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A Humanized Version of Foxp2 Affects Cortico-Basal Ganglia Circuits in Mice

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SUMMARY

It has been proposed that two amino acid substitutions in the transcription factor FOXP2 have been positively selected during human evolution due to effects on aspects of speech and language. Here, we introduce these substitutions into the endogenous *Foxp2* gene of mice. Although these mice are generally healthy, they have qualitatively different ultrasonic vocalizations, decreased exploratory behavior and decreased dopamine concentrations in the brain suggesting that the humanized *Foxp2* allele affects basal ganglia. In the striatum, a part of the basal ganglia affected in humans with a speech deficit due to a nonfunctional *FOXP2* allele, we find that medium spiny neurons have increased dendrite lengths and increased synaptic plasticity. Since mice carrying one nonfunctional *Foxp2* allele show opposite effects, this suggests that alterations in cortico-basal ganglia circuits might have been important for the evolution of speech and language in humans.

For a video summary of this article, see the Paper-Flick file available with the online Supplemental Data.

INTRODUCTION

Identification of the genomic differences that underlie the phenotypic traits that set humans apart from their closest relatives among the primates is important from an evolutionary, medical and a cultural perspective (Enard and Pääbo, 2004; Varki et al., 2008). The sequencing of human, chimpanzee and rhesus macaque genomes has accomplished the first step toward this goal by cataloguing the ∼20 million genomic changes that occurred on the human evolutionary lineage (Mikkelsen et al., 2005). The current challenge, therefore, is not to identify human-specific genomic features, but to distinguish the small number of features that may have phenotypic consequences from the vast majority of functionally neutral features. Since crosses between humans and chimpanzees are not possible, this can only be achieved by the analysis of additional information such as the extent of evolutionary conservation among primates, population variation among humans and disease associations. Examples of such work include the identification of a noncoding RNA gene that evolved rapidly on the human lineage (Pollard et al., 2006), evidence for positive selection in genes involved in primary microcephaly (Gilbert et al., 2005) and evidence for positive selection in *FOXP2* (Enard et al., 2002; Zhang et al., 2002), a gene involved in speech and language disorders (Lai et al., 2001; MacDermot et al., 2005). Once such candidate features are identified, the next challenge is to test whether they are indeed involved in any human-specific phenotype. This is obviously a difficult task since genetic manipulations of humans or chimpanzees are impossible and the mouse is the only mammal into which genetic changes can currently be efficiently introduced and their effects tested. Although human disease mutations have been successfully

modeled in the mouse (Cox and Brown, 2003) it is unclear whether the supposedly slight phenotypic effects of human-specific evolutionary changes can be modeled and recognized in the mouse. Here we establish a mouse model and study the functional consequences of evolutionary changes that affected the transcription factor FOXP2 in humans.

Individuals that are heterozygous for *FOXP2* alleles that carry a missense mutation (R553H) affecting the forkhead DNA binding domain of the protein, a nonsense mutation (R328X) or disruptions of the gene by a chromosomal rearrangement suffer from a developmental impairment especially affecting speech and language (Lai et al., 2001; MacDermot et al., 2005; Vargha-Khadem et al., 2005). Analyses of the evolution of the *FOXP2* gene in primates identified two amino acid substitutions (T303N, N325S), which became fixed on the human lineage after its separation from the chimpanzee and which appear to have been subject to positive selection (Enard et al., 2002; Zhang et al., 2002). It has been hypothesized that these substitutions underwent selection due to effects on some aspects of speech and language (Enard et al., 2002; Zhang et al., 2002). Conveniently, FoxP2 in chimpanzees differs from Foxp2 in mice by only one conservative amino acid substitution (D80E). Thus, the wild-type mouse Foxp2 protein can be regarded as a model for the ancestral version of the human FOXP2 protein and compared to a partly "humanized" version in which the two amino acid replacements have been introduced (Figure 1A).

RESULTS

Generation of Mice

The two nucleotide substitutions that occurred during human evolution are both located in exon seven of the *FOXP2* gene. We introduced these two substitutions into the orthologous exon of the mouse *Foxp2* gene by homologous recombination in embryonic stem (ES) cells derived from C57BL/6 mice (Figure 1A and Figure S1 available with this article online). This "humanized" mouse allele (*Foxp2hum*) segregates in Mendelian ratios (chi-square = 1.44, n = 636, p = 0.49) and *Foxp2hum/hum* mice seem healthy, and are as fertile and as long-lived (data not shown) as their wild-type littermates. This is in stark contrast to mice homozygous for knock-out or nonfunctional mutant alleles of *Foxp2*, which die 3–4 weeks after birth (French et al., 2007; Fujita et al., 2008; Groszer et al., 2008; Shu et al., 2005). Thus, the *Foxp2hum* allele is generally functional in mice.

We also crossed mice carrying *Foxp2hum* to mice ubiquitously expressing Cre recombinase to generate a *Foxp2ko* allele in which exon 7 is deleted (Figure 1B). This is expected to lead to a truncated Foxp2 protein of 291 amino acids. We did not analyze homozygous *Foxp2ko/ko* mice in any detail, but similar to mice homozygous for an allele with a nonsense mutation (S321X) in exon 7 (Groszer et al., 2008) they show reduced *Foxp2* mRNA levels and an absence of truncated Foxp2 protein (Figure S2). This is likely due to a combination of nonsense-mediated RNA decay (Groszer et al., 2008) and instability of truncated proteins (Vernes et al., 2006). Heterozygous *Foxp2wt/ko* mice show intermediate levels of Foxp2 protein (Figure S2) and can thus be used to assess the consequences of reduced Foxp2 expression. This may be useful since effects caused by the
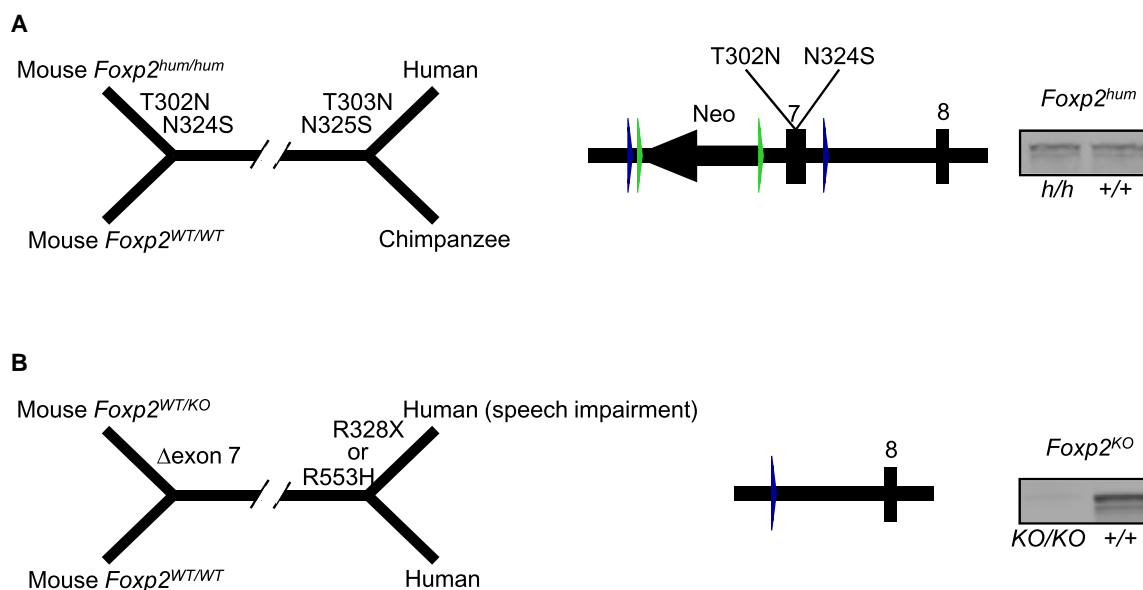
Figure 1. Introduction of Human FOXP2 Substitutions into Mice

(A) Since the human and chimpanzee lineages diverged, human FOXP2 changed at two amino acid positions (T303N and N325S). Only one other amino acid substitution separates humans and chimpanzees from mice (D80E). We generated a *Foxp2* knock-in allele (*Foxp2*hum) of the endogenous mouse *Foxp2* gene. *Foxp2*hum carries the substitutions T302N and N324S which are orthologous to the human substitutions. Its neomycin resistance cassette is flanked by FRT sites (green arrows) and exon 7 which carries the substitutions is flanked by loxP sites (blue arrows). Equal amounts of Foxp2 protein are detected in embryonic brains of mice homozygous for *Foxp2*hum (h/h) and homozygous for the Foxp2 wild-type allele (+/+). See Figure S1 for further details.

(B) A nonfunctional *Foxp2*ko allele was generated from the *Foxp2*hum allele using Cre-mediated recombination in order to model the R328X nonsense mutation (MacDermot et al., 2005) and the R553H missense mutation (Lai et al., 2001) in humans with speech impairment. This leads to an absence of Foxp2 protein in *Foxp2*ko/ko embryos and intermediate levels of Foxp2 in *Foxp2*ko/wt mice (Figure S2).

*Foxp2*hum allele may conceivably be due to impaired Foxp2 function in the mouse background that could be functionally similar to a reduced amount of protein. Furthermore, since humans that are heterozygous for nonfunctional FOXP2 variants exhibit speech and language deficits (Lai et al., 2001; MacDermot et al., 2005), these mice may model FOXP2 effects that lead to speech and language impairments in humans (Groszer et al., 2008). Hence, these mice can be used to assess whether phenotypic changes in *Foxp2*hum/hum mice might be related to the evolution of speech and language.

A Comprehensive Phenotypic Screen

Foxp2 is expressed in the brain (Campbell et al., 2009; Ferland et al., 2003; Lai et al., 2003; Takahashi et al., 2003) as well as in a wide variety of other tissues (Lai et al., 2001; Shu et al., 2005, 2007). For example, it has been proposed to play a role in combination with Foxp1 during the development of the lung and the esophagus (Shu et al., 2007). Thus, the *Foxp2*hum allele may have effects in multiple organs. To investigate which physiological systems might be affected by the human amino acid substitutions we analyzed a total of 60 animals, 15 of each homozygous genotype and sex, derived from 17 litters of heterozygous parents in a large standardized phenotypic screen at the German Mouse Clinic (Gailus-Durner et al., 2005) (see Supplemental Data S2 for a detailed description) that assesses morphological traits (Fuchs et al., 2000), hearing, vision, bone morphology and density (Fuchs et al., 2006), various clinical-chemical parameters and hematological parameters, including

leukocyte subpopulations and classes of immunoglobulins, metabolism, lung function, blood pressure and heart function. We also studied 24 neurological parameters (Schneider et al., 2006) including forepaw grip strength and nociception as well as motor coordination and motor learning, which were assessed on an accelerating rotarod over 3 consecutive days. Sensorimotor behavior was examined by the acoustic startle response and the prepulse inhibition of the startle response. Locomotor activity, exploration, novel object recognition and frequency of contact with group members was assessed using the modified hole board (Ohl et al., 2001). Finally, 31 tissues were analyzed histologically. Many known sex differences were identified in these tests. For the vast majority of tests, no significant *Foxp2*hum effects were found (Table S1). There were, however, two exceptions: First, several measurements indicated a reduced exploratory behavior on the modified hole board (see below) and this was also evident from a reduced forward locomotor activity in the neurology screen (ANOVA, n = 60, p < 0.05). Second, in electrocardiograms *Foxp2*hum/hum mice had lower R-wave amplitudes (ANOVA, n = 39, p < 0.01). To test if these results are robust, we analyzed heart function and behavior on the modified hole board in a second batch of 60 mice. These animals were derived from a different ES cell clone and had the Neomycin cassette (Figure 1A) removed by FLPe recombination between FRT-sites flanking the Neomycin cassette (Supplemental Data S1). No effect of the *Foxp2*hum allele on R-amplitude (ANOVA, n = 59, p = 0.7) was seen in these animals suggesting that *Foxp2*hum has little or no effect on heart function. However, we

again found several measurements indicating reduced exploratory behavior of *Foxp2^hum/hum* mice (Table S2). Importantly, we found no significant interaction between genotype and batch for any parameter, suggesting that the effect of *Foxp2^hum* is independent of the ES cell clone and the presence of the Neomycin cassette (Table S3).

On the modified hole board (Figure S3), *Foxp2^hum/hum* mice from both batches traveled significantly shorter distances (ANOVA, n = 109, p < 0.001) at a significantly lower mean velocity (p < 0.001), made fewer turns (p < 0.01), crossed fewer lines (p < 0.01), entered the hole board less often (p < 0.01), explored fewer holes (p < 0.01) and stayed closer to the wall (p < 0.05) than their wild-type littermates (Figure S3 and Table S5). Since *Foxp2^hum/hum* mice perform as well as their wild-type littermates on the rotarod and show no other signs of motoric impairments (Table S1), their altered behavior can be interpreted as a slightly reduced exploratory activity in a novel environment. The effect also seems to be relatively specific for exploratory behavior since we did not find any behavioral effects in a light-dark box or in an elevated plus maze, two tests assessing anxiety-related behavior (Table S2).

To put these results into perspective, we compared 25 mice heterozygous for the *Foxp2^ko* allele to 23 wild-type littermates in the primary phenotypic screen (Table S4). We found that *Foxp2^wt/ko* mice reacted less to a clicking sound (p < 0.001), show slightly impaired motor learning on the rotarod (less improvement over trials at day 1: p < 0.01), had a higher amount of fat mass (p < 0.05) and a lower amount of lean mass (p < 0.05), consumed more food (p < 0.05), assimilated more energy (p < 0.05), had a higher respiratory rate during activity and at rest (p < 0.05), a lower pulse rate (p < 0.05), slightly higher plasma Ferritin (p < 0.05) and lower plasma inorganic phosphorus (p < 0.05) concentrations and higher proportions of CD62L CD8a+ and CD4+ and CD8+ T cells (p < 0.05). Although we did not replicate these results in a second batch of animals, these results indicate that reduced expression of Foxp2 has several subtle, but significant effects on multiple organs other than the brain. Interestingly, although measures of forward locomotor activity of *Foxp2^wt/ko* mice were reduced on the modified hole board, other parameters, such as a longer exploration of an unfamiliar object (ANOVA, n = 48, p < 0.05), suggest a slightly increased exploratory behavior of *Foxp2^wt/ko* mice (Table S4). Indeed, several parameters differ significantly between *Foxp2^wt/ko* mice and *Foxp2^hum/hum* mice, indicating increased exploratory behavior in *Foxp2^wt/ko* mice and decreased exploratory behavior in *Foxp2^hum/hum* mice (Figure 2 and Table S5).

In summary, we find a reproducible and specific effect of the *Foxp2^hum* allele on mouse exploratory behavior, but no significant effect on almost 300 other measurements assessing a variety of physiological systems. This indicates that the *Foxp2^hum* allele affects predominantly the brain. Furthermore, this does not seem to be a simple loss-of-function effect of the *Foxp2^hum* allele in the mouse since *Foxp2^wt/ko* mice show different and partly opposite effects.

Foxp2^hum Reduces Dopamine Levels

Given that the phenotypic screen indicated that the *Foxp2^hum* allele affects the brain rather than other organ systems, we further investigated the effects of the humanized Foxp2 in this
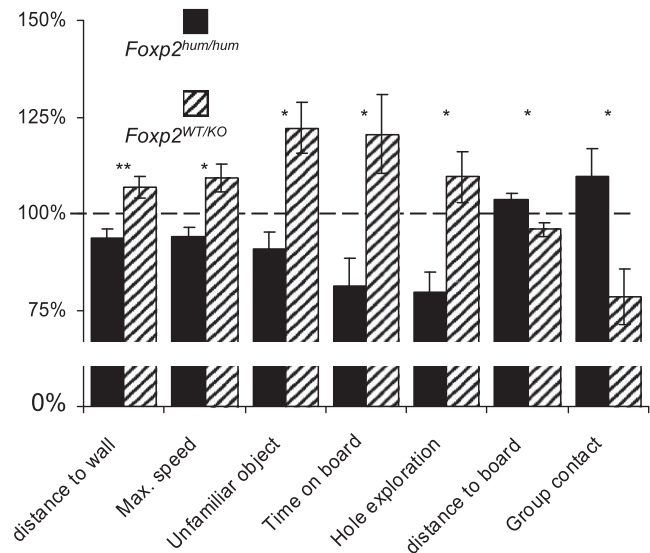

Figure 2. Different Exploratory Behavior of *Foxp2^hum/hum* and *Foxp2^wt/ko* Mice on the Modified Hole Board
All parameters that differ significantly between *Foxp2^wt/ko* (striped bars) and *Foxp2^hum/hum* mice (solid bars) are shown (see Table S5 and Supplemental Data S3 for details). Means (±SEM) are normalized to wild-type levels of the respective littermates (dashed line). Asterisks represent significance levels of 0.05 (*) and 0.01 (**).

organ. We first compared the brains of at least one *Foxp2^wt/wt* and one *Foxp2^hum/hum* mouse at embryonic day 16.5 (E16.5), postnatal day (P) 1, P10, P20 and 3 months with respect to gross anatomy and Foxp2 protein expression and found no differences (Figures S4–S6). Expression patterns agreed well with published results, i.e., expression and nuclear localization of Foxp2 was observed in a subset of postmitotic neurons in the striatum, the thalamus, cortical layer VI, the cerebellum (Purkinje cells), and several other areas (Ferland et al., 2003; Lai et al., 2003; Takahashi et al., 2003).

Next, we analyzed the tissue concentrations of four major neurotransmitters (glutamate, serotonin, dopamine and GABA) in five brain regions (frontal cortex, cerebellum, caudate-putamen, nucleus accumbens and globus pallidus) from 10 male *Foxp2^hum/hum* mice and 10 male *Foxp2^wt/wt* littermates. We found a reduction in dopamine concentrations in all regions (repeated-measure ANOVA, p < 0.001) in *Foxp2^hum/hum* mice but no effects for any of the other neurotransmitters (Table S6). When comparing neurotransmitter levels between 10 male *Foxp2^wt/ko* and 10 male *Foxp2^wt/wt* littermates in the same way, we find an increase in dopamine levels in all regions (repeated-measure ANOVA, p < 0.05; Figure 3) as well as an increase in serotonin levels especially in the nucleus accumbens (Table S6).

Hence, similar to the patterns observed for exploratory behavior, *Foxp2^hum/hum* and *Foxp2^wt/ko* mice show opposite effects on their dopamine levels. These effects could be causally linked since increasing extracellular dopamine levels pharmacologically or genetically tends to increase exploratory behavior and vice versa (David et al., 2005; Viggiano et al., 2003). However, tissue levels of dopamine largely reflect the storage pool of dopamine (Gainetdinov et al., 1998). Further studies are
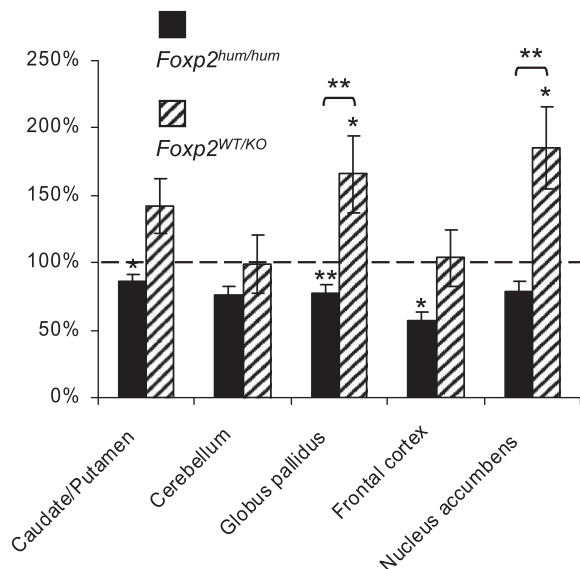
Figure 3. Brain Dopamine Concentrations in *Foxp2^hum/hum* and *Foxp2^wt/ko* Mice

Mean postmortem dopamine concentrations (±SEM) relative to wild-type litter-mates (dashed line) are given for *Foxp2^hum/hum* (solid bars) and *Foxp2^wt/ko* (striped bars) mice in different brain regions. Asterisks above columns indicate a significant difference to wild-type levels (Student's t test), and asterisks above brackets indicate a significant difference between *Foxp2^hum/hum* and *Foxp2^wt/ko* mice (see also Table S6).

needed to clarify effects on extracellular dopamine levels in *Foxp2^hum/hum* and *Foxp2^wt/ko* mice as well as the underlying mechanism. However, since Foxp2 is not expressed in dopaminergic neurons (Wijchers et al., 2006) any mechanism is probably indirect. In this regard, it is interesting that Foxp2 is expressed in many medium spiny neurons (Ferland et al., 2003; Lai et al., 2003; Scharff and Haesler, 2005; Takahashi et al., 2003), which are the major targets of dopaminergic neurons, and make up over 90% of neurons in the striatum. For several reasons they may be of relevance with respect to the role of Foxp2 in speech and language development. First, the striatum is structurally and functionally affected in patients heterozygous for the FOXP2-R553H substitution that impairs speech and language (Liegeois et al., 2003; Vargha-Khadem et al., 1998; Watkins et al., 2002b). Second, medium spiny neurons show abnormal function in mice that are heterozygous for a substitution equivalent to this human mutation (Foxp2-R552H) (Groszer et al., 2008). Third, in birds, a knock-down of FoxP2 in a striatal nucleus (A, area X) impairs vocal imitation (Haesler et al., 2007). Therefore, we decided to further investigate the effects of *Foxp2^hum* on medium spiny neurons.

Foxp2^hum Increases Dendritic Length

We isolated striatal neural precursor cells from *Foxp2^hum/hum* embryos and their *Foxp2^wt/wt* littermates and compared their proliferation and differentiation in vitro. The two genotypes did not differ significantly in cell growth or survival rate during proliferation (Supplemental Data S6). However, 7 days after differentiation, *Foxp2^hum/hum* cells positive for the neural marker TUBB3

had neurites, i.e., outgrowths from the cell body that may represent axons as well as dendrites, that were on average 80% longer than in *Foxp2^wt/wt* cells (Mann-Whitney U, p < 0.01; Figure 4A). In contrast, the size of the cell bodies was not significantly different between genotypes (data not shown).

In order to test the in vivo relevance of this finding, we stained adult brains using the Golgi-Cox method and measured the total dendrite length of three medium spiny neurons each in nine *Foxp2^wt/wt*, six *Foxp2^hum/hum* and six *Foxp2^wt/ko* mice. We found that *Foxp2^hum/hum* mice had dendritic trees that were on average 22% longer than *Foxp2^wt/wt* mice (Mann-Whitney U, p < 0.05) and *Foxp2^wt/ko* mice (p < 0.05; Figure 4B). *Foxp2^wt/ko* mice tended to have neurons with shorter dendrites when compared to their *Foxp2^wt/wt* littermates, but this difference was not significant (Mann-Whitney U, p = 0.145). Thus, *Foxp2^hum* increases the length of the dendritic trees of medium spiny neurons in vitro as well as in vivo.

Foxp2^hum Increases Long-Term Synaptic Depression

Medium spiny neurons integrate glutamatergic input from the cortex and dopaminergic input from the midbrain and generate output that contributes to the control and selection of appropriate behaviors via cortico-basal ganglia circuits (Graybiel, 2008) and the strength of corticostriatal synapses are important for acquiring and altering behaviors (Berretta et al., 2008). In order to investigate whether *Foxp2^hum* influences plasticity of corticostriatal synapses, we recorded membrane potentials of medium spiny neurons in acute tissue slices and studied the long-term depression (LTD) of their depolarization after high-frequency stimulation of cortical fibers. Current-voltage relation-ships and resting membrane potentials did not differ between *Foxp2^hum/hum* and *Foxp2^wt/wt* neurons (Figure S7), indicating that they do not differ grossly in their physiology. However, LTD was almost twice as strong in *Foxp2^hum/hum* neurons compared to *Foxp2^wt/wt* neurons (repeated-measures ANOVA, n = 17, p < 0.05; Figure 5). In stark contrast, it has been reported (Groszer et al., 2008) that LTD in mice heterozygous for the R552H mutation, which corresponds to the R553H mutation implicated in human speech and language deficits, is almost absent in striatal neurons. Thus, whereas *Foxp2^hum* increases synaptic plasticity in medium spiny neurons, a nonfunctional *Foxp2* allele has the opposite effect.

Foxp2^hum and Striatal Gene Expression

Genome-wide gene expression patterns were analyzed in striatal biopsies from embryos (E16.5), young animals (P15-P21) and adults (3 month old) in a total of 30 *Foxp2^hum/hum* mice and their 23 *Foxp2^wt/wt* littermates using high-density oligonucleotide arrays (see Supplemental Data S8 for details). The significance of results was assessed by generating 1,000 data sets where genotype labels were randomly permutated. This is a conservative approach that is robust against violations of analysis assumptions, for example the assumption that the expression levels of genes are independent of each other (Tusher et al., 2001). At a threshold of p < 0.001 (F-test after correcting for batch, age and sex effects), the expression of 34 genes differs between *Foxp2^hum/hum* and *Foxp2^wt/wt* mice in the observed data (Figure S8A), while on average four genes differ at this threshold
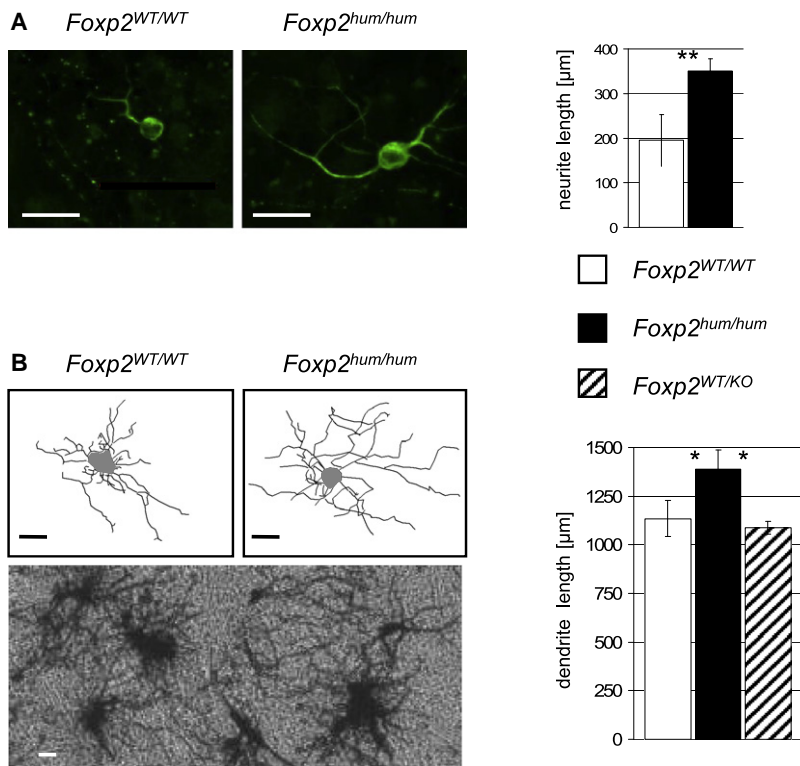
A *Foxp2^{WT/WT}*   *Foxp2^{hum/hum}*

B *Foxp2^{WT/WT}*   *Foxp2^{hum/hum}*

□ *Foxp2^{WT/WT}*

■ *Foxp2^{hum/hum}*

▨ *Foxp2^{WT/KO}*

Figure 4. *Foxp2^{hum}* Increases the Length of Dendritic Trees

(A) *Foxp2^{hum/hum}* and *Foxp2^{wt/wt}* striatal neurons stained for the neuronal marker beta–III-tubulin (green) after 7 days of differentiation. Plotted are averages (±SEM) of three individuals for each of which the total neurite length of five neurons was measured. Asterisks indicate a significant difference (Mann-Whitney U, p < 0.01) between the 15 neurons of each genotype.

(B) Golgi-Cox staining and representative drawings of medium spiny neurons in vivo. Plotted are averages (±SEM) of six (nine for *Foxp2^{wt/wt}*) individuals for each genotype of which the total dendritic tree of three neurons has been measured. Asterisks indicate a significant difference (Mann-Whitney U, p < 0.05) between the neurons of each genotype. Scale bars represent 25 μm.

−0.18; permutation test p = 0.2). Interestingly, among the 106 genes with higher expression in *Foxp2^{wt/ko}* and lower expression in *Foxp2^{hum/hum}*, 24 are known to be preferentially expressed in medium spiny neurons expressing the D1 dopamine receptor (Heiman et al., 2008), whereas only one would be expected by chance (permutation test p < 0.01; Figure S8B). In contrast, no such effect is seen for genes preferentially expressed in medium spiny neurons expressing the D2 dopamine receptor (Supplemental Data S8). Since *Foxp2* itself is preferentially expressed in the former cells (Heiman et al., 2008), this suggests that among the two major known subtypes of medium spiny neurons that differ e.g., in their axonal projections and their electrophysiological properties (Kreitzer and Malenka, 2008), *Foxp2* primarily affects D1 positive medium spiny neurons .

Foxp2^{hum} Alters Vocalization

Finally, to assess whether *Foxp2^{hum}* impacts vocalization, we recorded ultrasonic vocalizations emitted by pups when placed outside the nest (Ehret, 2005) at day P4, P7, P10, and P13 from 32 *Foxp2^{hum/hum}* mice and 39 *Foxp2^{wt/wt}* littermates. Using a semi-automated procedure to extract single calls and a general linear model (GLM) with the variables postnatal day, genotype, sex, litter and weight we found no significant differences in the number of calls emitted per minute or in the duration of intervals between calls (GLM, n = 71, p > 0.4; Figure S9A). To analyze the structure of calls (Figure 6A), we assigned them to one of four categories: (1) calls shorter than 50 ms with no frequency jumps; (2) calls longer than 50 ms with no frequency jumps; (3) calls with frequency jumps; and (4) remaining sounds, which were not analyzed (see Figure S9B for spectrographic displays of call types). The first call type, which was the most frequent, showed no difference between the two genotypes with regard to the number of calls, the duration of calls and five other parameters (Table S7). However, *Foxp2^{hum/hum}* animals, they had a significantly lower start peak frequency (p < 0.001), and lower mean (p < 0.01), minimum (p < 0.01) and maximum (p < 0.001) peak frequencies (Figure 6B). In addition, the slope of the calls declined less in frequency (p < 0.01, Figure 6B) and were locally
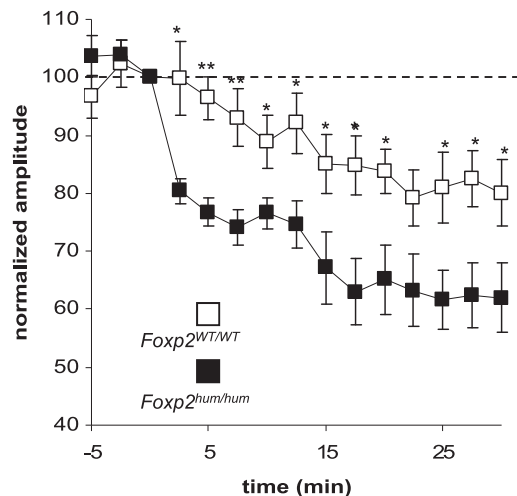
in the permutations (i.e., a false discovery rate of 12%). Among the 1,000 permuted data sets, 36 show 34 or more genes to differ (i.e., permutation test p < 0.05). In the promoters of the genes that are higher expressed in *Foxp2^{hum/hum}* mice, Foxp2 binding motifs (Wang et al., 2003) are enriched (permutation test p < 0.05; e.g., the motif TATTTAT occurs on average 2.6 times in such genes and 1.8 times in other genes), indicating that some of these genes may be primary targets of the humanized version of Foxp2. In conclusion, the analysis shows that *Foxp2^{hum}* has a significant effect on gene expression patterns in the striatum. It should be noted, however, that although many animals were analyzed, the expression of relatively few genes were found to be significantly affected by *Foxp2^{hum}* and the expression of those that are affected differ by no more than 30% from wild-type levels (Figure S8A). Two evolutionary amino acid substitutions in a transcription factor are not necessarily expected to cause major changes in gene expression and it is certainly possible that the subtle expression changes observed is sufficient to cause the phenotypic effects seen in the animals. However, we can obviously not exclude that the *Foxp2^{hum}* allele causes more pronounced changes in expression patterns at other time points during striatal development, or in other parts of the brain.

We also analyzed gene expression in striatal biopsies from 12 *Foxp2^{wt/ko}* and their 6 *Foxp2^{wt/wt}* littermates (P15–P21). *Foxp2* expression in the former mice is reduced to 68% of that in their wild-type littermates in agreement with what is seen in embryonic brains (Figure S2). When *Foxp2^{wt/ko}* and *Foxp2^{hum/hum}* mice (P15-P21) are compared to their wild-type littermates, gene expression tends to be affected in opposite directions (Spearman rank correlation of effect sizes across genes =

Figure 5. *Foxp2^hum* Increases Long-Term Depression in Medium Spiny Neurons

Mean ± SEM amplitudes normalized to baseline levels at time 0 are shown from *Foxp2^hum/hum* (n = 8) and *Foxp2^wt/wt* neurons (n = 9). Following 20 min baseline stimulation, three high-frequency tetani were applied (100 Hz, 3 s) separated by 30 s. Asterisks indicate significantly different means (Student's t test) between *Foxp2^hum/hum* (open squares) and *Foxp2^wt/wt* (filled squares) neurons.

less modulated (p < 0.01). Analyses using a second batch of animals that originated from a different ES cell clone, confirmed these findings except for the parameter local modulation (Table S8 and Supplemental Data S9). Further, similar nonsignificant tendencies were observed for calls longer than 50 ms (data not shown), whereas calls with frequency jumps lasted longer (ANOVA, n = 149, p < 0.05), had longer gaps (p < 0.05) and started (p < 0.01) and ended (p < 0.05) with higher peak frequencies in *Foxp2^hum/hum* mice than in their wild-type littermates (Table S9 and Figure S9C).

Mice homozygous for nonfunctional *Foxp2* alleles produce much fewer isolation calls than their wild-type littermates (Fujita et al., 2008; Groszer et al., 2008; Shu et al., 2005), but given that these animals suffer from severe developmental deficits and die around 3 weeks after birth, this finding may not represent specific effects of Foxp2 on mouse vocalizations (Groszer et al., 2008). It has been suggested that mouse pups heterozygous for nonfunctional *Foxp2* alleles have mild developmental delays and produce fewer ultrasonic calls (Fujita et al., 2008; Shu et al., 2005), but these observations could not be verified in another study (Groszer et al., 2008). Notably, studies of mouse pups with nonfunctional *Foxp2* alleles have not identified differences in the structural properties of calls (Groszer et al., 2008; Shu et al., 2005). Hence, the *Foxp2^hum* allele affects ultrasonic isolation calls of mice subtly but specifically and does so in a way different from nonfunctional *Foxp2* alleles.

DISCUSSION

A Mouse Model for Human Evolution?

To the best of our knowledge, our analysis of *Foxp2^hum* mice represents the first investigation of amino acid substitutions of

potential relevance for human evolution in an animal model. This raises the question whether such genetic changes can be reasonably modeled in a mouse. One concern is that phenotypic effects elicited in the mouse could simply represent an inability of the human gene product to function in the mouse background. A complete inability of the *Foxp2^hum* allele to function in the mouse would be equivalent to a knockout of *Foxp2*. Since *Foxp2^hum/hum* mice are fertile and healthy, whereas mice homozygous for nonfunctional *Foxp2* alleles die within 3–4 weeks after birth (French et al., 2007; Fujita et al., 2008; Groszer et al., 2008; Shu et al., 2005), *Foxp2^hum* certainly functions in the mouse, at least with respect to major effects with obvious phenotypic consequences. Furthermore, when the effects seen in *Foxp2^hum/hum* mice are compared to mice heterozygous for nonfunctional *Foxp2* alleles they are either not observed in the latter mice (e.g., altered vocalization) or show opposite effects to those seen in such mice (e.g., exploratory behavior, dopamine levels, long-term depression). Hence, it seems unlikely that the effects seen in *Foxp2^hum/hum* mice are caused by a simple reduction in biological activity of *Foxp2^hum* in the mouse background.

Given that *Foxp2^hum/hum* mice are generally healthy, how can any specific phenotypic effects be found if they exist? Since FOXP2 is expressed in many organs (Lai et al., 2001; Shu et al., 2001) and *Foxp2^hum* could have effects in any number of these, it is crucial to perform a comprehensive phenotypic screen where the functions of many organ systems are assessed in order not to bias results to organs or behaviors which may a priori be deemed interesting. An analysis of many organ systems is also crucial in order to assess the extent to which effects detected are specific to an organ system or may be secondary, especially if they are subtle as may be expected for evolutionary innovations that occurred over short time scales. Thus, we analyzed almost 300 different phenotypic parameters in the mice. None of them produced any evidence for effects of the *Foxp2^hum* allele in any organ system except the central nervous system. This suggests that the two amino acid substitutions that occurred on the human evolutionary lineage specifically affected the brain in the mouse. We therefore focused the further analyses on this organ.

Of special interest is obviously if any of the effects detected in the *Foxp2^hum/hum* mice might have something to do with any aspect of speech and language in humans. Since humans heterozygous for a nonfunctional *FOXP2* allele show speech and language impairments (Lai et al., 2001; MacDermot et al., 2005; Vargha-Khadem et al., 2005), a comparison with *Foxp2^wt/ko* mice may be helpful as they may recapitulate aspects of speech and language impairment. Thus, traits affected in opposite directions in *Foxp2^wt/ko* and *Foxp2^hum/hum* mice are of potential interest as candidates for being involved in aspects of speech and language evolution. We find that exploratory behavior, dopamine levels, striatal gene expression patterns and striatal synaptic plasticity are all affected in opposite directions in *Foxp2^hum/hum* and *Foxp2^wt/ko* mice respectively in mice heterozygous for a nonfunctional *Foxp2* allele (Groszer et al., 2008). As argued in detail below, some of these effects could model aspects relevant for speech and language in humans.
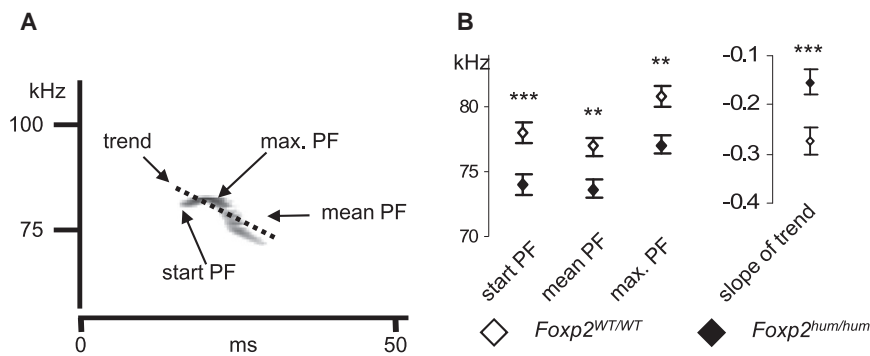
A

B

◇ *Foxp2^WT/WT*   ◆ *Foxp2^hum/hum*

Figure 6. *Foxp2^hum* Affects the Structure of Pup Isolation Calls
(A) Examples of acoustic parameters analyzed illustrated for typical call.
(B) Plots of acoustic parameters differing between genotypes. Measurements are averaged across days and individuals (±SEM). The peak frequency (PF) refers to the frequency with the maximum amplitude in each analyzed time window of 0.21 ms. Asterisks indicate significant differences between genotypes (**: < 0.01; ***: < 0.001).

Relevance of Ultrasonic Vocalization

The fact that *Foxp2^hum* influences ultrasonic vocalization of pups in a specific and reproducible way is of obvious interest. However, it is important to note that this influence is subtle and within the range of normal variation among mice. A relevant question is also to what extent mouse vocalization can be compared to human speech. All terrestrial mammals produce their vocalizations by an air stream from the lungs that passes the larynx and generates sounds. In most animals, oscillations of the vocal folds and/or specific structures of the vocal folds generate the sound through oscillations (Fitch, 2000; Hammerschmidt and Fischer, 2008; Lieberman, 2006). Ultrasonic vocalizations in rodents are also produced by the larynx (Roberts, 1975a) but they are thought to derive from an aerodynamic whistle rather than vibrations of vocal cords (Roberts, 1975b). Nevertheless, the basic neurological and muscular systems necessary for vocalizations probably overlap to a large degree in mice and humans (Hammerschmidt and Fischer, 2008; Jürgens, 2002). Indeed, some neural circuits important for vocalization are even conserved in fish (Bass et al., 2008). Hence, the fact that *Foxp2^hum* influences the structure of isolation calls in the mouse, especially since this effect is not accompanied by physiological effects outside the brain, supports the hypothesis that the two amino acid substitutions that occurred during human evolution affect aspects of speech and/or language.

However, we currently cannot exclude that very subtle changes in lung function or larynx morphology that could be irrelevant for speech and language evolution can be responsible for the effects on call structures, despite the fact that we find no indication that e.g., lung function is influenced by *Foxp2^hum* in adults. Furthermore, it is important to remember that vocalizations of mice as well as most other terrestrial mammals are considered to be innate. Humans share innate vocalizations like grunts, cries, and screams with other animals, but in addition humans have an unmatched ability to learn vocalizations (Egnor and Hauser, 2004; Hammerschmidt and Fischer, 2008). The acquisition and extensions of neural circuits making voluntary control of vocalizations possible is thought to be a hallmark in the evolution of human speech (Jürgens, 2002; Krubitzer, 2007). Since little is known about the neurological and anatomical basis of mouse vocalizations, it is an open question if some neural circuits homologous to the ones making voluntary vocalizations possible in humans would be affected in the *Foxp2^hum/hum* mice. Hence, more studies will be needed to clarify to what extent mouse vocalizations can model aspects of human speech evolution.

As argued below, it will be especially important to clarify any functional relationship to *Foxp2^hum*-dependent effects on cortico-basal ganglia circuits.

Cortico-Basal Ganglia Circuits and Speech and Language

The fact that *Foxp2^hum* affects dopamine levels, dendrite morphology, gene expression and synaptic plasticity of medium spiny neurons indicates that it impacts cortico-basal ganglia circuits where medium spiny neurons in the striatum receive contextual information from the cortex and reward signals from dopaminergic neurons and send integrated signals to brain stem structures and the cortex (Graybiel, 2008). Several lines of evidence indicate that cortico-basal ganglia circuits could be relevant for speech and language (Lieberman, 2002; Lieberman, 2006; Ullman, 2001). For example, reduced dopamine release in the striatum is positively correlated with speed and accuracy of phonological processing (Tettamanti et al., 2005), activation of a part of the striatum plays a crucial role in lexical-semantic control (Crinion et al., 2006), and patients with Huntington's disease show - dependent on the striatal subregions affected - impairments in the retrieval of lexical information and the application of combinatorial rules (Teichmann et al., 2008). Furthermore, cortico-basal ganglia circuits and their dopaminergic modulations are crucial for song learning in birds, which is thought to resemble aspects of vocal learning in humans (Hara et al., 2007; Jarvis, 2004). This is supported by the recent finding that when FoxP2 expression is knocked down in basal ganglia of songbirds, vocal imitation is impaired (Haesler et al., 2007). Furthermore, individuals heterozygous for a nonfunctional *FOXP2* allele show structural effects and functional impairments in the striatum (Vargha-Khadem et al., 2005; Vargha-Khadem et al., 1998) supporting a role for FOXP2 in cortico-basal ganglia circuits with respect to speech and language.

In conclusion, it is possible that the effects on cortico-basal ganglia circuits seen in the *Foxp2^hum/hum* mice model aspects of speech and language evolution in humans. It will now be important to further explore the mechanistic basis of these effects and their possible relationship to phenotypic differences between humans and apes. Currently, one can only speculate about the role these effects may have played during human evolution. However, since patients that carry one nonfunctional *FOXP2* allele show impairments in the timing and sequencing of orofacial movements (Alcock et al., 2000; Watkins et al., 2002a), one possibility is that the amino acid substitutions in

FOXP2 contributed to an increased fine-tuning of motor control necessary for articulation, i.e., the unique human capacity to learn and coordinate the muscle movements in lungs, larynx, tongue and lips that are necessary for speech (Lieberman, 2006). We are confident that concerted studies of mice, humans and other primates will eventually clarify if this is the case.

EXPERIMENTAL PROCEDURES

Generation of Mice

Mice carrying the $Foxp2^{hum}$ allele were generated by Ozgene (Bentley, Australia) from two C57BL/6 ES cell clones that had integrated the vector via homologous recombination. Whereas the first line (clone 5H10) was used for initial analyses, the second one (clone 5H11) was used for testing the reproducibility of the results from the first line. To this end, some mice were generated that carried a $Foxp2^{hum}$ allele in which the Neomycin resistance cassette had been removed by crossings with a FLPe deleter strain. Mice carrying the $Foxp2^{ko}$ allele were generated by crossing chimeric mice (clone 5H11) to a B6 Cre deleter strain (Ozgene, Bentley, Australia). and subsequent crosses to remove the Cre transgene. All animal work was performed in accordance with governmental and institutional ethical guidelines.

Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde in PBS and brains were either paraffin embedded (E16.5) or cryopreserved (P1, P10, P20, and adults). Sections were immunostained with antibodies against FOXP2 (HPA000382 Atlas Antibodies, Stockholm, Sweden or ab16046, Abcam, Cambridge, UK).

Neurotransmitter Measurements

Biopsies were taken from frozen brain slices of 10 male $Foxp2^{hum/hum}$ mice and 10 male $Foxp2^{wt/wt}$ littermates as well as from 10 male $Foxp2^{wt/ko}$ mice and 10 male $Foxp2^{wt/wt}$ littermates. Neurotransmitter levels were measured by high-performance liquid chromatography and normalized to the protein content of the sample to determine their concentrations. We used an ANOVA with brain regions treated as repeated-measures and genotype as factor to determine significant differences between genotypes separately for each strain and neurotransmitter. Differences between $Foxp2^{wt/ko}$ mice and $Foxp2^{hum/hum}$ mice were identified by a significant interaction between strain and genotype in a combined analysis, again using brain regions as repeated-measures.

Neuronal Cell Culture

Striatal neural precursor cells were dissected from ~E14-old embryos, expanded for 4 weeks as three-dimensional (neurosphere) cultures during which proliferation and cell survival was measured. Differentiation was induced via replacement of expansion media by media containing 1% FCS and 5 μM forskoline on precoated poly L-lysine coverslips. After 7 days cells were stained with DAPI and antisera against the neural marker TUBB3. For three cultures per genotype, we measured neurite length of five neurons each.

Golgi Staining

Brains were isolated from six 12-week-old $Foxp2^{hum/hum}$ mice and six wild-type littermates (derived from the 5H11 line in which the Neomycin cassette had been removed) and six 12-week-old $Foxp2^{wt/ko}$ mice and three wild-type litter mates. Golgi-Cox staining was performed using the FD Rapid GolgiStain kit (FD NeuroTechnologies, Ellicott City, MD, USA) according to the manufacturer's protocol. Stained slices were sectioned at a thickness of 200 μm using a cryomicrotome (Microm Thermo Scientific, Walldorf, Germany). Medium spiny neurons were identified by their morphology using a Axiovert 200 microscope equipped with a xyz motorized stage and their dendrites were traced using Mercator software (Explora Nova, La Rochelle, France).

Electrophysiology

Acute brain slices were prepared from the brains of 15- to 27-day-old $Foxp2^{hum/hum}$ mice and their wild-type littermates (all derived from the 5H11 line in which the Neomycin cassette had been removed) and medium spiny neurons in the striatum were recorded at a holding potential of −70 mV. Excitatory medium spiny neuron afferents were stimulated to yield largest EPSC amplitudes without eliciting an action potential or inducing direct stimulation. Continuous stimulation was performed at a frequency of 0.5 Hz. Following 20 min. of baseline stimulation, we applied 3 tetani of 3 s duration and a frequency of 100 Hz separated by 30 s. LTD was then measured for 30 min. Data points were calculated every 30 s by averaging the last 15 EPSCs. LTD data was analyzed using a repeated-measures ANOVA with genotype as fixed factor.

Gene Expression Analysis

Total RNA from striatal biopsies of 13 $Foxp2^{hum/hum}$ embryos (E16.5) and 12 $Foxp2^{wt/wt}$ littermates (prepared in two separate batches), 11 $Foxp2^{hum/hum}$ mice (P15, P18, P21) and 6 $Foxp2^{wt/wt}$ littermates, 12 $Foxp2^{wt/ko}$ mice (P15, P18, P21) and 6 $Foxp2^{wt/wt}$ littermates as well as 6 $Foxp2^{hum/hum}$ mice (3 month old) and 6 $Foxp2^{wt/wt}$ littermates was labeled and hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Expression levels were calculated using Bioconductor (Gentleman et al., 2004) and custom CDF files (Dai et al., 2005). Genotype-dependent effects were assessed after correcting for batch, age and sex effects using multiple regression. Using Cohen's D estimate of effect size, we correlated genotype-dependent effects with functional annotations (Prüfer et al., 2007), putative FOXP2 targets in a human neuroblastoma cell line (Vernes et al., 2007), the number of putative Foxp2 binding motifs (Wang et al., 2003) 5 kbp upstream and 2 kbp downstream of the transcription start site and genes differently expressed in D1 and D2 positive striatal cells (Heiman et al., 2008). The significance of the genotype-dependent effects was in each case assessed against at least 300 permutations of genotype labels. All primary expression data are available at the NCBI GEO database (accession number GSE13588).

Vocalization

For each recording at P4, P7, P10, and P13, a pup was selected randomly, placed on a cotton pad in a plastic beaker, weighed and recorded for 2 min (3 min for P13) in a soundproof plexiglas box. Calls were counted using the AVISOFT Recorder 2.97 (Avisoft Bioacoustics, Berlin). For the analysis of call structure, we visually inspected all recordings to ensure that the automated sampling routine selected only calls of mouse pups and calculated spectrograms. We submitted the resulting spectrograms to a custom software to extract a set of acoustic parameters (Table S7). To eliminate an overrepresentation of subjects with high vocal activity, we randomly selected a maximum of 10 calls per subject and recording day. The mean values per subject and recording day were analyzed using a general linear mixed model (SPSS 13.0), with day (P4, P7, P10, and P13) as within-subject factor, weight as covariate and genotype, sex, and litter as fixed between-subject factors.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, nine tables, nine figures, and a video summary and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00378-X.

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