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Axon responses of embryonic stem cell-derived dopaminergic neurons to Semaphorins 3A and 3C

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Abstract

Class 3 Semaphorins are a subfamily of chemotropic molecules implicated in the projection of dopaminergic neurons from the ventral mesencephalon and in the formation of the nigrostriatal pathway (NSP) during embryonic development. In humans, loss of mesencephalic dopaminergic neurons leads to Parkinson's disease (PD). Cell replacement therapy with dopaminergic neurons generated from embryonic stem cells (ES-TH⁺) is being actively explored in models of PD. Among several requisites for this approach to work are the adequate reconstruction of the NSP and the correct innervation of normal striatal targets by dopaminergic axons. In this work, we characterized the response of ES-TH⁺ neurons to Semaphorins 3A, 3C, and 3F, and compared it with that of tyrosine hydroxylase-positive neurons (TH⁺) obtained from embryonic ventral mesencephalon (VM-TH⁺). We observed that similar proportions of ES-TH⁺ and VM-TH⁺ neurons express Semaphorin receptors Neuropilin 1 and 2. Furthermore, the axons of both populations responded very similarly to Semaphorin exposure: Semaphorin 3A increased axon length, and Semaphorin 3C attracted axons and increased their length. These effects were mediated by Neuropilins, since addition of blocking antibodies against these proteins reduced the effects on axonal growth and attraction, and only TH⁺ axons expressing Neuropilins responded to the Semaphorins analyzed. The observations reported here show phenotypic similarities between VM-TH⁺ and ES-TH⁺ neurons, and suggest that Semaphorins 3A and 3C could be employed to guide axons of grafted ES-TH⁺ in therapeutic protocols for PD.

Keywords

Semaphorins; dopamine neurons; embryonic stem cells; neuropilin; collagen gels

INTRODUCTION

Embryonic stem (ES) cells give rise to cell types from the three embryonic germ layers. Among them, dopaminergic neurons are widely studied due to their potential in cell therapy for the neurodegenerative disorder Parkinson's disease (PD; Lindvall and Kokaia, 2006). Two main protocols have been developed to differentiate dopaminergic neurons from ES cells *in vitro*.

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One uses stromal cells (Barberi et al., 2003; Kawasaki et al., 2000; Shintani et al., 2008), and the other is a multi-step protocol including the formation of embryoid bodies and the use of growth factors (Kim et al., 2002; Lee et al., 2000). In both cases, the obtained tyrosine hydroxylase-expressing neurons (ES-TH⁺) resemble the A9 subtype which corresponds to the dopaminergic neurons from the substantia nigra pars compacta (SNc) in the ventral midbrain, assessed by the expression of markers such as Nurr-1, Pitx3, Lmx1b, Foxa2, RALDH1, calretinin and GIRK2 (Barberi et al., 2003; Diaz et al., 2009; Lin et al., 2005; Rodriguez-Gomez et al., 2007; Roy et al., 2006; Zhao et al., 2004).

SNc dopaminergic neurons project to the dorsolateral striatum forming the nigrostriatal pathway (NSP) which has an important role in the control of movement. In humans, the specific loss of SNc neurons leads to PD. Successful transplantation protocols for cell therapy of PD will require considerable knowledge regarding the identity of the neurons obtained from ES cells, and their capacity to maintain a phenotype similar to the endogenous dopaminergic populations upon transplantation (Isacson et al., 2003). Another aspect to consider is the behavior of dopaminergic axons in response to guidance cues, as projection to their normal target is desirable to re-establish the lost pathway (Mendez et al., 1996).

During development of the nervous system, axons project to their targets following stereotypical routes guided by molecular cues secreted by, or expressed at the surface of surrounding cells (Yamamoto et al., 2002). Some guidance molecules implicated in the projection of dopaminergic neurons to form the NSP are the Ephrin tyrosine kinase receptors (Sieber et al., 2004; Yue et al., 1999), the Slit proteins (Bagri et al., 2002; Lin et al., 2005), Netrin-1 (Lin et al., 2005; Livesey and Hunt, 1997), and the class 3 Semaphorins (Sema) 3A, 3C, and 3F (Hernandez-Montiel et al., 2008). The effect of some of these guidance molecules has been studied on ES-TH⁺ neurons; Netrin-1 enhanced axon growth while Slit1 and Slit3 impaired it (Lin and Isacson, 2006).

Class 3 Semaphorins are secreted proteins involved in the guidance of neuronal and nonneuronal cells, and they interact with receptor complexes formed by Plexins and Neuropilins (Koncina et al., 2007; Neufeld et al., 2007; Pasterkamp and Kolodkin, 2003; Suzuki et al., 2008). Using explants of ventral mesencephalon (VM) we previously observed that Sema3A increases the growth of TH⁺ axons, Sema3C attracts them and increases their length, whereas Sema3F repels them (Hernandez-Montiel et al., 2008), suggesting that these proteins are involved in promoting and directing dopaminergic projections during the formation of NSP. In the present study, we analyzed the response of growing axons of ES-TH⁺ neurons, obtained by a five-stage differentiation protocol, to the same molecules. We show that ES-TH⁺ neurons express the receptors Neuropilin1 (Npn1) and Neuropilin2 (Npn2) in a proportion similar to that of embryonic ventral mesencephalic dopaminergic neurons (VM-TH⁺). Moreover, under the same experimental conditions, ES-TH⁺ and VM-TH⁺ axons increase their length or are attracted, in response to specific Semaphorins. These results reveal a functional chemotropic resemblance between both assayed TH⁺ neurons, and suggest that class 3 Semaphorins could be employed to promote or guide axons of ES-TH⁺ neurons upon their transplantation in therapeutic approaches for treatment of Parkinson's disease.

MATERIAL AND METHODS

Animals

Wistar rat embryos at 13.5 and 14.5 days of gestational age (E13.5 and E14.5) were used in accordance with the regulations of the Mexican government regarding the use of laboratory animals for research purpose (NOM-062-ZOO-1999) and following *The Guide for the care and use of laboratory animals* of the Institute of Laboratory Animal Resources, U.S. National Research Council. Pregnant dams were euthanized by cervical dislocation by trained personnel

with a minimum of distress for the animal. The day of detection of vaginal plug was considered E0.5.

Embryonic Stem Cell Differentiation

R1 ES cells were differentiated into TH⁺ neurons using the five-stage protocol as previously described (Diaz et al., 2009; Diaz et al., 2007). ES cells were expanded undifferentiated (stage I) in the presence of 1000 U/ml of leukemia inhibitory factor (Chemicon, Temecula CA, USA). Subconfluent ES cultures were detached from the culture plate by trypsin-EDTA (Gibco, Invitrogen, Carlsband CA, USA) treatment, and seeded on bacterial culture dishes to allow formation of embryoid bodies (stage II). After four days in culture, embryoid bodies were transferred to tissue culture dishes and incubated for 7–11 days with serum-free medium supplemented with insulin, transferrin, selenite, and fibronectin (ITSFn) to select nestin-positive neural precursor cells (stage III). To expand the nestin-positive population (stage IV), cells were detached with trypsin and the cell suspension was plated on 24-well plates and incubated with N2 medium (Gibco, Invitrogen) supplemented with 10 ng/ml fibroblast growth factor (FGF-2) (Peprotech, Rocky Hill NJ, USA), 100 ng/ml FGF-8b (Peprotech), and 100 ng/ml recombinant human sonic hedgehog (Shh; R&D systems, Minneapolis MN, USA) for 4–6 days. Final differentiation was induced by incubation with N2 medium supplemented with 200 μ M ascorbic acid (Sigma, St Louis MO, USA) and without growth factors (stage V).

Collagen Gel Co-cultures

Human embryonic kidney (HEK) 293 cells were mock-transfected or transfected with expression vectors for Sema3A, Sema3C, or Sema3F using the FuGene reagent (Roche, Indianapolis, IN, USA). Cells were detached with trypsin-EDTA 24 hrs after transfection, washed, and re-suspended in a collagen solution extracted from rat tail at 7000 cells/ μ l. Drops of 1 μ l of this suspension were dispensed onto a culture dish and polymerized for 40 min at 37°C, 5% CO₂ as described (Hernandez-Montiel et al 2008). For co-cultures, differentiated ES cells at day 3 of stage V were trypsinized and re-suspended in the collagen solution at a final concentration of 300 cells/ μ l. Drops of 35 μ l of the collagen-ES cell suspension mix were placed over the cell clusters of HEK293 cells included in mini-gels, prepared as described above. The new gel was allowed to polymerize for 40 min at 37°C, 5% CO₂, and then N2-supplemented DMEM-F12 medium containing 0.2 mM ascorbic acid was added to each co-culture to allow neurons to develop their processes for 48 h. For dissociated embryonic ventral mesencephalic (VM) co-cultures, tissue from E13.5 or E14.5 embryos was obtained in Hank's solution (Gibco, Invitrogen) and dissociated as described previously (Hernandez-Montiel et al., 2008). Briefly, VM portions were treated with trypsin and partially dissociated using a Pasteur pipette. The resulting suspension was centrifuged, re-suspended in collagen solution, and placed over a cell cluster of HEK293 cells previously included in a small collagen gel as described above for co-cultures with differentiated ES cells. To block Neuropilins, co-cultures were incubated for 2 days with antibodies specific for Npn1 and/or Npn2 (R&D systems) at a concentration of 5 μ g/ml and 3 μ g/ml, respectively, or with purified normal goat IgG (R&D systems), at equivalent concentrations.

Immunostaining

Cultures were fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS), washed extensively with PBS and blocked with 5% pre-immune goat or horse serum in PBS. Gels were incubated with mouse monoclonal anti-TH (Sigma), rabbit anti-TH (Pel-Freez, Rogers AK, USA), mouse anti- β tubulin III (Covance, Berkeley, CA, USA), goat anti-Npn1 (R&D systems) or goat anti-Npn2 (R&D systems) antibodies in 0.1% Triton X-100/PBS. After extensive PBS washes, primary antibodies were detected with Alexa-Fluor secondary antibodies (Invitrogen,

Carlsband CA, USA). Immunostained co-cultures were mounted in Fluoromont-G medium (Southern Biotech Birmingham, AL, USA) and observed using confocal microscopy.

Statistical Analyses

The mean $\pm$ S.E.M. is presented for each experimental set, performed in 3–6 independent experiments. Table I shows the number of axons measured in each figure. The number of cells expressing Npn1 or Npn2 was compared between VM-TH⁺ and ES-TH⁺ cells, and analyzed with the Student's t-test using the SAS statistical program (SAS institute Inc; Cary, NC, USA). For axon outgrowth experiments, TH⁺ axons were measured in confocal images using the ImagePro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). The data obtained for each condition were analyzed by the Student's t-test using the SAS statistical program. For axon orientation experiments, the angles of growth of TH⁺ axons were measured in confocal images using the ImagePro Plus software. Axons in each condition were categorized in three possible angle ranges as depicted in Fig. 3A, and the frequency of axons in each angle category was determined. Only TH⁺ neurons that were clear of the HEK293 clusters were considered and therefore, dopaminergic cells located above the clusters were not quantified. These data sets were evaluated by ANOVA, and means were compared by the least square mean method using the SAS statistical program.

RESULTS

ES-TH⁺ neurons express the Semaphorin co-receptors Neuropilin1 and Neuropilin2

TH⁺ neurons were generated from ES cells by a five-stage differentiation protocol, yielding 32 ± 4 % (mean $\pm$ S.E.M., n=6) of neurons that express TH (not shown), in agreement with previous reports (Diaz et al., 2009; Diaz et al., 2007; Lee et al., 2000). We examined the expression of Semaphorin co-receptors Npn1 and Npn2 in differentiated ES cells at day 3 of stage V. This maturation stage has been previously used for transplantation of ES-TH⁺ neurons into the striatum of hemiparkinsonian rats to revert motoric symptoms (Kim et al., 2002; Rodriguez-Gomez et al., 2007). Differentiated ES cultures were fixed and immunostained for TH/Npn1 or TH/Npn2. Similar studies were performed in partially dissociated rat ventral mesencephalic (VM) cells from E13.5, a stage in which TH⁺ neurons are growing and can respond to Sema3C and 3F (Hernandez-Montiel et al., 2008). Dissociated VM cells grew in small cell clusters that contain TH⁺ neurons in a less compact distribution than in a VM explant. Among VM-TH⁺ cells, 77.2% of the neurons expressed Npn1 while 48.3% expressed Npn2 (Fig. 1A, C). In differentiated ES cultures, TH⁺ neurons were also positive for Npn1 and Npn2 (Fig. 1B, C); Npn1 was also present in a higher proportion of TH⁺ neurons (82%) than Npn2 (58.6%). When we compared the percentage of TH⁺ cells expressing either one of the Npn receptors, no differences were observed between ES-TH⁺ and VM-TH⁺ (Fig. 1C). Interestingly, in VM and ES cultures, a β -tubulin III⁺ TH[−] neuronal population also expressed Npn1 and Npn2 (Fig. 1D, VM not shown).

Sema3A and 3C increase VM-TH⁺ and ES-TH⁺ axon length

Expression of Npn1 and Npn2 in ES-TH⁺ neurons raised the possibility that their axons could respond to class 3 Semaphorins. To compare the responses of VM-TH⁺ and ES-TH⁺ axons to Semaphorins, we prepared gels that included HEK293 cells and either VM-TH⁺ or ES-TH⁺ neurons. Mock-transfected HEK293 cell clusters, or clusters expressing Sema3A, 3C, or 3F were co-cultured with dissociated TH⁺ neurons derived from VM or ES cells (Fig. 2 A, B). Based on our previous results, E13.5 VM cultures were exposed to Sema3C and 3F, and E14.5 cultures were exposed to Sema3A (Hernandez-Montiel et al., 2008). Figure 2 (C, D, I) shows that Sema3A increases by 35% the axon length in VM-TH⁺ cultures as compared with control cultures (mock-transfected HEK293 cells), while in VM-TH⁺ cultures treated with Sema3C, an increase of 25% was observed (Fig. 2 K). ES-TH⁺ cultures treated with Sema3A or 3C,

showed more dramatic increases in axon length: Sema3A caused an increase of 64% (Fig. 2 E, F, J), and Sema3C an increase of 78% over control values (Fig. 2 L). Moreover, addition of recombinant Sema3A to the culture, in the absence of HEK293 cells, caused an increase in axon length of 50% (Fig. 2 G, H, O). No effect, however, was observed when VM-TH⁺ or ES-TH⁺ neurons were co-cultured with HEK293 cells transfected with a Sema3F expression vector (Fig. 2 M, N).

Sema3C attracts ES-TH⁺ axons

To evaluate the chemotropic effects of Semaphorins on ES-TH⁺ axons, and to compare them with those on VM-TH⁺, we performed collagen gel co-culture experiments where the angles of growing TH⁺ processes, relative to the location of the HEK293 clusters, were measured and grouped within three possible categories as shown in Fig. 3A. Control co-cultures with mock-transfected HEK293 cells showed a random distribution of TH axons (Fig. 3). An increase in the frequency in the 0°–60° range reveals an attractive effect, while an increase in the 121°–180° range indicates a repulsive effect. Sema3C attracted both VM-TH⁺ and ES-TH⁺ axons, since increases were observed in the percentage of TH⁺ axons in the 0°–60° category in comparison with control cultures (Fig. 3D & 3E). No attractive or repulsive effects on TH⁺ processes were detected in Sema3A or Sema3F co-cultures (Fig. 3B, C, F, G).

Effects of Sema3A and 3C on ES-TH⁺ axons are mediated by Npn1 and Npn2

To test if the observed effects of Sema3A and Sema3C on ES-TH⁺ axons were specific and mediated by interaction with the co-receptors Npn1 and Npn2, we co-cultured ES-TH⁺ neurons with HEK293 cell aggregates expressing Sema3A and added a Npn1 blocking antibody to the culture medium (Chauvet et al., 2007). Since Npn1 mediates responses to Sema3A (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997), we expected a reduction in the axon length. In this new set of experiments, we observed again the growth-promoting action of Sema3A relative to control cultures (Fig. 4A, B & I). Addition of anti-Npn1 antibody, but not of control immunoglobulins, reduced axonal growth to control levels (Fig. 4C, D & I). Responses to Sema3C are mediated through both Npn1 and Npn2 co-receptors (Chen et al., 1997; Takahashi et al., 1998). We therefore tested if neutralizing Npn antibodies prevented the growth increase and attraction due to Sema3C. Hence, co-cultures of ES-TH⁺ cells with HEK293 cell aggregates expressing Sema3C in the presence or absence of anti-Npn1 and anti-Npn2 antibodies were evaluated. We observed that addition of both anti-Npn1 and anti-Npn2 antibodies reduced the axon length-enhancing effect of Sema3C (Fig. 4E–G & J), while control immunoglobulins did not (Fig. 4 H & J). When we evaluated the orientation of TH⁺ axons in Sema3C-exposed cultures in the presence of anti-Npn1 and anti-Npn2 antibodies, we also observed that the chemotropic effect of Sema3C was abolished (Fig. 4K).

To further analyze the specificity of ES-TH⁺ axon responses to Semaphorins, we performed double immuno-staining (TH/Npn1 or TH/Npn2; Fig. 5A–H) in collagen gel co-cultures to quantify independently the responses of TH⁺ axons expressing or lacking Neuropilins. Differentiated ES cultures exposed to mock-transfected (control), Sema3A- or Sema3C-expressing HEK293 cell clusters were cultured for 48 h and length and orientation of the different axon populations were measured. Among TH⁺ axons, Npn1⁺ axons significantly increased their length in response to Sema3A, while no increase was observed in Npn1[−], Npn2⁺ or Npn2[−] axons (Fig. 5I). In the case of cultures exposed to Sema3C (Fig. 5 A–H), TH⁺ axons expressing Npn1 and Npn2 significantly increased their length, and also showed significant attraction towards HEK293 aggregates (Fig. 5J–L), while no responses in length or angle of growth were observed in either Npn1[−] or Npn2[−] axons. Altogether, these results show that both, the increase in axon length induced by Sema3A and 3C, and the change of angle of growth induced by Sema3C, are mediated by neuropilin co-receptors.

DISCUSSION

In this work we show that ES-TH⁺ neurons express the Semaphorin co-receptors Npn1 and Npn2 in proportions similar to those of partially-dissociated embryonic VM-TH⁺ neurons. Moreover, ES-TH⁺ neurons show the same response as VM-TH⁺ neurons when exposed to Semaphorins 3A or 3C. In both types of neurons, *Sema3A* and *3C* increased axon length whereas only *Sema3C* had attractive properties. The use of neuropilin-blocking antibodies and the analysis of responses of TH⁺/Npn1⁺ and TH⁺/Npn2⁺ axons to *Sema3A* and *Sema3C* confirmed that these effects are specific and are mediated by Npn1 and Npn2 in accord with the known specificity of these Semaphorins for their receptors. On the other hand, *Sema3F* did not produce any effect on either type of neurons. Hence, the responses of ES-TH⁺ and partially-dissociated VM-TH⁺ axons to *Sema3A*, *3C*, or *3F* were similar.

In previous studies, we observed similar responses to Semaphorins of TH⁺ axons of ventral midbrain neurons but some differences with the present study were detected. Using rat embryonic ventral midbrain explants we showed, in agreement with the present data, that *Sema3A* increases the length of TH⁺ axons of E14 explants, and that *Sema3C* attracts and increases the length of TH⁺ axons of E13 explants (Hernandez-Montiel et al., 2008). *Sema3F* was found to have a repulsive effect on TH⁺ axons of E13 explants, which contrasts with the lack of effect of this guidance molecule on VM-TH⁺ axons in the present study. The main difference between our previous report and the present study is that in the former, non-dissociated ventral midbrain explants were used and here we worked with partially-dissociated cultures. A possible explanation for this differential behavior regarding *Sema3F* is that extracellular matrix components and/or other cellular elements present in the explant influence the response to these guidance cues. Upon dissociation, these elements could be affected or eliminated, thus altering the response to the guidance molecules. In dissociated cultures, there is a fraction of TH⁺ cells that does not express Npn receptors and there are also non-dopaminergic neurons that are Npn immunoreactive. Interaction between these neuronal types might determine responses of VM-TH⁺/Npn[−] neurons to Semaphorins. Axons of dissociated VM-TH⁺ neurons, however, can respond directly to *Sema3A* and *Sema3C* and their response is blocked by Npn-blocking antibodies.

The effect of other chemotropic molecules on TH⁺ neurons produced from ES cells has been reported recently, albeit using a different protocol to generate dopamine neurons. Netrin1, a chemotropic molecule that enhances and attracts TH⁺ neurite outgrowth from embryonic ventral mesencephalic explants (Lin et al., 2005), also enhances growth of ES-TH⁺ neurites but it does not have guide their growth (Lin and Isacson, 2006). Slit1 and Slit3, two chemotropic proteins expressed in the VM and striatum (Marillat et al., 2002), are repellent for ventral mesencephalic dopamine neurons (Lin et al., 2005) and also impair ES-TH⁺ neurite outgrowth (Lin and Isacson, 2006). No guidance activity, however, was observed for ES-TH⁺ neurons. Overall, these results reveal similarities and differences in the responses of VM-TH⁺ and ES-TH⁺ neurons to axon guidance cues. The differences could be due to interactions between the different axon types present in explant cultures, as suggested by our results. In addition, it has been postulated that ES-TH⁺ neurons might not be developmentally equivalent to VM-TH⁺ neurons on the basis of the steps they follow to their final differentiation (Parmar and Li, 2007; Tropepe et al., 2001). The differences in the response to axon guidance molecules, therefore, could be due to the stage of development of each neuronal population. Whereas E13.5 VM-TH⁺ axons do not respond to *Sema3A*, the ES-TH⁺ neurons at the stage used in this work responded equally to *Sema3A* and *Sema3C*, therefore resembling more the behavior of E14.5 VM-TH⁺ neurons. Hence, the differences in developmental stages of the embryonic and the in vitro-differentiated TH⁺ neurons must be taken into consideration when studying these populations. Another aspect to consider is the expression of Neuropilins by dopaminergic neurons: in our initial quantification (Fig. 1) made in adherent cultures, the percentage of ES-

TH⁺ cells positive for Npn1 was 82%, and the proportion of Npn2⁺ dopamine neurons was 58%. Whereas the percentage of Npn1⁺ dopaminergic cells remained unchanged after collagen gel co-cultures (82%, Table I), the Npn2⁺ population increased considerably to reach 86% of TH⁺ neurons. This suggests that neuronal maturation or different culture conditions influence Npn2 expression, which could result in higher responsiveness to Sema3C. The data presented here, however, reveal very consistent responses to Semaphorins by VM-TH⁺ and ES-TH⁺ neurons.

Other studies have reported similarities between VM-TH⁺ and ES-TH⁺ neurons. ES-TH⁺ neurons generated from neural precursors using the five-stage protocol express progenitor or mature dopaminergic markers found in mesencephalic cells such as En1, Lmx1a, Lmx1b, Foxa2, Pitx3, RALDH1 and calretinin (Diaz et al., 2009; Rodriguez-Gomez et al., 2007; Smidt and Burbach, 2007). These ES-TH⁺ neurons have the ability to restore dopaminergic function in hemiparkinsonian rats (Kim et al., 2002; Rodriguez-Gomez et al., 2007) to levels comparable to those observed after transplantation of ventral mesencephalic tissue (Bartlett and Mendez, 2005; Dunnett et al., 1989; Yurek and Fletcher-Turner, 2004). Furthermore, ES-derived dopaminergic neurons generated by a different differentiation protocol also express ventral mesencephalic dopaminergic markers (Barberi et al., 2003). These resemblances, in addition to the similar responses to guidance cues already mentioned, suggests that ES-derived TH⁺ neurons are phenotypically very close to those generated *in vivo* in the ventral midbrain.

The use of natural or induced pluripotent cells for restitution therapy has gained wide interest as a possible alternative treatment for neurodegenerative conditions such as Parkinson's disease (Soldner et al., 2009; Wernig et al., 2008). The success of such an approach will depend among other things on a solid understanding of ES differentiation, the characteristics of the dopamine neurons generated, and their behavior after transplantation into the brain of model animals. Our study adds evidence of the dopaminergic character of ES derived TH⁺ neurons, namely, their expression of Npn1 and Npn2 receptors and their response to class 3 Semaphorins, knowledge that could help devise better transplantation procedures. Hence, Sema3A and 3C have the potential to serve as tools to achieve the goal of correct re-innervation of the striatum by intranigally transplanted ES-TH⁺ cells.

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Fig. 1. ES-TH⁺ neurons express Npn1 and Npn2 in a proportion similar to VM-TH⁺ neurons
Partially dissociated E13.5 VM (A) or differentiated ES cells cultured until day 3 / stage V (B) were double immunostained for TH/Npn1 or TH/Npn2. VM-TH⁺ and ES-TH⁺ express Npn1 and Npn2 (A, B). In both ES and VM cultures the percentage of TH⁺ neurons expressing Npn1 was larger than that expressing Npn2 (C). There was no difference, however, in the fraction of the cells expressing Npn1 or Npn2 between VM and ES dopamine neurons (C). Triple immunostaining of differentiated ES cultures for TH, β-tubulin III, and Npn1 or Npn2 showing that TH-negative neurons present in the culture express Npn1 and Npn2 (D, arrows). Images representative of 4 independent experiments averaged in C. Scale bars: A & B, 10 μm; D, 20 μm.

Fig. 2. Semaphorins 3A and 3C increase axon length of ES-TH⁺ neurons

VM and differentiated ES cells included in collagen gels were co-cultured with mock- or Semaphorin vector-transfected HEK293 cells. (A) Phase contrast micrograph showing a co-culture of a HEK293 cell cluster and partially-dissociated differentiated ES cells included in a collagen gel. (B) Low magnification micrograph of a co-culture stained with Hoechst, showing nuclei of HEK293 clusters (dotted line), and ES cells (arrows). ES clusters detected over the HEK293 aggregates (arrowheads) were not measured. (C, D, I) Partially-dissociated E14.5 VM cells exposed to mock-transfected HEK293 cells (control, CT) or to Sema3A and quantification of TH⁺ axon length. Partially-dissociated E13.5 VM exposed to Sema3C (K) showed enhanced axonal growth, whereas Sema3F (M) had no effect. Differentiated ES cultures exposed to HEK293 cells transfected with Sema3A (E, F, J), Sema3C (L), or Sema3F (N), or alternatively incubated with recombinant Sema3A-Fc (O). TH⁺ axon length was measured in images obtained by confocal microscopy. Representative images for Sema3A and recombinant Sema3A treated cultures are shown (C-H) with their respective controls. In I-L and O, a significant difference was found between control and Semaphorin-treated cultures (* p<0.05). Arrows in C-F indicate the direction where HEK293 aggregates were located. Scale bars A=100μm, B= 200μm, C-H = 20 μm.

Fig. 3. Semaphorin 3C attracts ES-TH⁺ axons

(A) Diagram to illustrate angle classification of TH⁺ neurons. TH⁺ cells were immunostained and the angle of growth of each axon, relative to the location of clustered HEK cell was determined. The frequency of axons with angles falling in each of the three ranges 0°–60°, 61°–120° and 121°–180° was determined. E14.5 VM (B), E13.5 VM (D, F) or cultures of ES differentiated to TH⁺ neurons (C, E, G) were partially dissociated and co-cultured in collagen gels with mock-transfected HEK293 (CT), or HEK cells transfected with expression vectors for Semaphorin 3A (B, C), Semaphorin 3C (D, E) or Semaphorin 3F (F, G). Panels B, D, and F show the results for VM-TH⁺ whereas C, E, and G include data from ES-TH⁺ neurons. In D and E (Semaphorin 3C), an

increase in the proportion of processes falling between 0° – 60° was found when compared with control (CT) condition (* $p<0.05$). Scale bar = 20 μm .

Fig. 4. Antibodies against Npn1 and Npn2 abolish Sema3A and 3C effects

ES cultures differentiated to TH⁺ neurons were partially dissociated and co-cultured in collagen gels with mock-transfected HEK293 cells (CT, panels A & E), or cells transfected with expression vectors for Sema3A (B-D) or Sema3C (F-H). Arrows indicate the direction of HEK293 aggregates. Anti-Npn1 antibodies were added to Sema3A-exposed cultures (C); anti-Npn1 plus anti-Npn2 antibodies were added to Sema3C exposed cultures (G). To test for specificity, goat immunoglobulins (Ig) were added to Sema3A- or Sema3C-exposed cultures at equivalent concentrations (D, H). As described in legends of Fig. 2 and Fig. 3, axon length and angle of growth were measured (I-K). Brackets indicate statistical differences between bars (* $p < 0.05$). Scale bars = 20 μM.

Fig. 5. TH⁺ axons expressing Npn1 and Npn2 are responsive to Semaphorins 3A and 3C
Differentiated ES cells were co-cultured for 48 hours with mock- or Semaphorin vector-transfected HEK293 cell aggregates, and then double immunostained for TH / Npn1 or TH / Npn2. Representative images of ES-TH⁺ control cultures immunostained for Npn1 (A-B) or Npn2 (E-F), and cultures exposed to Sema3C stained for Npn1 (C, D), or Npn2 (G, H) are shown. Arrowheads in B, D, F & H indicate TH⁺/Npn-negative axons. Length of TH-expressing axons that were positive or negative for Npn1 and Npn2, was measured in confocal microscope images. In Sema3A-treated cultures (I) an increase in length was only observed in Npn1-expressing axons, while in Sema3C-treated cells (J) Npn1- and Npn2-expressing axons showed significant increases in length, as compared with control cultures (*p<0.05). The

frequency of angle of axon growth of control and Sema3C-treated cultures falling in each of the three ranges 0° – 60° , 61° – 120° and 121° – 180° relative to the location of the HEK293 cell clusters was determined. Npn1⁺ (K) and Npn2⁺ (L) axons showed an increase in the frequency of axons falling in the 0° – 60° range (color code in K also applies to L). Brackets indicate differences between bars (* $p < 0.05$). Scale bars = 20 μm .

Table I

Number of axons measured in all Semaphorin-exposed cultures

Fig. 2. Effect of Semaphorins on axon length of ES-TH⁺ and VM-TH⁺ neurons

	Control/Sema3A	Control/Sema3C	Control/Sema3F	Control/Sema3A-Fc
VM-TH ⁺	92/102	60/93	27/50	NA
ES-TH ⁺	54/35	54/37	54/33	30/41

Fig. 3. Effect of Semaphorins on direction of ES-TH⁺ and VM-TH⁺ axons

	Control/Sema3A	Control/Sema3C	Control/Sema3F
VM-TH ⁺	56/71	69/95	28/39
ES-TH ⁺	68/23	68/46	68/26

Fig. 4. Effect of anti-Npn1 antibodies on axon growth of Semaphorin 3A-treated ES-TH⁺

	Control	Sema3A	Sema3A + anti-Npn1	Sema3A +Goat Ig
ES-TH ⁺	76	54	96	92

Fig. 4. Effect of Anti-Npn1 plus Anti-Npn2 antibodies on axon growth of Semaphorin 3C-treated ES-TH⁺

	Control	Sema3C	Sema3C+ Anti- Npn1/Npn2	Sema3C+ Goat Ig
ES-TH ⁺	75	70	116	80

Fig. 4. Effect of Anti-Npn1 plus Anti-Npn2 antibodies on axon direction of Semaphorin 3C-treated ES-TH⁺

	Control	Sema3C	Sema3C+Anti Npn1/Npn2	Sema3C+Goat Ig
ES-TH ⁺	60	77	106	91

Fig. 5. Effect of Semaphorins on axon growth of ES-TH⁺ expressing Npn1 and Npn2 receptors

	Npn1 ⁺	Npn1 ⁻	Npn2 ⁺	Npn2 ⁻
Control	60	9	78	7
Sema3A	35	7	85	7
Control	75	17	102	9
Sema3C	82	24	44	16

Fig. 5. Effect of Semaphorins on angle of axon growth of ES-TH⁺ expressing Npn1 and Npn2 receptors

	Npn1 ⁺	Npn1 ⁻	Npn2 ⁺	Npn2 ⁻
Control	75	17	102	9
Sema3C	78	18	39	12

Abbreviations: ES-TH⁺: dopaminergic neurons differentiated from embryonic stem cells. VM-TH⁺: dopaminergic neurons obtained from embryonic ventral mesencephalon. NA: not applicable. Npn1: neuropilin-1. Npn2: neuropilin-2. Sema: Semaphorin.