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Effect of selegiline on neural stem cells differentiation: a possible role for neurotrophic factors

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ABSTRACT

Objective(s): The stimulation of neural stem cells (NSCs) differentiation into neurons has attracted great attention in management of neurodegenerative disease and traumatic brain injury. It has been reported that selegiline could enhance the morphologic differentiation of embryonic stem cells. Therefore this study aimed to investigate the effects of selegiline on NSCs differentiation with focus on the role of neurotrophic factor gene expression.

Materials and Methods: The NSCs were isolated from lateral ventricle of C57 mice brain. The cells were exposed to selegiline in nano to micromolar concentrations for 24 hr or 72 hr. In order to assay the effect of selegiline on NSCs differentiation into neurons, astrocytes and oligodendrocytes, immunocytochemical techniques were utilized. Samples were exposed to specific antibodies against neurons (β tubulin), astrocytes (GFAP) and oligodendrocytes (OSP). The expression of BDNF, NGF and NT3 genes was investigated using Real-Time PCR.

Results: Our findings revealed that selegiline increased NSCs differentiation into neurons at 10^{-7} and 10^{-8} M and decreased the differentiation into astrocytes at 10^{-9}, while oligodendrocyte did not significantly change in any of the used concentrations. In addition data analyses showed that selegiline increased BDNF, NGF and NT3 gene expression at 24 hr, but did not change them in the other time of exposure (72 hr) except 10^{-7} M concentration of selegiline, which increased NT3 expression.

Conclusion: Our results indicate selegiline induced the differentiation of NSCs into neurons and in this context the role of neurotrophic factors is important and should be considered.

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Introduction

Neural stem cells (NSCs) possess therapeutic potentials for treatment of pathological processes following central nervous system (CNS) disorders or injuries. NSCs are known to have a key role in regeneration, learning and memory (1). Moreover NSCs are the more appropriate cells for studying therapeutic and neurotoxicity effects of various factors in CNS (2). NSCs are multipotent cells that have the ability to self-renew and differentiate into neurons, astrocytes or oligodendrocytes (1). However, there are many obstacles that could not be fully overcome by NSCs transplantation alone. Combining complementary strategies might be required to advance NSC-based treatments to the clinical stage.

These cells have been used in various models of neurodegenerative diseases and CNS injuries such as spinal cord injury (3), cerebral ischemia (4), Parkinson's disease (5), traumatic brain injury (6), and neuropathy (7). It has been reported that neural stem cells are mostly differentiated into astrocytes (8-10). This phenomenon leads to the induction of allodynia which is the main problem in NSCs transplantation (11). Therefore, management of NSCs differentiation attracts the most attention.

On the other hand, it has been reported that selegiline could enhance the morphologic differentiation and survival of embryonic dopaminergic neurons (12). Selegiline a selective inhibitor of monoamine oxidase B (MAO-B) has been widely used to treat Parkinson's disease since the 1970's (13). Furthermore, this drug has been reported to induce neuronal phenotype and neurotrophins expression in the mouse embryonic stem cells (14). Selegiline has also been known as an antioxidant and neuroprotective agent (15). These survival-promoting functions might be related to its properties such as induction of free radical scavenger enzymes, antiapoptotic molecules or neurotrophic factors (16, 17). Also other investigators reported that selegiline enhances nerve growth

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factor (NGF) synthesis (18) as well as glial cell line-derived neurotrophic factor (GDNF) mRNA expression in nigrostriatal and mesolimbic dopaminergic pathways (19).

Several lines of evidence suggest that NSCs can be stimulated and reactivated by various factors like brain-derived neurotrophic factor (BDNF) (20), fibroblast growth factor (FGF-2) (21) and insulin like growth factor-1 (IGF-1) (22).

Literature review indicates that, the effect of selegiline on NSCs differentiation and neurotrophic factors gene expression has not yet been investigated. Therefore in the present study we were interested to evaluate the effect of selegiline on differentiation and neurotrophic factors gene expression in NSCs isolated from adult mouse subventricular zone (SVZ).

Materials and Methods

Cell culture procedures

Neural stem cells (NSCs) were isolated from adult C57 mouse SVZ in accordance with the Johansson *et al* protocol. Briefly, the lateral wall of the lateral ventricles were dissociated in 0.7 mg/ml hyaluronic acid, 0.2 mg/ml kynurenic acid, and 1.33 mg/ml trypsin in HBSS with 2 mM glucose at 37 ˚C for 30 min. Then the cells were centrifuged at 200 g for 5 min, resuspended in 0.9 M sucrose in 0.5× HBSS, and centrifuged for 10 min at 750 g. The cell pellet was resuspended in 2 ml of culture medium and centrifuged at 200 g for 7 min, followed by washing in DMEM/F12. Cells were proliferated in medium consisting of 20 ng/ml EGF, 20 ng/ml bFGF, B27 supplement, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in DMEM-F12 medium (Figure 1 a, b). The 0.05% Trypsin-EDTA was used to dissociate the neurospheres to single cells (23).

Their stemness was verified by anti-nestin immunoreactivity. In addition, this protocol has been approved in our previous study in which the isolated cells expressed the self-renewal genes (24).

Experimental treatment

The cells were grown at a density of 1000/cm^2 on either glass cover slips coated with poly-L-lysine and laminin for differentiation or on cell culture dishes for neurotrophic factors gene expression assay. Then the cells were exposed to selegiline in nano to micromolar concentrations in a culture medium containing 1% fetal calf serum (FCS) for 72 hr. This medium is known to be necessary to induce the differentiation of NSCs to neurons, astrocytes and oligodendrocytes (2).

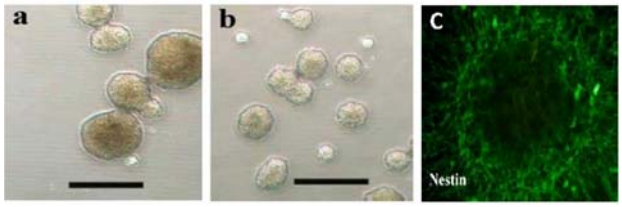

Figure 1. Phase contrast and fluorescent illustration of primary (a) and secondary (b) neurospheres. The nestin immunoreactivity is shown in a neurosphere (C). Scale bar = 200 µm

Trypan blue exclusion test

Cells were harvested with trypsin and centrifuged at 1000 rpm for 10 min. Then small aliquot of the cell suspension was diluted with an equal volume of 0.4% Trypan blue solution (Sigma). Cells with a damaged cell membrane (necrotic cells) stained blue, whereas cells with an intact plasma membrane (healthy cells) remained unstained. Different concentrations of selegiline (10^{-9} - 10^{-7} M) were used for viability assay. All experiments were performed in triplicate and repeated at least three times (2).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 1 hr then washed with phosphate buffered saline (PBS). Primary antibodies were diluted in PBS containing 0.3% Triton X-100 and 0.5% Bovine serum albumin (BSA, Boehringer Mannheim, Germany). Cells were incubated with the following primary antibodies: Mouse anti-nestin (1:100, ab6142), rabbit anti-oligodendrocyte (1:100, ab7474), rabbit anti-Glial fibrillary acidic protein (GFAP, 1:500, ab7260), and rabbit anti β-tubulin III (1:500, ab18207) overnight in a humid chamber at 4 ˚C. Cells were then rinsed with PBS and incubated with secondary FITC (AP308F, Chemicon, for nestin) or Texas-red conjugated secondary antibody for 1 hr at room temperature (1:200, Goat Anti-Rabbit IgG, ab6719). After rinsing with PBS, coverslips were mounted onto slides with Vectashield HardSet Mounting Medium (Vector Laboratories, Inc, Burlingame, CA, USA). Hence images were captured using an Olympus IX71 fluoresce microscope (Olympus, Tokyo, Japan), equipped with DP72 digital camera (Olympus, Tokyo, Japan). All experiments were performed three times in triplicate.

Extraction of total RNA, cDNA synthesis and Real-Time PCR

Total RNA was extracted from the cells using total RNA extraction kit (Bioflux-Bioer, Japan-China) according to the manufacturer's protocol. Reverse transcription was done by easy cDNA synthesis kit (Pars Tous, Iran) with 1 µg RNA adding 1 µl random 6-mer and appropriate amount of DEPC-treated water up to 10 µl. Then mixture incubated at 65 ˚C for 5 min and chilled on ice. Finally 10 µl RT premix was added and reverse transcription was performed at 25 ˚C for 10 min, 50 ˚C for 60 min, and reaction was stopped at 70 ˚C for 10 min. Real-time PCR was carried out by Corbett Rotor Gene 6000 Real-Time PCR system (Corbett Research, Australia) and SYBR

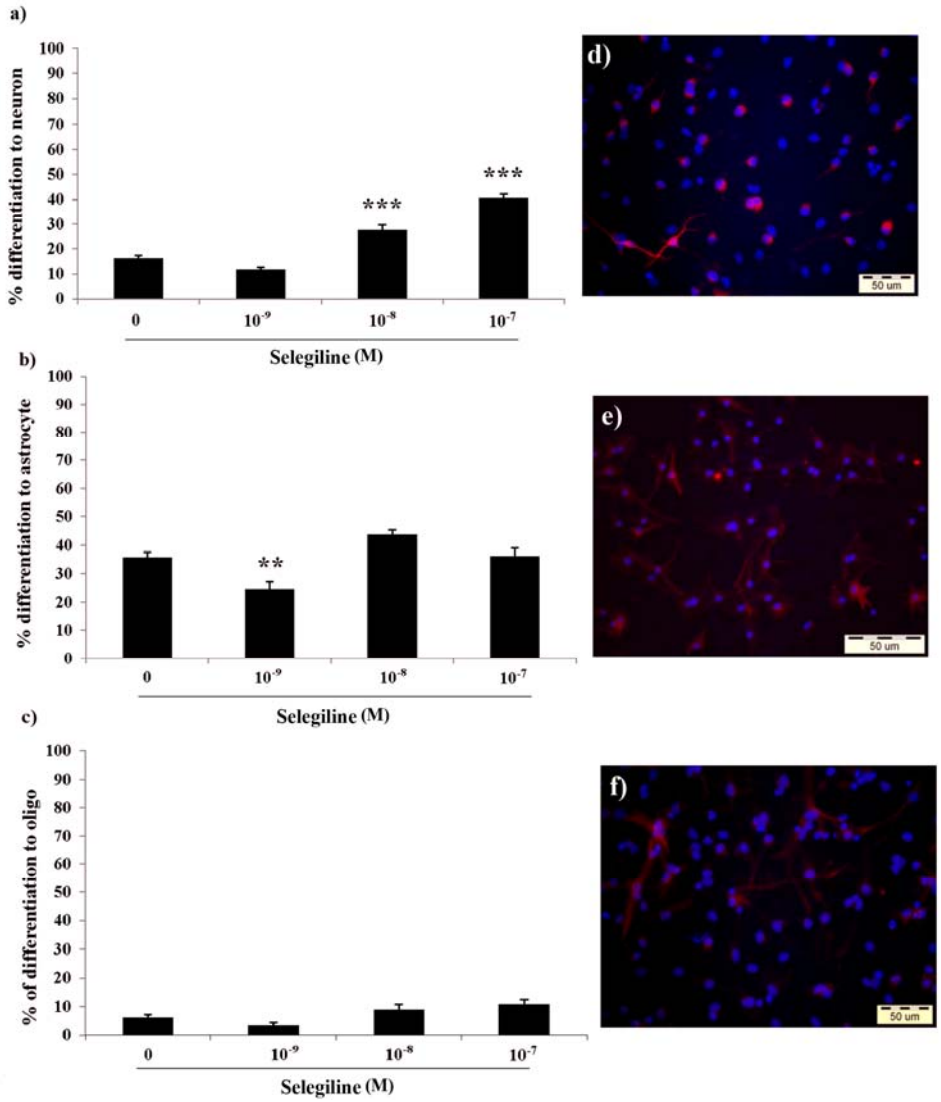

Figure 2. Neural stem cells differentiation. Quantification of differentiation of NSCs into neuron (a), astrocyte (b) and oligodendrocyte (c) after 72 hr exposure to different concentrations of selegiline. The data represent the mean ± sem percent of differentiation. The illustrations represent the β tubulin positive cells as neuron marker (d), GFAP positive cells as astrocyte marker (e) and OSP positive cells as oligodendrocyte marker (f). Experiments were performed in triplicate and *P*-values <0.05 were considered to be significant in all analyses. ** *P*<0.01, *** *P* <0.001 in comparison with the control group (0 selegiline concentration). M=Molar

Green Real-Time PCR Master Mix (Pars Tous, Iran). Total volume was 20 µl containing 2 µl cDNA, 10 µM forward Primer (1.2 µl), 10 µM reverse Primer (1.2 µl), 2X SYBR Green PCR master mix (10 µl), 50X ROX dye (0.4 µl) and dH2O (5.8 µl). Conditions for PCR were: denaturation at 95 ˚C for 10 min, 40 cycles of 30 sec 95 ˚C, 57 ˚C for 45 sec, 72 ˚C for 45 sec, and final extension at 72 ˚C for 5 min. The housekeeping gene beta-actin was used as internal control. Gene expression ratio was obtained by the Pfaffl method using PCR efficiency of each gene and ΔCT values.

All PCR products were analyzed by melting curve of the rotor-gene and on a 2% agarose gel with ethidium bromide staining. The primer sequences were as follows:

BDNF forward: 5'-CAGCAATTGTGTGGTCAGTG-3',
Reverse: 5'-ACAAGAGACCACAGCAAGAC-3', 183 bp
NGF forward: 5'- GTGAGGTGCATAGCGTAATG-3',
Reverse: 5'- CTGTGTCAAGGGAATGCTGA-3', 156 bp
NT3 forward: 5'- ACGGAGGAAACGCTATGCAG-3',
Reverse: 5'- CCCGAATGTCAATGGCTGAG -3', 107 bp
Beta actin forward: 5'-CTTGGGTATGGAATCCTGTG-3',
Reverse: 5'-ACTGTGTTGGCATAGAGGTC-3', 96bp

Data analysis

The obtained data from the immunocyto-chemistry and gene expression ratio, were defined as the mean±SEM. Experiments were performed in triplicate. One-way analysis of variance (ANOVA) followed by Tukey's test was used to analyze the

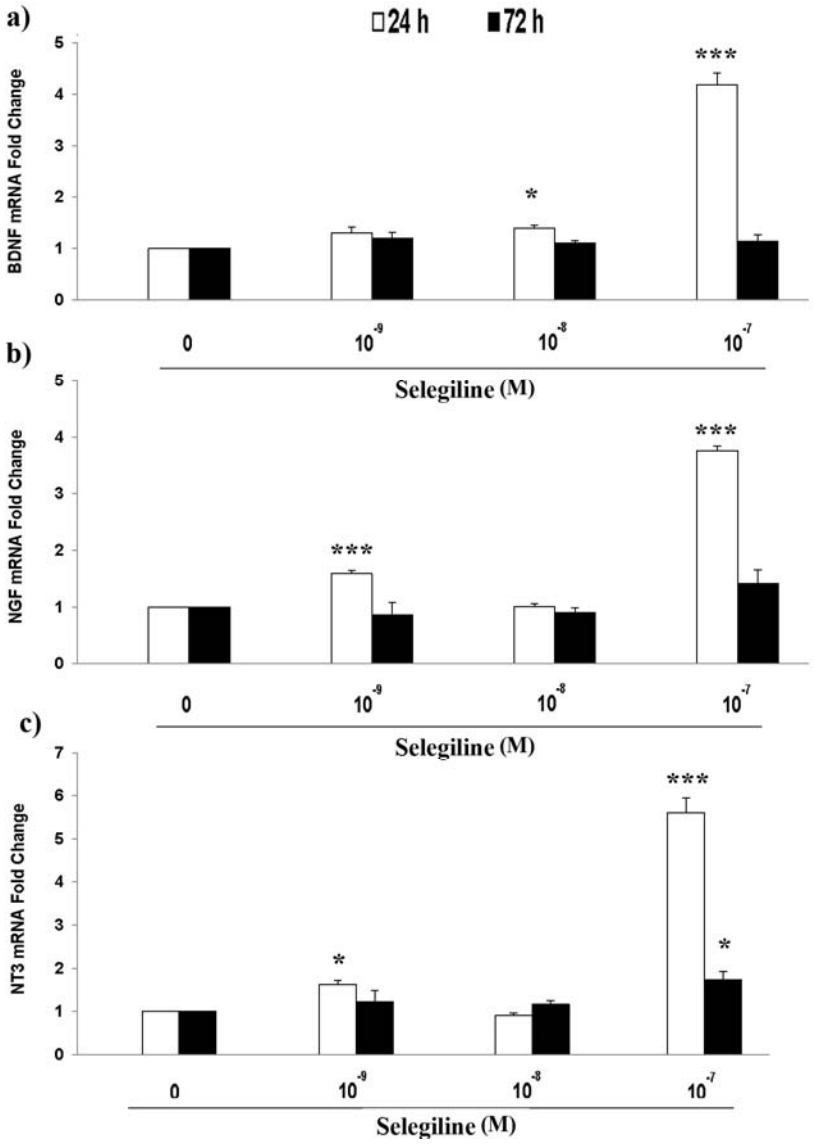

Figure 3. Data of Real-Time PCR regarding BDNF (a), NGF (b) and NT3 (c) gene expression after 24 or 72 hr exposure to selegiline. Experiments were performed in triplicate and *P*-values <0.05 were considered to be significant in all analyses. * *P*<0.05, *** *P* <0.001 in comparison with the control group (0 selegiline concentration). M=Molar

statistical significance in comparisons. The *P*-values <0.05 were considered to be significant in all analyses.

Results

Effects of selegiline on NSCs differentiation

At the beginning of the experiments all cells in the control and exposure were nestin positive, confirming their proliferative and undifferentiated status (Figure 1c). Immunocytochemical analysis showed that selegiline increased the beta tubulin positive cells (neuron, 10^{-8} and 10^{-7} M, Figure 2 a) and decreased the GFAP positive cells (Astrocyte, 10^{-9} M, Figure 2b), compared to vehicle treated cells. In addition, the results indicated that differentiation into oligodendrocyte did not change after exposure to selegiline (Figure 2c).

Effect of selegiline on BDNF, NGF and NT3 gene expression

The results obtained from Real-Time PCR indicated that selegiline increased BDNF, NGF and NT3 gene expressions after 24 hr of exposure. Data analysis revealed that neurotrophic factors gene expression was significantly greater in 10^{-7} M concentration of selegiline. However, our findings illustrated that the cells that were exposed to selegiline for 72 hr did not show a significant change in neurotrophic factors gene expression except 10^{-7} M concentration of selegiline, which represents an increase in NT3 expression (Figure 3).

Discussion

Our findings revealed that selegiline significantly

increased and decreased NSCs differentiation into neuron and astrocyte compared to the control, respectively, and the percent of differentiation to oligodendrocytes did not change. In addition data analysis showed that selegiline increased BDNF, NGF and NT3 gene expression at 24 hr but did not change them in the other time of exposure (72 hr) except 10^{-7} M concentration of selegiline, which represents an increase in NT3 expression. Our results were in agreement with those of Esmaeili *et al*, who reported that selegiline induced neuronal phenotype and neurotrophins gene expression in embryonic stem cells (14). Beside Mizuta *et al* investigated the effects of selegiline on induction of neurotrophic factors in cultured mouse astrocytes. They showed that treatment with 2 mM selegiline for 24 hr, increased the contents of NGF, BDNF, and GDNF in the culture medium. (25). This observed effect of selegiline may be related to its inhibitory effect on MAO B, which leads to dopamine augmentation, because Ohta *et al* indicated that exposure to ropinirole, D2 dopamine agonist, for 24 hr stimulates the synthesis/secretion of neurotrophic factors, including NGF, BDNF, and GDNF in cultured mouse astrocytes (26). Consistent with our results, Shimazu *et al* reported that selegiline enhanced NGF and BDNF concentration significantly in the cultured mouse astrocytes after 24 hr of exposure. It is worth noting that the minimum effective concentration of selegiline in their study was 5×10^{-4} M, but the most effective concentration in this evaluation was 10^{-7} M (27). It seems that the type of cell lines has a critical role in this difference because according to our results the 10^{-6} M or higher concentrations were found to be toxic.

Furthermore i*n vivo* studies reported that 2 weeks of treatment with selegiline (10 mg/kg) significantly increased BDNF levels in the anterior cingulate cortex (28).

There is accumulating evidence suggesting the role of neurotrophic factors especially BDNF in NSCs differentiation (29-31). In this regard Huang *et al* determined the effects of the controlled release BDNF on rat NSCs and they reported that the length of processes was markedly longer and the differentiation percentage of NSCs into neurons was much higher in the BDNF-collagen gel group (29). Consistent with their results, Yang *et al* compared the behavior of NSCs from the spinal cord of adult rats at the neurosphere level after the respective addition of the BDNF daily. Their results suggested that the BDNF increased the differentiation percentage of adult NSCs into neurons (30), which supports our findings.

Regarding the role of NGF in NSCs differentiation, Yi *et al* suggested that NGF stimulates the NSCs differentiation into neurons, a requirement for successful integration into the damaged central nervous system (32).

The role of neurotrophic factors in promotion of neuroprotection has been widely documented. Neurotrophic factors as endogenous substances are known to have key roles in different neuronal properties such as regulation of neuronal survival, differentiation, and synaptic plasticity (33). The effects of these agents on the CNS are relevant to various neurodegenerative diseases. For example, previously the role of BDNF and NT-3 in amyotrophic lateral sclerosis, and BDNF in Parkinson's disease, has been reported (34). There is an association between neuroprotection and neurotrophic factors levels in CNS. In this regard riluzole as an antiexcitotoxic and neuroprotective agent enhanced the synthesis of NGF, BDNF and GDNF in cultured mouse astrocytes (35). This neuroprotection may exert, at least in part, via stimulation of neurotrophic factors.

As mentioned before, selegiline is found to have antioxidant and neuroprotective properties (16, 17). Tang *et al* demonstrated that intrastriatal injections of selegiline (1.25 mg and 2.5 mg) significantly enhanced GDNF mRNA expression in the striatum, whereas the same concentrations of selegiline did not affect monoamine oxidase B (MAOB) activity (19). The observed effect was in agreement with Tatton and Chalmers-Redman who indicated that the neuroprotective effect of selegiline was independent of MAOB inhibitory activity (36).

Conclusion

Selegiline induced the differentiation of NSCs into neurons and it seems that this effect probably has been exerted through induction of neurotrophic factors.

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