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Effects of selegiline, a monoamine oxidase B inhibitor, on differentiation of P19 embryonal carcinoma stem cells, into neuron-like cells

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Abstract Selegiline, the irreversible inhibitor of monoamine oxidase B (MAO-B), is currently used to treat Parkinson's disease. However, the mechanism of action of selegiline is complex and cannot be explained solely by its MAO-B inhibitory action. It stimulates gene expression, as well as expression of a number of mRNAs or proteins in nerve and glial cells. Direct neuroprotective and anti-apoptotic actions of selegiline have previously been observed in vitro. Previous studies showed that selegiline can induce neuronal phenotype in cultured bone marrow stem cells and embryonic stem cells. Embryonal carcinoma (EC) cells are developmentaly pluripotene cells which can be differentiated into all cell types under the appropriate conditions. The present study was carried out to examine the effects of selegiline on undifferentiated P19 EC cells. The results showed that selegiline treatment had a dramatic effect on neuronal morphology. It induced the differentiation of EC cells into neuron-like cells in a concentration-dependent manner. The peak response was in a dose of selegiline significantly lower than required for MAO-B inhibition. The differentiated cells were immunoreactive for neuron-specific proteins, synaptophysin, and β-III tubulin. Stem cell therapy has been considered as an ideal option for the treatment of neurodegenerative diseases. Generation of neurons from stem cells could serve as a source for potential cell therapy. This study suggests the potential use of combined selegiline and stem cell therapy to improve deficits in neurodegenerative diseases.

Keywords Neuronal differentiation · Embryonic carcinoma cells · Neuron specific markers · Synaptophysin · β-III tubulin

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Introduction

Selegiline (deprenyl) a neuroprotective pharmacological drug (Fedchenko et al. 2008), was one of the first adjunct therapies in clinical neurology (Ebadi et al. 2002). It has been used to irreversibly inhibit monoamine oxidase B (MAO-B) in Parkinson's disease (PD) and Alzheimer's disease (AD). Selegiline has been demonstrated to exert anti-apoptotic, neuroprotective effects on a number of in vitro and in vivo models in a dose significantly lower than required for MAO-B inhibition (Palfi et al. 2006). Other researches have shown that the drug can induce expression of a number of genes (Tatton 1996; Fedchenko et al. 2008). Selegiline reduces PC12 cell apoptosis by inducing new protein synthesis (Tatton et al. 1994). Induction of

nerve growth factor (NGF) mRNA expression in cultured rat cortical astrocytes followed by a corresponding increase in NGF protein content was reported by this drug (Semkova et al. 1996). Selegiline blocks apoptosis and regulates the expression of apoptosis-related genes (Xu et al. 1999). This agent has a trophic effect on cultured neurons (Iwasaki et al. 1994). Also, it can improve signs of senility (Knoll 1983). A research in tissue culture and animal models has shown that selegiline can reduce neuronal apoptosis through a mechanism(s) that does not require MAO-B inhibition (Tatton 1996). Selegiline is efficient and a potent inducer for differentiation of bone marrow stem cells into neuronal phenotype (Ghorbanian et al. 2010).

Embryonal carcinoma (EC) and embryonic stem (ES) cells are developmentally pluripotent cells. These cells can differentiate into all cell types under the appropriate conditions. Retinoic acid (RA) induces the pluripotent EC and ES cells to differentiate into various lineages (Rohwedel et al. 2000). Bain et al. showed that RA enables differentiation of ES cells into neuron-like cells (Bain et al. 1995). In vitro differentiation of mouse ES cells into neuron-like cells demonstrated using nerve growth factor (Wobus et al. 1988). Dimethylsulfoxide (DMSO) also was reported to induce differentiation of these cells into neurons (Dinsmore et al. 1996). Lithium chloride has been shown to increase mRNA levels of genes encoding synaptophysin and the 160-kDa neurofilament protein (Schmidt et al. 2001). Selegiline induces neuronal phenotype and neurotrophin gene expression in ES cells (Esmaeili et al. 2006).

P19 cells are murine EC cells that have been used as a model system for studying early embryonic development and differentiation. P19 cells can be induced to differentiate with RA into cell types similar to those derived from neuroectoderm (MacPherson and McBurney 1995). The neurons obtained in these cultures are irreversibly postmitotic, show a typical neuronal morphology and exhibit a number of markers characteristic of CNS neurons such as neurofilament proteins (MacPherson et al. 1997), synaptophysin, and the brain-specific III tubulin (Falconer et al. 1992).

Stem cell therapy has been considered as an ideal option for the treatment of diseases such as PD. A recent investigation reported that ES cell therapy improves neurologic disorders (He et al. 2003). Neuron and synapse loss are important features of the neuropathology of PD (Hou et al. 2010) and AD (Rutten et al. 2005; Bailey and Lahiri 2010). Selegiline, used clinically in PD, has multiple pharmacological effects which make it a good candidate to treat neurotoxicity (Davidson et al. 2007).

Because selegiline induces neurotrophin gene expression and fetal neuron differentiation, as well as neuronal phenotype in ES and BMS cells, it may have the same effects on EC cells. Based on these findings, we investigated the effect of selegiline on in vitro differentiation of P19 EC cells into neuron-like cells. Samples were analyzed to determine what influence this drug may have on neuron-specific markers and neuronal morphology.

Materials and Methods

Cell culture and viability test. The studies reported here were carried out with the P19 line of murine EC stem cells (McBurney 1993). Undifferentiated P19 cells were grown in α-MEM (Gibco-BRL, Carlsbad, CA, 11900073) supplemented with 15% fetal bovine serum, penicillin (50 μg/ml), and streptomycin (50 μg/ml). The cells were kept in a humidified cell culture incubator at 37°C under 5% CO_2 with close control of pH. The viability of EC cells during the experiment was determined by the trypan blue dye exclusion method (Freshney 1994). The cells were removed from the dishes using trypsin/EDTA, and the isolated cells were centrifuged at 1,000 rpm for 10 min. The pellet was resuspended in PBS and stained with trypan blue (0.4%) dye. After 5 min at room temperature, viable and dead (blue stained) cells were counted using a hemocytometer under a microscope using a ×40 magnification. The percentage of the viable cells (PVC) was estimated, plotted against the dose used in the study, and subjected to linear regression analysis. Different concentrations of selegiline (was provided as a gift from Zahravi Pharmaceutical Company, Tabriz, Iran) were used for viability assay (10^{-6}–10^{-11} M). The assay was repeated at least five times.

Dose–response evaluation. We determined the dose–response relationship for percentage of neuronal induction (PNI). The optimal doses of selegiline that induced neuronal differentiation were 10^{-8}–10^{-10} M. The peak response for neuronal induction was at 10^{-8} M, which was used for further investigation. Cresyl violet was used to count the differentiated neurons.

Cresyl violet staining. Adherent cells were fixed in 70% ethanol for 10 min at room temperature and dehydrated as described for immunostaining (in 95% ethanol/5% acetic acid for 20 min at −20°C), then washed twice in PBS for 10 min. Cells incubated for 3–10 min in staining solution: 0.25% cresyl violet, 0.8% glacial acetic acid, 0.6 mM sodium acetate (Fraichard et al. 1995). Cells were washed in PBS, four times 10 min each, and then mounted for examination. PNI was determined from direct counts of stained cells. Five microscopic fields under ×40 magnification were randomly picked to count neuron-like (cresyl violet-stained) cells, and mean values were calculated for each group.

Differentiation induction protocol. Undifferentiated EC cells were dissociated into single-cell suspensions and then cultured in hanging drops to induce embryoid body (EB) formation at an initial cell density of 500 cells per 20-μl drop. EBs were cultured on 0.1% gelatin-coated petri dishes containing cover slip in α-MEM supplemented with 3% FBS. Neuronal differentiation induction of EBs was achieved by RA (5×10^{-7} M) as a positive control (control^{+}), and no inducer as a negative one (control^{-}). In the treatment group, 10^{-8} M concentration of selegiline was used to induce EBs differentiation to neuronal lineage.

Immunoflourescence. The following primary antibodies were used in this study: mouse anti-synaptophysin monoclonal antibody (Abcam, San Francisco, CA, ab8049) to identify the mature neurons and mouse anti β-III tubulin monoclonal antibody (Abcam, ab7751). FITC-conjugated anti-mouse IgG (Sigma, St. Louis, MO, F9137) was used as a secondary antibody.

For immunoflourescence, cells were cultured in 12-well plates. The cells were grown on a cover slip, fixed in 4% paraformaldehyde, rinsed three times with PBS, permeabilized with 0.3% Triton X-100 in PBS for 30 min at 37°C. Following three washes with PBS, the cells were incubated with 10% normal goat serum (Sigma, G9023) in PBS for 30 min, to block nonspecific binding. Afterwards, the cells were incubated with the relevant primary antibody for 60 min, followed by incubation with the secondary antibody for 30 min. All antibodies were used at the same dilutions (1:1,000). Cover slips were mounted with 70% glycerol in PBS. Several controls for immunostaining were used, and the primary antibody was omitted.

Formalin-fixed paraffin-embedded sections of chicken embryo with neural tube were used as positive control. Embryos were fixed in formalin, dehydrated through an ethanol series into xylenes and then embedded in paraffin and parasagittally sectioned at 5 μm. Slides were dewaxed in xylenes, rehydrated through decreasing concentrations of ethanol, and washed in PBS. All slides were treated with 2% sodium borohydride (Sigma, S9125), for 30 min followed by incubation in 0.1 M glycine to quench endogenous formalin-related fluorescence (Jagla et al. 2000). Antigen retrieval was done enzymatically. Details of all antibodies used are in the previous section.

Statistical analysis. The statistical analysis of the data was done using SPSS. The data were tested for normality using the S-K test. Analysis of variance followed by Duncan test was used to compare the data among the groups of PNI and PVC. The data were presented as means and standard deviation of the means.

Results

Viability assay. Linear regression analysis was done for the cell viability data in which the logarithm of dose against PVC was used. Table 1 shows PVC at 10^{-10} M, and higher concentrations of selegiline were significantly lower than that in the untreated group (control^{-}). There was a significant difference between the control^{-} and 10^{-6}–10^{-10} M, and no significant difference between the control^{-} and 10^{-11} M. PVC at 10^{-6}–10^{-8} M was significantly lower than that in the RA-treated group (control^{+}), while at 10^{-11} M was significantly higher. The table shows no significant difference between control^{+} and 10^{-9} and 10^{-10} M. Significant differences existed between all different concentrations of selegiline with each other.

Neuronal differentiation of EC cells by selegiline. The statistical differences among the groups used in the study showed that PNI was significant between 10^{-8}–10^{-10} M concentrations of selegiline and both controls (RA and control^{-}). Table 1 show that the PNI at 10^{-10} M and higher concentrations of selegiline were significantly more than the control^{-} and less than control^{+}. Significant differences existed between 10^{-8}, 10^{-9}, and 10^{-10} M. The peak response was at 10^{-8} M, which was used for further investigation to study the EC cell-derived neuron-like cells.

Cresyl violet staining. Figure 1 shows the morphology of the differentiated EC cells using cresyl violet. This stain is commonly used for identifying the basic neuronal structure. Selegiline-treated EBs were plated in gelatin-

Table 1. The viability assay and neuronal induction of the P19 EC cell line to different concentrations of selegiline

Groups	Concentration (M)	PVC mean±SD	PNI mean±SD
Sel	10^{-6}	29. 8±3.5*·***	ND
Sel	10^{-7}	39.2±4.5*·***	ND
Sel	10^{-8}	56. 5±2.6*·***	89±4.4**·****
Sel	10^{-9}	65.1±3.2*	82±5.3**·****
Sel	10^{-10}	69.7±2.8*	71.3±5.9**·****
Sel	10^{-11}	74.3±4.6***	ND
RA	5×10^{-7}	66.9±3.8	94.3±2.2
Cont.$^{-}$	0	76.5±3.3	11.4±2.3

The data present the means and the standard deviation of the means of the PVC and the PNI in different concentrations used in the study. Selegiline induced the differentiation of P19 EC cells at a higher mean than that of the untreated group (control^{-}). *RA* as control^{+}

Sel selegiline, *RA* retinoic acid, *ND* not done, *Cont.*$^{-}$ control^{-}

*$P<0.05$, vs. Cont.$^{-}$; **$P<0.05$, vs. Cont.$^{-}$; ***$P<0.05$, vs. RA; ****$P<0.05$, vs. RA

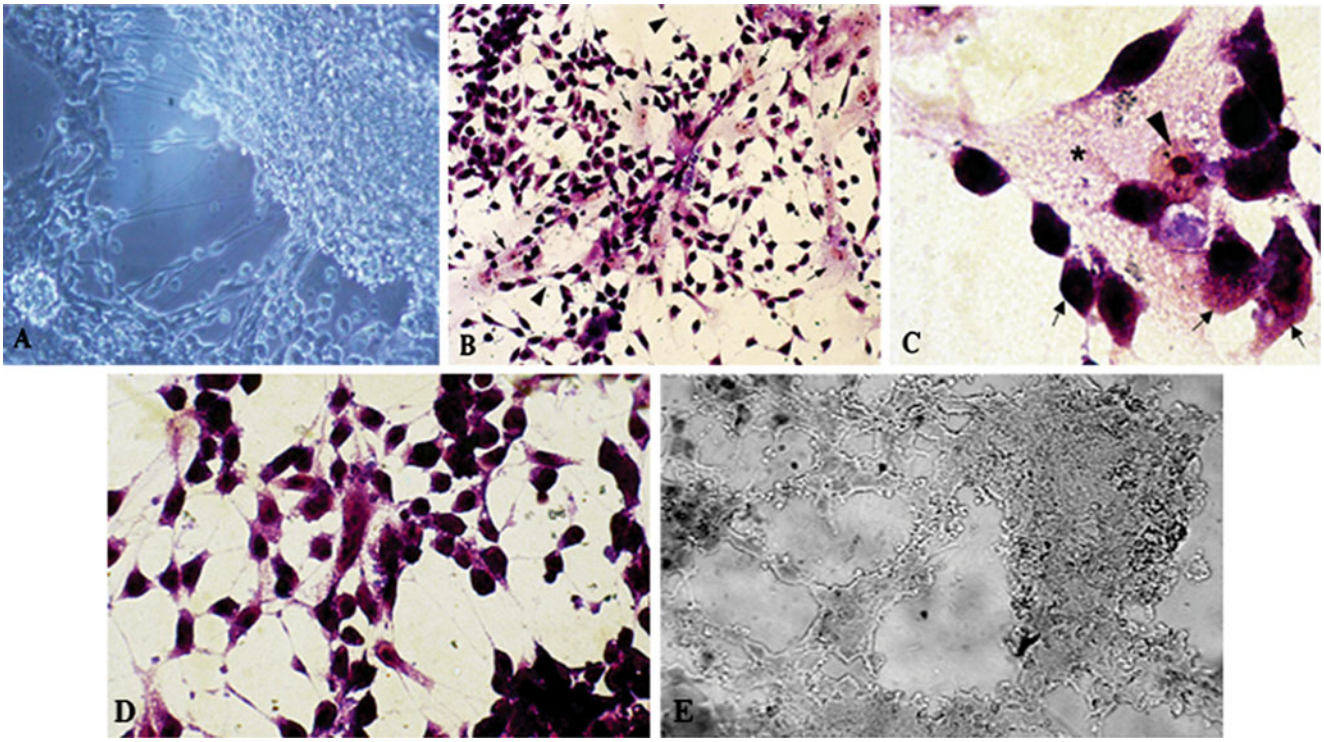

Figure 1. Photomicrographs show P19 EC cells differentiated into neurons, using selegiline as inducer. (*A*) After about 2 wk, cells showed the characteristic morphology of neuronal cells (soma and neurites). Neurons have formed aggregates with areas of lower density of neuronal cell bodies in between. The aggregates are interconnected by fine processes. Cresyl violet was used to stain the differentiated neurons. (*B*) Neuronal-like cells formed a cellular network on top of the monolayer (*arrow*). The *arrowheads* indicate the neuronal extension. (*C*) Neuron-like cells show Nissl bodies in their cytoplasm (*arrow*). The *asterisk* indicates a non-neuronal cell with distinctive nucleus and nucleolus (*arrowhead*). (*D*) P19-derived neurons induced to differentiate with 5×10^{-7} M retinoic acid, as positive control. (*E*) P19 cells treated without drug, as negative control. (*A*, *B*, *E*) ×10, (*C*) ×100, (*D*) ×40.

coated dishes, and then allowed to attach and form outgrowth cultures. Proliferative cells spread around it in a cell monolayer structure. Selegiline treatment had a dramatic effect on neuronal morphology (Fig. 1*A*). Cresyl violet staining was used for the detection of Nissl body in the cytoplasm of neurons (Fig. 1*C*). Neuron-like cells formed a cellular network on top of the monolayer (Fig. 1*B*). Figure 1*D* shows P19-derived neurons induced to differentiate with 5×10^{-7} M retinoic acid, as positive control. Figure 1*E* shows P19 cells treated without drug, as negative control.

Detection of neuron-specific proteins. To characterize post-mitotic neurons, expression of neuron specific proteins, β-III tubulin, and synaptophysin were tested using specific antibodies. Immunoflourescence evaluation of the differentiated P19 cells showed that these cells were immunoreactive to synaptophysin and β-III tubulin after selegiline treatment (Fig. 2*C*, *D*). Neurons positive for β-III tubulin or synaptophysin were detected either as individual cells or in small aggregates. Chicken embryo neural tube was examined for a positive control condition (Fig. 2*A*, *B*).

Discussion

In this study, we investigated the induction of neural differentiation of P19 EC cells by a neuroprotective pharmacological drug, selegiline. About 2 wk after induction by selegiline, subsets of the cells showed morphology of neurons. Our results documented that the neural differentiation of mouse EC P19 cells, was further accelerated by the addition of selegiline into media in a dose-dependent manner. Morphological and cell biological changes were followed during selegiline induced neural differentiation. The neurons obtained in these cultures showed a typical neuronal morphology and exhibited a number of markers characteristic of neurons such as synaptophysin and β-III tubulin.

The cells of many embryonal carcinoma lines differentiate if cultured at high density (McBurney 1976) or as aggregates (Martin and Evans 1975). However, differentiation of P19 cells can be induced if aggregates are exposed to nontoxic concentrations of a number of drugs. The drugs most effective in inducing differentiation of P19 cells are RA (Jones-Villeneuve et al. 1982) and DMSO (McBurney and Rogers 1982). Finley et al. have shown that within 2–

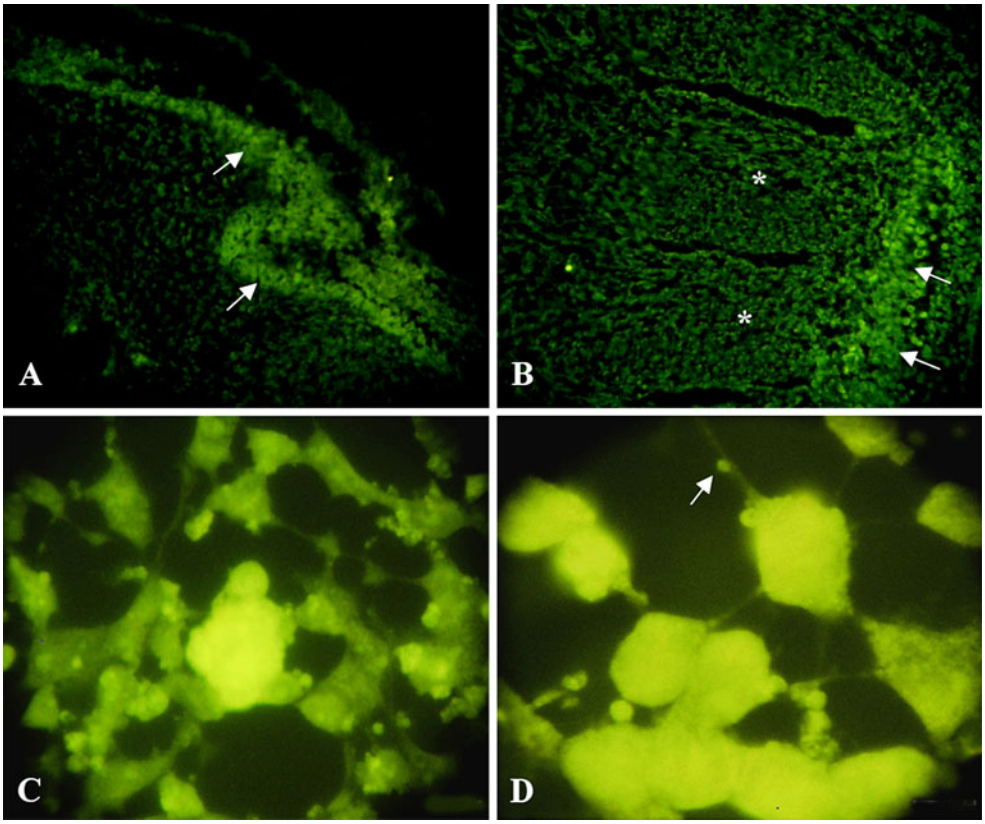

Figure 2. Fluorescence micrographs illustrating the expression of neuronal markers. Chicken embryo neural tube was examined for a positive control condition: (*A*) *arrows* show β-III tubulin-positive cells in the neural tube, (*B*) *arrows* show synaptophysin-positive cells in the neural tube, *asterisks* indicate somites. P19 cells treated with selegiline show immunoreactivity for β-III tubulin, a classic marker for differentiating neurons (*C*), and synaptophysin, a reliable marker of nerve terminal differentiation (*D*). Synaptophysin concentrated in a brightly fluorescent spot (*arrow*). (*A*, *B*) ×4, (*C*) ×40, (*D*) ×100.

3 wk after induction by RA, pluripotent EC cells, P19, and totipotent ES cells pass from a nearly (P19) or totally (ES) uncommitted phenotype to one that strongly resembles mature neurons (Finley et al. 1996). In vitro differentiation of mouse ES cells into neuron-like cells was reported using nerve growth factor (Wobus et al. 1988). This is the first report to our knowledge about the induction of neuronal differentiation of EC cells by selegiline. In order to be driven to neural lineage, EC cells cultured under non-adherent conditions that support the formation of EBs. Resulting EBs were plated on standard tissue culture dishes and then exposed to selegiline. The cultures continued without selegiline for more than 1 mo.

Direct neuroprotective and anti-apoptotic actions of selegiline have previously been observed in vitro and in vivo, in a dose significantly lower than required for MAO-B inhibition. Selegiline reduces the secretion of neurotoxin products in a concentration-dependent manner (Klegeris and McGeer 2000). We used cell viability and dose response assay to decide the range of neuronal induction concentration (10^{-6}–10^{-11} M). The best selegiline concentration to neural cell induction of P19 cells was 10^{-8} M. The cells generated an extensive network of processes during the 2–3 wk after induction. In our earlier investigations using selegiline, this drug could induce neuronal morphology as well as neurotrophin gene expression in ES cells at the concentration of 10^{-8} M (Esmaeili et al. 2006). Selegiline has been shown to increase neuronal survival and to alter protein synthesis and gene expression in astrocytes or PC12 cells independently of MAO-B inhibition (Tatton et al. 1994). Tatton et al. reported that selegiline reduced both cell death and internucleosomal DNA degradation in a concentration-dependent manner and was effective at concentrations too low to inhibit MAO ($<10^{-9}$ M) (Tatton et al. 1994). At concentrations as low as 0.0001 and 0.001 μM, selegiline significantly increased the number of surviving cells under serum-free and hypoxic conditions, respectively (Xu et al. 1999). Selegiline (10 pM–1 nM) induced NGF mRNA expression in cultured rat cortical astrocytes followed by a corresponding increase in NGF protein content (Semkova et al. 1996). It reduces neuronal death at concentrations too small to cause MAO-B inhibition (Tatton 1996).

Embryonic stem (ES) and embryonal carcinoma (EC) cells may provide information that is pertinent one to the other. Both of these cell types share the general properties of pluripotent stem cells in that they exhibit unlimited self-renewal and can give rise to derivatives of all three embryonic germ layers. They can be considered as complementary tools to study the developmental mechanisms. In 1984, Andrews et al. reported that compared to human ES cells, human EC cells seem to have less differentiation capacity in vivo and are usually aneuploid.

Therefore, because of their karyotype, EC cells are not suitable for clinical applications (Andrews et al. 1984). However, Passier et al. reported that after differentiation, EC cells are no longer malignant; they therefore became not only a useful model for the study of development, but were also of interest to oncologists testing differentiation–induction as therapy for teratocarcinoma (Passier and Mummery 2003).

The P19 mouse EC line is an excellent widely used model to analyze regulation of neuronal development and differentiation. P19 cells have a normal male karyotype (McBurney and Rogers 1982). They are widely available, easily cultured without feeders, and do not usually require special growth conditions. In addition, the neurons derived from this cell line have been successfully used in transplantation studies in several mouse models (Astigiano et al. 2005). Undifferentiated P19 cells are amenable to genetic manipulations such as transfection and establishment of stable clonal cell lines expressing introduced genes (Bain et al. 1994).

Here, a simple and efficient strategy is proposed to serve as a basis for neurodifferentiation studies in vitro. To create the basis for further experimentation, we first tested whether the cells of the mouse EC cell line P19 also acquire a neural phenotype after treatment by selegiline. The differentiated cells were then used in the transplantation studies in our experiments (data not shown).

In accord with the earlier work of others, we showed that selegiline could induce neuronal markers, β-III tubulin and synaptophysin in differentiated EC cells. It has been previously demonstrated that neurons derived from ES cells by selegeline induction, were immunoreactive for synaptophysin (Esmaeili et al. 2006). In this study, the formation of synapses between P19-derived neurons in mature cultures has been documented by immunoreactivity for synaptophysin. One of the most extensively studied synaptic proteins is synaptophysin (Masliah and Terry 1993). Synaptophysin is a reliable marker of nerve terminal differentiation (Sortwell et al. 1998). It has been previously demonstrated that P19-derived neurons form synapses in vitro and are immunoreactive for synaptophysin (MacPherson et al. 1997). The expression of this protein correlates with synapse formation (MacPherson et al. 1997). Previous studies showed that reduced synaptic activity causes detrimental effects on synapses and memory (Tampellini et al. 2010). A treatment that could restore or preserve synaptic connections between neurons could be of great potential utility in treating AD (Bailey and Lahiri 2010). Bailey et al. demonstrated that rivastigmine treatment in primary cortical cell culture model cannot only preserve neurons, but preserve neuronal morphology and synaptic markers that are vital for normal neuronal function (Bailey and Lahiri 2010). Also, lithium chloride was reported to increase mRNA levels of genes encoding synaptophysin and the 160-kDa neurofilament protein (Schmidt et al. 2001).

In our previous study the presence of nestin, a marker of neural progenitor cells has been observed in ES cell-derived neuron induced by selegiline (Esmaeili et al. 2006). Although this neuroepithelial marker was not directly tested here, the neuronal population indeed appears stronger in the selegeline conditions than untreated cultures. Also, positive staining of β-III tubulin showed differentiation of P19 EC cells into neurons. The β-III tubulin is a classic marker for differentiating neurons (Rak et al. 2011). Expression of brain-specific III tubulin in stem cell-derived neurons has been shown in previous studies (Falconer et al. 1992; Kompisch et al. 2010).

Conclusion

Our results documented that the neuronal differentiation of mouse P19 EC cells, was induced by a neuroprotective pharmacological drug, selegiline. The differentiated cells showed a typical neuronal morphology and exhibited a number of markers characteristic of neurons such as synaptophysin and β-III tubulin. An understanding of the molecular mechanism of selegeline may contribute to the development of new therapies for neurodegenerative diseases. These data suggest that selegiline may have utility in neurodegenerative diseases due to its effects on synapse formation.

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