Induction of A9 dopaminergic neurons from neural stem cells improves motor function in an animal model of Parkinson's disease

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Neural stem cells (NSCs) are widely endorsed as a cell source for replacement strategies in neurodegenerative disease. However, their usefulness is currently limited by the inability to induce specific neurotransmitter phenotypes in these cells. In order to direct dopaminergic neuronal fate, we overexpressed Pitx3 in NSCs that were then exposed to E11 developing ventral mesencephalon (VM) in explant culture. This resulted in a significant potentiation of dopaminergic differentiation of the cells. When transplanted into the 6-hydroxydopamine lesioned Parkinsonian rats, these cografts of VM and Pitx3 overexpressing NSCs resulted in a significant restitution of motor function. In addition, there were greater numbers of Girk2 positive A9 neurons in the periphery of the transplants that were NSC derived. This demonstrates that given the correct signals, NSCs can be induced to become dopaminergic neurons that can differentiate into the correct nigrastriatal phenotype required for the treatment of Parkinson's disease.

Introduction

The development of dopaminergic (DA) neurons of the ventral mesencephalon (VM) has been extensively studied owing to their degeneration in Parkinson's disease (PD). In the adult, these neurons can be divided into those located in the substantia nigra (A9 neurons), the ventral tegmental area (A10 neurons) and the retero-rubral field (A8 neurons) (Bjorklund and Lindvall, 1984). Selective degeneration of A9 DA neurons in the substantia nigra, and subsequent denervation of the dorso-lateral striatum, leads to the characteristic motor symptoms of PD (Dauer and Przedborski, 2003). One novel treatment for this disease is the transplantation of DA neurons ectopically into the striatum. Although the transplantation of VM tissue from

fetal sources has been shown in the best cases to mediate significant functional recovery, due to ethical and practical difficulties, the widespread implementation of this approach is unlikely (Bjorklund *et al.*, 2003).

One proposed alternative is to transplant DA neurons from neural stem cells (NSCs) isolated from the developing VM. It is well established that these cells can be isolated and expanded *in vitro* to give rise to neurons, astrocytes and oligodendrocytes (Gage, 2000). However, their ability to retain their DA phenotype following expansion is limited (Caldwell *et al.*, 1998; Chung *et al.*, 2006). Modifications to standard culture conditions such as lowered oxygen, addition of ascorbic acid or cytokines (Studer *et al.*, 2000; Storch *et al.*, 2001; Yan *et al.*, 2001; Chung *et al.*, 2006)

have had some success, but this seems to be critically dependent on donor age and expansion time *in vitro* (Studer *et al.*, 1998). Therefore the limiting factor of this approach is to generate sufficient number of DA neurons for transplantation.

Recent studies have focused on identifying the factors necessary for normal DA development. These include FGF8, sonic hedgehog and Wnt1 (Smidt and Burbach, 2007). In addition, a number of transcription factors necessary for DA neuron development have been identified. These include Pitx3, Nurr1, En-1, En-2, Lmx1a, Lmx1b, Msx1 and Ngn2 (Smidt and Burbach, 2007). Pitx3 is a paired like homeobox protein expressed exclusively within the CNS in DA neurons of the substantia nigra zona compacta and ventral tegmental area (Nunes *et al.*, 2003). In mice, which lack detectable levels of Pitx3 transcripts (Rieger *et al.*, 2001), A9 DA neurons degenerate, while A10 DA neurons are less affected (Reiger *et al.*, 2001; van den Munckhof *et al.*, 2003; Smidt *et al.*, 2004; Zhao *et al.*, 2004). Furthermore, tyrosine hydroxylase (TH) expression is lost specifically in the substantia nigra in Pitx3-null mice (Maxwell *et al.*, 2005). These data show that Pitx3 is critical for A9 DA neuron specification and survival (Simeone, 2005).

We overexpressed *Pitx3* using *Sin pgk* lenti viral vectors in neural progenitor cells (NPCs) from the E14 rat VM and assessed the ability of these to differentiate into DA neurons when co-cultured with an E11 VM explant. We also assessed whether these Pitx3 expressing NPCs could survive, differentiate, integrate into the host striatal network and improve motor function in the rat 6-OHDA model of PD. Furthermore, we assessed the distribution, maturity and subtype specification of the DA neurons *in vivo* and observed a large increase in the number of A9 specific DA neurons. Therefore we propose that this may be a useful strategy to generate large numbers of A9 DA neurons for cell replacement therapy in PD.

Methods
RT-PCR
Validation of the known genes involved in DA neuron development was done by RT-PCR. (See Supplementary Fig. 1A for primer sequences). Total RNA was isolated with TRIzol (Invitrogen, UK). RNA was subjected to RT-PCR with Superscript II (Invitrogen, UK). The reaction mix (20 µl) contained 200 µM dNTP, 1 µg of RNA, 0.5 µM of each primer at 42°C for 60 min. DNA was amplified by an initial incubation at 95°C for 5 min followed by 30–35 cycles of 95°C for 30 s, 65°C for 40 s, 72°C for 30 s and a final extension at 72°C for 5 min.

Neurosphere cultures and lentiviral gene delivery
The VM and cortex were dissected from E14 Sprague Dawley embryos, incubated in accutase for 15 min at 37°C and washed 2 × in DMEM. Single cells were plated at a density of 200 K cells/ml in growth media—DMEM/Ham's F12 (7:3 Gibco), PSF (1%),

B27 (2% Gibco), epidermal growth factor (EGF) (20 ng/ml), fibroblast growth factor 2 (FGF-2) (20 ng/ml) and heparin (5 µg/ml). Cells were fed every 2 days and passaged on the seventh day, the time when lentiviral vectors were used to transduce the cells.

Self-inactivating lentivirial vectors containing cDNA encoding Pitx3 or Nurr1 under the PGK promoter were generated as previously described (Naldini *et al.*, 1996). For infection neurospheres (NS) were dissociated following accutase incubation and cells were resuspended in growth media containing the virus (2TU/cell) for 24 h with polybrene (8 ug/ml) at a density of 5×10^6 cells/ml. Following this incubation the cells were re-suspended in growth media at 200 K/ml and expanded for a further 7 days with feeding every 2 days.

Explant cultures
E11 embryos were collected from SD rats expressing GFP under the beta-actin promoter (Okabe *et al.*, 1997). GFP$^+$ VM or forebrain tissue was plated directly onto a 0.2 um membrane inserts (Griener Bio One). One millilitre of basic culture medium consisting of Basal Medium Eagle (BME), Earle's balanced salt solution (EBSS)/Horse serum (2:1:1) supplemented with 0.1% D-glucose and 1 mM L-glutamine was placed below the membrane insert. Following 24 h incubation, one transfected neurosphere was plated beside, but not directly in contact with the E11 explant (estimated total cell number 8×10^5). Groups consisted of NS from cortex or VM, non-transduced or transduced with Nurr1 or Pitx3. Explant cultures were allowed to differentiate for 7 DIV with half of the media being replaced every 2 days. After 7 DIV, half of the explant cultures were fixed for immunocytochemistry in 4% paraformaldehyde (20 min). The remaining explant cultures were used for HPLC to determine potassium induced dopamine release using a concentration of 72 mM KCl (Dalley *et al.*, 2002).

6-OHDA lesion and behavioural testing
Female Sprague Dawley rats (Harlan, UK) received a 4 ul injection of 6-hydroxydopamine (6-OHDA) (6 mg free base/ml 0.1% ascorbate in saline) into the left medial forebrain bundle (MFB) as previously described (Ostenfeld *et al.*, 2000). Three weeks post lesion met-amphetamine (2.5 mg/kg) induced rotation was measured and animals with ⩾8 turns per minute were included in the study.

Stepping test and adjusting steps
Assessment of forelimb akinesia and spontaneous motor activity was evaluated as described by Olsson *et al.* (1995). The tests monitoring initiation time, stepping time and step length were preformed on a wooden ramp with a length of 1.1 m connected to the rats home cage. A smooth surfaced table with a width of 0.9 m was used for the test measuring adjusting steps (Olsson *et al.*, 1995). All testing was performed during daytime. *Initiation time, step time and step length:* the rat was held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. In addition, one forelimb was fixed allowing the other to be analysed. Initiation time was measured from the start of the experiment until the rat started movement using the free forelimb. Stepping time was measured from initiation of movement until the rat reached the home cage and step length was calculated by dividing the length of the ramp by the number of steps required for the rat to run up the ramp.

Each test was repeated twice on three consecutive days, and during each test both paws were monitored.

Adjusting steps

The rat was held in the same position as described above and was moved slowly sideways across the surface. The number of adjusting steps was counted for both paws, in both forehand and backhand directions. The test was repeated twice each day for three consecutive days and the average number of adjusting steps was calculated.

Cell transplantation

Three weeks post lesioning animals were stratified into groups ($n = 7$ animals per group) based on behavioural scores. Aliquots of 10 spheres and one intact E11 VM, or 10 NS or one intact VM, were prepared individually per animal. Tissue was loaded in 5 ul DMEM/B27 supplemented medium and loaded into a 5 ul zero dead volume, plunger-in-the-needle SGE glass microsyringe incorporating a 30-gauge stainless steel blunt cannula (Fischer Scientific, UK). Each animal received a unilateral, 5 ul graft of one E11 GFP$^+$ VM and 10 Pitx3-neurospheres (VM + Pitx3-NS) or one E11 GFP$^+$ VM and 10 control NS (VM + NS cont) into the striatum at the following co-ordinates; $A/P = +0.6$ $-L = 2.8$ and $V = -4.8$ mm, with the incisor bar set at -2.3. Control groups consisted of one E11 VM alone, 10 Pitx3-NS alone and lesion alone. The injection rate was 1 ul/min and the syringe was left in place for an additional 2 min. Behavioural testing was carried out at 4, 6 and 8 weeks post transplantation.

Tissue preparation and immunohistochemistry

At 8 weeks post-transplantation, animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde. Brains were removed, post fixed overnight and dehydrated in 30% sucrose in 0.1M phosphate buffer. For immunohistochemistry, 40 μm free floating sections (1 in 6 series) were quenched for 5 min in 10% H_2O_2, 10% methanol and washed $3 \times$ in tris buffered saline (TBS). Sections were blocked with 3% NGS in TBS-Triton (TBST, 0.2%) for 1 h. Sections were incubated in primary antibodies made up in 3% NGS/TBST, overnight followed by a 2 h incubation in secondary antibodies made up in 1% NGS/TBST. For peroxidase-based reactions, a biotinylated secondary was used followed by incubation with a strepavidin-horseradish peroxidase complex (ABC Elite Kit, Vectastin; Vector Laboratories) in tris non-saline (TNS) for 1 h. The sections were then exposed to di-amino-benzidine (0.5 mg/ml; Sigma plus 1% H_2O_2) For fluorescent immunohistochemistry, the quenching step was omitted. For a list of antibodies used see Supplementary Method 1.

Quantification and statistical analysis

The total numbers of tyrosine hydroxylase (TH)$^+$ cells within the transplant were quantified using a modified 2D stereological sampling protocol with the Olympus C.A.S.T grid stereological system (Denmark). Cells were counted in every sixth coronal section per animal and Abercrombie (1946) corrected. To accurately access the percentage of TH$^+$/Girk2$^+$ cells in the peripheral area of the graft, random visual fields, immunohistochemically labeled with TH and Girk2 were sampled using confocal microscopy (Leica, LASAF). Five animals were sampled per group, with three random visual fields being taken within five sections containing the graft per animal. Care was taken to sample only within the peripheral area of the graft. The same procedure was used to assess the percentage of TH$^+$/Girk2$^+$/GFP$^-$ also within the periphery of the graft.

Imaging and statistics

All fluorescent images were acquired using a Leica, FW4000 and a Leica LASAF laser-scanning microscope. For low magnification images of the graft and for cell morphological analysis LUCIA G software was used. The methods employed in the morphological study were adapted from Thompson *et al.* (2005). Within this study three animals were used per transplant group, with five sections containing grafts being analysed per animal. Five randomly selected fields were selected in the central and peripheral area of the grafts until an n number of 50 neurons had been selected. Once all images were collected and the images blinded, LUCIA G software was used to analyse cell body area and primary neurite branching within each transplant group. Behavioural data were analysed by two way ANOVA, followed by Neumann-Keuls test where appropriate with significance set as $P < 0.05$. Immunohistochemical results of TH$^+$ neurons, TH/Girk2$^+$ cells and TH/Girk2$^+$/GFP$^-$ cells per peripheral area of graft and cell morphological analysis were compared using a Student's two-tailed *t*-test.

Results

We have previously shown that E14 VM, upon expansion, loses its ability to differentiate into DA neurons in 7 days (Caldwell *et al.*, 1998). In order to examine why, we carried out RT-PCR analysis of the key factors involved in DA neurogenesis to determine if they are downregulated upon expansion. We found that TH, Pitx3, Lmx1b, SHH and FGF8 are expressed in primary VM tissue but are significantly downregulated after 7 days expansion in EGF and FGF2 (Supplementary Fig. 1).

To assess whether transfection with Pitx3 and Nurr1 could increase DA differentiation from expanded VM, we expressed these using lentiviral vectors in NS from the VM and cortex.

Signals from the E11 VM explants are required to induce DA differentiation from Pitx3 and Nurr1 over-expressing NS

Following expansion for 7 days *in vitro*, VM derived NS dissociated to a single cell suspension and virally transduced, to overexpress either Nurr1 or Pitx3. Expression of these factors alone did not generate dopamine neurons (data not shown). This suggests that although these factors are necessary for DA differentiation (Wagner *et al.*, 1999; Smidt *et al.*, 2004), they are not sufficient. One possibility is that other inductive signals present in the VM, are missing. To address this possibility we co-cultured NS with explants of E11 VM, which should contain multiple signals necessary for DA induction.
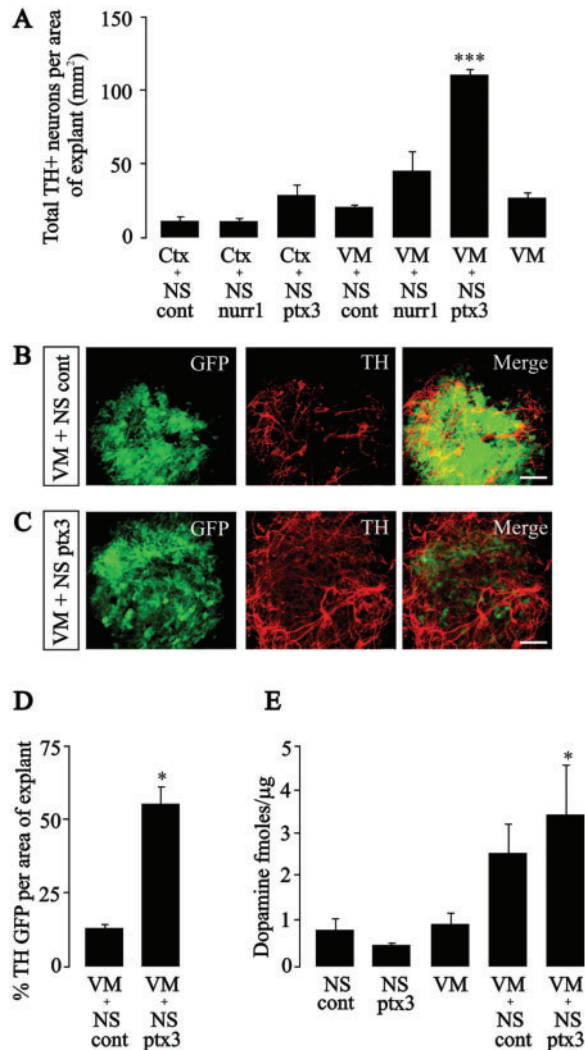
Fig. 1 EII VM induced dopamine neuron differentiation in neurospheres overexpressing Pitx3. (**A**) Total numbers of TH^{+} neurons per mm^2 of culture. Explant cultures containing VM + NSptx3 co-cultured with an EIIVM gave rise to significantly more total TH^{+} neurons when co-cultured for 7 DIV compared with all other groups. Pitx3 or Nurr1 over expressing NS derived from the cortex, co-cultured with an EII VM did not increase total TH^{+} neurons. (***$P < 0.0001$ versus all other groups; one-way ANOVA with fishers *post hoc* test). (**B** and **C**) Confocal images of VM derived TH^{+} neurons (B) (TH[+]/GFP[+]) and neurosphere derived TH^{+} neurons (C) (TH[+]/GFP[−]) found throughout explant cultures in both VM + NScont and VM + NSptx3, respectively. Scale bar = 100 µm. (**D**) Furthermore, VM + NSptx3 explant cultures contained significantly more NS derived TH^{+} neurons (TH[+]/GFP[−]) when compared with VM + NScont explant cultures. *$P < 0.01$. (**E**) Reversed phase HPLC showed a significant increase in dopamine release form VM + Pitx3 NS group compared with Pitx3 NS explant cultures, *$P < 0.01$.

When an E11VM and Pitx3 NS (hereafter referred to as VM + NS ptx3) were co-cultured there was a dramatic increase in the numbers of DA neurons in the culture when compared with control NS, co-cultured with the E11VM (hereafter referred to as VM + NS cont), (Fig. 1A). In cultures of an E11 VM and Nurr1 NS (hereafter referred to

as VM + NS nurr1) or VM alone, there was no increase in DA neuron number (Fig. 1A). This dramatic effect was specific to NS derived from the VM, as NS derived from the cortex transduced with Nurr1 or Pitx3 and co-cultured with E11 VM, displayed no increase in DA differentiation (Fig. 1A). Furthermore, explant cultures replacing E11 VM with E11 forebrain resulted in no increase in DA differentiation (data not shown) indicating the regional specificity of this effect.

Pitx3 significantly increases the number of NS derived DA neurons within explant cultures

Although the co-culture of Pitx3 expressing NS with an E11 VM, led to a 6-fold increase in the number of DA neurons, these neurons could be either NS or VM derived. In order to distinguish between VM and NS derived DA neurons, we co-cultured NS together with an E11 VM from a rat, which constitutively expresses GFP (Supplementary Fig. 2). Using this approach, DA neurons derived from the VM were GFP-positive (GFP[+]), while those derived from the NS were GFP-negative (GFP[−]). Both TH[+]/GFP[+] and TH[+]/GFP^{−} neurons were found throughout the explant cultures (Fig. 1B and C). However NS derived DA neurons (TH[+]/GFP[−]) were predominately located in the peripheral area of the explant cultures (Fig. 1B and C), while those derived from the VM (TH[+]/GFP[+]) were predominantly located in centre of the explants (Fig. 1B and C). In order to determine the percentage of NS derived DA neurons within each group, the numbers of TH[+]/GFP^{−} neurons were counted. In VM + NSptx3 cultures 54.98% ± 8.09 of the total dopaminergic neurons were TH[+]/GFP[−] indicating that they had differentiated from the NS, while in VM + NS cont, 13.15% ± 1.67 the total dopaminergic neurons were TH[+]/GFP[−] (Fig. 1D). These data show that NS virally transduced to express Pitx3 and co-cultured with E11VM, give rise to over half of the total DA neurons within explant cultures.

In order to determine if the DA neurons derived from the Pitx3 expressing NS were functionally active, we carried out reversed phase HPLC analysis for dopamine within the explant cultures. Addition of KCl to the culture medium resulted in the most dopamine being released in the VM + NSptx3 group compared with the VM alone group (Fig. 1E). None of the conditions resulted in the release of noradrenaline (data not shown).

Pitx3 induces behavioural recovery in 6-OHDA model of PD

As the co-culture of VM + NSptx3 efficiently generates high numbers of DA neurons *in vitro*, we sought to determine if this approach could improve behavioural recovery in the rat 6-OHDA model of PD.

Animals displaying a full lesion of the nigrostriatal pathway (>8 ipsilateral turns post-methamphetamine) were stratified into five groups, four of which received one of the following transplants into the striatum; one E11 GFP$^+$ VM and 10 Pitx3 expressing NS (VM + NSptx3) per animal, one E11 VM and 10 control NS (VM + NScont), one E11 VM alone or 10 Pitx3-expressing NS alone. To control for non-specific effects of the lesion, a control group of animals who received the 6-OHDA lesion but not a transplant was also included. Behavioural assessment was carried out 3 weeks post lesion (prior to transplantation) and at 4, 6 and 8 weeks post transplantation. First, methamphetamine-induced rotation behaviour was assessed. All groups that received a VM graft, showed significant behavioural recovery in this test (Supplementary Fig. 3).

In order to test non-drug-induced motor function, a stepping test was used to measure forelimb akinesia (Olsson *et al.*, 1995). As expected 3 weeks after the 6-OHDA lesion, the performance of the right paw (contralateral to the lesion) was significantly impaired compared with the left paw (Fig. 2 and Supplementary Fig. 4). No significant difference was seen pre-lesion. Following the lesion, all groups displayed a short latency in the initiation of movement using the left paw (<2 s) (Supplementary Fig. 4A). However, the right paw was significantly slower 3 weeks post lesion (Fig. 2A). Following transplantation, significant recovery in initiation time 4 weeks after grafting was observed only in the VM + NSptx3 group and was sustained throughout the survival period of the animals (Fig. 2A and A′). Left paw stepping time was similar in all treatment groups following lesion, with the right paw being significantly slower (Fig. 2B, B′ and Supplementary Fig. 5B). Stepping time was significantly reduced only in animals with VM + NSptx3 grafts at 4, 6 and 8 weeks post-transplantation (Fig. 2B and B′). Furthermore, in measurements of step length, all 6-OHDA lesioned animals required more time to mount the ramp with their right (contra-lateral) paw. However, only animals with the VM + NSptx3 grafts significantly increased their step lengths to values not significantly different from the left paw by 4 weeks post transplantation (Fig. 2C, C′ and Supplementary Fig. 4C).

Finally the number of adjusting steps in both the forehand and backhand direction was counted. Left paw performance was similar in all five groups in the backhand and forehand directions and was consistent over all the test sessions (Supplementary Fig. 5A and B). Again the lesion-induced deficit seen in the right paw in both directions significantly improved only in animals with the VM + NSptx3 grafts 4 weeks after transplantation (Fig. 3A, B, A′ and B′). The improvement in steps taken in the backhand direction was maintained between 4 and 8 weeks post grafting (Fig. 3B and B′) whereas steps in the forehand direction continue to improve with time (Fig. 3A and A′).

When taken together these results show that transplantation of VM + NSptx3 mediated significant and sustained functional recovery in the 6-OHDA model of PD.

Pitx3 expressing NS survive and differentiate into DA neurons *in vivo*

In order to determine if the improved motor function seen in animals transplanted with VM + NS ptx3 was due to enhanced survival of DA neurons within the transplant, we carried out immunohistochemistry to assess the numbers of DA neurons surviving within the graft after 8 weeks. All groups that were transplanted with an E11 VM contained significant numbers of DA neurons in the graft site. In the two groups which received a transplant of VM + NSptx3 or VM + NScont there was a significant increase in the numbers of DA neurons within the transplant site compared with VM alone, and there was no difference in the total number of DA neurons in the VM + NScont and the VM + NSptx3 groups (Fig. 4A). There were no DA neurons surviving in the animals that received a transplant of NSptx3 alone (data not shown).

Although there was no significant difference in the numbers of DA neurons surviving between the VM + NSptx3 and VM + NScont, there appeared to be a differential distribution of DA neurons within the grafts. In the VM + NSptx3 group there were significantly more DA neurons located in the periphery (Fig. 4B and C), whereas in the VM + NScont group, more were located in the centre (Fig. 4B and C). The vast majority of these neurons were A9-DA neurons as assessed by double staining for TH and the A9-specific marker Girk2 (Thompson *et al.*, 2005) (Fig. 4D). These data show that although the absolute numbers of DA neurons generated *in vivo* from VM + NSptx3 or VM + NScont is the same; their spatial location within the graft is different.

Pitx3 gives rise to more mature TH$^+$ neurons within the periphery of the graft

We next wanted to determine if there were any morpho-logical differences in the neurons located at the periphery of the graft in the VM + NSptx3, VM + NScont or VM alone groups. We analysed the number of primary neurite branches from the cell body, and the somal area and this analysis revealed that DA neurons in the VM + NSptx3 group had significantly more primary neurite branches and a greater somal area compared with those in the VM + NScont group and VM alone group (Fig. 5A–D). Within the VM + NSptx3 group there was also a high degree of variability in the maturity of dopaminergic neurons located within the centre compared with the periphery of the graft, with those in the periphery being of a more mature dopaminergic phenotype (Fig. 5C and D).

Pitx3 gives rise to increased numbers of A9 DA neurons in the peripheral area of the graft

The fact that there were greater number of DA neurons located in the periphery of the grafts in the VM + NSptx3
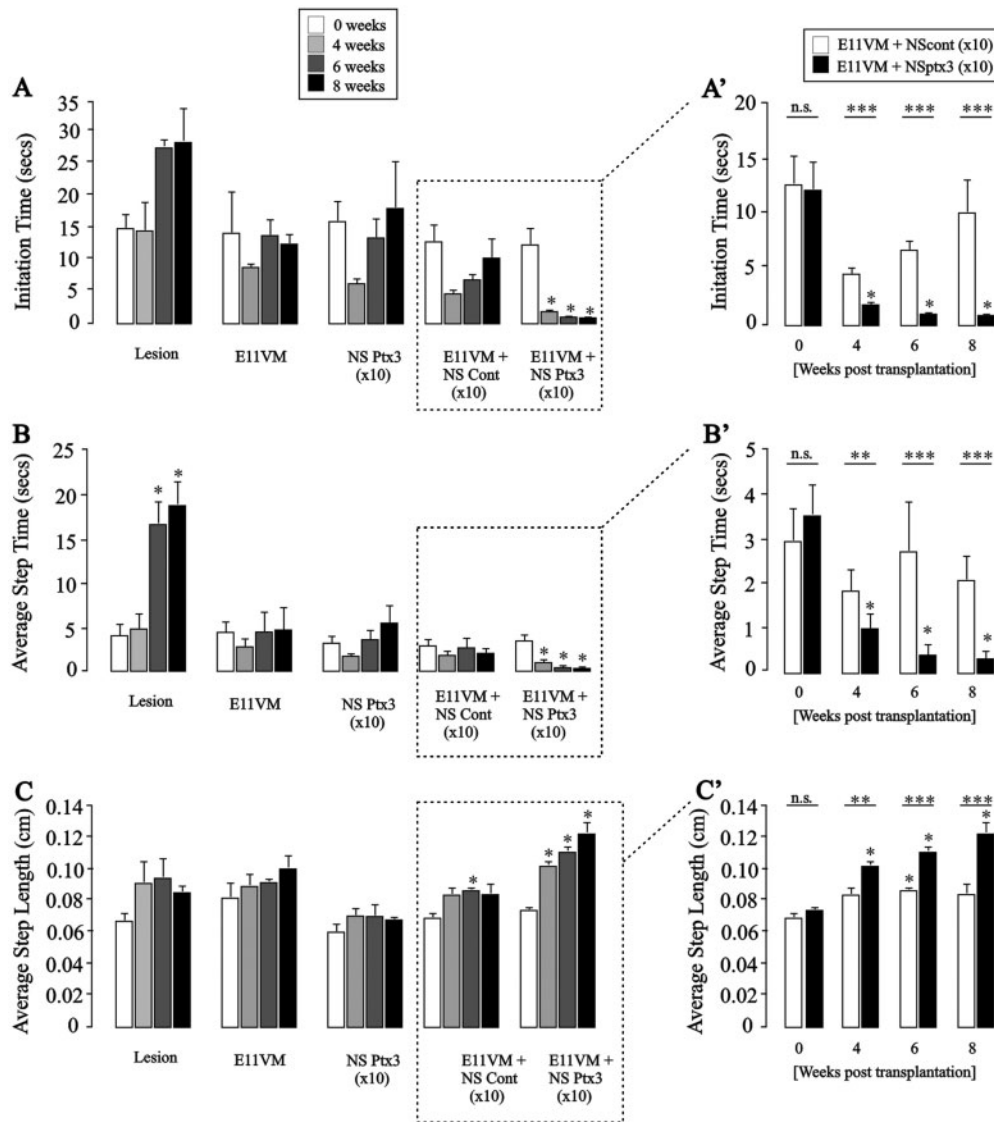
Fig. 2 Co-grafts of VM + NSptx3 promote a full and sustained functional recovery in the 6-OHDA lesioned rat as assessed by the stepping test. The mean initation time (s), the average step time (s) and the average step length (cm), were assessed 3 weeks post lesion just prior to transplantation (0 Weeks). Following transplantation, these parameters were assessed at 4, 6 and 8 weeks. (**A**) In measurements of initiation time, only animals that received a transplant of VM + NSptx3 showed a significant improvement in step initiation time at 4, 6 and 8 weeks post transplantation when compared with 0 weeks. (**A′**) Boxed area in A, illustrates the significant difference in initiation time in animals that received a transplant of VM + NScont and VM + NSptx3. The VM + NScont group did not display any significant functional improvement over time whereas the VM + NSptx3 showed a significant improvement at all time points that were assessed after transplantation, reaching a level where it was not different from the unaffected paw initiation time. Furthermore, there was a significant difference between the VM + NScont and the VM + NSptx3 at 4, 6 and 8 weeks following transplantation. (**B**) In measurements of the average step time, only animals that received a transplant of VM + NSptx3 showed a significant improvement in step time at 4, 6 and 8 weeks post transplantation when compared with the step time at 0 weeks. (**B′**) Boxed region in B, illustrates the significant difference between the VM + NScont and VM + NSptx3 groups; only the VM + NSptx3 group showed a significant improvement at all time points that were assessed after transplantation, reaching a step time that was not different from the unaffected paw. Again, there was a significant difference between the VM + NScont and the VM + NSptx3 at 4, 6 and 8 weeks following transplantation. (**C**) In measurements of the average step length, only the VM + NSptx3 group showed a significant increase 4, 6 and 8 weeks post transplantation when compared with 0 weeks. (**C′**) Boxed region in C, illustrates the significant difference between the VM + NScont and VM + NSptx3 groups; only the VM + NSptx3 group showed a significant improvement at all time points that were assessed after transplantation, reaching a step length that was not different from the unaffected paw. Again, there was a significant difference between the VM + NScont and the VM + NSptx3 at 4, 6 and 8 weeks following transplantation. ($n = 7$ per group; $*P < 0.05$ versus 0 weeks; $***P < 0.001$ VM + NSptx3 versus VM + NScont; n.s. = not significant).
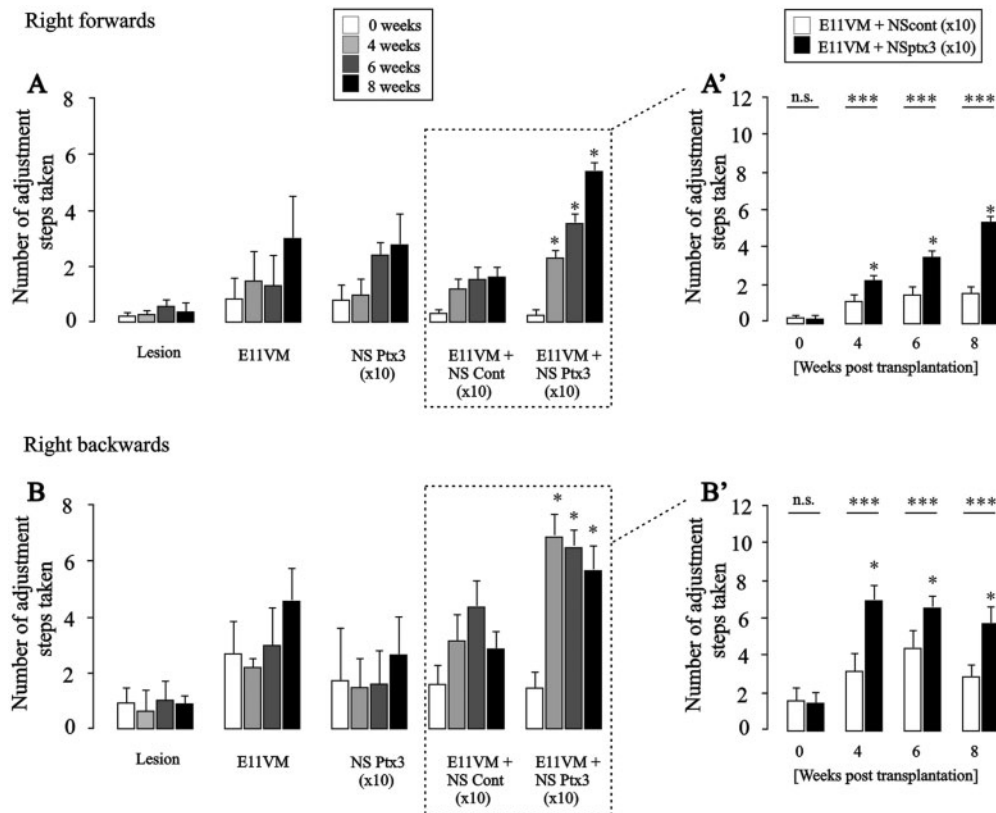
Fig. 3 Co-grafts of VM + NSptx3 promote a full and sustained functional recovery in the 6-OHDA lesioned rat as assessed by the adjustment test. (**A** and **B**) In measurements of the number of adjustment steps taken in the forward and backward direction, respectively, only the VM + NSptx3 group showed a significant increase in the numbers of these steps at 4, 6 and 8 weeks post transplantation when compared with 0 weeks. (**A′** and **B′**) Boxed region in A and B, respectively, illustrates the significant difference between the VM + NScont and VM + NSptx3 groups; only the VM + NSptx3 group showed a significant increase in the numbers of steps taken at all time points that were assessed after transplantation. Animals that received an VM + NSptx3 transplant recovered 66% of their motor function when compared with the unaffected paw, whereas the VM + NScont recovered 20%. ($n = 7$ per group; $^*P < 0.05$ versus 0 weeks; $^{***}P < 0.001$ VM + NSptx3 versus VM + NScont; n.s. = not significant).

group and only this group showed significant behavioural recovery, suggested that these grafts might contain significantly more A9 specific DA neurons than the VM + NScont grafts (Fig. 6A and F–H). To test this hypothesis more closely, we counted the total number of $TH^+/Girk2^+$ in these transplants. In the periphery of the VM + NSptx3 grafts, 1729 ± 85.62 of all TH^+ neurons were $Girk2^+$, whereas, in the periphery of the VM + NS cont grafts, 924 ± 55.89 of all TH^+ neurons were $Girk2^+$ (Fig. 6A). These data revealed that there were significantly greater numbers of neurons of the A9 DA lineage, in the peripheral area of the VM + NSptx3 transplants, and thus available to form synaptic contacts with the host striatum, when compared with the VM + NScont group. In addition, both groups also contained $TH^+/Girk2^-$ neurons within the periphery of the grafts indicating that they may belong to A10 subtype of DA neurons (Fig. 6C–H). In order to test this we carried out TH and calbindin double staining. Results show that whilst there were some $TH^+/Calbindin^+$ neurons located in the periphery of all transplants, the majority were located in the centre (Supplementary Fig. 6).

Pitx3 significantly increased number of NS derived A9 DA neurons within peripheral area of graft

As there was a significant increase in numbers of $TH^+/Girk2^+$ neurons in the periphery of the VM + NSptx3 grafts, we sought to determine whether these neurons differentiated from the E11VM or from the Pitx3-NS. This was possible as the E11 VM within all transplant groups was GFP^+. When we examined the relative numbers of DA neurons that were derived from the E11VM or from the NS, we found that $74.43\% \pm 9.61$ of all $TH^+/Girk2^+$ neurons in the periphery of the VM + NSptx3 grafts were GFP^- indicating that they were NS derived (Fig. 6B). Interestingly only $25.94\% \pm 2.459$ of all $TH^+/Girk2^+$ neurons within the peripheral part of the E11 + NScont group were GFP^- (Fig. 6B). In the periphery of the VM + NSptx3 grafts there were relatively low numbers of $GFP^+/TH^+/Girk2^+$ neurons (Fig. 6I–O), whereas in the periphery of VM + NScont grafts, most $TH^+/Girk2^+$ neurons co-expressed GFP, indicating they were derived from
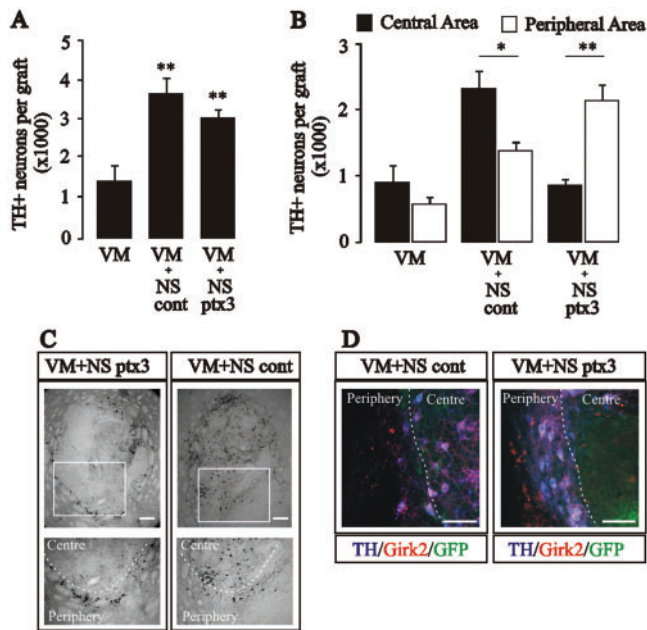
Fig. 4 Transplants of VM and either NScont or NSptx3 generate large numbers of TH$^+$ neurons *in vivo*, but with differential distributions in the graft site. (**A**) Counts of the total numbers of TH$^+$ neurons in the grafts of animals that received a transplant of an VM alone, VM + NScont or VM + NSptx3; there were significantly more TH$^+$ neurons observed within the two groups receiving VM + NS transplants compared with VM alone group, but no significant difference in the total numbers of TH$^+$ neurons between the VM + NScont and the VM + NSptx3 groups (**$P < 0.01$). (**B**) There was a significant difference in the spatial distribution of TH$^+$ neurons within grafts consisting of VM + NScont or VM + NSptx3. Significantly more TH$^+$ neurons were located in the graft centre than its periphery in the VM + NScont, whereas grafts of VM + NSptx3 contained significantly more TH$^+$ neurons in their periphery than centre (*$P < 0.05$, **$P < 0.01$). (**C**) Photomicrographs of VM + NScont and VM + NSptx3 grafts showing the differential distribution of TH$^+$ neurons. The boxed region is shown in higher power below each picture with the dashed line indicating the difference between the centre and the periphery of the grafts. Scale bar = 50 μm. (**D**) Although many TH$^+$ neurons VM + NScont and VM + NSptx3 grafts co-expressed Girk2 (indicating that they were A9 DA neurons), many more of these were present in the periphery of the VM + NSptx3 grafts when compared with VM + NScont grafts (See Fig. 6). TH = blue, Girk2 = red, GFP = green. Scale bar = 50 μm).

the VM (Fig. 6P–V). In addition, the majority of TH$^+$/Girk2$^+$ neurons in periphery of the VM + NSptx3 grafts, showed strong nuclear expression of Pitx3 (Supplementary Fig. 7).

Discussion

We show here that over expression of Pitx3 in VM derived NPC increases dopamine differentiation *in vitro* when exposed to neurotrophic signals from an early developing VM. When transplanted into a 6-OHDA model of PD,

these virally transfected NS survive, differentiate into a mature DA phenotype and integrate well into the host striatal network thus improving motor function. We also show that over expression of Pitx3 increases the number of substantia nigra (A9) DA neurons within the peripheral area of the graft.

In the initial phase of the study, we attempted to induce dopamine neurogenesis in NSCs at a time when transcription factors associated with their development are down regulated. We over expressed either Nurr1 or Pitx3 and found that neither factor alone was sufficient to elicit a positive effect. Therefore, we co-cultured NS expressing either transcription factor with an E11 rat VM and observed an increase in dopamine neurogenesis in vitro. This developmental stage equates to E9.5 in the mouse as the rat conceptus implants a day and a half later than the mouse resulting in a longer gestation period of one and a half days (Butler and Juurlink, 1987). This particular timepoint represents the stage in development when appropriate signals for the birth of dopamine neurons are present (Wagner *et al*., 1999; Smidt *et al*., 2004). In order to distinguish NS from VM derived TH positive neurons, we employed an explant culture system where the VM was GFP+, while the NS was not. Interestingly, only co-culture with Pitx3 overexpressing NS resulted in a significant increase in TH positive neurons, but this was not the case in the Nurr1 overexpressing group. Indeed it has previously been shown that Nurr1 alone is not sufficient to induce TH in NSCs, they need to be in contact with astrocytes from E16 VM (Wagner *et al*., 1999). Therefore, it is possible that astrocytes from older embryos elicit the correct signals and that E11 is too young. In support of this, this developmental age is prior to the neuronal-glial switch having taken place (Kessaris *et al*., 2007), hence the E11 VM is likely to be producing different signals than E16 VM astrocytes. Interestingly, in the Wagner study, cells overexpressing Nurr1 needed to be in direct contact with the astrocytes or separated by a microporous insert to affect TH expression. This was also the case in this study; conditioned medium from E11 VM was unable to elicit the same effect on Pitx3-NS, indicating that the required signal(s) is not diffusible and is in fact contact mediated or highly labile. Interestingly, if we co-cultured Pitx3-NS with E11 forebrain this did not result in TH differentiation, indicating that the necessary signal(s) are regionally specified. This is also in agreement with Wagner *et al*., 1999 who showed that astrocytes from other brain regions could not mimic the effects on dopamine differentiation in Nurr1 overexpressing cells. Moreover, if Pitx3 was overexpressed in the cortex and co-cultured with E11 VM, this does not result in TH differentiation in this brain region. This again highlights the importance of regional specification as has been described previously for developing brain in terms of neuronal/glial differentiation and transcription factor expression after expansion in vitro (Hitoshi *et al*., 2002; Ostenfeld *et al*., 2002a; Parmar *et al*., 2002).
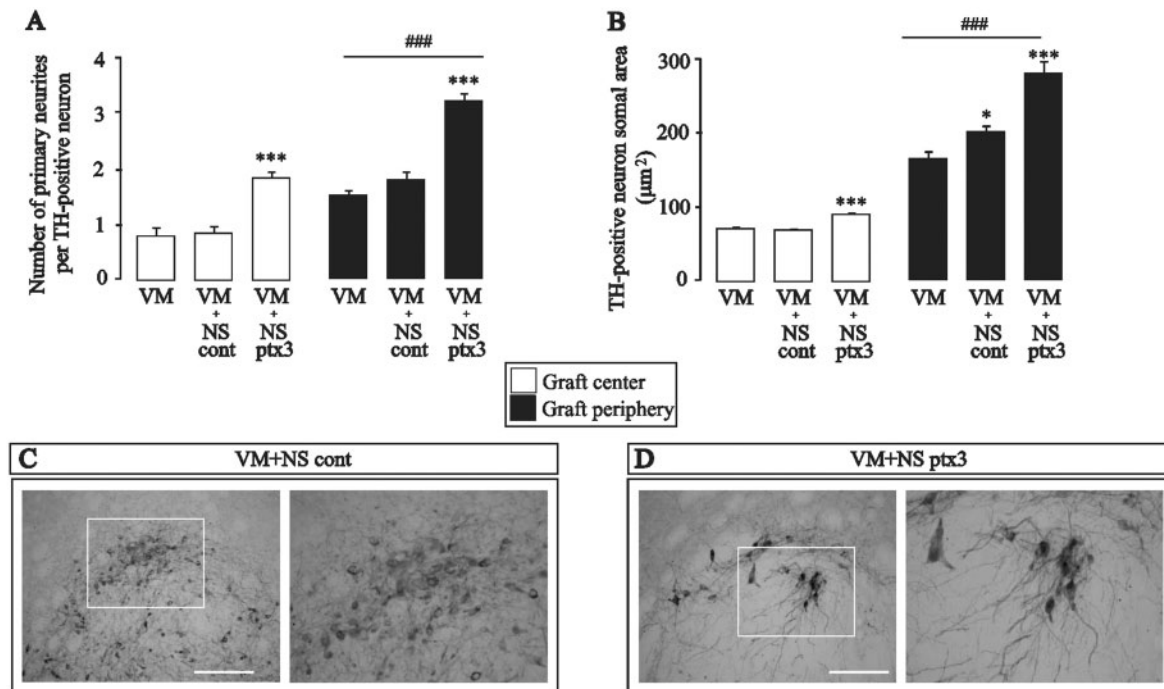
Fig. 5 TH+ neurons in VM + NSptx3 grafts are morphologically more mature than those in VM + NScont grafts. Somal area and the numbers of primary neurites of TH neurons were assessed in VM alone, VM + NScont and VM + NSptx3, in both the centre and periphery of the transplant. (A and B) Centrally and peripherally located TH+ neurons within the VM + NSptx3 group have significantly greater numbers of primary neurite branches and increased somal area, respectively, compared with the TH+ neurons located in either region of the VM alone or VM + NScont transplants. However, the TH neurons in the periphery of the VM + NSptx3 grafts are more mature than in the centre, indicating that more morphologically mature TH+ neurons are located in the peripheral area of these grafts. *$P < 0.05$ versus VM alone; ***$P < 0.001$ versus VM alone and VM + Nscont; ###$P < 0.001$ versus central TH neurons. (C and D) Photomicrographs of VM + NScont and VM + NSptx3 grafts, respectively, showing the increased morphological differentiation of TH+ neurons in VM + NSptx3 grafts. The boxed regions in C and D, is highlighted to the right of their respective pictures. Scale bar = 50 μm.

DA phenotype was confirmed by KCl evoked dopamine release *in vitro*, which demonstrated that the greatest amount of dopamine was produced in the VM + NS ptx3 group. We show here that over half of the TH positive neurons were non-GFP and hence neurosphere derived in the VM + NSptx3 group. This was reduced by 40% in the VM + NScont group. However, this indicates that the developing VM can produce signals itself, which induce TH expression in control NS but the effect of the VM is significantly enhanced in the presence of Pitx3. Indeed, Pitx3 is switched on later in development than many other transcription factors involved in dopamine neuronal development and therefore may be more important in the specification and maintenance of the TH phenotype (Maxwell *et al.*, 2005) in particular the A9 subtype (Chung *et al.*, 2006). Interestingly, in recent studies overexpressing Pitx3 in ES cells, there was no significant increase in the overall number of TH neurons. However, there was a significant increase in the number of midbrain A9 specific TH neurons suggesting that over expression of Pitx3 promotes the production of a true midbrain dopamine phenotype (Maxwell *et al.*, 2005; Chung *et al.*, 2006).

On the basis of these results we transplanted VM + NS ptx3 into the 6-OHDA rat model of PD in order to assess their functional efficacy and differentiation potential. VM alone, VM + NS cont and Pitx3-NS alone served as controls. Amphetamine induced rotation demonstrated that all animals grafted with VM had decreased rotation scores. Amphetamine induced rotation monitors a hyperkinetic or stereotype motor behaviour in response to activation of supersensitive dopamine receptors. In contrast, the stepping test provides a more direct measure of the underlying motor deficit analogous to limb akinesia seen in human PD (Olsson *et al.*, 1995) and as such have been proposed as a highly useful tool for more detailed analysis of the functional efficacy of neural transplants in the 6-OHDA model. Indeed we show here that the VM + NS ptx3 group have improved initiation time, stepping time and step length compared with any of the other treatment groups. In addition, the number of adjusting steps was significantly improved in VM + NSptx3 group in both directions. The improvement was less pronounced in the forehand than the backhand direction. Interestingly, this is the same result as has been reported when E14 VM was transplanted into the substantia nigra and striatum in the same animal model
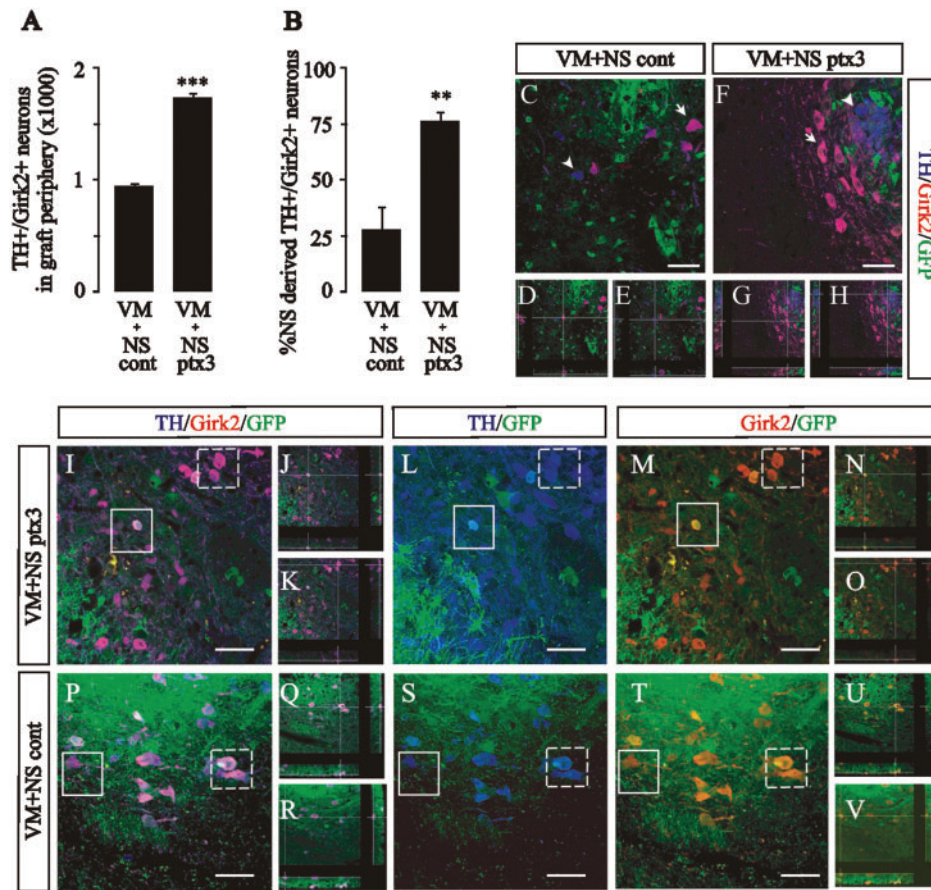
Fig. 6 Greater numbers of Neurosphere derived A9 dopamine neurons in the periphery of the VM + Pitx3 NS graft. (**A**) VM + NSptx3 group had significantly more TH$^+$/Girk2$^+$ cells located in the peripheral area of the graft compared with VM + NS cont group. ***$P < 0.001$. (**B**) A greater proportion of TH$^+$ neurons were neurosphere derived (TH$^+$/GFP$^-$) in the VM + NSptx3 graft than in the VM + NScont graft, Student's *t*-test, ***$P < 0.001$. (**C–E**) Confocal image of TH$^+$ (blue)/Girk2$^+$ (red) neurons (arrow) and TH$^+$ (blue)/Girk2$^-$ neuron (arrowhead) located in the peripheral part of the VM + NScont graft and (**F–H**) in a VM + NSptx3 graft. (**I–K**) NS derived (GFP$^-$) TH$^+$/Girk2$^+$ and VM derived (GFP$^+$) TH$^+$/Girk2$^+$ neurons within peripheral area of VM + NSptx3 group J. Boxed area in I, VM derived (GFP$^+$) TH$^+$/Girk2$^+$ neuron, (**K**) Dashed boxed area in I, NS derived (GFP$^-$) TH$^+$/Girk2$^+$ neuron. (**L**) NS derived TH$^+$ (GFP$^-$) neurons and VM derived TH$^+$ (GFP$^+$) with the majority of TH$^+$ being NS derived. (**M**) (same field as L), Girk2$^+$/GFP$^+$ and Girk2$^+$/GFP$^-$ neuron, (**N**), Boxed region in M, VM derived Girk2+/GFP+ neuron, (**O**), Dashed boxed region in M, NS derived Girk2$^+$/GFP$^-$ neuron. (**P**) NS derived (GFP$^-$) TH$^+$/Girk2$^+$ and VM derived (GFP$^+$) TH$^+$/Girk2$^+$ neurons within the peripheral area of VM + NS cont group. (**Q**), Boxed area in P, VM derived (GFP$^+$) TH$^+$/Girk2$^+$ neuron, (**R**) Dashed boxed area in P, NS derived (GFP$^-$) TH$^+$/Girk2$^+$ neuron. (**S**) NS derived TH$^+$ (GFP$^-$) neuron and VM derived TH$^+$ (GFP$^+$) neuron with the majority of TH$^+$ being VM derived. (**T**) (Same field as S), Girk2$^+$/GFP$^+$ and Girk2$^+$/GFP$^-$neuron, (**U**) Boxed region in T, VM derived (GFP$^+$) TH$^+$/Girk2$^+$ neuron, (**V**) dashed box in T, NS derived (GFP$^-$) TH$^+$/Girk2$^+$ neuron. Scale bar = 100 μm.

as used here (Olsson *et al.*, 1995). However, we did not see an improvement in these motor tests with E11 VM alone or VM + NS cont as has been reported for E14. This may be because E11 is younger in developmental terms and therefore may not contain so many TH neurons. In support of this a recent study re-examining the ontogeny of dopamine neurons has shown that the majority are probably born on E12 (Gates *et al.*, 2006), which is 1 day later than the tissue used here.

Quantitative stereology revealed that there was greater numbers of surviving TH neurons in the VM + NS cont and VM + NSptx3 groups than VM alone. This demonstrates that the NS themselves are having a tropic effect on the VM tissue. Indeed Yasuhara *et al.*, 2006 have shown that

transplantation of human NSCs exerts neuroprotection in an animal model of PD. Furthermore, addition of supernatent from human NSCs protects SH-SY5Y cells or fetal rat VM dopamine neurons from 6-OHDA toxicity in vitro (Yasuhara *et al.*, 2006), and NS increase the survival of DA neurons when grafted with E14 VM in the 6-OHDA lesioned rat (Ostenfeld *et al.*, 2002b). There was also a difference in the distribution of the DA neurons within the transplants. There were significantly more TH neurons in the periphery of the VM + NSptx3 grafts compared with the other groups. In fact, in the case of the VM alone group, there was equal distribution in the centre and periphery of the graft, while in the VM + NS cont grafts, there were more TH neurons in the centre than in the periphery.

Furthermore, morphological analysis of the TH neurons in the graft periphery revealed that there was greater primary branching and TH cell body area was greatest in the VM + Pitx3 group. Girk2 is a G protein-coupled inward rectifying current potassium channel type2 and has been shown to define a ventral population of dopamine neurons (Mendez *et al.*, 2005; Thompson *et al.*, 2005). Indeed a greater proportion of the peripheral neurons in the VM + NSptx3 group were also Girk2 positive. This is in agreement with two previous studies demonstrating that a higher proportion of the TH/Girk2 co-expression in transplanted dopamine neurons and a peripheral distribution of these neurons in the graft-host interface in the striatum (Mendez *et al.*, 2005; Thompson *et al.*, 2005). This suggests that the localization and greater number of TH/Girk2 positive cells in the VM + NSptx3 group results in a greater interaction with the host striatum which ultimately results in better functional recovery as shown here. Interestingly, we saw the reverse distribution of calbindin positive neurons, in that they were predominately located in the centre of the transplant also in line with previous studies (Mendez *et al.*, 2005; Thompson *et al.*, 2005). Thompson and colleagues have injected cholera toxin B (CTB), into the dorsolateral striatum, and found the CTB$^+$/TH$^+$ cells were almost exclusively located in the periphery of the graft and are Girk2$^+$. However, when they inject CTB in the frontal cortex, the majority of CTB$^+$/TH$^+$ cells are located in the centre of the graft and are calbindin positive. This provides evidence that the axonal outgrowth from the two cell types is differentially regulated and that the correct subtype of dopamine neurons is required for innervation of the dorsolateral striatum (Thompson *et al.*, 2005).

This present study highlights the importance of the correct type of DA neuron and its specific location with the graft. These parameters may be critical in influencing the therapeutic potential of stem cell derived dopamine neurons. This study provides evidence that it is possible to induce a DA phenotype in NSCs that have lost their ability to produce dopamine, a phenomenon that occurs after short-term expansion (Caldwell and Svendsen, 1998). This shows that Pitx3 is important in specifying an A9 phenotype when exposed to the correct signals from the developing VM. Indeed recent studies have provided evidence that it is the DA neurons from the substantia nigra zona compacta (A9) and not the ventral tegmental area (A10) that provide therapeutic value in cell replacement in PD (Mendez *et al.*, 2005; Thompson *et al.*, 2005). In support of this a recent autopsy study demonstrated that A9 (Girk2 positive) neurons, represented 40–50% of the surviving DA neurons in two PD patients who had successfully benefited from VM allografts (Mendez *et al.*, 2005). Hence, strategies aimed at increasing this population of A9 neurons could have major implications for the treatment of PD.

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