Secretome of Undifferentiated Neural Progenitor Cells Induces Histological and Motor Improvements in a Rat Model of Parkinson’s Disease

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ABSTRACT

Parkinson’s disease (PD) is a progressive neurodegenerative movement disorder that results from the death of dopamine (DA) neurons. Over recent years, differentiated or undifferentiated neural stem cells (NSCs) transplantation has been widely used as a means of cell replacement therapy. However, compelling evidence has brought attention to the array of bioactive molecules produced by stem cells, defined as secretome. As described in the literature, other cell populations have a high-neurotrophic activity, but little is known about NSCs. Moreover, the exploration of the stem cell secretome is only in its initial stages, particularly as applied to neurodegenerative diseases. Thus, we have characterized the secretome of human neural progenitor cells (hNPCs) through proteomic analysis and investigated its effects in a 6-hydroxidopamine (6-OHDA) rat model of PD in comparison with undifferentiated hNPCs transplantation. Results revealed that the injection of hNPCs secretome potentiated the histological recovery of DA neurons when compared to the untreated group 6-OHDA and those transplanted with cells (hNPCs), thereby supporting the functional motor amelioration of 6-OHDA PD animals. Additionally, hNPCs secretome proteomic characterization has revealed that these cells have the capacity to secrete a wide range of important molecules with neuroregulatory actions, which are most likely support the effects observed. Overall, we have concluded that the use of hNPCs secretome partially modulate DA neurons cell survival and ameliorate PD animals’ motor deficits, disclosing improved results when compared to cell transplantation approaches, indicating that the secretome itself could represent a route for new therapeutic options for PD regenerative medicine.

INTRODUCTION

Parkinson’s Disease (PD) is the second most common chronic neurodegenerative disorder in the elderly, and it is estimated to affect about 1% of population over 60 years of age [1, 2]. Clinically, PD is mainly characterized as a motor disease. The diagnosis currently available depends on the identification of cardinal features, such as bradykinesia, muscular rigidity, postural instability, and tremor that show a nonlinear progression during the development of the disease [3, 4]. These motor deficits are the result of the progressive loss of dopamine (DA) neurons in the nigrostriatal pathway, particularly in the substantia nigra pars compacta (SNpc), leading to the reduction of DA levels in the striatum [3, 5, 6]. Despite pharmacological advances, the current treatments do not address the etiology of the disease and the degenerative process is not avoided [7–10]. Therefore, new strategies based on the use of stem cells, such as neural stem cells (NSCs), have emerged as an alternative therapy for PD [11, 12]. NSCs are multipotent stem cells isolated from fetal and adult nervous system tissues with the ability to self-renew and differentiate into specialized functional neurons and glial cells, making them an interesting source of cells for neuronal repair after injury or disease [12–14]. In fact, different reports have already shown that the transplantation of NSCs display therapeutic effects, such as the capacity to protect and regenerate damaged DA neurons, as well as the potential to improve function in animal models of PD. For instance, Harrower and colleagues [15] showed a reliable long-term survival and integration of transplanted NSCs in the striatum of rats lesioned with 6-hydroxydopamine (6-OHDA). According to the authors, an increase in dopaminergic (DAergic) fiber densities and synapse formation was observed. Similarly, another study demonstrated that the transplantation of adult NSCs (expanded from the subependymal zone) in the striatum of 6-OHDA-lesioned rats...
led to a functional recovery in these animals, a fact that was positively correlated with DA transporter immunoreactivity re-establishment in the host tissue [16]. Even though, growing evidence suggests that the effects mediated by stem cell transplants are not associated with the generation of new neurons or glial cells [17, 18]. In fact, stem cells can secrete a wide panel of bioactive agents (e.g., growth factors, cytokines, and [micro]vesicles), which is defined as secretome. The latter is believed to be important in the modulation of several biological processes, such as cell survival, proliferation and differentiation, immunomodulation, antiapoptosis, and stimulation of tissue adjacent cells [18–20]. Currently, there are no studies regarding the application of NSCs secretome per se in animal models of PD, nor its effect on DAergic neuronal cell populations. Nevertheless, some reports have suggested them as neurotrophic-factor secreting cells [21]. Indeed, different studies showed that important neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), stem cell factor (SCF), and insulin growth factor (IGF), were found to be increased after NSCs transplantation, thereby supporting NSCs mediated effects [21–24]. Having this in mind, it would be interesting to further explore the impact of the human progenitor cells (hNPCs) secretome on the reversion of PD, when compared to more conventional approaches like cell transplantation. Following this, we analyzed the effects of hNPCs secretome on DA neurons survival and motor function of a 6-OHDA-rat model of PD, comparing it with the outputs obtained from animals containing the factors secreted by hNPCs was collected, centrifuged at a rate of 1.0 mm, DV = 7.4 mm) and into appropriate cages, under standard controlled conditions (12-hour light/12-hour dark cycles; room temperature (RT) at 22–24°C and 55% humidity; food and water ad libitum). For surgical procedures, animals were anesthetized intraperitoneally (i.p.) with ketamine (75 mg/kg) plus medetomidine (0.5 mg/kg), placed on a stereotaxic frame (Stoelting, Wood Dale, IL, https://www.stoeltingco.com/), and unilaterally injected using a 30-gauge needle Hamilton syringe (Hamilton, Bonaduz, Switzerland, https://www.hamiltoncompany.com/), with either vehicle (0.2 mg/ml of ascorbic acid in 0.9% NaCl; sham group, n = 9) or 6-OHDA hydrochloride (Sigma, H4381, St. Louis, MO, http://www.sigmaaldrich.com/portugal.html; 6-OHDA group, n = 15) directly into the medial forebrain bundle (MFB; coordinates related to Bregma: AP = −4.4 mm, ML = −1.0 mm, DV = −7.8 mm [30]) at a rate of 0.5 μl/min. Sham animals received 2 μl of 0.2 mg/ml of ascorbic acid (Sigma, A1968) in 0.9% NaCl and the 6-OHDA animals were injected with 2 μl of 6-OHDA hydrochloride (4 μg/μl) with 0.2 mg/ml of ascorbic acid in 0.9% NaCl. The needle was left in place for 4 min after each injection to avoid any backflow. The PD model with 6-OHDA injections into the MFB was chosen due to their relevance to study anti-PD properties of novel therapies, such as cell replacement strategies or the application of new drugs or cell-free therapeutic tools (e.g., hNPCs secretome) [31–33]. Animals presenting more than 100 rotations in the apomorphine-induced turning behavior (rotameter test) were considered having a complete lesion, as previously described by our group [34].

Surgical Treatment: Injection of hNPCs and hNPCs Secretome

Five weeks after 6-OHDA injections, the animals received hNPCs transplants or hNPCs secretome. After anesthesia administration, animals were unilaterally injected, as described in previous section, with either vehicle (Neurobasal A medium; 6-OHDA control group, n = 5), sterile saline (sham group, n = 9), hNPCs (n = 5), or hNPCs CM (n = 5) directly in the SNpc (coordinates related to Bregma: AP = −5.3 mm, ML = −1.8 mm, DV = −7.4 mm) and into four striatum coordinates (coordinates related to Bregma: AP = −1.3 mm, ML = 4.7 mm, DV = −4.5 mm, and −4.0 mm; AP = −0.4 mm, ML = 4.3 mm, DV = −4.5 mm, and 4.0 mm; AP = 0.4 mm, ML = 3.1 mm, DV = −4.5 mm, and −4.0 mm; AP = 1.3 mm, ML = 2.7 mm; DV = −4.5 mm, and −4.0 mm [30]). 6-OHDA-control group received 4 μl of Neurobasal A medium in the SNpc and 2 μl in each coordinate of striatum at a rate of 0.5 μl/min. Cell transplanted groups received 200,000 cells in SNpc and 50,000 cells in each coordinate of striatum. CM-injected animals received 4 μl in the SNpc and 2 μl in each coordinate of striatum at a rate of 0.5 μl/min. The needle was left in place for 4 min after each injection to avoid any backflow.

Behavioral Assessment

Behavioral analysis was performed 3 weeks after 6-OHDA injections for the PD model characterization and after treatments at 1, 4, and 7 weeks following surgeries (Fig. 1). Motor coordination and balance of the animals was evaluated using the Rotorod test. The skilled paw reaching test (staircase test) was used to assess the independent forelimb extension and grasping skills. Finally, the extension of DA depletion was evaluated using the apomorphine-turning behavior test (rotameter test). Understanding that apomorphine is a strong DA agonist,
the continuous overstimulation of the DAergic system could lead to an inadequate interpretation of the treatments impact on the functional outcomes. Therefore, this test was only used to select the animals that were truly injured upon 6-OHDA lesions [35–37]. All behavioral tests were performed as previously described [30, 34].

TH Immunohistochemistry

After 13 weeks (including the development of the lesion and consequent treatment) animals were sacrificed with sodium pentobarbital (Eutasi, 60 mg/kg, i.p.; Ceva Saúde Animal, Algés, Portugal, http://www.ceva.pt/) and processed as free-floating sections. Striatal and mesencephalon coronal sections, 50 μm thick, were obtained using a vibratome (Leica, VT1000S, Wetzlar, Germany, http://www.leicabiosystems.com/) and then with the streptavidine-biotinylated secondary antibody (ThermoFisher Scientific, c, A11008) during 2 h at RT. All sections were incubated with the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI, Sigma, D9505) in 50 ml of Tris-Cl 0.05 M (pH = 7.6) with 12.5 μl of H2O2. Sections were then mounted on superfrrost slides and allowed to dry in the dark. After 24 h, thionin counter-coloration was performed and the sections were mounted using entellan (Merck Millipore, AB152, Billerica, MA, http://www.merckmillipore.com/PT/en?bl=1) diluted in 0.01 M PBS with 2% of NBCS (1:2,000). Afterward, sections were sequential incubated during 30 min at RT with a biotinylated secondary antibody (ThermoFisher Scientific, TP-125-HL) and then with the streptavidine-peroxidase solution (ThermoFisher Scientific, TP-125-HL). The antigen visualization was performed using 25 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, D5905) in 50 ml of Tris-Cl 0.05 M (pH = 7.6) with 12.5 μl of H2O2. Sections were then mounted on superfrrost slides and allowed to dry in the dark. After 24 h, thionin counter-coloration was performed and the sections were mounted using entellan (Merck Millipore, 1079610500). To ensure a representative sampling among the animals, four identical TH-labeled slices covering the entire mesencephalon were chosen, including all the portions of the SNpc. Using a brightfield microscope (Olympus, BX51, Tokyo, Japan, https://www.olympus-global.com/), the boundaries of SNpc area were drawn. The delineation of this region was performed through identification of anatomic standard reference points and with the help of the rat brain atlas [38]. Counting of total TH-positive cells in the SNpc area was performed on both hemispheres, and the data presented as the percentage (%) of remaining TH-positive cells in the lesioned side compared to the control side. All the analysis was performed under blind conditions.

Striatal Fiber Density Measurement

TH-immunostained striatal sections (four sections per animal) representing the coordinates of injection sites within the striatum were photographed (x1 objective) using brightfield illumination (Olympus, SZX16). All image analysis was completed using the ImageJ software (National Institute of Health, v1.48, Bethesda, MD, https://imagej.nih.gov/ij/). The optical density of TH-positive fibers was measured by densitometry, as previously described [30, 39]. The data are presented as percentages of the contralateral striatum (intact side).

Immunostaining

Striatal and mesencephalon coronal sections (including SNpc) were obtained and processed as described above. As first approach, sections were permeabilized with 0.3% PBS-T for 10 min. Then, the sections were blocked with a solution of 10% NCBS in 0.01 M PBS for 30 min. After that, the samples were incubated overnight at 4°C with the primary antibodies namely, rabbit TH for DA neurons detection (1:1,000) and with the mouse Human Nuclear Antigen (HNA, 1:200; Merck Millipore, MAB1281) for hNPCs detection. Sections were afterward incubated with the secondary antibodies: Alexa Fluor 594 goat anti-mouse (1:1,000; ThermoFisher Scientific, A11005) and Alexa Fluor 488 goat anti rabbit (1:1,000; ThermoFisher Scientific, A11008) during 2 h at RT. All sections were incubated with the nuclear counterstain 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI, 1:1,000; Sigma). Finally, the slides were mounted in Immu-Mount (Thermo Scientific) and observed at a confocal point-scanning microscope (Olympus, FV1000).

Untargeted Mass Spectrometry Proteomic Analysis: IDA and SWATH Acquisitions

Three biological replicates of hNPCs CM were first concentrated (x100) using a 5 kDa cut-off concentrator (Vivaspin,
Phenotypic Characterization of 6-OHDA Lesions

To evaluate the functional integrity of the DAergic system, after 6-OHDA injections (Fig. 2A), and therefore select the animals that were truly injured, the rotameter test was performed at the end of behavior assessment (RotaRod and staircase tests). Three weeks after the 6-OHDA injections, statistical analysis revealed differences in the number of apomorphine-induced turning rotations in the 6-OHDA-injected animals when compared to the sham group ($t_{(12)} = 9.398, p < .0001$; Fig. 2B). In addition, also the motor performance of the animals was affected by the 6-OHDA injections. Regarding motor coordination and balance, measured by the rotaterod test, it was found to be impaired in animals injected with 6-OHDA ($t_{(19)} = 4.034, p < .001$; Fig. 2C). In the staircase test, performed to assess the forelimb use and skilled motor function, we also observed that the 6-OHDA-injected animals were remarkably affected when compared to sham animals ($F_{(1,22)} = 48.727, p < .0001, \eta_p^{\text{partial}} = .689$; Fig. 2D).

Injection of hNPCs CM Reduces the Motor Deficits of 6-OHDA-Lesioned Animals

To address the effects of hNPCs transplantation and its CM (i.e., secretome) in 6-OHDA-injected animals, the motor performance was evaluated at 1, 4, and 7 weeks after treatment by using the RotaRod and staircase tests, as previously described [30]. Regarding motor coordination and balance (assessed by the RotaRod test), the results showed a significant effect for the factor treatment ($F_{(3,98)} = 20.034, p \leq .0001, \eta_p^{\text{partial}} = .770$) and for the factor time (weeks; $F_{(3,96)} = 2.824, p \leq .05, \eta_p^{\text{partial}} = 0.136$), but no interaction between the factors ($F_{(9,54)} = .751, p = .661$). When we compared CM-injected animals with cells-transplanted animals, we observed a significant improvement of motor coordination performance promoted by the hNPCs CM when compared to hNPCs-injected group ($p \leq .05$, Fig. 3A). Similar results were also observed in the staircase test, used to assess the forelimb use and the fine motor coordination of the animals. Indeed, after CM injection, statistical analysis revealed a significant effect for the treatment ($F_{(3,98)} = 21.740, p \leq .0001, \eta_p^{\text{partial}} = .765$) and for the factor time ($F_{(3,96)} = 21.740, p \leq .0001, \eta_p^{\text{partial}} = .765$), but no interaction between these two factors ($F_{(9,54)} = .672, p = .645$). Comparing the animals injected with hNPCs CM with the untreated group (6-OHDA), a post hoc analysis revealed that the injection of hNPCs CM led to a significant improvement on the success rate of eaten pellets ($p \leq .05$, Fig. 3B). In addition, we were also able to observe that hNPCs CM-injected animals presented a better motor performance than those transplanted with hNPCs ($p \leq .05$, Fig. 3B). Finally, the hNPCs treatment did not lead to any motor improvements, in both tests, when compared with the untreated group.

Injection of hNPCs CM Restores TH Deficits

To analyze the effects of the 6-OHDA injections, as well as the resulting treatments with hNPCs CM or hNPCs cell transplantation, histological analyses for TH were performed. From the results, we observed that there was a significant decrease of DA neurons after the injection of 6-OHDA into the MFB (Fig. 4A–4D). After treatment, statistical analyses

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**RESULTS**

**Statistical Analysis**

Data evaluation for animal behavior tests after 6-OHDA injections was performed using an independent Student’s $t$ test (RotaRod and Rotameter tests), and repeated measures ANOVA if an evaluation along time was desired (Staircase test). After treatments, the behavior and histological data were analyzed using a one-way ANOVA to compare the mean values for the four groups. If an evaluation along time was required (RotaRod and Staircase tests), a mixed design factorial ANOVA was performed. Multiple comparisons between groups were accomplished through the Bonferroni statistical test. The significance value was set as $p \leq .05$ and all the results are presented as mean ±SEM (standard error of the mean).

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The injection of hNPCs CM most likely played a role in the DA neurons survival, leading to a significant higher number of TH-positive cells in the SNpc when compared with the untreated group 6-OHDA (-0.05, Fig. 4E). The same observations were also found in the striatum, by assessing TH-positive fibers through densitometry analysis (Fig. 4F–4I). Statistical analysis (F(3,23) = 91.258, p ≤ .0001, $\eta^2 = .942$) revealed that hNPCs CM was able to increase the TH expression levels when compared to the untreated group 6-OHDA (p ≤ .05, Fig. 4I). To emphasize that, it was observed that the treatment with hNPCs led to a small increase of TH-positive cells in the SNpc, even though nonsubstantial, and no differences were found in the striatum when compared with the untreated group. Moreover, an immunostaining against HNA was performed to identify the transplanted hNPCs in both SNpc and striatum, as it is a specific marker for human cells. Results showed that hNPCs were only found in one animal in the SNpc (Supporting Information Fig. S1) 8 weeks post transplantation.
To identify potential molecules in the hNPCs secretome that could be involved in the prosperous responses of the secretome-injected animals, we characterized the latter through a nontargeted proteomic analysis based on a combined mass spectrometry (MS) approach. From these results, it was possible to identify 595 proteins (according to the UniProtKB/Swiss-Prot classification; Supporting Information Table S2) in the hNPCs secretome, from which 401 proteins were common to the 3 replicates (Fig. 5A). From these, we focused on specific proteins with actions in CNS, finding important molecules in the context of PD (Fig. 5B-5C; highlighted in Table 1).

Discussion
In this study we used a well-defined rat model of PD, induced by a unilateral injection of 6-OHDA into the MFB [34]. This model leads to the DA neurons degeneration mimicking the appearance of the main motor deficits associated with the PD [34, 42]. As shown in the rotameter test (Fig. 2B), 6-OHDA-injected animals displayed an intense turning behavior when compared with the sham group, indicating a clear decline in the functional integrity of the DAergic system. Moreover, we verified that the motor function of these animals was also affected, which is in agreement with previous reports [30, 43, 44]. In fact, the animals presented impairments in motor coordination and balance, as assessed by the rotarod test (Fig. 2C), and in the skilled motor function addressed by the staircase test (Fig. 2D). Regarding the effects of hNPCs CM and hNPCs on motor coordination and balance (assessed by the rotarod test), we observed that the CM injection led to a significant improvement of the motor performance of the injected animals when compared with the hNPCs-transplanted group (Fig. 3A). Similar outcomes were also seen in the staircase test, in which we verified that the injection of hNPCs secretome improved the success rate of eaten pellets in the CM-injected animals when compared with the untreated group 6-OHDA and to those transplanted with hNPCs (Fig. 3B). In addition to this, we also observed that the administration of the hNPCs secretome significantly increase SNpc and striatal TH-positive neurons and fibers, respectively (Fig. 4), when compared with the untreated group 6-OHDA. This improvement is most likely mediating the positive motor functional gains that were observed. Such evidences were not observed in hNPCs-transplanted animals, probably due to the low rate survival of cells upon transplantation, as we observed the presence of them in just one animal (Supporting Information Fig. S1). In fact, hNPCs have been described as a potential stem cell.
source for the treatment of neurological disorders, including PD. Thus, to further understand which molecules present in the hNPCs secretome could be involved in the observed results, a nontargeted proteomic approach by MS was performed. In addition to the well-known neurotrophic factors such as GDNF, BDNF, SCF, among others, our proteomic analysis revealed the secretion of important neuroregulatory candidates, such as 14-3-3 proteins, PEDF, Galectin-1, Cystatin C, Clusterin, GDN, SEM7A, and Cadherin-2 (Table 1), with important roles on the migration, differentiation, and neuroprotection mechanisms both in vitro and in vivo [45–55]. Concerning (for instance) GDN, although its role in PD remains undiscovered, studies have described this molecule as a modulator of neuronal survival and neurite outgrowth [52]. From these, PEDF was found to have important actions in the context of PD [47, 56]. As stated by Falk and colleagues [57], PEDF is not only neurotrophic but also neuroprotective in both 6-OHDA and rotenone primary midbrain culture models of PD. Evidence of neuroprotective action of this neuroregulatory molecule has been linked to its capacity for incite the activation of nuclear factor NF-κB signaling cascade, allowing NF-κB to act as a transcription factor that induces the expression of genes that are crucial to neuronal protection and survival, such as BDNF and GDNF [56]. Such evidence reinforces the importance of BDNF in PD, as it has been suggested that the downregulation of BDNF expression in the SNpc can be one of the initial steps at PD onset, which leads to an increased sensitization of DA neurons [30, 58]. Still, Zheng and colleagues [59] have also suggested that PEDF can decrease mitochondria-derived reactive oxygen species generation, and subsequently down-regulate VEGF-A expression, possibly through the inhibition of the janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) activation. In addition, Yasuda and colleagues [60] have recognized that PEDF is a promoter of protective effects on various neuronal populations, including DA neurons, indicating that an acute damage induces a rise in PEDF levels in the CNS, thereby supporting the hypothesis that PEDF acts as a natural PD neuroprotective factor that has been correlated with motor performance ameliorations. DJ-1, also identified in hNPCs secretome, is a multifunctional protein deeply linked to PD, and the loss of its function is thought to result in the onset of PD [61, 62]. DJ-1 is a stress sensor and its expression is increased upon various stresses, including oxidative stress [61, 63, 64], modulating signaling pathways critical to cell survival [65, 66]. Moreover, studies showed that the administration of DJ-1 protein prevented DAergic cell death and restored locomotion in a 6-OHDA-rat model of PD, suggesting DJ-1 as a possible pharmaceutical target for PD [67, 68]. In addition to DJ-1, we also found TrxR1, Prdx1, and SOD enzymes, which have
been reported as important antioxidant agents and modulators of oxidative stress [69–71]. For instance, Arodin and colleagues [72], using a Caenorhabditis elegans (C. elegans) model, concluded that in the absence of TrXR1, DA neurons were significantly more sensitive to 6-OHDA, suggesting that this molecule is important for neuronal survival in DA-induced cell death. Regarding Prdx1, its overexpression in a DAergic neuronal cell line has shown to revert the effects of 6-OHDA, acting as ROS scavenger that led to DA neurons cell survival and protection [73]. Similarly, SOD enzymes are often regarded as the first line of defense against ROS [70, 74]. Fibronectin for instance, has also been described to exert neuroinflammatory and neuroprotective roles [75]. Evidence showed that this glycoprotein could bind integrin and growth factor receptors (such as IGF-1 receptor) to transactivate intracellular signaling events, such as the PI3K/AKT pathway, leading to the increase of growth factor-like neuroprotective actions [75]. Indeed, accumulating evidence suggests that the normal functioning of the PI3K/AKT pathway ensures the neuroprotective defense in degenerating DA neurons, by limiting apoptosis, preventing microglia activation and neuroinflammation, as well as preventing ROS accumulation, thereby keeping oxidative stress levels under control [76]. Interestingly, the results of a study about the impact of Cadherin on PI3K/AKT activation suggested that Cadherin-2 is indeed involved in the activation of this signaling cascade in DA neurons, being triggered by the glial cell line-derived neurotrophic factor (GDNF; of pivotal importance for DA neuronal maintenance and development) [54]. Finally, Dickkopf 3, was another molecule identified in the secretome of hNPCs, which has been described as an important modulator of DAergic neuronal differentiation through the Wnt/β-catenin signaling pathway [77]. Still, as previously stated by Teixeira and colleagues [25], all the mentioned proteins have already been identified in different cell secretomes by distinct proteomic-based techniques, supporting the results stated by our proteomic analysis. Hereupon, our findings suggest that the modulation effect in DA neurons triggered by hNPCs secretome could be related with the presence/expression of several secreted factors, as those discussed above. This is in line with previous secreteome studies from our lab, which demonstrated similar effects in the same PD model, as well as in the proteomic profile, by using a different stem cell population like human mesenchymal stem cells [30]. Nevertheless, further research is required to carefully define which molecules are imperative for the stem cells secretome-mediated neuroprotective and regenerative properties. Elucidation of the implicated molecular pathways is also a crucial step toward improving our understanding of the secreted factor profile and its clinical utility.

**Table 1.** Combined list of reproducible proteins with neuroregulatory potential

| Accession number | Accession name | Protein Name_UNIPROT recommended |
|------------------|----------------|---------------------------------|
| P19022           | CADH2_HUMAN    | Cadherin_2                       |
| P10909           | CLUS_HUMAN     | Clusterin                        |
| P10134           | CYTC_HUMAN     | Cystatin-C                       |
| Q9UBP4           | DKK3_HUMAN     | Dickkopf-related protein 3       |
| P02751           | FINC_HUMAN     | Fibronectin                      |
| P09382           | LEG1_HUMAN     | Galectin-1                       |
| P07093           | GDN_HUMAN      | Glia-derived nexin (GDN)         |
| P36955           | PEDF_HUMAN     | Pigment epithelium-derived factor|
| Q06830           | PRDX1_HUMAN    | Peroxiredoxin-1                  |
| Q99497           | PARK7_HUMAN    | Protein deglycase Dj-1           |
| O75326           | SEM7A_HUMAN    | Semaphorin-7A (SEM7A)            |
| P00441           | SODC_HUMAN     | Superoxide dismutase [Cu-Zn]     |
| P04179           | SODM_HUMAN     | Superoxide dismutase [Mn, mitochondrial]|
| Q16881           | TRXR1_HUMAN    | Thioredoxin reductase 1, cytoplasmic |
| P62258           | 1433E_HUMAN    | 14-3-3 protein epsilon           |
| P27348           | 1433T_HUMAN    | 14-3-3 protein theta             |
| P63104           | 1433Z_HUMAN    | 14-3-3 protein zeta/delta        |

**CONCLUSION**

The findings of this study demonstrated that the injection of hNPCs secretome enhanced the densities and fibers of TH-positive cells, a fact that probably explains the improved behavioral performance of secretome-injected animals when compared to the untreated group 6-OHDA and those transplanted with cells. The presence of important neuroregulatory molecules within the secretome such as PEDF, Dj-1, Cadherin-3, anti-oxidant proteins, among others, might play a role in the observed outcomes. In fact, it has been described that they are involved in different therapeutic mechanisms, including DA neurons cell survival and protection, opening in this way, new therapeutical opportunities for PD. Overall, our results strongly suggest that the use of the secretome per se may be considered as a possible tool for the treatment of PD, as the secretome was able to better modulate DAergic neuronal survival and animal behavior performance when compared to cell transplantation.

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**AUTHORS CONTRIBUTIONS**

M.P.B.: designed and performed most of the experiments, collected and analyzed the data, and drafted the manuscript; F.G.T.: performed the stereotaxic surgeries, helped with the data interpretation and with manuscript writing; S.I.A. and B.M.: performed and collected the data regarding the proteomic analysis. L.A.B.: provided study material, and helped with the manuscript writing. A.J.S.: conceived and financially supported the study, participated in its design and coordination, and helped with the manuscript writing. All authors read and approved the final manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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