Published in final edited form as:

Nat Neurosci. 2010 November ; 13(11): 1345–1347. doi:10.1038/nn.2658.

Fezf2* directs the differentiation of corticofugal neurons from striatal progenitors *in vivo

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Abstract

In the developing cerebral cortex, cell-extrinsic and cell-intrinsic signals govern the establishment of neuron subtype-specific identity. Here we show that, within the niche of the striatum, the expression of a single transcription factor, *Fezf2*, is sufficient to generate corticofugal neurons from progenitors fated to become medium spiny neurons. This demonstrates that a specific population of cortical projection neurons can be directed to differentiate outside of the cortex by cell-autonomous signaling.

The mammalian cerebral cortex contains an unparalleled diversity of neuron types. Glutamatergic projection neurons represent the majority of cortical neurons, and they include many subtypes, each with distinct anatomical and functional properties¹. Corticofugal (CFu) projection neurons are one subpopulation of cortical projection neuron that reside in deep layers Vb and VI of the cortex. They connect the cortex to its subcortical and subcerebral targets in the thalamus, brainstem and spinal cord¹. The generation of CFu neurons in the embryo is a multistep process that begins with the fate specification of neural progenitors to acquire cortical (dorsal) identity at the expense of ventral progenitor identity. Among other genes, this requires expression of *Pax6* and *Emx2* (refs. 2,3). In addition, cortical progenitors are specified to a glutamatergic fate, which is dependent on the expression of *Pax6*, *Ngn1* and *Ngn2* (ref. 4). Finally, the differentiation of individual populations of projection neurons is controlled by neuronal subtype-specific genes, which have only begun to be identified. By comparing expression profiles of pure populations of corticospinal motor neurons (CSMN) and callosal projection neurons (CPN), we previously identified genes that define these projection neuron subtypes^{5,6}. Among these, the transcription factor *Fezf2* is necessary for the specification of CSMN and related

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AUTHOR CONTRIBUTIONS

P.A. and C.R. conceived the experiments and wrote the manuscript. P.A. supervised the project and C.R. executed all the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Note: Supplementary information is available on the Nature Neuroscience website.

populations of subcerebral projection neurons, and it is sufficient to specify CFu projection neurons during late corticogenesis⁷⁻⁹. This suggests that *Fezf2* alone might be able to instruct projection neuron subtype-specific development from neural progenitors of a different cell fate, within neurogenic niches of other CNS regions.

Here, we investigated whether the ectopic expression of *Fezf2* in neural progenitors of the striatum would be sufficient to instruct the generation of CFu projection neurons, bypassing typical requirements for early cortical progenitor patterning and glutamatergic specification. We injected a *Fezf2*^{GFP} expression vector (*Fezf2* combined with an internal ribosomal entry site (IRES) and *GFP*) in the lateral ventricle under ultrasound guidance at embryonic day (E) 12.5 and directionally electroporated it *in utero* into neural progenitors of the lateral ganglionic eminence (LGE). Electroporated embryos were allowed to develop until E18.5. LGE progenitors normally give rise to GABAergic medium spiny neurons (MSN) of the striatum and interneurons of the olfactory bulb¹⁰. They do not produce cortical projection neurons. *In situ* hybridization for *Fezf2* showed that it is not normally expressed within the LGE and in other regions of the developing and postnatal striatum (Supplementary Fig. 1 and ref. 9). We found that *Fezf2*^{GFP}-electroporated LGE progenitors gave rise to neuronal progeny that migrated within the developing striatum, similarly to non-electroporated MSN (Fig. 1). However, despite being located within the niche of the striatum, *Fezf2*^{GFP}-neurons expressed proteins that are specific to CFu neurons of the cerebral cortex. These included SOX5, typically expressed in all CFu neurons; TBR1, a gene expressed at high levels in layer VI, in a subset of layer V CFu neurons and at lower levels in upper layer cortical neurons; BHLHB5 (BHLHE22), a transcription factor that early in development labels subcerebral projection neurons, including CSMN; and ZFPM2 (FOG2), normally expressed in layer VI cortical neurons ($n = 4$) (see Supplementary Methods for primary references). In contrast, none of these genes were expressed in MSN developing in the unelectroporated contralateral hemisphere (Fig. 1). Quantification of the percentage of *Fezf2*^{GFP}-electroporated neurons expressing CFu neuron markers showed that $82.8 \pm 3.1\%$ expressed TBR1 ($n = 4$) and $41.1 \pm 5.2\%$ expressed BHLHB5 ($n = 2$), a gene that at this age can distinguish subcerebral projection neurons of layer Vb from CFu neurons of layer VI. The data support a model by which, among the CFu neuron classes, *Fezf2* instructs the birth of both subcortical projection neurons of layer VI and subcerebral projection neurons of layer V. *Fezf2*^{GFP}-electroporated neurons were also positive for *Vglut1*, which they expressed at levels comparable to those in neocortical projection neurons at this age, indicating that they had acquired a glutamatergic identity (Supplementary Fig. 2). To determine whether, in addition to molecular properties of CFu neurons, *Fezf2*^{GFP}-electroporated neurons developed aspects of neuronal pyramidal morphology typical of cortical projection neurons, we let mice electroporated with either *Fezf2*^{GFP} or control *GFP* develop postnatally (until postnatal day 15) and analyzed neuronal morphology (Supplementary Fig. 3a). We found that $63.8 \pm 0.9\%$ of *Fezf2*^{GFP} neurons showed pyramidal-like morphology with a distinct primary process that resembled an apical dendrite ($n = 4$) (Supplementary Fig. 3b,c). This corresponded to three times as many pyramidal-like neurons as in control *GFP*-electroporated brains, and it was accompanied by a nearly three-fold decrease in the number of neurons with stellate-like morphology (typical of MSN) (Supplementary Fig. 3b,c). The data indicate that a distinct fraction of the *Fezf2*^{GFP} neurons can cell-autonomously acquire

morphological features of cortical projection neurons. These results motivated us to investigate long-distance axonal connectivity by the electroporated neurons. Not surprisingly, given their ectopic location, many *Fezf2^{GFP}* neurons projected to the substantia nigra, however, by postnatal day 15, we observed extensive innervation of the thalamus and the cerebral peduncle (Supplementary Fig. 4a–e). Considering the central role of the environment in axon guidance and target innervation, it is noteworthy that several of the *Fezf2^{GFP}*-positive neurons (albeit not all of them) were able to reach targets that are appropriate for layer VI (namely, thalamus) and V (namely, cerebral peduncle) CFu projection neurons.

To determine whether, in response to ectopic *Fezf2* expression, LGE progenitors undergo a full change of fate or, rather, give rise to neurons of mixed lineage identity, we performed *in situ* hybridization on electroporated brains for *Meis2*, *Islet1* and *Drd2*, three genes that label the majority of MSN and are absent from the cerebral cortex (Fig. 2a; see Supplementary Methods for primary references). Examination of the striatum in the *Fezf2^{GFP}*-electroporated hemisphere revealed distinct areas that were depleted of *Meis2*, *Islet1* and *Drd2* expression (Fig. 2a). We found that TBR1-positive CFu projection neurons occupied these spaces, exactly bordering and not overlapping with neighboring MSN (Fig. 2a). The absence of *Drd2* expression suggests that these neurons may not be able to respond to dopaminergic nigrostriatal input. In addition, and in contrast to GABAergic MSN, *Fezf2^{GFP}*-expressing neurons did not express GAD67, an enzyme necessary for GABA synthesis (Fig. 2b). These data indicate that expression of *Fezf2* in LGE progenitors caused a complete switch of fate to generate glutamatergic CFu projection neurons at the expense of GABAergic MSN. At least two mechanisms may account for these results: (i) *Fezf2* might instruct ventral neural progenitors (for example, expressing *Gsh2* and *Mash1* (refs. 2,3)) to differentiate directly into CFu neurons; alternatively, (ii) *Fezf2* might first instruct LGE progenitors to adopt a cortical progenitor identity (for example, initiate expression of *Pax6* and *Ngn2*) before differentiating into CFu neurons. We examined these possibilities and found the former to be true. Two days after electroporation, *Fezf2^{GFP}*-electroporated LGE progenitors did not express either *Ngn2* (Fig. 3a) or PAX6 (Fig. 3b). In addition, they did not express TBR2, a marker of cortical intermediate progenitors (Fig. 3c). On the contrary, they maintained expression of GSH2 and *Mash1* (Supplementary Fig. 5a,b). Finally, a time course of analysis for TBR1 expression showed that this postmitotic cortical marker appeared as early as 2 d ($3 \pm 1.5\%$ of neurons; $n = 2$) and 4 d ($16 \pm 2.3\%$ of neurons; $n = 2$) after electroporation, with $82.8 \pm 3.1\%$ of neurons expressing TBR1 at E18.5 ($n = 4$). These results indicate that LGE progenitors did not need to adopt cortical progenitor identity to generate CFu projection neurons in response to *Fezf2*, and support the possibility that *Fezf2* may act postmitotically.

Together, the data demonstrate that a single transcription factor, *Fezf2*, is sufficient to instruct progenitors of GABAergic MSNs to switch fate and generate a specific population of glutamatergic projection neurons of the cerebral cortex: corticofugal projection neurons. This effect is independent from the expression by LGE progenitors of *Pax6* and *Ngn2*, which in the cortex are centrally important for the generation of glutamatergic projection neurons⁴. *Ngn2* in particular was also recently found to be sufficient to ectopically instruct glutamatergic identity¹¹. A large number of neurons acquired molecular features of CFu

neurons, and a subpopulation also developed pyramidal-like morphology and connectivity to CFu-appropriate subcortical and subcerebral targets. This switch of fate occurred despite the location of the newly generated CFu neurons within the striatum, a niche that does not normally host development of cortical projection neurons. Despite this ectopic location, these neurons survived and could mature in the postnatal striatum. Previous transplantation experiments have shown that basal ganglia progenitors retain the ability to generate different types of neurons if removed from their own niche and exposed to extracellular signals of other neurogenic regions^{12–14}. Our experiments now demonstrate that cell-autonomous signals that drive neuron type-specific development in the cortex can override extracellular niche restrictions and instructions over progenitor cell fate. These findings support a model in which key transcription factors might be used to direct the differentiation of clinically relevant neuron types for future therapeutic benefit.

Supplementary Material

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Acknowledgments

We thank J. Macklis (Harvard University) for reagents and insightful advice; E. Abdel-Azim, G. Srubek-Tomassy, F. Zhang and S. Lodato for their comments; R. Hevner, (University of Washington), C. Lois (M.I.T), K. Campbell (University of Cincinnati) and Q. Ma (Harvard Medical School) for generous sharing of antibodies, probes and expression vectors; and A. Meleski, A. Merlino and Z. Trayer-Gibson for technical support. This work was partially supported by grants from the National Institutes of Health (NS062849), the Spastic Paraplegia Foundation, the Harvard Stem Cell Institute and the Harvard NeuroDiscovery Center to P.A.; C.R. was partially supported by a Milton-Safenowitz postdoctoral fellowship from the ALS Association.

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Figure 1.

LGE progenitors generate neurons with corticofugal molecular properties in response to *Fezf2*. *Fezf2*^{GFP}-expressing neurons located in the striatum express SOX5, TBR1, BHLHB5 and ZFPM2 (arrows), which are absent from the contralateral striatum (arrowheads). Ctx, cortex; LV, lateral ventricle; Str, striatum; CC, corpus callosum. Scale bars, 500 μm.

Figure 2. *Fezf2^{GFP}*-electroporated neurons do not express markers of MSN. **(a)** Coronal sections from contralateral and *Fezf2^{GFP}*-electroporated striatum showing absence of *Meis2*, *Islet1* and *Drd2* from TBR1-expressing CFu neurons. **(b)** *Fezf2^{GFP}*-expressing neurons do not express *Gad67* (consecutive sections). Ctx, cortex; LV, lateral ventricle; Str, striatum; CC, corpus callosum. Scale bars, 500 μ m and, in magnifications of boxed areas, 100 μ m.

Figure 3.

Fezf2 instructs the generation of CFu neurons from LGE progenitors without expression of cortical progenitor-specific genes. (a) Consecutive coronal sections showing that *Fezf2*^{GFP}-expressing LGE progenitors do not express *Ngn2*. (b,c) Confocal analysis showing that *Fezf2*^{GFP}-expressing LGE progenitors do not express PAX6 and TBR2. Ctx, cortex; LV, lateral ventricle; Sept, septum. Scale bars, 500 μm and, in magnifications of boxed areas, 100 μm.