CNTNAP2 is a direct FoxP2 target in vitro and in vivo in zebra finches: complex regulation by age and activity

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Mutations of FOXP2 are associated with altered brain structure, including the striatal part of the basal ganglia, and cause a severe speech and language disorder. Songbirds serve as a tractable neurobiological model for speech and language research. Experimental downregulation of FoxP2 in zebra finch Area X, a nucleus of the striatal song control circuitry, affects synaptic transmission and spine densities. It also renders song learning and production inaccurate and imprecise, similar to the speech impairment of patients carrying FOXP2 mutations. Here we show that experimental downregulation of FoxP2 in Area X using lentiviral vectors leads to reduced expression of CNTNAP2, a FoxP2 target gene in humans. In addition, natural downregulation of FoxP2 by age or by singing also downregulated CNTNAP2 expression. Furthermore, we report that FoxP2 binds to and activates the avian CNTNAP2 promoter in vitro. Taken together these data establish CNTNAP2 as a direct FoxP2 target gene in songbirds, likely affecting synaptic function relevant for song learning and song maintenance.

Keywords: CNTNAP2, EMSA, FoxP2, gene expression, luciferase assay, QPCR, songbird, spiny neurons, striatum, vocal learning, zebra finch

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Mutations of the transcription factor FOXP2 cause Developmental Verbal Dyspraxia (DVD), a severe speech and language disorder (Lai et al. 2001). Patients with FOXP2 mutations have profound deficits in productive and perceptive aspects of speech and language, and perform particularly poorly in word repetition tasks (Watkins et al. 2002). Interestingly, learning of other motor tasks is less (Alcock et al. 2000) or not affected (Watkins et al. 2002). The disorder is inherited in a monogenic, autosomal dominant way. This makes it possible to study the causality between FOXP2 mutations and DVD and provides an entry point to start addressing the molecular underpinnings of human language.

Towards this end, over 800 candidate direct Foxp2 target genes have been identified in large scale screens in mice and humans (Mukamel et al. 2011; Roll et al. 2010; Spereti et al. 2007; Vernes et al. 2007, 2008, 2011). Among them is contactin-associated protein-like 2 (CNTNAP2), a member of the neurexin protein family (Suedhof 2008; Vernes et al. 2008). CNTNAP2 is implicated in diverse human disorders such as autism, schizophrenia, cortical dysplasia focal epilepsy (CDFE), specific language impairment (SLI), epilepsy, Gilles de la Tourette syndrome and attention deficit hyperactivity disorder (ADHD; Rodenas-Cuadrado et al. 2014), some of which affect language (e.g. autism, Tourette and CDFE). How CNTNAP2 function relates to these pathologies is not yet well understood. CNTNAP2 is relevant for the clustering of voltage gated potassium channels at the juxtaparanodes of myelinated axons (Girault et al. 2003; Horresh et al. 2008; Poliak et al. 1999), but also plays important roles in neuronal migration, network formation, and the growth and maintenance of dendritic spines (Anderson et al. 2012; Gdalyahu et al. 2015; Penagarikano et al. 2011; Rodenas-Cuadrado et al. 2014). Knockdown of Cntnap2 in primary neuron cultures leads to a decrease in dendritic branching, spine head width and synaptic strength (Anderson et al. 2012). Loss of function in mice (Cntnap2−/−) results in fewer striatal interneurons and a lower spine density in cortical layer 5b neurons compared to litter-mate controls (Gdalyahu et al. 2015; Penagarikano et al. 2011). The decrease in spine density occurs because existing spines are eliminated, not because newly generated spines fail to be stabilized (Gdalyahu et al. 2015). Studying CNTNAP2 as a potential target of FoxP2 in postembryonic and adult songbirds holds the promise to illuminate which of the above functions are relevant for song learning and song maintenance.

Songbirds have shed light on the importance of striatal FoxP2 expression for learned vocalizations (Adam et al. 2016; Haesler et al. 2007; Heston & White 2015; Teramitsu & White 2006; Thompson et al. 2013; Wohlgemuth et al. 2014). Experimental alteration of FoxP2 levels in the striatal song nucleus Area X of zebra finches leads to decreased spine density and impairs the learning and production of song (Haesler et al. 2007; Heston & White 2015; Murugan et al. 2013; Schulz et al. 2010). We hypothesized that CNTNAP2 is a direct FoxP2 target gene in songbirds for the following reasons: (1) CNTNAP2 is a target of FOXP2 in humans (Vernes et al. 2008). (2) CNTNAP2 affects spine density in mice (Gdalyahu et al. 2015) as does FoxP2 in zebra finches (Schulz et al. 2010), raising the possibility that the observed effect of FoxP2 on spines is mediated via CNTNAP2. (3) Expression levels of CNTNAP2 and FoxP2 in adult zebra finches are both higher in the striatum than in Area X (Haesler et al. 2004; Panaitof et al. 2010; Thompson et al. 2013). Here we demonstrate
that FoxP2 can bind to and regulate the CNTNAP2 promoter in vitro and that CNTNAP2 expression is correlated to FoxP2 expression in Area X of juvenile and adult males. A FoxP2 knockdown can lead to a correlated CNTNAP2 down-regulation, suggesting that the striatal regulation of CNTNAP2 by FoxP2 is the result of a direct interaction.

Methods

Nomenclature
The FoxP2 nomenclature follows the convention proposed in Kaestner et al. (2000): FOXP2 in Homo sapiens, Foxp2 in Mus musculus and FoxP2 in all other species, including zebra finches (Taeniopygia guttata). For all genes we use the italicized letters to refer to the gene or cDNA and roman type to refer to the protein.

Subjects
All experiments were performed in accordance with the guidelines of the governmental law (TierSchG). 39 male zebra finches were used in this study. Prior to the experiments, animals were housed in large free flight aviaries under a 12h:12h light:dark-cycle with food and water provided ad libitum. Birds were sexed on the day of hatching via polymerase chain reaction (PCR) as previously described (Adam et al. 2014). At the beginning of the experiments, all birds (except those that underwent surgery) were isolated in sound attenuated recording boxes overnight to monitor morning vocal activity. Birds that did not sing were either overdosed with isoflurane immediately after the lights went on in the morning or 2 h later. Birds in the singing group were allowed to sing undirected song and were overdosed after they sang more than 500 motifs within 2 h after their first motif of the day. The first motif had to be sung within 2 h after the lights went on. Vocalizations were continuously monitored and recorded using the Sound Analysis Pro software SAP2011 (Tchernichovs et al. 2000).

Injection of lentiviral vectors
Injections of lentiviral vectors mediating a FoxP2-knockdown were performed as described previously (Adam et al. 2016; Haesler et al. 2007). In brief, at post hatch day 23 (day 23) male zebra finches were injected into Area X with two different lentiviral vectors, in one hemisphere with the vector carrying one of two FoxP2-knockdown constructs (shFoxP2-f or shFoxP2-h), in the other hemisphere with the vector carrying one of two FoxP2-knockdown constructs (shFoxP2-f or shFoxP2-h), in the other hemisphere with a non-silencing control construct (Haesler et al. 2007). Injections (approximately 200 nl per site) were placed into eight sites in each hemisphere. Injection side and order as well as the choice of the injection side were randomized. After surgery the animals were transferred to their home cages and allowed to grow up normally in the presence of their biological parents and siblings. At day 35 birds were overdosed, the brain was quickly extracted and cut into acute slices. Subsequently, microbiopsies of Area X were taken and stored individually at −80°C.

Electrophoretic mobility shift assay
HEK293T cells were transfected with pcDNA4-Foxp2V5-HISB (Haesler et al. 2007) or empty vector and lysates were affinity purified using the HIS-V5 tag. One microgram of purified Foxp2 protein and 0.8 ng of DIG labeled CNTNAP2 probe (oligo sequence 5′-TATTATTTATTATGACTTACATCTTTGATTATGACT-3′, Foxp2 binding sites are indicated by bold letters) were incubated in binding buffer (20 mM Hepes KOH (pH 7.6), 30 mM KCl, 1 mM EDTA (pH 8), 0.2% Tween-20, 10 mM (Na2HPO4, 1 mM DTT) for 15 min at room temperature. For the competition assay 200 ng of unlabeled probe were added to the reaction. For the supershift assay, 1 μg protein was preincubated with 0.5 μg anti-V5 antibody prior to the binding reaction. Separation of protein–DNA complexes was carried out on a 4% polyacrylamide Tris/Glycine/EDTA gel.

Luciferase assays
The zebra finch cell line G266 (Itoh & Arnold 2011) was seeded in a 96-well plate at 2 × 104 cells/well and transfected with 30 ng pGL4.13-CNTNAP2-Luc, 30 ng pGL4.13-CNTNAP2-Luc, 125 ng of pcDNA3.1-FoxP2-Flag (Mendoza et al. 2015) or empty vector. Forty-eight hours after transfection, Luciferase and Renilla activity were measured in a plate reader (Tecan, GENios, Männedorf, Switzerland) using the Dual Glo Luciferase Kit (Promega, Fitchburg, Wisconsin, USA). Mean background from wells not transfected with Luciferase or Renilla expressing vectors was subtracted from all other wells. Luciferase activity was calculated as Relative Luciferase Activity (Luciferase RLU/Renilla RLU).

Microbiopsies, RNA-extraction and cDNA synthesis
All RNA samples were generated for a previous study (Adam et al. 2016), in which the FoxP2 expression data were used in conjunction with another target, the reelin receptor VLDLR. Microbiopsies were taken as previously described (Adam et al. 2016; Olias et al. 2014). Briefly, brains were frozen at −80°C in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and cut sagittally into 200 μm sections on a cryostat. Microbiopsies (1 mm diameter) of Area X were excised and stored individually at −80°C. Remaining sections were stored in 4% (v/v) parafomaldehyde solution and inspected under the microscope for proper targeting of the biopsy.

In the case of virus-injected animals, Area X from both hemispheres was sampled, in the case of unmanipulated birds, only one hemisphere per bird was sampled. The side was chosen randomly. Total RNA from properly targeted microbiopsies of birds with a FoxP2 knockdown was extracted from individual biopsies. For all other animals, properly targeted microbiopsies of one hemisphere were pooled. In all cases total RNA was extracted using the RNAX kit (Macherey-Nagel, Düren, Germany). Complementary DNA (cDNA) synthesis was carried out using random hexamer primers and 180 ng or 40 ng total RNA for the pooled or individual microbiopsies samples, respectively. Reverse-transcriptase free reactions were included to control for genomic DNA contamination. All cDNAs were diluted with nuclease free water (10-fold for pooled, fivefold for individual microbiopsies).

Quantitative Reverse Transcription PCR
qRT-PCR reactions were run in duplicates in a total reaction volume of 20 μl. Five microliter of diluted cDNA were added to 15 μl reaction mix containing 10 μl 2x KAPA SYBR FAST Universal QPCR Mix (Peqlab, Erlangen, Germany), 10 pmol of each primer (18 pmol in the case of FoxP2 and ROX (50 μM, Peqlab) as a reference dye. Reverse-transcriptase free samples as well as no template controls were included to test for DNA-contamination and contamination of reagents, respectively. An inter-run calibrator (IRC) was always included, if samples were run on different plates. The efficiency of each primer pair was checked prior to all measurements using 10-fold dilution series over six orders of magnitude. The efficiency of all primer pairs ranged within 2±10%. We used the following primer pairs: FoxP2 (5′-CCTGGCTGTGAAA GCGTTTG-3′/5′-ATTTGACCCGGACACTGAGC-3′) (Haesler et al. 2007), HMBS (5′-GCAGCATTTGGTCATCAACAG-3′/5′-TGTTGCTTCC CTTGCTCAG-3′) (Haesler et al. 2007), GFP (5′-AGAACAGGCATACAG GTGAAC-3′/5′-GAGGGCAAGGTCAGTGTCCA-3′) (Adam et al. 2016) and CNTNAP2 (5′-GAGGGCAAGGTCAGTGTCCA-3′/5′-GAATGCGAC TCTAGTCCAGTGC-3′). Reactions were run on a MX3005P system (Agilent, Santa Clara, California, USA) using the following temperature program: 10 min at 95°C for 1 cycle, 40 cycles at 95°C, 30 seconds at 95°C, 30 seconds at 65°C and 30 seconds at 72°C. The mean C for each sample was derived from the run data and used to calculate relative gene expression for the gene of interest (GOI); FoxP2 or CNTNAP2. We used HMBS as a reference gene, as it is the most stable of all tested potential reference genes for our experiments (Adam et al. 2016; Haesler et al. 2007). We used the
following formula to calculate the relative expression of our GOIs:

\[
\text{Relative Exp}_{\text{GOI}} = \frac{e^{(C_{\text{GOI}} - C_{\text{REF}})}}{e^{(C_{\text{REF}} - C_{\text{GOI}})}}
\]

Relative expression values were averaged per animal and hemisphere.

Individual biopsies of virus-injected animals were screened for GFP expression as a marker for successful infection prior to running all other assays. Only cDNA from GFP-positive biopsies were used to measure the expression of FoxP2, CNTNAP2 and HMBS. Eighty-four percent of all injected birds expressed GFP in both hemispheres.

Search for transcription factor binding sites in the CNTNAP2 promoter

We utilized the CNTNAP2 promoter sequence that we used in the Luciferase experiment (genomic location: chr2:31 216 312–31 217 544, WashU taeGut3.2.4/taeGut2) to predict transcription factor binding sites by means of three databases – Jaspar (Matheiler et al. 2016), Patch (Matys et al. 2006) and MatInspector (Cartharius et al. 2005) – using the default settings. We merged all results into one table and then retained only matches of transcription factors with a zebra finch homolog including FoxP2 binding sites (Nelson et al. 2013) in the regulatory regions of the zebra finch gene (ENSTGUG0000001794). We identified several FoxP2 binding sites, in particular in the 5′-UTR-region of the gene. To test whether the FoxP2 protein binds to these sites, we conducted electrophoretic mobility shift assays (EMSA). We designed a 46 bp probe containing three binding sites approximately 350 bp upstream of the start-codon (genomic location: chr2:31 216 574–31 216 619, WashU taeGut3.2.4/taeGut2). Adding FoxP2 protein to the labeled probe resulted in an upwards shift of the band (Fig. 1a, ‘ps’), which was abolished by the addition of unlabeled probe (‘competitor’). Pre-incubation with an antibody against FoxP2-V5 led to a supershift of the band (‘ss’), confirming that FoxP2-protein caused the initial shift.

We further utilized luciferase reporter assays to test if binding of FoxP2 to the CNTNAP2 promoter changed its transcriptional activity. We performed these experiments on a zebra finch cell line G266 transfected with a pGL4.13 plasmid containing the SV40 promoter, in contrast, FoxP2 significantly enhanced transcription via the CNTNAP2 promoter.

Results

FoxP2 is directly enhancing CNTNAP2 expression in vitro

A prerequisite for CNTNAP2 to be a FoxP2 target gene is that it has FoxP2 binding sites in its regulatory regions and that FoxP2 can regulate CNTNAP2 expression by binding to these motifs. To investigate this in zebra finches, we screened for FoxP2 binding sites (Nelson et al. 2013) in the regulatory regions of the zebra finch CNTNAP2 gene (ENSTGUG0000001794). We identified several FoxP2 binding sites, in particular in the 5′-UTR-region of the gene. To test whether the FoxP2 protein binds to these sites, we conducted electrophoretic mobility shift assays (EMSA). We designed a 46 bp probe containing three binding sites approximately 350 bp upstream of the start-codon (genomic location: chr2:31 216 574–31 216 619, WashU taeGut3.2.4/taeGut2). Adding FoxP2 protein to the labeled probe resulted in an

CNTNAP2 and FoxP2 expression are positively correlated in Area X of juvenile males

FoxP2 expression is lower in Area X of adult males than in juvenile males (Haesler et al. 2004; Thompson et al. 2013)
Figure 2: CNTNAP2 expression is strongly and positively correlated to FoxP2 expression in Area X of non-singing juveniles.

(a) FoxP2 mRNA expression levels in Area X are significantly higher in juvenile males (50–52 days, mean 50 days, no fill, n = 15) than in adult males (>120 days, 123–968 days, mean 297 days, black fill, n = 9). Expression levels were measured by qRT-PCR from microbiopsy samples of Area X of juvenile and adult males that did not sing prior to sacrifice. (b) CNTNAP2 mRNA expression levels are significantly higher in adults than in juveniles. (c) Expression of CNTNAP2 was significantly and positively correlated with FoxP2 expression in non-singing juveniles, (d) but not in non-singing adults. Each dot represents one individual; the dashed lines around the fitted line denote the 0.95 confidence interval. Gene expression was normalized to the reference gene HMBS and the mean of the adult group was set to 1. Upper case letters denote adult males, lower case letters juvenile males.

Additional text:

and is downregulated by undirected singing in both age groups (Teramitsu & White 2006; Teramitsu et al. 2010). If FoxP2 directly regulated CNTNAP2 in Area X of zebra finches we would expect to see positively correlated changes in CNTNAP2 mRNA levels. To test our hypothesis, we measured the expression levels of both genes in Area X of juvenile and adult males. To exclude the influence of undirected song on this dataset, we made sure that males did not sing on the day of sacrifice. After taking microbiopsies from Area X we performed qRT-PCR. The results confirmed our previous finding that FoxP2 levels in Area X are lower in adult birds (>120 days, 123–968 days, mean 297 days, n = 9) than in juveniles (50–52 days, mean 50 days, n = 15; Mann Whitney U-test, U = 120, P = 0.0009774; Fig. 2a; Haesler et al. 2004). The opposite was the case for CNTNAP2 expression, which was significantly higher in adults compared to juveniles (Mann Whitney U-test, P = 0.002737; Fig. 2b). This direction of regulation suggested that FoxP2 may be repressing CNTNAP2 expression in Area X of male zebra finches. If this were the case, we expected to find a negative correlation between the expression levels of FoxP2 and CNTNAP2 in individual birds. However, FoxP2 and CNTNAP2 levels were strongly positively correlated in juvenile individuals (Linear Model, R² = 0.467, adjusted R² = 0.4261, F₁,13 = 11.39, P = 0.004973; Fig. 2c). Interestingly, this was not the case in adult birds (Linear Model, R² = 0.009593, adjusted R² = 0.1319, F₁,7 = 0.0678, P = 0.8021; Fig. 2d). How can we reconcile the finding that CNTNAP2 expression is enhanced by FoxP2 in juvenile non-singing males with the fact that levels of CNTNAP2 expression are not linked to levels of FoxP2 expression in non-singing adults? Another, unidentified, age-dependent factor might enhance...
CNTNAP2 expression in adult AreaX and this could cause the significantly higher CNTNAP2 mRNA levels in adult males in comparison to juvenile males. To address this possibility, we searched in silico for transcription factor binding sites on the CNTNAP2 promoter to narrow down the list of potential transcription factors affecting CNTNAP2 expression in adult non-singers. We therefore only took transcription factors into account that are expressed in adults but not in juveniles (Hilliard et al. 2012, unpublished own data), (see Methods for details). We identified 76 transcription factors, which are only expressed in adult AreaX and have predicted binding sites in the CNTNAP2 promoter (Table S1, Supporting information).

**Methods**

To causally link our finding that CNTNAP2 expression is positively correlated with FoxP2 expression, we experimentally reduced FoxP2 levels in AreaX of juvenile males using lentiviral vectors as described previously (Adam et al. 2016; Haesler et al. 2013; Schulz et al. 2010). We expected to see a downregulation of CNTNAP2 mRNA levels positively correlated to the decrease of FoxP2. Lentiviral vectors mediating a FoxP2 knockdown were injected into AreaX of one hemisphere of juvenile males at day 23, while a control construct was injected into the contralateral hemisphere. After confirming that FoxP2 was downregulated on the knockdown hemisphere compared to the control hemisphere (Wilcoxon signed rank test, \( W = 28, P = 0.01563, n = 7 \); Fig. 4a), we measured the expression of CNTNAP2 in all hemispheres. CNTNAP2 levels were reduced in four of seven knockdown hemispheres when compared to the control hemisphere of the same animal (Wilcoxon signed rank test, \( W = 18, P = 0.1422 \); Fig. 4b). Moreover the expression of CNTNAP2 was positively correlated to the FoxP2 expression levels in both, the control (Linear Model, \( R^2 = 0.6817 \), adjusted \( R^2 = 0.6018 \), \( F_{1,6} = 10.711, P = 0.02215 \); Fig. 4c) and the knockdown group (Linear Model, \( R^2 = 0.5686 \), adjusted \( R^2 = 0.4823 \), \( F_{1,6} = 6.5911, P = 0.0502 \); Fig. 4d) as indicated by the significant positive correlation between the expression of the two genes. We concluded that FoxP2 can indeed regulate CNTNAP2 expression in vivo.

**Discussion**

In this study, we investigated whether CNTNAP2 is a direct FoxP2 target gene in songbirds as reported for humans (Vernes et al. 2008). We found that FoxP2 protein directly bound to and regulated the CNTNAP2 promoter. In non-singing juvenile zebra finches CNTNAP2 and FoxP2 expression were positively correlated, which was also the case in adult males when they were singing vigorously. In the same vein, an experimental knockdown of FoxP2 led to lower CNTNAP2 levels in four of our seven birds, even though this difference did not reach statistical significance. The regulatory relationship between FoxP2 and CNTNAP2 may contribute to the behavioral and neuroanatomical changes observed after knockdown of FoxP2 in male songbirds, like the previously observed effect on striatal spine density (Schulz et al. 2010).

**CNTNAP2 expression in zebra finches can be regulated by FoxP2**

We found CNTNAP2 expression levels in AreaX to be lower in non-singing juveniles than in non-singing adult male zebra finches. While age-related upregulation of CNTNAP2 was also reported in LMAN of zebra finches (Condro & White 2014; Panaitof et al. 2010) and in cultured murine pyramidal
neurons (Varea et al. 2015), Panaitof et al. (2010) did not find expression differences between juvenile and adult Area X. The discrepancy between our findings and those by Panaitof et al. (2010) might be due to the fact that we used qRT-PCR whereas Panaitof et al. (2010) performed in situ hybridizations, methods with different dynamic ranges. Additionally, Panaitof et al. (2010) normalized the CNTNAP2 expression in Area X to the expression in the surrounding striatum, so that a concomitant upregulation of CNTNAP2 in the striatum could obscure the upregulation of CNTNAP2 in Area X.

In our study, FoxP2 expression was positively correlated with CNTNAP2 expression in zebra finches. The direction of regulation differs in human neuron-like cell line, where overexpression of FoxP2 leads to downregulation of CNTNAP2 expression (Vernes et al. 2008). Likewise, in the developing human cortex CNTNAP2 is expressed highest in layers with low FOXP2 expression and vice versa (Vernes et al. 2008). One explanation for these tissue differences could be that transcription factors binding to the same site can repress or enhance a gene depending on the presence of other transcription factors (Diamond et al. 1990). Different cofactors exist in different cell lines and tissues, influencing the direction of regulation. Examples for such cofactors could be other FoxP-family members (FoxP1 and FoxP4), Nkx2.1, CtBP1, PIAS1, members of the NuRD chromatin-remodeling complex and members of the FoxO-subfamily, all known to interact with FoxP2 and to modulate its ability to regulate target gene expression (Chokas et al. 2010; Estruch et al. 2016; Li et al. 2004; van Boxtel et al. 2013; Zhou et al. 2008). Furthermore, all of these cofactors are expressed in Area X of juvenile as well as adult male zebra finches (unpublished own data, Hilliard et al. 2012). Especially the FoxO-family members are promising candidates because FoxO3 and FoxP1 are known to bind to the same enhancers. Through this interaction FoxP1 is able to regulate specific FoxO3 target genes (van Boxtel et al. 2013). The same kind of interaction might occur between FoxP2 and FoxO4 binding sites, which are very similar to the FoxP1 and FoxO3 binding sites, respectively (Mathelier et al. 2016).

Tissue specific differences determining the direction of regulation are also likely since the negative correlation of FOXP2 and CNTNAP2 levels in human cortex (Vernes et al. 2008) is not mirrored in developing dorsal thalamus and striatum of humans where both genes can be co-expressed strongly (Alarcon et al. 2008; Teramitsu et al. 2004). Furthermore, in the zebra finch brain CNTNAP2 and FoxP2 expression overlap in the striatum, Purkinje cell layer and the optic tectum, whereas in LMAN and nidopallium CNTNAP2 is expressed but FoxP2 is not. In general, CNTNAP2 has a much wider expression pattern in the songbird brain (Condro & White 2014; Panaitof et al. 2010) than FoxP2 (Haesler et al. 2004; Teramitsu et al. 2004). In areas where both FoxP2 and CNTNAP2 are co-expressed, as is the case in Area X, our data show that FoxP2 can positively influence CNTNAP2 expression (e.g. in juveniles and singing adults); however, FoxP2 expression does not always have this effect, shown by the
absence of a relationship in non-singing adults. In the latter case, FoxP2 might not bind to the CNTNAP2 promoter or if it does, additional factors prevent its transcriptional regulation of CNTNAP2.

In summary, FoxP2 is clearly not the only transcription factor regulating CNTNAP2 expression. To search for additional ones expressed in Area X we used an in silico approach and identified 76 transcription factors with predicted binding sites in the CNTNAP2 promoter. These transcription factors are good candidates to mediate the age-dependent upregulation of CNTNAP2 expression. Further experiments are needed to address whether the candidates from this list actually bind to the CNTNAP2 promoter in vivo. The candidate gene with most binding sites in the CNTNAP2 promoter was SP3, a transcription factor known to repress the SP1 mediated activation of the human D1A receptor gene (Yang et al. 2000). Interestingly, D1A is regulated by FoxP2 in zebra finches (Murugan et al. 2013). Being regulated by FoxP2 and SP3 might thus be common to a subset of FoxP2 target genes.

Taking our findings and published data together, FoxP2 does regulate CNTNAP2 in Area X of male zebra finches, yet the relationship is more complicated than a simple ‘more is more’ dependency. We propose that during periods of enhanced plasticity, such as in juvenile males and during undirected singing in adult males, FoxP2 influences CNTNAP2 expression in a linear manner. However, in the ‘default state of the adult motor system’ (Brainard & Doupe 2013), when the anterior forebrain pathway is not injecting variability into the motor pathway, the influence of FoxP2 on CNTNAP2 might be overridden by other factors, e.g. miRNAs or other transcription factors.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Table S1 Predicted transcription factor binding sites in the zebra finch CNTNAP2 promoter.