Characterization of axon guidance cue sensitivity of human embryonic stem cell-derived dopaminergic neurons

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A B S T R A C T
Dopaminergic neurons derived from human embryonic stem cells will be useful in future transplantation studies of Parkinson's disease patients. As newly generated neurons must integrate and reconnect with host cells, the ability of hESC-derived neurons to respond to axon guidance cues will be critical. Both Netrin-1 and Slit-2 guide rodent embryonic dopaminergic (DA) neurons in vitro and in vivo, but very little is known about the response of hESC-derived DA neurons to any axonal guidance cues. Here we examined the ability of Netrin-1 and Slit-2 to affect human ESC DA axons in vitro. hESC DA neurons mature over time in culture with the developmental profile of DA neurons in vivo, including expression of the DA neuron markers FoxA2, En-1 and Nurr-1, and receptors for both Netrin and Slit. hESC DA neurons respond to exogenous Netrin-1 and Slit-2, showing an increased responsiveness to Netrin-1 as the neurons mature in culture. These responses were maintained in the presence of pro-inflammatory cytokines that might be encountered in the diseased brain. These studies are the first to evaluate and confirm that suitably matured human ES-derived DA neurons can respond appropriately to axon guidance cues.

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Introduction
Parkinson's disease (PD) is characterized by neurodegeneration of the midbrain substantia nigra (SN) where loss of dopaminergic (DA) neurons leads to deterioration of motor control (Savitt et al., 2006). Pharmacological interventions are initially effective but decline with advancing disease. There remains a large unmet need for late-stage treatment options (Jankovic, 2006). Transplantation of dopaminergic neurons into patients to reinervate the target areas in the striatum has been clinically explored but trials have been halted since 2001 as some patients showed an increase in dyskinesias after treatment.

Given the advances in pluripotent stem cell technology and difficulty of obtaining the necessary numbers of viable dopaminergic neurons from aborted fetuses, the resumption of transplantation trials will most assuredly be carried out using DA neurons derived from pluripotent stem cells, such as human embryonic stem cells (hESC) or human induced pluripotent stem cells. These clinical trials will only proceed however after critical questions concerning the molecular and biological maturation of hESC DA neurons in vitro are assessed. How do hESC DA neurons mature over time in culture? Is there an ideal stage in maturation in which hESC-derived DA neurons respond to biological cues that control connectivity? Which guidance cues are important?

While members of the Netrin, Slit, Semaphorin, Ephrin and Shh families have been shown to affect embryonic DA axons in vivo (Hammond et al., 2009; Hernandez-Montiel et al., 2008; Lin et al., 2005) or in vitro (Bagri et al., 2002; Kolk et al., 2009; Torre et al., n.d.; Yue et al., 1999), we have focused in these initial studies on the Netrin and Slit families.

Native rodent DA neurons respond to Netrin-1 and Slit-2 in vitro, attracted by Netrin-1 and repelled by Slit-2 (Lin et al., 2005). When tested on mouse ESC (mESC) DA neurons, Slit-2 prevented outgrowth, however, whereas Netrin-1 promoted outgrowth, it failed to have its signature guidance effect, suggesting that DA neurons derived from ES cells may be inherently different from native DA neurons (Lin and Isacson, 2006). No guidance cues have ever been tested on human ESC DA neurons.

Here we show that the molecular and biological time course of maturation of hESC DA neurons in vitro occurs consistently, and follows the known developmental biology of fetal DA neurons (Lee et al., n.d.; Perrier et al., 2004). In a subset of cells, we observe the natural sequence of floor plate induction, and the expression of the dopaminergic neuronal markers En1 (Simon et al., 2004), Nurr1 (Wallen et al., 1999) and FoxA2 (Kittappa et al., 2007), all considered appropriate markers of DA neuronal induction from ES cells (Perrier et al., 2004; Sasai, 2005; Schulz et al., 2004; Yan et al., 2005; Yang et al., 2008). In response to guidance cues we find that hESC DA neurons are attracted to Netrin-1, and repelled by Slit-2 attaining peak responsiveness to these cues over time in culture, confirming that the timing of DA transplant is an important variable for future studies using hESC-derived neurons in therapy.
Results

The maturation of hESC DA neurons over time in culture

The induction of hESC cells to DA neurons was carried out by differentiation of the cells to the neuroectodermal lineage by co-culture with MS5 cells, followed by replating at approximately day 28 (D28) and further patterning in the presence of SHH (200 ng/ml) and FGF8b (100 ng/ml) (Perrier et al., 2004). The neuronal marker doublecortin (DCX) (Fig. 1A,D,G,J) showed an increase in staining intensity over time, as did the DA neuron marker tyrosine hydroxylase (TH) (Fig. 1M–P). Glial fibrillary acidic protein (GFAP), which marks astrocytes, was not detected by immunochemistry (Fig. 1Q–T). Co-staining for Nestin (a marker of neuronal precursors) and DCX allowed visualization of the newly generated neurons in the cultures which were always first detected in the vicinity of Nestin+ rosettes (Fig. 1C,F), i.e., circular rings of elongated neuroepithelial cells, thought to be “mini neural tubes” (Elkabetz et al., 2008) (Fig. 1C,F,I,L; yellow arrow). The number of rosettes that contained TH+ neurons increased over time from 45% at D28 to 56% at D42. DA release was detected in these cultures by HPLC at D42 (Supp. Fig. 1).

To further characterize differentiation over time in the hESC cultures, the expression of 14 genes was examined by qPCR using material from undifferentiated ES cells, cells on D28 of neural induction, and on D35, D42 and D49 (or 7, 14, and 21 days after patterning with SHH and FGF8b). In addition neurons differentiated from hESC months apart, in 5 different cohorts, were pooled for these experiments.

Three pluripotency-related genes Oct3/4, Tert, Nanog, all decreased significantly during the course of differentiation (Fig. 2G). Some genes are expressed both in early and later stages of midbrain development so a change in overall expression level in the cultures over time was not expected. For these genes, we additionally examined their expression by ISH/IF for TH to show the spatial relationship between the gene of interest and developing DA neurons. The HMG box transcription factor Sox2 (Fig. 2A), a marker of both stem cells and of the DA neuron zone of proliferation (Bonilla et al., 2008), did not change over time by qPCR, but by ISH/IF was highly expressed both at the center of the rosettes (Fig. 2A*; white arrowhead) and closely interspersed with, but not co-expressed in TH+ DA neurons (Fig. 2A*; white arrows show TH+ DA neuron not co-labeled for Sox2). DA neurons arise directly from the midline...

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**Fig. 1.** Formation of rosettes and the induction of neurons in cultures of hESC cells over time. hESC cells were co-stained for DCX (doublecortin) and nestin (progenitors; rosettes) to examine the relationship between newly generated neurons (DCX) and their progenitors (Nestin), and counterstained for DAPI. TH, a marker of DA neurons, K67 (blue) and GFAP a marker of astrocytes were also examined. A–L (same fields) and M–T (different fields); D28 (day of addition of SHH and FGF8b): top row, D35; second row, D42; third row, D49; fourth row. Yellow arrows point to rosettes. Scale bar (A–T; 200 um).
midbrain DA neurons (Davis and Joyner, 1988; Kittappa et al., 2007; for the development and/or survival of DA neurons and which mark midbrain epithelium) (Crossley et al., 1996; Simon et al., 2004). Are normally expressed in both differentiating DA neurons and en-1 (Fig. 2B, C) showed steady levels of expression over time (both are normally expressed in both differentiating DA neurons and midbrain epithelium) (Crossley et al., 1996; Simon et al., 2004). More interestingly, by ISH/IF, FoxA2 was detected surrounding the center of the rosettes and co-expressed in TH+ DA neurons (Fig. 2B, B; yellow arrows show co-expression in Fig. 2B*), as expected, and en-1 was highly expressed in cells just adjacent to and in TH+ neurons on D42 (Fig. 2C, C; white arrowheads and yellow arrows, respectively). Nurr1, expressed in young midbrain DA neurons before and after the onset of TH, increased significantly over time in culture as measured by qPCR (Fig. 2D), and by ISH/IF was highly expressed both in TH+ (dopaminergic precursors; white arrowhead in Fig. 2D*) and in TH+ neurons emanating from the rosette (yellow arrows; Fig. 2D*). Further there is increased expression of ALDH1A1, a marker of embryonic substantia nigra DA neurons (McCaffery and Drager, 1994), the particular DA neurons that are necessary for the motor control lost in Parkinson’s patients (Fig. 2K).

Later markers of DA neurons such as gamma synuclein (γSyn) and the vesicular monoamine transporter VMAT (Hansson et al., 1998), showed significant increases over time in culture by qPCR (Fig. 2L,J), both over hES cells and hES cultures on D28, suggesting further maturation of the DA neurons. Finally, we examined whether non-DA neurons were prominent in our cultures by measuring the levels of GAD67 (for GABAergic neurons), HBB (for motor neurons), TPH1 (for serotonergic neurons), and GAPD (for glial cells) (Fig. 2E, F, L, M). None of these genes showed significant increases in expression up to D42 in culture, either over baseline ES cell levels or compared to ES cells grown for 28 days in culture.

By ISH/IF, a few identifiable GAD67 or HBB positive cells could be seen at D42, and these cells were not TH+ (Fig. 2E,F; white arrowheads). However, the expression level of each of these genes rose marginally over time suggesting that neuronal contaminants may increase over time in culture, but that there is a significant period during which TH+ neurons predominate over, moto-, serotonergic and GABAergic neurons (Fig. 2E, F, L, M) with TH levels measured by qPCR 10–100 fold more elevated over these other neuronal cell types (all normalized to GAPDH).

The expression of markers over time in hESC cultures. (A–M; qPCR, (A–F and A′–F′) combined ISH/IF of markers for neuronal differentiation, floor plate synthesis, DA, GABAergic and moto-neuron development TH in green; mRNA for named gene in red. Bars in graphs (A–F), dark bars show levels of TH, gray bars, level of named gene over time. (G–M) bars show level of named gene over time. mRNA was examined from samples taken from 5 different courses of differentiation. Samples were measured in triplicate. Shown is standard error of the mean for all samples. * significantly different from D28, p < 0.05, † significantly different from ES, p < 0.05 by ANOVA and post hoc Tukey HSD. Yellow arrow, co-expression of TH and gene of interest, white arrowhead, expression of gene of interest in non-dopaminergic cell, white arrow, expression of TH, in the absence of the gene of interest. Scale bar (A′–F′; 200 um; A,B′–D′; 70 um; C′ 50 um).

Responses of hESC DA neurons to guidance cues

We next compared the ability of D42 hESC-derived DA neurons and native rodent midbrain DA neurons to respond to guidance cues in vitro using explants from ventral embryonic midbrain, or aggregated hESC co-cultured in a collagen gel (Hyms et al., 1995) with COS cells secreting Ntn-1 or Slt-2. Cultures were fixed and stained for TH, and only TH+ axons were considered for outgrowth analysis. Under these conditions, rat DA neurons showed a robust and significant response to both Ntn-1 and Slt-2 (Fig. 3A–C and J, left bars; Student t-test p < 0.05 for Ntn-1, p < 0.01 for Slt-2), consistent with a previous report (Lin et al., 2005). When aggregates of hESC containing DA neurons were used in this assay, we obtained an identical response: hESC DA axons were significantly attracted by Ntn-1 and repelled by Slt-2 (Fig. 3G–I and J, right bars; Student t-test p < 0.01). Like rat neurons (Lin et al., 2005), outgrowth of hESC DA neurons in response to Ntn-1 was blocked by the addition of an antibody to the Ntn receptor DCC (Supp. Fig. 2).

Under identical conditions used for rat or human neurons, neither native mouse DA neurons nor mESC DA neurons showed a significant response to Ntn-1 or Slt-2 (Fig. 2D–F, middle bars; not significant) (Supp. Fig. 3 and see Discussion).

Assessing the time course over which hESC DA neurons gain the ability to respond to guidance cues (Jankovic, 2006) and understanding the developmental profile of DA neurons in culture may facilitate future use of these cells in trials of transplantation therapy for PD. To this end, we first examined the hESC cultures for expression of the Netrin and Slt receptors, as measured by qPCR and combined ISH/IF.

The expression of DCC, the Ntn-1 receptor that mediates attraction, increased significantly over time compared to naive hESC and hESC that had not been patterned by SHH and FGF8b (Fig. 4A). When examined by ISH/IF, all TH+ neurons appeared to express DCC, whereas non-TH+ neurons showed much lower expression levels (Fig. 4A*; co-labeled cells marked by yellow arrows in Fig. 4A*).

UNC5A–D are thought to mediate repulsion to Ntn-1. Unc5a did not increase over time but was detected at low levels in many TH+ DA neurons (Fig. 4B, B′; yellow arrows), but not all (white arrow). At D42 Unc5b showed low level expression in some TH+ neurons but not in most (Fig. 4C*), whereas Unc5d was not detectable in TH+ neurons (Fig. 4E). In contrast, Unc5c was highly expressed in the cultures, increasing significantly over time (Fig. 4D) with high-level expression at D42 in some DA neurons (Fig. 4D*, yellow arrows), and very high-level expression in non-DA neurons (Fig. 4D*, white arrowhead). Some DA neurons did not express Unc5c at levels above background (Fig. 4D*; white arrows) (see Discussion).

Robo1 and Robo2, both receptors that mediate the repulsive effects of Slt-2, had very different expression patterns. Robo1 was not detectable in TH+ DA neurons at D42 and did not change over time (Fig. 4F, F′), whereas Robo2 increased over time and was highly expressed in all TH+ DA neurons (Fig. 4G, G′, G″). Both Robo1 and 2 bind to Slit ligands, however analysis of mutant mice has suggested that in some contexts they might mediate distinct types of biological responses of the axons (Andrews et al., 2006). For comparison all TH+ neurons express TH (Fig. 4H).

Time-dependent changes in neurite outgrowth properties of human ESC DA neurons

We examined the ability of Ntn-1 and Slt-2 to affect the outgrowth of hESC DA neurons taken from the cultures at different stages of differentiation (on D35, 42 and 49). Although many TH+ neurons were present at D35, significant directional axon growth responses to Ntn-1 were not detected (ANOVA p > 0.05, followed by Tukey HSD; Fig. 5A–C and P, left bars; Ntn-1 NS). However Slt-2 did effectively block outgrowth of hESC DA neurons at this time point (ANOVA p < 0.01, Tukey HSD p < 0.01). By D42, the DA neurons in hESC aggregates responded significantly to both Ntn-1 and Slt-2 (Fig. 5D–F, and P, both p < 0.01 by Tukey HSD). The difference in outgrowth between proximal and distal axons at D42 is highlighted in Fig. 5 (J–O). In controls, axons grow from both sides of the explants.
In the presence of Netrin-1, there was distinct and significant growth in the proximal quadrant compared to controls (Fig. 5K,N). In the presence of Slit-2, TH+ axons showed very little growth towards the COS cells and some significant growth away (Fig. 5L,O). By D49, the response to both Netrin-1 and Slit-2 was still significant (Fig. 5G–I; and P, both $p < 0.01$, Tukey HSD). Thus, hESC DA neurons are responsive to Slit-2 by D35 in culture but gradually acquire responsiveness to Netrin-1 over time during differentiation in vitro. This may represent the normal development of DA neurons, or it may reflect these particular in vitro conditions (see Discussion).

Axon growth in the presence of pro-inflammatory cytokines

In animal models of PD and in human patients, transplanted DA neurons may be subjected to pro-inflammatory cytokines that could alter the responses of DA neurons to guidance cues. We assessed the response of D42 hESC DA neurons in the outgrowth assay in the presence of the pro-inflammatory cytokines IL1β, IL6, TNFα and TGFβ3 (Fig. 6A–O) and found that they did not block the ability of hESC neurons to respond to either Netrin-1 or Slit-2 (Fig. 6; compare E, H, K, N to B). IL-6 and its receptor are expressed in many brain regions (Gadient et al., 1995) and were suspected of playing an important role in midbrain DA regenerative axon sprouting in vivo (Parish et al., 2002). A more systematic examination was therefore made for IL6. Under these conditions IL-6 itself induced a statistically significant outgrowth response (Student’s t-test, Fig. 6P; left bars, $p < 0.01$), and when IL-6 was added to Netrin-1, the outgrowth was greater than with Netrin-1 alone (Fig. 6P; middle bars, $p < 0.05$). As well, the outgrowth in the presence of Netrin-1 plus IL6 was greater than with IL6 alone (Fig. 6P, $p < 0.01$). Thus, IL-6 has a modest outgrowth promoting effect that synergizes with the Netrin-1 response. In addition, IL-6 did not reverse the inhibitory effect of Slit-2 (Fig. 6P, right bars, $p < 0.01$). These results indicate that cytokines do not impair responses to Netrin-1 or Slit-2.

Discussion

Our studies show that the differentiation of human ESC to DA neurons can be replicated with high fidelity and that these neurons show robust responses to the axonal guidance cues Netrin-1 and Slit-2.
The induction of DA neurons from hESC cells is reproducible and stable over time

Over time and across different cultures we detect the expression of markers indicating a loss of pluripotency, the genesis of DA neurons, and the lack of generation of substantial numbers of some common neuronal types. hESC DA neurons can only be useful as transplantable material if one can reliably predict the time course over which cells mature in vitro, and define the optimal stage for transplantation, based on molecular and biological properties. Our experiments suggest that hESC undergo a steady maturation as they differentiate into DA neurons. By D42 in culture, the expression of the stem cell markers Oct, Tert, and Nanog were decreased significantly. Over this time period, the DA markers including En-1, FoxA2, and Nurr1 were highly expressed in the cultures as a whole and in many TH+ neurons examined by combined ISH/ICC. The DA neuron markers Nurr1, VMAT and γsyn were all significantly increased by D42. By this age TH had increased to a level that was 10–100 fold higher (normalized to GAPDH) than that of any other neuron-specific marker measured. However, although the expression of DA markers continued to rise through D49 in vitro, we also observed increased expression of markers for contaminating neurons at this time point. Our data suggest that under the differentiation conditions used here, D42 may be an optimal time for harvesting DA neurons from hESC cells.

Finally, the expression of two markers suggests that at least a portion of the induced DA neurons were substantia nigra DA neurons; first Aldh1, which is almost exclusively expressed by SN but not VTA DA neurons in the embryo (McCaffery and Drager, 1994) increased over time in these cultures. Second, it has been reported that Robo2 is primarily expressed in the SN and not the VTA (Marillat et al., 2002), thus the high levels of Robo2 in these cultures is consistent with at least a portion of these DA neurons being SN DA neurons.

hESC DA neurons respond robustly to guidance cues

Previous studies showed that midbrain DA neurons respond to both Netrin-1 and Slit-2 in vitro (Lin and Isacson, 2006; Lin et al., 2005), whereas ES DA neurons from mouse showed no guidance response to Netrin-1 (Lin and Isacson, 2006) but they are repelled by Slit-2. The lack of a guidance response to Netrin-1 suggests that native neurons and ESC DA neurons might be fundamentally different at least in their in vitro capabilities. Our data suggest that this is unlikely because neither native mouse nor mouse ESC DA neurons, tested under multiple growth conditions, showed significant responses to Netrin-1 or Slit-2 in vitro. The minor differences between our results and those of Lin et al. (Lin and Isacson, 2006) with regard to Slit-2 (we observed no response of mESC to either Netrin-1 or Slit-2) (Supp. Fig. 3) may be due to variations in the differentiation protocols used.
On the other hand, although Lin et al were able to confirm outgrowth promoting effects of Netrin-1, consistent with our results they did not observe any directional attractive effects of Netrin-1 (Lin and Isacson, 2006). Thus mouse DA neurons from both ES cells and fetal tissues of different ages fail to show a directional response to Netrin-1, suggesting that mouse ES cells are poor surrogates for human ESC in evaluating guidance response and targeting in vitro. Previous studies have suggested that mouse neurons in other brain regions also fail to show significant responses to guidance cues in vitro, suggesting that present culture conditions are not adequate to support guidance cue responses for many mouse neuron subtypes (e.g. (Okada et al., 2006). It is unlikely that mouse DA and mESC DA neurons are fundamentally different from human and rat DA neurons (as both Netrin 1 and Slit2 show guidance defects in mice in vivo; (Bagri et al., 2002; Li et al., in preparation), but when compared side by side in this assay, the ability of mouse neurons to respond to guidance cues is severely diminished compared to human and rat.

Importantly though, human ESC-DA neurons responded similarly to rat DA neurons, showing significant outgrowth in response to Netrin-1 and repulsion by Slit-2, with the responsiveness to Netrin-1 changing as the cells matured in culture. These results were observed in hESC DA neurons from multiple independent differentiation courses. The ability of hESC-derived neurons to respond appropriately to guidance cues therefore provides a novel bioassay with which to measure the biological maturity of hESC DA neurons in culture.

**Human ESC maturation compared to normal development of DA neurons**

For hESC DA neurons to be useful in transplantation therapy, it is important to identify stages at which they exhibit properties optimal for reinnervation of the damaged host striatum. In the fetal rat, that age is E12–15 when optimal yields of functional DA neurons for transplantation can be obtained (Barker et al., 1995; Dunnett, 1991; Torres et al., 2008). An E12 rat fetus corresponds approximately to a human embryo at about 33–37 days post-ovulation (Muller and O’Rahilly, 1987); we speculate that the 35–42 days that hESC neurons spend in culture after being removed from the feeder layer and

![Fig. 5. Response of hESC DA neurons to Netrin-1 and Slit-2 over time, in vitro. COS cells secreting a control plasmid (row 1), chicken Netrin-1 (row 2), or human Slit-2 (row 3), are marked by dashed yellow lines. DA neurons are stained with an antibody to TH (green). In all figures, outgrowth is marked by arrows, lack of growth by arrowheads. J–O shows higher power views of D42 explants (explants marked by red dashed line) showing proximal outgrowth (J–L) and distal outgrowth (M–O) in the same explant. Quantitation of outgrowth (ANOVA p < 0.5 with Tukey HSD) (P), left bars; D35, middle D42, right D49 with control (CTL); black bars, Netrin-1 (Net); light gray, Slit-2 (Slit); dark gray). * significantly different from control, p < 0.05, ** significant, p < 0.01 Data are represented as +/- SEM. Scale bar (A–I) 200 um, (J–O) 100 um.
allowed to pass from pluripotency to further stages of commitment might correspond to a maturation that approximates that stage of human fetal development. Interestingly, we observed a rise to peak outgrowth responses of hESC DA neurons to guidance cues during this period, at 42 days after induction, mirroring the relative optimal age for fetal donor cells using native rat tissue (E12–15 rat, corresponding

![Image]
roughly to human 33–37 days). Thus, the observed changes in the responsiveness of ES cells in vitro may represent a normal developmental peak in the ability to respond to guidance cues. Over this time period we also began to see an increase in the expression of Unc5 family genes, in particular Unc5c, a putative repulsive receptor for Netrin-1. Although Netrin-1 still exerted significant attraction at d49 we did notice an increase in the number of non-responding explants by d49 and it may be that the expression of Unc5c in some DA neurons at later ages in culture increases the variability of responses to Netrin-1 at older ages. This response of hESC DA neurons to guidance cues provides an additional criterion to monitor the differentiation of hESC DA neurons in vitro and to determine if they are mimicking their native counterparts in vivo, and may thus act as appropriate replacements for diseased DA neurons in PD patients.

**hESC DA neurons can still respond to guidance cues in the presence of cytokines**

Inflammatory cytokines have pleiotropic effects in the central nervous system, but their potential effects on neuron health or function after transplantation therapy is not well understood (Chen and Palmer, 2008). Inflammatory cytokines can inhibit spinal cord regeneration (Nakamura et al., 2003), and TNFα is implicated in the progression of PD (Sriram et al., 2002). Conversely, cytokines promote axon elongation after injury (Temporin et al., 2008) and outgrowth from NGF induced PC12 cells (Ihara et al., 1996), and can act as survival and differentiation factors for DA neurons (Guán et al., 2001; Ling et al., 1998). We show firstly that the addition of IL6 potentiates control outgrowth. Netrin-1 however was still able to stimulate growth significantly over the increased control outgrowth in the presence of IL6. Slit-2 remained inhibitory under these conditions. Thus the response to the guidance molecules Netrin-1 and Slit-2 are not impaired in the presence of pro-inflammatory cytokines. Further studies are needed to determine if inflammatory signaling in the degenerating brain alters outgrowth and connectivity of transplanted dopaminergic neurons.

In addition to providing an important bioassay, the in vitro analysis of axonal guidance and targeting, our data confirm that that hESC DA neurons respond appropriately to the Netrin-1 and Slit-2 guidance cues in vitro, and that they do so in the presence of pro-inflammatory cytokines they might encounter in the diseased brain. This supports the continued contemplation of clinical transplantation of these neurons. Guidance cues have multifaceted effects on neuronal migration (Hamasaki et al., 2001; Tanaka et al., 2008), survival (Furne et al., 2008), dendrite sprouting (Suli et al., 2006), and axonal fasciculation (Braisted et al., 2000). Documenting the ability of Netrin-1 and Slit-2 to affect the axons of DA neurons derived from hESC –3 days depending on cell density. In some experiments, cells were not passaged during differentiation in order to maintain larger, more compact colonies for removal into the directional outgrowth assay.

**Mouse ES cell maintenance and differentiation**

E14 mouse ES cells were maintained as a monolayer culture on gelatinized plastic in GMEM with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate, 100 μM beta-mercaptoethanol, and LIF. Cells were passaged every 2 days by trypsinization and a split ratio of 1:5. Mouse ES cells were differentiated according to Barberi et al. (2003). Briefly, mES cells were plated on a confluent layer (80,000 cells/cm²) of irradiated MS5 cells in serum replacement medium (SRM) consisting of Knockout DMEM, 15% Knockout Serum Replacement, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 100 μM beta-mercaptoethanol. Cells were maintained in SRM for 8 days and then switched to N2 media consisting of DMEM/F12 supplemented with 2 mM L-glutamate, 0.1 mM sodium pyruvate, and 100 μM beta-mercaptoethanol. Cells were maintained in N2 media for 8 days and then switched to N2 media consisting of DMEM/F12 supplemented with 2 mM L-glutamate, 0.1 mM sodium pyruvate, and 100 μM beta-mercaptoethanol.

**Explant culture**

Ventral midbrain (VM) explants were microdissected from embryonic day 14 (E14) (plug = D0) timed-pregnant Sprague-Dawley (Charles River) or E13 CD1 mice (bred in-house). ESC derived neural colonies were taken on day 35, 42, and 49 (hESC), or D8 and 11 (mESC). All were cultured with COS cell aggregates expressing Netrin-1 or Slit-2 in 3-D collagen gels (rodent explants cultured in DMEM/F12 (10% heat-inactivated FBS, penicillin/streptomycin) for 2 days). hESC and mESC DA neurons were cultured in their respective differentiation media for 3 days. The cytokines IL1b (4 ng/ml), IL-6 (50 ng/ml), TNFα (1 ng/ml), or TGFβ (10 ng/ml) (PeproTech, Rocky Hill, NJ), were added once at the time of explantation. All cultures were fixed with 4% PFA and stained as described previously (Hynes et al., 1995) with an anti-TH antibody.
(1:600, from Chemicon) to visualize dopaminergic neurons. In some cases human or mouse recombinant Ntr1 (company) was added once at the time of explant.

**COS cell aggregates**

COS cells (80% confluent in 6-well dishes) were transfected with 1 ug chick Ntrin-1, human Slit-2 cDNA (1 ug), or control vector with 3 μl FuGene 6 transfection reagent. 12–16 h later the cells were trypsinized, re-suspended in 90 μl DMEM/10% PBS and 20 μl drops were placed on the lids of 10 cm dishes, which were then inverted over dishes containing PBS. In each experiment one set of aggregates was stained with an anti-myc antibody to confirm expression of the guidance cue cDNA.

**Immunofluorescence**

Cells grown on glass coverslips were fixed with 4% PFA for 10 min, blocked (30° RT) in tris-buffered saline (TBS)/0.3% triton-x100/3% donkey serum, and incubated overnight (ON, 4°) with primary antibody. After washes in TBS/0.3% triton-x100 cells were incubated with secondary antibody (ON, 4°). Cells were washed and counterstained with DAPI (Calbiochem) and the coverslips attached to glass slides using PVA-DABCO.

**Combined in situ hybridization and immunofluorescence (ISH/IF)**

Six colonies were assembled in a collagen gel, fixed with 4% PFA, cryoprotected and embedded. Frozen sections were collected and the slides prehybridized for 1 h at RT, hybridized with 300 μg chick Netrin-1, human Slit-2 cDNA (1 ug), or control vector with TSATM plus Cy3 (Perkin Elmer). Cells were counter stained with DAPI, mounted with gel-mount medium, and imaged using an SLM-510 confocal microscope.

**Quantitation**

The number of TH+ axons in each explant was defined by the number of fluorescent pixels above a determined threshold (measured using Image J software, NIH, Bethesda, MD) in an area oriented towards (proximal) or away (distal) from the COS cells. Results from multiple experiments were pooled and analyzed using a Student t-test or a two-way ANOVA with post-hoc Tukey HSD.

**qPCR**

Total RNA was harvested (RNeasy Mini Kit (Qiagen)), and cDNA reverse-transcribed (Superscript III first-strand synthesis (Invitrogen)) with oligo (dt) primers. After 14 cycles of preamplification of 214 ng of cDNA using TaqMan PreAmp Master Mix (Applied Biosystems) and gene specific TaqMan primers (Applied Biosystems, see Supplementary Table 1), cDNA was diluted 1:5 in TE, and triplicate measures were made using a 96 x 96 Dynamic Array Chip (Fluidigm). Each sample had a no reverse-transcriptase control, and human fetal brain polyA mRNA (Clontech) was used to create standard curves for each primer set. Expression of each gene was normalized to GAPDH, and relative expression compared to non-differentiated hESCs is reported.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2010.07.004.

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