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Supplemental Information

Direct Reprogramming of Resident NG2 Glia into Neurons with Properties of Fast-Spiking Parvalbumin-Containing Interneurons

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Figure S1
Related to Figure 1

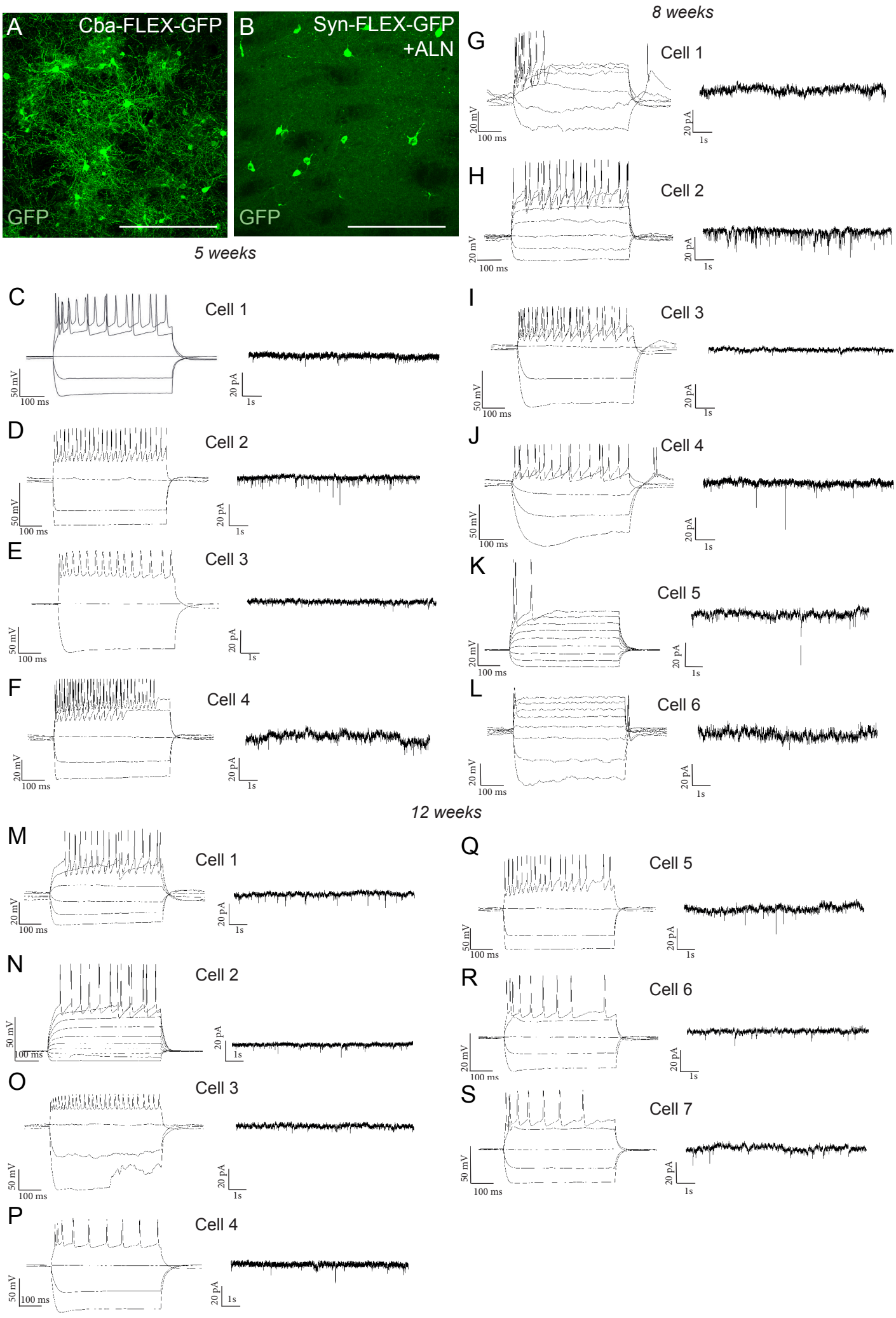


Figure S1 - Related to Figure 1

(A-B) Confocal images showing GFP+ cells in the striatum of mice injected either with (AAV5) Cba-FLEX-GFP, labelling NG2 glia (A), or (AAV5) SYN-FLEX-GFP, labelling neurons (B); (C-S) Individual traces from whole-cell recordings of reprogrammed neurons, 5 (C-F), 8 (G-L) and 12 (M-S) weeks post injection. Current-induced action potential (AP) and post-synaptic activity (current, in pA) of reprogrammed cells showed that the ability to induce repetitive AP preceded the ability to form post-synaptic contacts. After 5 weeks, only cell 2 (D) showed post-synaptic activity with event threshold of >5pA. (In cell 4 the baseline was too unstable to distinguish any synaptic events). All cells recorded showed repetitive AP after current injections. At 8 weeks, 3 cells out of 6 cells (cell 2,4 and 5 - H, J and K, respectively) showed synaptic events whereas 5 out of 6 showed repetitive spiking. At 12 weeks, 6 out of 7 cells showed postsynaptic activity (cell 3 did not show postsynaptic activity since events were not clear enough to be detectable for analysis). Scale bars A-B: 200 μm.

Figure S2
Related to Figure 2

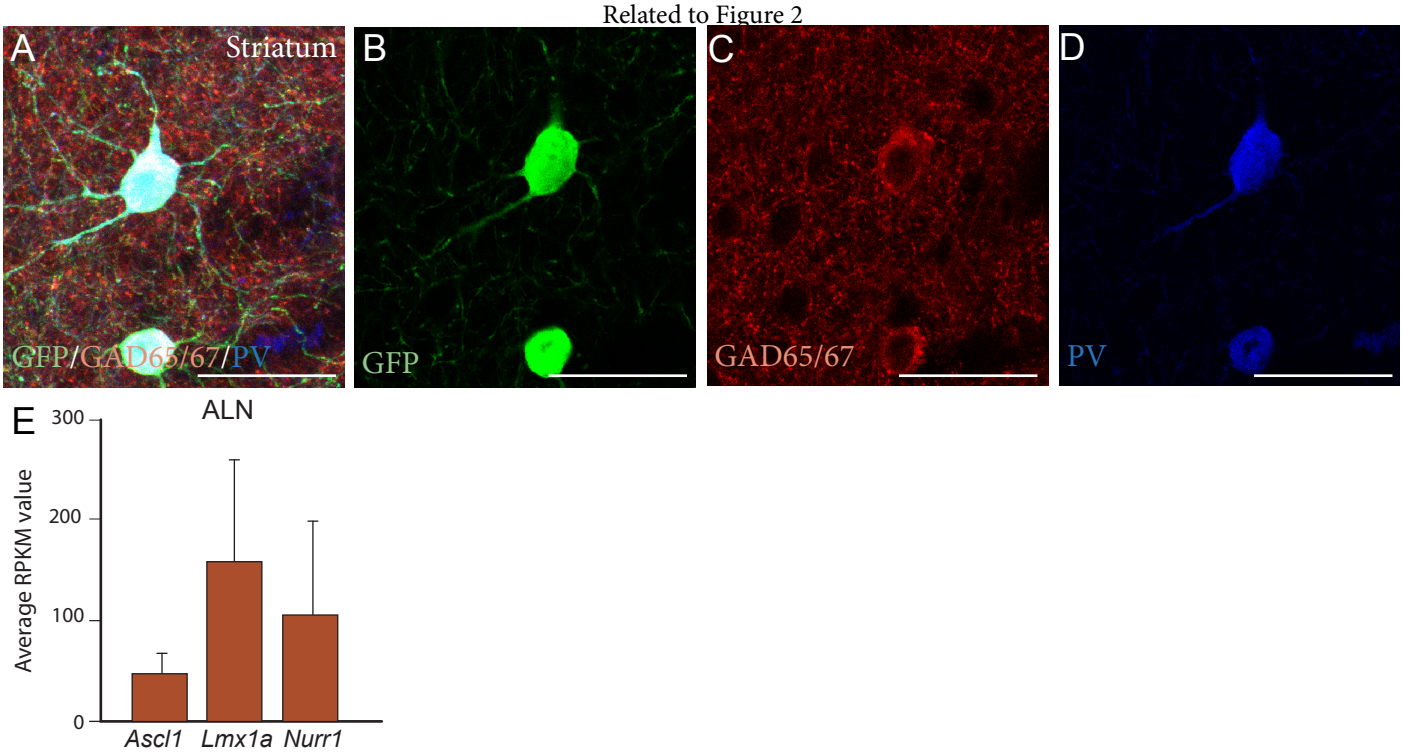


Figure S2 - Related to Figure 2
(A-D) Confocal images showing Parvalbumin-positive reprogrammed neurons (A, B and D) in the striatum of a mouse injected with SYN-GFP + ALN, expressing the striatal marker GAD65/67 (C); (E) The reprogramming factors ALN are detected at high levels in nuclear-GFP-expressing neurons, as demonstrated by RNAseq of neurons isolated by LCM (LCM-seq; n=12-65 cells collected from n=2-3 brains). Data presented as MEAN +/- SEM. Scale bars A-D: 25 µm.

Figure S3
Related to Figure 3

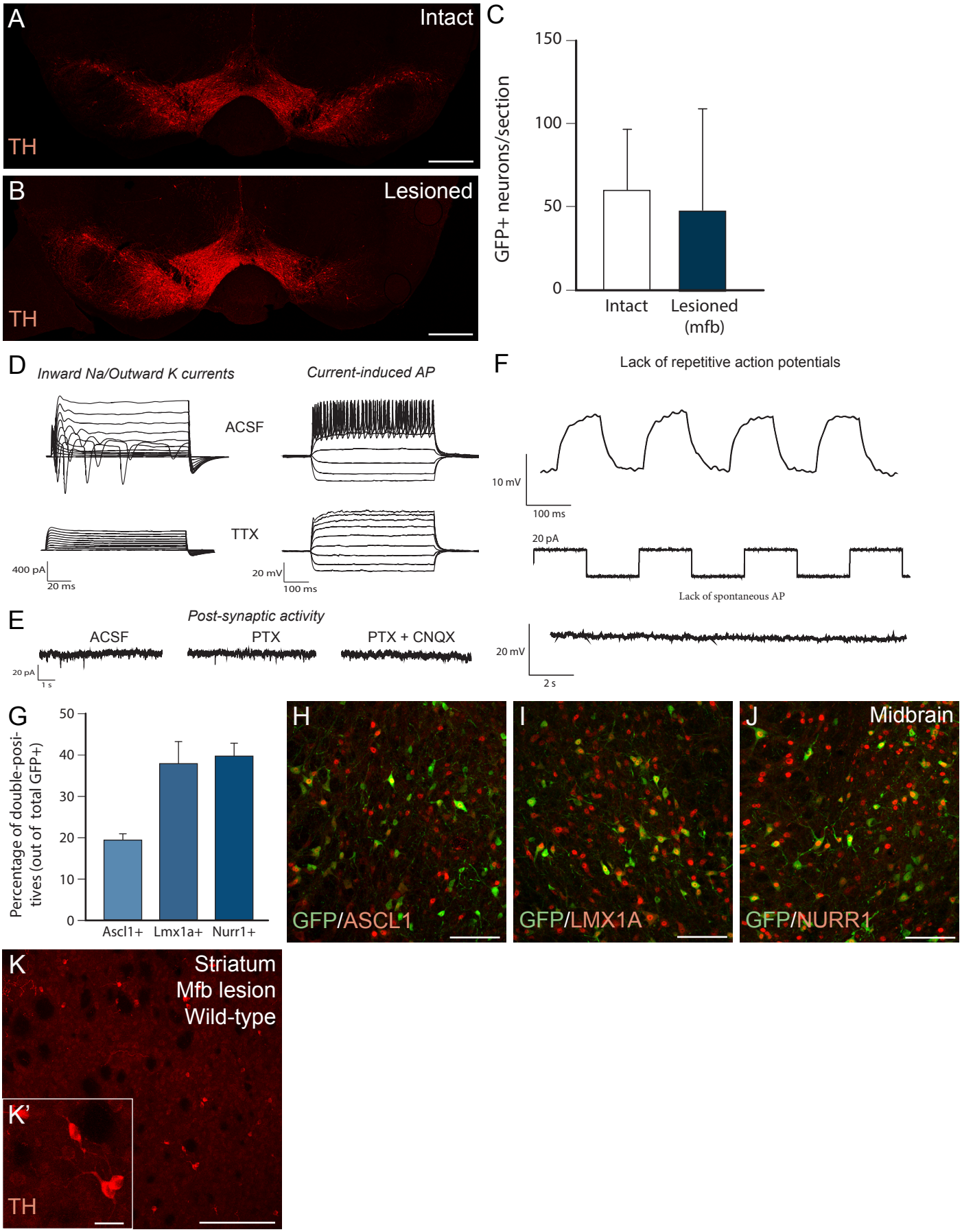
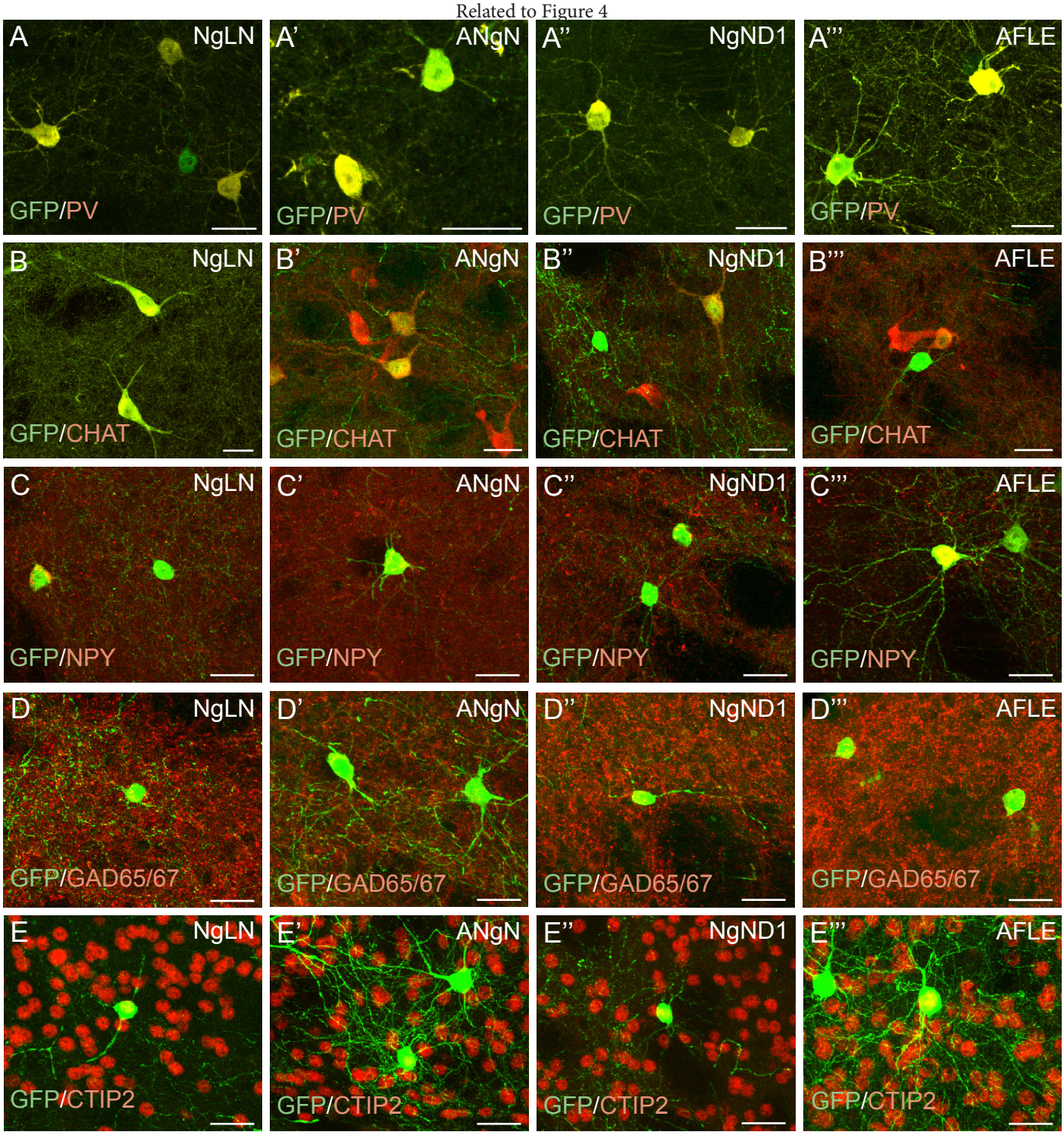


Fig. S3 – Related to Figure 3.
Tyrosine hydroxylase (TH) staining of the Subtantia Nigra showing a unilateral denervation on the right side, in a medial fore-brain bundle (mfb)-lesioned animal (B) but spared cell bodies in an intact animal (A); (C) Quantification of *SYN*-GFP+ neurons revealed no significant difference between intact and lesioned group (mean number of GFP+ cells/section was 60 ± 12,71 for intact and 48 ± 24,63 for lesioned striatum; P value = 0,6494; n=8 brains for intact and n=6 brains for lesioned); (D) Repro-grammed neurons expressed inward sodium and outward potassium currents at depolarizing steps, which could be blocked with the blocker of voltage-gated sodium-channel, tetrodotoxin (TTX) that also blocked AP; (E) Postsynaptic activity of reprogrammed neurons was seen with whole-cell patch clamp in both lesioned and intact brain, and this could be blocked with picrotoxin (PTX) and CNQX; (F) iNs showed no electrophysiological traits of DA phenotype such as repeated AP after small current injections or spontaneous AP; (G) Quantifications on sections from brains injected with ALN in the midbrain revealed the presence of overlap in percentages of cells expressing each one of the three markers (n=5 brains); (H, I, J) Confocal images showing the presence of *SYN*-GFP+ cells that co-label with Ascl1 (H), Lmx1a (I) or Nurr1 (J); (K) Confocal image shows TH+ cells in the the striatum of a wild-type lesioned mouse, which has not received any viral injection. Data presented as Mean+/-SEM. Scale bars A, B: 500 μm; H-J: 100 μm; K: 200 μm; K': 25 μm.

Figure S4
Related to Figure 4



(A-E) Images show the presence of GFP+ cells in the striatum of NG2-CRE mice in which different factor combinations of genes were used for reprogramming. These GFP+ cells co-label with the markers PV (A, A', A'', A'''), CHAT (B, B', B'', B'''), NPY (C, C', C'', C'''), GAD65/67 (D, D', D'', D''') and CTIP2 (E, E', E'', E''') when the gene combinations NgLN, ANgN, NgND1 and AFLE are used. All scale bars: 25 μm.

Table S1: Electrophysiological parameters for the different types of reprogrammed neurons. Related to Figure 2.

Cell-type B (reminiscent of FSI) shows hyperpolarized resting RMP, similar firing frequency and input resistance as their endogenous counterpart but does not yet show all the properties of mature FSI (Povysheva et al 2013, Kawaguchi 1993). Cell type C in our manuscript has greater amplitude of afterhyperpolarization (AHP) after spike compared to the other groups, a greater input resistance and more depolarized RMP, that is similar to LA neurons (including cholinergic IntNs). It is still not in the exact range of endogenous counterpart (Kawaguchi 1993). Cell type D has a longer duration of AP compared to FS and long duration of afterhyperpolarization (time to peak of AHP in this table) that is similar to PLTS neurons (NPY-containing). Yet, for the other parameters, this cell type cannot be identified as a specific IntN subtype (n=1 for cell type A, n=8 for cell type B, n=3 for cell type C and n=4 for cell type D).

	Celltype A	Celltype B	Celltype C	Celltype D
RMP (mV)	-77 ± 0	-63,75 ± 2,82	-54 ± 4,34	-72 ± 2,45
Input resistance (MΩ)	110 ± 0	204 ± 27,6	730 ± 101,5	291 ± 70,34
Firering frequency (Hz)	28 ± 0	43,25 ± 4,09	28,67 ± 7,69	19 ± 2,08
AP duration, (ms, Spike width at half amplitude)	0,7 ± 0	0,61 ± 0,04	0,77 ± 0,12	1,05 ± 0,13
AHP amplitude (mV)	15 ± 0	10,5 ± 1,21	13 ± 1,73	7,02 ± 0,87
Time to peak of AHP (ms)	9,9 ± 0	6,15 ± 0,23	7,03 ± 0,55	12,25 ± 1,82
N of cells recorded	1	8	3	4

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning and Viral vector production

Cre-inducible AAV5 vectors were created by inserting the cDNA for the genes of interest [1] in a reverse orientation flanked by two pairs of heterotypical, antiparallel LoxP (FLEX) sequences [2]. GFP was expressed under the control of a synapsin promoter, and *Smarca1a*, *Ascl1*, *Lmx1a*, *Nurr1*, *Neurogenin2*, *NeuroD1*, *FoxA2*, or *En1* were all regulated by a ubiquitously expressed chicken beta actin (cba) promoter. All constructs were sequenced prior to use. These Cre-inducible AAV5 vectors were produced according to [3] and each vector used individually or in mixture used at a 5% dilution in PBS.

Animals and surgery

All animals that were considered as dopamine (DA) lesioned in this study had a DA denervation in the substantia nigra pars compacta of >75% in comparison to the intact side (optical density analysis).

For *in vivo* reprogramming experiments in the striatum, the viral solution was injected at the following coordinates: AP = +1.0, ML = -2.0, DV = -2.7 relative to bregma, tooth bar = flat. For *in vivo* reprogramming experiments in the midbrain, the viral injections were performed at the coordinates: AP = -3.1, ML = -1.1 and DV = -4.0 relative to bregma, tooth bar = flat. For total 6-OHDA lesion experiments, 0.5 µl of 6-OHDA (3.2 µg/µl) was injected into the medial forebrain bundle (mfb) at the following coordinates: AP = -1.0, ML = -1.3 and DV = -4.75 relative to bregma, tooth bar = flat.

Immunohistochemisty

At the chosen experimental endpoints, animals were transcardially perfused with ice-cold 4% PFA, dissected and post-fixed for 12 hours in 4% PFA. In the following day, the brains were put in 25% sucrose for 12 hours, frozen and then cut on a microtome at 35µm of thickness in 8 series.

Sections were stained using standard protocols and the following antibodies: GFP (chicken, Abcam ab13970, 1:1000), TH (rabbit, Millipore ab152, 1:1000), parvalbumin (mouse, Sigma P3088, 1:2000), ChAT (goat, Chemicon ab144p, 1:200), NPY (rabbit, Immunostar 22940, 1:5000), Darpp32 (rabbit, Abcam ab40801, 1:1000), GAD65/67 (rabbit, Abcam ab49832, 1:1000), vGlut1 (rabbit, Synaptic systems 135303, 1:1000), Tbr1 (rabbit, Abcam ab31940, 1:300), CTIP2 (rat, Abcam ab18465, 1:1000), Calretinin (rabbit, Abcam ab702, 1:200). For fluorescent stainings, Alexa 488 (goat anti-chicken, Life Technologies A11039, 1:500), biotinylated antibodies (Jackson BA2001, BA1000, BA9500 and BA4001, 1:200) and Cy3 Streptavidin (Jackson 016-160-084, 1:200) were used as secondary antibodies.

RNA sequencing of nuclear GFP positive cells isolated by laser capture microdissection (LCM-seq)

For LCM, a Leica DM6000R/CTR6500 apparatus was used and cells captured with the Leica LMD7000 system under fluorescence light at 40X objective magnification. Before the session, slides were air dried for 10 minutes inside the microscope set up. To minimize contamination by surrounding tissue, only cells with clear nuclear GFP were collected using a close cutting outline. Laser power was kept at a minimum and light exposure reduced by closing the fluorescence shutter after drawing cell outlines. Relative humidity for all experiments was between 20 and 31 % and the temperature range was between 20 and 26 °C. An average of 33 cells were collected by gravity in each cap of 0.2ml PCR tubes. After the session, 5 µl lysis buffer (0.2% Triton X-100, with 2 U ml recombinant RNase inhibitor, Clontech) was added to each cap. Samples were spun down in a table top centrifuge (VWR) for 5-10 seconds, quickly labeled, sealed with parafilm (Pechiney Plastic Packaging) and snap frozen on dry ice. Samples were stored at -80 °C. Library preparation for sequencing was carried out using the Smart-seq2 protocol [4] with some modification as described in [5]). The quality and concentration of sequencing libraries was measured with an Agilent 2100 Bioanalyzer using the High Sensitvity DNA kit (Agilent). Equal amounts of the indexed libraries (a total of 15 samples) were pooled and sequenced in one lane. Samples were sequenced using an Illumina HiSeq2000 sequencer and reads were 43 bp in length. RNA-seq reads were mapped to the mouse reference genome mm10 (Ensembl version 78) by employing STAR (version 2.4.1) [6] with parameter -- outSAMstrandField intronMotif enabled. The number of uniquely mapped reads was calculated using featureCounts (version 1.4.6) [7] with default parameters. To quantify and normalize the expression of genes/ transcripts, Cufflinks and Cuffnorm (version 2.2.1) [8] was used with parameter - library-norm-method geometric. Quality control was conducted, with cutoffs of at least 1 million reads and 70% mapping ratio to the mm10 genome. All the RNA sequencing data generated in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO: GSE101091.

Electrophysiology

Mice were killed by an overdose of pentobarbital and brains were rapidly taken out and coronally cut on a vibratome at 275μm. Slices were transferred to a recording chamber and submerged in a continuously flowing artificial cerebrospinal fluid (ACSF) solution gassed with 95% O_2 and 5% CO_2 at 23°C. The composition of the ACSF was (in mM):126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4-H_2O, 1.3 $MgCl_2$-$6H_2O$, and 2.4 $CaCl_2$-$6H_2O$, 22 NaHCO3, 10 glucose adjusted to pH 7.4.
Whole-cell patch-clamp recordings were made using Multi-clamp 700B (Molecular Devices), and signals were acquired at 10kHz using pClamp10 software and a data acquisition unit (Digidata 1440A, Molecular Devices). Input resistances and injected currents were monitored throughout the experiments. Borosilicate glass pipettes (3–7 MΩ) for patching were filled with the following intracellular solution (in mM): 122.5 potassium gluconate, 12.5 KCl, 0.2 EGTA, 10 Hepes, 2 MgATP, 0.3 Na_3GTP and 8 NaCl adjusted to pH 7.3 with KOH as in [9]. For biocytin filling, 1mg of biocytin salt was added to 1ml of internal solution. GFP-positive cells with neuronal morphology were patched and slowly filled with biocytin-containing internal solution for 20 min before slowly removing the electrode. Sections were fixed in 4 % paraformaldehyde overnight and stained with streptavidin-Cy3 (1: 600 in KPBS-T, 2h), for a morphological overview of reprogrammed cells.
Resting membrane potentials were monitored immediately after breaking-in in current-clamp mode. Thereafter, cells were kept at a membrane potential of -60mV to -80mV, and 500ms currents were injected from -20pA to +90pA with 10pA increments to induce action potentials (AP). Inward sodium and delayed rectifying potassium currents were measured in voltage clamp at depolarizing steps of 10mV. Spontaneous postsynaptic activity was recorded in voltage-clamp mode at -70mV. For distinguishing the inhibitory postsynaptic events the membrane potential was set to 0mV for inhibitory events and -70mV for excitatory. Off line analysis of spontaneous excitatory post-synaptic currents (EPSC) and inhibitory post-synaptic currents (IPSC) were performed using a threshold-event detection (>5pA) in Clampfit analysis program. CNQX, an AMPA antagonist and Picrotoxin (PTX), the $GABA_A$ receptor antagonist was added to the bath at the concentration of 20 and 50μM respectively (Tocris biosciences) to distinguish glutamatergic and GABAergic events.

Microscopy
Figures 3A, 3B, S3A and S3B are the result of the capture and stitching of individual images using a Leica TCS SP8 laser-scanning confocal microscope and corresponding software.
For non-confocal imaging, a Leica inverted Microscope (DFC360FX + DMI 6000B) was used.

Quantifications and statistical analysis
For quantification of GFP+ cells in intact and lesioned animals, neurons were counted in whole striatum and expressed as average of cells/section. Three striatal sections were counted per animal (n=8 animals for intact and n=6 animals for lesioned) (spanning from Bregma 1.7 to -0.26, as in [10]). Comparisons between the conditions lesioned and intact were made in Prism Graph-Pad using unpaired t-tests.
To calculate the conversion efficiency *in vivo*, the number of GFP+ cells was counted in sections from brains injected either with (AAV5)-Cba-FLEX-GFP (n=3) or (AAV5)-Syn-FLEX-GFP (n=9), and the number of neurons was compared with the number of NG2 glia cells found, and expressed as a percentage. A total of 3 sections per brain was analysed.
Quantification of different markers expressed in GFP+ neurons in the striatum, obtained with ALN (n=9) or with different combinations of factors (n=3 per combination) was done by counting the number of double-positive cells in relation to the total GFP+ cells found in two fields in the striatum. Two sections per animal were analysed. Differences between different conditions were analysed by using unpaired t-tests in Prism Graph-pad.
For quantifications of co-labelling of GFP/ASCL1, GFP/LMX1A and GFP/NURR1 in the midbrain region, two sections per animal (n=5) were analysed in each of three independent stainings. A rectangular region of interest was selected and cells within this area were counted. The mean number of double-positive cells per animal found in the three stainings was then compared.
For quantification of TH+ cells found in the striatum of lesioned animals, the number of TH+ cells per section was counted in Lesioned and Lesioned + ALN groups of animals (n=6 animals per group, n=6 sections per animal). All quantifications were performed in blind.
For membrane intrinsic properties (capacitance, input resistance, resting membrane potential and synaptic activity) one-way ANOVA was used with Bonferroni post-hoc test for multiple comparisons. N of cells compared for capacitance: n=6 (8w), n=7 (12w), n=7 (endogenous, i.e. MSNs and interneurons); for input resistance n=6 (8w), n=7 (12w), n=7 (endogenous); RMP n=6 (8w), n=7 (12w), n=7 (endogenous); for synaptic activity n=6 (8w), n=7 (12w), n=4 (5w), n=5 (endogenous).

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