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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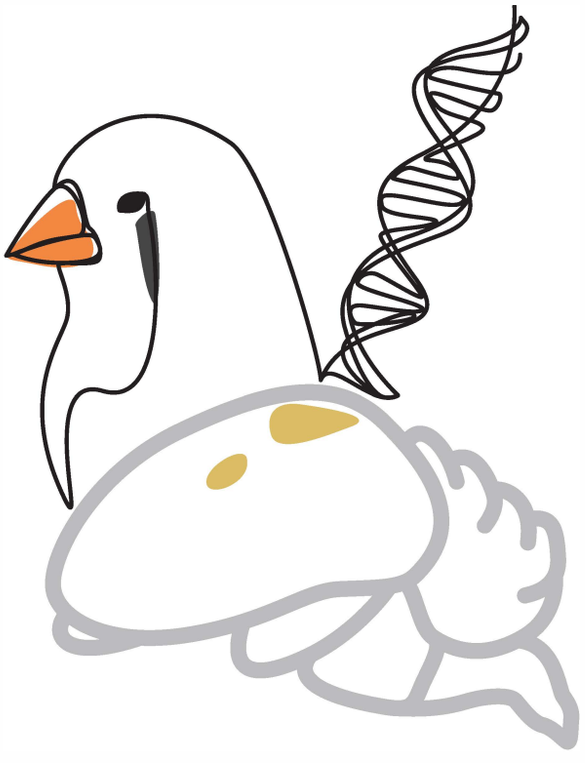
General Introduction



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).

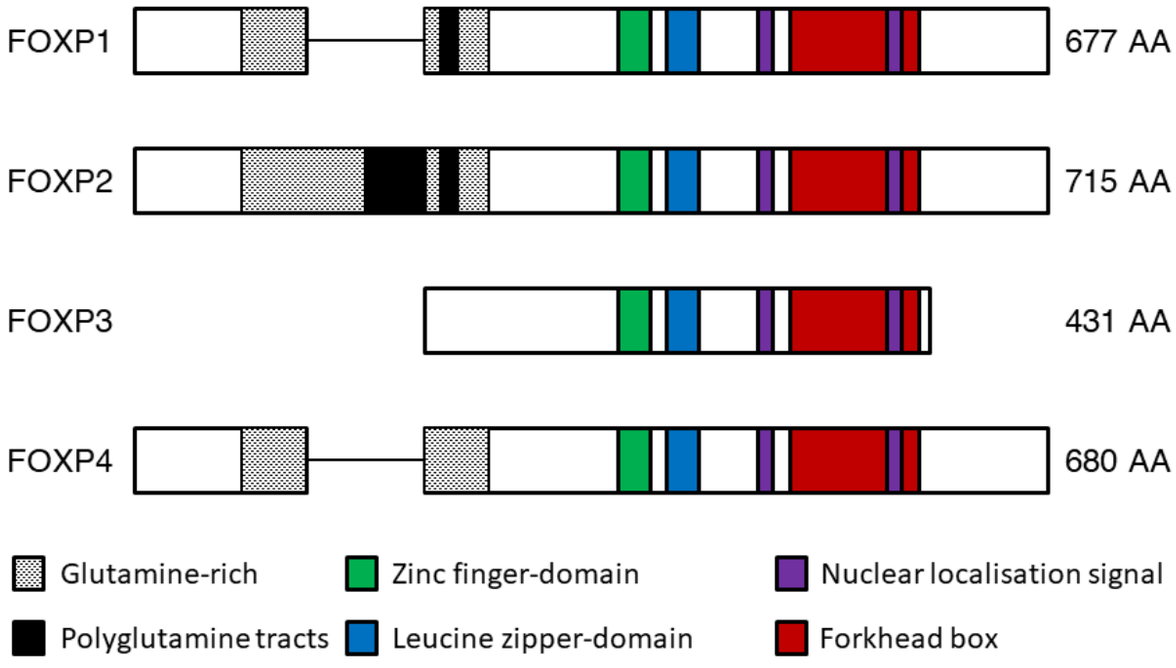


Figure 1: *Homologous structures of human main isoforms of FoxP transcription factors. All known FoxP members contain highly similar zinc-finger and leucine-zipper domains followed by nuclear localisation signals prior to and embedded in the shared forkhead box domain. Glutamine-rich regions have been 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)

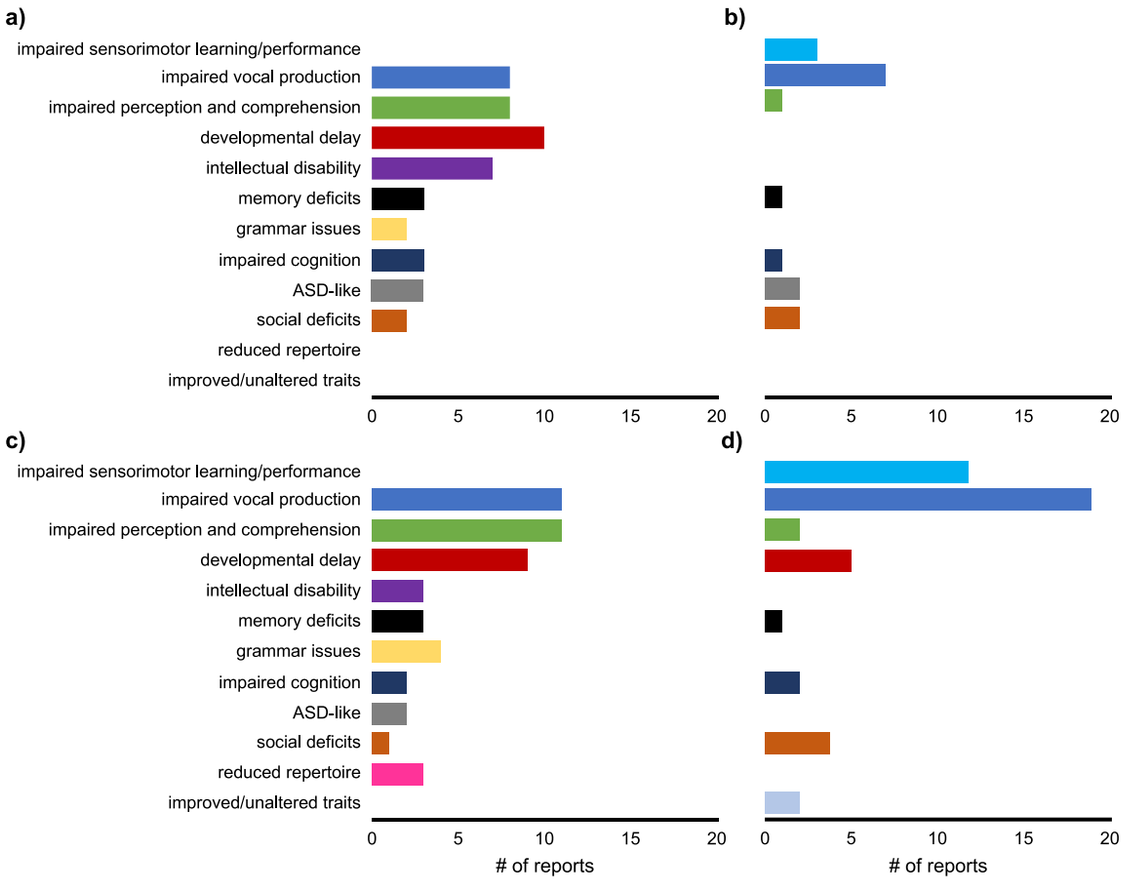


Figure 2: *Overview of documented phenotype categories in studies that assessed behavioural traits conducted on human FOXP1 (a; N =13) or FOXP2 (c) mutations (N = 14), and altered expression or modified proteins of FOXP1 (b; N = 7) or FOXP2 (d; N = 25) in model organisms. Observations were categorised 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).

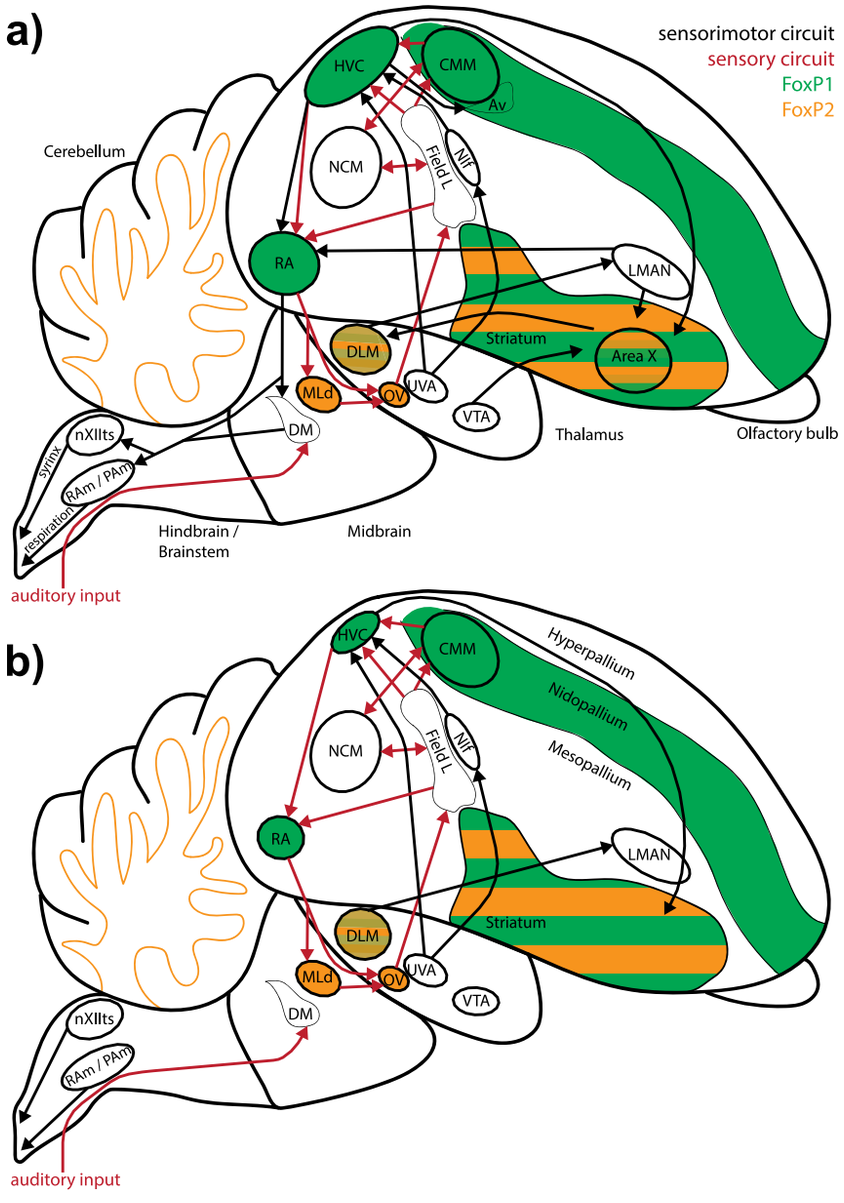


Figure 3: *Sagittal schematics of adult male (a) and female (b) zebra finch brains. Right is anterior, up is dorsal. Nuclei implicated in song learning, auditory perception or song production are outlined and named. The sensorimotor circuit which is crucial for song learning is marked with black arrows, 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.

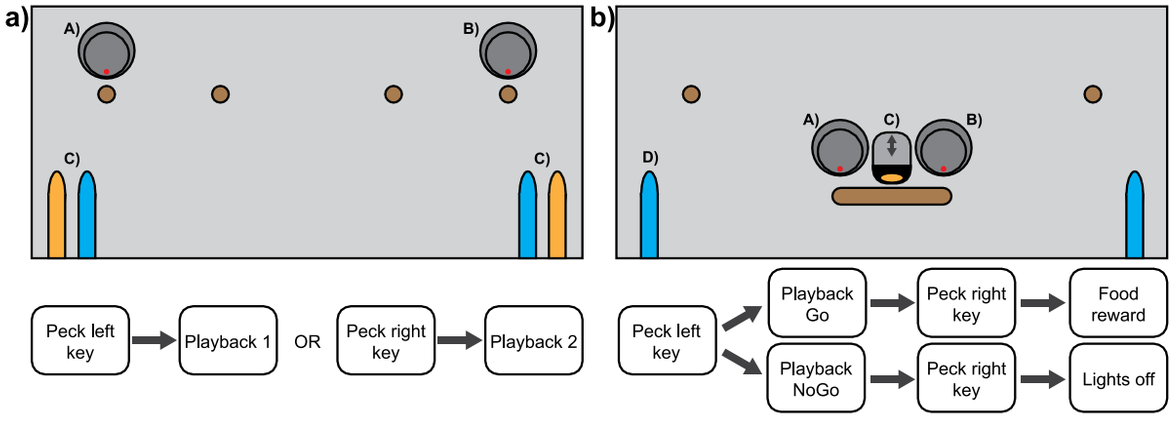


Figure 4: *Schematic drawing of the cage layout (top) and phases of the operant test paradigms used (bottom) in this thesis. During preference tests (a), birds could choose to receive one of two possible playback types by pecking either of the two keys (A, B)*

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.

Chapter 2

Effects of cortical FoxP1 knockdowns on learned song preference in female zebra finches

Chapter 2: Effects of cortical FoxP1 knockdowns on learned song preference in female zebra finches

Fabian Heim, Simon E. Fisher, Constance Scharff, Carel ten Cate, Katharina Riebel

Abstract

The search for the molecular underpinnings of human communication has recently focused on genes encoding forkhead-box transcription factors, as rare disruptions of FOXP1, 2 and 4 have been linked to disorders involving speech and language deficits. In male songbirds, a tractable model for vocal learning, experimentally altered expression levels of these transcription factors impair song learning and singing. The relative contributions of altered auditory processing, motor function or auditory-motor integration to the deficits observed after FoxP1, 2 or 4 manipulations in songbirds are not known. To examine the potential effects on auditory learning and development, this study focused on female zebra finches (*Taeniopygia guttata*) that do not sing but develop song memories, which can be assayed in adults via operant preference tests. More specifically, it was tested whether the relatively high levels of *FoxP1* expression in forebrains areas implicated in female song preference learning are crucial for the development and/or maintenance of this behaviour. Juvenile and adult female zebra finches received localised FoxP1 knockdowns in HVC (proper name) or the caudomedial mesopallium (CMM), forebrain areas important for auditory processing in female zebra fiches. Irrespective of target site and whether the knockdown took place before (juveniles) or after (adults) the sensitive phase for song memorisation, all groups preferred their tutor's song. However, adult females receiving HVC FoxP1 knockdowns showed weaker motivation to hear song and weaker song preferences than sham-treated controls, while no such differences were observed after knockdowns in CMM or in juveniles. In summary, FoxP1 knockdowns in the cortical song nucleus HVC appear to reduce the rewarding qualities of tutor songs but not their recall.

Introduction

The discovery of associations between developmental speech and language impairments and rare heterozygous mutations of the Forkhead box protein P2 (*FOXP2*) gene (Lai *et al.*, 2001) initiated investigations into the neurogenomic and molecular bases of vocal learning (reviewed in Fisher and Scharff, 2009, Deriziotis and Fisher, 2017). Mutations of *FOXP1,* a paralogue of *FOXP2*, are associated with a variable and multifaceted neurodevelopmental syndrome with a wide range of phenotypes including increased incidences of autism spectrum disorder and/or cognitive impairments which often include speech and language impairments (Sollis *et al.*, 2016, 2017). Moreover, heterozygous loss-of-function variants in another paralogue, *FOXP4*, were recently implicated in a novel developmental disorder characterised by speech/language problems and variable congenital abnormalities (Snijders Blok *et al.*, 2021). The homology between *FoxP* genes, and especially *FoxP1* and *FoxP2*, across vertebrates (Mazet *et al.*, 2003; Hannenhalli and Kaestner, 2009) spurred comparative research on the functions of these transcription factors in suitable animal models. Because the sensory and motor learning circuits that mediate avian vocal learning are well characterised (Nottebohm *et al.*, 1990; Mooney, 2009; Condro and White, 2014), bird song learning can also contribute to the understanding of sensorimotor and auditory processes of human speech acquisition, particularly since parallels between vocal learning in songbirds and humans span the range from behavioural to molecular similarities (Doupe and Kuhl, 1999; Jarvis, 2019; Bruno *et al.*, 2021). This allows for localised knockdown studies of gene expression as a means to identify neuromolecular underpinnings of vocal learning. Prior work has shown that knockdowns of FoxP1, FoxP2 and FoxP4 in Area X, a nucleus in the basal ganglia, impair song learning in juvenile male zebra finches (Haesler *et al.*, 2007; Norton *et al.*, 2019). Vocal learning requires song memorisation, sensory feedback and motor practice, and brain-expressed FoxP proteins might influence any one or a combination of these underlying mechanisms. Indeed, FoxP1 knockdowns in HVC (acronym used as a proper name) impair song learning in male juvenile zebra finches (Garcia-Oscos *et al.*, 2021). Song production is a pronounced sexual dimorphism and only males sing, but both sexes memorise tutor song as juveniles and as adults prefer to hear the songs they were exposed to early in life (Clayton, 1988; Houx and ten Cate, 1999; Riebel *et al.*, 2002; Riebel, 2003a, 2009). Song preference learning thus provides an opportunity to investigate tutor song memorisation independent of motor learning (Riebel *et al.*,

2002). *FoxP1* could be involved in this process because it is highly expressed in male and female HVC and caudomedial mesopallium (CMM) (Teramitsu *et al.*, 2004; Chen *et al.*, 2013; Mendoza *et al.*, 2015). Both brain areas are involved in song memory, auditory perception and auditory learning (Bell *et al*., 2015; Roberts *et al*., 2017; Soyman and Vicario, 2017; Inda *et al*., 2020). Other auditory areas which play important roles for song memory, preference and discrimination such as NCM or NCL show no elevated FoxP1 expression in comparison to the surrounding tissue (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015).

HVC receives input from auditory areas including the CMM (Bauer *et al.*, 2008), and projects to Area X and other areas of the song motor pathway (Mooney, 2000; reviewed in Prather *et al.*, 2017). HVC and CMM show increased neural activity in male and female zebra finches during playback of conspecific song, and this neural activity is highest in response to familiar over unfamiliar song (Terpstra *et al.*, 2004, 2006; Nick and Konishi, 2005; Kojima and Doupe, 2007; Ross *et al.*, 2017; Ruijssevelt *et al.*, 2017). Lesions of HVC in female canaries (Brenowitz, 1991; Del Negro *et al.*, 1998) or CMM in female zebra finches (MacDougall-Shackleton *et al.*, 1998) also impair conspecific song preference. The involvement of CMM and HVC in song preferences of female songbirds led us to hypothesise that the high *FoxP1* expression in these areas is important for auditory learning. By investigating this hypothesis in female zebra finches, a songbird in which females do not sing, auditory learning and memory maintenance can be studied independently of vocal motor development (Riebel *et al.*, 2002; Gobes and Bolhuis, 2007).

To test whether *FoxP1* expression in HVC or CMM is required for females to recognize and learn to prefer particular songs, FoxP1 was knocked down in these areas either before (as juveniles) or after (as adults) the sensitive period for song preference learning (Riebel, 2003b, 2009). Subsequently, auditory memories were tested in operant preference tests (Riebel, 2000; Holveck and Riebel, 2007). If expression of *FoxP1* in HVC and/or CMM were required for song memory formation or recall, then knockdown and control groups should differ in their motivation to hear song, the consistency of choice and their preference strength for tutor song. Any differences between age groups and knockdown target areas can thus inform us about locally or temporally-transient functions of FoxP1 in the development and maintenance of learned auditory preferences.

Material and Methods

Subjects and housing

Subjects were 96 female zebra finches from the breeding colony at the Freie Universität Berlin. In the colony, breeding pairs were housed in 180 x 50 x 50 cm steel-wire cages with solid floors, wood chip bedding and equipped with a nest box and nesting material. Mobile perches from different materials and water baths were provided as enrichment. Most subjects (N = 79) were raised by their biological parents and stayed with them until 90 days post hatching (dph). The remaining females (N = 17) were also raised by their biological parents but moved to foster parents at age 15 dph, i.e. before the sensitive phase for song memorisation (Roper and Zann, 2006), where they remained until 90 dph. All birds were provided with *ad libitum* water, cuttle bone, and tropical seed mix (Teurlings, Dordrecht) supplemented once a week with hardboiled egg and sprouted seeds. Bird rooms had a 12H:12H light:dark regime with a simulated dawn and dusk phase of 30 minutes each. Relative humidity was kept between 40 and 60%, and the ambient temperature was set to 22°C. Birds received surgery at different ages (details below) but were always moved within a month (range 14 – 30 days) after surgery to Leiden University (The Netherlands) for behavioural testing. At Leiden University, birds were housed in groups of two to six individuals in cages of 120 x 90 x 90 cm until testing started. Enrichment was provided in the form of swinging perches, a mirror and a water bath, and bedding consisted of a sand grit mixture. Birds were kept under a 13H:11H light:dark schedule with a simulated dawn and dusk phase of 15 minutes each. Temperature was kept between 19°C and 22°C, with a relative humidity of 45 to 52%. Birds had constant access to water, cuttle bone, and tropical seed mix (Beduco, Schoten) supplemented once a week with hardboiled egg and freshly grated apple or carrot.

Treatment groups

The four treatment groups were defined by when (as juveniles: 23 +/- 2 dph or adults: 210 +/- 124 dph) and where (HVC or CMM) they received the FoxP1 knockdown and labelled accordingly: HVC juvenile, HVC adult, CMM juvenile, CMM adult (for details see ‘virus generation’ and ‘surgery’ paragraphs). Due to a logistic cap on how many birds could be bred and treated simultaneously, not all experiments could be in parallel. To prevent the timing of breeding from influencing the outcomes of the comparisons between treated females and matched controls, fledging females were assigned to a

treatment and a matched control group on an alternating basis (assigning sisters to matched treatment and control groups wherever possible) until a sample size of N = 2 x 12 was reached for a treatment and its matching control group. Newly fledged females were then assigned to the next treatment and matched control group in the order of HVC adult, HVC juvenile or CMM adult and last CMM juvenile.

Virus generation

Viral particles for injection were produced in Berlin as previously described (Haesler *et al.*, 2007; Norton *et al.*, 2019). In total, three different constructs were prepared. Two of these constructs lead to expression of short-hairpins decreasing the expression of *FoxP1* via RNA interference and GFP as a marker of transduction. Two different shRNAs were used to reduce the impact of off-target effects on the behavioural analyses (Rossi *et al.*, 2015; Song *et al.*, 2015). The third construct was a control construct which only leads to GFP expression. The sequence of the two short-hairpin constructs is as follows:

	Hairpin sequence, see also Norton *et al.*, 2019
Construct 1 ("*shY31*")	5'-CCCCTATGCAAGCAATGCACCCAGTGCATG TCAAAGAAGAACCATTAGACCCAGATGAAA-3'
Construct 2 ("*shKRAK*")	5'-CCAGATGAAAATGAAGGCCCACTATCCTTAGTGACAACAGCCAACCACAG-3'

The experiments were run with one of 7 virus batches for each knockdown construct and one of 5 virus batches for the control construct, respectively. Each virus batch was used on an average of 4 birds (range 2 – 6) yielding an average of 6 (range 3 – 9) different virus batches per age/area treatment group plus matched control. This allowed maximal spread of virus batches across treatments and to obtain similar numbers of experimental and control animals within the same batch (for details see supplementary table 2). In every cohort, similar numbers of experimental and control subjects (ranging from 1 – 12 per virus batch) were reared together and received treatment or control injections with viral constructs of one batch within 7 to 14 days. Additionally, the two different knockdown shRNAs were divided equally among the cohorts to reduce the impact of off-target effects on the subsequent behavioural analyses (Song *et al.*, 2015).

Stereotaxic Surgery

Prior to surgery, birds were caught individually from their home cage and weighed, and then received Rimadyl as analgesic (Pfizer, New York, 5ml per gram bodyweight) intrapectorally, after which they were immediately returned to their home cage for 30 min until the analgesic took effect. The animals were then transferred in a mobile bird cage to the injection lab where they were anaesthetised with isoflurane (Dräger, Lübeck) via a beak mask. The initial level of isoflurane was between 3 and 4% (depending on the bird's weight) and was subsequently lowered to 1.5 to 2% at a flowrate of 1l of O_2 per minute.

As soon as a bird was deeply anaesthetised, it was fixed in a stereotactical apparatus (myNeurolab, St. Louis) connected to an injector (M-152, Narishige, London). All feathers at the back of the skull were removed with blunt tweezers and the area was sterilised with 70% EtOH. Subsequently, an approximately 4 mm horizontal incision was placed into the skin to allow for a longer vertical cut of the skin of approximately 1 cm. Within this opening, a rectangular piece (approximately 1.5 x 1.5 mm) of the skull bone was dissected and pushed under the surrounding skin to prevent it from drying out. The opening in the skull was located around the bifurcation of the midsagittal sinus which was optically determined after bone removal. The dura mater was kept in place and only punctured locally with the injection glass capillary (30 µm tip), which was used to inject 0.25 µl of virus with a titre of > $1x6^{10}$particles per µl bilaterally into each injection site (Table 1) based on coordinates determined by previous injections using FluoSpheres (F8842, Thermo Fisher Scientific, Waltham) diluted 1:10 in 1x phosphate buffered saline (HVC: Fig. 2e, CMM: Fig. 2k).

Injection coordinates	Juvenile HVC	Adult HVC	Juvenile CMM	Adult CMM
Anterior/Posterior	0 / 0.15	0/0.2	1.4/1.5	1.5/1.6
Medial/Lateral	1.9	2	1.1/1.0/0.9/0.8	1.2/1.1/1.0/0.9
Dorsal/Ventral	0.35/0.25	0.4/0.25	0.65	0.7

Table 1: *Coordinates for viral injections [mm]. Medial/Lateral coordinates are indicated as negative and positive for the left and right hemisphere, respectively.*

After each injection, the glass capillary was kept in place for 30s to allow pressure to normalise around the injection site before moving to the next site. For each operating session, the first injected hemisphere was chosen pseudo-randomly and subsequently left and right hemispheres were injected alternatingly. After the injections, the bone

was moved back into place and the skin incision was closed by overlapping its edges and gluing it with Collodion (nitrocellulose, Sigma-Aldrich, St. Louis). As soon as the incision site was fully closed, isoflurane was reduced to 0% and the oxygen level increased to 2% to cancel anaesthesia until the bird was fully awake (range = 39 – 103 minutes, average = 57 min after anaesthesia was initiated). Birds were then returned to the colony and checked every hour during the rest of the day. Adult subjects were returned to all-female aviaries (2 x 2 x 3 m, with N = 15 – 30 birds per aviary). Juveniles were returned to their (foster-) parents and siblings and remained in their family group until 90 dph to be then moved to all-female aviaries. All birds were seen to move, eat, fly and socialise within one hour after surgery and were behaviourally indistinguishable from non-operated birds the day after surgery.

Stimulus songs

Following the Leiden lab's established protocol for song preference testing (e. g. Riebel, 2000; Riebel *et al.*, 2002; Holveck and Riebel, 2010, 2014), stimulus sets consisting of the song of the female's father and an unfamiliar male were assembled as follows: songs of all (foster-) fathers were recorded when the pair was not breeding. For recording, males were first transferred individually from their home cage (90 x 35 x 45 cm) to a recording cage (40 x 30 x 40 cm) in a sound attenuated chamber (60 x 60 x 80 cm) in the afternoon to acclimatise. Recordings started the following morning until several long bouts of song were obtained. If a bird did not sing during the first morning it was kept in the recording chamber for an additional morning. Song was recorded with cardioid microphones (ME 64, Sennheiser, Wedemark-Wennebostel) mounted in front of the cage at a 20 cm distance from the perches and written directly onto a hard disk (Aardvark Direct Pro Q10 soundcard, Middlefield, sampling rate 44.1 kHz, 16 bits) using SAP software v. 2011 (Tchernichovski *et al.*, 2000) with automatic energy detection settings for 2 – 10 kHz, detection limits between 3 – 60 s and a buffer of 5 s. Recordings were screened using spectrograms (sample rate 44.1 kHz, FFT size 1024 bits, step size 0.1 µs, frequency resolution 0.0001 Hz, time resolution 0.1 ms, 20 kHz bandwidth, Blackman window, produced with the software Syrinx 2.6h, John Burt, University of Washington, Seattle) to visually identify the most frequent motif of each male, defined as the most common sequence of syllables in ten song bouts. For each male, a song with four to seven repetitions was selected. The songs of the females' respective (foster) fathers served as 'familiar' stimuli, while the songs of other fathers

were used as 'unfamiliar' stimuli. Familiar-unfamiliar stimulus sets were formed by matching pairs of songs that were as similar as possible in the number of syllables (average +/- standard deviation 4.8 +/- 0.6) and motif repetitions (5.4 +/- 0.9), as well as in overall song duration (Fig. 1a and b). The selected songs ranged from 5.0 – 6.87 s in duration (average: 5.72s +/- 0.51s) but within matched pairs total duration did not differ by more than 5.2 %. Where possible (N = 63/96 birds), each stimulus set was used for the daughters of both males that contributed the songs. Playbacks for the remaining 33 birds (juvenile HVC: 1 controls, 3 knockdowns; adult HVC: 6 controls, 5 knockdowns; juvenile CMM: 4 controls, 2 knockdowns; adult CMM: 5 controls, 7 knockdowns) consisted of the respective females' fathers' songs and the unfamiliar song which matched best in length and number of elements and motifs. This design ensured that each song was equally often (and in the same combination) offered as familiar and as unfamiliar song (for complete list and details, see supplementary table 1).

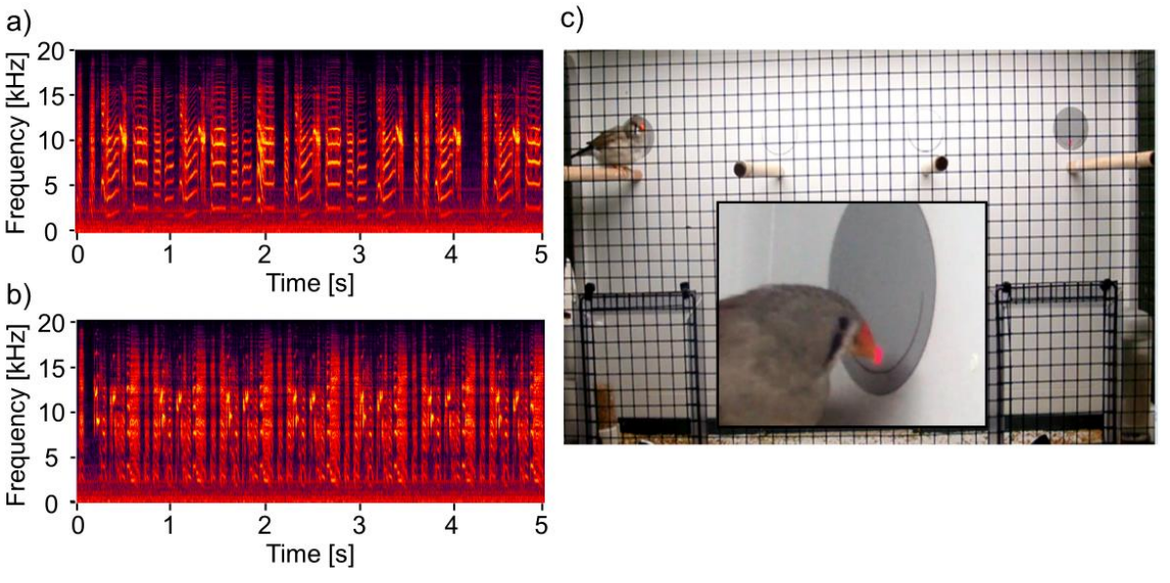


Figure 1: *An example of a stimulus set used for playback and the testing setup. a) & b) two spectrograms (frequency over time) of two song stimuli used in the preference test. Colour levels indicate local power distribution, the brighter the colour the higher the power at a specific location. c) shows a female zebra finch in the operant preference test setup. The top right shows the grey pecking disk with one of the red LED lights, the second pecking key is located behind the female on the outer left perch. The central inlay shows a close-up of the female pecking one of the keys.*

Operant song preference tests

Behavioural testing started 14 – 28 days after the birds had been transported to Leiden University (The Netherlands). Birds from the juvenile groups were between 104 – 130 days old and those from the adult groups 121 – 505 days old. For preference testing a validated operant song preference paradigm was used (Riebel, 2000; Holveck and Riebel, 2007), taking advantage of the positively reinforcing qualities of song. Briefly, females learned that pecking the operant keys elicited song playbacks. Key-pecking was voluntarily, and throughout testing, birds had *ad libitum* access to water, food and grit. To start training, individual females were moved into an experimental cage (the 'Skinner box', see Fig. 1c) in one of 20 sound attenuated chambers (min. size 2.4 x 1.4 x 2.3 m) between 3 – 5 pm. The experimental cages (70 x 30 x 45 cm) were made from wire mesh but for the floor and a solid plywood back panel with two integrated pecking keys (a 5 cm diameter piezoelectric plate with an embedded 5 mm diameter red LED at the bottom). The pecking keys were connected to custom made control devices (Leiden University electronics workshop) containing Oki MSM6388 soundchips (Tokyo) which were individually controllable via laptops (Sony Vaio E series, Sony, Minato) from outside the training chambers via custom software (Leiden University electronics workshop). The laptops that were connected to the Skinner boxes controlled the playbacks from a loudspeaker (Vifa 10BGS119/8, Viborg) suspended from the ceiling at 1 m above the centre of the cage. The custom written software kept a data log of occurrence and time of each key peck and the associated playbacks. Sound amplitude of stimuli was adjusted to peak levels of 70 dB re 20 µPa (set to continuous fast measurements over 5 s, RION NL 15, Kokubunji) at the perches near the pecking keys.

To start training, females were first left to acclimatise to and explore the new environment for one to two days (41+/- 3h), as earlier work with this setup showed that about 30% of females discover that key pecking triggers song playback by autoshaping (e. g. Holveck and Riebel, 2014). Therefore, during this phase, the red LEDs on the pecking keys were switched on continuously and the setup was operational during hours with lights on to provide immediate feedback should a bird start pecking the keys. To avoid exposing the females to the test stimuli before testing started, pecking either key during this phase triggered playback of the song of an unfamiliar male zebra finch until the females were actively pecking each key at least ten times per day. A total of 46/96 birds reached this criterion during the initial combined acclimatisation and

autoshaping phase of one to two days (juvenile HVC: 7 of 12 birds in the control group, 6/12 Knockdown; adult HVC: 4/12 Control, 6/12 Knockdown; juvenile CMM: 7/12 Control, 7/12 Knockdown; adult CMM: 4/12 Control, 5/12 Knockdown). The birds that had not reached criterion at this stage (50/96) received two 20 min training sessions per day between 9:00 – 11:00 AM and 3:00 – 5:00 PM for a maximum of five training days. Training used stepwise shaping by the experimenter by rewarding the bird with song for approaching a key, moving their head towards the pecking key or touching the area around the key with their beak (Holveck and Riebel, 2014). Birds that had not reached criterion after five days (19/96) were returned to their home cages, and after a seven-day resting period moved back into the Skinner box setup to start the training cycle again. The birds that still had not reached criterion after two training sessions (9/96), received a second resting period and a third training cycle, now with seeds of Japanese millet (*Echinochloa esculenta*) fixed with clear tape on top of the pecking keys. All remaining birds reached criterion this way.

The actual preference tests started the day after the birds reached criterion. The Skinner box was now programmed such that pecking of one key resulted in playback of the song of a female's tutor (the father or foster-father which was present between 23 to 90 dph) or an unfamiliar song (the tutor song of another experimental female which was tested with the same stimulus combination in a matched-pairs design). This way each song was tested as a familiar song for one female and as an unfamiliar stimulus for another female. Assignment of stimulus songs was pseudo-random on the first day of testing and afterwards songs were swapped between the pecking keys every 24 hours (during lights off). This way, each stimulus was presented an equal number of times at either side of the cage during the four-day long preference tests, thus controlling for potential individual side preferences that could confound song preferences.

Brain extraction

After behavioural testing for preference, females were transferred into another behavioural experiment with a Go/Nogo paradigm (Chapter 3 of this thesis), to examine whether knockdowns affected auditory discrimination learning. After all experiments were completed, females were returned to their home cages where they were housed with 2 - 5 familiar females for at least one week. Between 3 – 5 pm on the day prior to brain extraction, birds were individually transferred into familiar sound attenuated

chambers. The next morning (between 6:30 and 6:50 AM), the birds were sacrificed with an overdose of isoflurane gas and subsequently their brains were extracted before lights went on in order to minimise activity dependent expression changes. Age at brain extraction for juvenile groups was between 179 – 210 dph and between 165 – 579 dph for adult groups. Hemispheres were separated along the midline and frozen in Tissue Tek Optimal Cutting Temperature Compound (OCT, Sakura, Leiden) on dry ice and stored at -80°C until they were moved on dry ice to the Language and Genetics Department at the Max Planck Institute in Nijmegen, the Netherlands, for further processing.

RNA extraction

To determine the extent to which the injection of shRNA reduced the FoxP1 expression, qPCR of the targeted tissue was performed. To do so, RNA was extracted from brain punches. For this, brain hemispheres were first embedded in OCT and sliced sagittally in a cryostat into 200 µm sections. For each section, HVC or CMM was manually punched out with biopsy punchers (0.35 – 0.75 mm diameter, WPI, Sarasota, USA) and immediately submerged in RNAlater (AM7021, Thermo Fisher Scientific, Waltham) to prevent RNA degradation. The remaining slice was fixed in 4% fresh, ice cold PFA to validate the punching site, see 'Validation' below.

Correctly punched tissue, determined by GFP fluorescence examined under a stereo microscope (Fig. 2f, l) and immunohistochemical profile (see below) was pooled by hemisphere and RNA was extracted with a column-based RNA extraction kit for low amounts of tissue following the protocol (RNeasy micro plus, Qiagen, Hilden). RNA concentration was quantified with a Bioanalyser RNA kit (Biorad, Hercules) system and extracted RNA was stored on -80°C until further use. After RNA extraction and quantification, 10 ng of each sample were used for reverse transcription. Superscript III enzyme kit (Thermo Fisher Scientific, Waltham) was used according to the manufacturer's protocol. cDNA was kept frozen on -20°C until further use. Thawed cDNA samples were diluted 1:5 in molecular grade water prior to qPCR. 2 µl of cDNA sample were mixed with 2 µl of molecular grade water, 5ul of iQ SYBR Green Supermix (Biorad, Hercules) and 0.5 µl of 600nM forward and reverse primers, respectively (for a detailed list of used primers, see supplementary table 3). The PCR cycling conditions were as follows: 300 s at 95°C, 40 cycles of 30 s 95°C, 30 s 60°C, 45 s 95°C. Hydroxymethylbilane synthase (HMBS) served as a housekeeping gene to normalise

gene expression (Olias *et al.*, 2014). All samples were run in triplicate to generate average Cq values.

Validation of correct targeting

The tissue surrounding the punch was examined for the correct location (Fig. 2d, j). Additionally, after each 200 µm slice used for punching, an 8 µm slice was cut for immunohistochemical detection of GFP expression in HVC or CMM. Thin 8 µm slices were thawed and fixed for 10 min in fresh, ice cold 4% PFA on 4°C, and kept in the dark at 4°C until further processing. After fixation, immunohistochemical staining for GFP, Hoechst, zRAlDH and FoxP1 was conducted to validate the injection site of the virus.

Immunohistochemistry

To validate the localisation of the injected virus construct, triple immunohistochemistry analyses was conducted on cryostat sections. Slices were fixed in 4% PFA in 1x PBS for 10 minutes at 4°C and blocked in 10% ROTI Histol (Carl Roth, Karlsruhe) solution between stainings. The following antibodies were used: a mouse monoclonal (JC12) antibody against FoxP1 (1:100, ab16645, Abcam, Cambridge), a goat antibody against zRalDH (1:50, sc.22591, Santa Cruz, Dallas) to delineate HVC, and a rabbit GFP antibody (1:100, ab6556, Abcam, Cambridge) to increase signal strength from virally transmitted GFP. Ultimately, slices were counterstained with Hoechst (Sanofi, Paris) and mounted for fluorescence imaging (Apotome, Zeiss, Oberkochen).

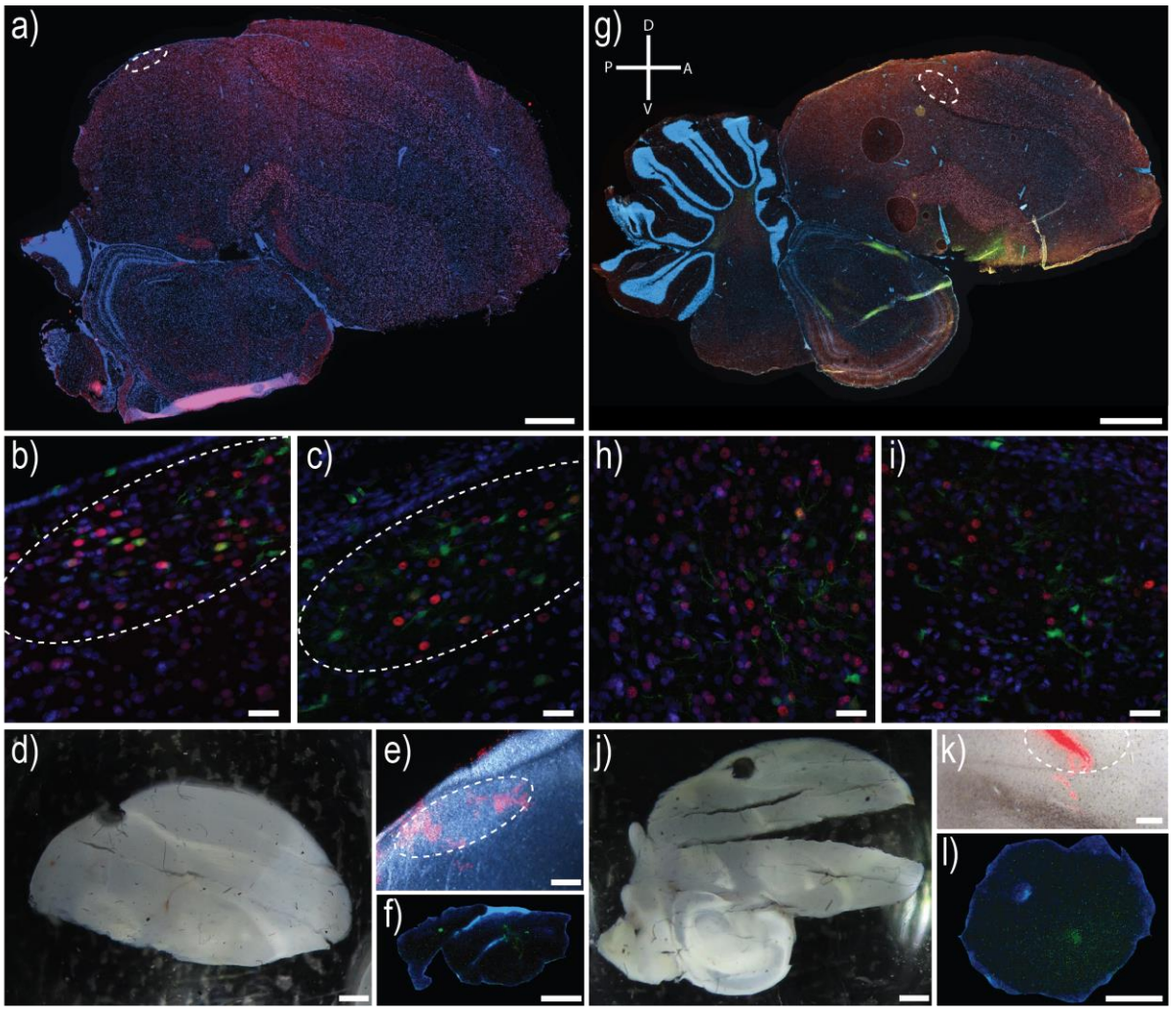


Figure 2: *Immunohistochemistry of an HVC (a – c) and CMM injected bird (g – i) after the experiments where completed. Note, that a successful expression of the knockdown construct does not eliminate FoxP1 immunostaining and that the intensity of GFP expression varies across cells and stainings. Shown are merged stainings of GFP (green), FoxP1 (red) and Hoechst (blue). a) Stitched sagittal overview indicating FoxP1 expression throughout the brain of an HVC injected bird, dotted line indicates HVC. b) Close-up of the injection site at HVC of a bird injected with the control construct, dotted line indicates HVC. c) Close-up of the injection site at HVC of a bird injected with a knockdown construct, dotted line indicates HVC. d) Validated punching site of HVC after experiments where completed. e) Dark-field image of previously injected fluorescent beads into HVC to validate coordinates used for viral injections. Dotted outline indicates HVC. f) Confirmation of GFP-fluorescence within the extracted tissue punch of HVC. g) Stitched sagittal overview indicating FoxP1 expression throughout the brain of a CMM injected bird, dotted line indicates CMM. h) Close-up of the injection site at CMM of a bird injected with the control construct. i) Close-up of the*

injection site at CMM of a bird injected with a knockdown construct. j) Validated punching site of CMM after experiments where completed. k) Bright-field image of previously injected fluorescent beads into CMM to validate coordinates used for viral injections. Dotted outline indicates position of CMM. l) Confirmation of GFP-fluorescence within the extracted tissue punch of CMM. Scalebars in a, d, g, j = 1000 µm; b, c, h, l = 20 µm; e, f, k, l = 200 µm.

Statistical analyses

The total amount of keypecks (activity) and the proportion of keypecks for the tutor song (preference strength) were used as primary response variables to gauge motivation to hear song and as a validated measure of song memorisation (Riebel, 2000; Terpstra *et al.*, 2006; Holveck and Riebel, 2007). Consistency of females' behaviour was assessed by calculating the repeatability of these two variables over the first and second block (day 1 – 2 and day 3 – 4 respectively) of the four-day preference tests. As these measures were highly repeatable across the two blocks (details see results), data from day 1 – 2 and day 3 – 4 were subsequently pooled to calculate one overall preference and one activity value based on the total keypecks during the 4-day tests for all subsequent analyses.

Preference strength for familiar versus unfamiliar song was calculated by dividing the total number of pecks for the familiar song by the total number of keypecks as follows: $\frac{\sum_{i-k} keypecks\ familiar\ song}{\sum_{i-k} keypecks\ total}$ (i = first, k = last day of the preference test). To ensure properties of normal distributions for analyses, all preference values were arcsine of the square root transformed (recommended transformation for proportional data that are bound between 0 and 1 centred around the mean) while keypecks were base 10 logarithmically transformed to adjust for the right skew of the data.

Subsequently, data were checked and confirmed to be normally distributed using Shapiro-Wilk tests (preference: $W(96) = 0.97$, $p = 0.06$; keypecks: $W(96) = 0.98$, $p = 0.08$, Shapiro and Wilk, 1965) in R v3.5 (R Development Core Team, 2011).

Keypecking was compared between knockdowns and controls per treated brain area (CMM, HVC) using generalised linear mixed models (glm2, v1.2.1 (Marschner, 2011)) based on Gaussian distributed data. Preference strength for tutor song in all treatment groups was likewise analysed separately for HVC- and CMM-injected groups, using GLM assuming a binomial distribution of data. To test whether preference strength

deviated from a 0.5 chance level, 0.5 was subtracted from each female's preference value (if females have no preference, proportions of pecks will be 0.5 for both the familiar and unfamiliar song). The values for the deviation from a 0.5 chance level were then used as response variable of a mixed linear model (lme4 (Bates *et al.*, 2015)) with only random effects (bird ID, virus batch) to test whether the intercept deviated significantly from 0 (model A) which corresponds to a significant preference for one song category. Subsequently, it was tested whether females with different treatment (knockdown or control) differed in preference strength by adding treatment as a fixed factor (model B). The next hypothesis assumed that age at FoxP1 knockdown has an effect by adding age at treatment (juvenile or adult) as an additional fixed factor and also investigated the impact of an interaction between age and treatment to account for behavioural variability (model C). Models to determine whether the efficiency of the FoxP1 knockdown was predictive of pecking activity or preference strength were also based on GLM with Poisson distributed data. These models included virus batch as random effect, relative FoxP1 expression in comparison to the respective matching control group, age and area of injection as well as their interaction as fixed factors. As the dependent variable, total number of pecks or preference strength for familiar song were included.

For post-hoc testing of the models, two sample t-Tests of controls and knockdowns of specific groups were conducted where necessary. Multiple t-Tests were corrected for false discovery rate (Benjamini and Hochberg, 1995). ANOVAs were conducted to determine the knockdown efficiency across treatment groups, as well as possible effects of knockdowns on learning speed. Post-hoc correction was conducted with Tukey's multiple comparison test (Tukey, 1949).

Ethical statement

All experimental procedures were 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) under REG 0019/15. All experiments at Leiden University were approved by the Animal Experimentation Committee at Leiden University (DEC license 14234) and by a license of the Ministry of Infrastructure and Environment (GGO license 14-097) in accordance with Dutch laws.

Results

Learning speed

There was no difference in how many days with or without training the birds needed in order to successfully peck the keys for song reward among any of the treatment groups (Knockdowns: mean +/- s.d. 3.7 +/- 3.4 days to criterion, Controls: 4.2 +/- 3.9 days to criterion, Two-way ANOVA $F(1,95) = 0.01$ $p = 0.98$). Neither treated area ($F(1,95) = 0.65$ $p = 0.42$) nor the birds' age during the injection ($F(1,95) = 2.1$ $p = 0.16$), nor an interaction between age and injected area ($F(1,95) = 0.001$ $p = 0.97$) affected the number of days to reach criterion.

Similar to the number of days, the required training sessions in case a bird did not start to peck the provided keys on its own did not differ between knockdowns and matched controls (Knockdowns: 3.4 +/- 5.6 sessions, Controls: 4.8 +/- 7.4 sessions, Two-way ANOVA $F(1,95) = 0.03$ $p = 0.86$). Neither were necessary training sessions affected by the treated area ($F(1,95) = 1.52$ $p = 0.22$) nor by the birds' ages at the time of injection ($F(1,95) = 1.37$ $p = 0.25$) or the interaction between age and injected area ($F(1,95) = 0.029$ $p = 0.87$).

Repeatability

The comparison of the first versus the second block (i.e. day 1 – 2 vs. day 3 – 4) of the actual preference tests showed females in both control and experimental groups to be consistent in their pecking activity and preferences. Total number of keypecks (Fig. 3a) and preference strength (Fig. 3b) for familiar song were highly repeatable between block 1 and block 2 (keypecks controls: Pearson's $r(48) = 0.84$, $p < 0.001$, knockdowns: $r(48) = 0.75$, $p < 0.001$; preference controls: $r(48) = 0.76$, $p < 0.001$, knockdowns: $r(48) = 0.75$, $p < 0.005$). Further analyses were thus conducted with the totals of days 1 – 4.

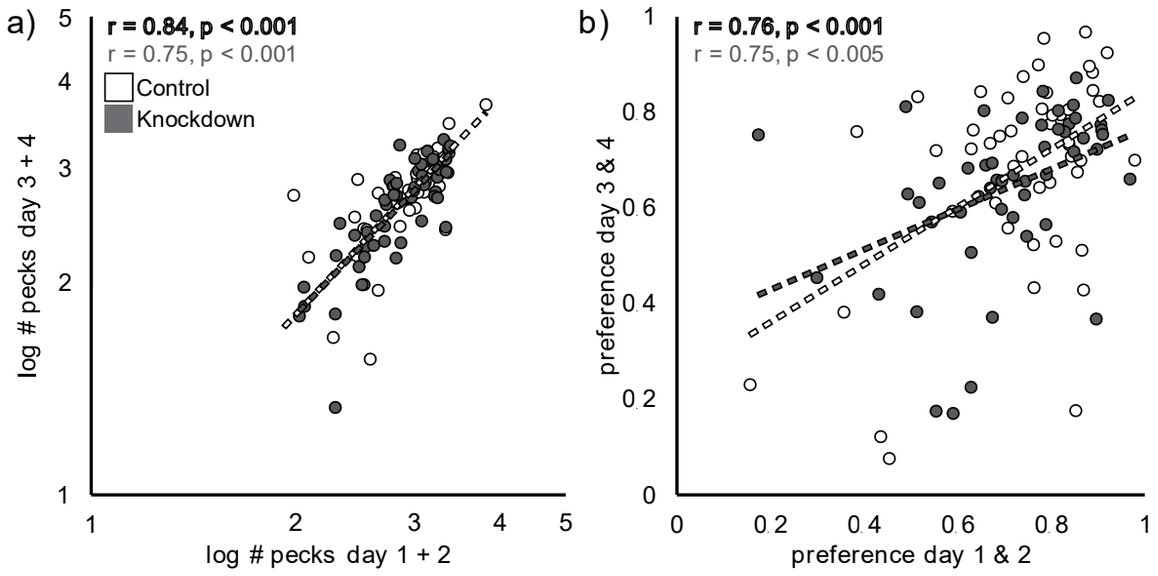


Figure 3: *Individual behaviour of birds was highly repeatable across preference testing days. Scatterplots for pecking (a) and preference (b) behaviour of all tested animals across days. Linear regressions across all controls or knockdowns are indicated by dotted lines.*

Pecking activity

During the four days of preference tests, females of all treatments (N = 96) initiated song playback by keypecking for a total of on average 1811 +/- 1514 times over the four-day testing period (range: 91 to 11,767, for group averages see Table 2). Birds that had received the viral FoxP1 knockdowns in CMM either as juveniles or adults did not differ from the controls in pecking activity (GLM total number of keypecks, see Table 3, Fig. 4a). The best fitting model for pecking behaviour of CMM injected birds contained neither treatment nor age and both factors contributed minimally to the model's weight. Birds that had received the viral FoxP1 knockdown injections into HVC showed a significant interaction of treatment and age in the best model fit (see Table 4, Fig. 4c) suggesting that both factors significantly contributed to the pecking activity, as models without both factors had a lower weight. Post-hoc analyses revealed that this effect resulted from adult birds with a FoxP1 knockdown in HVC pecking significantly fewer times than their matched controls (see Fig. 4c, two-sample t-Test $t(24) = 2.67$, $p = 0.015$). This difference was not observed in the females that had received knockdowns in HVC as juveniles (two-sample t-Test $t(24) = 0.22$, $p = 0.39$).

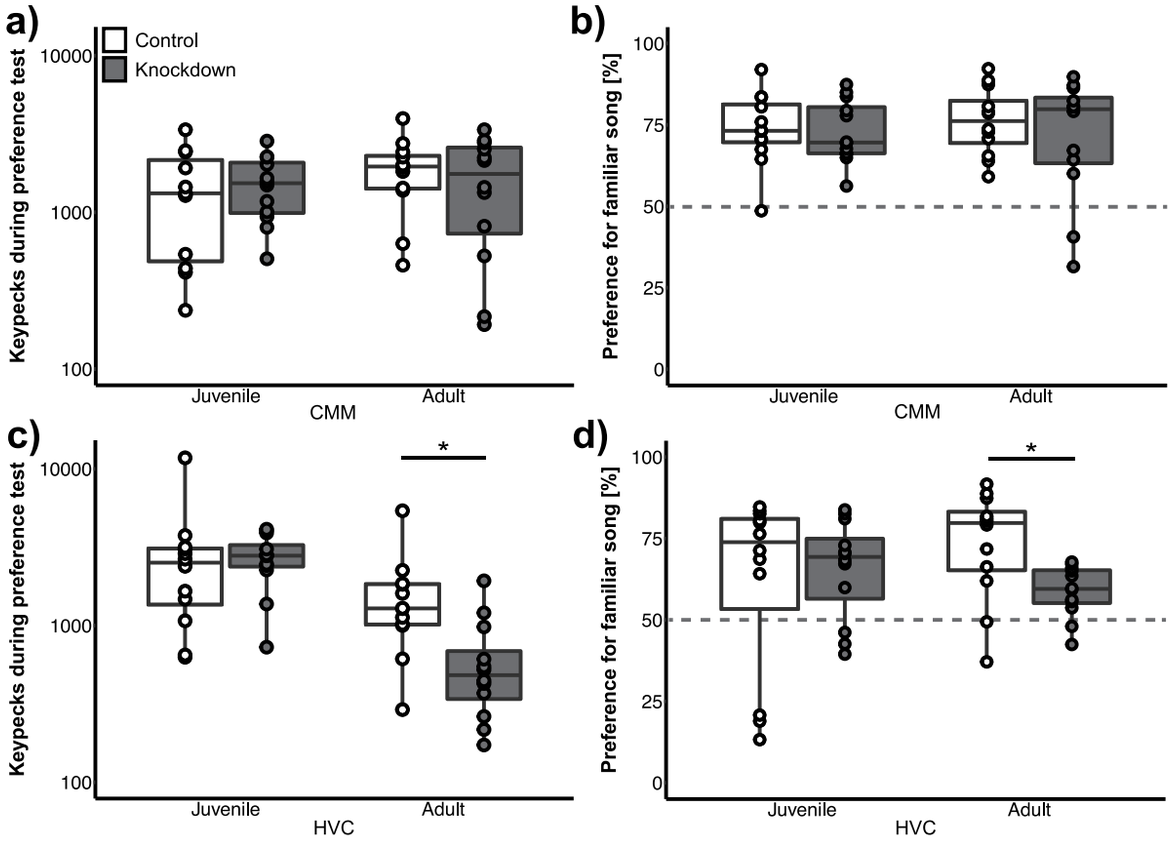


Figure 4: *Behavioural data of all birds during preference tests. Boxplots indicate the interquartile range from first to third quartile, the median is shown by the bold horizontal line while whiskers show the 1.5-fold interquartile range. Overlaying points depict data from individual birds. Chance level during the preference test is indicated by the dotted line. Significant differences of $p < 0.05$ are indicated by an asterisk. For detailed statistics on pecking behaviour refer to Tables 3 and 4. Statistics on preference behaviour are shown in Tables 5 and 6 for preference. a) Total number of keypecks (sum test-days 1 – 4) of birds injected into CMM with control or knockdown constructs. b) Preference strength for familiar song per treatment group of birds injected into CMM. c) Total number of keypecks (sum test-days 1 – 4) of birds injected into HVC with control or knockdown constructs. d) Preference strength for familiar song per treatment group of birds injected into HVC.*

Table 2: *Preference for familiar song and keypecking activity across different treatment groups. Pairwise comparisons for knockdowns and their matched controls are corrected for multiple testing.*

Area	Age	Treatment	Preference % +/- SD	t(24)	p	Keypecks +/- SD	t(24)	p
HVC	Juvenile	Control	62.1 +/- 7.6	0.37	0.367	2932 +/- 820	0.22	0.385
		Knockdown	65.4 +/- 4.2			2736 +/- 282		
	Adult	Control	73.1 +/- 4.6	**2.67**	**0.015**	1632 +/- 361	**2.45**	**0.024**
		Knockdown	58.7 +/- 2.2			641 +/- 140		
CMM	Juvenile	Control	73.7 +/- 3.1	0.2	0.387	1330 +/- 289	0.6	0.328
		Knockdown	72.8 +/- 2.7			1544 +/- 193		
	Adult	Control	76.1 +/- 2.9	0.9	0.261	1920 +/- 258	0.4	0.363
		Knockdown	76.4 +/- 5.3			1753 +/- 32		

Table 3: *GLM results for pecking activity of the groups that either received a viral knockdown or sham treatment in CMM. Shown are the results of model A including only bird ID and virus batch as random factors, model B which added treatment as a fixed factor and a model adding treatment and age as fixed factors (C). For all models, estimate, standard error (std. error) and the respective t and p values of the intercept and the included fixed factors are indicated. To find the best fitting model, the Akaike information criterion (AIC) and the weight of each model were calculated and models were ordered from best to worst fit.*

CMM pecking	**estimate**	**std. error**	**t value**	**p value**
Model A[1)] Total pecks ~ (1|Bird ID) + (1|Virus batch)				
Intercept	3.10	0.05	58.42	**< 0.001**
Model B: Total pecks ~ (1|Bird ID) + (1|Virus batch) + Treatment				
Intercept	3.09	0.09	36.50	**< 0.001**
Treatment: Knockdown	0.02	0.12	0.20	0.39
Model C: Total pecks ~ (1|Bird ID) + (1|Virus batch) + Treatment + Age/Treatment*Age				
Intercept	3.14	0.10	33.88	**< 0.001**
Treatment: Knockdown	0.03	0.11	0.30	0.38
Age: Adult	0.11	0.11	-1.06	0.23
Age*Treatment	0.07	0.23	-0.68	0.31
Models:	**AIC**	**∆AIC**	**weight**	
Model A	49.2	0.0	0.89	
Model B	53.7	4.5	0.09	
Model C	57.2	8.0	0.02	

[1)] bird ID and virus batch are included as random effects in all models. Significant p values are marked in bold.

Table 4: *GLM results for pecking activity of the groups that either received a viral knockdown or sham treatment in HVC. Shown are the results of model A including only bird ID and virus batch as random factors, model B which added treatment as a fixed factor and a model adding treatment and age as fixed factors (C). For all models, estimate, standard error (std. error) and the respective t and p values of the intercept and the included fixed factors are indicated. To find the best fitting model, the Akaike information criterion (AIC) and the weight of each model were calculated and models were ordered from best to worst fit.*

HVC pecking	**estimate**	**std. error**	**t value**	**p value**
Model A[1)] Total pecks ~ (1|Bird ID) + (1|Virus batch)				
Intercept	3.10	0.10	32.48	**< 0.001**
Model B: Total pecks ~ (1|Bird ID) + (1|Virus batch) + Treatment				
Intercept	3.23	0.15	20.96	**< 0.001**
Treatment: Knockdown	-0.21	0.20	-1.05	0.23
Model C: Total pecks ~ (1|Bird ID) + (1|Virus batch) + Treatment + Age/Treatment*Age				
Intercept	2.89	0.15	19.88	**< 0.001**
Treatment: Knockdown	-0.07	0.15	-0.57	0.34
Age: Adult	0.52	0.14	3.77	**< 0.001**
Age*Knockdown	0.61	0.15	4.43	**< 0.001**
Models:	**AIC**	**ΔAIC**	**weight**	
Model C	36.6	0.0	0.92	
Model A	42.1	5.5	0.06	
Model B	44.4	7.8	0.02	

[1)] bird ID and virus batch are included as random effects in all models. Significant p values are marked in bold.

Song preferences

Overall, females preferred the song of their tutors over unfamiliar song (mean preference for familiar song: 69 +/- 16% of all keypecks, see Table 2). Preferences for the familiar tutor song deviated significantly from chance (intercept significantly different from 0) in all treatment and control groups both in CMM (Fig. 4b, Table 5) and HVC injected females (Fig. 4d, Table 6). In HVC there was a significant age*area

interaction: adult knockdowns in HVC showed weaker preferences for familiar song than matched controls (Fig. 4d, t(24) = 2.45, p = 0.002) an effect that was absent in females that had received the knockdown in HVC as juveniles.

Table 5: *GLM results for preference for familiar song across different groups of birds injected into CMM. Shown are the null model (A), the Treatment model (B) and a model considering Treatment and Age (C). Indicated are estimate, standard error (std. error) and the respective t and p values of the intercept and the included fixed factors. To find the best fitting model, the Akaike information criterion (AIC) and the weight of each model were calculated and models were ordered from best to worst fit.*

CMM preference	**estimate**	**std. error**	**t value**	**p value**
Model A[1] Preference ~ (1|Bird ID) + (1|Virus batch)				
Intercept	2.71	0.60	4.54	**< 0.001**
Model B: Preference ~ (1|Bird ID) + (1|Virus batch) + Treatment				
Intercept	3.14	1.02	3.07	**0.002**
Treatment: Knockdown	-0.74	1.26	-0.59	0.56
Model C: Preference ~ (1|Bird ID) + (1|Virus batch) + Treatment + Age				
Intercept	2.83	1.11	2.55	**0.01**
Treatment: Knockdown	-0.74	1.27	-0.59	0.56
Age: Adult	0.74	1.27	0.59	0.56
Models:	**AIC**	**ΔAIC**	**weight**	
Model A	26.4	0.0	0.62	
Model B	28.1	1.7	0.26	
Model C	29.7	3.3	0.12	

[1] bird ID and virus batch are included as random effects in all models. Significant p values are marked in bold.

Table 6: *GLM results for preference for familiar song across different groups of birds injected into HVC. Shown are the null model (A), the Treatment model (B) and a model considering Treatment and Age (C). Indicated are estimate, standard error (std. error) and the respective t and p values of the intercept and the included fixed factors. To find the best fitting model, the Akaike information criterion (AIC) and the weight of each model were calculated and models were ordered from best to worst fit.*

HVC preference	**estimate**	**std. error**	**t value**	**p value**
Model A[1)] Preference ~ (1|Bird ID) + (1|Virus batch)				
Intercept	1.21	0.34	3.5	**< 0.001**
Model B: Preference ~ (1|Bird ID) + (1|Virus batch) + Treatment				
Intercept	1.34	0.50	2.66	**0.008**
Treatment: Knockdown	-0.24	0.69	-0.34	0.73
Model C: Preference ~ (1|Bird ID) + (1|Virus batch) + Treatment + Age				
Intercept	1.46	0.62	2.34	**0.02**
Treatment: Knockdown	-0.24	0.69	-0.34	0.73
Age: Adult	-0.24	0.69	-0.34	0.73
Models:	**AIC**	**ΔAIC**	**weight**	
Model A	55.7	0.0	0.65	
Model B	57.6	1.9	0.25	
Model C	59.4	3.7	0.10	

[1)] bird ID and virus batch are included as random effects in all models. Significant p values are marked in bold.

Validation of molecular knockdown and localisation of the virus construct

The knockdown efficiency varied from 10 – 70% across individual birds. Quantification of gene expression via qPCR showed that *FoxP1* expression in HVC and CMM was significantly lower in all treatment groups compared to controls across both hemispheres (Fig. 5, $F(1,96) = 176.57$, $p < 0.001$, Two-Way ANOVA with area and developmental stage as factors) independent of the injected hemisphere ($F(1,96) = 1.64$, $p = 0.2$) or area ($F(1,96) = 0.37$, $p = 0.54$). There was however a significant age*treatment interaction for the knockdown efficiency: birds injected into CMM as juveniles showing reduced knockdown efficiency ($F(1,96) = 5.07$, $p = 0.03$). Analyses

using immunohistochemistry showed localised expression of GFP that was restricted to the target areas of HVC (Fig. 2a-c) and CMM (Fig. 2g-i).

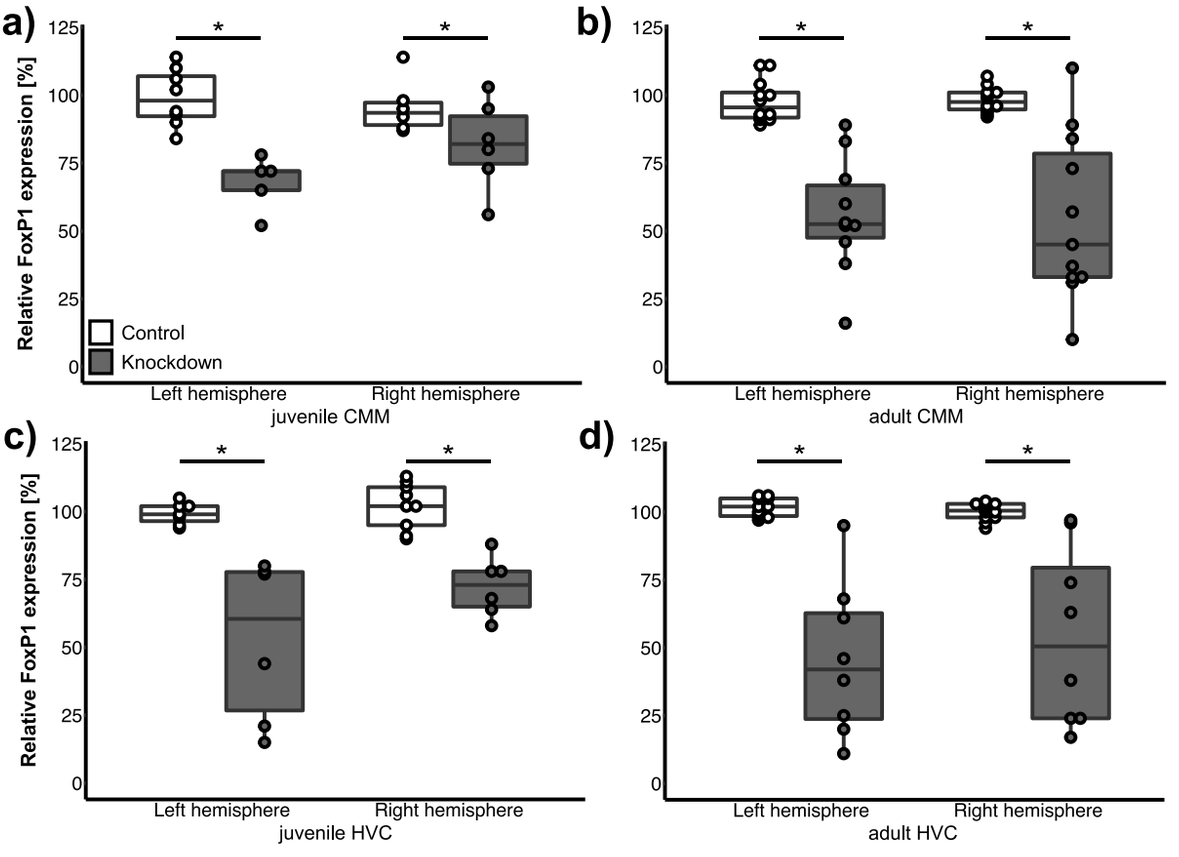


Figure 5: *Boxplots showing qPCR-based FoxP1 RNA expression levels separated by hemispheres relative to normalised expression of a stable house-keeping gene (Hydroxymethylbilane synthase, HMBS) in the respective sample. a) juvenile CMM, b) adult CMM, c) juvenile HVC, d) adult HVC. Boxes show range between first and third quartile, bold lines indicate median values while whiskers show 1.5 interquartile range. Despite large interindividual differences and variation between groups, FoxP1 expression was significantly reduced across all knockdown groups and hemispheres. Efficiency of the knockdown varied between 10 and 90% across left and right hemispheres of birds. At group level, knockdown efficiency varied between 18 and 55% in HVC and CMM of adults and juveniles. The right hemisphere of one bird which received the knockdown construct in CMM as an adult showed increased rather than decreased FoxP1 expression (+9%) in comparison to the controls. As the left hemisphere of this bird indicated a knockdown (-40%) the bird was not excluded from behavioural analyses.*

Despite significant differences on a group basis, some knockdown birds showed higher *FoxP1* expression than matched controls. This might be caused by interindividual variability in endogenous levels of expression of the gene (Chen *et al.*, 2013) as has been shown for *FoxP2* expression (Adam *et al.*, 2017; Kosubek-Langer and Scharff, 2020). Since gene expression can only be measured once per bird it is impossible to know what the birds' FoxP1 expression levels were before the knockdowns/controls were performed.

Prediction of behaviour during preference tests by FoxP1 expression levels

As the knockdown efficiency varied between individuals, it was tested whether higher knockdown efficiency led to stronger effects on keypecking activity and preference strength. The total number of pecks during the preference test did not correlate with knockdown efficiency (Fig. 6a, Pearson's $r(31) = 0.18$, $p > 0.05$). However, the preference strength for familiar song correlated with knockdown efficiency across all groups (Fig. 6b, Pearson's $r(31) = 0.5$, $p = 0.004$). The respective contributions of knockdown efficiency, age group and injected area as well as the interaction of the latter on number of pecks and preference strength were further modelled in GLMs (Tables 7 and 8). Total number of pecks by birds with FoxP1 knockdowns during the preference test was predicted by individual FoxP1 expression levels (Table 7, $z(32) = 36.61$, $p < 0.001$). Additionally, age at injection ($z(32) = -7.79$, $p < 0.001$) and area of injection ($z(32) = -1.31$, $p < 0.001$) contribute to this prediction. The interaction between these two factors was also significant ($z(32) = 3.17$, $p = 0.002$), which further supports behavioural changes occurring only in adult HVC knockdowns. Preference strength for familiar song during the test was also predicted by the relative FoxP1 expression (Table 8, $z(32)=3.54$, $p < 0.001$) but was neither affected by injected area nor age during injection.

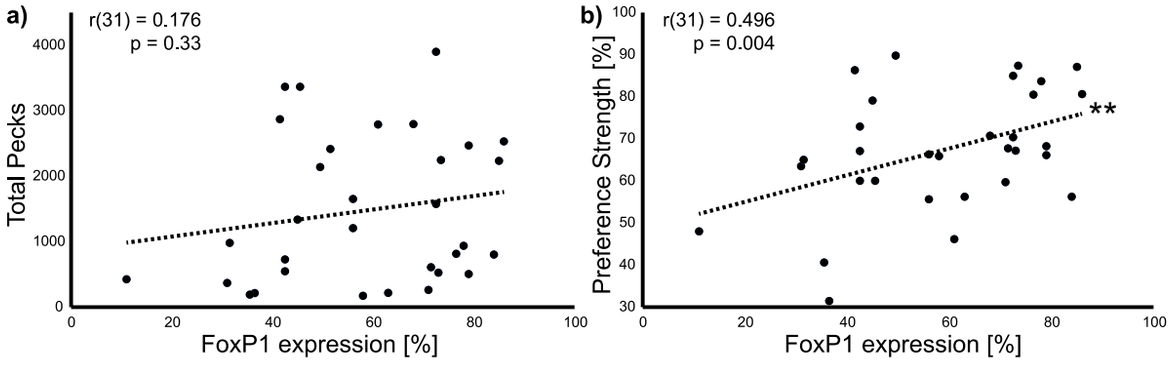


Figure 6: *Correlations between relative FoxP1 expression and behavioural measures. Individual points depict relative FoxP1 expression across both hemispheres in relation to a bird's behaviour. Dotted line indicates linear fit of data. a) relative FoxP1 expression in relation to total number of key pecks during the entire preference test. b) relative FoxP1 expression in relation to preference strength for familiar song.*

Table 7: *GLM results for the total number of pecks as dependent variable in relation to relative FoxP1 expression. Shown are the results of a model including virus batch as random factors, relative FoxP1 expression, injected area, and age during injection and the interaction of age and area as fixed factors as Poisson distributed data. Estimate, standard error (std. error) and the respective z and p values of the intercept and the included factors are indicated.*

Pecking vs. FoxP1	**estimate**	**std. error**	**z value**	**p value**
Intercept	6.34	0.27	23.72	**< 0.001**
Rel. FoxP1 expression	0.02	0.0004	36.61	**< 0.001**
Age: Juvenile	-1.5	0.02	-7.79	**< 0.001**
Area: HVC	-1.31	0.02	-66.2	**< 0.001**
Age*Area	1.79	0.57	3.17	**0.002**

*[1] virus batch is included as random effect. Significant p values are marked in bold. Full model: Total Pecks ~ (1|Virus batch) + FoxP1 expression + Age + Area/Age*Area.*

Table 8: *GLM results for the preference for familiar song as dependent variable in relation to relative FoxP1 expression. Shown are the results of a model including virus batch as random factors, relative FoxP1 expression, injected area, and age during injection as fixed factors as Poisson distributed data. Estimate, standard error (std. error) and the respective z and p values of the intercept and the included factors are indicated.*

Preference vs. FoxP1	**estimate**	**std. error**	**z value**	**p value**
Intercept	3.93	0.11	35.37	**< 0.001**
Rel. FoxP1 expression	0.006	0.002	3.56	**< 0.001**
Age: Juvenile	0.03	0.07	0.49	0.62
Area: HVC	-0.12	0.07	-1.71	0.09

[1)] virus batch is included as random effect. Significant p values are marked in bold. Full model: Preference ~ (1|Virus batch) + FoxP1 expression + Age + Area. Interaction between Age and Area was not significant and excluded from the model.

Discussion

This study tested for potential functional roles of FoxP1 in the development and maintenance of learned auditory preferences in female zebra finches. *FoxP1* expression was reduced by localised knockdowns in two forebrain areas, CMM and HVC, that both are part of the neural circuit supporting auditory learning. The development of a preference for tutor song was not affected by the reduction of FoxP1 levels reported here: females from all knockdown groups still preferred songs of their tutors over unfamiliar songs as adults. However, the treatment showed area-specific and age-specific effects on the reinforcing quality of (memorised) song: FoxP1 knockdown in adult HVC was associated with a lower motivation to elicit song playback (lower pecking activity) and a weaker preference for familiar song in experimental females versus sham-treated controls. No such differences were observed between treatment groups that had received the knockdown in HVC as juveniles or in CMM (independent of age) and their respective controls. Validation by qPCR analyses confirmed reduced *FoxP1* expression in the target regions in the knockdown groups compared to controls across all treatments. Knockdown efficiency did not correlate with the number of keypecks even though in a model it significantly predicted the number of keypecks which was also affected by age at injection and injected area. Preference strength was correlated to knockdown efficiency, but only predicted by knockdown efficiency, not by the other factors tested.

Age-specific knockdown effects could have arisen because higher receptor density and synaptic plasticity during development (Ribeiro and Mello, 2000; Wada *et al.*, 2004; Simonyan *et al.*, 2012) could have buffered potential effects of reduced FoxP1 in juvenile females. Knockdown buffering is also associated with mRNA decay induced transcription (Haimovich *et al.*, 2013) and possibly increased mRNA turnover in juveniles, non-specific responses to mRNA manipulation and off-target effects (El-Brolosy *et al.*, 2019). All of these could have contributed to phenotypic variation between the tested groups. However, in light of the large sample size and the use of multiple juvenile and adult control groups, systematic off-target effects are unlikely to explain the observed pattern of results. Similarly, potential implications of accidentally targeted tissue in HVC shelf underneath HVC can be excluded due to weak endogenous *FoxP1* expression in this area (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015).

It can be concluded that rather than impairing memory formation the knockdown of *FoxP1* expression in adults seems to affect how auditory input was processed within or relayed from HVC. The observed localised and age-specific effect in HVC aligns with the current understanding of a central role for this brain area in learned recognition and preference in female songbirds. Lesions of HVC in adult females interfere with the behavioural expression of learned song preferences in zebra finches (MacDougall-Shackleton *et al.*, 1998) and canaries (Del Negro *et al.*, 1998; Lehongre and Del Negro, 2011). The prediction that reducing FoxP1 expression in this area in juveniles would affect song preference learning could not be confirmed. In another study, FoxP1 knockdowns at day 35 in HVC of socially raised male zebra finches only impaired song development when the knockdown preceded auditory experiences (Garcia-Oscos *et al.*, 2021). This observation is consistent with the possibility that in male zebra finches, FoxP1 knockdowns in HVC affect the formation of appropriate vocal production memories, different from the present findings of effects on auditory memories in females.

CMM and HVC were tested specifically because both areas are involved in supporting memory, sensory feedback, and motor learning (Bell *et al*., 2015; Roberts *et al*., 2017; Soyman and Vicario, 2017; Inda *et al*., 2020), and typically show high *FoxP1* expression in juvenile and adult zebra finches (Fig 2a, g; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015). While HVC can be seen as a hub of auditory and motor input, relaying information and input from multiple sources in both the sensory and motor song circuit (Roberts *et al.*, 2012; Lynch *et al.*, 2013), studies of immediate early gene (IEG) expression have implicated CMM in tutor song memory (Bolhuis *et al.*, 2000; Terpstra *et al.*, 2006; Eda-Fujiwara *et al.*, 2016) because neuronal activity increases more after familiar than unfamiliar stimulus presentation. CMM neuronal activity is also associated with auditory perception and discrimination based on extracellular recordings in female (Inda *et al.*, 2020) and male zebra finches during passive playbacks (Woolley *et al.*, 2005) and Go/Nogo tasks (Bell *et al.*, 2015). In this study, preferences for tutor song were equally strong in CMM knockdowns and untreated control females, suggesting no functional role of FoxP1 in song preference and its acquisition.

The findings of this chapter imply a dosage dependent effect of FoxP1 on the reinforcing quality of the tutor song rather than its memorisation, which raises the question of how reduced *FoxP1* expression in HVC could have reduced the rewarding

qualities of song. FoxP1 knockdowns in mice modify the excitability of medium spiny neurons that express dopamine receptor 1 (Araujo *et al.*, 2015) which is relevant for motivational behaviours (Wise, 1989). Most dopamine receptors are highly expressed in HVC of juvenile zebra finches (Kubikova *et al.*, 2010) where blocked dopamine signals impair song copying (Tanaka *et al.*, 2018). Systemic activation of dopamine D2 receptors also affects female song preference (Day *et al.*, 2019b). From the data it cannot be concluded if and how FoxP1 influences the motivational system but the regulation of dopamine receptor expression by FoxP1 as a potential candidate mechanism for mediating feedback-based learning and memory is worth further investigation. The results from this study align with the idea of a dopamine driven system supporting rewarding qualities of tutor song perception during development. This system is either sufficiently plastic during development to compensate for reduced FoxP1 expression or it only depends on FoxP1 during maintenance but not during development. Once established, the reward system seems to be (partially) dependent on continuous Foxp1 expression in HVC as the local knockdown in the adults decreased both motivation and preference for hearing the songs that at this age are normally well consolidated and stable (Riebel, 2000, 2003a; Riebel *et al.*, 2009).

From the knockdown studies conducted to date in birds, a picture emerges that implicates several FoxP transcription factors in vocal behaviours and vocal learning. FoxP1 knockdown in the sexually dimorphic Area X of juvenile male zebra finches led to incomplete tutor song copying (Norton *et al.*, 2019). Local FoxP1 knockdowns in HVC of juvenile males suggest that reduced *FoxP1* expression in HVC before animals are exposed to tutors inhibits song learning but if these knockdowns occur after an initial learning period, the birds' ability to imitate tutor song is not altered (Garcia-Oscos *et al.*, 2021). Knockdowns of FoxP2 in Area X of juvenile male zebra finches altered song structure and learning (Haesler *et al.*, 2007) but these effects were weaker or absent in adult knockdowns where local knockdowns of FoxP2 abolish context dependent song variability but not the overall structure (Murugan *et al.*, 2013; Day *et al.*, 2019a). Notably, overexpression of FoxP2 in Area X also impairs juvenile song learning but alters production of learned song in adults as well (Heston and White, 2015; Day *et al.*, 2019a).

It should be noted that FoxP1 dimerises with itself and other FoxP transcription factors in regions of overlapping expression (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015), with potential consequences for transcriptional activity and DNA

binding (Wang *et al.*, 2003; Li *et al.*, 2004) resulting in different effects during developmental stages. Even though FoxP2 and FoxP4 in HVC and CMM are expressed at lower levels than FoxP1 (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015), manipulation of either one transcription factor could trigger an imbalance between monomers and dimers and lead to partially overlapping phenotypes (Norton *et al.*, 2019).

For now, combined evidence from expression and knockdown studies and the results presented here support functional involvement of FoxP1 in auditory processing and vocal production learning. Interesting perspectives could arise by comparing animal studies with phenotypic analyses of human *FOXP1* mutations associated with speech and language deficits (Sollis *et al.*, 2016, 2017) but also autism spectrum disorder (ASD) associated variation in sensory feedback processing (Marco *et al.*, 2011). Given that vocal learning progresses in stages and that FoxP1 and FoxP2 have diverse downstream functions (Vernes *et al.*, 2011; Mendoza and Scharff, 2017; Viscardi *et al.*, 2017), both perceptual and productive components of vocal learning could be affected independently or simultaneously.

In conclusion, this study shows that *FoxP1* expression in HVC of adult female zebra finches is involved in motivational behaviours. *FoxP1* expression in HVC might be related to auditory perception and not motor control alone as vocal production requires maintenance or retrieval of auditory information and feedback processing.

Conclusion

The effects of local lentiviral knockdowns of FoxP1 in either HVC or CMM of juvenile or adult female zebra finches on their preference for familiar conspecific songs were assessed. Females of all groups and irrespective of treatment preferred familiar song they heard early in life over unfamiliar song in operant preference tests. This implies that natural FoxP1 expression levels are not necessary to establish or maintain the memory of a specific song type. Additionally, the birds' ability to discriminate between two different auditory stimuli was not affected by the knockdowns. However, FoxP1 knockdowns in HVC of adult females resulted in fewer playback requests in comparison to their respective controls. The preference strength of adult HVC knockdowns towards familiar song was also weaker than that of respective controls. This suggests that FoxP1 might be implicated in the reward perception of auditory stimuli in adults. Even though successful knockdowns were confirmed across all

groups, local FoxP1 knockdowns in juvenile birds that still undergo developmental changes might be outweighed or overarched by neuronal plasticity. In summary, FoxP1 in HVC of adult females might contribute to reward perception when listening to previously memorised stimuli.

Appendix Chapter 2

Supplementary Table 1: *Test stimuli during preference tests of all birds. Bird IDs with an asterisk indicate playbacks that were used for multiple stimulus pairings.*

Bird ID	Pair	# Elements	# Motifs	Total duration stimulus song [s]	Difference duration stimulus set [%]
3089	1	5	6	5.56	1.83
g13r8*	1	5	6	5.46	
3740	2	6	7	5.59	2.19
p10r8	2	5	7	5.47	
4236*	3	6	5	5.87	0.17
4389*	3	5	5	5.88	
4396	4	4	7	5.5	0.36
4575	4	4	7	5.48	
4786*	5	4	5	6.8	1.02
5156*	5	5	5	6.87	
2137	6	5	5	5.83	0.68
4236*	6	6	5	5.87	
2804	7	5	5	5.85	1.39
4512	7	6	5	5.77	
4748	8	5	6	5.55	1.65
g13r8*	8	5	6	5.46	
5013	9	4	6	5.32	0.37
5141	9	4	6	5.34	
3653	10	5	*7*	5.16	0
5679	10	5	*5*	5.16	
5492*	11	4	5	5.29	5.2
4532	11	5	5	5.58	
5832	12	4	5	5.47	0.92
4217	12	5	5	5.42	
4479	13	4	5	5.48	0.9
5650	13	4	5	5.53	
4786*	14	4	5	6.8	1.02
5156*	14	5	5	6.87	

4170	15	5	5	5.59	4.93
4389*	15	5	5	5.88	
5788	16	5	3	5.04	0.8
5492*	16	4	4	5.00	
5804	17	5	5	6.46	1.41
4042	17	5	5	6.37	

Supplementary Table 2: *Viral constructs which were used during this study resulting in different virus batches distributed among the experimental groups. Indicated are the experimental condition they belong to, either control or knockdown, the serotype of one control construct and two knockdown versions of a short-hairpin construct, their respective production date and area, age group and number of birds they were injected into.*

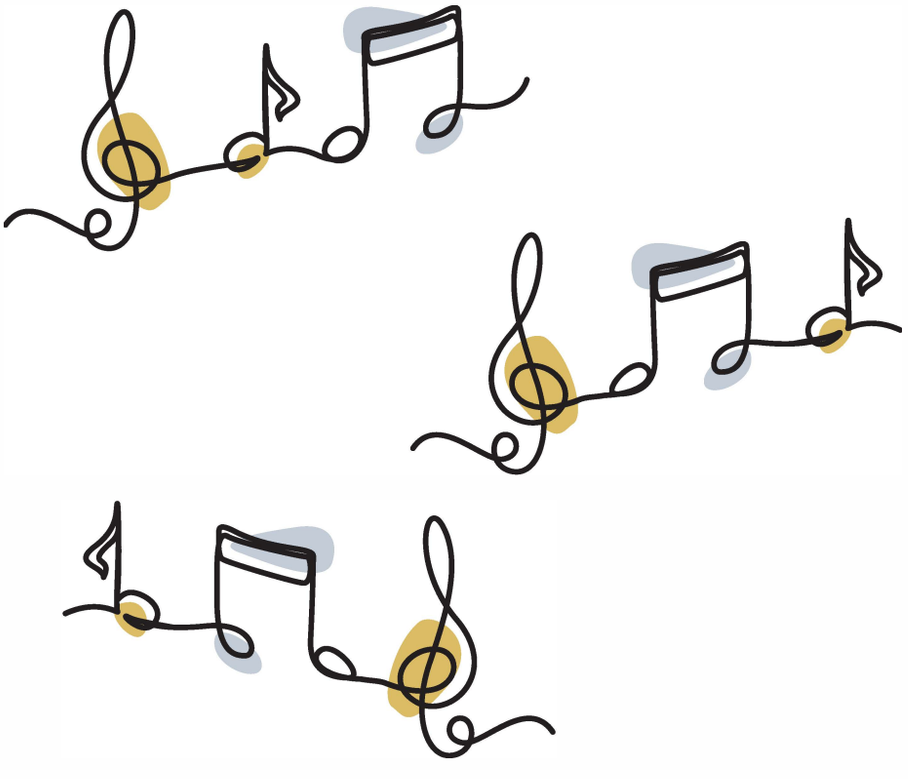
Experimental group	Serotype	Production date	Region	Age	Birds injected
Control	shCTRL	19.02.2016	CMM	Adult	2
Control	shCTRL	19.02.2016	CMM	Adult	1
Control	shCTRL	03.02.2017	CMM	Adult	4
Control	shCTRL	21.07.2017	CMM	Adult	6
Control	shCTRL	21.07.2017	CMM	Juvenile	12
Control	shCTRL	10.10.2014	HVC	Adult	11
Control	shCTRL	03.02.2017	HVC	Adult	1
Control	shCTRL	13.01.2017	HVC	Juvenile	5
Control	shCTRL	03.02.2017	HVC	Juvenile	5
Control	shCTRL	21.07.2017	HVC	Juvenile	2
Knockdown	shKRAK	10.10.2014	CMM	Adult	1
Knockdown	shKRAK	03.02.2017	CMM	Adult	2
Knockdown	shKRAK	21.07.2017	CMM	Adult	2
Knockdown	shKRAK	21.07.2017	CMM	Juvenile	6
Knockdown	shKRAK	10.10.2014	HVC	Adult	6
Knockdown	shKRAK	03.02.2017	HVC	Adult	1
Knockdown	shKRAK	13.01.2017	HVC	Juvenile	6
Knockdown	shY31	10.10.2014	CMM	Adult	2
Knockdown	shY31	19.02.2016	CMM	Adult	1
Knockdown	shY31	03.02.2017	CMM	Adult	1
Knockdown	shY31	21.07.2017	CMM	Adult	3
Knockdown	shY31	21.07.2017	CMM	Juvenile	6
Knockdown	shY31	10.10.2014	HVC	Adult	3
Knockdown	shY31	03.02.2017	HVC	Adult	1
Knockdown	shY31	21.07.2017	HVC	Adult	1
Knockdown	shY31	19.02.2016	HVC	Juvenile	1
Knockdown	shY31	13.01.2017	HVC	Juvenile	5

Supplementary Table 3: *Primers used for qPCR validation of knockdown in RNA extracted from zebra finch target regions HVC and CMM.*

Gene	Ensembl ID	Forward sequence	Reverse sequence	Amplicon Length
HMBS	ENSTGUG00000000010	5'-GCAGCATGTT GGCATCACAG-3'	5'-TGCTTTGCTC CCTTGCTCAG-3'	88 bp
FoxP1	ENSTGUG00000009872	5'-CGTTAAAGGG GCAGTATGGA-3'	5'-GCCATTGAAG CCTGTAAAGC-3'	130 bp

Chapter 3

FoxP1 knockdown in song system nuclei of female zebra finches does not affect song discrimination and the impact of different vocal parameters



Chapter 3: FoxP1 knockdown in song system nuclei of female zebra finches does not affect song discrimination and the impact of different vocal parameters

Fabian Heim, Constance Scharff, Simon E. Fisher, Katharina Riebel, Carel ten Cate

Abstract

Rare disruptions of FoxP1 have been implicated in a human neurodevelopmental disorder characterized by autism and/or intellectual disability with prominent problems in speech and language abilities. Avian orthologues of this transcription factor are evolutionarily conserved and highly expressed in specific regions of songbird brains, among those regions associated with vocal production learning and auditory perception. Here, the zebra finch was used as a model to investigate possible contributions of FoxP1 to auditory perception and stimulus discrimination. Juvenile and adult female zebra finches received a local lentiviral knockdown of FoxP1 in one of two brain areas, HVC (acronym used as proper name) or CMM (caudomedial mesopallium). The birds were then trained to discriminate between two stimulus songs in an operant Go/Nogo paradigm and, once they had mastered this, tested their ability to recognise and categorise altered versions of the training stimuli. The knockdown significantly reduced FoxP1 expression in the juvenile and adult knockdown groups but this did not affect discrimination learning and categorisation of the stimulus songs, or responses to songs modified in pitch, sequential order of syllables or by reversed playback. Subsequently, the full data set was used to assess the impact of different stimulus manipulations for song discrimination more generally. The findings from this study show that zebra finches can use multiple parameters for song discrimination, with a prominent role for spectral parameters, but also an effect of syllable sequence.

Introduction

Human spoken language, speech, is a complex form of vocal communication (Hockett and Hockett, 1960; Deacon, 1998; Pagel, 2017) for which genetic factors are known to make a significant contribution (Fisher *et al.*, 2003). While speech proficiency and related cognitive skills can be investigated with a variety of experimental approaches (Rodd *et al.*, 2005), the underlying molecular processes and the functional roles of relevant genes are difficult to examine in humans (Fisher *et al.*, 2003; Vernes and Fisher, 2009; Szalontai and Csiszar, 2013). One way to gain insights into neuromolecular pathways underlying speech and language is through comparative research in suitable animal models. Like humans, songbirds learn, imitate and modulate vocalisations (Nottebohm *et al.*, 1990; Doupe and Kuhl, 1999), processes that require auditory feedback (Kojima and Doupe, 2007; Mooney, 2018). Pallial songbird regions required for production and perception of song are functionally similar to mammalian, and especially human, cortical areas involved in production and perception of vocalisations, despite considerable neuroanatomical differences (Reiner *et al.*, 2004; Jarvis *et al.*, 2005, 2013; Dugas-Ford *et al.*, 2012; Stacho *et al.*, 2020). Furthermore, some of these functionally similar regions in songbirds and humans show convergent gene expression (Pfenning *et al.*, 2014). These behavioral, neural and genetic parallels between human speech and birdsong make songbirds a suitable animal model to study the neurobiological mechanisms underlying vocal learning and auditory perception (e. g. Nottebohm, 1971; Doupe and Kuhl, 1999; Prather *et al.*, 2009; Mason *et al.*, 2016), and might increase the understanding of neuromolecular aspects of pathways involved in human speech and language.

In this study, songbirds were used to examine the contributions of the transcription factor FoxP1 to the perceptual processing of vocalisations. The P subfamily (Shu *et al.*, 2001) of forkhead box transcription factors (FoxP) contains potential key players in the molecular basis of vocal production learning (Deriziotis and Fisher, 2013). FoxPs are found in a wide range of distantly related vertebrate species (Hannenhalli and Kaestner, 2009; Golson and Kaestner, 2017) pointing to conserved functions (Scharff and Petri, 2011). FoxP1, FoxP2 and FoxP4 show cross-species similarities not only in protein sequence, but also in expression patterns: in the brain they are active in regions which are hypothesised to be homologous across multiple species (Ferland *et al.*, 2003; Lai *et al.*, 2003; Haesler *et al.*, 2004; Rodenas-Cuadrado *et al.*, 2018). Notably,

FoxPs need to dimerise to be transcriptionally active and different FoxPs may form heterodimers in regions where they are coexpressed (Li *et al.*, 2004; Sin *et al.*, 2014; Castells-Nobau *et al.*, 2019) and may thereby contribute to partly overlapping phenotypes.

FoxP mutations have a variety of behavioural effects. Disruptions of one copy of the human FOXP2 gene are associated with a severe speech and language disorder (Fisher and Scharff, 2009) involving childhood apraxia of speech (CAS) and both expressive and receptive language impairments (Lai *et al.*, 2001; Morgan *et al.*, 2017), while mutations of *FOXP4* have also been associated with speech and language delays (Snijders Blok *et al.*, 2021). Rare mutations disrupting human *FOXP1* cause a neurodevelopmental syndrome including autism spectrum disorder and/or intellectual disability (Bacon and Rappold, 2012; Le Fevre *et al.*, 2013; Sollis *et al.*, 2016, 2017). Human *FOXP1* mutations are further associated with speech problems and language-related issues (Horn *et al.*, 2010). Putative perceptual impairments have been reported in some humans carrying heterozygous FOXP1 disruptions (e. g. Siper *et al.*, 2017). FoxP1 is also implicated in mouse vocal production where brain specific knockouts in pups reduce the number of isolation calls and delay their elicitation (Fröhlich *et al.*, 2017; Usui *et al.*, 2017a). A knockout of *Foxp1* in neocortical pyramidal cells and CA1/CA2 of the hippocampus in adult mice affects their ultrasonic vocalisations (Araujo *et al.*, 2017).

In mammals, neural sites of *FoxP1* expression include cortical pyramidal neurons, the hippocampus and the striatum (Ferland *et al.*, 2003; Tamura *et al.*, 2003, 2004; Hisaoka *et al.*, 2010). In zebra finches and other songbirds, *FoxP1* is highly expressed in the basal ganglia (including the striatal nucleus Area X, which is essential for song learning), the robust nucleus of the arcopallium (RA), the caudomedial mesopallium (CMM) and HVC (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015). Experimental knockdown of FoxP1, FoxP2 or FoxP2 in Area X of zebra finches results in phenotypically distinct but overlapping song impairments (Norton *et al.*, 2019). Alterations of FoxP2 expression levels in Area X impede copying of tutor song and interfere with developmental and social modulation of song variability (Haesler *et al.*, 2007; Murugan *et al.*, 2013; Heston and White, 2015), while knockdowns of FoxP4 in Area X of juvenile zebra finches lead to impaired tutor song imitation (Norton *et al.*, 2019). FoxP1 knockdowns in Area X of juvenile male zebra finches affect vocal

learning resulting in reduced motif similarity, altered syllable sequences and overall less similarity to tutor song. Given that *FoxP1* is active in zebra finch brain nuclei important for auditory perception (Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015), FoxP1 might be relevant for auditory processing as well.

In this study, it was examined whether experimental reduction of *FoxP1* expression in HVC and CMM of the female zebra finch brain affects auditory discrimination of songs in general and distinct song features specifically. To disambiguate auditory learning from song production learning, females were chosen because in zebra finches only male birds learn to produce a song (Immelmann, 1962; Zann, 1997), but both males (Adret, 1993; Houx and ten Cate, 1999b) and females (Miller, 1979b; Clayton, 1988; Riebel *et al.*, 2002) memorise songs heard early in life and prefer listening to them as adults. The expression pattern of FoxP1 is similar in both sexes (Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015), but the volume of HVC is significantly smaller in females than in males (Nottebohm and Arnold, 1976, Shaughnessy *et al.*, 2019)

HVC is hypothesised to be involved in auditory motor integration (Prather *et al.*, 2008) in zebra finches and other songbirds, such as canaries and starlings (Leitner and Catchpole, 2002; George *et al.*, 2005). Specifically, lesions of HVC in female canaries alter perception of conspecific songs (Brenowitz, 1991) and also lead to decreased immediate early gene expression in auditory areas such as CMM (Lynch *et al.*, 2013) which provides direct auditory input to HVC (Vates *et al.*, 1996; Coleman *et al.*, 2007; Bauer *et al.*, 2008; Schmidt and Wild, 2014). CMM has been linked to auditory processing in zebra finches such as song perception (Lampen *et al.*, 2014), tutor song memory (Terpstra *et al.*, 2006) and discrimination between trained and untrained stimuli (Bell *et al.*, 2015). Sound responsive neurons in CMM of zebra finches are specifically sensitive to amplitude, frequency and Wiener entropy (Inda *et al*., 2020). Auditory responses in CMM neurons also seem to be implicated in error detection during call playbacks (Beckers and Gahr, 2012) and song syllable discrimination (Elie and Theunissen, 2015). Additionally, song playback increases neural activity of CMM as measured by immediate early gene expression (Mello *et al.*, 1992; Terpstra *et al.*, 2006; Lynch *et al.*, 2018) and BOLD imaging (Van Ruijssevelt *et al.*, 2018) in female zebra finches. Given that HVC and CMM are implicated in perception and processing of auditory stimuli in both sexes, and show higher *FoxP1* expression than the

surrounding tissue, it can be hypothesised that *FoxP1* expression in these areas may be related to perception of song and other vocalisations in zebra finches.

The primary aim of this study was to investigate whether *FoxP1* expression in HVC and CMM contributes to the ability to discriminate zebra finch songs and to the perceptual processing of pitch, spectral structure and syllable sequence of zebra finch songs, taking advantage of the possibility to train zebra finches to perform in operant tasks based on playbacks of auditory stimuli. Prior research has shown that these birds are able to discriminate stimuli based on pitch (Nagel *et al.*, 2010), the overall spectral structure of syllables (Braaten *et al.*, 2006; Vernaleo and Dooling, 2011; Dooling and Prior, 2017; Lawson *et al.*, 2018) or the sequence of syllables (van Heijningen *et al.*, 2013; Chen and ten Cate, 2015; Chen *et al.*, 2015; Knowles *et al.*, 2018; ten Cate, 2018). In a study using the same birds as in the present study *FoxP1* expression was reduced in both target nuclei but only knockdowns in HVC of adult females were associated with a lower motivation to hear song and a weaker preference strength for familiar songs (see Chapter 2 of this thesis). As human *FOXP1* mutations are linked to a complex syndrome with diverse effects on cognitive tasks this study investigates whether FoxP1 knockdowns impair perceptual processing, by testing how manipulations of different parameters of zebra finch song affect the behaviour of the previously tested birds in operant discrimination tasks.

The birds were trained in an established operant Go/Nogo task (e.g. Scharff *et al.*, 1998; Ohms *et al.*, 2012; Kriengwatana *et al.*, 2016). Briefly, females were trained to discriminate between two unfamiliar songs by responding with key pecks to one song (Go-song) for a food reward and by withholding key pecks to a second song (Nogo song). Once the females mastered this discrimination, they received a series of test stimuli that were edited versions of the training stimuli. The sound manipulations consisted of either changes in pitch (fundamental frequency), rearrangements of the syllable sequence or reversed playbacks of the original song. After testing the females' responses to different stimuli, the extinction of the entrained discriminatory abilities were also examined. If localised high levels of *FoxP1* expression were required in developing or maintaining auditory discrimination and stimulus categorisation, females in the specific experimental group(s) were predicted to show reduced discrimination of training songs and/or test stimuli in knockdowns compared to controls.

Given the lack of clear differences among the groups, the behavioural data were pooled (for the control groups and where the knockdown did not affect the tested variables), resulting in a large data set that allowed a detailed examination and comparison of the discriminatory abilities of zebra finches with respect to pitch changes, syllable rearrangements and reversed playbacks. Further, this made it possible to relate the findings to those of earlier behavioural studies on the parameters involved in song discrimination in zebra finches (Braaten *et al.*, 2006; Nagel *et al.*, 2010; Vernaleo and Dooling, 2011; Lawson *et al.*, 2018; Prior *et al.*, 2018).

Material and Methods

Subjects, virus preparation and injections

All subjects (N = 96 females) were the offspring of domesticated zebra finches from the breeding colony at the Freie Universität in Berlin. They were the same individuals as described in a companion paper investigating the effects of local FoxP1 knockdowns on learned preference development (Chapter 2 of this thesis). All subjects were raised and housed with their siblings and parents (N = 79) or foster parents (N= 17 were cross fostered at 15dph) in steel-wire breeding cages (180 cm x 50 cm x 50 cm) until 90 dph. Subjects were assigned to four different treatment groups that were defined by where (HVC or CMM) and when (as juveniles: 21 - 29 dph or as adults: 98 - 490 dph) they received viral injections: HVC adult, HVC juvenile, CMM adult, CMM juvenile. Each knockdown group was also assigned a matched control group. To keep variation within treatment-matched comparisons low, young females were one by one pseudo-randomly assigned to each treatment and a matched control group (assigning sisters to a matched treatment and control groups wherever possible) until a sample size of N = 12 was reached for each of four particular treatments and the four matching control groups.

For a detailed description of the surgery, refer to Chapter 2 of this thesis. Briefly, viral constructs were injected bilaterally in a stereotaxic setup. Three different viral constructs with a GFP marker sequence were used: control (the construct with the GFP marker), and two constructs that additionally contained shRNA sequences with target sites in FoxP1 transcripts, to reduce local *FoxP1* expression (‘knockdown’). The rationale for employing two different shRNA constructs was to reduce the probability

of undetected off-target effects (Song *et al.*, 2015). The construct that only led to GFP expression was used to control for the effect of surgery, injection, virus infection and protein expression in the control birds.

After the injections, adult birds were moved to same sex aviaries (200 cm x 200 cm x 300 cm) while juveniles were returned to their home cages where they remained until 90 dph when they were also transferred to same sex aviaries. Birds were always housed in their home cages for at least 14 days after the procedure before they were transferred to Leiden (the Netherlands) for behavioural testing. At Leiden University, before and after behavioural testing, subjects were housed 2 – 6 individuals in cages of 120 cm x 90 cm x 90 cm (see Chapter 2).

Go/Nogo training and tests

All birds were trained as adults (juvenile groups: 124 – 159 dph, adult groups: 131 – 413 dph) in one of 12 sound attenuated chambers (at least 2.4 m x 1.4 m x 2.3 m) at the IBL at Leiden University. The chambers could be entered and observed via a door with a one-way mirror and were all identically equipped with an experimental cage (Skinner box, 70 cm x 30 cm x 45 cm, made of mesh wire with a solid back panel and floor) placed on a trolley at the long end. The Skinner box contained a food hatch in the back wall of the cage. Left and right of the hatch were piezoelectric sensors with red LED light indicators. A perch was placed in front of the hatch as well as the sensors, and additional perches were located at either side of the cage. Each cage was illuminated by a fluorescent tube emitting light at a daylight spectrum on a 13:11 hour light:dark schedule with dawn and dusk phases of 15 minutes each. The food hatch, lights and LED indicators were controlled by a custom build steering unit connected to a laptop (Sony Vaio E series, Sony, Minato) containing a sound chip (MSM6388, Oki, Tokyo) which could be accessed from outside the experimental chamber.

In the first phase of the Go/Nogo training (Fig. 1), birds were moved from their home cage into individual Skinner boxes. Water and grit were provided *ad libitum* with food being located behind an open hatch and freely available. During this stage, if a bird pecked one of the sensors, this would elicit a playback in the form of two song motifs from an unfamiliar zebra finch. The keys remained active and the food hatch open until 9 AM the following day when the hatch was closed to begin training. From this time onwards, a bird had to peck at either one of the two buttons next to the food hatch to

elicit a playback (same sound as before, for both buttons) and gain food access from the hatch for 20 seconds. After a bird was observed to peck regularly (feeding after the hatch opened in >7 of 10 trials), the next shaping phase was introduced. If the bird did not regularly feed after the food hatch was opened by a key peck, the hatch was opened in the evening at least one hour before lights off, and closed again the following morning until each bird had learned to peck the keys for access to food. Once the birds learned how to acquire food, access could be achieved any time during daylight.

During the next phase, birds were required to first peck the key to the left side of the hatch which would elicit the same playback as during previous shaping phases. In order to gain access to food for 20 seconds, birds had to peck at the right key within ten seconds after pecking the left key. As soon as the birds gained access to food in >75% of the initiated trials during three consecutive days, the Go and Nogo training stimuli replaced the initial stimulus. Both stimuli were now played back randomly. When a bird initiated a trial by pecking the left key and a Go stimulus was played, it was required to peck the right key within five seconds in order to be rewarded with ten seconds of food access. In case a Nogo stimulus was played, the birds had to refrain from pecking the right key until they could initiate a new trial via the left key after five seconds. If a bird pecked the right key within five seconds after a Nogo stimulus the lights were turned off for 12 seconds before a new trial could be initiated.

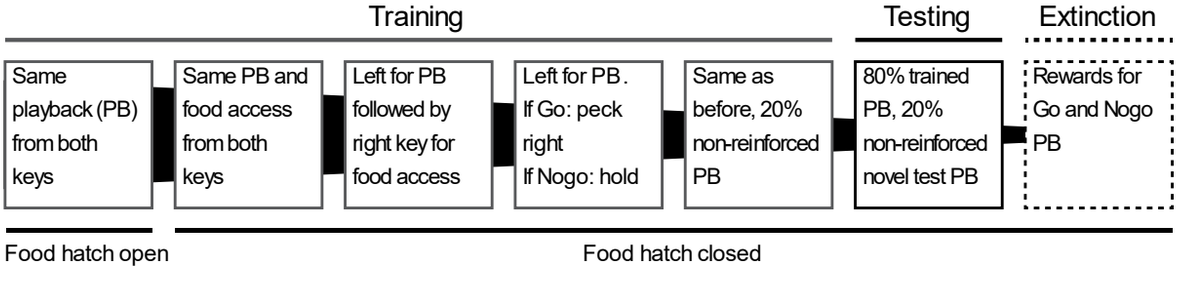


Figure 1: *Flow chart of the training and testing procedures during the experiments. Initially, the food hatch was open to allow unlimited food access while the birds could already elicit playback via the pecking keys. Over the course of the training, birds were conditioned to open the food hatch and respond in a sequence (first left to initiate the playback, then right to indicate a response and obtain a food reward) until novel and non-reinforced stimuli were tested. During the extinction period, the food hatch always opened after pecking the second key, irrespective of the stimulus' nature (Go or Nogo).*

To determine the birds' progress, the discrimination rate (DR) was calculated by dividing the proportion of Go responses to Go stimuli by the sum of the proportion of Go responses to Go stimuli and the proportion of Go responses to Nogo stimuli.
Once birds discriminated between Go and Nogo stimuli during >75% of trials over three consecutive days, the number of trials required to reach this stage were used as an estimate for learning speed and the next training phase began during which 20% of the initiated trials were not reinforced. This accustomed the birds to unrewarded stimuli before the actual test phase began. Test stimuli were introduced after a subject had performed for three days with a discrimination rate >75%.
During this phase, the test stimuli were played back at a rate of 20% among the trained Go and Nogo sounds. This testing phase lasted for six consecutive days. Responses to test stimuli were never reinforced. Playback of test stimuli was organized in a pseudo random fashion to generate similar playback rates for each stimulus. The number of responses per stimulus was measured and the response rate for each individual test stimulus was calculated by dividing the number of Go responses by the number of presentations of that particular test stimulus.
This testing period was followed by an extinction phase during which both Go and Nogo stimuli from the training phase were rewarded with ten seconds access to food. As soon as the discrimination rate between the two stimuli reached chance level (50%) during one entire day, subjects were moved back to their home cages.

Stimulus songs

All stimuli consisted of unfamiliar, undirected song recordings of birds from the breeding colony in Leiden. Song was previously recorded under standardised conditions in sound attenuated chambers with Ishmael software (v. 1.0.2, www.pmel.noaa.gov/vents/acoustics/whales/ishmael) directly onto the hard disc of a computer (CDX-01 soundcard, Digital Audio Labs, Chanhassen) with a microphone (MKH40, Sennheiser, Wedemark-Wennebostel) at 75 cm distance above the cage and a sampling rate of 44.1 kHz at 16bits. No bandwidth filters were applied. All playbacks were adjusted to a SPL of 70dB at the location where the bird would initiate the playback. Files were played back as .wav files from speakers (Vifa 10BGS119/8, Viborg) connected to custom made Skinner box devices, which were individually controlled via software written by the Leiden University electronics department. Stimuli

consisted of two repetitions of the same motif without introductory notes and were matched according to duration (1.6 - 1.9 s, mean = 1.71 s) and number of syllables (4 – 7, mean = 5.4). A motif was defined as the longest most common sequence within 5 song bouts. Go/Nogo training stimuli were selected from natural motif repetitions of multiple males and compiled into six different stimulus sets (see Fig. 2b for one exemplary training stimulus). The same stimulus sets were assigned to 3 – 10 matched pairs of knockdown and control females with similar occurrence of each stimulus during the different training periods. Experimental stimuli were modified versions of the training stimuli and are further referred to as test stimuli. Depending on the modified template (either 'Go' or 'Nogo'), they are referred to as 'TestGo' and 'TestNogo' stimuli. Stimulus sets with different pitch manipulations, reversals of the element sequence and stimuli played backwards (Fig. 2) were used. For pitch manipulations, all frequencies of a stimulus song were increased or decreased by 8% (Fig. 2c and 1d) using Praat v5.4 (Boersma and van Heuven, 2011, Praat Vocal Toolkit by Ramon Corretge). This level of change is close to the threshold for correct stimulus identification in zebra finches (Nagel *et al.*, 2010). For the sequence reversal, the syllable sequence within the trained songs was reversed (Fig. 2e, ABCD > DCBA, indicated as 'sequence reverse'). Further, backwards played songs (Fig. 2f, 'reversed playback') were used to determine whether knockdowns alter the ability of female zebra finches to recognise and categorise the spectral structure of song syllables (Okanoya *et al.*, 2000; Braaten *et al.*, 2006; Lawson *et al.*, 2018; Burgering *et al.*, 2019) while syllable number and their pitch remain unaltered.

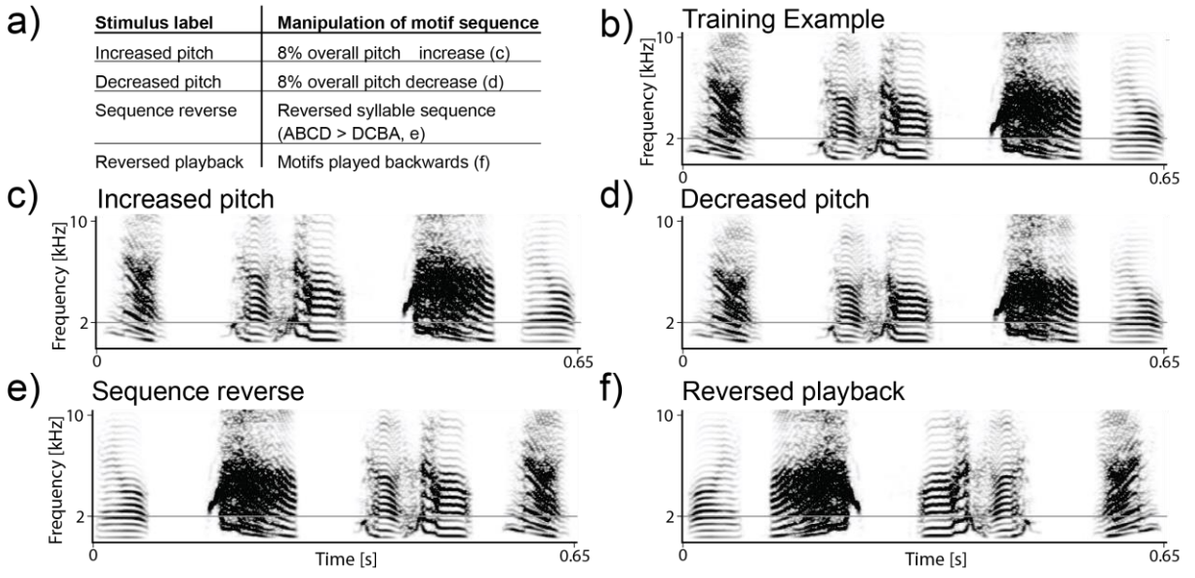


Figure 2: *Examples of a stimulus song the manipulations used for the test stimuli: a) Overview of stimulus definition and applied manipulations. b) spectrograms of an exemplary Go-stimulus. c-d) manipulations of the Go-stimulus used as test stimuli c) 8% pitch increase d) 8% pitch decrease, e) 'sequence reverse' with unchanged inter syllable intervals, f) 'reversed playback' (reversal of the entire original Go stimulus). The grey horizontal line marks 2000 Hz for better recognition of pitch changes.*

Brain extractions and knockdown validation

Two to three weeks after tests were completed, birds were sacrificed for brain extraction. The abundance of FoxP1 mRNA transcripts was determined via qPCR at the Max Planck Institute for Psycholinguistics in Nijmegen. Samples generated from HVC and CMM punches showed significantly lower *FoxP1* expression across both hemispheres of all knockdown groups in comparison to their respective matched controls (see Chapter 2 and supplementary Fig. 1).

Statistical analyses

All analyses were performed in R v3.5 (R Development Core Team, 2011). Normal distribution of data was tested and confirmed with Shapiro-Wilk tests (data not shown). Significance of discrimination rates between Go and Nogo stimuli was calculated with one sample t-tests. Multiple t-tests were corrected for false discovery rate (Pike, 2011). General linear model (GLM) analyses were performed assuming a Poisson distribution of the response and discrimination rates, and including individual bird identification number and injected virus batch as random factors. Modelling parameters were added as fixed factors one after another to determine the best fit.

Results

Training performance

During the training phase, all birds (N = 95) but one (juvenile HVC control) reached a discrimination rate (DR) >0.75 by one to five days after introducing the training Go and Nogo stimuli (mean ± standard error = 2.01 ± 0.21 days). Once a bird reached a DR > 0.75, the DR remained high or increased during the following training days. The number of days to reach DR > 0.75 did not differ between the knockdown groups and their controls and was not influenced by age, region or their interactions with the treatment (supplementary Table 1). The number of trials needed to reach DR > 0.75 was different for some groups (Fig. 3a): knockdown and control birds that received an injection into HVC as juveniles required more trials to reach DR > 0.75 than other groups (see the weak interaction between region and age, Table 1). Once birds had reached the training criterion, the DR between the Go and Nogo stimuli did not differ between any of the tested groups (Fig. 3b, supplementary Table 2). There was also no group-based effect on the response rates towards the trained Go (Fig. 3c) or Nogo stimuli (Fig. 3d, Supplementary Table 3) during the three days prior to the onset of test stimulus playbacks – the phase in which 20% of previously trained stimuli were not rewarded to simulate test conditions. The birds' response rates towards TestGo and TestNogo stimuli during the testing phase were analysed separately to prevent effect-masking (e.g. simultaneous reduction of Go and Nogo responses and thus no change in the discrimination rate).

Table 1: *GLM based analyses of the necessary trials until birds of various groups reached the training criterion of a discrimination rate > 0.75. Significant p values are marked bold.*

Trials to Training Criterion	**Estimate**	**std. error**	**t value**	**p value**
Model: 1|Virus batch + Treatment + Region + Age + Treatment*Region*Age[1]				
Intercept	787.5	194.26	4.054	**0.001**
Treatment: Knockdown	-117.5	274.72	-0.428	0.676
Region: HVC	-28.92	274.72	-0.105	0.918
Age: Juvenile	-237.5	274.72	-0.865	0.403
Treatment*Region	-72.17	388.52	-0.186	0.855
Treatment*Age	-28.33	388.52	-0.073	0.943
Region*Age	851.5	388.52	2.192	**0.047**
Treatment*Region*Age	430.58	549.45	0.784	0.447

[1]*All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model assumes Poisson distributed data and contains injected virus batch as random factor. All other parameters as equally weighted full factorials and tested for interaction.*

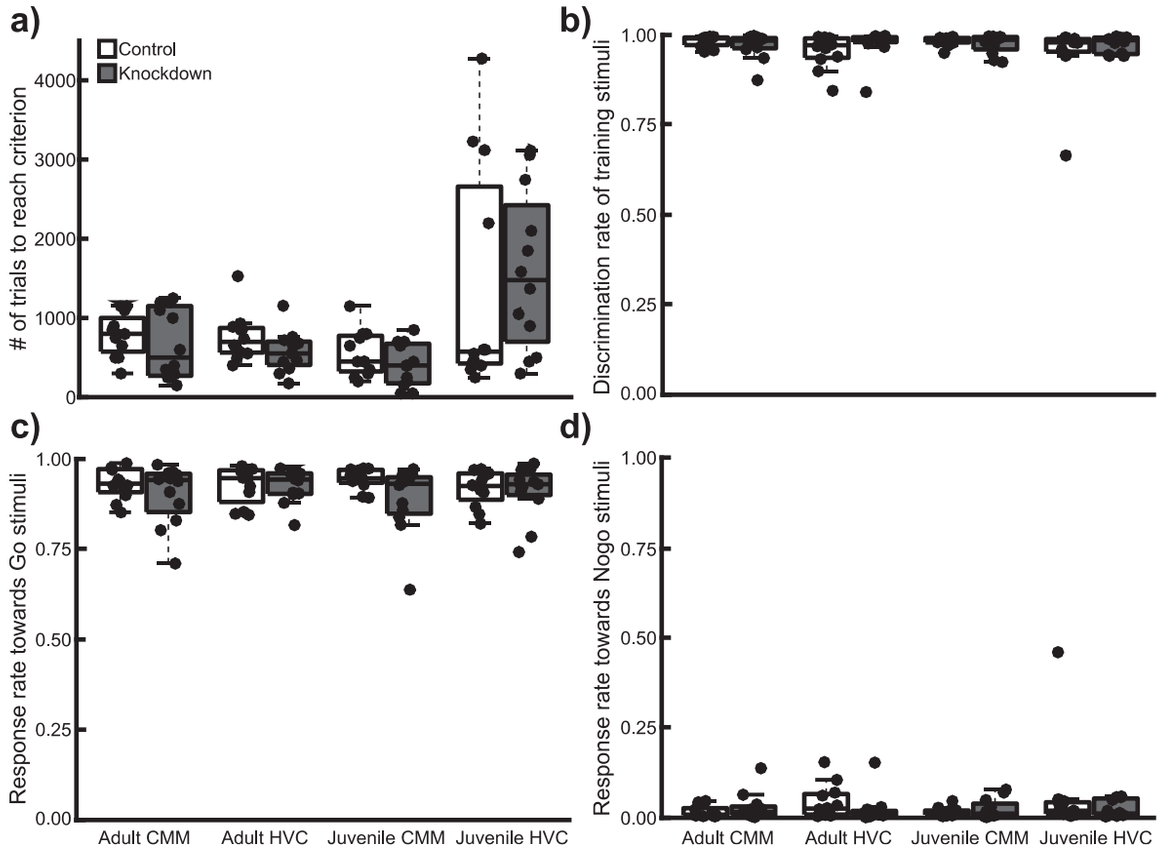


Figure 3: *Performance of all groups during the Go/Nogo task training period. Boxplots: box = range between 1st-3rd quartile; line within the box = median; whiskers = 1.5 interquartile range; data points depict individual birds. a) Number of trials required to reach training criterion was higher in birds receiving injections (control or knockdown) as juveniles but there was no systematic effect of area (HVC or CMM) or treatment (control or knockdown). b) Discrimination rates during the three days prior testing showing all groups discriminated equally well between the trained Go and Nogo stimuli c) response rates to Go and d) to Nogo stimuli during training.*

Performance to test stimuli

The discrimination rate of test stimuli did not differ between treatments, areas or age at injection (GLM, see supplementary Table 4). As Figure 4 shows, response rates differed to the same degree in all groups between TestGo and TestNogo stimuli with increased (Fig. 4a) or decreased pitch (Fig. 4b), sequence reverse (Fig. 4c) and reversed playback versions of the training stimuli (Fig. 4d). Thus, none of the stimulus manipulations resulted in a loss of discrimination. To analyse whether different stimuli gave rise to behavioural differences between any of the tested groups, separate GLM analyses were conducted for response rates to Go stimuli (see Table 2) and Nogo

stimuli (see Table 3). Separate models made it possible to analyse whether different stimuli affected the difficulty of the discrimination tasks resulting in either altered Go or altered Nogo responses. Additionally, the large difference between very high Go and very low Nogo response rates (see supplementary Figure 1) would have masked smaller effects particularly for the response rates to NogoTest stimuli which were consistently very low. The dependent variable was set as the birds' response rate to the respective stimulus category. Based on the AIC and its corresponding weight, model B was identified as the best fit for both the models of Go and those of Nogo responses. This model suggests that the various test stimuli had the largest impact on the birds' performance, whereas construct, targeted brain area or the birds' age during the injection did not contribute substantially to behavioural variation. The significant intercept throughout all models indicates that the tested birds behaved differently, but not in a systematic way relating to any of the experimental manipulations. All test stimuli of the Go type affected the birds' behaviour significantly (Table 2), whereas test stimuli of the Nogo type (Table 3) only showed higher false positive responses for stimuli played back in reverse.

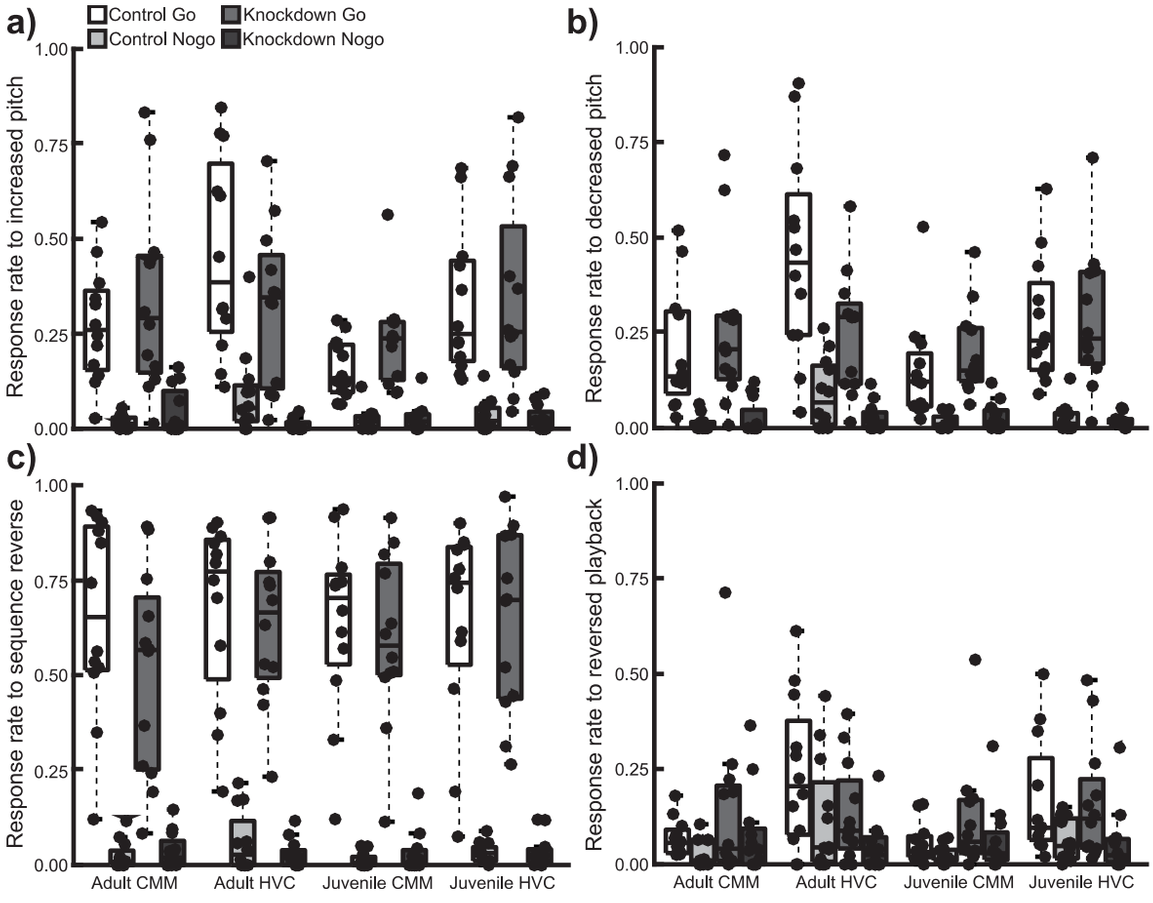


Figure 4: *Boxplots showing the proportion of Go responses (response rate) of all groups to TestGo and TestNogo stimuli. Boxes show range between first and third quartile, bold lines indicate median values while whiskers show 1.5 interquartile range. Birds responded in a similar manner to stimuli that were increased (a) or decreased in pitch (b). They showed the highest Go-response rates to TestGo stimuli with reversed syllables (c) and the lowest Go-response rates to TestGo stimuli played back in reverse (d).*

Table 2: *GLM based analyses of the Go-response rate of birds to TestGo stimuli during Go/Nogo-tasks. Different test types are included as different levels of a single factor. The best model was determined by Akaike's Information Criteria (AIC) analyses. Significant p values of the best fitting model are marked bold.*

Go response	**Estimate**	**std. error**	**t value**	**p value**
Model A[1]: 1|Virus batch + 1|Bird ID				
Intercept	0.323	0.02	16.14	$5.687*10^{-29}$
Model B: Test stimulus[2]				
Intercept	0.251	0.024	10.566	$\mathbf{1.026*10^{-17}}$
Test: Increased Pitch	0.049	0.021	2.312	**0.023**
Test: Sequence Reverse	0.362	0.021	17.249	$\mathbf{5.055*10^{-31}}$
Test: Reversed Playback	-0.124	0.021	-5.9	$\mathbf{5.572*10^{-8}}$
Model C: Test stimulus + Treatment[2]				
Intercept	0.258	0.028	9.1	$1.386*10^{-14}$
Test: Increased Pitch	0.049	0.021	2.312	0.023
Test: Sequence Reverse	0.362	0.021	17.249	$5.055*10^{-31}$
Test: Reversed Playback	-0.124	0.021	-5.9	$5.572*10^{-8}$
Treatment: Knockdown	-0.014	0.031	-0.434	0.666
Model D: Test stimulus + Treatment + Region[2]				
Intercept	0.222	0.031	7.27	$1.006*10^{-10}$
Test: Increased Pitch	0.049	0.021	2.312	0.023
Test: Sequence Reverse	0.362	0.021	17.249	$5.055*10^{-31}$
Test: Reversed Playback	-0.124	0.021	-5.9	$5.572*10^{-8}$
Treatment: Knockdown	-0.015	0.031	-0.492	0.624
Region: HVC	0.085	0.032	2.651	0.009
Model E: Test stimulus + Treatment + Region + Age[2]				
Intercept	0.242	0.034	7.143	$1.838*10^{-10}$
Test: Increased Pitch	0.049	0.021	2.312	0.023
Test: Sequence Reverse	0.362	0.021	17.249	$5.055*10^{-31}$
Test: Reversed Playback	-0.124	0.021	-5.9	$5.572*10^{-8}$
Treatment: Knockdown	-0.016	0.031	-0.51	0.611
Region: HVC	0.089	0.031	2.834	0.006
Age: Juvenile	-0.038	0.031	-1.199	0.234

Models:	AIC	ΔAIC	Weight
Model B	-209.58	0	0.6
Model C	-202.679	6.901	0.019
Model D	-201.59	1.089	0.348
Model E	-195.806	5.784	0.033
Model A	86.48	282.286	< 0.001

[1]The model assumes Poisson distributed data and includes individual bird identification number and injected virus batch as random factors. All other parameters are added as equally weighted fixed effects one after another. [2]Fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels.

Table 3: *GLM based analyses of the Go-response rate of birds to TestNogo stimuli during Go/Nogo-tasks. Best model is determined by AIC analyses. Significant p values of the best fitting model are in bold.*

Nogo Response	**Estimate**	**std. error**	**t value**	**p value**
Model A[1]: 1|Virus batch + 1|Bird ID				
Intercept	0.039	0.006	6.56	$2.807*10^{-9}$
Model B: Test stimulus[2]				
Intercept	0.031	0.007	4.506	$\mathbf{1.881*10^{-5}}$
Test: Increased Pitch	0.003	0.005	0.576	0.566
Test: Sequence Reverse	0.001	0.005	0.264	0.792
Test: Reversed Playback	0.029	0.005	5.339	$\mathbf{6.358*10^{-7}}$
Model C: Test stimulus + Treatment[2]				
Intercept	0.033	0.009	3.849	$2.149*10^{-4}$
Test: Increased Pitch	0.003	0.005	0.576	0.566
Test: Sequence Reverse	0.001	0.005	0.264	0.792
Test: Reversed Playback	0.029	0.005	5.339	$6.358*10^{-7}$
Treatment: Knockdown	-0.005	0.011	-0.462	0.645
Model D: Test stimulus + Treatment + Region[2]				
Intercept	0.026	0.01	2.566	0.012
Test: Increased Pitch	0.003	0.005	0.576	0.566
Test: Sequence Reverse	0.001	0.005	0.264	0.792

Test: Reversed Playback	0.029	0.005	5.339	$6.358*10^{-7}$
Treatment: Knockdown	-0.006	0.011	-0.506	0.614
Region: HVC	0.017	0.011	1.585	0.116
Model E: Test stimulus + Treatment + Region + Age^2				
Intercept	0.033	0.011	2.876	0.005
Test: Increased Pitch	0.003	0.005	0.576	0.566
Test: Sequence Reverse	0.001	0.005	0.264	0.792
Test: Reversed Playback	0.029	0.005	5.339	$6.358*10^{-7}$
Treatment: Knockdown	-0.006	0.012	-0.508	0.613
Region: HVC	0.017	0.012	1.59	0.115
Age: Juvenile	-0.014	0.011	-1.287	0.201
Models:	AIC	ΔAIC	weight	
Model B	-1186.098	0	0.788	
Model A	-1181.909	4.189	0.097	
Model C	-1177.094	4.815	0.071	
Model D	-1170.253	6.841	0.026	
Model E	-1162.688	7.565	0.018	

[1]*The model assumes Poisson distributed data and includes individual bird ID and injected virus batch as random factors. All other parameters are added as equally weighted fixed effects one after another. Different test types are included as different levels of a single factor.* ***[2]****Fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels.*

To assess how quickly birds can overcome the learned association which was established during training, Go and Nogo stimuli were both positively reinforced after completion of the testing phase. Subsequently, the number of days and necessary trials that the birds required to respond equally often both to Go and Nogo stimuli again was measured. There was no difference between the groups in the number of days (data not shown) or necessary trials (data not shown) until the birds responded equally often to the former Go and Nogo stimuli.

General behaviour in response to test stimuli

Since no overall effects of treatment, area of injection, or age were evident from the results of the statistical analyses (GLMs supplementary Tables 1 – 2 and Tables 1 – 4), all experimental groups were merged. The observed variability of response rates between different groups (Fig. 4, Tables 2 – 3) could not be assigned to a general effect caused by the injections. Thus the data of all tested birds were pooled to examine how female zebra finches differentiate among the test stimuli, and to assess their discriminatory abilities with respect to detecting the different types of changes to trained stimuli (Figure 5).

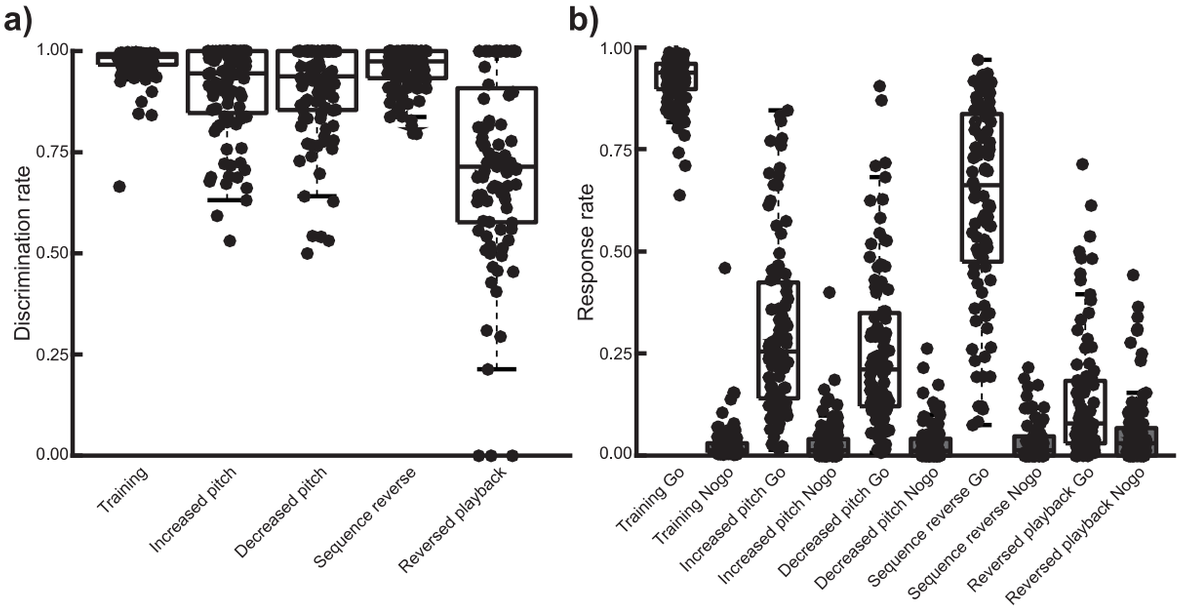


Figure 5: *Boxplots showing performance of all birds taken together, irrespective of age during injection, injected construct, or area. Boxes show range between first and third quartile, bold lines indicate median values while whiskers show 1.5 interquartile range. (a) Discrimination rate across all groups remains high after training but note the much lower response rate to reversed versus the other stimuli. (b) Response rates to the various TestGo stimuli are lower than to the training Go stimulus while no increased response can be seen to TestNogo stimuli.*

Across all groups, the birds discriminated between all versions of TestGo and TestNogo stimuli (Figure 5a) according to paired t-tests. The females discriminated stimuli with altered pitch, independent of the direction (8% increase: $t_{95} = 13.05$, $p < 0.0001$; 8% decrease $t_{95} = 12.31$, $p < 0.0001$), sequence reverse ($t_{95} = 24.39$, $p <$

0.0001) or reversed playbacks (t_{95} = 6.9, p < 0.0001). The DR of training stimuli remained the highest in comparison to test stimuli. While the DR of increased and decreased pitch test stimuli did not differ from each other, the birds' discriminatory performance among all test stimuli was best towards stimuli with reversed sequence and poorest to stimuli played back in reverse (Table 4).

Table 4: *GLM based analyses of the discrimination rate between all stimuli of all birds. Significant p values are marked bold.*

Discrimination Rate All Birds	**estimate**	**std. error**	**t value**	**p value**
Model: 1|Bird ID + Test Stimulus[1]				
Intercept	0.904	0.015	60.853	**$6.759*10^{-78}$**
Training	0.067	0.02	3.391	**0.001**
Test: Increased Pitch	0.002	0.02	0.077	0.939
Test: Sequence Reverse	0.051	0.02	2.601	**0.011**
Test: Reversed Playback	-0.221	0.02	-11.192	**$4.825*10^{-19}$**

[1]*All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels. The model assumes Poisson distributed data and contains bird ID as random factor. Stimulus type is included as a fixed factor with multiple levels. Test stimulus includes all stimuli that were played back to the birds, including the trained stimuli which are labelled as Training.*

In addition to small differences of the birds' DR of TestGo and TestNogo stimuli, larger differences occurred between the response rates to different stimuli. After playback of TestGo, response rates varied between stimulus types (Fig. 5b, Table 5). In line with the high DR of all presented stimuli, response rate to TestNogo stimuli remained significantly low. Responses to Nogo stimuli occurred in less than 4% of all trials. Birds responded with lower variance (p = 0.0025, t = 4.985, t-test) to different TestNogo stimuli (mean variance = 0.004) than to TestGo stimuli (mean variance = 0.042). Response rates to all versions of TestGo stimuli were significantly reduced in comparison to the trained Go stimuli. Response rates between increased or decreased pitch differed as well, albeit at a lower magnitude than between all other test stimuli as there was no interaction between the stimulus type (Go or Nogo) and the type of test

stimulus. The birds responded most to TestGo stimuli with sequence reverse and least to reversed playbacks.

Table 5: *GLM based analyses of the response rates towards all stimuli of all birds. The model further includes interactions between stimulus type (Go/Nogo) and both training and test stimuli. Significant p values are marked bold.*

Response Rate All Birds	**estimate**	**std. error**	**t value**	**p value**
Model: 1|Bird ID + Stimulus Type*Test Stimulus[1]				
Intercept	0.27	0.014	18.678	**$1.447*10^{-33}$**
Type: Nogo	-0.228	0.017	-13.54	**$6.69*10^{-24}$**
Training	0.659	0.017	39.077	**$2.449*10^{-60}$**
Test: Increased Pitch	0.049	0.017	2.879	**0.005**
Test: Sequence Reverse	0.362	0.017	21.476	**$3.122*10^{-38}$**
Test: Reversed Playback	-0.124	0.017	-7.346	**$7.001*10^{-11}$**
Type*Training	-0.66	0.024	-27.695	**$2.765*10^{-47}$**
Type*Test Increased Pitch	-0.045	0.024	-1.905	0.06
Type*Test Sequence Reverse	-0.361	0.024	-15.126	**$4.892*10^{-27}$**
Type*Test Reversed Playback	0.153	0.024	-27.695	**$2.765*10^{-47}$**

[1]*All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model assumes Poisson distributed data and contains Bird ID as random factor. Stimulus type and Test stimuli are included as fixed factors, each containing multiple levels.*

Discussion

This study hypothesised that local FoxP1 knockdown in HVC or CMM might impair auditory discrimination in female zebra finches since prior studies in human patients (Le Fevre *et al.*, 2013; Sollis *et al.*, 2016) and knockout mice (Araujo *et al.*, 2017; Fröhlich *et al.*, 2017; Usui *et al.*, 2017a) suggest that disrupted *FoxP1* expression or function could affect auditory perception and cognition. Furthermore, experimentally reduced *FoxP1* expression levels in HVC of these females (see Chapter 2) led to fewer playback requests and a reduced preference for familiar song. In juvenile males, FoxP1 knockdowns in Area X (Norton *et al.*, 2019) or HVC (Garcia-Oscos *et al.*, 2021) lead to impaired song learning which is dependent on auditory feedback. Consequently, this study tested if local FoxP1 knockdowns in HVC or CMM of female zebra finches affected the ability to discriminate songs and to categorise different song modifications. With the exception of one juvenile female in the HVC control group, all birds successfully discriminated their training stimuli, responded correctly in at least 80% of the training trials after a maximum of five training days, and retained or improved this discrimination rate for an additional six days of data acquisition before test stimulus playbacks started.

Learning speed, measured by the number of trials to reach a discrimination rate > 75% was only reduced in birds which received injections in HVC as juveniles (both knockdowns and controls). These birds required more trials than other groups before they reached the criterion indicating that they discriminated the initial Go and Nogo training stimuli. However, extinction of the trained discrimination was not affected by any treatment. It is possible that the virus injections in juvenile HVC could have mechanically damaged or otherwise altered HVC and/or the overlying hippocampus. Tissue damage might have resulted in effects on spatial learning (Bailey *et al.*, 2009) requiring more trials until the birds mastered the spatial left-right organisation of the paradigm. This interpretation would also be in line with the absence of effects on the actual discrimination and categorisation tasks: at the end of the training phase these birds showed the same discrimination and response rates as birds from other groups. However, no damage to the hippocampal tissue above HVC or in HVC itself could be detected during tissue histology.

Besides the necessary trials in order to learn the paradigm, learning and discrimination were unaffected by FoxP1 knockdown in HVC and CMM. This shows that even though

both brain regions strongly express *FoxP1* in juvenile and adult zebra finches (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015), an experimental reduction of FoxP1 levels in these brain regions does not impair processes underlying auditory discrimination at the times tested. The absence of group-specific effects is unlikely to have resulted from a misplaced or dysfunctional viral construct as several virus batches were used that resulted in a significant decrease of *FoxP1* expression in HVC of all knockdown groups (see Chapter 2 and supplementary Fig. 1). FoxP1 knockdowns were expected to have behavioural effects on some groups as a previous study using the same birds showed that adult females which received a FoxP1 knockdown in HVC were affected in the strength of their learned song preferences (Chapter 2 of this thesis). Moreover, during the preference tests, where song playback was the reward for the operant key pecking, the adult HVC knockdown group also showed a lower activity than the other groups. These behaviours are also predicted by the measured *FoxP1* expression levels in the target areas in relation to their respective controls.

As the tested factors of virus treatment, injection site or age did not systematically affect the measured responses to the test stimuli in this study, the merged data of all tested individuals was used to compare the discriminatory abilities of female zebra finches for the different test stimuli. The large sample size and variety of tests conducted allowed for a thorough analysis and comparisons with previous studies (Braaten *et al.*, 2006; Nagel *et al.*, 2010; Lawson *et al.*, 2018). Based on the discrimination rate between the various TestGo and TestNogo versions of training stimuli, birds discriminated stimuli altered in pitch and sequence reversed stimuli just as well as they discriminated the trained stimuli. The discrimination of reversed playback stimuli was poorer than that of training stimuli (although still significant), indicating that the birds paid more attention to the spectral structure of syllables than to syllable order or 8% pitch changes of a motif. However, pitch change is a quantitative measure change and its impact will therefore depend on the magnitude of change (Nagel *et al.*, 2010).

In addition to the discrimination rate, the birds' response rates to the different TestGo and TestNogo stimuli were analysed separately. The analyses of the response rates showed that the birds reduced their response to the TestGo stimuli in comparison to the trained Go stimuli, despite maintaining a high discrimination rate. Additionally, response rate to TestNogo stimuli remained low. The reduced responding indicates

that the birds distinguished test from training stimuli. The response rates varied more than the discrimination rates among the different test stimuli. Interestingly, the birds' response rates differed between stimuli with increased and decreased pitch. Birds responded more to stimuli with increased pitch. This difference was not detected previously (Nagel *et al.*, 2010), but the difference is small and there is a large overlap between the responses towards both pitch-shifted stimuli. The birds maintained the highest response rate to test stimuli with altered syllable order, consistent with the variable position of syllables during motifs (Lachlan *et al.*, 2016) and prior findings from preference tests suggesting that zebra finches recognised songs by their syllable composition despite the changed sequence (Riebel, 2000).

Our sequence reverse test songs contained the same song features, e.g. same pitch and acoustic fine structure of syllables, but in a different syllable order than in the training stimulus. This manipulation had a much smaller effect than reversed playback of songs, indicating that syllable order is less important for categorisation than the acoustic fine structure of the song syllables. Consistent with this, the females responded less to stimuli with increased or decreased pitch than to the training stimuli or the rearranged syllable sequence. Reversing the individual syllables changes the acoustic fine structure of songs the most in comparison to the other experimental stimuli, and also led to the lowest response rate during the experiments.

In summary, the findings from this study suggest that syllable sequence is less important with regard to song identification than overall pitch (at least with the 8% of pitch change during this study) or the structure of individual syllables. This observation may be related to the way juvenile male zebra finches modify their song when their tutor changes. They first adjust the pitch of an already learned syllable followed by a rearrangement of its position in a sequence (Lipkind *et al.*, 2017). Further, rearrangement of an already learned sequence of syllables in juvenile male zebra finches requires more time than they need to integrate an entirely new syllable (Lipkind *et al.*, 2013) hinting at different mechanisms for learning syllable structure and sequences. If females apply the same rules to categorise songs as young males apply during song learning, results from this study might point to potentially similar mechanisms in both sexes to recover sequential information from auditory stimuli. Different recognition mechanisms for pitch and sequence have also been suggested by neurophysiological research where single units in auditory cortex of starlings either

respond to the type of a motif or its pitch where neurons in CMM appeared to be of low selectivity (Meliza and Margoliash, 2012). In zebra finches, neural responses to song in HVC are of higher magnitude during conspecific playbacks with correct syllable sequence than during unaltered playbacks. (Soyman and Vicario, 2017).

Similar to this study, two previous experiments (Braaten *et al.*, 2006; Lawson *et al.*, 2018) found that zebra finches perceive stimuli played back in reverse as very different from non-reversed versions. Lawson *et al.* (2018) reported that recognition of playbacks with locally reversed syllables was reduced in zebra finches and canaries, but not budgerigars, while responses towards stimuli with reversed syllable sequence resembled those towards trained stimuli. However, this study also shows some notable differences from previous publications. Whereas Nagel *et al.* (2010) found that changing the pitch of songs affects their discrimination, they did not find a reduction in response rate to changed stimuli. This difference from the results of this study may be related to different experimental conditions. The present study employed a Go/Nogo paradigm while the prior study used a two-alternative forced choice design, which may reduce the tendency to refrain from responding during a trial as birds need to respond in order to avoid negative reinforcement. The study by Lawson *et al.* (2018) suggests that birds failed to recognise stimuli played back in reverse. However, the present study shows that, even though response to reversed playbacks is reduced, this manipulation apparently still provided some cues that allowed zebra finches to maintain the ability to categorise them correctly.

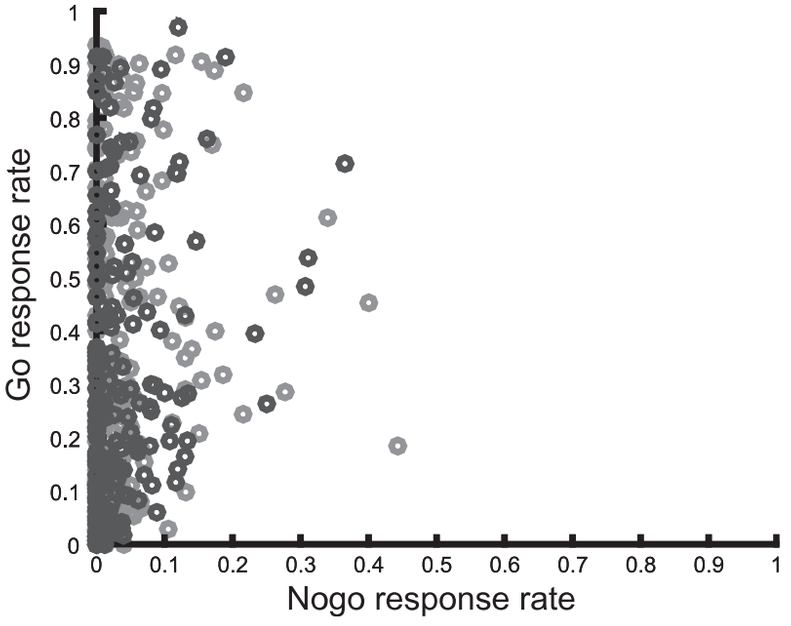
Despite the high and developmentally stable expression of *FoxP1* in HVC and CMM, brain regions which are both involved in perception and processing of song, a local knockdown in these areas did not affect discriminatory performance or any of the related features tested in this study. This preservation of function might conceivably result from individually variable expression or redundancy mechanisms around critical transcription factor families such as FoxPs, masking local knockdown effects. The underlying genetic framework of auditory guided behaviours such as auditory discrimination remains to be identified as it seems not to be influenced by natural *FoxP1* expression in HVC or CMM, or at least to be buffered sufficiently to overcome the effects of a local knockdown on the behaviours examined during this study. Further experiments will be needed to identify the molecular underpinnings of this fine-tuned auditory discrimination. Lastly, this study confirms that zebra finches rely more on

spectral features for song identification and discrimination than sequential information. However, this study also shows that syllable sequence does still contribute to the song identification process.

Conclusion

No behavioural differences were detected between controls and knockdowns of all groups during Go/Nogo tasks. Neither the learning speed nor the final discrimination rate of trained stimuli was affected by the knockdowns. The categorisation of novel playbacks which were modified versions of the initially trained stimuli did also not differ between controls and knockdowns. Subsequently, data from all groups were merged in order to assess the weight of different stimulus manipulations. Stimuli with reversed syllable sequence were categorised best whereas the females' performance towards stimuli played back in reverse was worst. Intermediate levels of correct categorisation were observed towards stimuli either increased or decreased in pitch. Taken together, the results from this Chapter suggest that local FoxP1 expression in HVC or CMM neither contributes to discrimination learning of novel stimuli nor to the categorisation of modified versions thereof. Female zebra finches are most sensitive towards the overall spectral envelope of a stimulus as suggested by their low performance towards reversed stimuli. Pitch changes of familiar stimuli are tolerated to a larger extent which correlates with pitch variability of male song during different social contexts. Syllable sequence seems to be the least important parameter of those assessed in this study suggesting that syllable position does not convey a large amount of information but that informational value of a certain song might be encoded in its spectral properties.

Appendix Chapter 3



Supplementary Figure 1: *Scatterplot of the Go response rates to Go and Nogo type stimuli of all birds. Control (light grey circles) and knockdown birds (dark grey circles) do not cluster separately. Response rates to Go stimuli are distributed along the entire potential behavioural scale whereas the Go response rate towards Nogo stimuli only occurs at low rates.*

Supplementary Table 1: *GLM based analyses of the necessary days until birds of various groups reached the training criterion of a discrimination rate DR > 0.75. Significant p values are marked bold.*

Days to Training Criterion	**estimate**	**std. error**	**t value**	**p value**
Model: 1|Virus batch + Treatment + Region + Age + Treatment*Region*Age[1]				
Intercept	2.333	0.248	9.399	**$3.675*10^{-7}$**
Treatment: Knockdown	-0.25	0.351	-0.712	0.489
Region: HVC	-0.333	0.351	-0.949	0.36
Age: Juvenile	-0.333	0.351	-0.949	0.36
Treatment*Region	0.333	0.497	0.671	0.514
Treatment*Age	-0.083	0.497	-0.168	0.869
Region*Age	-0.083	0.497	-0.168	0.869
Treatment*Region*Age	0.417	0.702	0.593	0.563

[1]All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model assumes

Poisson distributed data due to the large sample size and continuity of the response variable. Virus batch is a random factor. All other parameters were added to the model as equally weighted full factorials and tested for interaction.

Supplementary Table 2: *GLM based analyses of the discrimination rate between Go and Nogo stimuli during three days between reaching training criterion and the introduction of unrewarded stimuli. Significant p values are marked bold.*

Discrimination Training	**Rate estimate**	**std. error**	**t value**	**p value**
Model: 1|Virus batch + Treatment + Region + Age + Treatment X Region X Age*				
Intercept	0.983	0.013	78.517	**$8.653*10^{-19}$**
Treatment: Knockdown	-0.012	0.018	-0.684	0.506
Region: HVC	-0.026	0.018	-1.46	0.168
Age: Juvenile	-0.001	0.018	0.026	0.98
Treatment*Region	0.031	0.025	1.219	0.245
Treatment*Age	0.005	0.025	0.163	0.873
Region*Age	-0.008	0.025	-0.308	0.839
Treatment*Region*Age	0.002	0.035	0.066	0.948

[1]*All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model is based on Poisson distributed data and contains injected virus batch as random factor. All other parameters were added to the model as equally weighted full factorials and tested for interaction.*

Supplementary Table 3: *GLM based analyses of the response rates towards Go and Nogo stimuli during three days between reaching training criterion and the introduction of unrewarded stimuli. Significant p values are marked bold.*

Response Rate Training	**estimate**	**std. error**	**t value**	**p value**
Model: 1|Bird ID + 1|Virus batch + Type + Treatment + Region + Age + Type*Treatment*Region*Age[1]				
Intercept	0.932	0.017	55.569	**$7.645*10^{-17}$**
Type: Nogo	-0.915	0.024	-38.761	**$8.04*10^{-15}$**
Treatment: Knockdown	-0.03	0.024	-1.255	0.232
Region: HVC	-0.005	0.024	-0.21	0.837
Age: Juvenile	0.013	0.024	0.536	0.601
Treatment*Region	0.029	0.034	0.864	0.403
Treatment*Age	-0.027	0.034	-0.791	0.443
Region*Age	-0.022	0.034	-0.653	0.525
Treatment*Type	-0.042	0.033	1.261	0.229
Region*Type	0.031	0.033	0.94	0.364
Age*Type	-0.013	0.033	-0.398	0.697
Treatment*Region*Age	0.02	0.047	0.412	0.687
Treatment*Region*Type	-0.061	0.047	-1.287	0.221
Treatment*Age*Type	0.022	0.047	0.472	0.645
Region*Age*State	0.039	0.047	0.819	0.428
Treatment*Region*Age*Type	-0.03	0.067	-0.452	0.659

[1]*All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model assumes Poisson distributed data and contains Bird ID and injected virus batch as random factor All other parameters were added to the model as equally weighted full factorials and tested for interaction.*

Supplementary Table 4: *GLM based analyses of the discrimination rate of all test stimuli. Significant p values are marked bold.*

Response Rate All Birds	**estimate**	**std. error**	**t value**	**p value**
Model: 1|Bird ID + 1|Virus Batch + Treatment + Region + Age[1]				
Intercept	0.871	0.021	42.095	**$3.06*10^{-63}$**
Treatment: Knockdown	0.01	0.019	0.516	0.607
Region: HVC	0.003	0.021	0.158	0.875
Age: Juvenile	-0.008	0.022	-0.383	0.703

[1]All fixed factors consist of multiple levels where the first level is always used as a reference to compare the effect of all other levels, respectively. The model is based on Poisson distributed data and contains Bird ID and virus batch as random factors. Treatment (knockdown or control), target region (HVC or CMM) and age during injection (juvenile or adult) are included as equally weighted fixed factors.

Chapter 4

Transcriptomic investigations of age- and region-specific knockdowns in female zebra finches identify potential downstream networks of FoxP1

Chapter 4: Transcriptomic investigations of age- and region-specific knockdowns in female zebra finches identify potential downstream networks of FoxP1

Fabian Heim, Katharina Riebel, Carel ten Cate, Constance Scharff, Simon E. Fisher

Abstract

FOXP1 is a highly conserved transcription factor that regulates the expression of target genes in diverse species. Evidence from multiple sources suggests that FOXP1 is important for aspects of brain development and function. For example, humans with rare heterozygous disruptions of *FOXP1* have been diagnosed with intellectual disability and/or autism spectrum disorder, as well as speech and language deficits. The avian ortholog, *FoxP1*, is highly expressed in a subset of song-related nuclei in the brains of songbirds, and prior studies have employed experimental knockdowns of this gene in Area X, HVC and CMM of male or female zebra finches, to investigate potential links to behaviour. In particular, in the work described in earlier parts of this thesis, female zebra finches were injected with lentiviral knockdown constructs in HVC or CMM during two different developmental stages. The present chapter sought to use tissue samples from the targeted brain areas in these birds to identify putative molecular targets and pathways that lie downstream of FoxP1, via a transcriptomic approach based on next-generation RNA-sequencing. Differentially expressed genes between control and knockdown groups were analysed across the different brain areas and the different developmental stages of genetic manipulations. With the exception of *FoxP1* itself, no individual gene showed significant differences in expression in all groups of this study. Nonetheless, data from the different groups on differentially expressed genes, enriched GO terms, gene sets and local networks together point to possible links of *FoxP1* to retinoic acid signaling or SLIT-ROBO pathways, among others. Moreover, differentially expressed genes associated with FoxP1 knockdown showed an overrepresentation of candidate loci involved in autism spectrum disorder and intellectual disability, based on analyses of independent databases that collated likely risk genes. The expression profiling data from this study can offer new insights into neurogenetic networks that may be regulated by FoxP1, suggesting hypotheses for future investigation in a range of species and model systems.

Introduction

Studies in multiple species indicate roles of the FOXP1 transcription factor in aspects of brain development and function. In humans, rare variants that disrupt FOXP1 result in a neurodevelopmental syndrome involving a range of features including intellectual disability, autism spectrum disorder, and impairments in speech and language (Sollis *et al.*, 2016; Siper *et al.*, 2017). Orthologues of *FOXP*1 have been identified in highly similar form in many different vertebrate and invertebrate species where they are thought to regulate the expression of downstream target genes in the brain and other tissue (Mazet *et al.*, 2003; Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Hannenhalli and Kaestner, 2009; Lawton *et al.*, 2014; Viscardi *et al.*, 2017). The high degree of homology has made it possible to investigate implications of FOXP1 dysfunction in animal models (Takahashi *et al.*, 2009; Scharff and Petri, 2011; Deriziotis and Fisher, 2017; Co *et al.*, 2020a), often with a focus on consequence for vocal behaviours (Fröhlich *et al.*, 2017; Norton *et al.*, 2019). For example, heterozygous deletions of *Foxp1* disrupt mouse vocalisations (Araujo *et al.*, 2015), and forebrain-specific knockouts of the gene result in perturbed isolation calls of mouse pups (Usui *et al.*, 2017a). Consistent with these findings, other studies have shown that brain-wide homozygous deletions of mouse *Foxp1* reduce social interactions (Bacon *et al.*, 2015) and the rate of pup isolation calls upon removal of the mother (Fröhlich *et al.*, 2017).

As described in earlier Chapters of this thesis, and other recent studies, the contributions of FoxP1 to vocal behaviours have also been investigated in the zebra finch (*Taeniopygia guttata*), a songbird in which males learn their vocalisations by imitation of a tutor (Immelmann, 1962; Zann, 1997; Tchernichovski *et al.*, 2001)(Doupe and Kuhl, 1999; Scharff and Petri, 2011; Bruno *et al.*, 2021). It is of particular interest for this thesis that *FoxP1* is expressed in distinct nuclei in the brains of songbirds, including those known to be important for vocal learning, such as Area X in the striatum. Compared to the surrounding tissue, *FoxP1* expression is also elevated in the robust nucleus of the arcopallium (RA, a motor nucleus), the premotor area HVC, and the entire mesopallium, including the caudomedial mesopallium (CMM), which is a secondary auditory area (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015). With the exception of RA, *FoxP1* seems to be highly expressed in brain areas associated with tasks related to auditory perception and feedback. Notably, and despite the absence of Area X and a negligible state of RA in female zebra finches

(Nottebohm and Arnold, 1976), neural *FoxP1* expression patterns are highly similar between sexes of this species (Teramitsu *et al.*, 2004).

Genetic manipulations that reduce expression levels of *FoxP1* in the basal ganglia Area X of juvenile male zebra finches lead to impaired song learning, as do knockdowns of paralogues FoxP2 and FoxP4, albeit with differences in the nature of impairments (Haesler *et al.*, 2007; Murugan *et al.*, 2013; Norton *et al.*, 2019). FoxP2 overexpression in Area X of adult zebra finch males also alters song by increasing its variability (Day *et al.*, 2019a).

Knockdown of *FoxP1* expression levels in HVC of male juvenile zebra finches has also been shown to impair song learning, but only if the knockdown occurs prior to song exposure (Garcia-Oscos *et al.*, 2021). The experiments described in prior Chapters of this thesis showed that *FoxP1* knockdown in HVC of adult female zebra finches may disrupt rewarding qualities of conspecific song (Chapter 2), while the same manipulations made during earlier developmental stages, prior to song preference establishment, do not affect preference strength in adults or preference establishment for familiar song (Chapter 3). *FoxP1* knockdown in CMM of juvenile or adult females did not affect the birds' ability to establish or maintain a song preference (Chapter 2). Knockdowns of *FoxP1* in HVC or CMM in juvenile females prior to the onset of the sensory phase, or in adults well after closure of the vocal learning period, did not alter the birds' ability to discriminate different conspecific song stimuli or categorise altered versions of them (Chapter 3). These studies characterise behavioural consequences of manipulating *FoxP1* expression in particular brain structures, but they do not give information about the neurogenetic pathways that are regulated by the transcription factor. To gain insights at that level, it is necessary to integrate the employed knockdown strategy with a molecular screening technique, which is the subject of the current Chapter.

As a transcription factor, FoxP1 acts by forming homo- or heterodimers and multimers with other FoxP molecules (Li *et al.*, 2004; Sin *et al.*, 2014; Castells-Nobau *et al.*, 2019). Together, these complexes bind to DNA and modify expression levels of other genes – its downstream targets. Identification of target genes and their respective functions can provide insight into the molecular and cellular pathways that a transcription factor regulates. For transcription factors, like FoxP1, that have highly similar orthologues in multiple species (Hannenhalli and Kaestner, 2009), model systems can be used to

facilitate the identification of target genes. Depending on the underlying motivation of a study, experiments employ model systems ranging from cell-culture to analyses of tissue obtained from genetically modified animals, such as knockout mice, or zebra finches which underwent localised knockdowns.

To identify genes and pathways which are regulated by transcription factors such as FoxP1, multiple alternative molecular approaches are available. Chromatin immunoprecipitation (ChIP) methods make it possible to define the genomic interaction sites of a DNA-binding protein (Buck and Lieb, 2004; Park, 2009). These techniques involve cross-linking of DNA-binding molecules to the DNA, followed by immunoprecipitation of linked protein-DNA complexes with antibodies that specifically recognize the protein of interest. The precipitated complexes are then treated to remove the crosslinks, and the extracted DNA is analysed, for example by screening with arrays (ChIP-chip, Buck and Lieb, 2004) or via sequencing (ChIP-seq, Furey, 2012), to identify which genomic regions are enriched in the immunoprecipitated samples. The process yields knowledge of the genomic binding sites of the protein, and this information can be used to determine the identities of candidate target genes that it may regulate. However, the success of ChIP-based assays depends critically on the reliability of the antibody used for immunoprecipitation, and levels of enrichment can be subtle, making it difficult to identify differences between datasets. Additionally, the target epitope of the antibodies employed may be blocked by additional proteins.

For studies in which genetic manipulations are used to alter the levels of an important regulatory molecule, comparison of expression profiles represents a valuable strategy for characterizing downstream pathways. While ChIP uncovers primary targets of a transcription factor, expression profiling can identify both direct and indirectly regulated targets. These methods do not rely on binding sites of one specific protein but consider changes throughout the transcriptome (Pollack *et al.*, 1999). RNA is extracted, reverse transcribed, fragmented, amplified and can then be analysed with a number of techniques. In microarray-based expression profiling, the amplified fragments are tagged and applied onto a chip which contains complementary fragments based on the transcriptome of the species being studied. Tagged fragments binding their complementary strands on the chip are used to characterize the levels of expression of genes in the sample. However, due to the necessary preselection of complementary probe fragments, microarray analyses may be biased and are not optimal for covering

the entire transcriptome. These limitations have been largely overcome with the application of next-generation sequencing techniques to expression profiling (RNA-sequencing) allowing for quantification of transcript levels across the transcriptome in a less biased manner than arrays (Cloonan *et al.*, 2008; Wang *et al.*, 2009).

Genome wide changes due to knockdowns or other genetic manipulations could also be assessed by the identification of chromatin state changes. Chromosome conformation changes can provide insight about the status of DNA within the chromatin complex and possible differences between samples (Schmitt *et al.*, 2016). Based on the accessibility of a genomic region, putative target genes and genomic regions with increased or decreased accessibility due to a gene specific knockdown (for example) can be deduced. However, this approach requires relatively large amounts of DNA from the target tissue.

In mice, shRNA mediated *Foxp1* knockdown has been found to yield changes in the expression of genes with GO terms associated to neurogenesis, regulation of synapse organisation and nervous system development, in addition to the Notch signalling pathway which in turn might contribute to impaired differentiation of neural stem cells to astrocytes and neurons *in utero* and *in vivo* (Braccioli *et al.*, 2017). In mice with heterozygous knockout of *Foxp1* in medium spiny neurons, the development of subtype composition of these neurons is altered as determined by single cell RNAseq. *Foxp1* knockout reduces the occurrence of indirect pathway spiny neurons when compared to wildtype controls, possibly due to differential regulation of genes specific to spiny neuron subtypes. Additionally, *Foxp1* knockouts result in differential regulation of genes associated to autism spectrum disorders (ASD, Anderson *et al.*, 2020). Global *Foxp1* heterozygous knockout mice show significant overlap of differentially expressed genes to human neural progenitor cells (NPC) overexpressing *FOXP1*. When compared to gene expression in striatal or hippocampal tissue from mice with global heterozygous *Foxp1* knockouts, *FOXP1* overexpressing human NPCs show a larger overlap in gene expression with striatal tissue than with hippocampus as shown by module preservation in weighted gene coexpression network analyses. Gene expression in hippocampus of mice with global heterozygous *Foxp1* knockouts also highlights pathways linked to long term potentiation, synaptic signalling and spatial memory which are all relevant for learning (Araujo *et al.*, 2015). These findings are interesting given that global heterozygous *Foxp1* knockout mice demonstrated poor

learning during Morris water maze trials, less successful performance on T-Maze tasks, and reduced maintenance of long term potentiation, assessed via slice electrophysiology (Araujo *et al.*, 2017).

In this Chapter, I took advantage of the availability of zebra finches with *FoxP1* knockdowns in selective brain regions and distinct developmental stages, as generated in my prior thesis work, to help identify *in vivo* networks downstream of this transcription factor. I employed RNA sequencing analyses (RNAseq), since that made it possible to analyse expression levels of distinct genes and pathways directly with relatively low amounts of material at a larger dynamic range, covering the entire transcriptome and with improved detection of weakly expressed genes (Wang *et al.*, 2009; Zhao *et al.*, 2014). In the process of RNAseq, total RNA from different samples is extracted and reverse transcribed into cDNA which is further fragmented and purified prior to library generation. Following amplification of these libraries, the fragmented cDNA strands are sequenced and aligned to a reference genome. Based on the alignment, each fragment can be assigned to a coding or non-coding region of the target organism's genome. The number of assignments can then be used to determine and compare gene expression levels based on the number of fragments assigned to a specific region (= counts).

In this study, total RNA from the targeted brain areas of the different groups was extracted. Genes and pathways associated with knockdowns of *FoxP1* in general were investigated with the aim to identify affected genes and molecular pathways in the brain in general, but also how these genes and pathways are differentially affected in the context of age or developmental status (juvenile and adult groups) and local brain areas (HVC and CMM). Following the identification of unique and overlapping genes and pathways, the findings were compared to previous mouse studies focusing on downstream targets of Foxp1, and potential physiological and behavioural consequences that have been related to dysfunction of this important transcription factor.

Material and Methods

Test subjects

Subjects were 96 female zebra finches from the breeding colony at the Freie Universität Berlin. At Leiden University, birds were housed in groups until behavioural testing was started (Chapters 2 and 3 of this thesis). The four treatment groups were defined by when (as juveniles < 25 days post hatch (dph) or adults > 90 dph) and where (HVC or CMM) they received the *FoxP1* knockdown and labelled accordingly: HVC juvenile, HVC adult, CMM juvenile, CMM adult (for details see Method sections of Chapters 2 and 3). Each corresponding knockdown and control group consisted of 12 females.

Viral particles and injection

Viral particles were produced at the Freie Universität Berlin as described in Chapters 2 and 3. Birds were injected with one of two shRNA constructs complementary to *FoxP1* mRNA to knock down *FoxP1* expression. Both knockdown constructs also contained a GFP sequence to label successfully transduced cells. The two different knockdown constructs were employed to obtain an opportunity to filter for putative off-target effects induced by either one of the shRNAs (Song *et al.*, 2015). A similar construct which contained the sequence for GFP but no *FoxP1* targeting shRNA was used for control animals. The sequence of the two short-hairpin constructs was as follows (Norton *et al.*, 2019):

	Hairpin sequence
Construct 1	5'-CCCCTATGCAAGCAATGCACCCAGTGCATGTCAAAGAAGAACCATTAGACCCAGATGAAA-3'
Construct 2	5'-CCAGATGAAAATGAAGGCCCACTATCCTTAGTGACAACAGCCAACCACAG-3'

Viral particles were produced in seven batches for each knockdown construct, and five batches of control virus. Each virus batch was injected into both hemispheres of on average 4 birds (range 2 – 6). This corresponds to on average 6 different batches per treatment group (range 3 – 9) including matched controls. By merging samples from birds which received injections from different batches into larger control or knockdown groups, it was possible to control for batch-specific effects due to differences in titre or transduction-efficiency (for details see extended data of Chapter 2, Table 2-1). The injection procedure is described in detail in Chapters 2 and 3.

Briefly, viral constructs were injected bilaterally in one of the two target areas of a juvenile or adult female (see Chapter 2 Table 1 for injection coordinates in reference to the bifurcation of the midsagittal sinus). The injection site was closed with previously removed bone tissue, and the skin was sealed. After the surgical procedure, the birds were returned to their respective housing cages.

Brain extraction

After completion of behavioural experiments (preference tests and Go/Nogo tasks, described in Chapters 2 and 3 respectively), females were housed in their home cages with other familiar females for at least one week. Between 3 – 5 pm on the day before brain extraction, birds were individually transferred into familiar sound attenuated chambers used during the prior behavioural tests. In order to minimise activity-dependent expression changes, birds were sacrificed with an overdose of isoflurane gas before light onset on the next morning (6:30 – 6:50 AM). Birds of the juvenile groups were 179 – 210 days old and those of the adult groups 165 – 579 days old, respectively. Note that juvenile and adult refers to the developmental stage the birds received lentiviral injections while all behavioural experiments and subsequent tissue extractions where conducted in adult birds. Fresh hemispheres were separated along the midline and frozen in Tissue Tek Optimal Cutting Temperature Compound (OCT, Sakura, Leiden) on dry ice and stored at -80°C at the Language and Genetics Department at the Max Planck Institute in Nijmegen, the Netherlands.

Validation of injected area and extracted tissue

The injection site was validated immunohistochemically (Chapter 2) by staining with antibodies against FoxP1 and GFP, and counterstaining of nuclei with Hoechst (Thermo Fisher Scientific, Waltham USA). The target areas, HVC or CMM were extracted with biopsy punches of frozen brain slices. Correct placement of the biopsy punch site in HVC or CMM was validated visually under a stereomicroscope, and GFP-based fluorescence was documented in the extracted tissue punches.

RNA extraction

GFP-positive biopsy punches were submerged in RNAlater (Qiagen, Hilden) and pooled by hemisphere for each bird. At least 12hrs after punching, a column-based

RNA extraction kit was used to extract and purify total RNA according to the manufacturer's protocol (RNeasy micro plus, Qiagen, Hilden). RNA quality and concentration were determined with a Bioanalyser RNA kit (Biorad, Hercules). Extracted RNA was stored at -80°C until transcriptome sequencing.

Total RNA sequencing

RNA sequencing was performed in three batches. The first batch contained only samples from adult HVC knockdowns and their respective controls. The second batch included adult HVC and adult CMM knockdown and control samples, while the last batch consisted of knockdown and control samples from all targeted areas and ages (see Supplementary Table 1). Only a subset of samples from all birds (N = 104/192, 54%) fulfilled the necessary quality criteria for sequencing (> 0.4 µg total RNA, RNA integrity index > 7.2). Drop-out rate was distributed evenly among groups resulting in 5 to 9 samples per group (see Supplement). These drop-outs can be partly attributed to cases where tissue punches did not show fluorescence under the stereomicroscope or misplaced punching sites which led to fewer biopsy punches for RNA extraction. In a recent study on gene expression differences in brain nuclei of different birds, unrelated to the present work, no samples had to be dropped. However, because that study was limited to microarray analyses, the experiments only required one fourth of the RNA amount necessary for total RNAseq analyses (Ko *et al.*, 2021). As punched tissue samples in the current study were further preselected based on the correct site of the biopsy punch and presence of fluorescence, it is not possible to make meaningful comparisons of RNA yields to those in prior work. Library preparation and sequencing was performed by Novogene Co., Ltd. (Beijing). After enrichment with oligo(dT) beads and random fragmentation, libraries were constructed with 150 – 200 base-pair (bp) inserts. cDNA was synthesised using random hexamers and reverse transcriptase. The second strand was completed by nick-translation with a custom second-strand synthesis buffer provided by Illumina (San Diego, USA) containing dNTPs, RNAse H and *Escherichia coli* polymerase I. cDNA libraries then underwent purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment. Illumina HiSeq 2500 sequencers were used to produce > 20 million single-end, 50-bp reads.

Table 1: *Summary of all samples suitable for RNA sequencing. Samples are grouped in columns by treatment, targeted area, age during injection and the hemisphere from which RNA was extracted.*

Treatment	Control								Knockdown							
Target area	HVC				CMM				HVC				CMM			
Age group	Juvenile		Adult		Juvenile		Adult		Juvenile		Adult		Juvenile		Adult	
Hemisphere	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Samples	7	6	6	8	6	9	7	5	7	5	7	8	5	6	6	7

Gene expression analyses

Read counts ranged from 17.9 – 39.9 million reads per sample (mean: 24.2 ± 4 million reads per sample). One adult HVC control sample with significantly lower reads (bird ID: 5424; 2.48 million reads) was excluded from further data analyses. Quality control was conducted with FastQC (v0.11.9, Babraham Bioinformatics). Reads were aligned to the zebra finch Blue55 reference genome (NCBI assembly ID 5966711) using Rstudio (v1.3.1093) and the Rsubread package (v2.4.3) with standard settings (exception: indels = 10, count exon junctions). A BAM file was produced which included mapped (90.7 – 95.9 %, mean: 93.7 ± 1.2 %) and unmapped reads (see Figure 1a) to d) for controls and i) to l) for knockdowns). Multi-mapped reads were included in the analyses to cover potential splice variants. Rsubread was used to assign mapped reads (89.6 – 95.5 %, mean: 93.0 ± 1.4 %, see Figure 1e) to h) for controls and m) to p) for knockdowns) according to published annotations for the female zebra finch Blue55 reference genome. Counts were subsequently calculated according to protein-coding genes of the annotation file using standard settings of Rsubread with the exception of enabled counts of exon-exon junctions.

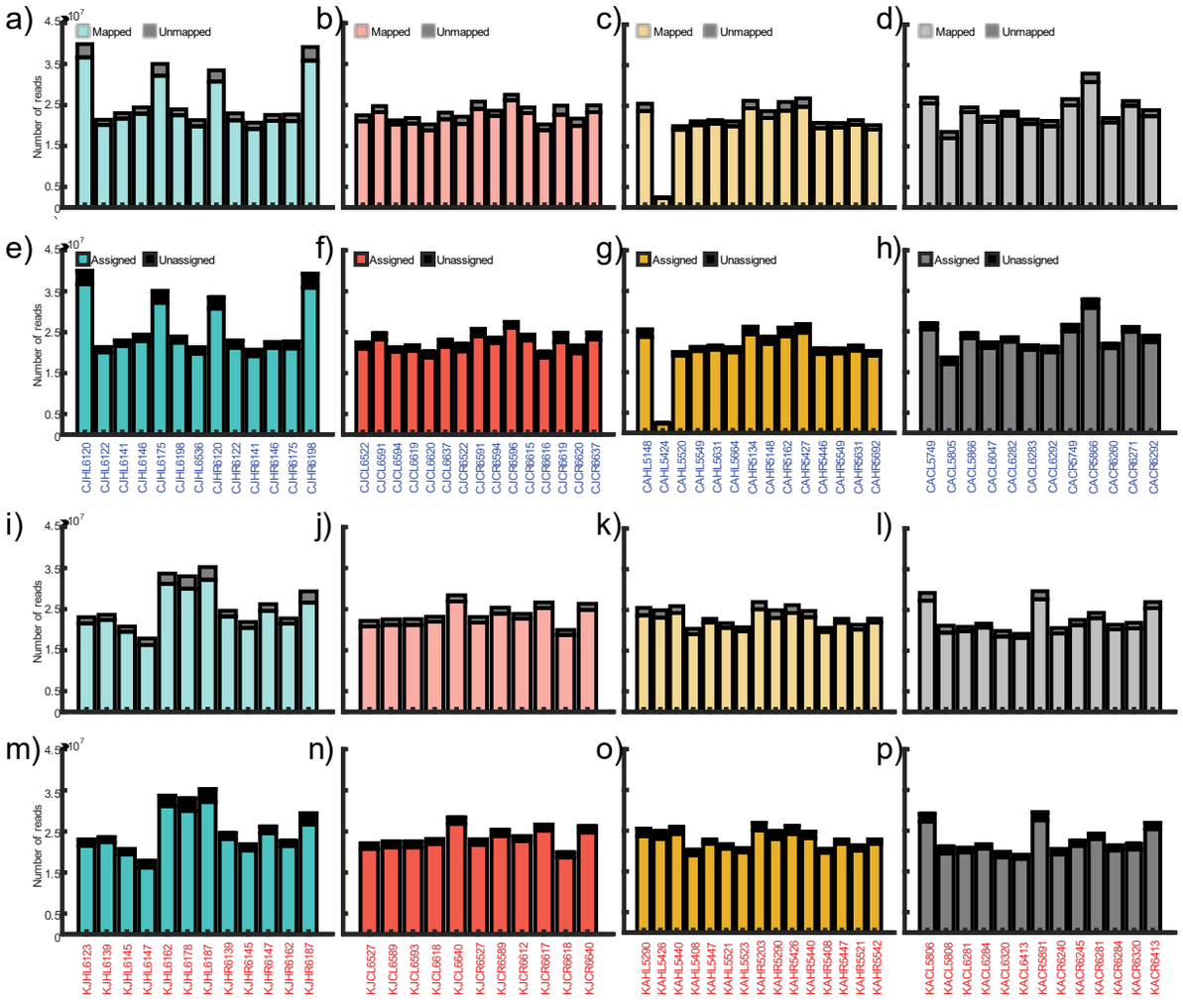


Figure 1: *Mapped and assigned reads per age group, injected area and treatment. Mapped and unmapped reads of juvenile control samples taken from HVC (a) or CMM (b) and adult control samples from HVC (c) or CMM (d). Note that one adult HVC control sample (CAHL5424) contained fewer reads than any other sample and was thus excluded from further analyses. Mapped and unmapped reads from knockdown samples taken from juvenile HVC (i), juvenile CMM (j), adult HVC (k) and adult CMM (l). The number of assigned reads is shown for control samples from juvenile HVC (e), juvenile CMM (f), adult HVC (g) and adult CMM (h). Assigned reads of knockdown samples is shown in the same order from m) to p). Individual sample IDs are indicated at the bottom and consist of a four-letter, four-digit code that is structured as follows: first letter = C for control or K for knockdown; second letter = J for juvenile or A for adult; third letter = H for HVC or C for CMM; fourth letter = L for left hemisphere or R for right hemisphere. Four numbers indicate the individual bird ID the sample was taken from. Hemispheres were not analysed separately during further analyses.*

Gene counts were normalised using reads per kilobase per million mapped reads (RPKM, Mortazavi *et al.*, 2008). Differentially expressed genes were limited to occurrences of >1 read per million mapped in at least two samples and visualised based on K-means clustering analyses.

Gene expression comparisons were conducted in Matlab release 2020a (Mathworks, Natick, USA) with the bioinformatics toolbox. Variance of read counts was identified by plotting the dispersion against the mean of the respective sample group. To determine statistical significance of gene expression differences, negative binomial models of the normalised read counts were conducted assuming a Poisson distribution, a constant variance link and a locally regressed non-parametric smooth function of the mean. Locally regressed modelling provided the best fit and was chosen for further analyses. To account for multiple testing of differentially expressed genes between control and knockdown samples, p-values were adjusted according to the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) considering a 10% false positive rate. Differences in gene expression were visualised as a Venn diagram to identify overlapping and exclusive genes identified for each group. As the female reference genome has a slightly lower coverage (82.5) than the latest reference genome of the male zebra finch (88.2, NCBI assembly ID 10005361), highly significant but unannotated loci were referenced to the male reference genome to identify potentially unannotated regions in the female genome. Additionally, annotations from an Affymetrix array containing predicted exon sequences from previous incomplete zebra finch genome assemblies (MPIO-ZF1s520811, Dittrich *et al.*, 2014) were used to cross-reference loci that were not annotated in the female reference genome.

Relative expression levels of *FoxP1*, based on RPKM differences between control and knockdown samples, were further compared with those determined during qPCR analyses of the same samples. Gene ontology enrichment (GO) and local network analyses were conducted based on avian/mammalian gene orthologues in STRING v11 (Szklarczyk *et al.*, 2019) considering only categories with a p-value <0.05 and corrected for false discovery rate (Benjamini and Hochberg, 1995). All assigned genes across all control and knockdown groups from this study were used as the background reference to control for brain- and area-derived enrichments. Unbiased gene set enrichment analyses was conducted with GSEA v4.1.0 (Subramanian *et al.*, 2005) including only genes with >1 read per million mapped in at least two control and

knockdown samples, respectively. Standard settings remained unchanged and the cut-off was set to a gene set size of 15. The gene set database was based on a complete list of human gene symbols (c2.all.v7.4.symbols) and data were permutated 1000 times by phenotype (control or knockdown). Annotation data of the identified genes were based on a chip annotation database integrated in the GSEA programme to identify human orthologues of the counts mapped in the samples of this study (Human Gene Symbol with Remapping MSigDB.v7.4). The overlaps of differentially expressed genes of this study and databases were tested for significance using a Chi-Square test including Yate's correction for continuity.

Results

Modelling of normalised read counts and identification of genes showing significant differences in expression

Read counts of this study were modelled best by a negative binomial distribution based on a local regression for control and knockdown samples respectively taken from juvenile HVC (Fig. 2a, b), juvenile CMM (Fig 2e, f), adult HVC (Fig 2i, j) or adult CMM (Fig. 2m, n). The relationship between local dispersion and means for samples from different regions and developmental stages did not differ between control (blue) and knockdown samples (red) from birds injected as juveniles in HVC (Fig. 2c), as juveniles in CMM (Fig. 2g), and as adults in CMM (Fig. 2o). However, dispersion of normalised reads of HVC from birds which received a control construct was positively biased (Fig. 2k) and thus these may have a possible underrepresentation of weakly expressed genes in comparison to their respective knockdown samples. The distribution of differentially expressed genes in knockdown samples is shown as volcano plots for samples from juvenile HVC (Fig 2d), juvenile CMM (Fig 2h), adult HVC (Fig 2l) and adult CMM samples (Fig 2p). The distribution of log2 fold changes of individual control (filled circles) and knockdown samples (open circles) across all groups (Fig. 2q) does not indicate a bias in fold change of one specific subgroup or treatment and thus comparable levels of gene expression changes between all groups. The relative expression of *FoxP1* in target areas of knockdown birds was comparable to qPCR data used previously to identify knockdown efficiency (Chapter 2). However, as determined by a two-way ANOVA, the variance differed between assessment methods ($p < 0.0001$), while no significant differences were evident for area and age during

treatment ($p > 0.05$) or the identified knockdown efficiency within individual groups ($p > 0.05$).

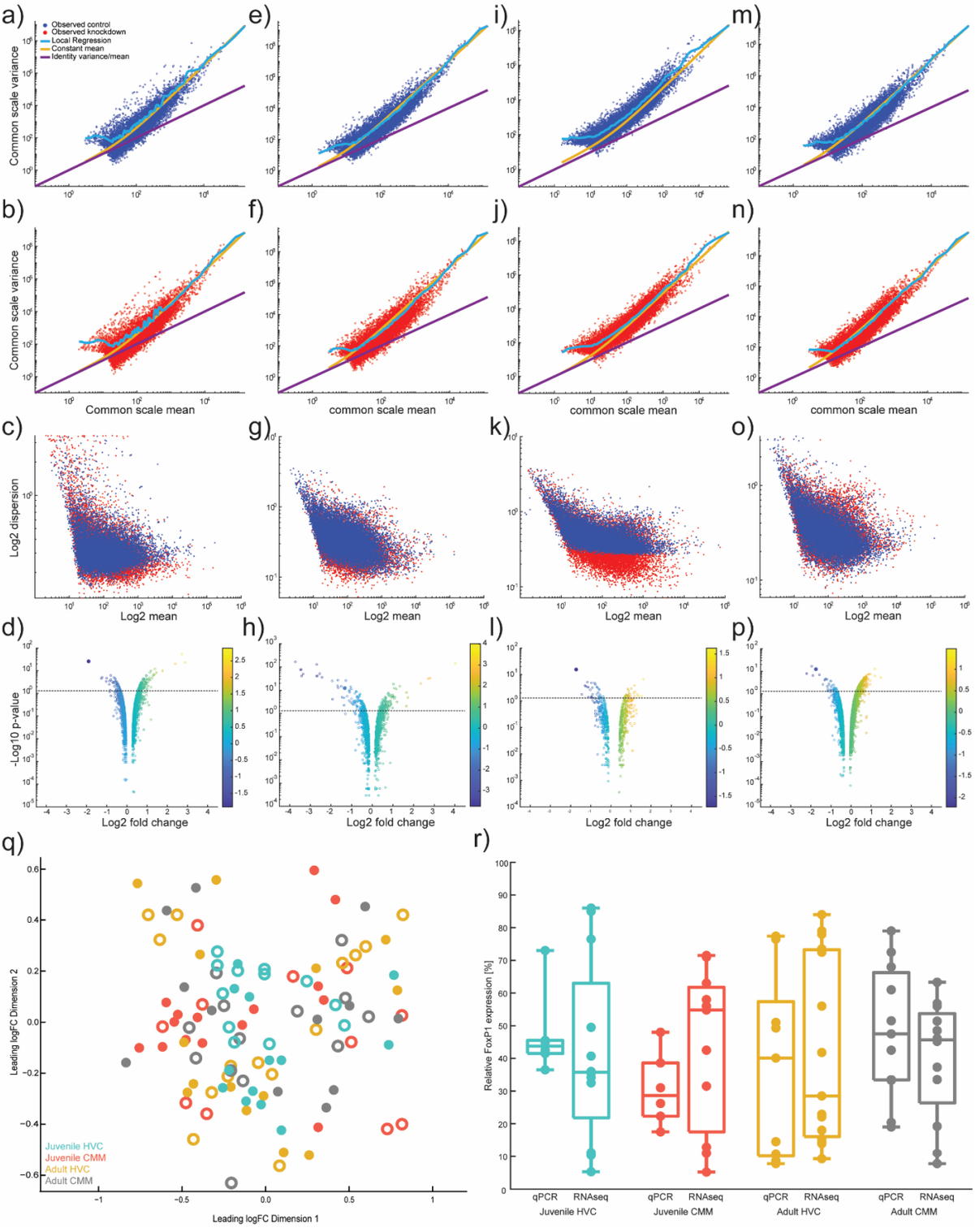


Figure 2: *Feature count properties, differentially expressed genes and their feature count distribution, and comparison between relative FoxP1 expression shown by qPCR and RNAseq. Each column symbolises one subgroup. a) – d) represent the data*

from juvenile HVC birds, e) – h) from juvenile CMM birds, i) – l) from adult HVC birds and m) – p) from adult CMM birds. a), e), i) and m) show the relation between a subgroups variance and mean for control samples, in blue. The same data are shown for knockdown samples in b), f), j) and n), in red. In order to specify the linkage type between the variance and mean, three approaches were taken. The purple line shows the results of assuming a linear correlation between variance and mean. The yellow curve shows the results of assuming the variance is a sum of the mean and a constant multiplied by the squared mean (yellow curve). Considering the variance as the mean read count variability, and applying a locally regressed smoothing function, results in the best fitting correlation (light blue curve). c), g), k) and o) show scatter plots of the log2 transformed dispersion and means of each subgroup's feature counts for control samples in blue and knockdown samples in red. In the subgroup of birds which received a control construct as adults in HVC, the dispersion dominates over the mean which suggests that more genes are highly expressed in the control when compared to the knockdown samples. d), h), l) and p) show volcano plots of all differentially expressed genes in the different subgroups. The dotted line indicates the significance threshold at an adjusted p-value of 0.05. Filled and enlarged circles indicate the differential expression strength of FoxP1. Colourbars symbolise the log2 fold change for each differentially expressed gene. q) shows two-dimensional scaling based on overall log2 fold changes of genes across all samples of this study, separated by colour. Filled circles indicate control samples, while open circles indicate knockdown samples. r) shows the relative FoxP1 expression levels in knockdown samples compared to control samples for a qPCR- and an RNAseq-based approach for all different subgroups. Relative expression levels differ significantly between results obtained by qPCR and RNAseq across all groups but neither between the target areas and developmental stages nor the assessment methods within each area and developmental stage (two-way ANOVA + TukeyHSD, method ($F=43.81$, $p < 0.0001$), subgroup ($F=2.65$, $p > 0.05$), method x subgroup($F=1.12$, $p > 0.05$).

Mapped genes across different groups

The number of identified genes based on read assignments varied between groups (Table 2, range 10,923 juvenile HVC control – 12,642 Adult HVC control).

Table 2: *Number of protein-coding genes per group and treatment (Control = Ctrl, Knockdown = KD) which were identified based on sequenced, mapped and assigned transcripts from total RNA sequencing data.*

Group	Juvenile HVC		Juvenile CMM		Adult HVC		Adult CMM	
Treatment	Ctrl	KD	Ctrl	KD	Ctrl	KD	Ctrl	KD
# of identified genes	10,923	11,068	11,439	11,140	12,642	12,530	11,841	11,409

In summary, counts were assigned to 13,695 protein-coding, annotated or predicted genes (Supplementary Table 2). This number corresponds to 84.5% of all 16,197 annotated or predicted protein-coding genes of the female zebra finch reference genome that was used for annotation. When non-coding genes and pseudogenes are also included, transcripts from 63.57% of 21,543 annotated segments of the reference genome were mapped and assigned in this study.

Dendrograms of differentially expressed genes

Hierarchical clustering of normalised reads from all genes identified across all samples of all groups results in a dendrogram which clusters all samples by age during treatment and the targeted area (Fig 3a). Samples generated from HVC of birds treated as adults were allocated in the most distant cluster in relation to the other three groups. Among the remaining three main clusters, juvenile CMM samples were the most distant, and juvenile HVC and adult CMM samples the closest. However, neither treatment nor hemisphere of the samples segregated in the overall hierarchical dendrogram, which is why samples from each subgroup of this analyses were then clustered separately. Samples from HVC of birds injected as juveniles form two main clusters to which both controls and knockdowns across both hemispheres contribute similarly (Fig. 3b). Samples from birds injected in CMM as juveniles cluster in two groups based on the normalised counts of all identified genes irrespective of hemisphere or treatment (Fig. 3c). Hierarchical clustering of normalised gene counts

of samples obtained from birds injected as adults in HVC (Fig. 3d) or CMM (Fig. 3e) results in three main clusters which neither segregate by hemisphere nor by treatment. In summary, hierarchical clustering suggests that local knockdowns during different developmental stages contribute differently to the transcriptome since the four treatment groups were well separated in the dendrogram that included all samples. However, when the groups are clustered separately, interindividual gene expression differences outweigh the effects caused by local knockdowns, as no clear discrimination of the used construct is evident at the transcriptome-wide level.

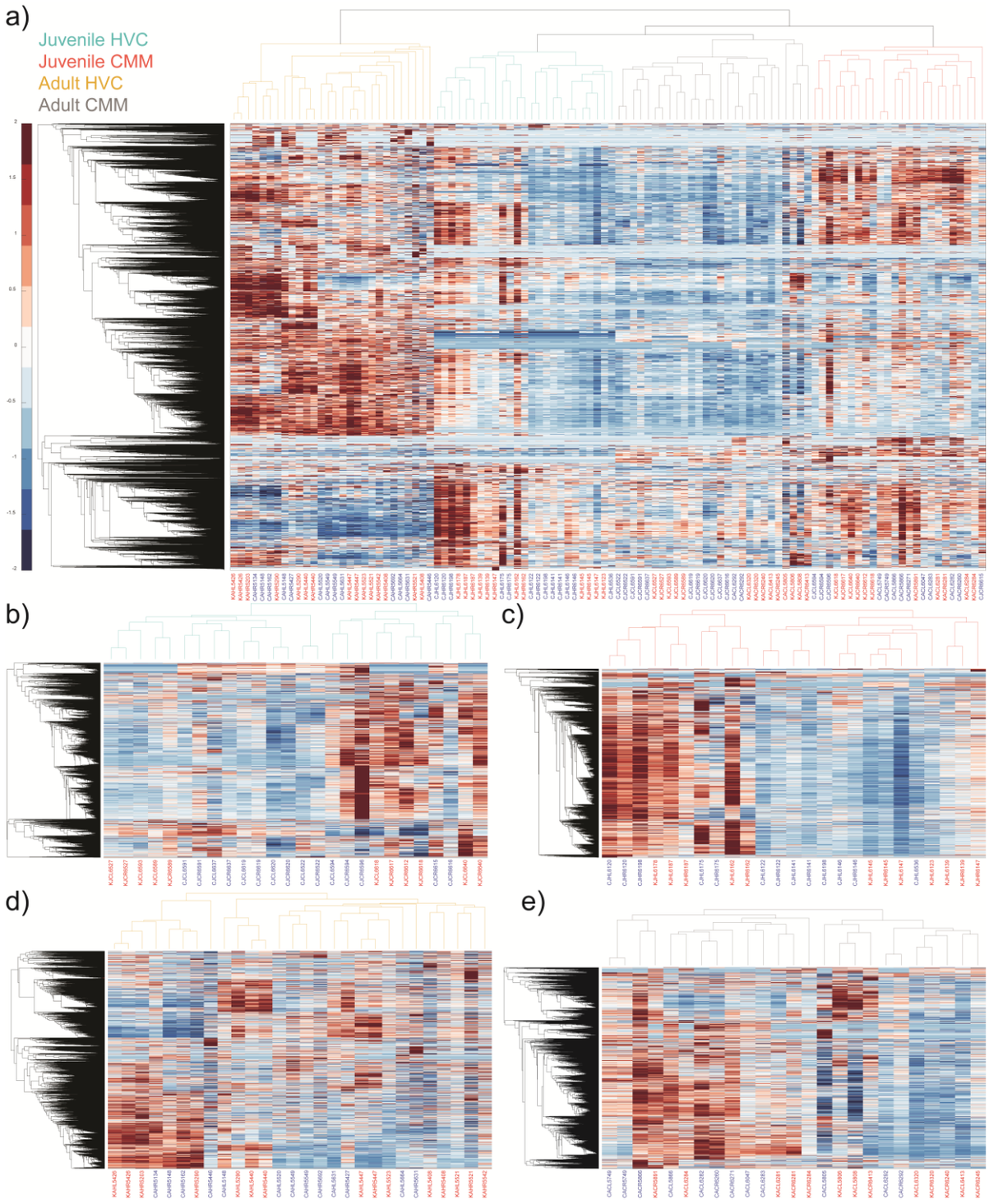


Figure 3: *Hierarchically clustered dendrograms of gene counts. Columns are clustered by sample ID, rows by gene ID. A heatmap encodes the standardised counts of the genes present in the dataset in relation to a mean of 0 and a standard deviation of 1. a) The dendrogram of the entire RNAseq data set shows clear clustering of knockdowns at different ages and in different areas as indicated by the colours of the dendrogram. b) to e) show individual dendrograms for each subgroup and indicate that,*

considered at the transcriptome-wide level, the within group effects of interindividual variability appear larger than effects of knockdowns. This is underlined by the close clustering of both hemispheres of one bird whenever data from both hemispheres were available. Even though some control and knockdown samples form subclusters, there is no clear overall distinction between treatments (i.e. control versus knockdown) within the dendrograms.

Differentially expressed genes

The number of genes which showed higher expression in response to *FoxP1* knockdown varied for the different subgroups, ranging from 26 (adult HVC) to 268 (adult CMM). (Figure 4a). Only a few such genes overlapped between different treatment groups, and there was no gene that overlapped between all groups (Figure 4a and b). The overlap was largest between the juvenile groups, where ten genes showed higher expression in response to *FoxP1* knockdown in both HVC and CMM. Four of these genes were annotated in the female zebra finch reference genome (*EXTL2*, *ASS1*, *THSD4*, *RP2*), while one gene was unannotated in the female reference genome (LOC100230755) yet recently annotated as coding for *ADAM33* in the male zebra finch reference genome. The last annotated gene overlapping between juvenile HVC and CMM samples codes for a tRNA (TRNAG-GCC). Four unannotated loci are also upregulated in both juvenile knockdown groups. Three loci, all unannotated, showed higher expression in response to *FoxP1* knockdown in both adult groups of this study, including one putative orthologue (LOC100222415) of *CYP2D14*, a cytochrome oxidase. One unannotated gene each overlapped between juvenile and adult HVC (LOC116806907) or CMM (LOC100230293) samples. According to previous microarray analyses (Dittrich *et al.*, 2014), the unannotated gene identified in both juvenile and adult CMM samples might be an orthologue of *CYB561*, a cytochrome oxireductase. One gene showed consistently elevated expression in response to *FoxP1* knockdown in three of the four subgroups (i.e. all except the adult HVC samples), annotated as NEK5, NIMA (never in Mitosis Gene A)-Related Kinase 5, encoding a serine/threonine-protein kinase.

Of the 26 genes that exclusively showed elevated expression in adult HVC knockdowns, *RPE65*, *PLXNB1* and *CUTA* may be of special interest, considering the prior literature. *RPE65* codes for retinoid isomerohydrolase; this protein is involved in

retinoic acid signalling, a pathway that has been linked to FOXP2 regulatory networks in previous studies (Van Rhijn and Vernes, 2015). *PLXNB1* expression has been reported in HVC (Lovell *et al.*, 2008) and the gene is implicated in the SLIT-ROBO signalling pathway (Xu and Fan, 2008; Hirschberg *et al.*, 2010; Schiweck *et al.*, 2015); variants of genes in this pathway have been associated with developmental dyslexia, expressive vocabulary in human infants, and performance on non-word repetition tasks in some studies (Hannula-Jouppi *et al.*, 2005; Bates *et al.*, 2010; Pourcain *et al.*, 2014; Mozzi *et al.*, 2016). Deletions of a human genomic region encompassing the ortholog *CUTA*, a CutA Divalent Cation Tolerance Homolog have been identified in humans with intellectual disability, hearing loss and delayed speech development (Writzl and Knegt, 2013) or absence of language (Zollino *et al.*, 2010).

Table 3 gives information on the ten most significant genes with elevated expression in each subgroup of this study. The entire set of significant genes is shown in Supplementary Table 3. In juvenile HVC knockdowns, *RGR, RLBP1* and *TUBAL3* showed significantly elevated expression as compared to controls. *RGR* and *RLBP1* encode members of the retinoid cycle (Saari *et al.*, 2001; Maeda *et al.*, 2003), which could be interesting in light of the putative link proposed between the dimerising partner of FoxP1, FoxP2 and retinoid related processes (Van Rhijn and Vernes, 2015). *TUBAL3* codes for a tubulin that may interact with the SLIT-ROBO signalling pathway according to the PathCards database (OMICS_07645). Among genes showing elevated expression in juvenile CMM knockdowns are *RP2*, encoding a protein implicated in retinitis pigmentosa in humans (Veltel and Wittinghofer, 2009), *IFI6*, encoding an interferon inducible protein, and *ROBO2*. In adult CMM knockdowns, genes with increased expression are associated to mitochondria, the respiratory complex and ribosomal actions as indicated by the top enriched cellular component GO terms (see Table 5, next section). Among these genes is *NDUFB1*, an oxireductase that shows reduced expression in the blood of early stage Alzheimer's disease patients (Lunnon *et al.*, 2017).

The number of genes showing significantly reduced expression in knockdown birds also varied across groups, ranging from 34 (adult HVC) to 120 (juvenile HVC) (Fig. 4c and d, Supplementary Table 3). Few genes overlapped between the treatment areas/stages, and *FoxP1* was the only to show significantly reduced expression across all groups (Fig. 4d). Knockdowns in both juvenile groups resulted in lower expression

for three protein-coding genes (*EIF2B5*, *SPOCK2*, *B2M*) and one unannotated locus (LOC10228369). Adult knockdowns of either area did not share any differentially expressed genes. Juvenile and adult knockdowns in HVC resulted in reduced expression of *IFI6*, encoding an interferon inducible protein and *SPINT1*, encoding a serine peptidase inhibitor. Notably, *IFI6* expression was significantly elevated in juvenile CMM knockdowns, raising the possibility that it may be differentially regulated by FoxP1 in different brain areas. In juvenile HVC and adult CMM two protein-coding genes (*ETFB*, *DHX33*) and four unannotated loci (LOC101234199, LOC100229421, LOC100224927, LOC116807667) overlapped between samples. *ETFB* is potentially linked to ASD according to the SFARI database which lists genes associated with this group of developmental disabilities. Although officially unannotated, it is thought that LOC100229421 may code for interferon-induced protein IFIT5 (Scalf, 2018), while LOC100224927 has been annotated in the male zebra finch genome as *OASL*, an oligoadenylate synthetase. All groups, with the exception of adult HVC knockdowns, overlapped in showing significantly reduced expression of *JCHAIN*, an immunoglobulin, as well as an unannotated locus LOC116809013 (Fig 4d).

Considering current knowledge on cognitive phenotypes associated with genetic manipulations of *FoxP1* in animals or disruptive variants in humans, some of the genes showing reduced expression in only one treatment group may be of special interest (Table 4 and Supplementary Table 3). In juvenile HVC knockdowns, such genes include the chromatin remodelling gene *ACTL6B*, human mutations of which result in intellectual disability, absence of speech or limited vocabulary (Bell *et al.*, 2019; Fichera *et al.*, 2019), and *DBN1,* encoding an actin-binding protein implicated in Alzheimer's disease in humans, and neuronal migration and synaptic plasticity in animal models (Shirao *et al.*, 2017). In juvenile CMM knockdowns, genes with significantly reduced expression included *SEMA3E,* encoding a semaphorin protein which forms complexes with plexins to regulate neuronal development, possibly via the SLIT-ROBO pathway (Xu and Fan, 2008; Schiweck *et al.*, 2015; Mata *et al.*, 2018). Genes exclusively downregulated in birds which received a *FoxP1* knockdown as adults in HVC are e.g. *PNMT*, *HRH1*, *TMEM233* and *TUBAL3* which is upregulated in birds of the juvenile HVC group. *PNMT* and *HRH1* are both implicated in the catecholamine pathway (Marley *et al.*, 1991; Kubovcakova *et al.*, 2004). In mouse astrocytes, homozygous knockouts of *HRH1* resulted in reduced anxiety and impaired novel object recognition

memory (Kárpáti *et al.*, 2019). Knockdown of *FoxP1* in CMM of adults resulted in reduced expression of *CHRNA10*, which encodes a subunit of a nicotinic acetylcholine receptor, and is implicated in auditory olivocochlear system development and function in mice (Vetter *et al.*, 2007). Deletion of the interferon regulatory factor *IRF1* in mice leads to cognitive impairments as demonstrated by reduced performance during water maze tasks (Mogi *et al.*, 2018). However, no cognitive impairments in female zebra finches which received a *FoxP1* knockdown could be identified during behavioural experiments of this thesis.

Table 3: *Top ten genes with most significantly elevated expression following local FoxP1 knockdowns in each subgroup. Gene symbols are followed by their average counts in knockdown and control samples, their respective log2 fold change (log2FC) and p-values adjusted by false discovery rate. In the last column, the gene name and an associated function are indicated. For loci which are not annotated in the reference genome, putative orthologues based on previous micro-array data* (Dittrich *et al.*, 2014) *are indicated.*

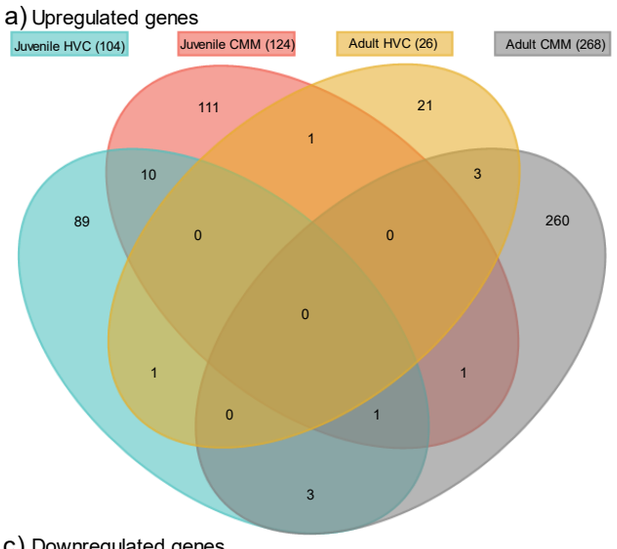
Juvenile HVC	**Counts KD**	**Counts Ctrl**	**log2FC**	**p adj.**	**Name and putative function**
FANCM	382.521	23.5654	4.0208	2.8E-142	FA complementation group M, cytogenetic instability, increased chromosomal breakage
RGR	80.3917	11.5054	2.80474	9.48E-34	Retinal g protein coupled receptor, retinaldehyde binding, retinitis pigmentosa
RLBP1	77.675	11.95	2.70044	4.27E-32	Retinaldehyde binding protein 1, component of visual cycle
TRNAE-UUC-2	60.2292	11.75	2.3578	9.45E-20	Transfer RNA glutamic acid
LOC100220024	25.6817	7.74308	1.72976	1.34E-05	Folate receptor gamma, FOLR3, cancer, innate immune system, endocytosis
LOC100230755	38.0108	12.0385	1.65876	1.22E-08	ADAM33, disintegrin and metalloproteinase domain-containing protein 33, cell-cell and cell-matrix interactions, neurogenesis
CHST9	18.9017	6.01692	1.65142	0.008931	Carbohydrate sulfotransferase 9, protein modification in golgi membrane, cell-cell interaction, signal transduction
TUBAL3	136.321	53.99	1.33624	9.41E-16	Tubulin alpha like 3, development slit-robo signalling, GTP binding
NEK5	27.3525	11.9677	1.19253	0.000712	Nima related kinase 5, transferase activity
LOC100228510	45.4667	21.2508	1.09729	8.49E-06	Sodium/hydrogen exchanger 2, ion exchange, cell volume regulation
Juvenile CMM					
LOC100224927	45.9546	6.20333	2.88909	4.82E-24	2’5’-oligoadenylate synthase 1, antiviral enzyme, also apoptosis, cell growth, differentiation, gene regulation
IFI6	1187.18	179.073	2.72892	2.91E-55	Interferon alpha inducible protein 6, apoptosis regulation

THSD4	58.2682	21.1693	1.46073	7.8E-09	Thrombospondin Type 1 Domain Containing 4, protein metabolism, metalloendopeptidase
TRNAG-GCC	167.5	64.1333	1.38502	1E-12	Transfer RNA glycine
ERP27	54.8536	21.5347	1.34893	1.72E-07	Endoplasmic reticulum protein, putative chaperone?
RP2	58.0673	23.0547	1.33267	1.4E-07	Rp2 activator of ARL3 GTPase, retinitis pigmentosa, folding of neuron specific tubulin isoforms, GTP binding
LOC101233947	19.3309	8.45533	1.19298	0.013185	Protein tilB homolog, LRRC6, DNAAF11, cilia motility
LOC100230755	29.2209	13.2607	1.13985	0.000164	ADAM33, disintegrin and metalloproteinase domain-containing protein 33, cell-cell and cell-matrix interactions, neurogenesis
ROBO2	392.557	178.265	1.13888	4.02E-09	Roundabout guidance receptor 2, axon guidance, cell migration, expressive language vocabulary in infants
AGR3	30.9036	15.0093	1.04192	0.000666	Anterior gradient 3, ER protein, protein folding, ciliary beat frequency
Adult HVC					
BGLAP	19.1887	7.42571	1.36965	0.030324	Bone gamma carboxyglutamate protein, osteoblasts, energy metabolism, calcium binding
CUTA	21.37	8.61643	1.31043	0.018327	Cuta divalent cation tolerance homolog, deafness
LPL	80.1173	35.7721	1.16328	0.000138	Lipoprotein lipase, heart, muscle and adipose tissue, receptor mediated lipoprotein uptake,
MMP2	36.538	16.6279	1.1358	0.009484	Matrix metallopeptidase 2, cleave ecm components, signal transduction
ENPP6	41.626	21.2114	0.97264	0.0305	Ectonucleotide pyrophosphatase, phosphodiesterase, neuropathy,
LOC100226434	59.9187	31.395	0.93247	0.017886	Hes5 like, transcription factor, brain development process, notch signalling
LOC100222415	56.8087	30.7921	0.88355	0.041241	Cytochrome p450 like, oxyreductase
VAMP1	126.771	69.4379	0.86843	0.003487	Vesicle associated membrane protein, synaptic vesicle docking, fusion presynaptic, spastic ataxia
PLXNB1	64.6407	35.5671	0.8619	0.022988	Plexin B1, slit robo signalling, semaphorine receptor
RPE65	70.1247	39.4414	0.83021	0.033594	Retinal pigment epithelium specific 65kda protein, vision
Adult CMM					
ND3	916.392	490.076	0.90296	2.94E-08	Mitochondrial NADH dehydrogenase, neurometabolic disorders
LOC100190731	445.739	243.702	0.87108	5.14E-08	Metallothionein, zinc ion binding
EPSTI1	41.7846	23.9875	0.80069	0.0007	Epithelial stromal interaction, cancer, lupus
LOC100190094	548.732	319.727	0.77926	2.22E-07	Metallothionein-i-like, MT4(?), zinc and copper ion binding, differentiation of stratified epithelia
COX7C	1101.5	646.551	0.76864	4.3E-06	Cytochrome oxidase subunit 7c, neurodegeneration
ATP5MPL	732.368	433.533	0.75643	1.53E-06	ATP synthase membrane subunit j
RPS29	280.552	166.643	0.75151	1.18E-06	Ribosomal protein s29, protein metabolism
HBAD	202.328	120.857	0.7434	1.81E-06	Hemoglobin subunit alphaD
NDUFB1	292.978	176.772	0.7289	2.32E-06	NADH Ubqiquinone Oxireductase Subunit 1, neuropathy, dysarthria
DIO2	87.9962	53.1717	0.72678	0.000033	Iodothyronine deiodinase 2, thyroid hormone metabolism

Table 4: *Top ten genes with most significantly reduced expression following local FoxP1 knockdowns in each subgroup. Gene symbols are followed by their average counts in knockdown and control samples, their respective log2 fold change (log2FC) and p-values adjusted by false discovery rate. In the last column, the gene name and an associated function are indicated. For loci which are not annotated in the reference genome, putative orthologues based on previous micro-array data* (Dittrich *et al.*, 2014) *are indicated.*

Juvenile HVC	**Counts KD**	**Counts Ctrl**	**log2FC**	**p adj.**	**Name and putative function**
JCHAIN	53.9292	584.981	-3.43925	8.15E-78	IgA and IgM factor, immune system
IFI6	578.643	3745.34	-2.69435	2.3E-130	Interferon Alpha Inducible Protein 6, apoptosis, innate immune system
LOC100229421	22.0233	138.795	-2.65585	1.83E-42	IFIT5, Interferon induced protein with tetratricopeptide repeats 5, tRNA binding, innate immune response
LOC100224927	16.8525	91.3369	-2.43824	1.07E-36	2'-5'oligoandeylate synthase 1, interferone induced, RNA degradation, reduced gene expression
LOC100224071	6.48667	24.6092	-1.92365	8.16E-05	Ovostatin-like(?), proteinase inhibitor
RSAD2	11.6642	30.1862	-1.3718	2.01E-05	Radical s adenosyl methionine domain containing 2, antiviral protein
FOXP1	55.3808	139.545	-1.33328	4.93E-13	Forkhead box transcription factor P1, intellectual disability, autism spectrum disorder
ETFB	208.16	445.645	-1.0982	4.25E-11	Electron transfer flavoprotein subunit beta, beta polypeptide, electron shuttling
ACTL6B	39.3208	84.0692	-1.09628	4.98E-09	Actin like 6b, intellectual developmental disorder with severe speech and articulation defects, cytoskeleton
DBN1	97.3458	201.215	-1.04754	1.1E-14	Drebrin 1, neuronal growth, Alzheimer, down syndrome
Juvenile CMM					
FOXP1	42.16	157.591	-1.90224	1.39E-26	Forkhead box transcription factor P1, intellectual disability, autism spectrum disorder
JCHAIN	10.6082	24.8373	-1.22733	7.51E-06	Joining of multimeric IgA and IgM
TNFSF13B	10.1591	20.71	-1.02756	0.000465	TNF superfamily, tumor necrosis factor, signalling receptor binding
TRNAE-CUC2	14.5455	29.25	-1.00787	2.14E-05	RNAgene, transfer RNA glutamic acid
SEMA3E	12.0636	22.1753	-0.87829	0.003138	Semaphorin 3E, axon guidance
LOC100232025	33.4455	56.0313	-0.74442	0.000387	Extracellular fatty acid-binding protein-like, immune?
SOX2	30.7546	49.03	-0.67287	0.001287	Stem-cell development
B2M	263.276	411.456	-0.64417	8.3E-06	Beta2Microglobulin, MHC heavy chain, antibacterial activity in amniotic fluid
FGFBP3	24.7791	38.1753	-0.62352	0.008708	Fibroblast growth factor binding protein 3, gpcr signalling
DPY19L3	35.2927	53.3673	-0.59659	0.003742	DPY-19 like C-mannosyltransferase 3, spermatogenic failure, podoconiosis
Adult HVC					
FOXP1	36.1647	119.288	-1.72179	3.49E-16	Forkhead box transcription factor P1, intellectual disability, autism spectrum disorder

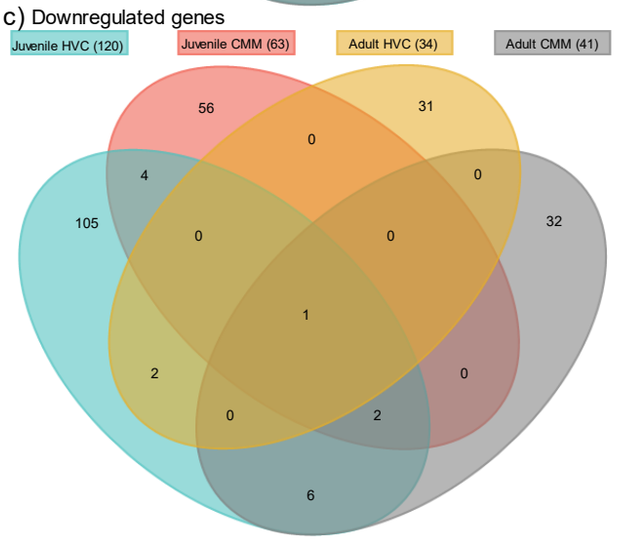
PNMT	5.44333	14.7936	-1.44241	0.016428	Phenylethanolamine n methyltransferase, catecholamine pathway
FOXJ1	10.7327	26.1686	-1.28583	0.000166	Motile cilia tf, left/right asymmetry, lupus
TMEM233	18.9307	42.3543	-1.16178	7.17E-06	Transmembrane protein 233, interferon induced,
DHTKD1	13.726	30.3729	-1.14587	0.000473	Dehydrogenase e1 and transketolase domain containing 1, mitochondrial, charcot marie tooth
MROH1	14.3007	31.1921	-1.1251	0.000494	Maestro heat like repeat family member 1, binding
C3	14.0433	28.5521	-1.02371	0.002424	Complement c3, inflammation and antimicrobial activity, gpcr signalling
PPL	37.25	72.9143	-0.96896	1.41E-05	Periplakin, desmosome component, cell growth
HRH1	9.35267	18.2821	-0.96699	0.035042	Histamine receptor h1, messenger, catecholamine release, neurotransmission, memory and learning
ZNFX1	9.10733	17.6557	-0.95503	0.047669	Zinc finger nfxtype containing 1, cancer, parotid disease
Adult CMM					
LOC100224927	6.35	29.3792	-2.20996	1.34E-08	2'-5'-oligoadenylate synthase-like protein 1
JCHAIN	45.4785	176.443	-1.95595	2.17E-17	Joining of multimeric IgA and IgM
LOC100229421	8.09	30.145	-1.89771	3.23E-07	Putative retinoic Acid and Interferon inducible Protein, viral RNA sensor
FOXP1	57.7454	194.766	-1.75396	2.31E-13	Forkhead box transcription factor P1, intellectual disability, autism spectrum disorder
TRNAG-GCC-2	7.30077	19.2958	-1.40217	0.002437	Glycine Transfer RNA
IRF1	21.58	53.4717	-1.30908	8.99E-05	Interferon regulating factor, viral response
TGM4	17.5331	42.8817	-1.29028	4.46E-05	Transglutaminase4, seminal tract in mammals
TRNAG-GCC	30.25	69.3958	-1.19791	0.000037	Glycine Transfer RNA
CHRNA10	8.62846	19.3392	-1.16435	0.046732	Neuronal acetylcholine receptor subunit alpha10, nAchR, olivocochlear sytem in auditory system
TNNI1	11.7777	26.14	-1.1502	0.020979	Troponin1, slow skeletal Muscle, but also corpus callosum in mice



b)

Juvenile HVC ∩ juvenile CMM
LOC116806890
LOC116806735
EXTL2
ASS1
LOC116807081
THSD4
LOC116807587
TRNAG-Gcc
RP2
LOC100230755
Juvenile HVC ∩ adult HVC
LOC116806907
Adult HVC ∩ adult CMM
LOC115490588
LOC115497561
LOC100222415
Juvenile CMM ∩ adult CMM
LOC100230293
Juvenile HVC ∩ juvenile CMM ∩ adult CMM
NEK5

Adult HVC exclusive
LOC100225683
BSG
LOC101233698
LOC100228369
CAVIN2
PHYHD1
RHBDL1
RPE65
LOC116806758
PLXNB1
VAMP1
LOC116807507
LOC100226434
ENPP6
MMP2
LPL
LOC115494492
LOC115491277
CUTA
BGLAP
LOC100218227



d)

Juvenile HVC ∩ juvenile CMM
EIF2B5
SPOCK2
LOC100228369
B2M
Juvenile HVC ∩ adult HVC
IFI6
SPINT1
Juvenile HVC ∩ adult CMM
LOC101234199
LOC100229421
LOC100224927
LOC116807667
ETFB
DHX33
Juvenile HVC ∩ juvenile CMM ∩ adult CMM
LOC116809013
JCHAIN
Juvenile HVC ∩ juvenile CMM ∩ adult HVC ∩ adult CMM
FoxP1

Adult HVC exclusive
PNMT
FOXJ1
LOC115495014
TMEM233
DHTKD1
MROH1
LOC115494495
C3
PPL
HRH1
ZNFX1
OLFM4
LOC100229516
TUBAL3
LOC115497572
ADSS1
LOC115495360
CYP19A1
LOC115495051
ENKD1
ME2
GPR26
C2CD4C
NRBP1
GPR78
KL
BOP1
VIPR1
LOC115497743
KCNK5
LOC101233348

Figure 4: *Overlapping and exclusive differentially expressed genes across all subgroups. a) shows a Venn diagram of genes with significantly elevated expression which are exclusively represented in one or shared across multiple subgroups. b) shows the list of genes corresponding to a). As a behavioural phenotype was identified for birds which received a knockdown in HVC as adults (see Chapter 2 of this thesis), genes which exclusively show elevated expression in these birds are listed separately. Similar to a), c) shows a Venn diagram of genes with significantly reduced expression, while d) lists the genes represented in c).*

GO terms and local network clusters

Due to the low number of overlapping genes which showed significantly increased or reduced expression across all experimental groups, GO and network terms were analysed separately for each region and developmental stage. The ten most enriched GO terms of each category and network terms are shown for genes with increased (Table 5) and reduced (Table 6) expression associated with *FoxP1* knockdown. In birds which received a knockdown as juveniles in HVC, no GO terms were significantly enriched, and only one network cluster associated with retinol metabolic process and retinol binding (ID: 9606_CL_25005) was enriched. Genes with elevated expression in juvenile CMM knockdown samples are represented by multiple GO terms of the biological processes (BP) and cell cycle (CC) categories. All three significantly enriched BP terms are related to cell adhesion. Seven of the ten most enriched GO terms of the CC category are related to plasma membrane or the extracellular matrix. Neuron projection (GO:0043005), postsynapse (GO:0098794) and syntrophin complex (GO:0016013) are also significantly enriched terms. Multiple enriched local network clusters based on genes with elevated expression in juvenile CMM samples represent functions related to cell adhesion, and there was also enrichment for networks related to voltage gated potassium channels (9606_CL_8930), interneuron migration (9606_CL_230) and growth response (9606_CL:2481). In adult HVC samples, enrichment was seen for one BP term related to bone trabecula morphogenesis (GO:0061430) and one term of the molecular function (MF) category corresponding to the membrane protein phosphatidylserine (GO:0001786). In this group of genes showing increased expression local network clusters are related to retinol metabolic processes and retinol-binding (9606_CL:24001), similar to juvenile HVC samples. Additionally, a local cluster implicated in mixed processes such as matrix metalloproteinases (9606_CL:907) is enriched across genes with elevated expression in adult HVC knockdown samples.

The top GO terms of genes showing increased expression in adult CMM knockdowns are related to ribosomal or mitochondrial processes across all three GO categories. The most enriched BP terms are linked to processes from e.g. translation (GO:0006412) to protein targeting to the endoplasmatic reticulum (GO:0045047) and protein localisation (GO:0072594). Six of ten CC terms are related to ribosomes, and four are implicated in mitochondrial processes. Out of ten MF terms, two are related to

ribosomal functions, six represent mitochondrial functions and cellular respiration, and the remaining two relate to structural molecule activity (GO:0005198) and proton transmembrane transporter activity (GO:0015078). Local clusters enriched in genes with increased expression following knockdown in adult CMM represent similar functions to the enriched GO terms, including e.g. ribosomal activities such as peptide chain elongation (9606_CL:14976) or mitochondrial complexes like the respirasome (9606_CL:22328). As the number of significantly enriched GO terms based on upregulated genes in adult CMM knockdowns was larger than in all other groups, the entire set of GO terms of this group is listed in Supplementary Table 4.

Compared to GO terms and local network clusters enriched in genes with increased expression in specific knockdown groups, few terms and networks were enriched in genes with reduced expression (Table 6). In this case, for juvenile HVC knockdowns there was enrichment for none of the GO terms and only one local cluster, with mixed associations including axonal growth inhibition (9606_CL:616). Genes with reduced expression in juvenile CMM knockdowns were enriched for one BP term on antibacterial humoral response (GO:0019731), CC terms related to mitochondrial processes such as mitochondrial proton-transporting ATP synthase complex (GO:0005753), and organelle membrane related processes such as e.g. organelle envelope (GO:0031967). Two enriched local clusters were related to oxidative phosphorylation (9606_CL:22327) and proton-transporting ATP synthase complex (9606_CL:22571). For genes with knockdown-related reductions of expression in birds that had been injected as adults, there were no enriched GO terms or local clusters.

In addition, GO terms and network clusters were assessed for genes exclusively regulated in birds which received a local *FoxP1* knockdown in HVC as adults, since this subgroup had shown a behavioural phenotype in Chapter 2 of this study. In this case, for genes with significantly increased expression, enrichment was seen for GO:0061430 which is associated to bone trabecula morphogenesis, and two local network clusters: 9606_CL_24001 retinol metabolic process, and retinol binding and 9606_CL_907 mixed, incl. activation of matrix metalloproteinases and dissolution of fibrin clot. For genes with significantly reduced expression, no GO terms or clusters were enriched. The only detected enrichment in this set of downregulated genes was attributed to a UniProt keyword (KW-0297) associated to G-protein coupled receptor

which is further linked to two MF GO terms G protein-coupled receptor activity (GO:0004930) and G protein-coupled receptor signalling pathway (GO:0007186).

Table 5: *Significantly enriched gene ontology (GO) terms and local clusters based on genes with increased expression following local FoxP1 knockdowns in each subgroup. GO terms and local clusters are based on the human orthologues of the genes found to be differentially expressed in this study. Analyses are based on data deposited in the string database (v11.0). The maximum ten most significant GO terms are clustered based on their affiliation to cellular components (CC), molecular functions (MF) or biological processes (BP). Each term is followed by the number of genes contributing to it as well as the total number of genes represented by each term and its respective false discovery rate (FDR).*

Juvenile HVC			
Local cluster	**Description**	**Gene counts**	**FDR**
9606_CL:24005	retinol metabolic process, and Retinol-binding	3/12	0.032
Juvenile CMM			
BP term			
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	11/101	1.32E-06
GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	8/65	6.65E-05
GO:0007155	cell adhesion	13/480	0.038
CC term			
GO:0005886	plasma membrane	38/2816	0.0177
GO:0016010	dystrophin-associated glycoprotein complex	3/16	0.0177
GO:0016021	integral component of membrane	36/2716	0.0177
GO:0031224	intrinsic component of membrane	38/2792	0.0177
GO:0045211	postsynaptic membrane	8/186	0.0177
GO:0016013	syntrophin complex	2/4	0.0216
GO:0016020	membrane	53/4968	0.0216
GO:0043005	neuron projection	16/869	0.0216
GO:0098794	postsynapse	9/334	0.0282
GO:0031226	intrinsic component of plasma membrane	15/866	0.0452
Local cluster			
9606_CL:2481	bZIP transcription factor, and Early growth response, N-terminal	3/12	0.02
9606_CL:6791	mixed, incl. Adherens junctions interactions, and Alpha-catenin	4/25	0.02
9606_CL:6796	Adherens junctions interactions	3/10	0.02
9606_CL:6813	Cadherin cytoplasmic region	2/2	0.021
9606_CL:8930	Voltage gated Potassium channels, and Phase 1 - inactivation of fast Na+ channels	3/17	0.0228
9606_CL:230	chemorepulsion involved in interneuron migration from subpallium to cortex and ovarian cumulus expansion	2/4	0.0289
Adult HVC			
BP term			
GO:0061430	bone trabecula morphogenesis	2/9	0.0345
MF term			
GO:0001786	phosphatidylserine binding	2/28	0.0442
Local cluster			

9606_CL:24001	retinol metabolic process, and Retinol-binding	2/18	0.0111
9606_CL:907	mixed, incl. Activation of Matrix, and Dissolution of Fibrin Clot Metalloproteinases	2/21	0.0111
Adult CMM			
BP term			
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	42/80	2.24E-40
GO:0045047	protein targeting to ER	43/88	2.24E-40
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	42/100	6.12E-38
GO:0006412	translation	56/293	3.72E-36
GO:0043043	peptide biosynthetic process	57/308	3.72E-36
GO:0006413	translational initiation	42/120	1.77E-35
GO:0000956	nuclear-transcribed mRNA catabolic process	44/158	9.39E-34
GO:0090150	establishment of protein localization to membrane	45/173	1.62E-33
GO:0006605	protein targeting	48/264	7.66E-30
GO:0072594	establishment of protein localization to organelle	48/311	5.43E-27
CC term			
GO:0044391	ribosomal subunit	42/125	1.14E-34
GO:0005840	ribosome	44/165	3.83E-33
GO:0022626	cytosolic ribosome	33/57	
GO:0005743	mitochondrial inner membrane	46/331	3.87E-24
GO:0098798	mitochondrial protein complex	38/201	6.53E-24
GO:0031966	mitochondrial membrane	52/489	8.31E-23
GO:0098800	inner mitochondrial membrane protein complex	28/94	4.88E-22
GO:0022627	cytosolic small ribosomal subunit	20/31	7.05E-21
GO:0015935	small ribosomal subunit	23/56	1.11E-20
GO:0070469	respirasome	23/64	1.19E-19
MF term			
GO:0003735	structural constituent of ribosome	45/126	2.33E-38
GO:0005198	structural molecule activity	49/405	3.86E-23
GO:0008137	NADH dehydrogenase (ubiquinone) activity	15/41	3.59E-12

GO:0016651	oxidoreductase activity, acting on NAD(P)H	17/70	8.04E-12
GO:0016491	oxidoreductase activity	33/437	4.46E-10
GO:0015078	proton transmembrane transporter activity	12/68	6.34E-07
GO:0019843	rRNA binding	9/48	2.43E-05
GO:0004129	cytochrome-c oxidase activity	5/6	2.48E-05
GO:0009055	electron transfer activity	9/61	9.60E-05
GO:0008121	ubiquinol-cytochrome-c reductase activity	3/4	4.40E-03
Local cluster			
9606_CL:14976	Peptide chain elongation	45/72	7.28E-47
9606_CL:14978	Peptide chain elongation	43/70	5.48E-45
9606_CL:14966	GTP hydrolysis and joining of the 60S ribosomal subunit, and Protein export	48/122	1.75E-43
9606_CL:14980	Peptide chain elongation	41/66	3.64E-43
9606_CL:14967	GTP hydrolysis and joining of the 60S ribosomal subunit, and Protein export	46/117	1.16E-41
9606_CL:14982	Peptide chain elongation	38/63	1.11E-39
9606_CL:14983	Peptide chain elongation	33/55	3.08E-34
9606_CL:14985	Viral mRNA Translation	30/50	4.73E-31
9606_CL:22327	Oxidative phosphorylation	33/109	1.54E-26
9606_CL:22328	respirasome	24/64	6.08E-21

Table 6: *Significantly enriched GO terms and local clusters based on genes with decreased expression following local FoxP1 knockdowns in each subgroup. GO terms and local clusters are based on the human orthologues of the genes found to be differentially expressed in this study. Analyses are based on data deposited in the string database (v11.0). GO terms are clustered based on their affiliation to cellular components (CC), molecular functions (MF) or biological processes (BP). Note that no significantly enriched GO terms or local clusters were identified in each of the adult subgroups. Each term is followed by the number of genes contributing to it as well as the terms' size and its respective false discovery rate (FDR).*

Juvenile HVC			
Local cluster	**Description**	**Counts/Size**	**FDR**
9606_CL:616	mixed, incl. Axonal growth inhibition, (RHOA activation) and Intermediate filament head, DNA-binding domain	4/28	0.0225
Juvenile CMM			
BP term			
GO:0019731	antibacterial humoral response	3/10	0.0216
CC term			
GO:0005753	mitochondrial proton-transporting ATP synthase complex	3/18	0.0165
GO:0005743	mitochondrial inner membrane	6/331	0.0417
GO:0019866	organelle inner membrane	7/370	0.0417
GO:0031966	mitochondrial membrane	8/849	0.0417
GO:0031967	organelle envelope	10/830	0.0417
GO:0098800	inner mitochondrial membrane protein complex	4/94	0.0417
GO:0070469	respirasome	3/64	0.0417
Local cluster			
9606_CL:22327	Oxidative phosphorylation	6/109	0.0009
9606_CL:22571	proton-transporting ATP synthase complex	3/20	0.0057
Adult HVC			
no enriched GO terms or local clusters			
Adult CMM			
no enriched GO terms or local clusters			

Gene set enrichment analyses

Due to the low number of overlapping differentially expressed genes between groups which suggests large variability, gene set enrichment analyses (GSEA) was performed to allow next to GO term analyses for an additional, less biased perspective on the putative implications of all genes which were identified based on the mapped and assigned reads of each sample. During GSEA analyses, normalised counts of all assigned genes filtered for low expression were ranked and weighted based on their log2fold-change. Subsequently the association of a gene to a specific pathway elevated this pathways' normalised enrichment score (NES), while no known pathway contribution of a gene respectively lowered it. As samples segregated by age during injection and injected area during hierarchical clustering, GSEA was performed separately for each group (Fig. 5, Supplementary Table 5).

For each GSEA, the 50 genes with increased or decreased expression which contribute most to the outcome of the gene set enrichment analyses are shown in matrix plots for juvenile HVC (Fig. 5a), juvenile CMM (Fig. 5b), adult HVC (Fig. 5c), and adult CMM samples (Fig. 5d). Note that the downregulated *FoxP1* in knockdowns of this study contributes most to gene sets enriched in these samples across all groups.

In samples from juvenile HVC, gene set enrichment scores are bimodally distributed (Fig. 5e). This distribution indicates comparable numbers of gene sets with high and low enrichment scores where high scores correspond to enrichments in control samples and low scores suggest enrichment in knockdown samples, respectively. However, no gene set was enriched significantly in this group. In samples from juvenile CMM (Fig. 5f) enrichment scores are negatively biased with three significantly enriched gene sets (FDR < 0.25) in knockdown samples. The significantly enriched gene sets consist of genes which are upregulated in an epithelial cell line after stimulation with serum (FDR = 0.22, NES = -2.04, Amit *et al.*, 2007), genes which are upregulated in a cell line derived from colon cancer after expression of FOXO3 (FDR = 0.21, NES = -1.95, Delpuech *et al.*, 2007) and genes which are downregulated in amyloidosis plasma cells in comparison to multiple myeloma cells (FDR = 0.23, NES = -1.93, Abraham *et al.*, 2005). No gene sets are enriched significantly in any of the adult groups, yet gene sets of adult HVC samples are biased towards negative enrichment scores (Fig. 5g) whereas adult CMM samples indicate a bias towards positive gene set enrichment scores (Fig. 5h).

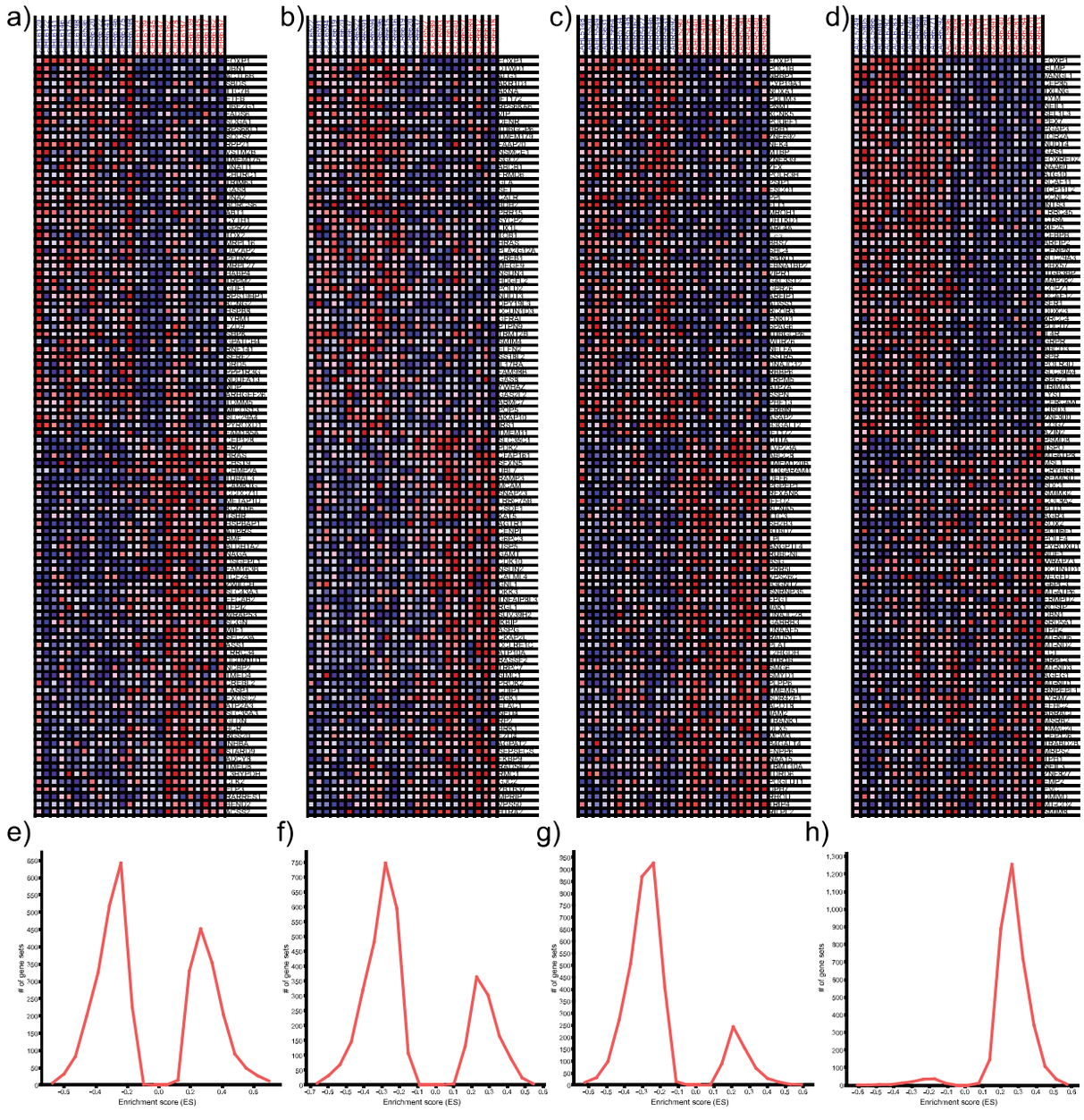


Figure 5: *Gene set enrichment analyses (GSEA) for each of the investigated subgroups. a) – d) show heatmaps of differentially expressed genes as they were ranked by GSEA based on their contribution to gene sets in samples from a) juvenile HVC, b) juvenile CMM, c) adult HVC and d) adult CMM (d). Each row represents one gene as indicated by its symbol on the right. Each column consists of the data generated from one sample as indicated at the top. Control samples are labelled in blue, knockdown samples in red, respectively. e) – h) show the respective enrichment scores for the number of gene sets identified in each subgroup consisting of samples from e) juvenile HVC, f) juvenile CMM, g) adult HVC or h) adult CMM. Negative scores*

represent enrichment in the knockdown samples while positive scores represent enrichment in controls.

Differentially expressed genes overlapping with a previous study of the striatum in heterozygous *FoxP1* knockout mice

64.5% (324) of all genes with increased expression and 46.1% (111) of all genes with decreased expression in this study (Supplementary Table 6) overlap significantly ($\chi^2(1, 104) = 1643.25$, $p < 0.001$) with genes previously identified in expression profiling experiments during a prior investigation of brain tissue samples from heterozygous *Foxp1* knockout mice (Araujo *et al.*, 2015). The authors of this study analysed differential gene expression in the striatum, the hippocampus and the neocortex of *Foxp1* heterozygous mice and compared the findings to consequences of *FOXP1* overexpression in human neural progenitor cells. The largest overlap of differentially expressed genes was identified between samples from mouse striatum and neural progenitors which suggests a higher level of module preservation in the striatum of mammals. Considering the direction of change, 21 genes consistently showed significant increases in expression and 18 genes consistently showed significant decreases in expression as a consequence of *FoxP1* knockout/knockdown across both studies (Table 7). However, in contrast to the overlap of genes irrespective of their direction of differential expression, neither upregulated ($\chi^2(1, 104) = 2.46$, $p > 0.05$) nor downregulated ($\chi^2(1, 104) = 0.93$, $p > 0.05$) genes of both studies significantly overlapped when analysed separately.

One of these overlapping genes shows significant increases in expression in both juvenile knockdown groups of this study and codes for Argininosuccinate synthetase 1 (*ASS1*). At least one human patient with a mutation in *ASS1* has also presented with speech delay (Lin *et al.*, 2019). Overlapping genes which show significant decreases in expression in both juvenile groups of this study are those coding for Beta-2-Microglobulin (*B2M*) and Testican-2 (*SPOCK2*). *B2M* expression increases with age in humans and mice, and artificially increased levels result in impaired performance in radial arm water mazes in mice whereas absence of B2M in mice leads to increased performance in the same type of maze (Smith *et al.*, 2015). SPOCK2 is a proteoglycan that is responsive to retinoic acid signalling in mice (Wei *et al.*, 2016). Another overlapping gene is *RPE65* of the retinoic acid signalling pathway which shows reduced expression in juvenile HVC knockdowns. Finally, two overlapping genes

(*MGST1*, *PTGR1*) contributing to the prostaglandin synthase pathway (Kelner *et al.*, 2000; Dick *et al.*, 2001) which in turn affects retinoic acid signalling (Ziboh *et al.*, 1975; Stock *et al.*, 2011) show increased expression in adult CMM knockdowns.

Table 7: *Genes with significant expression changes in this study which overlap in their direction with the findings of a previous study on differentially expressed genes in the striatum of heterozygous Foxp1 knockout mice (Araujo et al. 2015).*

Group	Regulation	Gene ID	Name and putative function
juvenile HVC & CMM	up	ASS1	Argininosuccinate Synthase 1, Citrullinemia
juvenile HVC	up	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A, tissue regeneration
juvenile HVC	up	GFRA1	GDNF Family Receptor Alpha 1, neuron differentiation
juvenile HVC	up	OAF	Out at first homolog, Spondylocarpotarsal Synostosis Syndrome
juvenile HVC	up	PDLIM4	PDZ and LIM Domain4, bone development, osteoporosis
juvenile HVC	up	PERP	P53 apoptosis effector related to PMP22, Keratinization, desmosome junctions
juvenile HVC	up	SCGN	Secretagogin, calcium binding
juvenile CMM	up	ENOX1	Ecto-NOX Disulfide-Thiol Exchanger 1, plasma membrane electron transport
juvenile CMM	up	MID1	Midline 1, multiprotein formation, midline abnormalities
juvenile CMM	up	PCDH7	Protocadherin 7, cell-cell recognition and adhesion
adult CMM	up	DIO2	Iodothyronine Deiodinase 2, thyroid hormone pathway
adult CMM	up	FBLIM1	Filamin Binding LIM Protein 1, cell adhesion to actin
adult CMM	up	MGST1	Microsomal Glutathione S-Transferase 1, prostaglandin and inflammation
adult CMM	up	PFDN1	Prefoldin subunit1, chaperone
adult CMM	up	PTGR1	Prostaglandin Reductase 1, inflammation
adult CMM	up	RPL22L1	Ribosomal protein l22 like1, sarcoma
adult CMM	up	RPL37A	Ribosomal protein l37a, 60S subunit part
adult CMM	up	SDC1	Syndecan1, cell binding and signalling
adult CMM	up	ABRACL	ABRA C-Terminal Like, cleft lip
adult CMM	up	AQP1	Aquaporin 1, ocular fluid movement
juvenile HVC & CMM	down	B2M	Beta2Micoglobulin, MHC complex
juvenile HVC & CMM	down	SPOCK2	SPARC osteonectin, extracellular matrix, calcium binding
juvenile HVC	down	ACBD7	Acyl-CoA Binding Domain Containing 7, lipid metabolism
juvenile HVC	down	CD59	CD59 molecule, cell lysis

juvenile HVC	down	CRISPLD1	Cysteine rich secretory protein lccl domain containing 1
juvenile HVC	down	CYTH1	Cytohesin 1, membrane trafficking
juvenile HVC	down	MOK	MOK protein kinase, cell growth and differentiation
juvenile HVC	down	PLP1	Proteolipid protein 1, myelin component, oligodendrocyte development, axonal survival
juvenile HVC	down	PROCA1	Protein interacting with cyclin a1, calcium ion binding, gaucher disease
juvenile HVC	down	PRR16	Proline rich 16, cardiomyopathy
juvenile HVC	down	RPE65	Retinoid isomerohydrolase RPE65
juvenile HVC	down	RPS6KL1	Ribosomal protein S6 Kinase Like 1, transferase
juvenile CMM	down	FGFBP3	Fibroblast growth factor binding protein 3, gpcr signalling
juvenile CMM	down	HOPX	HOP homeobox, cardiac development
juvenile CMM	down	PTPN9	Protein tyrosine phosphatase non-receptor-type9, cell growth and differentiation
juvenile CMM	down	SOX2	SRY-box transcription factor 2, cell fate determination
adult HVC	down	FOXJ1	Cilia production, left right asymmetry
adult CMM	down	CHSY3	Chondroitin sulfate synthase 3, glucosyl metabolism

Differentially expressed genes overlapping with the SFARI database on putative ASD risk genes

Across all age-groups and regions, six genes which showed significantly increased expression and 27 genes which showed significantly decreased expression in response to *FoxP1* knockdown are listed as putative ASD risk genes in the SFARI database (Table 8) resulting in a significant overlap ($\chi^2(1, 104) = 3.96$, $p < 0.05$) between the differentially expressed genes of this study and putative ASD risk genes. At the time of this study, the SFARI database listed 1011 genes which are scored at four different levels based on the available evidence of a gene's relevance for ASD ranging from S (syndromic, highest) to 3 (suggestive evidence, lowest). The overlapping genes with increased expression were only identified in one of the tested groups of this study. Next to *FoxP1* which (as expected) showed reduced expression in all groups, one gene, *ETFB* (Electron transfer flavoprotein subunit beta) had significantly lower levels in juvenile HVC and adult CMM samples. As an electron transfer protein, *ETFB* is involved in the energy metabolism in mitochondria and mutations are linked to multiple acyl-CoA dehydrogenase deficiencies (Schiff *et al.*, 2006) which can result in slight speech delay (Chautard *et al.*, 2020) and neurodevelopmental disorder (Pollard *et al*., 2010) in infants.

Next to these genes overlapping between the SFARI database and more than one subgroup of these analyses, genes found to be differentially expressed in one of this studies' subgroups include *ROBO2*, *PLXNB1* and two glutamate-receptor interacting proteins *GRIP1* and *GRID1*. Neuronal homozygous deletions of *GRIP1* in mice impair synaptic plasticity and inhibitory avoidance learning and memory (Tan *et al.*, 2020). *GRID1* homozygous knockout mice demonstrate decreased social novelty preference of conspecifics and impaired memory in context specific fear learning, as well as lowered motivation in a forced swim test when compared to controls (Nakamoto *et al.*, 2020).

Table 8: *Genes with significant expression changes in this study which overlap with genes listed in the SFARI gene database focused on autism candidate genes. According to SFARI the listed genes are associated with the listed phenotypes: neurodevelopmental disorder (NDD), epilepsy (EP), autism spectrum disorder (ASD), intellectual disability (ID), attention deficit hyperactivity disorder (ADHD), developmental delay (DD), schizophrenia (SCHZ), bipolar disorder (BIP), mental retardation (MR), Down syndrome (DS). SFARI provides a score for each gene, ranging from 3 (suggestive evidence) to 2 (strong candidate) and 1 (high confidence) up to S (syndromic).*

Group	Regulation	Gene ID	Association	Score
juvenile HVC	up	SATB1	ND, EP	1
juvenile HVC	up	HRAS	ASD	1
juvenile CMM	up	SOX5	ASD, ID, ADHD	1
juvenile CMM	up	CNTN5	ASD, ID, ADHD, DD, NDD	2
juvenile CMM	up	AGMO	ASD	3
juvenile CMM	up	CDH10	ASD	3
juvenile CMM	up	DLGAP1	ASD	2
juvenile CMM	up	RORA	ASD, EP	S
juvenile CMM	up	KCND2	ASD	3
juvenile CMM	up	GRIP1	ASD	2
juvenile CMM	up	MYO16	ASD	3
juvenile CMM	up	CDH9	ASD	3
juvenile CMM	up	LRRC4C	ASD	1
juvenile CMM	up	CSMD1	ASD, SCHZ, BIP	3
juvenile CMM	up	DMD	ID, ADHD, EP, ASD,	S
juvenile CMM	up	GRID1	ASD	2
juvenile CMM	up	AUTS2	ASD, MR, ADHD, EP	1
juvenile CMM	up	GATM	ID, EP, ASD	S
juvenile CMM	up	GPC6	ASD	3
juvenile CMM	up	DSCAM	ID, down syndrome	1
juvenile CMM	up	IL1RAPL1	ID, DD, EP, ASD	3

juvenile CMM	up	ATP10A	Conflicting reports	2
juvenile CMM	up	PCDH9	ID, ASD	3
adult HVC	up	ROBO2	DD, ASD	2
adult HVC	up	PLXNB1	ASD	2
adult CMM	up	AP1S2	EP, ASD, MR	S
adult CMM	up	NDUFA5	ASD	3
juvenile HVC & CMM, adult HVC & CMM	down	FOXP1	ID, ASD, MR	1
juvenile HVC & CMM	down	ETFB	ASD	2
juvenile HVC	down	ACTL6B	ASD, EP, ID, DD	S
juvenile HVC	down	BCAS1	ASD	3
juvenile HVC	down	ATP1A1	ASD, DD, ID	3S
adult CMM	down	BICDL1	ASD	3

Differentially expressed genes overlapping with the SysID database on putative risk genes for intellectual disability

Due to implications of human FOXP1 malfunctions in intellectual disability, differentially expressed genes of this study were also compared with the SysID dataset (Kochinke *et al.*, 2016) which collects genes associated to intellectual disability in humans. The database consisted of 2778 human genes at the time of this study of which 75 genes (see Table 9) overlapped significantly ($\chi^2(1, 104) = 26.63$, $p < 0.0001$) with differentially expressed genes in females which received local *FoxP1* knockdowns. Among the overlapping genes were *ACTL6B*, *ASS1*, *PLXNB1*, *RGR*, *SEMA3E* and *TUBAL3* which were previously listed in this Chapter as potentially interesting candidate genes that might contribute to the phenotypical consequences following FoxP1 malfunctions or altered expression levels. In addition to these previously mentioned genes, *ILRAPL1* is an overlapping gene which is significantly upregulated in juvenile CMM knockdowns. This gene encodes an interleukin 1 receptor accessory protein and its homozygous knockout in mice results in reduced dendritic spine density in cortical layer 2/3 and CA1 of the hippocampus. The same mice also show impaired spatial reference memory, working memory, fear learning and motor learning while they simultaneously present with increased social interaction when compared to controls (Yasumura *et al.*, 2014).

Table 9: *Genes with significant expression changes in this study which overlap with the SysID database on genes mutated in intellectual disability.*

Group	Regulation	Gene ID	Name and putative function
juvenile HVC	up	ALDH1A2	Aldehyde Dehydrogenase 1 Family Member A2, Retinoic acid synthesis
juvenile HVC	up	GLS	Glutaminase, Glutamate synthesis
juvenile HVC	up	HRAS	HRas Proto-Oncogene GTPAse, cell division
juvenile HVC	up	SATB1	Special AT-rich sequence-binding protein-1, Chromatin accessibility
juvenile HVC	up	UBR7	Ubiquitin Protein Ligase E3 Component N-Recognin 7, Ubiquitinylation
juvenile HVC	up	WRAP53	WD Repeat Containing Antisense To TP53, telomere synthesis
juvenile HVC	up	CHMP2A	Charged Multivesicular Body Protein 2A, chromatin modification
juvenile HVC	up	FH	Fumarate Hydratase, tricarboxylic acid cycle
juvenile HVC	up	RGR	Retinal G Protein Coupled Receptor, retinal conversion
juvenile HVC	up	SCGN	Secretagogin, calcium binding
juvenile HVC*	up	TUBAL3	Tubulin Alpha Like 3, SLIT-ROBO signalling
juvenile HVC	up	WWP2	WW Domain Containing E3 Ubiquitin Protein Ligase 2, ubiquitination
juvenile CMM	up	ADGRB3	Adhesion G Protein-Coupled Receptor B3, angiogenesis
juvenile CMM	up	AGMO	Alkylglycerol Monooxygenase, Kleefstra Syndrome 2
juvenile CMM	up	AUTS2	Activator Of Transcription And Developmental Regulator AUTS2, ASD
juvenile CMM	up	CNTN5	Contactin 5, nervous system development
juvenile CMM	up	DLGAP1	DLG Associated Protein 1, protein-protein interaction at synapses
juvenile CMM	up	DMD	Dystrophin, cytoskeleton
juvenile CMM	up	DSCAM	DS Cell Adhesion Molecule, nervous system development
juvenile CMM	up	GATM	Glycine Amidinotransferase, creatine biosynthesis
juvenile CMM	up	IL1RAPL1	Interleukin 1 Receptor Accessory Protein Like 1, synapse formation
juvenile CMM	up	KCND2	Potassium Voltage-Gated Channel Subfamily D Member 2, potassium channel
juvenile CMM	up	LRP1B	LDL Receptor Related Protein 1B, cellular metabolism
juvenile CMM	up	MID1	Midline 1, multiprotein structure formation
juvenile CMM	up	NPAS3	Neuronal PAS Domain Protein 3, transcription factor
juvenile CMM	up	NRG3	Neuregulin 3, tyrosine kinase receptor
juvenile CMM	up	PDE10A	Phosphodiesterase 10A, nucleotide phosphodiesterase
juvenile CMM	up	RORA	RAR Related Orphan Receptor A, nuclear hormone receptor
juvenile CMM	up	SLC35C1	Solute Carrier Family 35 Member C1, GDP-fucose transporter
juvenile CMM	up	SNTG1	Syntrophin Gamma 1, gamma-enolase trafficking to plasma membrane
juvenile CMM	up	SOX5	SRY-Box Transcription Factor 5, embryonic development
juvenile HVC, juvenile CMM	up	ASS1	Argininosuccinate Synthase 1, Citrullinemia
adult HVC	up	PLXNB1	Plexin B1, axon guidance
adult CMM	up	AP1S2	Adaptor Related Protein Complex 1 Subunit Sigma 2, clathrin recruitment
adult CMM	up	ASPA	Aspartoacylase, white matter maintenance
adult CMM	up	ATP5F1E	ATP Synthase F1 Subunit Epsilon, mitochondrial ATP synthase
adult CMM	up	ATP5PF	ATP Synthase Peripheral Stalk Subunit F6, mitochondrial ATP synthase
adult CMM	up	BOLA3	BolA Family Member 3, mitochondrial respiratory chain complex assembly
adult CMM	up	COX7B	Cytochrome C Oxidase Subunit 7B, mitochondrial respiratory chain
adult CMM	up	EEF1B2	Eukaryotic Translation Elongation Factor 1 Beta 2, guanine nucleotide exchange

adult CMM	up	GCSH	Glycine Cleavage System Protein H, methylamine group transfer
adult CMM	up	ISCA1	Iron-Sulfur Cluster Assembly 1, iron-sulfur cluster biogenesis
adult CMM	up	LYRM7	LYR Motif Containing 7, mitochondrial respiratory chain
adult CMM	up	MICOS13	Mitochondrial Contact Site And Cristae Organizing System Subunit 13, oxidative phosphorylation
adult CMM	up	NDUFA12	NADH:Ubiquinone Oxidoreductase Subunit A12, mitochondrial membrane respiratory chain
adult CMM	up	NDUFA2	NADH:Ubiquinone Oxidoreductase Subunit A2, mitochondrial membrane respiratory chain
adult CMM	up	NDUFA4	NADH:Ubiquinone Oxidoreductase Subunit A4, mitochondrial membrane respiratory chain
adult CMM	up	NDUFB8	NADH:Ubiquinone Oxidoreductase Subunit B8, mitochondrial membrane respiratory chain
adult CMM	up	NDUFS4	NADH:Ubiquinone Oxidoreductase Subunit S4, mitochondrial membrane respiratory chain
adult CMM	up	NPRL3	NPR3 Like, GATOR1 Complex Subunit, epilepsy
adult CMM	up	PSMG4	Proteasome Assembly Chaperone 4, chaperone
adult CMM	up	RPLP1	Ribosomal Protein Lateral Stalk Subunit P1, ribosome component
adult CMM	up	RPS23	Ribosomal Protein S23, ribosome component
adult CMM	up	SNAPIN	SNAP Associated Protein, vesicle docking and fusion
adult CMM	up	SOX2	SRY-Box Transcription Factor 2, embryonic development
adult CMM	up	SVBP	Small Vasohibin Binding Protein, neurodevelopmental disorder
juvenile HVC	down	ACTL6B	Actin Like 6B, cytoskeleton
juvenile HVC	down	ATP1A1	ATPase Na+/K+ Transporting Subunit Alpha 1, cation transportin ATPase
juvenile HVC	down	HEATR5B	HEAT Repeat Containing 5B
juvenile HVC	down	NDP	Norrin Cystine Knot Growth Factor NDP, Wnt/beta-catenin pathway activation
juvenile HVC	down	NDUFA1	NADH:Ubiquinone Oxidoreductase Subunit A1, mitochondrial membrane respiratory chain
juvenile HVC	down	NDUFA13	NADH:Ubiquinone Oxidoreductase Subunit A13, mitochondrial membrane respiratory chain
juvenile HVC	down	PIGP	Phosphatidylinositol Glycan Anchor Biosynthesis Class P, down syndrome
juvenile HVC	down	PLP1	Proteolipid Protein 1, oligodendrocyte development and axonal survival
juvenile HVC	down	PROCA1	Protein Interacting With Cyclin A1, calcium ion binding
juvenile HVC	down	SBDS	SBDS Ribosome Maturation Factor, ribosome biogenesis
juvenile CMM	down	ALG1	ALG1 Chitobiosyldiphosphodolichol Beta-Mannosyltransferase, oligosaccaride biosnythesis
juvenile CMM	down	SEMA3E	Semaphorin 3E, axon guidance ligand
juvenile HVC & CMM	down	EIF2B5	Eukaryotic Translation Initiation Factor 2B Subunit Epsilon, vanishing white matter
adult HVC	down	DHTKD1	Dehydrogenase E1 And Transketolase Domain Containing 1, amino acid degradation
adult HVC	down	FOXJ1	Forkhead Box J1, transcription factor
adult CMM	down	SPR	Sepiapterin Reductase, DOPA-responsive dystonia
juvenile HVC & CMM, adult HVC & CMM	down	FOXP1	Forkhead Box P1, transcription factor

Discussion

This study aimed to investigate the transcriptional differences following local *FoxP1* knockdowns in HVC and CMM of juvenile and adult female zebra finches. Even though not all the generated samples matched the quality criteria, the number of mapped and assigned reads was comparable between groups. Variance, mean and distribution of read counts as well as log 2fold changes did not differ visibly between the different treatment groups of this study. However, the dispersion of log2 fold changes across all genes identified in samples from birds injected in HVC as adults differed visibly between controls and knockdowns. Knockdown samples were more dispersed when compared to controls, which could be the result of variable knockdown efficiency across samples from this particular group. Different log2 dispersion does not result in read count bias when replicates within a group consist of unrelated or genetically distant samples as was the case in this study. However, dispersion is also affected by the presence of a large number of genes with a low count, which could be the case in this group as samples taken from adult HVC yielded the highest number of assigned genes which might be represented by a low number of counts (Yoon and Nam, 2017). Even though *FoxP1* knockdown efficiency varied across samples, *FoxP1* was the only gene to show significant reductions in expression in knockdowns of all groups when compared to their matched controls. Across all groups tested during this study, knockdown efficiency differed between previous qPCR analyses and the results from RNAseq, but this difference was not significant on the level of different subgroups. The assessment of relative expression levels during qPCR and total transcript counts during RNAseq analyses might account for this methodological difference. Besides *FoxP1*, no gene showed significantly altered expression across all the different knockdown groups. This suggests that region-specific but probably also interindividual differences outweigh common transcriptional changes across knockdowns. Substantive interindividual differences are also supported by the results from hierarchical clustering of samples, where samples clustered according to age during treatment and injected area but no further segregation between controls and knockdowns was visible on group level. Interindividual variability cannot be explained by activity-regulated genes as all samples were obtained in silence prior to light onset early in the morning, excluding immediate effects on different gene expression levels. However, variable knockdown efficiency and general variability in gene expression

levels between individuals with different degrees of relatedness could account for large interindividual differences.

Even though little overlap occurred between the groups of this study regarding differentially expressed genes, some GO terms and local network clusters were enriched in multiple groups. Additionally, genes with comparable functional implications were found to be differentially expressed in knockdowns injected in different areas during different developmental stages.

In all groups except for adult CMM, genes related to retinoic acid signalling, synthesis or other retinal proteins were among the genes showing the most significant increases in expression in response to *FoxP1* knockdown. Among the genes showing the most significant decreases in adult CMM knockdowns, one unannotated locus LOC100229421 is suspected to code for IFIT5 (Scalf, 2018), a retinoic acid and interferon inducible protein. Moreover, local network clusters related to retinoic acid signalling were enriched among genes showing increased expression in juvenile or adult HVC knockdowns. Taken together, these findings suggest that FoxP1 might be linked to retinoic acid signalling, possibly as a heterodimer with FoxP2 (Li *et al.*, 2004; Roeske *et al.*, 2014; Mendoza and Scharff, 2017) which is only weakly expressed in songbird HVC (Teramitsu *et al.*, 2004; Mendoza *et al.*, 2015) but has been shown to interact with the retinoic acid signalling pathway and thereby helps regulate neuronal differentiation (Devanna *et al.*, 2014; Van Rhijn and Vernes, 2015; Negwer and Schubert, 2017).

In addition to the putative gene *IFIT5* showing elevated expression in adult CMM knockdowns, other interferon-regulated genes are differentially expressed across knockdowns of all groups. Interferon signalling can be related to retinoic acid signalling as both pathways are linked and possibly potentiate each other (Pelicano *et al.*, 1997; Chelbi-Alix and Pelicano, 1999). Transcripts of interferon-related genes are among the most significantly reduced by *FoxP1* knockdown in samples of all groups except juvenile CMM where transcripts of one gene coding for an interferon-inducible protein is among the transcripts showing most significant increases. Next to the interferon signalling pathway, two more genes which show elevated expression in adult CMM knockdowns (*MGST1*, *PTGR1*) might indirectly contribute to retinoic acid related processes via prostaglandin signalling (Kelner *et al.*, 2000; Dick *et al.*, 2001). Prostaglandins have been shown to inhibit neuronal correlates of mate calling in frogs

(Schmidt and Kemnitz, 1989) and interact with retinoic acid signalling by suppressing retinoic acid synthesis (Stock *et al.*, 2011) which in turn stimulates prostaglandin production (Kim *et al.*, 2008).

Even though differentially expressed genes related to retinoic acid signalling or connected pathways were detected across all treatment groups, the behavioural changes following lentiviral *FoxP1* knockdowns were limited to adult HVC (Chapter 2). Perhaps FoxP1 and its contributions to retinoic acid signalling may be especially impactful in this area and developmental stage, as the retinoic acid synthesising enzyme zRalDH is highly expressed in HVC but not in CMM (Denisenko-Nehrbass *et al.*, 2000; Olson *et al.*, 2011) where only retinoic acid receptors are expressed (Roeske *et al.*, 2014). Dietary supplementation of retinoic acid (Wood *et al.*, 2008) or blockage of retinoic acid synthesis in HVC (Denisenko-Nehrbass *et al.*, 2000) during the critical learning phase of juvenile male zebra finches leads to more variable songs in adults.

Another possibly relevant group of genes showing differential expression in knockdown samples consists of loci related to SLIT-ROBO signalling. Among the most significant increases in expression in both juvenile and adult HVC knockdown samples was at least one gene associated to SLIT-ROBO signalling, and one gene of this pathway is also among the those showing the most significant reductions in juvenile CMM knockdown samples. Proteins of the SLIT-ROBO signalling pathway have been identified as downstream targets of human FOXP2 in vitro (Vernes *et al.*, 2007a; Konopka *et al.*, 2009), and binding partners of FoxP1 in zebra finches (Mendoza and Scharff, 2017). The SLIT-ROBO signalling pathway has been implicated in human language-related impairments (Hannula-Jouppi *et al.*, 2005; Bates *et al.*, 2010; Suda *et al.*, 2011; Pourcain *et al.*, 2014; Mozzi *et al.*, 2016) and its proteins show convergent substitutions and expression levels in vocal learning mammals (Wang *et al.*, 2015). Genes related to SLIT-ROBO signalling are also enriched in HVC of juvenile (45 days post hatch) and adult (100 days post hatch) male zebra finches (Shi *et al.*, 2021). Convergence between the avian and human orthologs of this pathway has been suggested based on differential regulation of *SLIT1* in RA of zebra finches and human laryngeal motor cortex (Pfenning *et al.*, 2014).

In addition to individual genes of specific pathways, the significant overlap with gene expression data from striatal neurons in mice with heterozygous knockout of *Foxp1* (Araujo *et al.*, 2015) further emphasizes that this transcription may regulate similar

molecular and cellular mechanisms in different species. Even though mice do not need to learn how to produce their vocalisations (Hammerschmidt *et al.*, 2012; Screven and Dent, 2019), female mice can discriminate contextual differences of male song (Hammerschmidt *et al.*, 2009; Chabout *et al.*, 2015) and develop preferences for specific songs by imprinting (Asaba *et al.*, 2014).

Next to overlaps with differentially expressed genes in mice following genetic *Foxp1* manipulations, significant subsets of genes which were differentially expressed in groups of this study are also listed as putative risk genes involved in autism spectrum disorder in the SFARI database or the SysID database on genes mutated in intellectual disability. This pattern is consistent with the involvement of *FOXP1* in phenotypes related to ASD and ID (Sollis *et al.*, 2016; Co *et al.*, 2020a).

Taken together, the potential regulation of genes related to retinoic acid, interferon, prostaglandin, SLIT-ROBO signalling, and orthologues of putative genes related to ASD-risk genes by FoxP1 in female zebra finches might enhance our understanding of the in vivo functions of this transcription factor in the songbird brain. The data presented here could be helpful for gaining new insights into how FoxP1 contributes to song motor control and auditory perception and memory in different brain areas during song production learning in male zebra finches (Norton *et al.*, 2019; Garcia-Oscos *et al.*, 2021) and perception in females (Chapter 2).

Another possibly relevant gene which shows significantly reduced expression in adult CMM knockdowns is *ETFB*. The protein that this gene encodes is implicated in energy metabolism of mitochondria, which could perhaps be related to the large amount of differentially expressed genes related to mitochondrial processes next to genes implicated in ribosomal processes in this group. This pattern of findings could be the result of biased knockdown-specific effects in CMM of adult birds, since differentially expressed genes related to energy metabolism in the mitochondria or ribosomal activity were also present in the other groups albeit at a smaller rate. Another possible contributory factor might be the different amounts of tissue that went into the RNA preparations of different groups. Tissue punches for HVC were placed at the dorsal edge of the brain resulting in lower amounts of tissue compared to CMM samples, where a biopsy punch was taken more centrally. However, as both juvenile and adult treated birds were sacrificed as adults, both groups should result in comparable differentially expressed genes unless the birds' age during the *FoxP1* knockdown

affects mitochondrial and ribosomal-related gene expression differentially. Interestingly, a recent study shows mitochondrial dysfunction in the striatum of heterozygous *Foxp1* knockout mice (Wang *et al.*, 2021) suggesting a possible contribution of altered energy supply and oxidative stress to *FoxP1*-related phenotypes.

In summary, gene expression analyses of samples generated from birds which received *FoxP1* knockdowns in HVC or CMM during different developmental stages show interesting convergences with previous studies on transcriptional differences following manipulations of this gene in other species and pathways relevant to FoxPs and vocalisation behaviours. Even though female zebra finches do not learn to produce a song of their own, FoxP1 might be implicated in similar pathways and mechanisms in both sexes. To further validate potential contributions of FoxP1 to pathways identified in this study, putative regulation of target genes should be experimentally verified. Overall, the expression profiling data from this study provide a valuable resource for further deciphering conserved roles of FoxP1 in vocalisation (and related) behaviours in diverse species, ranging from vocal learning in songbirds to speech and language in humans.

Conclusion

The data from this chapter suggest that, despite large interindividual and group-based differences in gene expression, the contributions of *FoxP1* to the regulation of specific pathways show some intriguing overlaps across the targeted brain regions and ages during treatment. The knockdown target itself, *FoxP1*, was the only individual gene to show significantly different expression across all the groups studied. However, analyses of differentially expressed genes with respect to enrichment of GO terms, gene sets and local networks identified a number of processes that had been previously associated directly or indirectly to FoxP1. Highlighted pathways include retinoic acid signaling or SLIT-ROBO signaling. A significant number of differentially expressed genes overlapped between this research and a study on striatal gene expression in FoxP1 knockout mice. Further, differentially expressed genes identified in this chapter overlap with databases on genes implicated in autism spectrum disorders or intellectual disability which are both associated with human FOXP1 mutations. Taken together, the results from this study can contribute to the

understanding of downstream effects which are influenced by *FoxP1* across different species and may also help to understand the molecular underpinnings of vocal learning at the basis of human speech and language.

Appendix Chapter 4

Supplementary Table 1: *Samples contributing to this analysis where sufficient RNA was obtained for RNAseq analyses. Individual bird ID encodes treatment group, target area, age group and hemisphere RNA was obtained from. shRNA type identifies the shRNA which was virally transduced in each bird. Batch date corresponds to the date each virus batch was produced and Seq. batch indicates the batch in which each sample was sent for total RNA sequencing.*

Bird ID	Treatment group	Age group	Target area	Hemi-sphere	shRNA type	Batch date	Seq. Batch
CJHL6120	Control	Juvenile	HVC	Left	shCtrl	13.1.2017	3
CJHL6122	Control	Juvenile	HVC	Left	shCtrl	13.1.2017	3
CJHL6141	Control	Juvenile	HVC	Left	shCtrl	13.1.2017	3
CJHL6146	Control	Juvenile	HVC	Left	shCtrl	3.2.2017	3
CJHL6175	Control	Juvenile	HVC	Left	shCtrl	3.2.2017	3
CJHL6198	Control	Juvenile	HVC	Left	shCtrl	3.2.2017	3
CJHL6536	Control	Juvenile	HVC	Left	shCtrl	3.2.2017	3
CJHR6120	Control	Juvenile	HVC	Right	shCtrl	13.1.2017	3
CJHR6122	Control	Juvenile	HVC	Right	shCtrl	13.1.2017	3
CJHR6141	Control	Juvenile	HVC	Right	shCtrl	13.1.2017	3
CJHR6146	Control	Juvenile	HVC	Right	shCtrl	3.2.2017	3
CJHR6175	Control	Juvenile	HVC	Right	shCtrl	3.2.2017	3
CJHR6198	Control	Juvenile	HVC	Right	shCtrl	3.2.2017	3
KJHL6123	Knockdown	Juvenile	HVC	Left	shKRAK	13.1.2017	3
KJHL6139	Knockdown	Juvenile	HVC	Left	shKRAK	13.1.2017	3
KJHL6145	Knockdown	Juvenile	HVC	Left	shY	13.1.2017	3
KJHL6147	Knockdown	Juvenile	HVC	Left	shKRAK	13.1.2017	3
KJHL6162	Knockdown	Juvenile	HVC	Left	shY	13.1.2017	3
KJHL6178	Knockdown	Juvenile	HVC	Left	shY	13.1.2017	3
KJHL6187	Knockdown	Juvenile	HVC	Left	shY	13.1.2017	3
KJHR6139	Knockdown	Juvenile	HVC	Right	shKRAK	13.1.2017	3
KJHR6145	Knockdown	Juvenile	HVC	Right	shY	13.1.2017	3
KJHR6147	Knockdown	Juvenile	HVC	Right	shKRAK	13.1.2017	3
KJHR6162	Knockdown	Juvenile	HVC	Right	shY	13.1.2017	3
KJHR6187	Knockdown	Juvenile	HVC	Right	shY	13.1.2017	3
CAHL5148	Control	Adult	HVC	Left	shCtrl	10.10.2014	1
CAHL5424	Control	Adult	HVC	Left	shCtrl	10.10.2014	1
CAHL5520	Control	Adult	HVC	Left	shCtrl	10.10.2014	2
CAHL5549	Control	Adult	HVC	Left	shCtrl	8.8.2015	2
CAHL5631	Control	Adult	HVC	Left	shCtrl	19.4.2013	3
CAHL5664	Control	Adult	HVC	Left	shCtrl	19.4.2013	3
CAHR5134	Control	Adult	HVC	Right	shCtrl	10.10.2014	1
CAHR5148	Control	Adult	HVC	Right	shCtrl	16.5.2014	1

CAHR5162	Control	Adult	HVC	Right	shCtrl	16.5.2014	1
CAHR5427	Control	Adult	HVC	Right	shCtrl	16.5.2014	1
CAHR5446	Control	Adult	HVC	Right	shCtrl	19.4.2013	1
CAHR5549	Control	Adult	HVC	Right	shCtrl	8.8.2015	3
CAHR5631	Control	Adult	HVC	Right	shCtrl	19.4.2013	3
CAHR5692	Control	Adult	HVC	Right	shCtrl	19.4.2013	3
KAHL5290	Knockdown	Adult	HVC	Left	shKRAK	10.10.2014	1
KAHL5426	Knockdown	Adult	HVC	Left	shKRAK	16.5.2014	1
KAHL5440	Knockdown	Adult	HVC	Left	shY	6.6.2014	1
KAHL5408	Knockdown	Adult	HVC	Left	shKRaK	10.10.2014	2
KAHL5447	Knockdown	Adult	HVC	Left	shY	10.10.2014	2
KAHL5521	Knockdown	Adult	HVC	Left	shKRAK	10.10.2014	2
KAHL5523	Knockdown	Adult	HVC	Left	shKRAK	10.10.2014	2
KAHR5203	Knockdown	Adult	HVC	Right	shRKAK	16.5.2014	1
KAHR5290	Knockdown	Adult	HVC	Right	shKRAK	10.10.2014	1
KAHR5426	Knockdown	Adult	HVC	Right	shKRAK	16.5.2014	1
KAHR5440	Knockdown	Adult	HVC	Right	shY	6.6.2014	1
KAHR5408	Knockdown	Adult	HVC	Right	shKRaK	10.10.2014	2
KAHR5447	Knockdown	Adult	HVC	Right	shY	10.10.2014	2
KAHR5521	Knockdown	Adult	HVC	Right	shKRAK	10.10.2014	2
KAHR5542	Knockdown	Adult	HVC	Right	shY	10.10.2014	3
CJCL6522	Control	Juvenile	CMM	Left	shCtrl	3.2.2017	3
CJCL6591	Control	Juvenile	CMM	Left	shCtrl	21.7.2017	3
CJCL6594	Control	Juvenile	CMM	Left	shCtrl	3.2.2017	3
CJCL6619	Control	Juvenile	CMM	Left	shCtrl	3.2.2017	3
CJCL6620	Control	Juvenile	CMM	Left	shCtrl	21.7.2017	3
CJCL6637	Control	Juvenile	CMM	Left	shCtrl	21.7.2017	3
CJCR6522	Control	Juvenile	CMM	Right	shCtrl	3.2.2017	3
CJCR6591	Control	Juvenile	CMM	Right	shCtrl	21.7.2017	3
CJCR6594	Control	Juvenile	CMM	Right	shCtrl	3.2.2017	3
CJCR6596	Control	Juvenile	CMM	Right	shCtrl	3.2.2017	3
CJCR6615	Control	Juvenile	CMM	Right	shCtrl	21.7.2017	3
CJCR6616	Control	Juvenile	CMM	Right	shCtrl	21.7.2017	3
CJCR6619	Control	Juvenile	CMM	Right	shCtrl	3.2.2017	3
CJCR6620	Control	Juvenile	CMM	Right	shCtrl	21.7.2017	3
CJCR6637	Control	Juvenile	CMM	Right	shCtrl	21.7.2017	3
KJCL6527	Knockdown	Juvenile	CMM	Left	shKRAK	21.7.2017	3
KJCL6589	Knockdown	Juvenile	CMM	Left	shKRAK	21.7.2017	3
KJCL6593	Knockdown	Juvenile	CMM	Left	shY	21.7.2017	3
KJCL6618	Knockdown	Juvenile	CMM	Left	shY	21.7.2017	3
KJCL6640	Knockdown	Juvenile	CMM	Left	shY	21.7.2017	3
KJCR6527	Knockdown	Juvenile	CMM	Right	shKRAK	21.7.2017	3
KJCR6589	Knockdown	Juvenile	CMM	Right	shKRAK	21.7.2017	3
KJCR6612	Knockdown	Juvenile	CMM	Right	shY	21.7.2017	3

KJCR6617	Knockdown	Juvenile	CMM	Right	shKRAK	21.7.2017	3
KJCR6618	Knockdown	Juvenile	CMM	Right	shY	21.7.2017	3
KJCR6640	Knockdown	Juvenile	CMM	Right	shY	21.7.2017	3
CACL5749	Control	Adult	CMM	Left	shCtrl	21.7.2017	2
CACL5805	Control	Adult	CMM	Left	shCtrl	14.2.2016	2
CACL5866	Control	Adult	CMM	Left	shCtrl	3.2.2016	2
CACL6047	Control	Adult	CMM	Left	shCtrl	21.7.2017	3
CACL6282	Control	Adult	CMM	Left	shCtrl	3.2.2017	3
CACL6283	Control	Adult	CMM	Left	shCtrl	3.2.2017	3
CACL6292	Control	Adult	CMM	Left	shCtrl	21.7.2017	3
CACR5749	Control	Adult	CMM	Right	shCtrl	21.7.2017	2
CACR5866	Control	Adult	CMM	Right	shCtrl	3.2.2016	2
CACR6260	Control	Adult	CMM	Right	shCtrl	3.2.2017	3
CACR6271	Control	Adult	CMM	Right	shCtrl	21.7.2017	3
CACR6292	Control	Adult	CMM	Right	shCtrl	21.7.2017	3
KACL5806	Knockdown	Adult	CMM	Left	shKRAK	10.10.2014	2
KACL5808	Knockdown	Adult	CMM	Left	shY	16.6.2014	2
KACL6281	Knockdown	Adult	CMM	Left	shY	21.7.2017	3
KACL6284	Knockdown	Adult	CMM	Left	shY	3.2.2017	3
KACL6320	Knockdown	Adult	CMM	Left	shKRAK	3.2.2017	3
KACL6413	Knockdown	Adult	CMM	Left	shKRAK	21.7.2017	3
KACR5891	Knockdown	Adult	CMM	Right	shY	21.7.2017	2
KACR6240	Knockdown	Adult	CMM	Right	shY	21.7.2017	3
KACR6245	Knockdown	Adult	CMM	Right	shY	21.7.2017	3
KACR6281	Knockdown	Adult	CMM	Right	shY	21.7.2017	3
KACR6284	Knockdown	Adult	CMM	Right	shY	3.2.2017	3
KACR6320	Knockdown	Adult	CMM	Right	shKRAK	3.2.2017	3
KACR6413	Knockdown	Adult	CMM	Right	shKRAK	21.7.2017	3

Supplementary Table 2: *Normalised read counts of all mapped genes across all samples of this study* [access via: https://doi.org/10.17026/dans-xux-y5ja].

Supplementary Table 3: *Differentially expressed genes for all subgroups of this study* [access via: https://doi.org/10.17026/dans-zg3-qvba].

Supplementary Table 4: *Extended list of enriched GO terms and local clusters based on significantly upregulated genes in adult CMM knockdowns* [access via: https://doi.org/10.17026/dans-zr3-eutj].

Supplementary Table 5: *Gene set enrichment analyses data for all subgroups of this study based on GSEA 4.11* [access via: https://doi.org/10.17026/dans-23p-7626].

Supplementary Table 6: *Differentially expressed genes across all subgroups of this study that overlap with a previous study by Araujo et al. (2015)* [access via: https://doi.org/10.17026/dans-2bj-ks3v].

Chapter 5

General discussion and Thesis summary

Chapter 5: General discussion and Thesis summary

The acquisition of human speech and language and as well as vocalisations in several animal groups relies on vocal production learning. This type of learning depends on perception and memory of auditory signals but also on comparisons of signals to an organism's own vocal output. Auditory processing of vocal input thus is a crucial component of vocal learning. Despite its relevance for vocal learning, the neural processing of auditory stimuli on cellular and transcriptional levels is not fully understood. This thesis aimed to shed light on the neurogenomic underpinnings of auditory perception in an important model species, the zebra finch. Zebra finches are songbirds that, like humans, are vocal learners. In this species, only males sing (Immelmann, 1962; Zann, 1997), but like males, juvenile female zebra finches establish a tutor song memory and learn to discriminate songs (Miller, 1979b; Clayton, 1988; Riebel, 2003b, 2009). This provides an opportunity to study the processes involved in vocal perception and processing separate from those of song production. In this thesis the role of FoxP1 has been examined. Disruptions in the human gene of FoxP1 which encodes a transcription factor are implicated in intellectual disability and/or autism spectrum disorders which are often accompanied by speech and language deficits. In order to investigate FoxP1's potential roles in auditory perception, female zebra finches received local lentiviral knockdowns as juveniles prior to song preference development or as adults well after development. In each age group, two different brain areas were targeted: the HVC (acronym used as a proper name) or the caudomedial mesopallium (CMM). The main hypotheses underlying this study were that altered FoxP1 expression in HVC or CMM of female zebra finches influences the development and maintenance of auditory memories and the birds' ability to learn the categorisation and discrimination of natural auditory stimuli.

In contrast to the initial hypotheses, lentiviral knockdowns of FoxP1 in female zebra finches did not impair the birds' abilities to establish a memory of a tutor's song and discriminate it from unfamiliar conspecific song. The knockdown neither affected the birds' ability to learn the discrimination of two unfamiliar songs nor their ability to categorise modified versions of the trained songs. However, the knockdown did affect the females' motivation to engage in operant behaviour to trigger song playbacks. The results of these experiments suggest that FoxP1 expression in the brain areas HVC or CMM of female zebra finches does not contribute to preference and memory establishment or its maintenance, nor to auditory discrimination learning or the

identification and categorisation of novel song stimuli. In order to elaborate on the processes and pathways influenced by the transcription factor FoxP1, total RNA was isolated from the targeted brain areas to draw further conclusions from differential gene expression analyses across the different groups of female zebra finches that were investigated during this thesis. Below, the different Chapters and experiments and the corresponding findings will be discussed and summarised.

Song preference learning in female zebra finches as a paradigm to understand the contributions of FoxP1 to auditory perception

Prior work has shown that knockdowns of FoxP1 in Area X or HVC of male zebra finches impair song learning (Norton *et al.*, 2019; Garcia-Oscos *et al.*, 2021). Since vocal learning relies on sensory components in addition to motor practice, *FoxP* manipulations might impair multiple features necessary for proper song imitation learning. Due to the high expression levels of *FoxP1* in male and female HVC and CMM (Teramitsu *et al.*, 2004; Chen *et al.*, 2013; Mendoza *et al.*, 2015) which are both involved in song memory, auditory perception and auditory learning (Bell *et al*., 2015; Roberts *et al*., 2017; Soyman and Vicario, 2017; Inda *et al*., 2020) it can be hypothesised that reduced expression of *FoxP1* in either of these areas impairs performance during auditory tasks as well. This would also correspond to the fact that both sexes are auditory learners but only males learn to produce a song. In order to study auditory learning independent of song learning, female zebra finches were chosen as a suitable model to study the impact of *FoxP1* expression on the perception and processing of conspecific song stimuli.

Is FoxP1 implicated in establishing and maintaining female song preference?

Examining song preference and its acquisition in female zebra finches (Riebel *et al.,* 2002) can provide insight into putative functions of brain expressed *FoxP1* on tutor song memorisation independent of motor learning. As juvenile females establish a memory for tutor song during a sensory phase (Clayton, 1988), the experiment described in Chapter 2 tested the hypothesis that undisturbed expression levels of *FoxP1* in HVC and CMM are necessary to establish and maintain memory for an adult tutor's song since *FoxP1* expression remains stable with age in female but also male zebra finches. In order to test the different levels of this hypothesis, behaviour of females which received a knockdown of FoxP1 as juveniles or adults in either HVC or

CMM was assessed in operant preference tests during which females could elicit playbacks of familiar or unfamiliar songs by pecking a respective key (Chapter 2). The birds' performance was predicted to be altered in animals which received a FoxP1 knockdown. In HVC, altered behaviour might be expected as a result from impaired processing of perceived stimuli. Potential behavioural differences in birds which received knockdowns to CMM might result from impairments in sensory perception or affected comparisons between memory templates and perceived stimuli. Depending on the exclusivity of an effect in either juvenile or adult birds, further implications on the importance of FoxP1 on memory establishment or maintenance might be deduced. Additionally, the participation rate during the preference tests, where song playback was the only reinforcing stimulus, would make it possible to assess the implication of *FoxP1* in the motivation to receive auditory feedback.

Reduced FoxP1 expression levels do not impair preference establishment or its maintenance

During the experiments on female preference behaviour following local FoxP1 knockdowns which are presented in Chapter 2 of this thesis, birds from all groups preferred their tutor's song over unfamiliar song. Behavioural changes could only be detected in females which received a knockdown in HVC as adults. Birds from this group showed a weaker preference strength for familiar song in comparison to matched controls and they also participated less in the operant test with respect to the elicited number of playbacks. As a successful knockdown could be validated for all experimental controls, misplaced or non-functional viral injections can be excluded as confounding factors of this age- and region-specific observation. Age- and region-specific functions of *FoxP1* expression in female zebra finches are further supported by a positive correlation of knockdown efficiency and preference strength. A basic modelling approach also identified knockdown efficiency as a predictor of preference strength. While knockdown efficiency and the number of elicited playbacks were not correlated, a modelling approach revealed knockdown efficiency, injected region and area as predictors of the number of playbacks elicited by the females.

Implications of FoxP1 expression in HVC for reward perception of song

Higher receptor density and increased synaptic plasticity in brain areas of the auditory pathway of juvenile compared to adult zebra finches (Ribeiro and Mello, 2000; Wada *et al.*, 2004; Simonyan *et al.*, 2012) might suggest increased flexibility to cope with and compensate local knockdowns and contribute to the observed effects being limited to adult females. As HVC can be seen as a hub that processes sensory information and simultaneously controls downstream projections, while CMM serves as an exclusively auditory area (Prather *et al.*, 2009; Bolhuis *et al.*, 2010; Ikeda *et al.*, 2020), the limitation of behavioural changes to HVC knockdowns might also hint toward an effect of FoxP1 on the reinforcing qualities of tutor song. In mice, Foxp1 knockdowns modify dopamine receptor 1 expressing cells (Araujo *et al.*, 2015). Dopamine, which is implicated in the motivation to express certain behaviours (Wise, 1989) might be specifically relevant in HVC where most dopamine receptors are highly expressed (Kubikova *et al.*, 2010). Blockage of dopamine signalling also impairs song copying in juvenile males (Tanaka *et al.*, 2018) and systemic dopamine D2 receptor activation affects female song preference (Day *et al.*, 2019b).

Based on these findings, it is possible that the perception of rewarding qualities of conspecific song might be influenced by a dopaminergic pathway including HVC of adult female zebra finches that is fine-tuned by *FoxP1* expression. A recent study on FoxP1 knockdowns in HVC of juvenile males further supports this hypothesis. Knockdowns of FoxP1 impair copying efficiency of tutor song if the knockdown occurs after tutor song presentation but not before (Garcia-Oscos *et al.*, 2021). The absence of any behavioural effects following knockdowns in CMM further implies that auditory perception per se is not impaired but its processing in HVC might be affected by FoxP1.

In summary, Chapter 2 shows that reduced *FoxP1* expression levels in HVC but not CMM of adult female zebra finches are implicated in feedback perception as it is manifested by the birds' motivation to elicit conspecific song playbacks. However, undisturbed *FoxP1* expression levels in either of the two areas are not necessary to identify and discriminate auditory stimuli or establish and maintain a learned preference.

Reduced *FoxP1* expression levels do not interfere with discrimination or categorisation of novel auditory stimuli

Chapter 3 of this thesis examined the implication of reduced levels of FoxP1 on the discrimination and categorisation of conspecific song stimuli by female zebra finches during Go/Nogo tasks. The aim of this Chapter was to identify whether FoxP1 knockdowns in HVC or CMM of adult or juvenile female zebra finches would impair their ability to learn to discriminate between auditory stimuli and to categorise altered versions thereof. Altered stimuli were modified in pitch, spectral structure or syllable sequence to identify the contribution of FoxP1 to the perception and weighting of these parameters. During operant training in the Go/Nogo task, control and knockdown birds which were treated in HVC as juveniles required more trials to achieve the same discrimination rate than birds treated in CMM or as adults. However, extinction of the learned discrimination was not affected in birds injected as juveniles in HVC or any other group. Minor tissue damage to the areas surrounding HVC might partly explain the increased number of training trials the birds of the juvenile HVC groups needed even though no such damage was visible during histology. An additional explanation of this area- and age-specific effect might be provided by putative damage to neighbouring parahippocampal structures (Bailey *et al*., 2009; Payne *et al*., 2021) in developing birds. Damage in this area could have resulted in prolonged spatial learning, as the Go/Nogo task also relies on a sequence of interactions with pecking keys at different locations.

Similar to the results from Chapter 2, the insights from Chapter 3 of this thesis suggest that general auditory perception and discrimination do not seem to be impaired by reduced *FoxP1* expression as birds of all groups were able to distinguish and categorise auditory stimuli during different operant tasks.

Food-rewards are equally motivating for control and knockdown birds

Even though no differences with respect to stimulus discrimination and categorisation were evident between any of the knockdown groups and their matched controls, the findings in Chapter 3 further support the implication of *FoxP1* expression in HVC of adult female zebra finches for the reinforcing qualities of hearing a familiar song. During preference tests in Chapter 2 where the only reward was the stimulus itself, females which received a knockdown in HVC as adults requested fewer playbacks in comparison to matched controls. In Chapter 3 during Go/Nogo tasks no such difference

between any of the knockdown and respective control groups was detected for food rewards that were provided after completing a successful Go-trial. This implies that positive reinforcement or stimulus categorisation and discrimination are not affected by reduced levels of FoxP1 in juvenile or adult HVC or CMM but that the perception of rewarding qualities of song is influenced by FoxP1 expression levels in HVC.

Spectral shape, pitch and syllable sequence of natural stimuli carry different informational content for female zebra finches

Since no behavioural difference during the Go/Nogo tasks could be assigned to local and age-specific knockdowns, data from all birds participating in this study were pooled. This large dataset provided insight into the weights of different properties and their involvement in the discrimination between two songs and allowed a comparison with previous studies on song discrimination in zebra finches (Braaten *et al.*, 2006; Nagel *et al.*, 2010; Vernaleo and Dooling, 2011; Lawson *et al.*, 2018; Prior *et al.*, 2018). Overall response rates towards altered and untrained test stimuli was reduced but the discrimination rate for all stimuli remained above chance which indicates that the tested females recognised all stimuli despite the conducted playback manipulations.

Response rate was highest towards stimuli with altered syllable sequence which is consistent with previous findings regarding the behaviour of female zebra finches during preference tests with switched syllable elements. When tested for song preference, females did not show a distinction between songs with switched or unchanged syllable sequence (Riebel, 2000). This suggests that potential informational value in zebra finch song is transmitted within individual syllables and their execution rather than their sequence. Altered pitch levels also had no large effect on the discrimination of song stimuli, even though the birds were more likely to respond to stimuli with increased rather than decreased pitch. Females might be biased to respond towards stimuli with increased pitch due to higher pitch levels of female directed song, which females prefer over undirected song (Chen *et al.*, 2017). Elevated levels of pitch might be more attractive to females as production of high pitched notes requires higher air sac pressure (Riede *et al.*, 2010). Entirely reversed stimuli affected discrimination the most even though the discrimination rate remained above chance level. This finding is in line with previous studies, where reversed playbacks also affected the birds' performance the most (Braaten *et al.*, 2006; Lawson *et al.*, 2018).

Taken together, these results suggest that variation in syllable sequence is tolerated more than an 8% pitch change or the spectral structure of zebra finch song. In turn, the overall spectral structure of a stimulus is of higher weight than the 8% pitch variation for identifying songs.

This may be related to findings that during song learning, juvenile male zebra finches first modify pitch to match a new template before they proceed to adjust the syllable sequence (Lipkind *et al.*, 2017). During this learning process, repositioning of a previously learnt syllable takes juvenile male zebra finches longer than learning a new syllable (Lipkind *et al.*, 2013). This suggests that at least during production learning, pitch modifications of previously learnt syllables or the integration of entirely new syllables are prioritised at the cost of syllable sequence adjustment.

Local FoxP1 knockdowns highlight age- and area-specific differences and similarities to previously identified processes and pathways

As shown in Chapter 4, *FoxP1* was determined by RNA sequencing analyses as the only gene showing significantly reduced expression across all experimental groups even though knockdown efficiency varied across individuals. No overlap of genes with increased expression associated with *FoxP1* knockdown could be detected across all groups indicating that gene expression was influenced differently in groups which received the knockdown construct at different ages and into different areas. Differential gene expression between controls and knockdowns was thus influenced by interindividual differences as well as age- and region-specific expression profiles. Considering that zebra finches used as laboratory animals are less inbred than e.g. mouse strains (Forstmeier *et al.*, 2007), individual differences within a single colony are to be expected.

Despite limited overlap between transcriptional changes of individual genes between the treatment groups, gene ontology (GO) terms (predicted or observed functional annotations of genes) and local networks were identified to be influenced by *FoxP1* knockdowns across multiple experimental groups of this study. These include retinoic acid signalling and synthesis which has been previously associated to FoxP1s binding partner FoxP2 (Devanna *et al.*, 2014). Genes from interferon and prostaglandin signalling pathways, which are also associated to retinoic acid, were also differentially expressed after local *FoxP1* knockdowns. Additionally, components of the SLIT-ROBO signalling pathway which has been tied to FOXP2 (Vernes *et al.*, 2007b) and variation

in language-related phenotypes in humans (Pourcain *et al.*, 2014) were differentially regulated following local FoxP1 knockdowns in all but samples from adult CMM in this study.

Next to specific pathways, a large overlap was detected between differentially expressed genes in this study and genes showing expression differences in the striatum of heterozygous *Foxp1* knockout mice as compared to wildtype animals from an earlier study (Araujo *et al.*, 2015). Differentially expressed genes in samples from female zebra finches also overlapped with genes listed in SysID and SFARI databases which collate information on putative risk genes for intellectual disability and autism spectrum disorder, respectively. Both phenotypes have been documented in human patients with FOXP1 mutations (Sollis *et al.*, 2016). Differential expression was also detected for genes implicated in mitochondrial function and cellular respiration. This finding overlaps with a recent study which reports impaired mitochondrial function in heterozygous Foxp1 knockout mice (Wang *et al.*, 2021).

Large variability between samples from different experimental groups in this study is evident from the lack of overlapping, differentially expressed genes across all groups. However, individual genes and pathways which have been previously linked to other FoxP genes in other species and phenotypes following FoxP manipulations or mutations indicate conserved functions of FoxP1 across species.

Conclusion

In summary, this thesis suggests that localised reduction of *FoxP1* expression in HVC or CMM of female zebra finches does not impair the establishment or maintenance of auditory memories of conspecific song nor the females' ability to discriminate or categorise auditory stimuli based on spectral or sequential features.

This was unexpected as *FoxP1* expression levels are elevated in corresponding nuclei of male and female zebra finches throughout development when compared to surrounding tissue and reduced expression levels of FoxP1 in HVC of juvenile male zebra finches have been shown to be of importance for song learning. There were however other effects: experimentally lowered *FoxP1* expression in HVC of adult female zebra finches reduced the rewarding qualities of song playback adding evidence to the hypothesis that FoxP1 is not implicated exclusively in fine motor learning and control but also contributes to sensory processing during vocal learning.

Even though *FoxP1* is consistently expressed throughout development and during adulthood of female zebra finches, no perceptual differences with respect to knockdowns during different developmental stages could be detected. Therefore, general auditory perception and processing of perceived stimuli in the brain areas tested in the experiments of this thesis do not seem to be influenced by the transcription factor FoxP1. However, a contribution of FoxP1 in HVC to motivational behaviours which are controlled by reward perception is highly likely, based on the observation that females which received a knockdown in HVC as adults request fewer song playbacks than their matched controls. This also implies that that impaired tutor song imitation after reduced *FoxP1* expression in brain areas of juvenile male zebra finches might be influenced by how these birds perceive or process internal or external feedback which is required to match their own to a given template.

The examination of genes, networks and pathways which differential expression after FoxP1 knockdowns might shed light on the question how this transcription factor leads to behavioural phenotypes related to vocalisations and cognition. This study links FoxP1 to pathways that have previously also been associated with FOXP2 including retinoic acid signalling and the SLIT-ROBO signalling cascade. Altered energy metabolism in different brain areas might also contribute to the observed phenotypes. Since only females which received a knockdown of FoxP1 in HVC as adults showed behavioural differences during the preference tasks of this thesis, FoxP1 manipulations might impair behaviour in a dosage dependent manner. Detectable differences in behaviour and cognition might thus be based on the knockdown efficiency where multiple pathways must be altered sufficiently during a specific developmental stage and in a certain brain area.

Ultimately, future research on sensory and processing implications of FoxP1 and other FoxP transcription factors is required in order to unveil their contributions to various stages and components of vocal learning beyond fine motor control. Although no effects of the FoxP1 knockdowns on memory or general auditory perception were observed during the experiments in this thesis, it became evident that FoxP1 also contributes to motivational behaviours in females in addition to song motor learning in juvenile male zebra finches. It remains unclear if auditory feedback perception and the rewarding qualities of tutor song are also affected by FoxP1 in juvenile males during song learning. Future studies in model organisms such as the zebra finch and further comparisons between sexually dimorphic males and females promise more insight in

the perceptual aspects of vocal learning without the overlay of vocal production. Investigations of this kind may ultimately enhance our understanding of the neurobiological basis of human speech and language.

Nederlands samenvatting

Zingen is zilver, horen is goud – De impact van lokale *FoxP1*-knockdowns op auditieve waarneming en genexpressie bij vrouwelijke zebravinken

Menselijke spraak en taal zijn unieke gedragskenmerken. Er is echter een aantal predisposities nodig om een taal te kunnen leren en spreken. Hierbij gaat het met name om vocaal leren, een eigenschap die slechts bij weinig diersoorten voorkomt. Zangvogels zijn het grootste taxon van vocaal lerende diersoorten, en vormen dus een diermodel om de principes van vocaal leren in het laboratorium te bestuderen. Onder de zangvogels zijn de zebravinken (*Taeniopygia guttata*) een veel bestudeerde soort. Er is veel bekend over hun neuronale circuit en als zangvogel bezitten zij geen gelaagde cortex, maar verschillende hersenkernen die gemakkelijk kunnen worden geïdentificeerd en dus gemanipuleerd. Ondanks dit verschil zijn de hersengebieden van zangvogels en de corticale gebieden van zoogdieren functioneel homoloog en is overlap in genexpressie aangetoond tussen de orthologen van mensen en zebravinken.

In het licht van deze functionele en transcriptionele gelijkenissen, zijn zebravinken zeer geschikt om de neuronale en moleculaire bijdragen te bestuderen aan complexe gedragingen die aan de basis liggen van vocaal leren. Juveniele mannelijke zebravinken leren hun zang van een volwassen mannelijke soortgenoot tijdens een kritische fase. Na deze fase blijft de aangeleerde zang onveranderd gedurende de rest van het leven van de vogel. In tegenstelling tot de mannetjes, leren vrouwelijke zebravinken geen zang produceren, maar onthouden ze de zang die ze vroeg in hun leven hoorden en ontwikkelen ze een voorkeur voor bekende zang ten opzichte van onbekende zang. Deze voorkeur kan aangetoond worden lang nadat de vogels volwassen zijn geworden en van hun vroegere leermeester zijn gescheiden.

Hoewel vrouwelijke zebravinken niet zingen, zijn hun hersenen vergelijkbaar met die van hun mannelijke soortgenoten. Enkele hersengebieden die verband houden met het leren van zang, zoals Area X of de robuuste kern van het arcopallium, zijn afwezig of kleiner bij vrouwtjes, maar de algemene hersenstructuur is vergelijkbaar.

Om de bijdrage van individuele genen en processen aan het leren van zang te bestuderen, moeten geschikte kandidaat-genen geïdentificeerd worden. Kandidaten

die het leren van zang waarschijnlijk beïnvloeden zijn bijvoorbeeld de forkhead-box-transcriptiefactoren van de p-familie (FOXP). Als transcriptiefactoren reguleren FOXPs de expressie van genen in het gehele genoom en kunnen zij dus een invloed hebben op diverse processen en mechanismen. Drie leden van deze familie, *FOXP1*, *FOXP2* en *FOXP4*, zijn in verband gebracht met spraak- en taalstoornissen, maar ook met andere cognitieve stoornissen waaronder autismespectrumstoornissen en intellectuele handicaps bij de mens. Interessant is dat de manipulatie van de expressieniveaus van deze transcriptiefactoren in hersengebieden die nodig zijn voor het leren van zang, het vermogen van mannelijke zebravinken om hun lied te leren van een mannelijke leermeester verandert. De invloed van FOXPs op vocale leereigenschappen bij niet-zingende vrouwtjes is nog niet onderzocht, ook al zijn de expressiepatronen van FoxP- transcriptiefactoren gelijk bij mannetjes en vrouwtjes.

Dit suggereert dat de expressie van FoxP-transcriptiefactoren ni*et al*leen gelinkt is aan het leren produceren van vocalisaties, maar ook kan bijdragen aan andere aspecten van vocaal leren, zoals geheugenvorming of auditieve discriminatie bij vrouwelijke vogels die niet leren zang te produceren. Vooral FoxP1 zou een rol kunnen spelen bij het leren van zang bij mannetjes en vrouwtjes, omdat dit gen bij jonge en volwassen vogels van beide geslachten opgereguleerd is in de hersengebieden die secundaire auditieve gebieden zijn of die auditieve informatie ontvangen van andere gebieden. Twee van deze gebieden zijn van speciaal belang vanwege hun functionele implicaties en verhoogde niveaus van FoxP1-expressie. Het gaat om HVC (eigennaam) en het caudomediale mesopallium (CMM). HVC is een premotorisch gebied dat de zangproductie regelt bij mannelijke zebravinken, maar bijvoorbeeld ook invloed heeft op copulatieverzoek of zangdiscriminatie bij vrouwelijke zangvogels. CMM is een secundair auditief gebied dat geactiveerd wordt door bekende zang en dat auditieve informatie verwerkt vooraleer die geprojecteerd wordt, bijvoorbeeld naar HVC.

Het doel van deze studie was om de functie van FoxP1 voor auditief geheugen, auditieve discriminatie en auditieve categorisatie te onderzoeken in de hersengebieden HVC en CMM van vrouwelijke zebravinken. Er werden lokale lentivirale knockdowns op basis van short hairpin RNAs geïnduceerd in juveniele of volwassen vrouwelijke zebravinken om de functionele implicaties van *FoxP1* vast te stellen tijdens vroege ontwikkelingsstadia, bijvoorbeeld wanneer de voorkeur voor bepaalde zang ontstaat en na afloop van deze kritieke periode bij volwassenen. Na de

beoordeling van gedragsverschillen aan de hand van operante voorkeurs- en Go/Nogo-taken, werd totaal RNA geëxtraheerd uit de doelgebieden om de succesvolle knockdown en differentieel gereguleerde genen, paden en netwerken na *FoxP1*-expressie-veranderingen te valideren.

Tijdens voorkeurstesten werd het duidelijk dat alle vrouwtjes, ongeacht hun leeftijd tijdens de knockdown of het doelgebied, de voorkeur gaven aan de zang van hun leermeester ten opzichte van onbekende zang. Vrouwtjes die als volwassene een knockdown van *FoxP1* in HVC kregen, vroegen echter minder om playbacks van beide typen zang en hadden een zwakkere voorkeur voor het bekende lied in vergelijking met hun controlegroep.

In Go/Nogo-taken werden de vrouwtjes getraind om verschillend te reageren op twee verschillende, onbekende soort-specifieke zangmotieven. Zodra de vrouwtjes geleerd hadden om afhankelijk van de stimulus-ID te reageren of niet te reageren, werden nieuwe stimuli geïntroduceerd die afgeleid waren van de oorspronkelijke trainingsgeluiden. De nieuwe stimuli werden gemanipuleerd op het gebied van de opeenvolging van syllaben, de toonhoogte of de spectrale structuur door het volledig omkeren van de playback. Tijdens de Go/Nogo-taken konden geen knockdown-gerelateerde afwijkingen worden vastgesteld. Leersnelheid, stimulusdiscriminatie of categorisatie van gemodificeerde stimuli verschilden niet tussen de controles en knockdowns. Daarom werden de gegevens van alle vogels samengevoegd in één grote groep om één omvangrijke dataset te verkrijgen met betrekking tot het vermogen van vrouwelijke zebravinken om verschillend gemanipuleerde stimuli te categoriseren op basis van eerdere discriminatietraining.

Vrouwelijke zebravinken scoorden het best bij stimuli met een veranderde syllabevolgorde en het slechtst bij stimuli die in omgekeerde volgorde werden afgespeeld. De vogels scoorden gemiddeld bij stimuli waarbij h*et al*gemene toonhoogteniveau was verhoogd of verlaagd. Dit suggereert dat voor vrouwelijke zebravinken de spectrale structuur en informatie in de zang van soortgenoten van groter belang zijn dan de sequentiële informatie gecodeerd in de syllabevolgorde.

Na de gedragsexperimenten werd de virale injectieplaats gevalideerd door immunohistochemie en werd het knockdown-effect gekwantificeerd met qPCR en RNAseq-analyses. Differentiële genexpressie op basis van totaal RNA-extracten

bracht ook genen en pathways aan het licht die eerder zijn geïdentificeerd in mensen met taalstoornissen. Daarnaast werden routes die eerder geassocieerd zijn met *FOXP2* verrijkt als gevolg van *FoxP1*-knockdowns. Dit is waarschijnlijk het gevolg van de dimerisatie van FoxP1 en FoxP2 en de daaropvolgende regulatie van genexpressie als een eiwitcomplex.

Hoewel gedragsveranderingen alleen konden worden vastgesteld bij vogels die als volwassen dieren waren behandeld in HVC, overlappen de gereguleerde genen, verrijkte paden en netwerken bij alle groepen die in deze studie zijn getest. Menselijke orthologen van specifieke genen en pathways waaraan zij bijdragen, zijn in verband gebracht met taalfenotypes bij mensen en suggereren dus dat *FoxP1* bij niet-zingende vrouwtjes invloed zou kunnen hebben op eigenschappen gerelateerd aan vocaal leren die niet uitsluitend nodig zijn voor het leren produceren van vocalisaties, maar ook betrokken zijn bij auditieve perceptie en auditief geheugen.

Samenvattend toont deze studie aan dat de gevolgen van lokale *FoxP1*-knockdowns in vrouwelijke zebravinken sterk variëren tussen vogels die verschillende leeftijden hadden tijdens de behandeling en tussen vogels waarbij verschillende gebieden behandeld zijn, maar ook dat de gevolgen in genexpressie vergelijkbaar zijn met de gevolgen bij individuen die vocalisaties leren produceren, wat suggereert dat de rol van *FoxP1* aan de basis van vocaal leren niet beperkt is tot het leren produceren van vocalisaties.

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Curriculum vitae

Fabian Heim was born in 1989 in Bad Mergentheim, Germany. He received his Abitur in 2008 at the Gymnasium Weikersheim. Following school, he studied Biology at the Eberhard Karls Universität Tübingen and obtained a Bachelor's degree in 2011. After that he moved to the University of Hohenheim where he completed his Master's degree with a major in zoology in 2014.

His Bachelor's thesis focused on the *Evolution and Genetics of Pristionchus pacificus' Hunting Behaviour* at the Max-Planck Institute for Developmental Biology in the department for Integrative Evolutionary Biology of Prof. Dr. Ralf Sommer. During his Master's, he worked as a field assistant with Blue Tits in German forests and arctic shorebirds in Alaska, USA, two projects in Prof. Dr. Bart Kempenaers' department at the Max-Planck Institute for Ornithology in Seewiesen, Germany. His Master's thesis on the *Analysis of dsRNA induced LTM-inhibition using CREB in Nasonia vitripennis (Hymenoptera: Pteromalidae)* was awarded as the Best Master's Thesis of the Agriculture and Science faculty at the University of Hohenheim in 2015.

After graduating in 2014, he began his PhD research at Leiden University in the Netherlands. He was supervised by his (co)promotores, Prof. Dr. Carel ten Cate and Dr. Katharina Riebel at Leiden University, Prof. Dr. Simon Fisher of the Language and Genetics department at the Max-Planck Institute for Psycholinguistics in Nijmegen, and Prof. Constance Scharff, PhD, at the Freie Universität Berlin, Germany. During his PhD he was trained in a multitude of methods by experts in their respective fields in the labs of his supervisors which he could frequently visit. His PhD research focused on the role of *FoxP1* expression in specific brain areas of female zebra finches for auditory perception and gene expression.

Currently he is working as a Postdoctoral scientist in the research group Neural circuits for vocal communication of Dr. Daniela Vallentin at the Max-Planck Institute for Ornithology in Seewiesen, Germany.