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What can mice tell us about Foxp2 function?

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Disruptions of the *FOXP2* gene cause a rare speech and language disorder, a discovery that has opened up novel avenues for investigating the relevant neural pathways. *FOXP2* shows remarkably high conservation of sequence and neural expression in diverse vertebrates, suggesting that studies in other species are useful in elucidating its functions. Here we describe how investigations of mice that carry disruptions of *Foxp2* provide insights at multiple levels: molecules, cells, circuits and behaviour. Work thus far has implicated the gene in key processes including neurite outgrowth, synaptic plasticity, sensorimotor integration and motor-skill learning.

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Introduction

The capacity to produce and understand language is uniquely human and an integral part of our society and culture. The turn of the century marked the beginning of a concerted effort to establish its neural underpinnings from a molecular perspective. This was initially driven by the discovery of genomic variants and gene disruptions that correlate with disorders of speech, language or reading [1•]. The first gene to be implicated in this way was *FOXP2* [2]. It encodes a transcription factor, mutations of which cause a severe neurodevelopmental speech and language disorder [3,4]. Identification of *FOXP2* and other candidate genes provided vital insight at the level of molecular mechanisms — the challenge is now to determine how the proteins they encode impact on the brain circuits that ultimately allow us to talk.

Contemporary findings from neurobiology and cognitive neuroscience indicate that speech and language skills depend on the activities of multiple sets of distributed neural circuits, both cortical and subcortical. It has been proposed that our unique human abilities arose through adaptive evolution of pre-existing systems (neural, physiological and anatomical) brought together in novel configurations [5,6]. This hypothesis is supported by existing molecular data. Thus far, genes that have been connected to aspects of speech and language have also been found in other species, often with surprisingly deep evolutionary histories [5]. Certain aspects of the neural infrastructure supporting spoken language may be particularly tractable for studying in an evolutionary framework. For example, learning to speak depends crucially on auditory-guided vocal learning; the acquisition of a vocal repertoire is based on hearing vocalisations of a conspecific. Thus, human speech may be partly built on modifications of ancestral brain networks involved in sensorimotor integration and motor-skill learning [7]. Against this background, it is valuable to investigate roles of genes like *FOXP2* by studying corresponding orthologues in other species. For discussion of songbird research, see the article in this issue by Wohlgemuth, Adam and Scharff. Here we focus on relevant findings from studying genetically manipulated mice.

FOXP2: first insights

FOXP2 was first implicated in speech and language through studies of a large pedigree, the KE family [8]. Around half the family members (15 people) carry a heterozygous *FOXP2* mutation, yielding an amino-acid substitution which interferes with the encoded protein's capacity to regulate target genes [2,9]. A growing number of other individuals and small families are being identified with mutations, chromosome rearrangements and deletions involving *FOXP2* (to date at least 34 people with 25 types of disruption, summarised in Refs [3,10•]). In all instances only one gene copy is affected, but in some cases the nature of the disruption means that effects of additional genes cannot be discounted.

A common feature associated with *FOXP2* disruptions is imprecise and inconsistent control of the co-ordinated sequences of movements required for fluent speech (developmental verbal dyspraxia, DVD or childhood apraxia of speech, CAS) [3,8]. Affected people make speech errors that can differ from one utterance to another and become worse with increasing complexity, suggesting problems with the brain's capacity to plan speech-related motor sequences. In addition to DVD/CAS, other expressive and receptive impairments also exist affecting both

oral and written language. When the genomic disruption specifically or primarily affects *FOXP2*, non-verbal aspects of cognition are relatively spared [3,4,11–13]. Neuroimaging studies of the KE family identified subtle but significant structural abnormalities in the caudate nucleus, inferior frontal gyrus (including Broca's area) and the ventral cerebellum [14,15]. Functional MRI has shown underactivation of the putamen, Broca's area [16,17] and the rolandic operculum [17] during language-based tasks. These regions are concordant with sites of high FOXP2 expression, which include subpopulations of neurons in the cortex, basal ganglia, thalamus, inferior olives and cerebellum [18]. These studies show that FOXP2 impacts on brain areas implicated in speech and motor control, supporting a framework where speech-related circuitry broadly overlaps with circuits required for other motor processes [8].

Mouse Foxp2 is highly similar to human FOXP2, with respect to neural expression pattern and sequence of the encoded protein [18,19•,20]. Whilst expression commences embryonically it also persists into adulthood [19•,20]. Several mouse lines have now been generated with disruptions of *Foxp2* (Table 1), including alleles

Table 1

Mouse lines with disruption of Foxp2

Mouse line	Reference	Genomic background	Disruption	Basic phenotypes
Foxp2-KO	Shu *et al*. [21]	Generated on 129 and then crossed to C57BL/6 resulting in a mixed background.	Exons 12–13 replaced by a neomycin cassette. Removes the FOX[a] domain yielding knockout mice	Homozygotes die by 3 weeks of age. Heterozygotes show mild developmental delay
Foxp2-R552H-KI	Fujita *et al*. [23]	Generated on 129 and then crossed to C57BL/6 resulting in a mixed background	Point mutation introduced into exon 14 using a knockin strategy resulting 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[b])	Homozygotes die by 3 weeks of age. Some heterozygotes show mild-moderate developmental delay
Foxp2-R552H-Enu	Groszer *et al*. [22••]	ENU-mutagenesis on BALB/c. Marker-assisted backcrossing to C3H or C57BL/6	Mice with a point mutation in exon 14 isolated from an ENU-mutagenesis screen[c]. Results in the Arg-to-His substitution seen in the KE family	Homozygotes die at 3–4 weeks of age. Heterozygotes are overtly normal
Foxp2-S321X	Groszer *et al*. [22••]	ENU-mutagenesis on C3H. Marker-assisted backcrossing to C57BL/6	Mice with a point mutation in exon 7 isolated from an ENU-mutagenesis screen. Results in a premature stop codon, shown to be equivalent to a null allele (no protein)	Homozygotes die at 3–4 weeks of age. Heterozygotes are overtly normal
Foxp2-N549K	Groszer *et al*. [22••]	ENU-mutagenesis on BALB/c. Marker-assisted backcrossing to C3H or C57BL/6	Mice with a point mutation in exon 14 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	French *et al*. [24]	Generated and maintained on C57BL/6 (NB. Cre lines can be of any background)	*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	Enard *et al*. [27••]	Generated and maintained on C57BL/6	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

[a] The FOX or forkhead box domain mediates the DNA-binding and transactivation properties of the Foxp2 protein [9].
[b] The murine Foxp2 protein is one amino acid shorter than the human protein, due to a difference in an N-terminal polyglutamine tract.
[c] ENU (*N*-ethyl-*N*-nitrosourea) is a compound used to induce mutations in the mouse genome. Screens can be carried out to identify interesting genetic changes or behavioural phenotypes [63].

Figure 1

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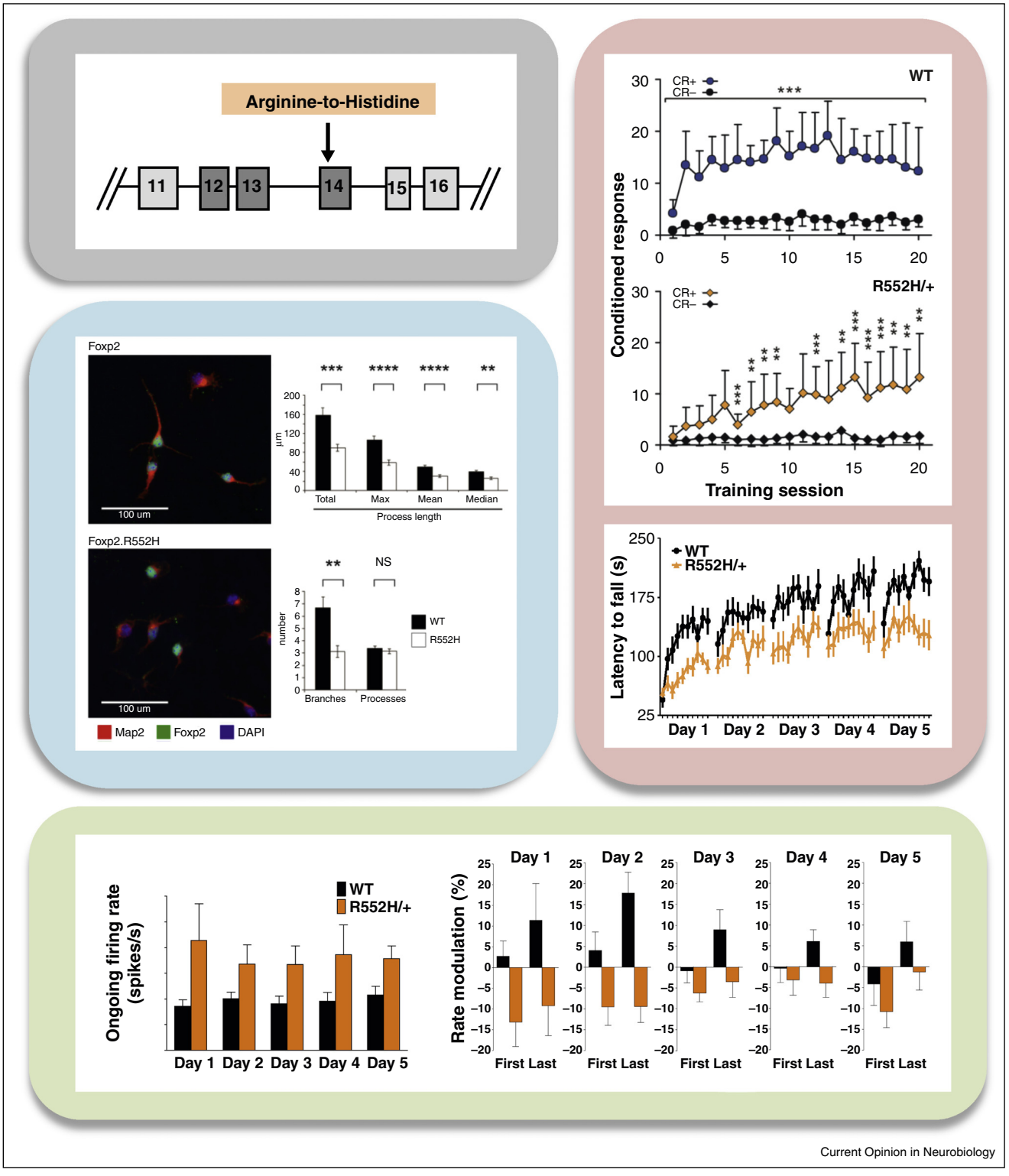

Recent examples of studies using *Foxp2-R552H-Enu* mice. (Grey box) This line has a missense mutation in exon 14 of the *Foxp2* gene; one of 3 exons which encode the DNA-binding domain (dark grey). The mutation causes an arginine to histidine substitution at position 552 which is equivalent to the change found in affected members of the KE family [22••]. (Blue box) At the cellular level, process length and branch number were reduced in neurites of primary cells isolated from the ganglionic eminences of E16 *Foxp2-R552H* homozygotes compared to wild-type littermates. Data represent the mean of 84 and 141 Foxp2-positive cells taken from *R552H* and wild-type embryos respectively. Error bars indicate SEM and

hat lead to a lack of Foxp2 protein (*Foxp2-KO* [21] or *Foxp2-S321X* [22••]) and those that recapitulate the missense mutation found in the KE family (*Foxp2-R552H-KI* [23] or *Foxp2-R552H-Enu* (Figure 1, grey box) [22••]). Homozygous mice that completely lack Foxp2, or that have only non-functional protein, are developmentally delayed and have severe motor impairments, dying at 3–4 weeks of age [21,22••,23,24]. Similar findings are also observed for compound heterozygotes that carry two different non-functional alleles on different gene copies [22••]. In contrast, heterozygous mice survive to adulthood and appear overtly normal [22••,24], although some studies have reported mild-to-moderate developmental delay [21,23] which might relate to differences in genomic background [25•].

In addition to examining effects of gene dysfunction, mice have also been used to assess potential phenotypic effects of human evolutionary changes. Although there are only three amino-acid substitutions distinguishing human FOXP2 protein from mouse Foxp2, two of these occurred after the human lineage diverged from the chimpanzee and have been speculated to represent adaptive changes [26•]. *Foxp2-Hum* (partially humanised) mice were engineered to carry the two amino acids found in humans. In this case homozygotes and heterozygotes are all healthy with normal fertility and lifespan [27••].

Deconstructing molecular networks

Large scale chromatin immunoprecipitation and expression profiling of midgestation embryonic mouse brain have uncovered gene networks regulated by Foxp2 during neural development, giving clues into the biological processes it affects [28]. This work identified many putative targets, particularly highlighting potential roles in modulating neurite outgrowth and synaptic plasticity [28], themes which also emerged in other studies [29–31]. Functional studies have confirmed the impact of Foxp2 disruption on neurite branching and length (Figure 1, blue box) [28,32]. Attention is now turning to how Foxp2 is itself regulated; for example one study reported that microRNAs (miR-9 and miR-132) repress Foxp2 expression, mediating effects on neurite outgrowth and radial migration of cortical projection neurons [33•]. The Foxp2-regulated gene, *CNTNAP2*, has been associated with neurodevelopmental disorders that involve language deficits [34], and even with variation in language development in the general population [35]. Other Foxp2 targets and interacting proteins have been implicated in autism spectrum disorders, schizophrenia, bipolar disorder, epilepsy and intellectual disability [10•,36,37]. One such target is *SRPX2*, mutations of which lead to epilepsy in speech-related areas of the brain and DVD/CAS [38•]. Recently, SRPX2 knockdown was shown to impair synaptogenesis and lead to reduced ultrasonic vocalisation of P7 mouse pups (see below for discussion of rodent vocalisations) [39••]. Together these data hint at common molecular and circuit mechanisms that cross diagnostic borders. However, it is often unclear whether regulation of targets is cell-type or brain-region specific, and what effect dysregulation has on the organism as a whole.

Foxp2 and sensory processing

Foxp2 is expressed in cortical and subcortical areas involved in sensory processing and integration. In the olfactory system expression is found in the glomerular layer of the olfactory bulb, the accessory olfactory bulb and the olfactory tubercle. Foxp2 is also expressed in the ascending auditory and visual relays as well as thalamic somatosensory areas. In the cortex, Foxp2 shows broad expression in layer VI and more restricted expression in layer V, where it is largely localised to the association and premotor areas [19•,20]. To date, work has concentrated mainly on the auditory system, presumably because of the importance of auditory processing for speech development. In mice, Foxp2 expression in the medial geniculate nucleus of the thalamus is dependent on auditory experience [40]. *Foxp2-R552H* heterozygotes (carrying the same mutation as the KE family) have auditory brainstem responses (ABRs) with longer latencies and smaller amplitudes than controls, which could reflect changes in the number or synchrony of activated neurons [41]. However, ABRs of *Foxp2-S321X* heterozygotes (with a half-dosage of functional Foxp2 protein) are not significantly different from wild-type littermates [41]. Both *Foxp2-R552H* and *Foxp2-S321X* heterozygotes showed slower learning of auditory-motor associations, assessed

Figure 1 Legend Continued p-values were calculated using ANCOVA followed by post hoc Sidak correction (****$P < 0.001$, ***$P = 0.001$, **$P < 0.01$) [28]. (Red box) At the behavioural level, learning of an auditory-motor association task was delayed in *Foxp2-R552H* heterozygotes compared to wild-type controls. Animals had to jump across a hurdle in the presence of a 12 kHz tone or remain where they were in the presence of a 7 kHz tone. CR+ and CR− represent jumps after the 12 kHz and 7 kHz tones respectively. Differences in CR+ and CR− rates were compared for each session (***$P < 0.001$, **$P < 0.01$), error bars indicate SD (top panel) [42•]. Latency to fall from the accelerating rotarod was significantly reduced in *Foxp2-R552H* heterozygotes compared to wild-type littermates (repeated measures ANOVA, $F_{1,19} = 9.87$, $P < 0.05$). Mice received 10 trials per day for five consecutive days. Error bars indicate SEM (bottom panel) [47•]. (Green box) At the electrophysiological level, *in vivo* recording uncovered significantly increased ongoing striatal activity in *Foxp2-R552H* heterozygotes compared to wild-type littermates (repeated measures ANOVA, $F_{1,9} = 5.54$, $P < 0.05$) (left panel). Wild-type animals also showed predominantly positive modulation of firing rate during running compared to inter-trial intervals, whereas *Foxp2-R552H* heterozygotes were significantly different and showed negative firing rate modulation (repeated measures ANOVA, $F_{1,9} = 7.68$, $P < 0.05$). First = trials 1 and 2, last = trials 9 and 10 (right panel). Error bars indicate SEM on both graphs [47•].
Figures were adapted with permission from prior publications [28,42•,47•].

via a conditioned avoidance paradigm using a shuttle-box, with more severe deficits for the *Foxp2-S321X* mice despite their normal ABRs (Figure 1, red box) [42•].

Contributions of Foxp2 to motor-skill learning and performance

Particularly noteworthy sites of Foxp2 expression are the cortico-striatal and cortico-cerebellar circuits involved in the acquisition and performance of motor-skills [18,19•,20]. In the cerebellum, expression is confined to Purkinje cells and deep cerebellar nuclei. It is also intense in the inferior olives of the medulla [19•,20,43]. In the striatum, Foxp2 is heterogeneously expressed and enriched in striosomes [19•,20,44]. Levels are higher in medium spiny neurons that express type 1 dopamine receptor (DRD1) than in those which express DRD2 [45]. Foxp2 is also found in the substantia nigra pars compacta, the ventral tegmental area and the subthalamic nucleus, pointing to possible roles in integrating motivation with motor output [19•,20]. As mentioned above, homozygotes for loss-of-function alleles of *Foxp2* show severe motor dysfunction and postnatal lethality whereas heterozygotes are viable, appearing largely normal [21,22••,23,24]. However, detailed analyses of *Foxp2-R552H* heterozygotes with normal baseline motor abilities uncovered learning deficits on accelerating rotarods and on voluntary running wheel systems [22••]. These effects on motor-skill learning and performance appear more generalised than those seen in humans with *FOXP2* disruptions, which seem to disproportionately disturb orofacial sequencing [4,8]. These differing observations may reflect species differences in Foxp2 function. However, more challenging tasks could reveal more pronounced deficits outside of the orofacial system in humans. Although they do not have limb dyspraxia [4], one study reported deficits in tapping rhythm in affected KE-family members [46]. Development of new behavioural tasks in humans and mice, or fine analyses of existing ones, would help identify what features of motor-skill learning are being affected, for example speed, accuracy or variability. It should then be possible to see if similar types of feature are consistently affected in speech and other motor-skills.

Targeted investigations of cortico-striatal circuits revealed increased striatal dopamine levels and reduced dendrite lengths in *Foxp2-KO* heterozygotes [27••], and impaired long-term depression (LTD) at cortico-striatal synapses of *Foxp2-R552H* heterozygotes [22••]. *In vivo* electrophysiological recordings in *Foxp2-R552H* heterozygotes during training on the accelerating rotarod, showed abnormally high striatal activity, which was aberrantly modulated when animals ran on the rod (Figure 1, red and green boxes) [47•]. Cortico-striatal functions have also been a major focus for understanding potential effects of partially humanizing this gene. In contrast to the findings from loss-of-function alleles, *Foxp2-Hum* homozygotes have increased cortico-striatal LTD, with no detectable differences in motor-skill learning [27••]. These mice also have longer dendrites in the cortex, striatum and thalamus but not in the cerebellum. It has therefore been postulated that the amino-acid changes which occurred in FOXP2 on the human lineage specifically affected cortico-striatal circuitry [26•,32].

In the cerebellum, *Foxp2-R552H* heterozygotes show subtle electrophysiological changes at parallel-fibre Purkinje-cell synapses [22••]. However, relatively little work has been done in this area. Region-specific Foxp2 deletion is now possible, for example through conditional knockout strategies using floxed alleles [24]. Such approaches will be important to decipher the roles this gene plays in different circuits, and to properly tease apart striatal and cerebellar abnormalities following Foxp2 loss [24].

Several papers have described early developmental roles for Foxp2 in the embryonic forebrain [48,49] and spinal cord [50•]. Given that expression persists into adulthood [19•], it seems likely that the protein also has regulatory functions in postnatal animals, perhaps throughout adult life. In songbirds, FoxP2 knockdown in striatal Area X of juvenile zebra finches results in inaccurate imitation of the song of a tutor [51]. A recent study [52••] extended the findings to adult birds, where reduced FoxP2 levels led to disrupted social modulation of song variability. FoxP2 depletion disrupted activity propagation through the anterior forebrain pathway (analogous to the mammalian basal ganglia–thalamocortical pathway), rendering it insensitive to DRD1-mediated signalling. In mice, the consequences of retaining Foxp2 expression during development, followed by selective knockdown in adulthood are not yet known.

What can vocalisations tell us?

Young mouse pups emit calls in the audible and ultrasonic range in situations such as thermal stress and separation from the mother. These calls are innate, not modulated by auditory feedback (mouse pups <10 days old are deaf), and thought to be an involuntary response to altered arousal state. Pup ultrasonic isolation calls (USIs) elicit retrieval by the mother [53] and homozygotes that lack functional Foxp2 emit very few compared to wild-type littermates [21,22••,23,54]. However, under conditions of elevated arousal (applying gentle pressure to the tail while lifting the animal) homozygotes do produce ultrasonic calls, albeit of lower intensity [22••,54]. Pups that are heterozygous for loss-of-function *Foxp2* alleles have been described as producing the same number of USIs as wild-types [22••,54], or a reduced number [21,23]. Where fewer USIs were reported, developmental delay was also observed in the heterozygous state [21,23]. In-depth studies of heterozygotes carrying Foxp2 disruptions (*Foxp2-S321X* and *Foxp2-R552H* lines) did not detect major differences in the acoustic properties of USIs

emitted by pups, as compared to wild-type littermates [54]. A study of *Foxp2-Hum* homozygotes reported that USIs of these partially humanized pups had lower frequency than controls but the number of calls was unchanged [27••]. Together, the data suggest that the reduced number of USIs described for some Foxp2 mouse mutants is secondary to other developmental problems, which are particularly severe in homozygotes with loss-of-function. Moreover, Foxp2 is not essential for ultrasonic vocalisation in mice.

A recent study [55•] compared 4-day-old male and female rat pups, finding that males emit more USIs, and that these USIs have lower frequency and amplitude. Males are retrieved preferentially by the mother and have higher Foxp2 levels in several brain regions. Apart from one report of a possible sex difference in Foxp2 expression in the adult rat cerebellum [56], this is the only study in rodents or humans to uncover sexual dimorphism in this regard although the issue has not been systematically examined at all developmental stages [18,19•,20]. To test how Foxp2 expression relates to USIs and order of retrieval, siRNAs were used at P0–P1 to knock down the gene. This resulted in a transient reduction in Foxp2 at P2, but by P4 male protein levels had returned to control levels and female protein levels were higher than that of controls. At P4, sex differences in the number and acoustic properties of USIs were no longer seen in siRNA treated animals and the order of retrieval was reversed [55•].

The same study reported that 4–5-year-old boys had less FOXP2 protein than girls (the opposite effect to rat pups) in BA44 of the left hemisphere, based on analysing this cortical region in postmortem tissue from five males and five females [55•]. The authors of the study postulated that higher FOXP2 expression in girls confers a linguistic advantage and/or makes them more communicative [55•], a claim that has gained widespread attention. However, there are grounds for caution. First, the sample size was small, and males and females differed in their ethnicity. Second, it is known from prior work that FOXP2 has a complex and dynamic expression pattern during foetal development and in postnatal brain, making it difficult to draw conclusions about the role of sex differences from analysing a single time point in one isolated brain region. Third, although girls appear to have some advantage in the early stages of language acquisition, after this point the existence of sex differences is contentious [57]. Fourth, while studies of innate USIs in rodents may be an informative readout of arousal state or motor function, such findings do not necessarily translate to vocal communication that is learned and socially motivated. Indeed, it is likely that largely different neural circuits underpin these two types of vocalisations [58]. Foxp2 mutant mice, or other rodent models, have yet to be studied with regard to the ultrasonic sounds produced by adult males in response to females or pheromones. These vocalisations have a rich structure [59] and there is some evidence that mice have a limited capacity to learn them, although this is still an area of much debate [60] (see article in this issue by Portfors).

Conclusions

Studies of FOXP2 orthologues in humans, mice, songbirds and other species have greatly increased our understanding of its neural functions. Neurite outgrowth and synaptic plasticity are two processes in which Foxp2 targets have been heavily implicated. These findings have been confirmed *in vivo* where Foxp2 depletion results in aberrant striatal plasticity as well as deficits in motor-skill learning and sensorimotor integration. Some differences in pup vocalisation have been reported and studies of the ultrasonic sounds emitted by adult rodents may prove interesting. To date, most Foxp2 mouse studies combined global genetic manipulation with either behavioural, morphological or electrophysiological analyses. Refinement of these approaches could provide significant advances. Genetic manipulations could be restricted to specific brain regions, cell types or developmental time points [24,61]. More detailed investigations of behavioural microstructure coupled with more complex analytical methods will also help explain the intricacies of an often subtle phenotype. A distinct advantage afforded by mice is the rapidly expanding range of genetic, molecular and electrophysiology tools available. Technology now exists to image and record neural activity in genetically defined circuits in behaving animals, and to optogenetically manipulate activity in these circuits [61,62]. Applying these techniques in mice with Foxp2 disruptions will fully exploit the benefits this species offers as an experimental system. Such approaches promise insights into underlying subcircuits in unprecedented detail, and may illuminate their contributions to speech and language in humans.

Conflict of interest statement

Nothing declared.

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