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

Branden J. Cord [b,1], Jie Li [a,1], Melissa Works [a], Susan K. McConnell [a], Theo Palmer [b], Mary A. Hynes [a,*]

[a] Department of Biology, Stanford University, Stanford, CA, USA
[b] Department of Neurosurgery, Stanford University, Stanford, CA, USA

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ABSTRACT

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.

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 reinnervate 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.

* Corresponding author. Department of Biology, Stanford University, 385 Serra Mall, Stanford, CA 94305-5020, USA. Fax: +1 650 725 9832.
E-mail address: mhynes@stanford.edu (M.A. Hynes).
[1] The first two authors contributed equally to this manuscript.

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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

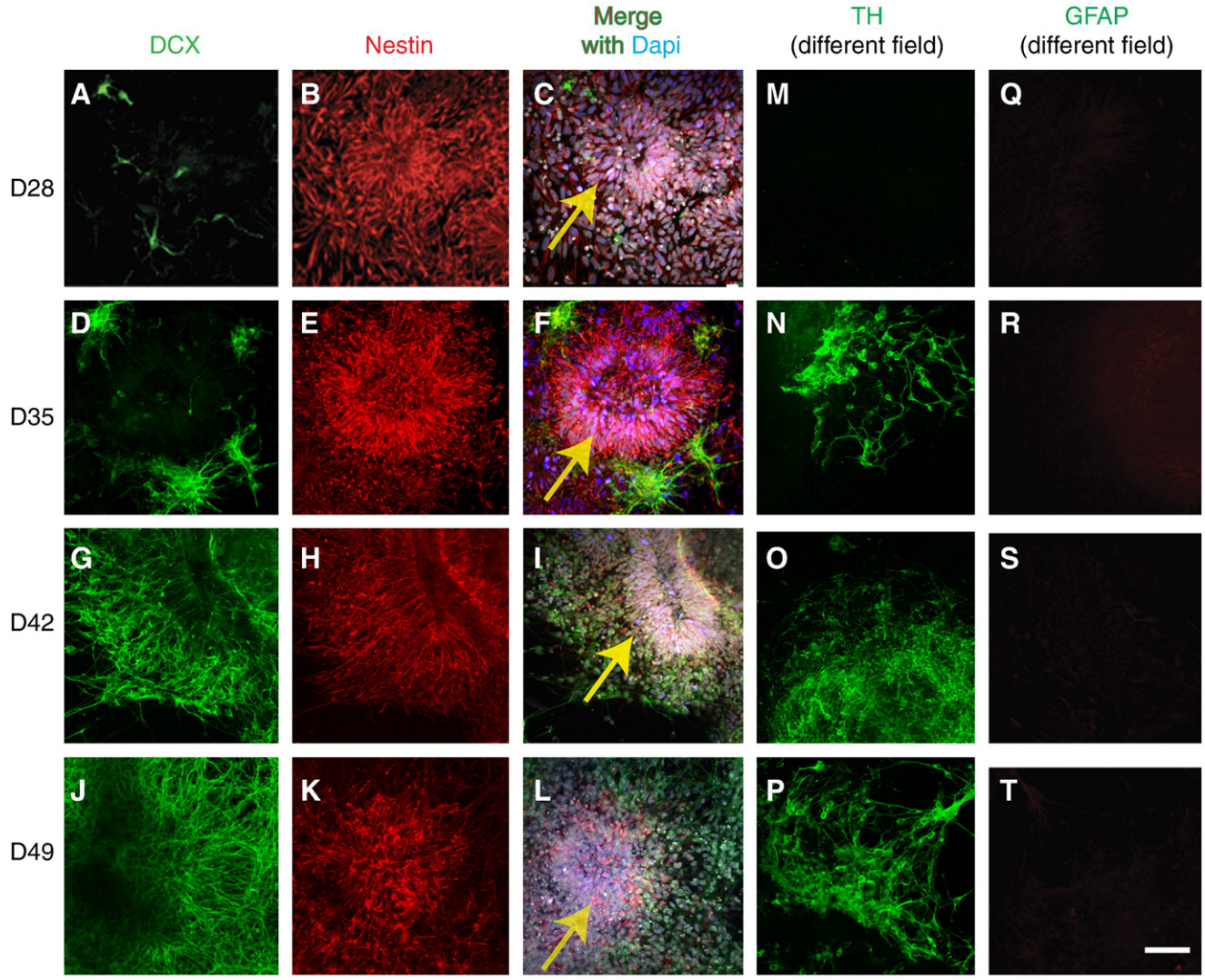
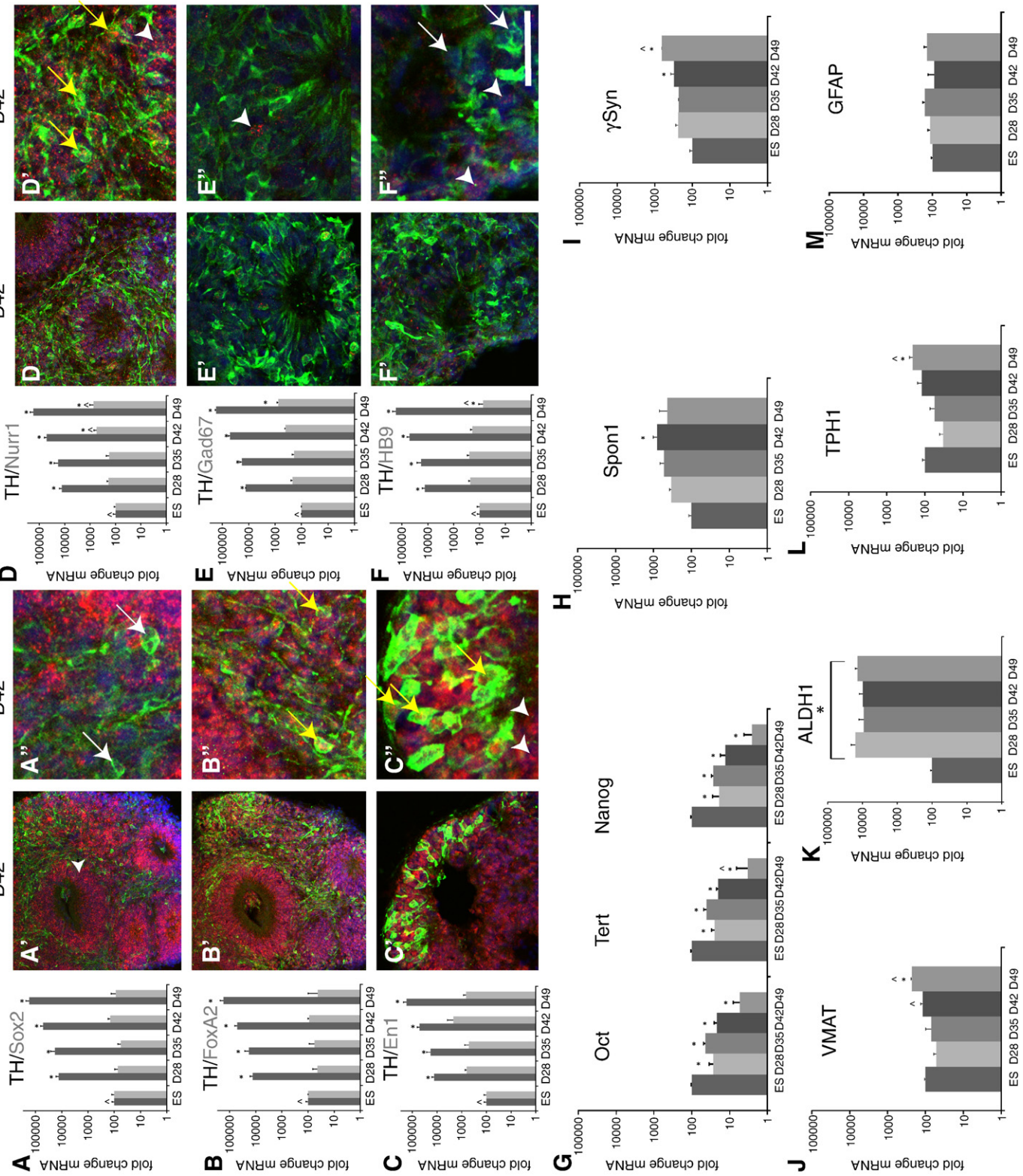


Fig. 1. Formation of rosettes and the induction of neurons in cultures of hESC cells over time. hESC cells were co-stained for DCX (doublecortin) and nestin (progenitors;rosettes) to examine the relationship between newly generated neurons (DCX) and their progenitors (Nestin), and counterstained for DAPI. TH, a marker of DA neurons, Ki67 (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).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TH/Gad67
TH/HB9
Oct
Tert
Nanog
Spon1
γSyn
VMAT
ALDH1
TPH1
GFAP
fold change mRNA
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D42
D42*
A' A'' B' B'' C' C'' D D' E' E'' F' F''

structure (the floorplate) (Bonilla et al., 2008; Kittappa et al., 2007), thus the presence of floorplate in these cultures is essential. *F-spondin* (*Spon-1*), a marker of floorplate (Klar et al., 1992), increased significantly over time in culture after induction by SHH and FGF8b (Fig. 2H), reaching a peak at D42 and decreasing by D49 thus mimicking the transient nature of the floorplate in vivo. *FoxA2*, *En-1 and Nurr-1*, members of a growing set of genes known to be necessary for the development and/or survival of DA neurons and which mark midbrain DA neurons (Davis and Joyner, 1988; Kittappa et al., 2007; Wallen et al., 1999), were examined by ISH/IF and qPCR. *FoxA2* 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 ALDH1, 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. 2I, 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), *HB9* (for motor neurons), *TPH1* (for serotonergic neurons), and *GFAP* (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 *HB9* 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).

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 (Hynes et al., 1995) with COS cells secreting Netrin-1 or Slit-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 Netrin-1 and Slit-2 (Fig. 3A–C and J, left bars; Student t-test $p<0.05$ for Netrin-1, $p<0.01$ for Slit-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 Netrin-1 and repelled by Slit-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 Netrin-1 was blocked by the addition of an antibody to the Netrin 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 Netrin-1 or Slit-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 Slit receptors, as measured by qPCR and combined ISH/IF.

The expression of *DCC*, the Netrin-1 receptor that mediates attraction, increased significantly over time compared to naïve 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 Netrin-1. *Unc5a* did not increase over time but was detected at low levels in many TH^+ DA neurons (Fig. 4B, B′, 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 Slit-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′, 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 Netrin-1 and Slit-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 Netrin-1 were not detected (ANOVA $p<0.05$, followed by Tukey HSD; Fig. 5A–C and P, left bars; Netrin-1 NS). However Slit-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 Netrin-1 and Slit-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

Fig. 2. 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 extracted from samples from 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–F″;70 um; C″ 50 um).

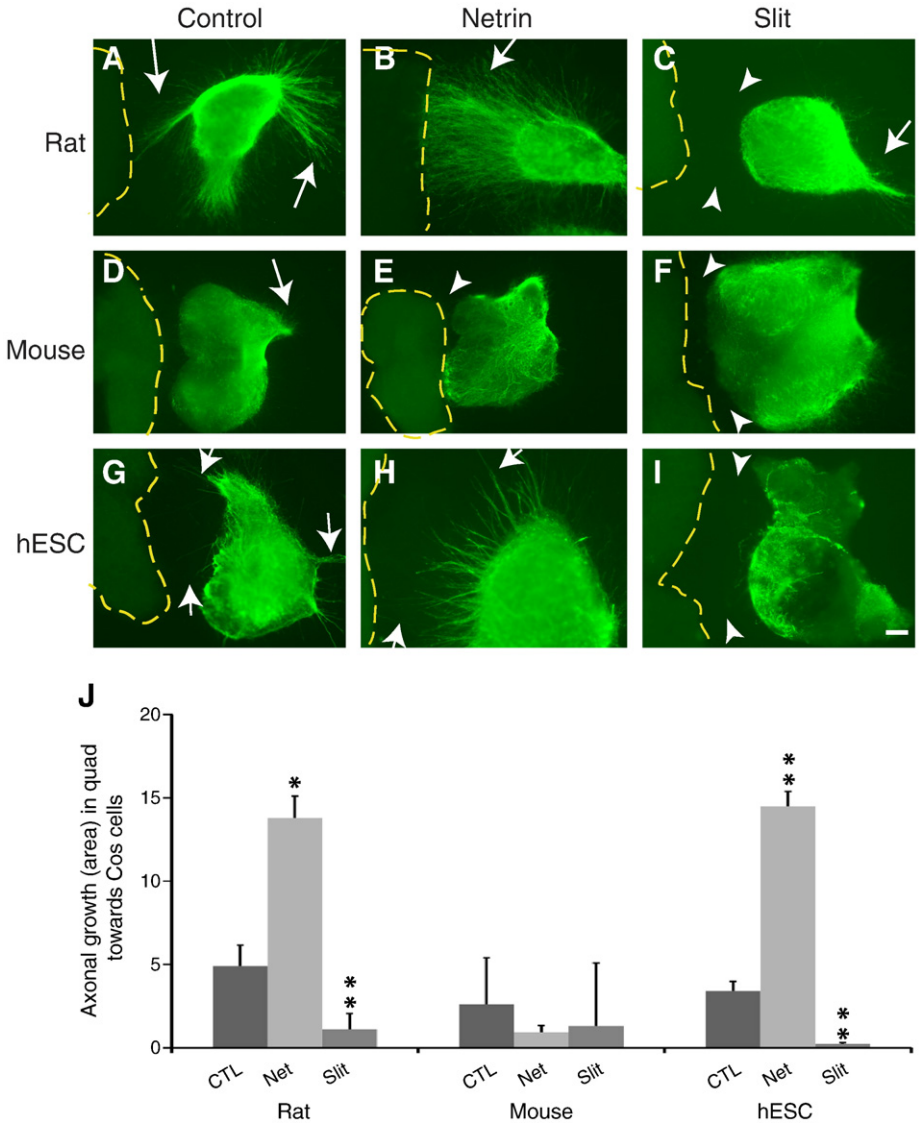

Fig. 3. Native rat and hESC DA neurons respond to Netrin-1 and Slit-2 in vitro. COS cells (secreting chicken Netrin-1 (column 2), human Slit-2 (column 3) or a control plasmid (column 1)) are marked by dashed yellow line. DA neurons are stained with an antibody to TH (green). In all figures, outgrowth is marked by arrows, lack of growth by arrowheads. Quantitation of outgrowth (J), left bars; rat ventral midbrain, middle bars; mouse ventral midbrain, right bars; hESC DA aggregates. Control (CTL); black bar, Netrin-1; light gray, Slit-2 (Slit); dark gray. * significantly different from control, $p<0.05$. ** significant, $p<0.01$by Student's t-test. Scale bar (A–I;250 um).

(Fig. 5J,M), but in the presence on 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 TGFβ (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.

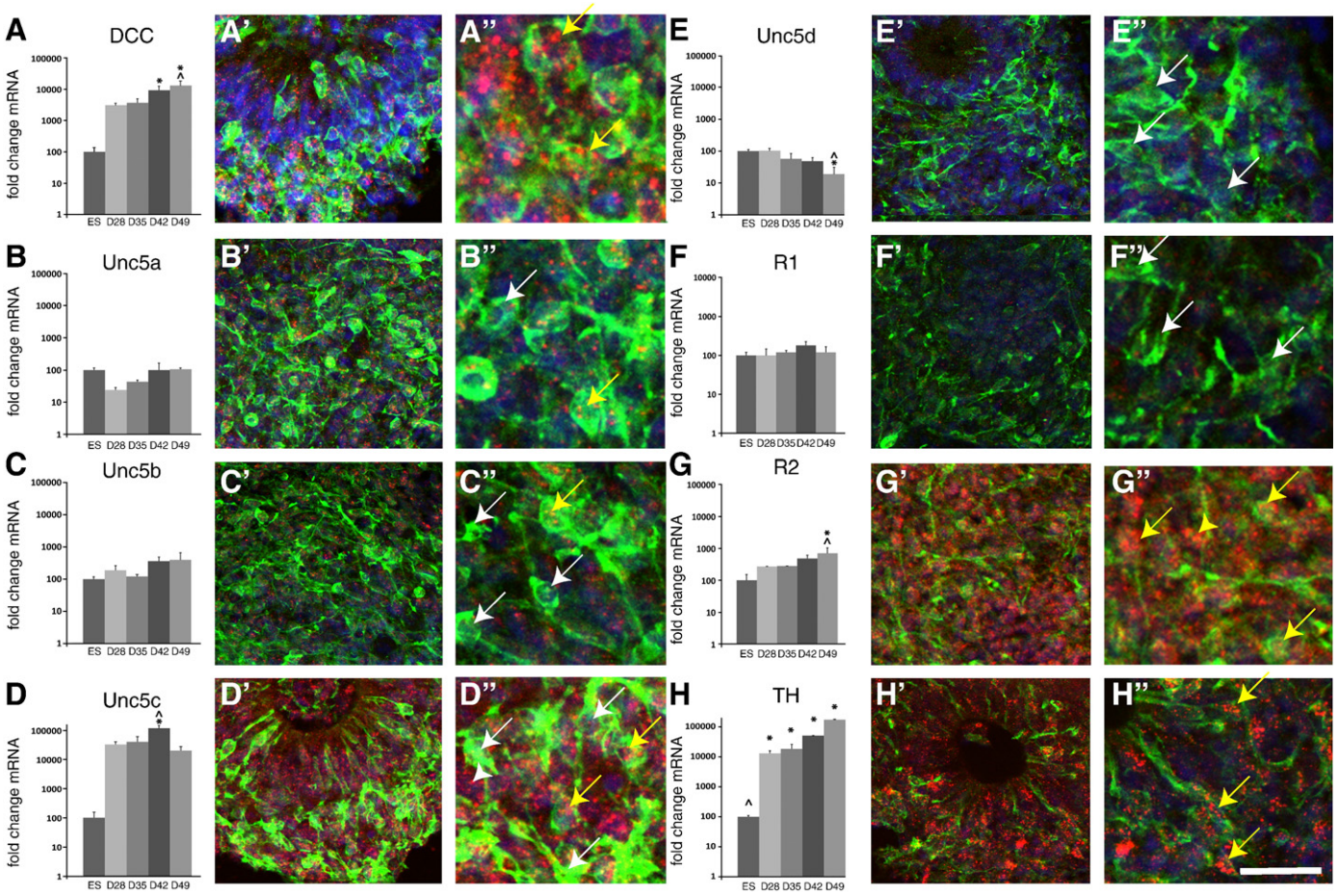

Fig. 4. Expression of Netrin-1 and Slit-2 receptors in hESC cultures. (A–H; qPCR), (A–H′ and A–H″) combined ISH/IF of receptors for Netrin-1 and Slit-2 (gene of interest in red); co-stained for TH in green. Bars in graphs (A–H) show level of named gene over time. mRNA was extracted from samples from taken from 5 different courses of differentiation. Samples were then measured in triplicate. Shown is standard error of the mean for all samples, pooled. **ES**, mRNA extracted from ES cells grown on a feeder layer, **D28**, cells grown in defined medium at the time of addition of SHH and FGF8b. **D35, D42 and D49**, 7, 14 and 21 days after addition of SHH and FGF8b. Measured by ANOVA and Neuman-Kuels. ^ significantly different from D28, $p<0.05$. * significantly different from ES, $p<0.05$. Yellow arrow, co-expression of TH and gene of interest, yellow 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′–H′, 200 um; A″–H″, 70 um).

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.

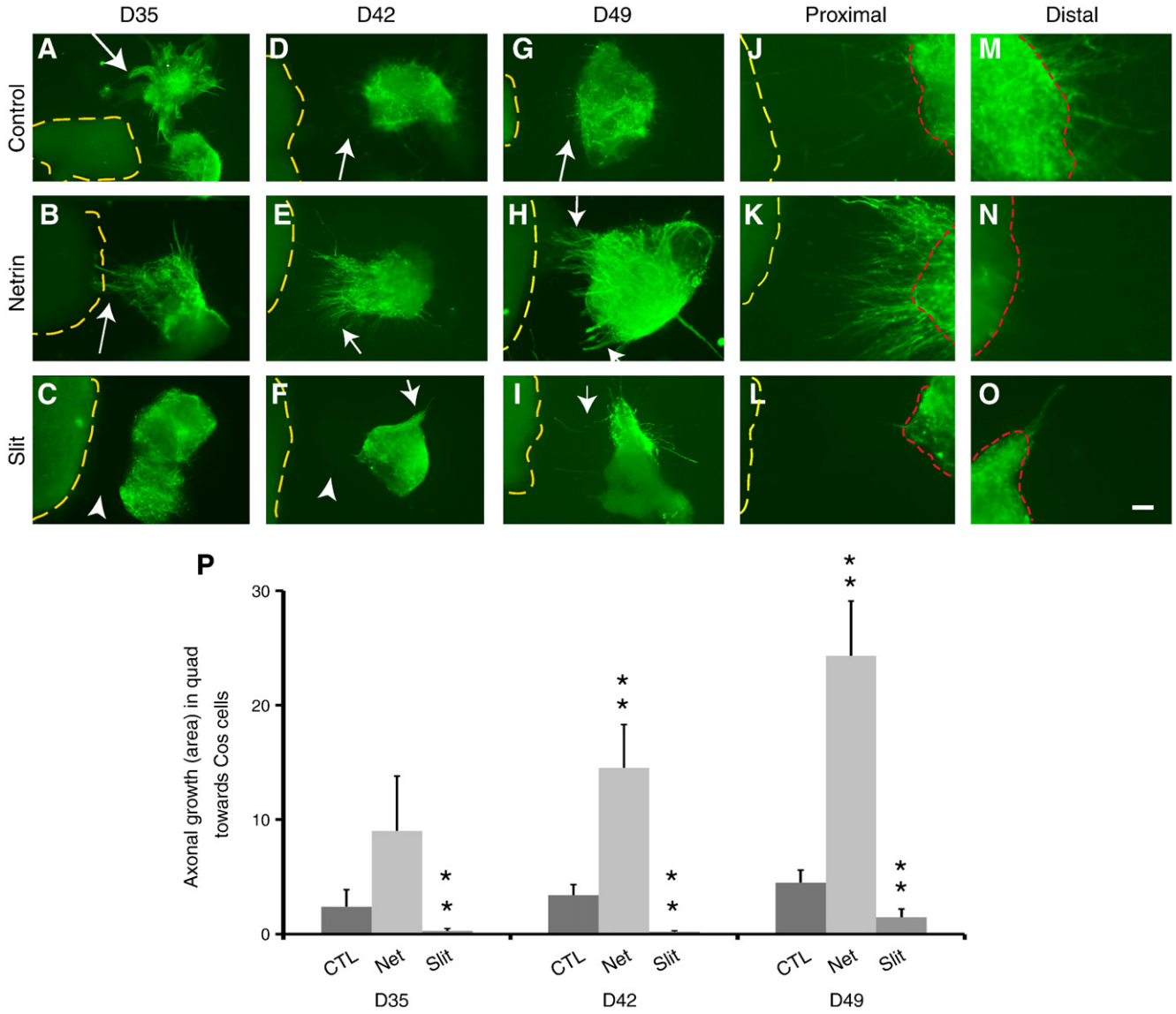

Fig. 5. Response of hESC DA neurons to Netrin-1 and Slit-2 over time, in vitro. COS cells secreting a control plasmid (row 1), chicken Netrin-1 (row 2), or human Slit-2 (row 3), are marked by dashed yellow lines. DA neurons are stained with an antibody to TH (green). In all figures, outgrowth is marked 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.

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 the 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

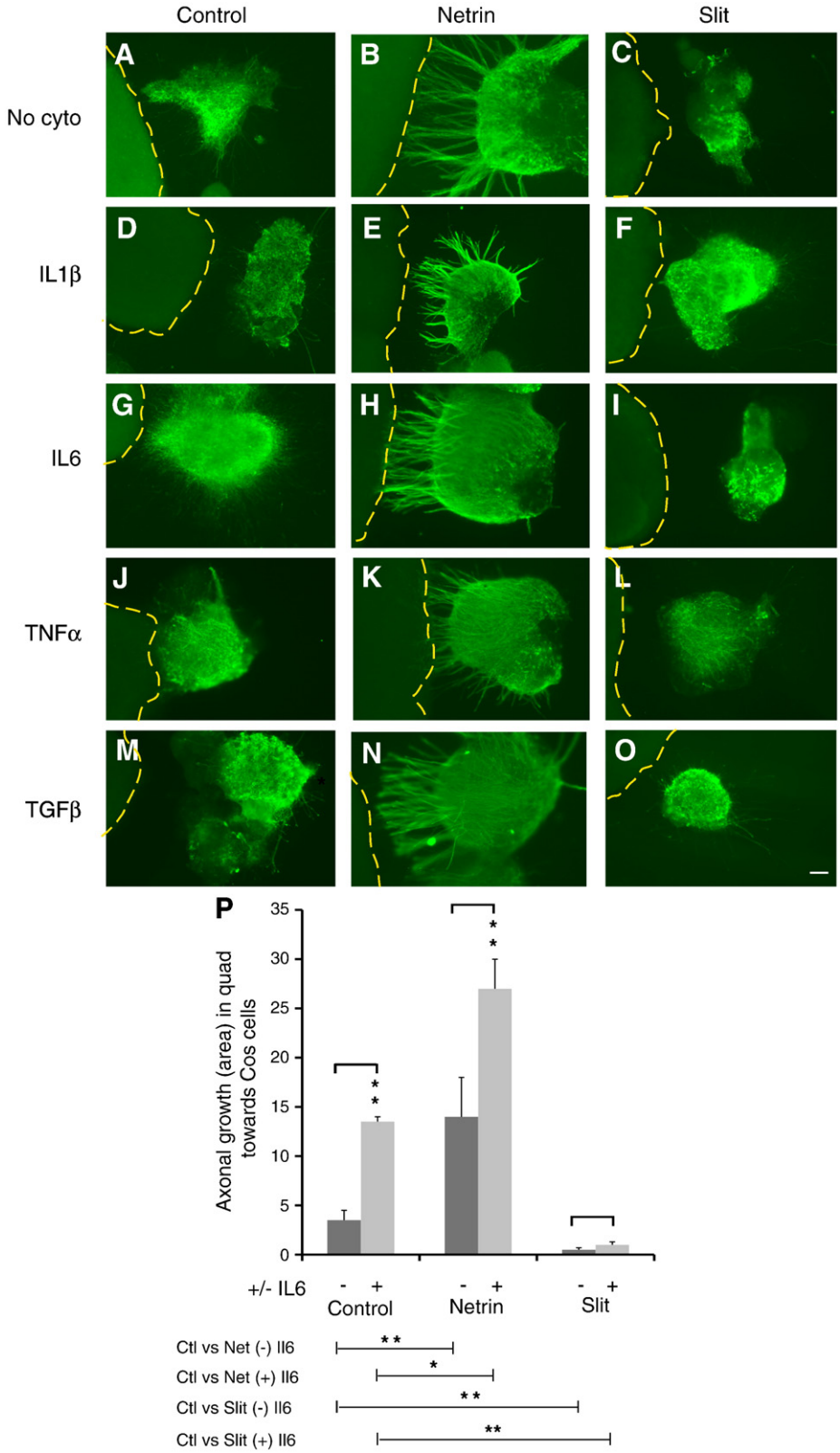

Fig. 6. hESC DA neurons respond to Netrin-1 and Slit-2 in the presence of pro-inflammatory cytokines. COS cells secreting a control plasmid (column 1), chicken Netrin-1 (column 2), or human Slit-2 (column 3), are marked by dashed yellow lines. DA neurons are stained with an antibody to TH (green). Quantitation of outgrowth (P), left bars; **D42** control +/− IL6, middle Netrin +/− IL6, right Slit +/− IL6. There was a significant difference between + and −IL6 for control (left; $p<0.01$), and for Netrin (middle; $p<0.05$), but not for Slit (right; NS). In addition both Netrin and Slit are significantly different from control in the absence of IL6 and in the presence of IL6 (bottom four horizontal bars) (Student t-test).

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

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 (Guan 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 represents a step toward a full characterization of how such cells might respond to guidance cues in vivo. Such information also predicts that the co-introduction of guidance ligands in vivo could serve as important adjunct therapeutics, for example to stimulate axonal targeting, branching or synapse formation in appropriate target regions. In the future it will be important to extend these findings to other families of guidance cues implicated in DA axon outgrowth including the Semaphorins (Hernandez-Montiel et al., 2008; Kolk et al., 2009; Torre et al., n.d.), the Ephrins (Yue et al., 1999) and Shh (Hammond et al., 2009).

Collectively these studies of the maturation of hESC derived DA neurons in vitro and their responses to guidance cues in vitro suggest that the dynamic changes in axon growth in response to guidance cues may impact the reinnervation of host tissue in animal models of PD and in the human brain. Our characterization of has revealed that many of the actions of guidance cues can be modeled in vitro. With this knowledge, we can begin to assess the optimal differentiation and maturation state of ES-derived DA neurons for transplantation in the treatment of Parkinson's disease.

Experimental methods

Human ES cell maintenance and differentiation

H9 human ES P25-60 (WiCell) cells were cultured and differentiated according to Perrier et al. (2004) with slight modifications. Cells were grown on matrigel coated dishes with mouse feeder cells (35,000 cells/cm^2), fed daily with DMEM/F12; 20% Knockout Serum Replacement (KSR), 2.6 mM L-glutamine, 0.1 mM non-essential amino acids, 100 μM beta-mercaptoethanol, and 8 ng/ml bFGF (all from Invitrogen) and passaged every 4–5 days using collagenase (1 mg/ml) and mechanical cutting.

For differentiation, hESCs were plated on a confluent layer (80,000 cells/cm^2) of irradiated MS5 cells in serum replacement medium (SRM) (Knockout DMEM:15% KSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 100 μM beta-mercaptoethanol). On day 16 the cells were switched to N2 media (DMEM/F12:2 mM L-glutamine, 20 nM progesterone (Sigma), 100 μM putrescine (Sigma), and 30 nM sodium selenite (Sigma)). At 28 days, cells were mechanically cut and replated onto poly-ornithine/laminin coated dishes and cultured in N2 medium plus 200 ng/ml SHH, 100 ng/ml FGF8b, 20 ng/ml BDNF, 20 ng/ml GDNF (all from R&D Systems), and 200 μM ascorbic acid (Sigma). On D42, supplements were changed to 20 ng/ml BDNF, 20 ng/ml GDNF, 1 ng/ml TGF-β3 (R&D Systems), 1 mM dibutyryl cyclic AMP (Sigma), and 200 μM ascorbic acid. Medium was changed every 1–3 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/cm^2) of irradiated MS-5 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-glutamine, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite. From days 5–12, media was supplemented with FGF8 (100 ng/ml) and SHH (200 ng/ml) (R&D systems). From days 8 to 12, media was additionally supplemented with bFGF (10 ng/ml). From days 12 to 15, previous supplements were exchanged for ascorbic acid (200 μM) and BDNF (20 ng/ml). During differentiation, mouse cells were fed every 2–3 days.

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 Netrin1 (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 Netrin-1, human Slit-2 cDNA (1 ug), or control vector with 3 µl FuGene6 transfection reagent. 12–16 h later the cells were trypsinized, re-suspended in 90 µl DMEM/10% FBS 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 counter-stained 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 300–400 ng/ml of RNA probe ON at 56 °C. Following washing with 2X SSC for 10 min, 0.2X SSC for 1 h and B1 (Tris/NaCl) for 15 min, the slides were blocked with B2 (B1 plus 1% blocking reagent, Roche) and then incubated ON (4°) with anti-dig-POD (Roche) and rabbit anti-TH antibodies (1:500) in B2. After washes with B1/0.1% TX100, slides were incubated with FITC conjugated goat anti-rabbit antibody for 1 h at RT. After triple washes, POD signal was amplified 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 96×96 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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