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Vera Medvedeva

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Université Pierre et Marie Curie

Ecole doctorale Cerveau Cognition Comportement

Institut du Fer à Moulin / Neurodevelopmental disorders

Characterization of Foxp2 functions in the mouse cortex

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Thèse de doctorat de Neurogenetics

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Dédicace

To my grandfather Leonid Michailovich Kolmak

Леониду Михайловичу Колмаку

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List of abbreviations

ADHD	attention-deficit/hyperactivity disorder
ASD	autism spectrum disorders
cKO	<i>Foxp2</i> cortical knockout mice, line <i>NEX-Cre Foxp2^{lox/lox}</i>
ChIP	chromatin immunoprecipitation
CSEA	cell-type-specific expression analysis tool
DIR	dopamine receptor type 1
DA	dopamine
DVD	developmental verbal dyspraxia
FTLD	frontotemporal lobar degeneration
GO	Gene Ontology
IP	immunoprecipitation
IPC	intermediate progenitor cells
m.o.	months old
mdThal	mediodorsal thalamus
mPFC	medial prefrontal cortex
NuAc	nucleus accumbens
RG	radial glial progenitors
SLI	speech and language impairment
SNP	single nucleotide polymorphism
USV	ultrasonic vocalizations
VTA	ventral tegmental area

Nomenclature: The gene and mRNA are referred to as *FOXP2* in humans, *Foxp2* in rodents, and *FoxP2* in other species (italicised) – for the encoded proteins, these same symbols are used, but they are not italicised (Fisher, 2007).

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Preface

While animals can develop rather sophisticated communicative systems and cognitive capabilities, human language and accompanying mental abilities are profoundly different from animals and constitute a uniquely human trait. Current hypotheses on human language and cognition imply the existence of dissociable mechanisms, which underlie human linguistic competence. Human language must therefore have evolved, similarly to other complex abilities, through qualitative and quantitative modifications of morphological traits and molecular networks that existed in our ancestors and were the object of natural selection (Pinker and Bloom, 1990; Fitch et al., 2010; Scharff and Petri, 2011).

According to the hypothesis of Chomsky, Fitch and Hauser (2002) the ‘faculty of language’ is based on three basic components: 1) a sensory-motor system which grossly underlies signaling and perception and in linguistics falls into the phonological domain, i.e. the organization of sound system of language 2) a conceptual-intentional system underlying concept formation, expression and interpretation, or in linguistic terms, semantics, which deals with meaning; and 3) a structure-generating system, or syntax, which provides the map between signals (phonology) and concepts (semantics). It is essentially the third component that allows inherent human communicative system, language, to be generative, recursive and virtually limitless and unique. The structure-generating system, however, may have its roots in abstract computational mechanisms and may have evolved for reasons other than language and separately from communicative systems (Chomsky, 1965).

A critical question is whether these components correspond to distinct brain circuits. Based on converging evidence from aphasic and imaging studies, Sakai et al. (2001) proposed a modular specialization of cortical Wernicke’s area, angular gyrus (Brodmann’s area) and Broca’s area which are directly related to phonological, semantic, and syntactic processing. There is further evidence for the involvement of other cortical areas (ex., inferior frontal regions or the left middle temporal regions) in language production, but imaging neuroscience has been unable so far to further discriminate linguistic components from other cognitive elements. Clearly, ‘the language system does

not stand alone, but interacts with other systems of perception, memory, and consciousness, as well as with the speech output system' (Sakai et al., 2001).

Furthermore communication, at least at the level of the first two components, signals and semantics, implies a strong social component. Social abilities indispensable for language acquisition include a capacity for imitation and vocal learning for the signaling and perception component (phonology), and mind-reading (theory-of-mind) abilities for concepts interpretation, i.e. semantics. These mechanisms are thought to exist in other species and a number of studies have already provided promising insights into the mechanisms of human social cognition (Fitch, 2009; Fitch et al., 2010).

Thus, interdisciplinary cooperation between linguists, evolutionary biologists and neuroscientists is required for understanding the faculty of language (Fitch et al., 2005; Poeppel et al., 2008).

FOXP2 was the first gene to be implicated in a speech and language disorder in humans. Efforts to characterize *FOXP2* functional roles at different levels reflect the challenges of investigating biological substrates for speech and language development and evolution. Clinically, *FOXP2* mutations were first associated with 'language grammar' deficiencies as well as higher cognitive and basic motor abnormalities in humans. Yet *FOXP2* expression is not exclusive to humans and is widely present in numerous brain regions and in peripheral organs during development and throughout adulthood. Hence, it is not specifically a 'speech' gene, but may have ancestral functions and may even have been selected for reasons other than language. As a transcription factor it controls the expression of a large number of targets. This complexity is increased by the existence of numerous splice variants which, as emerging evidence suggests, might be regulated at the translational level in an activity dependent fashion. Therefore *FOXP2* most likely subserves multiple complex roles in brain development and function.

Despite the complexity of *FOXP2* phenotypic manifestations, different recent lines of research have highlighted the role of *FOXP2* in brain structures underlying learning and communication: functional studies on patients carrying mutations in the gene, as described above; association of *FOXP2* transcriptional targets with pathologies characterized by strong language components, as for instance *Cntnap2*, involved in

speech development and ASD; and, characterization of *Foxp2* functions in vocal learning in animal models, notably songbirds.

The aim of the present work is to identify the functions of *FOXP2* in mouse cortical structures. Such functions may underlie ancestral mechanisms recruited and adapted to human speech and language during evolution.

In the Introduction I present studies supporting the hypothesis that *FOXP2* is a molecular entry point for the study of the biological basis of speech and language development and evolution. Further emphasis is put on *FOXP2* cortical and cognitive functions.

The first chapter is dedicated to the description of human clinical conditions associated with *FOXP2* mutation, as well as mental disorders closely associated with disturbances of *FOXP2* controlled molecular networks.

The second chapter describes the potential role of *FOXP2* in human evolution and reinforces the idea of *FOXP2* being a molecular window into speech, language and cognition.

The third chapter focuses on the function of *FoxP2* at the circuitry level, and argues why muroids along with songbirds constitute a valid neurobiological model for studying aspects of *FoxP2*-related phenotypes. This chapter also describes aspects of cortical morphology in relation to *Foxp2*-associated cognitive functions.

In the Context I summarize work done in our laboratory to elucidate the role of *Foxp2* in dopamine regulated circuits in the Nucleus Accumbens, which could be relevant for the regulation of specific social behaviours.

Introduction

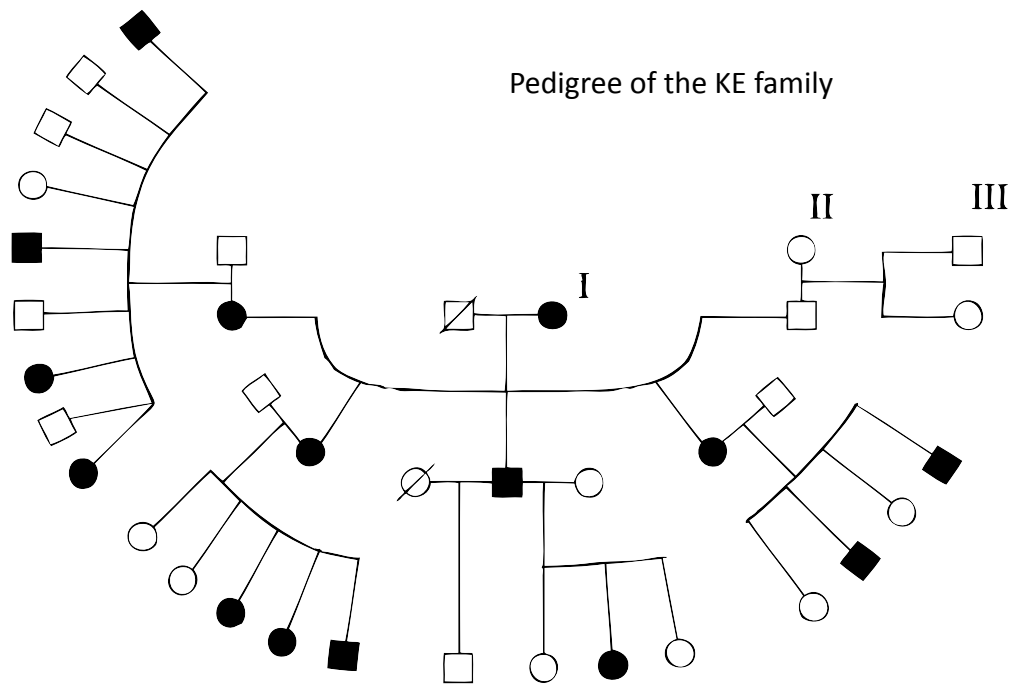
***FOXP2* deficiency causes a complex speech and language disorder**

The KE family comprised of three generations in which severe speech and language impairment (developmental verbal dyspraxia, DVD) segregated with an autosomal dominant mutation in the transcription factor *FOXP2*. The mutation is an arginine-to-histidine substitution (R553H) located in the forkhead DNA-binding domain, which disrupts DNA-binding and transactivation properties (Lai et al., 2001; Vernes et al., 2006; Mizutani et al., 2007), (**Fig.1**).

Although the monogenetic inheritance pattern of the disorder was evident at the time of its first description (Hurst et al., 1990), its nature and clinical diagnosis became a matter of polemics (Fletcher et al., 1990; Gopnik, 1990b), as the manifestation of the dysfunction and the exact language defect appeared to be rather complex.

Linguist M. Gopnik defined the disorder as a dysphasia – i.e. disturbed ability to communicate - due to inability of patients to infer general syntactic rules, like the generation of plurals, tenses, genders. This lack of general grammar rules was especially evident in spontaneous speech, writing or grammatical judgement; however, despite disturbed ability in usage of grammatical constructs, the affected individuals seemed to be able to assign their meaning correctly. Gopnik suggested that the defect was not a consequence of a general cognitive problem, but was specific to grammar domain ‘because the language skills that are not impaired are at least as complex as those which are’ (Gopnik, 1990a).

Investigations of the KE family by Vargha-Khadem et al. concluded that the most prominent phenotype of the disorder is a deficit in the language production-processing system rather than in grammar-processing or morphosyntactic rule formation. The disorder was defined as speech dyspraxia (from ‘praxis’- *greek* for process, activity), with a central defect in oro-facial motor movements; the extent of the deficit is such that a speech of some affected individuals is unintelligible for the naïve listener. These articulation problems are reflected in a relative immobility of the lower face and mouth and consequently affect oral and facial movements when motor actions are performed



Silent verb generation task, fMRI

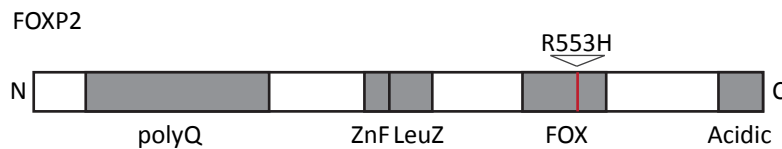
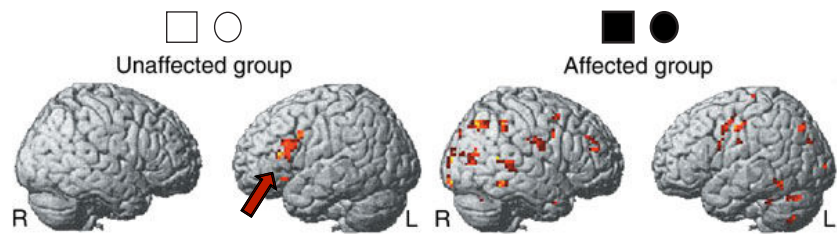


Figure 1. KE family pedigree: filled shapes - affected members, open shapes - unaffected members, circles-females, squares-males, / - deceased. Difference in cortical activation patterns of affected vs. unaffected family members during covert verb generation task, red arrow points to Broca's area. FOXP2 protein structure and location of causative mutation R553H: polyQ, polyglutamine tract; ZnF, zinc finger, LeuZ, leucine zipper; FOX, forkhead-box, Acidic, C-terminal acidic tail region domains. Modified from Watkins et al. (2002a), Liegeois et al. (2003) and Vernes et al. (2006).

simultaneously and sequentially (Vargha-Khadem et al., 1995). Accordingly the orofacial dyspraxia can be robustly detected using three tests: word repetition, non-word (i.e. meaningless words) repetition, and simultaneous and sequential oro-facial movements – all three essentially representing a measure of orofacial motor coordination (Vargha-Khadem et al., 1998; Watkins et al., 2002a). The motor phenotypes were instrumental to the precise assignment of affected and unaffected family members for genetic mapping and identification of the underlying FOXP2 mutation. The final confirmation of the causative genomic region came from an individual with a chromosomal translocation unrelated to the family (Lai et al., 2001).

However, intellectual and linguistic deficits are considered part of the condition; examination of affected KE family members using a battery of linguistic tests (13 tasks examining general language functions and 4 specifically addressing grammar) showed impairments in all but one: object naming. Impairments in language tasks included word and sentence repetitions, lexical decision (to define if the word is real), phoneme deletion and addition from words and non-words, non-word spelling and reading, rhyme production; grammatical tests addressed the ability to process inflections (i.e. knowledge of morphosyntactic rules), tense production and receptive knowledge of sentence embedding in the form of relative clauses (Vargha-Khadem et al., 2005).

Moreover, in addition to the pronounced language problems, the authors reported lower IQ scores in the KE family, and concluded that cognitive impairments are not confined to morphosyntax, but rather extend to verbal and non-verbal domain generally (Vargha-Khadem et al., 1995). Permanent lack of appropriate communication endured from early childhood might contribute to a slightly lowered IQ, a secondary effect to DVD. This idea is supported by Watkins et al. (2002a) stating that ‘a disorder may adversely affect the development of intelligence or of the skills required to maintain a given level of intelligence’

Given the potential presence of a cognitive component in the KE family disorder, it was relevant to dissociate it from the motor-articulation deficits, and to identify which brain structures were affected (Liegeois et al., 2003). Therefore a covert verb generation task was used: the participants were asked to generate the verb to a given noun in their

thoughts, without pronouncing it or performing any movement. While performing this task under functional magnetic resonance imaging (fMRI) monitoring, affected KE family members showed underactivation of Broca's area (classically associated with syntactic aspects of speech) and in Brodmann's area (semantic aspects), together with atypical patterns of overall cortical activation: higher and diffused activation of the right hemisphere as compared to the left, and posterior compared to anterior (**Fig.1**). In this task underactivation was also detected in striatal structures (right putamen/globus pallidus), interpreted as reflecting articulation planning impairments. Interestingly, the overt (spoken) verb tasks – generation and repetition – yielded largely similar misactivation patterns, suggesting that at least in these tests the phenotype largely relied on upstream cortical and striatal abnormalities (Liegeois et al., 2003). Consistently with fMRI observations, grey matter volume measurements in structural imaging studies revealed alterations in motor related structures with the most striking effects in striatal areas (caudate nucleus, putamen and globus pallidus), but also in cerebellum (ventral and posterior lobe); several cortical areas were affected morphologically as well: Brodmann's and Broca's areas, cingulate, sensorimotor, motor, posterior temporal, anterior insular, medial occipitoparietal and inferior frontal cortices (Vargha-Khadem et al., 1998; Watkins et al., 2002b; Belton et al., 2003). Comparison of affected KE-family members to patients with acquired Broca's aphasia (i.e. a collection of language disorders caused by damage to the brain, in this case a left hemisphere stroke) was employed to partially discriminate cortical-driven and striatal aspects of the performance: the difference between the two conditions was detected only within the tests of verbal fluency and lexical decisions, but surprisingly did not seem to lie within oral praxis or receptive and expressive language deficits (Watkins et al., 2002a).

Ackermann (2014) discussed fundamental differences in the profiles of speech motor deficits in verbal dyspraxia associated with *FOXP2* mutations *versus* Parkinsonian motor speech disorder, caused by basal ganglia dysfunctions. Acquired impairments in speech performance due to striatal damage are characterized by execution of orofacial movements with 'undershooting' gestures and disruptions of prosodic aspects of verbal utterances (i.e. rhythm, stress, and intonation), while communication disorders due to

fronto-opercular cortex or anterior insula damages in the language-dominant hemisphere resemble *FOXP2* phenotypes to much greater extent (Ackermann et al., 2014).

Hence, although it is difficult to conclude which language deficit in *FOXP2* deficient patients arises primarily, misarticulation or linguistic, at least in adults the primary problem may lie upstream of the motor system, and has a significant contribution from cortical dysfunctions.

Characterization of the KE family was followed by reports of other patients showing similar phenotypes with *FOXP2* loss-of-function mutations and disruptions of genomic regions residing in the vicinity of the gene (O'Brien et al., 2003; MacDermot et al., 2005; Lennon et al., 2007; Tomblin et al., 2009; Palka et al., 2012).

Disruptions of *FOXP2* chromosomal region (7q31) have been linked to a spectrum of neurodevelopmental disorders, such as autism and schizophrenia (as well as major depression, dyslexia, FTL, ADHD); these pathologies exhibit some level of association with *FOXP2* polymorphisms, often in parallel with the expressivity of language endophenotypes (Gong et al., 2004; Li et al., 2005; Sanjuan et al., 2005; Sanjuan et al., 2006; Padovani et al., 2010; Tolosa et al., 2010; Schaaf et al., 2011; Spaniel et al., 2011; Ribases et al., 2012; Wilcke et al., 2012; Li et al., 2013; Corominas et al., 2014; McCarthy-Jones et al., 2014). However, data on the direct genetic association between *FOXP2* and many of the aforementioned disorders remain controversial (Newbury et al., 2002; Gauthier et al., 2003; Laroche et al., 2008).

A molecular link between *FOXP2* and mental disorders has been established in functional genomics studies through a number of genes and pathways involved in several distinct mental pathologies (Spiteri et al., 2007; Vernes et al., 2007; Konopka et al., 2009; Vernes et al., 2011). Specifically, among the best described genes directly regulated by *FOXP2* are:

- *CNTNAP2* – a gene substantially enriched in frontal gray matter and associated with autism, Tourette's syndrome and severe recessive disorder involving cortical dysplasia and focal epilepsy (Vernes et al., 2008)
- *MET* - component gene involved in human temporal lobe development, associated with autism (Mukamel et al., 2011)

- *DISC1* - a leading candidate susceptibility gene for schizophrenia, bipolar disorder and recurrent major depression, which has been implicated in other psychiatric illnesses of neurodevelopmental origin, including autism (Walker et al., 2012).
- *SPRX2* - a gene responsible for speech dyspraxia and mental retardation which accompany a form of sylvian epilepsy (Roll et al., 2006), which independently links *SPRX2* to speech and language
- *uPAR* - an interaction partner of *SPRX2* which is associated with autism spectrum disorder (ASD) (Eagleson et al., 2010; Roll et al., 2010).

FOXP2 may be linked to autism and intellectual disability *via* interacting partners such as *FOXP1* (Bacon and Rappold, 2012) and *TBR1*. The latter is a recently described autism candidate gene (Deriziotis et al., 2014) and protein-interaction studies of ASD susceptibility genes have shown a direct interaction with *FOXP2* (Sakai et al., 2011; Corominas et al., 2014).

Thus, several lines of evidence strongly suggest that *FOXP2* is part of larger molecular networks underlying distinct language and social cognitive dysfunctions

To summarize, human studies on *FOXP2* gene function suggest that it is involved in complex motor phenotypes of speech and language processing. In addition, *FOXP2* role in both semantic and syntactic aspects of language is supported by pronounced defects in grammar rules formation and problems in lexical decision-making, in agreement with morphological abnormalities in the cortex including Broca's and Brodmann's area. Evidence for *FOXP2* role in phonological domain of language derives from recent studies on healthy individuals. *FOXP2* polymorphisms were shown to contribute to the normal inter-individual variability in hemispheric asymmetries and frontal cortex activation patterns for speech and language audio (dichotic listening task), and also visual (reading tasks) perception (Pinel et al., 2012; Ocklenburg et al., 2013). Cortical lateralization impairments, characteristic of *FOXP2* dysfunction, are well described in language and literacy *per se* and often diagnosed in autism and speech and language disorders (SLI) (De Fosse et al., 2004; Bishop, 2013). Co-morbidity of *FOXP2*-based language and molecular phenotypes with a variety of mental disorders, ASD in particular, hints to *FOXP2* involvement in more complex cognitive functions than simple processing of

certain aspects of language. In this respect Corballis (2004) suggested a link between FOXP2 and the mirror neuron system, which underlies many critical symptoms of autism. ‘Thus, while studies of *FOXP2* can offer insights into relevant neural pathways, it is not a ‘*gene for speech*’.’- Fisher (2007).

Foxp2 as an entry point to study molecular and neural networks contributing to cognitive aspects of speech and language

The discovery of *FOXP2* as the first gene involved in speech and language development led to studies on its role in human evolution. Using comparative analysis of human, chimpanzee, orangutan and mouse DNA sequences, two independent groups established that *FOXP2* is a remarkably conserved gene that was the object a selective sweep in the human lineage. Positive selection in the coding sequence is thought to have converged on two amino acids within exon 7 that distinguish humans from other primates, while only three amino acids substitutions differentiate mouse *Foxp2* from humans (Enard et al., 2002; Zhang et al., 2002) (**Fig. 2**). Further investigations suggested that selection pressure may have been exerted beyond the two human specific substitutions of the coding *FOXP2* region, on sites located near the gene (Ptak et al., 2009; Maricic et al., 2013). Genome-wide scans for positively selected genes during mammalian evolution identified only 5 transcription factors in the hominid branch with a selection rate comparable to *FOXP2* and none of them was associated with brain function, but with spermatogenesis and the immune system (Kosiol et al., 2008; Enard, 2011).

Enard proposed that a murine model, carrying two ‘human’ amino acid substitutions within *Foxp2*, could provide valuable information of the evolution of mammal circuits underlying speech and language in humans (Enard, 2011). Therefore, he and his colleagues have introduced the two human specific substitutions in the orthologous mouse locus, generating a homozygous knock-in *Foxp2* ‘humanized’ mouse (*Foxp2*-Hum) (**Table 1**) (Enard et al., 2009). Although *Foxp2* is expressed in vitally important organs such as heart, lung and guts (Shu et al., 2001), the exhaustive screen for almost 300 different phenotypic parameters did not produce any evidence for effects of the

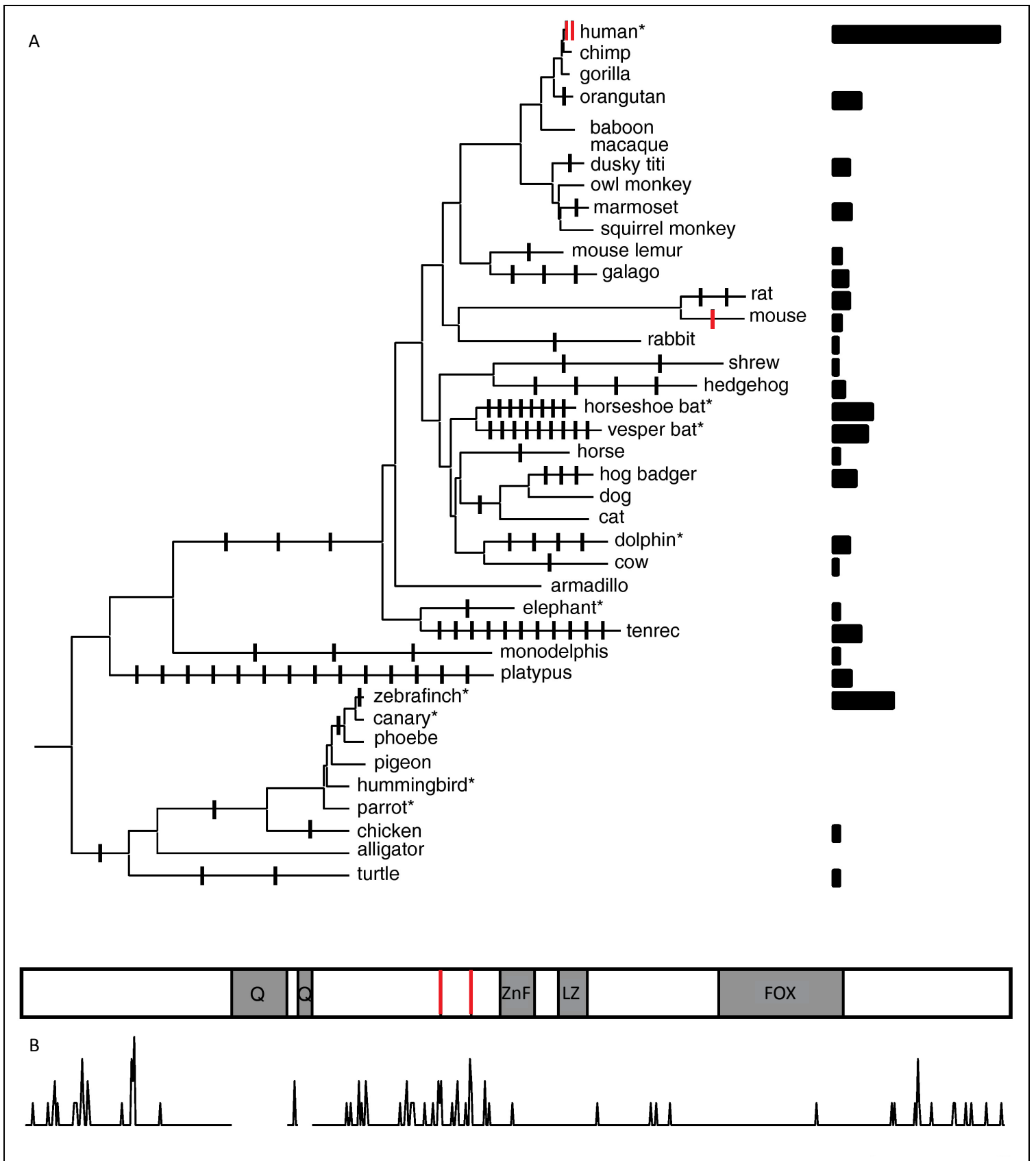


Figure 2. *FoxP2* evolution in vertebrates. **A**, Amino acid changes outside the polyglutamine tracts (Q) are mapped on the phylogeny of *FoxP2* sequences from vertebrates. The bars on the right side depict the ratio of amino acid changes to the length of the terminal branches. Asterisks indicate species with evidence for vocal learning. **B**, Amino acid changes in the tree are plotted for each position of the human *FOXP2* protein sequence. ZnF, zinc finger; LZ, leucine zipper; FOX, forkhead-box domains, the two human amino acid changes are shown as red lines. Adapted from Enard (2011).

*Foxp2*Hum allele in any organ system except the central nervous system. Here, anomalies were revealed in behavioral, electrophysiological, histological and molecular parameters within the brain. Furthermore, such anomalies were most prominent within cortico-striatal circuits (Enard et al., 2009; Reimers-Kipping et al., 2011; Schreiweis et al., 2014).

These findings are in agreement with the cortico-striatal core of phenotypes in affected KE-family members. The authors suggested that human-specific mutations in *Foxp2* in mice could model aspects of speech and language evolution in humans (Enard et al., 2009).

The neural circuits underlying uniquely human functions, involving abstract thinking and the cognitive mechanisms of language processing (as, for example, syntax), are located in the cortex. This brain structure expanded significantly in the human lineage relative to other primates, developing new regions in the frontal and parieto-temporal lobes. Cortical volume expansion is mechanistically based on increases in intercellular space and cell number, due to higher cortical neurogenesis, enhanced elaboration of dendritic trees and expansion of neuropil areas, especially in the prefrontal cortex (Geschwind and Rakic, 2013).

As discussed below (in *Foxp2 cellular functions, Foxp2 in the mouse cortex*), FoxP2 is strongly engaged in processes of neurite outgrowth and cortical neurogenesis (Spiteri et al., 2007; Vernes et al., 2007; Vernes et al., 2011; Tsui et al., 2013; Chiu et al., 2014); in this light it is interesting to examine how *FOXP2* role in cortical evolution is gradually emerging from comparative genomic studies:

When addressing evolution of the coding genome at the single nucleotide level it is accepted that brain-expressed genes are not specific for positive selection in human lineage (Wang et al., 2007; Geschwind and Rakic, 2013). However, *FOXP2* appears to be one of the salient exceptions, as inferred from Enard's studies described above, as well as from the analysis of Neanderthal and Denisovan genomes sharing the human-derived form of *FoxP2* right before the lineages split (Krause et al., 2007; Meyer et al., 2012). Many human brain-specific evolutionary accelerated changes lie within non-coding nucleotide stretches, i.e. enhancers and promoters (Capra et al., 2013). Visel et al. in their comparative study of conserved non coding regions relevant to telencephalic evolution,

found a dozen of elements in close and distant proximity of *FOXP2*, as well as many more close to the genes constituting *FOXP2* molecular network. Among these is the highly conserved transcription factor *POU3F2*, which has been suggested as candidate for having caused a recent selective sweep of *FOXP2* in the human lineage (Maricic et al., 2013; Visel et al., 2013). In a separate study using comparative epigenetic profiling of human, mouse and macaque corticogenesis to identify enhancers which gained activity in human evolution, *FOXP2*, although not identified as one of the hub genes, showed substantial connectivity to gain-enriched modules of co-expressed genes (Reilly et al., 2015).

Konopka et al. performed comparative analyses of transcriptomes from the telencephalon of human, chimpanzee and macaque telencephalon. They demonstrated a striking increase in transcriptional complexity specific to the human lineage in the frontal lobe, while genes expression in the caudate nucleus was conserved. In particular, the human prefrontal cortex is enriched in alternatively spliced genes involved in neuron projections, neurotransmitter transport, synapses, axons, and dendrites, as well as genes implicated in schizophrenia. Among the most differentially connected genes, this module contained *FOXP2*, and 13 genes that overlap with previously identified *FOXP2* targets, as well as its dimerization partner - *FOXP1* (Konopka et al., 2012).

Taken together comparative genomic data support the hypothesis that *FOXP2* was actively recruited in the formation of the most evolved structures of the human cortex during hominin evolution.

If the human telencephalon is a structure, providing unique capacities, how could we approach its origins? Insights might come from the application of the concept of ‘deep homology’, stating that new structures do not arise *de novo*. Instead there is a *continuum* of events which follows an evolutionary constraint imposed by pre-existing genetic regulatory circuits, which were initially established in early metazoans. Classical examples of deep homology are the re-appearance of eyes in widely divergent organisms, based on similar genetic components such as ciliary phototransduction and melanogenic pathways, or tetrapod limbs and fish fins which are both based on deeper homology in the network of Hox genes (Shubin et al., 2009). When evolutionary constraints are further restrained by the structure, composition and dynamics of the developmental system (the

fusion of these two fields is also called ‘evo-devo’ concept) there is even less room for deep phenotypic variability. The concepts of ‘deep homology’ and ‘evo-devo’ are classically applied to evolution of structures and forms, but could be adapted to neural circuits and behaviours (Robinson et al., 2008; Scharff and Petri, 2011). In the case of *FoxP2*, genomic sequence, developmental expression pattern and molecular network are conserved in distant mammals (such as human and mice) (Lai et al., 2003)(**Fig.3**). Within this framework mouse *Foxp2* could serve as an entry point to study ancestral substrates underpinning complex aspects of FOXP2-dependent human cognition.

The study of animal models provides insights into conserved FoxP2 functions

The role of FoxP2 in development

The speech and language deficits in individuals carrying genetic aberrations of *FOXP2* clinically manifest already in childhood (Hurst et al., 1990; Vargha-Khadem et al., 1995). The presence of developmental vocal learning deficits is supported by performance differences in tests of repetition of words with known meaning between adult affected KE family members and patients with acquired aphasia: the latter, who had a normal cognitive and linguistic development prior to the trauma leading to aphasia, performed significantly better than the former (Watkins et al., 2002a; Vargha-Khadem et al., 2005).

Histological examinations of postmortem brains support *Foxp2* role in nervous system formation, in human and mice, and the patterns of expression are highly conserved throughout development in both species (**Fig. 3**). Functional genomics data on *Foxp2* targets obtained on embryonic human and mouse tissue supports and further elucidates mechanisms of *Foxp2* role in CNS formation (discussed in more detail below in *FoxP2 cellular functions*) (Spiteri et al., 2007; Vernes et al., 2007; Vernes et al., 2011).

Foxp2 specifically plays a role in cortical development, affecting distinct stages of neuronal maturation (Clovis et al., 2012; Tsui et al., 2013; Chiu et al., 2014)(*Foxp2 in the mouse cortex*, **Fig.7**). Complete deletion of *Foxp2* in mice is lethal: homozygous *Foxp2* mutant mice display reduced weight gain, aberrant manifestation of developmental reflexes and die within 4 weeks after birth (Groszer et al., 2008), **Table 1**.

Mouse embryo:

Human newborn:

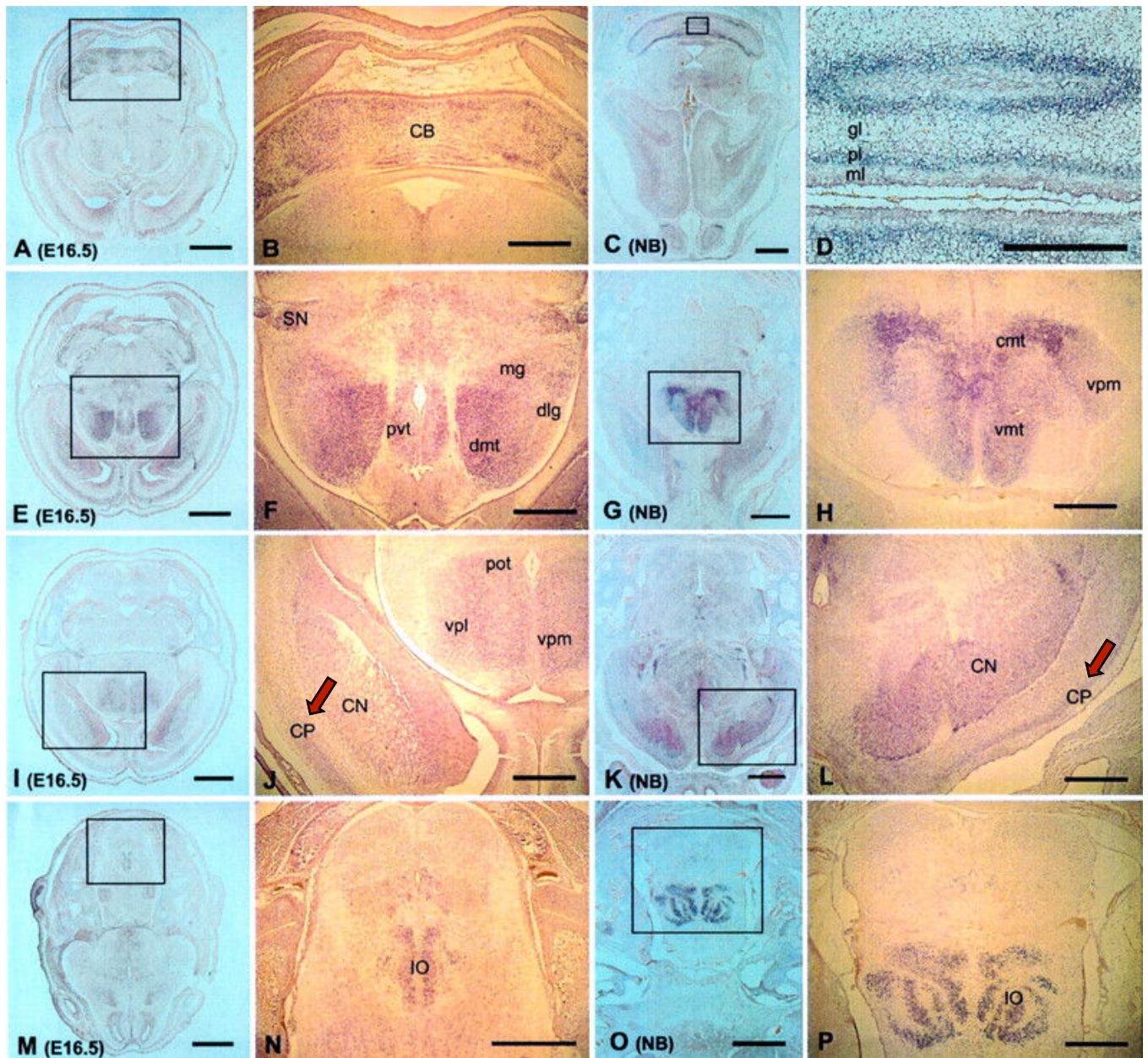


Figure 3. *Foxp2* mRNA expression in the embryonic mouse brain at E16.5 and in the newborn human. Sequential transverse sections, from anterior to posterior. Cortical plate (CP) *Foxp2* distribution is marked with a red arrow. Adapted from Lai et al. (2003).

FoxP2 conserved role in the ontogenesis of nervous system is illustrated by its expression in zebrafish, with a well preserved pattern in pallium and subpallium - telencephalic structures (Bonkowsky and Chien, 2005). In songbirds, a model which is extensively used in *FoxP2* research due to its vocal learning abilities, *FoxP2* is expressed in pallium, striatal and thalamic-like structures throughout development, with a prominent expression in Area X – a structure which intermingles striatal and pallidal components and is actively involved in vocal performance (Teramitsu et al., 2004). In Area X *FoxP2* mRNA show increased expression during a particular segment of time when young birds actively learn how to imitate song from a tutor (Haesler et al., 2004), decreases upon brain maturation as song acquires more stereotypy (Thompson et al., 2013) and is necessary for adequate song acquisition (Haesler et al., 2007).

Comparing *FoxP2* expression patterns in vocal-learners and non-learners in-between avian, reptilian and mammals, Haesler et al (2004) proposed that FoxP2 is an ancient transcription factor involved in shaping of cerebral architecture of striatal sensory and sensory-motor circuits to generate a permissive environment for vocal communication evolution and development of vocal learning. This hypothesis is in accordance with data arising from primate research and from a variety of human language disorders, suggesting that striatal control underlies the entrainment and automatization of speech motor patterns during speech acquisition, while mature verbal communication requires less striatal processing capacities and relies on the left-hemisphere peri- or subsylvian cortex (Ackermann et al., 2014).

Activity dependent function of FoxP2 in mature brain: evidence for a role in social behaviour and vocalizations

FoxP2 expression persists throughout development into the mature adult brain (Ferland et al., 2003), suggesting activity-dependent function. Evidence for the role of *FoxP2* in the adult brain is primarily coming from the study of songbirds and mice, and it appears to be tightly linked to the ability to learn and to modify the accuracy of socially-driven vocalizations upon involvement of the dopaminergic system (Murugan et al., 2013).

In zebra finches song learning and modulation is mediated by loops involving a set of nuclei homologous to cortex(pallium)/striatum/thalamus - like nuclei, which share many characteristics with mammalian cortico-striatal circuitry (Fisher and Scharff, 2009) (**Fig.4**). The organization of the precise timing signal for motor control during singing is thought to be governed by premotor (HVC) and motor (RA) pallial nuclei. RA establishes a direct connection with vocal motor neurons in the brainstem. This direct motor cortex connections may enable voluntary vocal control and vocal-learning abilities in particular species in distinct animal clades (**Fig.2**); it is absent in monkeys, but present in humans and have recently been described in mice (Jurgens, 2002, 2009; Arriaga and Jarvis, 2013). Before being transferred to downstream motor structures, the timing signal undergoes a social context-dependent modulation in the divergent striatum-thalamo-cortical loop (**Fig. 4**), where Area X is a prominent hub. Area X shows lower *Foxp2* mRNA and protein levels in the absence of social context (Miller et al., 2008). For zebra finch males the social context is defined by the presence of the female to whom structured and well organized song is presented (directed song), while greater variability (i.e. the song is more enriched in motifs, syllable types and frequency range) is observed when males are singing alone (undirected song). *FoxP2* knockdown in Area X of adult birds abolishes refinement of the directed song (**Fig. 5**) by rendering the timing signal insensitive to modulation by dopamine receptor 1 (D1R) and dramatically decreasing the levels of dopamine-regulated neuronal phosphoprotein DARPP-32, a key dopamine signaling regulator (Teramitsu and White, 2006; Murugan et al., 2013).

In mice, the first evidence for *Foxp2* activity-dependent expression came from studies of plasticity of auditory thalamic nuclei (MGN) , where *Foxp2* is upregulated after a high volume white noise stimulation (Horng et al., 2009). Analysis of four major neuromodulators in *Foxp2* mutant mice showed that dopamine (DA) levels are dramatically affected in cortico-striatal structures, and serotonin levels in nucleus accumbens (NuAc) (Enard et al., 2009). In striatum and cortex nearly all *Foxp2*⁺ neurons co-express DARPP-32 (Hisaoka et al., 2010; Vernes et al., 2011). With Cedric Mombereau in our team (Mombereau et al., submitted) we assessed the potential contribution of *Foxp2* to DA signaling using pharmacological stimulation with cocaine, a

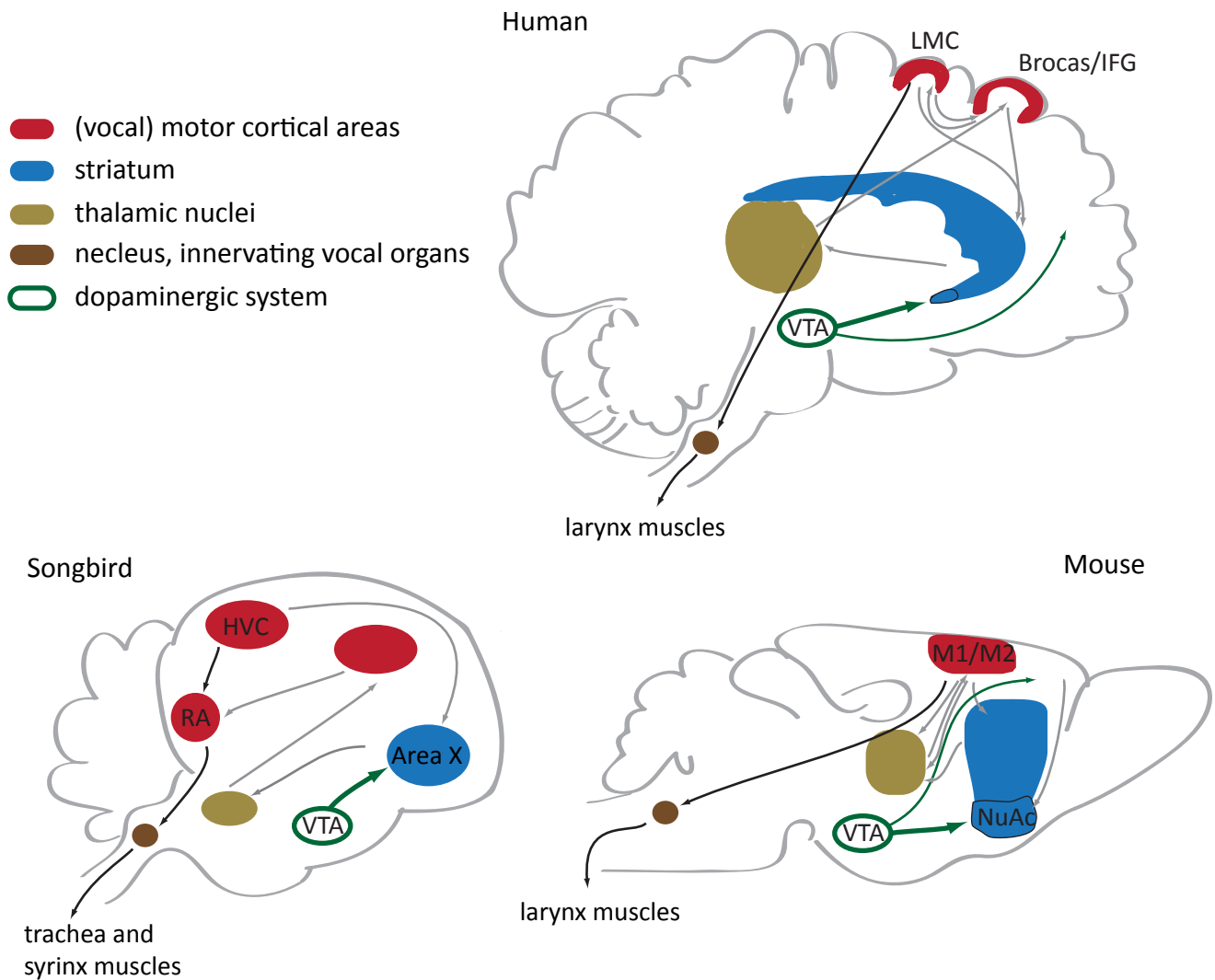


Figure 4. Brain circuitries involved in vocalizations in vocal-learning species and mice. Remarkably, all the depicted forebrain structures express *FoxP2*. Black arrows indicate cortical projections that within one synaptic switch innervate vocal organs – this direct motor connection is thought to be exclusive for vocal learning species. LMC, laryngeal motor cortex; IFG, inferior frontal gyrus; VTA, ventral tegmental area; M1, primary motor cortex; M2, secondary motor cortex. Figure is modified from Arriaga et al. (2012).

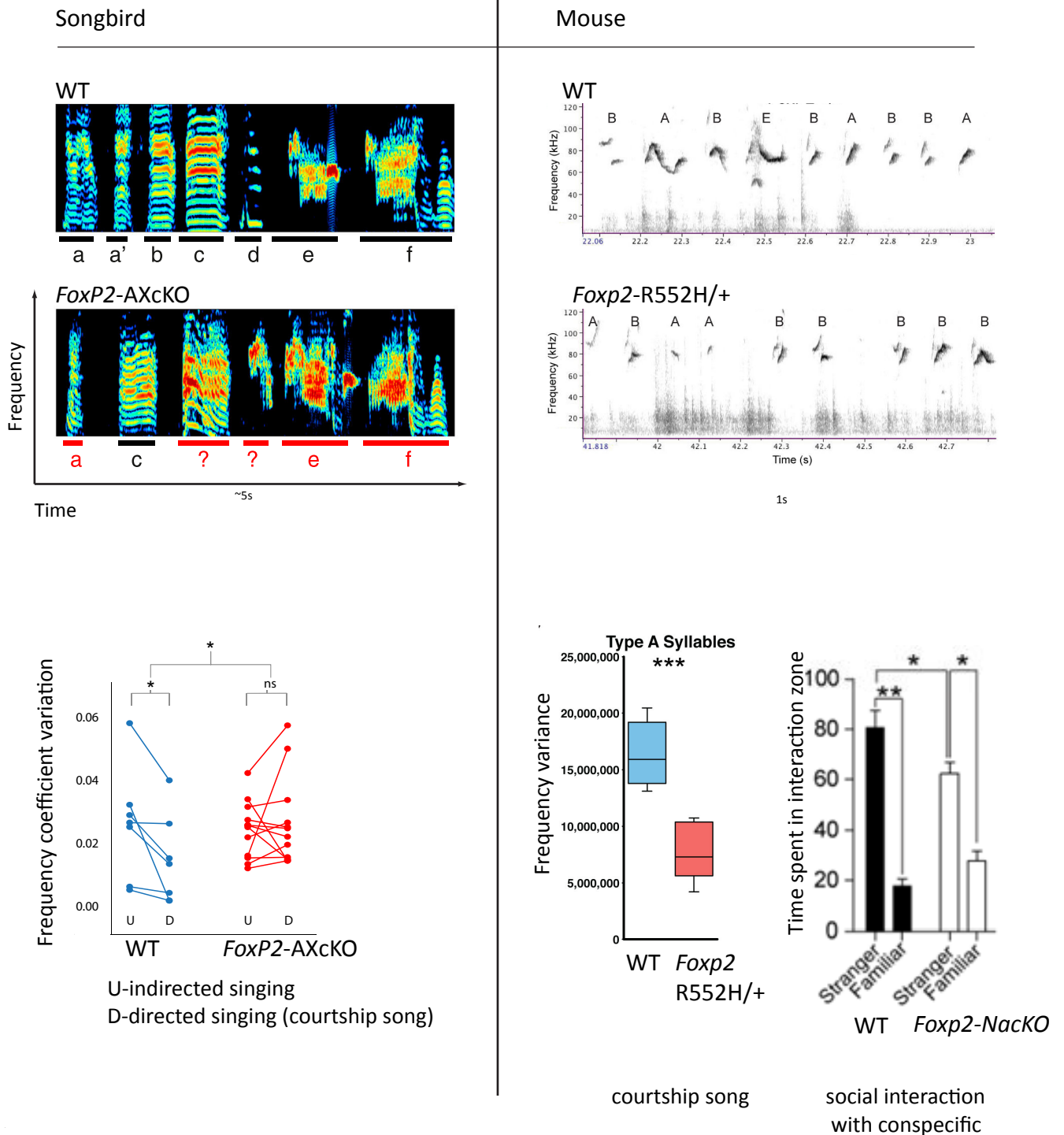


Figure 5. Vocalizations structure and social behaviors impaired in FoxP2 deficient animals. Upper panels depict typical sonograms obtained from animals under normal conditions and after *FoxP2* disruption. Bottom graphs illustrate frequency alterations in a courtship song of both species as well as diminished social interest revealed in 3-chamber test on NuAc knockout (*Foxp2-NacKO*) male mice. *FoxP2-AXcKO*, *FoxP2* shRNA knockdown in Area X; *Foxp2-R552H/+*, heterozygous mutant, carrying Foxp2 an amino acid substitution identical to the one found in the KE family. Adapted from Arriaga (2011), Mombereau et al. (submitted) and Murugan et al. (2013).

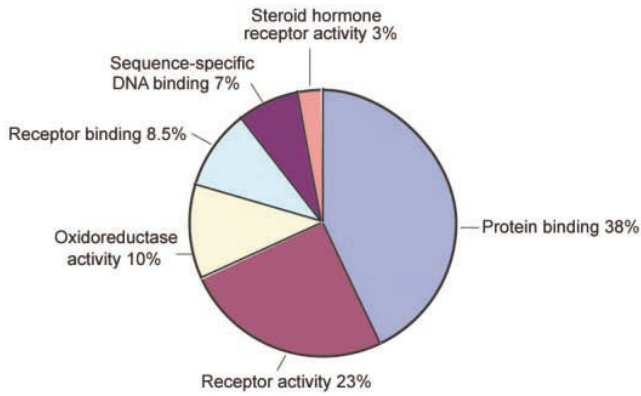
drug which recruits dopaminergic pathways. After stimulation of *Foxp2* heterozygous mice, we localized a specific underactivation pattern to D1R⁺ neurons of NuAc (ventral striatum nuclei). *Foxp2* protein levels in WT NuAc showed significant downregulation after one cocaine injection, correlating with cocaine-induced locomotor behavior attenuation in conditional *Foxp2*-NuAc knockdown mice. As NuAc is a structure particularly implicated in reward-associated learning and *Foxp2*-NuAc knockdown mice showed a decreased preference specifically for social situations (time spent in the chamber with conspecific WT) (**Fig. 5**, **Fig. 10** and **Context**), our study suggests that *Foxp2* regulates DA-mediated social reward signaling.

Some evidence exists for speech and language modulation by DA in humans. Neurological and psychiatric studies indicate that dopamine receptor antagonists disrupt vocal motor control and lead to the development of uncontrolled laryngeal spasms. Voice, articulation, phonological processing and syntactic complexity deterioration in Parkinsons disease patients - a disorder of dopaminergic neurons loss - is the most striking example of DA-dependent voice and speech problems. Similarly, alterations in DA levels or in nigrostriatal dopamine release are linked to speech related defects as diverse as vocal tics in Tourette's syndrome, auditory hallucinations in schizophrenia and stuttering (Simonyan et al., 2012).

FoxP2 cellular functions

The identification of FOXP2 transcriptional targets has provided valuable insights into regulated cellular processes. High-throughput approaches such as FoxP2 chromatin-immunoprecipitation, coupled with gene expression profiling in cell cultures transfected with *FoxP2* variants, or mouse and human embryonic material have been used (Spiteri et al., 2007; Vernes et al., 2007; Konopka et al., 2009). Differentially expressed direct and indirect targets have been functionally annotated using ontology categories defining specific biological processes. Gene Ontology (GO) categories characteristic for developmental FOXP2 targets in humans (**Fig. 6**) largely overlap with mouse GO categories. Detailed analysis of mouse data complements with the categories of neurogenesis, neuron projection morphogenesis, cell localisation functions as well as synaptic plasticity and spine formation (Vernes et al., 2011). These functions are

CNS in vivo molecular function



CNS in vivo biological function

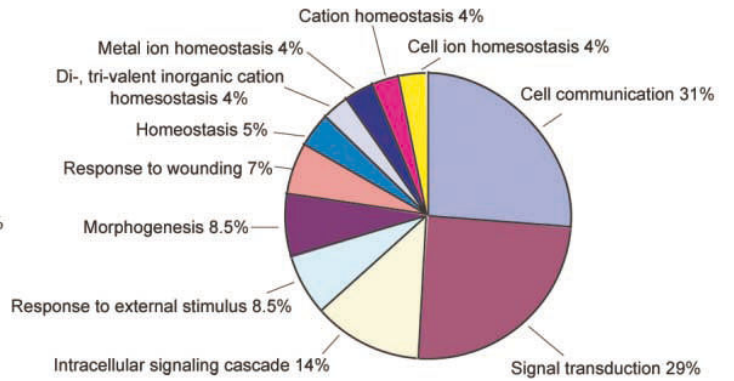


Figure 6. Gene Ontology categories of FOXP2 regulated genes in embryonic human frontal cortex and basal ganglia. Adapted from Spiteri et al. (2007).

supported by studies of *Foxp2* mutant mice *in vivo* with pronounced effects on development and function of cortico-striatal circuits (Groszer et al., 2008; Enard et al., 2009; Schulz et al., 2010; Chiu et al., 2014).

Foxp2 role in cortical neurogenesis is well described in mice, where *Foxp2* ectopic misexpression in cortical progenitors impairs *in vivo* radial migration, neurite maturation and synaptogenesis (Clovis et al., 2012; Sia et al., 2013), and affects progenitors type transitions during neuronal differentiation (Tsui et al., 2013; Chiu et al., 2014)(**Fig.8**). *FoxP2* specific role in neurogenesis is further supported by studies in chicken and zebra finch (Rousso et al., 2012; Thompson et al., 2013).

In zebra finches, the major functional modules associated with singing and regulated by *FoxP2* relate to synaptic plasticity (more specifically, the suppression of postsynaptic plasticity) *via* *FoxP2* interconnection with genes linked to MAPKK and NMDA receptors function, actin cytoskeleton regulation, and tyrosine phosphatase (Hilliard et al., 2012).

Cocaine stimulation of heterozygous mice (*Foxp2*-R552H/+ -Enu, **Table1**) revealed neuronal activity alterations in NuAc mediated by genes involved in calcium signaling, among which voltage-dependent calcium channel alpha 1G (*Cacna1g*) has been genetically associated with ASD, providing further support for the role of *Foxp2* in social behavior (Mombereau et al., submitted).

Mouse models in Foxp2 research: motor learning and ultrasound vocalizations

Foxp2 expression pattern is highly conserved across four species of muroid rodents: two species of singing mice (*Scotinomys teguina* and *S. xerampelinus*), their close relative the deer mouse (*Peromyscus maniculatus*), and more distantly related laboratory mouse *Mus musculus* (Campbell et al., 2009).

Foxp2 distribution in the mouse brain is rather broad and extends to lower cortical layers, striatum, mesolimbic and nigrostriatal dopaminergic systems, thalamic somatosensory areas, Purkinje cells of cerebellum and inferior olivary complex, olfactory system and ascending auditory and visual relays (**Fig. 7**) (Ferland et al., 2003; Campbell et al., 2009). This expression pattern suggests that *Foxp2* is unlikely to regulate circuits selectively governing verbal or vocal functions, but rather specifically interacts with genetic factors that define relevant circuits and pathways. The relevant functional

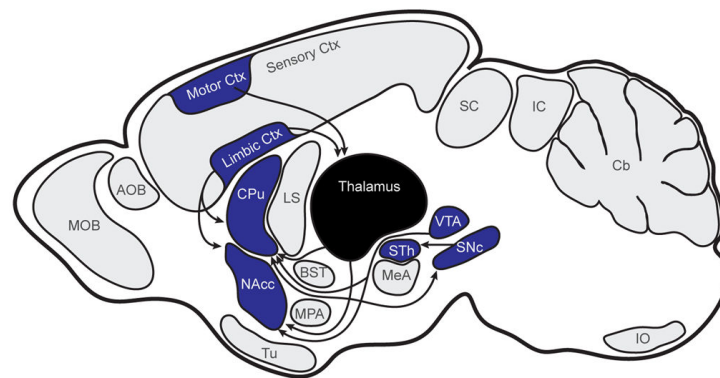


Figure 7. Overview of *Foxp2* expression in the adult mouse brain. Highlighted structures comprise the circuitry of striatal modulation of fine motor response, suggesting a role in motivational and integrative circuits, rather than in the direct regulation of motor output. AOB, accessory olfactory bulb; BST, bed nucleus of stria terminalis; Cb, cerebellum; CPU, caudate putamen; Ctx, cortex; IO, inferior olivary complex; LS, lateral septum; MeA, medial amygdala; MOB, main olfactory bulb; MPA, medial preoptic area; NAcc, nucleus accumbens; SNc, substantia nigra pars compacta; STh, subthalamic nucleus; Tu, olfactory tubercle; VTA, ventral tegmental area. Adapted from Campbell et al. (2009).

circuitry in adult mice has been suggested to lie within networks controlling fine motor output, multimodal sensory processing and sensorimotor integration (Campbell et al., 2009).

A concise overview of the existent genetically modified *Foxp2* mouse models and their basic phenotypes has been provided in a recent review by French and Fisher (2014), **Table 1.**

Motor learning

Because of the known relevance of cortico-striatal motor circuitry for auditory guided vocal communication (Wohlgemuth et al., 2014), supported by high conservation of striatal circuitries among vertebrates, most attempts to experimentally access *Foxp2* functions in mice have used motor learning paradigms. The heterozygous loss-of-function model, bearing KE family etiological mutation: *Foxp2*-R552H/+ -Enu (**Table 1**), manifests motor learning impairments in three distinct assays: voluntary-controlled running wheel, automatically accelerated rotarod and auditory-motor associations learning (accessing auditory-motor integration impairments potentially relevant for speech acquisition); the latter approach also revealed a worse learning curve in another heterozygous model - *Foxp2*-S321X/+. Striatal electrophysiological abnormalities were observed in *Foxp2*-R552H/+ mice, including reduced cortico-striatal long-term depression (LTD) in brain slices, and abnormally high ongoing striatal activity along with dramatic alterations of striatal plasticity during motor skill acquisition *in vivo* (Groszer et al., 2008; French et al., 2012; Kurt et al., 2012).

Foxp2 humanized mice (*Foxp2*-Hum) a model which effectively manifests gain-of-function profile, show enhanced striatal LTD compared to WT and *Foxp2* heterozygote mice (Enard et al., 2009; Reimers-Kipping et al., 2011). Furthermore, humanized mice demonstrate faster proceduralization of action sequences in conditional T-maze paradigm with spatial cues. This learning paradigm accesses declarative (place-based) learning transitions to procedural (response-based) learning, and reveals the speed of information transfer from dorso-medial to dorso-lateral striatum (Schreiweis et al., 2014).

Table1. Genetically modified mouse lines (modified from French and Fisher (2014)).

Mouse line	Disruption	Basic phenotypes
Foxp2-KO	Removed the FOX domain yielding knockout mice.	Homozygotes die by 3 weeks of age. Heterozygotes show mild developmental delay, but normal performance in Morris water maze memory and learning paradigm.
Foxp2-R552H-KI	Knockin point mutation in an Arg-to-His substitution in the FOX domain of the encoded protein. This substitution is found in affected members of the KE family (R553H).	Homozygotes die by 3 weeks of age. Some heterozygotes show mild-moderate developmental delay.
Foxp2-R552H-Enu	Mice with Arg-to-His substitution seen in the KE family, isolated from an ENU-mutagenesis screen.	Homozygotes die at 3-4 weeks of age. Heterozygotes are overtly normal.
Foxp2-S321X	Mice with a point mutation, isolated from an ENU-mutagenesis screen. Results in a premature stop codon, shown to be equivalent to a null allele (no protein). This mutation is found in a second family segregating SLI.	Homozygotes die at 3-4 weeks of age. Heterozygotes are overtly normal.
Foxp2-N549K	Mice with a point mutation isolated from an ENU-mutagenesis screen. Results in an Asn-to-Lys substitution in the FOX domain.	Homozygotes survive into adulthood (3-5 months age) with severe motor problems. Heterozygotes are overtly normal.
Foxp2-Flox	LoxP sites inserted around exons 12-14 to facilitate Cre-mediated removal of the FOX domain.	When crossed to the global Sox2-Cre line, homozygotes die at 3-4 weeks of age and heterozygotes are overtly normal.
Foxp2-Hum	Knockin strategy used to modify exon 7 and to introduce flanking LoxP sites. Results in 2 changes (T302N and N324S), where the amino acids found in the mouse are substituted for the orthologous human amino acids, partially humanizing the encoded protein.	Both homozygous and heterozygous humanized mice are overtly normal. Removal of exon 7 using a global Cre line results in a knockout phenotype. Homozygous knockouts die postnatally and heterozygous knockouts are overtly normal.

Interestingly, genetic manipulation of single drosophila ortholog - *FoxP* - seems to be responsible for operant self-learning abnormalities in this species, pointing to deeply ancient FoxP protein family mechanisms, underlying clue-based motor sequence acquisitions (Mendoza et al., 2014).

Ultrasound vocalizations

Campbell et al (2009) compared *Foxp2* brain distribution in two species of singing mice, characterized by highly complex and structured social vocalizations which require tight control of facial musculature, and related species, including lab mice. In this study the authors were looking for the morphological parameters which could explain the particular vocalization capabilities of singing mice, however no evident differences between species were detected, but only minor random fluctuations within limbic forebrain and cortex. Thus, the function of *Foxp2* in vocal communication does not seem to extend to mouse species differences in articulatory and acoustic complexity and is fundamentally conserved.

Mus musculus elicits two types of calls: audible, which often signals a distress situation, and ultrasonic vocalizations (USVs). USVs study is a rapidly developing field and a promising tool for analysis of communicative and social behaviour alterations in laboratory mice (Ey et al., 2011; Ey et al., 2013). Holy and Guo (2005) demonstrated that a mouse USV can be not only recognised as a song enriched with multiple syllables types, but also has non-random structure of syllable sequences, organised into motifs and phrases. Although mouse songs are not as elaborate as birds ones (**Fig 5**), it is generally accepted that mice are able to modify their song structure according to the social context to the extent that it becomes possible to talk about ‘mouse syntax’ and ‘prosody’ (Guo and Holy, 2007; Lahvis et al., 2011; Chabout et al., 2012; Chabout et al., 2015).

Whether mice are able to imitate and learn specific vocal patterns like songbirds and humans is a matter of debate (Arriaga and Jarvis, 2013; Portfors and Perkel, 2014). It is clear, however, that pup vocalizations are innate since newborn are deaf for half of their maturation period, and likely represent an isolation signal in order to elicit maternal care. The structure of this calls changes through development, changing frequency

characteristics, call length and phrasal structure and overall becoming more complex (Musolf and Penn, 2012).

Speech and language impairment in the KE family is a neurodevelopmental disorder. Therefore, initial studies in *Foxp2* mouse models focused on pups vocalizations. Complete absence of functional *Foxp2* in homozygous mice correlated with dramatic decrease in pup vocalizations along with loss of weight, severe motor abnormalities, lack of spontaneous activities, and delay of developmental reflexes maturation. Thus, the absence of vocalizations could have been due to somatic weakness and general arousal problems. Indeed, when homozygous pups were stimulated by tail lifting - a stressful condition - both WT and mutants emitted comparable number of calls (audible clicks and USV). Heterozygous *Foxp2*-R552H-Enu pups exhibit normal USV rates with normal acoustical parameters (call duration, power, minimal and maximal frequencies) along with overall normal developmental parameters; heterozygous *Foxp2*-R552H-KI and -KO mice showed reduced rate of calls accompanied with weight reduction and developmental delays. Thus, the general conclusion is that *Foxp2* partial loss-of-function does not affect innate vocalization production (Shu et al., 2005; Fujita et al., 2008; Groszer et al., 2008; Gaub et al., 2010).

Homozygous humanised mouse pups were studied through several developmental stages, which also covered later stages than those studied for heterozygous mutants. An initial analysis showed that normal basic call parameters, such as number of calls and interval duration, were unaffected. However, further analyses were based on structural classification in syllables types, distinguishing short and long-lasting calls with no frequency jumps (i.e. 'simple' calls, the prevalent types) and calls with frequency jumps ('complex' calls). All call types showed differences in various acoustic parameters (including mean, minimum and maximum of the frequency), however in opposite directions: while simple calls scored lower, calls with frequency jumps were higher in *Foxp2*-Hum *versus* WT. In addition, complex calls duration was significantly increased in humanised mice (Enard et al., 2009).

Altogether, it is difficult to compare results obtained in different *Foxp2* models due to varying experimental designs and confounding factors of developmental delays. Enard's study, however, suggests that *Foxp2* gain-of-function mutation might have changed fine

acoustic parameters in mouse vocalisations. However, it is difficult to conclude whether these changes relate to innate vocalisations or to learned and modifiable ones, since the analysis in the study covered a rather long developmental period. The ecological significance of these changes remains unknown.

To date, the role of *Foxp2* in adult mouse vocalizations is less well studied. Arriaga analyzed *Foxp2*-R552H male mice singing in response to female urine, sometimes designated as courtship song (Arriaga, 2011). The song analysis in this study distinguished simple calls with no frequency jumps (type A), and eleven more types of calls with varying frequency jump architecture. Call rate, repertoire and fractions of calls did not change in comparison to WT littermates. However, when the focus was shifted to spectral characteristics of the most abundant type A syllables, multiple frequency parameters appeared to be altered: starting, minimum, $\frac{1}{4}$ calls frequencies were significantly higher in heterozygous animals (however, mean and maximum frequency did not differ); in addition, a number of characteristics effectively reflecting frequency range distribution were decreased in heterozygotes. The frequency alterations were opposite to those observed in *Foxp2*-Hum pups, which is in concordance with previous studies (Enard et al., 2009).

The presence of frequency range alterations in *Foxp2* mouse mutants resembles songbird striatal *FoxP2* knockdown in Area X during directed singing, although, in opposite direction (**Fig. 5**).

Notably, *FoxP* knockdown in drosophila alters courtship behaviour and renders courtship male ‘pulse song’ (i.e. a vibration of a wing for production of trains of pulses) faster with more variable inter-pulse intervals, longer pulse song bouts and generally reduced percentage of time spent singing. These alterations occur together with motor coordination problems. Nevertheless, the findings suggest a deep functional homology with vertebrates in courtship communication behaviour (Lawton et al., 2014).

Foxp2 in the mouse cortex

The mammalian neocortex consists of six main layers of neurons which differ in cellular morphology, connectivity and physiological properties. Upper layers (L2/L3), contain pyramidal neurons which project intracortically; layer 4 contains pyramidal and

granule neurons and serves as sensory information relay as it receives a bulk of thalamocortical axons; layer 5 is an output layer with a variety of projection targets, including corticobulbar and corticospinal projections; cortical layer 6 contains the largest diversity of morphological cell types with not well defined functionality, receiving and sending projection to the thalamus and within the cortex (Briggs, 2010; Kwan et al., 2012). Layer 6 is remarkably enriched in *Foxp2* through all the areas of the cortex, in both human and mice; *Foxp2* is sparsely present in layer 5 as well (Ferland et al., 2003; Campbell et al., 2009; Hisaoka et al., 2010). In the developing cortex, *Foxp2* is expressed in cortical progenitor cells throughout neurogenesis (Ferland et al., 2003; Takahashi et al., 2003; Tsui et al., 2013).

Cortical development occurs in inside-out fashion, with lower cortical layer arriving first and upper cortical neurons gradually migrating through the lower ones. Maturation of cortical neurons starts after embryonic developmental day 11 (E11) in mice from multiple classes of neuronal progenitors. These are collectively classified into Radial Glial progenitors (RGs) and Intermediate Progenitor Cells (IPCs). RGs are located in the lower cortical compartment – the ventricular zone (VZ) - and through asymmetrical cell divisions give rise to postmitotic neurons, astrocytes and oligodendrocytes, as well as to IPCs. IPCs reside in the adjacent upper compartment - subventricular zone (SVZ) – and through symmetrical divisions IPCs generate postmitotic neurons (prevalently of upper layers). Early postmitotic neurons form a layer of cortical plate (CP) which will subsequently differentiate into lower cortical neurons, followed by incoming later-born upper cortical neurons. The formation of six-layered cortex is roughly over at E17 and at birth (~E20) the major classes of cells in the cortex are established (Guillemot et al., 2006; Molnar et al., 2006; Molyneaux et al., 2007; Merot et al., 2009; Shoemaker and Arlotta, 2010; Kwan et al., 2012; Sun and Hevner, 2014).

Foxp2 expression in cortical progenitors is low in comparison to postmitotic neurons. *Foxp2* loss-of-function in RGs has been shown to inhibit their transition to IPCs, partially by enhancing RGs proliferation, which leads to inhibited production of neurons. Ectopic expression of gain-of-function *Foxp2*-Hum gene demonstrates reversed phenotype with enhanced genesis of IPCs and neurons. Remarkably, overexpression of endogenous *Foxp2* in mouse progenitors does not impair their subtype specification, but it does alter

timing of migration and neurite outgrowth of developing neurons (Clovis et al., 2012; Tsui et al., 2013; Chiu et al., 2014) -**Figure 8**.

The functional role of Foxp2 role in mouse cortical development was recently illustrated by the study of another human language-associated gene, SPRX2: in the cortex Foxp2 regulates excitatory synapse density *in vitro* and *in vivo*, as well as the amount of pups USV by controlling the expression of SPRX2 (Sia et al., 2013).

In the adult cortex *Foxp2* is expressed in the subset of glutamatergic projection neurons. Interestingly, all Foxp2⁺ projection neurons coexpress DARPP-32 (dopamine- and cAMP-regulated phosphoprotein 32 kDa), a key DA signaling regulator. *Foxp2* is not expressed in interneurons (Hisaoka et al., 2010) - **Figure 9**.

Through postnatal development all Foxp2⁺ neurons within layer 6 colocalize with Tbr1, which further points to the glutamatergic nature of these neurons (Hisaoka et al., 2010). Tbr1 is a transcription factor which is crucial for layer 6 development as it defines the major efferent (corticothalamic and contralateral cortical) and afferent (thalamocortical and serotonin fibers) innervations; Tbr1^{-/-} mutant mice display massive cortical malformations (Hevner et al., 2001). Throughout cortical lamination process, Tbr1, in a gradient overlap with other transcription factors, promotes the identity of lower layer corticothalamic projecting neurons (Bedogni et al., 2010; McKenna et al., 2011; Kwan et al., 2012). In addition, Tbr1 was shown to interact with Foxp2 through its T-box domain (Sakai et al., 2011; Deriziotis et al., 2014)

Mature layer 6 contains a morphologically heterogeneous and functionally less well characterized population of glutamatergic neurons, which can be roughly divided into 3 classes: primary sensory corticothalamic cells (PCT), non-primary corticothalamic cells (NCT) and other neurons of variable morphology and projection pattern. PCT interconnect with layer 4 and sample the information arriving from primary sensory thalamic nuclei through their dendritic branches in layers 4 and 6; NCT send efferents to association ('secondary') thalamus, participate in reciprocal circuits with layer 5 and also send long lateral projections within layer 6 and sometimes to upper layers. Both cell types furthermore receive inputs and send outputs to various cortical and sub-cortical areas, such as claustrum or superior colliculus, which indicates further heterogeneity within the two subclasses and suggests a broader spectrum of projection areas and corresponding

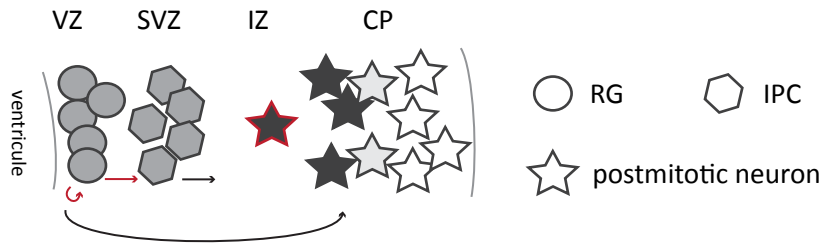
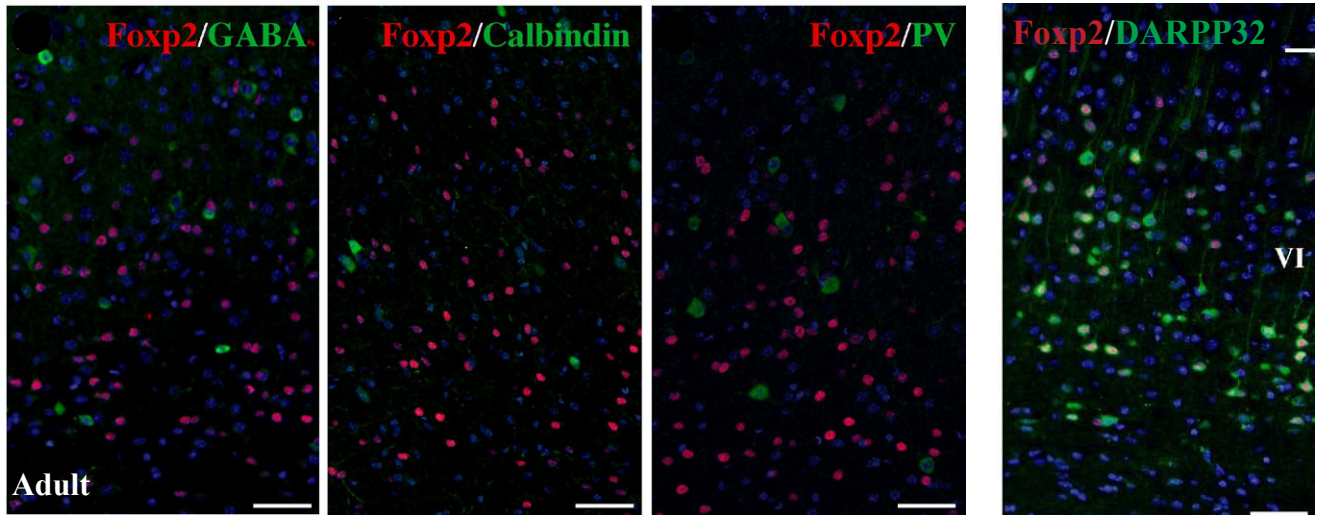


Figure 8. *Foxp2* in cortical neurogenesis. *Foxp2* expression levels in different types of cells are reflected by shades of grey. Red color marks the processes shown to be altered in *Foxp2* misexpression studies. IZ-intermediate zone, where neuronal migration occurs; SVZ, subventricular zone; VZ, ventricular zone; CP-cortical plate; RG, radial glial progenitor; IPC, intermediate progenitor cells.

Foxp2 in interneurons



Foxp2 in dopaminoreceptive cells

Foxp2 in projection neurons

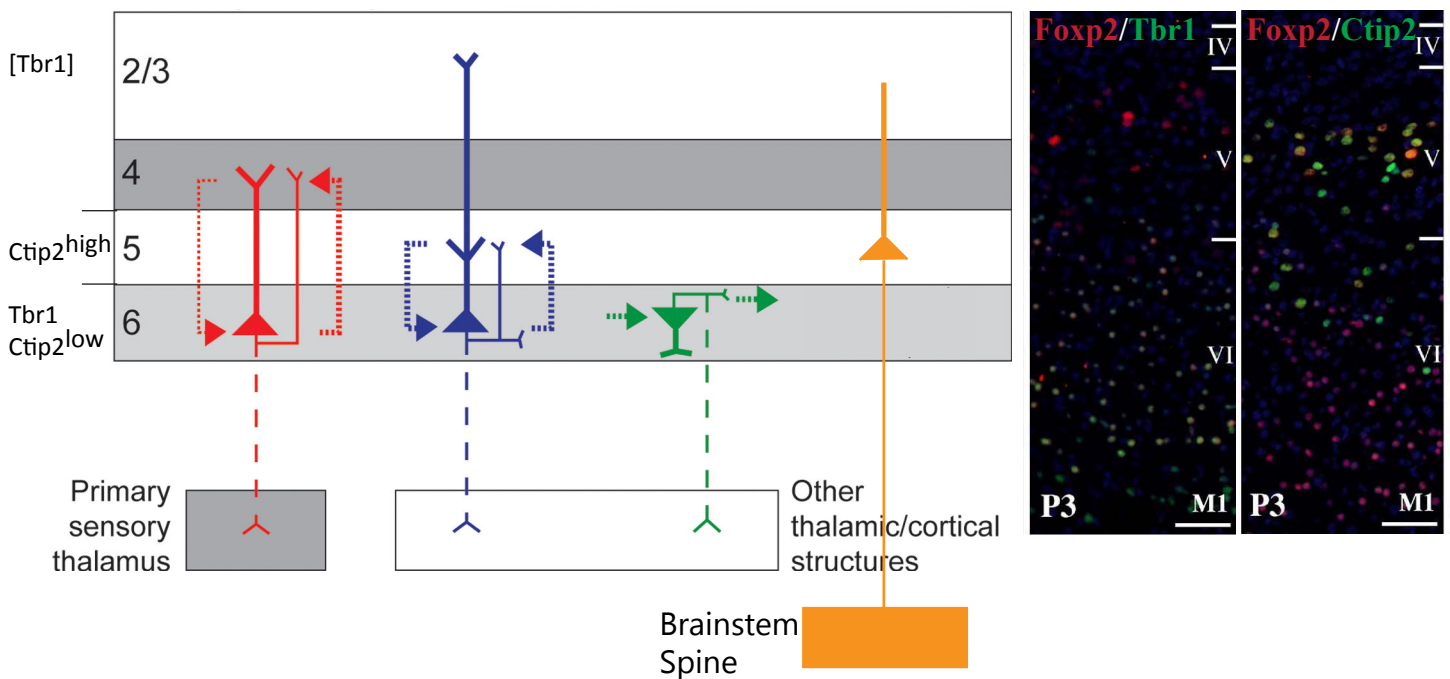


Figure 9. *Foxp2* is expressed in the deep layers of the cortex. *Tbr1* and *Ctip2*^{low} expression in the layer 6 marks the neurons of corticothalamic projection fate, *Ctip2* is highly expressed in layer 5 subcerebral projection neurons, *Tbr1* in upper cortical layers plays a role in callosal connectivity (McKenna et al., 2011; Srinivasan et al., 2012). PV, parvalbumin; P3, postnatal day 3; M1, primary motor cortex. Modified from Hisaoka et al. (2010) and Briggs (2010).

functional roles. The variable class of glutamatergic neurons is constituted by morphologically distinct cell types with upside-down, horizontal, bent-over apical morphologies that can project locally or to contralateral cortical areas, and non-pyramidal spiny excitatory neurons that can project to thalamus (Briggs, 2010; Thomson, 2010). Within this substantially heterogeneous population *Foxp2*⁺ is expressed in 63% of pyramidal cells (78% of *Foxp2*⁺ population), 60% of bipolar spiny neurons (10.5% of *Foxp2*⁺), 2% of inverted pyramidal neurons (5.3% of *Foxp2*⁺) and 2% of local circuit neurons of primary motor cortex (6.2% of *Foxp2*⁺); these proportions do not change in the cortex of *Foxp2*-Hum mice (Reimers-Kipping et al., 2011). The precise identity of *Foxp2*⁺ layer 6 neurons remains to be determined. While it is difficult to assume which exact circuitries *Foxp2* could be regulating, its pervasive expression across functionally diverse areas of neocortex suggests a critical role in sensory information integration with inputs from the central relay of thalamus and motor outputs from the basal ganglia and cerebellum (Campbell et al., 2009).

Layer 5 manifests sparse *Foxp2* expression in the rostral dorsomedial forebrain in premotor and medial motor cortices, and weaker expression in cingulate and prelimbic parts. In the caudal cortex primary somatosensory hind- and forelimb compartments express *Foxp2* as well (Campbell et al., 2009; Hisaoka et al., 2010).

In developing mouse cortex subcerebral (ex., corticobulbar or corticospinal) projection neurons of layer 5 are identified by high expression of the transcription factor *Ctip2* (Arlotta et al., 2005; McKenna et al., 2011); all *Foxp2*⁺ cells in postnatal animals express this ‘marker’ (Hisaoka et al., 2010). One particularly relevant circuit possibly related to *Foxp2* neurons in layer 5 involves corticobulbar projecting neurons of the mouse motor cortex. A subpopulation of these neurons is thought to form a motor-control output, connecting cortex and laryngeal muscles (**Fig. 4**). The existence of this connection together with singing-dependent activation of motor cortex (and anterior dorsal striatum) supports the hypothesis that mice may voluntarily modulate and acquire socially-relevant vocalizations (Arriaga et al., 2012). Whether these particular neurons also express *Foxp2* remains to be investigated.

Deep cortical layers have recently gained attention in light of functional genomics studies on ASD. Core ASD pathophysiological mechanisms have been suggested to reside within the cortex, and the major cellular processes involved are thought to be those of synaptic formation and function of neuronal projection, and, at molecular level, of transcription regulation (Gilman et al., 2011; Rubenstein, 2011; Voineagu et al., 2011; Ecker et al., 2012; Ben-David and Shifman, 2013). Recent studies found an enrichment of genetic variants associated with ASD in cortical projection neurons with one of the most important points of convergence in layers 5 and 6 (Parikshak et al., 2013; Willsey et al., 2013).

These results were further validated in the mouse. Existing collections of translation profiles of distinct mouse brain cell-types allowed Xu et al. (2014) to map ASD-linked genes against actively translated mRNAs in distinct cell types (cell-type-specific expression analysis, CSEA); this approach resulted in specific enrichment within lower cortical layers and striatum. In addition, *de novo* Tbr1 mutations in sporadic autism were shown to disrupt the protein functions, among which its interaction with Foxp2. It has been therefore suggested that in areas of the brain with overlapping expression patterns, such as in glutamatergic layer 6 neurons, the Tbr1-Foxp2 interaction may result in coordinated regulation of common downstream targets, which possibly involve several ASD candidate genes (Sakai et al., 2011; Deriziotis et al., 2014).

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Several lines of investigation have linked FoxP2 to language and a variety of mental disorders. These findings reinforce the hypothesis that the development of intelligible vocal communication requires not only basic vocalization abilities but also the acquisition of social skills. The most prominent brain structures governing the process of language ontogenesis may lie within compartments of cerebral cortex and striatum, with the striatal components mediating the timing of motor skills performance and acquisition, and the cortex providing fine upstream control of the process. FOXP2-dependent social reinforcement of vocal communication performance may be mediated by its integration with dopaminergic pathways.

Context

Foxp2 has a crucial function in neural circuits mediating social motivation.

In our laboratory we have investigated the role of Foxp2 in ventral striatal medium spiny neurons which are part of forebrain circuits with crucial functions in motivation and reward processing (O'Doherty et al., 2004; Voorn et al., 2004; Yin and Knowlton, 2006). Dopaminergic (DA) neurons projecting from the midbrain play critical roles in these processes by modulating neuronal excitability and synaptic plasticity (Bromberg-Martin et al., 2010). In striatum and cortex nearly all Foxp2⁺ neurons co-express DARPP-32 and *Foxp2* heterozygous mutant mice show significantly increased striatal DA levels (Enard et al., 2009; Enard, 2011).

To assess the potential contribution of Foxp2 to DA related signaling and reward associated learning, we used the psychostimulant drug cocaine, which exerts potent locomotor-stimulating and rewarding effects by enhancing DA transmission. Following injection of a single dose of cocaine (15 mg/kg.), we found that drug-induced locomotor activity was significantly attenuated in *Foxp2R552H/+* and *Foxp2S321X/+* animals as compared to wild-type littermates, suggesting a decreased sensitivity to acute stimulation of DA receptors. Impairment in reward-associated learning by Foxp2 deficiency was evaluated in the conditioned place preference: we found that *Foxp2R552H/+* mice spent significantly less time than wild-type littermates in an environment previously associated with cocaine administration.

Using a well characterized signaling mechanism by which cocaine exerts its long-term behavioral effects specifically in D1R⁺ medium spiny neurons (extracellular-signal-regulated kinase, ERK) (Girault et al., 2007; Bertran-Gonzalez et al., 2008) we mapped specific underactivation to NuAc in *Foxp2R552H/+* mice after single cocaine injection.

DA neurons in the ventral tegmental area and their main target regions in the NuAc and prefrontal cortex, which normally mediate the associative learning of natural rewards, are considered to be major sites of action of cocaine (Luscher and Malenka, 2011). NuAc is particularly implicated in reward-associated learning. It is thought that phasic DA release in the NuAc assigns motivational value to biologically relevant stimuli resulting in the selection and production of adaptive motor output. We therefore explored the

contribution of Foxp2 expression in the NuAc to reward-associated learning. To this end we bilaterally deleted *Foxp2* in the NuAc in adult mice (*Foxp2*-NAcKO).

We found that unlike *Foxp2* heterozygous mutant animals, *Foxp2*-NAcKO mice showed normal motor learning behavior on the accelerating rotarod (**Fig. 10A**). However, similarly to *Foxp2* heterozygotes, the *Foxp2*-NAcKO displayed a significantly attenuated locomotor response following a single cocaine administration (**Fig. 10C**) as well as impaired cocaine conditioned place preference. These results suggested that Foxp2 in the NuAc of adult animals is important for cocaine-induced responses and behavioral adaptations.

Since a neuronal activity-dependent role for FoxP2 previously emerged from studies in songbirds (**Fig. 5**) (Teramitsu and White, 2006; Haesler et al., 2007), we tested whether a single cocaine injection altered Foxp2 expression in the mouse striatum. Using quantitative PCR and immunoblots on striatal tissue samples one hour following cocaine administration, we detected a downregulation of Foxp2 mRNA and protein levels in the NuAc but not in the dorso-lateral and dorso-medial striatum. This finding was supported by *in vitro* stimulation with glutamate and D1R agonist. These data suggest that Foxp2 functions as a DA and glutamate-regulated transcriptional modulator in NuAc in medium spiny neurons.

Synaptic transmission in NuAc D1R⁺ neurons in *Foxp2*R552H/+ mice is enhanced and occludes LTP, consistent with their deficits in reward-associated learning. This also agrees with *in vivo* multielectrode recordings in *Foxp2*R552H/+ mice which detected an abnormally high basal firing activity of medium spiny neurons possibly resulting from enhanced neuronal excitability (French et al., 2012). DA mediated reward signaling in the NuAc is also crucially involved in social behaviors such as attachment, social play or social interactions in diverse rodent species (Insel and Fernald, 2004).

We assessed *Foxp2*-NAcKO mice and control littermates in a three-chamber task which evaluates the interest for social approach and social novelty. In this test *Foxp2*-NAcKO mice interacted significantly less time with the novel mouse as compared to controls. In contrast, the control cohort of Foxp2 cortex-specific knockout mice (*Foxp2* cKO) did not show differences in the three-chamber social behavior test (**Fig. 10D**).

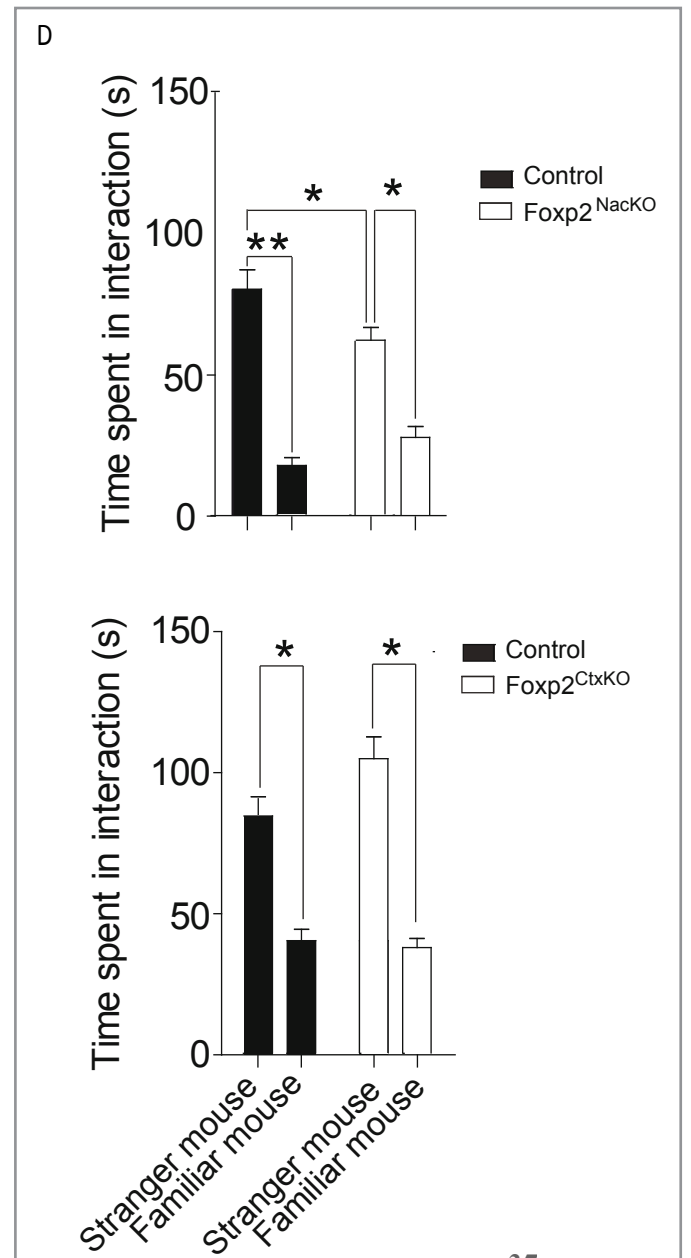
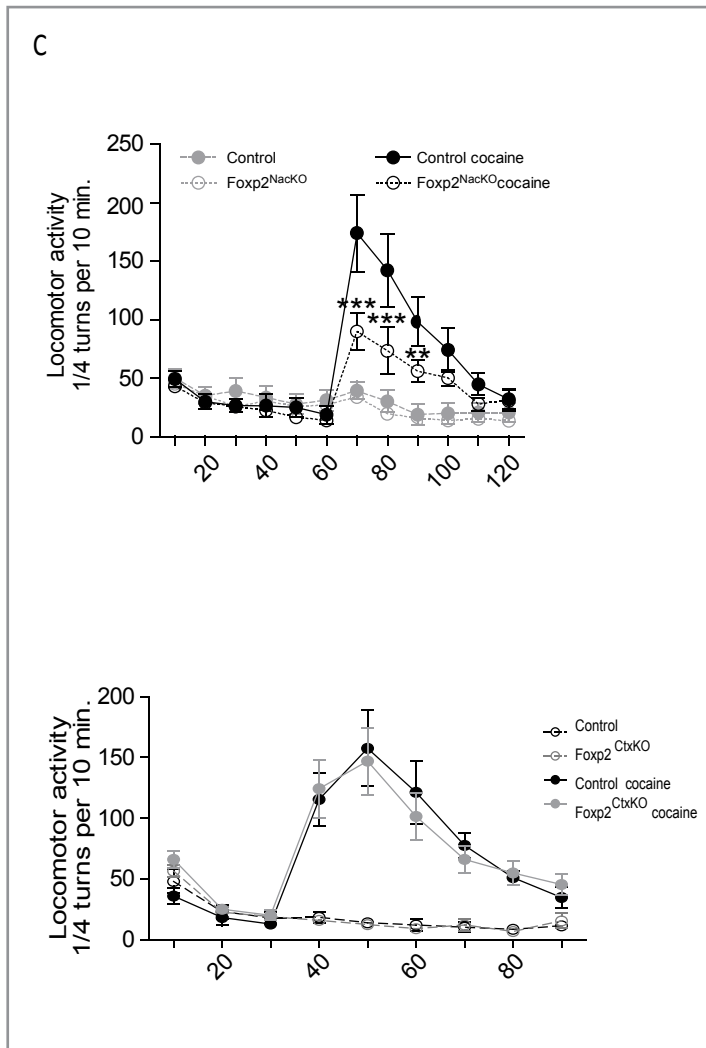
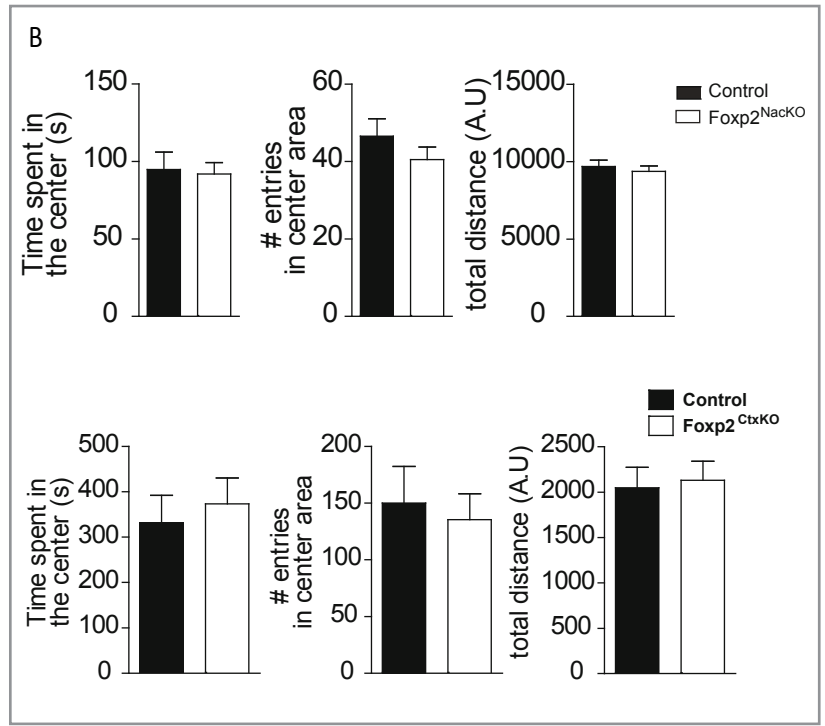
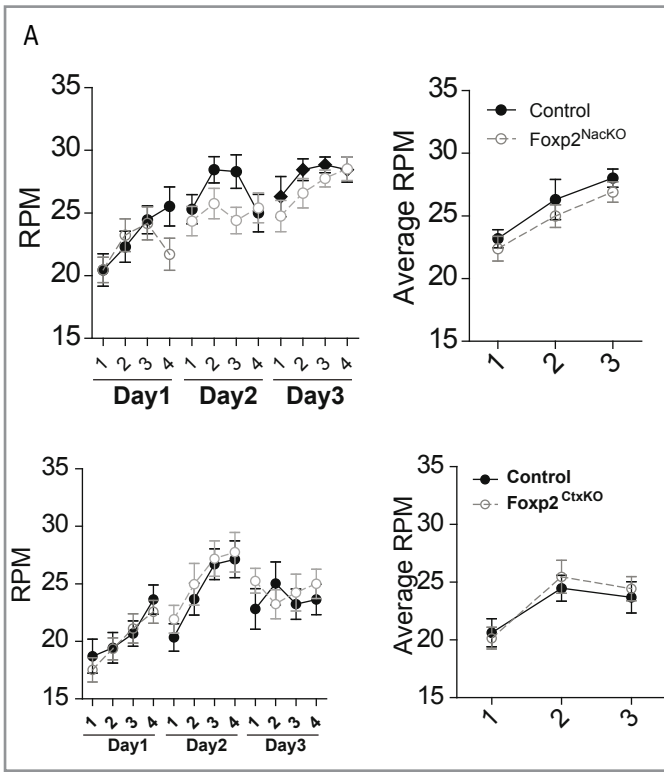


Figure 10. *Foxp2* in NuAc is involved in social behavior and reward processing. **A**, *Foxp2*-specific knockout mice in the NuAc (*Foxp2*^{NacKO}) and in the cortex (here *Foxp2*^{CtxKO} = cKO) do not exhibit motor learning deficits on the accelerated rotarod. Left panel: trial to trail performance, right panel: daily performance (RPM, rotations per minute). (*Foxp2*^{NacKO} n = 13 to 17 per group; RMANOVA). **B**, *Foxp2*^{NacKO} and cKO mice do not display deficits in the open-field paradigm, such as time spent in the central area, the number of entries and the distance traveled (*Foxp2*^{NacKO} n = 14 to 19 per group; two-tailed Student's t-test). **C**, Reduced acute cocaine (15 mg/kg) induced locomotor activity in *Foxp2*^{NacKO} mice (n = 15 to 19 per group, two-way repeated measures ANOVA, Bonferroni post hoc), but not in cKO. **D**, Three-chamber social interaction test: *Foxp2*^{NacKO} mice spent less time interacting with unfamiliar mice than controls (right panel) (n = 11 to 17 per group, two-way repeated measures ANOVA, Bonferroni post hoc), while cKO mice display no abnormalities in this test (n = 8 per group, two-way ANOVA, Bonferroni post hoc). (Mombereau et al., submitted)

To further assess social motivation in *Foxp2*-NAcKO mice we employed a social conditioned place preference test. We found that *Foxp2*-NAcKO mice did not display a preference for an environment previously associated with social interaction as compared to an environment in which they had been isolated. These deficits appear not due to increased anxiety or general impairments in novelty detection, since mutant mice performed normally in open-field tests (**Fig 10B**) or novel object recognition.

Taken together these results indicated that *Foxp2* in the NuAc supports socially motivated behaviors in adult mice. However *Foxp2* deficiency was not associated with general social deficits but rather might have impaired specific social behaviors.

In summary in this study we demonstrated that *Foxp2* in D1R⁺ medium spiny neurons in the NuAc is required for reward-associated synaptic plasticity and social behavior. These neurons are part of evolutionary highly conserved neuronal networks for social decision making and might have been recruited for speech and language development (O'Connell and Hofmann, 2012). Recent studies in autism spectrum disorder suggest that reward processing abnormalities specific to social stimuli in the first few years of life might impair the later development of complex social cognition and language (Chevallier et al., 2012). Thus *Foxp2* transcriptional regulation in neuronal circuits mediating reward signaling might significantly contribute to social brain development and function.

-Mombereau et al, submitted.

Aims

Converging evidence suggests that cortical FOXP2 may be crucial for vocal communication and generally cognitive and social traits in vertebrates. However, at the onset of this study, no functional characterization of FOXP2 role in the cortex has been carried out. Although recent investigations have shed light on *Foxp2* cellular functions in early cortical development, its role in postmitotic and mature cortical neurons remains to be elucidated.

The aim of this thesis was to obtain mechanistic insights into the role of *Foxp2* in the adult cortex at the behavioral, molecular and circuit level. Therefore, the following strategy was chosen:

Morphological and behavioural characterization of cortical *Foxp2* conditional knockout mice

- Generation of *Foxp2* cortex-specific knockout mice (*Foxp2*-cKO)
- Analysis of gross cortical morphology, projections and cytoarchitecture of adult animals
- Analysis of developmental milestones and adult basic behavior
- Investigation of the response of *Foxp2*-cKOs to dopamine and/or serotonin-system stimulations
- Screen for the social behavior abnormalities in a sensitive resident-intruder paradigm
- Analysis of the structure of adult vocalizations in the function of the variable social-context

Identification of cortical *Foxp2* transcriptional targets

- Molecular profiling of direct and indirect *Foxp2* targets in the whole cortex tissue and in the lower cortical projection neurons and of adult *Foxp2* heterozygous mice.
- Validation of relevant targets in *Foxp2*-cKO mice

Materials and Methods

Mice

All mouse lines were on C57BL/6 (J and NTac) background; for all the experiments only adult mice were used: age varied from 3 m.o. – 5 m.o. Animals were maintained under a 12h light/dark cycle. Food and water were supplied *ad libitum*. Experiments were performed in accordance with French (Ministère de l’Agriculture et de la Forêt, 87–848) and European Economic Community (EEC, 86–6091) guidelines for the care of laboratory animals.

Cortical *Foxp2* homozygous knockout (cKO) mice were generated by crossing *Foxp2^{lox/lox}* (French et al., 2007) and *Nex-Cre* transgenic (Goebbels et al., 2006) mice. In this model *NEX-Cre* is expressed in glutamatergic projection neurons of the dorsal telencephalon shortly before generation of the first postmitotic cortical neurons (Goebbels et al., 2006; Belvindrah et al., 2007) which leads to excision of *Foxp2* exons 12-14, encoding for the DNA-binding domain in both alleles (French et al., 2007). *NEX-Cre* expression is restricted exclusively to the CNS. Complete *Foxp2* cortical ablation was observed in both genders.

Animals for TRAP experiments were obtained by crossing *Ntsr1-eGFP-L10a* (Gong et al., 2010) with *Foxp2* heterozygous line *Foxp2^{S321X/+}* (Groszer et al., 2008). The *Foxp2^{S321X/+}* line carries a premature stop codon close to the human R328X nonsense mutation found in a second family segregating FOXP2-related speech and language deficits (MacDermot et al., 2005). *Foxp2^{S321X}* homozygous mice show reduced quantities of *Foxp2* messenger RNA (likely due to nonsense-mediated RNA decay), absence of *Foxp2* protein, and no detectable truncated product. Thus, S321X is effectively a null allele, implying a similar mechanism for the human R328X mutation (Groszer et al., 2008); here the line is designated as *Foxp2* heterozygous mutant: *Foxp2^{+/-}*. *Ntsr1-eGFP-L10a* is a transgenic line which carries eGFP fused with ribosomal protein L10a under control *Ntsr1* gene promoter. *Ntsr1⁺* cells constitute a subpopulation of cortico-thalamic projection neurons of the lower cortical layers, substantially overlapping with *Foxp2⁺*

neurons and allowing for cell-specific immunoprecipitation of translating polysomes in adult *Ntsr1-eGFP-L10a Foxp2^{+/-}* (BacTRAP) mice.

Histological analysis

Perfusion. Mice were rapidly anesthetized with pentobarbital and perfused transcardially with saline solution, followed by 4% (w/v) paraformaldehyde PFA in 0.1M PB, pH 7.5 (PB-PFA 4%). Brains were removed and postfixed overnight in the PB-PFA 4%, then cryoprotected in 10% sucrose-PB solution and fast-frozen in Isopentane, -50°C. Brains were cut in cryostat (CryoStar™ NX70, Thermo Scientific).

Nissl staining was performed with 0.025% Thionine dye on 30µm-thick sections.

Immunofluorescence protocol was the following: sections (10µm) were rinsed in PBS and subjected to antigen retrieval in 10mM citrate buffer with 0.05% Tween20, pH 6, for 10m in ~100°C. After cooling down sections were permeabilized in PBS containing 0.25% Triton (PBS-T) for 5m, then blocked in PBS-T with 0.2% Gelatin from porcine skin for 1h. Slices were incubated overnight at 4°C with primary antibodies: goat anti-Foxp2 N16 (Santa Cruz, 1:500); rabbit anti-Tbr1C (a gift from Robert F. Hevner, Washington University, Seattle, USA) 1:2500; rat anti-Ctip2 (Abcam, 1:500) in blocking solution. The next day, sections were washed 2 times in PBS-T, blocked for 5m and incubated for 2h at RT with species-specific fluorophore (Cy5, Cy3 or 488)-coupled secondary antibodies (Jackson Immunoresearch). Sections were washed 3 times in PBS-T, stained with Hoechst in PBS and mounted in Vectashield (Vector Laboratories).

Image analysis. Analysis of cytoarchitecture was performed on male adult mice.

Pictures were acquired with NanoZoomer scanner (Hamamatsu, Japan), at 20x resolution. After conversion into Tiff, 8-bit format with ImageJ plugin NDPI tools, pictures were analyzed using ICY software (de Chaumont et al., 2012b).

Brain regions were identified using mouse brain atlas (Paxinos and Franklin, 2007). In cortical thickness analysis sagittal sections levels corresponded to the following lateral coordinates: section level 1= 3.36mm, section level 2=1.56mm, section level 3=1.08mm (see *Results*, **Fig.16**). In cell counting analysis sagittal section levels corresponded to the following lateral coordinates: section level 1= 3.6mm, section level 2=1.32mm, section level 3=1.2mm. Coronal sections levels correspond to the following bregma coordinates:

section level 4=+0.26mm, section level 5=+0.14mm, section level 6=+0.62mm (*Results*, **Fig.17**).

Cortical thickness was measured using linear ROI, each ROI positioning was anchored to particular morphological features of the area (**Fig.17**). Cells were counted with a help of ICY Spot Detector plugin (Olivo-Marin, 2002), applying the following parameters: for Tbr1-scale 4 (13 pixels), sensitivity 160 and for Ctip2-scale 4 (13 pixels), sensitivity 90. These Spot Detector settings resulted in highly sensitive Tbr1⁺ cells detection, comprising almost all the visible spots (**Fig.17**, Tbr1 counting, top right panel); and Ctip2^{high} cell population detection - a marker of cortical sub-cerebral projecting neurons of layer 5 (McKenna et al., 2011) (**Fig.17**, Ctip2 counting, top right panel).

Tbr1-Foxp2 colocalization was estimated with a help of ICY Colocalizer protocol, using the following parameters: Colocalizer Max distance 8, Spot Detector Foxp2 - scale 4, sensitivity 250, Spot Detector Tbr1-scale 4, sensitivity 160. An example of ICY working protocol with the parameters for this experiment is shown in **Figure 11**. In the *Results* section **Figure 19** illustrates the efficiency with which this protocol recognizes and overlaps the signal.

Analysis of projections: brain stereotaxic injections

Labeling of anterograde projections from mPFC was accessed with AAV virus expressing cytoplasmic GFP. To restrict viral expression to cortical glutamatergic neurons I have chosen AAV-CAG-lox-STOP-lox-GFP virus and injected it either in *Nex-Cre; Foxp2^{lox/lox}* (cKO) or a WT littermate – *Nex-Cre; Foxp2^{wt}*, males. mPFC stereotaxic coordinates according to Paxinos and Franklin (2007) were the following: x=2.2, y= 0.3, z= 2.2.

Retrograde labeling from medial nucleus of the thalamus was performed with red retrograde beads. Stereotaxic coordinates were x=-1, y=0.3, z=3.3. Protocol was modified from Parnaudeau et al. (2013).

Animals were perfused 3 weeks after the surgery; brains were cut on coronal 60µm-thick sections and analyzed after immunofluorescence staining with Foxp2 antibody (see *Histological analysis* section).

Spot Detector block

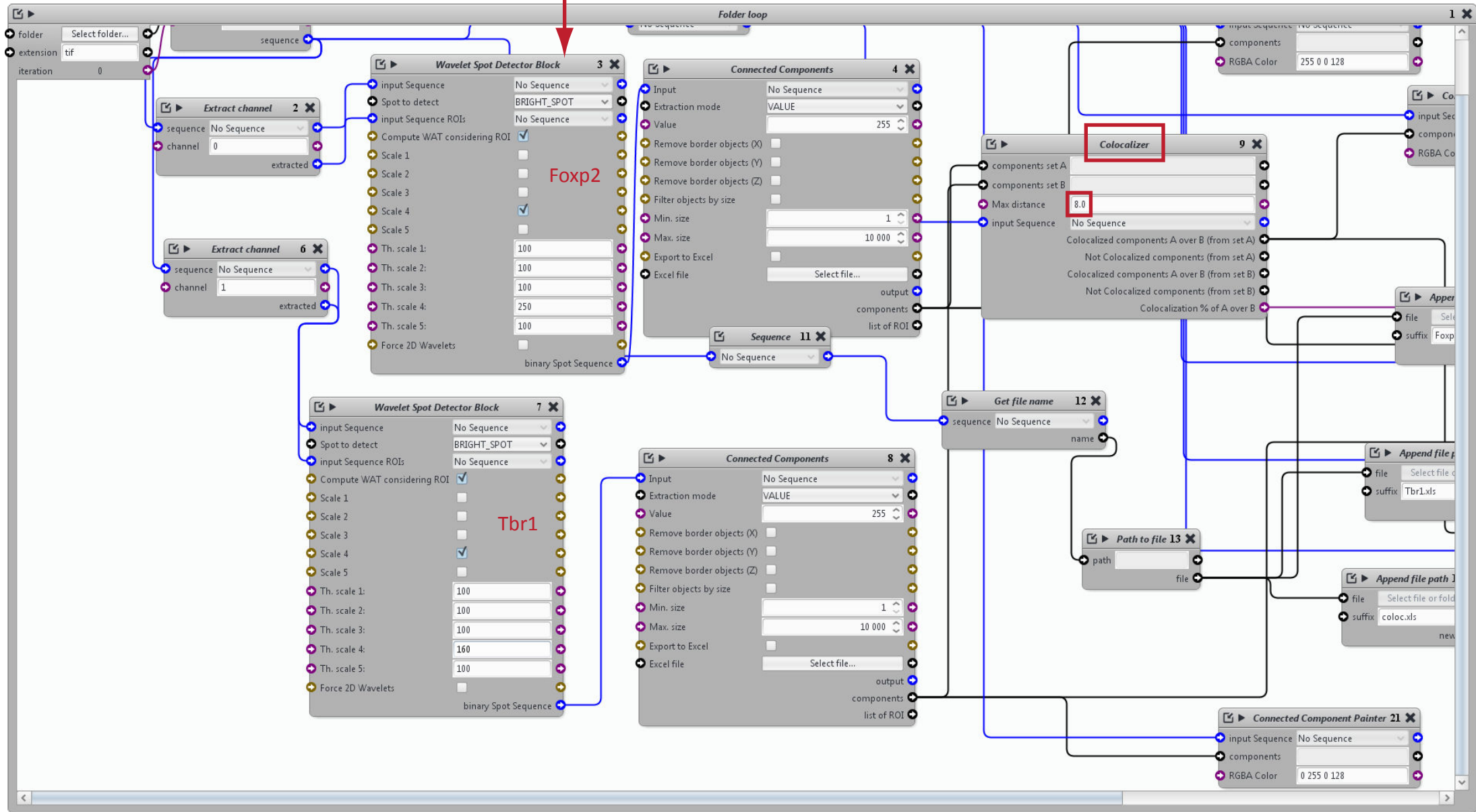


Figure 11. ICY Colocalizer protocol adapted for Tbr1-Foxp2 colocalization.

Expression analysis

Full cortical tissue was obtained by midline coronal segmentation on rostral and caudal parts of fresh brain tissue, followed by dissection of cortical hemispheres from diencephalon, basal ganglia structures and hippocampus. Brain punches preparations were previously described (Valjent et al., 2010). Punches were collected from adult male and female mice at the level of rostral cortex (frontal association - primary somatosensory cortex), caudal cortex (auditory cortex) and striatum. Lower cortical layers punches were collected from male mice using 0.75mm punch of Brain punch set (Stoelting Co, Chicago, USA) – rostral cortical areas included mPFC, motor and primary somatosensory parts (bregma +1.7 to -0.94mm), caudal areas included caudal motor and primary somatosensory parts, secondary somatosensory, parietal association, auditory and visual cortices (bregma -1.06 to -2.7mm).

RT-PCR and qPCR. Total RNA was extracted using TRIzol reagent (Ambion, Life technologies), cDNA was generated using random hexamers (Invitrogen, Life technologies) and Superscript III Reverse Transcriptase (Invitrogen, Life technologies) following manufacturer protocols. qPCR was performed on the Agilent Mx3005P real-time PCR machine by using SYBR green master mix (Applied Biosystems, ABI). HPRT1 was used as an endogenous control for normalization (Hickey et al., 2012). Relative gene expression was determined using comparative CT method as described (Livak and Schmittgen, 2001). Specific interexonic primers were designed using Primer3 (Rozen and Skaletsky, 2000) and the sequences (5'-3') were the following: Foxp2 forward TAGACCTCCCTTCACTTATGCAA, reverse TCCACACTGCTCCTTTAACATTT, Mint2 forward TGGCATCATTTCCAAGCTTTG, reverse CTTAGCAGCCTTCTGCATCC. Equal efficiency of target and endogenous reference primers were verified using standard curve where the difference between the absolute value of slopes between target amplification and endogenous reference amplification was <0.1. Specificity of DNA products was verified with dissociation curves, agarose gel electrophoresis and sequencing.

Immunoblotting. Lysate preparation from brain punches and western blot were performed according to previously described procedures (Valjent et al., 2010). Equal amounts of protein (50µg) were separated by SDS–polyacrylamide gel electrophoresis

before electrophoretic transfer onto nitrocellulose membranes (Hybond Pure, Amersham, Orsay, France) membranes. Membranes were incubated for 1h at RT in a blocking solution of TBS-T (0.01%) with 1 or 5% fat-free powdered milk. Membranes were then incubated overnight at 4°C with primary antibodies (goat anti-Foxp2 N16, Santa Cruz, 1:500; rabbit anti-Tbr1C 1:2500, R. Hevner gift; rabbit anti-Mint2 1:1000, Sigma; mouse anti-Actin 1:10000, Millipore). Secondary antibodies were IgG IRdye800CW-coupled or IgG IRdye700DX-coupled (Rockland Immunochemicals, Gilbertsville, PA). Fluorescence was analyzed at 680 and 800nm using the Odyssey infrared imager (Li-Cor, Lincoln, NE).

Quantitative blots were performed on 40µg of total protein in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, and 150 mM NaCl) with protease inhibitors (Complete protease inhibitors cocktail tablets, Roche Diagnostics), on PVDF membranes (Immobilon-P, Millipore). Proteins intensity was quantified using ICY ROI Statistics.

Behavioral tests

Tests were run on both genders; adult mice were 3-5 months old. All experiments, with exception of Open field, were performed during light cycle.

Neonatal behavior development was evaluated as previously described (Wu et al., 1997; Lim et al., 2008), and included the following measurements:

- Rooting (measurements started at P3). Measures tactile reflex and motor coordination. Filament of cotton was gently stroked 3 times along the side of the head of the pup. Pup responded by turning the head toward the filament.
- Forelimb grasp (P4). Measures muscular strength. The pup was held against a wire suspended over a cage with 5cm of shavings. If the pup fell immediately the test would be repeated once. Test was repeated daily until the pup could hold more than 1s for 2 consecutive days.
- Surface righting (P3). Measures body righting mechanisms, strength and coordination. Monitored: time to flip over to the abdomen with all 4 paws touching the surface, after 30s the test was terminated. Measured daily until pup did the task in less than 1s.

- Auditory startle (P7). Auditory reflex measure. Monitored: reaction on handclap at distance ~10cm. Mouse responded with quick involuntary jump. Repeated daily until the mouse responded correctly for 2 consecutive days.
- Activity: open field transversal (P8). Mouse was placed at the centre of 13cm field circle and time to move off the circle was measured. If the mouse did not move out of the circle in 30s, test would be terminated.
- Air righting (P8). The mouse was held upside down over a cage containing 5cm of shavings. After the mouse was released the response of landing right side up with all 4 feet was noted.
- Ear twitch (P7). Tactile reflex. The cotton tip of an applicator was pulled out and the end was twisted to form a fine filament. The filament was gently brushed against the tip of the ear 3 times. Mouse responded by fluttering the ear. Test was repeated daily until the mouse responded correctly for 2 consecutive days.

Startle response and pre-pulse inhibition were measured exactly as previously described (Spooren et al., 2004).

Open field and locomotor activity. The test was done during the first hours after the light turns off in the animal facility (8 PM) with at least one hour of habituation to the room. Mice were placed at the corner of a 40 x 40 x 30 cm open field (white Plexiglass on the sides and the floor) illuminated with 600 lux for 30 min. The total distance traveled in the whole arena, time spent in the center (20 x 20 cm) and velocities were measured using an automated videotracking system (Viewpoint, Lyon, France).

Elevated plus maze. A mouse was placed in the center area of the maze with its head directed toward a closed arm. Mice are allowed to move freely about the maze for 10 min. The distance traveled, the number of entries into each arm, the time spent in each arm, and the percent of entries into the open arms were measured using an automated video tracking system. The number of head-dips and rearings were scored manually. The protocol is based on Komada et al. (2008).

Accelerating rotarod. A computer-interfaced rotarod (LE8200, Bioseb, Chaville, France) was set to accelerate from 4 to 40 rpm over a 300s period. Mice were trained for three consecutive days, with one daily session consisting of four trials separated by 1h

resting periods. Trials ended when mice fell from the rod or after 300s. The highest speed reached (rpm) was analyzed.

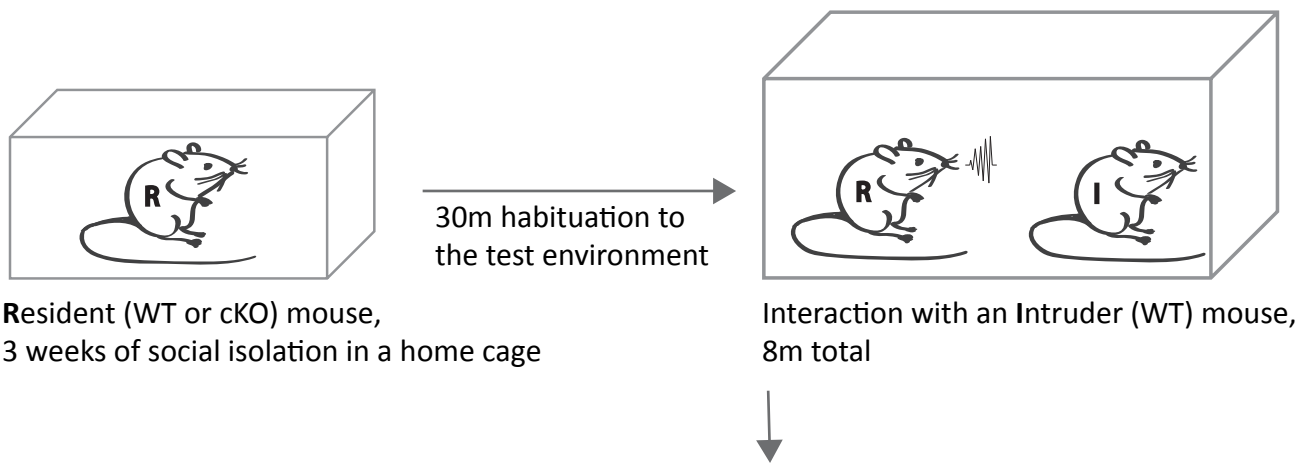
Cocaine-induced locomotor activity sensitization. Cocaine induced locomotor activity was automatically recorded in cylindrical boxes (Imetronics). Mice were habituated to injections and the test apparatus for 1.5h for three consecutive days before the experiment. During these sessions, mice were injected with three intraperitoneal saline injections before being placed in the apparatus as well as 30 and 90m afterwards. On day four, the first sensitization induction day, mice were injected with saline at the beginning of the session and received a single cocaine injection (15 or 20 mg/kg) 30 minutes later. Locomotor activity was recorded during the entire duration of the session (1.5h). Cocaine injections were repeated for 4-6 consecutive days for ‘sensitization induction’. After 5-7 days of withdrawal, when no procedures were applied to the animals, mice received the last ‘challenge’ cocaine injection. The protocols were based on numerous studies (Kalivas and Duffy, 1993; Williams and Steketee, 2005; Lhuillier et al., 2007; Barik et al., 2010).

Social interactions

Male-male interaction was assessed as described before (de Chaumont et al., 2012a) in a resident-intruder paradigm, where the tested mouse was a resident (or isolated host-mouse) and intruder (or social visitor) was a WT conspecific. Automated social behavior scoring was performed using ICY plugin Mice Profiler, on the 4 (2-6) minutes of 8m of total video - **Figure 12**. This work was performed in collaboration with Sylvie Granon, Université Paris-Sud 11, Orsay, France and Arnaud Cressant, Brain@vior SAS, Saint-Prest, France.

Manual blind scoring for paw control and index of aggressiveness was performed according to Coura et al (2013), on the total 8-minutes video.

Female-female interaction testing was performed according to D’Amato and Moles (2001): resident (test) females were single housed for 3 days before an experiment. On first and second days of separation animals were habituated to the test environment for 15 minutes; on the test day animals were habituated to the environment for 5 minutes before presentation of an intruder - a WT female. Interaction was performed in the resident home cage and recorded for 3 minutes.



1. USV analysis (Fig. 13, Fig. 26)

2. Analysis of 4 minutes of social interactions using automated screening approach of ICY Mouse Profiler:

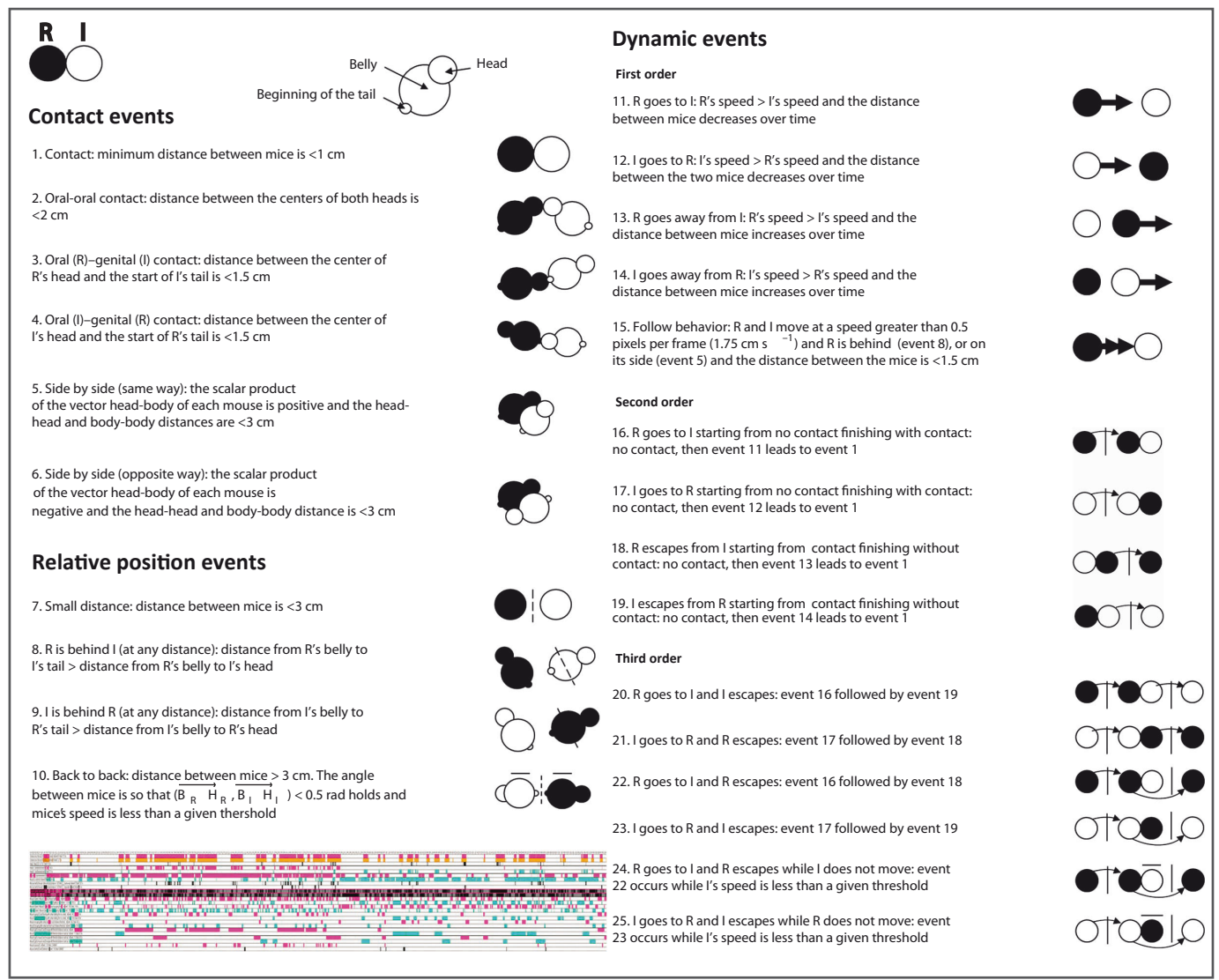


Figure 12. Experimental procedure for male-male social interaction analysis. After social isolation period, resident tested mouse (R) is exposed to a WT conspecific - an intruder (I), their interaction is recorded and screened with ICY Mouse Profiler software, followed by manually scoring blind for genotype for verification. The diagram of social interaction repertoire analyzed by ICY is adapted from de Chaumont (2012a), and on the left bottom corner depicts all the labeled events with two WT mice over the 8m. of experimentation.

Male-female interaction testing was based on Scattoni et al. (2011). Male resident tested mice were single housed for 5 days before an experiment. On third and fourth days of separation animals were habituated to the test environment for 15 minutes; on the test day animals were habituated to the environment for 5 minutes before presentation of an intruder - a WT female. Interaction was performed in the resident home cage and recorded for 5 minutes. Intruder females were superovulated by an injection of pregnant mares serum gonadotropin (PMSG) (5U per mouse), and 48h later, of Chorulon (5U per mouse), 12h later females were presented to the resident males. Hormones we purchased from Laboratoire INTERVET, France.

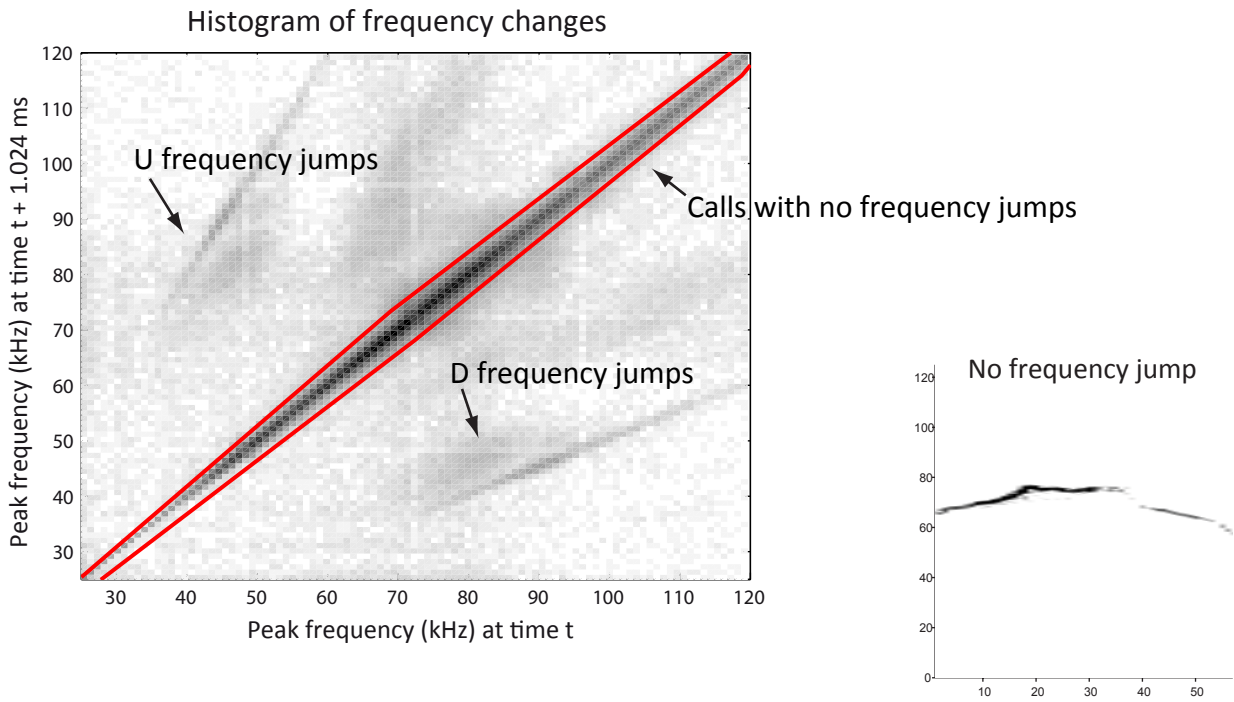
Manual scores for female-female and male-female interaction were obtained in collaboration with Susan Maloney and Joseph Dougherty, Washington University in St.Louis, MO, United States.

Ultrasonic vocalizations (USV)

USVs were recorded during social interaction experiments. A condenser ultrasound microphone was placed above the experimental chamber high enough for receiving angle of the microphone to cover the whole area of the test cage. USV were recorded under sampling frequency of 250 kHz;16-bit format. All recording hardwares and softwares were from Avisoft Bioacoustics, Germany.

Raw digital audio data was converted to voltage units given peak-to-peak Voltage of the UltraSoundGate hardware at 10 dB gain (± 0.25 mV). Spectrograms of recordings were prepared using a FFT size of 512 samples, with 50% overlap, multiplied by a Hamming window, which provides a temporal resolution of 1.024 milliseconds and a frequency resolution of 488.3 Hz. Two filter frames were used for subsequent analysis: bandpass filter 25-120 kHz (according to Holy and Guo (2005)) and 40 – 120 kHz (an optimal noise thresholding for the current dataset) – **Figure 13**. Locations of ultrasonic calls were detected using the method of Holy and Guo (2005), using the custom *whistimes()* function in MATLAB with the following parameters: minimum spectral purity: 0.2, maximum spectral discontinuity: 1.0, minimum duration: 5 ms, minimum intercall pause: 30 ms (**Fig. 26A** in *Results*).

25 -120 kHz band pass filtered data:



40 -120 kHz band pass filtered data:

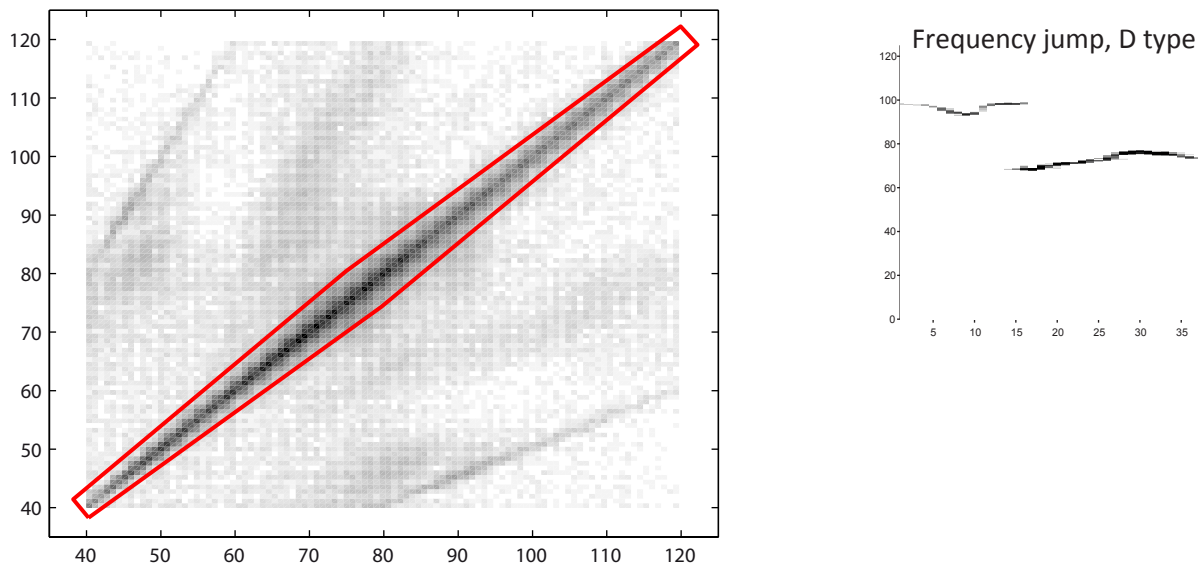


Figure 13. Classification of calls by frequency jumps according to Holy & Guo (2005). On the histograms the frequencies of pooled vocalization data are plotted at one time point versus the consecutive time point (+1.024ms), revealing distinct populations of syllables with and without frequency jumps. Syllables with frequency jumps can be classified by direction of frequency change: either Upward (U) or Downward (D). The filtering threshold of 40 kHz efficiently diminishes the background noise. On the right - example sonograms of the classified syllable type.

Spectral characteristics of the pitch were determined by finding the FFT (fast Fourier transform) frequency with peak power in each time window over a call, for all calls. From this can be determined the mean pitch and pitch range, as well as the presence of pitch jumps of differing sizes. **Figure 26B** in *Results* section shows an example ultrasonic call, and overlaid is the position of the pitch over time.

In order to segment the spectrogram into phrasal units, a range of inter-phrase gap sizes was tested. This range uses the peak of the distribution of pause times as an estimate of the true mean, and ranges cut-off values out to 5 standard deviations from this value (approximately 0.1–1.5s.). The actual values of the cutoffs differed slightly in each experiment (male-female, female-female, male-male) since the cut-offs are determined from the empirical distribution of pauses.

To understand group differences in usage of the frequency spectrum, power spectral density (PSD) was estimated from the FFT using the periodogram method. Periodograms were computed as $(|X(k)|^2)/(N \cdot F_s)$ where the scaling $1/(N \cdot F_s)$ (N =FFT size, F_s = sampling rate) ensures conservation of power by Parseval's theorem ($\sum x(t)^2 \Delta t = 1/(N \cdot F_s) (\sum_k |X(k)|^2)$, where $\Delta t = 1/F_s$, and k is frequency indexed from $0:N-1$). Two-sided periodograms (i.e., including negative frequencies) were converted to one-sided by doubling the values for frequencies with negative equivalents, resulting in a real-valued spectrum for $N/2+1$ frequencies in standard units of $V^2 \text{Hz}^{-1}$. Periodograms were time averaged within each call and over all calls for each animal. **Figure 26C** in *Results* section shows the estimated PSD over the population of males, recorded in male-female interaction experiment.

USV Statistical analysis

For comparisons between the groups means of single variables (fractional calls comparisons, mean and range of the pitch, phrase pause length, correlations between pauses and duration times, a chi-square statistic for Markov transition probabilities) simple t-tests were employed.

Comparison of averaged power spectra (represented as estimated power spectral densities, PSD in units of $\text{Amplitude}^2 \text{Hz}^{-1}$) were performed using a bootstrapping procedure, with the test statistic in question being the Kolmogorov-Smirnov D statistic, which is the maximum magnitude of difference between two normalized cumulative

distributions. The test and null distributions were compared by three possible decision criteria: a) if there is no overlap between the confidence intervals of D_{null} and D_{test} , reject the null hypothesis that the spectra come from the same underlying population of spectra (most conservative). b) allow some overlap between the intervals, and compute a probability of overlap as the number of bootstrap samples that occur in the region of overlap divided by the total, if $p_{\text{overlap}} < 0.05$, reject the null hypothesis c) compute the probability of observing the mean of D_{test} or greater in the D_{null} distribution - this procedure is analogous to computing D directly from their means instead of bootstrapping the test samples (least conservative).

USV analysis was performed in collaboration with Michael Rieger and Joseph Dougherty, Washington University in St. Louis, MO, United States.

Cell type-specific mRNA purification by translating ribosome affinity purification (BacTRAP)

Cell type specific polysomal purification was performed in BAC-transgenic mice carrying ribosomal protein L10 fused to eGFP under cell-type specific promoter. eGFP tagged ribosomes are affinity purified with eGFP antibodies. Thus, if upon tissue harvest ribosomes are maintained on the mRNAs that they are translating, purification of the cell-type-specific tagged ribosomes also yields cell-type-specific translated mRNAs (Heiman et al., 2014)-**Figure 14**.

Mice (males, 3 months old) were decapitated and the fresh brains were immediately processed for the BacTRAP experiments. BacTRAP protocol was based on Heiman et al. (2014), with slight modifications:

Both cortical hemispheres from one animal were dissected from diencephalon and basal ganglia structures, hippocampus and a tip of a rostral cortex, and processed in 2mL of tissue-lysis buffer. 30 μ l of the supernatant (S20) was collected as an Input sample immediately before immunopurification (IP). Affinity matrix was prepared with Dynabeads Protein G (Life Technologies, Novex) - 50 μ l per sample, and anti-GFP rabbit serum (Life Technologies, Molecular Probes) -10 μ l per sample, PBS washing and blocking steps were omitted and beads were washed exclusively in a low-salt buffer. IP

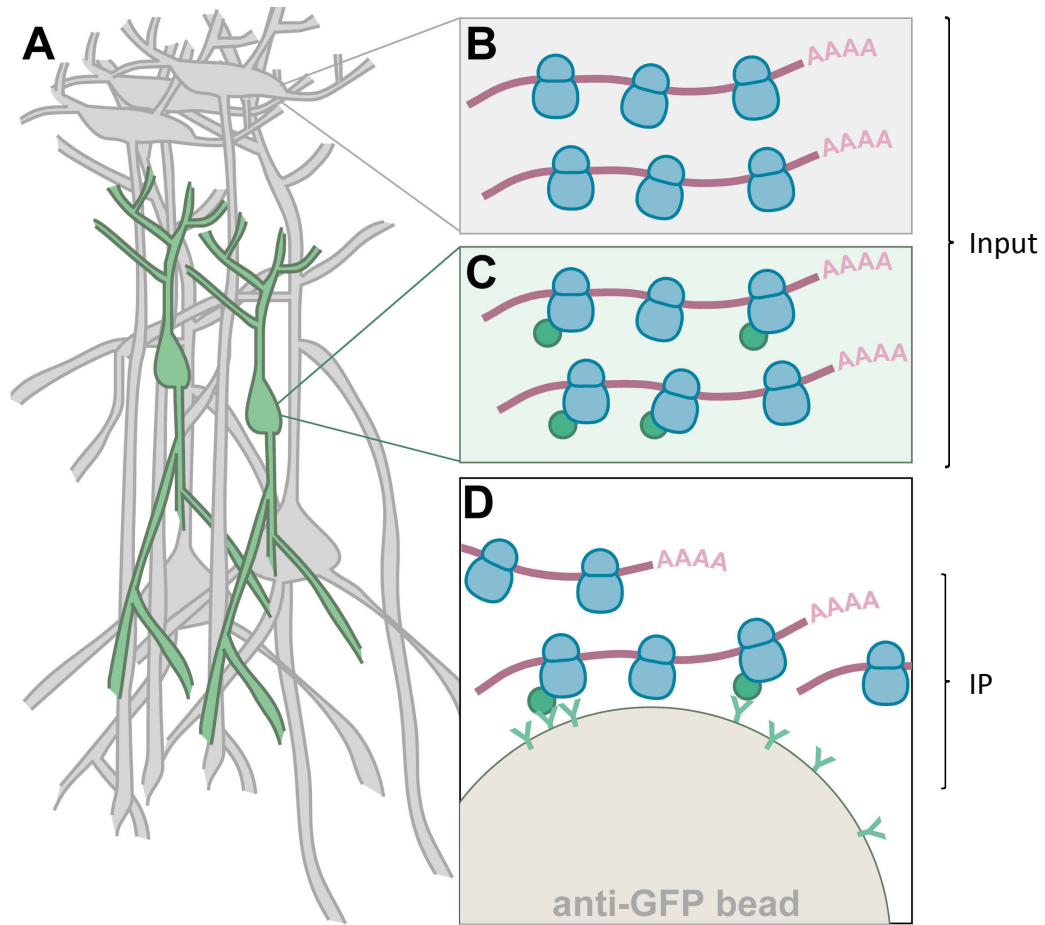


Figure 14. The translating ribosome affinity purification (TRAP). **A**, The cell type of interest - $Ntsr1^+$ neurons of the cortex in this study - is expressing the eGFP-L10 transgene. **B**, Translating polysomes from non-targeted cells do not have an eGFP tag on the ribosomes, while those originating from $Ntsr1^+$ cells do (**C**). Lysis of all cells releases both tagged and non-tagged polysomes, and only the tagged ones are captured during immunoprecipitation (IP) – **D**. Subsequent mRNA analysis from the IP fraction allows analyses of the actively translated pool of mRNA in $Ntsr1^+$ cells, while RNA purification from the Input fraction represents the whole transcriptome of the harvested tissue (cortex). Adapted from Heiman et al. (2014).

lasted for 4-6h, on ice. Buffers composition differed from Heiman et al. in concentrations of HEPES-KOH (10mM) and MgCl₂ (5mM). RNA quantity was assessed with Quant-iT RNA Assay kit (Life Technologies, Invitrogen). RNA yield for 1 animal varied between 1.2-5.7 ng, RNA quality was assessed using Bioanalyzer, the RIN \geq 7.8.

RT-qPCR analysis was performed as in *Expression analysis* section. Primers for Ntsr1 were used from PrimerBank (ID: 9055296a1), Cux1 primer sequences were: forward CTCGGCAGGTCAAAGAGAAG, reverse AGTTTGCTCCATGGTTTTGG.

Library preparation for RNA sequencing was performed using the Smart-seq2 protocol (Picelli et al., 2014). Sequencing was done on an Illumina HiSeq1500, using the Rapid Mode. The mapped reads were counted against an adapted gtf (version GRCm38.74). Only reads that mapped to a single gene were counted.

Statistical analysis was performed in R (mainly DESeq2, Bioconductor). The two major pairwise comparisons, Input control against IP and wildtype against heterozygous, led to similar results (i.e. approximately the same number of significant calls). Descriptive analysis included principal component analysis and sample-wise clustering based on normalized counts. The samples could be clearly and correctly separated by RNA source. Likelihood ratio test was used to determine the significance of a genotype factor in the model and a full model (i.e. with control for RNA source) was fitted against the count data. The p-value was adjusted for multiple testing (Benjamini--Hochberg FDR correction).

Library preparation, sequencing and statistical analysis were performed in collaboration with Christoph Ziegenhain, Beate Vieth and Wolfgang Enard at Ludwig Maximilians University, Munich, Germany.

Results

1. Generation and characterization of *Foxp2* cortex-specific homozygous knockout mice

Foxp2 deletion in the developing and mature postmitotic cortical neurons was obtained through generation of *Nes-Cre; Foxp2^{lox/lox}* (cKO) mice (**Fig.15** and *Materials and Methods*).

1.1. Cortical *Foxp2* ablation does not affect gross cortical morphology

In order to analyze the morphology of the cortex in adult cKO mice, I first used thionine staining (Nissl staining) on sagittal brain sections. I evaluated individual cortical layers based on differences in cell size and packing density and measured cortical thickness in 17 regions. These analyses did not reveal major abnormalities, suggesting that cKO mice do not show gross morphological alterations (**Fig.16**). Therefore I sought to examine cortical morphology in more detail, studying specific markers of cortical projection neurons.

In the cortex *Foxp2* is expressed in lower cortical layers, prominently in layer 6. Here it is co-expressed with *Tbr1*, a transcription factor which identifies cortico-thalamic projection fate of lower layer neurons; and in prefrontal, motor and somatosensory areas of layer 5, coexpressing *Ctip2^{high}*, a marker of subcerebral projection neurons (Hisaoka et al., 2010; McKenna et al., 2011). Cytoarchitecture of layer 5 and layer 6 was not altered upon *Foxp2* cortical deletion (**Fig.18, Fig.20**).

Tbr1 is a transcription factor, which regulates regional and laminar identity of mature cortical projection neurons (Bedogni et al., 2010; Zeisel et al., 2015) and physically interacts with *Foxp2* (Deriziotis et al., 2014). In order to know if *Foxp2* absence may directly influence the pool of *Tbr1⁺* cells, I counted the number of *Tbr1⁺* cells in layer 6 of cKO animals in different cortical areas (**Fig. 17**). None of the examined areas showed difference between cKO and WT littermates upon comparison of raw cells numbers or cell density estimate per area (**Fig. 18**). These results are supported by western blot

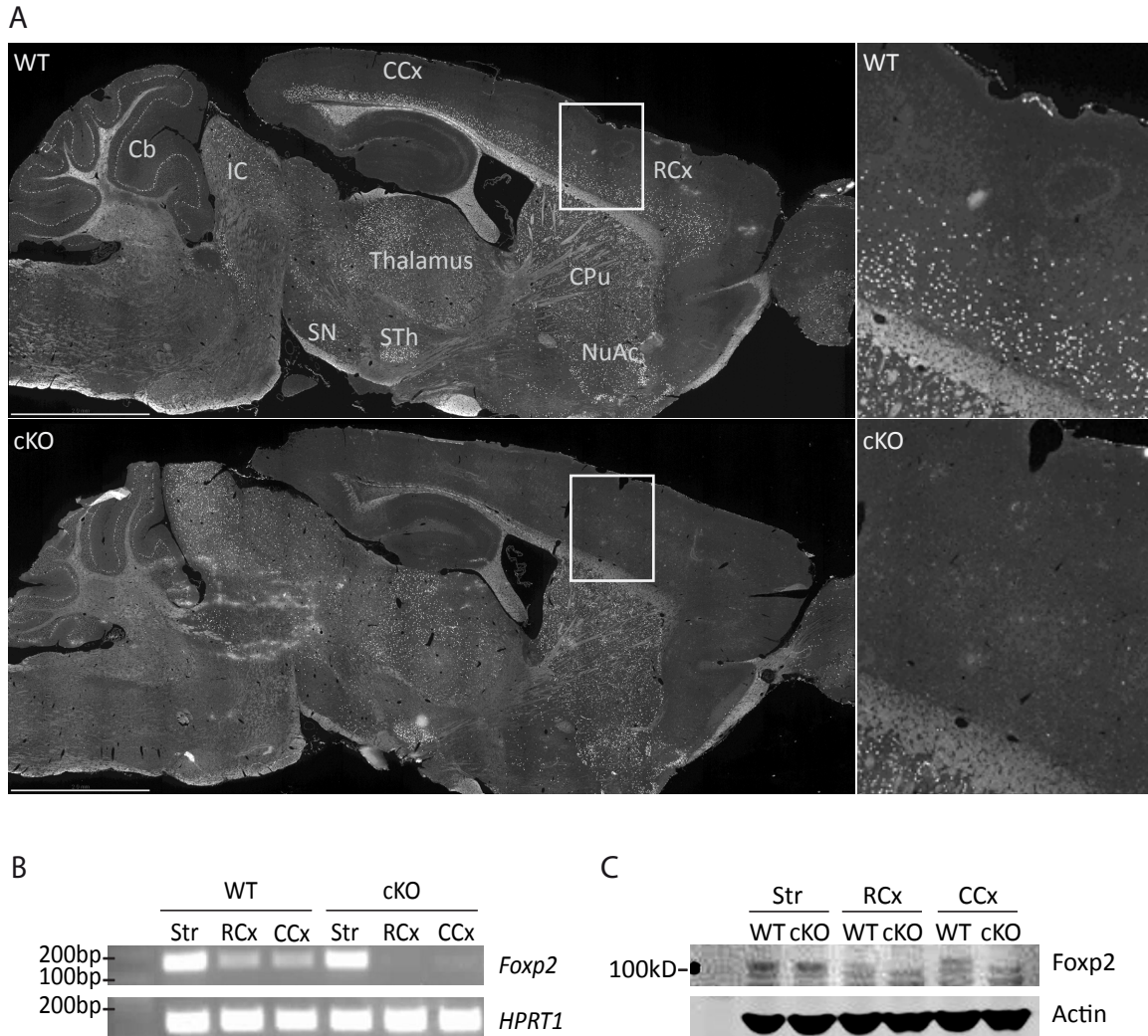


Figure 15. Cortex-specific *Foxp2* deletion in the *Nex-Cre; Foxp2^{lox/lox}* mouse (cKO) line. **(A)** Representative immunohistochemical images, scale bars 2mm. **(B)** Reverse transcription PCR and **(C)** western blot on punches collected from striatum (Str), rostral cortex (RCx) and caudal cortex (CCx). Cb, cerebellum; CPU (Str), caudate putamen; IC, inferior colliculus; SN, substantia nigra; STh, subthalamic nucleus; NuAc, nucleus accumbens.

analysis of lower cortical layers punches, where no difference in Tbr1 protein intensity was observed between the genotypes (caudal cortex, n=7, two-tailed t-test: $t(12)=0.2594$, $p=0.7997$).

Tbr1 is increasingly expressed in a caudal-rostral gradient with highest expression in prefrontal cortical areas. Therefore, in order to evaluate whether all Foxp2⁺ cells of layer 6 adapt the fate of cortico-thalamic projection Tbr1⁺ neurons in the adult animals, I have used an unbiased automatic quantification method. When quantifying cellular colocalization of these transcription factors, I found that only ~70% of Foxp2-positive neurons in layer 6 expressed Tbr1, and ~55% of Tbr1⁺ cells were Foxp2⁺ (**Fig. 19**). Upon evaluation of each cortical area examined (**Fig. 17**), it appeared that none of them showed 100% overlap of coexpression, as opposed to previous reports (Hisaoka et al., 2010). These results suggest that Tbr1⁺/Foxp2⁺ double positive cells in the adult cortex are a subpopulation within the heterogeneous layer 6. In addition to cortico-thalamic neurons, some Foxp2⁺ neurons may comprise cortico-cortical projection neuronal populations, suggesting a partial separation of Tbr1⁺ and Foxp2⁺ cortical circuits.

Foxp2 expression in a subpopulation of layer 5 pyramidal projection neurons has been identified in the medial prefrontal, motor and primary somatosensory cortical areas (Campbell et al., 2009; Hisaoka et al., 2010). Layer 5 pyramidal projections in these areas have been recently implicated in fundamental social behaviors. In the medial prefrontal cortex layer 5 neurons have been directly involved in neuronal circuits mediating social rank among male mice (Wang et al., 2011). In the primary motor cortex, subpopulations of layer 5 neurons are activated during courtship song production and establish monosynaptic projections to brainstem laryngeal motor neurons in the nucleus ambiguus. Such direct projections between motor cortex and laryngeal brainstem nuclei are thought to present a vocal control circuit specific to species with vocal learning (Arriaga et al., 2012) (**Fig.4**). The presence of such vocal control circuit led to the suggestions, that mice are able to modify their communicative vocalizations (USV), similar to other vocal learning species such as birds and humans (Arriaga and Jarvis, 2013).

In light of USV modulation impairments of *Foxp2* cKO which will be discussed below in section 1.6, and given the possibility that these layer 5 motor cortex neurons could

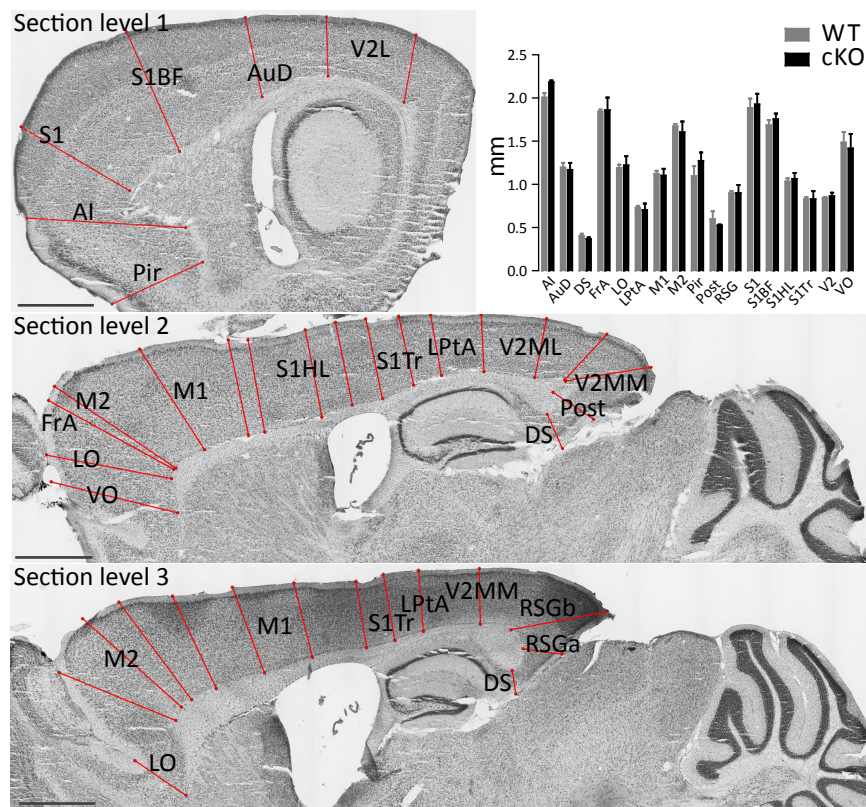
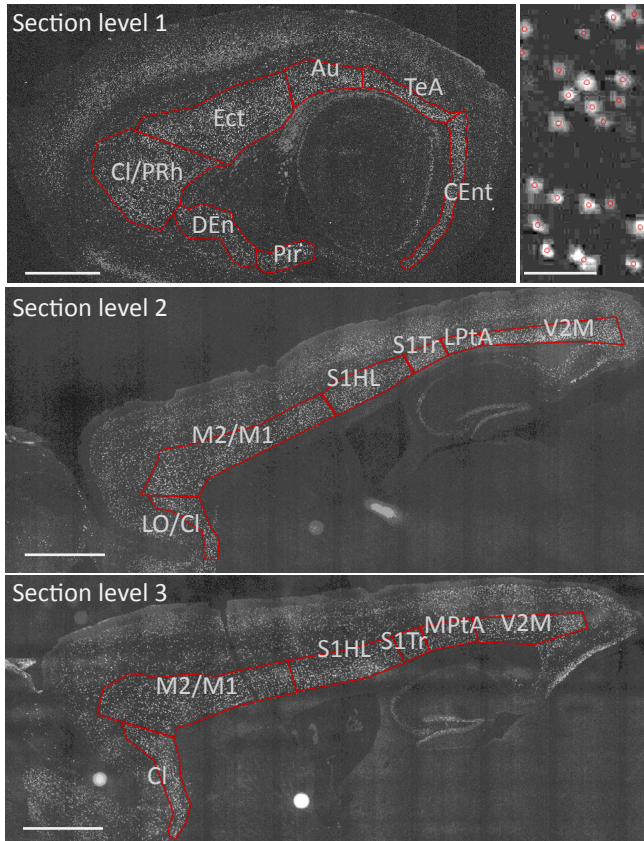
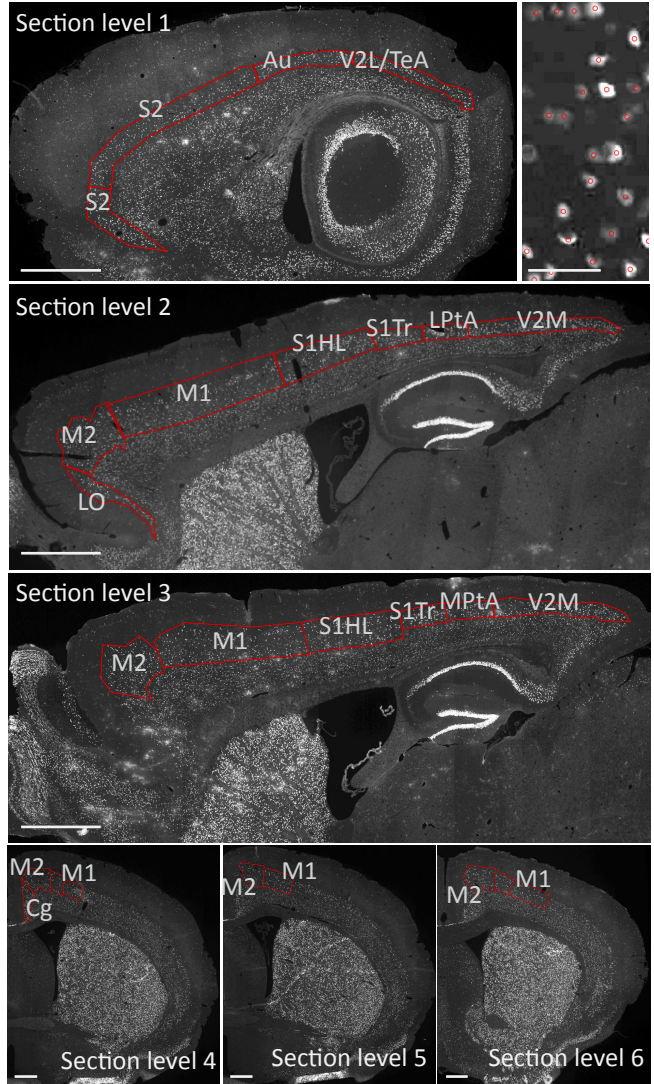


Figure 16. Cortical thickness measurements, Nissl staining. Measurements are performed on 3 different sagittal sectioning levels, red lines represent ruler (ROI) positions, scale bars 1mm. Brain areas determination and abbreviations are taken from the ‘Mouse Brain Atlas’ (Franklin and Paxinos, 2007). N=3-4, 2 way ANOVA: genotype $F(1,80)=0.42$. n.s., data expressed as mean +SEM.

Tbr1 counting



Ctip2 counting



Coronal

Figure 17. Cells counting. Tbr1⁺ counting is performed in layer 6 on 3 different sagittal sectioning levels; Ctip2⁺ counting is performed in layer 5 on 3 different sagittal (section levels 1-3) and coronal (section levels 4-6) sectioning levels. Red polygons define counting areas, scale bars 1mm. Top right panel of each scheme depicts cell detection efficiency by ICY Spot Detector, scale bar 50μm.

Tbr1

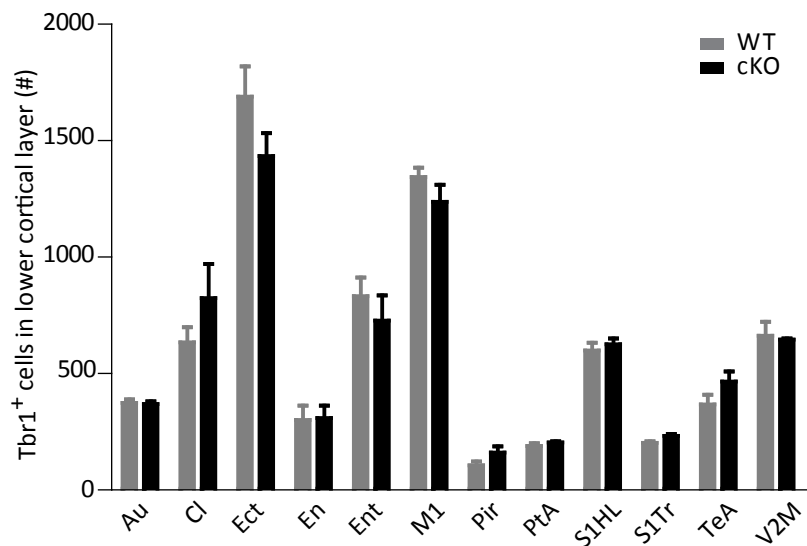
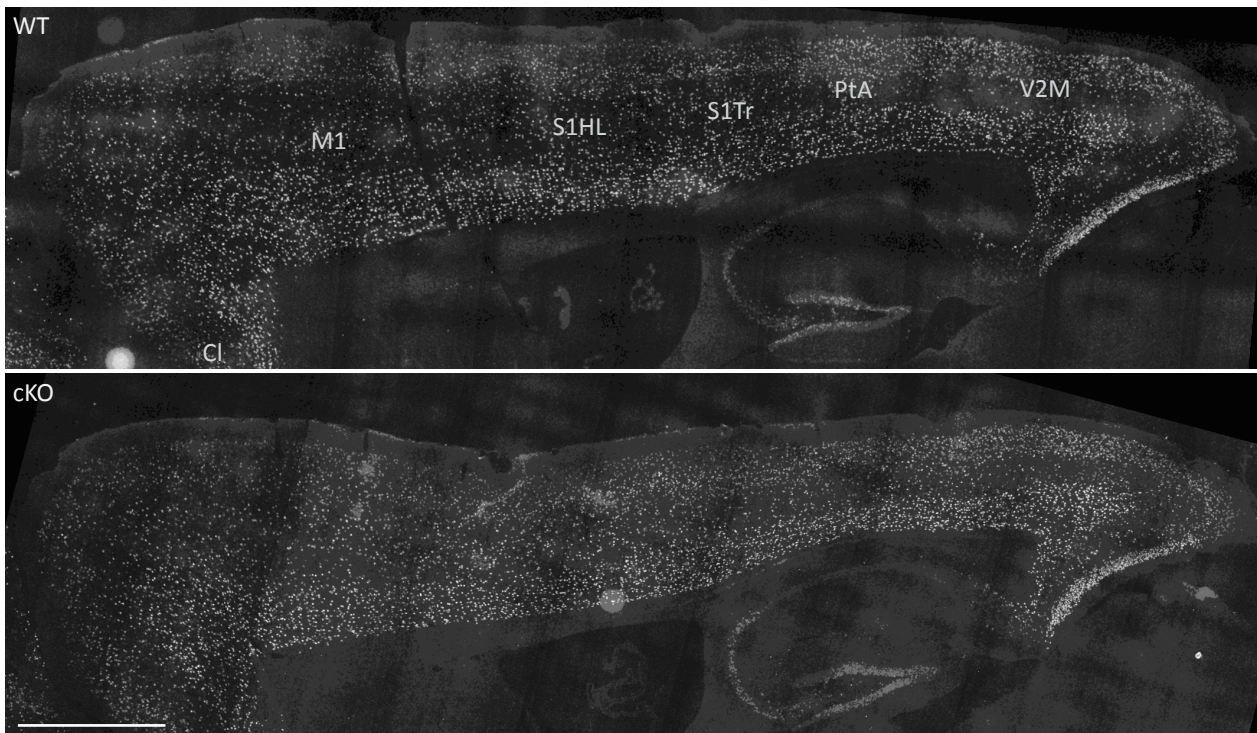
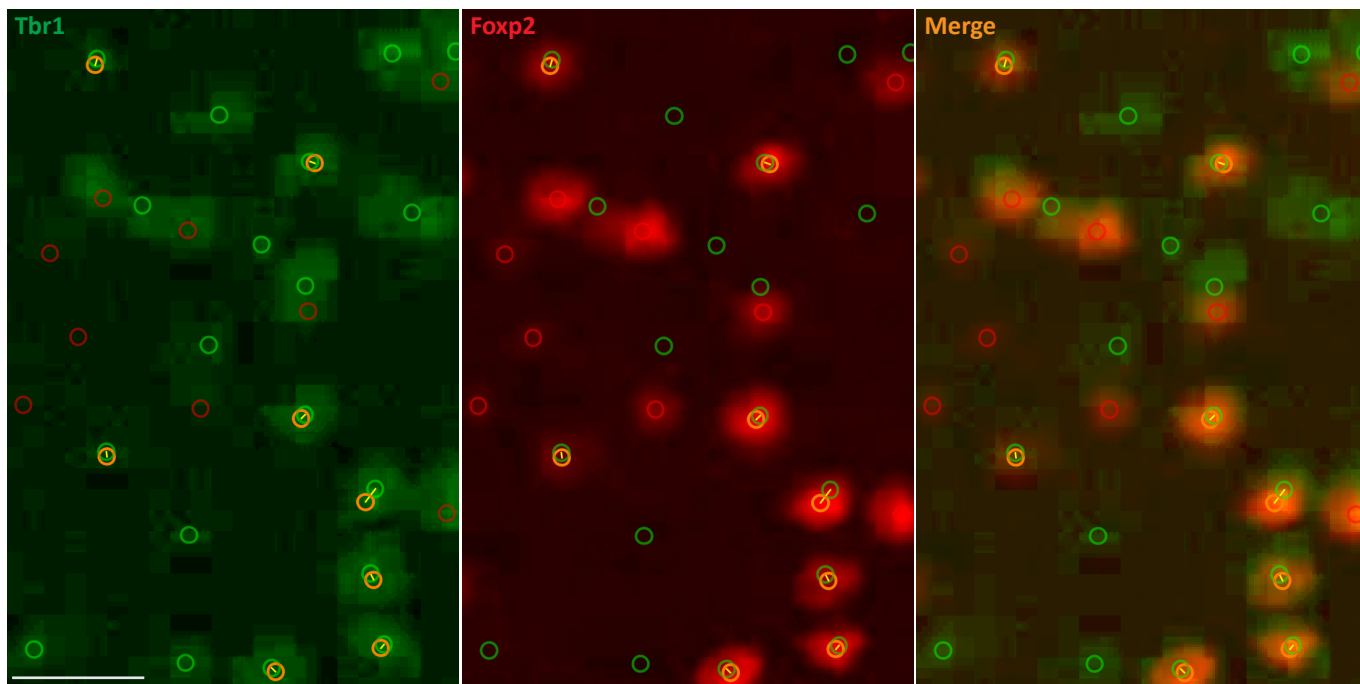
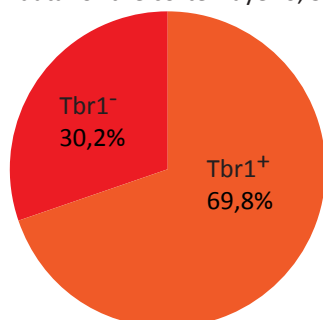


Figure 18. Tbr1 immunostaining and quantification. Quantification is performed in layer 6 on 3 different sagittal sectioning levels (see *Materials and Methods*), $n=4-3$ (exception Pir $n=2-4$), 2 way RMANOVA: genotype $F(1,50)=1.63$, n.s. Data shown are mean +SEM. Scale bars 2mm. Identification of brain areas and corresponding abbreviations according to the ‘Mouse Brain Atlas’ (Franklin and Paxinos, 2007).



Foxp2⁺ population:
colocalization with Tbr1,
mean data for the cortex layer 6, SEM \pm 3,2%



Tbr1⁺ population:
colocalization with Foxp2,
mean data for the cortex layer 6, SEM \pm 2,2%

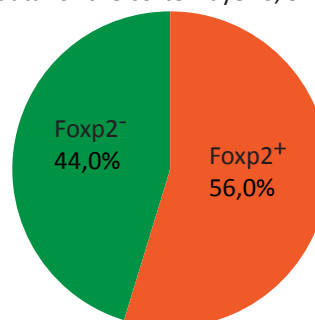


Figure 19. Tbr1 and Foxp2 colocalization in the cortical layer 6. Upper panel illustrates the precision level of with which ICY Colocalization protocol recognizes and overlaps single cells. Scale bar 50 μ m. Quantification is performed in 3 different sagittal sectioning levels of WT animals (**Fig. 17**), n=4.

Ctip2

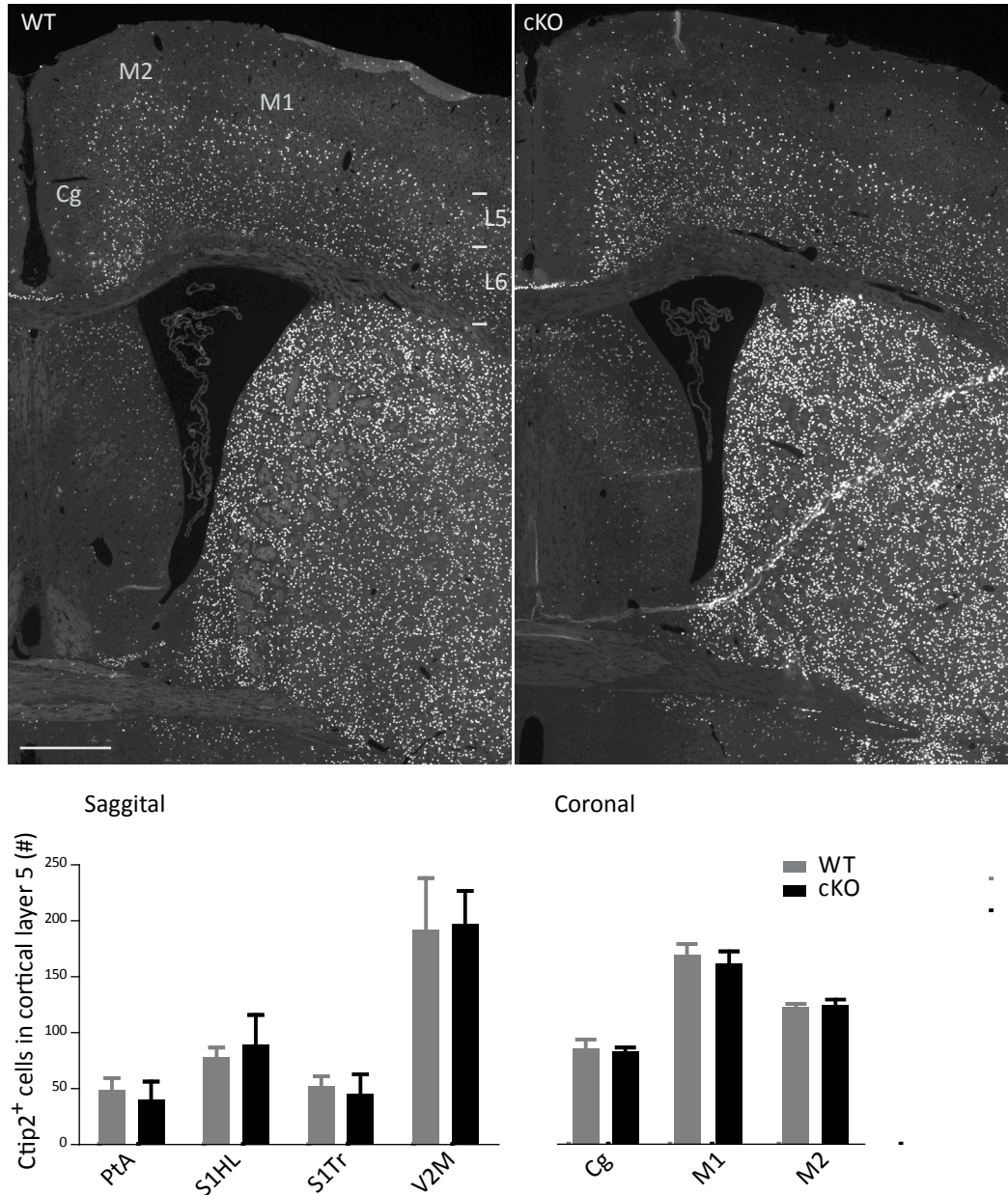


Figure 20. Ctip2 immunostaining and quantification. Counting is performed in layer 5 on 3 different sagittal and coronal sectioning levels (see *Materials and Methods*), 2 way RMANOVA: sagittal $n=3$, genotype $F(1,12)=0$, n.s.; coronal: $n=5$, genotype $F(1,16)=1.20$, n.s. Data presented as mean +SEM. Scale bar 1mm. Identification of brain areas and corresponding abbreviations according to the ‘Mouse Brain Atlas’ (Franklin and Paxinos, 2007).

belong to a $Foxp2^+/Ctip2^+$ population, I counted $Ctip2^+$ cells in layer 5 of $Foxp2$ cortical knockout mice. Coronal section levels were chosen to represent the motor cortex areas which were described to contain these cortical layer 5 motor neurons directly projecting to the nucleus ambiguus (**Fig.17** (coronal sections), **Fig. 4**)(Arriaga et al., 2012). There was no difference in $Ctip2^+$ cells raw number, cells number normalized to Hoechst staining or cell density per area in primary and secondary motor cortices, nor in the cingulate cortex. Similarly, analyses of $Ctip2^+$ cell numbers in sagittal sections showed no alterations - **Figure 20**.

Of note, although Hisaoka et al.(2010) report that all layer 5 $Foxp2^+$ neurons express $Ctip2$ in motor and primary somatosensory cortex during postnatal development, my own observations , as well as other studies (Tomassy et al. (2010)), suggest that in adult mice $Foxp2^+/Ctip2^-$ as well as $Ctip2^+/Foxp2^-$ populations in these areas are present. Thus, $Foxp2$ neurons of layer 5 constitute heterogeneous population with potentially distinct projections.

1.2. *Foxp2* cKO animals do not show gross projection abnormalities

Studies of FOXP2 targets *in vitro* and from heterogeneous mouse and human tissue suggest, that one key function of FOXP2 is the regulation of genes involved neurite outgrowth and axonal guidance (Vernes et al., 2011). Therefore I studied the connectivity of layer 6 projections to the thalamus in the adult cortex of cKO animals. The mPFC significantly contributes to a variety of social and DA-signaling dependent behaviors (Carr and Sesack, 2000; Vanderschuren and Kalivas, 2000; Li et al., 2014). In particular layer 6 projections to the mdThal have been involved in social dysfunctions in several neuropsychiatric disorders (Parnaudeau et al., 2013; Delevich et al., 2015).

Therefore I performed anterograde (from mPFC) and retrograde (from mdThal) tracing experiments (see *Materials and Methods*).

These experiments revealed no differences in mPFC – mdThal connectivity between cKOs and WT littermates - **Figure 21**. These data suggest that *Foxp2* knockout during embryonic development may have no major impact on axon pathfinding of lower cortical projection neurons. However, further studies are needed to detect potential roles of $Foxp2$ in the refinement of neuronal connectivity in their target areas.

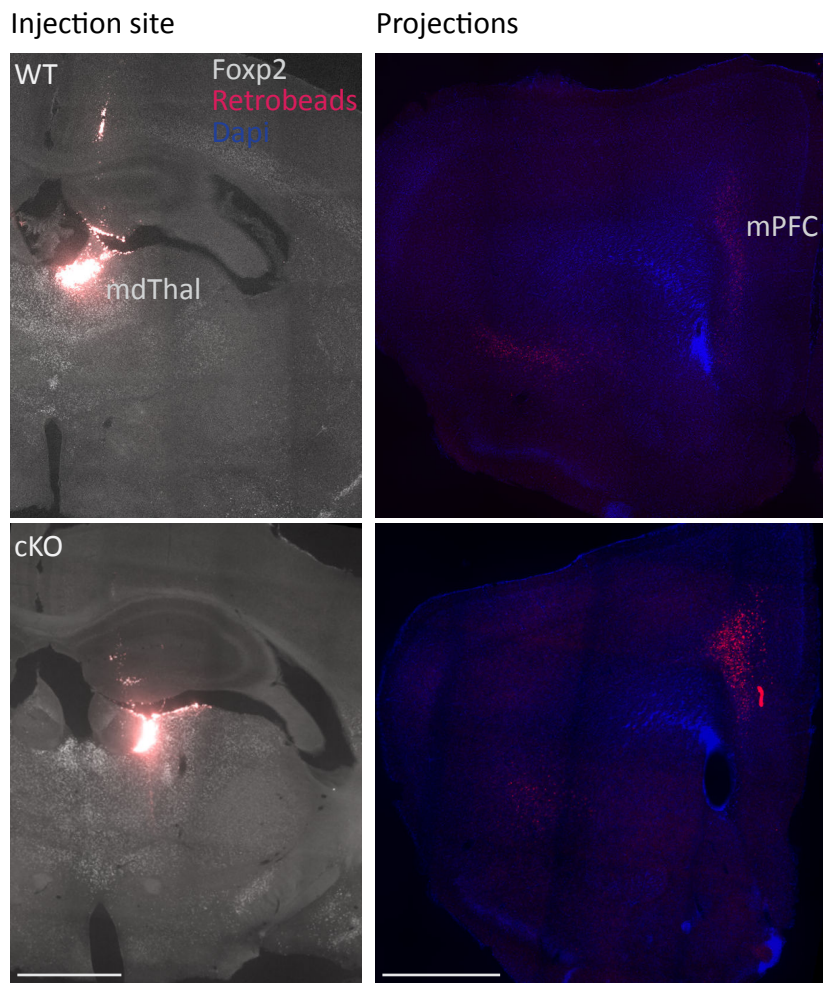
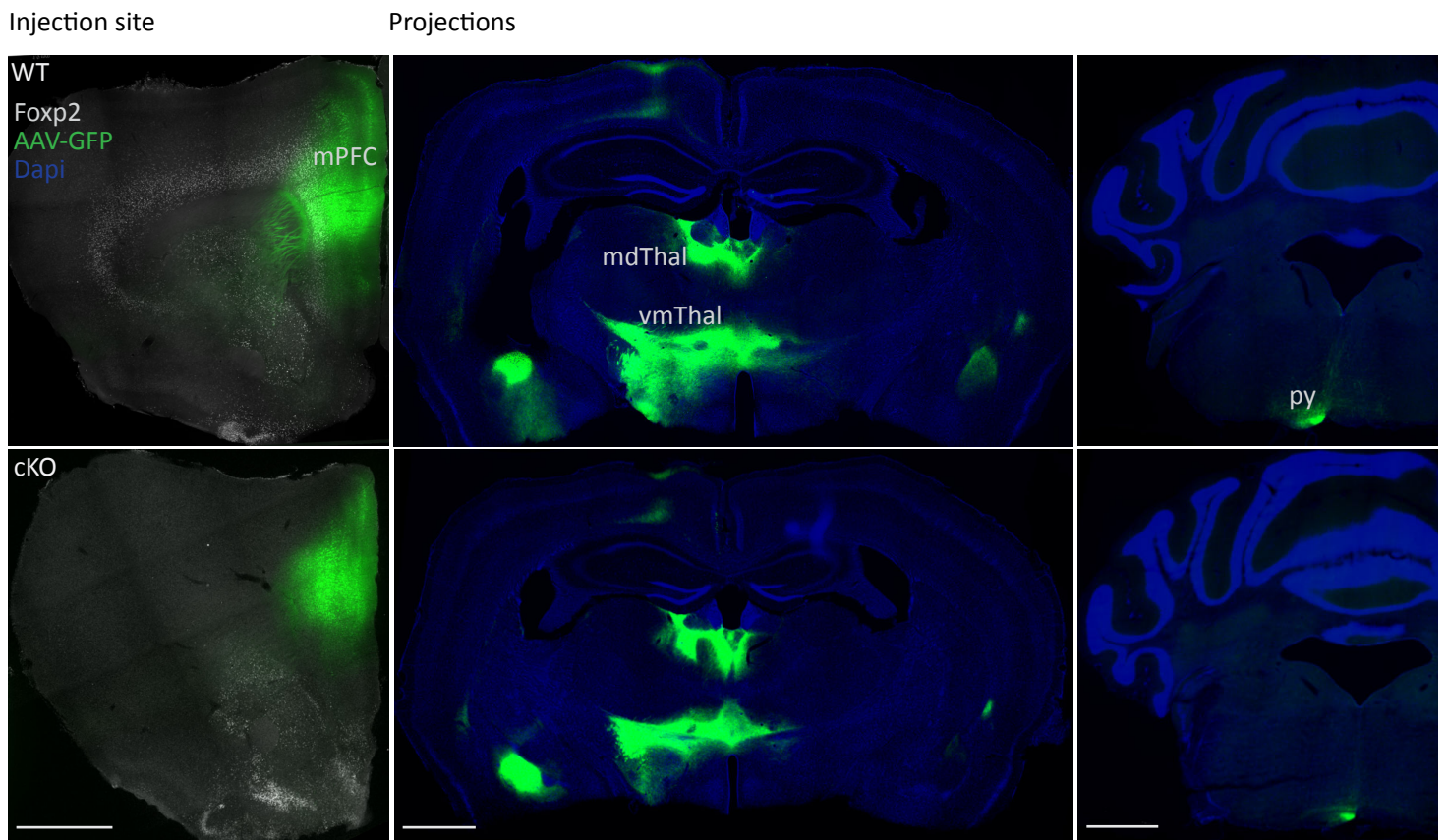


Figure 21. Foxp2 cKO projection from prefrontal cortex to mediodorsal thalamus appears indistinguishable from WT littermates. Scale bars 2mm. mdThal, mediodorsal thalamus; vmThal, ventromedial thalamus; py, pyramidal tract, projections from layer 5 corticobulbar neurons.

1.3. Postnatal development of *Foxp2* cKO mice and WT littermates is indistinguishable

Previous studies revealed significant impairments in weight gain and postnatal developmental in different strains of homozygous and certain heterozygous *Foxp2* mutant mice (Shu et al., 2005; Fujita et al., 2008; Groszer et al., 2008). The abnormal growth trajectories of these animals may affect general activity and be an important confounder for behavioral studies. For instance, vocalization abnormalities of pups may represent indirect consequences due to different developmental stages of *Foxp2* mutants mice and WT littermates (Groszer et al., 2008; Gaub et al., 2010). Therefore I have evaluated the postnatal development of cKO mice using weight measurements (**Fig. 22**) and a battery of developmental reflexes and behaviors presenting postnatal milestones (**Table 2**). I found no difference in postnatal growth and maturation between cKOs and WT littermates, indicating that the development of mutant mice was unaffected.

1.4 The role of cortical *Foxp2* in DA signaling related behavior

In cortex and striatum nearly all *Foxp2*⁺ neurons co-express DARPP-32, a key DA signaling regulator and *Foxp2* heterozygous mice show significantly increased striatal DA levels (Enard et al., 2009; Hisaoka et al., 2010; Vernes et al., 2011). These neurons are part of forebrain circuits with crucial functions in motivation and reward processing. Using neuropharmacological and behavioral investigations in *Foxp2*-R552H/+ mice, we found significant deficits in DA-related signaling, electrophysiology and reward-associated behavior. To dissociate the contributions of cortical and striatal circuits to these specific phenotypes, we employed conditional knockout mice. We found that *Foxp2* knockout in the NuAc mediated these specific deficits in DA-related signaling and social reward-associated behavior (**Fig. 10**) (Mombereau et al., submitted).

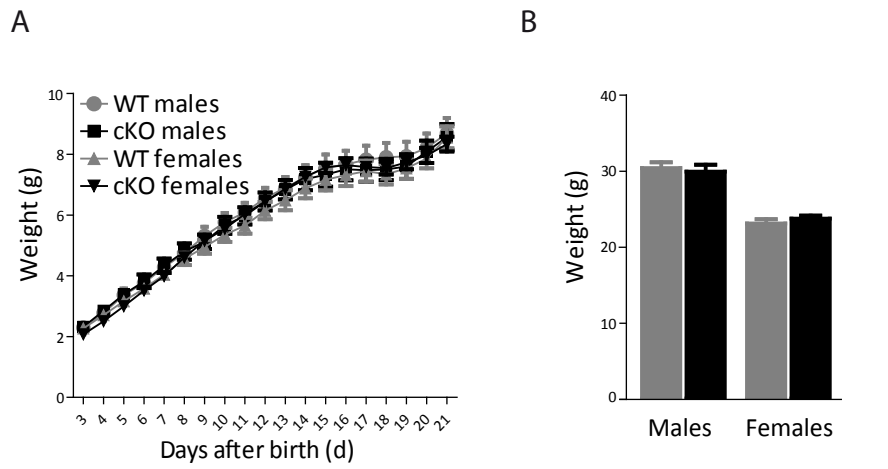


Figure 22. Normal weight gain of cKO animals throughout postnatal development. **A**, Weights of neonatal animals, n=10-11, 2 way RMANOVA: genotype-gender $F(3.684)=0.28$, n.s. **B**, Weights of adult animals, n=8-12, 2 way ANOVA: genotype $F(1.35)=0.02$, n.s. Data are presented as mean \pm SEM.

Table 2. Day of first performance of developmental behaviors and milestones

		WT	cKO
Rooting	males	4.1±0.3	4±0.3
	females	4.1±0.5	3.5±0.2
Forelimb grasp	males	7.1±0.4	7.7±0.4
	females	7.9±0.4	7.8±0.4
Surface righting	males	8.6±0.5	8.9±0.5
	females	9±0.5	9.5±0.4
Auditory startle	males	8.6±0.7	8.5±0.6
	females	9.7±1.5	9±0.4
Activity (open field)*	males	9.8±0.6	10.7±0.9
	females	9.97±0.8	12.5±1
Air righting	males	10.6±0.5	11.1±0.4
	females	11.7±0.4	11.7±0.5
Ear twitch	males	11.2±0.4	11.2±0.3
	females	11.9±0.2	12.1±0.3
Eye opening	males	13.5±0.3	13.2±0.1
	females	13.6±0.3	13.6±0.2

Data shown are mean ± SEM; n=11-13, except for Auditory startle n=3-6;

2 way ANOVA: genotype: Rooting $F(1.46)=0.91$, n.s., Forelimb grasp $F(1.46)=0.47$, n.s., Surface righting $F(1.46)=0.57$, n.s., Auditory startle $F(1.14)=0.26$, n.s., Activity (open field) $F(1.45)=4.54$, * $p=0.04$, Air righting $F(1.46)=0.32$, n.s., Ear twitch $F(1.45)=0.32$, n.s., Eye opening $F(1.46)=0.32$, n.s., Bonferroni post hoc tests: difference between genotypes within males and females groups are not significant for all tests.

Before addressing behavioral alterations in cortical knockout mice in more detail, I have evaluated the performance of adult cKO animals, males and females, in a variety of tests, accessing general neurological deficits.

Open field activity measures locomotor activity parameters and is used as a preliminary test to determine motor and anxiety deficits. Anxiety is determined by the time of exploration of in the open-field (center versus periphery) and locomotor activity is measured by determining the velocity and the amount of traveled distance. None of these parameters were altered in *Foxp2* cKO mice (males: n=8, total distance travelled $t(14)=0.08$, $p=0.9381$; velocity in the center $t(14)=0.4$, $p=0.6952$; time spent in the center $t(14)=0.05$, $p=0.6231$; females: n=9, total distance travelled $t(16)=0.2462$, $p=0.8086$; velocity in the center $t(16)=0.3694$, $p=0.7167$; time spent in the center $t(16)=0.6155$, $p=0.5469$) (**Fig. 10B**).

The elevated plus maze measures fear and anxiety. Time spent exploring open and closed arms of the plus-shaped platform is measured. In addition, the recording of ethologically relevant measures of exploration and risk assessment such as head-dips and rearing behaviors increases the sensitivity of this test (Rodgers and Cole, 1993). All the parameters of this test stayed normal in *Foxp2* cKO animals (males: n=8, time spent in the open/center/closed arms $F(2,42)=1.45$, $p=0.2451$; head-dips number $t(14)=1.660$, $p=0.1191$; rearings number $t(14)=1.249$, $p=0.2323$; females: n=10, time spent in the open/center/closed arms $F(2,54)=0.05$, $p=0.9493$; head-dips number $t(18)=0.3063$, $p=0.7629$; rearings number $t(18)=1.79$, $p=0.09$).

Sensorimotor gating is a neurological process that provides central nervous system inhibition in response to overwhelming sensory stimuli. It is thought to rely on different brain structures including cortex and NuAc, but is largely automated and engages attention. Stimulus overload and social perception abnormalities which lead to cognitive fragmentation in schizophrenic patients correlate well with deficiencies in sensorimotor gating (Braff and Geyer, 1990; Wynn et al., 2005). This function is accessed by a Prepulse Inhibition test (PPI) which relies on auditory stimuli perception, and measures how the weaker (prepulse) stimuli inhibits the subsequent strong stimuli (pulse). This test can be applied across species. In mice the response is measured by the magnitude of a 'jump' of acoustic startle reflex. Since the stimulus is auditory, the hearing is accessed

before the test. This behavior did not reveal any abnormalities in both sexes of *Foxp2* cKO animals (males: n=8, hearing 72-120dB $F(5,70)=0.26$, $p=0.9341$; PPI $t(14)=1.574$, $p=0.1378$; females: n=9, hearing 72-120dB $F(5,80)=1.14$, $p=0.3445$; PPI $t(16)=1.391$, $p=0.1834$).

Abnormal motor-skill learning in the rotarod paradigm has been previously described in *Foxp2* heterozygous mice, in agreement with substantial alterations of synaptic plasticity in associated cortico-striatal circuits (Groszer et al., 2008; French et al., 2012; Kurt et al., 2012). However the contribution of *Foxp2* in cortical circuits to these abnormalities in rotarod learning remained largely unknown. In order to test whether this paradigm of motor skill learning is dependent on cortical *Foxp2* I assessed this behavior in automated accelerating rotarod paradigm identical to the one used in *Foxp2* heterozygous animals (Groszer et al. (2008)). Over three consecutive days of training *Foxp2* cortical knockout mice demonstrated no motor learning deficiencies (males: n=9-8, rpm/16 trials $F(15,225)=0.63$, $p=0.8476$; females: n=15-14, rpm/12 trials $F(11,297)=0.82$, $p=0.6178$). (**Fig. 10A**) The result indicates that *Foxp2* expression in the cortex is dispensable for rotarod learning. Furthermore it suggests that abnormalities in *Foxp2* expressing striatal circuits may play a key role in motor learning, at least in this particular paradigm.

To further address behavioral alterations in cortical knockout mice following pharmacological DA stimulations I studied cocaine sensitization. Behavioral sensitization is the consequence of drug-induced neuroadaptive changes in a circuit involving dopaminergic, glutamatergic and GABAergic connections between VTA, NuAc, PFC and amygdala. While the immediate response to learning of task to obtain the drug is strongly mediated by DA release in the NuAc, the reward-associated learning process and relapse behavior are thought to be more strongly associated with DA release in prefrontal and allocortical brain regions (Vanderschuren and Kalivas, 2000; Kalivas, 2007; Luscher and Malenka, 2011). Thus, to access potential cortex-mediated responses to cocaine stimulation I employed a prolonged incremental 'learning paradigm', which consisted of induction phase (5-6 days), withdrawal period and a 'relapse', represented by a challenge cocaine injection.

Mice response to cocaine injections was measured by locomotor activity in cylindrical boxes. In agreement with Mombereau et al. no difference was observed on the first day of cocaine administration (**Fig. 10C**) and on the challenge day. However, cKO mice tended to display a shorter latency to maximize hyperlocomotor response during induction phase (**Fig. 23**). This result suggests a contribution of cortical *Foxp2* circuits to DA signaling dependent behaviors.

1.5. Social interaction defects in *Foxp2* cortical knockout mice

Patients with *FOXP2* mutation - KE family- are not autistic but have deficits in specific social domain (that is speech and language) without known correlate in mice. Therefore, I reasoned that *Foxp2* might be involved in subtle social behaviors which are not captured by currently used tests. To evaluate whether complex, social behaviors are affected in *Foxp2* cKO animals, I employed a recently developed automated video tracking and analysis of social interactions (Mice Profiler (de Chaumont et al., 2012a; Coura et al., 2013)). This computational algorithm allows the robust detection of interactions without much loss of continuity of the tracking (for instance, changing location of head and tail position following body contact or longer immobility), which permits to detect sequences of events and to identify not previously studied correlations (**Fig. 12**).

Male-male interaction dyads in a resident-intruder paradigm (resident being either cKO or littermate WT; and an intruder being unknown WT) were analyzed for 29 types of social behaviors. We observed that the following behaviors were decreased (n=9-10, two-tailed t-test) in cKO-WT dyads when compared to WT-WT dyads:

- count and duration of oral-genital contacts, initiated by the resident (p<0.05)
- count and duration the following events, initiated by the resident (p<0.05)
- count of the chasing events, initiated by the resident (resident goes to intruder and a speed of the resident is higher than that of an intruder, the distance between mice decreases over time) (p<0.05)
- duration of close (<1cm) distance (p<0.05)

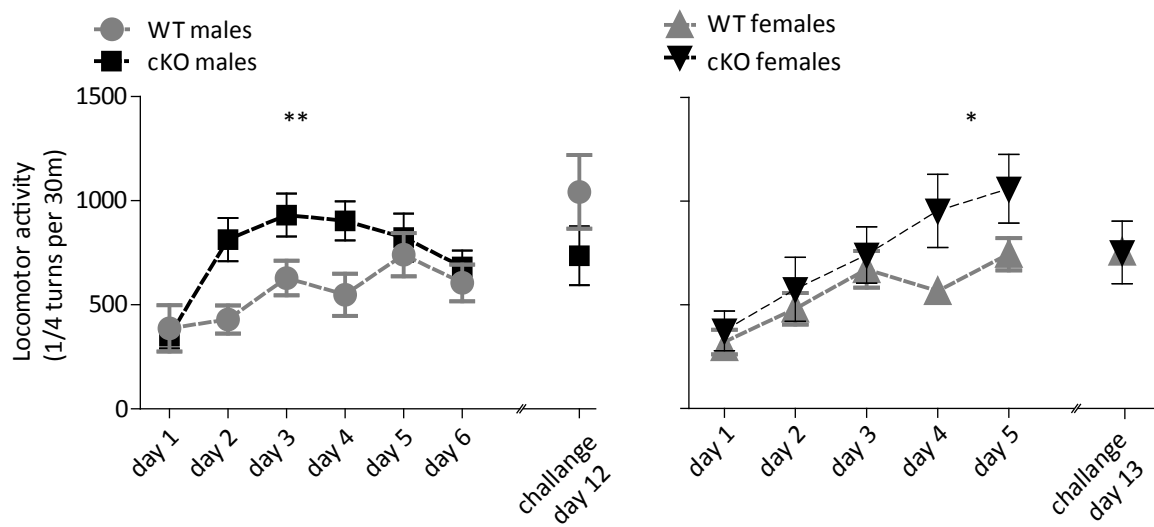


Figure 23. Sensitization to cocaine. Males (20mg/kg): sensitization induction n=15-14, 2 way RMANOVA: time-genotype interaction $F(5.135)=3.72$, $**p=0.0035$; sensitization expression on a challenge day n=12, $t(22)=1.351$, n.s. Females (15mg/kg): sensitization induction n=10, 2 way RMANOVA: time-genotype interaction $F(4.72)=3.38$, $*p=0.136$; sensitization expression on a challenge day n=10, $t(18)=0.011$, n.s. Data are presented as mean \pm SEM.

- mice next to each other, positioned the same way (head –head, tail-tail) at the small (<3 cm) distance-showed a trend to be decreased in occurrence as well (p=0.058)

Interestingly, certain behaviors appeared to be increased in cKO-WT dyads:

- duration of oral-genital contacts, initiated by an intruder (p<0.05)
- time an intruder is positioned behind the resident – approaches significance (p=0.075)
- the duration of back to back posture (p=0.065)

For detailed graphical representation of events occurrence over the time see **Figure 24A**.

To independently study these potential deficits in social interaction, I performed off-line manual blind scoring of video recordings. I indeed found a decrease in the social interaction (combined following and contact events) in cKO-WT encounters as compared to WT-WT (**Fig.24B**). Furthermore I studied dominance and aggressiveness. As dominance index, I scored the number of times the resident placed its forepaw on the head or on the back of the intruder ('paw control')(Coura et al., 2013). I found that cKOs residents initiated significantly fewer such close contact paw control events than WT's residents (**Fig. 24B**). However aggressiveness (defined as the number of times the resident circled around, bit, and rattled its tail in front of the intruder) was nearly absent in WT and cKOs residents. These data support social approach deficits of cKO males detected in Muse Profiler screening.

The behavioral repertoire can be presented in the form of transitional behavioral graphs (**Fig. 25**). These graphs represent the probability of a transition from one event to another and depict orders of events in a timely manner using stop behavior as reference, decision-making point (de Chaumont et al., 2012a). The events showing distinct probabilities reflect the increased social avoidance of *Foxp2* cKO animals (higher probabilities for escape behaviors, lower - for following). Moreover we detected an abnormal reciprocal reaction of WT intruders to the presence of resident cKO conspecifics (ex., increased probability for stopping or for oral-genital contact when cKO is passive).

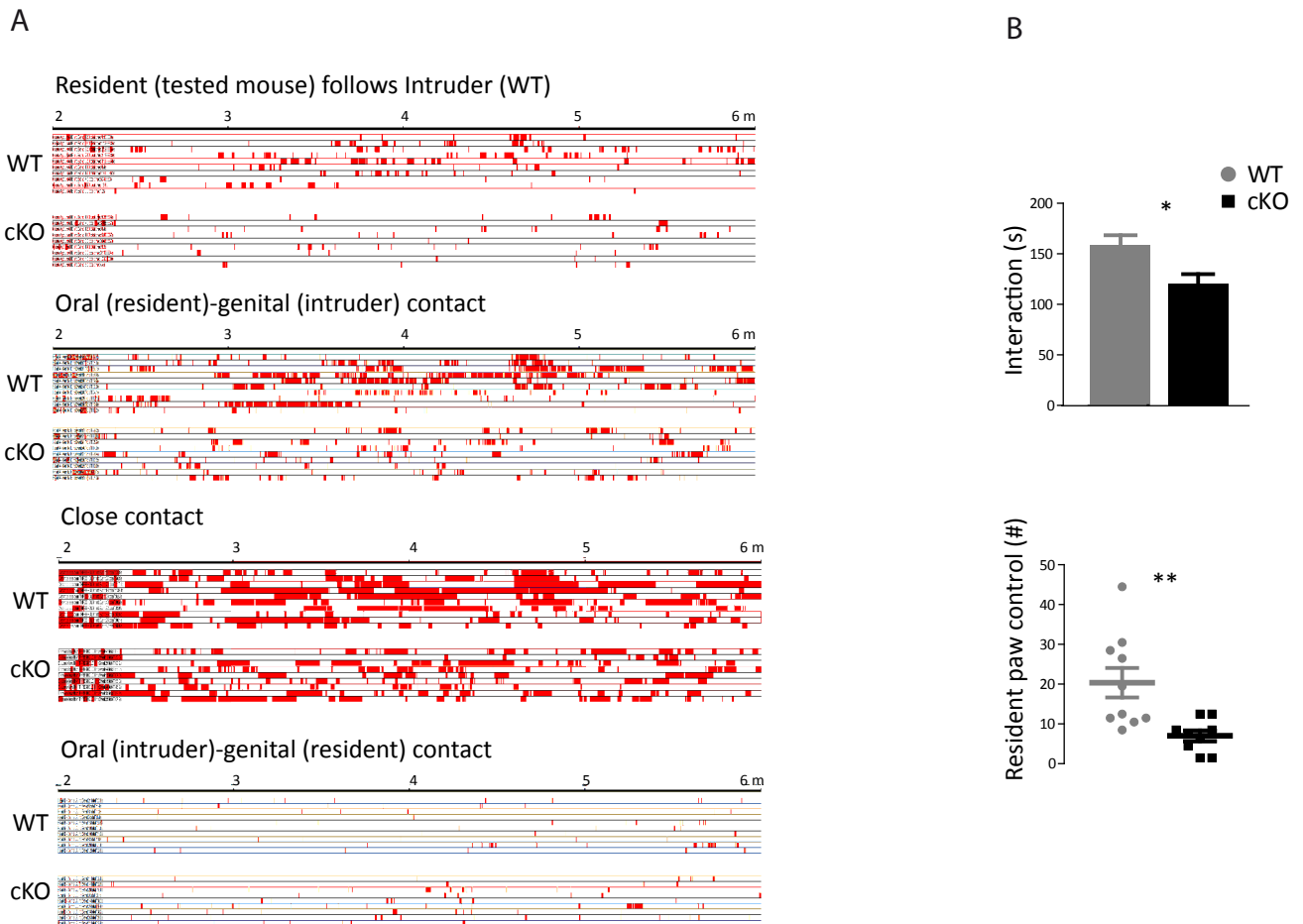


Figure 24. Social behavior alterations in *Foxp2* cortical knockout mice in male-male interaction test. **A**, Chronograms of significantly different events, depict the presence and duration of events for individual mice, analyzed for 4 minutes. (Two-tailed t-test: resident follows intruder, events number $t(17)=2.342$, $p=0.0316$, duration $t(17)=2.885$, $p=0.0103$; oral (resident)-genital (intruder) contacts, events number $t(17)=2.585$, $p=0.0193$, duration $t(17)=2.762$, $p=0.0133$; close contacts, events number $t(17)=0.4659$, n.s., duration $t(17)=2.131$, $p=0.0480$; oral (intruder)-genital (resident) contacts, events number $t(17)=1.714$, n.s., duration $t(17)=2.177$, $p=0.0439$.) **B**, Manual blind score for the duration of social interaction (following and close contact) $t(17)=2.729$, $*p=0.0143$, and paw control in a close contact $t(17)=3.225$, $**p=0.005$; data shown are mean \pm SEM. $N=9-10$.

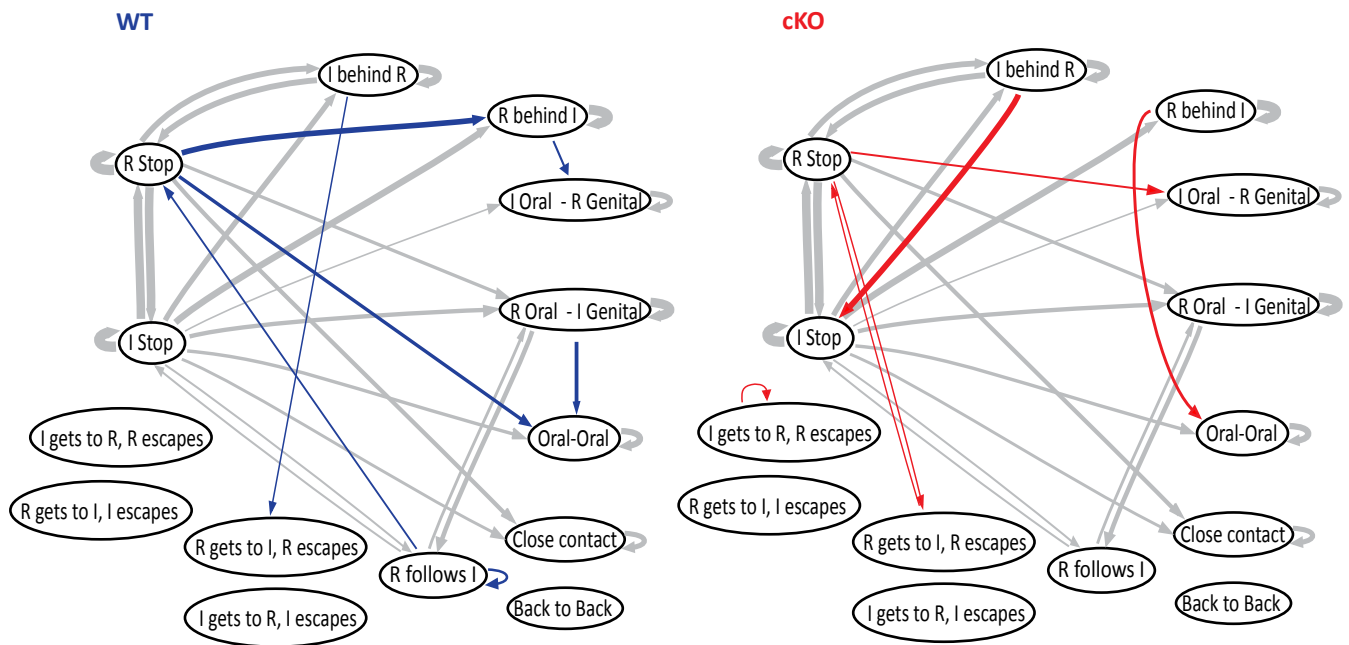


Figure 25. Transitional behavioral graphs: the arrows represent the string of events; the thickness of the arrow is proportional to the probability that the event occurs; grey arrows-events common for all the dyads of males tested, blue arrows - events which are specific for WT-WT dyads, red arrows - events which are specific for cKO-WT dyads; R-resident males, I-intruder males. N=9-10.

The preliminary manually obtained data on social female-female and male courtship interactions did not suggest abnormalities. Female-female and male-female behaviors were manually scored in a panel of social (sniffs, allogrooming, following and chasing), non-social (self-grooming, digging, cage exploration) and sexual (mount counts) behaviors.

Altogether, these results suggest cortical *Foxp2* involvement in regulation of social approach behavior of male mice, as well as reciprocal perception by the WT conspecific. Besides the direct reaction on withdrawal behavior of cKOs, the latter could be a result of a more specific communication deficit of cKO mice, for instance, ultrasonic vocalization alterations.

1.6. The role of cortical *Foxp2* in modulating ultrasonic vocalizations (USVs)

Mice produce ultrasonic whistle-like vocalizations during mixed-sex and same-sex interactions and are thought to be modulated by emotional/motivational mechanisms governed by the limbic system (Ehret, 2005). In the mixed-sex interactions USVs are emitted by males and represent secondary sexual marks, which are of interest from an evolutionary point of view (Hammerschmidt et al., 2012; Musolf and Penn, 2012). In same-sex interactions, the highest number of ultrasonic vocalizations is obtained in resident-intruder paradigms, which are emitted preferentially by the residents (D'Amato and Moles, 2001; Chabout et al., 2012). The vocal repertoire used is roughly similar in all three social paradigms (Guo and Holy, 2007; Scattoni et al., 2011).

Individual calls emitted during the social interactions are of different complexity: the majority are more stereotypic USVs of a single frequency, while others are more complex harmonic calls and with frequency jumps. The ethological significance of these different types is unknown and they are perhaps involved in signaling hierarchy, territory, social recognition and reproductive fitness. Mouse USV studies are complicated by the existence of a variety of call classification approaches. As summarized in the Mouse Ultrasonic Vocalizations workshop (Bourgeron et al., 2012), to date there are three ways of classification: 1) gathering call types together until only two categories are left (with or

without frequency jumps), 2) subjectively splitting ultrasonic vocalizations in a dozen of call types, 3) objective classification through cluster analysis to define call categories. The latter employs automatic comparisons within a study, and is less subject to bias.

We have recorded and analyzed adult USV emitted in resident-intruder paradigms during social male-male and female-female, and during courtship male-female interactions. The analysis was based on several parameters (**Fig. 26**) and automatic classifications schemes, previously utilized in *Foxp2* studies.

Call rate and fractions of call types. Overall call rate did not differ significantly between genotypes in any of the assays. We classified calls according to Holy & Guo (2005) which yields two major groups – simple and complex calls, without or with frequency jumps respectively (**Fig. 13**). This classification did not reveal changes in the relative proportion of calls.

Next, we employed a call analysis according to Arriaga et al. (Arriaga, 2011; Arriaga et al., 2012) which classifies complex calls with jumps of minimum 10 kHz or greater and further considers the direction of the frequency change from down (D) to up (U) or *vice versa*, yielding simple D or simple U, or sequences of DD, DU, UD, DDU, UDUD and the remaining groupings which fall under the 'remainder' class. Analyzing male-female dyads, this classification detected an increase in the number of D class calls and a trend for a decrease of the DU class in cKO-WT as compared to WT-WT interactions (**Fig. 27**). In female-female interactions, we detected a marginally increased fraction of DD calls in cKO-WT dyads (n=12-11, 25-120kHz: $t(21)=2.065$, $p=0.0515$). In male-male interactions, we found a trend for an increase in the U class (n=9-10, 40-120kHz: $t(17)=2.067$, $p=0.0543$) in cKO-WT dyads.

Another classification of calls was adapted from Enard et al. (2009) study of *Foxp2* humanized mouse pups. The classification relied on call duration and required a pitch jump to be at minimum 50% of the value before the jump. This method revealed a slight decrease of class 4 calls (i.e. short in duration with jumps) in male-female dyads (**Fig. 27**).

Mean frequency and range of the pitch. We explored the average of the dominant frequency (pitch) across calls (**Fig. 26B**). Pitch jumps were assessed according to Holy &

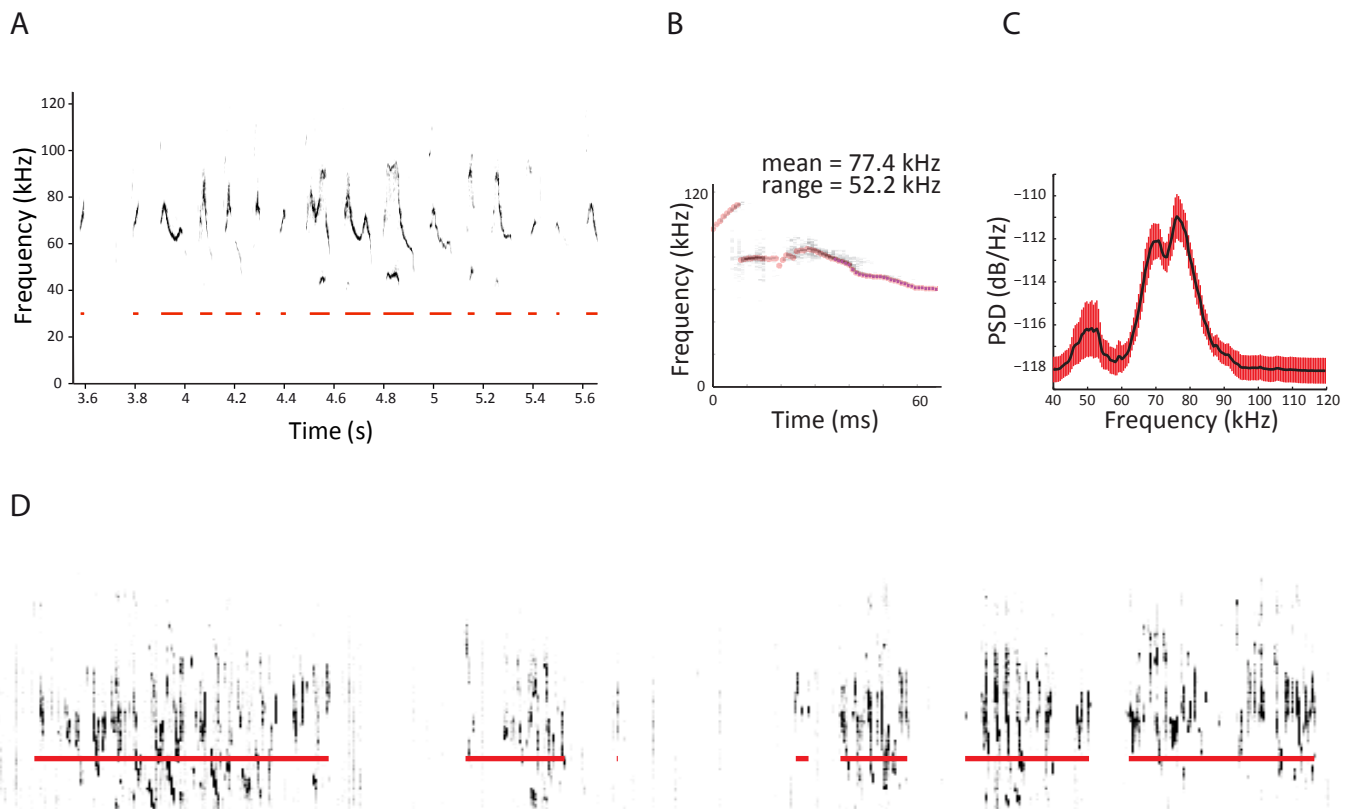


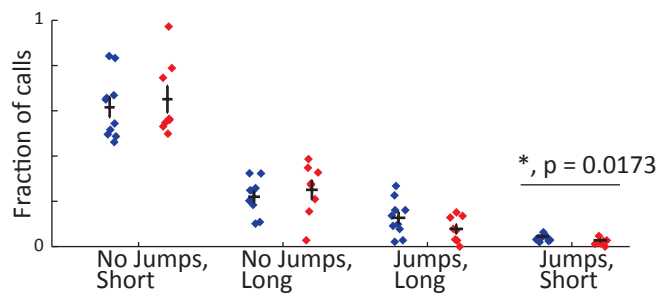
Figure 26. Ultrasound vocalization analysis. **A**, Example sonogram showing ultrasonic calls from male – female interaction. Red lines delineate call start and end times. **B**, Ultrasonic call example; measurement of the pitch (frequency at maximum power) in each time bin in the spectrogram. Pitch determination allows to calculate specific call features such as mean and range. **C**, Power Spectral Density (PSD) displays the used vocalization power across the frequency spectrum. Black line represents the mean for eight recordings, the red bars represent SEM. **D**, Sonogram segmented into phrases using the determined gap cut-off value.

Male-Female interaction

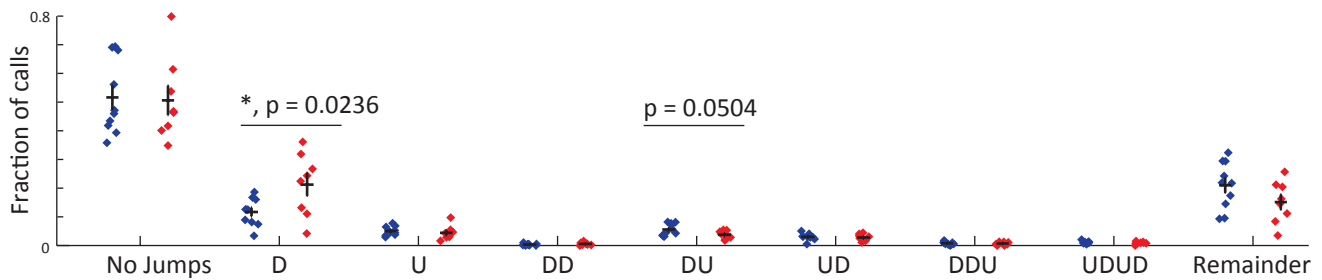
40-120kHz

◆ WT
◆ cKO

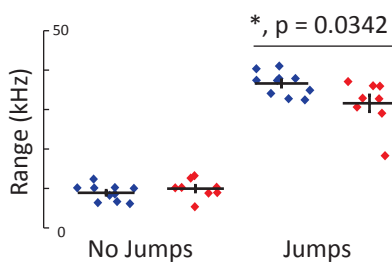
Enard et al. classes



Arriaga et al. classes



Holy & Guo classes



Calls with jumps, classified by the size of the jump

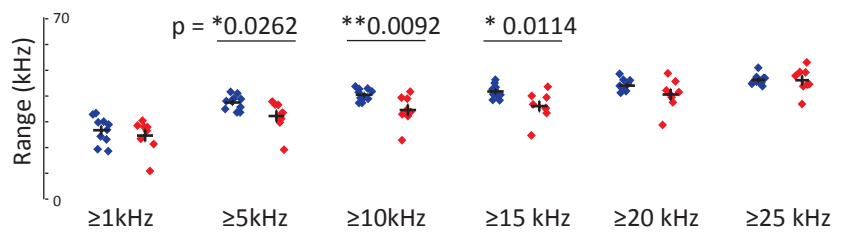


Figure 27. Courtship song abnormalities of *Foxp2* cKO males. Summary of differences in USV modulation according to different classification schemes. N=8-10, two-tailed t-test: fraction of calls: Enard et al., short jumps $t(16)=2.666$; Arriaga et al., D: $t(16)=2.499$, DU: $t(16)=2.111$; pitch range: Holy & Guo calls with jumps: $t(16)=2.316$; calls with jumps $\geq 5\text{kHz}$ $t(16)=2.450$, $\geq 10\text{kHz}$ $t(16)=2.960$, $\geq 15\text{kHz}$ $t(16)=2.858$.

Guo (2005). In complex calls emitted during male-female interactions, when pitch jumps were assessed according to the classification proposed by Holy & Guo (2005), we observed a 14% decrease in the average of pitch range (**Fig. 27**). Then, we analyzed the complex calls using an unbiased method which classifies calls in discrete bins defined by the size of the frequency jump. We could confirm a reduction in pitch range average for calls with frequency jumps 5 to 10, 10 to 15, and 15 to 20 kHz in size (**Fig. 27**). Conversely, frequency mean across courtship calls was not significantly different in calls from cKOs compared to WT littermates. Analysis of the calls emitted during female-female (n=12-10) and male-male (n=6-8) did not reveal differences in either pitch mean or range.

Power spectral density. The animal's PSD estimate represents its individual usage of the USV frequency spectrum across all calls (**Fig. 26C**). This parameter did not differ significantly between genotypes. A general observation however suggests that there is an increase of low-frequency calls lacking pitch jumps in a 50 kHz peak in the spectrum in male-male interactions. A caveat to this finding is the low number of calls in male-male interactions as compared to male-female and female-female, in agreement with observations by Holy & Guo (2007).

Temporal Analysis. No change in average call duration across analyses was observed; generally the overall male - male calls were slightly shorter in mean duration (~30 ms) compared to male-female and female- female (~50 ms). Moreover, no genotype difference between the mean pause between constituent syllables of a phrase was observed across all gap cut-offs (see *Materials and Methods*). To determine if temporal elements predict each other within a phrase, three correlations were computed: a) the correlation of pause time to subsequent call duration b) the correlation of duration time to subsequent duration time c) the correlation of pause time to subsequent pause time. Correlations were only performed if at least one phrase of several syllables in length or several short phrases were present in the recordings and that was only a concern for the male-male data. Thus, the data were permuted 10^4 times to generate an empirical distribution of Pearson's R values. The correlations were significantly different from random chance in the case of correlation between call duration time and subsequent call

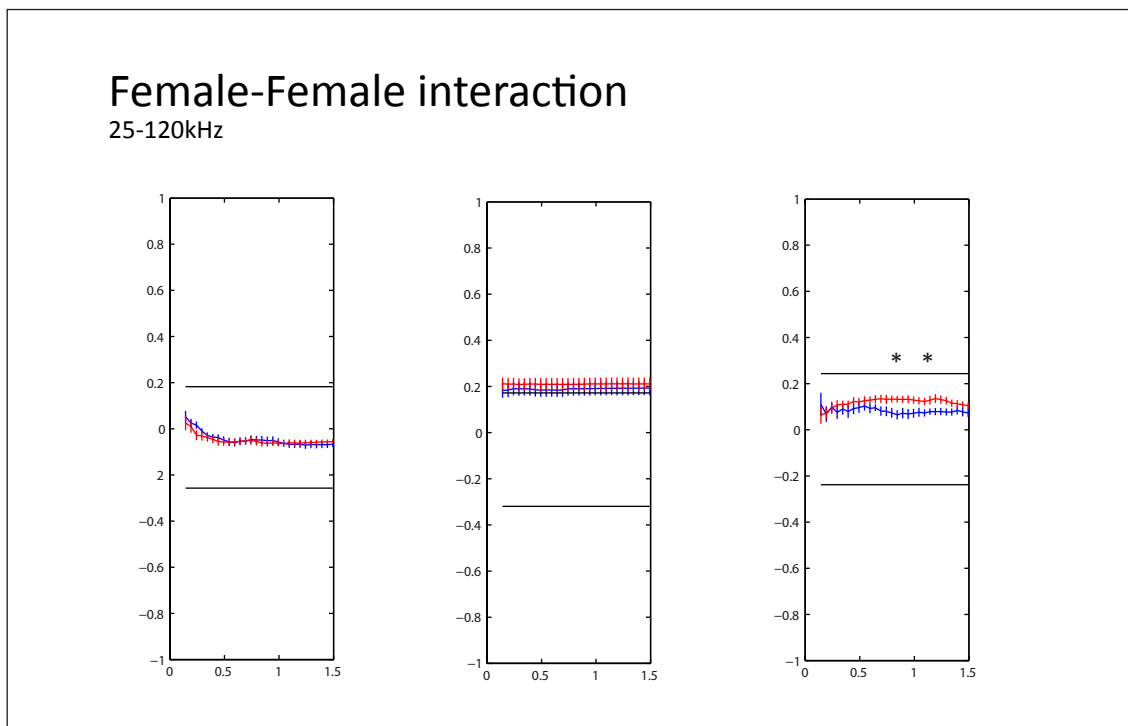
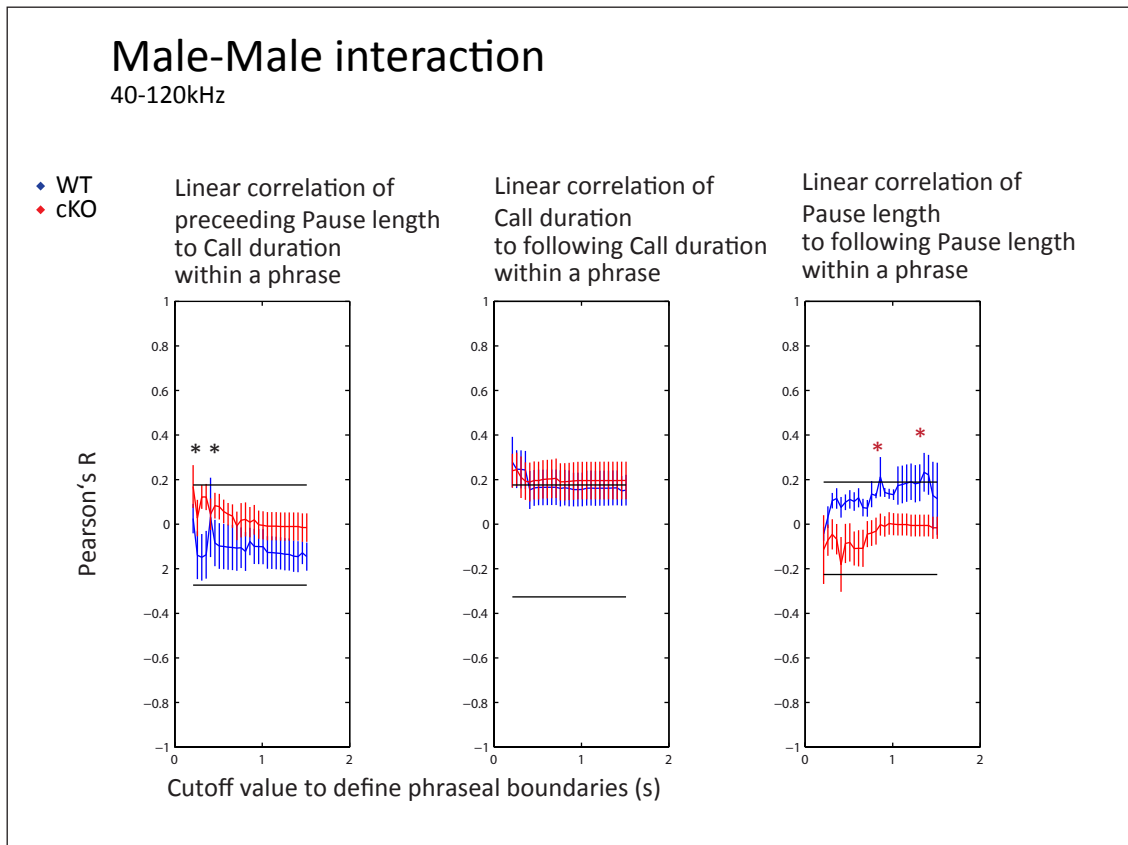


Figure 28. USV abnormalities of *Foxp2* cKO animals, detected during same-sex interactions.

Male-male interaction: correlation of pause to call duration, inter-phrasal gap cut off 0.3099s, n=6-8, two-tailed t-test: $t(12)=2.482$. * $p=0.0286$; 0.3599s $t(12)=2.266$. * $p=0.0428$; correlation of pause to pause length, inter-phrasal gap cut off 0.8599s $t(12)=2.250$. * $p=0.044$; 0.9099s $t(12)=2.712$. * $p=0.0189$; 1.3599s $t(12)=2.549$. * $p=0.0255$; 1.4099s $t(12)=2.379$, * $p=0.0348$.

Female-female interaction: n=12-10, two-tailed t-test: correlation of pause to pause length - range of gap cut off values ~0.7-1.3s demonstrate significant or trending differences, $p=0.0924$ - 0.0336.

Lines represent the boundaries of random chance correlation as determined by $n=10^4$ permutations with $\alpha=0.05$. Red asterisks mark the values differing between the genotypes while being correlated in nonrandom manner. Data presented as mean \pm SEM.

duration time – for all three resident-intruder tests. Genotype differences were detected in social male-male and female-female dyads, but not during courtship vocalizations, in correlation of pause to following pause duration, which were significantly different from random only in male-male interactions (**Fig. 28**).

Collectively these data suggest that cortical *Foxp2* contributes to fine call modulation of male courtship and social vocalizations. The nature of these modulations seems to depend on the social context: sub-types of female cue-driven complex syllables are shifted in their fraction and frequency range tuning in cKO males, while social asexual male-male and female-female cKO vocalizations appear to be differentially modulated on the level of phrasal timing organization.

2. Molecular profiling of lower cortical neurons in *Foxp2*^{+/-} mice.

In order to study *Foxp2* molecular targets (direct and indirect) in the subpopulation of *Foxp2*-expressing lower cortical layers neurons, I employed BacTRAP technology (**Fig. 14**), followed by deep sequencing. BacTRAP is a sensitive technique which allows for the *in situ* profiling of an entire cell's mRNA population, thus more closely matching the protein content than the total RNA gene expression studies (Heiman et al., 2014).

For these experiments I have used WT and *Foxp2*^{S321X/+} (*Foxp2*^{+/-}) mice crossed with the *Ntsr1*-eGFP-L10 BacTRAP line. *Foxp2*^{+/-} mice recapitulate the haploinsufficient conditions found in humans, making them a more relevant model for comparison to human studies. Moreover, this allowed to use a more simple F1 intercross with the *Ntsr1*-eGFP-L10 BacTRAP line. Patch clamp studies of *Foxp2* heterozygote mutants performed in our laboratory demonstrated severe electrophysiological deficits, indicating *Foxp2* haploinsufficiency in layer 6 cortical neurons (LeMagueresse et al., unpublished).

Ntsr1 has previously been shown to be specifically expressed in cortico-thalamic neurons as compared to cortico-cortical in layer 6 (Velez-Fort et al., 2014). To validate the *Ntsr1*-eGFP-L10 BacTRAP line for the molecular profiling of *Foxp2*⁺ neurons we first established that eGFP is coexpressed with *Foxp2* in the cortex (**Fig. 29A,B**). Next, I confirmed that *Foxp2* deficiency does not alter *Ntsr1* expression (**Fig. 29D**) and thus the amount of eGFP-tagged polysomes should be similar in WT and *Foxp2*^{+/-} mice. The

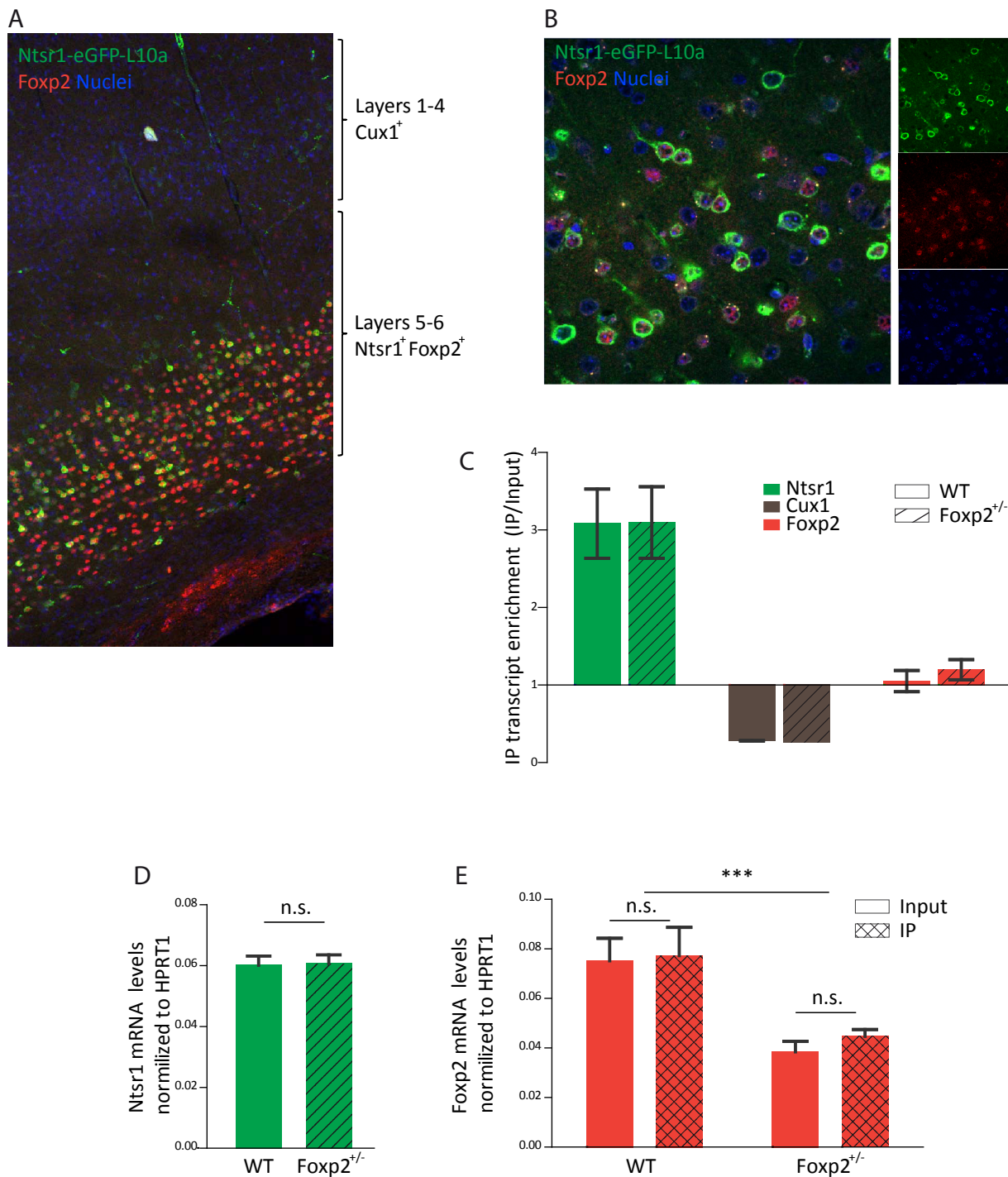


Figure 29. Validation of the *Ntsr1* BacTRAP line. **A**, *Ntsr1*-eGFP-L10a is expressed in low cortical layer neurons where its expression pattern overlaps with *Foxp2* and does not overlap with an area of *Cux1* expression. **B**, Confocal immunofluorescence colocalization of eGFP-L10a transgene with *Foxp2*: ~90% of *Gfp*⁺ cells express *Foxp2*, and ~60% of *Foxp2*⁺ are *Gfp*⁺. **C**, *Ntsr1* BacTRAP experiments yield an enrichment for lower cortical layer markers. **D**, *Ntsr1* mRNA levels are not influenced by *Foxp2* genotype in immunoprecipitated (IP) fractions, two-tailed t-test $p=0.9085$. **E**, Reduced *Foxp2* mRNA levels in IP and input fractions from *Foxp2*^{+/-} mice. 2 way ANOVA post hoc Bonferroni test; ***= $p<0.001$. *Ntsr1* $n=6$, *Foxp2* $n=4$, *Cux1* $n=2$, data shown are mean \pm SEM.

efficiency of BacTRAP protocol used in this study was demonstrated (**Fig. 29C**) by the following: high enrichment of *Ntsr1* transcript in the pull-down (IP) as compared to the input fraction; depletion of *Cux1*, a gene preferentially expressed in upper cortical layer neurons (Molyneaux et al., 2007); moderate enrichment in *Foxp2* transcript, which correctly represents *Foxp2* mRNA levels in WT and heterozygous animals in immunoprecipitated fraction (**Fig. 29E**).

For the sequencing experiments I have collected RNA from cortices of adult male BacTRAP *Foxp2*^{+/-} mice and their wild type littermates under baseline conditions. Deep sequencing of transcripts yielded 65 differentially expressed transcripts, among which 23 showed enrichment in the IP fraction (mRNAs expressed the cortico-thalamic *Ntsr1*⁺ neurons) (**Table 3**) and 42 transcripts were enriched in the input fraction, representing the total mRNAs from the whole cortex (**Table 4**).

Examination of the list of transcripts in the IP fraction revealed that *Foxp2* was well present in lower layer cortical neurons and demonstrated an expected ~two-fold diminution in the heterozygous mice. Semaphorins - signaling molecules crucial for axon guidance - have been previously shown to be directly regulated by FOXP2 (Spiteri et al., 2007; Vernes et al., 2011), and are represented in our gene list by *Sema3e*. Genetic polymorphisms of KIAA0319 have been recently associated with reading disability (dyslexia) and with altered brain activation in language-related temporal brain areas (Pinel et al., 2012). The presence of differentially regulated contactin genes (*Cntn2* and 1) is of interest with respect to ASD phenotypes which are known to be largely underlined by synaptic activity and plasticity dysfunctions (Zuko et al., 2013). Finally, *Mint2* (a.k.a. *Apba2/X11L*) – a gene directly associated with autism, schizophrenia and mental disability (Kirov et al., 2008; Babatz et al., 2009; Voineagu et al., 2011) - was downregulated in cortico-thalamic *Foxp2*^{+/-} neurons. Therefore, *Foxp2* deficiency in the cortico-thalamic neurons affects regulation of genes involved in neurodevelopmental disorders, affecting social behavior and cognition.

Interestingly, genes involved in neuroinflammation and/or confined to astrocytic expression or differentiation can be found in abundance the input fraction (ex., *Vcam1*, *Mfge8*, *Itih3*, *Gprc5b*) but also in IP (ex., *Slc1a4*). Interpretation of these findings is

difficult as it may represent reactive changes following *Foxp2* deletion in neurons (since *Foxp2* is specifically expressed in neurons and not astrocytes), but also may indicate changes in identity of cells during corticogenesis.

To identify principal biological themes regulated by *Foxp2* I used the WebGestalt on-line tool, which was previously employed for the identification of FOXP2 regulated genes in transfected cell lines (Vernes et al., 2007). GO categories are divided into three broad classifications: cellular compartment, biological process and molecular functions. Over-representation analysis of differentially expressed genes in IP fraction with regard to ‘biological process’ yielded genes involved in biological regulation, response to stimulus, developmental process and cell communication. Within ‘cellular components’ we found genes encoding membrane-bound proteins and related to cell projection. ‘Molecular functions’ showed enrichment for protein binding and ion binding (**Fig. 30**). The enrichment of *Foxp2* regulated genes in these GO categories is in agreement with previously described categories in human and mouse *FOXP2* functional genomics studies (Spiteri et al., 2007; Vernes et al., 2007) (**Fig. 6**). This suggests that our circuit specific BacTRAP analysis may provide a set of *Foxp2* regulated cortical transcripts.

While for biological categorization GO analysis employs functional gene annotation, an analogous *in silico* tool exploiting gene expression data was recently elaborated, that is Cell type-Specific Expression Analysis (CSEA) (Xu et al., 2014). CSEA utilizes generated BacTRAP mouse lines profiles of dozens of targeted cell populations to identify candidate cell types enriched by transcripts of interest. Mapping of differentially expressed sequence names versus CSEA transcripts library has correctly revealed lower cortical layers as a cell-type of origin for IP fraction (**Fig. 31B**). In addition we found an enrichment of transcripts associated with astrocytes, oligodendrocytes and immune cells in the input fraction (**Fig. 31C**).

CSEA was originally developed for identification of candidate cell populations likely to be disrupted in disorders with unclear cellular mechanisms and highly heterogeneous genetic background, such as autism. Remarkably, when mapping cortical transcriptomic data from human ASD patients, CSEA revealed astrocytes and immune cell transcripts to be overrepresented at multiple stringency thresholds (pSI) levels, as well as moderate

Table 3. Differentially expressed genes between *Foxp2*^{+/-} and WT mice (IP fraction)

	Transcript enrichment in IP vs Input	Transcript enrichment in <i>Foxp2</i> ^{+/-} vs WT	P value
Acsl3	1.01407286	0.7860931	0.0127
Slc1a4	1.04123165	0.70697033	0.0212
Mint2/Apba2	1.02240207	0.68290715	0.0218
Rab3b	1.2096002	1.11877066	0.0251
Cntn1	1.23971603	0.85858403	0.0265
Foxp2	1.52609271	0.67817002	0.0278
KIAA0319	1.24635128	0.70777727	0.0369
Cyp7b1	1.29541266	0.53896148	0.0369
Stt3a	1.35456103	0.80042048	0.0369
Sema3e	2.09680708	0.73145629	0.0406
Acp2	1.15309812	0.75328616	0.0417
Cntn2	1.06560793	0.70030917	0.0532
BC017158	1.26026183	0.67511481	0.0575
Tbc1d1	1.00079652	1.36456625	0.0651
Ktn1	1.028798	0.73589627	0.0651
Slc4a10	1.45617215	0.79353985	0.0651
Adpgk	1.20564821	0.73803636	0.0714
Tmx3	1.15037341	0.85621456	0.0720
Hs2st1	1.02009124	0.75753486	0.0784
Tmem255a	1.17038347	0.69378034	0.0784
Rmdn2	1.11143866	0.67713267	0.0928
Fstl5	1.43019584	0.44949249	0.0987
Ubxn8	1.44432628	0.77290629	0.1000

P values, Benjamini Hochberg FDR used for multiple testing correction, n=3/group.

Table 4. Differentially expressed genes between *Foxp2*^{+/-} and WT mice (Input fraction)

	Transcript enrichment in IP vs Input	Transcript enrichment in <i>Foxp2</i> ^{+/-} vs WT	P value
Htra1	0.55520996	0.65560911	0.0031
Gpr37l1	0.38641821	0.67309288	0.0061
Itih3	0.60080117	0.5137048	0.0061
Mlc1	0.60542382	0.70446946	0.0061
Mfge8	0.60623612	0.64581648	0.0063
Gprc5b	0.35560318	0.66779827	0.0100
Slc7a10	0.7431149	0.67541473	0.0100
Atp1a2	0.7567537	0.68522763	0.0127
Al464131	0.7641163	0.67031223	0.0136
Scd1	0.61641516	0.64916433	0.0143
Vcam1	0.91474572	0.63510791	0.0166
Gm15453	0.59211148	1.2942531	0.0212
Lcat	0.66901282	0.65763893	0.0218
Gjb6	0.40720043	0.67859654	0.0243
Abca2	0.86043168	0.68403698	0.0243
Timp4	0.419637	0.63965177	0.0251
Ppap2b	0.56287117	0.74342537	0.0263
Prep	0.51362544	0.54329494	0.0265
Aldh1a1	0.63401517	0.72838826	0.0277
Olfm3	0.92121022	0.76084015	0.0347
Ntsr2	0.390808	0.76096463	0.0367
Rpl9-ps6	0.47794989	1.47024187	0.0367

Cd63	0.29896304	0.73541669	0.0532
Fcrls	0.86966109	0.81184882	0.0651
Aqp4	0.430756	0.76162603	0.0714
Oaz2-ps	0.44999562	1.21879718	0.0720
Serpine2	0.70043359	0.72996592	0.0720
Slc16a2	0.99950719	0.76624651	0.0728
Scg2	0.84039173	0.85983315	0.0832
Pon2	0.43460501	0.64289368	0.0872
Btbd17	0.65798086	0.61954502	0.0872
Lamp2	0.87027633	0.83161705	0.0872
Golm1	0.53884316	0.71214152	0.0901
Elovl2	0.3297804	0.66178743	0.0928
Araf	0.7069658	1.08564073	0.0928
Tmem33	0.98280827	0.74839883	0.0949
Gfra1	0.76935355	0.78639682	0.0978
Stk25	0.79141222	1.16206217	0.0978
Elovl5	0.64339441	0.7268123	0.0994
Negr1	0.71873115	0.88293372	0.0994
Tril	0.63243762	0.749812	0.1000
Gabrb1	0.94662201	0.8061493	0.1000

P values, Benjamini Hochberg FDR used for multiple testing correction, n=3/group

enrichment for oligodendrocytes. Lower cortical neurons transcripts were represented in the human transcriptomic data as well as among genetic disruptions associated with ASD (Xu et al., 2014). These findings are supported by recent human ASD studies, employing functional genomics approaches. Using brain expression profiles from human brains across developmental time windows and in the adults the authors showed the enrichment

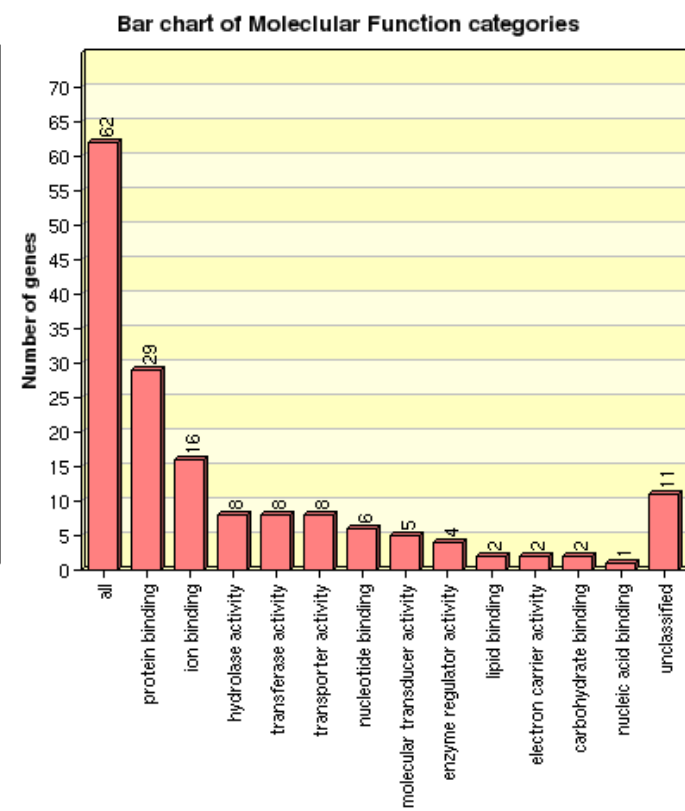
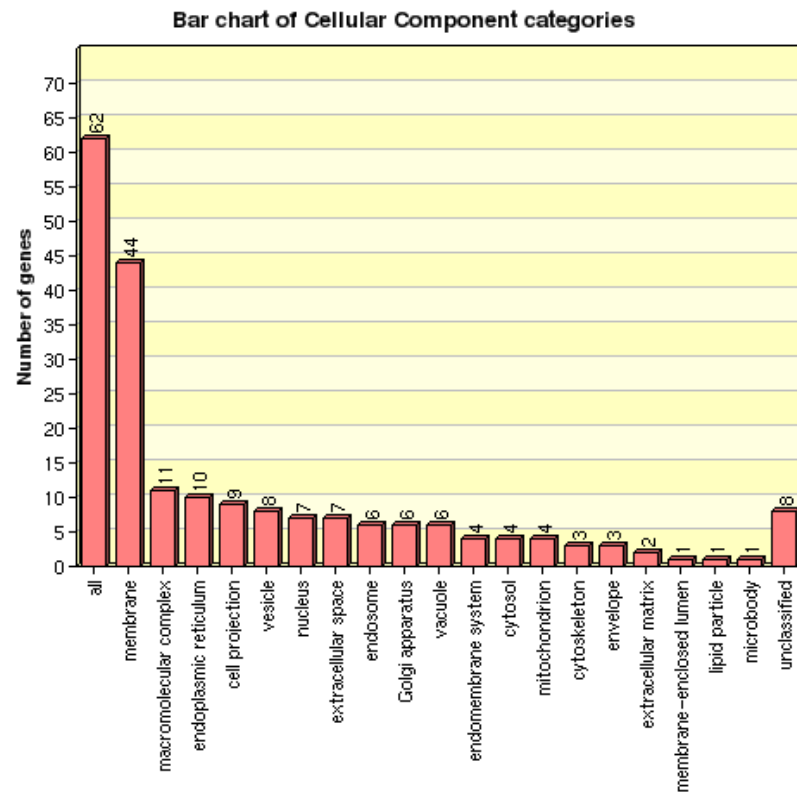
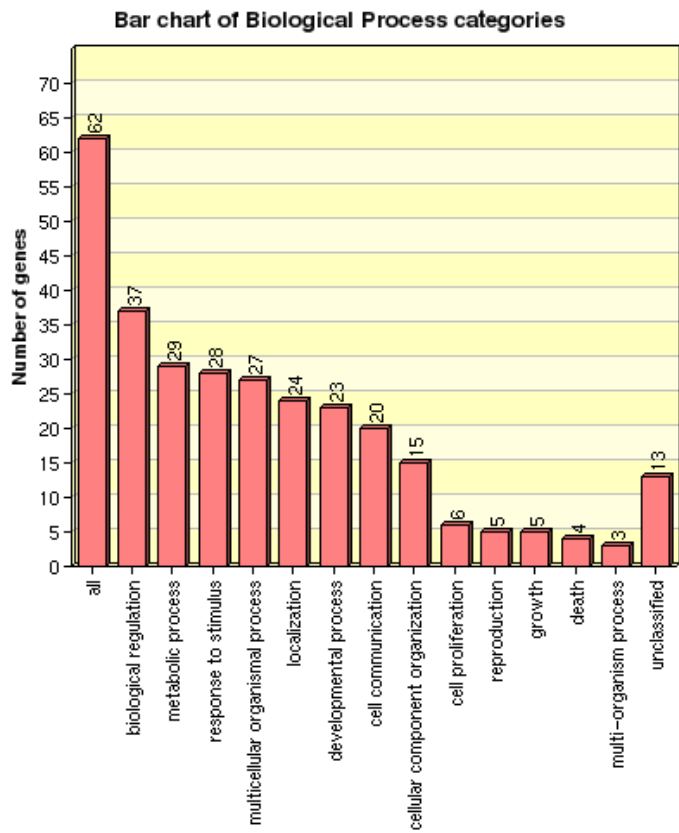
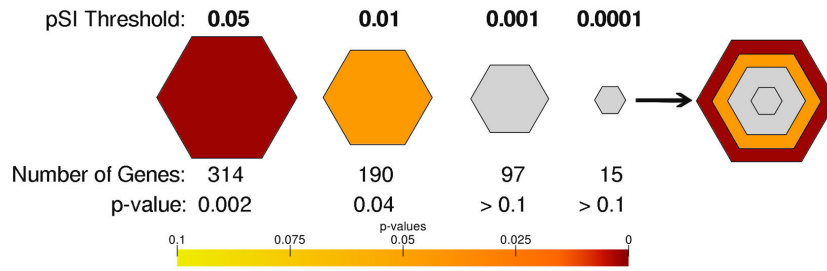
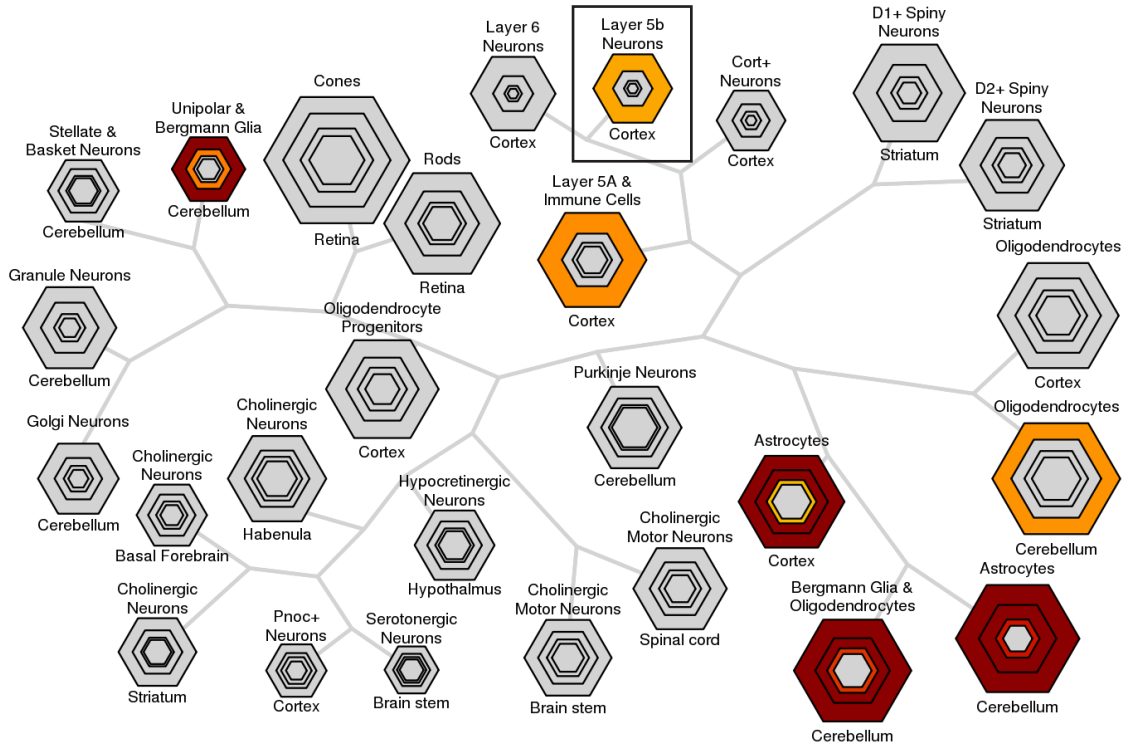


Figure 30. GO categories of differentially expressed genes in the cortex of *Foxp2*^{+/-} and WT mice.

A



B



C

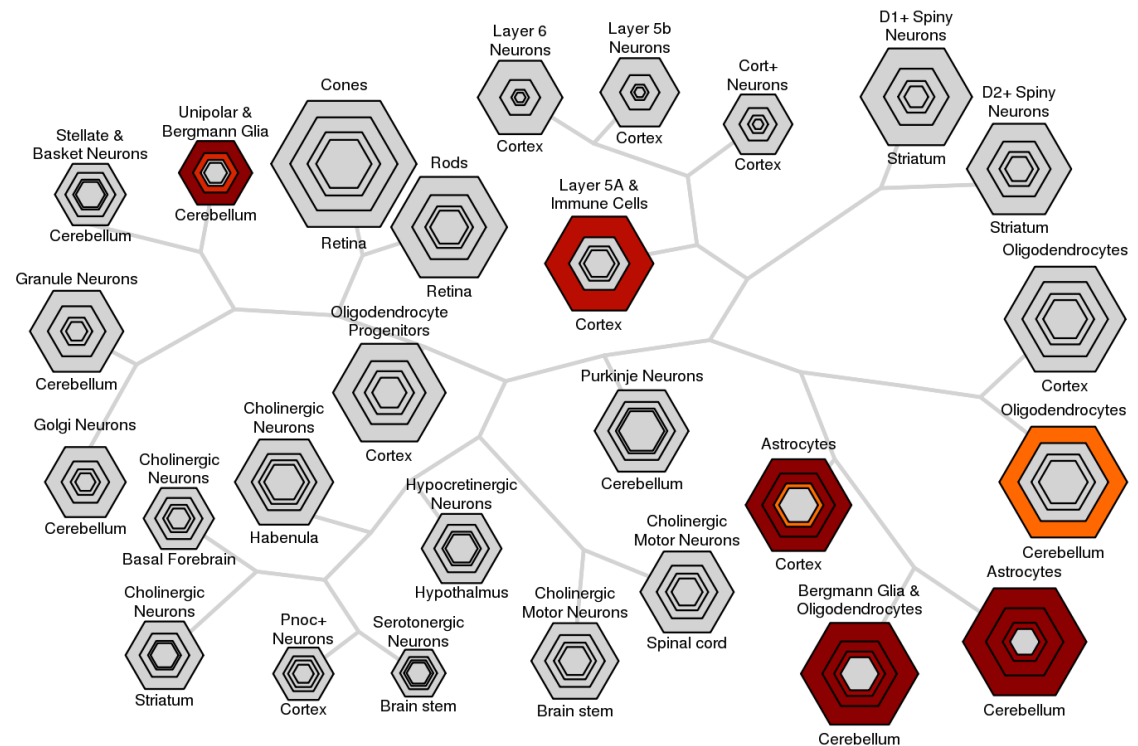


Figure 31. Cell type specific expression analysis (CSEA) of differentially expressed genes in *Foxp2*^{+/-} vs WT cortices. **A**, An example of a single plot: the size of the polygon reflects the number of uniquely expressed transcripts specific for each cell type at different stringency thresholds (pSI, enrichment score value); the color code is of Fisher's exact test p values for CSEA enrichment. Hierarchical ontogenetic clustering diagram of cell types is at the basis of CSEA; cell types enriched by *Foxp2*-dependent cortical transcripts specific for IP and Input fractions (**B**) and for Input fraction only (**C**). Lower layer neurons of the cortex correctly represent the IP fraction, while immune cells, astrocytes and oligodendrocytes – specific transcripts appeared to be altered through the whole cortical tissue in *Foxp2* heterozygous animals.

in mid-gestation frontal cortex in lower layers (Voineagu et al., 2011; Parikshak et al., 2013; Willsey et al., 2013).

The analysis of BacTRAP data is ongoing, but the preliminary outcome of molecular profiling of *Foxp2*⁺/*Ntsr1*⁺ cortical projection neurons together with expression analysis of the whole cortex of *Foxp2*-deficient animals strongly indicate genes and cellular processes involved in social cognition and behavior.

3. Autism related gene *Mint2* is downregulated in the cortex of *Foxp2* cKO mice

Neurexins and neuroligins are synaptic adhesion molecules connecting pre and postsynaptic neurons. Alterations in neurexins have been strongly implicated in a wide range of neurodevelopmental disorders and numerous studies directly link these proteins to both autism and schizophrenia (Feng et al., 2006; Szatmari et al., 2007; Yan et al., 2008; Ching et al., 2010; Dabell et al., 2013; Van Den Bossche et al., 2013; Hu et al., 2014). *Mint2* is a neuronal adaptor that binds to proteins essential for synaptic vesicle exocytosis and to intracellular domains of Neurexin 1 (NRXN1) (Biederer and Sudhof, 2000; Ho et al., 2006). *Mint2* has been directly linked to ASD and schizophrenia through its binding partner NRXN1, but also directly in studies of copy number and sequence variants associated with the disorder (Kirov et al., 2008; Babatz et al., 2009).

The extensive examination of behavioural phenotypes of *Mint2* KO mice by Sano et al. (2009) revealed characteristic deficits in motivated approach behaviours combined with an absence of anxiety, sensorimotor gating and locomotor abnormalities. *Mint2* KO mice showed subordinate behaviours under competitive conditions (decrease of food intake under food restriction conditions, while physical factors stayed normal) with wild type littermates. Moreover social approach and following behaviours in resident intruder paradigm was significantly decreased in these mutants.

These phenotypes were remarkably similar to those I observed in *Foxp2* cKO males. For these reasons, I validated potential differences of *Mint2* expression in *Foxp2* cKOs. Lacking a reliable antibody for immunohistochemistry I attempted to detect differences at mRNA and protein level from the cortex using qPCR and western blot analysis. Indeed,

mRNA and protein levels were slightly, but significantly decreased in the caudal cortex of *Foxp2* deficient mice (**Fig. 32**).

These results suggest that *Mint2* is aberrantly expressed in the cortex of *Foxp2* cKO mice, and this alteration is possibly contributing to the social abnormalities observed in these animals.

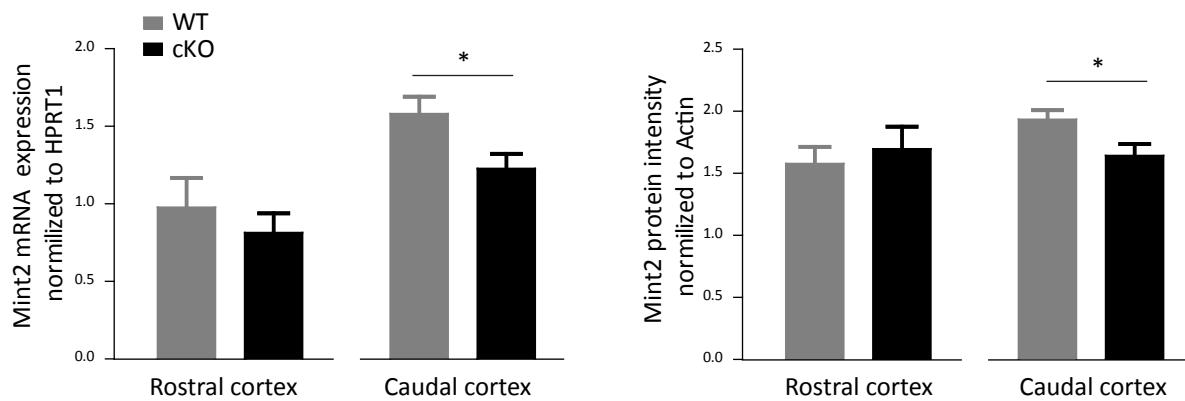


Figure 32. Mint2 downregulation in the cortex of *Foxp2* cKO males. Left panel: qRT-PCR results on the full rostral and caudal cortices, two-tailed t-test: rostral n=6, $t(10)=0.7229$, n.s., caudal n=5-6, $t(9)=2.427$, * $p=0.0381$. Right panel: western blotting protein intensity measurements, performed on the punches from the lower cortical layers, two-tailed t-test rostral n=7, $t(12)=0.5216$, n.s., caudal n=7, $t(12)=2.432$, * $p=0.0316$. Data shown are mean + SEM.

Discussion

The identification of FOXP2 mutations causing abnormal speech and language development has provided a molecular entry point for studying the evolutionary roots of this human specific trait. However *Foxp2* is widely expressed and the identity of molecular and cellular substrates of selection for speech and language related *Foxp2* regulated functions is entirely unknown.

In this context I used a hypothesis-generating approach by characterizing mice carrying *Foxp2* deletions in the cortex. I present the results from behavioral, molecular and morphological screening of *Foxp2*-related alterations in mouse models and discuss how they may contribute to generate new, experimentally approachable hypotheses on the role of this gene in the developing and mature cortical neurons.

Cortical cytoarchitecture in *Foxp2* mutant mice

Tbr1 and *Ctip2* are transcription factors which define the projection fate and laminar and regional specification of maturing cortical neurons. The cortices depleted of these factors demonstrate cortical malformations and alterations in molecular identity, neuronal positioning and projections (Hevner et al., 2001; Arlotta et al., 2005; Bedogni et al., 2010; McKenna et al., 2011; Srinivasan et al., 2012). Both *Ctip2* and *Tbr1* are coexpressed with *Foxp2* in the postnatal developing cortex (Hisaoka et al., 2010).

Here I demonstrated that in adult brains cortical thickness as well as number and layer specific expression patterns of *Tbr1* and *Ctip2*^{high} –positive cells in lower cortical layers are not affected by homozygous cortical *Foxp2* deletion (**Fig. 16, 18 and 20**). For *Ctip2*, one possible interpretation is that *Foxp2* regulation in postmitotic neurons occurs downstream of this transcription factor, as partially suggested by the fact that in striatum *Ctip2* binds to the proximal promoter region of *Foxp2* (Desplats et al., 2008).

The levels of *Tbr1* protein are not affected by *Foxp2* depletion *in vivo* (*Results*). However, *Tbr1* functional activity has been shown to be disrupted by two mutations in the DNA binding T-box domain, which have been identified in sequencing studies of

sporadic autism and are likely to be causal. These mutations prevent interaction with Foxp2. One of these disruptions could underline axon outgrowth phenotypes, hinting on TBR1/FOXP2-mediated axonogenesis (Sakai et al., 2011; Deriziotis et al., 2014). Tbr1 is expressed in maturing and adult neurons of the cortex and colocalizes with Foxp2 in layer 6, thus it is tempting to speculate on the existence of a Foxp2⁺/Tbr1⁺ cell population underpinning social traits within a language-related circuitry. Moreover, our observation that these two transcription factors are only partially coexpressed (**Fig.19**) may suggest that subpopulations of Foxp2 expressing cortical neurons are involved in microcircuits that are more relevant to social information processing.

Foxp2 in cortical progenitors has been suggested to regulate transition from proliferation to differentiation, as well as postmitotic neuronal migration and neurites maturation (Clovis et al., 2012; Chiu et al., 2014). In the adult cortex Foxp2 influences the volume of dendritic trees as has been found in Foxp2 humanized (Reimers-Kipping et al., 2011) and Foxp2-KE (LeMagueresse, unpublished) mice. Foxp2 role in axonogenesis has been repeatedly predicted in functional genomics studies (Spiteri et al., 2007; Vernes et al., 2011).

Nonetheless, I did not observe any gross projection abnormalities in the specific circuit of glutamatergic mPFC-thalamic efferent projections (**Fig.21**). Several explanations are possible: starting from the limitations of this particular experimental set up which did not distinguish among specific Foxp2⁺ populations in the cortex, to the uncorrect developmental time of examination (adult male mice) when such abnormalities could have been determined.

It is also possible that Foxp2-mediated axonal growth is pre-established in neuronal progenitor cells and thus subcortical projection fate cannot be altered in postmitotic neurons. Instead, the traits affected in maturing neurons could be more specific to dendritic outgrowth. Furthermore, more subtle phenotypes affecting including synaptic formation, strength and plasticity may be present (Groszer et al., 2008; Sia et al., 2013).

Prolonged cocaine administration alters locomotor responses of *Foxp2* cortical knockout mice

We have shown that single cocaine administration attenuates locomotor activity upon *Foxp2* disruption in the NuAc, but not in the cortex (Mombereau et al., submitted). This finding delineates the role of NuAc in *Foxp2*-mediated DA signaling, which immediately recruited in response to reward-associated stimulation (like cocaine). However, during the reward-associated learning OR upon repeated drug administration, the cortex along with other structures is gradually engaged in the circuitry underlying motivation and addiction, and thus, plays a role in the ‘drug sensitization’ process (Kalivas, 2009). Sensitization to cocaine is taking place already after the second drug administration (Valjent et al., 2010), however classical protocols employ several days to robustly elicit addictive behaviors. I assessed locomotor activity response to cocaine in *Foxp2* cortical knockout mice and observed a significant alteration during the sensitization induction phase, but not in sensitization expression, i.e. after withdrawal on the challenge day (sensitization expression) (**Fig. 23**).

Several mechanistic explanations for these observations are possible.

Cocaine exerts its reinforcement action through DA overflow, and to a lesser extent, through modulation of the serotonergic neurotransmission system (Andrews and Lucki, 2001; Sora et al., 2001). Given that altered serotonin levels were detected in NuAc in *Foxp2* mutant mice (Enard et al., 2009), it cannot be excluded that the observed effect could be due to a serotonergic component. It is known that 5HT (serotonin receptor) is expressed in mPFC (a key cortical structure underlying cocaine sensitization) of deep layer neurons projecting to VTA and serotonergic nucleus (raphe) (Vazquez-Borsetti et al., 2009). Additionally, my own observations confirm that *Foxp2* is expressed in layer 5 (containing NuAc and VTA projection neurons) of the mPFC (Campbell et al., 2009).

It has been hypothesized that an increase in excitatory glutamatergic transmission from the mPFC to subcortical regions - VTA and NuAc - is involved in both induction and expression of sensitization (Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000; Steketee, 2005; Pascoli et al., 2012). However, the induction of sensitization could be specifically associated with an enhancement in cortical-mediated excitation of the VTA (Almodovar-Fabregas et al., 2002; Luscher and Malenka, 2011). D1R mPFC-VTA

connection is involved in temporal control and movement execution through the time (Narayanan et al., 2012). Given that motor movements of the KE family members are affected upon sequential (time-framed) performance and, moreover, timing abnormalities were suggested to be at the core of the disorder (Alcock et al., 2000), deeper investigation of this circuitry could an interesting point for further insights into *Foxp2* functions.

Cocaine sensitization is a model for drug addiction behaviors in rodents. Addiction can be seen as a form of strong habit formation, which is underlined by information processing flow in striatum: ventral to dorsal, which mediates motivational processes (Pierce and Kalivas, 1997; Kalivas, 2009), but also medial to lateral striatum, which are implicated in habitual and compulsive aspects of drugs administration (Porrino et al., 2007; Steiner, 2010). Enhancement of locomotion (ambulation along the wall of the activity chamber without interruption for extended periods of time) is considered to be one of several forms of stereotypies which develop during chronic psychostimulant treatment (in Steiner 2010: Cotterly et al., 2007; Unal et al., 2009). Behavioral stereotypies can be interpreted as a selection and switching dysfunction of motor programmes in cortico-basal ganglia-cortical loops (at Stainer 2010: Redgrave and Gurney, 2006). The link between *Foxp2* and the information flow within striatal areas has been recently demonstrated. The switching process between dorsomedial and dorsolateral striatum has been shown to be at the core of accelerated switch between declarative (conscious, goal-directed) and procedural (unconscious, habit-formation) learning in humanized *Foxp2* mice. This ability was accompanied by alterations of DA levels and cortico-striatal plasticity within the two distinct areas of the striatum (Schreiweis et al., 2014). Alterations in cortico-striatal plasticity and associated habit formation (i.e. motor learning) are prominent phenotypes of *Foxp2 mutant* animals (Groszer et al., 2008; French et al., 2012) and is further supported by research in our laboratory with respect to impaired synaptic transmission specifically in ventral striatal (NuAc) medium spiny neurons (Mombereau, submitted). While collectively this data draw attention primarily to striatal activity alterations on the background of *Foxp2* deficiency in the whole brain, locomotor activity enhancement in *Foxp2* cortical knockout animals (**Fig.23**) could be indicative of DA-dependent cortical-driven, presynaptic plasticity alterations in the striatum. Of note, cortical projection neurons of lower layers show hyper excitable

phenotype in *Foxp2* heterozygous mice (LeMagueresse, unpublished). Moreover, the enhanced ‘learning’ curves in the sensitization induction phase, but not during expression, remarkably resemble the learning pattern of *Foxp2* humanized mice in declarative-procedural paradigm, when the differences were only visible in the acquisition phase, but not during overtraining (Schreiweis et al., 2014). Given that many aspects of goal-directed behaviors are mediated by PFC (Hasselmo, 2005; Amodio and Frith, 2006) and that humanized *Foxp2* specifically affects cortico-striatal circuits (Reimers-Kipping et al., 2011), the possibility of an implication of cortical *Foxp2* in mechanisms underlying declarative learning and habit-formation is intriguing.

Reduced social approach behaviors in animals with *Foxp2* cortical deficiency

DA mediated reward signaling in the NuAc is crucially involved in social behaviors such as attachment, social play or social interactions in diverse rodent species (Insel and Fernald, 2004). Thus we assessed *Foxp2* NuAc knockout mice in a three-chamber task which evaluates the interest for social approach and social novelty. In the social approach test control mice spent significantly more time in a chamber containing an unfamiliar mouse as compared to a chamber with an unfamiliar object whereas *Foxp2* NuAc knockout did not display such preference; moreover, *Foxp2* NuAc knockout mice did not show preference for the unfamiliar mouse *versus* the familiar (**Fig. 10D**). Lack of social motivation in NuAc KO animals was further confirmed in social conditioned place preference test (Mombereau et al., submitted).

In contrast, *Foxp2* cKO mice did not show differences in the three-chamber social behavior test (**Fig. 10D**). This could have indicated either that cortical *Foxp2* was not involved in general social deficits, or that social difficulties controlled by the cortex could have been of more subtle nature and impair only specific social behaviors.

Social cognition abnormalities are sensitive to misbalance in DA, norepinephrine and cholinergic systems in PFC and could be detected in social interaction tasks upon manual or automated scoring of more fine-grained features (Coura et al., 2013; Woehr and Scattoni, 2013).

Therefore I assessed cKO in three types of social interaction dyads: same sex male-male and female-female, and courtship interaction, male-female. Manually obtained data on social female-female and male courtship interactions did not suggest abnormalities (*Results*). Male-male interactions, however, were subjected to automated screening for 29 types of social behaviors and that approach revealed a number of subtle but significant alterations (**Fig. 24, 25**). Overall resident cKO mice tended to reduce the time of the close distance and showed less following and chasing behaviors. Moreover, they demonstrated higher probability of escape behaviors when a WT conspecific was trying to approach or interact and seemed to display changes in decision-making (as seen by occurrence of stop behaviors) in the situation when conspecific was located behind (**Fig. 25**).

The absence of social abnormalities in male-female and female-female tests could indicate different sensitivities of the tests used. In this context it maybe noteworthy that *Foxp2* expression in sex-specific manner and its influence in communicative vocal behaviors have been described (Hamson et al., 2009; Bowers et al., 2013; Bowers et al., 2014).

While the behavioral repertoire of cKO males does not change, the difficulties they experience in the presence of another male mouse could lie within the domain of behavioral flexibility. In rodents flexibility is accessed in distinct paradigms entailing shifts between rules, strategies, or attentional sets and is dependent on neural circuits incorporating mPFC and NuAc. Whereas general impairments of flexibility are associated with D2 receptor activity, more complex forms, like strategies shifts, rely on DA signaling within D1R circuitry (Haluk and Floresco, 2009). Since D1R is enriched in lower cortical layers and colocalize with *Foxp2* in the cortex (Hisaka et al., 2010) and NuAc (Mombereau et al., submitted) the attention-set shifting approaches could be beneficial for clarifications of cortical deficiencies in *Foxp2* mutant animals (Colacicco et al., 2002).

In order to confirm close contact interactions differences I have blindly scored for the decrease in paw control shown by cKO males. Besides close approach deficits, dramatic reduction in this behavior (**Fig. 24B**) could indicate decrease in dominance and aggressivity (Coura et al., 2013) - behaviors that are at the base of hierarchical and territorial establishment. Territorial behaviors have a profound impact on animal survival

and reproductive success - the driving force of evolution. Dominant status determination between two mice have been associated with the synaptic strength of mPFC inputs and can be robustly measured with the tube test (when two mice compete for the exit from the tube)(Wang et al., 2011). it is important to take into account that while social behavior is often studied in pairs under artificial conditions it may be insufficient to capture group hierarchy. Models that include third-order interactions have been recently elaborated and give promising and more accurate descriptions of the group interactions (Shemesh et al., 2013).

Finally, oro-genital contacts exhibited by tested cKO mice were reduced (**Fig. 24A**). Oro-genital contacts represent a measure of interest in urinary scents (containing steroidal pheromones) from other mice. Scent behaviors is an etiologically valid measure of communication in mouse species and are used to access social motivation and olfactory communication abnormalities. It often correlates with other forms of communication, such as USV during male-female interaction (Silverman et al., 2010; Wohr et al., 2011; Wohr and Scattoni, 2013). As opposed to human (vocal) communication, mouse communication systems could be preferentially based on other sensory modalities (like, olfaction), thus studies on *Foxp2*-dependent communication could be deepened in this perspective.

Modulation of vocal communication in mice carrying a cortical *Foxp2* deletion

Wild type conspecifics spent longer time in oro-genital contact with cKO males, than with their WT littermates (**Fig. 24A**). Additionally, two other behaviors showed a trend for augmentation: time the WT intruder was spending behind cKO resident and back-to-back posture (*Results*). The positioning behind the resident, followed by the high probability of stop behavior (**Fig. 25**), and oro-genital sniffing, may be explained as an enhanced interest towards the socially abnormal partner. Back-to-back is a posture which could reflect a risk-prone behavior and is potentially associated with an enhanced vigilance pattern (de Chaumont et al., 2012a). This altered perception by an interaction partner suggests that cortical *Foxp2* may be involved in biologically relevant social functions, which may incorporate distinct forms of communication, such as USV.

To explore the USV structure of *Foxp2* cKO animals we have analyzed vocalizations recorded during all 3 types of social interactions. It has been previously reported that USV abnormalities in adult mice do not display dramatic changes in basic parameters neither upon cortical lesions, nor in *Foxp2* heterozygous mice (Arriaga, 2011) or in mice which lack large parts of the cortex (Hammerschmidt et al., 2015). Thus, based on previous reports we have assessed several parameters: including syllable classifications, phrasal temporal structure and power of vocalizations through the frequency spectrum (**Fig. 13, 26**).

We observed that vocalizations were modified in different dimensions in function of the social context: male courtship songs were modified at the level of distinct syllable types, while the song emitted during same sex social interactions showed a tendency for temporal organization differences within a phrase, but not within the syllables types (**Fig. 27, 28**). These observations suggest two intriguing possibilities 1) *Foxp2* during song production may act in activity-dependent manner, as it does in Area X in songbirds during singing (Teramitsu and White, 2006) or in NuAc after cocaine stimulations (Mombereau et al., submitted), 2) mouse vocalizations could be voluntarily modified. The latter is supported by a recent study on male courtship song variation in dependence of female proximity: the song is more elaborate when male is stimulated with female pheromones, but female itself is not present (more distant) (Chabout et al., 2015).

Male courtship song alterations of *Foxp2* cortical knockout animals is interesting to compare to results obtained on *Foxp2*^{R552H/+} male mice (Arriaga, 2011). Therefore, in our analysis, among other calls classifications, we have also used the one employed in this study. The altered spectral characteristics of *Foxp2* heterozygous males were detected in the most prevalent type of calls without jumps (type A in Arriaga, 2011), whereas we did not observe any difference in cKO's simple calls. However, we did notice similar tendencies in frequency range (Arriaga: 'singing over a more restricted frequency band') of more complex calls with jumps of types D (or B), DU (E), DDU (G), UDUD (H). This exact data is not presented because in a course of the analysis we realized that the decreased frequency range is in fact specific to the complex calls with jumps of particular 'height' (5-20kHz)(**Fig. 27**) and thus, the differences of range in the

complex calls with these frequency jumps are revealed across all the other classifications. Arriaga has also revealed that specific spectral characteristics of simple calls (starting, $\frac{1}{4}$ and minimum frequency) are increased in heterozygous *Foxp2* mutants, while we found these tendencies to be decreased and, again, only in the complex calls - types D(B), DD(D), DU(E), DDU (G) (data not shown because these fine call's characteristics are collectively falling into the parameter 'range', which is described just above). Arriaga's analysis of frequency range is restricted to the type A calls, thus we do not know whether complex syllables were affected in *Foxp2*R552H/+ males.

The discrepancy between *Foxp2* heterozygous and cKO animals in regulation of the range of simple calls is interesting and suggests that modulation of simple calls is not under *Foxp2*-dependent cortical control. Another possibility is that this difference may derive from experimental protocol: males in Arriaga study were exposed to female urine, while cKO males were recorded during direct interaction with females. We know that complexity of calls modulation varies under these two conditions (Hanson and Hurley, 2012; Portfors and Perkel, 2014; Chabout et al., 2015).

While Arriaga reports no significant differences in proportion of calls types, we registered an increase in the type D and decrease of DU in cKO. The change of complex calls fraction was also detectable in Enard's classification (short calls with jumps) (**Fig.27**). Guo and Holy (2007) demonstrated that percentage of D and (H)DU (H from 'high') syllables is different in male songs in response to female or male urine presentation. When wild type male mice sung to male stimuli, they demonstrated a significant increase in D and decrease in HDU percentage, similar to alterations we observed in *Foxp2* cKO males courtship song. This observation raises the question, whether *Foxp2* cKO males modulate song differently when exposed to females. Importantly, the motivation for sexually oriented vocalizations was not altered in cKO, since they produced a high amount of calls comparable with WT littermates. Call rate is a crucial parameter that distinguishes female-oriented *versus* male-oriented USVs (Guo and Holy, 2007). Thus, the defect is unlikely due to failure of olfactory discrimination of female cues.

Overall, these subtle alterations of male sexually-driven vocalizations suggest, that it would be important to study social interaction in more detail in freely interacting group

housed colonies to identify behavioral parameters which more robustly may reflect the impact of *Foxp2* on for instance mate preference (Pasch et al., 2011; Musolf and Penn, 2012).

Cortical *Foxp2* may play a role in territorial, hierarchical and/or social recognition vocalizations during same-sex interactions (Musolf and Penn, 2012; Portfors and Perkel, 2014). Our data on male-male and female-female interactions suggest possible alterations of correlation patterns of call and pause duration within a phrase (**Fig. 28**). Biological significance of these alterations should be confirmed; however, duration (and frequency) modulations have been shown to be important parameters for acoustical recognition in mice (Liu et al., 2003; Uematsu et al., 2007).

RNA profiling results suggest deregulation of genes involved in social cognition and behavior

The model used for RNA profiling in the cortex is rather different from the one employed in behavioral and morphological experiments: instead of homozygous cortical knockout, we have utilized the cortices from *Foxp2* heterozygous animals. The nonsense mutation in these mice effectively leads to monoallelic *Foxp2* expression, and thus allows for studying the consequences of *Foxp2* half-dose effect. This mutation is similar to the one described in a second family with SLI (MacDermot et al., 2005). We have performed patch clamp experiments in our team on Ntsr1-GFP⁺ neurons. They showed a strongly abnormal hyperexcitability of mature cortico-thalamic neurons in the cortex of *Foxp2* heterozygous mutant mice (LeMagueresse, unpublished). Thus these mice represent a valid model to identify *Foxp2* regulated gene networks. .

The RNA differentially expressed in the cortex of *Foxp2* heterozygous males specifically in lower cortical layers of cortico-thalamic projection neurons and overall the cortex was subjected to GO analysis for the general functional annotation and CSEA tool for cell type specific enrichment.

GO analysis revealed differentially expressed genes forming biological themes which have been described in previous FOXP2 functional genomics studies (**Fig. 30**). Transcripts from functional groups such as ‘developmental process’, ‘cell proliferation’, ‘reproduction’, ‘growth’, ‘death’ and ‘cell projection’ are consistent with the role of

Foxp2 in corticogenesis. Findings related to this well characterized Foxp2 function could reflect Foxp2 dosage diminution in cortical progenitor cells in Foxp2^{+/-} mice, and constitute a useful internal control for our experimental protocol.

Differentially expressed genes appear to be enriched in neurons of lower cortical layers and non-neuronal cellular population: astrocytes, oligodendrocytes and immune cells (**Fig. 31**) While the latter seem to be related to both fractions, input and IP, neuronal markers are prevalently restrained to IP (**Fig. 31B**). While further validation of these results is necessary, primarily by enhancing of the sampling size (n=3) and subsequent *in situ* validations, these observations is prone to the speculation on the influence of cortical Foxp2 on cell identity specification, which could occur in corticogenesis. This is possible as well that these alterations were secondary to Foxp2 alterations, and reflect 'environmental' reaction to molecular or cellular disbalances within the brain.

Nonetheless, it is worth to mention that Hillard et al. (2012), when analyzing gene modules enriched during singing in songbirds, noticed that all of them were enriched in astrocyte markers. While it is difficult to suggest an interpretation for the exact role for astrocytes in these behaviors, it is known that astrocytes respond to neuronal activity and can be involved in cognitive impairments (Halassa and Haydon, 2010). Also, the enrichment in immune response, activated microglia, and neuroglial markers was noted in neurological disorders, particularly in ASD (Voineagu et al., 2011; Xu et al., 2014; Zeidan-Chulia et al., 2014).

Among interesting genes downregulated in the cortex of Foxp^{+/-} males was Htra1. This gene is expressed in caudal cortex during embryogenesis (Elsen et al., 2013), and is regulated by the same microRNAs as *Foxp2*, thus suggesting that they may together constitute a network important for cellular specification in cortical neurogenesis (Clovis et al., 2012). The functional engagements of Htra1 lie within maturation, development and radial glia proliferation and, together with astrocyte markers, represents a signature of sleep deprivation and following cognitive abnormalities (De Luca et al., 2004; Thompson et al., 2010; Nigro et al., 2012; Hines et al., 2013).

Of note, Tbr1 and Ctip2, both did not show any misregulation in Foxp2^{+/-} cortices, which is in agreement with morphological data obtained on *Foxp2* cKO.

One of the most relevant genes differentially regulated in IP fraction was *Mint2* (*Apba2*) (**Table 3**). *Mint2* is directly associated with ASD and schizophrenia (Kirov et al., 2008; Babatz et al., 2009) and shows signs of adaptive gene-culture co-evolution within human species (Williamson et al., 2007; Laland et al., 2010).

I have examined its expression in the cortices of *Foxp2* homozygous mutants – cKO. The downregulation levels in the cortex of cKO were comparable to those in *Foxp2*^{+/-} (**Table 3**)(**Fig. 32**). These results suggested that *Foxp2*-dependent regulation of *Mint2* may happen in postmitotic neurons, during later phases of cortical maturation. Also, diminution of *Mint2* could contribute to social behavior abnormalities observed in *Foxp2* cortex-deficient mice, since the gene was shown to be involved in motivational and social mouse behavior (Sano et al., 2009).

It would be important to assess other molecular alterations observed in the BacTRAP study in cKO. With this respect it is also important to keep in mind that the crucial differences may not be visible on the single gene levels (only), but rather engage the functional categories as a whole, as it was shown for dorsomedial and dorsolateral striatum of homozygous *Foxp2* humanized mice (Schreiweis et al., 2014)

General conclusions

1. *Foxp2* is co-expressed with *Tbr1* in subpopulations of layer 6 neurons. It suggests a differential effect of *Foxp2* deficiency on specific microcircuits involved in social behavior and cognition.
2. *Foxp2* homozygous deletion in early postmitotic neurons does not cause changes in gross neuronal morphology or projections in lower layer cortical neurons
3. Mice carrying cortical *Foxp2* deletions show subtle abnormalities in social approach behavior.
4. Mice carrying cortical *Foxp2* deletions display changes in ultrasonic vocalizations in male-female interactions.
5. Cortical *Foxp2* deficiency causes dysregulation of *Mint2*, a gene involved in autism in humans.

Future directions

The aim of this study was to generate new hypothesis about the function of cortical Foxp2 in social cognition and behavior. Morphological, behavioral and molecular analyses suggest that Foxp2 loss of function in the mouse cortex causes subtle phenotypes. Based on the present study, I propose following experiments to provide further insights into cortical Foxp2 functions:

Morphological studies

One strategy to describe exact morphological, projection and electrophysiological properties of Foxp2⁺ neurons in the cortex could be relying on the recently described cortical enhancers located in proximity to *FOXP2* (Visel et al., 2013). By cloning of putative enhancers to the reporter construct, followed by *in utero* electroporation in the developing mouse cortex (Wang and Mei, 2013), it could be possible to label and analyze Foxp2⁺ neurons specifically.

Identification and analysis of layer 5 cortical neurons, projecting to the larynx. This can be achieved by retrolabeling of laryngeal muscles with a multisynaptic tracer (Arriaga, 2011; Arriaga et al., 2012). While injection of the tracer to the two most exposed muscles of the vocal organ is by now achievable, the tracer which could satisfy the required safety and timing conditions is under investigation. The next steps after successful labeling of layer 5 motor cortical layers would be a confirmation of their Foxp2-positivity, followed by laser capture microdissection to investigate their molecular properties after socially-directed singing and dopaminergic stimulation.

Molecular studies

ChIP experiments to define direct Foxp2 cortical targets. In order to determine, which genes may represent direct as compared to indirect Foxp2 transcriptional targets, chromatin immunoprecipitation experiments are needed. The crucial condition for successful ChIP experiments is a specific antibody with a high affinity to the protein of interest in functional conformation (Foxp2 bound to DNA). In order to obtain such an antibody I have designed and produced two clones of potential peptides exposed within

Foxp2 N-terminal domain. The obtained antibodies are currently under analysis. Their high affinity to Foxp2 protein was demonstrated by the IP results; however immunohistochemistry did not confirm their specificity. As alternative we may generate mice carrying a tagged *Foxp2* allele.

Mint2 in an interesting candidate Foxp2-dependent regulation in the function of social behavior. While the analysis of BacTRAP sequencing results is ongoing, *Mint2* is the first gene revealed to be directly associated with ASD and thus, gives a promising mechanistic insights into social abnormalities observed in *Foxp2* cKOs. Therefore, it could be interesting to first, study its expression overlap with Foxp2, followed by characterization of affected neurons and targeted rescue experiments performed *in vivo* and *in vitro*.

Behavioral studies

Higher order social interactions in Foxp2 cKO mice could be beneficial using the recent advances in behavioral analysis in social groups (Shemesh et al., 2013).

Brain structures involved in Foxp2-cortical activity dependent circuitry could be revealed by pharmacological and social stimulation followed by immediate early genes mapping in Foxp2 cKO and heterozygous animals. As Foxp2 largely colocalizes with D1R in mesolimbic and social reward system (Campbell et al., 2009; O'Connell and Hofmann, 2011b, a) the approach could be combined with BacTRAP molecular profiling of affected structures. D1-bacTRAP mice are an available well established model (Heiman et al., 2008).

Behavioral tests assessing timing perception and performance abnormalities in VTA-mPFC circuits. Tests of impulsivity and temporal control paradigms are well established for the laboratory mice (Loos et al., 2009; Narayanan et al., 2012).

Playback of Foxp2 cKO male's USVs to wild type females could be useful to determine ecological significance of the subtle differences in vocalizations observed during male-female interactions in the current work. This paradigm is well established for the rodents (Uematsu et al., 2007; Willuhn et al., 2014).

Publications supporting the thesis

Medvedeva VP, Rieger MA, Mombereau C, Ziegenhain C, Vieth B, French CA, Nicod J, Cressant A, Benecke AG, Enard W, Fisher SE, Granon S, Dougherty JD and Groszer M. Altered social behavior in mice carrying a cortical Foxp2 deletion. *In preparation*

Mombereau C, Meye FJ, **Medvedeva V**, Ghosh T, David EB, Planchon M, Garcia M, French CA, Enard W, Fisher SE, Girault JA, Hervé D, Ragoussis J, Shifman S, Mameli M, Groszer M. Foxp2 in the nucleus accumbens regulates reward signaling and social behavior. *Submitted*

Kraushar M, Viljetic B, Wijeratne S, Thompson K, Jiao X, Pike J, **Medvedeva V**, Groszer M, Kiledjian M, Hart R, and Rasin MR. Thalamic WNT3 secretion spatiotemporally regulates the neocortical ribosome signature and mRNA translation to specify neocortical cell subtypes. *Journal of Neuroscience, accepted*

Altered social behavior in mice carrying a cortical Foxp2 deletion

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ABSTRACT

Genetic disruptions of the forkhead box transcription factor FOXP2 in humans cause an autosomal-dominant speech and language disorder. FOXP2 expression pattern and genomic structure are highly conserved in distant vertebrates. Here we began to characterize mice carrying a homozygous cortical Foxp2 deletion. The gross morphological architecture and postnatal development of mutant mice was undistinguishable from wildtype littermates. However, unbiased behavioral profiling of adult mice demonstrated abnormalities in specific social behaviors such as approach behavior towards conspecifics as well as reciprocal responses of wildtype interaction partners. Furthermore, mutant mice showed alterations in specific acoustical parameters of ultrasonic vocalizations (USV), and the type of modulation differed in function of social context.

Gene expression profiling of cortical pyramidal neurons in Foxp2 mutants revealed the dysregulation of Mint2, a gene involved in approach behavior in mice and autism spectrum disorder in humans. The study suggests that cortical Foxp2 regulates social behaviors in mice and provides a rational basis for mechanistic studies of ancestral cortical functions of this gene that may have been recruited during human evolution.

The paper is to be submitted by the defense date (17/6/2015)

Title: Foxp2 in the nucleus accumbens regulates reward signaling and social behavior

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Abstract:

The transcription factor FOXP2 has been implicated in speech and language development. The neuronal expression patterns of FOXP2 are highly similar across different vertebrate species suggesting conserved roles in neural circuits underlying social cognition and behavior. Here we demonstrate that Foxp2 in medium-sized spiny projection neurons in the nucleus accumbens in mice regulates dopamine-related signaling, reward-associated synaptic plasticity and social behavior. These results suggest that Foxp2 has crucial functions in neural circuits mediating social motivation. In humans these circuits may make important contributions to speech and language development.

Introduction

Speech and language are acquired in the first years of life without formal instructions in the context of close social interactions ^{1, 2}. The biological basis of this capacity and its evolutionary history remain largely unknown. Genetic analyses of speech and language disorders are beginning to provide valid entry points to study underlying cellular and molecular mechanisms in model systems ^{3,4}.

Several rare mutations of the gene encoding the forkhead-box transcription factor FOXP2 have been implicated in a speech and language disorder ⁵. Initially a heterozygous *FOXP2* missense mutation had been identified in the three generation KE family ⁶. The mutation yields a substitution (R553H) within the forkhead-box protein domain which in turn disrupts DNA binding. Affected family members show impaired learning of orofacial motor sequences (developmental verbal dyspraxia) and wide-ranging receptive and expressive language deficits. Neuroimaging studies of KE family members have pointed to abnormalities in cortico-basal ganglia-thalamo-cortical circuits involved in learning and producing complex motor behaviors. The presence of core deficits in neuronal circuits upstream from the motor system and their impact on speech and language development in the KE family however remain key areas of investigation ⁷.

The FOXP2 protein is highly conserved and is expressed in homologous brain regions in humans and mice, notably in striatal medium-size spiny neurons (MSNs), cortical layer VI and few layer V projection neurons, cerebellar Purkinje cells and thalamic nuclei ⁵. Throughout the striatum, levels of Foxp2 are high in type 1 dopamine receptor expressing (D1R⁺) MSNs and low in D2R⁺ MSNs ⁸. This expression pattern emerges

during embryogenesis and is maintained in the adult brain suggesting roles for Foxp2 in both, neuronal development and function.

Functional studies indicate that FOXP2 has transcriptional suppressor activities and regulates the expression of genes involved in various processes including neurite outgrowth and synaptic plasticity⁸⁻¹¹. Several direct transcriptional FOXP2 target genes are strongly implicated in neurodevelopmental syndromes such as autism spectrum disorder (ASD) or specific language impairment (SLI) suggesting its participation in gene networks with important functions for social cognition and behavior¹²⁻¹⁵.

To study Foxp2 functions we previously employed several mutant mouse lines. The *Foxp2-R552H* line carries a point mutation which is identical to the one found in the KE family while the *Foxp2-S321X* line is effectively a knockout¹⁶. In both lines, homozygous mutants show severe growth deficits and die about 3-4 weeks of age whereas heterozygotes develop normally without overt morphological brain abnormalities. Initial studies in these mice focused on dorsal striatal circuits due to their established functions in motor behavior. Electrophysiological recordings from *Foxp2-R552H* heterozygous mice (*Foxp2^{R552H/+}*) revealed a deficient long-term depression in the dorsal striatum, in line with their impairments in learning to run on rotating wheels and rods¹⁶.

In the present study we investigated the role of Foxp2 in ventral striatal MSNs which are part of forebrain circuits with crucial functions in motivation and reward processing¹⁷⁻¹⁹. Dopamine (DA) neurons projecting from the midbrain play critical roles in these processes by modulating neuronal excitability and synaptic plasticity²⁰. In striatum and cortex nearly all Foxp2⁺ neurons co-express DARPP-32 (dopamine- and cAMP-

regulated phosphoprotein 32 kDa), a key DA signaling regulator and *Foxp2* heterozygous mutant mice show significantly increased striatal DA levels ^{21, 22}.

Results and Discussion

To assess a potential contribution of *Foxp2* to DA related signaling and reward-associated learning, we used the psychostimulant drug cocaine, which exerts potent locomotor-stimulating and rewarding effects by enhancing DA transmission. Following injection of a single dose of cocaine (15 mg/kg; i.p.), we found that drug-induced locomotor activity was significantly attenuated in *Foxp2*^{R552H/+} and *Foxp2*^{S321X/+} animals as compared to *wild-type* littermates, suggesting a decreased sensitivity to acute stimulation of DA receptors (Figure 1A and 1B). To study a potential role of *Foxp2* in reward-associated learning we employed a conditioned place preference test which provides an indirect measure of cocaine reward. We found that *Foxp2*^{R552H/+} mice spent significantly less time than *wild-type* littermates in an environment previously associated with cocaine administration, suggesting that *Foxp2* deficiency impairs reward-associated learning (Figure 1C).

A well characterized signaling mechanism by which cocaine exerts its long-term behavioral effects is the phosphorylation and activation of extracellular signal-regulated kinase (ERK) signaling specifically in D1R⁺ MSNs ^{23, 24}. To assess D1R⁺ MSN specific Erk activation in different striatal regions, *Foxp2*^{R552H/+} mice were crossed to bacterial artificial chromosome (BAC) transgenic mice expressing enhanced green fluorescent protein in D1R⁺ MSNs (*Drd1a::eGFP*). Following a single cocaine injection the number of phospho-Erk⁺ D1R⁺ MSNs in the dorso-lateral (DLS) and dorso-medial striatum (DMS) were similar

in *Foxp2*^{R552H/+} mice as compared to *wild-type* littermates. In contrast, the number of phospho-Erk⁺ MSNs in the nucleus accumbens (NAc) was significantly lower in *Foxp2*^{R552H/+} mice as compared to *wildtypes* (Figure 1D and 1E), suggesting that DA related signaling in D1R⁺ MSNs in the NAc depends on two functional *Foxp2* alleles.

DA neurons in the ventral tegmental area and their main target regions in the NAc and prefrontal cortex, which normally mediate the associative learning of natural rewards, are considered to be major sites of action of cocaine²⁵. The NAc is particularly implicated in reward-associated learning. It is thought that phasic DA release in the NAc assigns motivational value to biologically relevant stimuli resulting in the selection and production of adaptive motor output. We therefore explored the contribution of *Foxp2* expression in the NAc to reward-associated learning. To this end we deleted *Foxp2* in the NAc in adult mice carrying *Foxp2*^{lox/lox} alleles (*Foxp2*^{NAcKO}) (Figure 1F). Bilateral stereotaxic injections of adeno-associated virus (AAV) expressing Cre recombinase/green fluorescent fusion protein (AAV-CreGFP) yielded a ~90% reduction of *Foxp2*⁺ neurons in the NAc as compared to controls injected with AAV-GFP (Figure 1-figure supplement 1). We found that unlike *Foxp2* heterozygous mutant animals, *Foxp2*^{NAcKO} mice showed normal motor learning behavior on the accelerating rotarod (Figure 1-figure supplement 2). However, similarly to *Foxp2* heterozygotes, the *Foxp2*^{NAcKO} mice displayed a significantly attenuated locomotor response following a single cocaine administration as well as impaired cocaine conditioned place preference (Figure 1G and Figure 1H). As an additional control we studied mice carrying a cortex-specific *Foxp2* deletion (*Nex-Cre*^{+/-}; *Foxp2*^{lox/lox} or *Foxp2*^{CtxKO}) (Figure 1I). *Foxp2*^{CtxKO} mice and control littermates did not show differences in motor learning (Figure 1-figure supplement 3) or locomotor responses following a single

cocaine administration (Figure 1J). These results suggest that Foxp2 in the NAc of adult animals is important for cocaine-induced responses and behavioral adaptations.

A neuronal activity-dependent role for FoxP2 previously emerged from studies in songbirds. Here FoxP2 is highly expressed in Area X, a basal ganglia nucleus required for vocal learning. FoxP2 expression in Area X correlates with song plasticity and FoxP2 knockdown causes song learning deficits^{26,27}. We tested whether a single cocaine injection altered Foxp2 expression in the mouse striatum. Using quantitative PCR and immunoblots on striatal tissue samples one hour following cocaine administration, we detected a downregulation of Foxp2 mRNA and protein levels in the NAc but not in the DLS or DMS (Figure 2A and Figure 2B). Furthermore time course experiments showed that the downregulation of Foxp2 protein in the NAc following cocaine administration was transient, and returned to pre-injection levels within three hours (Figure 2A). To further assess the signaling mechanisms of Foxp2 downregulation, we co-stimulated striatal neurons in primary cultures with glutamate and D1R agonist. Whereas neither glutamate nor D1R agonist alone was sufficient to alter Foxp2 protein levels, co-administration significantly decreased them (Figure 2C). These data suggest that Foxp2 functions as a DA and glutamate-regulated transcriptional modulator in NAc MSNs.

To gain further mechanistic insights into the roles of Foxp2 in NAc functions we investigated synaptic transmission and plasticity. Modulation of synaptic strength at excitatory inputs onto MSNs has been shown to be crucial for NAc functions during reward and cocaine-evoked behavioral adaptations. A single cocaine injection potentiates excitatory synapses specifically onto D1R-MSNs in the NAc and occludes the expression of long-term potentiation (LTP). Depotential of cortical afferents restores synaptic

transmission as well as cocaine-induced behavioral adaptations, suggesting that cortico-D1R MSN LTP has a critical role in reward-associated learning²⁸. Therefore we studied whether Foxp2 deficiency affects excitatory transmission and plasticity onto D1R-MSN by employing *Foxp2*^{R552H/+}; *Drd1a*::eGFP mice for whole-cell patch clamp recordings from eGFP-positive D1R MSNs. We found that high-frequency stimulation (HFS) of excitatory afferents onto NAc D1R-MSNs elicited LTP in *wild-type* animals, whereas HFS was insufficient to produce LTP in *Foxp2*^{R552H/+} animals (Figure 3A). This failure of LTP expression might be either due to its impaired induction or because synapses are already maximally potentiated and consequently LTP is occluded. To further differentiate between these possibilities, we determined the ratio of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) to N-methyl-D-aspartate (NMDA) receptor (AMPA/NMDAR ratio) mediated evoked excitatory postsynaptic currents (EPSCs). We detected a significantly increased AMPAR/NMDAR ratio in *Foxp2*^{R552H/+} mice suggesting enhanced glutamatergic synaptic strength (Figure 3B). Recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs) revealed significantly increased amplitudes and a decreased frequency in *Foxp2*^{R552H/+} mice (Figure 3C). Given the postsynaptic Foxp2 expression, these results suggested an increased number of AMPA receptors and already enhanced synaptic strength in *Foxp2*^{R552H/+} mice. In line with the reduced frequency of mEPSCs, the number of synaptic spines on D1R MSNs in *Foxp2*^{R552H/+} mice was significantly reduced as compared to *wild-type* littermates (Figure 3D). In summary these experiments demonstrate that synaptic transmission in NAc D1R-MSNs in *Foxp2*^{R552H/+} mice is enhanced and occludes LTP, consistent with their deficits in reward-associated learning. This also agrees with *in vivo* multielectrode recordings in

Foxp2^{R552H/+} mice which detected an abnormally high basal firing activity of MSNs possibly resulting from enhanced neuronal excitability ²⁹.

The enhanced transmission in *Foxp2* heterozygous mutants may furthermore indicate an abnormal transcriptional regulation of neuronal activity regulated genes and an impaired neuronal homeostasis. To identify transcriptional alterations which might be involved in these physiological deficits, we performed mRNA profiling of NAc tissue from *Foxp2*^{S321X/+} mice and *wild-type* littermates one hour following single cocaine or saline injections. We identified 23 genes that were significantly upregulated in *Foxp2*^{S321X/+} mice in baseline (saline) conditions and became significantly upregulated in *wildtypes* following cocaine treatment (Figure 4A and 4C). Gene Ontology (GO) analysis showed that genes involved in calcium signaling pathways were significantly over-represented among these 23 upregulated transcripts, whereas no significant pathway over-representation was found for downregulated genes (Figure 4B and Figure 4-supplement figure 1). Among the calcium signaling-related genes, the T type, voltage-dependent calcium channel alpha 1G subunit (*Cacna1g*) has been genetically associated with autism spectrum disorder (ASD) ³⁰, while Cornichon homolog 3 (*CNIH3*) is an AMPAR binding protein that promotes receptor trafficking and synaptic transmission ³¹. Abnormal calcium channel signaling also emerged recently from genome-wide association studies as potential major contributor to the pathogenesis of several psychiatric disorders including ASD, further supporting the biological relevance of our observations ³². Quantitative PCR analysis of four calcium signaling-related transcripts confirmed their upregulation in *Foxp2*^{S321X/+} mice in baseline conditions (Figure 4D). Taken together the mRNA profiling experiments suggest an

abnormal expression of neuronal activity related genes in the NAc of *Foxp2* mutants which is consistent with disturbed neuronal homeostasis and synaptic plasticity.

DA signaling in the NAc has critical roles in the motivating and rewarding aspects of fundamental social behaviors such as parental care or mating^{33, 34}. We assessed maternal and sexual behavior in *Foxp2*^{NAcKO} animals. We found no significant differences between primiparous *Foxp2*^{NAcKO} females and controls in the latency to retrieve their pups when removed from the nest (Figure 5A). Likewise when sexually naive *Foxp2*^{NAcKO} or control males were mated with females, no difference in the number of vaginal plugs was observed suggesting similar sexual motivation (not shown).

DA mediated reward signaling in the NAc is also crucially involved in social behaviors such as attachment, social play or social interactions in diverse rodent species³³. We assessed *Foxp2*^{NAcKO} mice and control littermates in a three-chamber task which evaluates the interest for social approach and social novelty. In the social approach test control mice spent significantly more time in a chamber containing an unfamiliar mouse as compared to a chamber with an unfamiliar object whereas *Foxp2*^{NAcKO} animals did not display such preference (Figure 5B). When the object was replaced with an unfamiliar mouse, *Foxp2*^{NAcKO} mice did show a preference for the novel mouse compared to the familiar mouse, though they interacted significantly less time with the novel mouse as compared to controls (Figure 4B). In contrast *Foxp2*^{CtxKO} mice and control littermates did not show differences in the three-chamber social behavior test (Figure 5C). To further assess social motivation in *Foxp2*^{NAcKO} mice we employed a social conditioned place preference test. We found that *Foxp2*^{NAcKO} mice did not display a preference for an environment previously associated with social interaction as compared to an environment

in which they had been isolated (Figure 5D). These deficits appear not due to increased anxiety or general impairments in novelty detection, since mutant mice performed normally in open-field tests (Figure 5-figure supplement 1) or novel object recognition tasks (Figure 5-figure supplement 2). Taken together these results indicate that Foxp2 in the NAc supports socially motivated behaviors in adult mice. However Foxp2 deficiency is not associated with general social deficits but rather might impair specific social behaviors.

In summary we demonstrate that Foxp2 in D1R⁺ MSNs in the NAc is required for reward-associated synaptic plasticity and social behavior. These neurons are part of evolutionary highly conserved neuronal networks for social decision making and might have been recruited for speech and language development ³⁵. Recent studies in autism spectrum disorder suggest that reward processing abnormalities specific to social stimuli in the first few years of life might impair the later development of complex social cognition and language ³⁶. Thus Foxp2 transcriptional regulation in neuronal circuits mediating reward signaling might significantly contribute to social brain development and function.

Materials and methods:

Mice:

Foxp2^{R552H/+}, *Foxp2*^{S321X/+} and *Foxp2*^{lox/lox} animals were generated as previously described^{16, 37}. All mouse lines were on a C57BL/6J background. For electrophysiological experiment, BAC *Drd1a::eGFP* mice³⁸ were crossed with *Foxp2*^{R552H/+} mice. Cortical *Foxp2* knockout mice were generated by crossing *Foxp2*^{lox/lox} and *Nex-Cre* transgenic mice³⁹. Animals were maintained under a 12 h light/dark cycle, temperature was 22 ± 1°C and humidity 60 ± 5%. Food and water were supplied ad libitum. Animals were tested at the age of four to seven months. Experiments were performed in accordance with French (*Ministère de l'Agriculture et de la Fo rêt*, 87–848) and European Economic Community (EEC, 86–6091) guidelines for the care of laboratory animals.

Viral Infection:

Foxp2^{lox/lox} mice were stereotactically injected with adeno-associated virus expressing eGFP-Cre recombinase fusion protein or eGFP only (control animals) (AAV2/9.hSynap.HI.GFP-Cre.WPRE.SV40 and AAV2/9.hSynapsin.EGFP.rBG, Penn Vector Core, University of Pennsylvania, Philadelphia, PA) eighteen days before the start of behavioral procedures. AAV2/9 (0.3 µl) was infused at a rate of 0.1 µl/min via an infusion needle positioned in the nucleus accumbens [Paxinos coordinates: anteroposterior (AP), +1.8 mm; mediolateral, ±1.1 mm; dorsoventral, -4.5 mm].

Behavioral tests:

Cocaine-induced locomotor activity.

Cocaine induced locomotor activity was automatically recorded in cylindrical boxes (Imetronics). Mice were habituated to injections and the test apparatus for 2h for three consecutive days before the experiment. During these sessions, mice were injected with three saline injections (NaCl 0.9%; i.p.) before being placed in the apparatus as well as 60 and 120 minutes afterwards. On day four, mice were injected with saline at the beginning of the session and received cocaine injection (15 mg/kg; i.p.) 60 minutes later. Locomotor activity was recorded during the entire duration of the session (two hours).

Conditioned place preference to cocaine.

The conditioned place preference apparatus consisted of a Plexiglas Y-shaped apparatus (Imetronic) located in a soundproof testing room with low luminosity as described before⁴⁰. Briefly, two of the three arms of the apparatus were used for the conditioning and distinguished by different patterns on floors and walls, separated by a central neutral area. Time spent in each arm was measured using an electronic interface. On day one, mice were allowed to explore both arms for 18 min. These data were used to separate animals into

groups with equal biases for each side. From day two on, control and cocaine treated animals were paired with the environment over six days, each day for 25 minutes. The control group received injections (0.9% NaCl; i.p.) in both arms, whereas the treated group received cocaine injections (15 mg/kg; i.p.) in one and saline in the other arm. On the test day, mice were allowed to freely explore both arms and the time spent in each arm was recorded for eighteen minutes. Data were expressed as the time spent in the drug-paired arm minus time spent in the saline-paired arm.

Open field:

Mice were placed in the center of a 40 x 40 x 30 cm open field (white Plexiglass on the sides and the floor) illuminated with 600 lux for 30 min. The total distance traveled in the whole arena and time spent in the center (20 x 20 cm) was measured using an automated videotracking system (Viewpoint, Lyon, France).

Novel object recognition:

The object-recognition task consisted of two phases: the training phase and the testing phase. Before training, all mice were habituated to the experimental apparatus (a white square open field 50 x 50 x 50 cm) for 3 consecutive days with 15 min in the absence of objects. During the training phase (15 min), mice were placed back into the apparatus with two identical objects placed 20 cm apart. After a 24h retention interval, mice were placed back into the apparatus for the testing phase. Two objects were again present, but one of the familiar objects was now replaced with a novel one. Mice were again allowed to freely explore the environment and the objects for 15 min. All testing and training sessions were videotaped and analyzed by an individual blind for the genotype. A mouse was scored as exploring an object whenever any part of its body other than the tail was touching the object or whenever it was within 1 cm of the object and facing it. Measurement of the time exploring each object was recorded and expressed as the percentage of time spent exploring the novel object relative to the total time spent exploring both of the objects (discrimination index). The objects used in the test were previously screened in order to avoid any intrinsic preference.

Social interaction (three chamber test):

Sociability and preference for social novelty were measured in a three-chamber box (each chamber 300 × 150 × 150 mm) with a door (50 × 50 mm) in each delimiting wall. The time spent in interaction with objects or mice were measured by an individual blind for the genotype. The test is divided into four phases. During the habituation phase, the test mouse was placed into the central chamber, with the two doorways closed for 5 minutes. Afterwards the doors were opened allowing the mouse to visit either the side compartment containing an unfamiliar adult C57BL/6J mouse enclosed in a wire cage or the other compartment containing an empty wire cage (sociability phase). The time spent in interaction with each wire cage during 5 minutes was recorded. During the third phase

(social novelty), the test mouse was maintained in the chamber containing the social counterpart and a new unfamiliar C57BL/6J mouse was placed in the empty wire cage in the opposite compartment. The test mouse was then allowed to freely investigate the familiar or unfamiliar mouse. Again the time spent in interaction with each wire cage during five minutes was recorded and analyzed by an individual blind for the genotype.

Pup retrieval paradigm:

Maternal behavior was assessed using a pup retrieval paradigm as described ⁴¹. Pups were separated from their mother for 1h and kept warm in another cage. The mother was removed from the home cage and concomitantly three pups were placed in each corner of the home cage. The test started when the female was reintroduced into the home cage and the latency to retrieve each pups was measured by an observer blind for the genotype.

Social conditioned place preference:

Social conditioned place preference was conducted as described ⁴². Pairs of *Foxp2*^{NacKO} littermates or control littermates were housed together for three weeks, then socially isolated for 24h in individual clean cages containing normal bedding and nesting material. The next day, social conditioning started over three consecutive days with two conditioning sessions per day. Conditioning sessions consisted in reuniting or socially isolating pairs of mice for 20 min in one peripheral compartment of the three-chamber box containing either some aspen bedding or alpha dry bedding (Serlab, France). Each pair of mice was kept the same throughout the experiment and each mouse was reunited with the same familiar cagemate in order to assess the reward value of interacting with a familiar partner and to limit agonistic behaviors. As unconditioned control group mice were socially isolated during conditioning sessions and were reunited with their respective cagemate once per day for 20 min in a novel cage containing some normal bedding in order to receive an equivalent amount of experience with the unconditioned stimulus (social interaction) as the conditioned groups. All variables associated with the conditioning procedure were randomized and counter-balanced across the unconditioned and conditioned groups of mice. In order to decrease the anxiogenic effect of exposure to the three-chamber box, mice were allowed to explore the apparatus for 15 mins on the test day without the presence of conditioning cues. For the test session, mice were individually place in the central compartment of the apparatus and the time spent in each compartment containing the conditioned stimulus (bedding) was automatically measured using a videotracking system (Viewpoint, Lyon, France).

Accelerating Rotarod:

A computer-interfaced rotarod (LE8200, Bioseb, Chaville, France) was set to accelerate from 4 to 40 rpm over a 300s period. Mice were trained for three consecutive days, with one daily session consisting of four trials separated by 1h resting periods. Trials ended when mice fell from the rod or after 300s. The highest speed reached (rpm) was analyzed.

Electrophysiology:

Three-to four-weeks old *Drd1a-eGFP*; *Foxp2*^{R552H/+} and *Foxp2*^{+/+} mice were anesthetized (Ketamine/Xylazine) for slice preparation. Coronal 300- μ m slices were prepared in bubbled ice-cold 95% O₂/5% CO₂-equilibrated solution containing (in mM): cholineCl 110; glucose 25; NaHCO₃ 25; MgCl₂ 7; ascorbic acid 11.6; Na⁺-pyruvate 3.1; KCl 2.5; NaH₂PO₄ 1.25; CaCl₂ 0.5 and then stored at room temperature in 95% O₂/5% CO₂-equilibrated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124; NaHCO₃ 26.2; glucose 11; KCl 2.5; CaCl₂ 2.5; MgCl₂ 1.3; NaH₂PO₄ 1. Slices were kept at 32–34°C in a recording chamber superfused with 2.5ml/min ACSF. Visualized whole-cell voltage-clamp recording techniques were used to measure holding current and synaptic responses of D1R⁺ EGFP positive MSNs of the nucleus accumbens shell with the help of an upright microscope connected to a an LED source at 490 nm (Olympus France; CoolLed, UK). Currents were amplified, filtered at 5 kHz and digitized at 20 kHz and recorded at a holding potential of -70 mV (IGOR PRO, Wavemetrics, USA). Access resistance was monitored by a step of -10 mV (0.1 Hz) and experiments were discarded if the access resistance increased more than 20%. Miniature EPSCs were recorded in the presence of tetrodotoxin (0.5 μ M). The frequency, amplitudes and kinetic properties of these currents were then analysed using the Mini Analysis software package (Synaptosoft, Decatur, GA, USA). Synaptic currents were evoked by stimuli (60 μ s) at 0.1Hz through a glass pipette placed 200 μ m from the patched neurons. The internal solution for long-term plasticity experiments contained (in mM): 140 K-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Na₃GTP and 10 sodium creatine-phosphate. The internal solution for recordings of mEPSC and AMPA:NMDA ratio contained (in mM): 130 CsCl, 5 KCl, 10 HEPES, 1.1 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Na₃GTP and 10 sodium creatine-phosphate. All experiments were carried out in the presence of picrotoxin (100 μ M). LTP was induced by using the following HFS protocol: 100 pulses at 100Hz repeated 4 times at 0.1Hz paired with depolarization at 0mV²⁸. AMPA:NMDA ratios of evoked-EPSC were obtained by AMPA-EPSC +40 mV/NMDA-EPSCs at +40 mV as previously described⁴³. All drugs were obtained from Abcam (Cambridge, UK) and Tocris (Bristol, UK) and dissolved in water, except for TTX (citric acid 1%). Online/offline analysis was performed using IGOR-6 (Wavemetrics, USA) and Prism (Graphpad, USA). Compiled data are expressed as mean \pm s.e.m. Significance was set at p = 0.05 using Student t-test and the Kolmogorov-Smirnov (KS) test.

Spine analysis:

Drd1a-eGFP; *Foxp2*^{R523H/+} and *Drd1a-eGFP*^{+/-} mice were anesthetized and perfused transcardially with 40 ml of 1.5% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.5. Brains were post-fixed in 1.5% paraformaldehyde solution for 1h and sliced (150 μ m) using a vibratome (Leica). Slices were labeled by ballistic delivery of fluorescent dye DiI (Molecular Probes) as described⁴⁴. Primary dendrites from Drd1⁺ MSNs from the NAc shell were analyzed using confocal microscope (Leica SP5) with an oil immersion lens (x63). Z-stacks of 0.2 μ m depth intervals were used to reconstruct dendrites using 'Neuronstudio' software⁴⁵. Spine density was calculated on 30 to 40 μ m length sections of primary dendrites by an observer blind for the genotype.

Neuropharmacological experiments in primary striatal cultures:

Experiments were performed essentially as described earlier ⁴⁶. Timed-mated Swiss Webster dams were anaesthetized with pentobarbital (Day of plug: E0.5) and embryos were removed at E14.5. The ganglionic eminence (GE) was isolated and cells were suspended in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA), 500 nM L-glutamine, 60 µg/ml penicillin G and 25 µM β-mercaptoethanol (Sigma Aldrich, St.Louis, MO). 1,000,000 cells were plated into Nunc-six-well plates coated with 50 µg/ml poly-D-lysine (Sigma Aldrich). Following 10 and 90 minutes of pharmacological treatment, striatal neurons were lysed in 1% SDS (v/v), 1 mM sodium orthovanadate for Western blot analysis.

Immunohistochemistry:

Immunochemistry experiments were performed as described earlier ²³. Briefly, mice were rapidly anesthetized with pentobarbital and perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.5 (PB-PFA 4%). Brains were removed and postfixed overnight in the PB-PFA 4%. Free-floating vibratome sections (30 µm) were rinsed twice in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3% H₂O₂ and 10% methanol, and rinsed three times for 10 min each in TBS, permeabilized in 0.2% Triton X-100 in TBS (20 min) and rinsed three times in TBS. Slices were incubated overnight at 4°C with primary antibodies: Foxp2 (N16, Santa Cruz, 1:1000); ERK (diphospho-Thr-202/Tyr-204-ERK1/2, Upstate, 1:1000), 0.1 mM NaF was included in all buffers. The next day, sections were rinsed three times for 10 min in TBS and incubated for 45 min at RT with rabbit Cy3-coupled (1:750, Jackson Laboratory) or goat A555 (1:750, Invitrogen) secondary antibodies. Sections were rinsed for 10 min twice in TBS and twice in Tris buffer (0.25 M Tris) before mounting in Vectashield (Vector Laboratories). Brain regions were identified using a mouse brain atlas (Paxinos and Franklin, 2001), sections corresponding to the following bregma coordinates were taken (in mm): 1.54, rostral nucleus accumbens (NAc); 1.18, medial NAc and dorsal striatum; 0.86, caudal NAc.

Western blot, striatal tissue:

Western blot experiments were performed as described previously ⁴⁷. Mice were decapitated and heads immediately frozen in liquid nitrogen at indicated times following drug treatment. 210µm thick slices were obtained using a cryostat (Leica) and nucleus accumbens micropunches (1.4mm diameter) were collected. The tissue was homogenized in hot solution (maintained in a boiling water bath) of 1% (w/v) SDS and 1mM sodium orthovanadate, immediately sonicated and heat inactivated at 100°C for 5min. 60 µg protein were separated by SDS–polyacrylamide gel electrophoresis (4-12% w/v acrylamide) before electrophoretic transfer onto nitrocellulose membranes (Hybond Pure, Amersham, Orsay, France). Membranes were incubated for 1h at RT in a blocking solution of Tris-buffered

saline (TBS: 100mM NaCl, 10mM Tris, pH 7.5) with 0.05% (v/v) Tween-20 and 5% fat-free powdered milk. Membranes were then incubated overnight at 4°C with primary antibodies (Foxp2: N16, Santa Cruz, 1:500; anti-ERK1/2, 1:1000, Millipore, Molsheim, France). Secondary antibodies were IgG IRdye800CW-coupled or IgG IRdye700DX-coupled (1:4000, Rockland Immunochemicals, Gilbertsville, PA). Fluorescence was analyzed at 680 and 800nm using the Odyssey infrared imager and quantified using Odyssey software (Li-Cor, Lincoln, NE).

Gene expression analysis:

RNA isolation:

Total RNA from nucleus accumbens tissue micropunches was extracted 1h after cocaine or saline i.p. injection using the mirVana™ kit (Ambion) following the manufacturers protocol. RNA quantity was determined by a nanodrop 1000 (Thermo scientific), RNA quality (RIN>8) was verified with a Bioanalyzer (Agilent). 500 ng of total RNA was used for mRNA microarrays (Illumina BeadChip array MouseWG-6 v2) experiments following manufacturer protocols.

Data analysis:

For data analysis the background subtracted average signal intensity for each detected probe was used (Illumina array detection p-value<0.05). Samples were six biological replicates for *wild-type* saline, *wild-type* cocaine, *Foxp2*^{S321X/+} cocaine and five biological replicates for *Foxp2*^{S321X/+} saline. Probes not detectable in all arrays were removed and outlier probes were discarded before quantile normalization. A total of 16,253 probes representing 11,851 genes were used for statistical analysis (two-way ANOVA, factorial design). P-values were adjusted by FDR (Benjamini-Hochberg) to correct for multiple testing. For the cocaine effect, probes with an adjusted p<0.05 and fold change |FC|>1.11 were considered differentially expressed. For the *Foxp2*^{S321X/+} genotype effect, probes with p<0.05 and fold change |FC|> 1.11 were considered differentially expressed and included in the Venn diagram (Figure3) without multiple testing correction. Based on our previous expression profiling of striatal tissues from Foxp2 heterozygous and wildtype mice²², we expected that biologically relevant expression differences between genotypes in baseline conditions (saline) might be above the significance threshold following multiple testing correction in our array data. This is most likely due to cellular heterogeneity and sparseness of DA stimulated MSNs in baseline conditions. Further qPCR analysis confirmed expression differences between genotypes and cocaine (Figure 4). Two genes (1110007L15Rik, Mfge8) were upregulated in *Foxp2*^{S321X/+} saline conditions and downregulated in cocaine conditions and are not shown in Figure 3 for clarity. Over-representation analysis for the GO pathway terms was performed using DAVID⁴⁸, the Bonferroni method was used for p value adjustment.

GEO accession number of microarray data: GSE47457

Quantitative PCR validation:

Total RNA from nucleus accumbens tissue micropunches was extracted as above. cDNA was generated by using random hexamer (Invitrogen) and Superscript III Reverse Transcriptase (Invitrogen) following manufacturer protocols. qPCR was performed on the Agilent Mx3005P real-time PCR machine by using SYBR green master mix (Applied Biosystems, ABI). The real time PCR was programmed as: 95°C for 10 min., followed by 40 cycles of 95°C for 30s and 60°C for 1min. Dissociation protocol was run. Hprt was used as an endogenous control for normalization. Relative gene expression was determined by using the comparative CT method as described ⁴⁹. Equal efficiency of target and endogenous reference primers were verified by using standard curve where the difference between the absolute value of slopes between target amplification and endogenous reference amplification was <0.1.

Primer: Specific primers were designed using PrimerBank ⁵⁰ or Primer3 ⁵¹.

Transcripts	Forward primer (5' to 3')	Reverse primer (5' to 3')
Cacna1g	CTGCGGTCTGGACTATGAGG	TGTGATGACCTGGAAGATGG
Cnih3	CTCACCCCTGGGACTCAATGT	GTGTCCGCATTCATGACAAC
Calb2	TCTGGCATGATGTCCAAGAG	GCCTGAAGCACAAAAGGAAA
Camk2d	CTGGCACACCTGGGTATCTT	ATCCCAGAAGGGTGGGTATC
Foxp2	ATCCTGGAAAGCAAGCAAAA	TGAAGGCTGAGCAGATGTTG

Statistics:

Data are presented as means ± SEM. Student's t-tests were used for the analysis of studies with two experimental groups. One-way or two-way analysis of variance (ANOVA) with repeated measures were used and Bonferroni post hoc analysis when indicated. Main and interaction effects were considered significant at P < 0.05.

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Competing interests

The authors declare that no competing interests exist.

List of associated primary figures:

Figure 1-figure supplement 1

Figure 1-figure supplement 2

Figure 1-figure supplement 3

Figure 4-supplement figure 1

Figure 5-figure supplement 1

Figure 5-figure supplement 2

Figure legends:

Figure 1: Foxp2 in the NAc is required for cocaine induced locomotor activity and CPP. Reduced cocaine induced (15mg/kg,i.p.) locomotor activity in **(A)** $Foxp2^{R552H/+}$ and **(B)** $Foxp2^{S321X/+}$ mice as compared to *wildtype* (WT) littermates. ($Foxp2^{R552H/+}$: n = 8 to 11 per group; $Foxp2^{S321X/+}$: n = 8 to 17 per group, two-way repeated measures ANOVA, Bonferroni post hoc). **(C)** Attenuated cocaine conditioned place preference in $Foxp2^{R552H/+}$ mice (n = 11 to 13 per group, two-way ANOVA, Bonferroni post hoc). **(D)** Example of phospho-Erk immunoreactivity (white arrows) in *Drd1a::eGFP* MSNs in the nucleus accumbens following cocaine administration. Scale bar: 100 μ m **(E)** $Foxp2^{R552H/+}$ as compared to *wildtype* mice show decreased phospho-Erk immunoreactivity in *DIR*⁺ MSNs in the nucleus accumbens core and shell but not in the dorso-lateral (DLS) or dorso-medial (DMS) striatum 15 minutes after cocaine administration (n = 5 to 8 per group, two-way ANOVA, Bonferroni post hoc). **(F)** Example of AAV-CreGfp mediated *Foxp2* deletion in the nucleus accumbens ($Foxp2^{NacKO}$ mice). The white dotted line delimits the deletion area. Scale bar: 500 μ m **(G)** Reduced cocaine (15 mg/kg i.p.) induced locomotor activity in $Foxp2^{NacKO}$ mice (n = 15 to 19 per group, two-way repeated measures ANOVA, Bonferroni post hoc). **(H)** Attenuated cocaine conditioned place preference in $Foxp2^{NacKO}$ mice (n = 11 to 16 per group, two-way ANOVA, Bonferroni post hoc). **(I)** Immunohistochemistry shows cortical deletion of Foxp2 (green) in $Foxp2^{CtxKO}$ mice. **(K)** No difference in cocaine induced locomotor activity (15mg/kg, i.p.) between $Foxp2^{CtxKO}$ and controls (n = 7 to 8 per group).

Figure 1-figure supplements 1-3

Figure 1 – figure supplement 1: Foxp2 (red) immunohistochemistry on NAc tissue sections following AAV-CreGfp injection. Upper panels: Viral infection in *wild-type* mice shows no impact on Foxp2 expression. Lower panels: Foxp2 protein is largely absent following AAV-CreGfp injection in *Foxp2^{lox/lox}* mice. Scale bar= 200µm

Figure 1-figure supplement 2: (A) *Foxp2^{NacKO}* mice do not exhibit motor learning deficits on the accelerating rotarod. Left panel: trial to trail performance, right panel: daily performance (RPM, rotations per minute) (n = 13 to 17 per group; one-way repeated measures ANOVA). (B) *Foxp2^{NacKO}* mice do not display deficits in the open-field paradigm, such as time spent in the central area, the number of entries and the distance traveled (n = 14 to 19 per group; two-tailed Student's t-test).

Figure 1-figure supplement 3: (A) Cortex specific *Foxp2* knockout mice (*Foxp2^{CtxKO}*) do not show motor learning deficits on the accelerating rotarod, (n = 14 to 15 per group). (B) *Foxp2^{CtxKO}* mice do not display behavioral changes in the open field test (n = 8 per group) (mean+/-SEM, *P < 0.05, **P < 0.01).

Figure 2: Activity dependent regulation of Foxp2 in the NAc. **(A)** A single cocaine administration (15mg/kg, i.p.) in *wild-type* mice induces a transient downregulation of Foxp2 protein (left) in NAc tissue (n = 3 to 5 per group; one-way ANOVA, Bonferroni post hoc). Similarly 1 hour following cocaine injection, Foxp2 mRNA (right) in NAc tissue is decreased (n = 5 to 9 per group; two-tailed Student's t-test). **(B)** In tissue samples from the dorsal striatum 1h following a single cocaine administration, there were no detectable changes in Foxp2 protein (left, n = 7 to 8 per group; two-tailed student's t-test) and mRNA expression (right, n = 5 to 6 per group; two-tailed student's t-test). **(C)** In striatal neurons in primary cultures, co-administration of glutamate (0.3 μ M) and D1R agonist SKF 81297 (3 μ M) decreases Foxp2 protein levels 90 minutes following treatment. (n = 3 per group; mean \pm SEM, one-way ANOVA, Bonferroni post hoc) (mean \pm SEM, *P < 0.05,**P < 0.01,***P < 0.001).

Figure 3: Altered excitatory synaptic transmission and synaptic plasticity in D1R⁺ MSNs in the NAc in *Foxp2*^{R552H/+} mice. **(A)** Sample traces overlay of averaged (10 trials) traces of AMPAR EPSCs before (thin line) and after (bold line) and time versus amplitude plot indicating that HFS-induced LTP was abolished in D1R⁺ MSNs in *Drd1a::eGFP; Foxp2*^{R552H/+} mice (221±25.8% to 102.9±15.9%, Student's t-test, $t_{15} = 4.2$; $n = 8$ to 9 per group). **(B)** Bar graph of AMPA/NMDA ratio and sample traces of AMPA and NMDA-mediated currents recorded at +40 mV, showing higher AMPA/NMDA in D1R⁺ MSNs in *Drd1a::eGFP; Foxp2*^{R552H/+} mice (WT; AMPAR/NMDAR: 0.91 ± 0.15 ; *Foxp2*^{R552H/+}; AMPAR/NMDAR: 1.57 ± 0.23 , Student's t-test, $t_{15} = 2.48$; $P < 0.05$). Scale bars: 100ms and 20pA. ($n = 8$ to 9 per group, mean±/− SEM, * $P < 0.05$). **(C)** Sample traces, cumulative probability and mean values of amplitude and frequency for mEPSCs recorded from *Drd1a::eGFP; Foxp2*^{R552H/+} and *wild-type* mice indicating alterations in the amplitude and frequency of mEPSC (amplitude: WT, 26.6 ± 1.21 ; *Foxp2*^{R552H/+}, 33.5 ± 1.4 ; $t_{22} = 3.6$, $P < 0.01$; Frequency: WT, 1.1 ± 0.2 ; *Foxp2*^{R552H/+}, 0.35 ± 0.1 ; $t_{22} = 2.7$, $P < 0.05$; $n=10$ to 14 per group). **(D)** D1R⁺ MSNs in *Drd1a::eGFP; Foxp2*^{R552H/+} mice show reduced density of dendritic spines as compared to D1R⁺ MSNs in *Drd1a::eGFP* control littermates ($n = 3$ per group; mean±/−SEM, * $P < 0.05$).

Figure 4: Cocaine induced genes involved in calcium signaling are overexpressed in the NAc of *Foxp2* heterozygous mice in baseline conditions. **(A)** Venn diagram representing the overlap of differentially expressed genes in different experimental conditions. A set of 39 genes was differentially expressed (up and down) in *Foxp2*^{S321X/+} mice as well as following cocaine treatment (n= 5 to 6 per group, *p<0.0001, two-sided Chi-square test with Yate's correction; ns: not significant, p>0.05). **(B)** Among the 39 differentially expressed genes, the 23 upregulated genes were significantly overrepresented for the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway term 'calcium signaling'. Downregulated genes did not show significant over-representation for pathway terms (threshold: P=0.05, Bonferroni adjustment). **(C)** Relative fold changes of upregulated genes were plotted for different conditions with respect to *wild-type* saline. The fold induction of upregulated genes in *Foxp2*^{S321X/+} mice was positively correlated with their fold induction following cocaine treatment (Pearson correlation coefficient: *Foxp2*^{S321X/+} saline and *wild-type* cocaine=0.59; *Foxp2*^{S321X/+} cocaine and *wild-type* cocaine=0.71; *p<0.01). **(D)** Quantitative PCR validation of four upregulated genes involved in calcium signaling. (n = 4-6 per group, mean+/-SEM, two-tailed Student's t-test, *P < 0.05).

Figure 4-figure supplement 1

Figure 4-figure supplement 1: Relative fold change (FC) of the 16 genes downregulated in both, *Foxp2*^{S321X/+} mice in baseline (saline) conditions and in wild-type mice following cocaine injection. (Pearson correlation coefficients: *Foxp2*^{S321X/+} saline and wild-type cocaine =0.73; *Foxp2*^{S321X/+} cocaine and wild-type cocaine=0.89; *P < 0.01). Downregulated genes did not show significant enrichment for pathway terms.

Figure 5: *Foxp2* in the NAc regulates social behavior. **(A)** Maternal behavior of *Foxp2*^{NacKO} females in the pup retrieval paradigm is normal (n = 5 to 7 per group, two-way repeated measures ANOVA, Bonferroni post hoc). **(B)** Three-chamber social interaction test: *Foxp2*^{NacKO} mice did not show a preference to interact with unfamiliar mice as compared to unfamiliar objects (left panel) and spent less time interacting with unfamiliar mice than controls (right panel) (n = 11 to 17 per group, two-way repeated measures ANOVA, Bonferroni post hoc). **(C)** *Foxp2*^{CtxKO} mice display no abnormalities in three-chamber social interaction tests (n = 8 per group, two-way ANOVA, Bonferroni post hoc). **(D)** *Foxp2*^{NacKO} mice did not display social conditioned place preference (left panel), similar to unconditioned *Foxp2*^{NacKO} and control mice (middle panel) (n = 15 to 16 per group, two-way repeated measures ANOVA, Bonferroni post hoc). Example video track of social conditioned place preference experiment (right panel). (mean±SEM, *P < 0.05, **P < 0.01)

Figure 5-figure supplement 1

Figure 5-figure supplement 1:

Foxp2^{NacKO} mice show no deficits in a novel object recognition test. Similarly to controls, they display a discrimination index above chance level (red dotted line) (n = 14 to 19 per group; one-way repeated measures ANOVA; mean+/-SEM).

Figure 1

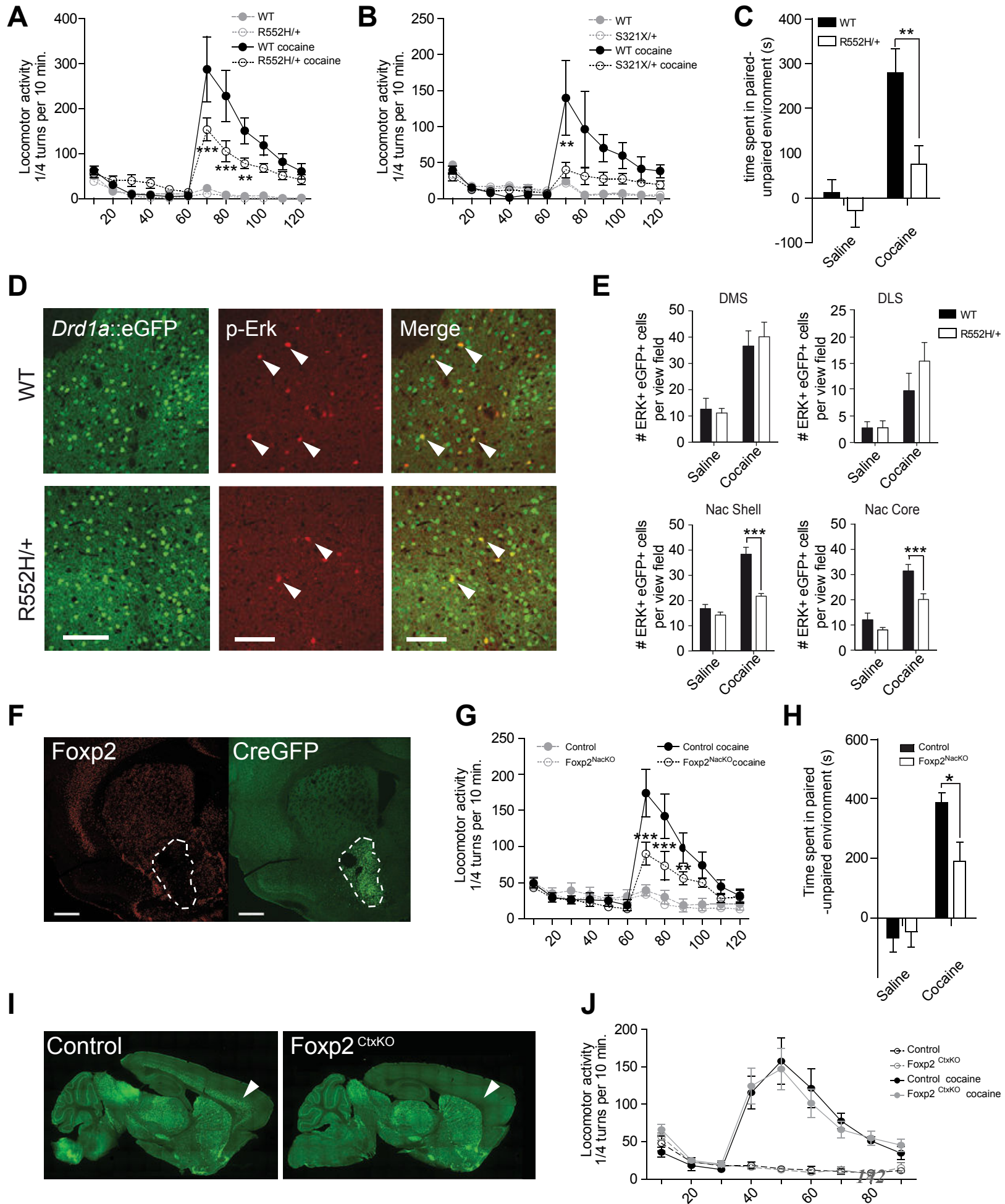


Figure 1 – figure supplement 1

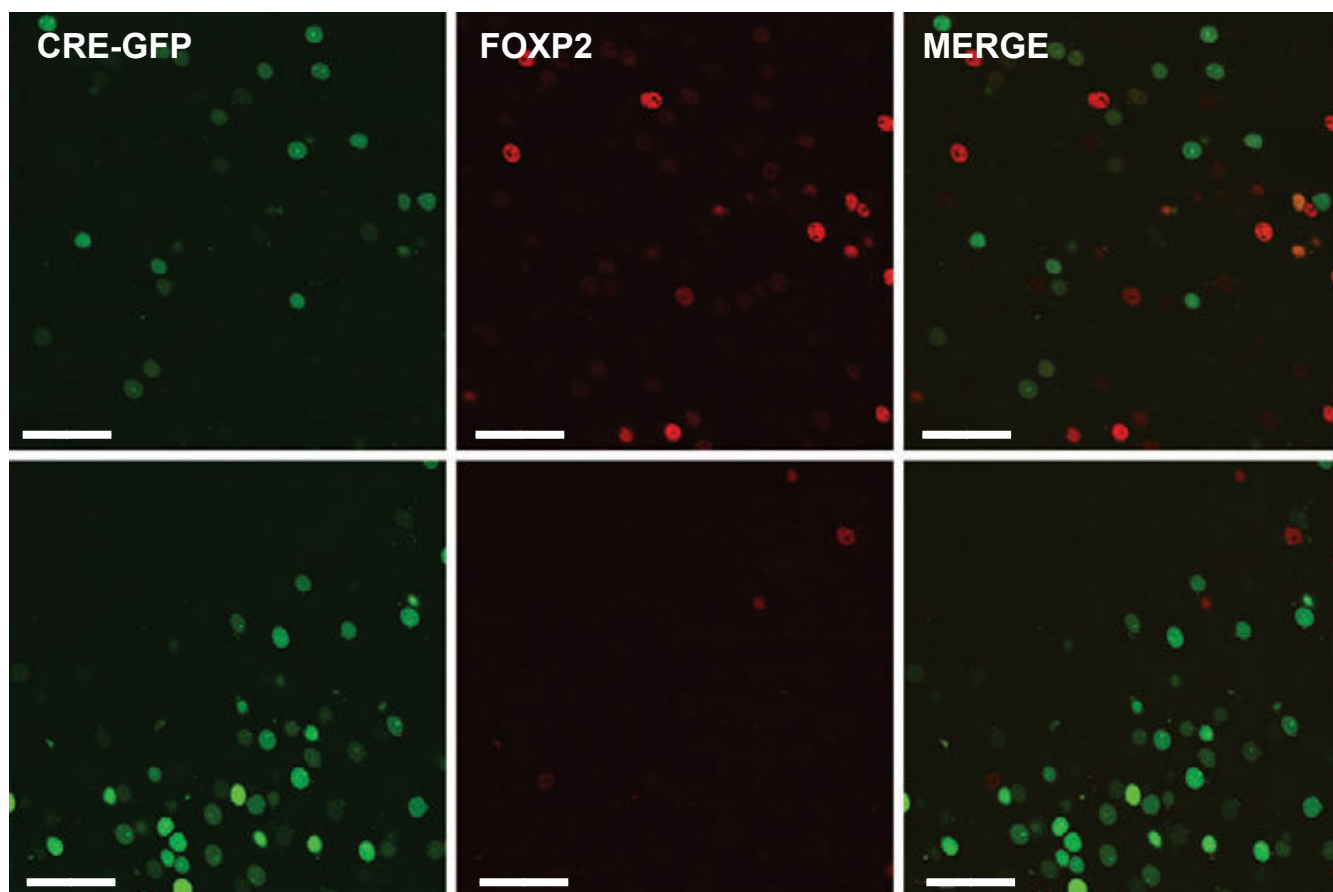
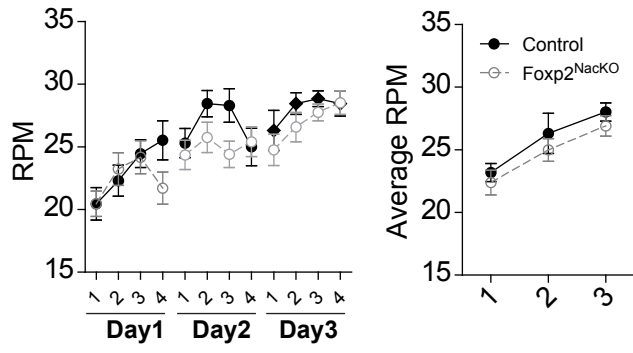


Figure 1 – figure supplement 2

A



B

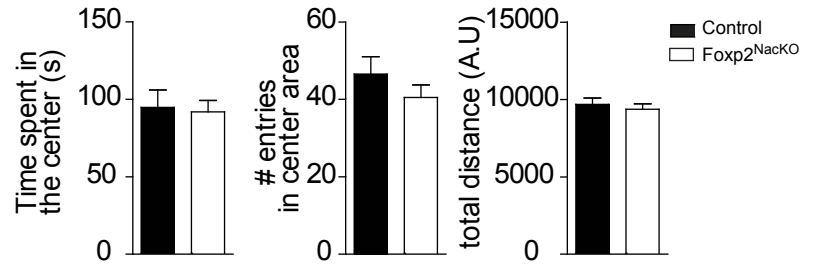
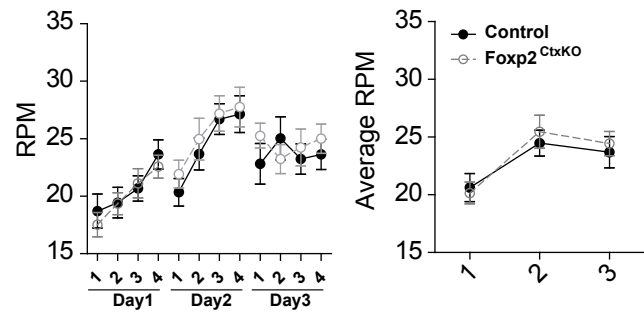


Figure 1 – figure supplement 3

A



B

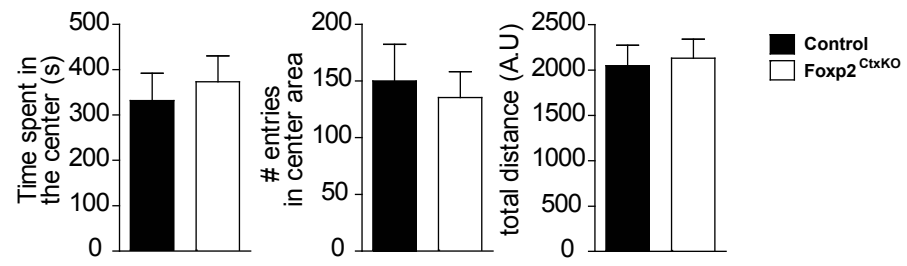


Figure 2

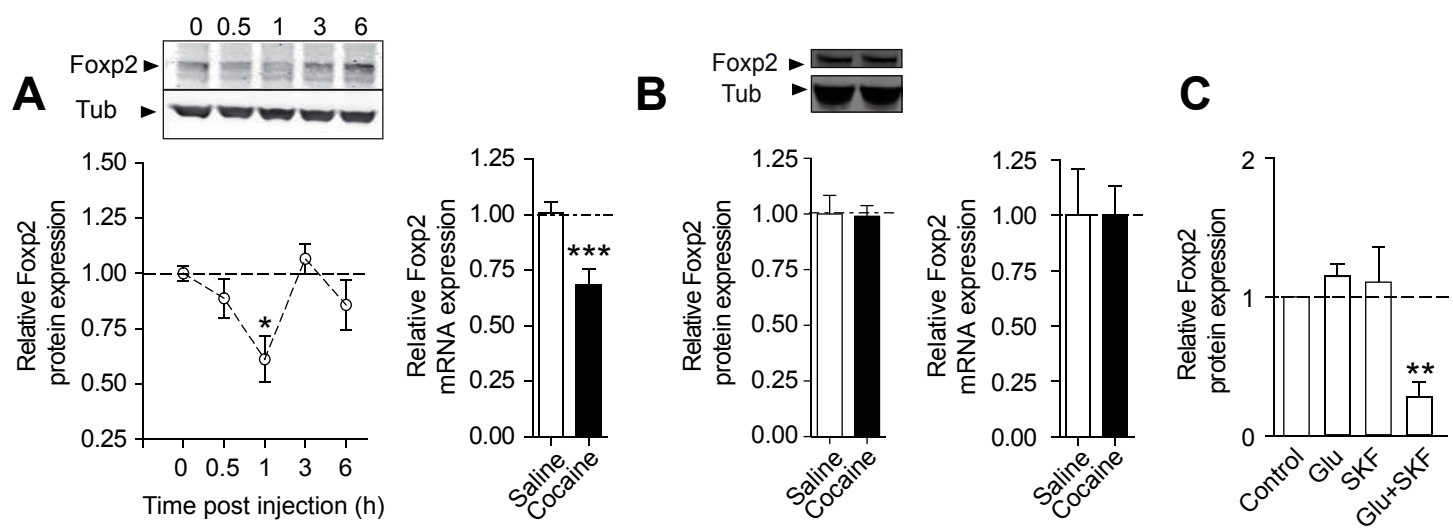


Figure 3

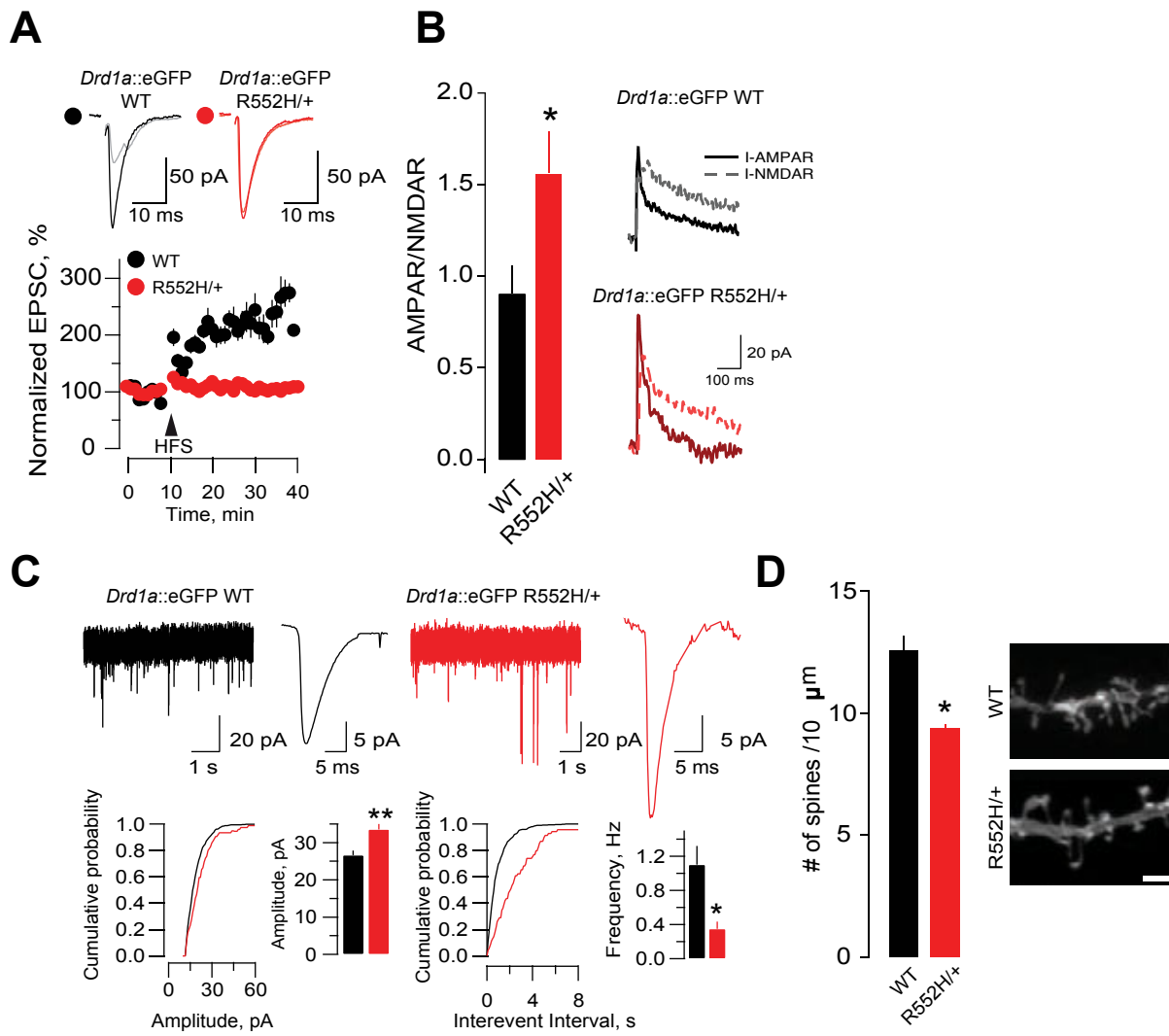


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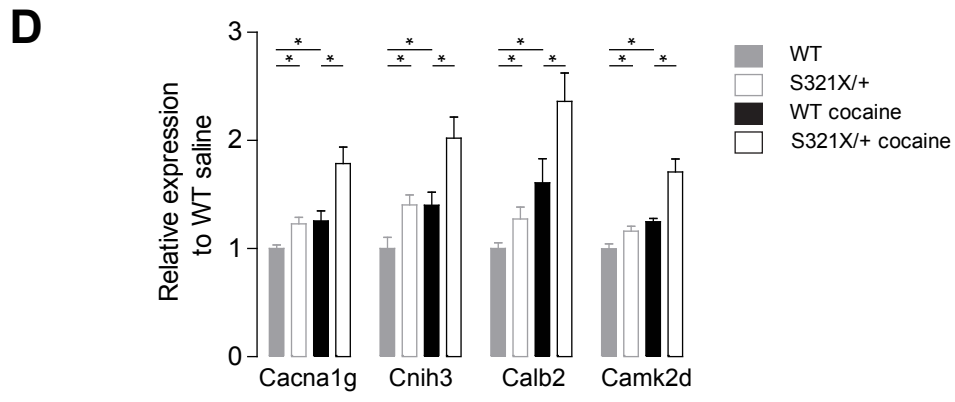
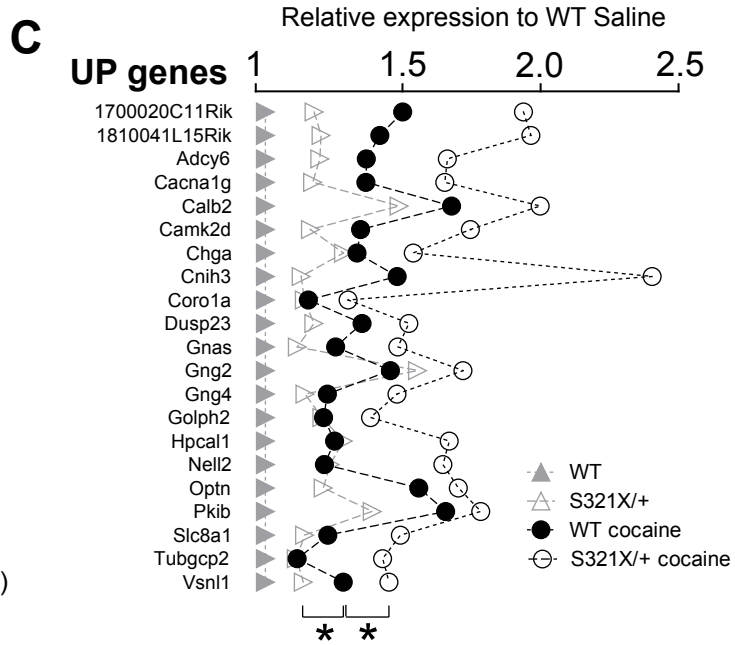
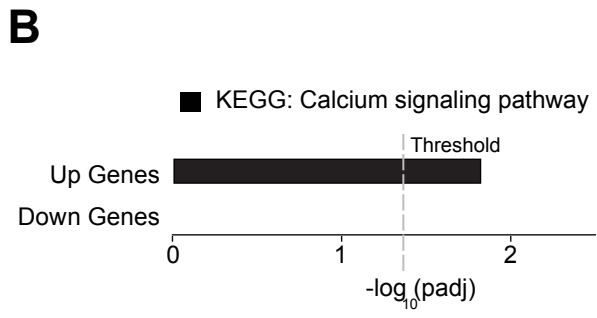
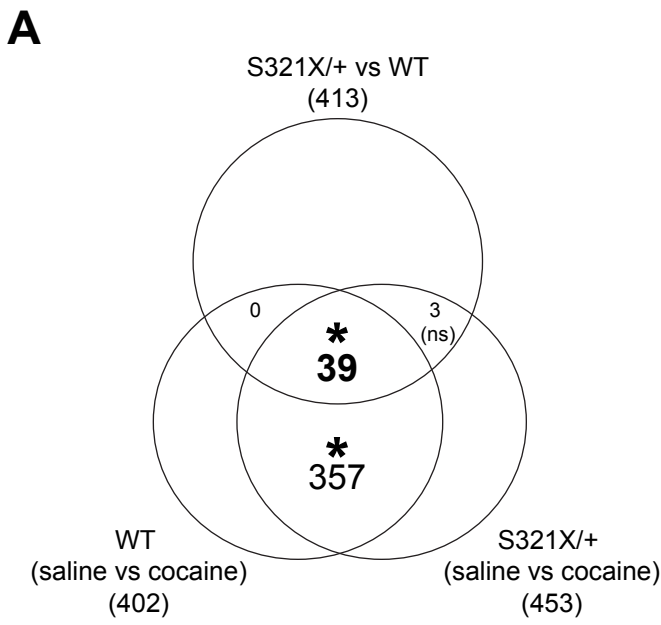


Figure 4 – figure supplement 1

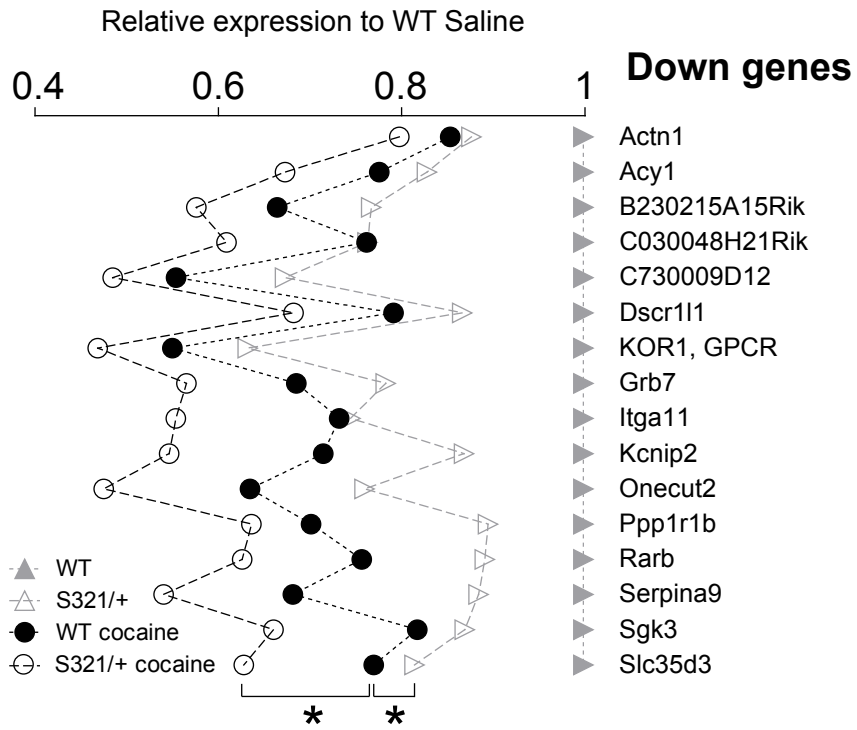
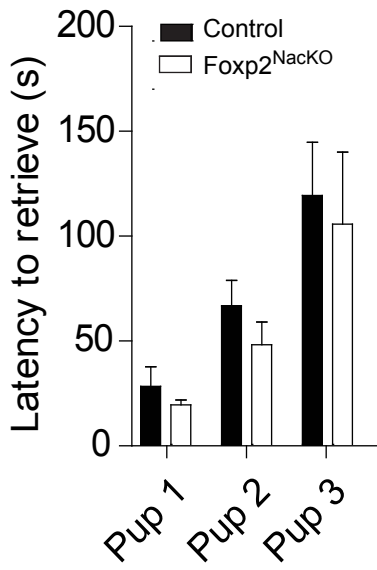
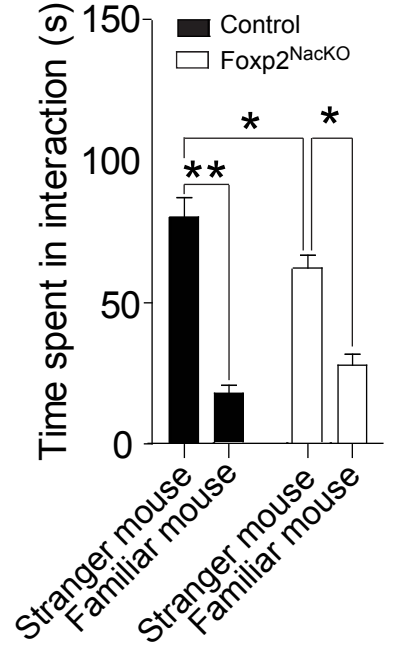
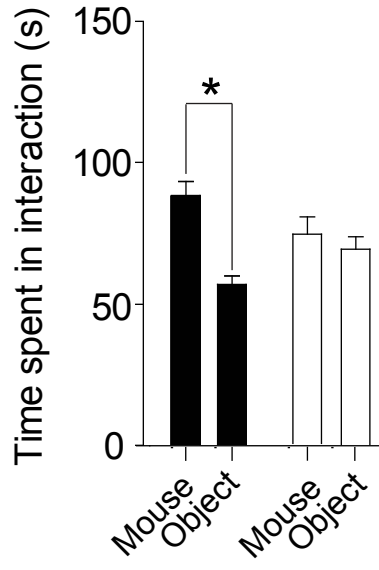


Figure 5

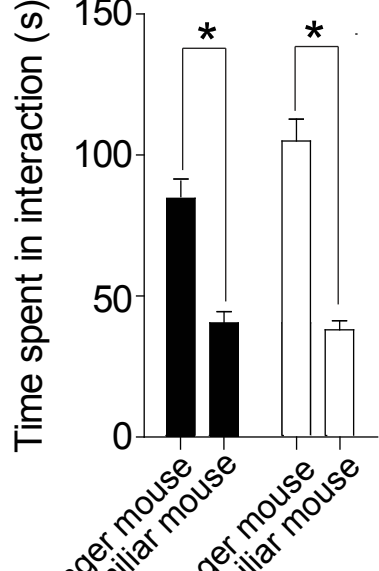
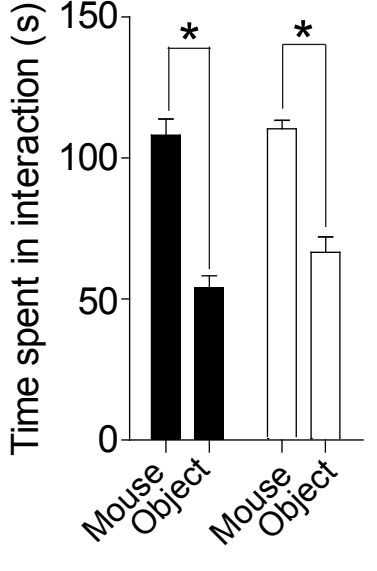
A



B



C



D

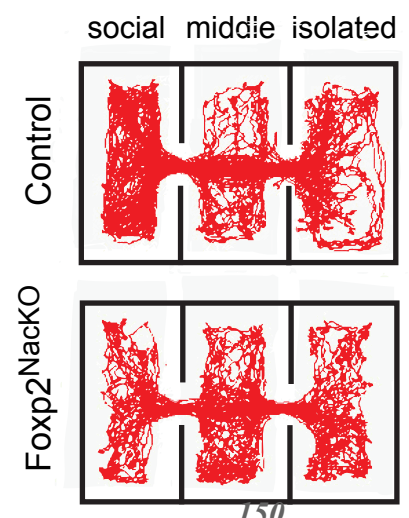
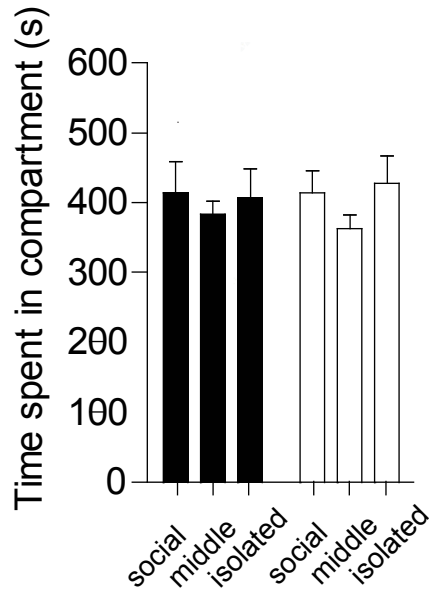
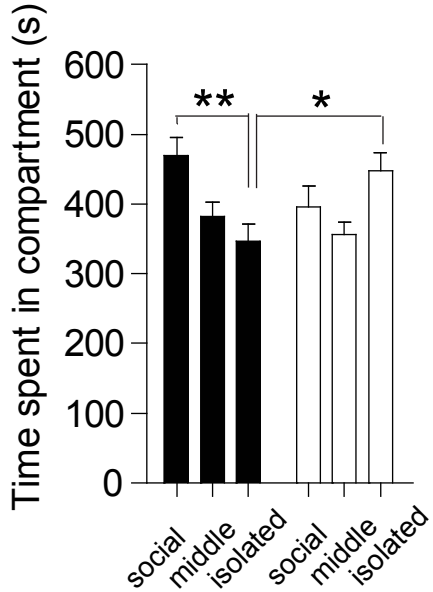
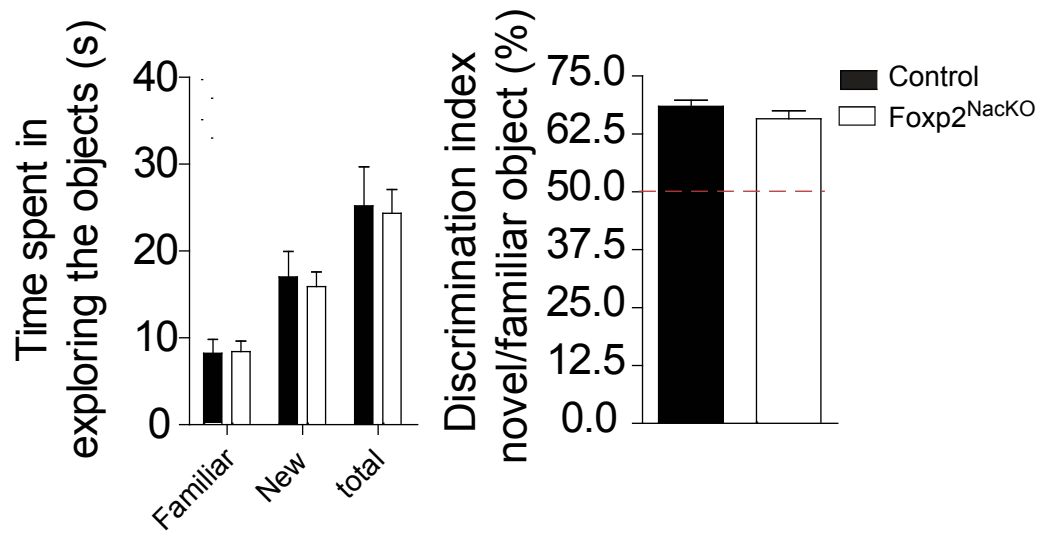


Figure 5 – figure supplement 1



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