reporting changes in other observed traits. Subsequently, the majority of animal studies document impaired vocal production after *FOXP1* (Figure 2b) or *FOXP2*

manipulations (Figure 2d). Impaired sensorimotor learning and/or performance are also often found to be altered in animal studies on behavioural consequences following FOXP1 or FOXP2 manipulations.

Table 1: *Summary of current reports on effects of FOXP1/2 mutations in humans and animal studies on altered gene expression levels, modified protein structure, systemic and conditional knockouts as well as local knockdowns. Reports are ordered by species, gene of interest and year of publication. Literature search was conducted in April 2021 via pubmed.gov with the following key-words in various combinations: foxp, foxp1, foxp2, forkhead-box, mouse, mice, mammal, human, mutation, songbird, behaviour, phenotype, vocal, learning. Studies were preselected for behavioural phenotypes. *Asterisk indicates exemplary studies for multiple investigations that have been conducted on various phenotypical aspects of the same subjects.*

Species	**Gene**	**Modification**	**Documented effects**	**Study**
Human mutations				
Human	*FOXP1*	Deletion including *FOXP1*	Developmental delay, impaired perception & comprehension	(Pariani *et al.*, 2009)
Human	*FOXP1*	Deletion exons 4-14, point mutation	Developmental delay, impaired vocal production, social deficits, intellectual disability	(Hamdan *et al.*, 2010)
Human	*FOXP1*	Deletion including *FOXP1*	Intellectual disability, impaired vocal production, grammar issues, impaired perception & comprehension	(Horn *et al.*, 2010)
Human	*FOXP1*	Deletion including *FOXP1*	Developmental delay, impaired vocal production	(Carr *et al.*, 2010)
Human	*FOXP1*	Deletion exons 6-13	Developmental delay, impaired vocal production, impaired perception & comprehension	(Le Fevre *et al.*, 2013)
Human	*FOXP1*	Point mutation	ASD, developmental delay, impaired vocal production, intellectual disability, impaired perception & comprehension, memory deficits	(Lozano *et al.*, 2015)
Human	*FOXP1*	Point mutation	Developmental delay, impaired vocal production, intellectual disability, impaired cognition, impaired perception & comprehension	(Sollis *et al.*, 2016)
Human	*FOXP1*	Altered splice site, frameshift, point mutations, in-frame deletions	Developmental delay, intellectual disability, ASD, memory deficits, grammar issues, impaired perception & comprehension, impaired cognition	(Siper *et al.*, 2017)
Human	*FOXP1*	Point mutation	Impaired vocal production, developmental delay, impaired perception & comprehension, memory deficits	(Urreizti *et al.*, 2018)
Human	*FOXP1*	Paracentric inversion including *FOXP1*	Impaired vocal production, intellectual disability, developmental delay, ASD, social deficits, impaired perception & comprehension	(Vuillaume *et al.*, 2018)
Human	*FOXP1*	Point mutation	Developmental delay, intellectual disability, impaired cognition	(Zombor *et al.*, 2018)
Human	*FOXP2*	Point mutation	Impaired vocal production, impaired cognition, grammar issues, reduced vocabulary, impaired perception & comprehension, memory deficits	(Hurst *et al.*, 1990) (Vargha-Khadem *et al.*, 1995) (Watkins *et al.*, 2002a)*
Human	*FOXP2*	Truncation	Impaired vocal production, impaired perception & comprehension	(MacDermot *et al.*, 2005)

Human	*FOXP2*	Translocation including *FOXP2*	Impaired vocal production, reduced vocabulary, impaired perception & comprehension	(Shriberg *et al.*, 2006)
Human	*FOXP2*	Translocation exons 1-2, deletion	Developmental delay, impaired vocal production, intellectual disability, impaired perception & comprehension	(Feuk *et al.*, 2006)
Human	*FOXP2*	Deletion including *FOXP2*	Developmental delay, impaired vocal production, grammar issues, reduced vocabulary, impaired perception & comprehension	(Zeesman *et al.*, 2006)
Human	*FOXP2*	Deletion including *FOXP2*	Developmental delay, impaired vocal production, memory deficits, impaired cognition, impaired perception & comprehension	(Lennon *et al.*, 2007)
Human	*FOXP2*	Deletion including *FOXP2*	ASD, developmental delay, impaired vocal production, intellectual disability, social deficits	(Žilina *et al.*, 2012)
Human	*FOXP2*	Deletion including *FOXP2*	Developmental delay, impaired vocal production, grammar issues, impaired perception & comprehension	(Palka *et al.*, 2012)
Human	*FOXP2*	Deletion including *FOXP2*	Developmental delay, impaired vocal production, impaired perception & comprehension	(Rice *et al.*, 2012)
Human	*FOXP2*	Deletion, point mutation	Developmental delay, impaired vocal production, impaired perception & comprehension	(Turner *et al.*, 2013)
Human	*FOXP2*	Rearrangement with breakpoint downstream of *FOXP2*	Developmental delay, impaired vocal production, impaired perception & comprehension	(Moralli *et al.*, 2015)
Human	*FOXP2*	Deletion exons 12-17, point mutations	Developmental delay, intellectual disability, ASD, memory deficits, grammar issues, impaired perception & comprehension	(Reuter *et al.*, 2017)
Animal gene modifications, knockouts, knockdowns				
Mouse	*Foxp1*	Decreased *FoxP1* expression due to Alpha Synuclein KO	Impaired vocal production	(Kurz *et al.*, 2010)
Mouse	*Foxp1*	Whole brain KO	Social deficits, impaired perception & comprehension, ASD-like behaviours, memory deficits, impaired cognition	(Bacon *et al.*, 2015)
Mouse	*Foxp1*	Conditional Nestin KO	Impaired vocal production	(Fröhlich *et al.*, 2017)
Mouse	*Foxp1*	KO in pyramidal neurons of neocortex and hippocampus	ASD-like behaviours, impaired vocal production, Social deficits, impaired sensorimotor learning and/or performance	(Araujo *et al.*, 2017)
Mouse	*Foxp1*	KO in forebrain	impaired vocal production	(Usui *et al.*, 2017a)
Zebra finch	*FoxP1*	Knockdown in HVC	Impaired vocal production, impaired sensorimotor learning and/or performance	(Garcia-Oscos *et al.*, 2021)
Zebra finch	*FoxP1* *FoxP2* *FoxP4*	Knockdown in Area X	Impaired vocal production, impaired sensorimotor learning and/or performance	(Norton *et al.*, 2019)

Mouse	*Foxp2*	Deletion exons 12-13	Impaired vocal production, impaired sensorimotor learning and/or performance, developmental delay	(Shu *et al.*, 2005)
Mouse	*Foxp2*	Human derived point mutation	Developmental delay, impaired sensorimotor learning and/or performance, impaired vocal production	(Groszer *et al.*, 2008)
Mouse	*Foxp2*	Human derived point mutation	Impaired vocal production, developmental delay	(Fujita *et al.*, 2008)
Mouse	*Foxp2*	Human derived point mutations	Impaired perception & comprehension	(Kurt *et al.*, 2009)
Mouse	*Foxp2*	Humanized gene, heterozygous KO	Social deficits, impaired vocal production	(Enard *et al.*, 2009)
Mouse	*Foxp2*	Human derived point mutations	Impaired vocal production	(Gaub *et al.*, 2010)
Mouse	*Foxp2*	Point mutations	Developmental delay, memory deficits, impaired cognition, impaired perception & comprehension, impaired vocal production	(Kurt *et al.*, 2012)
Mouse	*Foxp2*	Human derived point mutation	Impaired sensorimotor learning and/or performance	(French *et al.*, 2012)
Mouse	*Foxp2*	Humanized gene	*Accelerated learning of stimulus-response associations*	(Schreiweis *et al.*, 2014)
Mouse	*Foxp2*	Humanized gene	*Unaffected vocal production*	(Hammerschmidt *et al.*, 2015)
Mouse	*Foxp2*	Human derived point mutations	Impaired vocal production, developmental delay	(Gaub *et al.*, 2016)
Mouse	*Foxp2*	Human derived point mutation	Impaired vocal production, social deficits	(Chabout *et al.*, 2016)
Mouse	*Foxp2*	Heterozygous KO	Impaired vocal production	(Castellucci *et al.*, 2016)
Mouse	*Foxp2*	Knockdown in Purkinje-cells	Impaired sensorimotor learning and/or performance, impaired vocal production	(Usui *et al.*, 2017b)
Mouse	*Foxp2*	Heterozygous point mutation	Impaired sensorimotor learning and/or performance	(van Rhijn *et al.*, 2018)
Mouse	*Foxp2*	KO in Purkinje-cells, striatum, cortex	Impaired sensorimotor learning and/or performance	(French *et al.*, 2019)
Mouse	*Foxp2*	KO in cortex	Impaired vocal production, social deficits	(Medvedeva *et al.*, 2019)
Mouse	*Foxp2*	KO in cortex	Impaired sensorimotor learning and/or performance, impaired cognition	(Co *et al.*, 2020b)
Mouse	*Foxp2*	KO in Purkinje-cells, striatum, cortex; spontaneous deletion	Impaired vocal production	(Urbanus *et al.*, 2020)
Zebra finch	*FoxP2*	Knockdown in juvenile Area X	Impaired vocal production, impaired sensorimotor learning and/or performance	(Haesler *et al.*, 2007)
Zebra finch	*FoxP2*	Knockdown in adult Area X	Impaired vocal production, impaired sensorimotor learning and/or performance, social deficits	(Murugan *et al.*, 2013)

Zebra finch	*FoxP2*	Overexpression in juvenile Area X	Impaired vocal production	(Heston and White, 2015)
Zebra finch	*FoxP2*	Isoform/full length overexpression in juvenile Area X	Impaired vocal production, impaired sensorimotor learning and/or performance	(Burkett *et al.*, 2018)
Zebra finch	*FoxP2*	Overexpression in adult Area X	Impaired vocal production, impaired sensorimotor learning and/or performance	(Day *et al.*, 2019a)

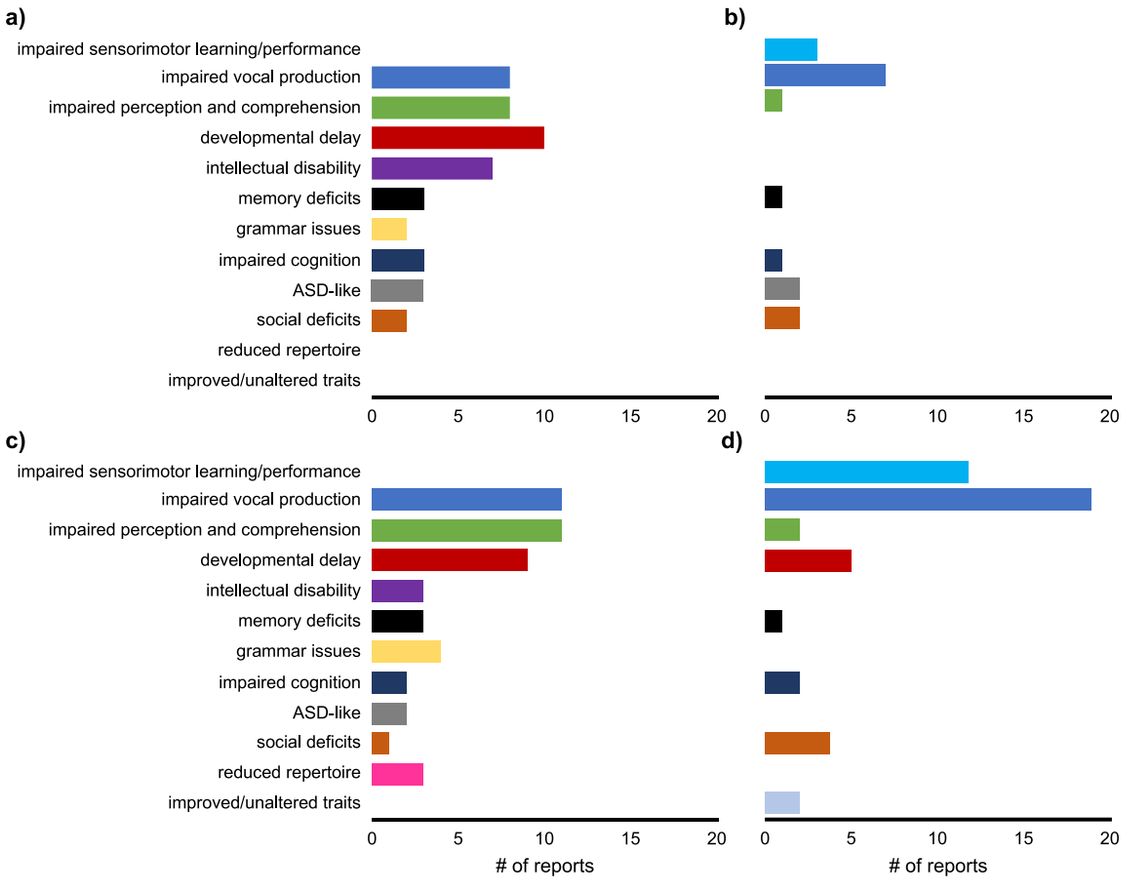


Figure 2: *Overview of documented phenotype categories in studies that assessed behavioural traits conducted on human FOXP1 (a; N =13) or FOXP2 (c) mutations (N = 14), and altered expression or modified proteins of FOXP1 (b; N = 7) or FOXP2 (d; N = 25) in model organisms. Observations were categorised to allow for more uniform grouping. Absent categories in b) and d) indicate features that are irrelevant in animal models or have not been tested. Impaired sensorimotor learning and/or performance has not been systematically tested in human participants (a, c).*

Putative functions of FOXP1 and FOXP2 in relation to vocalisation behaviour and learning have been prominently investigated in mice despite these animals being less competent vocal learners. For example, Araujo *et al.*, 2015 reported that vocalisations are disrupted in mice with a heterozygous deletion of the *Foxp1* gene (Araujo *et al.*, 2015). Mice with brain-specific homozygous deletions of *Foxp1* showed impairments in overall neuronal development and reduced social interaction and sensory integration in adults, potentially due to decreased neuronal excitability (Bacon *et al.*, 2015). In mouse pups with brain-wide homozygous deletion of *Foxp1*, the calling rate upon removal of the mother is reduced (Fröhlich *et al.*, 2017). These observations could

result from a decreased motivation to call, which is not necessarily due to motor effects but might be explained by altered perception of the mother's absence. Mice with homozygous deletions of *Foxp1* in cortico-hippocampal projections also vocalise less than controls and show altered cortical lamination (Usui *et al.*, 2017a) and subsequent deficits in long term potentiation in the hippocampus (Araujo *et al.*, 2017).

Systemic Foxp2 disruptions in mice link this transcription factor to altered and reduced vocalisations in pups and adults, motor control of locomotion as well as motor-skill learning (Shu *et al.*, 2005; Fujita *et al.*, 2008; Groszer *et al.*, 2008; Gaub *et al.*, 2010, 2016; French *et al.*, 2012; Castellucci *et al.*, 2016; Chabout *et al.*, 2016; Chen *et al.*, 2016). Fewer excitatory synapses and postsynaptic currents (Chen *et al.*, 2016), impaired synaptic plasticity in the striatum (Groszer *et al.*, 2008), overall increased neuronal activity which is less modulated during motor skill learning in the striatum and Purkinje-cells (French *et al.*, 2012, 2019), possibly due to increased GABAergic inhibition (Van Rhijn *et al.*, 2018) hint towards potential physiological mechanisms involving synaptic regulation which might underly the observed behavioural changes. Yet, conditional knockouts in the cortex, the striatum or Purkinje-cells do not result in altered pup vocalisations observed in systemic knockouts even though spontaneous deletions of *Foxp2* result in reduced pup USV calls and more click sounds that are suggested to be failed USV calls due to physiological impairments (Urbanus *et al.*, 2020). Further, auditory perception might be altered in mice carrying heterozygous human *Foxp2* mutations due to a disturbed synchrony between the cochlea and the auditory brainstem (Kurt *et al.*, 2009).

Cortex-specific *Foxp2* deletions in mice result in subtle vocalisation changes that depend on context (Medvedeva *et al.*, 2019; Co *et al.*, 2020b) while deletions in the Purkinje-cells, medium-spiny neurons in the striatum or the cortex impair performance and microstructure of behaviours during a lever-pressing task. Perturbances during locomotor learning result in lower performance rates of all deletion types when compared to controls, yet only deletions of *Foxp2* in the Purkinje-cells also impair unperturbed performance (French *et al.*, 2019). Cerebellar *Foxp2* knockdowns impair motor functions such as the righting reflex while early developmental knockdowns result in perturbed isolation calls of pups (Usui *et al.*, 2017a). Generally, results on the quality of vocalisations after *Foxp2* manipulations differ between studies based on differences with respect to the applied manipulations including different changes from point mutations to deletions, studies on homozygous or heterozygous specimen during

different developmental stages with systemic or region-specific changes. They range from wildtype-like vocalisations (Groszer *et al.*, 2008) to severely impaired vocal production following homozygous mutants with generally impaired development (Fujita *et al.*, 2008). Taken together, this variability with respect to vocalisations suggests that vocal phenotypes emerge from more severely affected physiological traits which are necessary to properly elicit vocalisations. Additional sex-differences and separate pathways underlying adult and pup vocalisations might also contribute to this variability (French and Fisher, 2014).

Partial humanisation of the mouse *Foxp2* gene (by introducing two amino-acid changes that distinguish human *FOXP2* from the chimpanzee orthologue) also might affect vocalisations (Enard *et al.*, 2009; Reimers-Kipping *et al.*, 2011) even though this could not be reproduced in a follow-up study (Hammerschmidt *et al.*, 2015). Mice that carry mutations matching those found in human *FOXP2*-associated disorders show reduced learning speed during auditory-motor association tasks (Kurt *et al.*, 2012) and altered electrophysiological properties of cells in brain regions associated with sensory processing and learning (Groszer *et al.*, 2008).

In songbirds, baseline expression levels of *FoxP1* and *FoxP2* are influenced by behaviours such as listening to or production of song. In zebra finches, dynamic FoxP2 downregulation in the basal ganglia follows after song practice (Teramitsu and White, 2006; Miller *et al.*, 2008; Thompson *et al.*, 2013; Heston and White, 2015). In male Bengalese finches, FoxP2 expression is absent in the mesopallium during both song production and while the bird does not sing, but increased in the cerebellum during both of these states (Chen *et al.*, 2013). In the same species, expression levels of *FoxP2* are not altered after singing while other songbirds show decreased expression of *FoxP2* in Area X after song production while *FoxP1* expression remains unchanged (Teramitsu and White, 2006; Chen *et al.*, 2013).

Localised knockdowns of FoxP1 via AAV driven expression of a short-hairpin RNA in HVC, a premotor area of juvenile male zebra finches lead to reduced tutor song imitation (Garcia-Oscos *et al.*, 2021) and FoxP2 knockdowns or overexpression in striatal Area X of juvenile male zebra finches (Haesler *et al.*, 2007; Murugan *et al.*, 2013; Burkett *et al.*, 2018; Norton *et al.*, 2019) impairs learning of vocalisations with phenotypic similarity to the speech characteristics of humans with *FOXP2* mutations (Scharff and Petri, 2011). Interestingly, knockdowns of FoxP1, FoxP2 (and also FoxP4) in Area X of juvenile male zebra finches result in overlapping yet distinct phenotypes,

based on analyses of multiple parameters of learned song. Song stereotypy of FoxP1 knockdowns was no different from tutor song and song deficits of these birds occurred in the fewest measurements in comparison to other knockdowns. Yet syllables from FoxP1 knockdowns could not be assigned to tutor syllables more often than syllables from other knockdowns. In contrast, FoxP2 knockdowns specifically impaired the birds' copying accuracy in addition to motif similarity based on comparisons with template song which the birds were supposed to learn (Norton *et al.*, 2019). *FoxP2* overexpression in Area X of zebra finches exacerbates song deterioration in deafened birds that lack auditory feedback (Day *et al.*, 2019a).

Although many animal studies focused on impaired vocal production, manipulations of *FOXP1* or *FOXP2* in animal models are often followed by various feedback-based behavioural changes such as altered perception and memory related traits across different test setups and species (Figure 2B). Observed developmental and behavioural changes range from faulty reproduction of song in zebra finches (Haesler *et al.*, 2007; Murugan *et al.*, 2013; Norton *et al.*, 2019; Garcia-Oscos *et al.*, 2021) to decreased stimulus-response associations as well as feedback-based motor performance in mice (French *et al.*, 2012; Schreiweis *et al.*, 2014). Despite a lack of specific research on the effects of manipulations of FOXP1 and 2 on perceptual tasks, observations from previous studies suggest that impaired auditory perception, processing and feedback may contribute to the impact of these transcription factors on vocal learning and production.

Do FoxP1 and FoxP2 have an impact on auditory perception?

Research on the contributions of *FOXP1* and *FOXP2* to speech and language acquisition or vocal learning in general has focused on traits relevant for vocal production such as orofacial movements or fine motor control (Vargha-Khadem *et al.*, 1998; Carr *et al.*, 2010; French *et al.*, 2012), coordination of complex vocalisations or verbal fluency (Watkins *et al.*, 2002a) and abnormalities in related brain structures (Watkins *et al.*, 2002b). However, auditory perception is an essential part of vocal learning (Gilbert *et al.*, 2009) and auditory feedback is crucial for speech acquisition and language learning (Simon, 1978; Jones and Munhall, 2003). This also holds true for song learning in birds (Konishi, 1965; Keller and Hahnloser, 2009; Tschida and Mooney, 2012), yet experimental studies on putative functions of FOXPs seldom critically assess auditory perception, feedback processing or memory establishment

and maintenance. Just like vocal motor control, these processes are necessary for vocal learning and even though not at the centre of studies often altered in comparison to control groups after genetic manipulations of orthologues *FOXP1* or *FOXP2* (Table 1, Figure 2).

Without studying the impact of *FOXP1* and *FOXP2* on auditory perception and sensory integration, their roles within the molecular framework of vocal learning cannot be fully understood. Sensory processing and experience driven response of sensory systems, known as perceptual learning are necessary to produce species-specific vocalisations during development and beyond the initial vocal learning phase. Vocal learning in general and more specifically in songbirds, consists of multiple levels. Different stages include stimulus reception by suitable sensory organs which will result in stimulation of sensory cells and ultimately stimulus perception on a cognitive level. During vocal learning in songbirds, sensory integration, auditory perception and feedback are crucial (Konishi, 1965; Brainard and Doupe, 2000; Prather, 2013; Soha, 2017; Elie *et al.*, 2019) for the establishment of a song template (Moseley *et al.*, 2017) or fine tuning of the motor program (Villain *et al.*, 2016; Rivera-Cáceres and Templeton, 2019). Ultimately, motor performance, the repertoire of vocalisations or the application of learnt rules rely on all previous steps in this vocal learning cascade. Effects of FOXP1 or FOXP2 malfunctions on initial steps of the learning process might affect later developmental stages since they are all intertwined and build upon each other.

Therefore, disturbance during any of these stages might ultimately result in a motor deficit. So far, consequences of manipulations of FOXP1 and FOXP2 have typically been investigated at the output levels of vocal learning, such as success of imitation learning in zebra finches (Haesler *et al.*, 2007; Norton *et al.*, 2019; Garcia-Oscos *et al.*, 2021), or vocal plasticity (Chabout *et al.*, 2016), vocal production frequency (Gaub *et al.*, 2010) and vocal development (Castellucci *et al.*, 2016) in mice. These studies did not allow conclusions to be drawn about whether impaired output was due to direct effects on motor performance or on other levels of vocal learning. However, the expression of *FOXP1* and *FOXP2* spans both motor and auditory areas and the phenotypes that result from impairments of these transcription factors encompass perception and production. Thus, an influence of these genes on multiple levels of vocal learning is more likely than an exclusive influence on either production or perception.

Vocal learning songbirds, such as zebra finches, are well suited to investigate the impact of FoxP1 or FoxP2 on the various stages of vocal learning. Similar to language acquisition, song learning in zebra finches involves multiple steps, from song memorisation via song practice during a subsong stage in juveniles (Doupe and Kuhl, 1999; Bruno *et al.*, 2021), with one of the main differences being that adult males only produce one song type with little variability which consists of multiple syllables within one motif that varies between individuals (Helekar *et al.*, 2000; Hyland Bruno and Tchernichovski, 2019). Even though zebra finches raised in isolation will produce a song, they require auditory input and feedback during this process in order to develop species-specific characteristics (Tchernichovski *et al.*, 2001). Disrupted auditory feedback transmission has been shown to alter song production even beyond the learning phase of songbirds (Sober and Brainard, 2009; Hoffmann *et al.*, 2012; Elie *et al.*, 2019).

In songbirds, the brain structures supporting auditory perception and vocal motor control are well described, as they can be labelled histologically which makes it possible to identify their contributions to various aspects of song learning (Scharff and Nottebohm, 1991; MacDougall-Shackleton *et al.*, 1998; Gobes and Bolhuis, 2007; Mooney, 2009). Thus, FoxP1 or FoxP2 can be locally manipulated with e.g. lentiviral knockdowns and their functions in songbird vocal learning can be further explored. Meanwhile it is necessary to also pay attention towards potential effects on sensory stimulation, stimulus perception and memory formation as well as its maintenance. This allows to evaluate if and how effects of these genes on perception and processing of auditory stimuli might eventually lead to changes in vocal production.

Studying song perception might shed light on implications of FOXP1 and FOXP2 in auditory traits

In order to study the effects of FoxP1 and FoxP2 on learning abilities and perception as well as on the processing of information, experiments have to be adapted towards skills related to perception and cognitive processing of auditory information. Such experiments often rely on operant tasks focusing on perceptual discrimination of auditory stimuli. As a common model for studying vocal learning, zebra finches have been tested for learned song preference (Miller, 1979a; Clayton, 1988; Houx and ten Cate, 1999a; Riebel, 2000). Over time, a number of sound discrimination paradigms have been established and validated such as Go/Nogo (e.g. Park *et al.*, 1985; Scharff

et al., 1998; Ohms *et al.*, 2012; Kriengwatana *et al.*, 2016) or two alternative forced choice tests (Burgering *et al.*, 2018, 2019) that can determine an individuals' abilities to discriminate and categorise auditory stimuli without relying on motor control.

Several studies have now reported song disturbances in juvenile zebra finches after experimental manipulations of *FoxP1* or *FoxP2* expression (Haesler *et al.*, 2007; Murugan *et al.*, 2013; Norton *et al.*, 2019; Garcia-Oscos *et al.*, 2021). This could have resulted from direct disturbance of vocal production. However, it is also possible that specifically during learning, nuclei with altered expression levels of *FoxP1* or *FoxP2* process auditory feedback or stimuli differently since overall motor-control during song does not seem to be affected in adult birds with FoxP1 or FoxP2 knockdowns.

This hypothesis is further supported by the findings that Area X is implicated in discrimination of familiar and unfamiliar song (Scharff *et al.*, 1998) and that adult FoxP2 knockdowns in this area lead to a lack of differences between song directed to a female and undirected song (Murugan *et al.*, 2013).

This would mean that following FOXP knockdowns adult song could deviate from the model because impairments in auditory learning and/or auditory feedback processing led to a template different from the original model. Thus, an altered template rather than impaired motor skill learning is causing the differences between model and pupil song. In consequence, impairments of different mechanisms can in principle lead to similar phenotypic effects on songs. Because of widespread downstream target genes which are regulated by FOXP transcription factors, multiple pathways are highly likely to be affected. To close this knowledge gap, experiments need to be designed which target the perceptual steps of vocal learning specifically.

Studying the contribution of FoxP1 to auditory perception in female zebra finches

Song preference learning in female zebra finches has several properties that recommend it as an experimental system to test for a functional role of FoxP1 and FoxP2 in auditory perception. Female zebra finches do not sing but like males form song memories early in life (Clayton, 1988; Houx and ten Cate, 1999b, 1999a; Riebel *et al.*, 2002). These early song memories lead to a preference for similar songs in adults (Riebel, 2000, 2003). Male and female brains exhibit anatomical differences in the song system (Fig. 3A and 3B) of zebra finches (Nottebohm and Arnold, 1976; Hamaide *et al.*, 2017; Shaughnessy *et al.*, 2019) but the expression patterns of *FoxP1*

and *FoxP2* across both sexes are similar in the brain structures that are shared by both sexes (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004).

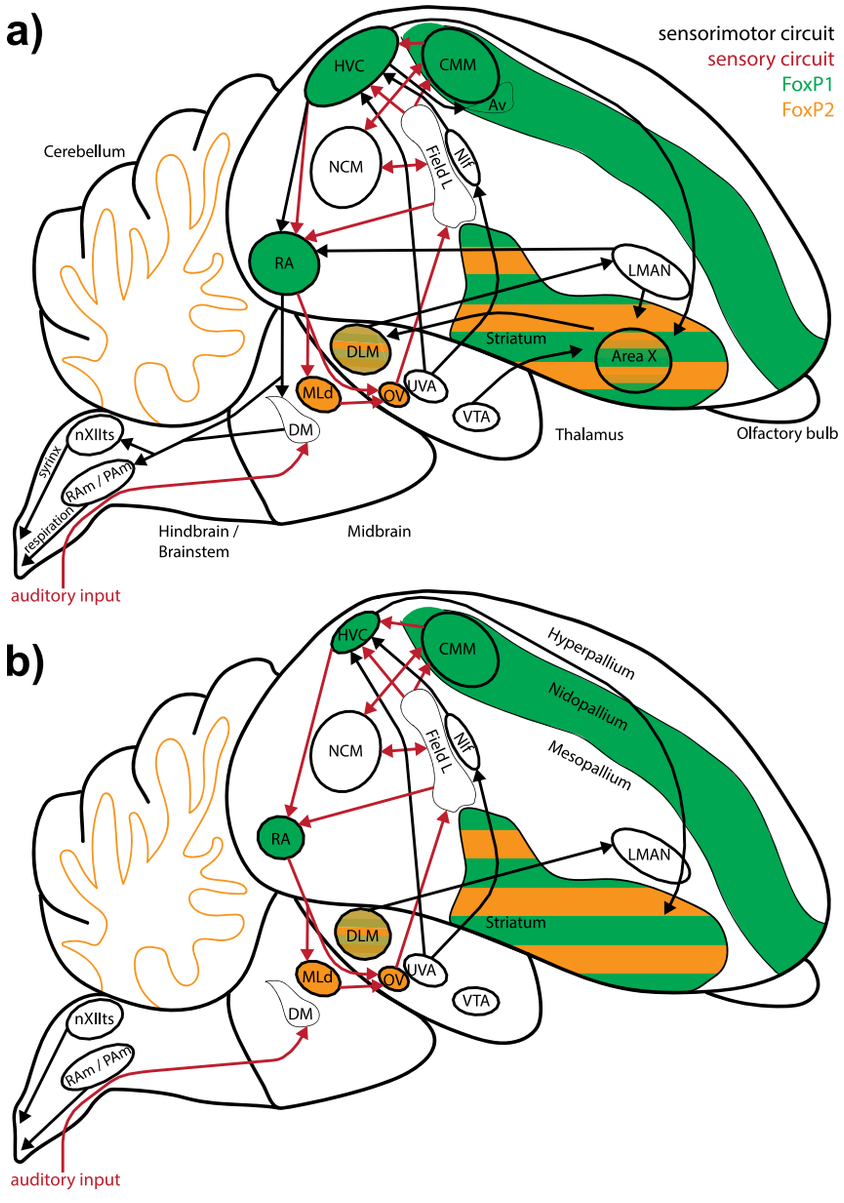


Figure 3: *Sagittal schematics of adult male (a) and female (b) zebra finch brains. Right is anterior, up is dorsal. Nuclei implicated in song learning, auditory perception or song production are outlined and named. The sensorimotor circuit which is crucial for song learning is marked with black arrows, while purely sensory connections are labelled in red. Gene expression of FoxP1 and FoxP2 is indicated by green and orange colour, respectively. Most prominently, females do not possess a functional representation of Area X and both HVC as well as RA are reduced in size. Note that the female brain has been investigated less thoroughly in comparison to the male brain. Undocumented pathways thus not necessarily indicate the absence of a projection.*

Song learning and production are supported by a well delineated sensorimotor circuit in the songbird brain (e.g. Bottjer *et al.*, 1984; Nottebohm *et al.*, 1990; Scharff and Nottebohm, 1991; Mooney, 2009; Moorman *et al.*, 2011; London, 2017). Starting during the late subsong stage during song learning, the premotor nucleus HVC connects to Area X (Kozhevnikov and Fee, 2007; Andalman and Fee, 2009; Kojima and Doupe, 2009) in the striatum (Figure 3a) and nucleus RA, a pre-motor nucleus which further transmits signals to downstream regions in the midbrain and brainstem, ultimately resulting in controlled breathing rates and song output (Iyengar *et al.*, 1999; Schmidt and Wild, 2014). Area X projects to the dorsolateral nucleus of the anterior thalamus (DLM) from where neurons project further (Goldberg and Fee, 2011) to the lateral magnocellular nucleus of the nidopallium (LMAN). From LMAN this sensorimotor circuit or anterior forebrain pathway (AFP) either propagates back to Area X or to RA, Another crucial pathway for song production is the song motor pathway (SMP) which, like the AFP, might be initiated by HVC (Mooney, 2000). HVC neurons projecting to RA (Hahnloser *et al.*, 2002) lead to a more direct song output which also shows less variability (Woolley and Doupe, 2008) and is mostly employed by males when singing to a female zebra finch (Burke and Schmidt, 2020).

Both the AFP and the SMP show pronounced sex differences in zebra finches (Figure 3). Area X is absent in females and the nuclei RA and HVC remain small in adult females that in zebra finches do not sing (Nottebohm and Arnold, 1976; Hamaide *et al.*, 2017). Brain regions responsible for auditory perception and processing exist in both sexes (Canopoli *et al.*, 2016; Boari and Amador, 2017; Shaughnessy *et al.*, 2019) and include the primary auditory area Field L, the sensorimotor nucleus interfacialis of the nidopallium (NIf) and downstream secondary auditory areas such as the caudomedial nidopallium (NCM) or the caudomedial mesopallium (CMM). Fewer projections have been investigated in the female brain (Figure 3B) so that the absence of a connection in Figure 3B does not necessarily mean an absent pathway.

Despite the pronounced song related behavioural and brain anatomical differences between male and female zebra finches, expression of *FoxP1* and *FoxP2* in the different nuclei of the song system is highly similar between sexes (Figure 3). With the exception of RA, areas related to motor control tend to show more prominent *FoxP2* expression while auditory areas express mostly *FoxP1*. Both transcription factors are

expressed in the striatum and DLM of both sexes (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015).

The premotor area HVC and the secondary auditory area CMM within the mesopallium that broadly express *FoxP1* stand out due to their similarity between sexes. RA also shows *FoxP1* expression in both sexes even though this nucleus is smaller in females (Nottebohm and Arnold, 1976). Next to the size difference, the dominant function for motor output of RA presumably excludes it from contributions to auditory related tasks in female zebra finches.

Aims and outline of this thesis

The aim of this thesis is to start uncovering the contributions of *FoxP1* to auditory perception by investigating the impacts of localised knockdowns of the gene in brain areas of female zebra finches. In order to study this, lentiviral knockdowns using short-hairpin RNAs were conducted in either HVC or CMM of juvenile or adult female birds. Juvenile females were treated at 23 days of age, prior to the onset of the sensory phase during which females establish a song memory (Clayton, 1988). Adults were subjected to a knockdown when they had reached at least 90 days of age, which is sufficient to establish a preference in females (Miller, 1979b; Clayton, 1988). Matched controls for each experimental group underwent sham surgeries and injections of control constructs, to determine exclusive effects caused by the knockdowns.

As adults, all groups were tested in two different experimental setups. For the first experiment (Chapter 2), females were transferred individually to sound attenuated chambers in cages set up for song preference tests (Figure 4a). In these tests, females could peck either one of two pecking keys to elicit a playback of a familiar or unfamiliar song. The number of times a female could elicit playbacks was not restricted, to allow for a quantification of the birds' motivation to listen to playbacks, as no other reward than song playback was provided during this task. The preference tests made it possible to assess multiple potential effects of local FoxP1 knockdowns in female zebra finches by comparing their performance with that of the control females. First, effects on memory establishment could be tested by FoxP1 knockdowns in either HVC or CMM of juvenile females. Second, potential impacts of local knockdowns of FoxP1 on maintenance of already established song memory could be assessed in adult birds. Lastly, general perception and behaviour towards two different stimuli could be evaluated during the preference tests, to determine whether local knockdowns lead to

behavioural differences beyond preference strength or the number of elicited playbacks.

After finishing the preference tests, the same birds (knockdowns or controls) were trained in a Go/Nogo paradigm (Chapter 3) in sound attenuated chambers (Figure 4b). Once the females had successfully discriminated between trained Go- and Nogo-song-stimuli, derivatives of the originally trained stimuli were introduced. These test stimuli made it possible to evaluate the females' abilities to assess the similarity of the novel song to two previously established categories. Test stimuli were pitch-modified, reversed in their syllable sequence or entirely reversed. Employing this paradigm, it was possible to investigate how local FoxP1 knockdowns in two different brain areas and during different developmental stages impacted on multiple learning parameters. First, the speed at which the females learnt to distinguish between positively and negatively reinforced stimuli could provide insights into how FoxP1 affects auditory discrimination learning. Second, the overall performance of birds towards training stimuli provides an overview of the impact of FoxP1 knockdowns on the general ability of birds to distinguish two songs. Third, categorisation of test stimuli allowed the identification of specific auditory cues which are important for stimulus discrimination and which may be potentially disturbed by local FoxP1 knockdowns. Finally, the extinction rate at which birds stopped performing according to the trained paradigm after both Go- and Nogo-stimuli were reinforced positively. In addition, the results of this experiment provided the opportunity to assess the relative impact of the different stimulus manipulations on song discrimination more generally.

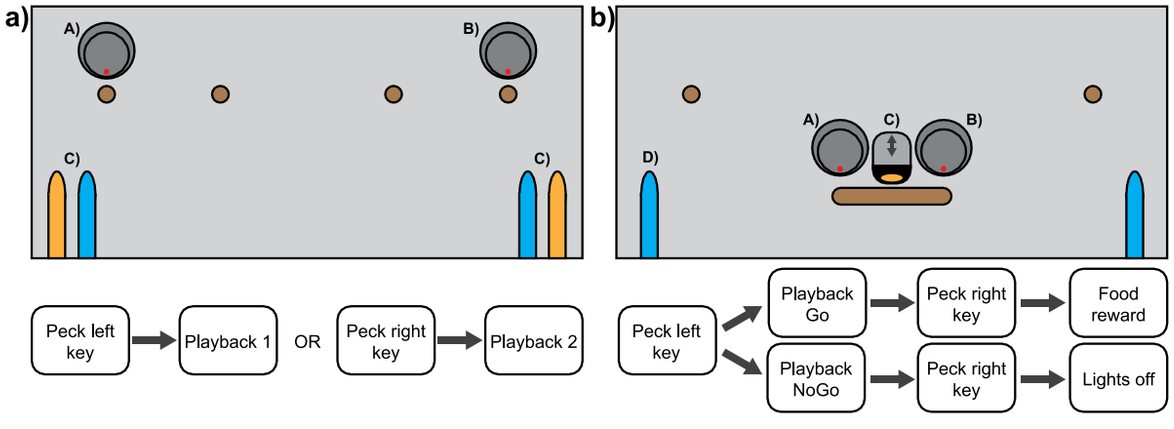


Figure 4: *Schematic drawing of the cage layout (top) and phases of the operant test paradigms used (bottom) in this thesis. During preference tests (a), birds could choose to receive one of two possible playback types by pecking either of the two keys (A, B)*

on opposite sides of the cage. On day one, the left key (A) would elicit familiar song playback while the right key (B) would elicit unfamiliar song playback. Playback identity was switched between the keys every 24 hours. Food (orange) and water (blue) (C) were available ad libitum on both sides of the cage. During Go/Nogo tests (b), birds were supposed to initiate a trial by pecking the left key (A) which elicited either a Go or a Nogo type playback. When presented with a Go-type playback, the bird was supposed to peck the right key (B) in order to obtain a food reward (orange) behind the food hatch (C). In case the presented stimulus was a Nogo-type playback, the bird was supposed to refrain from pecking the right key and initiate a new trial after a short waiting period. If the right key was pecked after a Nogo-type playback, the choice was negatively reinforced by brief lights off before the bird could reinitiate a trial. Participation in the paradigm was not limited to a particular number of trials and the only way to obtain food during this test. Water (D) was available ad libitum on both sides of the cage.

In order to identify potential genes and pathways affected by local FoxP1 knockdowns, RNA was extracted from the previously targeted brain areas and mRNA transcripts were sequenced using next-generation methods (Chapter 4). Gene expression analysis was performed to identify differentially expressed genes (DEG) linked to FoxP1 knockdowns. DEG were analysed on multiple levels with increasing specificity, starting with genes which showed generally altered expression after the knockdowns, independent of the birds' age during the injection or the target site in the brain. Subsequently, DEG specific for knockdowns in adults or juveniles and either one of the two areas were investigated. Further analyses of Gene Ontology (GO), local clusters, and gene set enrichment provided insight into molecular and cellular processes that might be most affected by FoxP1 knockdowns. Additionally, differentially expressed genes overlapped significantly with databases on previously identified genes implicated in autism spectrum disorders and intellectual disabilities. Together, this data validate previous findings on downstream effects of FoxP1 manipulations and give novel perspectives on downstream pathways regulated by this transcription factor.

In summary, in this thesis a wide range of methods was employed. First, local lentiviral knockdowns were used to decrease FoxP1 expression in HVC or CMM. Subsequently females' perceptual and behavioural performance was tested during operant tasks.

Following the behavioural assays, transcriptional profiles of neural tissue were analysed to shed new light on the pathways regulated by *FOXP1 in vivo* in the brain. This work focuses in particular on the contributions of local *FoxP1* expression in two brain regions of female zebra finches to auditory perception, memory establishment and maintenance, as well as auditory discrimination and categorisation. In Chapter 5 the findings of this thesis in relation to the current literature are summarised and discussed. The findings broaden the understanding of how FoxP1 is implicated not only in motor learning but also in auditory perception, and illuminate how this transcription factor may contribute to vocal learning and ultimately human speech and language.