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Dopamine receptor activation increases glial cell line-derived neurotrophic factor in experimental stroke

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ABSTRACT

Treatment with levodopa enhances functional recovery after experimental stroke but its mechanisms of action are elusive. Reactive astrocytes in the ischemic hemisphere are involved in mechanisms promoting recovery and also express dopamine 1 (D1) and dopamine 2 (D2) receptors. Here we investigated if the activation of astrocytic dopamine receptors (D1 and D2) regulates the expression of glial cell line-derived neurotrophic factor (GDNF) after combined in vitro hypoxia/aglycemia (H/A) and studied the expression of GDNF in the ischemic brain after treatment with levodopa/benserazide following transient occlusion of the middle cerebral artery (tMCAO) in the rat. Twenty-four hours after H/A, GDNF levels were upregulated in exposed astrocytes compared to normoxic control cultures and further elevated by the addition of the selective D1 receptor agonist (R)-(+)-SKF-38393 hydrochloride while D1 receptor antagonism by R(+)-SCH-23390 hydrochloride significantly reduced GDNF. No effect on GDNF levels was observed by the application of the D2 receptor agonist R(-)-2,10,11-trihydroxy-N-propyl-noraporphine hydrobromide hydrate or S-(-)-eticlopride hydrochloride (D2 receptor antagonist). After tMCAO, GDNF was upregulated in D1 expressing reactive astrocytes in the peri-infarct area. In addition, treatment with levodopa/benserazide significantly increased GDNF levels in the infarct core and peri-infarct area after tMCAO without affecting the expression of glial fibrillar acidic protein (GFAP), an intermediate filament and marker of reactive gliosis. After stroke, GDNF levels increase in the ischemic hemisphere in rats treated with levodopa, implicating GDNF in the mechanisms of tissue reorganization and plasticity and in L-DOPA enhanced recovery of lost brain function. Our results support levodopa treatment as a potential recovery enhancing therapy in stroke patients.

Introduction

Dopamine regulates a wide variety of physiological processes, and is important for almost all brain functions during development as well as in the adult brain (Kawashima et al., 2012), such as motor control and motor learning (Hosp et al., 2011). Reduced brain dopamine levels, on the other hand, can cause severe clinical symptoms resulting in neurological disorders such as Parkinson's disease (PD) as the most prevalent one (Calne and Sandler, 1970). Thus, symptomatic treatment with the dopamine precursor levodopa in combination with a peripherally acting decarboxylase inhibitor can effectively attenuate clinical symptoms of PD. Levodopa treatment combined with forced physical therapy has been investigated in a prospective double-blinded randomized clinical trial and significantly enhances motor function in stroke patients (Scheidtmann et al., 2001). Moreover, levodopa treatment enhances procedural motor learning paradigms in chronic stroke patients (Rosser et al., 2008).

In a reversed translational approach, we recently demonstrated that the administration of levodopa/benserazide improves functional recovery in rats after transient occlusion of the middle cerebral artery (tMCAO) (Ruscher et al., 2012). We also found that glial fibrillary acidic protein positive astrocytes in the peri-infarct region express dopamine 1 (D1) receptors and dopamine 2 (D2) receptors. Previous studies have reported the expression of functional dopamine receptors on astrocytes (Zanassi et al., 1999; Miyazaki et al., 2004) and other non-neuronal cells (Howard et al., 1998) which together with our findings suggest a functional role for D1 and D2 receptor activated mechanisms in these cells in addition to those activated by dopamine receptors located on neurons. In particular, reactive astrocytes defined by morphological changes and the expression of intermediate filaments secrete various factors that may contribute to tissue reorganization in response to injury (Sofroniew, 2009). Among those, it is the paracrine release of trophic factors that may stimulate mechanisms of neuronal plasticity after stroke (Cuello, 1997).

Glial cell line derived neurotrophic factor (GDNF) and its receptor complex GFRα-1/Ret are expressed in astrocytes and neurons (Lin et al., 1993; Schaar et al., 1993; Treanor et al., 1996). Treatment with

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GDNF has attracted attention as a possible treatment for patients with Parkinson's disease due to its protective and restorative effects on dopaminergic and motor neurons in experimental models and improved functional recovery in monkeys after 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (Gash et al., 1996). In experimental stroke research, previous studies have focused on the neuroprotective role of GDNF (Wang et al., 1997; Kitagawa et al., 1998). A transient increase in GDNF, GFRa-1 and c-Ret mRNA expression was observed in the rat hippocampus and cortex after tMCAO, respectively (Arvidsson et al., 2001). In addition, Miyazaki and colleagues reported that transient forebrain ischemia led to a transient increase of GDNF mRNA and that delayed expression of GDNF protein was observed in reactive astrocytes and surviving neurons in the hippocampus with a possible role for neuroprotection (Miyazaki et al., 2001).

Pharmacological studies in different in vivo or in vitro models have demonstrated that GDNF expression is partially dependent on dopaminergic signaling, mediated by either D1 or D2 receptor activation (Kinor et al., 2001; Ohta et al., 2003, 2010). Therefore, the aim of the present study was to examine the effect of levodopa/benserazide treatment on GDNF protein expression following tMCAO in the rat, in an experimental paradigm that improves recovery of lost function without affecting infarct size (Ruscher et al., 2012). To further elucidate the regulation of GDNF levels in the injured brain, GDNF expression was studied in primary cortical astrocytes exposed to combined hypoxia/aglycemia in response to the modulation of D1 and D2 receptor activity.

Materials and methods

Transient occlusion of the middle cerebral artery (tMCAO) and levodopa treatment studies

Transient MCAO (tMCAO) was induced in male Wistar rats by transient MCA occlusion for 120 min with a nylon thread introduced into the internal carotid artery and advanced to the MCA bifurcation under anesthesia (3% isoflurane in O_2 with spontaneous ventilation) and has been essentially described earlier (Ruscher et al., 2009, 2012). Rats were treated either with levodopa/benserazide (5 mg/kg body weight) or vehicle (saline) for 12 consecutive days starting on day 2 after tMCAO as described in Ruscher et al. (2012). For immunohistochemistry/immunofluorescence analysis additional rats were subjected to tMCAO (120 min) and were perfusion fixed on day 7 after the insult. Animal studies were approved by the Lund/Malmö ethical committee.

Preparation of cortical astrocytes

Primary cultures of astrocytes were prepared according to a modified method described previously (Ruscher et al., 2002). After 10 days, microglial cells were removed by shaking at 37 °C for 1 h (250 rpm). Astrocytes were resown in 6-well plates and grown until confluency and used for experiments on day 7 after plating.

Hypoxia/aglycemia (H/A)

Combined sublethal H/A for 10 min was performed as described previously (Ruscher et al., 2011). Duration of H/A has been evaluated in preliminary experiments. No cell damage has been observed (data not shown).

In vitro pharmacology

Immediately after H/A or normoxic control treatment, astrocytes were treated with the following D1 and D2 receptor agonists or antagonists for 24 h (final concentrations in parentheses): R(+)-SCH-23390 hydrochloride (SCH, 1 μM, D1 receptor antagonist), S-(-)-eticlopride hydrochloride (eti, 1 μM, D2 receptor antagonist), R(-)-2,10,11-trihydroxy-N-propyl-noraporphine hydrobromide hydrate (TPN, 1 μM, D2 receptor agonist), and (R)-(+)-SKF-38393 hydrochloride (SKF, 1 μM, D1 receptor agonist). Compounds were dissolved in DMSO and used in 1:100 dilutions. Final concentration of DMSO in the culture medium was 1%, which at this concentration was not toxic. For controls, only 1% DMSO was added to the culture medium. After 24 h, cells were harvested and proteins were extracted in a standard procedure as described previously (Ruscher et al., 2002).

Western blotting

Proteins were separated on a 10% SDS polyacrylamide gel. Thereafter, blocking was performed onto polyvinyldifluoride membranes using blocking buffer (20 mM Tris, 136 mM NaCl, pH 7.6, 0.1% Tween 20, 5% nonfat dry milk). After incubation overnight with a rabbit polyclonal antibody against GDNF (dilution 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) or monoclonal anti-GFAP (dilution 1:5000, Sigma-Aldrich) at 4 °C, membranes were incubated with respective secondary HRP-linked antibodies (Sigma-Aldrich, Germany; dilution 1:15000) and visualized by exposing the membrane to a CCD camera (LAS1000, Fujifilm, Japan) using a chemiluminescence kit (Millipore, UK). Membranes were stripped and reprobed for β-actin (Sigma-Aldrich, Germany, and diluted 1:50000). After densitometric analysis, GDNF and GFAP levels were calculated as percentage of β-actin expression.

Immunohistochemistry/immunofluorescence

Brain sections (thickness 30 μm) from 4% paraformaldehyde-perfused animals were washed with phosphate buffered saline (PBS). Blocking was performed with 5% normal swine or donkey serum in PBS supplemented with 0.25% Triton X-100 for 60 min. For immunohistochemistry, a rabbit polyclonal anti-GDNF (1:200, R&D Systems) and polyclonal swine anti rabbit biotinylated antibody (1:500, Dako) were used. The immunoreaction was visualized with avidin–biotin–peroxidase/DAB.

For co-localization of proteins, the following antibodies were used: rabbit polyclonal anti-GDNF (1:200, R&D Systems), monoclonal directly Cy3 conjugated anti-GFAP (1:5000, Sigma-Aldrich, MO), rabbit polyclonal anti D1 receptor and D2 receptor (1:250, Abcam) and mouse monoclonal anti nestin (1:500, Chemicon). After overnight incubation at 4 °C, cells were incubated with appropriate secondary antibodies (Cy5 conjugated donkey anti-mouse antibody, biotinylated donkey anti-goat antibody, both diluted 1:200, Jackson Laboratories). Sections exposed to the secondary biotinylated horse anti-goat antibody before were further incubated with an Alexa 488 streptavidin conjugate (1:200) at room temperature for 60 min. Fluorescent signals were visualized using a confocal microscopy system (LSM510 Zeiss, Germany).

Statistics

Analysis of GDNF in astrocyte cultures includes 3 independent experiments (cell culture preparations) with at least 4 independent cultures (one culture corresponds to one 6-well plate) including in total 24 individual cell cultures wells per condition. Number of animals included in GDNF analysis is stated in the respective figure legends. Quantifications of Western blots are presented as means ± SEM. Significances were tested by oneway ANOVA and Bonferroni correction using IBM SPSS Statistics 20.0 (IBM Svenska AB, Sweden).

Results

GDNF expression in the rat brain after transient MCAO

As shown in Fig. 1A, GDNF immunoreactivity (GDNF-ir) is evident in cell bodies and branches of neocortical neurons of layers II to VI in