Direct reprogramming of oligodendrocyte precursor cells into GABAergic inhibitory neurons by a single homeodomain transcription factor Dlx2

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Oligodendrocyte precursor cells (NG2 glia) are uniformly distributed proliferative cells in the mammalian central nervous system and generate myelinating oligodendrocytes throughout life. A subpopulation of OPCs in the neocortex arises from progenitor cells in the embryonic ganglionic eminences that also produce inhibitory neurons. The neuronal fate of some progenitor cells is sealed before birth as they become committed to the oligodendrocyte lineage, marked by sustained expression of the oligodendrocyte transcription factor Olig2, which represses the interneuron transcription factor Dlx2. Here we show that misexpression of Dlx2 alone in postnatal mouse OPCs caused them to switch their fate to GABAergic neurons within 2 days by downregulating Olig2 and upregulating a network of inhibitory neuron transcripts. After two weeks, some OPC-derived neurons generated trains of action potentials and formed clusters of GABAergic synaptic proteins. Our study revealed that the developmental molecular logic can be applied to promote neuronal reprogramming from OPCs.

NG2 glia, also known as oligodendrocyte precursor cells (OPCs or polydendrocytes), represent a fourth major glial population1 that self-renew throughout life and proliferate in response to various environmental cues. In the postnatal brain, their physiological fate is largely restricted to the oligodendrocyte lineage2,3 except for a small number of neurons in the adult hypothalamus and piriform cortex detected by genetic fate mapping of OPCs, and the production of protoplasmic astrocytes from OPCs in the embryonic ventral gray matter, which ceases after birth (reviewed in2–4). A subpopulation of OPCs in the neocortex arises from ventral progenitors in the embryonic medial and caudal ganglionic eminences, which also generate inhibitory neurons5–9. Dlx2 is a homeodomain transcription factor that is expressed in ventral telencephalic neural progenitors and is critical for the development of GABAergic neurons. Ventral neural progenitor cells also express the oligodendrocyte basic helix-loop-helix (bHLH) transcription factor Olig2, which is repressed as neural progenitors become committed GABAergic inhibitory neurons by sustained Dlx2 expression10,11.

Previous studies have shown that OPCs can be reprogrammed into neurons by a combination of neuronal transcription factors under specific conditions12–14. However, the mechanism underlying the fate conversion has not been examined. Our previous bioinformatic study revealed that in OPCs, compared with astrocytes and fibroblasts, the chromatin at key interneuron transcription factor genes is more accessible, with more active and less bivalent or repressive histone posttranslational modifications15. In this study, we show that forced expression of a single transcription factor Dlx2 in committed OPCs from the neocortex was sufficient to convert them into immature neurons within two days. Transcriptomic analysis of OPCs two days after Dlx2 transfection revealed upregulation of inhibitory but not excitatory neuronal genes. Subsequently, within 2 weeks Dlx2-transfected

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OPCs differentiated into purely GABAergic inhibitory neurons, some of which exhibited rapid non-adapting firing patterns and developed inhibitory synaptic puncta.

**Methods**

**Animals.** The NG2-CreER A BAC transgenic mice (NG2cre)\(^{16,17}\) have constitutive cre expression in OPCs and were maintained as a homozygous colony. Gad1-GFP knock-in reporter line (Gad1-GFP)\(^{18}\) was generated by inserting EGFP cDNA in-frame into exon 1 of the Gad1 gene and maintained as heterozygotes in a C57 background. NG2cre mice were crossed to Gad1-GFP to generate heterozygous NG2cre/Gad1-GFP mice. CD1 mice were obtained from Charles River (strain #022; RRID:IMSR_CRL:022). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Connecticut and carried out in accordance with the NIH and local guidelines and regulations.

**Primary cultures.** Postnatal cortical OPCs. For culturing postnatal OPCs, we modified the previously published sequential immunopanning purification procedure\(^{19}\). We used postnatal day 2–3 (P2–3) CD1 mice from Charles River Laboratory strain #022. After removal of meninges, the cortices were minced and incubated at 35 °C for 60 min in a papain solution (20 U/ml; Worthington). Subsequently, the tissue was triturated in a solution containing ovomucoid and bovine serum albumin (BSA) (Sigma Aldrich) and filtered through a 70-μm cell strainer (Falcon) to obtain a single cell suspension. The cell suspension was incubated in a petri dish coated with the O1 antibody (Gift from Dr. Steven Pfeiffer at University of Connecticut Health Center, 1:1 dilution)\(^{20}\) to eliminate oligodendrocytes, with subsequent incubation of the supernatant in a petri dish coated with rat anti-mouse platelet-derived growth factor receptor alpha (PDGFRα) antibody (CD140a, 2 μg/ml; BD Biosciences, RRID:AB_397117) to capture OPCs. Non-adhered cells were washed off with phosphate-buffered saline (PBS), and adhered OPCs were collected by trypsinization. Cells were centrifuged for 5 min at 1100 rpm and re-suspended in DMEM (Thermo Fisher Scientific) supplemented with Sato's supplements (DMEM-Sato's) and 50 ng/ml PDGF-AA (Peprotech). Cells were plated on 12-mm glass coverslips (Fisher) coated with 30 μg/ml of poly-d-lysine (Sigma-Aldrich) and 15 μg/ml of laminin (Sigma-Aldrich) or on 35-mm tissue culture dishes (Fisher Scientific) coated with 30 μg/ml of poly-l-lysine (PLL, Sigma-Aldrich). At 3 days post transfection (dpt), cells were trypsinized and re-plated onto a layer of astrocytes at 125,000 cells per 12-mm glass coverslip. Media was switched to neuronal differentiation media consisting of 1% N2 and 1% B27 supplements, 1% l-glutamine, and 1% penicillin and streptomycin in DMEM/F12 (all from Thermo Fisher Scientific) and supplemented with 50 ng/ml human brain-derived neurotrophic factor (BDNF, Peprotech). At 7 dpt, media was completely switched to neuronal maturation media consisting of Neurobasal A (Thermo Fisher Scientific) and supplemented with 2% B27, 1% Glutamax (Thermo Fisher Scientific), and 1% penicillin and streptomycin supplemented with 50 ng/ml human BDNF. Half of media was replaced with fresh media and BDNF every 3–4 days. On 10 dpt, 1% horse serum was added for astrocyte maintenance.

**Postnatal cortical astrocytes.** For culturing primary postnatal astrocytes, we isolated cortical astrocytes as described previously\(^{21}\). Briefly, we dissected and chopped the cortices of P1–P3 CD1 mice. Cortical pieces were transferred to a tube containing Hank's Balanced Salt Solution (Thermo Fisher Scientific) and 0.1% trypsin (Thermo Fisher Scientific) and incubated in 37 °C water bath for 15–30 min. Cells were collected by centrifuging for 5 min at 1100 rpm. Cells were resuspended in astrocyte media consisting of 10% horse serum (Hyclone) and 0.6% d-glucose (Thermo Fisher Scientific) in DMEM) and gently triturated into a single cell suspension. Cells were plated in T75 culture flasks coated with 30 μg/ml PLL. Medium was changed every 2–3 days. At 7–9 days post plating when astrocyte layer was confluent, flasks were shaken at 37 °C at 260 rpm overnight to detach microglia and OPCs. The next day, astrocytes were rinsed with PBS, trypsinized and frozen in astrocyte media with 10% DMSO and 50% horse serum for future use.

**Transfection.** Full-length mouse Dlx2 cDNA (NM_010054.2, from translation start site to the stop codon) was cloned into pCMV-IREs2-mCherry expression vector, which had been generated by replacing the EGFP coding region of pIREs2-EGFP vector (Clontech) with mCherry coding region (obtained from Dr. Hitoshi Gotoh, Kyoto Prefectural Medical University). OPCs were transfected 16 h after plating with pCMV-Dlx2-IREs2-mCherry (Dlx2) or control pCMV-IREs2-mCherry (mCherry) plasmid DNA using Lipofectamine 2000 (Invitrogen), and after 5 h, the medium was changed to fresh DMEM-Sato's medium with 50 μg/ml PDGF-AA.

For utilization of the Tol2 transposon system to achieve genomic integration of the Dlx2-IREs2-mCherry cassette, Dlx2-IREs2-mCherry was cloned into the cre-dependent Tol2 transposon donor vector pT2AL-loxP-STOP-loxP-ires2-mCherry vector (kindly provided by Dr. Koichi Kawakami, National Institute of Genetics, Mishima, Japan)\(^{22,23}\). OPCs were co-transfected 16 h after plating with Tol2-Dlx2-IREs2-mCherry (Tol2-Dlx2) or Tol2-IREs2-Cherry (Tol2-control) donor plasmids and pCAGGS-T2TP helper plasmid encoding transposase (provided by Dr. Koichi Kawakami) as described above.

**Immunofluorescence labeling.** Coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1 M sodium phosphate buffer for 10 min, rinsed three times with PBS and then blocked and permeabilized in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 5% normal goat serum (Thermo Fisher Scientific) or 5% normal donkey serum (Thermo Fisher Scientific) at room temperature (RT). The coverslips were then incubated with primary antibodies in blocking solution for 45 min. After three rinses, coverslips were incubated with the secondary antibodies in blocking solution for 30 min. Primary and secondary antibodies are listed in Table 1. Coverslips were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).
Electrophysiological recordings. Whole-cell patch clamp recordings were performed at room temperature on Dlx2- or control-transfected OPCs grown in culture 14 days after transfection (dpt). Coverslips were scanned to identify mCherry + cells that were GFP + or—using an Olympus BX51WI upright microscope with FITC and TRITC fluorescence and Nomarski optics. Whole-cell recordings were obtained using borosilicate glass electrodes having resistances of 3 to 5 MΩ filled with intracellular solution containing the following (in mM): 130 potassium methylsulfate, 10 KCl, 5 Tris-phosphocreatine, 10 HEPES, 4 NaCl, 4 Mg2ATP, and 0.4 Na4GTP. The pH was adjusted to 7.2 to 7.3 with KOH. The physiological extracellular solution contained the following (mM), 125 NaCl, 26 NaHCO3, 2.5 KCl, 1 NaH2PO4, 1.3 MgCl2, 2.5 CaCl2, and 12 glucose and was saturated with 95% O2 and 5% CO2. Membrane potentials were recorded during 500 ms current injections ranging from -50 to 250 pA in 10 pA increments. Data were acquired through a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 10 kHz, and sampled at 50 kHz.

RNA-sequencing (RNA-seq). RNA extraction, library construction, and sequencing. Immunopurified OPCs were transfected with either control mCherry or Dlx2 plasmid vector. At 2 dpt, transfected cells were isolated by fluorescence-activated cell sorting (FACS) for mCherry with the BD FACSAriaII (Becton Dickinson), using the 488 nm diode laser in the Flow Cytometry Facility (University of Connecticut, Storrs). Three biological replicates consisted of mCherry- and Dlx2-transfected OPCs that had been isolated from different litters of mice. The sorted cells were collected and stored in Trizol reagent (Invitrogen), and total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). Genomic DNA was removed using gDNA Eliminator spin-columns provided by the kit. RNA concentration and integrity of each sample were measured using an Agilent 2100 Bioanalyzer (Agilent), housed in the Center for Genome Innovation (University of Connecticut, Storrs). Samples with

| Antibody Source | Cat no. | RRID | Dilution |
|-----------------|---------|------|----------|
| BD Biosciences  | 558774  | RRID:AB_397117 | 1:250 |
| BioLegend       | 801202  | RRID:AB_10063408 | 1:3000 |
| Millipore Sigma | AB5320  | RRID:AB_91789 | 1:500 |
| Millipore Sigma | MAB50N  | RRID:AB_10807410 | 1:1000 |
| Millipore Sigma | AB5726  | RRID:AB_209341 | 1:1000 |
| Abcam           | ab75713 | RRID:AB_1310432 | 1:3000 |
| Millipore Sigma | MAB5406 | RRID:AB_2278725 | 1:3000 |
| Santa Cruz Biotechnology | sc-17319 | RRID:AB_661259 | 1:200 |
| Developmental Studies Hybridaoma Bank | Rat-401 | RRID:AB_2235915 | 1:8 |
| R&D systems     | AF1062  | RRID:AB_2236897 | 1:1000 |
| Santa Cruz Biotechnology | sc-36596 | RRID:AB_10844002 | 1:1000 |
| Synaptic Systems | 131 005 | RRID:AB_1106810 | 1:1000 |
| Neumab          | L114/3  | RRID:AB_2877608 | 1:100 |
| Millipore Sigma | MAB354  | RRID:AB_225365 | 1:100 |
| Synaptic Systems | 147 103 | RRID:AB_11041706 | 1:250 |
| Aves Laboratories | GP1202 | RRID:AB_2734732 | 1:500 |
| Rockland Immunochemicals | KCA379 | RRID:AB_2614911 | 1:1000 |
| Dr. Steven Pfeiffer* | N/A    | N/A | 1:1 |

Table 1. Antibodies used. *University of Connecticut Health Center, Farmington, CT.

RNA-sequencing (RNA-seq). RNA extraction, library construction, and sequencing. Immunopurified OPCs were transfected with either control mCherry or Dlx2 plasmid vector. At 2 dpt, transfected cells were isolated by fluorescence-activated cell sorting (FACS) for mCherry with the BD FACSAriaII (Becton Dickinson), using the 488 nm diode laser in the Flow Cytometry Facility (University of Connecticut, Storrs). Three biological replicates consisted of mCherry- and Dlx2-transfected OPCs that had been isolated from different litters of mice. The sorted cells were collected and stored in Trizol reagent (Invitrogen), and total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). Genomic DNA was removed using gDNA Eliminator spin-columns provided by the kit. RNA concentration and integrity of each sample were measured using an Agilent 2100 Bioanalyzer (Agilent), housed in the Center for Genome Innovation (University of Connecticut, Storrs). Samples with
an RNA Integrity Number ≥ 8 were processed. Ribosomal RNA was removed from samples using RiboGone® (Clontech). cDNA libraries were prepared with 100 ng total input RNA using the SMARTer Stranded RNA-Seq Kit (Clontech). Libraries were multiplexed and subjected to a total of 18 cycles of PCR amplification, and the amplified cDNA library was sequenced at the Center for Genome Innovation (University of Connecticut, Storrs, CT) using Illumina NextSeq 500. Barcoded and multiplexed samples were run on four lanes to generate four technical replicates of 150-nucleotide-paired end reads.

Data processing, mapping, and differential expression analysis. Illumina adapters were removed, and sample reads were trimmed using Trimomatic v0.39 (RRID:SCR_011848) and filtered based on a quality score ≥ 20. Trimmed sequences were aligned to the mouse reference genome (GRCm38 version 84) using HISAT2 v2.1.0 (RRID:SCR_015530). Output files from HISAT2 were sorted using SAMtools (RRID:SCR_013035), and counts were generated using HTSeq-count v0.8.0 (RRID:SCR_011867). To obtain normalized counts in fragments per kilobase per million (FPKM), we used TopHat2 v2.1.0 (RRID:SCR_013035) to align the trimmed sequences to the mouse reference genome, and Cufflinks v2.2.1 (RRID:SCR_014597) to estimate transcript abundance. To identify differentially expressed (DE) genes, count tables from HTSeq-count were fed into DESeq2. Differential expression analyses were conducted with R v3.2.2 (RRID:SCR_001905). Differentially expressed genes with p-val < 0.01 were considered significant.

Gene ontology (GO) enrichment analysis. GO enrichment analysis of the differentially expressed genes was performed to identify biological processes enriched in differentially expressed genes using the enrichGO function of clusterProfiler v3.6.2 (RRID:SCR_016884). The GO over-representation test was performed using the Benjamini & Hochberg Method, with the cut-off values for p and q set to 0.01 and 0.05, respectively.

Quantitative PCR. Immunopurified OPCs were transfected with either control mCherry or Dlx2 plasmid vector, with a transfection efficiency of 10–15%, and cultured as described above. The entire cell population, including transfected and un-transfected, was lysed at 2, 7, and 14 dpt, and total RNA was purified with the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to manufacturer’s protocol. RNA was reverse transcribed into cDNA using Superscript IV First-Strand Synthesis System (Invitrogen) with a mixture of random primers and oligo(dT) primers (Invitrogen), according to manufacturer’s protocol. SsoAdvanced Universal SYBR Green Supermix (BioRad) was used to perform qPCR in a BioRad CFX96 Touch Real-time PCR Detection System with 20 ng of cDNA and the primers listed in Table 2A,B. Three experimental replicates were used per condition.

Data analysis and statistical evaluation. Images of fluorescently labeled dissociated cells were acquired using a Leica SP8 confocal microscope (Advanced Light Microscopy Facility, University of Connecticut, Storrs) or Zeiss Axiovert 200 M. For quantification, images were captured from random fields on coverslips from a minimum of three separate experiments (8–16 male and female mouse pups per experiment) and immunolabeled cells in each field were counted. Quantification data are represented as means ± standard deviations. For RNA-seq, three biological replicates, consisting of 2 or more immunopanning experiments per replicate, were used per condition. For electrophysiological recordings, neurons were recorded from two separate experiments. Generation of graphs and statistical analysis of data was performed with GraphPad Prism (Version 7 and 8) software or R (v3.2.2, RRID:SCR_001905). Images were processed and analyzed using ImageJ (RRID:SCR_001935), and Adobe Photoshop (RRID:SCR_014199) was used to generate figures.

Results

Differentiation of Dlx2-transfected postnatal OPCs into GABAergic neuron-like cells. Dlx2 represses Olig2 during interneuron development and is not detected in OPCs.4,26 Previously we showed that OPCs in the postnatal SVZ or the forebrain parenchyma do not express detectable levels of the key interneuron homeodomain transcription factor Dlx2.26 To examine whether Dlx2 could alter the fate of OPCs that had been committed to the oligodendrocyte lineage, we transfected immunopurified OPCs from postnatal mouse cortex with either control mCherry (control) or Dlx2-mCherry (Dlx2) plasmid. One day after transfection (1 dpt), Dlx2-immunoreactivity was detected in Dlx2- but not in control-transfected OPCs (Supplemental Fig. 1A). As early as 2 dpt, 24.3 ± 4.9% of Dlx2-transfected cells had barely detectable Olig2 (Fig. 1A, arrowheads; Supplemental Fig. 2B) and had large cell bodies and longer processes (see bottom left mCherry + cell in Fig. 1B), while the remaining Dlx2-transfected cells exhibited weak Olig2 immunoreactivity. This was not due to transfection of Dlx2 into Olig2-negative non-OL lineage cells, since at 1 dpt > 97% of mCherry + cells showed no detectable immunoreactivity for PDGFRα and Sox10, respectively (Supplemental Fig. 2A–D). Furthermore, by 2 dpt, 76.0 ± 5.0% of mCherry + Dlx2-transfected cells expressed the neuronal cytoskeletal protein βIII-tubulin (Tuj1), while they continued to express the OPC antigen NG2 (Fig. 1B, arrows). By 7 dpt, 73.3 ± 6.6% and 55.6 ± 11.6% were negative for PDGFRα and Sox10, respectively, (Supplemental Fig. 2E–H), indicating that Dlx2-transfected cells had exited from the oligodendrocyte lineage. Thus, overexpression of Dlx2 in postnatal OPCs had caused them to switch their fate from oligodendrocytes to immature neurons.

By 14 dpt, Dlx2-transfected OPCs expressed microtubule-associated protein 2 (MAP2), known to be present in neuronal soma and dendrites, and glutamic acid decarboxylase (GAD67), which converts glutamate into GABA in inhibitory GABAergic neurons. In Dlx2-transfected cultures, there were clusters of MAP2 + GAD67 + cells, which were 30-fold more abundant than in control-transfected cultures (Fig. 1D–E), and 99.4 ± 0.4% of the...
MAP2 + cells expressed GAD67. There were occasional isolated GAD67 + MAP2 + cells in control-transfected cultures. These were likely to have arisen from contaminating neuroblasts in the subventricular zone (SVZ), which were present in up to 2% of the immunopurified OPCs. Consistently, at 2 dpt, 0.7% ± 0.3% of the mCherry + cells in control-transfected cultures expressed Tuj1 (Fig. 1C). These observations suggest that forced expression of Dlx2 in postnatal oligodendrocyte lineage-committed cells led them to switch their fate toward GABAergic inhibitory neurons.

Dlx2 induces GABAergic neuronal genes in OPCs within 2 days. To explore the molecular mechanism underlying the fate switch from OPC to GABAergic neurons by Dlx2, we compared transcriptomic profiles of control- and Dlx2-transfected OPCs after 2 dpt. Among the 740 significantly differentially expressed genes (p-adj < 0.01), 445 were upregulated and 295 were downregulated in Dlx2-transfected cells (Supplementary Table 1). Figure 2A shows a heatmap of the 50 most significantly differentially expressed genes ranked by p-adj values. Among the most significantly downregulated genes were Cspg4, encoding NG2, and the oligodendrocyte transcription factors Olig1 and Olig2. Gene ontology (GO) term analysis revealed that gliogenesis was the largest represented biological process with the largest number of downregulated genes (Fig. 2B). Other significantly downregulated genes in this GO category were Pdgfra (ranked 209 by p-adj) and Sox10 (ranked 705). Other biological processes enriched in downregulated genes were related to chromatin assembly and organization, with the majority of the genes in these groups encoding histone proteins, suggesting changes in the chromatin structure during neuronal reprogramming of OPCs.

| Primer name | Primer orientation | Primer sequence |
|-------------|--------------------|-----------------|
| (A) Dlx genes for Fig. 3B-F | | |
| Dlx1 | Forward | 5'-CTACAGTTGGGGTGACGAACACTCT-3' |
| | Reverse | 5'-TTCCAGGGCTCTTCTTC-3' |
| Dlx1as | Forward | 5'-GCAGACACTTGGTCGTTT-3' |
| | Reverse | 5'-CTCACTACGGTCGCTC-3' |
| Dlx2 | Forward | 5'-TCCTACTCCGGGCAAAAGACG-3' |
| | Reverse | 5'-GAGCACTGACCTGGGT-3' |
| Dlx5 | Forward | 5'-GGTTGAGAAGAGTCGACGAAACG-3' |
| | Reverse | 5'-ATTGAGATCCTCGAGA-3' |
| Dlx6 | Forward | 5'-GTGGGTATACTCGCTGCTCA-3' |
| | Reverse | 5'-GACTCAATCCCTGGCCTCC-3' |
| Dlx6as (Env2) | Forward | 5'-CAAGAGGGGGGGGATGGTTCA-3' |
| | Reverse | 5'-TGCCCATACGTGCCTGATC-3' |
| (B) Inhibitory neuron genes for Fig. 4G | | |
| Ccnd2 | Forward | 5'-CTTCTGCACTGGGGGAAACCAACC-3' |
| | Reverse | 5'-CTTCTGCACTGGGGGAAACCAACC-3' |
| Epha5 | Forward | 5'-TGAGAGAGAGACCTTGAGG-3' |
| | Reverse | 5'-TGATAGAGAGAGACCTTGAGG-3' |
| Erbb4 | Forward | 5'-GCCTGCTGAACTGGGTGG-3' |
| | Reverse | 5'-TCATAAGAGAGAGACCTTGAGG-3' |
| Gad1 | Forward | 5'-AGCAGAGGAGAAGAGACCAACC-3' |
| | Reverse | 5'-ACAGAGGAGAAGAGACCAACC-3' |
| Mef2c | Forward | 5'-ACGAGAGGAGAAGAGACCAACC-3' |
| | Reverse | 5'-AGCAGAGGAGAAGAGACCAACC-3' |
| Pdcd3 | Forward | 5'-GGAAACAGACAGAGACCAACC-3' |
| | Reverse | 5'-GGAAACAGACAGAGACCAACC-3' |
| Pvalb | Forward | 5'-GGCAGTCAGAGAAGAGACCAACC-3' |
| | Reverse | 5'-ATTCAGTCAGAGAAGAGACCAACC-3' |
| S1b1 | Forward | 5'-GGTCTCGGGGCAACCTGTAAGA-3' |
| | Reverse | 5'-GGTCTCGGGGCAACCTGTAAGA-3' |
| Sst | Forward | 5'-CCCCAGACCTCGCTAGTTTCC-3' |
| | Reverse | 5'-GGCTCAGAGGAGCATCCTCC-3' |
| St18 | Forward | 5'-CAGGGCAAGGCAAAAGCAG-3' |
| | Reverse | 5'-ACGAGGGGAAAGGAGCTCAG-3' |
| Vip | Forward | 5'-TACGAGAAATGGCCACACCTTA-3' |
| | Reverse | 5'-TGTCTTGGATGGCAGGAG-3' |

Table 2. Primers used for qPCR.
Among the significantly upregulated genes were those known to be expressed by immature neurons such as Dcx (Doublecortin) and Tubb3 (encoding βIII-tubulin) (Fig. 2A, Supplementary Table 1), as well as other genes important for neuronal function such as those encoding voltage-dependent K+ channels (Kcnq3 and Kcng1) and synaptic proteins such as Grin2a (NMDA receptor 2A) and Sytl4 (synaptotagmin-like protein 4). Among the most significantly upregulated transcription factors was Sp9, which regulates the expression of Fgf8 (fibroblast growth factor 8) implicated in telencephalic patterning27. GO analysis of the upregulated genes revealed an enrichment of genes that are important for neurogenesis and neuronal differentiation (Fig. 2C). Nine out of ten GO terms that were enriched in upregulated genes in Dlx2-transfected cells were related to axonal growth.

We also examined genes that regulate the cell cycle and are known to be expressed in OPCs28,29. The majority of the positive regulators of the cell cycle were downregulated in Dlx2-transfected cells compared to control-transfected cells (Supplemental Fig. 3A), with Ccnd1, encoding the G1/S-specific cyclin-D1, being the most strongly downregulated. This suggests that Dlx2-transfected OPCs had become post-mitotic as they differentiated into inhibitory neurons, consistent with previous reports that neuronal differentiation from neural progenitor cells is initiated through downregulation of the activity of cyclins and CDK proteins30,31.

**Upregulation of GABAergic but not glutamatergic neuronal genes.** We next further examined neuronal genes that were upregulated in Dlx2-transfected OPCs (Fig. 3A; Supplementary Table 1). There was a significant upregulation of Gad1 and its closely related gene Gad2, which encode GAD67 and GAD65, respectively, in GABAergic inhibitory neurons32,33, as well as upregulation of Slc32a1, which encodes vesicular GABA transporter (VGAT). However, we did not detect transcripts expressed by the major non-overlapping mature interneuron classes in the neocortex, Sst encoding somatostatin (SST), Pvab encoding parvalbumin (PV), and Vip encoding vasoactive intestinal peptide (VIP), while we saw an upregulation of Calb2 encoding calretinin, also known as calbindin-2, expressed by a subset of VIP+ and SST+ interneurons34. Within the Dlx family of interneuron transcription factors, Dlx1, and Dlx5, to a lesser extent, were upregulated. By contrast, we did not detect the excitatory neuron bHLH transcription factor Neurod113 in Dlx2- or control-transfected OPCs. Nor did we see upregulation of other key genes expressed by cortical excitatory neurons such as Slc17a7 encod-
ing vesicular glutamate transporter VGLUT135 or Tbr1 (T-box brain protein 1) encoding a transcription factor expressed in pyramidal neurons in the deep neocortical layers (Fig. 3A)36. These findings indicate that transient episomal Dlx2 misexpression in OPCs was sufficient to selectively upregulate inhibitory neuron transcription factor expression and initiate the program of differentiation towards GABAergic inhibitory neurons in 2 days.

Changes in chromatin modifiers during fate switch into GABAergic neurons. To predict potential changes to the chromatin architecture during the early phase of neuronal reprogramming of OPCs, we next examined the RNA-seq dataset at 2 dpt to see whether Dlx2 overexpression altered the levels of transcripts encoding chromatin modifying factors. Among histone deacetylases (HDACs), there was a 3.7-fold upregulation of Hdac11 (Supplementary Fig. 3B), which encodes a class IV histone deacetylase and is implicated in spinal motor neuron survival by interacting with the mRNA splicing machinery37. Notably, there was no statistically significant change in Ezh2, which encodes the catalytic subunit of the polycomb repressive complex 2 (PRC2) responsible for depositing the repressive trimethylation mark on histone 3 lysine 27 (H3K27) at many inhibitory neuron genes in OPCs15,38,39. Nor was there any change in expression of Kdm6b, which encodes H3K27 demethylase. Among other histone posttranslational modifiers, Kdm3a and 7a were significantly upregulated. Kdm3a demethylates mono- and dimethyl H3K9. It interacts with Neurogenin-2 and increases chromatin accessibility for NeuroD1 and Tubb2b during neuronal differentiation40. Kdm7a, which demethylates di-methyl H3K27 and H3K9, promotes neural differentiation by increasing FGF4 expression41. Interestingly, among the transcripts encoding ATP-dependent chromatin remodeling enzymes, chromodomain helicase DNA binding protein 3 (Chd3), implicated in cortical layer specification42, was significantly upregulated. DNA methylation also regulates chromatin structure and gene transcription through the deposition of methyl groups at CpG islands. Dnmt1, which encodes maintenance DNA methyltransferase essential for correct alternative splicing during OL differentiation43, was significantly downregulated in Dlx2-transfected OPCs (Supplemental Fig. 3C). By contrast, the level of Dnmt3a encoding de novo DNA methyltransferase was not changed in Dlx2-transfected OPCs. These changes triggered by Dlx2 overexpression could have caused nucleosome repositioning and facilitated activation or derepression of critical genes during differentiation of OPCs into GABAergic neurons.
To explore the mechanism of neuronal reprogramming from OPCs, we used qPCR to examine the temporal changes in transcription factor transcripts known to be critical for interneuron development in Dlx2-transfected cultures. We focused on other members of the Dlx family transcription factors, since they were upregulated in our initial RNA-seq analysis at 2 dpt and are known to be influenced by Dlx2. During development, Dlx2 is expressed early and transiently and directly activates Dlx5 and 6, which are critical for inhibitory neuron differentiation and maturation. Dlx1 expression is co-regulated with Dlx2. Its expression is transient in PV+ interneurons but sustained in SST+ interneurons. In Dlx2-transfected OPCs, Dlx1 expression peaked at 7 dpt, although it did not reach significance, likely due to large variability among samples. By 14 dpt, Dlx1 returned to a level only slightly above control (Fig. 3B). As predicted from developmental expression, the upregulation of Dlx5 expression in Dlx2-transfected OPCs was most prominent at 14 dpt, although a small increase was observed by RNA-seq at 2 dpt.
2 dpt (Fig. 3C). Dlx6 expression increased earlier than Dlx5, with peak expression at 7 dpt, and decreased but remained slightly elevated at 14 dpt (Fig. 3D).

Interestingly, two long non-coding RNAs (lncRNAs) in the Dlx family were highly upregulated (Fig. 3E–F and Supplementary Table 1). Dlx1as, which is an antisense lncRNA to Dlx1, has been shown to modulate Dlx1 expression.66–67 Dlx6as, an antisense lncRNA to Dlx6, also known as Dlx6os1 or Evf2, has been shown to promote neurogenesis by inhibiting DNA methylation at the ultraconserved enhancer of Dlx6/5.68 At 2 dpt, we detected a >100-fold upregulation of Dlx1as. Dlx6as transcript was also elevated early at 2 dpt. Both transcripts declined by 7 dpt.

Functional and morphological differentiation of OPC-derived GABAergic neurons. OPC-derived immature neurons generate action potentials. We noticed that episomal mCherry expression disappeared beyond the first week after transfection. To achieve persistent reporter expression in OPC-derived neurons, we transfected OPCs from NG2cre;Gad1-GFP mice6–18 with a cre-dependent Tol2 transposon donor vector Tol2-Dlx2ires2-mCherry (Tol2-Dlx2) or control Tol2-ires2-mCherry (Tol2-control) and the T2 transposase helper vector. This enabled identification of GABAergic neurons by GFP and Dlx2-transfected cells by mCherry expression. We detected Gad1-GFP in neuron-like cells as early as 3 dpt in Tol2-Dlx2-transfected cells, consistent with the early upregulation of Gad1 mRNA from our RNA-seq data. At 14 dpt, 93.6 ± 0.4% of mCherry + cells were also Gad1-GFP + in Tol2-Dlx2-transfected OPCs compared to 1.2 ± 1.1% in Tol2-control-transfected OPCs. Most of the latter remained in the oligodendrocyte lineage and expressed Olig2 (Supplemental Fig. 4A, arrowheads). The small number of Gad1-GFP + cells found in Tol2-control-transfected cultures were mCherry-negative (Supplemental Fig. 4B), also supporting the notion that they were likely to have arisen from untransfected neuroblasts in the original OPC culture.

Using the optimized method of sustaining reporter expression in Dlx2-transfected cells, we examined the excitability of mCherry + OPC-derived GABAergic neurons at 14 dpt using whole cell patch–clamp electrophysiological recordings. In current-clamp configuration, we examined whether cells could generate action potentials in response to a series of depolarizing current injections. When we recorded from Tol2-control-transfected mCherry + cells, we saw a graded voltage response but no action potentials in 8 out of 9 recorded cells (Fig. 4A–C). We categorized cells with this pattern of voltage change as type 1 cells. One cell in the control cultures exhibited a single action potential (categorized as a type 2 cell). We were unable to record from GFP + mCherry + cells in control OPCs as they were extremely rare and difficult to locate. When we examined mCherry + Gad1-GFP + cells in Tol2-Dlx2-transfected cultures, we observed a range of different types of responses (Fig. 4D). About half of the cells exhibited a single action potential (type 2 cells), and one-third of the cells displayed a burst of several action potentials that quickly adapted in the continuous presence of depolarizing current (type 3 cells), indicative of immature neurons. Notably, in 3 out of 15 cells we detected continuous non-adapting spiking trains of action potentials (type 4 cells). The heterogeneous action potential patterns likely reflect the transitional state of OPC-derived neurons from an electrically immature to a more mature state at 14 dpt after Dlx2 misexpression.

OPC-derived immature neurons develop GABAergic synaptic puncta. To look for morphological evidence of synapse formation in OPC-derived neurons, we immunolabeled for synaptic proteins. At 14 dpt, we observed clustering of VGAT at the junction between a GAD67 + axon and a MAP2 + GAD67 + dendrite in areas of high cellular density (Fig. 4E, arrowheads), indicative of an early stage of synapse formation. At 21 dpt, we saw clusters of VGAT and the inhibitory post-synaptic scaffolding protein gephyrin around the soma and proximal process of MAP2 + neurons derived from Dlx2-transfected OPCs, indicating the formation of morphological synapses (Fig. 4F, arrowheads). By contrast, none of the neurons in control-transfected cultures expressed VGAT. In Dlx2-transfected cultures we did not detect synaptic puncta that contained VGLUT1 or 2 and the excitatory (Fig. 4F, arrowheads). By contrast, none of the neurons in control-transfected cultures expressed VGAT. In Dlx2-transfected OPCs from NG2cre;Gad1-GFP mice6–18 with a cre-dependent Tol2 transposon donor vector Tol2-Dlx2ires2-mCherry (Tol2-Dlx2) or control Tol2-ires2-mCherry (Tol2-control) and the T2 transposase helper vector. This enabled identification of GABAergic neurons by GFP and Dlx2-transfected cells by mCherry expression. We detected Gad1-GFP in neuron-like cells as early as 3 dpt in Tol2-Dlx2-transfected cells, consistent with the early upregulation of Gad1 mRNA from our RNA-seq data. At 14 dpt, 93.6 ± 0.4% of mCherry + cells were also Gad1-GFP + in Tol2-Dlx2-transfected OPCs compared to 1.2 ± 1.1% in Tol2-control-transfected OPCs. Most of the latter remained in the oligodendrocyte lineage and expressed Olig2 (Supplemental Fig. 4A, arrowheads). The small number of Gad1-GFP + cells found in Tol2-control-transfected cultures were mCherry-negative (Supplemental Fig. 4B), also supporting the notion that they were likely to have arisen from untransfected neuroblasts in the original OPC culture.

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OPCs express a low level of Ascl1 (Supplementary Table 1)56,57, obviating the need for exogenous Ascl1. This is for the ability of Dlx2 alone to convert OPCs into inhibitory neurons without other transcription factors is that Dlx2 alone could reprogram OPCs into inhibitory neurons at an efficiency of 94%. One possible explanation prenatally but remains for several weeks when deleted at P1858,59.

finding that upon Olig2 deletion in OPCs, Sox10 expression disappears within a few days when Olig2 is deleted oligodendrocyte lineage than those that had newly differentiated from NPCs. This is supported by our recent 25% of OPCs are resistant to reprogramming by Dlx2 or take longer to be reprogrammed. The shutdown of dpt, but roughly 25% still contained detectable Olig2, PDGFRα, and Sox10 at 7 dpt, suggesting that roughly 25% of OPCs are resistant to reprogramming by Dlx2 or take longer to be reprogrammed. The shutdown of astrocyte genes.

Although the exact mechanism underlying the fate choice of OPCs after loss of Olig2 remains unclear, it is likely different intrinsic properties that facilitate reprogramming of OPCs into GABAergic neurons. Previous reports that used Dlx2 to reprogram non-neuronal cells into inhibitory neurons invariably required additional transcription factors, particularly Ascl1, which functions as a “pioneer” transcription factor. Pioneer factors alter the chromatin landscape around closed chromatin regions by recruiting chromatin remodeling complexes and enzymes that catalyze histone posttranslational modifications, thereby making the key neuronal genes accessible to reprogramming by Dlx2 to reprogram astrocytes into GABAergic interneurons from 35 to 93%55. By contrast, our results showed that Dlx2 alone could reprogram OPCs into inhibitory neurons at an efficiency of 94%. One possible explanation for the ability of Dlx2 alone to convert OPCs into inhibitory neurons without other transcription factors is that OPCs express a low level of Ascl1 (Supplementary Table 1)56,57, obviating the need for exogenous Ascl1. This is supported by our recent finding that in OPCs genes encoding the key critical transcription factors for inhibitory neurons exist in a more readily accessible chromatin state than those in astrocytes or fibroblasts15. This could also account for the selective activation of inhibitory but not excitatory neuronal genes. Whether the relatively de-repressed state of inhibitory neuron genes in OPCs is mediated by Ascl1 or other transcription factors is currently unknown.

We previously showed that conditional knock-out of Olig2 in postnatal cortical OPCs converts their fate into protoplastic astrocytes but not into neurons58,59. A similar fate conversion to astrocytes but not neurons occurs after deletion of HDAC3, which causes downregulation of Olig220. By contrast, we did not observe astrocyte differentiation from Dlx2-transfected OPCs, despite an early loss of Olig2 (Fig. 1, Supplemental Fig. 1B). Although the exact mechanism underlying the fate choice of OPCs after loss of Olig2 remains unclear, it is likely that loss of Olig2 in the presence of Dlx2 triggers a transcription factor network that represses STAT3 and other astrocyte genes.

In OPCs transfected with Dlx2, there was a gradual loss of oligodendrocyte lineage antigens from 2 to 7 dpt, but roughly 25% still contained detectable Olig2, PDGFRα, and Sox10 at 7 dpt, suggesting that roughly 25% of OPCs are resistant to reprogramming by Dlx2 or take longer to be reprogrammed. The shutdown of oligodendrocyte lineage proteins could take longer in some OPCs that are more “mature” and farther along the oligodendrocyte lineage than those that had newly differentiated from NPCs. This is supported by our recent finding that upon Olig2 deletion in OPCs, Sox10 expression disappears within a few days when Olig2 is deleted prenatally but remains for several weeks when deleted at P1858,59.

Partial recapitulation of developmental transcription network triggered by Dlx2 misexpression in OPCs. During interneuron development in the medial ganglionic eminence, Dlx1/2 first specifies GABAergic lineages by repressing Olig2. Subsequently, in postmitotic cells, Dlx1/2 promotes further neuronal differentiation by inducing Gad1/2 and Dlx5/6 (reviewed in53), as well as Sp9. These immediate targets of Dlx1,2,
as well as Dlx1, were all upregulated in OPCs after less than 48 h of exposure to Dlx2. We further used qPCR to examine the extent to which these secondary transcription factors were sustained in Dlx2-transfected OPCs. In our qPCR analysis, we could not attain the sensitivity obtained by RNA-seq at 2 dpt, since the OPC-derived neurons were too fragile for FACs purification at later time points, and many untransfected cells were mixed in the analyzed population. Nevertheless, the results from qPCR showed a trend for an early upregulation of Dlx1, though not statistically significant, and a significant upregulation of Dlx6 and 5, as well as significantly elevated and sustained expression of Gad1 through 14 dpt. Elevation of Dlx1 and Dlx6 was transient and declined by 14 dpt, whereas the most significant upregulation of Dlx5 expression occurred between 7 and 14 dpt.

Interestingly, two IncRNAs in the Dlx family of genes, Dlx6as and Dlx1as, were significantly upregulated at 2 dpt in our RNA-seq data, and this was further validated by qPCR, suggesting a greater magnitude of elevation of these genes compared to the other Dlx genes. The upregulation of both Dlx IncRNA transcripts was transient, and by 7 dpt they returned to a level that was not significantly above baseline levels. The steep early upregulation of the two Dlx IncRNA species could play a significant role in modulating the temporal profiles of the levels of Dlx1/5/6 genes. The specific mechanism of action of Dlx1as and Dlx6as in this context remains unclear, and reports of studies in different cell types vary. Dlx6as has been shown to recruit and form a complex with Dlx2 to activate the Dlx5/6 intergenic enhancer. Thus, it is possible that the elevated expression of Dlx6as is contributing to the elevated expression of Dlx 6 and 5 observed at 7 and 14 dpt, respectively. This is consistent with the report that knockdown of Dlx1as in adult SVZ cells decreased Dlx1 and Dlx2 expression. On the contrary, Dlx1as knock-out mice have higher Dlx1 expression and exhibit the phenotype of gain-of-function of Dlx1. If Dlx1as positively affects Dlx1 expression in OPC-derived neurons, the early induction of Dlx1as may have played a role in inducing Dlx1 expression, but the transient nature of Dlx1as expression may not have been sufficient to sustain Dlx1 expression.

Incomplete manifestation of interneuron class phenotypes in OPC-derived neurons. Dlx2-transfected OPCs did not exhibit the fully differentiated phenotype of any of the three major interneuron classes known to exist in the neocortex. RNA-seq data at 2 dpt did not detect upregulation of Pvalb, Sst, or Vip. By 14 dpt, we detected a small but significant increase in Sst transcript by qPCR, suggesting an incomplete differentiation toward SST+ interneurons. Interestingly, Sp9, which was found to be elevated at 2 dpt by RNA-seq, is known to promote the activation of Satb1 and St18, both of which were elevated at 14 dpt in qPCR analyses. Satb1 is critical for the survival and maturation of SST+ and some PV+ interneurons, and is characteristically expressed as one of the signature transcripts of SST+ interneurons (database by the Linmarsson lab, http://mousebrain.org/cellt ypes/).

During development, Dlx1 is expressed transiently in early PV+ interneuron-fated progenitors and is downregulated upon PV expression, whereas Dlx1 expression is sustained as progenitor cells mature into SST+ interneurons. The decline of Dlx1 beyond 7 dpt in OPC-derived neurons could be related to the emergence of a transcriptional signature of SST+ interneurons. However, electrophysiological recordings of OPC-derived neurons revealed that 3/15 cells exhibited a train of action potentials with an average firing rate of ~85 Hz upon current injection with no sign of adaptation. The range of action potential firing frequency was higher than that of the fastest firing SST+ neurons, but lower than that of mature PV+ neurons and more similar to immature PV+ neurons seen during the first postnatal week. The most common action potential firing pattern seen among Dlx2-transfected cells was several spikes of decaying amplitude after current injection followed by inactivity. This is often seen in reprogrammed neurons, suggesting a partially differentiated phenotype, possibly reflected by the density and type of Na+ and K+ channels. OPCs in different functional states have different Na+ channel densities, though they are generally too low to generate regenerative action potentials. Thus, types 2 and 3 firing patterns observed in Fig. 4 likely represent a gradient of neuronal differentiation from OPCs.

The health of the OPC-derived neurons began to deteriorate beyond 21 dpt under the culture conditions used, and we were unable to examine whether the cells developed more characteristic neurochemical and biophysical phenotypes of more mature interneuron classes over time. Since progenitor cells in the medial ganglionic eminence acquire competence to produce SST+ neurons earlier than PV+ class of interneurons, it is possible that the detection of Sst, Satb1, and St18 is reflecting the relatively early stage of interneuron maturation. The lack of detectable PV or SST immunoreactivity in OPC-derived neurons at 14 dpt is also consistent with their immature state. We have also noted that Lhx6, a critical secondary interneuron transcription factor, was not upregulated in Dlx2-transfected OPCs. Additional transcription factors such as Nkx2-1 may be needed in parallel with the transcriptional network that is activated by Dlx2.

Of note, our electrophysiological recordings were conducted from OPC-derived neurons that had integrated Dlx2-IRES2-mCherry into the genome. While beneficial for lineage tracing of transfected cells, sustained Dlx2 expression may have impeded full neuronal maturation. Future studies may be conducted with temporal control of gene Dlx2 expression to further promote maturation of OPC-derived interneurons.

Inputs from surrounding neurons, particularly those from excitatory neurons promote the survival of postmitotic immature interneurons. Thus, it is possible that further maturation of OPC-derived neurons in a recent in vivo study in which striatal OPCs were reprogrammed into neurons with multiple transcription factors may have been facilitated by the local in vivo environment. Our findings provide a logical framework for further testing strategies to promote interneuron reprogramming from OPCs under pathological conditions in which greater inhibitory neuron function is needed.
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Author contributions
L.B., A.N., and A.T. designed the experiments. L.B., H.S., T.N., and W.W. conducted the experiments. I.M. assisted with the bioinformatic analysis of RNA-seq data. Y.Y. provided the Gad1-GFP mice. L.B., H.S., A.T., and A.N. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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