Aromorphine induces trophic factors that support fetal rat mesencephalic dopaminergic neurons in cultures

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Abstract

Aromorphine, the catechol-derived dopamine D1/D2 receptor agonist, is currently in use as an antiparkinsonian drug. It has previously been reported that aromorphine was able to elicit expression of the enzyme tyrosine hydroxylase, a marker for DA neurons, in the fetal rat cerebrocortical cultures whilst in the presence of brain-derived neurotrophic factor. The present study demonstrated that treatment of fetal rat ventral mesencephalic cultures with aromorphine caused a marked increase in the number of dopaminergic neurons. The action of aromorphine can be mimicked by dopamine receptor (D1 and D2) agonists or blocked by preincubation with D1/D2 receptor antagonists. Incubation of recipient mesencephalic cultures with the conditioned medium derived from aromorphine-stimulated donor mesencephalic cultures elicited a 3.72-fold increase in the number of TH-positive neurons. Increased mRNA expression levels of brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor were also found in the aromorphine-treated mesencephalic cells along with concomitant protein expression increases in the conditioned medium. Moreover, the trophic activity observed could be partially neutralized by antibodies against either brain-derived neurotrophic factor or glial cell line-derived neurotrophic factor. Cultured fetal striatal cells, but not hippocampal cells, also responded to aromorphine treatment. The membrane filtration studies revealed that both <30 kDa and >50 kDa fractions contained trophic activities. The latter characterization distinguishes them from most known neurotrophic factors. These results suggest that the aromorphine-modulated development of dopaminergic neurons may be mediated by activation of the dopamine receptor subtypes D1 and D2 thereby increasing the production of multiple growth factors.

Introduction

Parkinson’s disease is characterized by progressive dopaminergic (DA) neuronal cell death in the substantia nigra, resulting in severe motor deficits. Several lines of evidence have suggested that many substances including growth factors support the survival and/or differentiation of dopaminergic neurons both in vitro and in vivo. These include brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4), fibroblast growth factor (FGF)-1, FGF-2, platelet-derived growth factor (PDGF), glial cell line-derived neurotrophic factor (GDNF) and various cytokines (Engele et al., 1991; Hama et al., 1991; Hyman et al., 1991; Lin et al., 1993; Hyman et al., 1994; Zhou et al., 1994). More recently, additional neurotrophic factors of the GDNF family have been discovered. Neurturin was purified from conditioned medium from Chinese hamster ovary cells using an assay based on its ability to promote the survival of sympathetic neurons in culture (Kotzbauer et al., 1996). Persphin was discovered by a cloning protocol using degenerate primer PCR with genomic DNA as a template (Milbrandt et al., 1998). Artemin was identified, by searching DNA databases (Balogh et al., 1998). Like GDNF, neurturin, persphin and artemin are survival factors for mesencephalic DA neurons as well as for other neuronal populations. Moreover, other as yet unidentified trophic factors with survival-promoting effects on DA neurons have been reported (Carvey et al., 1993; O’Malley et al., 1994; Ling et al., 1998; Zhou et al., 2000a). Thus, understanding the molecular mechanisms of action of known growth factors as well as their regulation, and searching for novel neurotrophic factors are the objects of considerable interest.

In previous studies on the induction of DA neurotransmitter phenotype in cultured fetal cerebrocortical cells, it was observed that dopamine (10 μM) in combination with BDNF was able to elicit expression of tyrosine hydroxylase (TH), a marker for DA neurons. Intriguingly, in the presence of BDNF, a tiny amount of exogenously added apomorphine (APO, e.g. 1 nM) was as effective as 10 μM dopamine (Zhou et al., 1996). Withdrawal of APO treatment, however, resulted in rapid loss of expression of DA phenotype in those cortical cells, suggesting the supporting roles of APO in DA neurotransmitter phenotype or the survival of DA neurons.

APO, the catechol-derived dopamine D1/D2 receptor agonist, is in therapeutic use as an antiparkinsonian drug. APO has been shown to be a highly potent iron chelator, a free-radical scavenger and an inhibitor of membrane lipid peroxidation both in vitro and in vivo (for review, see Youdim et al., 1999). APO has also been implicated in inhibition of brain and mitochondrial protein oxidation (Youdim et al., 1999). In vivo APO has been shown to protect against

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MPTP-induced striatal DA neurodegeneration in mice (Grunblatt et al., 2001). The neuroprotection observed with APO does not seem to be related to its DA agonist properties, instead it appears to be due to the antioxidant and free radical scavenging effects of the compound. What exact role APO plays in the growth of mesencephalic DA neurons has not yet been determined. In this study, it was sought to determine whether APO has a supporting effect on the cultured fetal DA neurons. It was found that APO-treated conditioned medium (CM) has trophic effects on DA neurons in fetal mesencephalic cultures, which may be mediated by BDNF, GDNF and other neurotrophic molecules.

Materials and methods

Mesencephalic assay culture
All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fetal ventral mesencephalons (VM) were obtained at embryonic day 14 (E14, where E0 is the day of vaginal plug) of gestation, immediately after the rats were killed with an overdose of barbital. The rat embryos were stored in ice-cold Hank’s balanced saline solution (Ca2+- and Mg2+ free, HBSS) until dissection.

After removal of meninges, the embryos were dissected out in HBSS and cut into 0.5 mm sections. Following washing, the tissue was incubated in HBSS containing 25 μg/mL trypsin and 0.5 mg/mL DNase at room temperature for 10 min. Tissue was then sequentially washed in HBSS containing DNase.

Rat mesencephalic cells were seeded into 96-well plates (Nunc, Roskilde, Denmark) precoated with poly L-lysine (10 μg/mL, Sigma, St. Louis, MO, USA) as described previously (Zhou et al., 1994). Cell viability in suspensions of dissociated cells was determined by the ability of viable neurons to exclude the dye trypan blue. The cells were plated at 105 viable cells/cm2 in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Gaithersburg, MD, USA) and Ham’s F12 (1:1), supplemented with 10% fetal calf serum (Life Technologies). Four hours later, cultures were switched to serum-free conditions, i.e. DMEM/Ham’s F12 (1:1) with addition of N2 (1:100, Life Technologies) and streptomycin/penicillin. It has been well established that the growth of astroglia is greatly inhibited under these conditions. APO-conditioned media and other molecules of interest (e.g. BDNF, GDNF) were added immediately after the medium was changed. Total volume of culture medium was 100 μL per well. Cells were incubated at 37 °C in a 95% air/5% CO2 humidified atmosphere and maintained for 24 h after switching to serum-free medium.

Preparation of conditioned medium
To prepare the conditioned medium (CM), fetal ventral mesencephalic cells were plated on a poly L-lysine-coated 35 mm-diameter dish (Nunc) and were grown in DMEM/F12 supplemented with N2 at cell density of 3 × 103/cm2 for 3 or 6 days. During that time, the VM cultures were treated twice with compound of interested, such as APO (Sigma), haloperidol (HAL, Sigma). The CM was collected and centrifuged at 1000 g to remove cell debris. They were then aliquoted and stored at −80 °C until use. The CM derived from fetal striatal or hippocampal cells were prepared in the same manner.

BrdU incorporation, immunohistochemistry and cell count
Immediately after fetal VM cells were plated, a solution containing 5-bromo-2’-deoxyuridine (BrdU, 50 μM final, Sigma) was added to the cell cultures. BrdU was added again when culture medium was changed to serum-free medium. Cells were washed and fixed as described previously (McTigue et al., 1998). The cells were then treated with 0.1% Triton X-100/phosphate-buffered saline for 2 min and then incubated in 2 N HCl for 2 h at 37 °C. They were washed and incubated in 0.15 M borate buffer (pH 8.5) during 10 min. After blocking in 5% normal goat serum, the cells were then subjected to immunostaining.

The procedure for immunohistochemistry has been fully described elsewhere (Zhou et al., 1994). Rabbit anti-TH antibody (1 : 5000, Chemicon, Temecula, CA, USA), GFAP (1 : 500, Sigma) and mouse anti-BrdU antibody (1 : 500, Sigma) were used. The peroxidase was visualized by incubation with diaminobenzidine (DAB) and intensified with nickel cobalt (Pierce, Rockford, IL, USA). The numbers of TH-positive, GFAP-positive, or BrdU-positive cells were counted in the entire surface area of a culture well, and were expressed as the percentages of immunoreactive cells found in paired cultures treated with CM from control cultures (H2O, used as the reference value, i.e. 100%). In some cases, the number of TH-positive cells and entire cell populations were counted in eight randomly chosen fields under 200 × magnification. Controls included samples without BrdU incorporation and samples without primary antibodies.

RNA isolation and RT-PCR
The cultured VM cells exposed to either APO (2 μM) or H2O were rinsed with 0.01 M PBS and harvested in denaturing solution at various time points and stored at −70 °C until use. The total RNA was isolated using a TOTALLY RNA isolation kit (Ambion, Austin, TX, USA). Single strand cDNA was synthesized from 1 μg total RNA in a reaction volume of 20 μL containing 50 pmol random hexamers (Life Technologies), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 50 mM DTT, 0.75 U RNasin (Promega, Madison, WI,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Amplified region</th>
<th>Expected size (bp)</th>
<th>Cycle conditions</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>Forward 5'-CACTCCGACCTGCCCCCGCC-3'</td>
<td>463–826</td>
<td>364</td>
<td>1 min at 94 °C, 1 min at 57 °C, 2 min at 72 °C, 25 cycles</td>
</tr>
<tr>
<td>BDNF</td>
<td>Reverse 5'-TCCCATATCTCCCGCTTTTTA-3'</td>
<td></td>
<td></td>
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<tr>
<td>GDNF</td>
<td>Forward 5'-ATGAAATATGGAGGATTGCT-3'</td>
<td>50–671</td>
<td>622 &amp; 544</td>
<td>45 s at 95 °C, 3 min at 52–59 °C, 1.5 min at 72 °C, 25 cycles</td>
</tr>
<tr>
<td>GDNF</td>
<td>Reverse 5'-ACGGTTAGCGGAATGCTTTT-3'</td>
<td></td>
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<tr>
<td>HPRT</td>
<td>Forward 5'-CTCGCTGGATACATATAAGCC-3'</td>
<td>257–626</td>
<td>370</td>
<td>1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C 23 cycles</td>
</tr>
<tr>
<td>HPRT</td>
<td>Reverse 5'-CTCGAAGTGTGCTTCTAGTC-3'</td>
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All PCR reactions were performed with hot-start.
American Type Culture Collection, Rockville, MD, USA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and either 200 U or no MMLV reverse transcriptase (Life Technologies). Reactions were incubated for 1 h at 37 °C, terminated by heating 5 min at 95 °C, and stored at −70 °C.

Specific oligonucleotide PCR primers designed to amplify BDNF and GDNF were as described previously (Zhou et al., 2000b). Primers were also designed to amplify regions of coding sequence for hypoxanthine phosphoribosyltransferase (HPRT) gene (Table 1). Negative control templates for each set of PCR reactions included H2O to show that no contaminants were introduced during reaction preparation. Amplification was performed with Mastercycler Gradient® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) in a volume of 20 µL containing 1–3 µL template, 25 pmol each primer and 1.25 U Taq polymerase (Promega, Madison, WI, USA) in 1 × PCR buffer, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 1.5 mM MgCl2 and 0.5 µCi [α-32P]-dATP. Cycle condition consisted of 5 min at 94 °C and N cycles that were listed in Table 1, followed by 72 °C for 7 min. The concentration of HPRT cDNA of each sample was adjusted to the same level before PCR amplification.

Seven-microliter aliquots of each PCR product from the amplification reactions were analysed on 1.5% agarose gel electrophoresis. The gels were fixed with 6% trichloric acid for 30 min, vacuum-dried and then exposed to X-ray film at −80 °C for 5 h. All PCR products obtained were sequenced using automated PCR sequencer (ABI Prism System, Perkin-Elmer, Shelton, CT, USA).

Measurement of BDNF and GDNF by ELISA

The CM from either the APO-stimulated or control VM cultures were collected at 3 days in vitro (DIV) and mixed with the protease inhibitor cocktail (1 : 25, Roche Diagnostics GmbH, Mannheim, Germany). The conditioned media were then concentrated up to 50-fold with Centricon filtration units (molecular weight cut-off, 3000; Millipore, Bedford, MA, USA). The protein concentrations of the samples were adjusted to the same level before 100 µL of each sample was applied into each well (Maxisorp® 96-well plate, Nunc) for the immunoassay. The concentrations of BDNF or GDNF were determined using BDNF or GDNF Emax® ImmunoAssay System (Promega) that are sensitive enough to detect respective trophic factor as low as 12.5 pg/mL. The procedures described in the manuals of the kits were followed in this study.

Molecular weight fractions

The CM of VM cultures treated with APO were fractionated into either less than or more than 5 kDa, less than or more than 30 kDa, and less than or more than 50 kDa fractions using Ultra-free MC centrifugal filtration units (Millipore) or Amicon filtration units (Amicon, Danvers, MA, USA). The fraction, that was < 5 kDa and contained exogenously added APO, was discarded. The residues in filtration units were washed with DMEM/F12 by repeated spins to ensure complete removal of APO. They were then recovered in DMEM/F12 supplemented with N2, and the volumes were adjusted in all cases such that concentration of proteins were the same before the media was added to the recipient VM cultures for bioassay.

Experiment treatment

GABA (Sigma) and dopamine receptor agonists (SKF-38393, quinpirole, Sigma) were added to the cultures starting from the day after plating out the culture (day 0) until the third day in vitro. Dopamine receptor antagonists (haloperidol, SCH-23390 and spiperone, Sigma) were applied to cultures at least 2 h before APO was added.

Antibodies against BDNF (gift of X.F. Zhou), or GDNF (PeproTech, London, UK) were applied to the VM cultures 4 h before APO-induced CM was added.

Statistical analysis

Statistical analysis used commercially available statistical software (GraphPad Prism v2.0, GraphPad Software Inc. San Diego, CA, USA). The data were submitted to either a one-way or a two-way analysis of variance (ANOVA) as indicated in the Results Section. Either the Dunnet test or the Student-Newman-Keuls test (as a posthoc test) was used to compare data samples from the control group with the different treatment groups, or between pairs of groups. Differences were considered significant only when P-values were <0.05.

Results

Effect of APO and CM from APO-stimulated VM culture on the number of mesencephalic DA neurons in vitro

Typically there were ~110 TH-positive neurons per well (96-well plate) in the untreated cultures 1 day after switching the cultures to serum-free medium. Under the conditions, TH-positive neurons deteriorated and a majority of them died off within 3 days. It was observed that addition of APO (2 µM) itself significantly increased the number of TH-positive neurons in VM cultures. The number of TH-positive neurons in APO-treated cultures was 2.86-fold that in untreated cultures (Fig. 1). In contrast, treatment with GABA (10 µM) did not affect the number of TH-positive neurons present in the cultures (GABA, 114.3 ± 8.7 cells per well; untreated, 100 ± 8.0 cells per well; P > 0.05, n = 3).

To determine whether this effect was due to direct action of APO or the effect was transferable, the CM of donor VM cultures incubated with APO were collected at 3 and 6 DIV. They were then separately filtered through 5 kDa filtration units repeatedly to remove any APO present in the medium. It was found that the number of TH-positive neurons in the recipient VM cultures increased up to 3.72-fold when exposed to the CM derived from the 3-day-old-donor VM culture, as compared to that treated with the control CM (Fig. 2).
suggesting that the action of APO can be mimicked by the APO-free CM taken from the APO-stimulated VM cultures. This result indicated that the CM from VM cultures incubated with APO, may contain soluble molecules which are correlated with the elevated number of TH-positive neurons. However, incubation of recipient VM culture with CM from 6-day-old donor VM cultures failed to affect the number of TH-positive neurons when compared with those treated with CM from the untreated donor culture (Fig. 2). Therefore the following experiments used the culture medium collected from 3-day-old donor VM culture throughout.

The promoting effect of the CM from the VM cultures incubated with APO on expression levels of TH in the recipient VM cultures was dependent on the concentration of APO present in the donor cultures. The maximal increase of TH-positive neurons was observed when recipient VM cultures were incubated with CM from donor VM cultures treated with 2 μM APO (Fig. 3). Higher concentration of APO (e.g. 5 μM) resulted in deterioration of donor cultures (data not shown) and 2 μM APO thus was employed in the rest of studies.

APO-induced CM promotes increase of VM TH-positive cells as well as overall cell populations

To determine whether APO treatment specifically promoted the TH expression in the cultures, the recipient VM cultures were incubated with the CM from APO-stimulated donor VM culture for 1 day and then fixed for TH immunohistochemical staining. It was observed that the total number of entire cell populations increased 1.74-fold as compared to control (Table 2). However, the average percentage of TH-positive neurons was increased 5.19-fold (Table 2). This suggested that APO-induced CM increased survival of entire cell populations in cultures but more specifically acted on DA neurons.

To investigate whether the elevated number of TH-positive cells in the culture resulted from promoted proliferation of neural progenitor cells and subsequent differentiation toward DA neurons, BrdU was used to label proliferating cells in the cultures. The cultures were fixed at 3 DIV for immunohistochemical analysis. It was found that direct APO treatment did not significantly increase the number of BrdU-positive cells (APO, 98.66 ± 17.61 cells per well; untreated, 84.66 ± 7.25 cells per well; P > 0.05, n = 3). Double-labelling of immunohistochemical staining revealed that no cells showed both TH- and BrdU-immunoreactivity in the cultures incubated with either control or APO-induced CM (data not shown). These findings suggest that treatment with APO-induced CM did not promote proliferation of mesencephalic progenitor cells but rather increased the survival of postmitotic DA neurons. It may also result from promoted TH expression in non-DA cells in the mesencephalic cultures.

Effects of fractions of conditioned medium from APO-stimulated VM cultures

To determine whether a protein(s) present in APO-induced CM is responsible for the elevated number of TH-positive neurons, the CM from VM cultures incubated with APO (2 μM) was boiled for 15 min. Alternatively, they were mixed with either sulphuric acid (final pH 2.0) or sodium hydroxide (final pH 12.0) and their pH was then

FIG. 2. (A) Dose–response curve of the APO-induced CM from donor VM cultures on the number of TH-positive neurons. The CM was prepared from the donor mesencephalic neuronal cultures by incubating the cultures for 3 or 6 days with or without APO. The CM were included in the recipient cultures of the E14 VM plated at 10^5 cells/cm^2. Cultures were fixed 24 h after media were switched to serum-free medium and the number of TH-positive neurons was counted. Data represent the mean ± SEM of triplicates from five to seven independent experiments. *P < 0.05. (B and C). Photomicrographs of cultured rat fetal mesencephalic cells from E14 rat embryos. The recipient VM cultures were maintained in the presence of 80% of the conditioned medium from control (B) or APO-stimulated VM cultures (C) and immunostained for TH. Scale bar, 50 μm.

FIG. 3. Dose–response curve of APO-induced conditioned medium on the number of TH-positive neurons. Mesencephalic cultures were established as described in Materials and methods section and supplemented with varying percentages of APO-induced conditioned medium from donor VM cultures treated with APO (0.002–2 μM). Control was the medium conditioned without APO. The number of TH-positive neurons present in the cultures was determined after 24 h. Data represent the mean ± SEM of triplicates from three to four independent experiments (two-way ANOVA, F_dose = 23.48, P < 0.001, F_CM = 15.93, P < 0.001).
The dissociated E14 VM were plated at 10^3/cm^2 in DMEM/F12 containing 10% fetal calf serum. The medium was changed to serum-free N2 medium with or without APO-induced CM 4 h after plating. The cultures were fixed 24 h later for TH immunohistochemistry. Numbers of TH-positive neurons and total cell populations were counted in 8 randomly chosen field under a phase-contrast microscope (200 x magnification) and expressed as number of cells/cm^2. Values represent the mean ± SEM, n = 6.

brought back to pH 7 after 5-minute incubation, this treatment was then followed by filtering through 5 kDa filtration membranes. As shown in Fig. 4, the activity was reduced, but not abolished, by either of these treatments, as was demonstrated by the fact that the numbers of TH-positive cells in the recipient VM cultures incubated with acid-treated, alkaline-treated CM or heat-inactivated CM were 34.9%, 63.88% of that in the recipient cultures exposed to the native APO-induced CM at 50% of total volume. This suggests a proteinaceous composition in the CM.

The APO-induced CM was further fractionated into 3 fractions, i.e. Fraction 1, 5–30 kDa, Fraction 2, 30–50 kDa, Fraction 3, >50 kDa, and subsequently their effects on the DA neurons of the recipient VM cultures were evaluated. It was found that these three fractions resulted in very different effects on the TH expression in the recipient VM cultures (F < 0.0001). Significant increases of TH-positive neurons were observed in the cultures incubated with Fractions 1 and 3 (P < 0.05), but not Fraction 2 (P > 0.05). These activities were also dose-dependent (F < 0.0001). HIGHEST activity was observed when 100% of Fraction 1 or 50% of Fractions 3 were used to culture VM (F = 5.777, P = 0.002). However, Fraction 2 had no significant effect on the number of TH-positive cells in recipient VM cultures at three concentrations tested.

**Stimulation with APO increased mRNA expression levels of BDNF and GDNF as well as their release in the CM**

BDNF and GDNF are well-studied neurotrophic factors for mesencephalic DA neurons both in vivo and in vitro (Beck et al., 1993; Lin et al., 1993; Mayer et al., 1993; Hyman et al., 1994; Zhou et al., 1994; Fawcett et al., 1995). To determine whether the treatment of APO in the VM cultures resulted in alteration of gene expression, the mRNA level of these neurotrophic factors was analysed. BDNF was the first to be examined in this setting. There were significant elevations of BDNF transcript (364 base pairs) at 10 min following addition of APO. It had returned to control levels at 2 h and thereafter (Fig. 6). RT-PCR analysis of RNA from the VM cultures using GDNF primers yielded a major band of the predicted size (of ~622 base pairs), as well as a minor band of ~544 base pairs. The levels of the two GDNF transcripts were significantly increased in the APO-treated VM cultures 10 min and 2 h after treatment. However, their expression had returned to control levels by 3 days after plating (Fig. 6).

By using an ELISA assay, both BDNF and GDNF proteins were detected in the VM culture media. The concentration of BDNF was increased 1.6-fold in the APO-stimulated CM as compared with the control (APO, 50.08 ± 12.3 pg/mL of the concentrated CM; control, 31.3 ± 7 pg/mL, n = 5). Similarly, the APO treatment also resulted in increased protein levels of GDNF of up to 2.56 fold (APO, 243.0 ± 22.3 pg/mL; control, 93.5 ± 16.7 pg/mL, n = 3) in the CM of mesencephalic cultures.

**Addition of antibodies against either BDNF or GDNF partially abolished the effect of APO-induced CM**

As the mRNA of BDNF and GDNF, as well as their protein levels were up-regulated following APO treatment, it was next investigated whether these trophic factors were responsible, either fully or in part, for the elevation of TH expression. APO-induced CM was incubated with one of the two antibodies, respectively, 4 h before they were used in VM recipient cultures. These antibodies recognize their respective antigens with a high degree of specificity and have been previously used for in vivo and in vitro studies neutralizing the biological activities of these endogenous growth factors (Zhou et al., 2000a, c). Their high specificities were verified as addition of individual antibody against BDNF or GDNF in the VM cultures neutralized the survival-promoting effect of respective human recombinant trophic factors as shown in Fig. 7. Moreover, irrelevant rabbit IgG (e.g. antibodies against Fos or microtubule-associated protein) had no effect on the survival of TH-positive neurons. It was observed that additions of individual antibody against either BDNF or GDNF reduced the APO-mediated increase in the number of TH-positive neurons by 20.9–23.3% (Fig. 7). Together, these data suggest that the APO-mediated effect was partially dependent on BDNF and GDNF produced by the mesencephalic cells.

**Pharmacological characterization of the APO-induced SPE on mesencephalic DA neurons**

**Effect of DA receptor agonists**

It has been shown that APO is able to affect cellular responses in the central nervous system via two subtypes of DA receptors, i.e. the D₁...
and D₂ receptors. Both D₁ and D₂ receptors are associated with neuronal elements in the adult rat substantia nigra and in cultured fetal rat DA neurons (Magal et al., 1993). It is not clear, however, whether DA receptors are involved in the APO-induced elevation of TH expression observed in this study. To determine whether D₁ receptors mediate this process, SKF-38393 (a specific D₁ receptor agonist) and quinpirole (a specific D₂ receptor agonist) were tested to mimic the effect of APO itself.

Donor VM cultures were exposed to either SKF-38393 or quinpirole at 0.001–10 μM for 3 days. It was shown that the maximal effect of quinpirole was achieved at 10 μM when the number of TH-positive neurons was increased to 1.69-fold that of control, whilst SKF-38393 was less effective than quinpirole (1.42-fold increase, Fig. 8A), indicating that D₁ and D₂ receptors may play important roles in the process.

Effect of DA receptor antagonists
To establish more precisely whether the supporting effect of APO was mediated via D₁ or D₂ receptors, the effectiveness of APO treatment was challenged by the dopamine specific receptor antagonists, haloperidol (nonselective), SCH-23390 (selective D₁), and spiperone (selective D₂). They were added to donor VM cultures 2 h before APO addition on day 0. The CM was collected on day 3 and filtered through a 5 kDa filteration unit to remove HAL and APO present in the medium. The recipient VM cultures were incubated with the filtered CM. The number of TH-positive neurons was determined at day 1. The results suggested that the effect of APO was completely blocked by 1 μM haloperidol (Fig. 8B). Moreover, the effect of the CM was decreased with addition of SCH-23390 (10 μM) or spiperone (10 μM), but neither completely abolished APO-mediated effect on TH expression in culture (Fig. 8B).

Effect of conditioned medium from fetal striatal or hippocampal cultures exposed to APO
To examine whether APO stimulation on fetal cells from other brain regions can produce similar transferable effect, the CM from either fetal, striatal or hippocampal cell cultures, which were incubated with APO for 3 days, were collected and tested in the same culture system. The recipient VM cultures were grown in the CM for 24 h. Incubation of the recipient VM cultures with the CM derived from the APO-treated fetal striatal cultures dramatically elicited a 4.79-fold increase in the number of TH-positive neurons.
positive cells as compared to control (Fig. 9). However, incubation of the recipient VM cultures with the CM from APO-treated fetal hippocampal cultures did not increase the number of TH-positive cells (10% CM, 110.75%, H2O, used as the reference value, i.e. 100%; F = 0.7393, P > 0.05). These results demonstrated that the effect of APO may be dependent on the presence of DA receptors (D1/D2) in the target cells. Moreover, non-DA innervated brain regions (such as hippocampus) may not be able to exert the effect specifically on DA neurons in response to APO stimulation.

Discussion

The observations in the present study indicate that APO increases the number of cultured mesencephalic DA neurons by enhancing biosynthesis of BDNF and GDNF, as indicated by up-regulation of mRNA and protein levels of these trophic factors. Furthermore, this effect may be involved in activation of the dopamine D1 and D2 receptors. Thus, the present study presents novel evidence demonstrating that APO is able to regulate neuronal growth of nigrostriatal system through multiple trophic factors.

Roles of neurotrophic factors

There is ample evidence indicating that neurotrophic factors, such as GDNF (Lin et al., 1993) and BDNF (Beck et al., 1993; Zhou et al., 1994), influence growth of developing and adult nigral DA neurons.

The questions are whether these growth factors are produced by cultured mesencephalic neurons and whether they are subsequently responsible for the APO-induced effect observed in this study. The results described here suggest that these growth factors are very likely to play important roles in this process. Their mRNA and protein levels were upregulated in the VM cultures stimulated with APO, as indicated by RT-PCR analysis and immunocytoassay. This is consistent with Kuppers’s observation that stimulation with dopamine or a dopamine D1 receptor agonist increased BDNF mRNA and protein in neuronal cultures (Kuppers & Beyer, 2001). Moreover, the antibody neutralization studies described here showed that preincubation of CM from APO-treated VM cultures partially abolished the increase in number of TH-positive neurons, suggesting that BDNF and GDNF were relevant to the activity of the CM. The molecular weight of most neurotrophic factors, except for GDNF, is less than 27 kDa. Thus presence of BDNF (26 kDa) may partially explain the trophic activity of the <30 kDa fraction observed in this study.
The molecular weight of the GDNF homodimer is about 32 kDa (Lin et al., 1993; Okragly & Haak-Frendscho, 1997). Therefore, it was assumed that the 30–50 kDa fraction would act as a trophic agent on DA neurons in the survival assay. The finding that this fraction failed to elevate the number of DA neurons in cultures suggested that it might not have contained GDNF. One possible explanation for this discrepancy is that the GDNF homodimer had fallen into the <30 kDa fraction in dialfiltration. This is due to the fact that its molecular weight (32 kDa) and the molecular weight cut-off of the filter (30 kDa) are so close to each other that the filter membrane failed to retain GDNF molecules. Although the data suggest that BDNF and GDNF may be responsible for the neurotrophic activity observed here, it does not preclude the possibility that other trophic factors are involved.

The present study also showed that the promoting effect of the CM from the APO-treated cultures was present in the 5–30 kDa fraction and the >50 kDa fraction. Intriguingly, the latter possessed higher activity than the former (containing BDNF, GDNF and other factors), as the promoting effect was more prominent when recipient VM cultures were incubated with the >50 kDa fraction at 50% of total volume (Fig. 5). This characterization distinguished itself from most known growth factors, suggesting that it may contain a novel dopaminergic growth factor(s).

It has been reported that APO treatment elevated mRNA level of nerve growth factor (NGF) in cultured astrocytes, raising the question of whether NGF is involved in the action of the CM (Ohta et al., 2000). Although the mRNA and protein levels of NGF in the VM cultures were not examined in this study, it is very likely that NGF is not relevant in this regard, as it has been well established that NGF possesses no trophic action on DA neurons in either in vivo or in vitro studies (Knusel et al., 1990a, b).

Astroglia may not be responsible for the observed up-regulation of TH expression

It is plausible that the molecules responsible for the increase in the number of TH-positive neurons observed here were released from mesencephalic cells that exclusively express D1 and D2 receptors (as judged by the evidence presented in this study). Moreover, they can also be produced by D1/D2-expressing striatal cells, the target of nigral DA neurons (Gerfen et al., 1990; Trovero et al., 1994). There is a large volume of evidence indicating that DA receptors are expressed in astrocytes (Bal et al., 1994). Therefore it is possible that APO acts on glia through DA receptors to produce the observed trophic effect. Indeed, APO treatment stimulates the synthesis and release of neurotrophic factors, such as NGF and GDNF, in astrocytes (Ohta et al., 2000). Dopamine or dopaminergic drugs are also able to induce the synthesis of various growth factors (e.g. fibroblast growth factor-2) in cultured astrocytes (Reuss & Unsicker, 2000; Reuss et al., 2000). If this were the case, however, then APO would not have been able to promote the number of TH-positive neurons, as well as the overall cell population in VM cultures, as they were incubated in serum-free media that is highly inhibitory to the growth of astroglia (<0.1% GFAP-positive cells were present in the VM cultures). Thus, we have postulated that astroglia do not seem to mediate the APO-induced activity and thus the molecules responsible for this effect are released by the mesencephalic DA neurons and/or other cell subpopulations and may act in an autocrine/paracrine manner.

Involvement of DA receptors

The APO-induced up-regulation of TH expression in the present study seems to be heavily dependent on activation of D1 and D2 receptors, but not GABA receptors, all of which are expressed in DA neurons and mesencephalic astrocytes. However, the observed effect was not due to the activation of GABA receptors, as GABA itself failed to promote the survival of cultured DA neurons in this study. Moreover, treatment of DA receptor agonists produced a similar effect to that of APO itself, and the effect of the media from APO-stimulated VM culture was partially blocked by either SCH-23390 or spiperone, but neither completely abolished the SPE in the recipient VM cultures. Furthermore, the media from APO-treated striatal cultures, but not hippocampal cultures, also possessed trophic activity. Taken together, these findings strongly support the notion that the D1 and D2 receptors play critical roles in mediating the trophic activity of APO. This observation, however, is not consistent with those reported by Ling et al. (Ling et al., 1998). They suggested that culture media conditioned by exposure to VM cultures in the presence of D1 receptor agonist pramipexole, but not D2 receptor agonists and antagonists, increased the growth and survival of DA neurons in recipient VM cultures (Carvey et al., 1997; Ling et al., 1998). Furthermore, they showed that a 35-kDa band as revealed by electrophoresis, which was present in normal cultures, was increased in pramipexole-incubated cultures. As the 30–50 kDa fraction showed no trophic activity in the present study, it is likely that APO promoted the TH expression in cultured DA neurons through distinct mechanisms of pramipexole action.

Although these pharmacological studies strongly suggest the importance of DA receptors in mediating the APO-induced activity, the present results do not rule out the involvement of receptors of known trophic factors, such as trkB and trkC. It has been shown that trkBTK+ and trkC mRNA levels in the striatum were increased in postnatal rats treated with the indirect dopamine agonist cocaine (Jung & Bennett, 1996a; Jung & Bennett, 1996b). The trkBTK+ effect was blocked by pretreatment with the D1 receptor antagonist, SCH23390, and was not affected by pretreatment with the D2 receptor antagonist, eticlopride. In contrast, trkC regulation may be mediated by independent stimulation of D1 and D2 receptors (Jung & Bennett, 1996b). As both fetal mesencephalic DA neurons and striatal neurons express trkB and trkC receptors, we postulate that APO may modulate receptors of trophic factors thereby influencing the growth and differentiation of mesencephalic and striatal neuronal cells.
Moreover, recent studies have demonstrated that D_{2R} receptor and somatostatin receptor SSTR5 interact physically via hetero-oligomerization to create a novel receptor which is the molecular basis for interaction between two different neurotransmitter systems (Rocheville et al., 2000). Whether APO acts on DA receptors as well as receptors of other systems requires further study.

The present study showed the potent effect of APO on the TH-expressing neurons in mesencephalic cultures. These neurons are most likely postmitotic as they are derived from E14 rat VM (Hanaway et al., 1972). Thus, the increased number of TH-positive neurons observed here could be the result of a growth factor-mediated survival-promoting effect. Alternatively, it is possible that the growth factors work synergetically with APO and/or DA (produced by mesencephalic dopaminergic neurons) to induce TH expression in the non-DA cells in the cultures. This hypotheses is supported by previous studies of these authors and others, demonstrating that growth factors, such as BDNF, FGF-1, FGF-2 or ciliary neurotrophic factor, in combination with DA/APO could elicit TH expression in cultured fetal brain cells (Magal et al., 1993; Du et al., 1995; Zhou et al., 1996). More detailed investigation is therefore needed to distinguish between these possibilities.

It has been demonstrated here that APO stimulated the synthesis of both BDNF and GDNF in mesencephalic as well as striatal neuronal cultures. These growth factors could either directly influence striatal neural cells or could be retrogradely transported to the substantia nigra (Anderson et al., 1995; Ferguson & Johnson, 1991; Mufson et al., 1994). It is possible therefore that APO could be a physiological factor regulating growth and differentiation in the nigrostriatal system. This notion is supported by recent findings that demonstrate the presence of the APO enantiomers in extracellular fluid collected from the rat striatum by microdialysis. Moreover, the concentrations of R(−)- or S(+)APO in brain tissue is significantly higher than the free APO measured in plasma (Sam et al., 1997), suggesting its involvement in maintaining brain function at least in adult rats.

It has been shown that administration of APO in patients with Parkinson’s disease reduces the need for orally delivered antiparkinsonian medication by a mean of 59%, and virtually eliminated ‘off’ time. There was also a significant reduction in dyskinesias and a markedly improved quality of life (Manson et al., 2001). Future studies may need to address the question of whether administration of APO could enhance biosynthesis and release of mesencephalic and striatal BDNF, GDNF and possibly other factors in vivo, through which it may be protecting nigral DA neurons against neurodegeneration.

In conclusion, the results reported here indicate that APO is a potent trophic substance on mesencephalic DA neurons and may play a pivotal role in the development of the nigrostriatal system, as well as maintaining its normal function in the adult. This effect may be mediated by activation of the DA receptor subtypes D_{1} and D_{2} thereby increasing the production of multiple growth factors. Identification of the factors present in the >50 kDa faction which elicited trophic action on DA neurons may be helpful to develop new methods of treatment for neurodegenerative diseases such as Parkinson’s disease.

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Abbreviations

APO, apomorphine; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; CM, conditioned medium; DA, dopamine/dopaminergic; DME, Dubecco’s modified Eagle’s medium; GDNF, glial cell line-derived neurotrophic factor; HAL, haloperidol; HBSS, Hank’s balanced saline solution; HPRT, hypoxanthine phosphoribosyltransferase; 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase; VM, ventral mesencephalic.

References


