Direct lineage reprogramming of postmitotic callosal neurons into corticofugal neurons in vivo

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Abstract

Once programmed to acquire a specific identity and function, cells rarely change *in vivo*[1]. Neurons of the mammalian central nervous system (CNS) in particular are a classic example of a stable, terminally differentiated cell type. With the exception of the adult neurogenic niches, where a limited set of neuronal subtypes continue to be generated throughout life[2,3], CNS neurons are only born during embryonic and early postnatal development. Once generated, neurons become permanently postmitotic and do not change their identity for the life span of the organism. Here, we have investigated whether excitatory neurons of the neocortex can be instructed to directly reprogram their identity postmitotically from one subtype into another, *in vivo.* We show that embryonic and early postnatal callosal projection neurons (CPN) of layer II/III can be postmitotically lineage reprogrammed into layer V/VI corticofugal projection neurons (CFuPN) upon expression of the transcription factor *Fezf2*. Reprogrammed callosal neurons acquire molecular properties of corticofugal projection neurons and change their axonal connectivity from interhemispheric, intracortical projections to corticofugal projections directed below the cortex. The data indicate that during a window of postmitotic development neurons can change their identity, acquiring critical features of alternate neuronal lineages.

Seminal work has challenged the idea that differentiated cells cannot change their identity and has underscored the powerful role played by transcription factors in instructing lineage reprogramming [4–6]. However, whether all cells possess the same ability to change identity is still unclear. In the central nervous system, neurons represent a quintessential example of a permanently postmitotic and differentiated cell type. Despite the tight control over maintenance of neuronal identity, it is not known whether neurons can be lineage reprogrammed to a new cell fate under appropriate stimuli.

Prior work has demonstrated that an entire mouse can be cloned via the transfer of nuclei of at least some neuron types into enucleated oocytes[7,8]. This work supports the argument that in the CNS neurons resist phenotypic changes without irreversible loss of genetic material and that epigenetic mechanisms that may be in place to maintain cell identity, can be reset under appropriate conditions. In support of this notion is the demonstration that spinal motor

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neurons can undergo a class switch if manipulated immediately after exit from the cell cycle[9–11].

To determine whether excitatory neurons of the cerebral cortex, one of the most complex regions of the CNS, can undergo direct lineage reprogramming after they have become postmitotic and whether this can occur *in vivo*, we have investigated if a lineage conversion between layer II/III callosal projection neurons and layer V/VI corticofugal projection neurons[12], can be instructed within the brain. Both of these neuronal subtypes are generated during development and do not regenerate in the adult[13].

The molecular programs controlling CFuPN development are emerging[12,14]. In particular, the transcription factor *Fezf2* is a powerful master gene necessary for the development of subcerebral projection neurons, a class of CFuPN[15–17], and whose overexpression is sufficient to confer corticofugal neuronal identity on neural progenitors fated to generate different neuronal populations[17,18].

Here, we developed two different approaches to ectopically express *Fezf2* in postmitotic layer II/III CPN. First, to determine whether at the earliest stages of postmitotic development CPN retain the ability to lineage reprogram, we have taken advantage of the *Cdk5r* gene promoter[19] to drive *Fezf2* expression in "young" postmitotic CPN. In the developing cerebral cortex, *Cdk5r* is restricted to migratory, postmitotic neurons[20,21]. To confirm the specificity of its minimal promoter, we co-electroporated *Cdk5r-DsRed* and *Nestin-eGFP* expression vectors in E13.5 cortical progenitors and determined their expression patterns. As expected, we found that eGFP was restricted to progenitors while DsRed was restricted to postmitotic CPN (Supplementary Fig. 1).

Cdk5r-Fezf2^{eGFP} or control *Cdk5r-Ctl^{eGFP}* expression vectors were then electroporated *in utero* into cortical progenitors at E14.5 and mice were sacrificed at E18.5, P7, P14 and P28. In both control and *Fezf2*-electroporated embryos, GFP-positive neurons were visible in the upper layers; however, neurons that expressed *Fezf2* showed a reproducible change in migration, with a subset of neurons located ectopically in the deep layers (Figure 1c). We thus concentrated our analysis only on GFP-positive neurons located within layer II/III. We first analyzed the molecular identity of the neurons generated. We found that ER81, CRYM, TLE4, CTIP2, ZFPM2 and *Clim1* – all markers of CFuPN excluded from layer II/III CPN[12,14,16,22] – were present in layer II/III of the *Cdk5r-Fezf2^{eGFP}* cortex (n=4) but not in control *Cdk5r-Ctl^{eGFP}*-electroporated cortex (n=4) at all ages examined (Fig. 1, 2, 3). Next we quantified at the single neuron level the percentage of GFP-positive neurons in layers II/III that expressed CFuPN and CPN proteins. We found that, at P7, CPN that had received *Fezf2* expressed ER81, a protein normally restricted to layer V[22] (49.18 +/− 7.7% versus 0.41 +/− 0.31% in *Cdk5r-Ctl^{eGFP}*); CRYM, a protein selectively expressed in CFuPN[14] (43.51 +/− 2.02% versus 0.36 +/− 0.36% in *Cdk5r-Ctl^{eGFP}*); TLE4, a protein restricted to corticothalamic neurons[12] (47.98 +/− 3.62% versus 0% in *Cdk5r-Ctl^{eGFP}*); CTIP2, a protein specific for subcerebral projection neurons and corticothalamic neurons[14] (19.90 +/− 2.61% versus 0.61 +/− 0.25% in *Cdk5r-Ctl^{eGFP}*); and ZFPM2, a protein only expressed in CFuPN[16] (2.70 +/− 1.01% versus 0% in *Cdk5r-Ctl^{eGFP}*). Notably, CUX1, a protein selectively expressed in upper layer CPN[12,23] was expressed in significantly fewer CPN that

had received *Cdk5r-Fezf2eGFP* compared to controls (34.92 +/− 3.02% versus 57.03 +/− 5.7% in *Cdk5r-CtleGFP*) (Fig. 1b). These results show that young postmitotic CPN are able to acquire molecular features of CFuPN.

In order to further assess the extent of these molecular changes, we investigated the combinatorial profile of expression of these subtype-specific proteins. We found that up to 75.6% of *Fezf2*-expressing CPN that expressed one CFuPN protein also co-expressed a second CFuPN marker. Specifically, *Fezf2*-expressing CPN co-expressed CRYM and ER81 (19.90 +/− 6.67%; n=4), CRYM and TLE4 (32.91 +/− 2.77%; n=4), CRYM and CTIP2 (11.59 +/− 0.99%; n=3), CTIP2 and ER81 (13.19 +/− 0.98%; n=4), and CTIP2 and TLE4 (15.47 +/− 0.53%; n=4) (Fig. 2a,b). Notably, a distinct fraction of *Fezf2*-expressing CPN that was negative for CUX1 (65.08 +/− 3.02%) was also positive for CRYM (33.58 +/− 0.88%; n=3) and CTIP2 (13.34 +/− 1.02%; n=4) (Fig. 2b), indicating that they had made a clear lineage choice. *Fezf2*-expressing CPN were also calculated to co-express CRYM, CTIP2 and ER81 (8.23 +/− 3.35%), CRYM, CTIP2 and TLE4 (9.37 +/− 2.22%) and to be positive for CRYM and CTIP2 but negative for CUX1 (8.30 +/− 3.28%) (Fig. 2c). These results demonstrate that, in an early period of postmitotic life, CPN of layer II/III are able to reprogram their molecular identity, acquiring combinatorial expression of CFuPN proteins and repressing CPN proteins.

Interestingly, reprogrammed CPN also expressed the transcription factor *Clim 1*, (Fig. 1c), which is not a direct target gene of *Fezf2* (Lodato *et al.*, unpublished data)*,* providing evidence that the reprogramming process goes beyond activation of a first line of *Fezf2* direct target genes to induce a broader program of CFuPN gene expression.

It is possible that reprogrammed CPN may not be stable and, over time, revert to their original identity or die. We therefore assessed the expression of ER81, CRYM and CUX1 in *Fezf2*-expressing CPN over time. ER81 remained expressed in *Fezf2*-expressing CPN at P14 (20.21 +/− 2.10%, n=2 versus 0% in *Cdk5r-CtleGFP*, n=3) and P28 (17.96 +/− 1.78%, n= 3 versus 0%, in *Cdk5r-CtleGFP* n=2) (Fig. 3a,b). Similarly, CRYM was maintained at P14 (16.99 +/− 3.74%, n=2 versus 0% in *Cdk5r-CtleGFP*, n=4) and P28 (17.74 +/− 0.83%, n=3 versus 0.29 +/− 0.29%, in *Cdk5r-CtleGFP* n=2) (Fig. 3a,b). Finally, we found that the percentage of CUX1-positive neurons remained low in the *Cdk5r-Fezf2eGFP*-targeted CPN population over time (Fig. 3a,b). Together, these data show that *Cdk5r-Fezf2eGFP*-electroporated, reprogrammed CPN retained CFuPN molecular identity more than four weeks after the initial expression of *Fezf2*. Importantly, there is a reduction in the fraction of *Fezf2*-expressing CPN that express ER81 and CRYM between P7 and P14, however these values stabilize after P14.

To investigate whether this decrease was due to cell death, we quantified the number of caspase-3 positive cells among GFP-expressing neurons, in both *Cdk5r-Fezf2eGFP* and *Cdk5r-CtleGFP* cortices at P7, P14 and P28. We found minimal cell death and no significant differences between groups (Supplementary Fig. 2a,b). We conclude that the reduction in the percentages of ER81- and CRYM-positive *Cdk5r-Fezf2eGFP*-expressing CPN observed by P14 is not due to cell death but rather to the inability to maintain expression of these

markers. In agreement, the percentage of CUX1-negative *Cdk5r-Fezf2^eGFP*-expressing CPN remained unchanged over time.

Together, the data indicate that upper layer II/III CPN can undergo an early postmitotic change of identity in response to cell autonomous signals instructive of a different neuronal lineage. Furthermore, a significant proportion of reprogrammed neurons maintain their new molecular identity in postnatal cortex.

We next investigated whether CPN can reprogram their molecular identity at later postmitotic ages, when they have already positioned in the upper layers and have acquired connectivity features of the CPN lineage. The reprogramming abilities of neurons at these later postmitotic stages of development have not been investigated before for any CNS neuron subtype.

We constructed a conditional expression vector where the *Fezf2* expression cassette was cloned in antisense orientation between two pairs of inverted LoxP sites (*DF-Fezf2^eGFP*). We electroporated cortical progenitors at E14.5 with *DF-Fezf2^eGFP* or control *DF-Ctl^eGFP* together with a 4-hydroxytamoxifen (TAM)-inducible form of *Cre* recombinase[25] (*CAG-ER^T2-Cre-ER^T2*) (Fig. 4a). Recombination was induced by injections of TAM at either E17.5, P3 or P21 (Fig. 4 and Supplementary Fig. 3 and 4). TAM administration at P3 and P21, resulted in robust recombination and *Fezf2* expression (95.24 +/− 0.43% and 93.32 +/− 1.99%, respectively) (Supplementary Fig. 4a,b). In parallel experiments, to increase nuclear plasticity, expression of *Fezf2* was paired with either administration of Valproic Acid (VPA) a histone deacetylase inhibitor[26], or with surgical transection of the corpus callosum (callosotomy) as a CPN-specific type of injury (Fig. 4a). Pups were sacrificed at P14, P28 and P42.

We found that CPN that began to express *Fezf2* at P3 were still able to reprogram their molecular identity and expressed several CFuPN genes. Similar results were obtained when *Fezf2* was induced at E17.5 (Supplementary Fig. 3 and data not shown). At P28, twenty five days after initial *Fezf2* expression, these neurons expressed ER81 (22.72 +/− 3%, n=3 versus 0.29% +/− 0.29% in *DF-Ctl^eGFP*, n=3); CRYM (8.94 +/− 3.46%, n=3 versus 0% in *DF-Ctl^eGFP*, n=5); *Opsin3* and *Crim1*, two late markers of subcerebral neurons[14]; and were notably able to induce transcription of the endogenous *Fezf2* gene (Fig. 4b,c). In addition, CPN expressing *DF-Fezf2^eGFP* downregulated expression of CUX1 (47.78 +/− 1.85%, n=3 versus 94.47 +/− 1.81% in *DF-Ctl^eGFP*, n=3) (Fig. 4b,c). Double marker analysis further showed that 33.56% of the CRYM-positive neurons co-expressed ER81 and 76.51% downregulated CUX1 (Fig. 4d and data not shown). By P28 we could not detect expression of the CFuPN markers CTIP2, TLE4 and ZFPM2 proteins, which at P7 are present in CPN reprogrammed early postmitotically (Fig. 1). These markers might only be expressed by reprogrammed neurons soon after initial expression of *Fezf2*, or P3 neurons might differ from early postmitotic neurons at P28.

The efficiency of reprogramming remained unaltered by VPA treatment (Fig. 4c). However, callosotomy increased the percentage of *Fezf2*-expressing CPN that was positive for CRYM and negative for CUX1 (Fig. 4c).

These findings indicate that postnatal CPN, despite having been postmitotic for several days still retain the ability to reprogram key aspects of their molecular identity *in vivo*. Although a significant proportion of CPN targeted at P3 could reprogram, the efficiency was lower compared to early postmitotic neurons, suggesting that a progressive restriction of fate occurs with time during the postmitotic life of the neuron. To test this possibility, we induced *Fezf2* expression in upper layer CPN at P21. By P42, we found that virtually all targeted CPN expressed exogenous *Fezf2* (82.52 +/− 14.77%). However neither upregulation of CRYM and ER81 nor downregulation of CUX1 was detected (Supplementary Fig. 4a,b,c,d and data not shown). The data indicate that CPN gradually lose their ability to reprogram their molecular identity and suggest the existence of a critical period of nuclear plasticity for postmitotic neurons.

Neuronal identity is defined as the combination of molecular, morphological and connectivity features. Layer II/III CPN and CFuPN have distinct patterns of axonal connectivity. CPN axons extend across the corpus callosum, with subpopulations maintaining collaterals to the ipsi- and contralateral striatum and to the frontal cortex[12,27,28]. CFuPN, to the contrary, project axons below the cortex, through the internal capsule to targets in the thalamus, pons and spinal cord[12]. To determine whether CPN can reprogram their axonal projections from callosal to corticofugal targets, we first examined the axonal connectivity of pups that had received either *Cdk5r-Fezf2^eGFP* or control DNA via electroporation at E14.5 and were sacrificed at E18.5, P7, P14, or P28 (Fig. 5). As expected, in *Cdk5r-Ctl^eGFP* control animals, GFP-positive axon bundles were present crossing the corpus callosum and no axons entered the internal capsule (Fig. 5b, left and upper panels). In contrast, in *Cdk5r-Fezf2^eGFP* mice, a reduction in GFP callosal axons was observed at E18.5 (Fig. 5b, right panels), and at P7, P14 and P28 distinct axon bundles had entered the internal capsule (Fig. 5c, lower panels and data not shown). In addition, axons of reprogrammed CPN, but not control CPN, were able to extend caudally to targets in the thalamus and the cerebral peduncle, and some axons reached the spinal cord (Fig. 5c, lower panels). Similar results were obtained when *Fezf2* was expressed in CPN using the *Doublecortin* promoter, which also restricts expression to early postmitotic neurons[19,29–31] (Supplementary Fig. 5). In all experiments axons that reached corticofugal targets were maintained at P28 (data not shown).

To determine whether axonal projections could be reprogrammed at a later stage, we followed GFP-positive axons from postmitotic CPN that had received *DF-Fezf2^eGFP* or control *DF-Ctl^eGFP* together with *CAG-ER^T2-Cre-ER^T2*, and were induced by TAM at E17.5. This induction scheme corresponds to *Fezf2* expression at a time when the bulk of CPN are still completing axonal extension across the corpus callosum and represents one of the latest time points available to investigate a change in axon pathfinding. By P14, GFP-positive axon bundles were present extending through the internal capsule and reaching the thalamus and cerebral peduncle of mice that received *DF-Fezf2^eGFP* but not in *DF-Ctl^eGFP* controls (Fig. 5d and data not shown). Together, the data indicate that CPN retain the ability to reprogram their axonal projections in response to a change of identity imposed postmitotically. The complexity of axon guidance decisions likely required to achieve such new long-distance connectivity provides further support of the molecular data indicating that

a series of neuron subtype-specific genes, rather than individual molecules, is regulated as a result of reprogramming.

Recent work has demonstrated that differentiated cell types can be lineage reprogrammed to acquire a new cellular identity upon expression of transcription factors of alternative cell fates[4–6,9–11,32]. In the CNS, neurogenic transcription factors can reprogram cultured postnatal astrocytes into neural progenitors and neurons[33–35]. Pericytes isolated from the human cerebral cortex have also been recently shown to reprogram into neurons[36]. However, to what extent neurons have the potential to reprogram their identity is largely unknown. Here, we provide the first demonstration that in the neocortex a conversion between two subtypes of neurons can occur during a temporal window of embryonic and early postnatal development.

The data indicate that critical features of neuronal subtype-specific identity, including the acquisition of a new molecular identity and the ability to extend axons to a new target, can still be changed postmitotically, even several days after initial fate-specification. These changes occurred despite the ectopic location of the CFuPN-like neurons and through the use of a single transcription factor, suggesting that higher reprogramming efficiency might be obtained in more instructive environments or by combining *Fezf2* with additional fate modifying signals. Importantly, the number of CPN able to reprogram is highest during the earliest stages of postmitotic development and, while this capacity persists at P3, it is eliminated by P21. This suggests that mechanisms are in place postmitotically to progressively restrict neuronal fate potential as neurons age. The work indicates that postmitotic neurons can undergo lineage reprogramming *in vivo* and paves the way for mechanistic studies aimed at enhancing the process of neuronal conversion by extending the period of neuronal reprogramming to the mature brain.

Methods

In utero electroporation

The *Cdk5r*, *Dcx* and *Nestin* minimal promoters used here have been previously described[19]. The *Cdk5r-Ctl^eGFP*, *Dcx-Ctl^eGFP*, *Cdk5r-Fezf2^eGFP* and *Dcx-Fezf2^eGFP* constructs were generated by subcloning an *IRES-GFP* or *Fezf2-IRES-GFP* cassette downstream of the *Cdk5r* and *Dcx* promoters. The *DF-Ctl^eGFP* and *DF-Fezf2^eGFP* constructs were generated by subcloning a *GFP* or *GFP-T2A-Fezf2* cassette between two pairs of inverted LoxP sites within the AAV-DIO-*ChR2-mCherry* construct described previously[37]. *CAG-ER^T2-Cre-ER^T2* has also been previously described[25]. In vivo electroporations were performed according to a previously published protocol[17,38]. Briefly, 800 µl of purified DNA (2µg/µl) mixed with 0.005% Fast Green in sterile PBS was injected *in utero* into the lateral ventricle of CD1 embryos (Charles River Laboratory) under ultrasound guidance (Vevo 770, VisualSonics). Five 35 volt pulses of 50 ms duration at 1 second intervals were delivered in appropriate orientation across the embryonic head using 1 cm-diameter platinum electrodes placed outside the uterus and a CUY21EDIT square wave electroporator (Nepa Gene). Embryonic TAM injections were performed as previously described[25]. P3 pups received a single dose of 0.5 mg of TAM (100 µl at 5 mg/ml) intra peritoneum (IP) and P21 mice received a single dose of 2 mg of TAM (200 µl at 10 mg/ml) IP. VPA-injected animals

received two doses of 0.5 mg each (100 μl at 5 mg/ml) IP at P5 and P7. All animal studies were approved by the Massachusetts General Hospital and Harvard University Institutional Animal Care and Use Committee and performed in accordance with institutional and federal guidelines.

Immunocytochemistry and *in situ* hybridization

Brains for immunocytochemistry were processed as previously described[14]. Primary antibodies and dilutions were as follows: rabbit anti-ZFPM2 antibody[16], 1:500 (Santa Cruz, sc-10755); rat anti-CTIP2[14] antibody, 1:1000 (Abcam, ab18465); rabbit anti-GFP antibody, 1:500 (Invitrogen, A-11122); rat anti-GFP antibody, 1:500 (Nacalai, 04404⁻84); rabbit anti-ER81 antibody[22], 1:2500 (gift of Silvia Arber); rabbit anti-CRYM antibody[14], 1:100 (Abcam, ab54669); rabbit anti-TLE4 antibody[12], 1:100 (Santa Cruz, sc-9525); rabbit anti-cleaved Caspase-3, 1:400 (Cell Signaling Technology, 9661S). Appropriate secondary antibodies were from the Molecular Probes Alexa series and the Vectastain ACB system (Vector Labs). Tissue sections were imaged using a Nikon 90i fluorescence microscope equipped with a Retiga Exi camera (Q-IMAGING) and analyzed with Volocity image analysis software v6.0.1 (Improvision). Confocal images were obtained using a Nikon A1 confocal (Eclipse Ti) and a Zeiss 700 Confocal. Images were collected and analyzed with the NIS-Elements imaging (Nikon) and Zen 2010 programs, respectively. Nonradioactive *in situ* hybridizations were performed on 40 μm vibratome sections mounted on superfrost slides (Fisher) using reported methods[39]. Riboprobes were generated as previously described[14]. cDNA clones for *Clim1, Opsin3, Crim1* and *Gfp* have been previously described[14,18]. cDNA clones to detect endogenous *Fezf2* transcript were generated by RT-PCR using the following primer pairs: 5′-TCTTCCTGCCCTGTACCAAC-3′ and 5′-CCGGTGTCCCAAGAAAGTTA-3′.

Callosotomy

Callosotomy was performed at P3. Pups were anesthetized on wet ice and their head immobilized. The non-electroporated hemisphere was exposed and a 30G needle was inserted below the cortex starting at lambda and immediately lateral to the midline. The corpus callosum was then sectioned between bregma and lambda. Pups were resuscitated by bringing their body temperature to physiological levels.

Cell quantification and connectivity

For quantification of CFuPN and CPN marker gene expression, anatomically matched sections from *Cdk5r-Ctl^eGFP* and *Cdk5r-Fezf2^eGFP* were selected and processed by immunohistochemistry for subtype-specific markers (P7: n=5 mice, 4,053 total neurons for *Cdk5r-Ctl^eGFP* and n=6 mice, 4,649 total neurons for *Cdk5r-Fezf2^eGFP*; P14: n=3 mice, 2,608 total neurons for *Cdk5r-Ctl^eGFP* and n=2 mice, 1,514 total neurons for *Cdk5r-Fezf2^eGFP*; P28: n=2 mice, 1,182 total neurons for *Cdk5r-Ctl^eGFP* and n=3 mice, 1,557 neurons for *Cdk5r-Fezf2^eGFP*), *DF-Ctl^eGFP* and *DF-Fezf2^eGFP* with TAM injection at P3 (n=6 mice for *DF-Ctl^eGFP* and for *DF-Fezf2^eGFP*, n=2 mice for *DF-Fezf2^eGFP* with callosotomy and n=3 mice for *DF-Ctl^eGFP* with callosotomy, *DF-Ctl^eGFP* with VPA and *DF-Fezf2^eGFP* with VPA; 1,761 total neurons for *DF-Ctl^eGFP*; 3,580 total neurons for *DF-*

Fezf2^{eGFP}; 2,271 total neurons for *DF-Ctl^{eGFP}* with callosotomy; 2,199 total neurons for *DF-Ctl^{eGFP}* with VPA; 1,243 total neurons for *DF-Fezf2^{eGFP}* with callosotomy and 2,175 total neurons for *DF-Fezf2^{eGFP}* with VPA) and *DF-Ctl^{eGFP}* and *DF-Fezf2^{eGFP}* with TAM injection at P21 (n=3 mice for *DF-Ctl^{eGFP}* harvested at P28 and P42 and for *DF-Fezf2^{eGFP}* harvested at P42 ; n=2 mice for *DF-Fezf2^{eGFP}* harvested at P28; 1,633 and 1,123 total neurons for *DF-Ctl^{eGFP}* harvested at P28 and P42, respectively and 3,021 and 2,035 for *DF-Fezf2^{eGFP}* harvested at P28 and P42, respectively). Only GFP-positive neurons located in layer II/III and within matched areas were quantified. All the results are expressed as the mean ± s.e.m. The paired, two-tailed *t* test was used for statistical analysis. For analysis of long-distance connectivity, electroporated embryos were sacrificed at E18.5, P7, P14 and P28 for processing. eGFP-positive axons where visualized by immunocytochemistry for GFP and analyzed in coronal sections of the brain and spinal cord at different rostrocaudal locations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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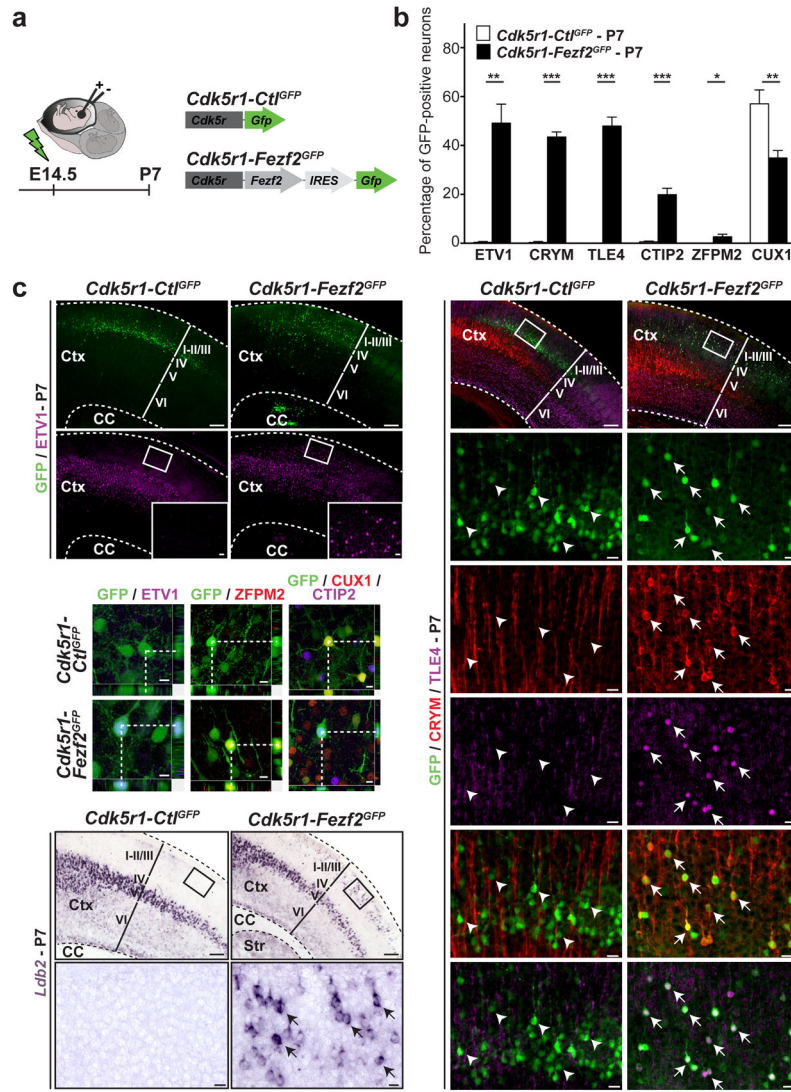
Figure 1. Expression of *Fezf2* in migratory, postmitotic CPN induces them to acquire molecular features of CFuPN

a, Schematic representation of the experimental approach. *Fezf2* or control vectors were electroporated at E14.5 and expression was restricted to CPN at the earliest stages of postmitotic development using the promoter of the *Cdk5r* gene. **b**, Quantification of the percentage of electroporated CPN expressing CFuPN-specific markers (ER81, CRYM, TLE4, CTIP2 and ZFPM2) and downregulating CPN-specific markers (CUX1). Results are expressed as the mean ± s.e.m. The paired, two-tailed *t* test was used for statistical analysis. * p<0.05; ** p<0.01; *** p<0.001. **c**, Immunocytochemistry and *in situ* hybridization for a panel of CFuPN and layer II/III CPN markers. Ctx, cortex; CC, corpus callosum; Str, striatum. Scale bars, 200 μm, 20 μm in high magnification panels and 10 μm in confocal images.
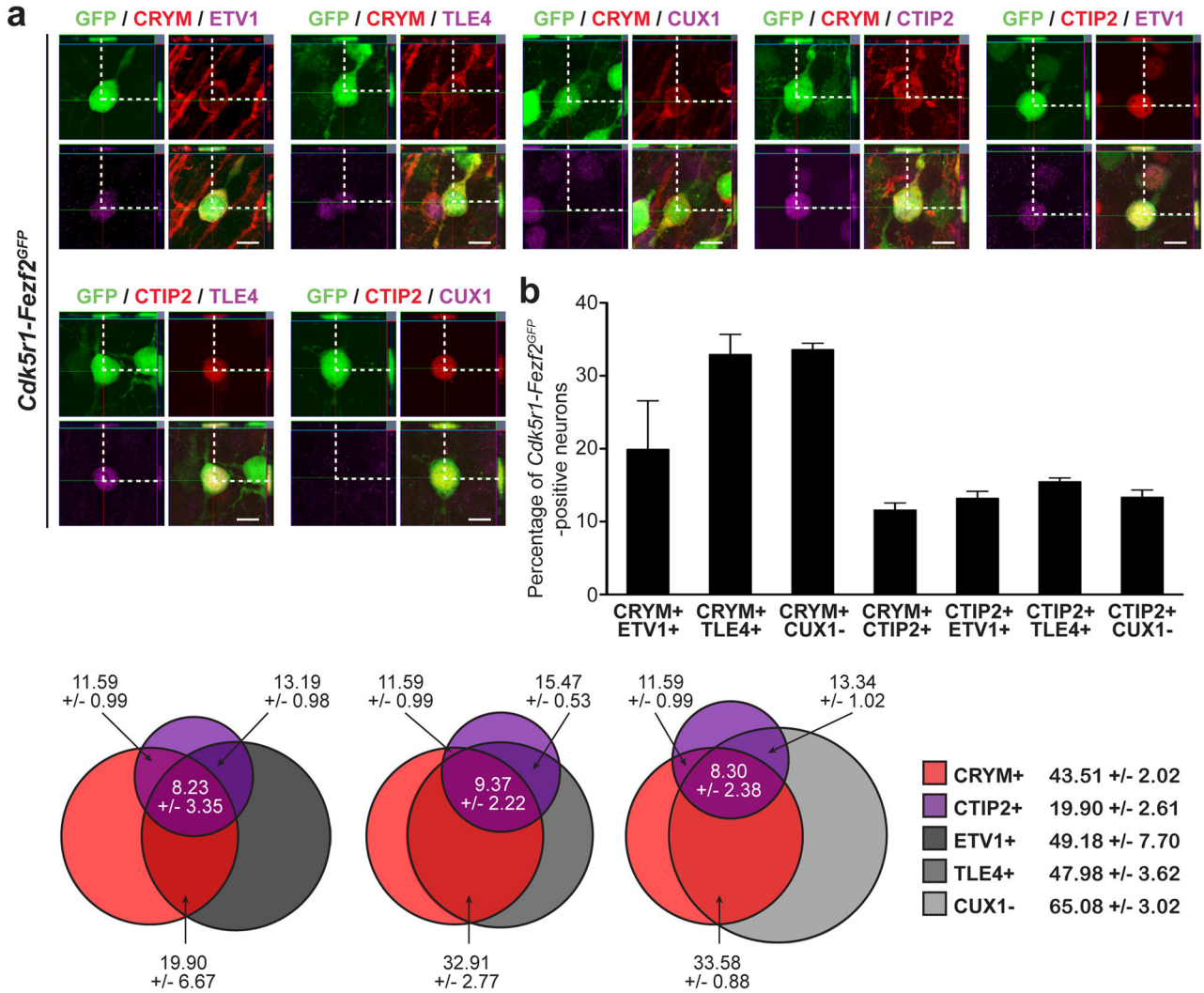
Figure 2. *Fezf2* expression in postmitotic CPN induces a program of CFuPN-specific genes
a, Confocal analysis showing that CFuPN-specific genes are co-expressed in *Cdk5r-Fezf2^{eGFP}*- electroporated CPN. Scale bars, 10 μm. **b**, Quantification of the percentage of *Cdk5r-Fezf2^{eGFP}*-electroporated CPN expressing a combination of two CFuPN-specific markers. Results are expressed as the mean ± s.e.m. **c**, Proportional Venn diagrams representing an estimate of the percentage of *Cdk5r-Fezf2^{eGFP}*-electroporated CPN co-expressing combinations of three CFuPN-specific markers.
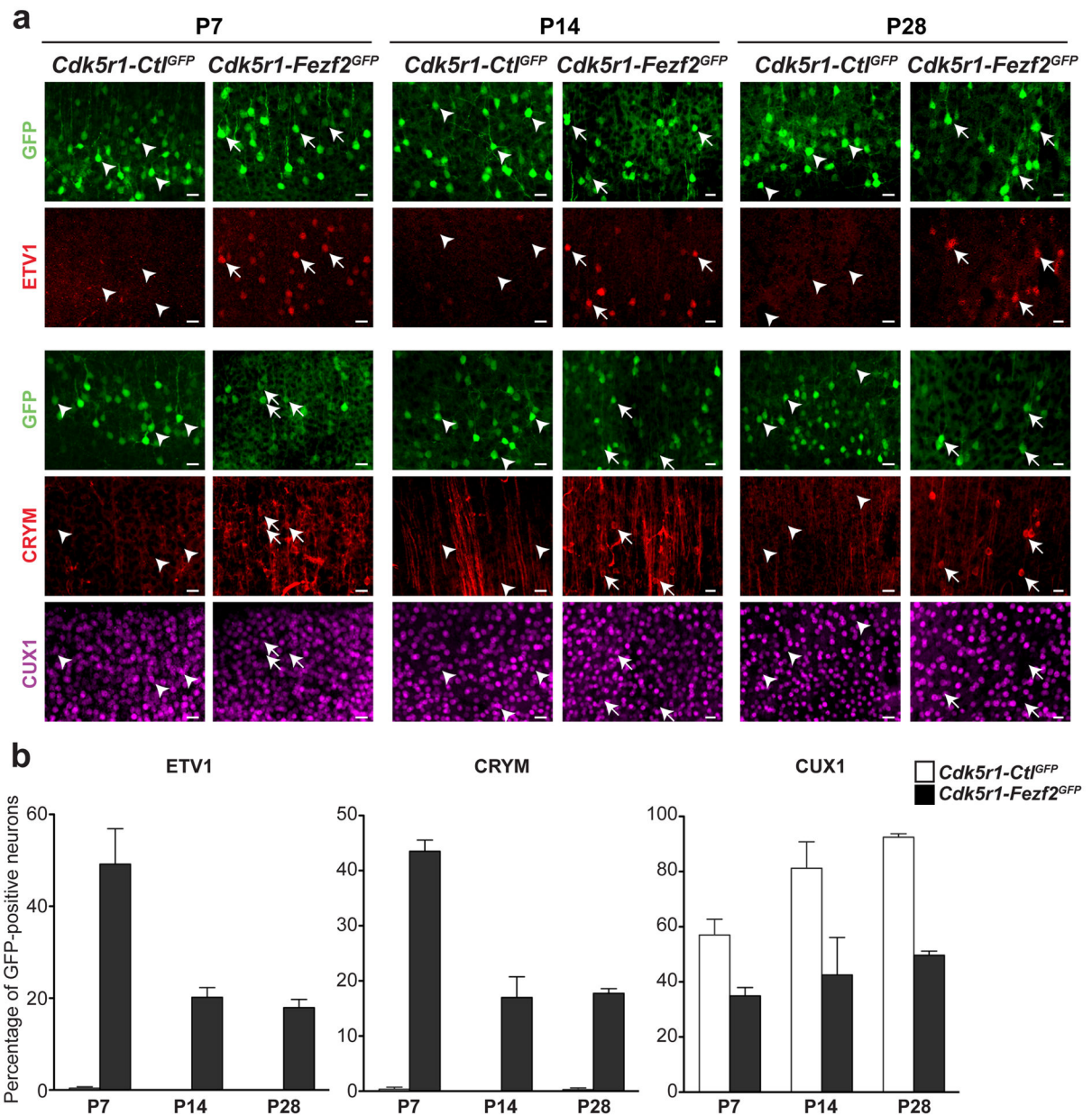
Figure 3. Reprogrammed CPN maintain stable expression of CFuPN markers
a, Immunocytochemistry for ER81, CRYM and CUX1 in *Cdk5r-Fezf2^eGFP*-electroporated
CPN versus control (*Cdk5r-Ctl^eGFP*) at P7, P14 and P28. Scale bars 20 μm. **b**,
Quantification of the percentage of *Cdk5r-Fezf2^eGFP*- and *Cdk5r-Ctl^eGFP*-electroporated
CPN maintaining ER81, CRYM and CUX1 expression at P7, P14 and P28. Results are
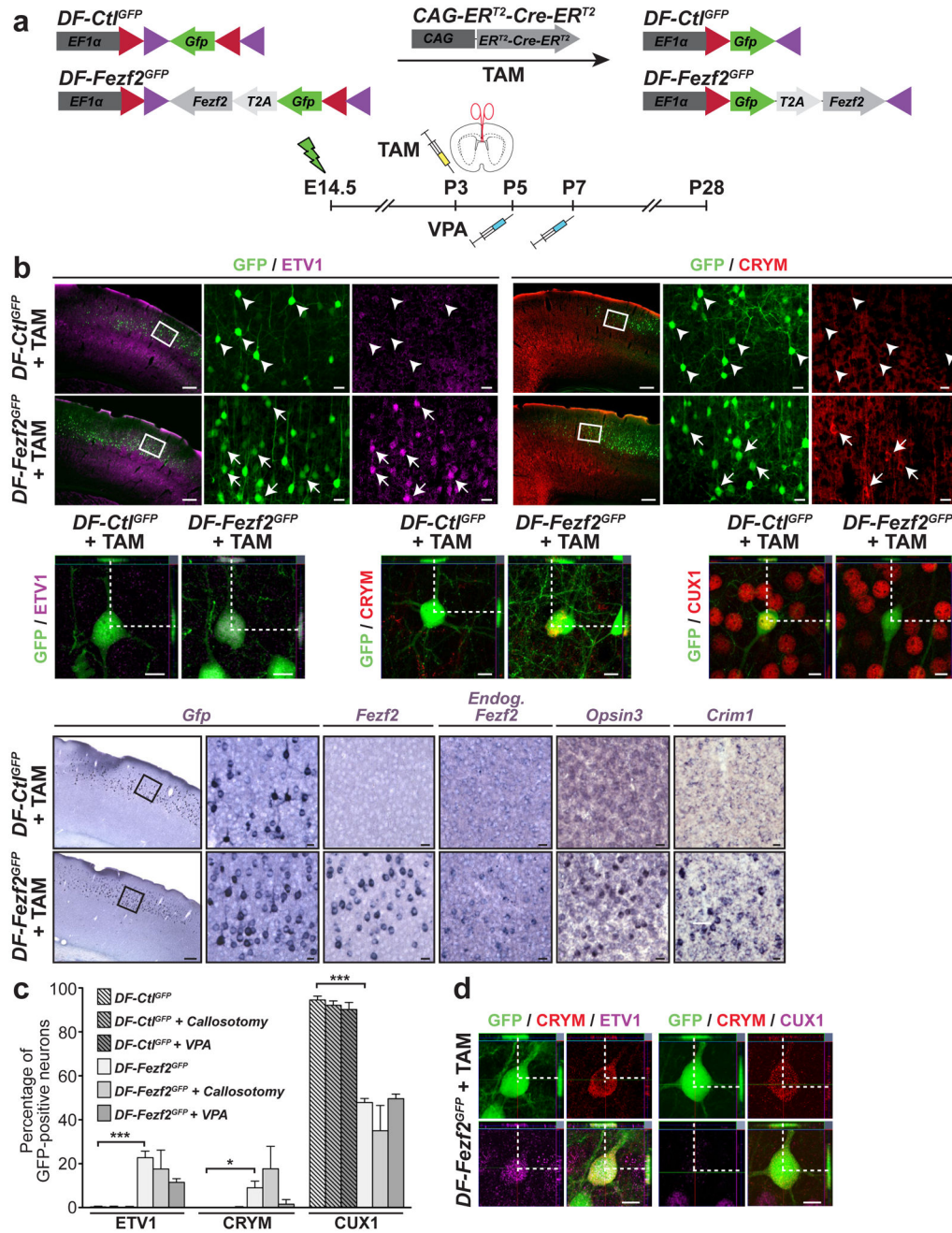expressed as the mean ± s.e.m.

Figure 4. Callosal projection neurons can undergo molecular reprogramming of subtype-specific genes in response to *Fezf2* expression at postnatal day P3

a, Schematic of the constructs used to conditionally express *Fezf2* at P3 and of experimental approach. CPN received *Fezf2* (or control DNA) alone or in combination with either VPA treatment or callosotomy. **b**, Immunocytochemistry and *in situ* hybridization on coronal sections of cortex. **c**, Quantification of the percentage of electroporated CPN expressing CFuPN and CPN proteins. Results are expressed as the mean ± s.e.m. The paired, two-tailed *t* test was used for statistical analysis. * p<0.05; ** p<0.01; *** p<0.001. **d**, Confocal analysis showing that a subset of *DF-Fezf2^eGFP*-electroporated CPN co-expressed CRYM

and ER81 and are CRYM-positive and CU7-negative. Scale bars, 200 μm, 20 μm in high magnification panels and 10 μm in confocal images.
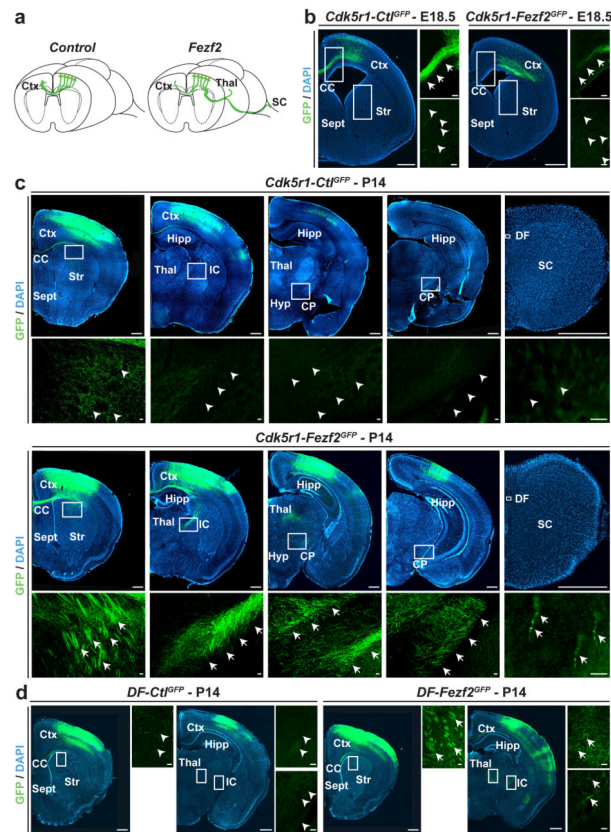
Figure 5. CPN can reprogram their axonal projections from callosal to corticofugal targets in response to *Fezf2*

a, Graphic representation of changes in axonal connectivity by layer II/III CPN in response to *Fezf2* versus control. **b**, GFP-positive CPN axons crossing the corpus callosum at E18.5 in *Cdk5r-Fezf2^eGFP* and *Cdk5r-Ctl^eGFP*-electroporated CPN. Callosal projections are greatly reduced in *Cdk5r-Fezf2^eGFP*- versus Cdk5r-*Ctl*^eGFP-electroporated CPN**. c**, At P14, GFP-positive axons are restricted to the corpus callosum in *Cdk5r-Ctl^eGFP*-electroporated CPN. By contrast, axons of *Cdk5r-Fezf2^eGFP*-electroporated CPN are also present in the internal capsule and extend caudally to the thalamus and the cerebral peduncle. A small number of GFP-positive axons are present in the dorsal funiculus of the spinal cord in *Cdk5r-Fezf2^eGFP*- but not in control-electroporated animals. **d**, GFP-positive axons at P14 of *DF-Fezf2^eGFP*- or *DF-Ctl^eGFP*-electroporated CPN that received TAM postmitotically at E17.5. Callosal axons are restricted to the corpus callosum in *DF-Ctl^eGFP* CPN while axons are also present within the internal capsule in *DF-Fezf2^eGFP* CPN. CC, corpus callosum; CP, cerebral peduncle; Ctx, cortex; DF, dorsal funiculus; Hipp, hippocampus; Hyp, hypothalamus; IC, internal capsule; Str, striatum; Sept, septum; Thal, thalamus. Scale bars, 200 μm and 20 μm in high magnification panels.