Brain Research 1668 (2017) 56–64

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/bres

Research report

Nogo-receptor 1 antagonization in combination with neurotrophin-4/5 is not superior to single factor treatment in promoting survival and morphological complexity of cultured dopaminergic neurons

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ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form 12 May 2017
Accepted 13 May 2017
Available online 20 May 2017

Keywords:
NEP1-40
Nogo-receptor 1 antagonization
Neurotrophin-4/5
Dopaminergic neurons
Ventral mesencephalon
Cell culture

ABSTRACT

Cell transplantation using ventral mesencephalic tissue is an experimental approach to treat Parkinson's disease. This approach is limited by poor survival of the transplants and the high number of dopaminergic neurons needed for grafting. Increasing the yield of dopaminergic neurons in donor tissue is of great importance. We have previously shown that antagonization of the Nogo-receptor 1 by NEP1-40 promoted survival of cultured dopaminergic neurons and exposure to neurotrophin-4/5 increased dopaminergic cell densities in organotypic midbrain cultures. We investigated whether a combination of both treatments offers a novel tool to further improve dopaminergic neuron survival. Rat embryonic ventral mesencephalic neurons grown as organotypic free-floating roller tube or primary dissociated cultures were exposed to neurotrophin-4/5 and NEP1-40. The combined and single factor treatment resulted in significantly higher numbers of tyrosine hydroxylase positive neurons compared to controls. Significantly stronger tyrosine hydroxylase signal intensity was detected by Western blotting in the combination-treated cultures compared to controls but not compared to single factor treatments. Neurotrophin-4/5 and the combined treatment showed significantly higher signals for the neuronal marker microtubule-associated protein 2 in Western blots compared to control while no effects were observed for the astroglial marker glial fibrillary acidic protein between groups, suggesting that neurotrophin-4/5 targets mainly neuronal cells. Finally, NEP1-40 and the combined treatment significantly augmented tyrosine hydroxylase positive neurite length. Summarizing, our findings substantiate that antagonization of the Nogo-receptor 1 promotes dopaminergic neurons but does not further increase the yield of dopaminergic neurons and their morphological complexity when combined with neurotrophin-4/5 hinting to the idea that these treatments might exert their effects by activating common downstream pathways.

1. Introduction

Cell transplantation of embryonic ventral mesencephalic (VM) cells has been proposed as a therapeutic intervention to meet the critical need of better treatment options for Parkinson's disease (PD) (Barker et al., 2013; Kordower et al., 1995; Stromberg et al., 2010). The major limitations in cell transplantation for PD are the poor survival and integration of the grafted dopaminergic neurons and the insufficient innervations of the host brain (Emgard et al., 1999; Karlsson et al., 2000). While foetal nigral tissue can be transplanted safely bilaterally into the caudate and putamen in patients with PD, it became evident that significant motor improvements are only achieved after grafting of a sufficient amount of VM tissue (Brundin et al., 2000; Hauser et al., 1999; Mendez et al., 2005). The findings that neurotrophic factors support neuronal survival and differentiation were rapidly implemented in strategies to improve the outcome of transplantations. Indeed, pre- or co-treatment of midbrain transplants with

Abbreviations: FFRT, free-floating roller tube; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; HS, horse serum; LINGO-1, leucine rich repeat neuronal protein 1; MAP2, microtubule-associated protein 2; NgR1, Nogo-receptor 1; NT4/5, neurotrophin-4/5; p75, low affinity nerve growth factor; PBS, phosphate buffered saline; PD, Parkinson's disease; TH, tyrosine hydroxylase; TrkB, tropomyosine receptor kinase B; TROY, tumor necrosis family member; VM, ventral mesencephalic.

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http://dx.doi.org/10.1016/j.brainres.2017.05.015
0006-8993/

neurotrophin-4/5 (NT4/5), brain derived neurotrophic factor or glial cell line-derived neurotrophic factor (GDNF) have been reported to improve functional recovery, to elevate numbers of dopaminergic neurons in the grafts and to increase the number of graft derived fibres growing into the host brain (Andereggen et al., 2009; Chaturvedi et al., 2006; Espejo et al., 2000; Haque et al., 1996; Mehta et al., 1998). In line with these observations, treatment of foetal midbrain cultures with either NT4/5 or GDNF led to an enhanced survival of dopaminergic neurons and GDNF promoted their morphological complexity (Lin et al., 1993; Meyer et al., 2001; Widmer et al., 2000). Importantly, Studer and colleagues proposed that based on their findings NT4/5 treatment is superior in increasing morphological differentiation of dopaminergic neurons as compared to administration of other neurotrophic factors (Hyman et al., 1994; Studer et al., 1995). Recently, a lot of attention in the field of neuronal repair was paid to Nogo-A, one of the most potent neurite growth inhibitor of the central nervous system (Gonzenbach and Schwab, 2008; Schwab and Strittmatter, 2014). Along with the detection of various new functions of Nogo-A and a better understanding of the complex signalling, interesting findings have been made in regard to the development of treatment options for PD. Thus, it has been shown that inhibition of Nogo-A signalling augmented survival of cultured dopaminergic neurons and resulted in functional improvement in parkinsonian mice and rats (Inoue et al., 2007; Seiler et al., 2016a). Similarly, we reported that Nogo-receptor 1 (NgR1) antagonization in midbrain cultures increased dopaminergic cell numbers and enhanced their morphological complexity (Seiler et al., 2013). Even though primary dissociated cultures of foetal midbrain are a valuable tool to study effects of various factors on dopaminergic neurons and especially on their morphology, organotypic foetal midbrain cultures have several advantages over dissociated cultures (Studer, 2001). So, the free-floating roller tube (FFRT) culture technique for foetal VM tissue allows for effective in vitro storage and thereby offers the possibility to expose donor tissue prior to transplantation to various treatments promoting survival and growth of dopaminergic neurons (Hoglinger et al., 1998; Meyer et al., 1998). Given that in clinical trials tissues from several foetuses need to be pooled to get a sufficient amount of dopaminergic neurons for transplantation, these properties are of significant importance. Hence, for the present study we hypothesized that a combined treatment with NT4/5 and NgR1 antagonization has supplementary beneficial effects on foetal VM cultures than single factor administration.

2. Results

2.1. Effects of NgR1 antagonization combined with NT4/5 administration on organotypic midbrain cultures

As expected from the results of our previous studies, treatment of midbrain cultures with either NEP1-40 or NT4/5 significantly augmented dopaminergic cell numbers per culture as compared to control (29% ± 11.2%, $t_{2.0/34} \leq 0.05$ and 47% ± 13.3%, $t_{2.9/41} \leq 0.01$ increase, respectively; Fig. 1). Likewise, the combined treatment significantly raised the TH positive cell number per culture (43% ± 19.6%, $t_{2.2/31} \leq 0.05$ increase compared to control; Fig. 1). However, the combined administration of NEP1-40 and NT4/5 did not significantly affect the number of TH positive cells per culture as compared to single factor treatment (13% increase compared to NEP1-40 and 4% decrease compared to NT4/5, F (2,43) = 0.40, p > 0.05; post hoc, combination vs. NEP1-40 p = 0.83 and vs. NT4/5 p = 0.98; Fig. 1, Fig. 2A). In line with this, the combined treatment did not significantly increase the TH positive cell densities as compared to single factor treatment (27% decrease compared to NEP1-40 and 4% decrease compared to NT4/5, F (2,43) = 0.88, p > 0.05; post hoc, combination vs. NEP1-40 p = 0.48 and vs. NT4/5 p = 0.98; Fig. 1, Fig. 2B). Moreover, only NEP1-40 treatment significantly increased the TH positive cell densities compared to control (45% ± 20.2%, $t_{2.4/34} \leq 0.05$ increase and 22% ± 10.9%, $t_{1.6/41} > 0.05$ and 18% ± 13.5%, $t_{1.2/31} > 0.05$ increase compared to control for NT4/5 and the combination, respectively). These results can be explained by the significant bigger sphere volume observed after NT4/5 and the combination treatment (32% ± 9.7%, $t_{3.1/41} \leq 0.01$ and 25% ± 10.2%, $t_{2.7/31} \leq 0.01$ increase compared to control), but not by NEP1-40 treatment (4% ± 11.2%, $t_{0.4/34} > 0.05$ increase compared to control). No significant increase in the sphere volume could, however, be detected after the combined treatment when compared to single factor treatments (21% increase compared to NEP1-40 and 7% decrease compared to NT4/5, F(2,43) = 1.96, p > 0.05; post hoc, combination vs. NEP1-40 p = 0.43 and vs. NT4/5 p = 0.89; Fig. 2C). The effect of the three treatment options on TH positive cell number was also reflected in the higher protein levels of TH as assessed by Western blotting. Specifically, TH levels in all the treated cultures displayed an increase of 77% ± 32.3% ($t_{2.0/13} = 0.06$; NEP1-40), of 83% ± 29.3%, ($t_{2.4/13} \leq 0.05$; NT4/5) and of 97% ± 40.0% ($t_{2.3/12} \leq 0.05$; combination) as compared to controls. No significant difference was found between the groups (22% increase compared to NEP1-40 and 16% increase compared to NT4/5, F(2,20) = 0.11, p > 0.05; post hoc, combination vs. NEP1-40 p = 0.89 and vs. NT4/5 p = 0.94; Fig. 3).

Analysis of the expression level of the neuronal marker microtubule-associated protein 2 (MAP2) displayed a significant increase after NT4/5 treatment (35% ± 9.5%, $t_{3.3/9} \leq 0.01$), but no effect could be observed after NEP1-40 administration (−7% ± 23.7%, $t_{0.4/8} > 0.05$) and the combined treatment (39% ± 36.6%, $t_{1.4/9} > 0.05$). No significant difference between the combined treatment and single factor treatments was detected (46% increase compared to NEP1-40 and 4% increase compared to NT4/5, F(2,8) = 0.83; post hoc, combination vs. NEP1-40 p = 0.49 and vs. NT4/5 p = 0.99; Fig. 4). On the other hand, none of the treatments showed significant differences on the level of the astroglial cell marker glial fibrillary acidic protein (GFAP; control vs. NEP1-40–4% ± 14.6%, $t_{0.2/13} > 0.05$, vs. NT4/5 15% ± 17.7%, $t_{0.7/13} > 0.05$, and vs. combination 34% ± 21.8%, $t_{1.3/13} > 0.05$ and combination 39% increase compared to NEP1-40 and 19% increase compared to NT4/5, F (2,21) = 1.1; p > 0.05; post hoc, combination vs. NEP1-40 p = 0.32 and vs. NT4/5 p = 0.75; Fig. 5).

2.2. Effects of NgR1 antagonization combined with NT4/5 administration on dopaminergic neurons in dissociated primary midbrain cultures

A similar effect of NEP1-40, NT4/5 and the combined treatment on TH positive cell numbers was observed in primary dissociated VM cultures. All three treatment options significantly increased the number of TH positive cells compared to control (42% ± 7.1%, $t_{5.5/20} \leq 0.0001$; 46% ± 11.1%, $t_{3.7/22} \leq 0.001$, and 40% ± 9.0%, $t_{3.9/22} \leq 0.001$, increase compared to control for NEP1-40, NT4/5 and the combination, respectively; Fig. 6A) but the combination did not result in a higher yield of TH positive cells compared to single factor treatments (3% decrease compared to NEP1-40 and 6% decrease compared to NT4/5, F(2,34) = 0.12; post hoc, combination vs. NEP1-40 p = 0.98 and vs. NT4/5 p = 0.88; Fig. 6B).

To investigate whether the combined treatment improves the TH positive cell morphology rather than cell numbers, we assessed the size of the soma, the number of TH positive primary neurites, as well as the length of the TH positive neurites. As expected from the results from our previous studies, treatment of midbrain cultures with NEP1-40 and NT4/5 significantly increased the soma size as compared to control (9% ± 3.5%, $t_{2.3/14} \leq 0.05$, and