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Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain

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ABSTRACT

Transplants of foetal dopamine neurons can be used to restore dopamine neurotransmission in animal models of Parkinson's disease (PD), as well as in patients with advanced PD. In these studies the cells are placed in the striatum rather than in the substantia nigra where they normally reside, which may limit their ability to achieve full restoration of motor function. Using a micro-transplantation approach, which allows precise placement of small cell deposits directly into the host substantia nigra, and foetal donor cells that express green fluorescent protein under control of the tyrosine hydroxylase promoter, we show that dopamine neuroblasts implanted into the substantia nigra of adult mice are capable of generating a new nigrostriatal pathway with an outgrowth pattern that matches the anatomy of the intrinsic system. This target-directed re-growth was closely aligned with the intrinsic striatonigral fibre projection and further enhanced by over-expression of glial cell-line derived neurotrophic factor in the striatal target. Results from testing of amphetamine-induced rotational behaviour suggest, moreover, that dopamine neurons implanted into the substantia nigra are capable of integrating into the host circuitry also at the functional level.

INTRODUCTION

Cell transplantation in Parkinson's disease (PD) is based on the idea that grafted dopamine (DA) neurons can be used to replace the intrinsic nigral DA neurons lost to disease. Clinical trials using transplants of DA neuroblasts, obtained from foetal ventral mesencephalon (VM), have given promising results although the clinical outcome has so far been highly variable (Lindvall & Bjorklund, 2004; Olanow & Fahn, 2006). In these trials the cells have been implanted into the striatum, *i.e.* in close proximity to the target cells, rather than in the substantia nigra (SN) where the DA neurons normally reside. Dopamine neurons grafted directly into the striatum are capable of providing an extensive functional re-innervation of the host striatum (Piccini *et al.*, 1999; Mendez *et al.*, 2005). In this ectopic location, however, the grafts are likely to be deprived of major afferent inputs that may be required for optimal regulation of DA neuron function during ongoing behaviour. This may explain why functional recovery obtained with intrastriatal DA neuron grafts is incomplete both in patients (Lindvall & Hagell, 2000) and in animal models of PD (Annett *et al.*, 1994; Winkler *et al.*, 2000).

In an ideal scenario, DA neuron grafts should be implanted into the SN to allow reconstruction along the entire nigrostriatal DA pathway. Attempts to re-establish a functional nigrostriatal connection from grafts placed in the SN, however, have so far met with limited success. In rodent experiments intranigral DA neuron grafts have shown no or very limited growth of axons along the nigrostriatal pathway toward the striatum (Nikkhah *et al.*, 1994a; Mendez *et al.*, 1996; Bentlage *et al.*, 1999; Mukhida *et al.*, 2001). Previous studies have suggested that the growth territory becomes non-permissive for outgrowing axons during post-natal development (Nikkhah *et al.*, 1995; Bentlage *et al.*, 1999), and that supply

of exogenous growth factors, such as glial cell line derived neurotrophic factor (GDNF) may be used to overcome this obstacle (Wang *et al.*, 1996; Wilby *et al.*, 1999).

Interestingly, studies of neural transplants in other areas of the adult CNS have shown that immature neuroblasts or young post-mitotic neurons can retain the capacity to extend axons in a target-specific manner over large distances in adult recipients (Bjorklund *et al.*, 1986; Wictorin *et al.*, 1990; Wictorin *et al.*, 1991; Wictorin *et al.*, 1992; Li & Raisman, 1993; Isacson *et al.*, 1995; Isacson & Deacon, 1996; Gaillard *et al.*, 2007). The failure in previous studies, therefore, to detect significant long-distance axon growth from intranigral DA neuron transplants could be due to the techniques used for graft implantation and visualization of the graft-derived axonal projections. Here, we have made use of donor tissue from a transgenic mouse in which Green Fluorescent Protein (GFP) is driven by the tyrosine hydroxylase (TH) promoter (Sawamoto *et al.*, 2001) and a micro-transplantation approach that allows very precise implantation of small deposits of cells into the SN in adult mice. The GFP expression allows unequivocal identification of the transplanted DA neurons and their axonal projections with high sensitivity within the host brain, even in the presence of a residual nigrostriatal projection. Using these tools we show that foetal DA neuroblasts implanted into the SN of adult mice are capable of extending axons along the nigrostriatal trajectory and establish a terminal network in striatal and limbic forebrain areas that closely matches that seen in the intact animal. Moreover, we show that this axonal re-growth can be further enhanced by the addition of trophic support provided by over-expression of GDNF in the striatal target. Finally, results from testing of rotational behaviour in response to amphetamine treatment

show that grafted DA neurons are capable of functional integration into the host basal ganglia circuitry when placed into the substantia nigra.

MATERIALS AND METHODS

Animals. Adult (25 – 35g), female NMRI mice (Charles River) were housed on a 12/12 h light/dark cycle with *ad libitum* access to food and water. All procedures were conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals at Lund University (Malmö/Lunds djurförsöksetiska nämnd).

VM cell suspensions. Timed pregnant mice [day of vaginal plug designated embryonic day 0.5 (E0.5)] were generated by crossing wild-type (wt), NMRI females with transgenic (+/-) males (mixed C57BL/6-NMRI background) expressing GFP under the control of the TH promoter (Sawamoto et al., 2001). At E12.5, embryos were removed from pregnant mothers after lethal exposure to CO_2, and $GFP^{+/-}$ and wt littermates were identified and separated under a fluorescence microscope. The VM was dissected free from each embryo as described previously (Dunnett & Björklund, 1992; Dunnett & Björklund, 2000), taking care to remove the meninges. Single cell suspensions were prepared from $GFP^{+/-}$ VM tissue as previously described (Nikkhah *et al.*, 1994b). Briefly, pooled VMs were incubated for 20 min at 37°C in HBSS without Ca^{2+} or Mg^{2+} ($HBSS^{-Ca2+/Mg2+}$, 0.1 M; Invitrogen) with 0.1% trypsin and 0.05% DNase before washing to remove trypsin and mechanical dissociation in $HBSS^{-Ca2+/Mg2+}$ with 0.05% DNase. The number of viable cells was estimated based on trypan blue (Sigma, St. Louis; MO) exclusion and after centrifugation (500x g, 5 min, 4°C), the supernatant was removed and the volume was adjusted to give a suspension of 1.0 X 10^5

viable cells/µl in $HBSS^{-Ca2+/Mg2+}$. The cells were stored on ice during the transplantation procedure.

Surgical procedures.

6-OHDA lesions: Adult mice received a unilateral lesion of the nigrostriatal dopamine system through injection of 6-OHDA (Sigma; 1.6 µg/µl free base in 0.9% w/v NaCl with 0.2 mg/ml L-ascorbic acid) using a stereotaxic apparatus (Stoelting, Germany) and a 5 µl Hamilton syringe fitted with a fine glass capillary (external diameter 60 – 80 µm). A total of 1.5 µl was delivered to the substantia nigra at the following anterior-posterior (AP), medial-lateral (ML) and dorsal-ventral (DV) co-ordinates (mm) relative to bregma or the dural surface: AP -3.0; ML -1.2; DV 4.5 (flat skull position). For all surgical procedures, induction and maintenance of anaesthesia was achieved using 2% isoflurane delivered as an inhalant in a 70:30 air/N2O mixture.

Transplantation and GDNF delivery: The transplantation procedure was based on a micro-transplantation protocol using glass capillaries attached to a 5 µl Hamilton syringe as described previously (Nikkhah *et al.*, 2000). At 4-6 weeks after the 6-OHDA lesion, intact (n=5) or lesioned (n=26) mice received a single, 1.5 µl injection of foetal TH-GFP VM suspension (1 x 10^5 cells/µl) into the substantia nigra at the following stereotaxic co-ordinates: AP -3.2; ML -1.4; DV -4.2; flat skull position. Cells were injected over 2 min and the capillary was left in place for another 2 min before slow withdrawal. Ten of the 6-OHDA lesioned animals also received intrastriatal injection of a recombinant adeno-associated viral vector carrying GDNF under control of the chicken β-actin promoter (rAAV2/5-CBA-

GDNF; infectious titre of 1.0 x 10^{12}) at the time of cell grafting, delivered at two sites (0.3 µl + 0.3 µl) on the dorso-ventral axis at the following co-ordinates: AP +1.0; ML -2.3; DV -3.2, -2.2; flat skull. A further three 6-OHDA lesioned animals received rAAV2/5-CBA-GDNF but no graft as controls. The rAAV2/5-CBA-GDNF vector was constructed using inverted terminal repeats from a serotype 2 background and serotype 5 capsid proteins according to previously described procedures (Eslamboli *et al.*, 2005). Animals were killed (lethal dose of pentobarbiturate, i.p.) at either 6 or 16 weeks (see supporting Table S1 for animal grouping).

Retrograde labelling: For retrograde labelling, three of the 6-OHDA lesioned, grafted mice received an intrastriatal injection of rhodamine-labelled microbeads (Lumafluor, FL) 15 weeks after intranigral grafting at two sites on the dorso-ventral axis (100 nl/site) at the following co-ordinates: AP +0.5: ML -2.6; DV -3.2 and -2.5; flat skull position. Survival time after the microbead injections was 1 week.

Motor asymmetry in the amphetamine-induced rotation test. Amphetamine-induced rotation was performed in automated rotational bowls (AccuScan Instruments, Columbus, OH) as described previously (Ungerstedt and Arbuthnott, 1970). Mice received an injection of D-amphetamine sulfate (5 mg/kg, i.p.) three weeks after 6-OHDA treatment. Full body rotations were recorded over a period of 60 min and data are expressed as net full body turns per min, with rotation toward the side of the lesion given a positive value. Animals displaying ≥ 5 turns per minute were selected as sufficiently impaired for inclusion in the rotational analysis. These animals were again tested using the same parameters at 8, 12 and 16 weeks after grafting.

Tissue processing and immunohistochemistry. Following a lethal does of pentobarbiturate (i.p.), animals were transcardially perfused with saline (0.9% w/v) followed by paraformaldehyde (PFA; 4% w/v in 0.1 M PBS). The brains were removed, post-fixed a further 2 h in 4% PFA and cryo-protected overnight in sucrose (25% w/v in 0.1 M PBS) before being sectioned on a freezing microtome (Leica, Germany). Coronal or horizontal sections were collected in 12 series at a thickness of 30 μm.

Immunohistochemical procedures were performed as has previously been described in detail (Thompson *et al.*, 2005). Briefly, free-floating sections were incubated with primary antibodies overnight at room temperature in an incubation solution of 0.1 M PBS with potassium (KPBS) containing 5% normal serum and 0.25% Triton X-100 (Amresco, USA). Secondary antibodies were diluted 1:200 in KPBS with 2% normal serum and 0.25% Triton X-100 and applied for 2 h at room temperature (approx. 22°C). Detection of the antibody conjugates was achieved using either a peroxidase-based reaction followed by precipitation of di-amino-benzidine (DAB; Sigma), or conjugation of a fluorophore (either directly to the secondary antibody or with a streptavidin-biotin amplification step where necessary). Slide mounted sections labelled with fluorescent markers were cover-slipped with PVA-DABCO (Peterson, 1999), DAB labelled sections were dehydrated in alcohol and xylene and coverslipped with DePeX mounting media (BDH Chemicals, UK). Primary antibodies, species raised in, clonality, supplier, catalogue number, immunogen used for production (recombinant full-length protein [rFLP] or peptide sequence and species) and dilution factors were as follows: rabbit polyclonal anti-calbindin D-28k (1:1000; Swant, Switzerland; Cat#CB38; rFLP, rat), rabbit polyclonal anti-dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32, 1:1000; Chemicon, Temecula, CA; Cat#AB1656;

CQVEMIRRRRPTPAM, human), rabbit anti-Girk2 (1:100; Alomone Labs, Jerusalem, Israel; Cat#APC-006; ELANRAEVPLSWSVSSKLNQHAELETEEEEKNPEELTERNG, mouse), chicken (1:1000; Cat#ab13970) or rabbit (1:20000; Cat#ab290) polyclonal anti-GFP (both Abcam, Cambridge; rFLP, *Aequorea victoria*), rabbit anti-TH (1:1000; Pel-Freeze Biologicals, Rogers, AR; Cat#P40101-0; SDS-denatured protein from pheochromocytoma, rat). Control experiments to confirm the specificity of the primary antibodies were as follows: Calbindin D28k, immunohistochemistry on brain sections from Calbindin D28k knockout mice; Girk2, pre-incubation of antibody with 3-fold concentration of control immunogen prior to immunohistochemistry on mouse brain sections; DARPP-32, comparison with known mRNA expression pattern in adult mouse brain; GFP, immunohistochemistry on brain sections from wild-type mice; TH, detection of 60k TH protein through western blot using rat striatal lysate. For fluorescent labelling: secondary antibodies were cyanine 2 (Cy2), Cy3, Cy5 (Jackson ImmunoResearch, Westgrove, PA) or biotin conjugated (Vector laboratories, Burlingame, CA); sections labelled with biotin conjugated antibodies were subsequently incubated (1 h) with Cy2, Cy3 or Cy5 conjugated streptavidin (1:200; Jackson ImmunoResearch). For DAB labelling: biotin conjugated goat anti-rabbit was used (Jackson ImmunoResearch) followed by incubation with peroxidase conjugated streptavidin (Vectastain ABC kit, Vector laboratories, Burlingame, CA). No significant non-specific signal was generated from the secondary antibodies, as determined by control experiments where the primary antibody was omitted from the immunohistochemical procedure.

Imaging and Schematics. All fluorescent images were captured using a Leica DMRE confocal microscope equipped with green helium/neon, standard helium/neon and argon

lasers. The images are presented as collapsed reconstructions of optical sections captured every 2 μm on the z-axis over a depth of approximately 20 μm within the tissue section, at a scan-rate of 200Hz, a resolution of 1024 x 1024 and with 2x oversampling for both the line and frame acquisition modes. The signal from each fluorophore was captured sequentially and the emission filter was adjusted to capture only the peak (<50nm) emission in order to avoid off-target bleed-through from other fluorophores. To provide low-magnification overviews of graft-derived fibre innervation throughout the host brain (Fig 2), GFP+ fibre patterns were accurately traced (Canvas version 9.04, Deneba Systems, USA) over digital photomontages of coronal sections immunohistochemically (DAB) labelled for GFP, using a Leica microscope with bright-field illumination and a dark-field filter. The low magnification images of GDNF immunohistochemistry in horizontal sections in Figure 6 were acquired using a high resolution scanner (Scanscope GL) and Imagescope v8.2 software. Objectives and numerical apertures used for all photographs are stated in the figure legends.

Cell counting. The number of dopamine neurons contained in each graft was estimated through extrapolation of the total number of GFP+ cells, detected by immunohistochemistry (DAB), counted in every 6^{th} tissue section. The method of (Abercrombie, 1946) was used to correct for the possibility of double-counting due to cells spanning more than one tissue section.

Statistics. A one-way ANOVA (with Dunnett's correction) was performed at each time-point in order to assess the significance of differences in mean amphetamine-induced rotational scores between each group. A maximum p value of 0.05 was assigned to indicate statistical

significance. Animals with no surviving graft, or significantly misplaced grafts, were excluded from the analysis (see supporting Table S1).

RESULTS

Structure and placement of intranigral grafts

At sacrifice (6 and 16 weeks after transplantation) the grafts appeared as well-demarcated, round or oval cell clusters located within, or immediately adjacent to, the SN, identifiable by their content of GFP+ neurons (Figs. 1a, 2g, 3a). In the animals not treated with GDNF, the surviving grafts (16/21; 3/5 in intact hosts and 13/16 in lesioned hosts) contained on average 1415 ± 268 GFP+ DA neurons (1 ± 0.2 % of total cells grafted) which corresponds to about 15 % of the number of DA neurons normally present unilaterally in the mouse SN-VTA complex (Bjorklund & Dunnett, 2007). Double immunostaining showed that the majority of the GFP+ cells in the grafts were also TH+ (Fig. 1b), which is consistent with the previous observation that >95% of the GFP expressing neurons in intrastriatal grafts of VM taken from the TH-GFP mice are also TH+ (Thompson *et al.*, 2005). The two principal DA neuron types normally present in the SN-VTA region, *i.e.,* the TH+/Girk2+ A9 neurons of the SN and the TH+/Calbindin+ positive A10 neurons of the VTA, were both present (Fig. 1c,d) and were arranged such that the Girk2+ neurons were distributed predominately within the periphery of the grafts with the calbindin+ neurons closer to the centre. In the host midbrain, some TH+ neurons remained in the SN, while the VTA remained relatively spared on the 6-OHDA-injected side (Fig. 1a).

Extension of axons along the nigrostriatal pathway

The GFP reporter allowed us to trace the graft-derived dopaminergic axonal outgrowth within the host brain with a sensitivity that permitted the detection of individual varicose terminal branches within the innervated territories. In 6-OHDA lesioned mice, GFP+ fibres were found to extend over long distances along the nigrostriatal pathway and the medial forebrain bundle (MFB) to give rise to widespread innervation in striatal and limbic areas of the forebrain (Fig 2). At 6 weeks post-grafting, GFP+ fibres could be detected throughout the normal target fields for A9/A10 DA neuron cell groups, including the striatum, nucleus accumbens, bed nucleus of the stria terminalis, amygdala and septum (Fig. 2b-e), reaching as far rostrally as frontal cortex and olfactory tubercle, at a distance of approximately 6-7 mm from the graft (Fig 2b, level1). Graft-derived fibres were found also in the host substantia nigra pars reticulata (Fig. 2g) and the ipsilateral and contralateral pontine nuclei, caudal to the graft (not shown). By 16 weeks, this innervation pattern had expanded further (Fig. 3) to cover larger areas of the forebrain including an elaborately ramified, fine beaded terminal network that extended throughout large parts of the striatum (Fig. 3b,c,e). The degree of innervation was greatest in ventral aspects of the striatum and became less dense dorsally, such that few fibres were seen in the dorsal and rostral parts of the striatum.

As seen in sections cut in the horizontal plane (Fig. 3b,d), the outgrowth of GFP+ fibres from the graft cell cluster was highly polarized and directed along the trajectory normally carrying the axons of the nigrostriatal and meso-limbic DA pathways. The GFP+ axons extending from the graft coursed as a tightly clustered fibre bundle within the medial aspect of the internal capsule and the adjoining MFB (Figs. 2f and 3b,d). At the level of the globus pallidus

the fibres diverged in rostral, lateral and caudal directions towards multiple forebrain target areas. On reaching the border of the caudate-putamen the elongated projections abruptly branched to give rise to a highly elaborated terminal network within the striatal grey matter (Fig 3e), while others continued further rostrally within the white matter bundles of the internal capsule.

Double staining for TH and GFP showed that graft-derived GFP+/TH+ fibres were often intermingled with the spared host-derived GFP-/TH+ axons, although they were rarely seen to project in apposition to each other (3f-g, 4b-d). Closer inspection of the GFP/TH double stained sections, however, revealed that the central core of the GFP+ fibre bundle did not overlap with the trajectory of the intrinsic nigrostriatal pathway, but coursed further laterally in a position corresponding to the reciprocal striatonigral pathway. To explore this possibility we used an antibody recognising DARPP-32, which is selectively expressed in the striatal projection neurons, to visualize the striatonigral axons, in combination with GFP. The GFP/DARPP-32 double stained sections revealed that the central portion of the GFP+ fibre bundle ran within the DARPP-32+ axon bundle, with many of them in ~~close~~ apposition to the surface of the DARPP-32+ fibres (Fig 4e-g).

The survival of the transplanted DA neurons, as well as the extent of fibre outgrowth into the host, was clearly influenced by the presence or absence of the intrinsic dopaminergic projections. In non-lesioned mice, the grafts (n=3) contained on average 909 ± 187 GFP+ neurons, *i.e.,* approximately half the number seen in the 6-OHDA-lesioned mice (1566 ± 333; n=13), and the GFP+ axonal outgrowth along the nigrostriatal pathway was less dense. In

these animals innervation was limited to some scattered fibres in the amygdala and the most caudal aspects of the striatum. Further rostrally, single GFP+ fibres were detected in the olfactory tubercle and the medial frontal and perirhinal cortex, but the overall density and extent of the GFP+ innervation in these forebrain areas was much less than in the 6-OHDA lesioned hosts (compare coronal sections in Fig. 2a with those in 2b).

Identification of the projecting neurons by retrograde tracing

To identify the cells of origin of the outgrowing axons we injected rhodamine-labelled microbeads as a retrograde axonal tracer into the striatum of three grafted 6-OHDA-lesioned mice, 15 weeks post-transplantation. Panel *a* in figure 5 illustrates the placement of the microbeads relative to the GFP+ graft in a representative animal. Bead-labelled, GFP+ cells were found in all three grafts. When examined in Girk2 stained sections, 9 of the 10 bead-labelled cells analysed were also Girk2+, and had a large, angular morphology (Fig. 5b) suggesting that the axons innervating the striatum were primarily derived from the A9 DA neurons within the grafts. The single bead-labelled, Girk2- cell observed was comparatively smaller and with a distinctly more spherical morphology (Fig. 5c).

Stimulation of graft-derived fibre outgrowth by over-expression of GDNF

At the time of intranigral cell grafting, a subset of the 6-OHDA lesioned animals received an intrastriatal injection of a recombinant adeno-associated viral (rAAV) vector encoding GDNF, driven by a constitutive (chicken β-actin) promoter (rAAV-CBA-GDNF). Immunohistochemistry for GDNF protein 16 weeks later showed robust GDNF expression throughout the striatum and the overlying cortex (Fig 6a). We also observed anterograde

transport of GDNF along the striato-pallidal and striatonigral pathways towards globus pallidus, entopeduncular nucleus and SN, and also in areas innervated by the transduced fronto-parietal cortex, most conspicuously in the thalamus (boxed area in Fig. 6a). Closer inspection revealed a diffuse GDNF staining along the entire fibre trajectory connecting the striatum with the graft site, and also within the graft itself (Fig. 6a).

Over-expression of GDNF in the host striatum had a clear impact on both survival and overall outgrowth of dopaminergic fibres from the intranigral grafts (compare Figs. 3 and 6). Surviving grafts were found in 10/10 GDNF treated animals (as compared to 16/21 animals without GDNF). Similar to the non-GDNF treated mice (Fig. 1) the grafts occurred as discrete cell deposits within or immediately adjacent to the host SN (Fig. 7). The grafts in the GDNF-treated animals contained on average greater numbers of DA neurons (2747 ± 417 GFP+ cells) than those in the non-GDNF treated group (1566 ± 333). The composition of DA neuronal subtypes within the grafts appeared similar, however, with approximately equal numbers of Girk2 and calbindin expressing GFP+ cells (Fig. 7b-f). The spatial distribution of GFP+ fibres throughout the striatum was similar in both groups, but the density of the innervation was markedly increased in the GDNF treated animals (*cf.* Figs 3a and 6b,d,e), particularly in the part of the striatum bordering on the globus pallidus (Suppl. Fig. 1). In the GDNF-treated animals the graft-derived fibre outgrowth closely resembled the structure of the intrinsic DA projection system, as was the case for grafted animals without GDNF treatment. There were some notable differences however. While in all animals without GDNF, the GFP+ fibre outgrowth was restricted to the normal boundaries of the nigrostriatal fibre trajectory, in the GDNF treated animals a subset of GFP+ fibres exited the nigrostriatal

pathway towards the GDNF expressing area in the medial thalamus (Fig. 6b and c). In addition, we observed signs of localized sprouting of GFP+ fibres, particularly within the globus pallidus, not present in animals without GDNF treatment. This was also evident at the level of the graft, where there was a greater degree of localized neuritic outgrowth in the GDNF treated animals.

Functional impact of intranigral grafts

The functional effect of the intranigral VM grafts was assessed by amphetamine-induced rotation before and at 8,12 and 16 weeks after grafting. All mice included in the study showed an amphetamine-induced rotational asymmetry of $\geq$ 5 turns/min prior to grafting. Both groups of grafted animals showed a marked improvement in rotational asymmetry, relative to the non-grafted controls, which was evident by 8-weeks after grafting (Fig. 8). The grafted animals that received intrastriatal injections of rAAV-GDNF showed a sustained and consistent improvement, and almost complete recovery in motor asymmetry at 12 and 16 weeks post-grafting, while the grafted animals without GDNF treatment showed more variable responses at these longer time-points (open and filled triangles respectively in Fig. 8). Non-grafted animals that received intrastriatal rAAV-GDNF alone did not show any sign of functional recovery.

DISCUSSION

The results show for the first time that foetal mouse DA neuroblasts allo-grafted into the host SN are able to generate a new nigrostriatal pathway in adult 6-OHDA-lesioned mice. Axonal

outgrowth along the nigrostriatal trajectory has previously been observed from xenogenic transplants, *i.e.* from human or pig cells grafted to the striatum or SN in lesioned adult rats (Wictorin *et al.*, 1992; Isacson *et al.*, 1995; Isacson & Deacon, 1996). In these studies antibodies recognizing human or pig, but not rat, neurofilaments were used to visualise the graft-derived neurites. Given the failure in earlier studies to obtain similar re-growth of graft-derived axons from cells obtained from foetal rodent donors (Mendez *et al.*, 1996; Bentlage *et al.*, 1999; Mukhida *et al.*, 2001), this raised the possibility that axonal growth inhibitory factors may operate poorly between species, *i.e.*, that the cells derived from human or pig donors may not recognise the growth inhibitory molecules present along the growth trajectory in adult hosts. The present data show that this is not the case.

The use of GFP as a surrogate marker of DA neuronal identity has allowed us to unambiguously detect graft-derived dopaminergic outgrowth patterns in mice with partial lesions of the intrinsic nigrostriatal pathway. In earlier allo-grafting studies the investigators have had to rely on TH immunohistochemistry in order to visualise graft-derived axons, and thus there was no basis for distinguishing between DA fibres of graft and host origin. This made it necessary to perform the grafting experiments in animals with complete lesions of the nigrostriatal pathway, achieved by injection of 6-OHDA directly into the MFB. In case axonal growth along the nigrostriatal pathway is facilitated by the presence of spared DA axons, animals with complete lesions may not provide the right conditions for re-growth to occur. Furthermore, there is a risk that injection of high doses of the toxin directly into the MFB may cause non-specific damage at the injection site, and disruption of axonal conduits along the growth trajectory. The use of fine glass capillaries to inject low doses of 6-OHDA

directly into the SN, as done here, minimises non-specific damage to local fibre tracts and may thus provide more favourable conditions for re-growth.

The axonal projection from the grafted DA neuroblasts was remarkably specific. The outgrowth of GFP+ axons from the graft cell cluster was clearly polarized; the vast majority of the GFP+ fibres exited the graft at the rostral pole and were oriented rostrally, along the intrinsic nigrostriatal fibre trajectory and the adjacent MFB. Moreover, the ramification of the fibres and the distribution of the GFP+ terminals in striatal, limbic and cortical areas matched closely the projection patters of the intrinsic midbrain DA neurons. The axons coursing along the nigrostriatal pathway, arranged in a loose non-ramifying bundle, were seen to branch profusely at the border between the globus pallidus and caudate-putamen, suggesting a direct influence of local cues on the ability of the growing axons to establish a new terminal innervation network in the denervated target. This is further supported by the observation that the extension and terminal distribution of the graft-derived GFP+ fibres was much more restricted in the non-6-OHDA lesioned hosts where the intrinsic DA projections were left intact. At the level of the SN, the projection of GFP+ dendrites into the SN pars reticulata highlights that also this component of the normal circuitry can be at least partially re-established through homotopic placement of the grafted DA neurons.

These observations suggest that axonal outgrowth and terminal innervation patterns from the grafted neurons are quite specifically regulated by interactions with the host growth environment. During normal development the growth of axons from the developing midbrain DA neurons rostrally along the MFB is determined by a combination of local directional

cues, associated with the growth substratum, and more long-distance chemoattractive influences present along the MFB and in the striatal primordium (Nakamura *et al.*, 2000; Gates *et al.*, 2004). Similar mechanisms may be involved in axonal pathfinding also in the adult brain. Axonal guidance factors, such as Netrins, Slits, and Ephrins, have been implicated in axonal pathfinding of midbrain DA neurons during development (Yue *et al.*, 1999; Sieber *et al.*, 2004; Lin & Isacson, 2006), although it remains unclear whether any of these signalling mechanisms function in DA axon growth or regeneration in the adult animal.

A particularly interesting possibility is that host axons present along the growth trajectory, either DA axons spared by the 6-OHDA lesion, or the axons of the reciprocal striatonigral projection, may assist the re-growing axons to reach their appropriate targets. In developing animals it is known that pioneer axons (that form connections when the distances are short), or early developing axons that grow in the opposite direction from the target area, can act as guides for the developing axons towards their targets (Klose & Bentley, 1989; McConnell *et al.*, 1989; 1994; Lin *et al.*, 1995; Hidalgo & Brand, 1997; Molnar *et al.*, 1998; Pittman *et al.*, 2008). Our observations suggest that similar mechanisms could operate also during axonal re-growth from grafted foetal neurons. Close inspection of the TH/GFP double-stained sections showed that graft-derived GFP+ fibres were intermingled with the spared TH+ axons along the nigrostriatal pathway and the MFB, which raises the possibility that the spared axons could promote axonal re-growth by secretion of diffusible chemoattractant or growth-promoting factors, such as the neurotrophic factor BDNF (Bustos *et al.*, 2004), known to be produced by the host DA neurons. Along these lines, previous studies have suggested that increased growth support along the nigrostriatal pathway provided by tissue bridge grafts

(Brecknell *et al.*, 1996; Wilby *et al.*, 1999; Chiang *et al.*, 2001), or a permissive environment provided by kainic acid lesions (Zhou *et al.*, 1996), can facilitate the growth of DA axons along the nigrostriatal pathway. The most striking association, however, was with the axons of the host striatonigral pathway where the outgrowing GFP+ DA fibres, were seen to course in apposition to the DARPP-32+ fibre bundles. Interestingly, the medium-sized projection neurons are known to be the prime source of GDNF in the striatum (Trupp *et al.*, 1997; Barroso-Chinea *et al.*, 2005). Striatum-derived GDNF, transported and expressed along the striatonigral pathway, may thus be able to create a growth-permissive environment for the graft-derived dopaminergic axons. GDNF is known to exert its growth stimulating effect by interaction with the GDNF receptor, GRFα1, which is expressed by the developing DA neurons. Recent data suggest that the GRFα1 receptor, when combined with GDNF, indeed can act as an attractant guidance molecule, as well as and a cell-adhesion factor, for axons that express the GDNF co-receptor Ret (Ledda *et al.*, 2002; Paratcha & Ledda, 2008).

GDNF has previously been shown to act as a powerful chemoattractant for outgrowing DA axons, both in organotypic co-culture (Schatz *et al.*, 1999; Jaumotte & Zigmond, 2005) and *in vivo* from intrastriatal or intranigral VM grafts (Rosenblad *et al.*, 1996; Wang *et al.*, 1996; Wilby *et al.*, 1999; Redmond *et al.*, 2009). Moreover, supply of exogenous GDNF to the striatum has been shown to provide a stimulus for axonal regeneration in the lesioned nigrostriatal system (Rosenblad *et al.*, 1998; Brizard *et al.*, 2006). Based on these observations we investigated the possibility to boost the long-distance axonal re-growth from intranigral DA neuron grafts by over-expression of GDNF in the striatum. Within the striatum, the cells most efficiently transduced by the AAV vector are the striatal projection

neurons. As a result, GDNF is expressed not only within the striatum, but is also transported along the striato-pallidal and striato-nigral pathways and delivered along its trajectory to the downstream striatal targets, including globus pallidus, entopeduncular nucleus and the SN. This anterograde transport made it possible for GDNF to act synergistically at three sites: in the SN to improve DA neuron survival at the graft site (by about 75%); along the axonal growth trajectory to promote the extension of the outgrowing axons toward the striatum; and locally in the striatum to provide a more wide-spread re-innervation of the striatal target. In these animals the more extensive striatal reinnervation was accompanied by a more consistent and near-complete reversal of motor asymmetry in the amphetamine rotation test. While an important proof-of-principle demonstration of integration at the functional level, it should be noted that amphetamine-induced rotation is one of the least stringent tests of behavioural recovery in transplantation experiments. Further studies, including tests of spontaneous motor function, will be needed to provide a more in-depth analysis of the potential for behavioural recovery that can be achieved through intranigral grafting.

The present data suggest that GDNF can act as a diffusible attractant to promote the re-growth of graft-derived axons over larger distances. This is further supported by the observations in mice where GDNF was delivered to parts of the thalamus (by anterograde axonal transport from cortical projection neurons transduced by the AAV-GDNF vector). In these animals the re-growing graft-derived fibres were seen to deviate from the nigrostriatal bundle to form a separate branch directed toward the area of GDNF expression. Since these areas of the thalamus normally receive a sparse DA innervation (Garcia-Cabezas *et al.*, 2008) this does not necessarily represent an anomalous projection, but rather an increased

innervation of a normally sparsely innervated target, similar to that which has been observed after addition of exogenous GDNF in slice cultures (Jaumotte & Zigmond, 2005).

In conclusion, the present results demonstrate for the first time the ability of intra-nigral DA neuron allografts to extend axons along the nigrostriatal pathway and establish an extensive terminal network in striatal and limbic forebrain regions, with a distribution that matches closely that seen in the intact animal. The grafts contained the normal complement of A9 (nigral, Girk2+) and A10 (VTA, calbindin+) neuronal subtypes, and the retrograde tracing experiment indicates that graft neurons projecting to the striatum were of the correct, nigral phenotype. These data suggest that DA neuron transplants can achieve a level of circuitry reconstruction than has hitherto been possible to obtain with DA neuron allografts only in neonatal recipients. The implication of these findings is two-fold. First, the results extend earlier observations, obtained in other parts of the CNS (Bjorklund *et al.*, 1986; Wictorin *et al.*, 1991; Li & Raisman, 1993; Gaillard *et al.*, 2007), showing that the adult brain retains a capacity for pathway reconstruction and repair that is much greater than previously realized, and that grafted fate-committed neuroblasts are capable of expressing their inherent capacity to grow long-distance, target specific projections also in the lesioned adult CNS. Secondly, the results suggest that the current approach to DA neuron replacement in PD, based on ectopic grafting to the striatum, may be possible to develop into a technique that will allow more complete circuitry reconstruction from neurons grafted homotopically to the nigra itself. This sets a new standard for DA neurons derived from stem cells, *i.e.*, to express not only a midbrain dopaminergic phenotype, but also possess the capacity to re-construct the nigrostriatal pathway when implanted homotopically into the host SN.

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
BDNF	Brain Derived Neurotrophic Factor
CBA	chicken β-actin
DA	Dopamine
DAB	di-amino-benzidine
DARPP-32	Dopamine and cAMP regulated phosphoprotein 32KD
GDNF	Glial Cell-line Derived Neurotrophic Factor
HBSS	Hank's Buffered Salt Solution
KPBS	Potassium Phosphate Buffered Saline
MFB	Medial Forebrain Bundle
PFA	Para-formaldehyde
PD	Parkinson's Disease
SN	Substantia Nigra
TH	Tyrosine Hydroxylase
VM	Ventral Mesencephalon
VTA	Ventral Tegmental Area

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FIGURE LEGENDS

Figure 1 - Placement and structure of intranigral grafts. Low magnification overview of a 6-OHDA lesioned mouse with an intranigral graft. (**a**) Placement of the grafts relative to the architecture of the intrinsic system, as viewed in a horizontal section through the midbrain DA cells groups (16 weeks post-grafting). The location of the three major DA neuron cell groups (A8, A9, and A10) are indicated on the intact side of the brain (left in the picture). TH (red) and GFP (green) have been visualized through immunohistochemistry, with coexpression in grafted DA neurons giving a yellow appearance. The boxed area from (**a**) is shown at higher magnification in (**b**), and illustrates the overlap between TH and the GFP reporter (boxed area in b also shown as single colour channels – **b´- b´´**; arrowhead identifies one of few GFP+/TH- cells). Grafts were composed of similar numbers of Girk2+ (**c**) and calbindin+ (**d**) DA neurons. The Girk2+ (red)/GFP+ double-labelled cells were located preferentially in the periphery of the grafts and had the typical large and angular morphology indicative of the A9 DA neuron type. The boxed area in (**c**) is shown as single colour channels (**c´- c´´**; arrow identifies a large Girk2+/GFP+ neuron in the periphery of the graft, arrow identifies a smaller Girk2-/GFP+ neuron). The calbindin+/GFP+ cells were located predominately towards the centre of the grafts and the small, rounded morphology is consistent with an A10 DA neuronal identity. The boxed area in (**d**) is shown as single colour channels (**d´- d´´´**; arrow identifies a calbindin+/GFP+ neuron, arrowhead identifies a larger calbindin-/GFP+ neuron towards the periphery of the graft). SNr, substantia nigra pars reticulata. Objective and numerical apertures: **a**, 10x/0.4 (montage of 3 separate images arranged on the horizontal plane); **b-d**, 20x/0.7. Scale bars: **a**, 500 μm; **b-d**, 100 μm.

Figure 2 – Patterns of dopaminergic fibre outgrowth from intranigral VM grafts.

GFP+ fibre patterns in coronal sections from representative intact and 6-OHDA lesioned mice with intranigral grafts (**a**, **b**; 6 weeks survival). Substantially fewer fibres were seen in the forebrain of intact hosts, relative to lesioned animals, particularly at the level of the striatum. Darkfield photographs of GFP+ fibres, as revealed through immunohistochemistry, depict: graft placed in the nigra with fibres projecting ventrally into the pars reticulata (**g**); fibres coursing within the internal capsule, parallel to the medial forebrain bundle in a position corresponding to the lesioned host nigrostriatal pathway (**f**); fibres at two levels of the striatum, including more sparsely at a rostral level (**c**) and also more extensively at a caudal level (**e**); and olfactory tubercle, where the bulk of fibres were seen at the more rostral levels examined (**d**). *, injection tract; AC, anterior commisure; Amy, amygdala; CPu, caudate putamen; FM, forceps minor; H, hippocampus; IC, internal capsule; ml, medial lemniscus; NAc, nucleus accumbens; OT, olfactory tubercle; Pir, piriform cortex; S, septum; SN, substantia nigra; SNr, substantia nigra pars reticulata; T, transplant. Objective and numerical apertures: **c**, 20x/0.6; **b-d**, 10x/0.4. Scale bar: 200 μm for (**c**) and (**d-g**).

Figure 3 – Anatomical re-construction of the nigrostriatal pathway. Darkfield photo-montages illustrating the pattern of graft-derived GFP+ fibre outgrowth in horizontal sections from a representative 6-OHDA lesioned animal, 16 weeks post-grafting (**a**). The approximate dorso-ventral level of each section (**1-3**) is indicated in the parasagittal schematic. The trajectory and structure of the GFP+ fibre outgrowth matched well the intrinsic system. The GFP+ outgrowth was highly polarized, exiting the rostral pole of the graft and coursing

parallel to the MFB en route to the forebrain (**b**). Boxed areas in (**b)** illustrate in (**d)** elongated fibres projecting through the nigrostriatal pathway, and in **(c)** and the highly ramified fibre patterns established in the striatum. The transition between these distinct patterns of fibre morphology was seen to occur abruptly at the palladial-striatal border (**e**). Dual immunohistochemistry for TH and GFP (red and green, respectively, in **f** and **g**) revealed that the grafted (GFP+/TH+; arrowheads) fibres commonly shared the same structure and territory as the host (GFP-/TH+) fibres throughout the nigrostriatal pathway (**f**) and within the striatum (**g**). Abbreviations: AC, anterior commisure; CPu, caudate putamen; GP, globus pallidus; IC, internal capsule; T, transplant. Objective and numerical apertures: **a-d**, 20x/0.6; e, 10x/0.4; **f-g**, 20x/0.7. Scale bar: **b**, 500 μm; **c-d**, 100 μm; **e**, 200 μm; **f-g**, 200 μm.

Figure 4 – **Anatomical relationship between graft-derived dopaminergic fibres and local fibre pathways in the host brain**. Immunohistochemistry for TH (red) illustrates dopaminergic nigrostriatal projections at a ventral level on the intact (**a**) and 6-OHDA lesioned (**b-d**) sides of the brain, as seen in the horizontal plane. Dopaminergic fibres originating from the graft (GFP+/TH+; arrows in **c**) were often intermingled with remaining host fibres (GFP-/TH+; arrowheads in **c**) along the nigrostriatal pathway (**b-d**). Parallel sections stained for GFP and DARPP-32 illustrate the pattern of graft-derived dopaminergic outgrowth (green) relative to that of the descending striatonigral projections (red; **e** – **g**, approximate position of these panels shown as dashed boxes in panel **b**). The GFP+ axons were seen to precisely follow the trajectory of DARPP-32+ fibre bundles throughout the projection territory (**e**) and individual GFP+ and DARPP-32+ fibres were often observed in apposition to one another, coursing in parallel trajectories over long distances (**f** – **g**).

Abbreviations: CPu, caudate putamen; GP, globus pallidus; mfb, medial forebrain bundle. Objective and numerical apertures: **a-g**, 20x/0.6 (**a**, **b**, **e** and **f** are photomontages of multiple images). Scale bars: **a**-**b**, 500 μm; **c**-**d**, 50 μm; **e**, 100 μm; **f**, 150 μm.

Figure 5 – Dopamine neuronal subtype specificity of target innervation. Fluorescent microbeads (red) were injected into the striatum 15 weeks after intranigral grafting in order to retrogradely label grafted dopamine neurons. Immunohistochemistry for DARPP-32 (blue) and GFP (green) in a section through the horizontal plane from a representative animal illustrates the placement of the beads, within the host striatum, relative to the GFP+ graft (**a**). The vast majority of bead labelled, GFP+ cells (9/10 cells in 3 animals) displayed characteristics indicative of A9 phenotype, including Girk2 expression (**b**). (**c)** The single bead labelled, GFP+ cell detected that did not express Girk2. Objective and numerical apertures: **a**, 20x/0.6 (photomontage of multiple images); **b**-**c**, 63x/1.2w. Scale bars: **a**, 500 μm; **b**, 50 μm.

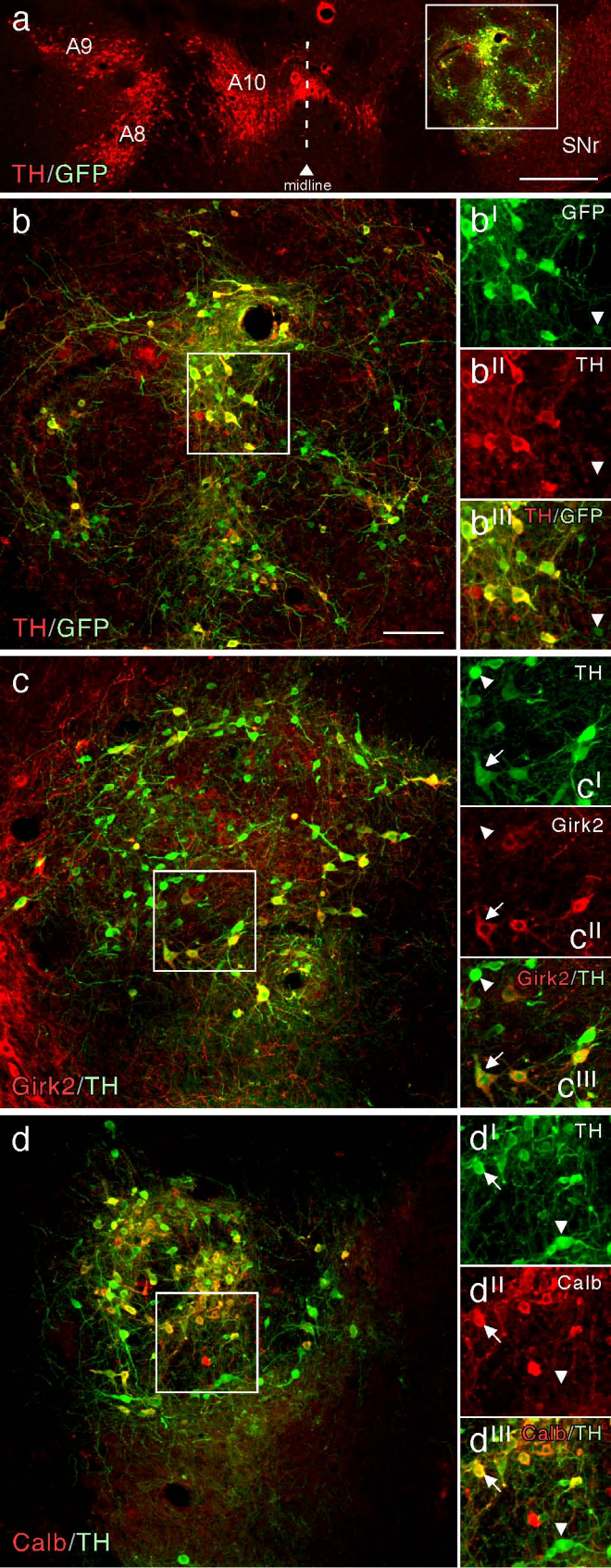
Figure 6 – Target-derived over-expression of GDNF facilitates innervation by grafted dopamine neurons. Sixteen weeks after injection of AAV-GDNF into the host striatum. Immunohistochemistry revealed a robust level of GDNF throughout the striatum and overlying cortex; and also within the efferent targets of striatal and cortical projection neurons, including globus pallidus and substantia nigra, as well as thalamus (**a**). The pattern of GFP+ fibre outgrowth from intranigral grafts in GDNF-treated animals is illustrated in darkfield photo-montages of horizontal sections through a representative animal (**b**). The dorso-ventral level of each section (**1**-**3**) is indicated on the schematic parasagittal section.

The GFP+ fibres extended from the graft throughout the nigrostriatal pathway to innervate various forebrain regions, including striatum, which contained areas of dense innervation from a highly ramified network of GFP+ fibres (**b**, **d**, **e**). Although the majority of GFP+ ve fibres projected within the normal territory of the nigrostriatal pathway, a subset of fibres in the GDNF treated animals were seen to depart this pathway to innervate GDNF expressing nuclei in the dorsal thalamus (**c**). Abbreviations: AC, anterior commisure; Amy, amygdala; CPu, caudate putamen; Cx, cortex; GP, globus pallidus; FR, fasciculus retroflexus; H, hippocampus; IC, internal capsule; MD, mediodorsal thalamic nuclei; MFB, medial forebrain bundle; NAc, nucleus accumbens; Pir, piriform cortex; Th, thalamus; T, transplant; v, ventricle. Objective and numerical apertures: **b-e**, 20x/0.6 (**b**, **d** and **e** are photomontage of multiple images). Scale bars: **b**, 1 mm.

Figure 7 – Placement and structure of intranigral grafts in GDNF-treated animals. Grafts in animals over-expressing GDNF were found within or immediately adjacent to the SN (**a**) (16 weeks post-grafting). Immunohistochemistry for TH (red) and GFP (green) illustrates graft placement in a representative animal (GFP+/TH+), in relation to the intrinsic DA neurons (GFP-/TH+). Expression of calbindin (red) and Girk2 (blue) identifies major midbrain DA cell groups (A8, A9 and A10) within the host and also in the GFP+ graft (**b**). As in the non-GDNF-treated animals the grafts contained similar numbers of calbindin+ A10 neurons (arrowheads, **c-f**), typically in the centre of the graft, and Girk2+ A9 neurons, distributed predominately within the periphery of the graft (arrows, **c – f)**. T, transplant. Objective and numerical apertures: **a-b**, 20x/0.7 (photomontage of 3 images arranged on the horizontal plane); **c-f**, 63x/1.2w. Scale bars: **a**, 500 μm; **c**, 50 μm.

Figure 8 – Functional impact of intranigral grafts. Rotational response to amphetamine (2.5 mg/kg, i.p.) was assessed prior to grafting, and at 8, 12 and 16 weeks after grafting. Intact control animals (filled circles; n=7) did not display any significant rotational bias throughout the test period. The 6-OHDA lesioned animals (open circles; n=6) and GDNF treated, but non-grafted 6-OHDA lesioned animals (filled squares; n=3) displayed a prominent rotational bias (10-15 turns/min) that was maintained at all time-points. Animals that received intranigral VM grafts, alone (open triangles; n=5) or in combination with GDNF (filled triangles; n=5), showed significant recovery in motor behaviour that was most pronounced and consistent in the animals that received grafts in combination with GDNF (*$p<0.05$ compared to 6-OHDA lesioned controls; one-way ANOVA at each time-point).

Supplementary Figure 1 – Graft-derived innervation of the host striatum can be augmented through GDNF over-expression. Darkfield images of immunohistochemistry for GFP illustrate graft-derived patterns of striatal innervation in horizontal sections from representative animals with (**b**, **d**, **f**) or without (**a**, **c**, **e**) striatal AAV-GDNF injection at the time of cell grafting. Animals that received GDNF treatment exhibited a substantially greater degree of GFP+ striatal innervation. Three levels spanning the dorso-ventral axis are shown, and the approximate position is indicated in the parasagittal schematic (**a**, **b** are ventral [**3**], **e**, **f** are dorsal [**1**]). Abbreviations: AC, anterior commisure; CPu, caudate-putamen unit; GP, globus pallidus. Objective and numerical apertures: **a-f**, 20x/0.6. Scale bar: 200 µm.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A9
A10
A8
SNr
TH/GFP
midline
b
TH/GFP
b^I
GFP
b^II
TH
b^III
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c
Girk2/TH
c^I
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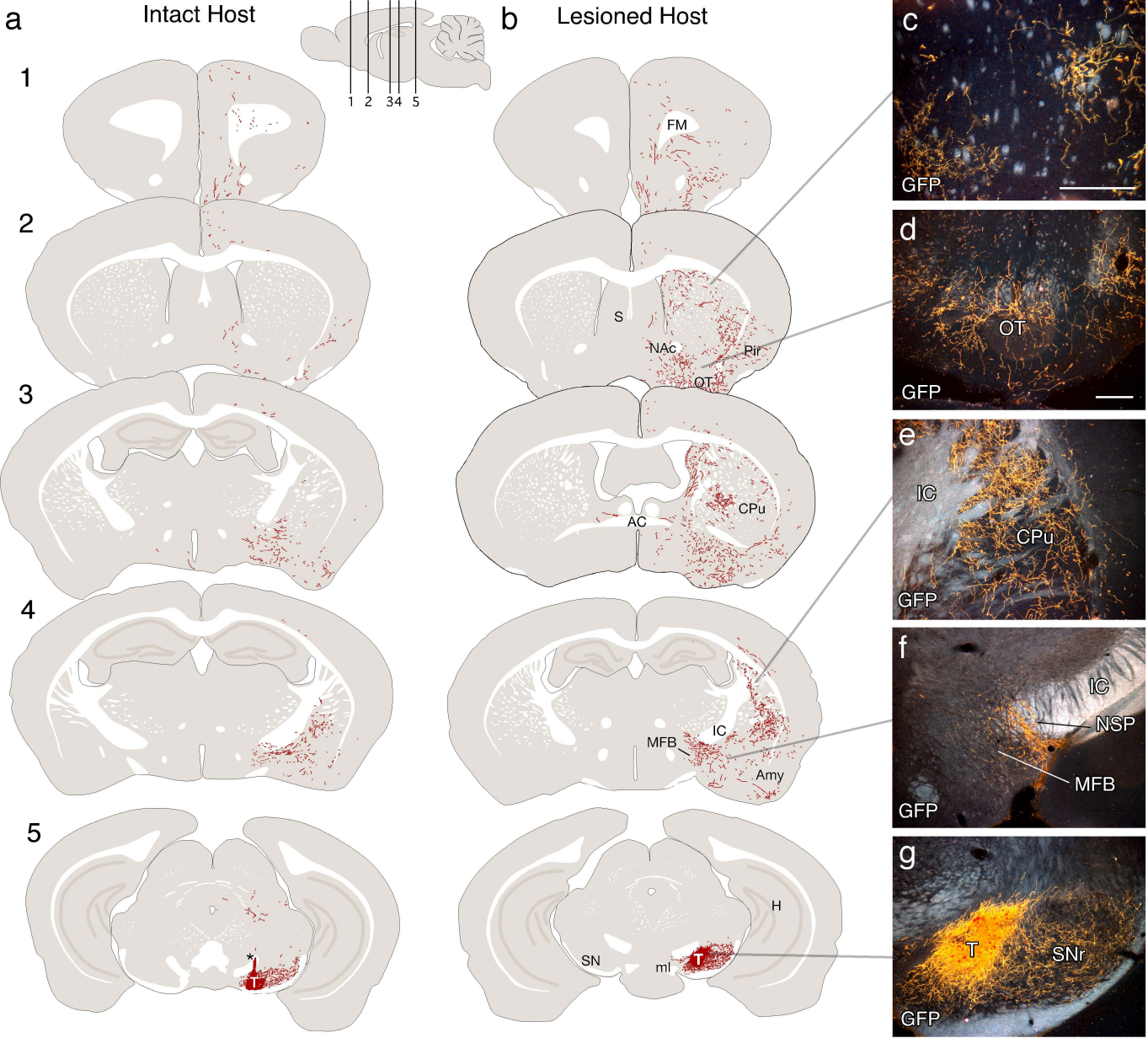
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Intact Host
1
2
3
4
5
1 2 34 5
T
b
Lesioned Host
FM
S
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CPu
MFB
IC
Amy
SN
ml
H
T
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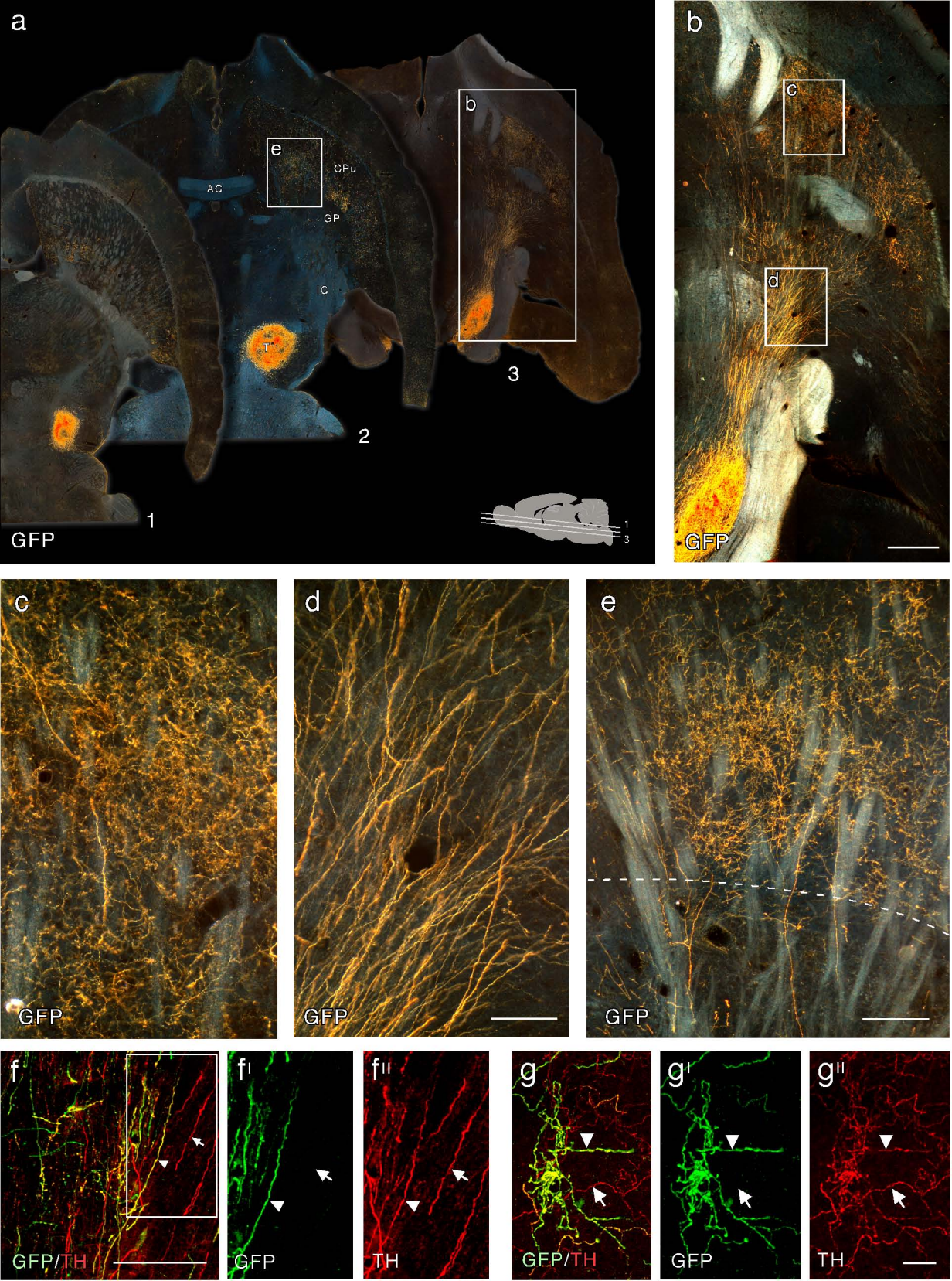
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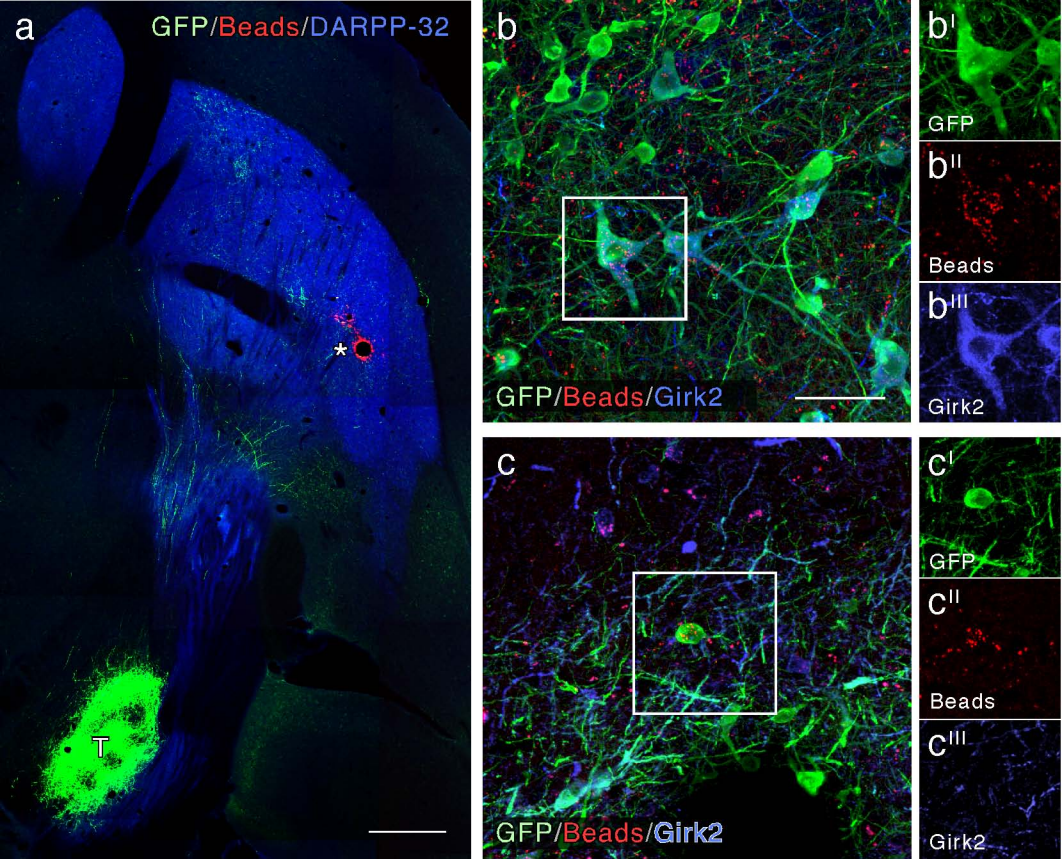
g
GFP/TH
gI
GFP
gII
TH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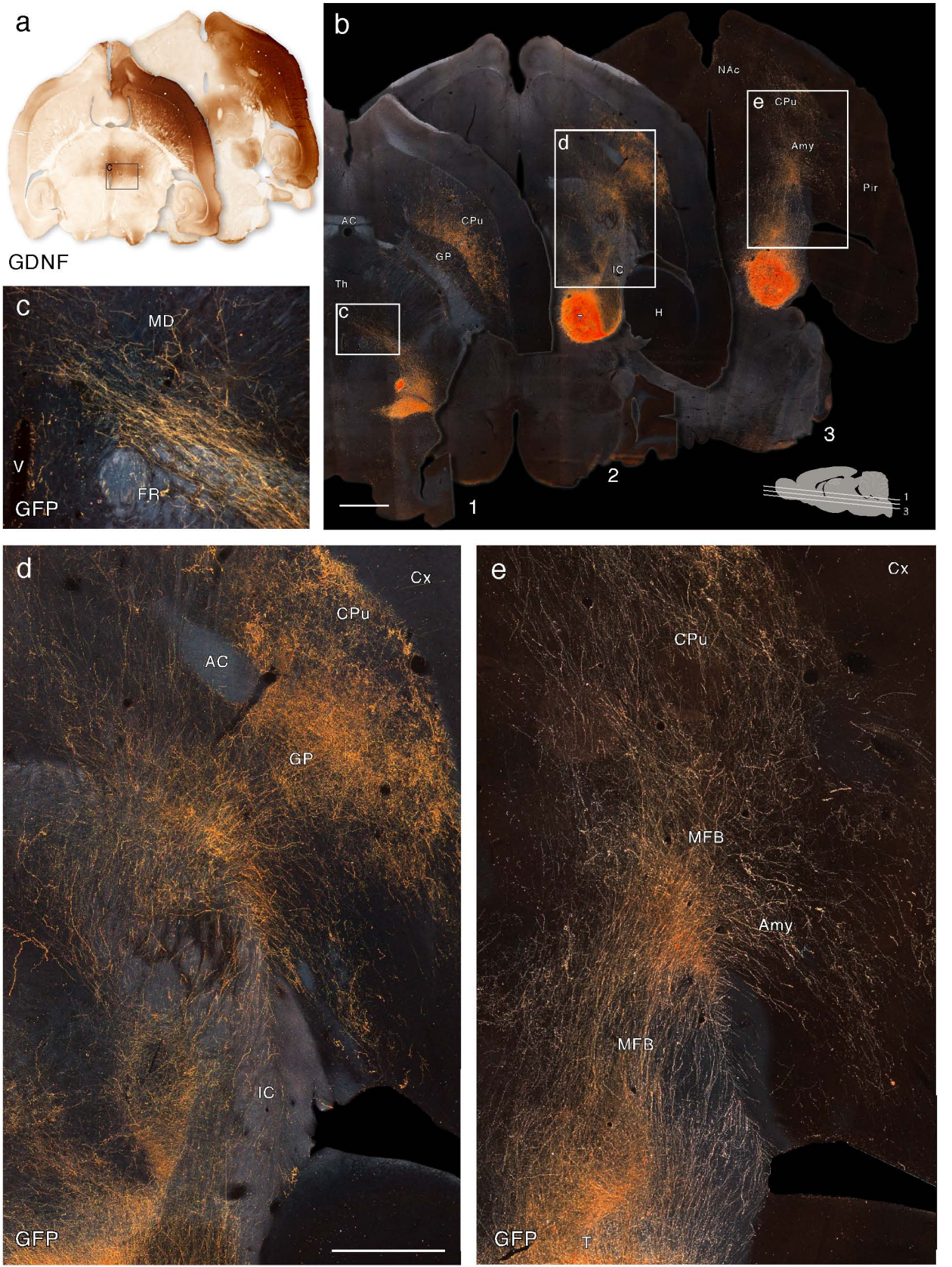
GFP/Beads/DARPP-32
*
T
b
GFP/Beads/Girk2
b'
GFP
b''
Beads
b'''
Girk2
c
GFP/Beads/Girk2
c'
GFP
c''
Beads
c'''
Girk2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GDNF
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NAc
e CPu
Amy
Pir
d
AC
CPu
GP
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Th
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3
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1
c
MD
V
FR
GFP
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Amy
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GFP
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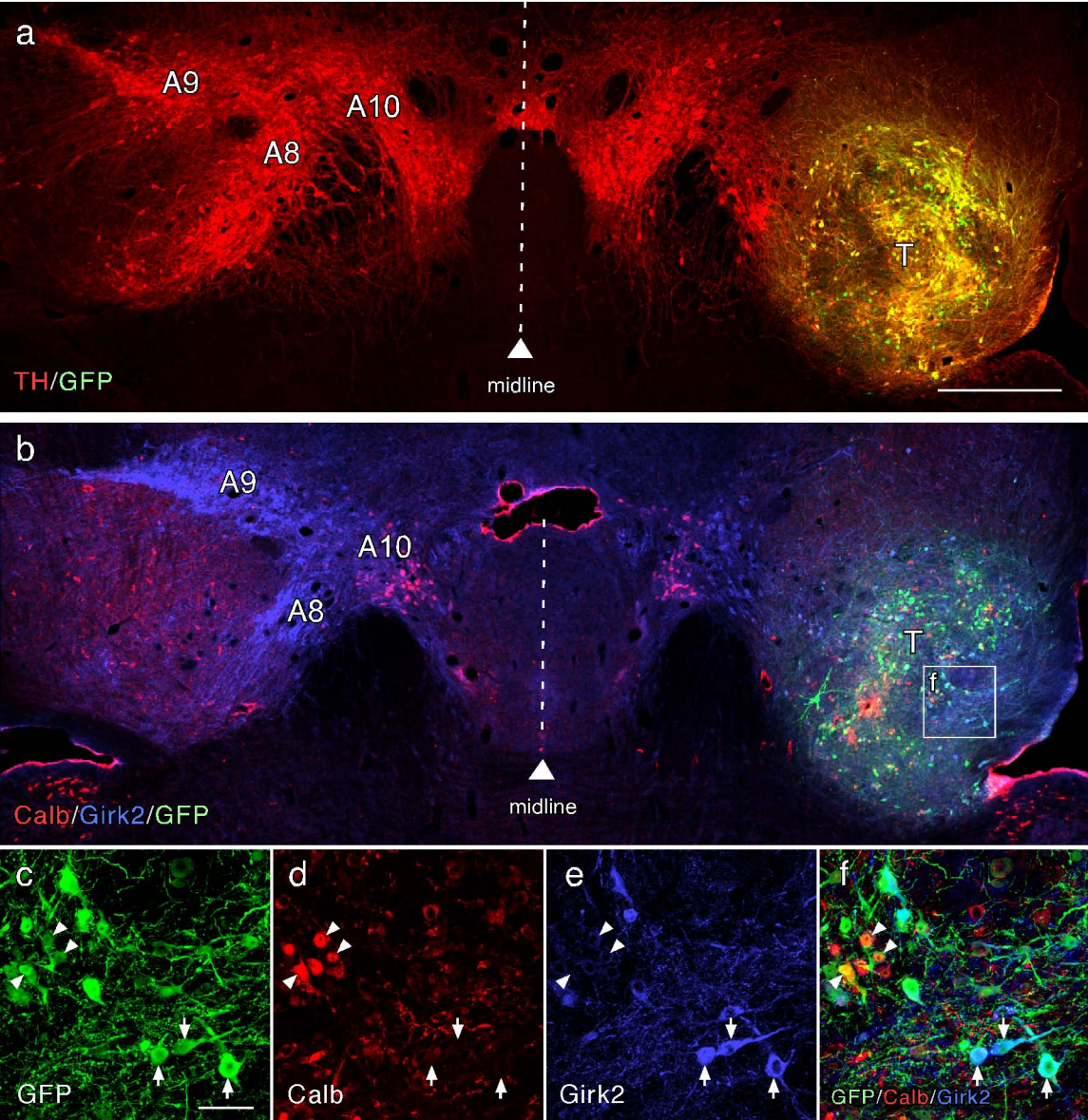
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A10
A8
T
TH/GFP
midline
b
A9
A10
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T
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Calb/Girk2/GFP
midline
c
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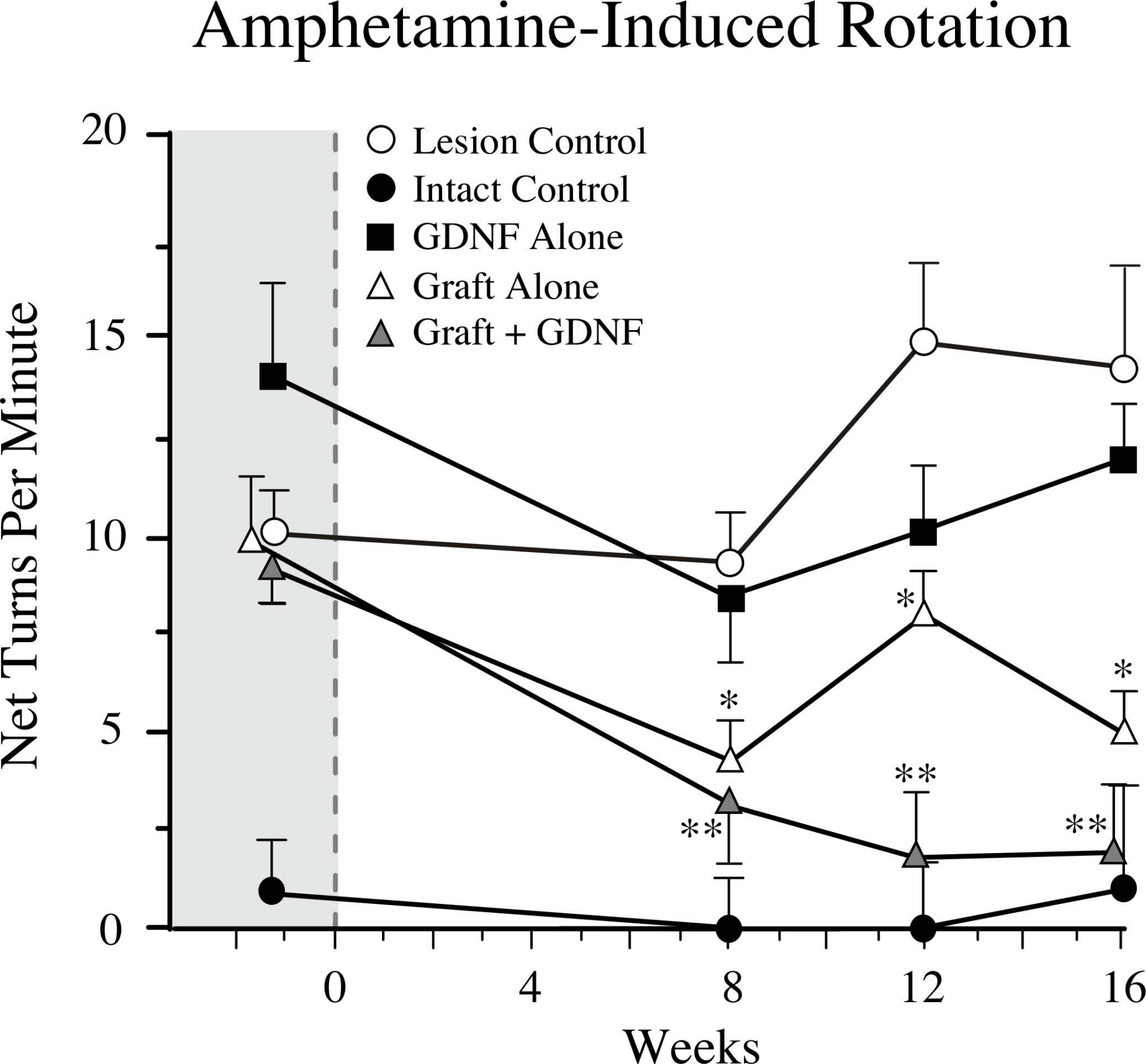
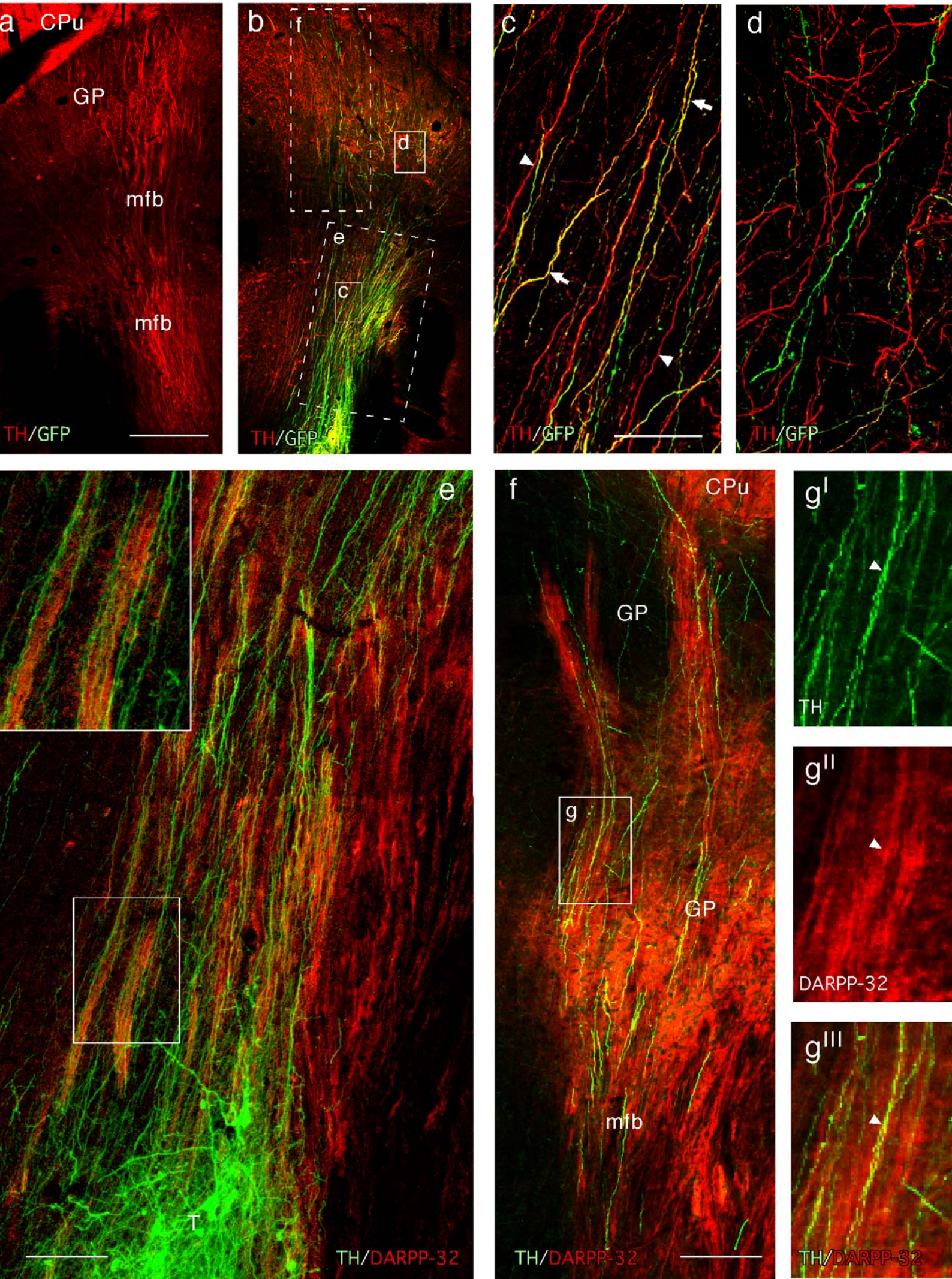
d
Calb
e
Girk2
f
GFP/Calb/Girk2


Amphetamine-Induced Rotation
Lesion Control
Intact Control
GDNF Alone
Graft Alone
Graft + GDNF
Net Turns Per Minute
20
15
10
5
0
0
4
8
12
16
Weeks
*
*
*
**
**
**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mfb
mfb
TH/GFP
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c
TH/GFP
c
TH/GFP
d
TH/GFP
e
T
TH/DARPP-32
f
CPu
GP
g
GP
mfb
TH/DARPP-32
gI
TH
gII
DARPP-32
gIII
TH/DARPP-32