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

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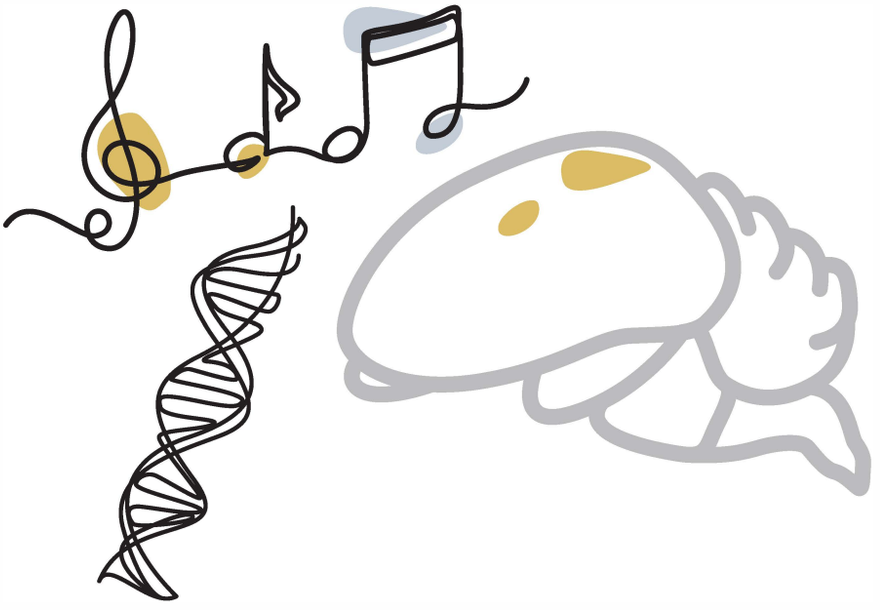
Chapter 5

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.