THE ROLE OF FOXP2 IN STRIATAL CIRCUITRY

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ISBN
978-94-6284-179-6

Design/lay-out
Bregje Jaspers, ProefschriftOntwerp.nl, Nijmegen

Print
ProefschriftMaken, www.proefschriftmaken.nl

THE ROLE OF FOXP2 IN STRIATAL CIRCUITRY

Proefschrift
ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op

donderdag 24 januari 2019

om 12:30 uur precies

door

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geboren op 25 november 1990

te Leiden

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Doctoral Thesis
to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on

Thursday, January 24, 2019,

at 12:30 hours

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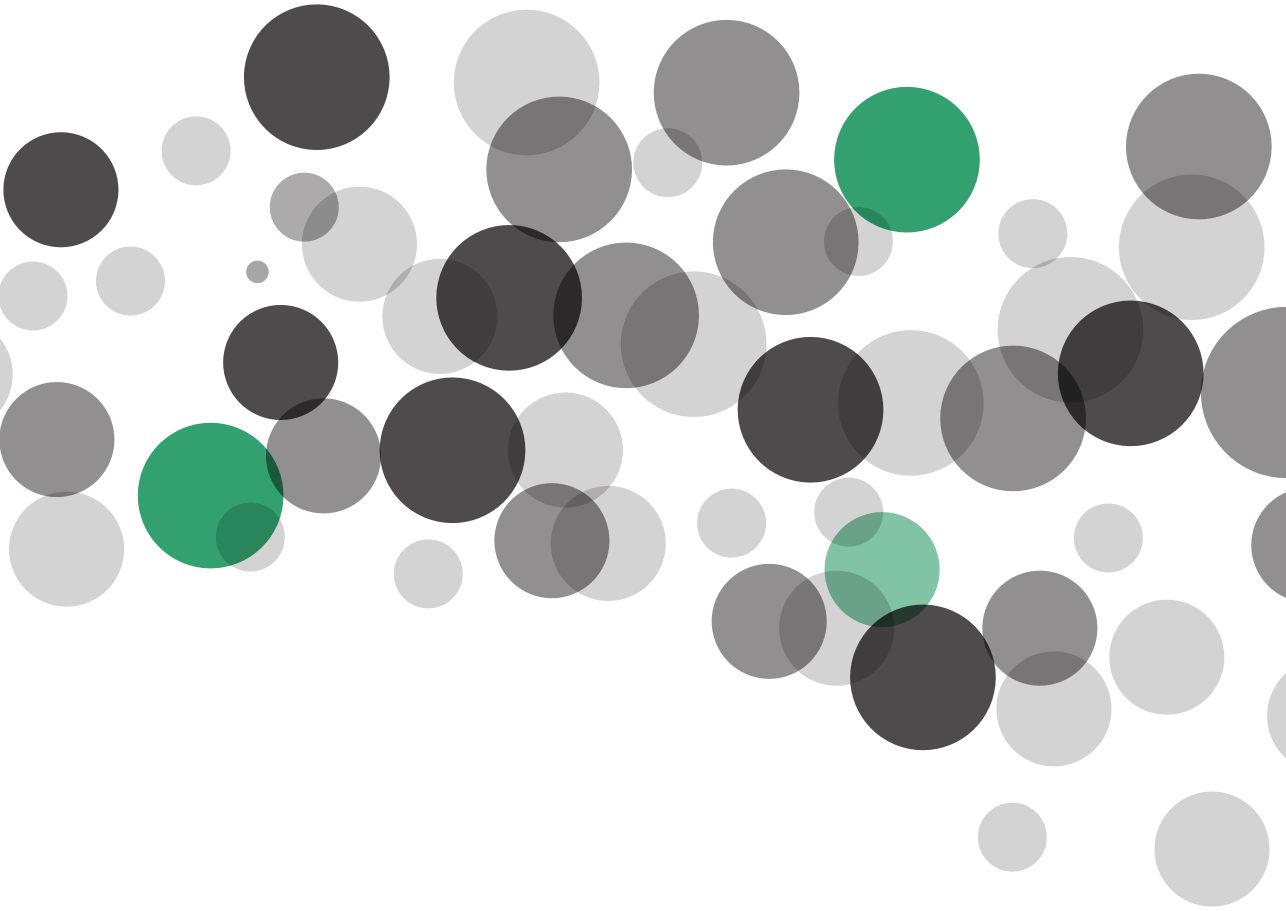
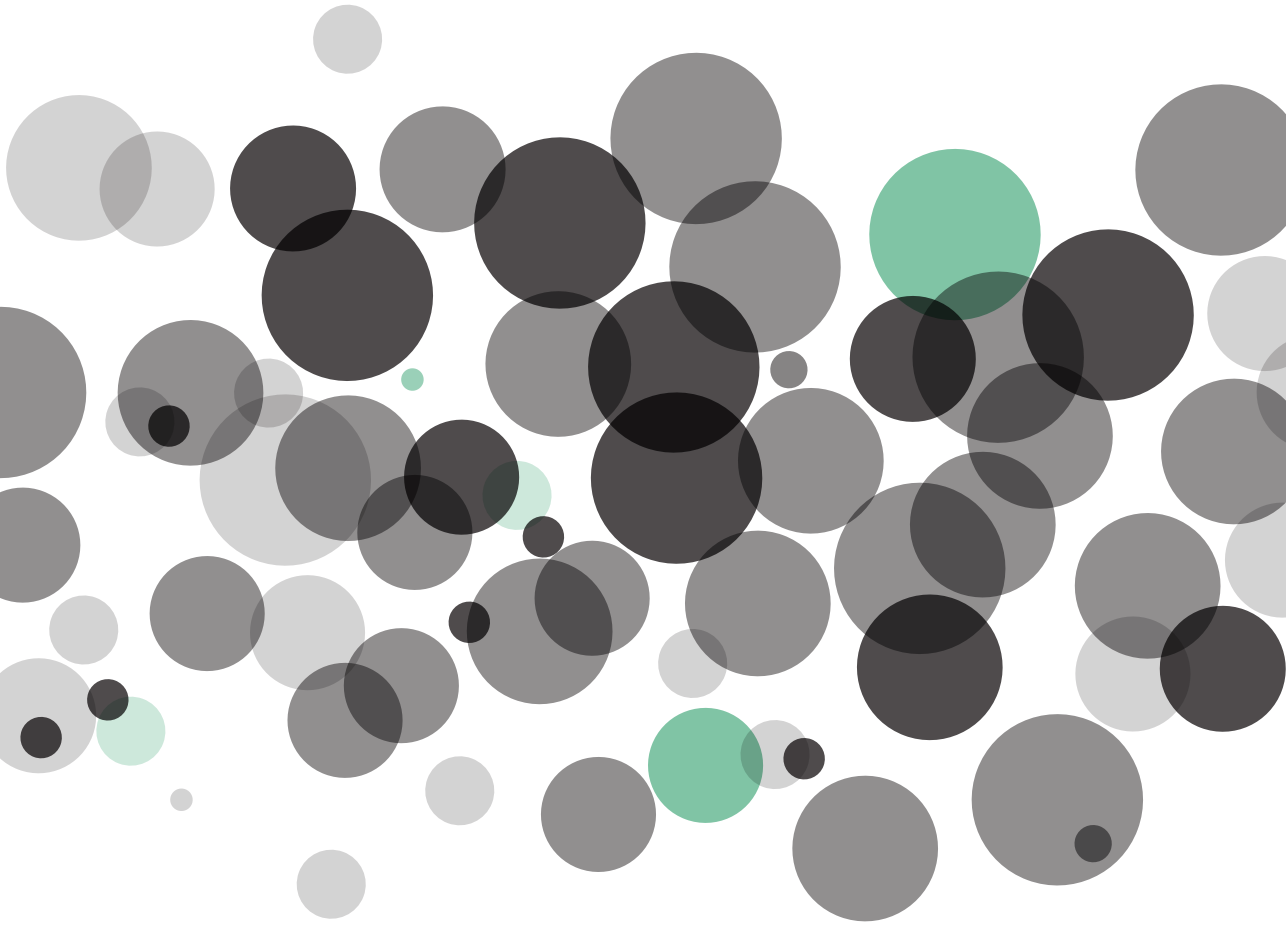


1.

Introduction



1.1 UNDERSTANDING COMPLEX MOTOR CONTROL IN THE BRAIN

Already since the earliest endeavors to study the brain, researchers have been fascinated by how activity in the brain enables us to interact with the world around us. For example, more than a century ago it was found that disruption of the afferent nerve leads to impairments in limb use (Mott, 1895). Studies on human participants have shown that movement is encoded by the brain, as it can be initiated by stimulation of certain brain areas (Penfield, 1937). The recent advances in brain imaging methods have enabled researchers to investigate the encoding of movement in the brain in high detail. In turn, this has led to investigation of the encoding of more complex movements in the brain. For example, a comprehensive assessment of brain regions involved in the movement of facial muscles has only been conducted recently (Krippl et al., 2015). The ability to produce speech and spoken language requires a high degree of control over the activation of facial musculature (Redle et al., 2015). Multiple brain regions involved in complex motor control, such as motor cortex, caudate/putamen and substantia nigra are crucial for speech motor control (Wildgruber et al., 2001), and are highly active when participants perform spoken language tasks (Gracco et al., 2005; Hervais-Adelman et al., 2015). Furthermore, in disorders which disrupt speech and spoken language skills, activity within these regions is affected (for review see (Liegeois et al., 2014)). Thus, detailed investigation of brain circuits involved in complex motor sequencing can reveal how human spoken language might be encoded in the brain.

It is known that disorders which impair basal ganglia circuit function result in impairments of complex motor sequencing and speech difficulties in humans, as well as deficits in complex motor sequencing in animal species such as fruitflies, mice and songbirds. In some cases, these disorders may have a monogenic origin, where heterozygous mutation leads to impairments in speech sequencing, accompanied by deficits in expressive and receptive language. Such monogenic disorders, while rare, can provide important avenues to investigate how impaired circuit function can result in behavioral impairments. One of these monogenic causes for impaired motor circuit function, which leads to a very recognizable phenotype, is FOXP2[1]-associated childhood apraxia of speech. In this chapter we will introduce the concepts explored in the different thesis chapters, and explain why FoxP2 is an ideal entry point to investigate how circuit dysfunction in the brain can lead to impairments in complex motor sequencing and speech / spoken language difficulties.

1 Throughout the thesis we will use the standard accepted nomenclature to refer to the gene/protein in different species: *FOXP2*/FOXP2 refers to humans, *Foxp2*/Foxp2 refers to mice and *FoxP2*/FoxP2 refers to other chordates.
Kaestner, K.H., Knochel, W., and Martinez, D.E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. Genes & development *14*, 142-146..

1.2.1 Brain circuits in complex motor sequencing behavior and speech production

The uniquely human ability to generate spoken language is one of the most complex movement paradigms in nature. Groups of muscles in the face, larynx (Ziegler and Ackermann, 2013) and tongue (Sanders et al., 2013) must work in concert to produce coherent speech patterns. Fluent production of speech reaches a speed of, on average, 5 syllables per second (Baese-Berk and Morrill, 2015), which necessitates fast coordination of activation and inactivation between these muscle groups. This is different from non-speech oral tasks, which generally are less complex and require lower spatiotemporal coordination of orofacial musculature (Bunton, 2008). In order to understand how such complex motor sequences necessary for speech are generated, we can explore how movement in general is encoded in the brain. Movement, and especially the generation of complex sequences of movement, is governed by the cortico-striato-thalamic circuitry (Doyon et al., 2003). Within this circuitry, movement is initiated in the cortex and activity is sent along downward projections toward the striatum, which in humans consists of the caudate and the putamen. Corticostriatal connections terminate on two major cell classes in the striatum: medium spiny neurons (MSNs) of the striatonigral direct pathway, and MSNs of the striatopallidal indirect pathway. The separation between these pathways and their involvement in the modulation of activity plays a key role in motor control and motor sequence learning (Calabresi et al., 2014). Regulation of striatal activity in these two projection pathways is thought to be highly important for complex motor sequence learning and execution of complex motor tasks, including the production of spoken language (Gracco et al., 2005; Simonyan et al., 2016).

1.2.2 The cortico-striato-thalamic circuit

Corticostriatal connections are excitatory and activate neurons of both the direct and indirect pathway (Shepherd, 2013). These two striatal cell classes can be distinguished by unique protein expression in addition to their downstream projection patterns (Gittis and Kreitzer, 2012). Direct pathway striatal MSNs express the dopamine 1 receptor (D1R), substance P and dynorphin (D1R-MSNs). By contrast, the indirect pathway MSNs express the dopamine 2 receptor (D2R), enkephalin and A2a adenosine receptors (D2R-MSNs). All striatal MSNs are GABAergic and send inhibitory projections to downstream targets, which ultimately affects activity in the substantia nigra (SNr) (Figure 1a). The direct projection of the D1R-MSNs inhibits the substantia nigra. By contrast, the striatopallidal projections of the D2R-MSNs inhibit the globus pallidus and subthalamic nucleus. Projections from these areas normally inhibit the SNr as well. However, indirect pathway activity modulates the inhibitory drive from these regions by increasing inhibition within the globus pallidus and STN. This way, increased D2R-MSN activity leads to disinhibition of the SNr. Additionally, MSNs are also able to regulate activity within the striatum, as D1R-MSNs and D2R-MSNs project unidirectionally to other D1R-MSNs or DR2-MSNs, respectively (Taverna et al., 2008). Increased activity in D1R- or D2R-MSNs therefore results in a negative feedback loop, which

affects the downstream inhibitory drive of the striatum (Saklayen et al., 2004). From the SNr, inhibitory projections are sent toward the thalamus, which ultimately affect thalamocortical drive. Activity within the entire cortico-striato-thalamic circuit can therefore be affected by changes in activity and downstream targeting of the striatum. This in turn directly affects motor activity within the cortex and motor behavior (Kravitz and Kreitzer, 2012). Activation of the direct pathway is necessary for the initiation of movement as well as the maintenance of movement paradigms and reinforcement of motor sequence learning. By contrast, activation of the indirect pathway governs switching of motor paradigms as well as the learning of novel motor sequences (Bateup et al., 2010). Concurrent activation of the direct and indirect pathway has been shown to occur *in vivo* when movement is initiated (Cui et al., 2013), which suggests tight regulation of activity within and between these pathways is essential for complex locomotion.

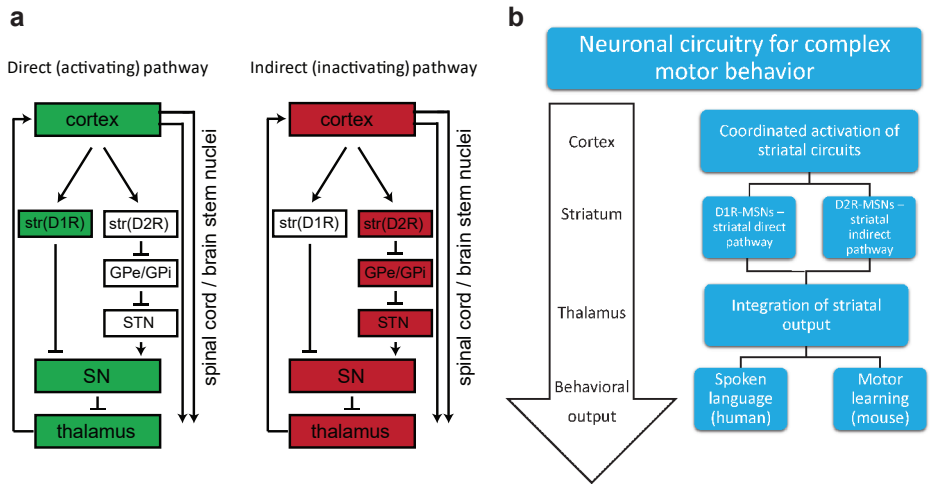


Figure 1: Pathways involved in the generation of coordinated movement.

(a) Two pathways can be distinguished at the level of the striatum and balanced activation of these pathways is necessary to generate complex motor circuitry output necessary for motor sequencing behavior. (b) Different brain circuits govern unique aspects of coordinated locomotion, which have to work in concert to result in the generation of complex behavioral output such as motor learning or spoken language.

1.2.3 Complex motor sequencing is affected often in disorders which affect cognitive function

Disruption of activity within the direct or the indirect pathway has been implicated in disorders where (complex) motor sequencing is affected. Neurodegenerative disorders such as Parkinson's disease (PD) or Huntington's disease (HD) lead to significant reductions in motor function, and areas such as the striatum and substantia nigra are among those most

significantly affected by PD or HD (Huot et al., 2007; Przedborski, 2005). Another aspect of disrupted motor sequencing which is often seen in individuals with autism spectrum disorder (ASD) or Tourette's syndrome is motor stereotypy. These stereotypies are described as rhythmic and repetitive movements without a clear purpose, and include hand flapping, arm waving, and rocking back and forth. Aberrant striatal morphology (Hollander et al., 2005) and disrupted activation of striatal circuits have been the focus of recent investigation into motor stereotypies (for review, see: (Peter et al., 2017). For example, in Tourette's syndrome, it is thought that aberrant activation of small groups of striatal MSNs could be responsible for stereotypical movement generation (Gittis and Kreitzer, 2012). Additionally, dysfunction of the autism-associated gene Neuroligin-3 (*NL-3*) (Levy et al., 2011; Sanders et al., 2011) has been shown to cause motor stereotypy in a mouse model with heterozygous *nl-3* knockout (Rothwell et al., 2014). However, individuals affected by ASD, Tourette's or PD often show cognitive dysfunction in addition to their motor phenotypes (Beitz, 2014; Cavanna and Seri, 2013; Payakachat et al., 2012). The effects of neurodevelopmental and neurodegenerative disorders on cognitive ability complicate investigation into the specifics of how motor sequencing and motor behaviors are encoded. A disorder in which only motor-circuit associated behaviors are affected would be an ideal tool to enable detailed investigation into striatum specific effects on motor behavior.

1.2.4 Speech as a complex motor sequencing challenge

The active vocalization of speech during spoken language production is a highly complex motor task, and can be described as the combination of vocalizations and muscle manipulations necessary to convey intelligible speech. For much of the research in this area, comprehension and production of spoken language have been thought of as intertwined processes (Macdonald, 2013). Traditional views focusing on classically defined speech comprehension and production centers such as Broca and Wernicke areas (Jurgens, 2002), have developed into contemporary cognitive models which recognize that spoken language depends on distributed circuits involving multiple additional brain regions (Tremblay and Dick, 2016). Undoubtedly the ability to produce meaningful speech requires an internal lexicon combined with an understanding of grammar and sentence structure (Giraudo and Dal Maso, 2016; Libben and Jarema, 2002; Marslen-Wilson and Warren, 1994). However, the complex motor sequencing aspects of speech production are probably not governed by such higher cognitive processes. The production of spoken language also involves the mechanical process of forming coherent motor patterns by the underlying facial musculature (Ziegler and Ackermann, 2013). This process could be governed by the same (or similar) pathways involved in the production of complex motor sequences in the brain, thus regulation of striatal activity could be required (Ackermann et al., 2014). Disentangling the cognitive and motor processes which are involved in the production of spoken language is an intriguing issue in the field of

language sciences. Investigation of neuronal pathways involved in the production of complex motor sequences can help understand how such aspects of speech production are encoded

1.3.1 FOXP2 and spoken language production

Impairments in speech production have been described in neurodevelopmental disorders (Koolen and de Vries, 1993; Mei et al., 2018; Morgan et al., 2018; Rodenas-Cuadrado et al., 2014; Rodenas-Cuadrado et al., 2016; Sia et al., 2013; Snijders Blok et al., 2018). Especially genetic disruptions which affect spoken language production are highly interesting as they provide a molecular stepping stone toward understanding the pathophysiology of speech impairment. In many neurodevelopmental disorders, speech impairment is part of a larger phenotype which includes ASD and intellectual disability. There are however individuals in which speech and/or language impairments may be seen without other developmental delays. Prominent forms of these disorders include specific language impairment (SLI, also known as developmental language disorder, DLD) and childhood apraxia of speech (CAS) (Bishop, 2006; Chen et al., 2017b; Reilly et al., 2014). The familial aggregation of developmental speech and language disorders suggests a genetic origin (Gopnik and Crago, 1991; Tallal et al., 2001; Tallal et al., 1989; Whitehouse, 2010). Currently, only a few genes associated with developmental speech and language disorders are known (Reader et al., 2014). Investigation of a familial case of CAS (the KE family) led to the identification of the transcription factor Forkhead box-protein 2 (FOXP2) as being extensively involved in speech production (Lai et al., 2001).

Individuals with heterozygous mutations in the gene *FOXP2* are affected by CAS. Their language production is severely impaired, especially in complex word production (Lai et al., 2001; MacDermot et al., 2005; Morgan et al., 2017; Watkins et al., 2002a). Although proficiency can be improved with intensive speech therapy, underlying problems remain present. CAS is described as 'a disorder of speech motor programming or planning that affects the production, sequencing, timing and stress of sounds' (Morgan et al., 2017). In people with heterozygous *FOXP2* mutations, CAS is the most prominent feature, but it is accompanied by wide-ranging impairments in expressive and receptive language, affecting spoken and written modalities (Morgan et al., 2017; Watkins et al., 2002a). Given that the phenotype associated with CAS suggests underlying impairments in the production of complex motor sequences, it is plausible that activity within the cortico-striato-thalamic circuitry might be altered in affected individuals. As mutations in *FOXP2* have been linked to CAS in multiple cases, investigation into how FOXP2 regulates neuronal cellular and circuit function can help us understand how the human brain enables production of proficient speech.

1.3.2 Mutations of *FOXP2*

In the KE family, a single nucleotide change within the gene *FOXP2* is thought to be responsible for the CAS in affected individuals (Lai et al., 2001) FoxP2 belongs to the Forkhead box family of transcription factors. Within this large group of proteins, the FoxP subfamily consists of 4 different proteins, of which only FoxP3 is not expressed in the brain (Lai et al., 2003; Takahashi et al., 2008a; Tamura et al., 2003). FoxP1, 2 and 4 are all localized to the nucleus (Li et al., 2004a; Spiteri et al., 2007; Vernes et al., 2006). Compared to *FOXP2*, mutations in *FOXP1* and *FOXP4* lead to different phenotypes. Mutation of *FOXP1* results in a more severe neurodevelopmental deficit with global developmental delay, ID and ASD, in addition to speech and language impairments (Bacon and Rappold, 2012; Bowers and Konopka, 2012; Hamdan et al., 2010; Sollis et al., 2017; Sollis et al., 2016). This suggests FOXP1 has a more essential role in brain development and function compared to FOXP2. A single case of heterozygous mutation of *FOXP4* has currently been described, in which the affected individual shows developmental delay as well as motor problems (Charng et al., 2016). However, in mouse models the function of FOXP4 has mainly been investigated in other regions than the brain. Homozygous knockout of *FOXP4* in mice leads to abnormal cardiac development and early embryonal death (Li et al., 2004b). Though brain expression of FOXP4 has been shown, it's function in the brain currently remains unresolved (Takahashi et al., 2008a; Teufel et al., 2003). Since the initial discovery of a *FOXP2* mutation responsible for CAS in the KE family, multiple independent mutations disrupting *FOXP2* in other cases/families have been described, all of which result in CAS (for review, see (Morgan et al., 2017)). Two disruptions of *FOXP2* which have received most attention. These are the R553H mutation found in the KE family, and the R328X mutation found cosegregating with CAS in the AD family (MacDermot et al., 2005). The R553H mutation affects the DNA-binding domain of *FOXP2* (Lai et al., 2001; Vernes et al., 2006), whereas the R328X mutation introduces an early stop codon in exon 7, yielding protein truncation and nonsense mediated RNA decay (Groszer et al., 2008; MacDermot et al., 2005). Mutation of *FOXP2* has been related to alterations in brain morphology and activity in individuals of the KE family. MRI comparisons between affected family members, unaffected siblings and controls show that grey matter density is reduced in the parts of the cortex, caudate/putamen and thalamus, which are all part of the canonical motor circuit (Watkins et al., 2002b). Furthermore, when participants perform a language task and activity is measured by fMRI, activity in the multiple regions of the canonical motor circuit is decreased in affected individuals of the KE family (Liegeois et al., 2003). This suggests that FOXP2 affects brain morphology and function in areas relevant for complex motor sequencing and speech production.

1.3.3 Evolutionary conservation of FoxP2 and face validity of animal models

FoxP2 is highly conserved throughout mammalian evolution, especially with regard to the sequence of the encoded protein (Campbell et al., 2009; Enard et al., 2002). Between humans and chimpanzees, two amino acid substitutions within exon 7 have been described (T303N and N325S). Only a single additional amino acid substitution (D80E) differentiates mouse *Foxp2* from chimpanzee *FoxP2* (Figure 2).

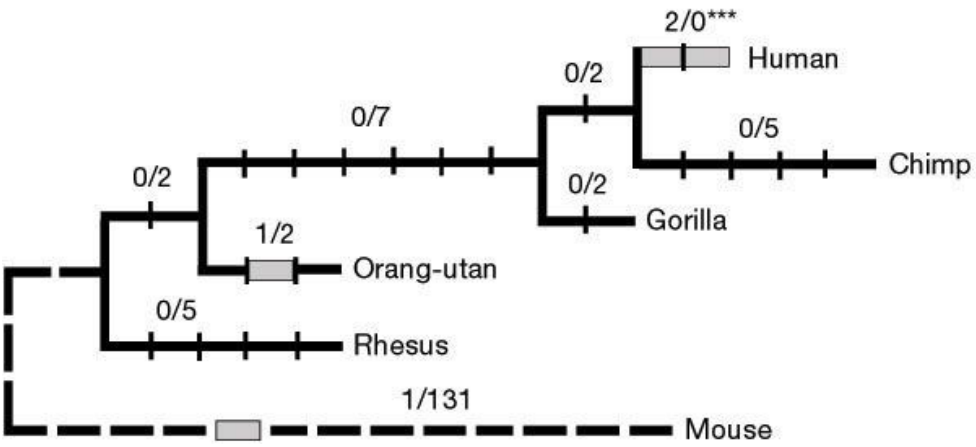


Figure 2: Conservation of Foxp2 between mouse and primates (From (Enard et al., 2002)).

FoxP2 is highly conserved between distantly related species, such as mouse, primates and humans. Black vertical bars denote both nucleotide changes. Grey squares show amino-acid changes.

Furthermore, *FoxP2* in other species shows remarkable overlap with mammalian *FoxP2* either in amino acid sequence, proposed function, or both. For example, the DNA-binding domain of Foxp2 is identical between songbirds and humans, and dysfunction in songbirds leads to impaired song production (Haesler et al., 2007). Even distant analogues of *FoxP2* seem to affect motor function. In *Drosophila melanogaster* (the common fruit fly) FoxP is the only member of the FoxP family (Lawton et al., 2014), and disruption of FoxP expression leads to aberrant coordinated movement (Mendoza et al., 2014), as well as disrupted cognitive function (DasGupta et al., 2014). Since speech production is a highly complex motor sequencing task (Ackermann et al., 2014), functional specialization of FOXP2 and its putative roles in regulation of motor circuit activity might have contributed to the development of spoken language on the human lineage (Enard, 2011). Nonetheless, complex motor sequence impairments are a common phenotype shared between different species with dysfunctions in *FoxP2.* This suggests that FoxP2 similarly affects brain function between species, including species without speech or even without vocalizations. This is an example of deep homology

in biology and it lends face validity to the use of different animal models to understand how FoxP2 influences brain activity and motor behaviors.

1.3.4 FoxP2 expression is restricted in animal and human brain

As *FoxP2* is essential in motor circuit function, we might expect the expression of this gene to be prevalent in neuronal circuits and neurons embedded within the motor circuits. The motor circuit is grossly similar between mammalian species, but markedly different between mammals, songbirds and insects. Intriguingly however, FoxP (for *Drosophila*) and FoxP2 are in all species that have currently been investigated expressed in neuronal structures known to be involved in locomotion. In Drosophila, FoxP expression is highly localized and especially high in the protocerebral bridge. This is a section of the insect brain central complex and is thought to be analogous to mammalian basal ganglia (Lawton et al., 2014). In songbirds FoxP2 expression is prevalent in the striatum, and highly expressed in the striatal vocal nucleus area X (Haesler et al., 2004; Teramitsu et al., 2004). Furthermore, FoxP2 expression in area X is dependent on singing behavior during development and in adulthood and changes in Foxp2 expression in area X affect the quality and stability of birdsong (Murugan et al., 2013; Rochefort et al., 2007; Teramitsu and White, 2006). In mammals, FoxP2 expression is high in many regions of cortico-striato-thalamic motor circuit, as well as in the cerebellum. In humans, FOXP2 expression is confirmed in the cortex, striatum, thalamus and cerebellum (Ferland et al., 2003; Lai et al., 2003; Teramitsu et al., 2004), with especially high expression in the caudate and putamen in humans and nonhuman primates (Lai et al., 2003; Takahashi et al., 2008b). This confirms that FOXP2 is well placed to impact on neuronal function in human motor circuits. Furthermore, expression patterns of Foxp2 have been assessed especially in detail in mice. Here, Foxp2 is highly expressed in deeper layers of the cortex. Layer VI shows Foxp2 expression throughout the cortex, whilst in layer V Foxp2 is highly expressed in the motor cortex and parts of the somatosensory cortex (Hisaoka et al., 2010). The excitatory neurons in these layers mainly identify as corticothalamic and corticostriatal projection neurons, and Foxp2 is suggested to have functions in regulation of activity in these neurons (Sia et al., 2013). As noted above, Foxp2 expression is notably high in the striatum, where it is primarily localized to D1R-MSNs (Fong et al., 2018; van Rhijn et al., 2018; Vernes et al., 2011). Foxp2 is typically absent from interneurons, both in cortex and striatum (Fong et al., 2018; Haesler et al., 2007; Hisaoka et al., 2010). The restricted expression of Foxp2 to D1R-MSNs suggests that this transcription factor might regulate striatal activity in a pathway specific manner. Furthermore, Foxp2 is also expressed in the substantia nigra, thalamus, and multiple subthalamic regions. Currently, it is not known if Foxp2 is expressed within specific cell populations in these areas or if Foxp2 is able to affect activity within these regions. Lastly, in the cerebellum Foxp2 is localized to Purkinje cells and deep cerebellar nuclei (Ferland et al., 2003; Fujita and Sugihara, 2012), and it has been shown that mutations of *Foxp2* affect cerebellar activity (French et al., 2018; Groszer et al., 2008). Though the cerebellum is not

part of the canonical motor circuit, it is involved in motor function, especially in coordination and smooth movement control (Manto et al., 2012; Zhang et al., 2014). Figure 3 shows an overview of Foxp2 expression in the mouse brain and how the relevant parts of the motor circuit are interconnected. The expression of Foxp2 in the cerebellum suggests Foxp2 might be involved in other aspects of motor control which are not governed by the canonical motor circuit. Combined, the high conservation of Foxp2 expression throughout mammalian evolution and the nature of this conserved expression pattern suggest that it has essential functions in the motor circuit.

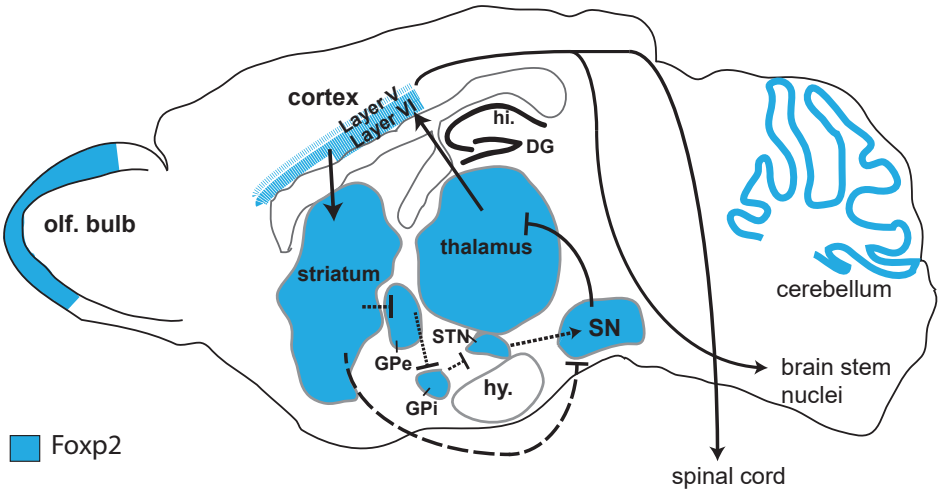


Figure 3: Foxp2 expression in mouse brain.

Also highlighted are the pathways described in Figure 1, which underlines the importance of both Foxp2 and the striatum within the cortico-striato-thalamic motor circuit.

1.4.1 Investigating Foxp2 function in cell and animal models

The previously described data from human MRI and fMRI studies shows that mutations in *FOX2* affect brain morphology and activity. To further study the mechanisms by which this gene affects brain function, animal models have been used in which FoxP2 function is affected. For example, multiple mouse models have been developed with mutations in *Foxp2* (French and Fisher, 2014). Two mouse models of disrupted Foxp2 function are of particular interest: mice with the R552H missense mutation, which equivalent to the human DNA-binding domain R553H mutation and mice with the S321X nonsense mutation, which is considered analogous to the loss of function R328X mutation found in the AD family (Groszer

et al., 2008). Furthermore, mice in which Foxp2 can be conditionally knocked out in specific cell populations have been developed, and these can be used to investigate brain region specific functions of Foxp2 (French et al., 2007). Lastly, mice have been developed in which the human specific T303N and N325S amino-acid substitutions have been introduced into mouse *Foxp2* (Enard et al., 2009). In songbirds, manipulation of FoxP2 expression has been achieved through ShRNA mediated interference (Haesler et al., 2007; Murugan et al., 2013). Mouse and songbird models of Foxp2 dysfunction have been used routinely to investigate how Foxp2 functions as a transcription factor, if mutation of *Foxp2* affects motor circuit function and whether this results in changes in motor and vocal behaviors in these animals.

1.4.2 FoxP2 regulates genes involved in synapse development and neuronal activity

As FoxP2 is a nuclear transcription factor, it has the possibility to regulate a plethora of genes and regulatory processes (Spiteri et al., 2007; Vernes et al., 2006; Vernes et al., 2011; Vernes et al., 2007). Chromatin immunoprecipitation followed by promoter microarray study (ChIP-chip) shows that Foxp2 is able to directly regulate expression of proteins involved in neurodevelopment (Spiteri et al., 2007; Vernes et al., 2011) as well as possibly proteins involved in synaptic activity (Vernes et al., 2011). In both songbird and mouse, dysregulation of FoxP2 function has been shown to affect neurodevelopment and synapse formation (Schulz et al., 2010; Vernes et al., 2011). Furthermore, it has been shown that Foxp2 regulates expression of genes involved in synapse maturation, and as such it seems that Foxp2 is an upstream regulator of neurodevelopmental processes. For example, Foxp2 regulates the expression of the Sushi Repeat-containing Protein X-lined 2 (SRPX2), the Myocyte Enhancer Factor 2C (MEF2C) and the Retinoic Acid receptor Beta (RARβ) (Chen et al., 2016; Devanna et al., 2014; Roll et al., 2010; Sia et al., 2013). Foxp2 normally represses the expression of MEF2C and SRPX2, whilst Foxp2 promotes RARβ expression. Introduction of the R552H mutation into rat primary cortical neurons leads to a loss of SRPX2 repression and increased generation of excitatory synapses (Sia et al., 2013). Heterozygous knockout of *Foxp2* leads to increased expression of MEF2C, which acts as a brake on corticostriatal synapse formation (Chen et al., 2016). Furthermore, a downstream effect of the dysregulation of MEF2C expression following *Foxp2* heterozygous knockout is a reduction in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) expression at the synapse (Chen et al., 2016). Lastly, *in vitro* cell models show that FOXP2 overexpression increases RARβ expression and leads to increased neurite outgrowth (Devanna et al., 2014). Both RARα and RARβ are highly expressed in mouse brain, but they show distinct expression patterns. There are multiple regions where expression of both overlaps, however, whilst RARβ is highly expressed in the striatum, RARα is absent from this region (Dolle, 2009; van Rhijn and Vernes, 2015). For both RARα and RARβ it is known that they affect synaptic plasticity (Chiang et al., 1998; Sarti et al., 2012). Blockade of synaptic transmission in cultured hippocampal neurons leads to increased GluA1 AMPAR subunit expression through a RARα dependent mechanism (Aoto et

al., 2008; Chen et al., 2014). RARα mediated synaptic scaling requires RARα expression at the synapse, whereas canonically RARs are known as nuclear transcription factors (Germain et al., 2006; Huang et al., 2014; Zhang et al., 2015b). Currently, it is unknown whether RARβ can modulate synaptic plasticity through synaptic mechanisms, as synaptic expression of RARβ has not been established. In RARβ knockout mice, induction of long-term depression (LTD) is impaired. However, this lack of LTD induction doesn't seem to be due to changes in pre- or postsynaptic expression of proteins involved in neurotransmission (Chiang et al., 1998). As FoxP2 regulates RARβ expression and LTD induction is impaired in mice with both the R552H and the S321X mutation (Groszer et al., 2008; van Rhijn et al., Chapter 3), establishing the relationship between FoxP2, RARβ and LTD could reveal whether the functions of RARβ in neuronal plasticity might depend on FoxP2.

1.4.3 Neuronal activity is altered by disruption of FoxP2 function

Multiple aspects of neuronal activity and plasticity are affected by mutations in *Foxp2*. As noted above, LTD I the dorsolateral striatum is impaired in mice with heterozygous R552H or S321X mutations of *Foxp2.* Furthermore, LTD is affected as well in mice in which the human lineage specific changes (T303N and N325S) to *Foxp2* have been introduced (Schreiweis et al., 2014). Interestingly, a subregion specific effect was observed. LTD in the dorsolateral striatum was increased, whilst dorsomedial LTD was reduced in these mice, which suggests Foxp2 might have striatal subregion dependent functionality. In addition, evidence from both heterozygous *Foxp2* mutant mice as well as the T303N/N325S mice suggests Foxp2 modulates excitatory synaptic strength in mouse striatum (Chen et al., 2016; Enard et al., 2009; Schreiweis et al., 2014; van Rhijn et al., 2018). Lastly, *in vivo* recordings from mice with the heterozygous R552H *Foxp2* mutation suggest that this transcription factor is important for correctly modulating behavior dependent activity (French et al., 2012). In songbirds, reduction of FoxP2 expression in area X by ShRNA mediated knockdown leads to aberrant signal propagation in the song circuit (Murugan et al., 2013). The song circuit includes cortical, striatal and thalamic regions in songbirds which are involved as well in complex motor sequencing with similarities to how motor skill learning in mouse and speech production in human are encoded (Brainard and Doupe, 2013). Currently research on the effects of disrupted FoxP2 function on neuronal activity has shown modulation of striatal excitatory activity. However, both the striatum in mammals and area X in birds are entirely comprised of inhibitory GABAergic MSNs (Carrillo and Doupe, 2004; Gittis and Kreitzer, 2012). The previously published data on gene expression which can be regulated by FoxP2 (Vernes et al., 2011) suggests that it is able to regulate inhibitory activity. Additionally, the expression of FoxP2 in inhibitory neurons in the striatum makes the R552H and S321X mouse models for Foxp2 dysfunction highly useful tools to investigate if this transcription factor affects inhibitory striatal function.

1.4.4 Behavioral effects of Foxp2 dysfunction

Mice with homozygous mutations in *Foxp2*, both the R552H DNA-binding mutations and the S321X loss of function mutation, are highly impaired in their development and motor functions. Homozygous mutant mice show dramatic reductions in growth rate by postnatal day 8 and do not survive past the third postnatal week (Groszer et al., 2008). By contrast, littermates with heterozygous *Foxp2* mutations are not impaired in their development, reproduce normally and do not show any overt impairments in natural motor behavior (French et al., 2012; Groszer et al., 2008). Behavioral impairments in heterozygous mice only become apparent when they are challenged on a task which requires motor skill learning and complex motor sequencing. *Foxp2* heterozygous mutant mice carrying either the R552H missense mutation or the S321X nonsense mutation have been subjected to an accelerating rotarod task. This task requires mice to increase their running speed on a rotating beam which increases rotation speed from 4 to 40 rpm over a 5-minute time course. Each mutation leads to a decrease in the latency to fall from the rotarod, as well as a reduction in learning rate (French et al., 2012; Groszer et al., 2008). Furthermore, in zebra finches, reduced FoxP2 expression has a large impact on song reproduction and song quality. During development, male zebra finches learn their song from an adult tutor (Gobes and Bolhuis, 2007). However, juvenile zebra finches in which FoxP2 expression is reduced by ShRNA interference show increased variability in song reproduction. This results in reduced song complexity and decreased performance in song learning (Haesler et al., 2007; Murugan et al., 2013). Though the behavioral data suggests Foxp2 is important in complex motor function, it is currently unclear if the alterations on the genetic, cellular and behavioral level following FoxP2 dysfunction are related. Unraveling how Foxp2 affects motor circuitry at the single-cell level can help us connect the genetic regulatory functions of Foxp2 to striatal cellular physiology and ultimately to how alterations in circuit function lead to behavioral changes.

1.5.1 Does FoxP2 affect striatal function at the single cell level?

In this thesis, we explore how Foxp2 affects activity at the level of the striatum and how this might relate to the behavioral phenotypes associated with heterozygous *Foxp2* mutations. Firstly, it has been suggested that Foxp2 is predominantly expressed in cells of the striatal direct pathway (Fong et al., 2018; Vernes et al., 2011). The dissociation between the direct and indirect pathway in the striatum has been shown to be important for motor skill learning, both in mice and songbirds (Calabresi et al., 2014; Farries et al., 2005). Therefore, we first asked the question if Foxp2 differently affects activity in striatal cell populations which govern the direct and indirect pathway. Furthermore, the presence of Foxp2 both during early development and in adulthood (Ferland et al., 2003) and its effects on neurodevelopment (Devanna et al., 2014; Vernes et al., 2011) prompted us to investigate whether it is involved in the development and maturation of striatal MSNs at the synaptic level. Next, the overlapping behavioral phenotype of the heterozygous R552H and S321X mutations in these two mouse

models, as well as in affected individuals with similar mutations, led us to ask if these different disruptions lead to similar effects on striatal activity in mouse brain. Furthermore, initial evidence of an interaction between Foxp2 and retinoic acid signaling (Devanna et al., 2014) made us assess whether interaction between retinoic acid signaling and Foxp2 might be informative for investigating how Foxp2 influences striatal development and synaptic activity. Lastly, we and others have extensively used mouse models to investigate Foxp2 dysfunction. However, the limited translational ability of results from mouse models has prompted us to examine if the phenotypes on the single cell level found in mouse striatum can be replicated in a human neuronal model for FOXP2 dysfunction. Taken together, all these questions resulted in a detailed investigation of FoxP2 function at the single cell level in the striatum and in human neurons, of which we present the results in this thesis.

1.5.3 Main questions and chapter summary

To answer the previously posed questions, we will use multiple mouse models for Foxp2 dysfunction and explore how this transcription factor affects striatal function at a single cell level in segregated striatal cell populations. Our ability to differentiate between different cell populations in mouse striatum through genetic fluorescent labeling enables us target specifically cells of the striatal direct and indirect pathways. We then use whole-cell patch clamp to investigate if mutations in *Foxp2* affect striatal excitatory and inhibitory activity. We furthermore aim to assess if the changes in activity we find are correlated with aberrant expression of proteins involved in synaptic activity. Lastly, we want to utilize our conclusions from the electrophysiological and molecular experiments to perform *in* vivo interventions aimed at amelioration of the motor skill learning deficits present in mice with heterozygous *Foxp2* mutations.

In **chapter 2,** we assess whether Foxp2 affects the striatum in a cell population specific manner and find that heterozygous loss of *Foxp2* function only affects striatal D1R-MSNs and that both excitatory and inhibitory activity are strongly affected by loss of *Foxp2* function during development and in adulthood. We are the first to show that Foxp2 affects inhibitory activity and provide a novel molecular mechanism by which *Foxp2* mutation leads to increased inhibitory drive in D1R-MSNs through increased presynaptic GABA production. Lastly, we correlate the increased striatal inhibitory activity with the motor skill learning deficits in *Foxp2* loss of function mice. In **chapter 3** we investigate if Foxp2 affects the maturation of excitatory synapses, by examining AMPA receptor subunit expression during striatal development. We demonstrate that maturation of AMPA receptor expression occurs between PND8 and 11 in the striatum, with a switch from calcium-permeable AMPAR expression to calcium-impermeable AMPAR expression that coincides with the start of coordinated locomotion. Reduced Foxp2 expression delays this maturation and leads to delayed emergence of coordinated locomotion. In **chapter 4** we question whether heterozygous nonsense mutation

and heterozygous DNA-binding mutation of *Foxp2* might differently affect striatal activity. We show that the way in which disruption of *Foxp2* affects striatal circuitry is mutation-specific. This could have important consequences for the genetic characterization of *FOXP2* mutations and how these might uniquely affect brain function. In **Chapter 5** we investigate the possible functions of retinoic acid signaling through RARβ as one of the latest targets found to be regulated by Foxp2. We review the current literature regarding the functions of RA signaling on neuronal activity, the functions of RARβ in the striatum and its relationship to disorders of movement**.** RARβ could be a novel regulatory protein with an important role in the striatal motor circuit. Lastly, in **chapter 6** we describe the possibility to generate human dopaminergic neurons which express FOXP2. We show that homozygous knockout of *FOXP2* in these human dopaminergic neurons leads to markedly similar deficits in excitatory activity to those we have seen in mouse striatal neurons with heterozygous loss of *FOXP2* function. These human dopaminergic neurons present a new tool which will generate novel opportunities to investigate function of FOXP2 on a genetic and cellular level.

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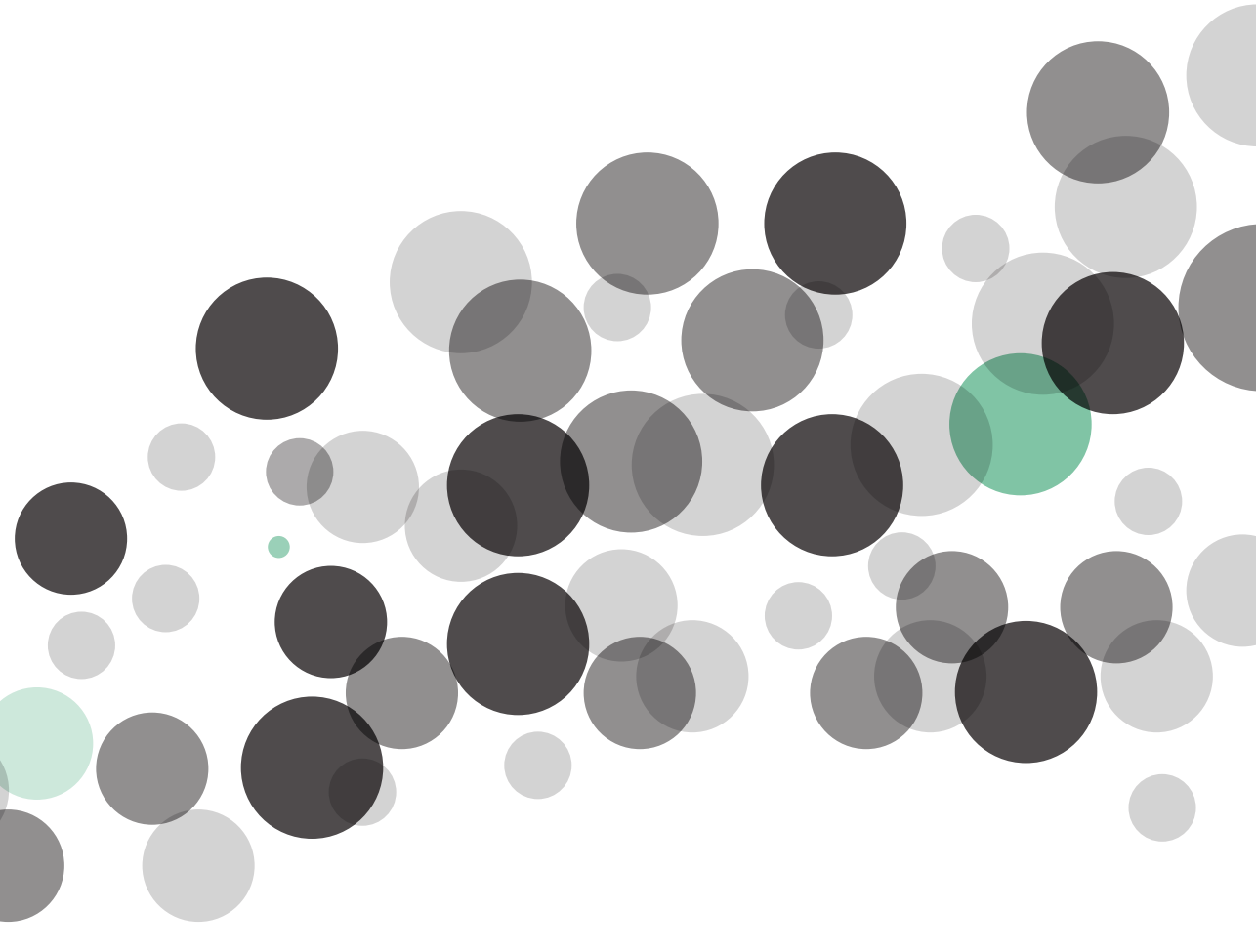
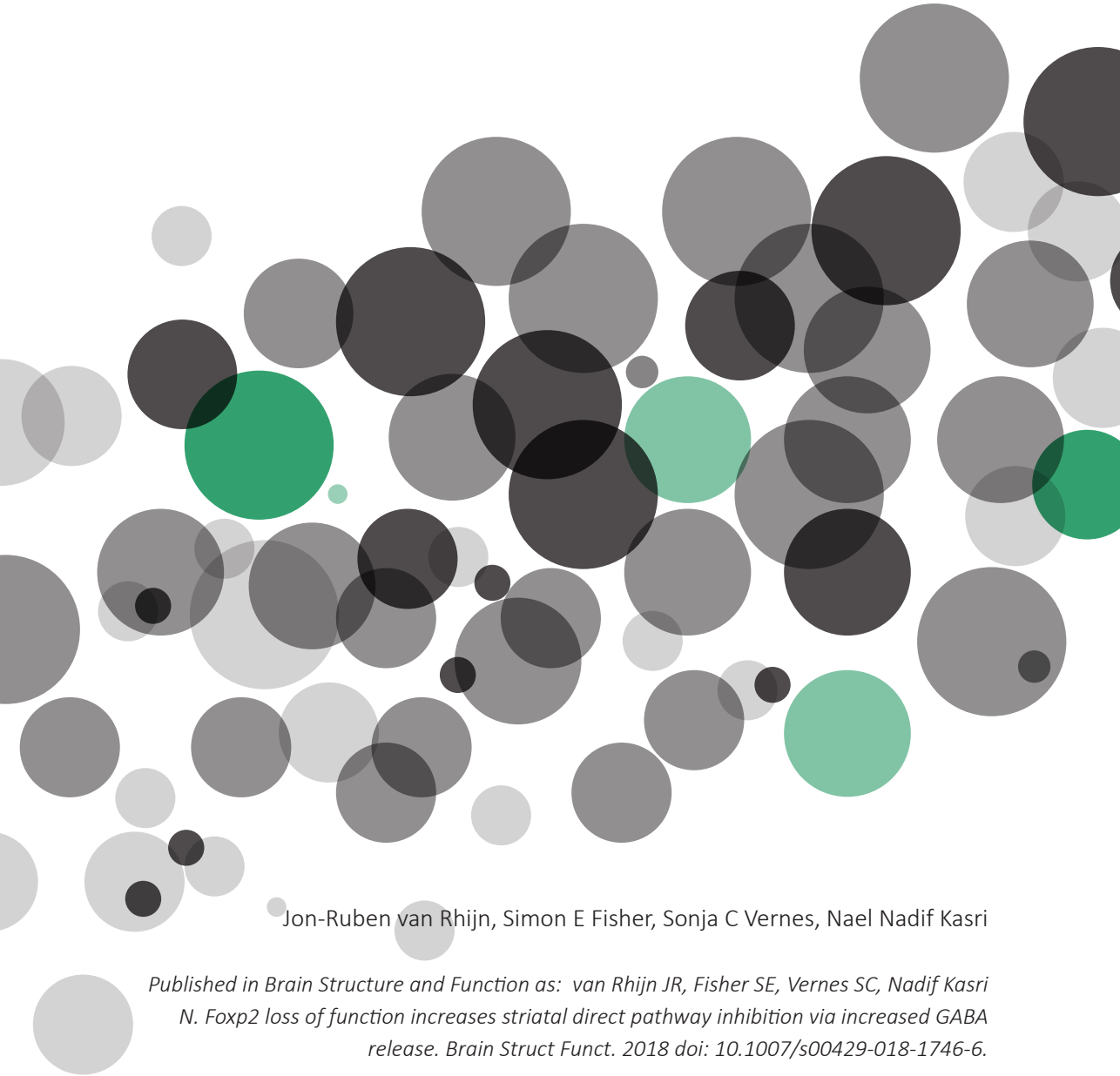


2.

Foxp2 loss of function increases striatal direct pathway inhibition via increased GABA release



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Published in Brain Structure and Function as: van Rhijn JR, Fisher SE, Vernes SC, Nadif Kasri N. Foxp2 loss of function increases striatal direct pathway inhibition via increased GABA release. Brain Struct Funct. 2018 doi: 10.1007/s00429-018-1746-6.

ABSTRACT

Heterozygous mutations of the Forkhead-box protein 2 (*FOXP2)* gene in humans cause childhood apraxia of speech. Loss of Foxp2 in mice is known to affect striatal development and impair motor skills. However, it is unknown if striatal excitatory/inhibitory balance is affected during development and if the imbalance persists into adulthood. We investigated the effect of reduced Foxp2 expression, via a loss-of-function mutation, on striatal medium spiny neurons (MSNs). Our data show that heterozygous loss of Foxp2 decreases excitatory (AMPA receptor-mediated) and increases inhibitory (GABA receptor-mediated) currents in D1 dopamine receptor positive MSNs of juvenile and adult mice. Furthermore, reduced Foxp2 expression increases GAD67 expression, leading to both increased presynaptic content and release of GABA. Lastly, pharmacological blockade of inhibitory activity *in vivo* partially rescues motor skill learning deficits in heterozygous *Foxp2* mice. Our results suggest a novel role for Foxp2 in the regulation of striatal direct pathway activity through managing inhibitory drive.

INTRODUCTION

Balanced neuronal activity between cortex, striatum and thalamus is essential for the generation of voluntary movements (Shepherd 2013). Imbalanced activity within the striatum is known to disrupt complex motor behaviors, such as the production of spoken language (Peach 2004; Square-Storer 1990). *FOXP2*, the first single gene linked to a speech and language disorder (Lai et al. 2001), is important for the correct execution of complex motor behaviors used for speech. Individuals with mutations in the *FOXP2* gene have problems executing coordinated sequences of orofacial movements, which impede their speech (diagnosed as developmental verbal dyspraxia or childhood apraxia of speech (CAS)), while their general cognitive functioning and other aspects of motor coordination are usually less severely affected(MacDermot et al. 2005; Morgan et al. 2017). Mice with heterozygous Foxp2 mutations display impairments in motor skill learning, shown by decreased performance on the accelerating rotarod (French et al. 2012; Groszer et al. 2008), suggesting that similar neurobiological substrates could underlie the behavioral phenotypes in human and mouse. *FoxP2* codes for a transcription factor (Devanna et al. 2014; Vernes et al. 2007; Vernes et al. 2006) and plays important roles during the early development of the central nervous system as well as in the postnatal brain(Spiteri et al. 2007; Vernes et al. 2011; Groszer et al. 2008). Mutations of this gene affect both cortical and striatal activity in human cases and animal models (French et al. 2012; Groszer et al. 2008; Liegeois et al. 2003). Of particular note, striatal long-term depression is affected in adult mice with heterozygous *Foxp2* mutations(Groszer et al. 2008; Enard et al. 2009), which suggests that Foxp2 regulates molecular mechanisms involved in synaptic plasticity. Additionally, evidence from *in vivo* recordings shows that *Foxp2* mutant mice display abnormal ongoing striatal activity and dysregulated firing rates during a motor-learning task (French et al. 2012). Lastly, Foxp2 has been reported to regulate genes involved in synapse formation (Sia et al. 2013; Vernes et al. 2011) and was recently shown to affect excitatory synaptic activity during early postnatal development through inhibition of the *Mef2c* gene (Chen et al. 2016).

Studies using mouse models to investigate the functions of Foxp2 have made use of two well described mutations which differentially affect Foxp2 and are similar to mutations described in patients with CAS. These mutations lead to either disruption of the DNA binding domain of Foxp2 or a stop-gain mutation in exon 7 and nonsense mediated decay of Foxp2 protein (MacDermot et al. 2005; Morgan et al. 2017). Though neurobiological mechanisms affected by these different mutations could differ, there is currently no data to suggest this. Moreover, heterozygous Foxp2 mice with either the DNA binding domain mutation or the loss of function mutation display similar impairments in motor skill learning (French et al. 2012; Enard et al. 2009; Groszer et al. 2008).

To date, investigations into the functions of Foxp2 in striatum have focused on how Foxp2 affects excitatory activity (Groszer et al. 2008; Enard et al. 2009; French et al. 2012; Chen et al. 2016; Schreiweis et al. 2014). Although the striatum receives numerous excitatory connections from the cortex (Shepherd 2013) and thalamus (Smith et al. 2009; Smith et al. 2004), it is itself entirely composed of inhibitory neurons (Kreitzer and Malenka 2008). GABAergic medium spiny neurons (MSNs) make up 95% of the striatum, and two major populations can be distinguished: MSNs that express either the D1 dopamine receptor (D1R-MSNs) or the D2 dopamine receptor (D2R-MSNs) (Gittis and Kreitzer 2012). These MSN populations differentially affect the downstream neural sites to which they ultimately project, and each regulate separate aspects of motor behavior (Calabresi et al. 2014; Surmeier et al. 2007; Gittis and Kreitzer 2012). D1R-MSNs innervate the direct pathway, which leads to increased activation of the cortico-striatal-thalamic motor circuit. In contrast, D2R-MSNs belong to the indirect pathway, inactivating this motor circuit. Balanced excitation and inhibition (E/I balance) of cells within both striatal pathways is crucial for the generation of complex motor behaviors (Schroll et al. 2015).

How Foxp2 affects neuronal function has been investigated in both early development and adulthood, but knowledge of how Foxp2 affects striatal circuits during (motor) development is lacking. This is especially important to address since E/I balance is dynamic. Changes in E/I balance during development are tightly regulated and have been described in multiple cell types in hippocampus (Liu 2004) and cortex (Zhang et al. 2011) of juvenile mice. A disrupted E/I balance during development can severely affect adult behavior (Peixoto et al. 2016). Indeed, aberrant E/I balance in striatal cells is known to lead to impaired motor learning in adult mice (Rothwell et al. 2014), similar to the deficits observed in adult mice with mutations in *Foxp2* (French et al. 2012; Groszer et al. 2008).

We examined the effects of reduced Foxp2 expression from early development into adulthood in the striatum, using a heterozygous mouse model for the stop-gain Foxp2 mutation (S321X). Foxp2 protein expression is absent in $Foxp2^{S321X/S321X}$ mice and reduced to intermediate levels in $Foxp2^{S321X/+}$mice (Groszer et al. 2008; Vernes et al. 2011). We provide evidence that Foxp2 plays a role in the regulation of striatal E/I balance, regulates inhibitory activity through repression of GAD67, and regulates inhibitory presynaptic strength of D1R-MSNs, but not D2R-MSNs. Lastly, we show that pharmacological blockade of striatal inhibition partially rescues the motor skill learning deficits observed in heterozygous *Foxp2* mutant mice. Taken together, our results reveal a developmental circuit defect caused by reduced levels of functional Foxp2, which suggests that E/I imbalances in striatal activity may contribute to (vocal)motor problems in humans with *FOXP2* mutations.

RESULTS

Reduced Foxp2 expression affects D1R-MSN excitatory synaptic inputs

Previous studies have suggested that Foxp2 is differentially expressed in D1R- versus D2R-expressing MSNs in the striatum (Vernes et al. 2011). To directly assess the expression of Foxp2 in D1R- and D2R-MSNs, we performed immunocytochemistry for Foxp2 on mice containing bacterial artificial chromosome (BAC)-TRAP GFP constructs (Heiman et al. 2008; Doyle et al. 2008) under the D1R or D2R promoter, which have been shown to faithfully label D1R- or D2R-expressing MSNs, respectively (Heiman et al. 2008). Upon investigation of expression in juvenile mice (PND11-14) we found that Foxp2 is expressed in nearly all striatal D1R-MSNs, in contrast to only a small fraction of D2R-MSN (Fig 1a).

We next investigated whether heterozygous *Foxp2* loss of function differentially affects striatal MSN subtypes at the synaptic level. To enable a targeted single-cell characterization of how reduced Foxp2 expression affects striatal activity, we crossed *Foxp2*$^{S321X/+}$ mice with (BAC)-TRAP D1R-GFP mice. We performed patch-clamp recordings on GFP-positive D1R-MSNs and non-GFP putative D2R-MSNs. Although Foxp2 expressing MSNs are spread throughout the striatum, we chose to focus on cells in the dorsolateral striatum, because of its connection to the motor cortex (Hunnicutt et al. 2016). Moreover, previous experiments regarding Foxp2 function have shown aberrant activity in dorsal striatum of heterozygous Foxp2 mutant/knockout mice (French et al. 2012; Groszer et al. 2008; Reimers-Kipping et al. 2011), and reduced motor skill learning suggests areas involved in motor control might be more severely affected by reduced Foxp2 expression. We measured excitatory synaptic strength through glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation by analysis of miniature excitatory postsynaptic current (mEPSC) amplitude and frequency. In D1R-MSNs of heterozygous postnatal day (PND) 10-14 juvenile mice, mEPSC amplitude was reduced, whereas mEPSC frequency was similar between genotypes (Fig 1c). No changes in D2R-MSN amplitude or frequency were observed (Fig 1d). Lastly, we measured AMPA/NMDA ratio in D1R-MSNs of juvenile *Foxp2*$^{+/+}$ and *Foxp2*$^{S32X/+}$ mice. AMPA/NMDA ratio is significantly increased in *Foxp2*$^{S32X/+}$ mice, which suggests that NMDA currents are decreased as well in addition to the previously observed reduction in AMPAR-mediated activity (Suppl Figure 1). These results show that reduced Foxp2 expression leads to decreased excitatory postsynaptic strength of only direct pathway MSNs, which is consistent with the predominant expression of Foxp2 in D1R-MSNs.

Inhibitory synaptic inputs are increased in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice

Physiological effects of heterozygous Foxp2 mutations have only been investigated in the context of excitatory synaptic transmission (Chen et al. 2016; Reimers-Kipping et al. 2011; Schreiweis et al. 2014).

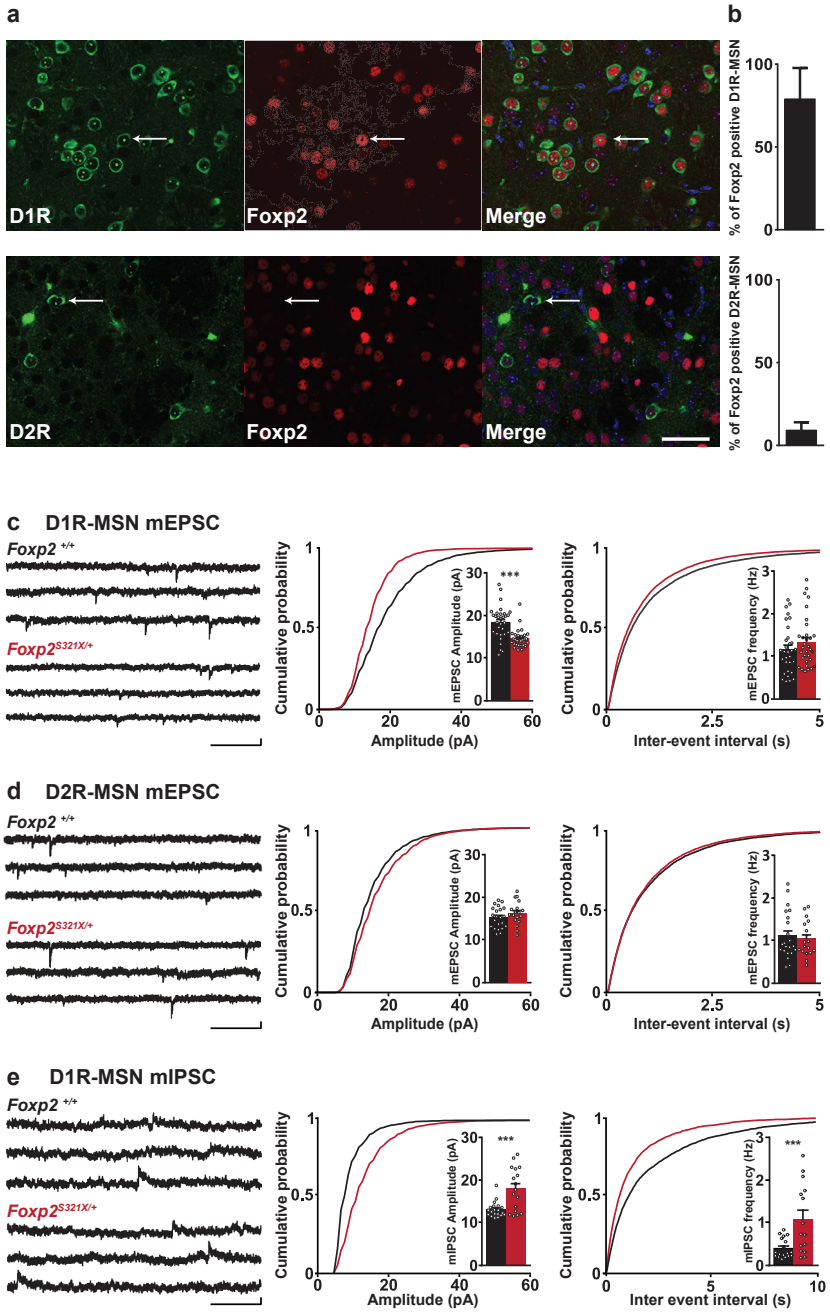

Figure 1: Foxp2 is predominantly expressed in D1R-MSNs in dorsolateral striatum and affects synaptic activity

(a) D1R-GFP, D2R-GFP and Foxp2 are expressed in a subset of striatal cells in juvenile (PND11-14) mice, arrows point to cells with overlapping D1R and Foxp2 expression (top row) or D2R expression without Foxp2 expression (bottom row). Scale bar 50µm. (b) 83.7 ± 16% of D1R-GFP positive cells express Foxp2, compared to 16.9 ± 4% of D2R-GFP

cells (N = 3 mice). (c) Striatal D1R-MSN mEPSC amplitude is decreased following reduced Foxp2 expression. Example of mEPSC activity in striatal D1R-MSNs from juvenile (PND14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Scale bar 200ms/20pA. Cumulative distribution of mEPSC amplitude (*Foxp2*$^{+/+}$ = 19.4±0.69pA, *Foxp2*$^{S321X/+}$ = 14.3±0.36 pA, *P*<0.0001) and frequency (*Foxp2*$^{+/+}$ = 1.12±0.1Hz, *Foxp2*$^{S321X/+}$ = 1.19±0.11Hz, NS) in striatal D1R-MSNs. *Foxp2*$^{+/+}$ N/n = 3/31, *Foxp2*$^{S321X/+}$ N/n = 3/32. (d) Example traces of mEPSC activity in striatal D2R-MSNs from juvenile (PND14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice Scale bar 200ms/20pA. Cumulative distribution of mEPSC amplitude (*Foxp2*$^{+/+}$ = 15.64±0.53pA, *Foxp2*$^{S321X/+}$ = 16.14±0.62pA, NS) and frequency (*Foxp2*$^{+/+}$ = 0.92±0.12Hz, *Foxp2*$^{S321X/+}$ = 0.89±0.1Hz, NS) in striatal D2R-MSNs. *Foxp2*$^{+/+}$ N/n = 3/22, *Foxp2*$^{S321X/+}$ N/n = 3/19. (e) Example traces of mIPSC activity in striatal D1R-MSNs from juvenile (PND14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Scale bar 200ms/10pA. Cumulative distribution of mIPSC amplitude (*Foxp2*$^{+/+}$ = 8.6±0.27pA, *Foxp2*$^{S321X/+}$ = 11.9±0.83pA, *P*<0.001) and frequency (*Foxp2*$^{+/+}$ = 0.18±0.036Hz, *Foxp2*$^{S321X/+}$ = 0.71±0.14Hz, *P*<0.01) in striatal D1R-MSNs. *Foxp2*$^{+/+}$ N/n = 3/20, *Foxp2*$^{S321X/+}$ N/n = 3/17. *** *P*<0.01. N: number of mice, n: number of cells. All data analyzed by two-sided students T-test.

Since E/I balance is important for the development and maintenance of neuronal circuitry, we examined the role of Foxp2 in striatal inhibition. Striatal inhibition is accomplished through extra-striatal as well as intra-striatal sources. From the cortex, GABAergic interneurons project to the striatum and provide inhibitory input (Melzer et al. 2017). However, corticostriatal GABAergic interneurons do not express Foxp2. We therefore expect differences in inhibitory activity between wild-type and *Foxp2*$^{S321X/+}$ mice through changes in intra-striatal inhibition, which is regulated through MSNs and striatal interneurons (Taverna et al. 2008; Lalchandani and Vicini 2013). We measured inhibitory activity only in D1R-MSNs, since unidirectional connections between D1R-MSNs are common, while connections between D1R-MSNs and D2R-MSNs are rare (6%) (Taverna et al. 2008). Though D2R-MSNs synapse on D1R-MSNs (27%) the lack of Foxp2 expression in D2R-MSNs, combined with the lack of an excitatory phenotype, suggests that D2R-MSNs cannot be cell-autonomously affected by Foxp2.

We measured miniature inhibitory postsynaptic currents (mIPSCs), which are mediated by GABA and reflect inhibitory synaptic strength. In D1R-MSNs of juvenile (PND10-14) *Foxp2*$^{S321X/+}$ mice, we found that mIPSC amplitude and frequency were increased compared to wild-type controls (Fig 1e). Our data show that reduced Foxp2 expression differentially affects excitatory and inhibitory synaptic strength. There is no compensation for the decreased excitatory activity, but rather this is aggravated by increased inhibition.

E/I imbalance persists in dorsolateral striatum of adult *Foxp2*$^{S321X/+}$ mice

In mice, Foxp2 is present during the entire lifespan (Ferland et al. 2003), and expression does not change strongly between juvenile and adult animals (Ferland et al. 2003; Takahashi et al. 2003). However, given that this gene is important for early neuronal development (Vernes et al. 2011; Chen et al. 2016), it is conceivable that functional effects of reduced Foxp2 expression differ between juvenile and adult animals. In previous studies the effects of Foxp2 mutations on striatal physiology have only been investigated in adult (French et al. 2012;

Groszer et al. 2008) or juvenile mice separately (Chen et al. 2016), and thus a developmental profile of synaptic changes due to reduced Foxp2 expression is lacking. We hypothesizethat the E/I imbalance present in D1R-MSNs of juvenile Foxp2S321X/+ mice might persist until adulthood, since adult Foxp2 heterozygous mice show clear deficits in motor skill learning. We measured the GABA/AMPA ratio as an index of E/I balance in juvenile (PND11, PND14,

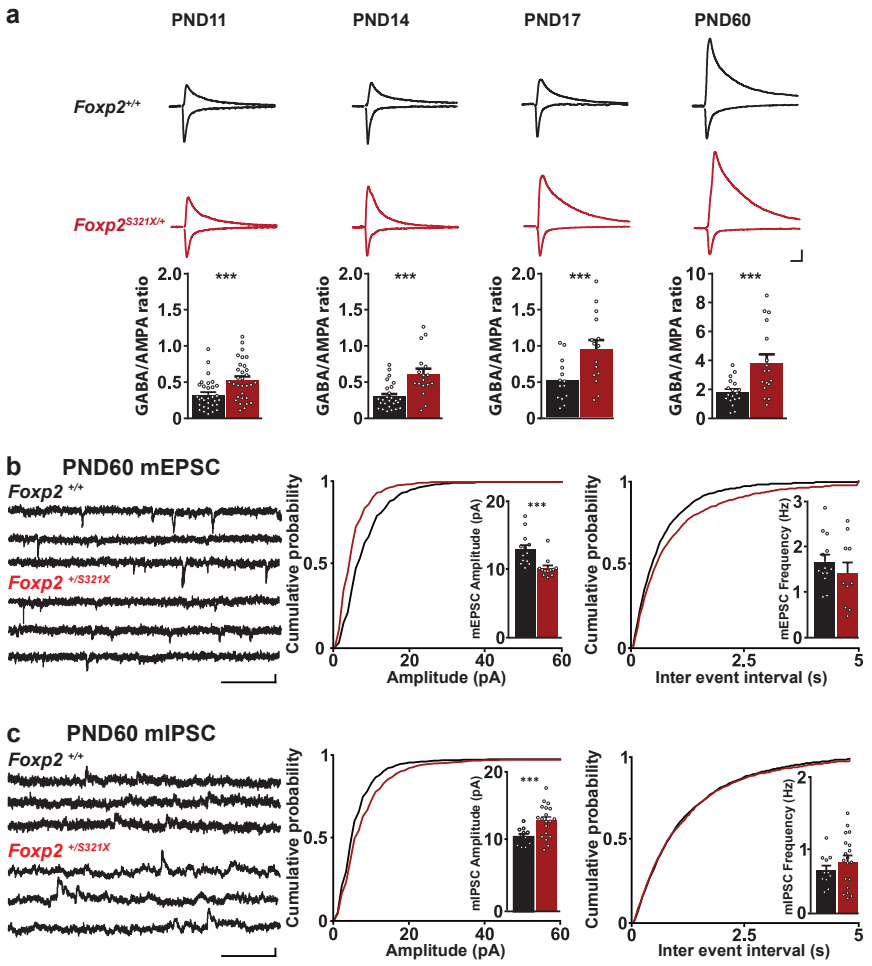

Figure 2: Decreased excitation and increased inhibition persist in adult mice with decreased Foxp2 expression

(a) Example traces show AMPA response (negative) and GABA response (positive) in D1R-MSNs of *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice during development and in adulthood. Scale bar 200/50pA. GABA/AMPA ratio in D1R-MSNs of *Foxp2*$^{+/+}$ (PND11 = 0.32±0.037, PND14 = 0.29±0.035, PND17 = 0.50±0.077, PND60 = 2.04±0.47) and *Foxp2*$^{S321X/+}$ (*Foxp2*$^{S321X/+}$ PND11 = 0.53±0.049, PND14 = 0.6±0.078, PND17 = 0.94±0.12, PND60 = 4.5±0.85), mice during

development and in adulthood (2-factor ANOVA (genotype X age) = P<0.001 for both factors). N: number of mice, n: number of cells. *Foxp2*$^{+/+}$ N/n = 3/31 (PND11), 3/26 (PND13), 3/14 (PND17), 3/18 (PND60), *Foxp2*$^{S321X/+}$ N/n = 3/31 (PND11), 3/26 (PND13), 3/15 (PND17), 3/17 (PND60). (b) Example traces of mEPSC activity in striatal D1R-MSNs from adult (PND60) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Cumulative distribution of mEPSC amplitude (*Foxp2*$^{+/+}$ = 12.9±0.64pA, *Foxp2*$^{S321X/+}$ = 10.2±0.4pA, P<0.01, two-sided students T-test) and frequency (*Foxp2*$^{+/+}$ = 1.68±0.16Hz, *Foxp2*$^{S321X/+}$ = 1.42±0.2Hz, NS, two-sided students T-test) in striatal D1R-MSNs. *Foxp2*$^{+/+}$ N/n = 3/13, *Foxp2*$^{S321X/+}$ N/n = 3/12. (c) Example traces of mIPSC activity in striatal D1R-MSNs from adult (PND60) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Cumulative distribution of mIPSC amplitude *(Foxp2*$^{+/+}$ = 11.36±0.41pA, *Foxp2*$^{S321X/+}$ = 12.9±0.82pA, P<0.01, two-sided students T-test) and frequency (*Foxp2*$^{+/+}$ = 0.67±0.07Hz, *Foxp2*$^{S321X/+}$ = 0.81±0.09Hz, NS, two-sided students T-test) in striatal D1R-MSNs.*Foxp2*$^{+/+}$ N/n = 4/11, *Foxp2*$^{S321X/+}$ N/n = 6/19. Scale bar in (b, c) 200ms/10pA. *** P<0.001.

PND17 as well as adult (PND60) mice, which comprises a developmental profile at ages around the critical time points for the emergence of motor coordination (Dehorter et al. 2011) and striatal synaptic integration and circuit formation in mice (Lee and Sawatari 2011). During development in wild type mice, the GABA/AMPA ratio increases sharply in D1R-MSNs (Fig 2a). Interestingly, both during development and in adulthood the GABA/AMPA ratio of D1R-MSNs was significantly higher in *Foxp2*$^{S321X/+}$ mice than in wild-type controls (Fig 2a), which indicates that the E/I imbalance we uncovered in juvenile mice indeed persists into adulthood. We subsequently measured mEPSCs and mIPSCs in D1R-MSNs of adult (PND60) *Foxp2*$^{S321X/+}$ mice to determine if the increased GABA/AMPA ratios in *Foxp2*$^{S321X/+}$ mice reflect persistent changes in excitatory and/or inhibitory synaptic strength. Our results show that the increased GABA/AMPA ratio in adult heterozygous mice is due to decreased mEPSC amplitude (Fig 2b) coupled with an increased mIPSC amplitude (Fig 2c). However, the increased mIPSC frequency we had observed in our juvenile mice was not present in adult mice (Fig 2c), which indicates that some form of compensation might be present. This compensation is however insufficient to return activity to baseline levels, and therefore we conclude that the changes in E/I balance are persistent into adulthood. Changes in inhibitory synaptic strength can indicate changes at either the pre- or the postsynapse, such as increased presynaptic neurotransmitter release or increased expression of postsynaptic GABA receptors, respectively. We therefore set out to assess the effect of reduced Foxp2 expression on striatal synapses at the molecular level.

Decreased Foxp2 expression leads to increased GAD67 expression around D1R-MSN somata

Foxp2 might modulate inhibitory activity by transcriptionally regulating genes involved in GABA signaling (Vernes et al. 2007; Vernes et al. 2011; Fujita et al. 2008). One target gene identified in an *in vivo* chromatin immunoprecipitation (ChIP)-chip screen for Foxp2 binding in mouse brain was *Gad* (Vernes et al. 2011), the gene that codes for GAD67, a key enzyme in the production of GABA at the synapse (Lau and Murthy 2012). By contrast, other genes involved in GABAergic activity, such as VGAT or GAD2, were not detected in this ChIP screen. Moreover, GAD2 expression has been shown to be unaltered in striatal tissue from

Foxp2 heterozygous knockout embryos (French et al. 2007). Based on these findings we hypothesized that reduced Foxp2 expression could lead to changes in GAD67 expression and thus contribute to aberrant GABAergic activity.

We compared GAD67 expression around D1R-MSN somata in the striatum of juvenile *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice (Fig 3a). GAD67 puncta surrounding striatal D1R-MSNs originate mostly from D1R-MSN - D1R-MSN pairs (Taverna et al. 2008) and to a lesser extent from extrastriatal GABAergic interneurons (Melzer et al. 2017) and striatal interneurons (Taverna et al. 2008). However, of these cells only D1R-MSNs express Foxp2. Thus, aberrant GAD67 expression levels can be related to changes in Foxp2 expression. This could either be through direct regulation of GAD67 expression by Foxp2 or by indirect effects of altered Foxp2 levels. Foxp2 is known to affect development of striatal cells in primary cell culture (Vernes et al. 2011) and impaired D1R-MSN development in vivo may account for changes in protein expression such as reduced GAD67 levels. We found that GAD67 expression was significantly increased around D1R-MSN somata in *Foxp2*$^{S321X/+}$ mice compared to wild-type controls (Fig 3b), whilst GAD67 was not changed around D2R-MSNs (Suppl Figure 2). Furthermore, protein expression analysis by western blot in dissected striatum from juvenile *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice showed GAD67 expression to be increased (Fig 3c).

To assess whether or not the increased GAD67 expression could be due to a general increase in expression of key components of GABA transmission, we quantified vesicular GABA transporter (VGAT) expression around D1R-MSN somata (Fig 3a). No change in VGAT expression was observed between *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice, which suggests that Foxp2 specifically regulates GAD67 but does not affect the number of synapses. The increase in GAD67 levels of mice with reduced Foxp2 expression is consistent with the hypothesis that Foxp2 normally acts to repress the transcription of *Gad1* and is supported by the prior ChIP-chip data (Vernes et al. 2011). Differences in GAD67 expression have been described as a cause for changes in presynaptic GABA content and inhibitory activity (Lau and Murthy 2012). Thus, reduced Foxp2 expression could lead to increased inhibitory drive of D1R-MSNs through increased GABA production.

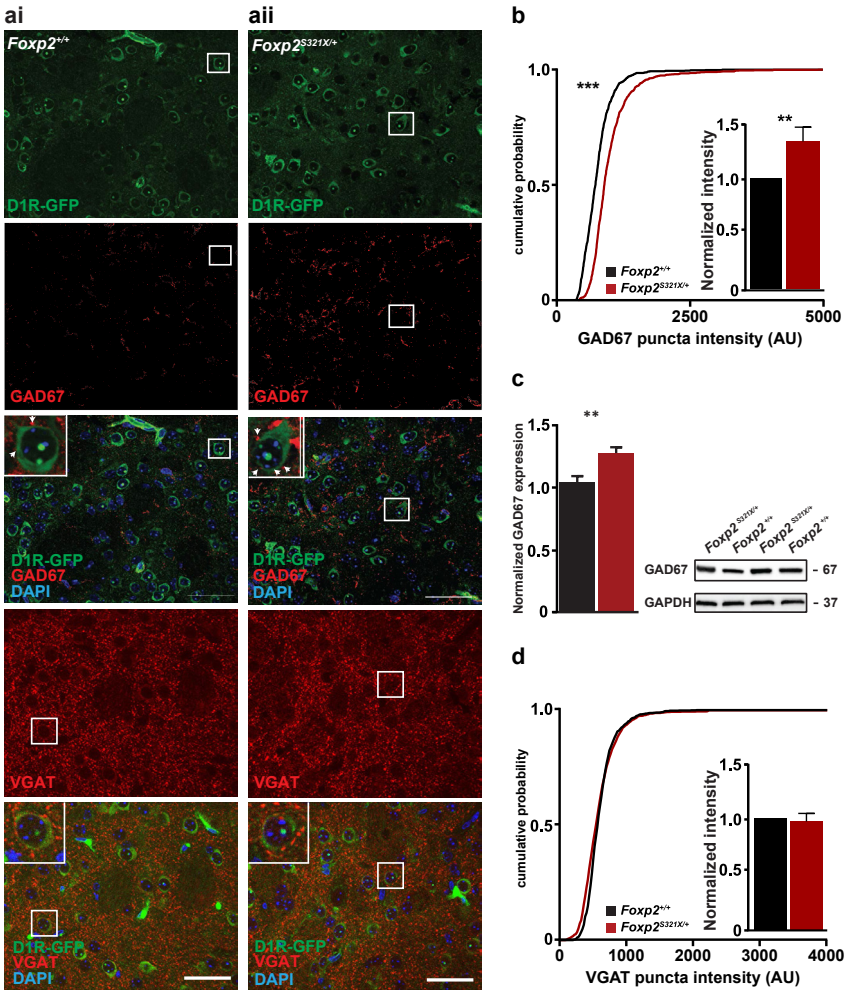

Figure 3: Increased GAD67 expression in *Foxp2*$^{S321X/+}$ mice (ai, aii)

Overview of GAD67 and VGAT expression levels in striatal slices of juvenile (PND10-14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Insets show puncta which surround D1R-MSN somata. These perisomatic puncta were used for intensity analysis, in order to restrict analysis to D1R-MSNs (b) Comparison of GAD67 expression (both cumulative distribution in arbitrary units (AU) and normalized expression) around D1R-GFP positive somata in dorsolateral striatum of juvenile (PND10-14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. (Normalized expression: *Foxp2*$^{+/+}$ = 1.0±0.04, *Foxp2*$^{S321X/+}$ 1.44±0.15, $P<0.05$, two-sided student's T-test). Kolmogorov-Smirnov (KS) test for cumulative distributions was used for the cumulative distribution data, $P<0.001$. N: number of animals, n: number of slices. *Foxp2*$^{+/+}$ N/n = 3/10, *Foxp2*$^{S321X/+}$ N/n = 3/10. (c) Quantification and representative western blot of GAD67 protein expression in juvenile *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice (*Foxp2*$^{+/+}$ 1.04 ± 0.05, *Foxp2*$^{S321X/+}$ 1.28 ± 0.04, *P<0.01*, two-sided students T-test). Expression is normalized to *Foxp2*$^{+/+}$ expression (*Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ N=6). (d) Comparison of VGAT expression (both cumulative distribution in arbitrary units (AU) and normalized expression) around D1R-GFP positive somata in dorsolateral striatum of juvenile (PND10-14) *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. (Normalized expression: *Foxp2*$^{+/+}$ = 1.0±0.03, *Foxp2*$^{S321X/+}$ 0.94±0.06, NS, two-sided student's T-test). KS test for cumulative distribution data: NS. N: number of animals, n: number of slices. *Foxp2*$^{+/+}$ N/n = 3/11, *Foxp2*$^{S321X/+}$ N/n = 4/14 * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Presynaptic GABA content is increased upon heterozygous loss of *Foxp2* function

As GAD67 levels directly correlate with presynaptic GABA production, we explored if the increased GAD67 levels following reduced Foxp2 expression lead to elevated presynaptic GABA concentration. Presynaptic GABA is stored in vesicles, and is released upon electrical or pharmacological stimulation of the neuron (Alabi and Tsien 2012)**.** A 10 second 10Hz stimulation protocol has been described that efficiently depletes the entire readily releasable GABA vesicle pool (RRP) (Maas et al. 2017; Chen et al. 2017). This depletion protocol can be used to compare the quantal content of the GABA RRP between D1R-MSNs of wild-type and *Foxp2*$^{S321X/+}$ mice. We show that this stimulation protocol indeed depletes the RRP in juvenile wild-type and *Foxp2*$^{S321X/+}$ mice (Fig 4a, b). However, in *Foxp2*$^{S321X/+}$ mice the average current transferred per stimulation, as well as the cumulative current transferred after 100 stimulations, was significantly increased compared to wild-type controls (Fig 4c). However, we did not observe a difference in the kinetics of release when release was normalized, which indicates that vesicle recycling was not affected in *Foxp2*$^{S321X/+}$ mice (Fig 4b).

Changes in GABA concentration at the synapse can affect synaptic strength and vesicle release probability (Olpe et al. 1994; Jensen et al. 1999). We therefore examined both excitatory and inhibitory paired pulse ratios (PPRs) in juvenile Foxp2$^{S321X/+}$ mice and littermate controls. No differences in excitatory PPRs were found between genotypes (Fig 4d). However, in contrast to the expected increase in inhibitory PPR Foxp2$^{S321X/+}$ mice showed a lack of inhibitory paired pulse depression, specifically at longer inter stimulus intervals (Fig 4e). The lack of inhibitory PPD can be explained increased GABA release per stimulation (figure 4a). If only a fraction of the total released GABA is necessary to saturate postsynaptic GABA, then reduction of vesicles released with subsequent stimulations would not lead to PPD, because enough GABA is still released to saturate the postsynaptic GABA receptors that are present.

Next, we sought to confirm the increased presynaptic GABA release pharmacologically, to exclude aberrant effects from recurrent stimulation. Local application of 500 mM sucrose for 10 seconds (Lipstein et al. 2017) efficiently induced vesicle exocytosis in juvenile Foxp2+/+ and Foxp2S321X/+ mice (Fig 4f). The total current transfer during sucrose application was increased by approximately 50% in D1R-MSNs f*Foxp2*$^{S321X/+}$ mice, similar to the increase in current transfer observed upon electrical stimulation (Fig 4f). Lastly, the increase in mIPSC amplitude (Fig 2b and 3h) in mice with reduced Foxp2 expression also suggests postsynaptic GABA receptor abundance might be increased. We used local application of GABA to investigate if postsynaptic $GABA_A$ receptor presence was affected by reduced Foxp2 expression. GABA application elicited a strong response in D1R-MSNs of both *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice (Fig 4g). No difference in peak response amplitude or total current transfer could be observed between genotypes (Fig 4g). Taken together, our data suggest that D1R-MSNs exhibit increased GABA content at the presynapse following reduced Foxp2 expression,

leading to a heightened quantal GABA release. This in turn leads to elevated inhibition of the striatal direct pathway.

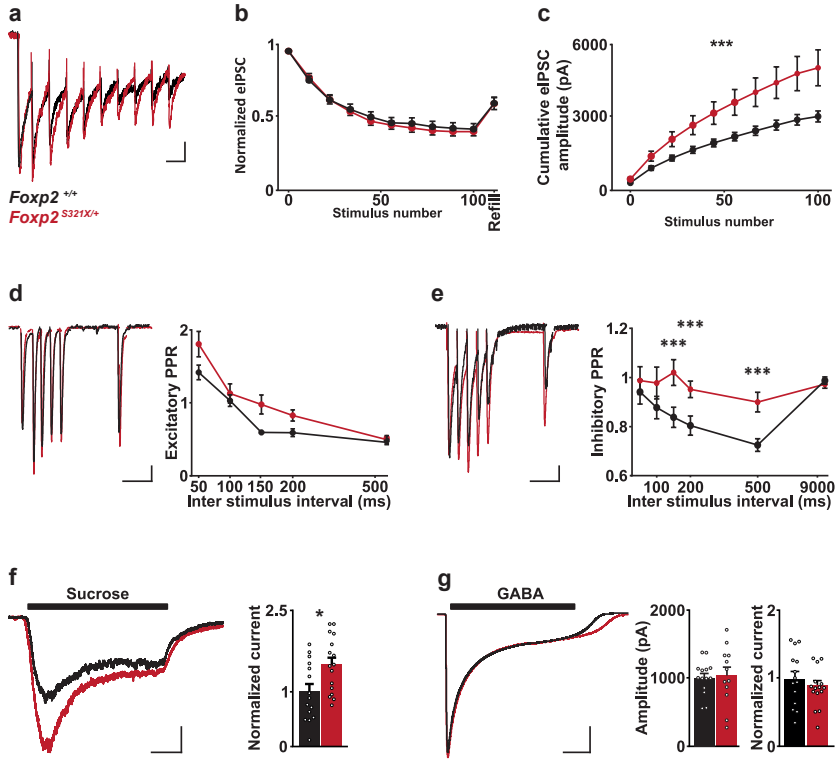

Figure 4: Presynaptic GABA content in juvenile (PND10-14) striatal D1R-MSNs is increased upon decreased Foxp2 expression.

(a) Example traces of vesicle depletion following train stimulation (10Hz, 100 stimuli) in *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ D1R-MSNs, every 10th response is shown. Scale 100ms/50pA. (b) Normalized (to first pulse) IPSC response during train stimulation. (c). Cumulative IPSC amplitude during train stimulation of D1R MSNs, (*Foxp2*$^{+/+}$ = intercept 294.24±85.3pA, cumulative 3112.9±286.4pA, *Foxp2*$^{S321X/+}$ = intercept 498.9±301.3 pA, cumulative 5142.1±484.6pA, *P*<0.001, two-sided students T-test) N: number of mice, n: number of cells. *Foxp2*$^{+/+}$ N/n = 3/16, *Foxp2*$^{S321X/+}$ N/n = 3/16 (D) Example trace of excitatory PPR at different inter stimulus intervals (ISI) in D1R-MSNs of *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Scale 100ms/50pA. Quantification of paired pulse ratio (PPR) (50, 100, 150, 200, 500ms: *Foxp2*$^{+/+}$ 1.573±0.08, 1.284±0.06, 0.958±0.02, 0.955±0.04, 0.856±0.02 vs *Foxp2*$^{S321X/+}$ 1.864±0.13, 1.357±0.1, 1.244±0.1, 1.131±0.06, 0.884±0.04, NS, Repeated measures ANOVA). *Foxp2*$^{+/+}$ N/n = 2/8, *Foxp2*$^{S321X/+}$ N/n = 3/11. (e) Same as (d) but for inhibitory PPR, (50, 100, 150, 200, 500ms: *Foxp2*$^{+/+}$ 0.936±0.05, 0.872±0.05, 0.832±0.04, 0.798±0.04, 0.718±0.03 vs *Foxp2*$^{S321X/+}$ 0.983±0.06, 0.973±0.07, 1.016±0.05, 0.947±0.03, 0.894±0.04. *P*<0.001 for 150, 200, 500ms, repeated measures ANOVA). *Foxp2*$^{+/+}$ N/n = 3/23, *Foxp2*$^{S321X/+}$ N/n = 3/20. PPR is normalized to the first pulse. (f) Example trace of postsynaptic inhibitory response to forced vesicle exocytosis during 10 second local application of 500mM sucrose. Scale 2s/50pA. Normalized (to wild-type) current transfer during 10 second sucrose application

(*Foxp2*$^{+/+}$ = 9.1*10^5±1.26*10^5 pA, *Foxp2*$^{S321X/+}$ = 13.4*10^5±1.6*10^{5}pA, *P*<0.01, two-sided students T-test). *Foxp2*$^{+/+}$ N/n = 2/15, *Foxp2*$^{S321X/+}$ N/n = 3/16. **(g)** Example trace of postsynaptic response during 10 second local application of 100µM GABA. Scale 2s/200pA. Quantification of peak amplitude and total current transfer during GABA application (*Foxp2*$^{+/+}$ = 1.00*10^3±66.7pA, *Foxp2*$^{S321X/+}$ = 1.04*10^3±125pA, NS, total current transfer *Foxp2*$^{+/+}$ = 4.8*10^6±3.4*10^{5}pA, *Foxp2*$^{S321X/+}$ = 5.4*10^6±5.5*10^{5}pA, NS, two-sided students T-test). *Foxp2*$^{+/+}$ N/n = 2/14, *Foxp2*$^{S321X/+}$ N/n = 2/12). * *P*<0.05, *** *P*<0.001.

Pharmacological manipulation of inhibition partially rescues motor skill learning deficits in *Foxp2*$^{S321X/+}$ mice

Because aberrant regulation of direct pathway inhibitory activity has been shown to produce motor skill learning deficits (Rothwell et al. 2014; Zhang et al. 2015), we next investigated whether blocking inhibitory activity might be an effective in vivo intervention. One of the most pronounced behavioral deficits displayed by mice with heterozygous mutations in *Foxp2* is decreased motor skill learning, shown by impaired performance on the accelerating rotarod (Groszer et al. 2008; French et al. 2012). Increased inhibition of the direct pathway as demonstrated herein could help explain why *Foxp2* mutations lead to impaired rotarod performance, since successful acquisition of this task is dependent on precise regulation of striatal activity. Cui and colleagues (Cui et al. 2008) showed that increases in presynaptic GABA content cause learning and memory deficits when present in hippocampal neurons. Intriguingly, they found that learning and memory improved dramatically after a low concentration intraperitoneal (IP) injection with picrotoxin (PTX), a compound that blocks $GABA_A$ receptor mediated inhibition (Cui et al. 2008). We therefore hypothesized that a low dose of PTX might be able to ameliorate the motor skill learning deficits present in the *Foxp2*$^{S321X/+}$ mice in a similar manner.

We first validated the presence of motor skill learning deficits in our *Foxp2*$^{S321X/+}$ mice by measuring their performance and learning rate on the accelerating rotarod during 5 consecutive training days and comparing them to littermate controls (Fig 5a, b). The impaired rotarod performance in adult *Foxp2*$^{S321X/+}$ mice that we observed is consistent with previous reports on *Foxp2* heterozygous mutant mice (French et al. 2012; Groszer et al. 2008). Next, we assessed a viable treatment dose. Injection of 1mg/kg PTX produced grand mal seizures in both *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice, whereas both 0.01mg/kg and 0.05mg/kg did not have any effect on rotarod performance (Suppl. Figure 3). An intermediate dose of 0.1mg/kg did not induce seizures, but had a notable negative effect on the rotarod performance of pre-trained wild-type mice (Suppl. Figure 4), whereas the rotarod performance of pre-trained heterozygous mice was not affectWe therefore injected wild-type and *Foxp2*$^{S321X/+}$ mice with 0.1mg/kg PTX 10 minutes prior to each training session and subjected them to the same motor learning paradigm as the vehicle-injected (DMSO) mice. Interestingly, this 0.1mg/kg PTX injection differentially affected rotarod performance of wild-type and *Foxp2*$^{S321X/+}$ mice. Both wild-type and Foxp2$^{S321X/+}$ mice still show an increase in performance and a positive

learning rate during sessions (Fig 5c,d). Treatment with PTX had a profound negative effect on rotarod performance in *Foxp2*$^{+/+}$ mice, whilst in *Foxp2*$^{S321X/+}$ mice, rotarod performance was significantly increased compared to mice without treatment. These opposite effects of PTX treatment resulted in a comparable performance of *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice when comparing average rotarod speed (RPM) at fall from the last 2 trials, (Fig 5e) with PTX treatment. This shows that decreasing inhibitory activity might be a viable method to ameliorate motor deficits induced by decreased expression of Foxp2 and corroborates our data that in mice with reduced Foxp2 expression the E/I balance is shifted towards increased inhibition.

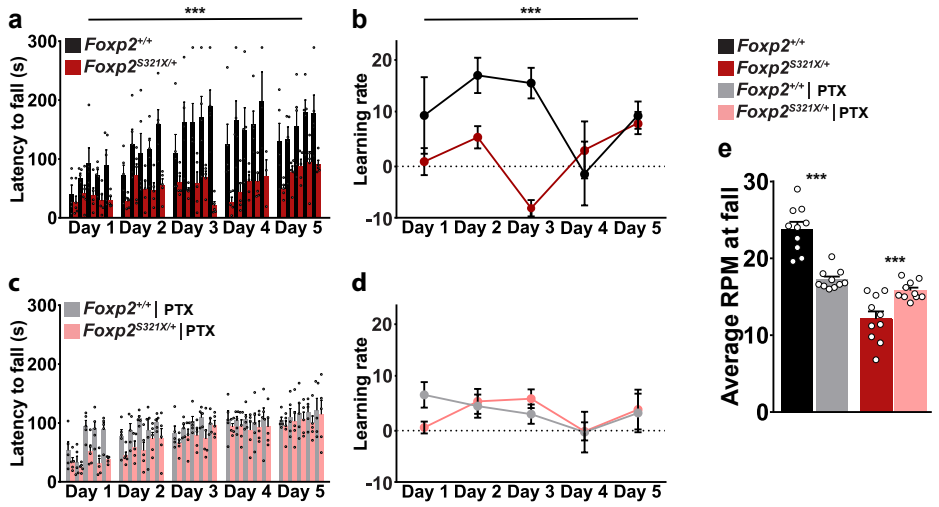

Figure 5: Pharmacological blockade of inhibition modulates rotarod performance and motor skill learning.

(a) *Foxp2*$^{S321X/+}$ mice show impaired motor skill learning, shown by the decreased latency to fall (in seconds) across training sessions (day 1-5: *Foxp2*$^{+/+}$ 75.3±9.7, 120.4±14.4, 164.7±13.6, 165.5±12.2, 160.7±10.8. *Foxp2*$^{S321X/+}$ 34.4±3.1, 52.5±7.4, 53.1±8.7, 55.0±8.15, 82.6±8.38. P<0.01, Repeated measured ANOVA). Each session consists of 5 trials of 5 minutes, during which the rotarod accelerated from 4-40rpm. (b) Both *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice show a positive learning rate during most sessions, with *Foxp2*$^{+/+}$ mice having a significantly higher learning rate (day 1-5: *Foxp2*$^{+/+}$, 10±7.5, 17.8±3.5, 16.3±3.00, -1.4±6.3, 10±2.7. *Foxp2*$^{S321X/+}$, 0.9±2.7, 5.7±2.2, -8.2±1.6, 3.2±5.6, 8.5±2.2. P<0.05, Repeated measures ANOVA), learning rate was calculated as: . (c,d) *Foxp2*$^{+/+}$ mice subjected to 0.1mg/kg intraperitoneal injection of PTX show decreased rotarod performance and learning rates, whereas these were increased in *Foxp2*$^{S321X/+}$ mice (Latency to fall: day 1-5: *Foxp2*$^{+/+}$ 71.7±12.4, 91.7±5.5, 96.4±4.7, 104.4±1.2, 114±3.2. *Foxp2*$^{S321X/+}$ 33.8±4.5, 59.8±5.7, 77.4±5.3, 91.8±1.1, 103.2±3.61. NS Learning rate: *Foxp2*$^{+/+}$ 7.2±2.5, 5±2.3, 3.4±1.9, -0.1±4.0, 3.6±3.8. *Foxp2*$^{S321X/+}$, 0.76±1.2, 5.9±2.5, 6.4±1.9, 0.0±1.8, 4.3±3.9. NS, Repeated Measures ANOVA). (e) Average RPM at which mice fail the accelerating rotarod task during session 4 and 5 in vehicle and PTX conditions (vehicle, *Foxp2*$^{+/+}$ 24.1±0.94 RPM, *Foxp2*$^{S321X/+}$ 12±0.96 RPM, P<0.001. PTX, *Foxp2*$^{+/+}$ 16.7±0.4RPM, *Foxp2*$^{S321X+}$ 15.3±0.372RPM, NS, two-sided students T-test). For all treatment conditions, N = 5 mice. *** *P*<0.001.

DISCUSSION

Mutations in *FoxP2* affect striatal circuitry both in human cases of speech/language disorder and in animal models of *FoxP2* dysfunction (Liegeois et al. 2003; Schulz et al. 2010; Groszer et al. 2008; French et al. 2012). Here we show that Foxp2 affects both excitatory and inhibitory striatal activity in a cell-specific manner during development and in adulthood. Foxp2 is predominantly expressed in striatal direct pathway D1R-MSNs. Decreased Foxp2 expression leads to reduced excitatory activity and increased inhibitory activity in D1R-MSNs. Molecular evidence suggests that the increase in inhibitory activity is due to a de-repression of GAD67 expression. The number of GAD67-positive puncta around the somata of D1R-MSNs increases when Foxp2 expression is reduced, which is accompanied by increased presynaptic GABA content and increased inhibition of the striatal direct pathway. Intriguingly, blocking inhibition with PTX results in a partial rescue of motor skill learning deficits in *Foxp2*$^{S321X/+}$ mice, whereas wild-type littermates show impaired motor skill learning after treatment.

Striatal excitatory connections are formed exclusively by projections from external sources (Hunnicutt et al. 2016). Subpopulations of cortical and thalamic projection neurons form excitatory connections to the striatum (Pan et al. 2010; Hintiryan et al. 2016), and these brain regions contain Foxp2-positive cells as well (Lai et al. 2003; Takahashi et al. 2003; Vargha-Khadem et al. 2005; Hisaoka et al. 2010; Sia et al. 2013). However, it is currently unknown if the cortical and thalamic neurons that express Foxp2 project to the striatum. Our data show that reduced Foxp2 expression decreases D1R-MSN mEPSC amplitude, without influencing mEPSC frequency or excitatory PPR. This suggests that only postsynaptic excitatory strength is affected, and excitatory inputs to the striatum are not affected by reduced Foxp2 expression. Furthermore, the lack of excitatory presynaptic changes in striatal MSNs indicates that excitatory cortical and thalamic cells which do express Foxp2 either do not project to MSNs in the dorsolateral striatum or that Foxp2 has no presynaptic function in these neurons.

Concurrent with the decrease in excitatory activity, we observed an increase in inhibitory activity of striatal D1R-MSNs. Gene ontology analysis following Foxp2-ChIP experiments (Vernes et al. 2011), which groups significantly regulated genes among common biological pathways, has suggested GABA signaling pathways are regulated by Foxp2. Striatal MSNs express both GAD67 and GAD65, two catalytic enzymes involved in the production of GABA (Laprade and Soghomonian 1999). To our knowledge, GAD65 has not been identified as a regulatory target of Foxp2, and mRNA levels of *Gad2* (the gene which codes for GAD65) are unaltered when Foxp2 expression is reduced (French et al. 2007). In contrast, the *Gad1* gene is clearly a regulatory target of Foxp2, shown by ChIP (Vernes et al. 2011), and we show that expression of its protein product GAD67 is increased around striatal D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. Interestingly, increased GAD67 expression levels have been causally linked

to increased presynaptic GABA content (Chao et al. 2010; Hibbert et al. 2004) and enhanced GABA transmission (Krishnan et al. 2015), both of which occur in D1R-MSNs from *Foxp2*$^{S321X/+}$ mice.

Spine formation and excitatory activity in striatal MSNs are affected in homozygous *Foxp2* knockout mice during early postnatal development, possibly through increased Mef2C expression. Mef2C is a transcription factor, which acts as a developmental brake on glutamatergic synapse formation and is regulated by Foxp2 (Chen et al. 2016). However, we show that decreased excitatory activity in D1R-MSNs is present in both juvenile and adult *Foxp2*$^{S321X/+}$ mice. Mef2C expression is virtually absent in adolescent mouse striatum (Chen et al. 2016). This suggests that the decrease in excitatory activity could be caused by impaired generation of glutamatergic synapses during early development, which can have lasting effects on physiology and behavior in adult mice (Harrington et al. 2016). Intriguingly, Mef2C, has been shown to regulate the activity of both excitatory and inhibitory synapses in cortex in a cell-autonomous way (Harrington et al. 2016). Knockout of Mef2C decreased excitation and increased inhibition in cortex, similar to the physiological changes that we show in striatal D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. Dysregulation of striatal Mef2C expression following heterozygous *Foxp2* loss of function could therefore be partially responsible for the striatal E/I imbalance that we measured.

Our findings show that reduced Foxp2 expression disrupts striatal E/I balance, which is dynamically regulated through pre-and postsynaptic mechanisms (Abbott and Nelson 2000; Bolshakov and Siegelbaum 1994; Bi and Poo 1998; Yang and Calakos 2013). Whilst the decreased excitatory activity seems to originate postsynaptically, our data suggest that reduced Foxp2 expression leads to increased presynaptic GABA production. D1R-MSNs form extensive connections toward the substantia nigra (SN), such that reduced activation of D1R-MSNs leads to reduced inhibition of the SN. Increased release of GABA could be a cell-autonomous mechanism to increase the inhibitory drive of D1R-MSN projections toward the SN. However, intra-striatal inhibition is governed by MSNs as well: D1R-MSNs project to other D1R-MSNs (Taverna et al. 2008). This means that a feedback loop could occur to increase inhibitory drive, which would result in stronger inhibition of striatal D1R-MSNs. Paired recording of striatal D1R-MSNs in *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ could help to determine whether such a feedback loop is present and if such a mechanism can negate the effect of the increased presynaptic GABA production in presynaptic terminals within the SN.

Furthermore, the striatal E/I imbalance following reduced Foxp2 expression is maintained throughout development and in adult mice. This can explain why impaired striatal plasticity and motor skill learning deficits are present in adult mice with heterozygous *Foxp2* mutations (French et al. 2012; Groszer et al. 2008). Interestingly, in a mouse model for neuroligin-3

(NL-3) dysfunction, known to produce similar behavioral and physiological phenotypes as mutation of *Foxp2*, adult re-expression of NL-3 rescues motor skill learning deficits (Rothwell et al. 2014). Restoration of E/I balance in adulthood could therefore be a viable strategy to ameliorate the motor learning deficits observed upon reduced Foxp2 expression. Modulation of GABAergic activity using $GABA_A$ antagonists has been shown to improve learning and memory in mouse models for cognitive disorders (Rueda et al. 2008; Cui et al. 2008) and phase I clinical trials are underway to test $GABA_A$ antagonists on people with Down syndrome (Contestabile et al. 2017). We show that modulation of GABAergic activity by partially blocking inhibitory activity increases motor skill learning in *Foxp2*$^{S321X/+}$ mice. Intriguingly, wild-type mice were adversely affected by the same PTX treatment, which indicates that successful modulation of GABAergic activity might be highly dose dependent.

Taken together, we show for the first time that reduced Foxp2 expression bidirectionally affects both excitatory and inhibitory activity of striatal direct pathway MSNs, throughout development as well as in adult mice. Partially blocking inhibitory activity in vivo might restore this E/I imbalance, and we found that this intervention had a positive effect on motor skill learning in mice with reduced Foxp2 expression. Restoring the E/I balance by pharmacologically modulating inhibitory activity might be a feasible therapeutic intervention for complex motor disorders.

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MATERIALS & METHODS

Mouse lines

The experimental procedures were approved by the Animal Ethics Committee of the Radboud University Nijmegen, under DEC application number 2014-098 (Nijmegen, the Netherlands) and conducted in accordance with the Dutch legislation. Every effort was made to minimize animal discomfort and the number of animals used.

The *Foxp2*-S321X line was maintained on a C57BL/6J background, and heterozygotes and wildtype littermates between PND11 and PND17 (juvenile) or PND55-65 (adult) were used for the immunofluorescent stainings and electrophysiological recordings. The generation, marker-assisted backcrossing and genotyping of this strain are fully described in (Groszer et al. 2008; Keays et al. 2006; Coghill et al. 2002). BACtrap mice carrying GFP under the D1R promoter (D1R-GFP) or D2R promoter (D2R-GFP) were originally generated by the GENSAT (Gene Expression Nervous System Atlas) (Gong et al. 2003) and backcrossed to C57BL6/J mice.

Electrophysiology

Experiments were conducted on 350µm thick coronal slices. Mice (PND11-17 or PND55-65) were sacrificed by decapitation following isoflurane anesthesia. Slices were cut using a vibratome (HM650V Thermo Scientific) in cooled (4°C) artificial cerebrospinal fluid containing (in mM): 87 NaCl, 11 Glucose, 75 Sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$), continuously oxygenated with 95%O2/5%CO2. Collection of slices started when the striatum became visible and slices were collected until the hippocampus was visible. After collection, slices were incubated at 32°C in oxygenated ACSF for at least 1h before recording. Slices were transferred to the recording setup 10 minutes prior to recording and incubated in recording ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 Glucose, continuously oxygenated and heated to 32°C. Patch pipettes (3.5 – 5.5 MΩ) were made from borosilicate glass capillaries and filled with intracellular solution containing: 115 CsMeSO3; 10 CsCl; 10 HEPES; 2.5 MgCl2; 4 Na2ATP; 0.4 NaGTP; 10 Na-Phosphocreatine; 0.6 EGTA, 10 QX-314. Activity was recorded using a Digidata 1440A digitizer and a Multiclamp 700B amplifier (Molecular Devices). Sampling rate was set at 20KHz and a lowpass 1KHz filter was used during recording. All recordings were conducted in the dorsolateral quadrant of the striatum.

Miniature postsynaptic currents

mEPSCs were recorded in the prescience of Tetrodotoxin (TTX, 1µM, Tocris) and Picrotoxin (PTX, 100µM, Tocris) at a holding voltage of -60mV. mIPSCs were recorded in the presence of

Tetrodotoxin (TTX, 1µM, Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 µM, Tocris) and (2R)-amino-5-phosphonovaleric acid (APV, 100 µM, Tocris) at a holding voltage of +10mV.

GABA/AMPA ratio

All stimulation experiments were conducted by stimulation of afferent corticostriatal and intrastriatal axons using a bipolar concentric stimulus electrode (FHC, Bowdoin, Maine) placed in the dorsolateral striatum. GABA/AMPA ratio was measured in the presence of APV (100µM). Cells were voltage-clamped at -60mV and a 1ms stimulus from a bipolar tungsten electrode was given to record the AMPA response. Subsequently cells were clamped at 0mV and the GABA response was measured.

Paired Pulse ratio

Excitatory PPR was measured in the presence of PTX (100µM) and APV (100µM) with voltage clamped at -60mV. Inhibitory PPR was measured in the presence of CNQX (5µM) and APV (100µM) with voltage clamped at -60mV. Stimulation strength was set to evoke an approximately 200 pA response to the first stimulus. Two 1 ms pulses were given with a 50 ms, 100 ms, 150 ms, 200 ms, 500 ms or 9000 ms (inhibitory PPR only) interval. PPR was calculated as the peak2/peak1 ratio after correcting for any residual current at the second pulse.

GABA

Vesicle depletion

1ms pulses were given at 10Hz for 10 seconds to entirely deplete the presynaptic GABA vesicle pool. After each 10 second stimulus train, cells were given 0.2Hz stimulations for 40 seconds to assess the recovery of the vesicle pool between each stimulus train. One recording consisted of 10 consecutive stimulus trains. Cells were recorded in the presence of CNQX and at a holding voltage of -60mV.

Compound application

Sucrose (500mM) or GABA (20µM) was applied using a pressure ejection system (PDES-2DX, NPI, Tamm, Germany). The injection pressure was set to 5psi/0.4 bar and injection duration was set to 10 seconds. Interinjection interval was set to 1 minute. Compounds were delivered using a micropipette positioned at 30µm from the target cell soma.

Immunofluorescence

Animals were sacrificed by decapitation and whole mouse brain was fixed in 4% paraformaldehyde (PFA) / 4% sucrose for 24 hours. 60 µm coronal sections including the striatum were cut using a vibratome (Leica VT1000S, Leica microsystems). Slices were transferred to 1x Phosphate buffered saline (PBS) for immunofluorescent staining. The

following antibodies were used: FoxP2 (Santa Cruz Sc-21069, 1:500), GAD67 (Millipore MAB5406, 1:200). Imaging was done using a Zeiss upright fluorescent microscope with apotome (Zeiss Axio Images, Oberkochen, Germany) using a 63X oil immersion objective. Images were analyzed offline using FIJI (Fiji is just imageJ) image analysis software.

Intraperitoneal injection

Foxp2$^{S321X/+}$ mice and wild-type littermate controls were injected intraperitoneally with either vehicle (DMSO) or 0.1mg/kg picrotoxin (Tocris, Bristol, UK). Injection was done by hand and mice were placed back in their home cage for 10 minutes following injection, after which mice were placed on the accelerating rotarod.

Accelerating rotarod

Foxp2$^{S321X/+}$ mice (6-8 weeks old) and wild-type littermate controls were placed on an accelerating rotarod (LE8200, Harvard apparatus) which increased rotation speed from 4 r.p.m. to 40 r.p.m. over a 5-minute period. Mice were trained for 5 consecutive days, with 5 trials per day. Latency to fall (in seconds or RPM at fall) was scored, and mice were placed back in their home cage for 5 minutes between trials.

Western blot

PND10-15 *Foxp2*$^{S321X/+}$ animals and wild-type controls were sacrificed by decapitation. The striatum was dissected from separated hemispheres, frozen in liquid nitrogen and kept at -80°C. Samples were homogenized in 200 µl of lysis buffer (50 mM Hepes pH 7.4, 140 mM NaCl, 0.1% Triton-X100, 1% Tween 20, 0.1% deoxycholate) containing protease inhibitor mix (Roche Diagnostics). Protein levels were assessed using BCA. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) at 200V for 30 minutes was carried out using a Mini-Protean system (Bio-Rad, USA). Protein (50 µg) was loaded in each lane with loading buffer (0.25 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.25% bromophenolblue, 4% beta-mercaptoethanol). After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (PVDF, Amersham, Hybond-P), using an electrophoretic transfer system (Bio-Rad, USA). The membranes were then blocked with 5**%** skimmed milk dissolved in TBS-tween 0.1% for one hour. The membranes were incubated overnight at 4 °C with the primary antibodies diluted in blocking buffer containing 1% skimmed milk dissolved in a TBS-Tween. The primary antibodies were the following: mouse monoclonal anti- bodies GAD67 (1:1000, Abcam), and GAPDH as a control (1:1000, Cell signaling). After being washed for one hour with 1% skimmed milk in TBS-T (0.05%), the membranes were incubated for one hour in the dark at room temperature with goat-anti-mouse secondary antibody (1:5000; Bio-Rad, Goat-anti-mouse HRP conjugated). The membranes were imaged using a Chemidoc Touch imaging system (Bio-rad, Hercules, CA) and the generated pictures were quantified using ImageJ

software. The levels of protein expression were normalized to GAPDH. Protein expression values are normalized to *Foxp2*$^{+/+}$ expression (relative intensity).

Statistics

Sample size was calculated assuming power of 0.8 and effect size d=0.8, data are acquired from at least 3 mice for each genotype. All data are shown as mean ± SEM. Analysis between two groups was done using students' T-test, analysis between multiple groups using repeated measures ANOVA. All statistical analysis was conducted in PRISM (Graphpad PRISM 7.00, Graphpad Software, San Diego, CA).

Acknowledgements

We thank Nathaniel Heintz and Myriam Heiman for kindly providing D1R-GFP and D2R-GFP BACtrap mice. We are grateful to Pelagia Deriziotis for backcrossing of the mouse lines used for this study. JRvR is supported by an award from the Donders institute PhD program, awarded to NNK and SCV. SCV is supported by a Marie Curie Career Integration Grant (PCIG12-GA-2012-333978) and by a Max Planck Research Group Award. SEF is supported by the Max Planck Society.

Contributions

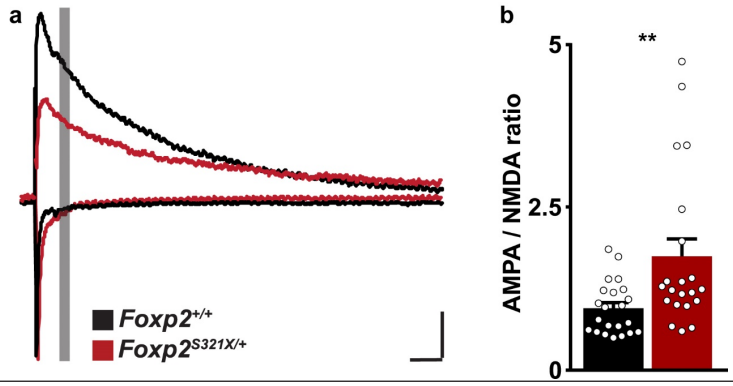
SCV, NNK conceived and supervised the study; SCV, NNK and SEF provided resources; JRvR, SCV, NNK designed the experiments; JRvR performed all experiments; JRvR, SEF, SCV, NNK wrote the manuscript

Compliance with ethical standards

JRvR is supported by an award from the Donders institute PhD program, awarded to NNK and SCV. SCV is supported by a Marie Curie Career Integration Grant (PCIG12-GA-2012-333978) and by a Max Planck Research Group Award. SEF is supported by the Max Planck Society.
The authors declare no competing financial interests,

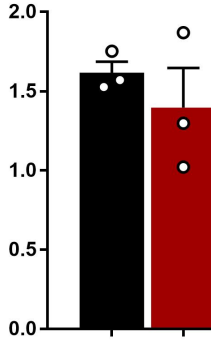
The experimental animal procedures were approved by the Animal Ethics Committee of the Radboud University Nijmegen, under DEC application number 2014-098 (Nijmegen, the Netherlands) and conducted in accordance with the Dutch legislation. Every effort was made to minimize animal discomfort and the number of animals used. No human participants were used in this study.

SUPPLEMENTARY FIGURES



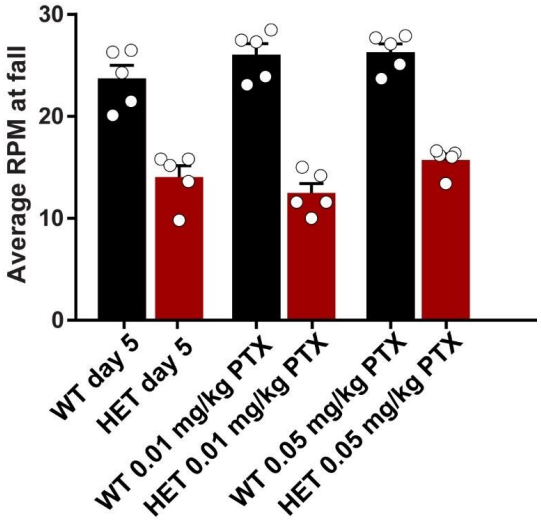
Supplementary figure 1 AMPA/NMDA ratio is increased in *Foxp2*$^{S321X/+}$ juvenile mice (PND11-13).

(a) Example traces of AMPA/NMDA measurement by stimulation at -60mV (AMPA) and +40mV (NMDA) in the presence of PTX to block GABAergic neurotransmission. The AMPA response was measured as the peak response, whereas the NMDA response was measured as the average (pA/ms) of the area between 60-65ms after the AMPA peak (grey shaded area). (b) AMPA/NMDA ratio (*Foxp2*$^{+/+}$ 0.94±0.09 N/n = 3/22, *Foxp2*$^{S321X/+}$ 1.75±0.27 N/n = 3/22). Scalebar = 100ms/50pA, ** = P<0.01. All data is reported as mean ± SEM. N/n = animals/cells



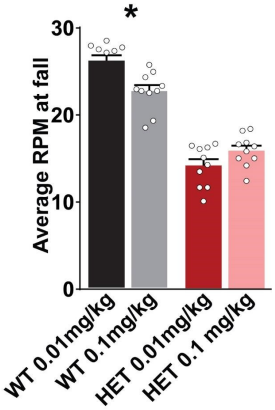
Supplementary Figure 2: The number of GAD67 puncta surrounding D2R-MSN somata is not changed.

We counted GAD67 positive puncta surrounding D1R-negative (putative D2R) MSNs. No differences between the number of GAD67 puncta per soma were found between *Foxp2*$^{+/+}$ or *Foxp2*$^{S321X/+}$ mice. (*Foxp2*$^{+/+}$ = 1.6±0.04 puncta/D2R-MSN soma, *Foxp2*$^{S321X/+}$ 1.4±0.136 puncta/D2R-MSN soma, NS, two-sided students T-test). *Foxp2*$^{+/+}$ N/n = 3/8, *Foxp2*$^{S321X/+}$ N/n = 3/9. N/n = mice/brain slices



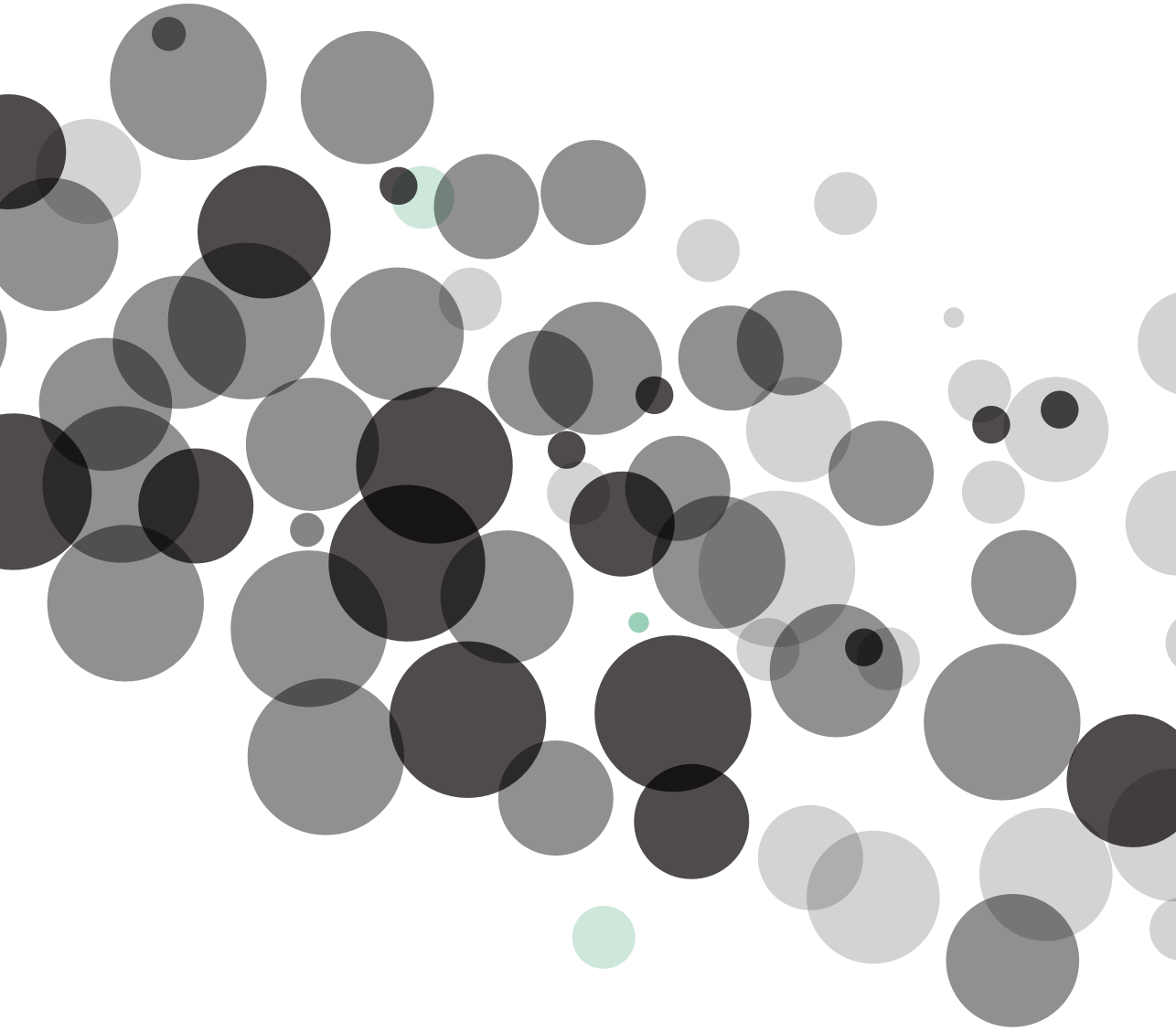
Supplementary Figure 3. Low dose (0.01 or 0.05 mg/kg) IP PTX injection does not affect rotarod performance of *Foxp2*$^{+/+}$ or *Foxp2*$^{S321X+}$ mice.

The last trial day (day 5) and 2 subsequent days (0.01mg/kg injection or 0.05 mg/kg) are shown. Average RPM at fall is not affected by injection and not different between experimental days (*Foxp2*$^{+/+}$ 23.6±1.28, 25.9±2.4, 26.2±0.81 N.S. *Foxp2*$^{S321X/+}$ 14±1.13, 12.5±0.92, 15.7±0.59, NS, 2 Factor ANOVA, factors experimental day and PTX treatment). N = 5.



Supplementary Figure 4. Pre-trained *Foxp2*$^{S321X/+}$ mice are not affected by PTX injection.

Average RPM at fall for pre-trained mice injected with either 0.01mg/kg PTX or 0.1mg/kg PTX. (*Foxp2*$^{+/+}$ 25.9±0.63, 23.2±0.71, P<0.05, *Foxp2*$^{S321X/+}$ 12.5±0.75, 14.04±0.58, NS, two-sided students T-test). N = 5.



3.

A fast shift from striatal Calcium-permeable AMPAR to Calcium-impermeable AMPAR expression coincides with the start of coordinated locomotion and is delayed in heterozygous *Foxp2* loss of function mice

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In preparation for submission

ABSTRACT

Maturation of excitatory synapses during postnatal development is highly spatiotemporally regulated. Heterozygous loss of *Foxp2* function leads to impaired striatal development as well as deficits in excitatory spine maturation. Furthermore, excitatory activity is reduced in these mice during development and in adulthood. Adult mice with heterozygous *Foxp2* mutations show motor skill learning deficits, however, no behavioral phenotype in juvenile animals with heterozygous loss of *Foxp2* function has been described. We describe a fast shift from immature calcium-permeable to mature calcium-impermeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) between postnatal day 9 and 11 in control animals. Critical period maturation of excitatory dorsal striatal synapses is thought to coincide with the start of coordinated locomotion. AMPAR maturation coincided with an increase in performance on the hanging bar test, a behavioral task which requires coordinated locomotion. AMPAR maturation and hanging bar performance are impaired in juvenile mice with heterozygous loss of *Foxp2* function mutations. Furthermore, we show that AMPAR trafficking and long-term depression (LTD) are impaired in adolescent heterozygous mice, which could contribute to the delayed AMPAR maturation. Taken together, our data show that AMPAR maturation is highly temporally regulated in the dorsal striatum and that this maturation is delayed in mice with a heterozygous loss of *Foxp2* function, affecting the initiation of coordinated locomotion during postnatal development in these mice.

INTRODUCTION

Rapid changes in synapse physiology and neuronal connectivity lead to the formation of synapses with mature receptor expression and mature neuronal activity (Lee and Sawatari, 2011; Zhang et al., 2011). A large proportion of synaptic maturation is dependent on external input during tightly constrained time windows known as critical periods in early postnatal life (Bassani et al., 2013; Kilb, 2012; Mackowiak et al., 2014). These critical periods coincide with developmental milestones. For example, the critical period for visual cortex maturation in mice coincides with eye opening (for review, see: (Hensch, 2005)). Aberrant temporal specificity of changes in synapse physiology during critical periods has been implicated in cognitive disorders such as autism and intellectual disability (Krishnan et al., 2015; Peixoto et al., 2016). Currently, there is only tentative evidence that similar critical periods are involved in the maturation brain circuits involved in complex movement.

The striatum is the principal brain region where excitatory inputs from cortex and thalamus, as well as dopaminergic inputs from the substantia nigra are integrated (Hunnicutt et al., 2016). This complexity of striatal connections has implicated it as a central hub involved in motor control (Kravitz and Kreitzer, 2012). It is known that striatal medium spiny neurons (MSNs) mainly express calcium permeable AMPA receptors (CP-AMPARs) that consist of homomers of the GluA1 subunit, during early postnatal development (figure 1a,b). During neuronal maturation, CP-AMPARs are replaced by calcium impermeable AMPA receptors (CI-AMPARs), that consist of heteromers of GluA1, and GluA2 or GluA3 (Henley and Wilkinson, 2016). In adult mice striatal MSNs predominantly express CI-AMPARs at the synapse (Bellone et al., 2011) (Fig. 1b). CP-AMPARs show inward rectification of responses to stimulation at positive membrane voltages, whilst CI-AMPARs show a linear relationship between responses to stimulation at negative and positive holding voltages (Figure 1c). Expression of CI-AMPARs is essential for mature excitatory striatal activity (Isaac et al., 2007). The expression of CI-AMPARs is essential for the ability of adult mice to perform complex motor tasks, as mice which lack the GluA2 subunit are highly impaired in coordinated locomotion (Christie et al., 2010; Jia et al., 1996). The shift from CP-AMPAR to CI-AMPAR expression at striatal synapses could be dependent on locomotion behavior. Maturation of striatal synaptic activity has been shown to coincide with the start of coordinated locomotion, which occurs between postnatal day 9-11 in mice (Dehorter et al., 2011). Furthermore, it is known that the shift from CP-AMPAR to CI-AMPAR expression occurs between the second and fourth postnatal week (Bellone et al., 2011). However, a more detailed time course of the shift between CP- and CI-AMPARs and the relation to the start of locomotion could further inform us how striatal maturation enables the emergence of coordinated locomotion.

Modulation of striatal circuit activity affects complex motor sequencing (Boecker et al., 2008; Desmurget and Turner, 2010; Kawashima et al., 2012) and is involved in the generation of speech (Ackermann et al., 2014; Ziegler and Ackermann, 2013). Impaired basal ganglia activity has been shown in speech disorders (Redle et al., 2015; Wildgruber et al., 2001), including childhood apraxia of speech (CAS). CAS is a condition characterized as 'a disorder of speech motor programming or planning that affects the production, sequencing, timing and stress of sounds' (Morgan et al., 2017). A genetic cause for CAS was discovered in a family in which more than half of the family members are affected with CAS. This family, known as the KE-family, shows heterozygous mutation by a single amino-acid change (R553H) of the DNA-binding domain of the transcription factor FOXP2 (Lai et al., 2001). (f)MRI investigation of affected individuals from the KE family, and comparison with unaffected family members and controls, has shown that striatal morphology and activity are affected by heterozygous mutation of *FOXP2* (Liegeois et al., 2003; Watkins et al., 2002). Furthermore, animal models in which Foxp2 function is disrupted have shown that striatal development and function is impaired. In mice with heterozygous loss of *Foxp2* function mutations synaptic maturation and excitatory activity are impaired (Chen et al., 2016; van Rhijn et al., 2018). Furthermore, FoxP2 has been shown to regulate genes involved in synapse formation such as SRPX2 (Sia et al., 2013) and VLDLR (DiBattista et al., 2015; Mendoza and Scharff, 2017) and mice with heterozygous mutations of *Foxp2* display aberrant motor skill learning as adults (French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018). The reduced striatal excitatory activity remains present in adult mice (van Rhijn et al., 2018), concomitant with a reduction in AMPAR expression in striatal MSNs (Chen et al., 2016). Currently, only in adult mice with heterozygous mutations in *Foxp2* motor skill learning impairments have been described. However, the morphological and physiological phenotypes suggest synapse development and maturation of striatal MSNs might be affected by mutations in *Foxp2*. These impairments could affect motor coordination at an early age. This makes Foxp2 dysfunction an exciting opportunity to investigate striatal AMPAR maturation and to link regulation of striatal excitatory activity to the emergence of coordinated locomotion.

We investigated the shift from CP-AMPAR to CI-AMPAR expression during early juvenile (PND 7-17) development and found that this shift happens between PND 9 and 11 in wild-type animals. We next used a *Foxp2* loss of function mouse model in which a STOP codon is inserted in exon 7 (S321X) to investigate if Foxp2 is involved in AMPAR maturation. In $Foxp2^{S321X/+}$ mice, the CP-AMPAR to CI-AMPAR shift is both more gradual and delayed compared to $Foxp2^{+/+}$ mice. We show that impaired AMPAR trafficking could underlie this delay, as internalization of CP-AMPARs and mGluR1 mediated LTD induction are impaired in $Foxp2^{S321X/+}$ mice. Lastly, we show AMPAR maturation coincides with the emergence of coordinated locomotion and that juvenile $Foxp2^{S321X/+}$ mice show reduced performance on a task which necessitates coordinated locomotion. Our data show for the first time that the emergence of coordinated

locomotion correlates with a shift from CP-AMPAR to CI-AMPAR expression and that Foxp2 is involved in striatal synaptic maturation and early coordinated locomotion by regulating AMPAR subtype expression.

RESULTS

Rectification index measures relative abundance of CP- and CI-AMPARs

AMPAR subunit composition shifts between immature and mature excitatory synapses. Immature synapses mostly contain CP-AMPARs, which are homomeric receptors comprised exclusively of the GluA1 subunit (Figure 1A-B) (Chan et al., 2003; Henley and Wilkinson, 2016). During development, these CP-AMPARs are replaced by CI-AMPARs which consist of heteromers of GluA1 and GluA2 (Figure 1a-b).

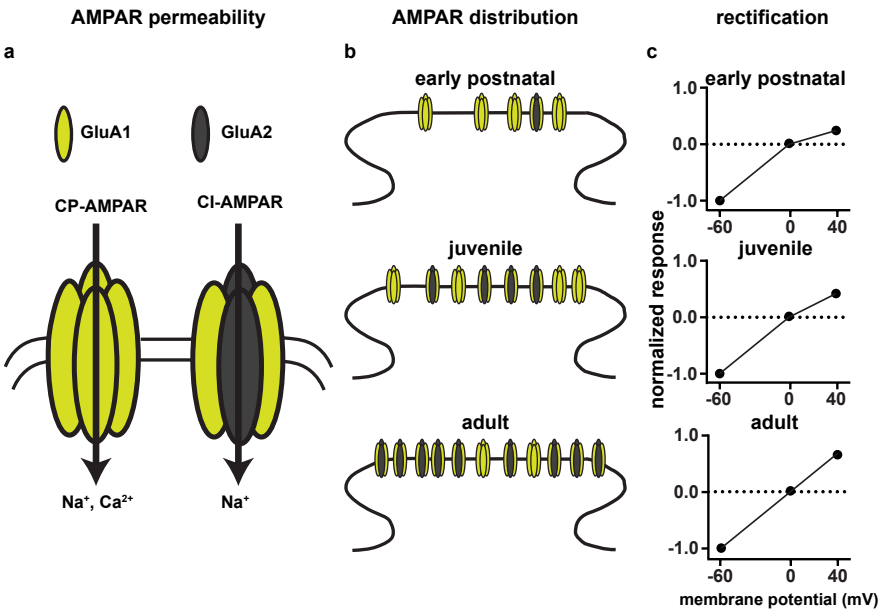

Figure 1: schematic overview of AMPAR development and GluA subunit properties.

(a) Overview of the differences between CP and CI-AMPARs. CP-AMPARS consist of homomeric GluA1 subunits are permeable to both sodium and calcium. CI-AMPARs include GluA1 and GluA2 subunits and are only permeable to calcium. (b) AMPA receptor expression in striatal excitatory postsynapses during different stages of development. Low abundance of CI-AMPAR expression and a high expression of CP-AMPARs early in development. During development, relatively more CI-AMPARs are expressed. Later in development and in adulthood most AMPARs are CI CI-AMPARs. (c) Inward rectification at positive membrane potentials during development. Early in development there is a strong inward rectification at positive membrane potentials (top). This inward rectification reduces (middle) during development and is no longer present during adulthood (bottom).

The ratio between CP- and CI-AMPARs can be calculated by the rectification index (RI). Rectification leads to a reduced response at positive membrane potentials (Figure 1c and methods). CP-AMPARs are responsible for this inward rectification at positive membrane voltages, as these are sensitive to polyamine block at positive membrane potentials, the method commonly used to investigate AMPAR composition (Bowie and Mayer, 1995; Donevan and Rogawski, 1995). By contrast, CI-AMPARS do not show inward rectification, instead they display similar responses to stimulation at negative and positive membrane potentials. Therefore, a RI larger than 1.0 indicates more synaptic CP-AMPARs, whilst a synapse which almost exclusively harbors CI-AMPARs has a RI of approximately 1.0 (Bellone et al., 2011).

AMPAR maturation is delayed in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice

We investigated how temporally constrained the shift in GluA subunit expression is in the striatum and if disruptions in Foxp2 might affect AMPAR maturation. Foxp2 is predominantly expressed in the striatal cell population which expresses the Dopamine type 1 receptor (D1R-MSNs) (van Rhijn et al., 2018; Vernes et al., 2011). In juvenile *Foxp2*$^{+/+}$ mice of postnatal day (PND) 7-9, immature RI is visible. However, already by PND 11-13 mature RI values of approximately 1.0 are measured in D1R-MSNs of *Foxp2*$^{+/+}$ mice. RI remains stable further during development, and in early adulthood (Figure 2a). By contrast, *Foxp2*$^{S321X/+}$ mice show a delay in the shift from CP-AMPAR to CI-AMPAR expression. RI values are equally high in PND 7-9 *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. However, at PND 11-13, the RI remains significantly higher in *Foxp2*$^{S321X/+}$ mice compared to wild-type littermates (Figure 2b compared to d). Despite the delay in AMPAR maturation, the RI in *Foxp2*$^{S321X/+}$ mice is comparable to the RI in wild-type mice by PND15 and values remain similar into adulthood. In comparison to the RI developmental profile shown by D1R-MSNs from *Foxp2*$^{+/+}$ mice, D1R-MSNs from *Foxp2*$^{S321X/+}$ show a much more gradual change in RI (Figure 2e). Whilst RI is significantly decreased between PND 7-9 and PND 11-13 in D1R-MSNs from *Foxp2*$^{+/+}$ mice, this decrease is only significant in *Foxp2*$^{S321X/+}$ mice when comparing PND 7-9 and PND 15-16.

To further corroborate our data, we used the CP-AMPAR selective antagonist 1-Naphthyl acetyl spermine trihydrochloride (NASPM) to extracellularly block CP-AMPARs (Figure 3a). At PND 11-13 D1R-MSNs from *Foxp2*$^{+/+}$ mice show a RI of 1.0, which suggests CP-AMPARs are absent, whilst the increased RI of D1R-MSNs from *Foxp2*$^{S321X/+}$ suggests CP-AMPARs still remain at the synapse. Blockade of CP-AMPARs should therefore not affect the response to stimulation in D1R-MSNs of *Foxp2*$^{+/+}$ mice, whilst it would reduce stimulus-response in *Foxp2*$^{S321X/+}$ mice. We bath applied NASPM to striatal slices of PND 11-13 mice and measured stimulus evoked response before, during and after NASPM application. Stimulus response was not reduced in D1R-MSNs of *Foxp2*$^{+/+}$ mice, but a reduction in response strength of 35% was seen in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice during NASPM application (Figure 3b, c). This further indicates CP-AMPARs are still present to a high degree in D1R- MSNs of *Foxp2*$^{S321X/+}$ mice, in contrast to *Foxp2*$^{+/+}$ mice in which CP-AMPARs have been exchanged for CI-AMPARs by PND 11.

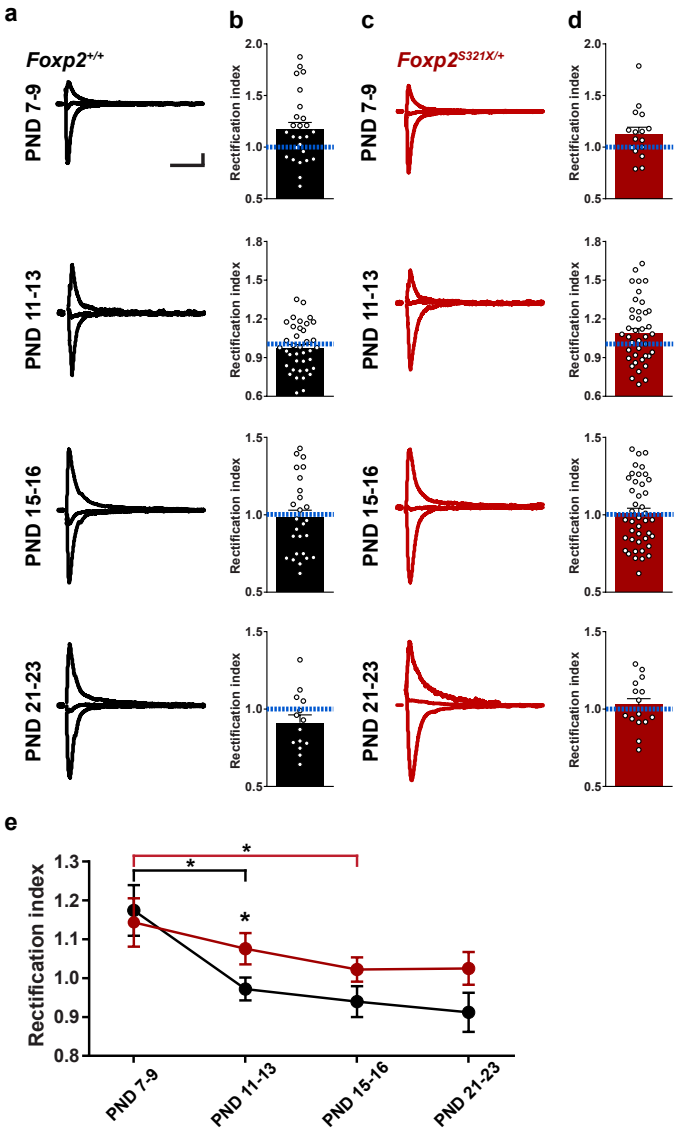

Figure 2: AMPA receptor maturation is delayed in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice.

(a) Representative traces of response to stimulation at -60, 0 and +40 mV during development in *Foxp2*$^{+/+}$ mice. (b) Rectification index: *RI* $\frac{\text{(peak current (-60mV))}}{\text{(peak current (+40mV))}}$ / 1.5 of D1R-MSNs of juvenile *Foxp2*$^{+/+}$ mice during development. (c), same as (a) but for *Foxp2*$^{S321X/+}$ mice. (d) same as (b) but for *Foxp2*$^{S321X/+}$ mice. scale bar in (a,c) 60ms/20pA Average RI values during development between genotypes: (*Foxp2*$^{+/+}$ / *Foxp2*$^{S321X/+}$ PND7-9 RI=1.18±0.07 / 1.13±0.06, NS, 11-13 RI=0.97±0.03 / 1.09±0.04, *P<0.05*, 15-16 RI=0.98±0.05 / 1.01±0.03 NS, 21-23 RI=0.91±0.05 / 1.03±0.04 NS). (e) Average RI during development in D1R-MSNs of *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. (Compare *Foxp2*$^{+/+}$ PND7-9 and PND 11-13: 1.18±0.07 / 0.97±0.03, *P<0.05*. Compare *Foxp2*$^{S321X/+}$ PND 7-9 and. PND 15-16: 1.13±0.06 / 1.01±0.03, *P<0.05.* all other comparisons between ages are NS) * = P<0.05. Statistical analysis of data in (e) by multiple ANOVA.

Regulation of CP-AMPAR expression is impaired in *Foxp2*$^{S321X/+}$ mice

As maturation of AMPAR expression is an active process which requires molecularly regulated AMPAR trafficking at the synapse, we next investigated the molecular mechanisms related to AMPAR trafficking that could be affected by heterozygous loss of Foxp2 function. One of the described mechanisms by which AMPARs are cycled at the synapse is through metabotropic glutamate receptor (mGluR) mediated endocytosis (Kelly et al., 2009; Sung et al., 2001; Zhang et al., 2008). In the dorsolateral striatum, this is governed through mGluR1, activation of which has modulatory effects on AMPAR expression depending on the brain region (Luscher and Huber, 2010; Wolf and Tseng, 2012). Commonly, mGluR1 activation induces LTD through a change in the CI:CP AMPAR ratio.

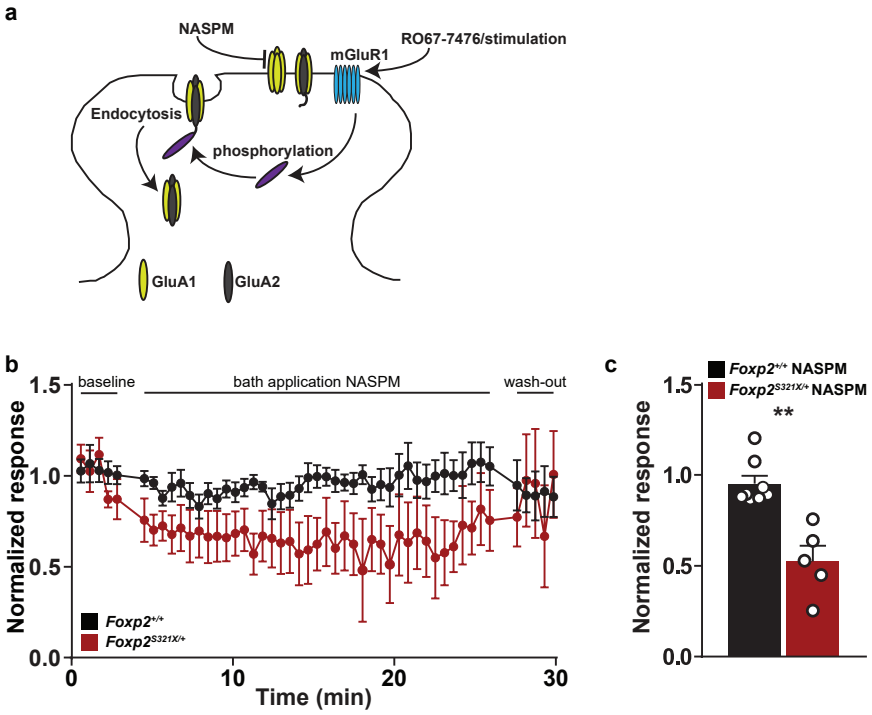

Figure 3: AMPAR block by NASPM in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice.

(a) Schematic overview of molecular mechanisms investigated in Figures 3 and 4. NASPM specifically blocks GluA1 receptors extracellularly, which impairs CP-AMPAR function. RO67-7476 is an mGluR1 specific agonist. mGluR1 activation leads to increased endocytosis of GluA2 containing AMPA receptors in striatal medium spiny neurons (see figure 4). (b,c) Bath application of NASPM reduces response to stimulation in D1R-MSNs of *Foxp2*$^{S321X/+}$ whereas it has no effect on D1R-MSNs of *Foxp2*$^{+/+}$ mice (average response during NASPM bath application *Foxp2*$^{+/+}$ = 0.95±0.04, N/n=3/8, *Foxp2*$^{S321X/+}$ NASPM = 0.52±0.08, N/n = 3/5, P<0.01. Normalized to baseline before NASPM application). N/n = mice/cells. ** = P<0.01 Statistical comparison by nonparametric Mann-Whitney U test.

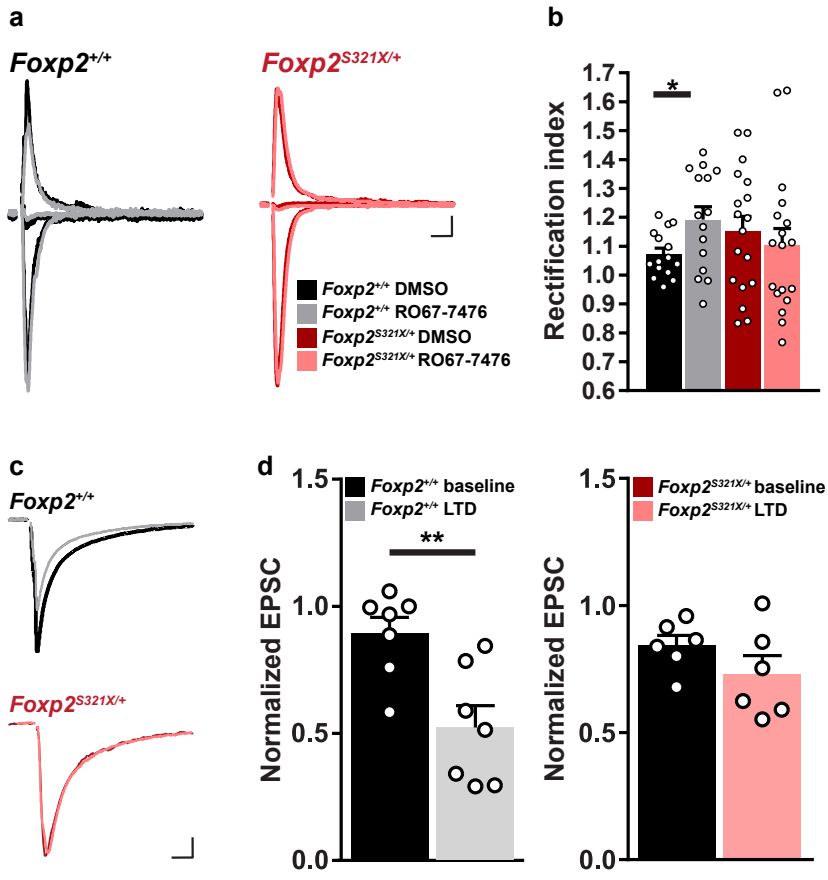

Figure 4: mGluR1 mediated endocytosis of AMPARs is impaired in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice

Endocytosis of CI-AMPARs leads to a relative increase in expression of CP-AMPARs, which results in an increase of the rectification index to values >1.0. (a) Representative traces of response to stimulation at -60mV, 0mV and +40mV in sham (DMSO) and RO67-7476 bath application, scale bar 20ms/20pA. (b) Application of the mGluR1 agonist RO67-7476 leads to endocytosis of CI-AMPARs in D1R-MSNs of *Foxp2*$^{+/+}$ mice, but has no effect on D1R-MSNs of *Foxp2*$^{S321X/+}$ mice (rectification index *Foxp2*$^{+/+}$ DMSO compared to RO67-7476: 1.072± 0.08 vs. 1.225±0.214, *Foxp2*$^{S321X/+}$ DMSO compared to RO67-7476: 1.153± 0.05 vs 1.104±0.06). (c) LTD cannot be induced in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. (c) Example trace of the response to stimulation before (dark line) and after (light line) LTD induction. (d). Average normalized response to stimulation (averaged to baseline before LTD) is reduced in D1R-MSNs *Foxp2*$^{+/+}$ mice after theta burst stimulation, whilst there is no different in response strength in D1R-MSNs from *Foxp2*$^{S321X/+}$ mice. (Foxp2$^{+/+}$ baseline 0.89±0.07, LTD 0.52±0.09, *P<0.05*. Foxp2$^{S321X/+}$ baseline 0.84±0.04, LTD 0.73±0.07, NS) * = *P<0.05*, Student's T-test

For example, in the ventral tegmental area (VTA), mGluR1 activation leads to endocytosis of CP-AMPARs and insertion of CI-AMPARs (Bellone et al., 2011). As CI-AMPARs are less conductive compared to CP-AMPARs, this results in LTD. However, in the dorsal striatum the effect of mGluR1 activation on CI:CP AMPAR ratios is unclear. It is suggested that mGluR1 agonist application in dorsal striatum induces endocytosis of CI-AMPARs (Figure 3a) (Ahn and Choe, 2010), in contrast to CP-AMPAR endocytosis in the VTA. We first investigated if mGluR1 activation in dorsal striatum indeed leads to changes in the CI:CP-AMPAR ratio, by measuring the effect of the synthetic agonist RO67-7476 on RI.

Our results show that bath application of RO67-7476 leads to an increase in RI in D1R-MSNs of wild-type mice (Figure 4a). The increase in RI upon mGluR1 activation suggests a relative increase in the abundance of CP-AMPARs at striatal excitatory synapses. This could be either through insertion of CP-AMPARS, endocytosis of CI-AMPARs, or an exchange of CI-AMPARs for CP-AMPARs. However, in *Foxp2*$^{S321X/+}$ mice this increase in RI was absent, which suggests that mGluR1 mediated AMPAR trafficking is impaired or occluded in these mice (Figure 4b). Impairments in AMPAR trafficking could mean that both endocytosis and/or insertion of AMPARs are impaired. However, occlusion would mean that mGluR1 activation normally leads to insertion of additional CP-AMPARs at the synapse, which in the case of the already high CP-AMPAR expression in *Foxp2*$^{S321X/+}$ mice would not additionally increase RI.

As CP-AMPARs are more conductive compared to CI-AMPARs, an exchange of CI-AMPARs for CP-AMPARs at the synapse would result in long term potentiation (LTP). This is the inverse of how LTD in the VTA is induced through an exchange of CP-AMPARs for CI-AMPARS. Alternatively, it is possible that mGluR1 activation in dorsal striatum exclusively leads to CI-AMPAR endocytosis but does not increase CP-AMPAR expression. This process results in LTD, as overall AMPAR expression would be reduced. We therefore activated mGluR1 through high-frequency stimulation (Atwood et al., 2014; Calabresi et al., 1992; Calabresi et al., 1994; Groszer et al., 2008) and measured if this leads to LTD induction in dorsal striatum. We were able to induce LTD in D1R-MSNs from *Foxp2*$^{+/+}$ mice, which suggests activation of mGlur1 leads to endocytosis of CI-AMPARs, without a concomitant increase in CP-AMPARs (Figure 4c, d). Next, we assessed if LTD could still be induced in D1R-MSN of *Foxp2*$^{S321X/+}$ mice. Though it is known that the R553H DNA-binding mutation of *Foxp2* affects striatal LTD in mice (Groszer et al., 2008), the mechanism which is affected and leads to the absence of LTD is unknown. Application of LTD stimuli to the striatum of *Foxp2*$^{S321X/+}$ mice failed to initiate LTD (Figure 4d) in D1R-MSNs of these mice. Additionally, the lack of an increase in RI following chemical mGluR1 activation in *Foxp2*$^{S321X/+}$ mice suggests the CI:CP-AMPAR ratio cannot be altered in these mice by mGluR1 activity. This could be due to impaired CI-AMPAR endocytosis. Alternatively, it is also possible CI-AMPAR expression is already reduced at the synapse, as it has been shown AMPAR expression in general is reduced in *Foxp2*$^{+/-}$ mice. This way, an effect of further reduction of CI-AMPAR might be occluded.

The emergence of coordinated locomotion is delayed in *Foxp2*$^{S321X/+}$ mice

The emergence of coordinated movement occurs during the second postnatal week. During this timeframe, mice go from uncoordinated limb movement to coordinated walking (Dehorter et al., 2011; Heyser, 2004). We have shown here that in wild-type mice AMPAR subunit expression undergoes changes between PND 9 and PND 11, resulting in mature CI-AMPAR expression. Therefore, we explored if this change in AMPAR expression might be correlated to behavioral changes in coordinated locomotion during the second postnatal week. This correlation would be suggestive of a specific critical period during striatal development related to motor behavior. Additionally, the delay in AMPAR subunit maturation we measured in *Foxp2*$^{S321X/+}$ mice suggests these mice could be delayed in the development of coordinated locomotion.

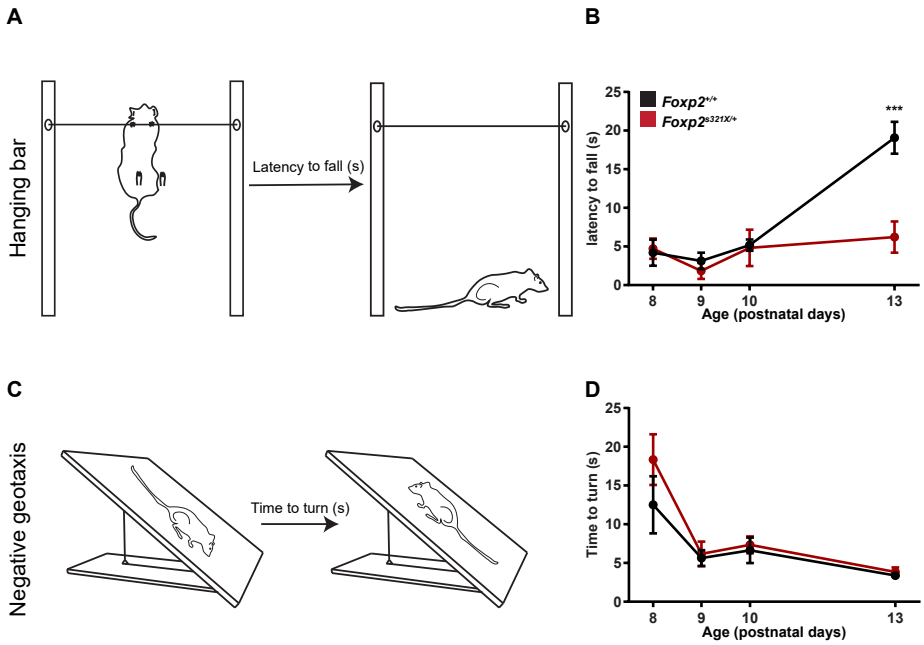

Figure 5: Hanging bar and negative geotaxis test performance is impaired in *Foxp2*$^{S321X/+}$ mice

(a) Schematic representation of the hanging bar setup. Beginning situation (left) and scoring paradigm (right). (c) Schematic representation of the negative geotaxis task. Beginning situation (left), and end situation (rig ht) are shown. The time it takes for the animal to complete the 180-degree turn is recorded. (b) latency to fall in seconds for the hanging bar test in *Foxp2*$^{+/+}$ or *Foxp2*$^{S321X/+}$ mice during the second postnatal week. (*Foxp2*$^{+/+}$ PND8, 9, 10, 13 (4.2±1.7, 3.1±1.1, 5.2±0.74, 19.1±2.1) *Foxp2*$^{S321X/+}$ (4.7±1.3, 1.84±1.1, 5.2±0.74, 6.2±2.0) *P<0.001* for PND13 (d) Time to turn in seconds for the negative geotaxis experiments in *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice during the second postnatal week. (*Foxp2*$^{+/+}$ PND8, 9, 10, 13 (12.5±3.7, 5.6±1.0, 6.6±1.7, 3.4±0.1) *Foxp2*$^{S321X/+}$ (18.3±3.3, 6.2±1.6, 7.3±1.1, 3.8±0.6)). **** = P<0.001*, 2-Way ANOVA with genotype and postnatal day as factors.

In order to assess coordinated locomotion development, juvenile *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice (PND 8-13) were subjected to a hanging bar test and negative geotaxis test. The hanging bar tests measures grip strength and coordinated locomotion (Figure 5a). Grip strength is a measure for skeletal muscle function and indicated not to be affected by *Foxp2* mutation (Enard et al., 2009). However, the ability for the mouse to use its hindlimbs to maintain position results in a better performance on this hanging bar test and this ability develops quickly during the second postnatal week (Aartsma-Rus and van Putten, 2014). The negative geotaxis test measures the ability for an animal to orient itself from an inverted (head down) position to a normal position when placed on an inclined surface (Figure 5b). This is especially difficult for very young animals but does not require a high degree of coordinated locomotion, which makes this test an ideal control for general motor impairment between *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice. Additionally, these tasks do not require any learning and are as such suited to investigate coordinated motor behavior at very young ages. At PND 8, *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice are unable to hold on to the hanging bar. Intriguingly, between PND 10 and PND 13 *Foxp2*$^{+/+}$ mice drastically increase their performance on the hanging bar test, whilst *Foxp2*$^{S321X/+}$ mice remain low in their performance (Figure 5c). Furthermore, we did not observe any differences in performance between *Foxp2*$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice on the negative geotaxis test. Both genotypes increased performance between PND 8 and PND 9 already, with no further reduction in time to turn between PND 9 and PND 13 (Figure 5d). This suggests that loss of Foxp2 expression affects the performance of juvenile mice on a specific set of tasks, which require coordinated locomotion. Together our data indicate that AMPAR maturation in the striatum and coordinated locomotion may be causally related.

DISCUSSION

Synaptic AMPAR composition changes drastically during development and mature AMPAR composition is essential for mature brain activity (Henley and Wilkinson, 2016). In the striatum, CP-AMPARs are predominantly expressed during early development, whilst CI-AMPARs show high expression in adult striatum (Bassani et al., 2013). However, the exact time-course by which these changes occur in the dorsal striatum is currently unclear. Here we show that AMPAR subunit composition at striatal D1R-MSNs changes quickly during juvenile development. However, this change is delayed and more gradual in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. Aberrant AMPAR trafficking at the synapse seems to underlie the impaired AMPAR maturation, as mGluR1 mediated receptor trafficking is absent in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. Furthermore, the change in AMPAR subunit expression is correlated with a critical period for the initiation of coordinated locomotion. The performance of wild-type mice on the hanging bar test increases during the same developmental time window in which a shift from CP-AMPAR expression to CI-AMPAR expression occurs. As *Foxp2*$^{S321X/+}$ mice are impaired

at the hanging bar test compared to wildtype mice at PND 13, the delayed AMPAR subunit composition switch during development might be associated with decreased coordinated locomotion.

Foxp2 has been shown to regulate molecular mechanisms involved in neurogenesis, neuronal development and network maturation (Vernes et al., 2011). For example, the expression of MEF2C (Chen et al., 2016) and SRPX2 (Roll et al., 2010) is affected by Foxp2: aberrant Foxp2 expression results in dysregulation of MEF2C and SRPX2, which affects synapse formation and development of cortical and striatal networks (Harrington et al., 2016; Sia et al., 2013). Though it is not known if Foxp2 is able to directly regulate AMPAR expression, intermediate targets can help understand how Foxp2 affects excitatory activity. The transient delay in AMPAR maturation could be due to other intermediate targets which are regulated by Foxp2 and affect AMPAR maturation, such as PICK1 (Xu et al., 2014).

Furthermore, a core regarding synaptic maturation during critical periods is the interdependence of maturation of neuronal processes and behavioral input. For example, the start of critical periods for synapse maturation in sensory cortices coincides with developmental milestones such as eye or ear opening (Hooks and Chen, 2007; Polley et al., 2013). It is therefore thought the initial reception of input by the auditory and visual sensory cortices is the main catalyst for the initiation of a critical period in development and synaptic maturation. However, the striatum does not directly receive sensory information. It has been shown that pharmacological blockade of striatal activity during, but not before or after, a critical period in adolescence leads to impaired performance on a motor task (Soiza-Reilly and Azcurra, 2009). Additionally, striatal NMDA receptor maturation has been shown to coincide with the initiation of coordinated locomotion as well, and the striatum shows a sudden development of mature activity during the start of coordinated locomotion (Dehorter et al., 2011). This suggests motor input to the striatum might be a catalyst for striatal development, similar to how sensory input signifies sensory cortex critical periods. However, it is not known if initial motor input to the striatum is delayed in *Foxp2*$^{S321X/+}$ mice. Nonetheless, the delay of coordinated locomotion initiation in *Foxp2*$^{S321X/+}$ mice could preface the slower AMPAR maturation.

Though mature AMPAR composition is described in detail in many brain regions, the relative expression of CP-AMPARs and CI-AMPARs is highly brain region dependent (for review see: (Henley and Wilkinson, 2016; Traynelis et al., 2010)). In areas such as the ventral tegmental area, hippocampus, and cortex, CI-AMPAR expression is high in mature synapses (Bellone et al., 2011; Ho et al., 2007; Liu and Cull-Candy, 2000; Murphy et al., 2012), whilst the high CP-AMPAR expression in cerebellar spiny stellate cells of adult mice is a notable exception (Liu and Cull-Candy, 2000). Furthermore, dynamic trafficking of AMPARs at the synapse is

essential for the induction of synaptic plasticity (Bassani et al., 2013; Diering et al., 2014; Esteban et al., 2003; Man, 2011), and dysregulated AMPAR subtype expression or AMPAR subunit trafficking is implicated in cognitive disorders, such as schizophrenia and addictive behavior (Bariselli et al., 2016; Bellone et al., 2011).

The hanging bar and negative geotaxis are both common behavioral paradigms to assess the development of motor circuitry in rodents during early postnatal life and juvenile development (Aartsma-Rus and van Putten, 2014; Heyser, 2004; Wells et al., 2016). In adult life however, basic motor function of *Foxp2*$^{S321X/+}$ is normal, but they show impaired motor skill learning function as adults by reduced performance on the accelerating rotarod (van Rhijn et al., 2018). Though we show that impaired AMPAR maturation in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice is transient, it is known that AMPAR expression is reduced in adult mice with a heterozygous *Foxp2* knockout mutation (Chen et al., 2016). This suggests impaired AMPAR trafficking is a persistent phenotype, which impacts both AMPAR maturation during development and AMPAR expression in adulthood. Intriguingly, both juvenile and adult mice with heterozygous *Foxp2* mutations display aberrant excitatory activity in the striatum, with reduced miniature excitatory postsynaptic current (mEPSC) amplitude and absence of striatal LTD (French et al., 2012; van Rhijn et al., 2018). It could very well be that the transient impairment in AMPAR maturation and the persistent deficit in AMPAR trafficking affect unique aspects of coordinated locomotion.

In addition to these deficits in AMPAR expression and excitatory activity, increased presynaptic inhibitory drive has been observed in D1R-MSNs of juvenile and adult *Foxp2*$^{S321X/+}$ mice (van Rhijn et al. 2018). Furthermore, intervention by blockade of inhibitory activity with picrotoxin in adult mice improves motor skill learning performance in *Foxp2*$^{S321X/+}$ mice. This suggests the delayed start of coordinated locomotion and the motor skill learning deficits in adult mice could alternatively be governed by separate neurobiological mechanisms, both affected by changes in Foxp2 expression. Designer receptors exclusively activated by designer drugs (DREADDS) could be used to spatiotemporally up- or downregulate excitatory or inhibitory activity in D1R-MSNs *in vivo* in both control and *Foxp2*$^{S321X/+}$ mice. This way, we would be able to investigate if manipulation of only excitatory or inhibitory activity could explain the motor phenotypes observed in mice with heterozygous *Foxp2* mutations.

Another way by which AMPAR trafficking is disrupted is through dysregulation of dopamine mediated activity, for example by exposure to drugs of abuse such as cocaine. Exposure to cocaine increases CP-AMPAR expression through interaction of the AMPAR subunit GluA2 with the protein PICK1 in the ventral tegmental area (Fiuza et al., 2017). This increase in CP-AMPAR expression is thought to affect motivation and goal directed behavior (Bariselli et al., 2016; Bellone and Luscher, 2006). Activation of mGluR1 in the VTA leads to insertion

of CI-AMPARs and a decrease in rectification. As such balanced activity of PICK1 interaction and mGluR1 activation is thought to be essential for glutamatergic synaptic function in the VTA (He et al., 2009; Wolf and Tseng, 2012). In contrast to the function of mGluR1 in the VTA, mGluR1 activity leads to GluA2 endocytosis rat dorsal striatum (Ahn and Choe, 2010). We show here that application of the mGluR1 agonist RO67-7476 leads to increased rectification in mouse D1R-MSNs in dorsal striatum, possibly through endocytosis of CI-AMPARs. This suggests that like rat dorsal striatum, the function of mGluR1 might be reversed between the VTA and the dorsal striatum in mice.

To conclude, we show here that heterozygous loss of *Foxp2* function leads to delayed maturation of Rs and that this delay might be correlate to early developmental deficits in motor coordination This would suggest an early phenotype related to disruptions in FOXP2 function could be present in human individuals but is transient and disappears early in postnatal development. Additionally, our data suggests that symptoms displayed early in life in neurodevelopmental disorders could be established through different neurobiological mechanisms than the adult phenotype. Which aspects of neurobiology are transiently or persistently affected by Foxp2 is an exciting new direction for research focused on the molecular mechanisms behind Foxp2 function.

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MATERIALS & METHODS

Mice

The experimental procedures were approved by the Animal Ethics Committee of the Radboud University Nijmegen, under DEC application number 2014-098 (Nijmegen, the Netherlands) and conducted in accordance with the Dutch legislation. Every effort was made to minimize animal discomfort and the number of animals used.

The Foxp2-S321X line was maintained on a C57BL/6J background, heterozygotes and wildtype littermates between PND7 and PND23 were used for electrophysiological recordings and mice between PND8 and 13 were used for behavioral experiments. The generation, marker-assisted backcrossing and genotyping of this strain are fully described in (Coghill et al., 2002; Groszer et al., 2008; Keays et al., 2006). BACtrap mice carrying GFP under the D1R promoter (D1R-GFP) were originally generated by the GENSAT (Gene Expression Nervous System Atlas) (Gong et al., 2003) and backcrossed to C57BL6/J mice. Both male and female mice were used in all experiments, and care was taken to use equal numbers of male/female mice for behavioral experiments.

Brain slices

Experiments were conducted on 350µm thick coronal slices. Mice (PND 11-21) were sacrificed by decapitation following isoflurane anesthesia. Slices were cut using a vibratome (HM650V Thermo Scientific) in cooled (4°C) artificial cerebrospinal fluid containing (in mM): 87 NaCl, 11 Glucose, 75 Sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$), continuously oxygenated with 95%O2/5%CO2. Collection of slices started when the striatum became visible and slices were collected until the hippocampus was visible. After collection, slices were incubated at 32°C in oxygenated ACSF for at least 1h before recording. Slices were transferred to the recording setup 10 minutes prior to recording and incubated in recording ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 Glucose, continuously oxygenated and heated to 32°C. Patch pipettes (3.5 – 5.5 MΩ) were made from borosilicate glass capillaries and filled with intracellular solution containing: 115 CsMeSO3; 10 CsCl; 10 HEPES; 2.5 MgCl2; 4 Na2ATP; 0.4 NaGTP; 10 Na-Phosphocreatine; 0.6 EGTA, 10 QX-314. Activity was recorded using a Digidata 1440A digitizer and a Multiclamp 700B amplifier (Molecular Devices). Sampling rate was set at 20KHz and a lowpass 1KHz filter was used during recording. All recordings were conducted in the dorsolateral quadrant of the striatum.

Rectification index

In order to calculate rectification index, AMPA receptor mediated responses to stimulus evoked neurotransmitter release are measured at different membrane holding potentials in the presence of 0.1mM spermine (Tocris bioscience, UK) in the patch pipette. Spermine

blocks CP-AMPARs at positive membrane potentials, without having an effect on the conductivity of CI-AMPARs. Response to stimulation at -60, 0 and +40mV was measured, and rectification index was calculated as follows: RI $\frac{(\text{peak current (-60mV)})}{(\text{peak current (+40mV)})}$ / 1.5 The 0mV response was used to correct stimulation artefacts of the AMPAR stimulation. The same recording ACSF was used as described above, and picrotoxin (100uM, Tocris bioscience) and APV (100µM, Tocris bioscience) were added to block GABA and NMDA mediated currents, respectively.

Extracellular CP-AMPAR block

1mM 1-Naphthyl acetyl spermine trihydrochloride (NASPM, Tocris bioscience) was added to the recording ACSF during stimulus response recording to block CP-AMPARs extracellularly. Stimulus response was normalized to the first 10 minutes of the recording, after which wash-in was started and NASPM was left in the recording ACSF for the further duration of the recording.

mGluR mediated AMPAR endocytosis

We first measured rectification index as described previously. Subsequently, 6µM of the mGluR1 specific agonist RO67-7476 (Tocris bioscience) was applied to the recording ACSF for 30 minutes, and rectification index was measured again in D1R-MSNs of the same brain slice as the baseline measurement. ACSF was exchanged for fresh ACSF between brain slices and only one experiment was conducted per brain slice.

LTD induction

LTD induction experiments were conducted by stimulation of afferent corticostriatal and intrastriatal axons using a bipolar concentric stimulus electrode (FHC, Bowdoin, Maine) placed in the dorsolateral striatum. Baseline evoked responses were established for 15 minutes prior to LTD induction, and average baseline response was set to 200 pA. LTD was induced by applying 4 theta burst stimulations of 1 second at 100Hz frequency, with a 10 second inter burst interval. LTD was recorded for at least 20 minutes after induction. Cells with a series resistance of >25MΩ after recording were discarded.

Hanging bar

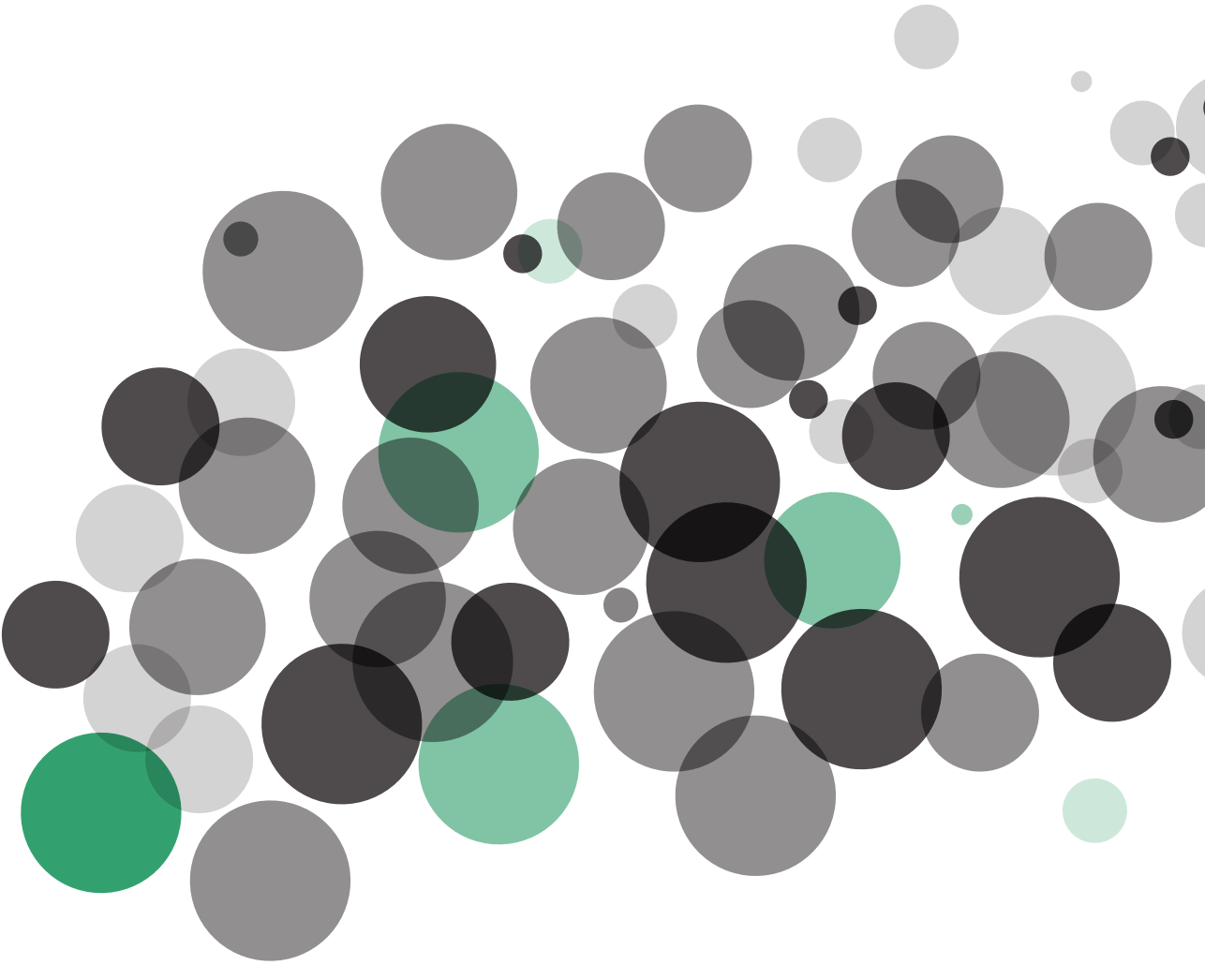
Foxp2$^{+/+}$ and *Foxp2*$^{S321X/+}$ mice were tested at different timepoints (Postnatal day 8, 9, 10, 13, 17, adult) for hanging bar performance. The hanging bar was designed as shown in Figure 4A. In short, a H profile with a wire placed between two vertically set bars was used to suspend the mice by their forelimbs. Immediately when the mouse was placed, a stopwatch was started and the time until either the mouse dropped from the wire or manages to reach one of the sides of the H profile was recorded. If a mouse manages to reach the side of the H profile, time was recorded as 100 seconds, which was the maximum hanging time recorded if mice were unable to fully complete the task. Lower times indicate worse performance.

Negative geotaxis

Mice from the same age groups as used for the hanging bar test were also subjected to negative geotaxis. For the experiment, animals were placed facing downward on a ramp covered with a cloth mesh surface which was set at a fixed 45° angle. The time for the mice to turn 180 degrees (from facing downward to facing completely upward with the entire body) was scored. Mice which rolled down during turning were scored as failures and their time to task completion was set as 1 second higher than the highest value for the respective group.

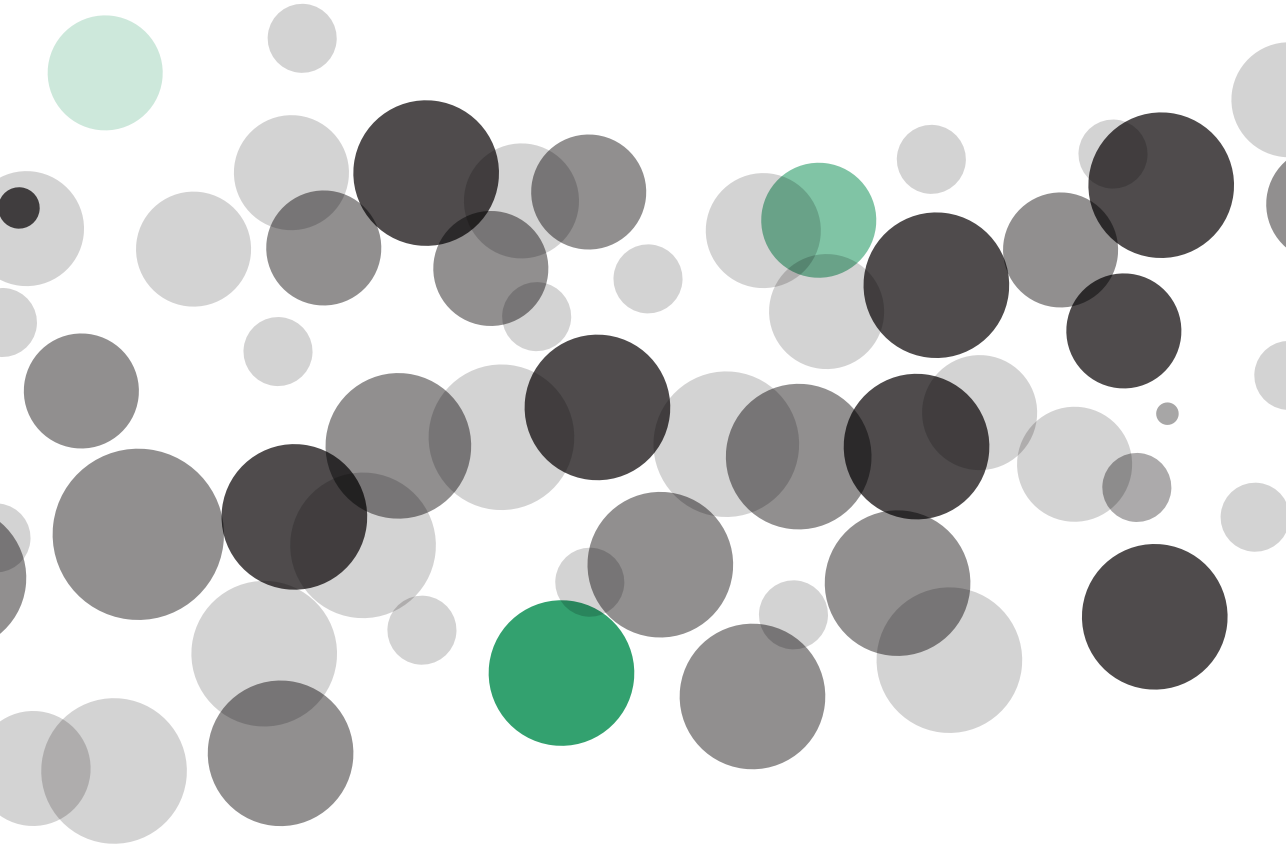
Statistics

Comparisons between two groups were made with the Student's T-test. Comparisons between multiple groups were conducted with repeated measures ANOVA. Normality was confirmed before analysis. Statistical analysis was conducted in Graphpad PRISM 7.04 (La Jolla, CA, USA). All experiments were conducted in at 3 mice for each genotype, with at least 2 cells per mouse for each electrophysiological measurement



4.

Heterozygous DNA-binding domain mutation of *Foxp2* impairs striatal inhibition



Jon-Ruben van Rhijn, Simon E Fisher, Sonja C Vernes, Nael Nadif Kasri

ABSTRACT

Heterozygous mutations of *FOXP2* lead to childhood apraxia of speech (CAS) in humans and motor skill learning impairments in mice. Examples of mutations which disrupt FOXP2 function include missense mutations which affect the DNA-binding domain and nonsense mutations leading to premature stop-codons and protein truncation. Cell and animal models have been used to investigate how FoxP2 affects cellular function. Two of the most well-studied etiological FOXP2 mutations that have been implicated in CAS are a missense mutation in the DNA-binding domain found in a large multigenerational pedigree (R553H mutation, the KE family), and a nonsense mutation observed in a smaller family (R328X mutation). Mouse models carrying these mutations (Foxp2-R552H matching the FOXP2-R553H mutation, and Foxp2-S321X as a close analogue of the FOXP2-R328X mutation) have been characterized at molecular, physiological and behavioral levels. However, direct comparison of these mutations has been limited to genetic and behavioral experiments, where overlap in phenotypes has been observed (Estruch et al., 2016; Gaub et al., 2016; Gaub et al., 2010; Vernes et al., 2006). By contrast, the neurobiological mechanisms affected by these mutations have only been investigated separately using different methods. We previously found that heterozygous S321X mutation of *Foxp2* leads to decreased excitatory and increased inhibitory activity in dopamine type-1 receptor containing medium spiny neurons (D1R-MSNs) in the striatum. To investigate if the R552H DNA-binding mutation of *Foxp2* similarly affects striatal D1R-MSNs, we assessed striatal excitatory and inhibitory activity in heterozygous R552H mice. Our data show that the R552H mutation, in contrast to the S321X mutation, does not affect excitatory activity. Furthermore, the R552H mutation leads to a reduction of inhibitory (GABA receptor-mediated) activity in striatal MSNs. Lastly, *in vivo* blockade of GABAergic activity aggravates the motor skill learning difficulties present in heterozygous R552H mice, whilst the same treatment leads to improved motor performance in S321X mice. Taken together, our data suggest that physiological consequences of Foxp2 dysfunction may be mutation specific. Detailed understanding of how unique mutations in *FoxP2* affect striatal function is therefore necessary for possible therapeutic interventions.

INTRODUCTION

The development of striatal circuits and striatal neuronal activity are commonly affected in neurodevelopmental or neurogenerative disorders which affect motor control (for review, see (Robinson and Gradinaru, 2018)). The striatum is part of the cortico-striato-thalamic motor circuit, which is essential for complex motor sequencing (for review, see(Shepherd, 2013)). Within the striatum, two main populations of GABAergic cells can be distinguished, which differ in their projection sites and as such regulate separate aspects of motor control (Calabresi et al., 2014; Surmeier et al., 2007). These are medium spiny neurons which either express the dopamine receptor type 1 (D1R-MSNs) or type 2 (D2R-MSNs) (Gittis and Kreitzer, 2012). D1R-MSNs innervate the striatal direct pathway, which projects to the substantia nigra (SNr), such that activation leads to upregulation of cortico-striato-thalamic circuit activity. By contrast, D2R-MSNs project through the indirect pathway, via the globus pallidus and subthalamic nucleus, to the SNr and thereby inhibit SNr activity (Kravitz et al., 2010; Kravitz and Kreitzer, 2012). Complex motor disorders generally show one or both cortico-striato-thalamic pathways to be affected, and behavioral phenotypes can often be correlated with specific circuit dysfunction (Bariselli et al., 2016; Rothwell et al., 2014).

Investigation of monogenic disorders which present with impairments in complex motor sequencing is an invaluable tool to further understand how the striatum governs motor learning. One monogenic disorder with prevalent changes in striatal function is heterozygous mutation of the transcription factor Forkhead box protein 2 (FOXP2) (Lai et al., 2001), which leads to Childhood apraxia of speech (CAS). CAS involves problems with mastering the complex coordinated sequences of orofacial and mouth movements that are crucial for proficient speech ((Lai et al., 2003; MacDermot et al., 2005), for review see (Morgan et al., 2017)). Multiple mutations have been described for *FOXP2* such as missense and nonsense mutations, as well as frameshifts, translocations and deletions (Morgan et al., 2017). Several missense mutations in *FOXP2* have been found affecting different residues of the DNA binding domain (Laffin et al., 2012; Lai et al., 2001; Reuter et al., 2017). The first of these DNA-binding domain disruptions was originally described cosegregating with CAS in all fifteen affected members of the three-generation KE family (Lai et al., 2001). In affected family members, the R553H mutation leads to disrupted function of the forkhead domain, including aberrant cellular localization and absence of DNA binding ability, which disrupts transcription factor function (Vernes et al., 2006). The first nonsense mutation of *FOXP2* was subsequently described in a smaller family in which the proband, his affected sister and the mother are all affected by CAS (MacDermot et al., 2005). In this family, the mutation yields a stop codon in exon 7 (R328X) that is predicted to lead to protein truncation before the Forkhead domain (Figure 1). Nonsense mediated decay and absence of protein expression in case of homozygous mutation have been shown in a mouse model carrying an S321X variant,

which is similar to this mutation (Groszer et al., 2008). Different *FOXP2* mutations have been described since then, which lead to similar disruptions of FOXP2 function (Estruch et al., 2016; Morgan et al., 2017). Initial comparison of brain morphology and activity using (f)MRI between affected KE family members, unaffected siblings and unrelated controls has revealed reductions in striatal grey matter density (Watkins et al., 2002) and reduced striatal activation during a language task (Liegeois et al., 2003) in affected individuals. These changes in brain morphology and activity prompted further investigation of FoxP2 function in animal models.

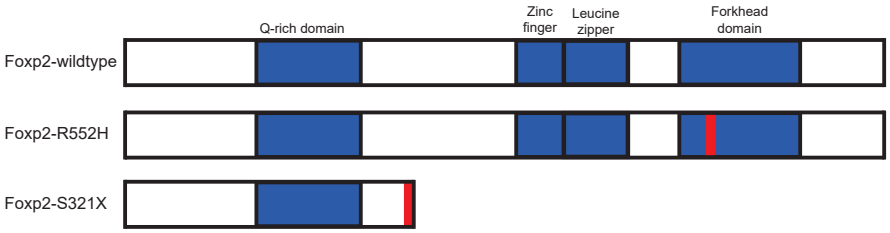


Figure 1: schematic representation of important Foxp2 domains and the R552H and S321X mutation.

The R552H mutation falls within the DNA binding domain, and yields a protein with absent transcriptional ability. The S321X mutation results in truncation of the protein and nonsense mediated decay. Foxp2-S321X is not detectable on western blot analysis (Groszer et al., 2008)

Animal model systems ranging from *Drosophila*, to mice and songbirds, have been used to study behavioral and cellular effects of disrupted function for FoxP2 orthologs (here we use the unified nomenclature for Forkhead-box proteins by (Kaestner et al., 2000)). Behavioral phenotypes in animal models suggest that complex motor sequencing is impaired. For example, song production in zebra finch (Haesler et al., 2007) is impaired when FoxP2 expression is reduced, and mouse models carrying heterozygous missense or nonsense mutations show impaired motor skill learning (French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018). These effects on complex motor behaviors suggest that Foxp2 is involved in the regulation of activity within the cortico-striatal-thalamic motor circuit. Evidence from *in vitro* and *in vivo* investigations does show that heterozygous *Foxp2* mutation leads to aberrant striatal plasticity and activity in mouse (French et al., 2012; Groszer et al., 2008) and aberrant signal transmission in songbirds (Murugan et al., 2013). The same neuronal circuits are thought to be important for both these complex motor behaviors in animals, and spoken language production in humans (Nudel and Newbury, 2013).

As Foxp2 is a transcription factor, disruption of its function can have widespread consequences for neurodevelopment, morphology and neuronal function. Firstly, primary striatal culture of

mice homozygous for the R552H mutation (a change that is directly equivalent to the R553H mutation in human *FOXP2*) has shown that this mutation impairs neuronal development (Vernes et al., 2011). Additionally, assessment of synapse development and synapse morphology in striatal tissue of mice with both heterozygous and homozygous knockout of *Foxp2* has shown that synapse maturation is impaired (Chen et al., 2016). Furthermore, short hairpin RNA (shRNA)-mediated knockdown of *Foxp2* in a song-related striatal nucleus (area X) of the zebra finch reduces spine density (Schulz et al., 2010). In *in vitro* cell models, primary neuronal cultures and brain tissue, it has been shown that both missense and nonsense mutations of *Foxp2* lead to loss of transrepression capacities, and *in vitro* overexpression of these Foxp2 variants leads to aberrant protein localization (Estruch et al., 2016; Fujita et al., 2008; Mizutani et al., 2007; Sollis et al., 2017; Vernes et al., 2006). Furthermore, it has been shown that brain morphology is altered in both homozygous R552H mutant mice (Groszer et al., 2008) and affected KE family members(Watkins et al., 2002). Foxp2 has been shown to regulate multiple genes important for neurodevelopment, such as MEF2C (Chen et al., 2016; Harrington et al., 2016), SRPX2 (Roll et al., 2010; Sia et al., 2013) and RARβ (Chandra et al., 2017; Devanna et al., 2014; Liao et al., 2005), among others. Thus, it seems that the mouse R552H mutation as well as *Foxp2* nonsense and knockout mutations affect similar neurobiological mechanisms. The R552H mutation leads to a loss of DNA-binding capacity of Foxp2, but the protein is still expressed. This is in contrast to the S321X mutation, which results in a reduction of Foxp2 expression, but all the Foxp2 which remains expressed is fully functional. Both these mutations have been shown to affect downstream regulation of DNA targets, with consequences for neurodevelopment (Spiteri et al., 2007; Vernes et al., 2011). Furthermore, *in vivo* investigations of mouse models of Foxp2 dysfunction show that the R552H and the S321X mutation similarly affect motor behavior. Both mice heterozygous for the R552H and the S321X *Foxp2* mutation show aberrant motor skill learning by decreased performance on the accelerating rotarod (French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018).

Interestingly, impairments in neuronal function have been assessed differently in the mouse model for the R552H mutation and the mouse model for the S321X mutation. In $Foxp2^{R552H/+}$ mice, the focus has been on changes in synaptic plasticity *ex vivo* and behavior-dependent regulation of striatal activity *in vivo*. $Foxp2^{R552H/+}$ mice display a lack of long-term depression (LTD) in the striatum (Groszer et al., 2008), as well as increased baseline striatal activity and aberrant modulation of synaptic activity when presented with a motor skill task (French et al., 2012). However, in $Foxp2^{S321X/+}$ mutant mice and $Foxp2^{+/-}$ knockout mice, research has focused on changes in synaptic strength and molecular mechanisms underlying changes in excitatory and inhibitory activity in the striatum (Chen et al., 2016; van Rhijn et al., 2018, Chapter 3). In these mice, which both display reduced Foxp2 expression (approximately half of the normal dosage of wild-type animals), decreased excitatory activity and increased inhibitory activity

have been shown in striatal MSNs, but *in vivo* investigations of neuronal function have not been conducted. Moreover, changes in excitatory or inhibitory activity have never been assessed at the single cell level in *Foxp2*$^{R552H/+}$ mice.

We therefore set out to comprehensively assess whether striatal D1R-MSNs of *Foxp2*$^{R552H/+}$ mice show similar effects on excitatory and inhibitory activity as do striatal D1R-MSNs of *Foxp2*$^{S321X/+}$ mice. Furthermore, we investigated the effect of blockade of GABAergic activity *in vivo* on motor skill learning *in Foxp2*$^{R552H/+}$ mice, as this intervention ameliorates the motor skill learning deficits of *Foxp2*$^{S321X/+}$ mice (van Rhijn et al. 2018). Our data indicate that the overlapping behavioral phenotype present in the *Foxp2*$^{S321X/+}$ and *Foxp2*$^{R553H/+}$ mice is established through different neurophysiological mechanisms. This finding could have important consequences for the development of therapeutic interventions and the translation of these to clinical practice.

RESULTS

Excitatory activity is not affected by a heterozygous *Foxp2* DNA-binding domain mutation
Foxp2 is known to be predominantly expressed in D1R-MSNs (Fong et al., 2018; van Rhijn et al., 2018; Vernes et al., 2011), and the effects of the S321X mutation are constrained to D1R-expressing MSNs in the striatum (van Rhijn et al., 2018). Therefore, we chose to select D1R-MSNs in the striatum of *Foxp2*$^{R552H/+}$ for physiological recording. In order to enable targeted recording of only D1R-MSNs, we crossed mice which express GFP under the D1R promoter through a Bactrap construct (D1R-GFP) with *Foxp2*$^{R552H/+}$ mice. Bactrap-D1R-GFP is known to faithfully label D1R expressing cells in mouse brain (Heiman et al., 2008) and can thus be used to target D1R-MSNs for live cell measurements. We recorded miniature excitatory postsynaptic currents (mEPSCs) in striatal D1R-MSNs of juvenile (PND 10-14) *Foxp2*$^{R552H/+}$ mice and wild-type littermate controls to measure the contribution of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) to synaptic strength. Neither mEPSC amplitude or frequency (Figure 2a,b) were affected by the DNA-binding domain disruption of the R552H mutation. Thus, AMPAR-mediated synaptic strength is not changed in D1R-MSNs of *Foxp2*$^{R552H/+}$ mice. This is in sharp contrast to the decrease in mEPSC amplitude shown in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice and suggests that striatal excitatory activity is uniquely affected by separate *Foxp2* mutations.

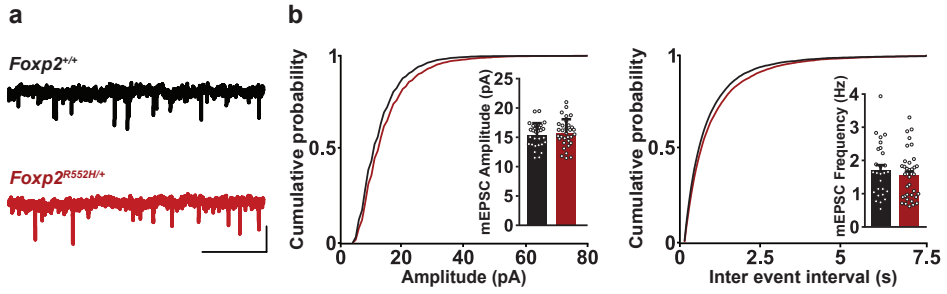


Figure 2: Normal excitatory activity in juvenile *Foxp2*$^{R552H/+}$ mice.

(a) representative traces of mEPSC activity in D1R-MSNs from juvenile (PND14) *Foxp2*$^{R552H/+}$ mice and *Foxp2*$^{+/+}$ littermate controls. (b) Average and cumulative amplitude and frequency of mEPSC events in D1R-MSNs indicate no difference in AMPA receptor mediated excitatory activity amplitude *Foxp2*$^{+/+}$ = 14.22 pA, *Foxp2*$^{R552H/+}$ = 15.37 pA, NS, frequency *Foxp2*$^{+/+}$ = 1.32 Hz, *Foxp2*$^{R552H/+}$ = 1.67 Hz, NS. N/n = mice/cells. *Foxp2*$^{+/+}$ 3/29, *Foxp2*$^{R552H/+}$ 3/32. Scale bars in (a) are 2 seconds / 10pA. Statistical comparison by two-sided student's T-test. *** P<0.001

Foxp2$^{R552H/+}$ mice show decreased striatal direct pathway inhibition

In *Foxp2*$^{S321X/+}$ mice the decrease in excitatory activity is accompanied by an increase in GABAergic inhibitory activity, which results in disruption of the excitatory/inhibitory balance of the striatal direct pathway (van Rhijn et al., 2018). Since excitatory synaptic strength is not altered in *Foxp2*$^{R552H/+}$ mice, we next investigated whether inhibitory activity could still be affected by the R552H mutation. To measure if inhibition was changed, we used an evoked stimulation protocol. We assessed the AMPA receptor specific response at -60mV membrane potential and the GABA receptor specific response at -0mV, which is the reversal potential of AMPA receptors and excludes the AMPA signal from the recording. Single bipolar stimulations were given through a stimulus electrode placed in the dorsolateral quadrant of the striatum. The emergence of inhibitory GABAergic activity is delayed compared to glutamatergic activity during development. Therefore, we measured GABA/AMPA ratio both during early juvenile development (PND11) as well as at a later time window (PND17). At PND11, no difference in GABA/AMPA ratio was observed between *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice (Figure 3a), and the GABAergic response to simulation was low (Figure 3a). However, a significant decrease in GABA/AMPA ratio was measured at PND17 in *Foxp2*$^{R552H/+}$ mice compared to *Foxp2*$^{+/+}$ controls (Figure 3b). Our mEPSC data shows that AMPA-mediated activity is not different between *Foxp2*$^{R552H/+}$ mice and *Foxp2*$^{+/+}$ controls during the second postnatal week. Therefore, this reduction in GABA/AMPA ratio can only be attributed to a reduced GABAergic response. The reduced GABA/AMPA ratio is in contrast to the previously measured increase in GABA/AMPA ratio in *Foxp2*$^{S321X/+}$ mice (van Rhijn et al., 2018). As such, both excitatory and inhibitory activity seem to be differently affected by the R552H mutation compared to the S321X mutation. The

lack of a change in excitatory activity combined with a decrease in inhibitory activity suggests striatal D1R-MSNs are more excitable in *Foxp2*$^{R552H/+}$ mice.

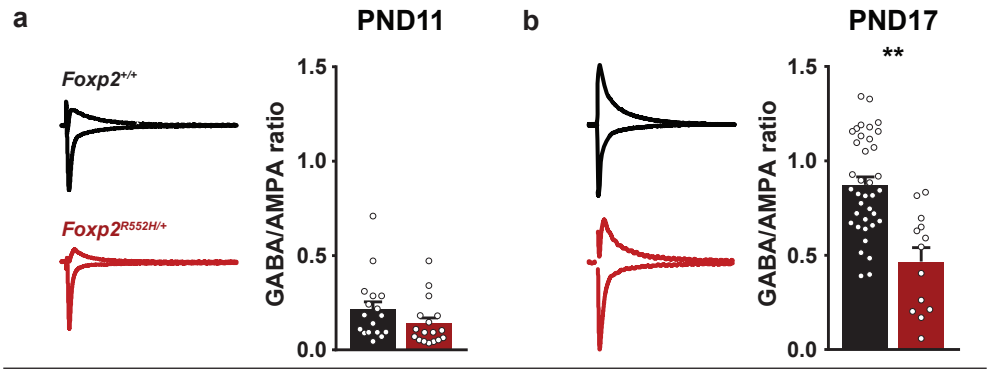


Figure 3: Decreased GABA/AMPA ratio in juvenile *Foxp2*$^{R552H/+}$ mice.

(a) GABA/AMPA ratio measured at early juvenile development (PND11) and late juvenile development (PND17). Early development shows only a very weak response to activation of GABAergic systems (*Foxp2*$^{+/+}$ = 0.21, *Foxp2*$^{R552H/+}$ = 0.14, NS). (b) At PND17, GABAergic responses are strong in wild-type animals, whereas a reduced GABA/AMPA ratio is shown in heterozygous *Foxp2*$^{R552H/+}$ mice (*Foxp2*$^{+/+}$ = 0.87, *Foxp2*$^{R552H/+}$ = 0.46) N/n= 3/17 for PND11 (both *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$), 5/37, PND17 *Foxp2*$^{+/+}$, 3/13 *Foxp2*$^{R552H/+}$. Statistical comparison by two-sided student's T-test. *** P<0.001

Motor skill learning deficits in *Foxp2*$^{R552H/+}$ mice are aggravated by inhibitory blockade

Changes in striatal activity following *Foxp2* mutations have already been correlated with aberrant motor behavior present in both *Foxp2*$^{R553H/+}$ (French et al., 2012) and *Foxp2*$^{S321X/+}$ mice (van Rhijn et al., 2018). Thus, there are overlaps in motor deficits observed in *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice, despite the different effects these mutations seem to have on striatal function at the neurophysiological level. We have previously shown that intervention by *in vivo* blockade of GABAergic activity using a low intraperitoneal (IP) dose of picrotoxin (PTX) ameliorates motor skill learning impairments in *Foxp2*$^{S321X/+}$ mice (van Rhijn et al., 2018). By contrast, wild-type mice are adversely affected in their motor skill learning by the same low dose of PTX. We therefore tested whether blockade of GABAergic activity affects motor skill learning as well in *Foxp2*$^{R552H/+}$ mice. Firstly, we confirmed the presence of a motor skill learning deficit in *Foxp2*$^{R552H/+}$ mice (French et al., 2012). We furthermore subjected our control animals to vehicle injection of 0.1mg/kg 0.01% dimethylsulfoxide (DMSO) 10 minutes before each accelerating rotarod session. This allowed us to directly compare the measurements in our control group with the PTX treatment group. *Foxp2*$^{R552H/+}$ and wild-type littermate controls of age 6-8 weeks were subjected to 5 daily sessions of accelerating rotarod training for 5 consecutive days. During each session, rotarod speed increased from 4-40 rpm over a course of 5 minutes and the latency to fall (in seconds) was recorded. We measured both

average performance per session as well as learning rate (Figure 4a,b). Though all animals were able to improve their performance during and between training days, the latency to fall was significantly reduced in *Foxp2*$^{R552H/+}$ mice compared to *Foxp2*$^{+/+}$ littermates in all sessions, corroborating previous published results (French et al., 2012).

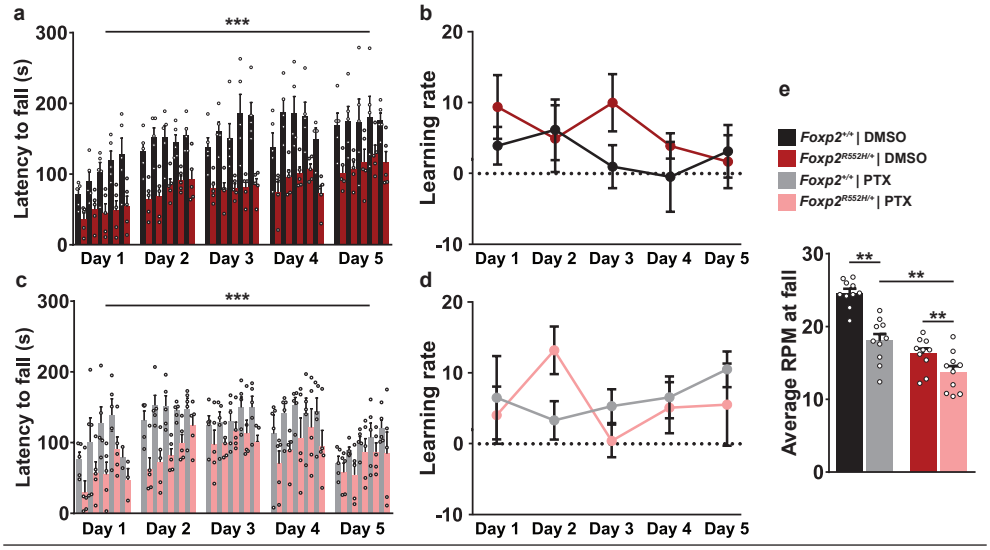


Figure 4: Pharmacological blockade of inhibition impairs motor skill learning both in *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice

(a) *Foxp2*$^{R552H/+}$ mice show impaired motor skill learning compared to *Foxp2*$^{+/+}$ mice, shown by the increased latency to fall (in seconds) across training sessions (day 1-5: *Foxp2*$^{+/+}$ 102.2, 147.2, 163.6, 169.7, 174.8 *Foxp2*$^{R552H/+}$ 47.0, 80.1, 80.6, 90.1, 114.2 *P*<0.01, 2-factor ANOVA). Each session consists of 5 trials of 5 minutes, during which the rotarod accelerated from 4-40rpm. (b) Both *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice show a positive learning rate during most sessions, with learning rate not being significantly different between genotypes. Learning rate was calculated as: $Learning\ rate = \frac{latency\ to\ fall\ (session\ 5\text{-}session\ 1)}{number\ of\ trials}$. (c,d) Both *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice subjected to 0.1mg/kg intraperitoneal injection of PTX show decreased rotarod performance and learning rates, compared to sham treatment. (Latency to fall: day 1-5: *Foxp2*$^{+/+}$ 108.1, 145.7, 136.7, 138.8, 97.7 *Foxp2*$^{R552H/+}$ 64.4, 94.5, 107.1, 102.9, 78.2). (e) Average RPM at which mice fail the accelerating rotarod task during session 4 and 5 in sham and PTX conditions (sham, *Foxp2*$^{+/+}$ 24.5 RPM, *Foxp*$^{R552H/+}$ 16.8RPM, P<0.001. PTX, *Foxp2*$^{+/+}$ 18.2RPM, *Foxp2*$^{R552H/+}$ 14.1RPM, P<0.05, two-sided students T-test). For all treatment conditions, N = 5 mice. *** *P*<0.001.

Next, we IP injected *Foxp2*$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice with 0.1 mg/kg PTX 10 minutes before each training session. We previously established that this concentration of PTX induces a notable reduction in motor skill learning in *Foxp2*$^{+/+}$ animals from carrying a S321X mutation, but general motor behavior is unaffected. Motor skill learning remained significantly impaired between Foxp2$^{+/+}$ and *Foxp2*$^{R552H/+}$ mice after PTX treatment (Figure 4c,d). Moreover,

comparison of average latency to fall between sham-injected and PTX-injected mice of the *Foxp2*$^{R552H/+}$ group shows PTX injection had a significant negative effect on motor skill learning, similar to the effect of PTX in wild-type control mice (Figure 4e). This is opposite from the effect PTX has on motor skill learning in *Foxp2*$^{S321X/+}$ mice, where PTX injection significantly improved accelerating rotarod performance. This opposite effect of the same *in vivo* intervention confirms that neurobiological mechanisms involved in motor skill learning are affected differently by the S321X or the R552H mutation.

DISCUSSION

Striatal morphology and function are affected by heterozygous mutation of the transcription factor *FoxP2* in humans (Liegeois et al., 2003; Watkins et al., 2002) as well as animal models (Chen et al., 2016; French et al., 2012; Groszer et al., 2008; Schulz et al., 2010). Multiple different mutations of *FOXP2* are known, which affect FOXP2 in distinct ways (Morgan et al., 2017). Two of the most well-studied are; a missense mutation which disrupts the DNA binding domain (Lai et al., 2001), and a nonsense mutation which leads to loss of protein function, protein truncation and nonsense mediated decay (MacDermot et al., 2005). Intriguing differences between these mutations on the neurobiological level have been observed, and it has been suggested that the R328X and the R553H mutations could differently affect FOXP2 function (Estruch et al., 2016; Vernes et al., 2006). However, a direct comparison of the neurophysiological effects these mutations have on striatal activity has not been conducted. Here we show for the first time in mouse models that the neurophysiological phenotype of the heterozygous *Foxp2-R552H* missense mutation differs from the effect of heterozygous *Foxp2-S321X* nonsense mutation (table 1). The heterozygous R552H mutation does not affect excitatory activity. Furthermore, a decreased GABA/AMPA ratio in D1R-MSNs of juvenile (PND17) *Foxp2*$^{R552H/+}$ mice shows GABAergic activity is reduced. This produces an inverse phenotype from that described in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice, where excitatory activity is decrease and inhibition is increased (van Rhijn et al., 2018). Pharmacological manipulation of neuronal activity *in vivo* by IP injection with PTX negatively affects motor skill learning in *Foxp2*$^{R552H/+}$ mice, in contrast to the known beneficial effect of IP PTX injection on motor skill learning in *Foxp2*$^{S321X/+}$ mice (van Rhijn et al., 2018).

The molecular mechanisms affected by the S321X mutation (as well as other loss of function alleles) have been investigated in detail, and multiple pathways involved in neurodevelopment and neuronal activity have been implicated (Devanna et al., 2014; Vernes et al., 2011). Heterozygous *Foxp2* loss of function mutations leads to reduced striatal AMPA receptor expression (Chen et al., 2016) as well as an increase in GAD67 (Glutamic acid decarboxylase 67, one of the principal enzymes for the production of GABA) expression in D1R-MSNs (van

Rhijn et al., 2018). Interestingly, *Foxp2*$^{S321X/+}$ mice do show similar impairments in LTD to those observed in *Foxp2*$^{R552H/+}$ mice (van Rhijn et al., Chapter 3), which suggests at least a degree of overlap in neuronal functions which are affected by both mutations. Though *in vivo* data from *Foxp2* knockout or loss of function mice is lacking, the changes in excitatory and inhibitory activity suggest *in vivo* baseline activity in the striatum could be decreased in *Foxp2*$^{S321X/+}$ mice.

Currently, it is not known if the R552H mutation leads to dysregulation of the same profile of downstream targets as for the S321X mutation, or if it might show unique effects (for example due to the existence of a mutant product that can bind to and interfere with function of wild-type Foxp proteins). Though both *Foxp2*$^{S321X/S321X}$ and *Foxp2*$^{R552H/R552H}$ mice show gross neurodevelopmental impairments (Fujita et al., 2008; Groszer et al., 2008), the data we present here suggest that the R552H mutation may, at least to some extent, uniquely affect the regulatory function of Foxp2 in striatal MSNs. Therefore, a comparison between expression profiles of brain tissue from mice with wildtype *Foxp2, Foxp2-S321X* mutation and *Foxp2-R552H* mutation might provide additional evidence that these mutations uniquely affect *Foxp2* function. Differences between the profile of how regulatory targets are affected in mice with the R552H and the S321X mutation could offer important novel insight for functional follow-up.

Proteins in the FoxP family can dimerize through interaction of their leucine zipper domains, and homo- or heterodimerization of Foxp1/2/4 has been posited as necessary for the DNA binding of these transcription factors (Bacon and Rappold, 2012; Li et al., 2004; Mendoza and Scharff, 2017). The S321X mutation results in simple reduction of Foxp2 expression, decreasing to availability of Foxp2 for interaction with other Foxp proteins. However, the R552H mutation solely disrupts the DNA binding domain, without disturbing the potential for Foxp2 to dimerize (Li et al., 2004). Another effect of both the S321X and R552H mutation that has been shown *in vitro* in cell models which overexpress Foxp2 with these mutations, is that nuclear localization is impaired (Mizutani et al., 2007; Sollis et al., 2017; Vernes et al., 2006), however, the significance of this result for the *in vivo* function of Foxp2 is currently unclear. Since the homo/heterodimerization of Foxp2 with other Foxp proteins is probably essential for its regulatory function (Li et al., 2004), interaction between Foxp2-R552H and wild-type Foxp2 could lead to competitive inhibition. This might be a major difference between the S321X and the R552H mutation, as the S321X mutation leads to reduced Foxp2 expression in general, but should not affect wild-type Foxp2 (Groszer et al., 2008; Vernes et al., 2006). The possible competitive inhibition between Foxp2-R552H and wild-type Foxp2 might reduce the availability of wild-type Foxp2 even more strongly than a general reduction in functional protein through the S321X mutation. Alternatively, homodimers of Foxp2-R552H only or wild-type Foxp2 and Foxp2-R552H could have different regulatory targets compared

Table 1: phenotypes described for heterozygous R552H and S321X/knockout mutations

Phenotype	*Foxp2*-S321X | *Foxp2* knockout	Reference	*Foxp2*-R552H	Reference
Excitatory activity D1R-MSNs	Decreased	(Chen et al., 2016; van Rhijn et al., 2018)	Unchanged (*ex vivo*) /increased (*in vivo)*	This manuscript / (French et al., 2012)
Inhibitory activity D1R-MSNs	Increased	(van Rhijn et al., 2018)	Decreased	This manuscript
Long-term depression	Absent	(van Rhijn et al., Chapter 3)	Absent	(Groszer et al., 2008)
Synapse maturation	Delayed	(Chen et al., 2016)	Unknown	N/A
Neurite outgrowth	Unknown	N/A	Impaired (homozygous)	(Vernes et al., 2011)
Motor skill learning	Negatively impaired	(French et al., 2012; van Rhijn et al., 2018)	Negatively impaired	(French et al., 2012; Groszer et al., 2008), This manuscript
Effect of GABA blockade on motor skill learning	Positive	(van Rhijn et al., 2018)	Negative	This manuscript

to homodimers of wild-type Foxp2 only. Lastly, Foxp2-R552H can probably still interact with Foxp1 and Foxp4 and form dimers with these proteins as well, in regions of co-expression. Expression of both Foxp1 and Foxp2 has been shown in mouse striatum as well as songbird brain (Chen et al., 2013; Teramitsu et al., 2004). At the single cell-level, Foxp1 has been confirmed to be expressed similarly in both D1R-MSNs and D2R-MSNs, whilst Foxp2 is mainly expressed in D1R-MSNs. This suggests there is a population of D1R-MSNs in which Foxp1 and Foxp2 are both expressed, but probably this is a subpopulation of all D1R-MSNs (Fong et al., 2018). Because of the possibility that such interactions can occur, Foxp2-R552H might have much more complex effects on gene regulation compared to the S321X mutation and is an interesting exploratory tool to investigate how dimerization of Foxp2 is involved in neuronal function.

We show that motor skill learning impairments due to mutations in *Foxp2* can arise through unique mutation-dependent effects on neurobiology. Therefore, we think that a detailed understanding of how distinct types of mutations in the same gene affect neurobiological mechanisms in monogenic disorders is necessary to fully decipher gene-behavior correlations. Disruption of the DNA-binding domain of Foxp2 oppositely affects striatal activity compared to a simple loss of Foxp2 function. Moreover, blockade of GABAergic activity in mice carrying $Foxp2^{R552H/+}$ or $Foxp2^{S321X/+}$ mutations inversely affects motor performance. Similar to the shared motor skill impairments in mouse models of Foxp2 dysfunction (Enard et al., 2009; French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018), all heterozygous disruptive mutations of *FOXP2* so far found in humans result in speech and language impairment (Morgan et al.,

2017). However, it could be that these mutations do differently affect behaviors, but the current methods used to characterize the phenotype are not able to show these differences in mice. Tasks which require increased or decreased direct pathway activity might reveal behavioral differences between *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice. Lastly, mutation dependent phenotypes have been described in other neurodevelopmental disorders, for example in individuals with different mutations of MECP2 (Baker et al., 2013) and SHANK3 (Zhou et al., 2016). Taken together, our results highlight that even when the affected gene is known, care should be taken not to suggest intervention before the neurobiological mechanisms affected in the specific individual are completely understood. The current ability to generate human neurons which express Foxp2 (van Rhijn et al., Chapter 6) would enable us to investigate patient-specific neuronal phenotypes. This could be a highly interesting follow up investigation and would help to both validate the unique effects of different *FoxP2* mutations and investigate patient-specific neurobiological mechanisms which are affected.

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MATERIALS & METHODS

Mice

The experimental procedures were approved by the Animal Ethics Committee of the Radboud University Nijmegen, under DEC application number 2014-098 (Nijmegen, the Netherlands) and conducted in accordance with the Dutch legislation. Every effort was made to minimize animal discomfort and the number of animals used.

The Foxp2-R552H line was maintained on a C57BL/6J background, heterozygotes and wildtype littermates between PND11 and PND17 were used for electrophysiological recordings, and adult mice between 7 and 9 weeks were used for behavioral experiments. The generation, marker-assisted backcrossing and genotyping of this strain are fully described in (Coghill et al., 2002; Groszer et al., 2008; Keays et al., 2006). BACtrap mice carrying GFP under the D1R promoter (D1R-GFP) were originally generated by the GENSAT (Gene Expression Nervous System Atlas) (Gong et al., 2003) and backcrossed to C57BL6/J mice. Both male and female mice were used in all experiments, and care was taken to use equal numbers of male/female mice for behavioral experiments.

Whole-cell patch clamp

Experiments were conducted on 350µm thick coronal slices. Mice were sacrificed by decapitation following isoflurane anesthesia. Slices were cut using a vibratome (HM650V Thermo Scientific) in cooled (4°C) artificial cerebrospinal fluid containing (in mM): 87 NaCl, 11 Glucose, 75 Sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$), continuously oxygenated with 95%O2/5%CO2. Collection of slices started when the striatum became visible and slices were collected until the hippocampus was visible. After collection, slices were incubated at 32°C in oxygenated ACSF for at least 1h before recording. Slices were transferred to the recording setup 10 minutes prior to recording and incubated in recording ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 Glucose, continuously oxygenated and heated to 32°C. Patch pipettes (3.5 – 5.5 MΩ) were made from borosilicate glass capillaries and filled with intracellular solution containing: 115 CsMeSO3; 10 CsCl; 10 HEPES; 2.5 MgCl2; 4 Na2ATP; 0.4 NaGTP; 10 Na-Phosphocreatine; 0.6 EGTA, 10 QX-314. Activity was recorded using a Digidata 1440A digitizer and a Multiclamp 700B amplifier (Molecular Devices). Sampling rate was set at 20KHz and a lowpass 1KHz filter was used during recording. All recordings were conducted in the dorsolateral quadrant of the striatum.

Miniature postsynaptic currents

mEPSCs were recorded in the prescience of Tetrodotoxin (TTX, 1µM, Tocris) and Picrotoxin (PTX, 100µM, Tocris) at a holding voltage of -60mV.

GABA/AMPA ratio

Stimulation experiments were conducted by stimulation of afferent corticostriatal and intrastriatal axons using a bipolar concentric stimulus electrode (FHC, Bowdoin, Maine) placed in the dorsolateral striatum.

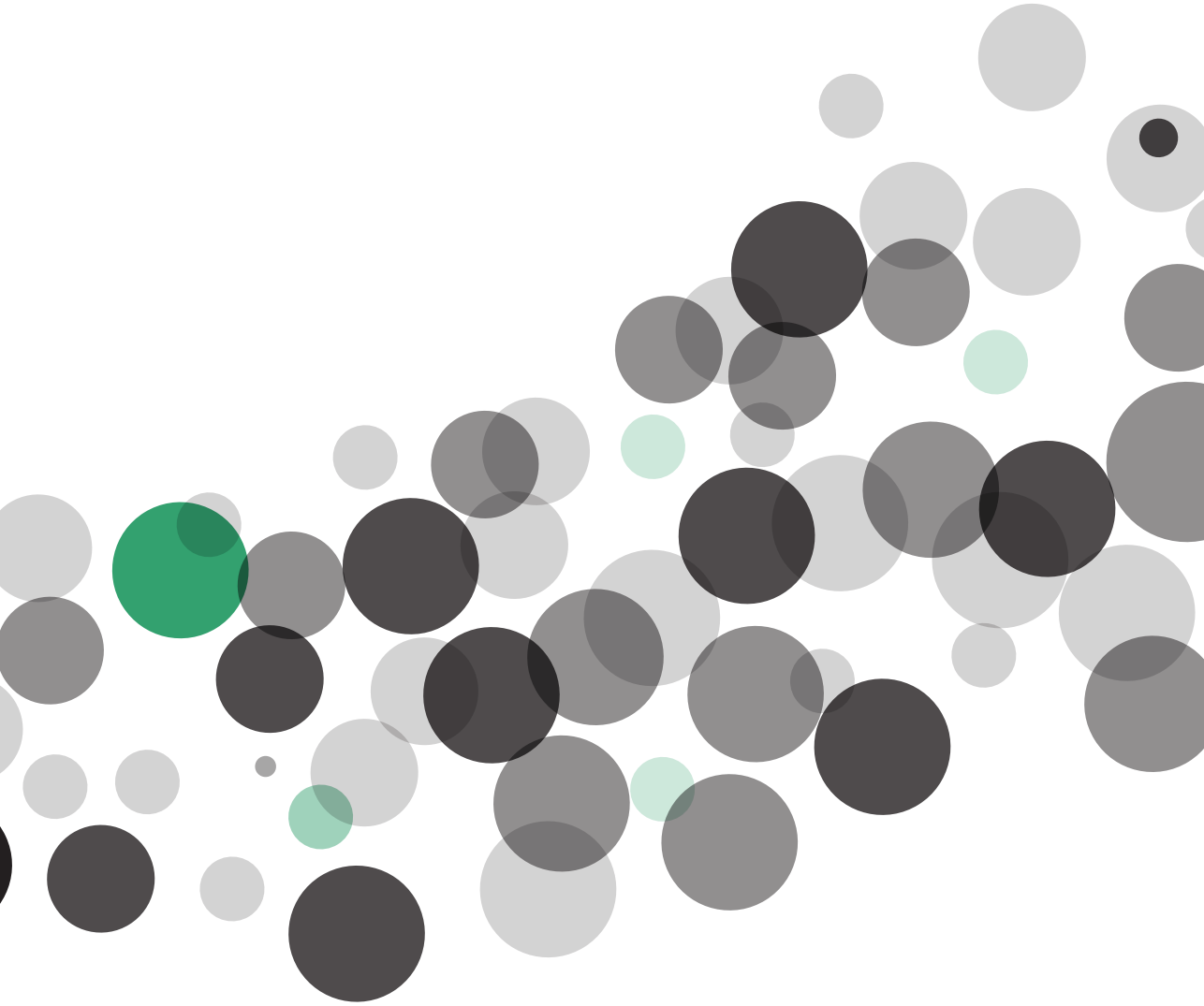
GABA/AMPA ratio was measured in the presence of APV (100µM). Cells were voltage-clamped at -60mV and a 1ms stimulus from a bipolar tungsten electrode was given to record the AMPA response. Subsequently cells were clamped at 0mV and the GABA response was measured.

Intraperitoneal injection

Foxp2$^{R552H/+}$ mice and wild-type littermate controls were injected intraperitoneally with either vehicle (DMSO) or 0.1mg/kg picrotoxin (Tocris, Bristol, UK). Injection was done by hand and mice were placed back in their home cage for 10 minutes following injection, after which mice were placed on the accelerating rotarod.

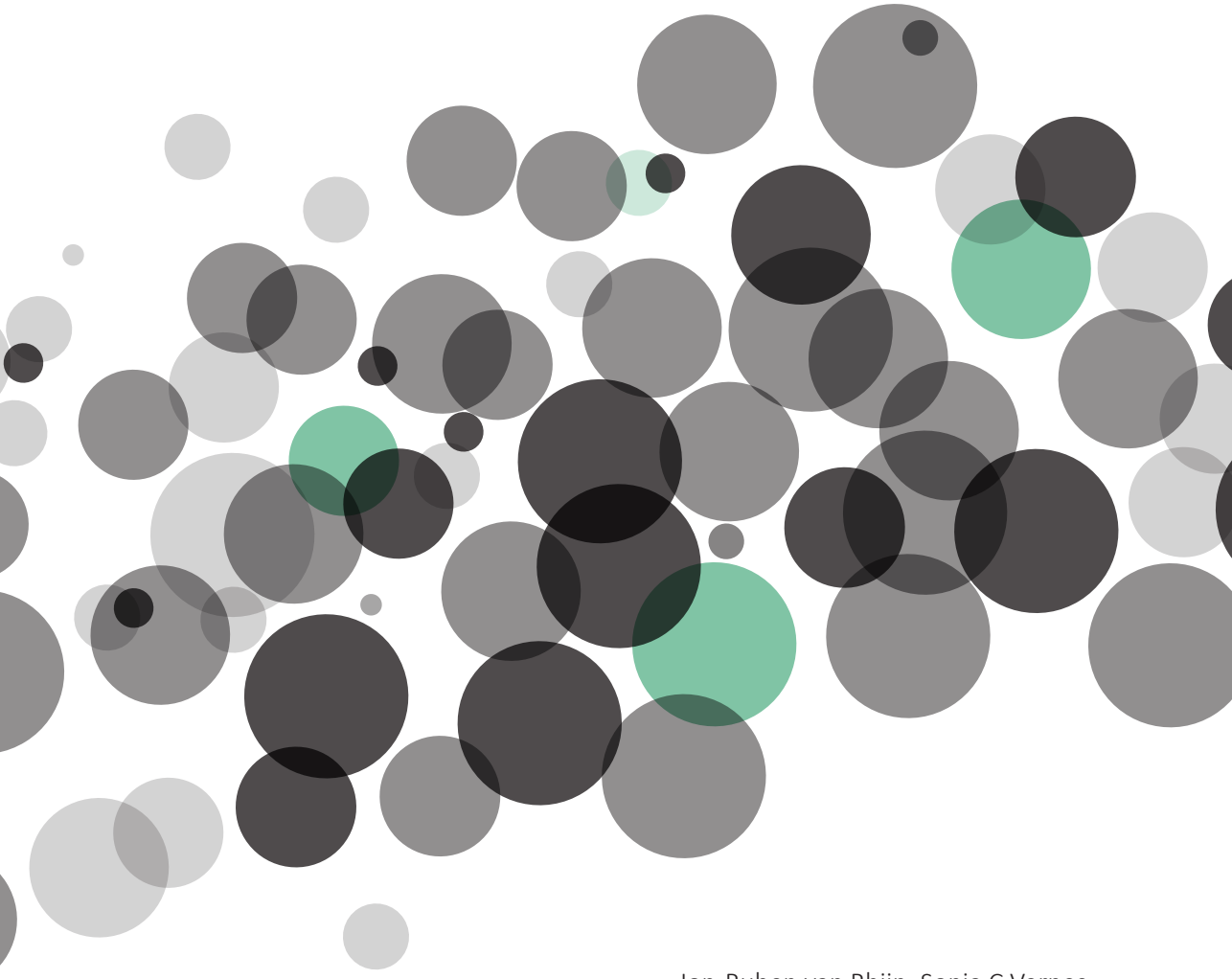
Accelerating rotarod

Foxp2$^{R552H/+}$ mice (6-8 weeks old) and wild-type littermate controls were placed on an accelerating rotarod (LE8200, Harvard apparatus) which increased rotation speed from 4 r.p.m. to 40 r.p.m. over a 5-minute period. Mice were trained for 5 consecutive days, with 5 trials per day. Latency to fall (in seconds or RPM at fall) was scored, and mice were placed back in their home cage for 5 minutes between trials.



5.

Retinoic Acid Signaling: A New Piece in the Spoken Language Puzzle



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Published as: van Rhijn JR and Vernes SC (2015) Retinoic Acid Signaling:
A New Piece in the Spoken Language Puzzle.
Front. Psychol. 6:1816. doi: 10.3389/fpsyg.2015.01816

ABSTRACT

Speech requires precise motor control and rapid sequencing of highly complex vocal musculature. Despite its complexity, most people produce spoken language effortlessly. This is due to activity in distributed neuronal circuitry including cortico-striato-thalamic loops that control speech–motor output. Understanding the neuro-genetic mechanisms involved in the correct development and function of these pathways will shed light on how humans can effortlessly and innately use spoken language and help to elucidate what goes wrong in speech-language disorders. *FOXP2* was the first single gene identified to cause speech and language disorder. Individuals with *FOXP2* mutations display a severe speech deficit that includes receptive and expressive language impairments. The neuro-molecular mechanisms controlled by *FOXP2* will give insight into our capacity for speech–motor control, but are only beginning to be unraveled. Recently FOXP2 was found to regulate genes involved in retinoic acid (RA) signaling and to modify the cellular response to RA, a key regulator of brain development. Here we explore evidence that FOXP2 and RA function in overlapping pathways. We summate evidence at molecular, cellular, and behavioral levels that suggest an interplay between FOXP2 and RA that may be important for fine motor control and speech–motor output. We propose RA signaling is an exciting new angle from which to investigate how neuro-genetic mechanisms can contribute to the (spoken) language ready brain.

SPEECH AND SPOKEN LANGUAGE

Speech is the primary modality by which humans use language, and human orofacial morphology is uniquely suited to the production of intricate vocalizations needed for spoken language (Lieberman, 2007). The orofacial musculature is one of the most complex muscle systems in the body and in order to successfully produce meaningful speech these muscles must be controlled and coordinated in rapid sequences involving distributed neuronal circuitry. This motor activity is generated in several neural loops that select appropriate actions and generate the necessary motor patterns. One crucial circuit, the cortico-basal ganglia loop, sends activity from the motor cortex to the striatum (a component of the basal ganglia) where activity is integrated. Subsequently, outputs from here modulate activity in several thalamic nuclei. Activity from the thalamus is then sent back to the motor cortex, where a specialized population of output neurons organizes the complex thalamocortical inputs (Kravitz and Kreitzer, 2012; Calabresi et al., 2014). These cortical output neurons send the information, via the pyramidal tract, to motor neurons directly controlling muscle tissue. These neurons are either located in the spinal cord (controlling limb and body movements), or in the brainstem's cranial nerve nuclei (controlling facial and vocal tract movements). An illustration of the cortico-basal ganglia loop (in the rodent brain) is given in **Figure 1A**. Proper connectivity within this pathway is necessary to enable the precise outputs needed for orofacial muscle control.

The striatum can be seen as a central hub within the motor pathway, making it one of the most intriguing regions in which to investigate properties of motor circuitry and orofacial control. Striatal activity is especially important for fine motor behavior and motor skill learning (Doyon et al., 2003) and cortical and subcortical circuitry, including the striatum, has been established as highly important for speech–motor control (Lieberman, 2002). Furthermore, increased activation of the basal ganglia (which incorporates the striatum) has been shown via functional brain imaging (fMRI) in specific speech–motor language tasks (Wildgruber et al., 2001; Booth et al., 2007). Lastly, morphological changes in the striatum have been described in individuals with speech problems such as stuttering (Craig-McQuaide et al., 2014) and non-fluent aphasia (Ogar et al., 2007).

The principal cell type in the striatum is the medium spiny neuron (MSN), which makes up approximately 98% of all striatal cells (Kemp and Powell, 1971; Huang et al., 1992; for review, see Kreitzer and Malenka, 2008). MSNs can be further divided into two categories of neurons that have different connectivity and opposing functions: dopamine receptor type 1 (D1R) and dopamine receptor type 2 (D2R) expressing cells (**Figure 1A**). D1R expressing MSNs connect to thalamic nuclei via the "direct pathway" which results in excitation of the motor cortex. D2R expressing MSNs form an "indirect pathway" that connects to the thalamus via multiple

subcortical regions leading to inhibition of the thalamus and thus reduced cortical input (**Figure 1A**), (Albin et al., 1989; Kravitz and Kreitzer, 2012; Calabresi et al., 2014).

This balance between excitation (resulting in more movement) and inhibition (less movement) is crucial for coordinated motor function (Calabresi et al., 2014) including fine orofacial motor control. In order to unravel the fundamental components that enable humans to effortlessly use spoken language, we will need to understand the neuro-genetic mechanisms involved in establishment, function, and maintenance of speech–motor pathways.

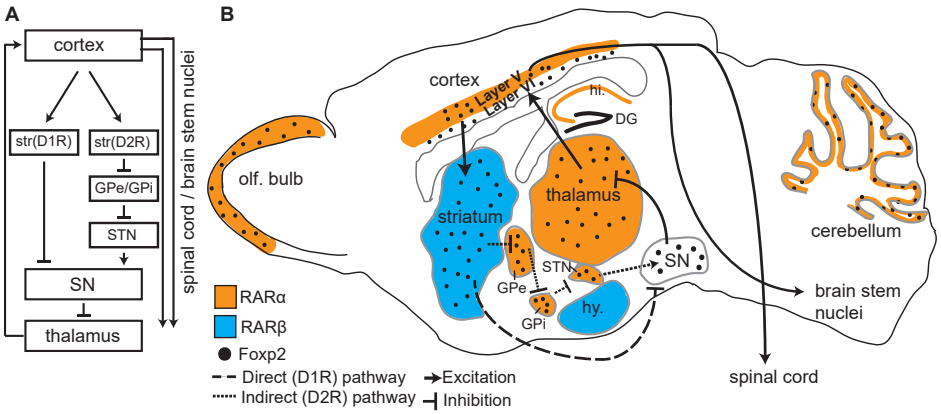

Figure 1: Foxp2 and retinoic acid receptors (RARs) show overlapping expression patterns in motor associated circuitry.

(A) An overview of the direct and indirect pathways represented in the sagittal view showing connectivity between different regions. Dopamine receptor type 1 (D1R) and Dopamine receptor type 2 (D2R) expressing cells in the striatum are separated to highlight direct and indirect pathways. (B) Sagittal Schematic of the mouse brain showing that Foxp2, RARα, and RARβ are all expressed in motor associated circuitry. RARα and RARβ are expressed in distinct regions, but each receptor partially overlaps with Foxp2. RARα and Foxp2 can be found in deep layers of the cortex, thalamus, subthalamic nucleus (STN), the internal (GPi) and external (GPe) globus pallidus, cerebellum, and olfactory bulbs (OB). Foxp2 and RARβ overlap in the striatum. RARα shows non-overlapping expression in the hippocampus (hi.), RARβ in the hypothalamus (hy), and Foxp2 in the substantia nigra (SN). Connectivity between regions involved in motor processing (including outputs to brain stem nuclei and spinal cord) is shown by solid lines. The direct (excitatory) and indirect (inhibitory) pathways, which are the two outputs from the striatum, are shown by dashed lines.

SPOKEN LANGUAGE AND FOXP2

A breakthrough in speech and language genetics came with the identification of the first gene to cause a speech/language disorder: *FOXP2* (Lai et al., 2001). Mutations in *FOXP2* were found in a large pedigree known as the KE family (Hurst et al., 1990; Fisher et al., 1998; Lai et al.,

2001). Affected family members were diagnosed with a severe speech impairment known as developmental verbal dyspraxia (also known as childhood apraxia of speech; OMIM: 602081) and carried a mutation in one copy of their *FOXP2* gene. In addition to speech impairments, affected family members demonstrated receptive and expressive language problems (Watkins et al., 2002a). Although rare, *FOXP2* mutations have been found in a number of unrelated families and individuals with similar speech/language phenotypes (MacDermot et al., 2005; Feuk et al., 2006; Shriberg et al., 2006; Lennon et al., 2007; Palka et al., 2012; Rice et al., 2012; Zilina et al., 2012; for review, see Bacon and Rappold, 2012). In depth investigations of the KE family phenotype indicated a severe impairment in orofacial praxis tasks (Vargha-Khadem et al., 1995; Lai et al., 2001; Watkins et al., 2002a). In addition, impairments in language production tasks (e.g., phoneme addition, word repetition) were found between control and affected individuals (Vargha-Khadem et al., 1995). Different aspects of speech are thus impaired in KE family members (Watkins et al., 2002a). Orofacial praxis deficits underlie impaired lexicon building and subvocal (internal) speech representations which can affect irregular verb grammar (Doyon et al., 2003) and rule based grammar learning (Ullman, 2001). Thus, some of the language impairments in the KE family could be related to the core speech production deficits observed.

FOXP2, and its murine homolog Foxp2, are found across many regions of the developing and postnatal brain (FoxP2 will be used when referring to both species). Intriguing is the high expression of FoxP2 throughout the mouse and human cortico-striato-thalamic motor circuitry (Lai et al., 2003). During early development FoxP2 is broadly expressed in these regions, but in later developmental and postnatal stages expression becomes more restricted (**Figure 1B** depicts Foxp2 expression in the postnatal mouse brain). In adults, Foxp2 is limited to deep layer cortical neurons (layer 5 motor cortex and layer 6 throughout; Ferland et al., 2003; Morikawa et al., 2009; Hisaoka et al., 2010; Tomassy et al., 2010; Reimers-Kipping et al., 2011; Tsui et al., 2013). Within the striatum, Foxp2 is highly expressed in both types of MSN, though more commonly in D1R MSNs compared to D2R neurons (Vernes et al., 2011). Corresponding with its expression pattern, imaging studies have shown humans with *FOXP2* mutations display structural and functional differences in motor areas. Affected members of the KE family showed structural gray matter volume differences in the motor cortex and striatum (Watkins et al., 2002b). Furthermore, functional imaging studies showed an underactivation of the striatum and altered cortical activation (including speech/motor areas such as the left anterior insular cortex) during word generation and word repetition tasks (Liegeois et al., 2003).

Converging evidence from FoxP2 expression pattern studies and phenotypic characterization of human mutations suggests that FOXP2 may play an important role in the development of the speech–motor pathway. The high expression of Foxp2 in a specific subset of neurons (D1R

MSNs) in the striatum indicates a functional specificity related to motor tasks requiring the striato-thalamic connections of the direct pathway. Malfunctions within this pathway could ultimately affect aspects of the motor circuitry related to fine motor control and contribute to the observed speech–motor deficit in humans.

FOXP2 AS A MOLECULAR ENTRY POINT INTO SPEECH-MOTOR PATHWAYS

FoxP2 is a transcription factor; its molecular function is to regulate the expression of other genes, switching them on or off in a temporally and spatially controlled manner. FoxP2 has been shown to regulate 100s of different genes involved in processes crucial to brain development and function, ranging from neurogenesis and migration, to neurite outgrowth and synaptic activity (Spiteri et al., 2007; Vernes et al., 2007, 2011; Konopka et al., 2009; Devanna et al., 2014). Recently, evidence has suggested that FOXP2 regulates a number of genes involved in the retinoic acid (RA) signaling pathway (Devanna et al., 2014). RA is a vitamin-A derivative essential to mammalian development. Disruption of the RA signaling pathway (caused by genetic disruptions or dietary deficiencies) can have severe consequences during development and adulthood (Holson et al., 1997; Krezel et al., 1998)

Retinoic acid induces genetic and morphological changes in cells. When neuronal precursors (cells that generate neurons during development) differentiate into neurons they switch on genes normally found in mature neurons, stop dividing and grow long processes known as neurites (Siegenthaler et al., 2009; Korecka et al., 2013). We previously compared how neuron-like cells with or without FOXP2 responded to RA and found that cells showed stronger genetic and morphological changes in response to RA if FOXP2 was present (Devanna et al., 2014). In addition we discovered that FOXP2 changed the expression of RA receptors – proteins that directly control the cellular response to RA (Devanna et al., 2014). Of particular interest, FOXP2 upregulated retinoic acid receptor β (RARβ) and a number of other genes involved in transport or modification of RA were also transcriptionally regulated (e.g., RORβ, CRABPII, and ASCL1). These experiments suggest an intriguing link between FOXP2 and the RA pathway, in which FOXP2 seems to contribute to or modify the cellular response to RA.

Given the importance of the RA pathway for development, this raises new questions about how FOXP2 might mediate its effects on brain and neural circuit development. Could the relationship between FOXP2 and the RA pathway be relevant for (1) normal motor circuitry development and function, and/or (2) effects of FOXP2 dysfunction in patients? To address these questions, we need to understand how FoxP2 and the RA pathway might interact, and in what way FoxP2 mutations might affect the RA pathway on a cellular, functional and behavioral level.

RA, FOXP2 AND MOTOR BEHAVIOR

Retinoic acid is a key compound during embryogenesis, affecting a multitude of critical developmental pathways. Precise control of RA levels is essential for normal brain development as either an excess or a deficiency of RA results in widespread adverse effects on the brain.

Gestational treatment of rats with excess RA results in behavioral deficits in learning, memory and motor function (Holson et al., 1997). Rats treated with excess RA displayed poor generalized motor control including impairments in the 'righting reflex' (the ability to return to upright position), and the ability to sit only on the back paws. In addition, gestationally treated adult rats showed problems with learning and memory, such as decreased learning rates in a water filled T maze (Butcher et al., 1972; Holson et al., 1997). Rats lacking dietary vitamin A (of which RA is a metabolite) also perform poorly on motor learning and motor performance tasks (Carta et al., 2006). Furthermore, mice engineered to lack a key facilitator of RA signaling (RARβ) develop severe locomotion deficits and are highly impaired on motor learning tasks (Krezel et al., 1998).

The displayed motor deficits are similar to phenotypes observed in mouse models of Foxp2 dysfunction. Mouse models of two well characterized patient mutations of FOXP2 have been created that have comparable phenotypes. One mouse model reflects the R553H missense mutation found in the KE family (Lai et al., 2001). The second mouse model mirrors an early stop codon in exon 7 introduced by a nonsense mutation that leads to a loss of FOXP2 protein in an independent family with speech/language disorder (MacDermot et al., 2005; Groszer et al., 2008). Mice that have a homozygous Foxp2 mutation show severe general motor impairments, reminiscent of animals treated with excess RA. However these Foxp2 homozygous mutants do not survive beyond 3–4 weeks after birth, possibly due to a requirement for Foxp2 in other organs such as the lungs or heart (Groszer et al., 2008). In mice where a single copy of Foxp2 is affected (as per the heterozygous state of the mutations observed in patients) general motor control is normal but motor learning is impaired (Groszer et al., 2008; French et al., 2012). This more subtle phenotype closely resembles the motor learning phenotype observed in RA deprived rats (Carta et al., 2006). For an overview of the different phenotypes exhibited by Foxp2 mutation, RAR mutation, and RA treatment, see **Table 1**.

FOXP2 AND RA SIGNALING AFFECT NEURONAL FUNCTION

In addition to the behavioral deficits, vitamin A deprivation/supplementation adversely affects striatal development and function. Cells in the developing lateral ganglionic eminence (the

precursor region of the striatum) do not differentiate into the appropriate neuronal subtypes when RA signaling is blocked (Toresson et al., 1999; Chatzi et al., 2011). However restoring RA levels rescued this phenotype and resulted in normal differentiation into appropriate neuronal cell types (Chatzi et al., 2011). Separately, mice engineered to knockout the *RAR*β gene display gross morphological striatal defects including impaired neurogenesis and deficits in acquiring proper neuronal identities (Liao et al., 2008). Lastly, chronic postnatal vitamin A supplementation has been linked to oxidative cell toxicity in the striatum (de Oliveira et al., 2007).

Foxp2 also contributes to striatal cell morphology and function. Foxp2 mutant neurons exhibit reduced neurite growth and branching in primary striatal cultures (Vernes et al., 2011) and the *in vivo* striatum displays aberrant neuronal activity. Mice with a heterozygous Foxp2 mutation showed unusually high activity in the dorsomedial striatum during active motor behavior (French et al., 2012). This suggests striatal cells can no longer properly modulate their activity following input from motor areas when lacking Foxp2. Moreover, the increased striatal activity normally seen when animals perform motor learning tasks was absent in mutant mice. Instead, a decrease

in firing rate was seen, again suggesting aberrant modulation of responses to cortical and/or thalamic input (French et al., 2012). Additionally, extracellular measurements on striatal brain slices from heterozygous Foxp2 mutant animals show these cells fail to respond to induction of long term depression (LTD; Groszer et al., 2008). An inability to induce long term plasticity [either LTD or long term potentiation (LTP)] has debilitating consequences as scaled activity (plasticity) is necessary for circuits to properly regulate their input and output. Synaptic long term plasticity changes underlie information storage and are necessary for learning and memory (Novkovic et al., 2015; Zhu et al., 2015). Interestingly, in the striatum, synaptic plasticity has been strongly linked to motor learning (Dang et al., 2006; Kreitzer and Malenka, 2007). Defects specifically related to striatal LTD and LTP are known to affect procedural motor learning and the acquisition of new motor paradigms (Gubellini et al., 2004).

Aberrant induction of synaptic scaling has also been found in mice following acute RA depletion, which results in a complete lack of hippocampal LTP or LTD (Misner et al., 2001). This phenotype was specific to RA depletion and was reversible, as vitamin A supplementation rapidly restored normal synaptic plasticity (Misner et al., 2001). At a molecular level, RA signaling is mediated by the action of RA receptors (RARs; RARα, RARβ, and RARγ) and similar plasticity defects have been shown for mice lacking RARα (Sarti et al., 2012) or RARβ (Chiang et al., 1998). Hippocampal cells from these mice fail to establish LTD when subjected to low frequency stimulation – the paradigm necessary to induce LTD in the hippocampus. By contrast, excess RA induced the reverse effect in cultured hippocampal slices, where

increased excitatory activity was observed (Aoto et al., 2008). It is not yet known if RA signaling affects synaptic plasticity in the striatum. However, the similarity in synaptic activity phenotypes between Foxp2-, RARα-, and RARβ-deficient animals (albeit focusing on different brain regions) does indicate these transcription factors may play a role in similar intracellular pathways regulating neuronal activity and synaptic plasticity.

The aforementioned plasticity (LTD and/or LTP) deficits in Foxp2, RARα, and RARβ mutant animals suggests an improper reaction of neuronal circuits to changes in external input. Induction of LTD or LTP leads to a decrease or an increase, respectively, in the amount of glutamate receptors (of the AMPA- receptor class) at the synaptic membrane (Seidenman et al., 2003; Briand et al., 2014; for review, see Luscher and Huber, 2010). This change in AMPA receptor abundance modifies the response strength of a cell when it is excited. The change in stimulus–response strength is transient, and in time the normal AMPA receptor distribution will be restored, returning synaptic responses to normal levels. RA treatment of hippocampal cultures has shown an increase of AMPA receptors on the cell surface (Aoto et al., 2008), but no data on the striatum is currently present. The shared synaptic plasticity defect following disruption of RA signaling pathways or Foxp2 mutation does suggest that they both may influence receptor abundance or localization at the synapse in the striatum, an intriguing area for further study.

A thorough investigation of the mechanisms leading to LTD and LTP deficits resulting from RA/RAR and Foxp2 malfunction will be necessary to understand if they function in the same pathways. Understanding the molecular mechanisms underlying striatal function, especially related to complex motor circuitry function, will lead to a better understanding of striatal speech– motor control.

MOLECULAR LINKS BETWEEN RARB AND FOXP2

Retinoic acid receptors canonically function as transcription factors, regulating genes responsible for directing normal embryogenesis and brain development. Interestingly, FoxP2 and RARs share some of the same target genes (Balmer and Blomhoff, 2002; Delacroix et al., 2010; Devanna et al., 2014). RARs are highly expressed in the brain (Krezel et al., 1999) and are present throughout embryonal development (Mollard et al., 2000), postnatal development (Wei et al., 2011), and in adults (Krezel et al., 1999; Zetterstrom et al., 1999). Notably high expression of RARs can be found throughout the motor circuitry, including cortical, striatal, and multiple thalamic regions (Krezel et al., 1999), (**Figure 1B**). We focus on two key receptors found in the motor circuitry: RARα and RARβ. RARα is found in layer 5 of the cortex and in the thalamus – both regions that overlap with murine Foxp2 expression (Krezel et al.,

1999; Zetterstrom et al., 1999; Ferland et al., 2003; Lai et al., 2003; Hisaoka et al., 2010). Interestingly, Foxp2 only overlaps with RARα in the motor cortex layer 5, because Foxp2 expression is largely restricted to layer 6 of other mature cortical areas. RARβ is strongly expressed only in the striatum, another site where Foxp2 expression is highest (**Figure 1B**). Notably, FOXP2 has been shown to directly drive RARβ expression in human cells (Vernes et al., 2007; Devanna et al., 2014), although this is yet to be shown in the striatum. This high level of overlap, combined with shared target genes and molecular interactions, strongly supports interplay between FoxP2 and RARs in motor pathways.

CONCLUDING REMARKS

In addition to its canonical role during embryogenesis, studies described here suggest RA signaling plays a specific role in the development and function of striatal motor circuitry and may link to FoxP2 function. Disruption of the RA pathway results in strikingly similar phenotypes to FoxP2 mutation on multiple levels, which suggests a potential mechanistic interaction.

TABLE 1 | Overview of phenotypes described in Foxp2 mutation, retinoic acid receptor (RAR) mutation and RA excess/depletion treatments.

	Deficit	Foxp2 mutation		RA receptor mutation	RA excess/ depletion
		Homozygous	Heterozygous		
Development	Embryogenesis defects	–	–	–	+
	Lethality	++	–	+	++
	Aberrant basal ganglia development	++	+	++	++
Cellular	Basal ganglia cell identity defects	NT	–	+	++
	Decreased neurite growth and branching	++	NT	NT	NT
	Aberrant neuronal activity in striatum	++	++	–	+
	Unable to induce LTD	NT	++	++	NT
	Unable to induce LTP	NT	NT	++	NT
Behavior	General motor control deficits	++	–	++	++
	Motor learning deficits	N/A (postnatal lethality)	+	++	++ (postnatal treatment)
	Spatial learning deficits	N/A (postnatal lethality)	NT	+	+

–, no effect; +, mild effect; ++, strong effect; N/A, not applicable; NT, not tested.

FoxP2 and RARs can regulate some common target genes, affect similar cellular phenotypes and show highly overlapping expression patterns in the cortico-striato-thalamic motor circuitry. In the striatum, aberrant function of Foxp2 and RA signaling contributes to altered development and, in the case of mutations of mouse Foxp2, altered synaptic plasticity similar to that seen in the hippocampus of RARα mutant animals. Given that RARβ is predominantly expressed in the postnatal striatum, it seems likely that its disruption will also affect striatal

plasticity, however, this is yet to be experimentally determined. Lastly, animals with mutated Foxp2 or RA signaling defects show comparable motor control/learning impairments. Thus at multiple levels (molecular, cellular, circuit, and behavioral) there is evidence that interplay between FoxP2 and RA signaling may facilitate proper development and function of motor circuitry. This evidence from mice is strengthened by findings in songbirds which show both FoxP2 and RA influence song learning by acting in circuits that have parallels with human vocal-motor pathways (Haesler et al., 2007; Wood et al., 2008). In the future it will be of great value to understand if these signaling cascades interact to influence neuronal mechanisms related to song learning or speech–motor control, and if RA signaling deficits are involved in aberrant speech–motor development in humans. The capacity for human speech and spoken language is dependent on multiple molecular and neural building blocks. With the link between FoxP2 and RA signaling, a new block has been suggested, giving us new opportunities to investigate the evolution and development of the (spoken) language ready brain.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Donders Institute, Radboud University Nijmegen, a Marie Curie career development grant awarded to SCV and by the Max Planck Society. We would like to thank Moritz Negwer for valuable comments on the manuscript.

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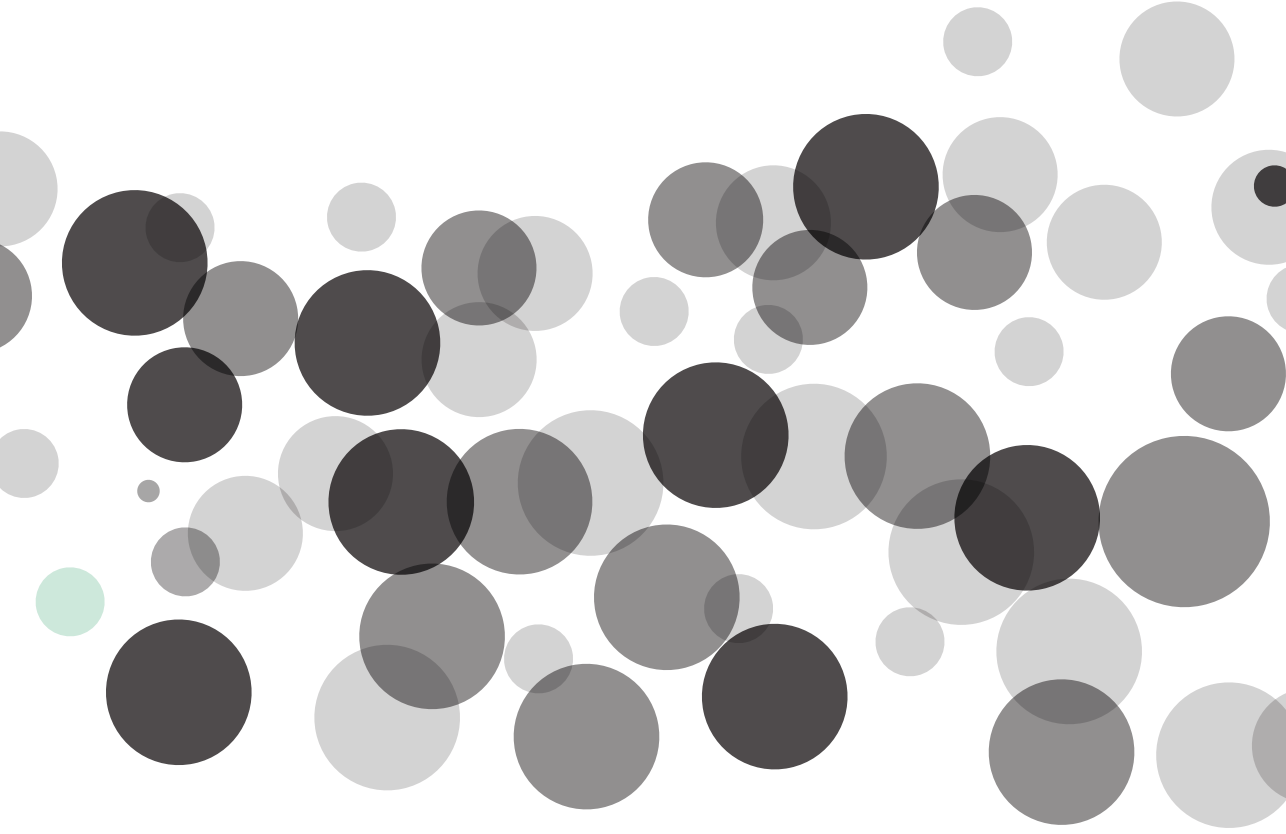
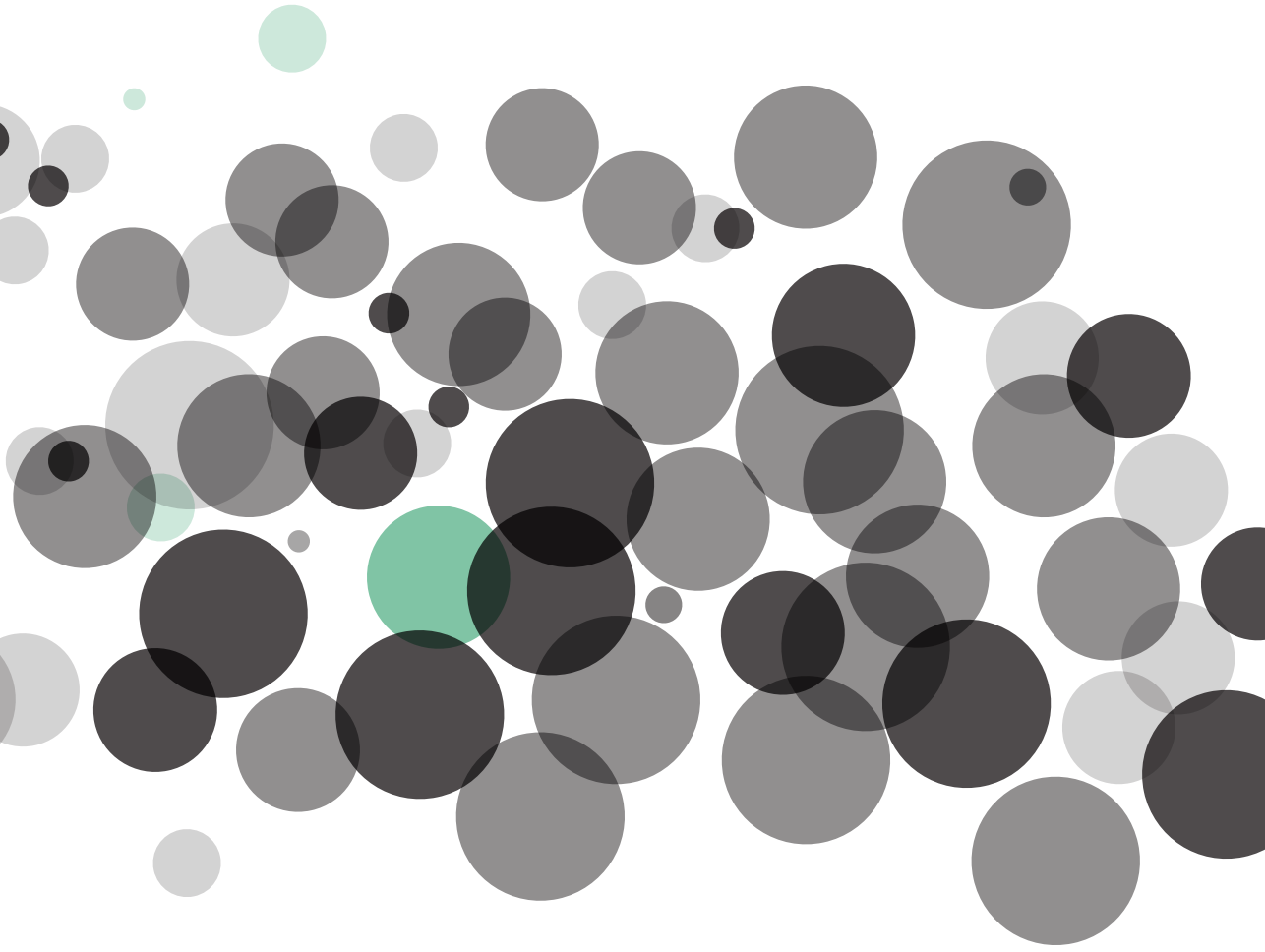
CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



6.

Reduced excitatory activity in a human dopaminergic model for FOXP2 homozygous deletion



Jon-Ruben van Rhijn, Yan Shi, Monica Frega, Dirk Schubert,
Tomislav Maricic, Svante Pääbo and Nael Nadif Kasri

ABSTRACT

Heterozygous mutations of the transcription factor *FOXP2* in humans lead to childhood apraxia of speech (CAS). FOXP2 is highly expressed in GABAergic and dopaminergic neurons throughout the basal ganglia, including expression in the striatum, substantia nigra and multiple thalamic nuclei. fMRI data from individuals with CAS shows that basal ganglia activity is affected by heterozygous mutation of *FOXP2*. In mouse models of Foxp2 dysfunction, aberrant striatal development and impaired striatal activity and plasticity have been established at the single-cell level. This commonality of how FoxP2 dysfunction affects striatum across species suggests human and mouse FoxP2 might affect the same neurobiological pathways. In order to investigate the effect of *FOXP2* mutation in human neurons at single-cell resolution, we developed a protocol to generate human excitatory induced dopaminergic neurons (DA iNeurons), via controlled differentiation of human induced pluripotent stem cells (hiPSCs). We confirmed the dopaminergic identity of these cells as well as the expression of FOXP2 in mature DA iNeurons. Next, we used an isogenic *FOXP2* knockout line (*FOXP*$^{-/-}$) to compare excitatory activity between control and *FOXP2* knockout neurons. *FOXP2*$^{-/-}$ DA iNeurons showed reduced excitatory activity both on the single cell and network level. The effects of *FOXP2* mutation on excitatory activity are similar to findings in mouse models for FoxP2 dysfunction and suggest human and mouse FoxP2 regulate excitatory striatal activity through the same neurobiological pathways.

INTRODUCTION

The production of spoken language is a uniquely human feature, which depends on higher cortical function, as well as subcortical circuits (Lieberman, 2002, 2007). Generation of the complex motor sequences necessary for speech requires rapid control of the relevant musculature, achieved through strict regulation of activity within cortico-striato-thalamic circuitry (Ackermann et al., 2014; Barbas et al., 2013; Booth et al., 2007; Jurgens, 2002; Ziegler and Ackermann, 2013). Moreover, neurodevelopmental disorders which dysregulate cortico-striato-thalamic circuit function severely impair complex motor sequencing in both humans and animal models (Shepherd, 2013). With speech production being a specialization of complex motor sequencing (Ackermann et al., 2014), a number of disorders that disproportionately affect speech production have been described (Deriziotis and Fisher, 2017; Newbury and Monaco, 2010). One of these is disruption of the transcription factor *FOXP2* (Fisher et al., 1998; Lai et al., 2001; Lai et al., 2003; MacDermot et al., 2005; Morgan et al., 2017). Mutation of *FOXP2* was first described for the KE-family, in which half of the family members are affected by childhood apraxia of speech (CAS), accompanied by wide-ranging problems with expressive and receptive language (Lai et al., 2001). CAS is defined as 'a disorder of speech motor programming or planning that affects the production, sequencing, timing and stress of sounds'(Morgan et al., 2017). Investigations into FOXP2 protein function have shown that this transcription factor is able to regulate many genes, including genes involved in neurodevelopment and synaptic function (Spiteri et al., 2007; Vernes et al., 2011). Furthermore, investigation of brain morphology and activity in KE family members and controls has revealed that grey matter volume is reduced (Watkins et al., 2002) and brain activity is altered in affected KE family members in areas relevant for complex motor sequencing. Overactivation of the head of the caudate nucleus was seen during a word repetition task (Vargha-Khadem et al., 1998), and underactivation of the putamen and thalamus in a different study during a covert verb generation task (Liegeois et al., 2003).

Animal models for FoxP2 dysfunction (we use here the standard nomenclature from (Kaestner et al., 2000)) have been used to investigate how this transcription factor is involved in the regulation of motor sequencing and motor skill learning (Campbell et al., 2009; French et al., 2012; Gaub et al., 2010; Groszer et al., 2008; Haesler et al., 2007; Murugan et al., 2013). It has been shown that mutations and/or localized knockdowns of *FoxP2* lead to morphological and functional changes in the striatum in mice (Chen et al., 2016; French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018) and in the striatal song nucleus area X in zebra finch (Murugan et al., 2013; Schulz et al., 2010). Foxp2 affects both excitatory and inhibitory activity in the striatum. In mice with a heterozygous *Foxp2* nonsense mutation, excitatory activity is reduced and inhibitory activity is increased in Dopamine receptor type 1 expressing striatal medium spiny neurons (D1R-MSNs) (van Rhijn et al., 2018). It has been shown that heterozygous knockout

of Foxp2 leads to changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor abundance (Chen et al., 2016) yielding a reduction of excitatory activity. Furthermore, expression of GAD67, one of the main enzymes involved in the synthesis of GABA, is increased in mice with heterozygous Foxp2 loss of function. This leads to increased presynaptic GABA content and can explain the increased inhibitory drive of D1R-MSNs (van Rhijn et al., 2018).

Three amino-acid substitutions distinguish the human FOXP2 protein from its mouse ortholog. Two of these changes occurred on the hominid lineage after splitting from that of chimpanzees and are present in exon 7 of *FOXP2* (T303N and N325S). These evolutionary changes have been hypothesized to confer unique properties to human FOXP2 that may be relevant for its involvement in speech and language production (Enard et al., 2002; Zhang et al., 2002). The substitutions have been shown as present in older hominids (Neanderthals and Denisovans (Krause et al., 2007)), and highly detailed investigation of FOXP2 has suggested that no other changes to FOXP2 which can convey additional functionality occurred later during human evolution (Atkinson et al., 2018). The third change is a conservative change exclusive to the mouse evolutionary lineage and is present towards the start of *Foxp2* (D80E) (Enard et al., 2002). Mice have been generated in which normal Foxp2 has been substituted with a version of the gene which encodes the T303N and N325S human substitutions (Enard et al., 2009). Assessments of cell morphology have shown that synapse density and striatal MSN complexity are increased in these partially 'humanized' mice (Chen et al., 2016; Enard et al., 2009; Reimers-Kipping et al., 2011). Furthermore, both long term potentiation (LTP) and long-term depression (LTD) can be more readily induced in striatal brain slices of the 'humanized' mice (Schreiweis et al., 2014). Lastly, on the behavioral level these amino-acid substitutions have been associated with faster switching from declarative to procedural learning (Schreiweis et al., 2014) as well as to changes in exploratory behavior (Enard et al., 2009). These findings suggest that incorporation of the human amino-acid substitutions is able to modify functionality of mouse Foxp2.

The recent advancements in the generation of human neurons from induced pluripotent stem cells (iPSCs), have made it possible to investigate genetically mediated neurodevelopmental disorders using human cellular models (Brennand et al., 2011; Chailangkarn et al., 2012; Chailangkarn et al., 2016; Linda et al., 2018; Tamburini and Li, 2017). FOXP2 is expressed in distributed areas the basal ganglia, both in humans and mice (Campbell et al., 2009; Lai et al., 2003). For the striatum, it is known that FoxP2 is expressed highly in GABAergic medium spiny neurons, which are a category of dopamine neuron (Fong et al., 2018; van Rhijn et al., 2018; Vernes et al., 2011). Furthermore, FoxP2 is also expressed in areas where almost exclusively excitatory dopaminergic neurons are present. For example, FoxP2 expression has been shown in parts of the substantia nigra pars reticulata (SNr) and throughout the substantia nigra pars compacta (SNc), though it is currently unknown in which cell populations in these areas FoxP2

is predominantly expressed (Campbell et al., 2009; Lai et al., 2003). In order to investigate the functions of FOXP2 in excitatory human neurons, we generated a human iPSC derived culture of excitatory DA neurons, hereafter referred to as DA iNeurons using a protocol adapted from (Sundberg et al., 2013). Neuronal identity was confirmed by immunofluorescent staining and we furthermore confirmed that mature DA iNeurons express FOXP2. We used this protocol to generate neurons from a control cell line and a cell line with homozygous deletion of FOXP2 (*FOXP2*$^{-/-}$) generated by CRISPR/Cas9 mediated genome editing. Control and *FOXP2*$^{-/-}$ DA iNeurons showed comparable active and passive intrinsic properties. Further electrophysiological analysis revealed a clear reduction in excitatory activity in the *FOXP2*$^{-/-}$ neurons at the single-cell and network level. Our data therefore suggest that homozygous deletion of *FOXP2* in human neurons affects synaptic maturation and neuronal network development. Investigation of FOXP2 function in a human neuron model is an important step towards understanding how FOXP2 might regulate brain circuits involved in the generation of spoken language and if this might differ from the conserved roles Foxp2 has played in complex motor skill learning during evolution.

RESULTS

DA iNeurons are viable and express FOXP2 in mature DA iNeurons

To assess the effects of homozygous deletion of FOXP2 on DA iNeurons, we first developed a differentiation protocol to produce excitatory DA iNeurons from human iPSCs (Figure 1a). Our protocol is adapted from a previously published protocol which reliably produces functional DA iNeurons (Sundberg et al., 2013). Supplementation with DAPT was used to promote differentiation toward a DA lineage. For a detailed list of the compounds used during iPSC culturing and differentiation, see table 1. At days in vitro (DIV)55, DA iNeurons show a mature complexity by staining of dendrites with MAP2 (Figure 1b). Reconstruction of individual stained neurons further highlights that these neurons show multiple dendrites, which span >100 µm within the culture dish (Figure 1c). DA identity of the iNeurons was confirmed at DIV55 by tyrosine hydroxylase (TH) staining (Figure 1d), and all neurons in the DA iNeuron culture express TH. Furthermore, at DIV55, at least 60% of the DA iNeurons express FOXP2, as shown by co-expression of TH and FOXP2 (Figure 1d). These data demonstrate that our differentiation protocol generates DA iNeurons with mature complexity similar to DA neurons from mouse primary culture (Gaven et al., 2014), and that FOXP2 is expressed in these neurons. This expression pattern of FOXP2 shows similarities to the distributed expression of FOXP2 in dopaminergic neurons in the mouse, zebra finch and human, where the sparse labeling of cells within for example the SNr and SNc suggests specific cell populations express FoxP2. (Campbell et al., 2009; Lai et al., 2003; Teramitsu et al., 2004) Subsequently, we used this differentiation protocol to investigate the effect homozygous deletion of *FOXP2* on neuronal activity.

Both *FOXP2*$^{+/+}$ and *FOXP2*$^{-/-}$ iNeurons show comparable active and passive intrinsic properties

Homozygous knockout or mutation of FOXP2 is known to affect neurodevelopment in vitro in primary striatal cell cultures (Vernes et al., 2011) and mouse brain tissue (Groszer et al., 2008). Furthermore, mice with heterozygous Foxp2 mutations develop normally, but display aberrant neuronal activity during development as well as in adulthood.

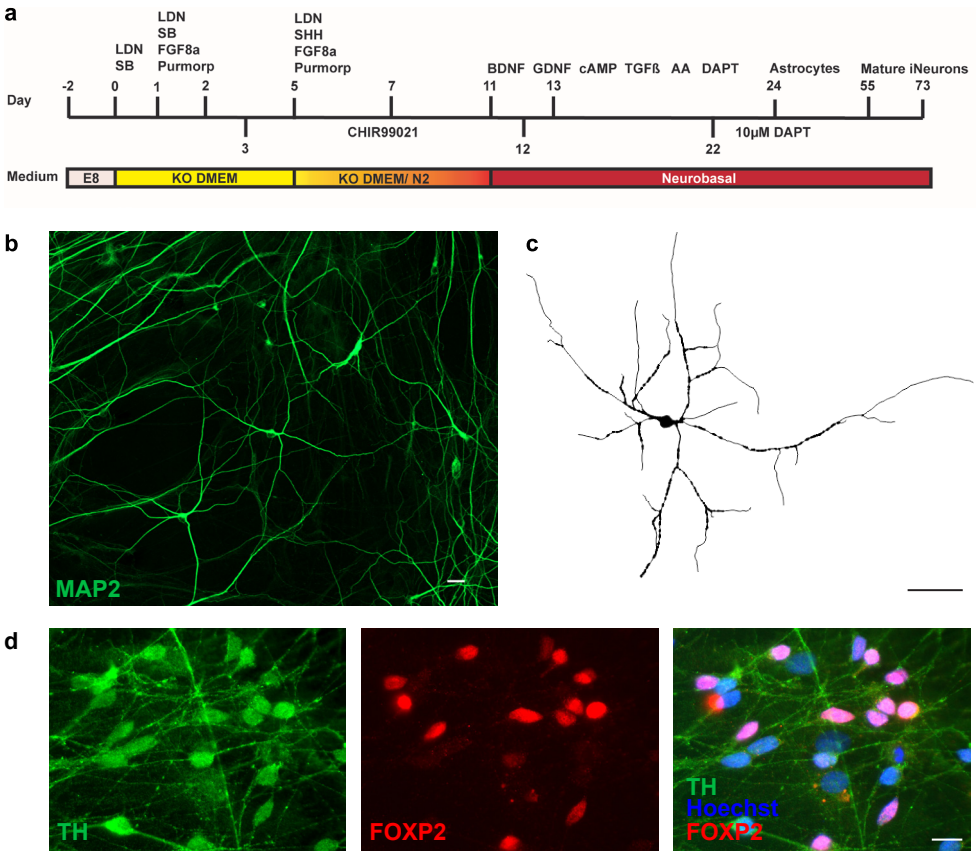


Figure 1: iPSCs differentiated into DA iNeurons express TH and FOXP2.

(a) Timeline for differentiation of iPSCs toward a dopaminergic lineage. For details on the protocol see methods. (b) MAP2 staining of dopaminergic neurons shows high complexity and >100µm neurite length. (c) reconstruction of a single dopaminergic neuron including all dendrites. The axon is not visible with MAP2 staining. (d) FOXP2 is expressed in a subset of dopaminergic iNeurons, as shown by the incomplete overlap between HOECHST (which stains all nuclei) and FOXP2. (e) Dopaminergic neuron identity is confirmed by Tyrosine hydroxylase expression, and dopaminergic neurons express FOXP2 shown by overlap between FOXP2 and TH. Scalebar for b,d,e = 20µm.Scalebar for c = 50µm

By contrast, mice with homozygous Foxp2 mutations show severe developmental impairments and die between the third and fourth postnatal week (Groszer et al., 2008). This suggests cellular physiology might be severely impaired by homozygous FOXP2 knockout. We therefore assessed initially if intrinsic membrane properties might be affected by homozygous FOXP2 knockout in our human DA iNeuron model. Intrinsic properties can be divided into active properties and passive properties. Active properties describe the possibility of neurons to generate strong action potential firing when presented with a depolarizing current, whereas passive properties give a measure of the excitability and maturity of a cell (Planert et al., 2013). In order to investigate FOXP2 dysfunction in human DA iNeurons we used an isogenic line generated from our control line in which FOXP2 was homozygously deleted through a disruption of exon 7, which leads to a lack of the encoded protein, and hence loss of function. Loss of FOXP2 function has been previously described in multiple cases of FOXP2 associated CAS (MacDermot et al., 2005; Morgan et al., 2017)**.** Reported cases include nonsense mutations that lead to insertion of a STOP codon in exon 7 of FOXP2, such as the R328X mutation, predicted to result in protein truncation and nonsense mediated decay. Reduction of Foxp2 expression has been shown in mice carrying a similar (but not identical) mutation (S321X) (Groszer et al., 2008).

To investigate active and passive intrinsic properties, we used a current-voltage protocol in which depolarizing current steps of increasing current amplitude were given until reliable action potential firing was elicited (Figure 2a). We measured intrinsic properties of DA iNeurons at DIV55 and found that both control and *FOXP2*$^{-/-}$ DA iNeurons are able to generate action potentials (Figure 2b). Active and passive properties were extracted from either a 10 mV square voltage step (cell capacitance of membrane resistance, Figure 2d,e) or from the I-V traces (resting membrane potential, max AP amplitude and AP threshold, Figure 2f,g,h). Intrinsic properties were comparable for the different lines at DIV55, with a similar resting membrane potential, action potential threshold, membrane resistance and cell capacitance. We did not observe any changes in intrinsic properties, which suggests that DA iNeurons lacking any FOXP2 expression are generally healthy and develop into fully functional neurons.

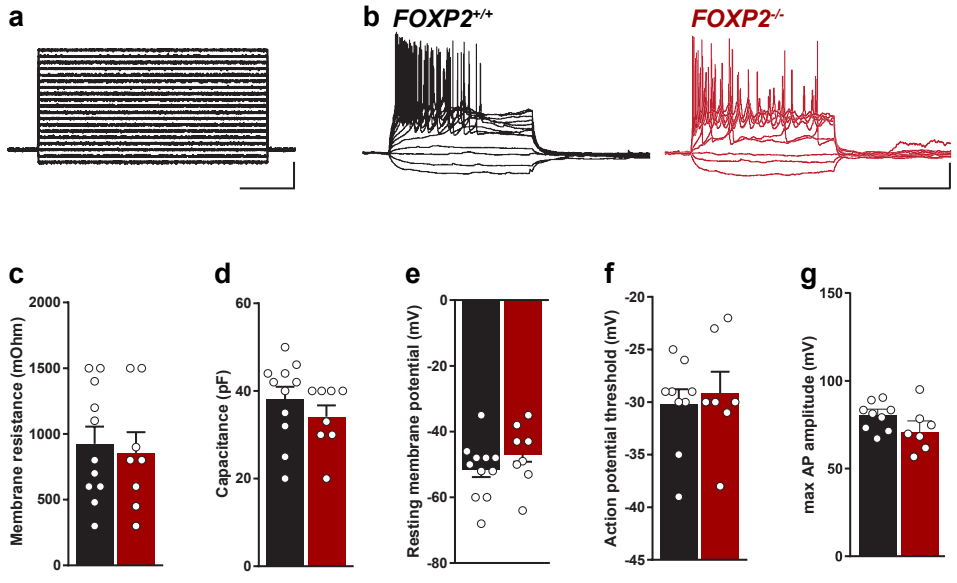


Figure 2: Intrinsic properties are similar between *FOXP2*$^{+/+}$ and *FOXP2*$^{-/-}$ DA iNeurons

(a) Current-voltage protocol. A two second square step of fixed current size was given. Step size increased with each consecutive recording. Scalebar = 500ms/20pA. (b) Example trace of action potentials generated by *FOXP2*$^{+/+}$ and *FOXP2*$^{-/-}$ DA iNeurons at DIV55, scalebar = 20mV/1sec. (c-g) Membrane resistance, cell capacitance, resting membrane potential, action potential threshold and maximum action potential amplitude for *FOXP2*$^{+/+}$ and *FOXP2*$^{-/-}$ iNeurons (Resistance: *FOXP2*$^{+/+}$ 925.5±130 MΩ, *FOXP2*$^{-/-}$ 856±156 MΩ, NS. Capacitance: *FOXP2*$^{+/+}$ 38.2±2.8 pF, *FOXP2*$^{-/-}$ 34.1±2.6 pF, NS. RMP: *FOXP2*$^{+/+}$ -51.6±2.6mV, *FOXP2*$^{-/-}$ -46.9±3.3 mV, NS. AP threshold: *FOXP2*$^{+/+}$ -30.2±1.4 mV *FOXP2*$^{-/-}$ -29.1±2.0 mV, NS, max AP amplitude: *FOXP2*$^{+/+}$ 82.0±11.2 mV *FOXP2*$^{-/-}$ 72.3±17.3mV NS).

Excitatory activity is reduced in human DA iNeurons with reduced FOXP2 expression

Heterozygous and homozygous *Foxp2* loss of function leads to decreased excitatory activity in mouse striatal neurons (Chen et al., 2016; van Rhijn et al., 2018). Therefore, we next investigated if spontaneous synaptic activity might be impaired in human DA iNeurons with homozygous *FOXP2* deletion. In mice, excitatory activity is reduced both in neurons from juvenile (postnatal day 10-15) as well as adult (postnatal day 55-60) mice (van Rhijn et al., 2018). Activity is furthermore only decreased for those MSNs which would normally express FOXP2, which predominantly are D1R-MSNs. In mice excitatory input onto D1R-MSNs is mainly achieved through corticostriatal connections (Hunnicutt et al., 2016; Smith et al., 2004) However, the DA iNeurons we generate are excitatory and form a network *in vitro*. Therefore, an effect of FOXP2 on excitatory neurotransmission would likely be aggravated. To investigate if homozygous *FOXP2* deletion affects excitatory activity in human DA iNeurons, we measured spontaneous excitatory postsynaptic currents (sEPSCs) and compared *FOXP2*$^{+/+}$ neurons with isogenic *FOXP2*$^{-/-}$ neurons. Two developmental time points (DIV55 and DIV73,

Figure 3a,c) were chosen to investigate if differences in activity between genotypes would persist during maturation. sEPSC amplitude was reduced in cells with homozygous *FOXP2* deletion (Figure 3b,d) whereas sEPSC frequency was increased (Figure 3c,f). The decreased amplitude and increased frequency are persistent and present at both DIV55 and DIV73. Though the decreased sEPSC amplitude is in line with the data obtained from mice with heterozygous and homozygous loss of Foxp2 function, such mice did not show an increase in sEPSC frequency. This difference might be explained by the fact that presynaptic excitatory striatal connections are not established by cells which express FoxP2 *in vivo*. sEPSC frequency is mostly affected by release probability and presynaptic strength (Planert et al., 2013), and our data suggest that FOXP2 might affect presynaptic mechanisms as well in DA iNeurons, in addition to its effects on the postsynapse, both in DA iNeurons and mouse D1R-MSNs.

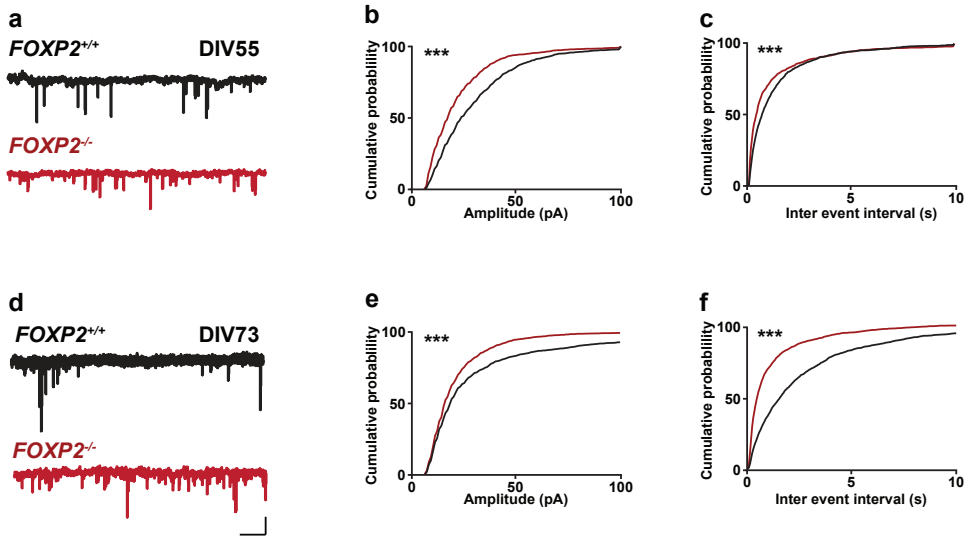


Figure 3: *FOXP2*$^{-/-}$ DA iNeurons show reduced sEPSC amplitude and increased sEPSC frequency.

(a) Example traces of sEPSC activity at DIV55. (b) cumulative graph of sEPSC amplitude (Average: FOXP2$^{+/+}$ 27.0±4.5pA, FOXP2$^{-/-}$ 19.4±3.1 P<0.01) and frequency (Average: FOXP2$^{+/+}$ 0.86±0.15Hz, FOXP2$^{-/-}$ 1.93±0.8 P<0.01) at DIV55 . (d) Same as (a) but for DIV73. (e) cumulative graph of sEPSC amplitude (Average: FOXP2$^{+/+}$ 32.11±8.7 pA, FOXP2$^{-/-}$ 20.36±2.72 pA P<0.01) and frequency (Average: *FOXP2*$^{+/+}$ 1.88±0.36 Hz, *FOXP2*$^{-/-}$ 5.27±1.4 Hz *P*<0.01) at DIV73. ***=P<0.01, Kolmogorov-Smirnoff nonparametric test. Scalebar = 10pA/1sec.

Network activity is impaired in human iNeurons with reduced FOXP2 expression

The change in sEPSC amplitude and frequency shows that excitatory inputs are affected by homozygous FOXP2 deletion. Intriguingly, our cells show both a reduction in amplitude (suggesting reduced excitatory input) and an increase in frequency (suggesting increased

excitatory input). Because of this, it is unclear how the changes at the single cell level affect the general network activity. We therefore investigated if a network phenotype is present in human DA iNeuron cultures with homozygous FOXP2 deletion. To investigate the roles of FOXP2 at the network level, we cultured FOXP2+/+ and FOXP2-/- human DA iNeurons on multi-electrode arrays (MEAs). This enabled us to measure population activity, as each MEA is equipped with a high number of electrodes which can measure action potential generation at the single spike level. We measured activity in DA iNeurons from both the FOXP2+/+ and FOXP2-/- lines in a single multi-well MEA. Network activity was measured at both DIV55 and DIV73 (Figure 4a,b). Activity was highly reduced in the FOXP2-/- lines, shown by a strong reduction in the mean firing rate (Figure 4c,e). Intriguingly, the increased sEPSC frequency shown at the single cell level is not visible at the network level, which suggests that increased sEPSC frequency due to homozygous FOXP2 deletion does not lead to an overall increased network activity. Furthermore, burst duration did not seem to be affected (Figure 4d,f), which suggests that FOXP2 deficient cells still form a well-connected network in which correlated activity can propagate through the culture (Bisio et al., 2014; Frega et al., 2017).

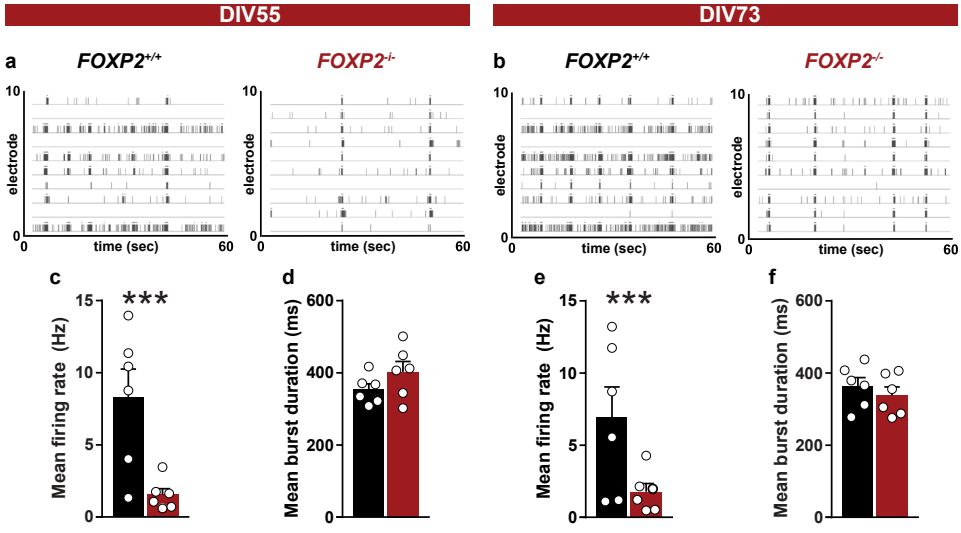


Figure 4: Homozygous *FOXP2* deletion leads to impaired network activity.

(a) Raster plot of 1 minute of spontaneous activity recorded on a 6 well MEA device of either *FOXP2*$^{+/+}$ (left) or *FOXP2*$^{-/-}$ (right) DA iNeurons at DIV55 and DIV73. Each horizontal line is a single electrode. Each vertical line displays a single recorded event. Reduced activity can be seen as a reduction of events recorded. (b) Quantitative analysis of mean firing rate and burst duration between *FOXP2*$^{+/+}$ and *FOXP2*$^{-/-}$ cells at DIV55 and DIV73 (Mean firing rate DIV55: *FOXP2*$^{+/+}$ 9.6±1.95Hz *FOXP2*$^{-/-}$ 1.34±1.04Hz, *P*<0.01. Mean firing rate DIV73 *FOXP2*$^{+/+}$ 7.13±2.12Hz, *FOXP2*$^{-/-}$ 1.6±0.6, *P*<0.04. Burst duration DIV55 *FOXP2*$^{+/+}$ 353±16.3ms, *FOXP2*$^{-/-}$ 402.4 NS., Burst duration DIV73 *FOXP2*$^{+/+}$ 363.3±24.4ms *FOXP2*$^{-/-}$ 338.1±23.2 NS)

DISCUSSION

FoxP2 is a transcription factor with high conservation across species. However multiple amino acids which could potentially affect FoxP2 function distinguish between mouse and human orthologs. In order to investigate if human and mouse FoxP2 affect the same neurobiological processes, we studied human iPSCs with either intact *FOXP2* or homozygous *FOXP2* deletion. We subsequently generated human DA iNeurons which express FOXP2 and characterized neuronal activity at a single-cell and network level. Our data show reduced excitatory sEPSC amplitude, similar to the decrease in excitatory synaptic strength shown in D1R-MSNs from mice with a heterozygous loss of Foxp2 function mutation. Intriguingly, whilst in mice the frequency of excitatory events is not affected, sEPSC frequency is increased in human *FOXP2*$^{-/-}$ DA iNeurons. This finding suggests that FOXP2 affects both pre- and postsynaptic mechanisms. We show for the first time that FOXP2 is important for network activity *in vitro*. Homozygous FOXP2 knockout leads to a reduced firing rate, whereas bursting behavior seems to not be affected by a lack of FOXP2 expression.

Different *FOXP2* mutations, including nonsense mutations, have been found in individuals with CAS (Morgan et al., 2017). Despite variation in mutation type, all individuals with etiological heterozygous mutations in FOXP2 show similar behavioral phenotypes, with CAS accompanied by wide-ranging language impairments. Furthermore, mice with heterozygous mutations in Foxp2 display impaired motor skill learning, and behavioral phenotypes are conserved between mutations (French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018). However, it seems that different mutations of FOXP2 can uniquely affect underlying neurobiological mechanisms. For example, the missense R553H mutation of the KE family leads to an increase in striatal activity *in vivo* (French et al., 2012) and *in vitro* (van Rhijn et al., Chapter 3) in the *Foxp2*$^{R552H/+}$ mouse model of this mutation. This is in contrast to the decreased excitatory activity shown for the S321X nonsense mutation (van Rhijn et al., 2018) and Foxp2 knockout (Chen et al., 2016). Investigation of a human *FOXP2*$^{R553H/+}$ cell line would be an intriguing addition to the emerging view that different FoxP2 mutations have unique effects on cellular function.

Both Foxp2 knockouts as well as missense mutations have been shown to affect neurodevelopment. In mouse primary cultures, *Foxp2*$^{R552/R552H}$ leads to gross impairments in neurite outgrowth and neurodevelopment (Vernes et al., 2011). Similarly, homozygous knockout of *Foxp2* leads to reduced excitatory spine density of MSNs. In affected individuals from the KE family changes in gray matter density are seen for a number of regions, including the caudate/putamen and the cerebellum, where FOXP2 is highly expressed (Watkins et al., 2002). Our control human DA iNeurons form a functional neuronal network *in vitro* and FOXP2$^{-/-}$ neurons show similar intrinsic properties to control neurons. This finding suggests

that general neuronal development is not grossly impaired, even with complete loss of FOXP2 expression. However, smaller deficits on the level of synapse development could still be present, and in fact are suggested by our single cell electrophysiology data. An in-depth characterization of control and affected neurons through immunofluorescent staining and morphological reconstruction will unveil exactly how neurodevelopment in human neurons is affected by homozygous *FOXP2* deletion. Furthermore, mice with heterozygous knockout of Foxp2 show a reduction in AMPA receptor expression (Chen et al., 2016), which would be a logical explanation for the reduction in excitatory amplitude that we observed in our human DA iNeurons. The physiological effects that absence of FOXP2 expression has on excitatory activity indicate that excitatory pre- and postsynapses are affected.

FOXP2 expression is high in a range of different types of neurons throughout the brain (Ferland et al., 2003; Hisaoka et al., 2010; Takahashi et al., 2003). Research on the physiological effects of Foxp2 mutations has largely focused on striatal GABAergic MSNs, and these show a clear reduction in excitatory event amplitude following heterozygous Foxp2 loss of function mutation (van Rhijn et al., 2018). However, the increased excitatory event frequency that we observed in our human DA iNeurons with homozygous *FOXP2* knockout is absent in mouse MSNs with heterozygous mutations. This can possibly be explained by the difference in neuronal identity between the cells. Our DA iNeurons are glutamatergic, which contrasts with the GABAergic identity of MSNs. Striatal MSNs only receive excitatory connections from the cortex and the thalamus. Though there are cortical and thalamic cells which express Foxp2, it unclear whether these cell populations project to the striatum (Hunnicutt et al., 2016; Smith et al., 2004). The lack of a change in excitatory event frequency in mouse striatal D1R-MSNs suggests that presynapses which terminate on striatal D1R-MSNs are probably not affected by changes in Foxp2 levels. In contrast to this, our human DA cells form an excitatory network with excitatory pre- and postsynaptic connections between cells which normally express FOXP2. The measured increase in excitatory activity, combined with the reduction of sEPSC amplitude in FOXP2 knockout DA iNeurons, suggests that FOXP2 affects both pre- and postsynaptic mechanisms in these cells. Alternatively, the increased frequency could be a cell-autonomous response to a reduction in excitatory amplitude following FOXP2 knockout, to restore the balance of excitatory activity within the network. Compensatory neurobiological mechanisms have been described in the context of neurodevelopmental disorders (Marder and Goaillard, 2006; Nelson and Valakh, 2015), and the homogenous network established by DA iNeurons could enable compensation for the effects of FOXP2 knockout on neuronal activity. Further investigation of the mechanisms which lead to the changes in excitatory amplitude and frequency would give novel insights into how FOXP2 affects homeostasis and how different neuronal networks are affected by disruptions of this transcription factor.

Importantly, the use of iNeurons from a human background enables us to investigate possible human-specific functions of FOXP2. Human FOXP2 is distinct from orthologs of other animal species (Enard et al., 2002). It has been suggested that the T303N and N325S amino-acid substitutions specific to the hominind lineage might convey unique functionality to FOXP2 (Enard, 2011). In the past, mice have been generated that express Foxp2 with these human substitutions. Mice which express a partially 'humanized' Foxp2 show increased neuron complexity, increased plasticity, changes in exploratory behavior and a faster switch from declarative to procedural learning (Reimers-Kipping et al., 2011; Schreiweis et al., 2014). Such findings suggest evolutionary modification of the ways that FOXP2 can regulate neuronal activity. Furthermore, it is known that FOXP2 exhibits human specific regulation of genes involved in brain development compared to chimpanzee FoxP2 (Konopka et al., 2009). Though the mouse data suggests unique properties of FOXP2 in cell physiology, these data lack translational ability to the human situation. Our DA iNeurons provide a unique opportunity to generate cells from a human lineage which express FOXP2 and investigate these on a genetic, morphological and functional level. Furthermore, the successful generation of DA iNeurons from human material also enables the generation of functional neurons from individuals who carry CAS-associated mutations in *FOXP2*. These could be used to investigate whether different mutations of *FOXP2* or other CAS related genes uniquely affect neuronal function in human neurons and might provide a platform to investigate possible therapeutic treatments *in vitro* which restore neuronal activity to control conditions. Though we are currently constrained to the generation of glutamatergic cells, loss of *FOXP2* function clearly affects these neurons. A next step should be to generate inhibitory neurons with FOXP2 expression, as this transcription factor strongly affects inhibitory activity as well (van Rhijn et al., 2018). This combination of excitatory and inhibitory cells which express FOXP2 will be a powerful tool to further understand how the gene affects neuronal function.

Lastly, the ability to generate human neurons which express FOXP2 is of interest as well from an evolutionary perspective. The ability to generate *FOXP2* with specific mutations through CRISPR/Cas9 editing enables the generation of human cell lines, which recapture ancestral states of FOXP2 in evolution of our ancestors. In a similar fashion to the partially 'humanized' mice it is possible to generate a cell line where the T303S and N325S substitutions have been reverted. This would result in DA neurons in which the FOXP2 protein is the same as that of chimpanzees (Enard et al., 2002). Investigation of differences in FOXP2 function between control human neurons and these 'ancestralized' neurons can show us the commonalities and differences between human FOXP2 and orthologs of closely related species. How FOXP2 might have changed during evolution and which role it might play in our ability to produce speech and language is still a largely unresolved issue. Our human DA iNeurons could be a valuable tool for providing novel insights into the neuronal processes which have enabled emergence of spoken language during human evolution.

To conclude, we have for the first time generated human functional neurons which endogenously express FOXP2. In these neurons, FOXP2 regulates excitatory activity and loss of its expression by homozygous knockout leads to a marked reduction of excitatory activity. This recapitulates the excitatory phenotype found in mice with heterozygous loss of Foxp2 function, validating DA iNeurons as a novel tool to study FOXP2. The ability to culture cell lines from individuals with mutations in FOXP2, as well as to generate cell lines which can recapture ancestral evolutionary states of this gene, will be a powerful way to further help us understand how it is involved in the uniquely human ability to produce spoken language.

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MATERIALS & METHODS

Genome editing

IPSC lines which express either *FOXP2*$^{+/+}$ or *FOXP2*$^{-/-}$ were generated from a control IPSC line (A161A) by the research group of Svante Pääbo (MPI for Evolutionary Anthropology, Liepzig, Germany). In short, one 80% confluent well with a control IPSC line A161A of a one six-well plate was treated with Accutase for 15 minutes and cells were filtered through a 40 um sieve. DNA was added to cells and electroporated with Nucleofector 2b Device (Lonza) using B-16 program. Electroporation mixture contained ~1 million cells, plasmid pSpCas9n(BB)-2A-Puro (PX462) V2.0 (Addgene #62987), dsDNA encoding for gRNA (1ug), donor ssDNA (200pm) and Scr7 (1uM). Cells were treated with puromycin (1ug/mL) for two days to select for cells transfected with DNA.

Loss of function mutations on both alleles were generated in sequence. First, one mutation was introduced (heterozygous). Positive clones were expanded and sequenced. Subsequently, the second mutation was introduced in a previously selected positive clone. Two different target sequences were designed, one for each allele. Target sequences and corresponding DNA donor sequence for genome editing are shown in Table 1.

Table 1:Target and donor sequences:

Target sequence 1	GATGCTTTGGAAGTGTTGG
Target sequence 2	GTCTAAGTGCAAGACGAGAC
Donor sequence 1	CATTCACTATGGAATGATGAGTTATTGGTGGTGATGCTTTGGAAGTGGTGGAGGAGGTAGTCGAGGAGGAATTGTTAGTAGTGAGGTCTAGCCC;
Donor sequence 2	ACCAATAACTCATCATTCCATAGTGAATGGACAGTCTTCAGTTCTAAATGCAAGACGAGACAGGTAAATCTCATGAGCTTTATTCTATATTTATCTATT.

Generation of dopaminergic iNeurons

Frozen iPSCs were thawed and reprogrammed to a dopaminergic lineage as outlined in Figure 1A according to a protocol modified from (Sundberg et al., 2013). In short, iPSC colonies were split into single cells by Accutase treatment for 5 min at 37°C 2 days before the start of differentiation and 2.0*10^4 cells were plated on vitronectin-coated plates in E8 medium supplemented with 1x RevitaCell (Thermo Fisher). From DIV0 to DIV5 E8 medium was replaced with knockout DMEM supplemented with 15% knockout serum replacement, 1x GlutaMAX 100U/ml, Penicillin-Streptomycin (100U/ml) and 1x MEM-NEAA. β-Mercaptoethanol (0.1mM) was added to the stock medium fresh before each medium change. Between DIV6 and 9, KO-DMEM was gradually replaced by 25%, 50%, 75% and 100% N2 medium (DMEM/F2 with 1x N2 supplement). From DIV12 iNeurons were cultured in neurobasal medium supplemented

with 1x B27 and 1x Glutamax, and half the medium was replaced every two days. Cells were passaged by dissociation with Accutase at 100% confluency. At DIV20, immature iNeurons were dissociated to single cell state and $2.0*10^4$ cells were re-plated on poly-L-ornithine (Sigma, 50µg/ml) and mouse laminin (Sigma 10µg/ml) coated coverslips or MEAs. From DIV24, cells were co-cultured with rat astrocytes prepared according to (Frega et al., 2017) on a 1:1 ratio to promote maturation. For the concentrations and timepoints of the small molecules and growth factors which were added during culturing, see Table 2.

Table 2: Compounds and small molecular used during differentiation and maturation of Dopaminergic iNeurons.

Compound	DIV when added	Concentration	Company
LDN-193189	DIV 1-11	10µM	Stemgent inc.
SB431542	DIV 1-5	10µM	Stemgent inc.
Purmorphamine	DIV 2-11	10µM	Stemgent inc.
FGF-8a	DIV 2-11	100ng/ml	R&D system
CHIR99021	DIV 3-12	3µM	Stemgent inc.
SHH(C24II)	DIV 6-12	100ng/ml	R&D system
BDNF	DIV 12-22	20ng/ml	Peprotech
GDNF	DIV 12-22	20ng/ml	Peprotech
cAMP	DIV 12-22	0.5mM	Enzo Life Science
TGFβ3	DIV 12-22	2ng/ml	Millipore
Ascorbic acid	DIV 12-22	200µM	Sigma
DAPT	DIV 12-22	10nM	Millipore
DAPT	DIV 22-end maturation	10µM	Millipore

Immunocytochemistry

Coverslips containing at least $2.0*10^4$ cells were immersion-fixated in 4% paraformaldehyde/sucrose solution for 15 minutes at room temperature. Non-specific antibody binding was prevented by incubating the cells with 10% normal donkey serum or normal goat serum (blocking solution) at RT for 1 hour. Nuclei were stained with Hoechst and the coverslips were mounted with DAKO fluoromount medium. Primary antibodies used were Tyrosine Hydroxylase (TH-16, Sigma, 1:500) and Foxp2 (Sc-21069, Santa Cruz, 1:500). Imaging was done using a Zeiss upright fluorescent microscope with apotome (Zeiss Axio Images, Oberkochen, Germany).

Neuronal reconstruction

Wide field fluorescent images of the fixated and MAP2-labelled hiPSC derived dopaminergic neurons were taken at 20x magnification using ApoTome microscopy (Zeiss Axio Imager Z1/Z2). The images were stitched using Fiji (Fiji Is Just ImageJ) 2017 software. Subsequently

digital 3D reconstructions of neuronal morphology were created from the MAP2 images using Neurolucida 360 (Version 2017.01.4, Microbrightfield Bioscience). An overlay drawing was made of the somatodendritic morphology of dopaminergic neurons. Only dopaminergic neurons with at least two primary dendrites and at least one dendritic branch point were selected for the reconstruction.

Single-cell electrophysiology

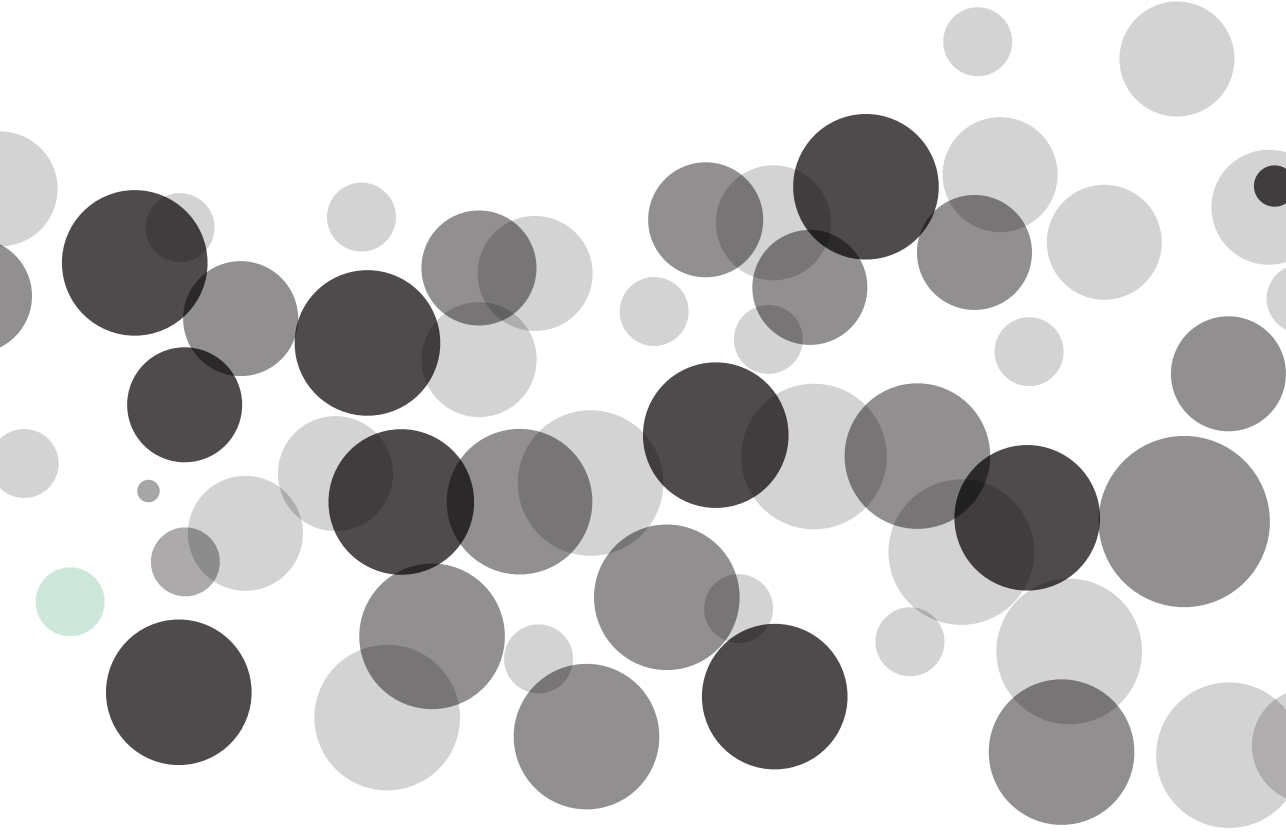
Intrinsic properties and spontaneous excitatory postsynaptic currents (sEPSCs) were measured in dopaminergic iNeurons cultured on glass coverslips. Activity was measured at DIV55 and DIV73. At the day of measurement, coverslips were transferred to the recording setup and incubated in recording artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 1.25 NaH_2PO_4, 3 KCl, 26 $NaHCO_3$, 10 glucose, 2 $CaCl_2$, and 1 mM $MgCl_2$ continuously oxygenated with 95% O_2/5% CO_2. The recording bath temperature was kept constant at 32°C. Patch pipettes (5–7 MΩ) were made from borosilicate glass capillaries with filament and an inner diameter of 0.86mm (Fine Science Tools, Heidelberg, Germany). Patch pipettes were filled with intracellular solution containing (in mM): 130 K-Gluconate, 5 KCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2ATP, 0.4 Na_3GTP, 10 Na-phosphocreatine, 0.6 EGTA (PH 7.2-7.3, 285-295 mOsm). sEPSC, cell capacitance and membrane resistance were recorded at -60mV membrane potential. Resting membrane potential, action potential amplitude and action potential threshold were recorded by I-V curve. sEPSC activity was analyzed using minianalysis 6.0.2. software (synaptosoft). All other parameters were extracted using Clampfit 10.7 (Molecular devices, San Jose, CA).

Micro-electrode array measurements

Network activity was measured using a 6-well multi-electrode array (MEA) device (Multichannel systems, Reutlingen, Germany). MEAs contained 60 TiN/SiN planar round electrodes (30 µm diameter; 200 µm center-to-center inter-electrode distance). Dopaminergic iNeurons were cultured as described above and spontaneous activity was measured at DIV55 and DIV73. Medium was refreshed at least 24 hours before the measurement. At the start of the measurement, the MEA was transported from the incubator to the setup and placed in a separate incubation chamber, continuously perfused with 95% O_2/5% CO_2 and kept at 37°C. Baseline activity was measured for 10 minutes after the MEA was placed in the recording chamber. When baseline acitvity was stable, recording was started, and 20 minutes of spontaneous activity was recorded. After recording, the MEA was immediate placed back in the incubator at 37°C degrees. Activity was quantified using a customized software package in MATLAB (SpyCode, (Bologna et al., 2010)).

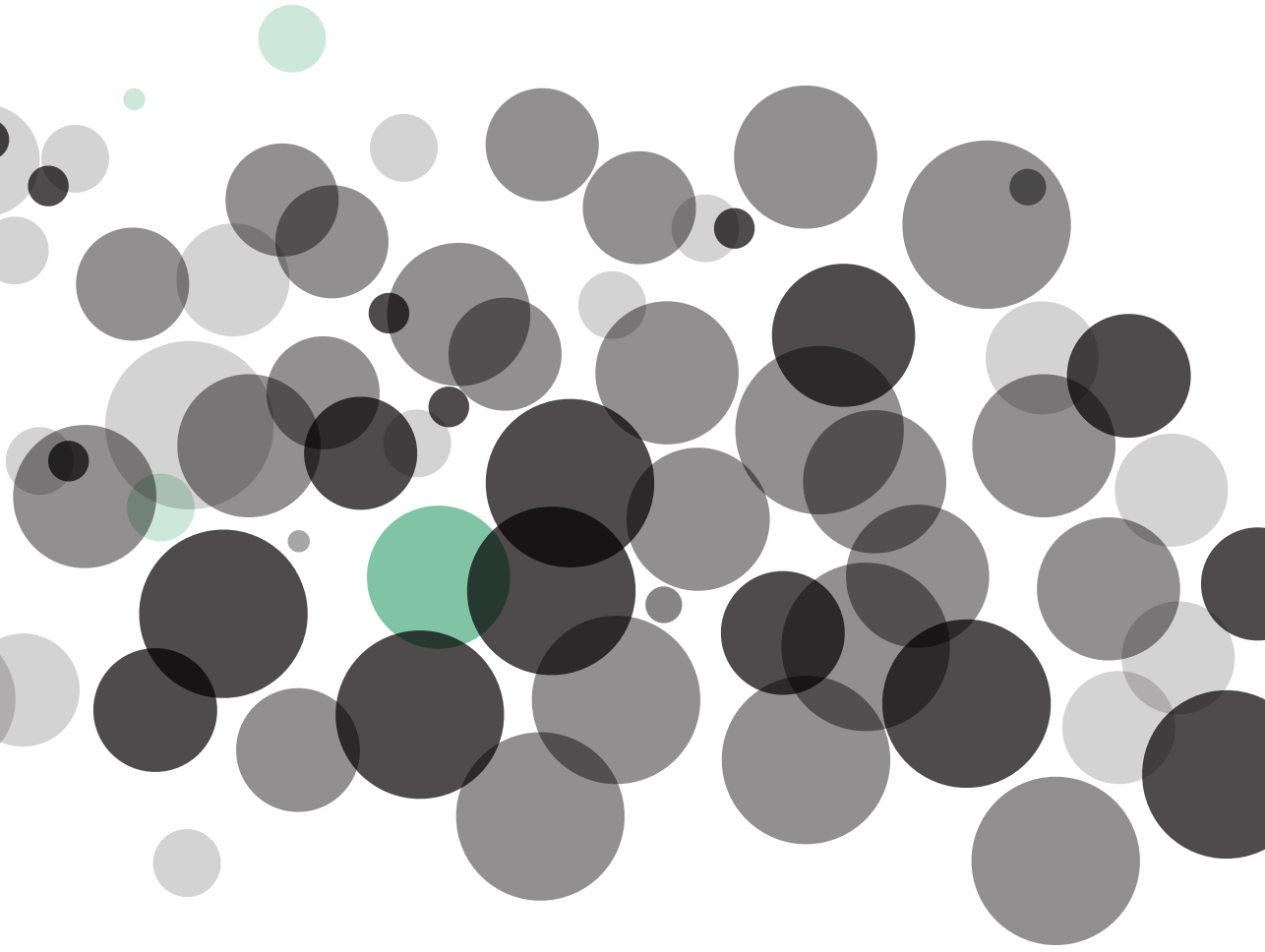
Statistics

All statistical analysis was conducted between two groups (*FOXP2*$^{+/+}$ or *FOXP2*$^{-/-}$) and average data was analyzed by students' T-test. Cumulative data was analyzed by Kolgomorov-Smirnov test (KS-test). Significance was set at $P<0.05$. All data is represented as average ± SEM.



7.

Discussion



FOXP2 AND ITS FUNCTION IN REGULATION OF STRIATAL ACTIVITY

The transcription factor FoxP2 is involved in motor skill learning in rodents, and speech and spoken language production in humans. Heterozygous mutation of *FOXP2* leads to childhood apraxia of speech (CAS) in humans. Previous research has shown that mutations in *Foxp2* affect striatal activity and lead to impaired motor behavior in animal models. However, a detailed investigation of how Foxp2 regulates striatal activity on the single cell level has not been conducted before. In this thesis, we have shown that Foxp2 regulates both excitatory and inhibitory activity specifically in dopamine receptor type-1 expressing medium spiny neurons (D1R-MSNs). Nonsense mutation of *Foxp2* results in a persistent decrease of excitatory and an increase of inhibitory activity, disrupting striatal E/I balance. Blockade of inhibitory activity *in vivo* results in amelioration of the motor skill learning deficit in these mice **(chapter 2).** Next, we have shown that Foxp2 is involved in the early postnatal maturation of striatal glutamatergic neurotransmission, as nonsense mutation of Foxp2 leads to delayed maturation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). Furthermore, we provided evidence that that impaired excitatory receptor trafficking might underlie impairments in long term depression known to be present in striatum of both Foxp2 missense and nonsense mutation mice **(chapter 3).** We further provided the first evidence that different mutations of Foxp2 differently affect striatal activity. Comparison of the effects of the nonsense mutation described in **chapter 2** and missense mutation **(chapter 4)** on striatal D1R-MSNs revealed that they inversely affect striatal D1R-MSN activity. Missense mutation Foxp2 leads to decreased inhibition, and *in vivo* blockade of inhibition aggravated the motor skill learning impairments in these mice. Next, we investigated if retinoic acid signaling, through regulation of transcription factor RARβ by Foxp2, is possibly a novel mechanism through which Foxp2 affects striatal activity. We comprehensively analyzed the current literature on retinoic acid (RA) signaling and concluded that regulation of striatal retinoic acid receptor expression and function are highly involved in motor skill learning, striatal neurodevelopment and striatal plasticity **(chapter 5)**. This overlaps with the current knowledge on Foxp2 and its involvement in regulation of striatal development activity. The ability of Foxp2 to regulate RARβ suggests interaction between these two transcription factors may have a key function in striatal circuit function. Lastly, we generated human dopaminergic neurons and used these to assess how dysfunction of human FOXP2 affects neuronal activity **(chapter 6)**. We show that FOXP2 is expressed in excitatory induced dopaminergic neurons (DA iNeurons) and that homozygous deletion of FOXP2 leads to reduced excitatory activity and impaired network function in these cells. In this chapter, we will discuss these findings and where we think future research into FoxP2 should be aimed.

The function of FOXP2 at the level of human speech and spoken language

The first possible genetic underpinnings for speech were described by studies of the KE family, in which more than half of the family members were described as affected by developmental verbal dyspraxia (DVD): 'difficulty in organizing and coordinating the high speed movements necessary to produce intelligible speech' (Hurst et al., 1990). After the clinical description of the speech impairment present in this family, additional investigation into affected family members by linguists led to the proposal that they have expressive and receptive deficits in usage of grammatical rules (Gopnik and Crago, 1991). This led some researchers to suggest that affected family members have impaired higher order cognitive processing, resulting in specific deficits in spoken language production and comprehension. The possible genetic nature of the disorder in turn fueled a discussion that genes for uniquely human traits such as spoken language or grammar might exist. Though it has been shown that both verbal and nonverbal IQ are lower in affected individuals compared to unaffected family members of the KE family (Vargha-Khadem et al., 1995), nonverbal IQ is much more preserved compared to verbal IQ. Advancements in genetic screening reignited the investigation of this family and unveiled mutation of the gene *FOXP2* as the genetic cause of their disorder (Fisher et al., 1998; Lai et al., 2001). From subsequent research into *FOXP2*-associated disorders, and related speech problems in other cases, the description of DVD or (the currently more used) childhood apraxia of speech (CAS) has been refined: 'a disorder of speech motor programming or planning that affects the production, sequencing, timing and stress of sounds' (Morgan et al., 2017). This description places the speech impairment at the core of the spoken language difficulties displayed by individuals with mutations in *FOXP2.* As such, it may be argued that the grammar, sentence, and phonological impairments could be secondary consequences of impaired control over the motor sequencing programs which enable speech production. Since the initial discovery and characterization of *FOXP2*, functional studies of various kinds have been conducted to investigate whether and how mutations in this gene affect the brain. Imaging of brain structure and recordings of brain activity in human participants have unveiled that mutations in *FOXP2* lead to alterations in brain anatomy and function (Liegeois et al., 2003; Watkins et al., 2002). Convergent findings especially implicate the caudate and putamen, which has prompted detailed investigation of analogues of this region in animal models for FoxP2 dysfunction. In animal studies, striatal impairment has been shown to be a key factor in Foxp2 dysfunction (French et al., 2012; Groszer et al., 2008; Haesler et al., 2007; Schulz et al., 2010). The results obtained from the initial genetic and functional investigations in both human individuals and animal models show clearly the striatal activity is affected by mutations in *FoxP2*. In order to better understand how Foxp2 regulates striatal activity, we set out to investigate changes in striatal physiology following *Foxp2* mutation at the single-level. This detailed assessment of how Foxp2 regulates striatal activity can provide novel insight into the complex regulation of circuit activity underlying motor sequencing in animals and ultimately the generation of speech/spoken language in humans. This lack of a detailed

physiological analysis on the function of Foxp2 in the striatum was the starting point for the work presented in this thesis.

Foxp2 affects striatal direct pathway E/I balance during development and in adulthood

Until now, all research into how FoxP2 regulates striatal activity has focused on extracellular recordings *in vivo* (French et al., 2012) and *in vitro* (Groszer et al., 2008), and intracellular recordings of excitatory activity (Chen et al., 2016; Schreiweis et al., 2014). However, these investigations lack detail on the striatal cell populations affected by Foxp2. Furthermore, no investigation of the effect of Foxp2 on inhibitory activity or if Foxp2 affects striatal activity during development has been conducted. The distinction between striatal cell populations is highly relevant, as suggestions that FoxP2 expression is segregated between MSNs of the striatal direct and indirect pathways have been made (Fong et al., 2018; Vernes et al., 2011). Furthermore, heterozygous knockout of Foxp2 affects many genes with preferential expression in direct pathway MSNs (Enard et al., 2009; Heiman et al., 2008). This distinction between the direct and indirect pathway is highly relevant for how striatal activity is involved in the generation of complex motor sequences (Kravitz and Kreitzer, 2012; Kreitzer and Malenka, 2008). Generally, neuronal activation of the direct pathway leads to increased activity of the movement circuit and is involved in initiation and maintenance of motor sequences. On the other hand, activation of the indirect pathway leads to decreased activity in the movement circuit and these neurons are mainly involved in switching behavior and adaptation (Andre et al., 2011; Calabresi et al., 2014).

In this thesis, we have shown that mutations of Foxp2 affect striatal activity in a cell population specific manner. We and others quantitatively established that Foxp2 is predominantly expressed in direct pathway MSNs (Fong et al., 2018; van Rhijn et al., 2018; Vernes et al., 2011), we are the first to show that heterozygous mutation of *Foxp2* specifically affects activity in D1R-MSNs (Figure 1a). Since Foxp2 did not affect activity in D2R-MSNs, this demonstrated that the regulation of activity is only disrupted in the direct pathway mice with heterozygous Foxp2 mutations. Balanced excitation of MSNs within both the direct and indirect pathway is important for motor circuit function and disruptions in pathway specific regulation of activity have been implicated in motor dysfunction (Andre et al., 2011; Rothwell et al., 2014). To study how aberrant Foxp2 function affects striatal activity on the single-cell level, we used mice in with a bacterial artificial chromosome (BAC)-eGFP construct was expressed under the D1R-promoter. This enabled identification of D1R-MSNs in *ex vivo* brain slices. We crossed these mice to two different mouse models of Foxp2 dysfunction. The R552H mouse model has a substitution in the DNA- binding domain of *Foxp2*, directly matching the mutation described in the KE family. This substitution disrupts DNA-protein interaction of Foxp2 and leads to impaired transcriptional activity (Vernes et al., 2006). The S321X mutation, similar to a R328X mutation described in another family with CAS, (MacDermot et al., 2005) leads

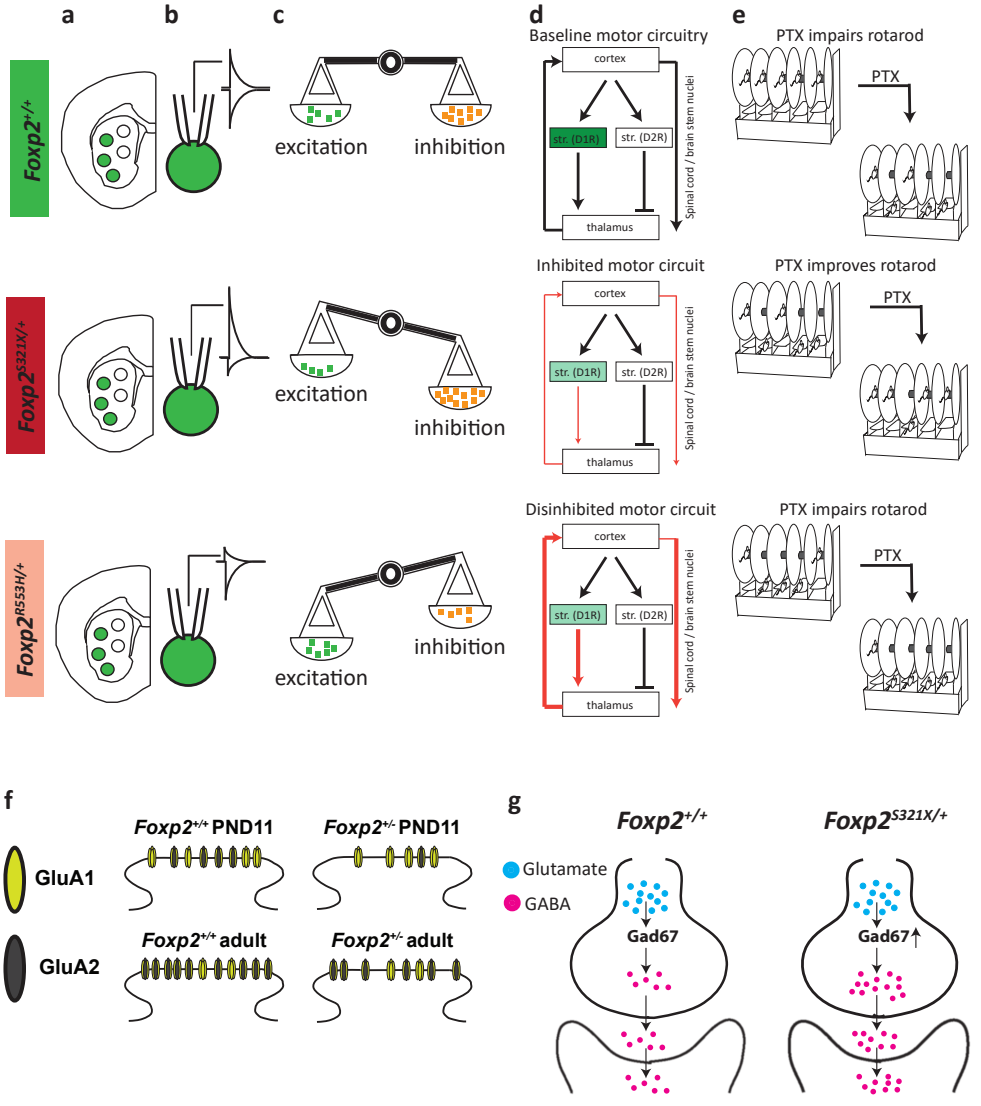


Figure 1: Foxp2 affects D1R-MSN E/I balance, schematic overview of main findings.

(a) Foxp2 is predominantly expressed in D1R-MSNs and we have shown Foxp2 only affects activity in these cells in the striatum. (b) Inhibitory and excitatory activity are affected by Foxp2 mutations, with different effects dependent on the specific mutation. (c) These effects lead to dysregulation of E/I balance in D1R-MSNs. (d) The motor circuit in *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ is inversely affected by the changes in the striatum. (e) Though motor behavior impairments are shown in both genetic backgrounds, in vivo blockade of activity differently affected motor learning skills. (f) AMPAR maturation and AMPAR expression are affected by the S321X mutation. (g) GAD67 production and presynaptic GABA content are increased in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice

to loss of function, protein truncation and nonsense mediated decay of Foxp2, which results in a reduction of Foxp2 expression (Groszer et al., 2008). On the behavioral level, mice with the R552H mutation were shown to exhibit clear reductions in motor skill learning (French et al., 2012; Groszer et al., 2008). Though a similar behavioral phenotype was suggested for mice with the S321X mutation, this mutation seemed to result in a less severe phenotype on motor skill learning (French et al., 2012). We confirmed the presence of similar motor skill learning deficits as in *Foxp2*$^{R552H/+}$ mice in *Foxp2*$^{S321X/+}$ mice, with reduced accelerating rotarod performance and impaired motor learning. Therefore, we can conclude that the R552H/R553H and the R328X/S321X mutations lead to similar behavioral phenotypes: in human individuals to CAS, and in both mouse models to motor skill learning impairments.

It is well established that Foxp2 affects striatal activity from previous research (Chen et al., 2016; Enard et al., 2009; French et al., 2012; Groszer et al., 2008; Liegeois et al., 2003; Murugan et al., 2013; Schreiweis et al., 2014). However, detailed investigation of how striatal activity is affected was lacking until now. Furthermore, previous evidence has shown that heterozygous mutation of *Foxp2* (both the R552H mutation and the S321X mutation) affects striatal activity in adult mice (French et al., 2012; Groszer et al., 2008). However, in humans, FOXP2-associated CAS typically manifests already during early childhood (Morgan et al., 2017), which suggests the gene has functions earlier in life as well. Additionally, FoxP2 expression has been confirmed in 9-weeks post conception human fetal tissue and mouse tissue from embryonic day 13.5 onwards and continues throughout life (Ferland et al., 2003; Fong et al., 2018). Furthermore, homozygous mutation of *Foxp2* strongly affects neurodevelopment and the generation of mature neurons in mice (Chen et al., 2016; Vernes et al., 2011). Despite this evidence that Foxp2 functions during development, we are the first to show that Foxp2 affects striatal activity from as early as postnatal day (PND) 7 in mice and continues to affect striatal activity throughout development until adulthood. This is especially relevant as heterozygous mutation of *FoxP2* already leads to a clear phenotype in humans early in life whilst in mice effects of heterozygous *Foxp2* mutation on behavior have only been investigated during adulthood. Therefore, we have used the Foxp2-S321X mouse model to assay the functions of Foxp2 through different stages of postnatal development on the physiological and behavioral level **(Chapter 2 and 3)**. Early in development, reduced Foxp2 expression affects the maturation of AMPARs in the striatum (Figure 1f). During development, striatal glutamatergic synapses change their ratio of calcium-impermeable (CI) AMPARs to calcium-permeable (CP) AMPARs (CI:CP-AMPAR ratio) (Bellone et al., 2011). Mature striatal synapses predominantly express CI-AMPARs, and the possibility to change the CI:CP-AMPAR ratio is essential as well for induction of long-term depression (LTD) and long-term potentiation (LTP) (Man, 2011). We are the first to show the temporal specificity by which this switch occurs in the striatum, where in wild-type D1R-MSNs juvenile CI:CP AMPAR ratios are present until PND 9, but mature CI:CP ratios are present at PND11. This switch

coincides with the emergence of coordinated locomotion, which suggests fast maturation of AMPAR expression is necessary for motor circuit function. We have shown in *Foxp2*$^{S321X/+}$ mice that the emergence of an adult CI:CP-AMPAR ratio is delayed, as is the emergence of coordinated locomotion. Furthermore, in control mice, activation of the mGluR1 pathway leads to endocytosis of CI-AMPARs in the striatum, which results in LTD. In *Foxp2*$^{S321X/+}$ mice this mechanism is impaired, as there is no change in the relative expression of CP-AMPARs nor can LTD be induced in these mice. Intriguingly, we have also shown that excitatory activity is reduced throughout development in *Foxp2*$^{S321X/+}$ mice. This finding corroborates previously published data showing that AMPAR expression is reduced in juvenile mice with a heterozygous *Foxp2* knockout mutation (Chen et al., 2016). We suggest that this aberrant AMPAR expression already arises early in development. Though the CI:CP-ratio does eventually show adult expression in *Foxp2*$^{S321X/+}$ mice, the reduced excitatory activity in adult mice as well as the lack of LTD suggest that regulatory mechanisms which govern AMPAR expression remain affected in *Foxp2*$^{S321X/+}$ mice throughout life. This suggests Foxp2 might have unique functions in the regulation of D1R-MSN excitatory and striatal plasticity during development and adulthood, with different consequences for motor behavior.

In addition to showing reduction of excitatory activity during development and in adulthood, we have also investigated if Foxp2 affects inhibitory striatal activity. Is has previously been shown that Foxp2 can regulate targets involved in inhibitory activity (Vernes et al., 2011). However, the consequence of this regulation, and the effect of Foxp2 dysfunction, on striatal inhibition was not explored to date. We have shown that Foxp2 regulates inhibitory activity in D1R-MSNs by repression of GAD67 expression, which was a ChIP target in (Vernes et al., 2011) (Figure 1b,f). GAD67 is one of the principal enzymes for the production of GABA. Changes in GAD67 expression strongly impact on inhibitory activity. For example, *Gad1* (the gene which codes for GAD67) knockout mice show highly reduced inhibitory synaptic strength (Lau and Murthy, 2012). *Foxp2*$^{S321X/+}$ mice show an increase in GAD67 expression, as well as an increase in inhibitory synaptic strength. Our detailed assessment of how the S321X mutation leads to increased inhibitory activity has established that GABA content at striatal D1R-MSN presynapses is increased. Combined with the previous data on excitatory activity, this means that the direct pathway is much more strongly inhibited in *Foxp2*$^{S321X/+}$ **(chapter 2)**. However, it is currently unknown if this increased inhibition directly related to the motor skill learning impairments in these mice. Therefore, we manipulated inhibitory activity *in vivo* in *Foxp2*$^{S321X/+}$ mice, which is the first time that possible therapeutical intervention to ameliorate Foxp2-associated behavioral phenotypes in mice has been reported. Blockade of GABAergic inhibitory activity in the entire brain by intraperitoneal injection of the noncompetitive GABA antagonist Picrotoxin leads to impaired motor skill learning in wild-type mice. By contrast, the same treatment leads to marked increase in motor skill learning in *Foxp2*$^{S321X/+}$ mice compared to vehicle control (Figure 1e). This clearly shows that Foxp2 normally regulates inhibitory

activity, at least in the striatum but possibly also in other brain regions where it is expressed. The difference in affinity to inhibitory blockade between wild-type mice and *Foxp2*$^{S321X/+}$ mice furthermore suggests that the concentration of picrotoxin that we injected leads to partial blockade of inhibitory activity. This novel mechanism by which Foxp2 can regulate E/I balance at the single cell level generates new opportunities to unravel how this transcription factor is involved in the generation of complex activity within the motor circuit. The ways that Foxp2 affects other brain regions downstream of the striatum is an exciting new avenue which can build upon the results we obtained.

Most of our work has been conducted in the *Foxp2*-S321X loss of function mouse model. However, previous results which show Foxp2 affects striatal activity have mostly been obtained in mice heterozygous for the R552H DNA-binding domain mutation. As these two mutations differently affect Foxp2 function, they might influence striatal activity differently. Moreover, the physiological data currently available on these mutations has been amassed using different methods. Previous data on *Foxp2*$^{R552H/+}$ mice has been obtained through extracellular measurements of activity, whilst we have assessed the effect of the S321X mutation at the single cell level **(chapter 2)**. To enable a direct comparison of these two different mutations, we used the same single cell methods used in brain slices from *Foxp2*$^{S321X/+}$ mice to assess striatal activity *in vitro* in *Foxp2*$^{R552H/+}$ mice **(chapter 4)**. We show that the R552H leads to increased direct pathway function, whilst the S321X mutation produces the inverse phenotype. Intriguingly, our data suggest that the mechanisms through which these mouse mutations affect neuronal activity are different. The S321X mutation reduces excitatory activity, possibly through a reduction in AMPAR maturation and impaired AMPAR trafficking, and increases inhibition by increased presynaptic GABA content. By contrast, the R552H mutation does not affect excitatory activity, and instead leads to a decrease in inhibitory activity in D1R-MSNs. This mechanistic finding suggests that these mutations inversely affect the output of the striatal direct pathway (Figure 1c,d). The S321X mutation leads to increased inhibition of D1R-MSNs as well as reduced excitability. D1R-MSNs are in turn less prone to activation and therefore there is a reduction in inhibitory drive down to the substantia nigra (SNr). By contrast, the reduced inhibition due to the R552H mutation leads to increased inhibitory drive from the striatum toward the SNr (Figure 1c). This is contrary to the current state of knowledge in the field, as the fact that both mutations produce similar behavioral phenotypes in humans and mouse models (French et al., 2012; Groszer et al., 2008; Kurt et al., 2012; Nudel and Newbury, 2013; van Rhijn et al., 2018), suggested that they likely affect the same neurobiological mechanisms in a similar way. Though the behavioral phenotype is the same in these mouse models, the inverse effects on striatal activity between *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice suggested that blockade of *in vivo* inhibitory activity could differently affect motor performance in these mice. Indeed, in *Foxp2*$^{R552H/+}$ mice, blockade of inhibitory activity aggravates the motor skill learning impairment, whilst the same treatment

in *Foxp2*$^{S321X/+}$ mice leads improved motor skill learning compared to vehicle controls (Figure 1e). Our data confirm that aberrant striatal activity as a consequence of Foxp2 dysfunction contributes to the behavioral phenotype observed in mutant mice. Furthermore, changes in striatal activity seem to have predictive value toward which therapeutical intervention may be most effective. However, it is still an open question how both increased and decreased striatal inhibition can lead to similar impairments in motor skill learning. Our results thus add complexity to models of how Foxp2 affects striatal activity, opening up new areas for future research. Further molecular genetic studies may help to establish whether mutations specifically affecting the DNA binding domain generate novel transcriptional targets which might help explain the mechanistic differences from other loss of function alleles.

Taken together, We show that Foxp2 only affects the striatal direct pathway, during early development as well as in adulthood. Furthermore, we show that Foxp2 affects both excitation and inhibition, however the direction in which they are affected is mutation dependent. The mouse R552H mutation leads to decreased inhibition, whilst the S321X mutation results in both decreased excitation and increased inhibition. For the S321X mutation, we have provided evidence that deficits in AMPAR maturation and trafficking could underlie the aberrant excitatory deficit, whilst increased presynaptic GABA content is likely to explain the increased inhibitory drive. Despite the different effects on striatal activity, both mutations lead to a change in the striatal direct pathway E/I balance and result in similar behavioral deficits in motor skill learning. However, the effect of manipulation of inhibitory activity *in vivo* on behavior is mutation dependent. The novel role of Foxp2 in inhibition as well as its possible contributions to AMPAR maturation during early development give insights into how Foxp2 is involved in the development and maintenance of the striatal motor circuit, whilst the mutation specific effects provide new opportunities for future research into how Foxp2 regulates striatal activity as a transcription factor.

Looking outside the striatum – conditional knockout of Foxp2 in movement circuity areas
The striatum is one of the central hubs which modulates activity from the cortex and attunes activity in downstream regions such as the SNr and thalamus (Kravitz and Kreitzer, 2012). In wild-type mice, reduction of inhibitory activity in the brain *in vivo* by intraperitoneal injection of the GABA receptor blocker negatively affects motor behavior in a dose dependent manner **(chapter 2, 3)** (Buckett, 1981; Kamal, 2012; Kryzhanovskii et al., 1989). However, we have shown that blockade of inhibitory activity in *Foxp2*$^{S321X/+}$ mice positively affects motor skill learning **(chapter 2, figure 5).** Contrary to the positive effects of GABA blockade in *Foxp2*$^{S321X/+}$ mice, GABA blockade aggravates the motor skill learning deficits in *Foxp2*$^{R552H/+}$ mice **(chapter 4, figure 3)**. Further investigation into manipulation of the movement circuit is an essential step toward translation of our findings to the human situation. From our data, it is plausible that the regulatory function Foxp2 has on striatal activity also affects downstream brain

areas. A next step in Foxp2 research will be to investigate to which extent Foxp2 affects the entire movement circuit. Foxp2 is expressed in other areas of the movement circuitry as well, including lower layer cortex and multiple (sub)thalamic nuclei (Ferland et al., 2003; Lai et al., 2003; Takahashi et al., 2008; Yin et al., 2017). Furthermore, Foxp2 is expressed in cerebellar Purkinje cells as well as deep cerebellar nuclei (Ferland et al., 2003; Fujita and Sugihara, 2012). Though the cerebellum is not part of the canonical movement circuit, it is highly involved in smooth and precise execution of motor programs (Manto et al., 2012; Zhang et al., 2014). This means that Foxp2 may affect motor circuits at multiple levels: either by modulation of activity within a brain region by Foxp2 (as shown for the striatum), or its effects on connections between brain regions. Spatially restricted modulation of Foxp2 expression by conditional knockout of Foxp2 (French et al., 2007) will be an essential step to understand its relative contributions in cortex, striatum or thalamus to normal movement circuitry function (French et al., 2018). Investigation of motor behavior and neuronal physiology in mice with spatially restricted Foxp2 expression can reveal how Foxp2 regulates different areas of the motor circuit necessary for complex motor behavior.

Modulation of motor behavior by manipulation of movement circuit activity

In addition to spatially restricted manipulation of Foxp2 expression in mice, targeted manipulation of activity within the movement circuit will be an invaluable step to understand how complex motor sequencing in mice is encoded in the brain. Such studies might also ultimately shed light on circuits that are recruited in humans towards proficient speech. Since we now know how Foxp2 dysfunction affects striatal direct pathway activity in *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mutant mice, we can make informed decisions how circuit activity should be manipulated in such mouse models to ameliorate motor skill learning deficits.

In the past years, multiple molecular genetic tools have become available to manipulate neuronal activity in a cell population specific manner. Optogenetics relies on activation of light sensitive ion channels to activate or inactivate neurons. Channelrhodopsin (ChR2) is a light sensitive cation channel, and activation of this channel with blue light (~470nm) leads to increased neuronal activation (Arenkiel et al., 2007; Lin et al., 2009). By contrast, the chloride channel halorhodopsin (NpHR) can be activated by yellow (~520nm) light and such activation leads to increased inhibition of neuronal activity (Zhang et al., 2007; Zhao et al., 2008). High temporal and spatial specificity are easy to achieve with this method, but *in vivo* use of optogenetic tools requires implantation of optic fibers, which is an invasive procedure. However, after implantation, it is feasible to use optogenetic stimulation in freely moving animals and rotarod experiments are routinely combined with this method (Bonin et al., 2016; Gutierrez et al., 2011). Thus, it should be possible to manipulate activity with high spatial and temporal specificity during a motor learning task. Another approach to manipulate neuronal activity *in* vivo is via the use of designer receptors exclusively activated

by designer drugs (DREADDs). hM3Dq and hM4Di are the most commonly used DREADDs used to respectively increase excitability (Alexander et al., 2009) or presynaptic inhibition (Armbruster et al., 2007; Zhu and Roth, 2014). These DREADDs are activated by Clozapine N-oxide (CNO), and *in vivo* injection of CNO rapidly and transiently affects neuronal activity and behavior in mice which express DREADDs (for review see (Smith et al., 2016)). Recently, DREADDs expressed specifically in direct- or indirect pathway neurons have been used to investigate motor performance in a mouse model for Parkinson's disease (Alcacer et al., 2017). DREADDs can be delivered through viral transfection and use of a D1R or D2R promoter can restrict DREADD expression to cells of either the direct or indirect pathway. Mouse models carrying the R552H or the S321X *Foxp2* mutations would be an ideal tool to investigate the effect of spatially restricted modulation of activity. We have shown **(in chapter 2 and 4)** that blockade of inhibitory activity by intraperitoneal injection of picrotoxin brain affects motor skill learning. Furthermore, modulation of activity in the direct and indirect pathway, either through optogenetics or chemogenetics, has clearly observable effects on motor behavior (Alcacer et al., 2017; Freeze et al., 2013). Using DREADDs, we would be able to restore the aberrant activity in direct pathway MSNs of *Foxp2*$^{S321X/+}$ and *Foxp2*$^{R552H/+}$ mice. This approach would allow us to investigate whether restoration of striatal activity is sufficient to ameliorate *Foxp2* mutation induced motor skill learning impairments or if other Foxp2-expressing brain regions provide a substantial contribution to the motor skill learning phenotype.

Interestingly the different effects of the R552H and the S321X mutations on striatal activity in these mouse models mean that we would have to use mutation specific approaches to manipulate activity. The reduced inhibition in mice with the R552H mutation can be ameliorated by increase of inhibitory drive in D1R-MSNs, whilst the phenotype in the S321X mice necessitates a reduction of inhibitory activity, possibly combined with an increase in excitability of D1R-MSNs. It would be intriguing to investigate whether these bidirectional manipulations lead to the same amelioration of motor skill impairments in *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice, respectively. Additionally, selective manipulation of either excitatory or inhibitory activity in D1R-MSNs of *Foxp2*$^{S321X/+}$ mice can help to give insights into the relative contributions of excitation and inhibition to the behavioral phenotype. Lastly, both optogenetics and DREADDs can be used to highly efficiently activate or inhibit cell populations *in vitro.* This way, it is possible to activate or inhibit D1R-MSNs in the striatum and measure how changes in striatal activity contribute to downstream activity in other areas of the motor circuit in wild-type cells as well as cells with disrupted Foxp2 function.

Disentangling the unique effects of different *FoxP2* mutations on activity and behavior

In addition to the previous investigations of the R552H and S321X mutation on the genetic and functional level, we have investigated the effect of these different FoxP2 mutations on striatal activity **(chapter 2 and 4)**. On the genetic level, the R553H mutation of the KE family

affects the DNA-binding domain of human FOXP2 (Lai et al., 2001), and the R328X mutation of a second smaller family leads to loss of FOXP2 function, protein truncation and nonsense mediated decay (Groszer et al., 2008; MacDermot et al., 2005). Despite this difference in how the mutations affect FoxP2 at the molecular level, both mutations lead to CAS in humans and motor skill learning deficits in mice, with highly similar phenotypes (French et al., 2012; Groszer et al., 2008; van Rhijn et al., 2018). The key difference between the R328X and the R553H mutation is that the R328X mutation leads to protein truncation of the mutated product. This reduces overall protein expression by approximately half in the case of a heterozygous mutation (Groszer et al., 2008), but the function of the remaining wild-type FoxP2 is not affected. By contrast, the R553H mutation leads a loss of transcription factor function, but the protein is still expressed. The transcription factor function of FoxP2 requires dimerization, either by homodimer formation between Foxp2 proteins, or by heterodimerization of Foxp2 with FoxP1 or FoxP4 (Li et al., 2004). This dimerization is established through the leucine zipper domain, which is not altered in FoxP2 with the R552H/R553H DNA binding domain mutation. Therefore, it is plausible that mutant FoxP2 can interact with wild-type FoxP1/2/4, and lead to altered function of these dimers. Additionally, *in vitro* cell model studies have shown that overexpression of FOXP2-R553H disrupts the nuclear localization (Mizutani et al., 2007; Sollis et al., 2017; Vernes et al., 2006), though this phenotype has not yet been confirmed in mouse or human brain. The possibility for FOXP2-R553H to still interact with other proteins could convey additional functionality to this variant of FOXP2, and a difference in regulatory properties between FOXP2-R553H and FOXP2-S321X has been suggested (Vernes et al., 2006).

We have investigated excitatory and inhibitory activity mouse models for both these mutations. The *Foxp2*$^{R552H/+}$ mouse model is similar to the KE family DNA-binding domain mutation (Groszer et al., 2008), whilst the *Foxp2*$^{S321X/+}$ is comparable to the heterozygous loss of Foxp2 function in another small family (Groszer et al., 2008). These mutations differently affect striatal activity, with decreased inhibition of direct pathway activity in de *Foxp2*$^{R552H/+}$ mice, whilst inhibition is strongly increased following heterozygous Foxp2 loss of function. On the molecular level, it is known that the R328X and R553H mutations differently affect Foxp2 function. The R328X mutation results in a reduction of Foxp2 expression. However, gene regulatory function is retained in the Foxp2 that is still expressed. This means that although reduced Foxp2 expression can have a major impact on regulation of gene expression, the targets which can be regulated in cells from *Foxp2*$^{S321X/+}$ mice should not be altered, whilst Foxp2-dependent regulation is abolished in homozygous mutant material (Vernes et al., 2006; Vernes et al., 2011). On the other hand, the R553H mutation affects the ability of Foxp2 to regulate gene transcription, but Foxp2-R553H remains expressed. This mutation might however lead to aberrant regulation of Foxp2 transcription through interaction of mutant Foxp2 with other Foxp2 proteins. As dimerization is necessary for the transcriptional

function of Foxp2 (Li et al., 2004). As such, the R553H mutation could have a different effect on transcriptional regulation compared to the R328X mutation, due to competitive effects on wild-type Foxp function. Possible different effects on gene regulation between the R328X and R553H mutation have been suggested from *in vitro* data (Estruch et al., 2016; Vernes et al., 2006). Our data suggests that the interaction between Foxp2-R553H and other Foxp proteins leads to unique transcriptional regulation of targets involved in synaptic function. Targeted investigation of the interactome in MSNs of *Foxp2*$^{R552H/+}$ mice and wild-type controls can shed light on how this mutation uniquely affects Foxp2 function and explain the different phycological phenotypes in D1R-MSNs of *Foxp2*$^{S321X/+}$ and *Foxp2*$^{R553H/+}$ mice.

Intriguingly, the decrease in inhibition we show in D1R-MSNs of Foxp2-R552H mice is in line with previously conducted *in vivo* activity measurements, where an increase in baseline activity was observed (French et al., 2012). Moreover, the increased negative modulation of activity when mice were presented with a motor task suggests that behavior dependent activity might be regulated by Foxp2 as well (French et al., 2012). Our findings *in vitro* furthermore corroborate the increase in baseline activity, as we show decreased inhibition in D1R-MSNs of *Foxp2*$^{R552H/+}$ mice. By contrast, similar measurements of *in vivo* baseline activity or task-dependent activity modulation have not been conducted in *Foxp2*$^{S321X/+}$ mice. From our data, we can conclude that baseline activity in the striatum should be decreased *in vivo* in *Foxp2*$^{S321X/+}$ mice, due to the increased inhibitory drive in the direct pathway. Measurement of baseline and task-dependent modulation of activity in *Foxp2*$^{S321X/+}$ mice in turn could provide evidence that the R552H and S321X mutations differentially affect Foxp2 function *in vivo*. Nonetheless, the inverse changes in striatal activity between both mouse models result in similar impairments in motor skill learning. Investigation of the motor skill learning deficits by use of behavioral paradigms which specifically necessitate control of direct pathway activity might reveal subtle differences in behavioral deficits between genetic backgrounds. For example, investigation of step size variation, which is governed by regulation of striatal direct pathway inhibition (Rothwell et al., 2014), might be informative with regard to mutation specific differences in striatal direct pathway activity.

Until now it has been suggested that the neurobiological mechanisms affected by nonsense and DNA-binding domain mutations of Foxp2 are likely to be the same. The different genetic mutations result in the same behavioral impairments in human and mouse (French et al., 2012; Groszer et al., 2008; Morgan et al., 2017; van Rhijn, 2018). However, our data indicates this presumption stems from an incomplete understanding of the neurobiological pathways affected by FoxP2. This conclusion could have important consequences for translation of results found in animal models for genetic disorders to the human situation, as care should be taken not to generalize effects found for one mutation to other mutations within the same gene.

A role for FOXP2 beyond movement disorders?

Language impairment has been associated with other neurodevelopmental disorders, including schizophrenia (Radanovic et al., 2013; Stephane et al., 2007) and ADHD (Mueller and Tomblin, 2012). Genetic evidence has provided tentative links between intronic single nucleotide polymorphisms (SNPs) in Foxp2 and these disorders. Case-control studies have suggested that multiple SNPs are associated with schizophrenia (Li et al., 2013; Rao et al., 2017; Sanjuan et al., 2005; Sanjuan et al., 2006; Tolosa et al., 2010) and ADHD (Demontis et al., 2017). This might indicate that common SNPs which affect FOXP2 function (as opposed to rare coding mutations that have large disruptive effects on protein properties) can increase risk of a different phenotype from CAS. However, it should be noted that large genome-wide screens did not report any association between SNPs in FOXP2 and schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Though FoxP2 is predominantly expressed in brain regions involved in the motor circuitry, the protein is also found in parts of the brain which are not involved in complex motor sequencing, as well as other regions of the body outside of the nervous system. Within the brain, in addition to the expression in striatal D1R-MSNs, FoxP2 is present as well in the ventral tegmental area (VTA) and the nucleus accumbens (NAc) (Campbell et al., 2009). From human and mouse data, we know that striatal activity is increased in affected family members as well as *Foxp2*$^{R552H/+}$ mice. However, no change in morphological of functional organization of the VTA or NAc was reported in members of the KE family, and the function of Foxp2 in these areas has not been investigated in mouse models of Foxp2 dysfunction. As such it is unknown if Foxp2 can affect activity in these brain regions to a similar extent as in the striatum. Changes in activity in the VTA and the NAc have been shown in individuals with schizophrenia, where increased activity has been associated with auditory hallucinations (Rolland et al., 2015). Furthermore, it has already been shown that auditory-motor association learning is impaired in the R552H and S321X mouse models (Kurt et al., 2012). However, it is not known if deficits in auditory perception could underlie this phenotype. We could use conditional knockout of Foxp2 to selectively manipulate expression in the VTA or NAc and investigate *in vivo* how Foxp2 might be involved in non-motor behaviors.

Furthermore, areas such as the dorsal striatum additionally govern other aspects of behavior outside complex motor sequencing. Reduced dopamine signaling in the dorsal striatum decreases the motivation to repeat a task until a reward is received (Palmiter, 2008), and impairs reward based learning (Higa et al., 2017). Intriguingly, striatal dopamine concentration is suggested to be increased in *Foxp2*$^{+/-}$ mice (Enard et al., 2009). A large screen of spontaneous behavior in *Foxp2*$^{+/-}$ mice has shown that exploratory behavior is increased in these mice. This suggests that non-motor behavior might be affected by FoxP2 loss of function as well. Despite these indications, evoked behaviors dependent on motivation and reward-based learning have not been investigated in mouse models for Foxp2 dysfunction.

Behavioral paradigms to investigate motivation and reward-based learning in mice are well established (Drew et al., 2007; Higa et al., 2017; Palmiter, 2008) and can be readily implemented for investigating Foxp2 function. The previously mentioned ability to measure *in vivo* neuronal activity combined with conditional knockout of Foxp2 in cell types or brain regions involved in specific behaviors will be a powerful tool to investigate if Foxp2 regulates non-motor behaviors as well. Many of the brain regions where Foxp2 is present are canonically associated with motor circuit function. However, the current knowledge on non-motor behaviors governed by the striatum, combined with high Foxp2 expression throughout the entire striatum (Fong et al., 2018) invites further exploration of Foxp2-associated behavioral phenotypes. Results obtained from such an investigation can inform us about possible non-speech related symptoms present in individuals with disrupted FOXP2 and could broaden the clinical FOXP2-associated phenotype.

Towards a human neuron model for speech impairment

Mouse models are a valuable tool to investigate neurodevelopmental disorders with a genetic origin and have provided us the means to assess Foxp2 on the cellular, circuit and behavioral level. However, there are potential human specific aspects of FOXP2 function which we cannot investigate in a mouse model. Human FOXP2 differs from FoxP2 of any other species, as two amino acid changes within exon 7 (T303N and N325S) have occurred only in the hominid lineage, at the latest prior to the Neanderthal/Denisovan split. These might convey unique function to FOXP2, as has been suggested from mouse models in which these substitutions were introduced (Enard et al., 2009; Reimers-Kipping et al., 2011; Schreiweis et al., 2014). Striatal neurons from mice with 'humanized' Foxp2 show increased complexity (Enard et al., 2009) and plasticity (Schreiweis et al., 2014). Based on differences between mouse and human FoxP2, it has been proposed that human FOXP2 exhibits specific functionality which helps to enable the highly complex facial muscle movements necessary to produce coherent speech (Enard et al., 2002; Konopka et al., 2009). Detailed investigation of these human specific functions necessitates a human model of FOXP2 function. In the last decade, human neuronal models have become a staple of functional investigation in neurodevelopmental disorders (Chailangkarn et al., 2012; Tamburini and Li, 2017). Currently, multiple different types of neurons can be generated, including cortical excitatory and inhibitory upper layer neurons (Frega et al., 2017), hippocampal neurons (Sarkar et al., 2018) and excitatory dopaminergic neurons (Sundberg et al., 2013). We have shown that the excitatory dopaminergic neurons, generated by a protocol adapted from (Sundberg et al., 2013) express FOXP2 and form a coherent neuronal network **(chapter 6)**. This has enabled us to for the first time investigate how mutation of FOXP2 affects neuronal activity in human neurons. We show that human FOXP2 similarly affects excitatory activity as mouse Foxp2, with reduced excitatory activity in dopaminergic iNeurons which express FOXP2. Furthermore, we used multi-electrode arrays to for the first time investigate if mutation of FoxP2 affects neuronal network formation. Though

network activity is impaired, it seems homozygous deletion of FOXP2 in human neurons does not overtly impact neurodevelopment or network formation. However, more detailed analyses of the effect of FOXP2 knockout on neurodevelopment, neuronal morphology and synapse maturation *in vitro* should be conducted in the future.

An important consideration for comparison of current data on the role of FOXP2 in human neurons and data obtained from mouse brain is the mismatch in neuronal identity. The function of FOXP2 has been explored in depth in striatal GABAergic MSNs. However, the dopaminergic neurons we generated have a glutamatergic identity, which means they are not striatal in origin. FoxP2 is expressed in the mammalian brain in other areas than striatum as well such as SNr and thalamus, where excitatory dopaminergic are present. Though currently it is unknown is FoxP2 affects activity in excitatory dopaminergic cells in mouse or human brain, our data shows that *in vitro* FOXP2 is required for normal formation of excitatory neuronal networks. However, in order to directly compare mouse and human data, inhibitory striatal MSNs have to be generated from induced pluripotent stem cells (iPSCs). Multiple protocols exist which results in differentiation of iPSCs into neurons with an MSN identity (Noakes et al., 2015; Stanslowsky et al., 2016; Yuan et al., 2015). The ability to generate inhibitory cells which express FOXP2 would be an incredible asset. Though we know that excitatory postsynaptic activity is affected in mouse MSNs, formation of an excitatory/inhibitory network *in vitro* will enable us to assess if excitatory activity is affected as well in human neurons of similar identity. Next, with inhibitory MSNs we can verify whether human FOXP2 regulates inhibitory activity through similar neurobiological mechanisms as mouse Foxp2. Inhibitory neurons would also enable us to verify if therapeutic interventions aimed at modulation of inhibitory activity affect human dopaminergic neurons with mutations in *FOXP2*.

Another avenue for exploration of human specific features of FOXP2 concerns tracing the evolution of FOXP2 on a genetic level. It has been suggested that human FOXP2 differently regulates targets *in vitro* compared to chimpanzee FoxP2, including targets related to central nervous system development (Konopka et al., 2009). However, conclusions about potentially unique functions of the human version of FOXP2 from this research remain limited. The generation of human neurons, which express FOXP2 also enables us to utilize novel molecular methods such as CRISPR/Cas9 to generate human neuron lines with specific alterations of FOXP2. This way we can generate human neurons in which either the human specific amino acid changes are eliminated, and/or we can introduce the additional single amino acid change (D80E) which occurred specifically in mice. This approach could help to answer whether changes to FoxP2 during evolution have uniquely affected mouse or human FoxP2 function at the genetic and physiological level. Revealing how FoxP2 function has changed during evolution can help us understand how FOXP2 has contributed to the emergence of capacities

for spoken language in our species. Specialization of FOXP2 function during human evolution is one of the mechanisms through which this transcription factor may have contributed to the uniquely human ability to acquire and use spoken language (Enard et al., 2002).

CONCLUDING REMARKS

The work presented in this thesis reveals that FoxP2 regulates striatal circuit activity only in the striatal direct pathway and the behavioral effects of blockade of inhibitory activity provide the first evidence for possible therapeutic intervention. Manipulation of specific cell populations and brain areas is the next important step to understand how Foxp2 regulates the motor circuitry more broadly in addition to its role in the striatum. The ability to generate human neurons which express FOXP2 will enable us to investigate human-specific functions of FOXP2 in unprecedented detail. Our novel data on Foxp2 also generates many new questions. We think follow-up investigation of the potential mutation specific effects of DNA-binding domain and loss of *Foxp2* function mutations on motor circuit physiologic is highly important. It has been suggested that DNA-binding domain mutation of FOXP2 generate a more complex phenotype compared to stop-gain mutations (Kurt et al., 2012; Mizutani et al., 2007; Sollis et al., 2017; Vernes et al., 2006), possibly through interaction with wild-type FoxP proteins. A direct comparison of *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice on the genetic, physiological and behavioral level will help to understand how these mutations uniquely affect motor circuit function. Furthermore, our evidence that Foxp2 also regulates inhibitory activity in the striatum shows the complex functions it may play in E/I balance. This validates the need for further research into the function of FoxP2 in the entire motor circuit. Current evidence shows the intricate role Foxp2 has in the regulation of striatal development and activity. However, FoxP2 is also expressed in other areas of the motor circuit and additionally cells which express Foxp2 generate projections outside of the brain region where they reside. This necessitates a thorough investigation of Foxp2 in other areas of the motor circuit, to further disentangle its complex role in motor circuit function. Lastly, we think that this thesis has shed a novel light on how FoxP2 is involved in the regulation of motor circuit activity. Dysfunction of FoxP2 produces highly recognizable phenotypes in mice, songbirds and humans, which impinge on impairments in the generation of complex motor sequences. In mice, these are important for motor skill learning and seem to play a role in the complexity of innate vocalizations (Castellucci et al., 2016; Chabout et al., 2016). In songbirds, complex motor sequencing is necessary for song reproduction, variability and quality (Olveczky et al., 2011). The role of FoxP2 in the circuit which underlies the ability to generate such behaviors shows that it is intricately involved in the development and function of brain circuits necessary for the generation of complex motor sequences, making it well-placed to contribute to human capacities for proficient spoken language.

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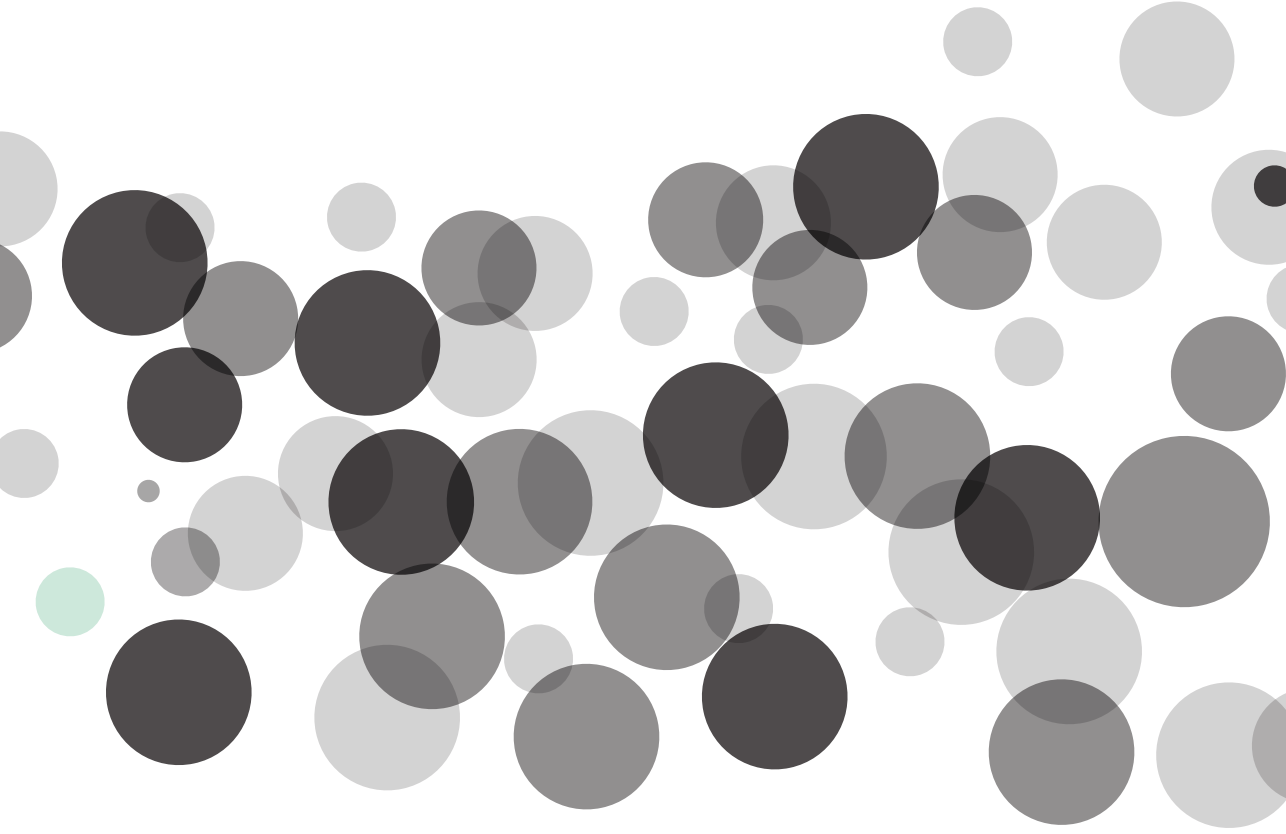

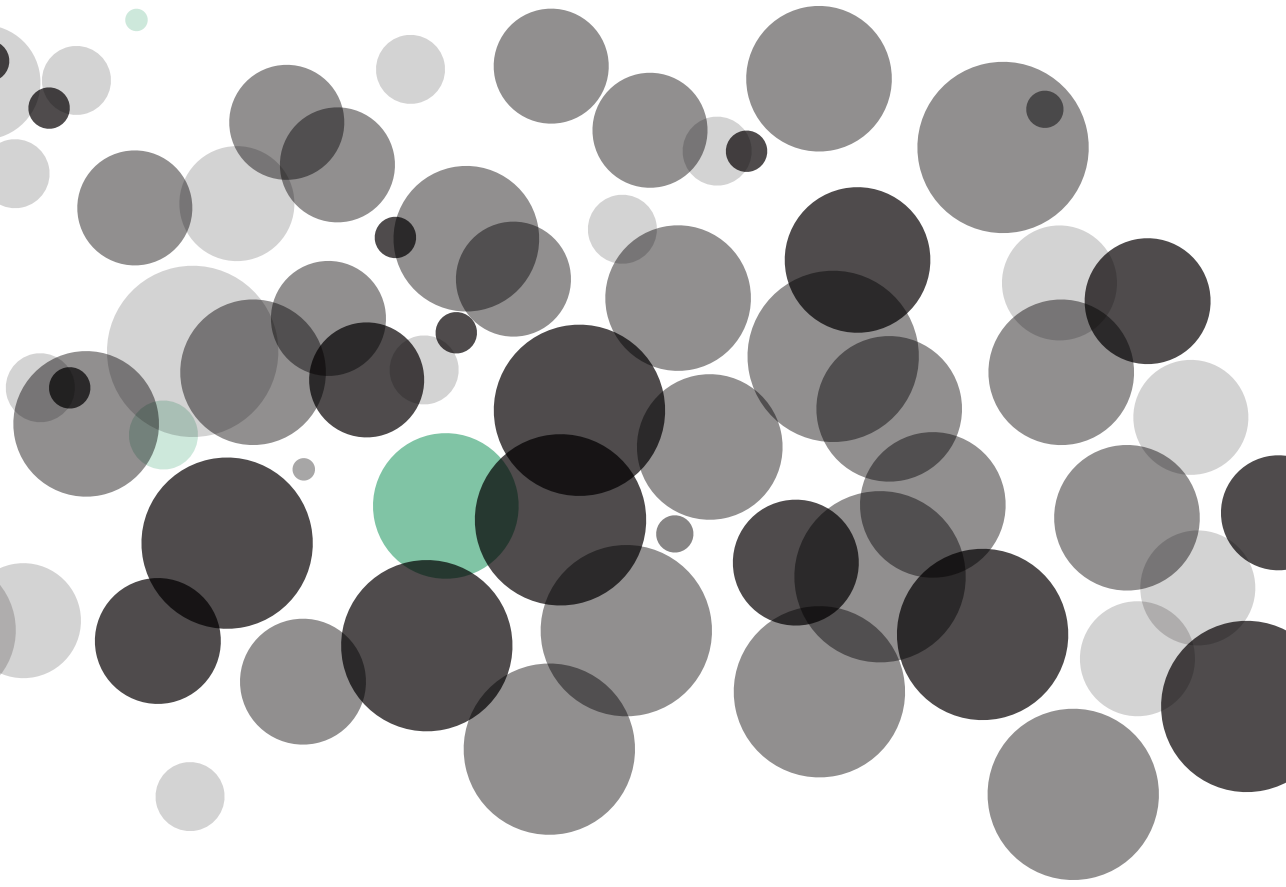

NEDERLANDSE SAMENVATTING

Communiceren door gesproken taal is een unieke menselijke eigenschap en lijkt heel vanzelfsprekend. Al vanaf de geboorte maken we geluidjes. Tijdens het eerste levensjaar nemen ons begrip van geluiden en onze vaardigheden om geluiden te maken snel toe, en na een jaar kunnen we onze eerste woordjes zeggen. Daarna leren we snel meer woorden, en tegen de tijd dat we naar de basisschool gaan kunnen we simpele gesprekjes houden. Het leren praten is een proces waar onderzoekers erg geïnteresseerd in zijn, en we weten dat je hersenen veel informatie verwerken tijdens het leren van een taal. Maar net zo belangrijk is de fysieke mogelijkheid om spraak te produceren. Je kan alle woorden kennen die er bestaan, maar als je ze fysiek niet uit kunt spreken, zul je nooit kunnen communiceren via gesproken taal.

Om duidelijk te kunnen praten moet de aansturing van de spieren in je gezicht, mond en tong goed werken. Activatie van deze spieren wordt mede geregeld door specifieke hersengebieden. Spraakstoornissen kunnen optreden wanneer dit proces is verstoord. Een van deze stoornissen is de taalontwikkelingsstoornis verbale apraxie bij kinderen (childhood apraxia of speech, CAS). Bij veel mensen met CAS is dit probleem te wijten aan mutatie van het gen *FOXP2*. Mensen met mutaties in dit gen hebben problemen met het uitspreken van moeilijke woorden, terwijl ze taal prima begrijpen. Alleen met logopedie kunnen ze leren om zich verstaanbaar te maken. Door de hersenactiviteit van mensen zonder mutatie te vergelijken met die van mensen met een mutatie in *FOXP2* kunnen we beter begrijpen hoe de hersenen de fysieke kant van taal regelen (zoals de fijne motoriek in het gezicht, de tong en de keel).

In mijn onderzoek heb ik muismodellen gebruikt om te onderzoeken hoe mutaties in *Foxp2* de activiteit van individuele hersencellen beïnvloeden. Eerder is al aangetoond dat FoxP2 de hersenactiviteit beïnvloedt bij mensen en muizen, en dat ook het gedrag van muizen verandert als *Foxp2* is gemuteerd. Muizen kunnen niet praten, maar ze kunnen wel moeilijke opdrachten uitvoeren waarbij activatie van dezelfde hersencellen nodig is als die bij mensen belangrijk zijn voor spraak. Mutatie van *Foxp2* zorgt ervoor dat muizen deze opdrachten minder goed uit kunnen voeren. Hoewel bekend is dat mutaties in *Foxp2* de hersenactiviteit beïnvloeden, was nog niet bekend hoe Foxp2 dit precies doet. Mijn onderzoek richt zich op het een specifiek hersengebied, het striatum, waar Foxp2 aanwezig is in een deel van alle cellen. Het striatum is heel belangrijk voor het aansturen van complexe bewegingen bij muizen, maar ook voor het aansturen van spraak bij mensen. Ik heb onderzocht hoe mutatie van *Foxp2* leidt tot verandering van activiteit in deze cellen en of deze veranderingen de bewegingsproblemen bij muizen en de spraakproblemen bij mensen kunnen verklaren.

In **hoofdstuk 2** laat ik zien dat de activiteit in het striatum is verstoord als er minder Foxp2 aanwezig is. Excitatie (activatie) is lager, terwijl inhibitie (inactivatie) hoger is. Dit leidt tot een verstoring in het evenwicht tussen het activeren en inactiveren van de celpopulatie in het striatum waar Foxp2 aanwezig is. De juiste balans tussen activatie en inactivatie van deze cellen is belangrijk, omdat ze signalen sturen naar andere hersengebieden en betrokken zijn bij het initiëren van beweging. Het omkeren van de verhoogde inactivatie (door de inactivatie deels te blokkeren) zorgt ervoor dat muizen met minder mutatie weer beter complexe bewegingsopdrachten uit kunnen voren. Het medicamenteus herstellen van de balans van activatie en inactivatie kan dus interessant zijn in het kader van therapeutische interventie.

Mutaties in *Foxp2* beïnvloeden de activiteit van hersencellen in zowel jonge als volwassen dieren. Tot op heden was het nog niet bekend of ook processen die betrokken zijn bij de ontwikkeling van volwassen hersenactiviteit beïnvloed worden door Foxp2, omdat dit proces nog niet is onderzocht in muizen met mutaties in *Foxp2*. In **hoofdstuk 3** onderzocht ik of Foxp2 een invloed heeft op bepaalde kritieke processen tijdens de ontwikkeling van het striatum. Tijdens de ontwikkeling wordt de aanwezigheid van verschillende soorten membraanreceptoren sterk gereguleerd. Ik ontdekte dat mutaties in *Foxp2* ervoor zorgen membraanreceptoren voor volwassen cellen pas later tijdens de ontwikkeling van de cel aanwezig zijn. Mijn data laten ook zien dat de aanwezigheid van deze receptoren samenvalt met het starten van gecoördineerde beweging. Jonge muizen met minder Foxp2 kunnen minder goed simpele opdrachten uitvoeren waarvoor ze gecoördineerd moeten kunnen bewegen. Foxp2 heeft dus een effect op hersenactiviteit en op de uitrijping van de cellen tijdens de ontwikkeling.

Een van de essentiële functies van Foxp2 is het aan- en uitzetten van de expressie van andere genen, waarvoor het aan DNA moet kunnen binnen. In **hoofdstuk 4** beschrijf ik een andere mutatie van *Foxp2* dan de mutatie waardoor minder Foxp2 aanwezig is. Deze mutatie zorgt ervoor dat Foxp2 niet meer aan DNA kan binden, maar de gemuteerde variant van *Foxp2* blijft wel aanwezig in de cel. Ook in mensen met CAS wordt deze mutatie gevonden. Mijn onderzoeksresultaten laten zien dat deze mutatie een ander effect heeft op activiteit van hersencellen dan mutaties die ervoor zorgen dat Foxp2 minder aanwezig is. In plaats van verhoogde inhibitie, is de inhibitie bij deze mutatie juist verlaagd. Daardoor zijn cellen actiever in plaats van minder actief. Desondanks leidt deze mutatie wel tot dezelfde verminderde bewegingsvaardigheid in muizen. Ook mensen met deze verschillende mutaties vertonen dezelfde taalontwikkelingsstoornis. Dit suggereert dat mutatie-afhankelijke verschillen in hersenactiviteit kunnen leiden tot dezelfde stoornis, en dat we nog steeds niet helemaal begrijpen hoe Foxp2 precies werkt.

Foxp2 reguleert de expressie van heel veel genen in de hersenen. Een van die genen is de retinolzuur-beta-receptor (RARβ), en regulatie van RARβ door Foxp2 speelt mogelijk een rol bij de hersenontwikkeling en regulering van hersenactiviteit. In **hoofdstuk 5** bespreek ik vanuit de literatuur hoe RARβ en Foxp2 hersenactiviteit beïnvloeden en of ze mogelijk invloed hebben op dezelfde processen in de hersenen. Er zijn ook diverse aanwijzingen dat de regulatie van RARβ door Foxp2 hersenactiviteit betrokken bij beweging kan beïnvloeden. Meer onderzoek naar het samenspel tussen Foxp2 en RARβ kan een waardevolle stap kan zijn om beter te begrijpen hoe Foxp2 complexe hersenactiviteit reguleert.

Recent zijn er nieuwe technieken ontwikkeld waarbij hersencellen gemaakt kunnen worden van menselijk celmateriaal. In plaats van het uitvoeren van een hersenbiopsie (wat onmogelijk is) nemen we huidcellen van mensen en maken we daar eerst stamcellen van. Van deze stamcellen maken we daarna hersencellen. In **hoofdstuk 6** heb ik menselijke hersencellen gemaakt uit stamcellen om te onderzoeken hoe FOXP2 activiteit in menselijke cellen beïnvloedt. Ik laat zien dat we werkende hersencellen kunnen maken die FOXP2 in zich hebben. Deze cellen heb ik vervolgens vergeleken met cellen waarin FOXP2 door een mutatie niet aanwezig is. Menselijke cellen zonder FOXP2 zijn minder actief dan cellen met FOXP2, net als in de muis. Deze methode biedt nieuwe mogelijkheden om te onderzoeken welke eigenschappen van FOXP2 hetzelfde zijn in mens en muis.

De nieuwe data die ik in dit proefschrift presenteer zijn een bijdrage aan het onderzoek naar hoe complexe bewegingen (inclusief de mogelijkheid om te praten) worden aangestuurd door de hersenen. Mutaties in *Foxp2* veroorzaken kleine veranderingen in de balans tussen activatie en inactivatie van hersencellen. Dit heeft een grote invloed op het succesvol uitvoeren van bewegingsopdrachten door muizen. Gebalanceerde activatie en inactivatie blijkt dus een van de belangrijkste processen is voor een gezonde werking van processen belangrijk voor complexe aansturing van de spieren. Als we meer te weten komen over de mechanismes die aan deze balans ten grondslag liggen, en hoe deze te beïnvloeden zijn, kunnen we nieuwe mogelijkheden voor therapeutische interventie ontwikkelen voor mensen waarbij deze balans is verstoord.

SUMMARY IN ENGLISH

Communication trough spoken language is a uniquely human behavior, and seems very natural to us. As soon as we are born, we start making sounds. Within the first year of our life our understanding of and ability to make sounds rapidly increases and this leads us to utter our first word. Then, quickly, we learn to say more words, and when we were at kindergarten age we could already have simple conversations. The acquisition of language is a process that has fascinated many researchers, and it is known that learning a spoken language requires a large amount of information processing. However, just as important is the physical ability to produce speech. You can know all the words in the world, but if you lack the capacity to physically speak them you will not be able to communicate using spoken language.

To be able to speak clearly, you need good control over the muscles in your face, mouth and tongue. The activation of these muscles is controlled by specific areas in the brain. Speech related disorders can arise when the control over these muscles is impaired. One such disorder is childhood apraxia of speech (CAS). In many cases of CAS, the disorder is directly attributed to the mutation of the gene *FOXP2*. Individuals with mutations in this gene are less able to clearly pronounce complex words, though their language comprehension is normal. They require extensive speech therapy in order to speak understandably. By comparing brain activity between individuals with and without mutations in *FOXP2*, we can better understand how the brain directs the physical side of language (i.e. the fine motor movements in the face, tongue and throat).

In my research I have used mouse models to investigate how mutations in *Foxp2* affect activity in single brain cells. It has already been shown that activity in both human and mouse brains is changed when *Foxp2* is mutated, and that this also affects behavior in mice. Though mice do not speak, they can perform complex movement tasks, which require the same brain activity as speaking does in humans. In mice with mutations in *Foxp2*, the performance on these tasks is impaired. Though it was known that Foxp2 affects brain activity, it was not established how mutations of *Foxp2* lead to changes in brain activity. My investigation focuses on a brain region called the striatum, where Foxp2 is present in a specific cell population. These cells are involved in control of brain activity necessary for complex movement in mice and speaking in humans. Throughout my research, I have investigated by which mechanisms mutations in Foxp2 affect activity in these cells and if the changes in activity can explain the impaired movement behavior in mice and possibly the childhood apraxia of speech in humans.

In **Chapter 2,** I show that activity in the striatum is disrupted if less Foxp2 is present. Excitation (activation) of cells is decreased, whilst inhibition (inactivation) of cells in increased. This leads to disbalance of activation and inactivation of the cell population in the striatum in

which Foxp2 is expressed. Balanced activation and inactivation of these cells is important, as this population sends signals to other brain regions and is involved in initiation of movement. I therefore investigated if the impaired complex movement in these mice can be corrected by blockade of inactivating signals in the brain. This indeed improves walking performance in mice with less Foxp2 expression, which might be a mechanism to explore for possible therapeutic intervention.

Though mutations in Foxp2 affect neuronal activity in both young and adult mice, it was not known if Foxp2 also affects the maturation of striatal neurons during development. I therefore investigated if reduced Foxp2 expression affects hallmarks of maturation in striatal neurons. In **chapter 3**, I show that reduced expression of Foxp2 delays the maturation of membrane receptors involved in the activation of neurons. Mature receptor function is essential for mice to start with coordinated movement, and in mice with reduced Foxp2 expression the development of coordinated movement is delayed. From this I conclude that Foxp2, next to its effect on activity, also affects the maturation of striatal neurons.

As Foxp2 regulates expression of other genes, this ability to bind to DNA is essential for its function. In **Chapter 4** I explored a different mutation of *Foxp2* from mutations which reduce Foxp2 expression. This mutation only affects a specific part of Foxp2, which results in an inability of Foxp2 to bind to DNA. This specific mutation is found as well in individuals with CAS. However, compared to the mutation described in chapter 2 and 3, this mutated version of Foxp2 is still present in the cell. My results show that the effect of this mutation on activity in the striatum is opposite from that of mutations which reduce Foxp2 expression. The mutation which impairs DNA-binding leads to reduced inhibitory activity of cells which express Foxp2. These cells are thus more active. However, this increased activity results in the same changes in movement behavior as reduced Foxp2 expression, and individuals with this mutation also display CAS. This suggests different changes in neuronal activity can result in the same change in behavior, and that our understanding of how Foxp2 regulates brain activity is still incomplete.

Foxp2 regulates the expression of many different genes in the brain. Recently, regulation of the retinoic acid receptor beta (RARβ) emerged as a possible mechanism by which Foxp2 can affect brain development and neuronal activity. In **chapter 5**, I discuss the current literature on the function of RARβ and Foxp2 in regulation of activity in the brain and compare the similarities and differences in their function. Furthermore, I suggest that investigation of how Foxp2 regulates RARβ and how this interaction affects activity related to control of complex movement is an intriguing opportunity to better understand how Foxp2 influences complex activity in the brain.

In the past years multiple methods to generate functional neurons from cellular material taken from humans have been established. Instead of taking a brain biopsy (which is impossible), we can take skin cells, make them into stem cells and generate brain cells from those. In **chapter 6**, I have used human stem cell derived neurons to investigate how FOXP2 affects neuronal activity in human cells. I show that we are able to generate neurons with FOXP2 expression and compare these to neurons in which FOXP2 is made completely absent through mutation. The lack of FOXP2 expression in human neurons leads to a similar decrease in activity as we found in mouse neurons with reduced FOXP2 expression. This method opens new possibilities to investigate which functions of FOXP2 might be conserved between mouse and human brain.

Taken together, the data presented in this thesis increase our understanding of how complex movement (including word production) is governed by the brain. Mutations in Foxp2 lead to small changes in balanced activation and inactivation, which profoundly affects execution of complex movement tasks. This shows that balanced activation and inactivation is essential for normal function of brain processes necessary for complex muscle control. Increased knowledge about the mechanisms underlying balanced neuronal activity, and how these can be affected, opens new avenues for possible therapeutic intervention in disorders where the neuronal activity balance might be upset.

DANKWOORD

Na een flinke periode van noeste arbeid is het proefschrift dan eindelijk daar. Tijdens mijn promotieonderzoek heb ik heel veel geleerd van enorm veel mensen. Jullie zijn te veel om op te noemen, maar hierbij toch een poging.

Bedankt **Nael, Simon** en **Sonja,** voor jullie steun, betrokkenheid en supervisie tijdens mijn PhD. **Nael,** bedankt voor je ondersteuning tijdens dit project. Ik heb heel veel geleerd over hoe je een project van pril begin naar het eindpunt brengt. Je feedback en correcties waren altijd heel waardevol. Ook de praktische begeleiding was erg fijn en we hebben veel goede gesprekken over mijn project gehad. **Sonja,** your feedback on all my writing, presentations and posters was always spot on. Discussing with you about both the style and substance of my thesis chapters was invaluable, and made them into the best logical stories they could be. **Simon,** thank you for your input and guidance, especially during the last months of my PhD. When we had to finish very quickly you were always there with the right feedback to speed everything along and make the last few months go very smoothly.

Next I would like to thank the reading committee: **Tansu Celikel, Catherine French** and **Wolfgang Enard,** for their willingness to read and judge my thesis. Thank you for your comments, the time you invested to read and the advice that you gave.

Hoewel supervisors degenen zijn die het project dragen, was mijn PhD natuurlijk nooit succesvol geweest zonder alle geweldige labmanagers, PhDs en postdocs van Moleculaire Neurofysiologie en Neurogenetics of Vocal Communication.

Dirk, ik heb veel aan onze gesprekken over diepgaande elektrofysiologie gehad tijdens mijn PhD en heb altijd erg fijn met je samengewerkt. Nu ben je mijn supervisor in mijn postdoc, en ik hoop op nog vele interessante discussies in de toekomst. **Astrid,** bedankt voor al je hulp en ondersteuning in het lab, en voor je geduld als ik weer eens iets vergeten was te bestellen of de bench niet goed had opgeruimd. Het is altijd fijn om met met je samen te werken en nu in mijn postdoc hoop ik dat te mogen blijven doen. **Martijn,** jouw vriendschap zorgde ervoor dat ik me snel welkom voelde in het lab toen ik met mijn PhD begon. We hebben veel fijne discussies gehad over diepgaande onderwerpen, of het nou elektrofysiologie of soms ook niet-werkgerelateerd was. Veel van jouw adviezen over elektrofysiologie zal ik altijd meedragen. **Vivien**, thank you for your hospitality in letting a novel electrophysiologist work on your setup. It was always nice to discuss science with you. **Marco**, **Stephanie**, I thoroughly enjoyed our time together in the lab. All of you together were pretty much the 'core team' of the MNP lab, and the team that set up what the lab is now. All of you left your mark on my progress as a scientist and I am glad for that.

Of course I was not alone in the 'new group of students'. **Yan-Fen** and **Jori**, you started around the same time as me. I learned a lot from your input on my work from a behavioral or a more molecular perspective, thank you for that. **Monica** and **Britt**, talking about the more engineering parts of electrophysiology has always been a joy with you. I've learned a lot from you, and I hope you have learned about the more biological parts of electrophysiology form me. **Katrin**, technically you have been in the lab for longer than I have, or at least you 'started' before me. I always value your molecular viewpoint on my work and your critical view on my data. **Moritz,** you were the first PhD to start in the lab with a similar background and outlook on his project as I had. Our discussions in the electrophysiology lab, be it on science, music, or life and philosophy in general never cease to make me happy. I hope we can continue this trend in the coming years. Also thank you for being my paranymph together with **Kai**, I couldn't wish for better people to be the wingmen during my defense. Thanks to everyone of the MNP lab for all for the time I've had here during my PhD working with you. Thank you for all the involvement in your projects I've had, and the involvement you've had in mine. Since I am now pretty much still in the same office but as a postdoc, I can't forget to include the people who've 'just joined', **Eline, Elly, Shan**. Though you've joined after my PhD, I'm now enjoying our time working together during my time as a postdoc with Dirk.

I have enjoyed the molecular work I have done at the Max Planck Institute for Psycholinguistics, as well as the group meetings and department meetings there. Therefore, I would like to thank everyone from the Language and Genetics department and the Neurogenetics of Vocal Communication group. Especially **Kai** and **Paolo**, as I very much valued their input regarding the more molecular aspects of my work. **Kai**, we are still working together to finish our joined project, and our discussions about how to fit all the detailed molecular work and the electrophysiology together are always good. **Paolo**, you have taught me the very first ropes of genotyping and PCR outside of a university course, thank you for that. The NVC group meetings were a good way for me to learn how to bring electrophysiology to a molecular lab, and to try to explain it to you all. The nice discussions we have had when I was working on my thesis chapters definitely helped to improve this finished product and for that I will be forever grateful.

Work is not always serious of course, and therefore I would like to thank everyone from the MNP, CNP, NVC and L&G groups for all the fun times we have had as well. Either on the more serious side (retreats, conferences) or at the more fun events we did (de Efteling, canoeing and Pieterburen spring to mind as the most memorable). And let's make more memories, serious and pleasant, in the years to come.

Mijn dankwoord zou niet compleet zijn zonder de mensen van het Centraal dierenlaboratorium te vermelden. Dierverzorgers, assistenten, managers en welfare officers, zonder jullie zou

mijn onderzoek nooit succesvol zijn geweest. **Karin**, toen ik begon was jij het absolute opperhoofd van het CDL, het begin en het einde van al het dieronderzoek (voor mij in elk geval). Jij hebt mij wegwijs gemaakt in de praktische kant van dierverzorging. **Daphne** en **Mariëlle**, na alle veranderingen van personeel zijn jullie altijd de mensen geweest waar ik op terug heb kunnen vallen. Voor logistiek, hoe alles nu weer veranderd was met een update van Ivention, maar ook soms een reminder dat genotyperingen nog moesten gebeuren. Bedankt daarvoor.

Een ander belangrijk onderdeel van de PhD is voor mij altijd het begeleiden van studenten geweest, en dit heb ik altijd met veel plezier gedaan. **Emma**, jij kwam bij mij in een erg pittige fase voor het project. bedankt voor je inzet en betrokkenheid. **Jorryt**, de iDISCO technieken die jij hebt opgezet in het lab worden nu veelvuldig gebruikt, dus je hebt zeker een stempel op het lab gedrukt. **Guido**, jij hebt mij laten zien wat doorzettingsvermogen kan zijn, binnen en buiten het werk. **Marina**, het was heel leuk om aan een ander project dan mijn eigen project te werken met je, en je de fijne kneepjes van slice elektrofysiologie bij te brengen.

Naast collega's wil ik natuurlijk ook andere mensen noemen die in de afgelopen jaren een bijdrage hebben geleverd bij het succesvol 'overleven' van mijn PhD. **Gabrielle**, **Els**, we zijn met z'n vieren al een aardige tijd bordspelletjes aan het spelen en vechten elkaar nog steeds de tent niet uit. Ik hoop dat we dit nog vele jaren met elkaar mogen doen, en van elkaars gezelschap mogen genieten en op mogen laden voor de werkweek. Naast bordspelletjes en gezelschap is ook dansen met jullie, en iedereen waar ik ooit mee gedanst heb, voor mij een hele fijne manier om weer op te laden. **Tessa**, bedankt dat je Femke en mij ooit in de balfolk hebt geintroduceerd. Dansen heeft mij zeker mijn PhD doen overleven, en ik ben heel dankbaar dat wij jouw mogen kennen. Naast dansen ook jij bedankt voor alle goede gesprekken en de fijne tijden die Femke en ik bij jou mogen hebben als we langskomen, en jouw gezelschap als je bij ons langskomt. We zien elkaar niet heel vaak, maar het is altijd fijn als we bij elkaar zijn. **Joris** en **Irene**, we wonen blijkbaar al sinds we in Ede wonen bij jullie om de hoek, maar hebben jullie pas afgelopen jaar mogen leren kennen. Gelukkig blijven we allemaal volgens mij nog wel even in Ede wonen, dus op naar meer dansjes, bordspellen en gezelschap!

Rita en **Herman**, papa en mama. Van jullie heb ik mijn interesse voor de natuur en alles wat leeft gekregen, naar mijn idee al van jongs af aan. Bedankt dat jullie mij hebben gesteund door mijn opleiding heen. Biologie was eigenlijk niet mijn eerste keuze, maar het is de beste keuze gebleken die ik ooit kon maken. Jullie hebben mee kunnen maken hoe mijn enthousiasme groeide en zich ontwikkelde naar hersenonderzoek. Daar ontspoorde het en was waar ik het over had als ik op bezoek was vaak niet meer te volgen voor jullie, en nu ligt dit boekje er.

Wimmie en **Rinze**, pa en ma Brouwer, jullie interesse naar wat ik doe was, is en blijft altijd aanstekelijk. Dat ik soms even moet stoppen met vertellen zodat pa het op kan schrijven alsof ik college geef laat alleen maar zien hoe groot jullie interesse is. Nu ik doorga in het hersenonderzoek zullen er nog meer en wildere verhalen volgen, want mijn enthousiasme zal nog wel even blijven en hopelijk dat van jullie ook.

De rest van de familie aan de van Rhijn en Brouwerskant, bedankt voor alle gezelschap en steun de afgelopen jaren. Ontspanning is ook belangrijk, en verjaardagen, familiedagen en dergelijke dragen daar ook zeker aan bij. Het is altijd fijn om samen te zijn, met wie van de broers, zussen, neven en nichten dat ook is. Opladen door fijne gesprekken is een goede motor om daarna weer de chaos van een PhD in te duiken. **Diede**, het is fijn om te merken hoe vaak we elkaar vinden in goede gesprekken als we bij elkaar zijn, ook al zijn onze levens zo verschillend. **Jesse**, hoe jij je weg vindt in de wetenschap is mooi om te zien, en soms ook een voorbeeld voor mij. Ik hoop dat ik ook soms een voorbeeld (of een voorbeeld hoe het niet te doen) ben geweest voor jou tijdens mijn PhD en dat kan blijven zijn in de toekomst. **Dylan** het is altijd fijn als jij erbij bent en ben ik blij dat ik je mijn jonge broertje mag noemen.

En als laatste natuurlijk de belangrijkste: **Femke**. Mijn partner in alles. Tegelijk een PhD doen is een wilde rit geweest, en deze rit gaat door nu we allebei doorgroeien in onze carrière. Wat een voorrecht is het voor mij dat ik me jouw man mag noemen. Wat is het fijn om iemand te hebben die begrijpt hoe raar het leven van een PhD'er is. Iemand die ook diep in de nacht een presentatie wil oefenen, of graag in slaap valt op een college over elektrofysiologie. Wat wij hebben komt nooit meer goed, en kan tegelijkertijd niet beter. Bedankt dat jij altijd mijn steun wilt zijn in alles, en dat we samen van onze beestenboel kunnen genieten. Echte liefde is: om 03:00 elkaars artikel verbeteren!

CURRICULLUM VITAE

Jon-Ruben van Rhijn werd op 25 november 1990 geboren te Leiden. Na zijn VWO diploma te hebben gehaald in 2008 is hij algemene biologie gaan studeren en haalde zijn Bachelordiploma biologie in 2011 aan de Vrije Universiteit Amsterdam. Hierna volgde een Research Master Neuroscience aan de Vrije Universiteit Amsterdam, waar hij zijn masterdiploma behaalde in 2013. Zijn promotieonderzoek heeft hij uitgevoerd op de afdelingen Cognitieve neurowetenschappen en Humane genetica aan het Radboudumc en het Max Planck Instituut voor Psycholinguistiek in Nijmegen onder supervisie van Nael Nadif Kasri, Sonja Vernes en Simon Fisher. Tijdens dit onderzoek heeft hij zich gespecialiseerd in cellulaire neurofysiologie op dier- en celmodellen. Hij ontdekte dat het gen Foxp2 de balans tussen activatie en inactivatie van hersencellen betrokken bij complexe bewegingen beïnvloedt.

Op dit moment past Jon-Ruben zijn ruime ervaring in de cellulaire neurofysiologie toe in een postdoctoraal onderzoek dat zich richt op de neurofysiologische achtergrond van schizofrenie. In dit onderzoek zal hij complexe elektrofysiologie op cel- en populatieniveau combineren met moleculaire technieken zoals CRISPR-CAS en het maken van geïnduceerde hersencellen uit menselijk materiaal.

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