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Anterograde delivery of brain-derived neurotrophic factor to striatum via nigral transduction of recombinant adeno-associated virus increases neuronal death but promotes neurogenic response following stroke

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Abstract

To explore the role of brain-derived neurotrophic factor for survival and generation of striatal neurons after stroke, recombinant adeno-associated viral vectors carrying brain-derived neurotrophic factor or green fluorescent protein genes were injected into right rat substantia nigra 4–5 weeks prior to 30 min ipsilateral of middle cerebral artery occlusion. The brain-derived neurotrophic factor–recombinant adeno-associated viral transduction markedly increased the production of brain-derived neurotrophic factor protein by nigral cells. Brain-derived neurotrophic factor was transported anterogradely to the striatum and released in biologically active form, as revealed by the hypertrophic response of striatal neuropeptide Y-positive interneurons. Animals transduced with brain-derived neurotrophic factor–recombinant adeno-associated virus also exhibited abnormalities in body posture and movements, including tilted body to the right, choreiform movements of left forelimb and head, and spontaneous, so-called ‘barrel’ rotation along their long axis. The continuous delivery of brain-derived neurotrophic factor had no effect on the survival of striatal projection neurons after stroke, but exaggerated the loss of cholinergic, and parvalbumin- and neuropeptide Y-positive, γ-aminobutyric acid-ergic interneurons. The high brain-derived neurotrophic factor levels in the animals subjected to stroke also gave rise to an increased number of striatal cells expressing doublecortin, a marker for migrating neuroblasts, and cells double-labelled with the mitotic marker, 5-bromo-2′-deoxyuridine-S′monophosphate, and early neuronal (Hu) or striatal neuronal (Meis2) markers. Our findings indicate that long-term anterograde delivery of high levels of brain-derived neurotrophic factor increases the vulnerability of striatal interneurons to stroke-induced damage. Concomitantly, brain-derived neurotrophic factor potentiates the stroke-induced neurogenic response, at least at early stages.

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of a trophic factor family called neurotrophins, and plays a major role in survival and differentiation during embryonic development of the nervous system (for review, see Davies, 1994). In the adult brain, ischaemic insults lead to marked increases of BDNF levels in cortical and hippocampal neurons (Lindvall et al., 1992; Takeda et al., 1993; Kokaia et al., 1995; Arai et al., 1996; Kokaia et al., 1996; Kokaia et al., 1998b), and it has been proposed that these changes of endogeneous BDNF levels might be a neuroprotective response (Lindvall et al., 1994). In agreement with this hypothesis, mice heterozygous for BDNF gene deletion exhibit larger cerebral infarcts as compared with wild-type animals after stroke (Endres et al., 2000), and blockade of endogeneous levels of BDNF by intraventricular infusion of TrkB-Fc fusion protein reduces the survival of several types of forebrain neurons following global forebrain ischaemia (Larsson et al., 1999).

To study the effect of increased BDNF levels on neuronal survival after stroke, several routes of delivery have been used. BDNF has been reported to protect against neuronal death following global forebrain ischaemia when delivered by intraventricular infusion (Beck et al., 1994) or by transplantation of genetically modified primary fibroblasts into the hippocampus (Ferrer et al., 1998). In addition, recombinant BDNF reduces the damage after hypoxia-ischaemia both in cell culture (Cheng & Mattson, 1994) and when infused intra-cerebroventricularly in the neonatal brain (Han & Holtzman, 2000; Han et al., 2000). Intraventricular (Schäbitz et al., 1997), intravenous (Schäbitz et al., 2000) and intraparenchymal (Yamashita et al., 1997; Yanamoto et al., 2000) infusion of BDNF also protect cortical neurons against stroke-induced damage. We have demonstrated that continuous BDNF delivery mediated by direct gene transfer to the striatum with recombinant adeno-associated viral (rAAV) vectors partially protects striatal neurons against stroke-induced death (Andsberg et al., 2002).
Recent experimental studies have indicated that BDNF can also influence neurogenesis in the adult brain. Intraventricular infusion of BDNF (Zigova et al., 1998; Pencea et al., 2001) or overexpression of the BDNF gene in the ventricular zone (Benraiss et al., 2001) increases the number of new neurons generated from the subventricular zone (SVZ) in several forebrain structures. Stroke leads to increased production of new striatal neurons from precursor cells in the SVZ (Arvidsson et al., 2002; Parent et al., 2002). Because the number of new striatal neurons showing long-term survival was small, we proposed that delivery of BDNF might be one approach to promote this neurogenic response (Arvidsson et al., 2002).

The objectives of the present study were twofold. First, to test whether striatal BDNF levels can be increased by intranigral transduction of rAAV carrying the BDNF gene. Endogeneous BDNF is transported efficiently in nigrostrial axons in the anterograde direction (von Bartheld et al., 1996; Altar et al., 1997; Smith et al., 1997). This route of delivery should therefore increase the BDNF content in the striatum without altering its structural integrity. Second, to explore whether continuous high intrastratal BDNF levels, obtained through anterograde transport from substantia nigra, can influence neuronal survival and neurogenesis after stroke.

Materials and methods

Vector production

The rAAV vectors used in this study (BDNF-rAAV and GFP-rAAV) were produced at the University of Florida Powell Gene Therapy Center, USA. Virus production followed a two plasmid adenovirus-free method, as reported in detail previously (Hauswirth et al., 2000). Briefly, the helper/packaging plasmid pOG (Grimm et al., 1998) that supplies all the necessary helper functions as well as rep and cap in trans was cotransfected by calcium phosphate precipitation with vector plasmid, plasmid cytomegalovirus promoter with chicken R-actin intervening sequence-BDNF (pCBA-BDNF), in near confluent 293 cells. After lysing the cells, rAAV was prepared by iodixanol centrifugation and hand-packed heparin column purification, as described previously (Zolotukhin et al., 1999).

Animals and experimental groups

All animal related procedures were conducted in accordance with local ethical guidelines and approved animal care protocols. Thirty-six male Wistar rats weighing approximately 210 g at the beginning of the experiment (Taconic M & B A/S, Ry, Denmark) were housed under 12 h light : 12 h dark cycle with ad libitum access to food and water. All animals were anesthetized by inhalation of 1% halothane and injected unilaterally in the substantia nigra with either BDNF-rAAV (n = 18) or GFP-rAAV (n = 18). At 4 weeks after transduction, four animals were tested for the effect of catecholamine inhibition, and another four rats were implanted with recording electrodes for monitoring cortical and striatal electroencephalogram (EEG) (n = 2 for BDNF-rAAV- and n = 2 for GFP-rAAV-transduced animals for both analyses). All animals were tested for spontaneous rotation. Four rats transduced with BDNF-rAAV were used for immunocytochemical assessment of BDNF levels prior to stroke, and another six rats (three transduced with BDNF-rAAV and three with GFP-rAAV) for determination of BDNF tissue levels with ELISA.

At 5 weeks after viral transduction, 15 rats were subjected to stroke using middle cerebral artery occlusion (MCAO; n = 7 for BDNF-rAAV and n = 8 for GFP-rAAV), and six rats transduced with GFP-rAAV were sham-operated. From the day after MCAO or sham, and for 1 week thereafter, all animals received daily injections (50 mg/kg, intraperitoneally (i.p.)) of 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU). At 1 week after MCAO, all animals were tested for spontaneous rotation, and at 2 weeks were evaluated immunocytochemically.

Viral injections

The BDNF-rAAV or GFP-rAAV vectors were injected under halothane anaesthesia in the right substantia nigra (2 μL, 0.5 μL/min) using a 10-μL Hamilton microsyringe fitted with a glass micropipette (outer diameter 60–80 μm). The injection coordinates were: 5.3 mm caudal and 2.0 mm lateral from bregma, and 7.0 mm ventral from brain surface, with tooth bar at −2.3 mm (Paxinos & Watson, 1997).

Middle cerebral artery occlusion

Stroke was induced by MCAO, as described previously (Andsberg et al., 1998; Kokaia et al., 1998a; Andsberg et al., 2002). The rats were first anaesthetized by inhalation of 3.5% halothane in N2O:O2 (70:30), and then intubated and ventilated artificially with inhalation of 1–1.5% halothane during the rest of the operation. The tail artery was catheterized for blood sampling and blood pressure recording, and 0.1 mL of heparin (300 IU/mL) was given. Mean arterial blood pressure, PO2, PCO2, pH and blood glucose concentration were monitored (Table 1), and body temperature was maintained at 37 ± 0.5 °C with a heating blanket. The middle cerebral artery (MCA) was occluded by an intraluminal filament technique (Kozumi et al., 1986; Zhao et al., 1994). In brief, the external carotid and common carotid arteries were ligated, and the internal carotid artery was temporarily closed by a microvascular clip. A small incision was then made in the common carotid artery, and a filament, which had a distal cylinder of silicon rubber, was inserted into the internal carotid artery through the common carotid artery to close the origin of MCA. Animals were then extubated and allowed to wake up to resume spontaneous breathing. The filament was withdrawn after 30 min. Sham-operated animals were treated identically, except that MCA was not occluded. The efficiency of MCAO was determined in GFP-rAAV-transduced animals at 1 h of reperfusion on the basis of neurological assessment (Bederson et al., 1986; Andsberg et al., 1998). Only animals with

Table 1. Physiological parameters in rats subjected to intranigral GFP-rAAV- or BDNF-rAAV transduction and middle cerebral artery occlusion or sham surgery

<table>
<thead>
<tr>
<th>Physiological parameters</th>
<th>Animal group</th>
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<tbody>
<tr>
<td></td>
<td>GFP-rAAV–sham</td>
</tr>
<tr>
<td>Glucose concentration (mm)</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.40 ± 0.02</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>37.4 ± 16.9</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
<td>107.7 ± 16.8</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>Start of occlusion</td>
<td>37.0 ± 0.4</td>
</tr>
<tr>
<td>Start of reperfusion</td>
<td>37.0 ± 0.5</td>
</tr>
<tr>
<td>After 1 h of reperfusion</td>
<td>36.3 ± 0.6</td>
</tr>
<tr>
<td>After 24 h of reperfusion</td>
<td>38.1 ± 0.2</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mmHg)</td>
<td>108.2 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SD. <sup>1</sup>Significantly different from GFP-rAAV–sham. <sup>2</sup>Significantly different from GFP-rAAV–MCAO, P < 0.05, ANOVA followed by Bonferroni–Dunn post-hoc test. All physiological parameters were measured just before the induction of ischemia, and temperature, in addition, at the start of reperfusion, and after 1 and 24 h of reperfusion. The BDNF-treated rats were not fasted before surgery due to their unstable physiological condition. However, their glucose value was still within the predefined range and below the level which is known to exaggerate ischemic damage (Li et al., 1994; Li et al., 1995).
pronounced gait disturbances and circling or walking to the left were included in the study. The behavioural abnormalities in BDNF–rAAV-transduced animals did not allow for this neurological assessment.

**Behavioural analysis**

Assessment of rotational behaviour was performed at 4 and 6 weeks after viral transduction (1 week before and 1 week after MCAO) in automated rotometer bowls (Ungerstedt & Arbuthnott, 1970). Spontaneous rotation was monitored for 30 min, and asymmetry scores expressed as mean net full 90° turns, with ipsilateral rotations assigned a positive value.

**Electrode implantation and EEG analyses**

The rats were anaesthetized and bipolar stainless-steel recording electrodes were implanted bilaterally into the striatum and cortex using a Kopf stereotaxic frame. Coordinates were, with tooth bar at −3.3 mm: 0.8 mm posterior to bregma; 2.6 mm lateral to midline; and on the cortical surface (cortex): 0.8 mm posterior to bregma; 5.0 mm lateral to midline; and 5.0 mm ventral from dura (striatum). On the next day, EEG recording and behavioural observation were performed for several hours on freely moving animals.

**Catecholamine depletion**

Rats were injected with the reversible inhibitor of dopamine and noradrenaline synthesis, α-methyl-para-tyrosine (AMPT; 250 mg/kg; i.p., Sigma). The motor behaviour of the animals was observed at 2, 4 and 8 h after drug administration.

**Immunocytochemistry**

The procedures used for immunocytochemical staining are described in detail elsewhere (Larsson et al., 2002). Briefly, the animals were deeply anaesthetized with pentobarbital and transcardially perfused with 50 mL of saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed, postfixed with the same fixative overnight, and then transferred to 20% sucrose solution at −20°C. Immunocytochemistry was performed on free-floating sections in potassium phosphate-buffered saline (KPBS). The following primary antibodies were used: neuronal-specific antigen (NeuN, 1:100, mouse monoclonal, Chemicon, Temecula, CA, USA), choline acetyltransferase (ChAT, 1:500; rabbit polyclonal, Chemicon), GTP (1:1000; rabbit polyclonal, Clontech, Palo Alto, CA, USA), neuropeptide Y (NPY, 1:1500; rabbit polyclonal, Sigma), GFP (1:1000; rabbit polyclonal, Clontech, Palo Alto, CA, USA), neuromelanin (1:500), biotinylated secondary antibody: horse antimouse (1:200 for NeuN) and biotinylated goat-antirabbit (1:200 for ChAT, NPY, BDNF and GTP, Vector Laboratories) in KPBS containing 0.25% Triton X-100 for 1 h. Sections were then rinsed, incubated in avidin–biotin complex (Elite ABC Kit, Vector Laboratories) for 1 h and developed by reaction with dianinobenzidine.

For double-label immunocytochemistry, sections were first treated with 1 M hydrochloric acid at 65°C for 30 min, and then rinsed and incubated for 36 h with BSA antibody together with NeuN, Hu, DCX or Meis2 antibodies and appropriate sera. Sections were subsequently rinsed and incubated for 2 h with Cy3-conjugated donkey antirabbit (1:400 for BSA, Jackson ImmunoResearch, West Grove, PA, USA) and biotinylated horse-antimouse (NeuN and Hu) or goat-antirabbit (DCX and Meis2) secondary antibodies (1:200, Vector Laboratories). After rinsing, sections were incubated for 2 h with Alexa 488-conjugated streptavidin (1:250, Molecular Probes, Eugene, OR, USA), mounted on glass slides and cover-slipped. When staining for Hu, the streptavidin step was preceded by tyramide amplification procedure (TSA biotin system, NEN, Boston, MA, USA).

**BDNF ELISA**

Measurement of BDNF protein levels was performed in tissue samples from substantia nigra and striatum (n = 3 for BDNF–rAAV- and n = 3 for GFP–rAAV-transduced animals) using BDNF Emax ImmunoAssay System (Promega, Madison, WI, USA) according to manufacturer’s recommendations. Briefly, each well of 96-well polystyrene ELISA plates (Nunc MaxiSorpTM) was coated with monoclonal anti-BDNF antibody (1:1000). On the next day after rinses with washing buffer, wells were incubated with Block & Sample (BS) buffer for 1 h. Tissue extract (duplicate) and BDNF standards (triplicate) were loaded to wells and incubated for 2 h. After several washes, each well was loaded with BS buffer containing chicken polyclonal anti-BDNF antibody (1:500) and incubated for 2 h. Horseradish peroxidase-conjugated secondary antibody (1:2000) in BS buffer was added and incubated for 1 h followed by several washes and incubation with TMB solution containing peroxidase substrate for 10 min. The reaction was stopped with 1 M phosphoric acid, and the absorbance at 450 nm was recorded on a plate reader (Anthos Labtec Instr., Salzburg, Austria). Standard curve of BDNF was determined for each ELISA plate and the values obtained from the absorbance reader were expressed as pg/mg tissue.

**Cell counting**

Numbers of NeuN-, NPY-, ChAT- and parvalbumin-positive neurons were quantified using stereological procedures (West et al., 1991; West, 1999) in every 20th (for NeuN and NPY) or 10th (for ChAT and parvalbumin) section throughout the striatum (Larsson et al., 2001; Andsberg et al., 2002). The image of the section was displayed on the screen of a computer using an Olympus BH-2 microscope and a CCD-IRIS colour video camera, both controlled by CAST-GRID™ software (Olympus, Denmark). The border of the striatum ipsilateral to MCAO was first marked at low magnification (×1.25 objective). Sampling and cell counting were then performed using ×100 (for NeuN) or ×40 objectives (for ChAT, NPY and parvalbumin).

The number of DCX-, BrdU–Hu– and BrdU–Meis2-immunopositive cells was quantified in the striatum (including SVZ) using a grid in an epifluorescence microscope. The number of neurons counted in six sections was pooled. Double-labelling was validated using a confocal laser scanning microscope (Leica) in one randomly chosen section from every other animal. A cell was considered as double-labelled when staining with both antibodies was colocalized in a minimum of three consecutive sections in a sequential z-series with 1-μm interval.

**Cell volume measurement**

To investigate if biologically active BDNF was released in the striatum in sufficient amounts to exert trophic effects on BDNF-responsive NPY-positive interneurons (Croll et al., 1994; Andsberg et al., 2002), mean cell volume was estimated using the rotator method (Weber et al.,

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Fig. 1. (A) Photomicrograph showing brain-derived neurotrophic factor (BDNF)-immunoreactivity in a coronal section from a rat subjected to BDNF–recombinant adeno-associated viral (rAAV) transduction unilaterally in the substantia nigra. Note the high BDNF immunoreactivity distributed equally throughout the whole striatum. (B) Levels of BDNF immunoreactivity in the striatum ipsi- and contralateral to GFP–rAAV (n = 2) and BDNF–rAAV (n = 4) transduction, as assessed using optical density measurements. (C–F) BDNF immunoreactivity in the substantia nigra contralateral to (C, E) and ipsilateral to (D, F) BDNF–rAAV transduction. (E, F) Enlargements of the areas marked by boxes in C and D, respectively. (G and H) Levels of BDNF protein as assessed by ELISA in (G) striatum and (H) substantia nigra of animals transduced with GFP–rAAV (n = 3) or BDNF–rAAV (n = 3). All values are mean ± SEM. Scale bar, 500 μm (C and D); 100 μm (E and F).
Quantification of BDNF immunoreactivity

Relative levels of BDNF immunoreactivity were measured in the striatum ipsilateral to rAAV transduction as mean optical densities by computerized image analysis using Image 1.52 software (Wayne Rasband, NIMH) in three sections from each animal. Background optical density, determined in a structure lacking BDNF immunoreactivity (corpus callosum), was subtracted from each measurement.

Statistical analysis

All comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni–Dunn post-hoc test. Significance was set at $P < 0.05$.

Results

Characterization of transgene expression and behavioural consequences

To study the efficacy of transduction in the substantia nigra and the distribution of anterogradely transported BDNF in the ipsilateral striatum, we used GFP and BDNF immunocytochemistry. It has been observed that, within 1–2 weeks after intraparenchymal transduction of rAAV-carrying genes, elevated levels of the corresponding proteins are detected immunocytochemically with further increases over subsequent weeks (Mandel & Kirik, unpublished observation). Significant staining was detected in the substantia nigra and striatum of all transduced animals (Fig. 1A, D and F), with a similar pattern and intensity for both transgenes (data not shown). In the substantia nigra, neurons of both the pars reticulata and compacta were intensely stained, and the neuropil was moderately immunoreactive. Some neurons in and around the injection tract were also GFP- and BDNF-immunoreactive. The striatum ipsilateral to the BDNF–rAAV nigral transduction was extensively stained with the BDNF antibody (Fig. 1A). The strong immunoreactivity in the neuropil was equally distributed in all directions with no detectable cellular staining. In animals transduced with GFP–rAAV, BDNF-immunoreactivity was not observed either in substantia nigra or striatum.

The semiquantitative analysis revealed about ninefold increase of BDNF immunoreactivity in the ipsilateral striatum of BDNF–rAAV-transduced rats, as compared with the striatum in GFP–rAAV-transduced animals (Fig. 1B). Similarly, ELISA showed more than a 20-fold increase of BDNF protein levels in the striatum ipsilateral to BDNF–rAAV transduction (Fig. 1G). There were no pronounced changes of BDNF protein levels in the contralateral striatum. The BDNF levels were 190-fold higher in BDNF–rAAV- as compared with GFP–rAAV-transduced nigra (Fig. 1H). There was also a 14-fold increase of BDNF levels in the contralateral substantia nigra, possibly due to transport of BDNF from transduced cells.

In order to determine whether the striatal BDNF which had been transported anterogradely following the nigral rAAV transduction was biologically active, the volume of NPY-positive neurons was measured in BDNF- and GFP-transduced animals subjected to MCAO, and in sham-treated rats injected with GFP–rAAV. The volume of NPY-positive neurons was 2.2- and 1.8-fold larger in the BDNF–rAAV-transduced animals subjected to MCAO as compared with GFP–rAAV-transduced nigra (Fig. 1I). There was also a 14-fold increase of BDNF levels in the contralateral substantia nigra, possibly due to transport of BDNF from transduced cells.

Fig. 2. (A–C) Photomicrographs of NPY-immunoreactive striatal interneurons in animals transduced with (A, B) GFP–recombinant adeno-associated virus (rAAV) or (C) brain-derived neurotrophic factor (BDNF)–rAAV and subjected to (A) sham treatment or (B and C) middle cerebral artery occlusion (MCAO). (C) Note the hypertrophy of NPY-positive neurons in animals transduced with BDNF–rAAV. (D) Volume of NPY-positive striatal interneurons, as measured using the rotator method, in animals transduced with GFP–rAAV ($n = 14$) or BDNF–rAAV ($n = 6$) and subjected to sham treatment ($n = 6$) or MCAO ($n = 14$). All values are mean ± SEM. *$P < 0.05$, one-way ANOVA followed by Bonferroni–Dunn post-hoc test. Scale bar, 100 μm (C).
The body weight of rats injected with BDNF–rAAV was lower than that of GFP–rAAV animals at 3 weeks after transduction, but was relatively stable thereafter (data not shown). Animals transduced with BDNF–rAAV also exhibited abnormalities in body posture and movements. The head and frontal part of the body were constantly tilted to the right (transduced) side with periodic choreiform movements of the left forelimb and head. Most BDNF–rAAV-transduced rats exhibited spontaneous, so-called ‘barrel’ rotation along their long axis. The rats also had problems with balance when rearing, and showed repeated head nodding movements. They were hyperactive and rotated spontaneously towards the transduced side, which was not observed in GFP–rAAV animals (Fig. 3A). The MCAO did not alter this spontaneous rotation (Fig. 3B). Despite the behavioural abnormalities, the BDNF–rAAV-transduced rats were able to drink, eat and groom themselves.

We hypothesized that seizure development had contributed to the observed behavioural abnormalities, and therefore EEG was recorded from the frontal cortex and striatum in four animals at 4 weeks after viral transduction. However, no seizure activity was detected and the rats did not loose consciousness, were responsive to touch and handling, and exhibited exploratory behaviour in the open-field. Moreover, the clonic movements of the forelimb were observed only contralaterally to the side of BDNF–rAAV transduction.

We also investigated whether the motor abnormalities could be due to effects of BDNF on the function of nigrostriatal neurons (e.g. striatal dopamine release). At 2 h after injection of the dopamine and noradrenaline synthesis inhibitor, AMPT, movements were slower in both GFP–rAAV- and BDNF–rAAV-transduced rats, and between 4 and 8 h, the hypokinetic effect of the drug became even more pronounced. However, the tilted posture and barrel rotation were still observed in the BDNF–rAAV-transduced animals.

**Effect of BDNF gene transfer on survival of striatal neurons after MCAO**

The stroke-induced neuronal damage in the striatum was first assessed by combining immunocytochemical staining with the neuron-specific marker NeuN and stereological procedures (Andsberg et al., 1998; Larsson et al., 1999; Larsson et al., 2001; Andsberg et al., 2002). Because more than 90% of striatal neurons are medium-sized spiny projection neurons (Kawaguchi et al., 1995), the loss of NeuN-positive cells after MCAO primarily reflects the death of these cells. The MCAO caused 64% and 71% reduction of the number of NeuN-positive cells in animals transduced with GFP–rAAV and BDNF–rAAV, respectively (Fig. 4A). The lesion was mainly localized to the dorsolateral striatum (Fig. 4C and D) (Memezawa et al., 1992; Andsberg et al., 1998). There was no significant difference in the survival of NeuN-positive cells between GFP–rAAV- and BDNF–rAAV-transduced rats.

In addition to medium-sized spiny projection neurons, the striatum contains three major classes of interneurons which can be distin-

![Fig. 3. Spontaneous rotation as assessed at 4 weeks after intranigral injection of GFP-recombinant adeno-associated viral (rAAV, n = 8) and brain-derived neurotrophic factor (BDNF)-rAAV (n = 7) vectors [A, before middle cerebral artery occlusion (MCAO)] and at 1 week after MCAO (B). ’–’ means rotation to the left and ’+’ means rotation to the right. Note the pronounced rotational asymmetry towards the injected (right) side in BDNF–rAAV-transduced animals, which is maintained after MCAO.](image)

![Fig. 4. (A) Total number of NeuN-stained cells in the striatum quantified using stereological procedures in animals transduced with GFP-recombinant adeno-associated virus (rAAV, n = 13) or brain-derived neurotrophic factor (BDNF)-rAAV (n = 6) and subjected to sham treatment (n = 6) or 30 min of middle cerebral artery occlusion (MCAO, n = 13). (B–D) NeuN-stained coronal sections from the central part of the striatum in representative animals from the different groups. The MCAO groups showed similar, uniform loss of NeuN-stained cells in the ipsilateral dorsolateral striatum (*). Values are means ± SEM. #P < 0.05, one-way ANOVA followed by Bonferroni–Dunn post-hoc test.](image)
guished on basis of their immunoreactivity to ChAT, parvalbumin or NPY (Kawaguchi et al., 1995). We quantified the ischaemic damage to these classes of interneurons. The MCAO caused a significant (27%) loss of parvalbumin-expressing interneurons in GFP–rAAV-transduced animals (Fig. 5A). This loss was further exaggerated (to 65%) in BDNF–rAAV-transduced rats. In agreement with previous reports (Chesselet et al., 1990; Uemura et al., 1990; Andsberg et al., 1998; Kokaia et al., 1998a; Andsberg et al., 2002), MCAO caused no significant reduction of the number of NPY- or ChAT-positive striatal interneurons in GFP–rAAV-transduced animals. However, in the BDNF–rAAV transduced animals subjected to MCAO, there was a 40% and 50% loss of NPY- and ChAT-positive interneurons, respectively (Fig. 5B and C).

**Effect of BDNF gene transfer on stroke-induced striatal neurogenesis**

We also assessed whether the elevated BDNF levels could influence the initial phase of stroke-induced striatal neurogenesis (Arvidsson et al., 2002). First, the number of DCX-positive cells was counted in the striatum ipsilateral to MCAO (Fig. 6). DCX is a marker of migrating neuroblasts, and in the adult brain is expressed mostly in the SVZ and rostral migratory stream with single cells detectable in the striatum (Nacher et al., 2001). Stroke triggers the migration of DCX-positive cells from the SVZ to the damaged striatum (Arvidsson et al., 2002; Parent et al., 2002). We observed here that at 2 weeks after the stroke, the number of DCX-positive cells in the GFP–rAAV-transduced animals had increased to more than 231% of that in sham-treated animals. Transduction with BDNF–rAAV led to a further increase in the number of DCX-positive striatal cells (to 522% and 233% of that in GFP–rAAV–sham and GFP–rAAV–MCAO groups, respectively, Fig. 6).

To confirm the neuronal phenotype of the new striatal cells, all animals were injected daily with BrdU for 1 week following MCAO, and were killed 1 week thereafter. BrdU is a thymidine analogue, which is incorporated into DNA during cell division. We double-immunostained sections with antibodies against BrdU and Hu, an early neuronal marker, which starts to be expressed in neurons soon after differentiation and remains in mature cells (Marsisch et al., 1994). The BrdU–Hu-double-labelled neurons (Fig. 7A–F) were mainly distributed close to the lateral ventricle, the corpus callosum and the anterior commissure. Many cells were also detected in the border zone between the damaged and the spared medial parts of the striatum. Quantification revealed increased numbers of BrdU–Hu-double-labelled striatal cells in the GFP–rAAV-transduced animals subjected to MCAO as compared with sham-treated rats (Fig. 7G). In the BDNF–rAAV-transduced animals, the number of BrdU–Hu-double-labelled striatal cells was further increased to 253% and 726% as compared with GFP–rAAV-transduced animals with and without MCAO, respectively.

In order to characterize the early phenotype of the new striatal neurons, we quantified the number of BrdU–Meis2-positive cells. Meis2 is a transcription factor which is expressed at high levels by striatal precursors during embryonic development, and also in the adult striatum (Toresson et al., 2000). Similar to BrdU–Hu-positive cells, BDNF–rAAV transduction significantly increased the number of BrdU–Meis2-double-labelled striatal neurons after stroke (Fig. 7H).

**Discussion**

The present study shows that injection of rAAV carrying the BDNF gene into the rat substantia nigra leads to increased production of BDNF by nigral neurons, and anterograde transport and release of high amounts of this neurotrophic factor in the ipsilateral striatum. Continuous supply of BDNF via this route does not protect striatal projection neurons from stroke-induced damage. On the contrary, anterogradely delivered BDNF exaggerates the loss of parvalbumin-positive interneurons and causes the death of resistant NPY- and ChAT-positive striatal interneurons following stroke. Concomitantly,
increased levels of intrastratal BDNF promote the initial phase of stroke-induced neurogenesis in the striatum.

Under normal conditions, approximately 14% of striatal BDNF has been transported anterogradely from the substantia nigra (Altar et al., 1997). Our immunocytochemical and ELISA data clearly document that after transduction of nigral cells with BDNF–rAAV, large amounts of BDNF are similarly transported in nigrostrial axons to the striatum. By using this natural route of BDNF delivery, homogeneous distribution of high levels of this neurotrophic factor is obtained throughout the striatum. The observed hypertrophy of NPY-positive neurons indicates that sufficient levels of BDNF are released in biologically active form. Moreover, the BDNF–rAAV-transduced animals stopped gaining weight, similar to that which has been reported previously with intracerebroventricular (Lapchak & Hefti, 1992; Sauer et al., 1993; Pelleymounter et al., 1995; Siuciak et al., 1996), supranigral (Altar et al., 1992; Martin-Iverson et al., 1994) and striatal (Altar et al., 1992) delivery of BDNF. These animals also exhibited abnormal motor behaviour, including choreiform movements, spontaneous circling and barrel rotation. Intracerebral infusion of BDNF can lead to behavioural seizures (Scharffman et al., 2002). However, the EEG recordings and administration of a catecholamine synthesis inhibitor provided no evidence that seizures or increased dopamine release, respectively, were responsible for the motor abnormalities. We hypothesize that the continuous delivery of BDNF leads to increased striatal glutamate levels, which induce the behavioural changes. In agreement, intrastratal injection of excitatory amino acids gives rise to increased locomotor activity, choreiform movements, contralateral turning behaviour and ipsilateral barrel rotation (Toth & Lajtha, 1989; Vecsei & Beal, 1991).

Several studies have demonstrated that delivery of exogenous BDNF counteracts MCAO-induced damage, predominantly in the cerebral cortex (Schäbitz et al., 1997; Yamashita et al., 1997; Schäbitz et al., 2000; Yamamoto et al., 2000; Zhang & Pardridge, 2001). BDNF also protects striatal neurons from excitotoxic lesions (Martinez-Serrano & Björklund, 1996; Bellemans et al., 1999; Perez-Navarro et al., 1999; Perez-Navarro et al., 2000; Gratacos et al., 2001). In accordance, we have previously observed that direct intrastratal injection of BDNF–rAAV vector at 4–5 weeks prior to the ischaemic insult used here leads to a small increase in the number of surviving projection neurons in the dorsolateral striatum, with more pronounced sparing of parvalbumin-containing interneurons (Andsberg et al., 2002). Interestingly, the survival of cholinergic interneurons following global forebrain ischaemia is dependent on endogenous BDNF (Larsen et al., 1999). Scavenging of endogenous BDNF by intraventricular injection of TrkB-Fc fusion protein causes approximately 50% reduction in the number of ChAT-positive interneurons in the dorsolateral striatum following a 30-min insult.

In contrast to these observations, we found here that anterograde delivery of BDNF to the striatum increased the ischaemic damage to several neuronal populations. BDNF aggravated the stroke-induced loss of parvalbumin-positive cells and also caused degeneration of NPY- and ChAT-positive interneurons, which was not seen in GFP–rAAV-injected animals subjected to MCAO. There was no significant effect of BDNF on the survival of striatal projection neurons. The paradoxically increased vulnerability of striatal interneurons, induced by the continuous anterograde delivery of BDNF, probably illustrates that the level and duration of route delivery determine the effect of this neurotrophin on neuronal survival. In accordance, the immunocytochemical analysis revealed that the BDNF levels were much higher and equally distributed throughout the whole striatum following intranigral as compared with the direct intrastratal injection of BDNF–rAAV, which gave rise to patchy appearance of BDNF immunoreactivity close to the injection site (Andsberg et al., 2002). Similarly, glial cell line-derived neurotrophic factor (GDNF)–rAAV transduction supports much higher levels of GDNF protein as compared with striatal transduction using the same vector (Kirik et al., 2000). We propose that low and moderate levels of BDNF are neuroprotective. However, when BDNF is delivered continuously in high amounts, exaggeration of excitotoxic mechanisms overrides the neuroprotective effect of BDNF and increases the vulnerability of striatal cells to ischaemic damage. Stroke leads to depolarization of corticostratial neurons and glutamate release, giving rise to excitotoxic damage to striatal neurons (Gagliardi, 2000; Sattler & Tymianski, 2001). It is tempting to speculate that the increased vulnerability of striatal neurons to ischaemic injury, mediated by the high, homogeneously distributed levels of BDNF, is due to the ability of this neurotrophic factor to enhance excitatory glutamatergic synaptic transmission (for review, see Lessmann, 1998). BDNF also increases glutamate release in cortical neurons (Akaneya et al., 1997; Carmignoto et al., 1997; Takei et al., 1997; Numakawa et al., 1999) and astrocytes (Pascual et al., 2001).

Our finding that BDNF can enhance neuronal vulnerability is consistent with reports demonstrating that BDNF increases oxidative stress-dependent cell death (Gwag et al., 1995) and potentiates the necrosis induced by oxygen–glucose deprivation or N-methyl-D-aspartate (NMDA) (Koh et al., 1995) in cultured cortical neurons, and also that intrahippocampal infusion of BDNF exacerbates the loss of CA3 pyramidal neurons induced by kainic acid (Rudge et al., 1998). Interestingly, the high-affinity neurotrophin receptor tyrosine-kinase inhibitor K252a enhances the survival of hippocampal neurons after exposure to high levels of free radicals (Goodman & Mattson, 1994). Moreover, it was recently reported that the lack of another neurotrophin, NT-3, in conditional knockout mice leads to reduced infarct volume after MCAO, whereas addition of NT-3 exaggerates the death of cultured cortical neurons after oxygen–glucose deprivation (Bates et al., 2002).

We have previously shown that 2 h of MCAO leads to neurogenesis in the SVZ, and that the new neurons migrate to the stroke-damaged part of the striatum, where they express morphological markers of striatal projection neurons (Ardvissi et al., 2002). The present data

**Fig. 6.** (A–D) Photomicrographs showing DCX-immunoreactivity in coronal sections through the dorsomedial striatum (A) contra- and (B) ipsilateral to the insult at 2 weeks after middle cerebral artery occlusion (MCAO) in animals injected with brain-derived neurotrophic factor (BDNF)–recombinant adeno-associated virus (rAAV). Note the large number of DCX-positive cells in the ipsilateral striatum (B; enlarged in C and D); distributed in a density gradient from the SVZ, bordered dorsally by the corpus callosum and medially by the lateral ventricle (LV). (E) Number of DCX-positive cells in the striatum ipsilateral to sham surgery (n = 6) or 30 min of MCAO (n = 13) in animals injected with GFP–rAAV (n = 13) or BDNF–rAAV (n = 6) in the substantia nigra. Values are mean ± SEM. *P < 0.05, one-way ANOVA, followed by Bonferroni–Dunn post-hoc test. Scale bar, 200 μm (A and B); 30 μm (C and D).

**Fig. 7.** (A–C) Consecutive 1-μm confocal images in z-dimension showing (A) Hu or (B) 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU) immunoreactivity separately or (C) as merged images. (D–F) Confoal 3D reconstruction of neurons from the striatum ipsilateral to middle cerebral artery occlusion (MCAO) showing (D) Hu and (E) BrdU immunoreactivity separately or (F) as a merged image. Reconstructed orthogonal images are presented as viewed in the x–z (top) and y–z (right) planes. Arrows indicate double-labelled cells. (G, H) Number of cells double-labelled with BrdU and (H) Hu or (H) Meis2 in the striatum ipsilateral to sham surgery (n = 6) or 30 min of MCAO (n = 13) in animals injected with GFP–rAAV (n = 13) or brain-derived neurotrophic factor (BDNF)–rAAV (n = 6). All values are mean ± SEM. *P < 0.05, one-way ANOVA, followed by Bonferroni–Dunn post-hoc test. Scale bar, 30 μm (A–C); 20 μm (D–F).
indicate that this neurogenic response is promoted by long-term BDNF delivery. In the BDNF-exposed ischaemic striatum, there was a marked increase in the number of DCX-immunoreactive migrating neuroblasts, and cells double-labelled with BrdU and early neuronal (Hu) and striatal neuronal (Meis2) markers. The BDNF-induced promotion of striatal neurogenesis following stroke is in agreement with previous observations that BDNF increases the number of neurons in embryonic striatal cultures (Ahmed et al., 1995), and that new neurons are recruited to the striatum after intraventricular injection of adeno-virus carrying the BDNF gene (Benraiss et al., 2001) or intraventricular administration of BDNF (Pencea et al., 2001). Thus, the increase in the number of new striatal neurons observed here could be explained either by this direct effect of BDNF on striatal neurogenesis or by BDNF modulating the stroke-induced generation of new neurons. The mode of action of BDNF during striatal neurogenesis remains to be explored. It has been shown that the majority of new neurons in the dentate gyrus and the striatum die within the first weeks after ischaemic and epileptic insults (Ekdahl et al., 2001; Arvidsson et al., 2002). At least in the dentate gyrus, this seems to occur through a caspase-mediated apoptotic mechanism (Ekdahl et al., 2001). One possibility could be that BDNF counteracts the degeneration of the newly formed neurons. In support of this hypothesis, BDNF knockout mice show increased apoptotic death of precursor cells in the dentate gyrus and the SVZ (Linnarsson et al., 2000). Furthermore, BDNF counteracts caspase-3 activation-dependent apoptotic cell death in the ischaemia–hypoxia model (Han et al., 2000).

In conclusion, we have found that transduction of nigrostriatal neurons with a BDNF–rAAV vector provides an efficient route for long-term delivery of this neurotrophic factor to the striatum. However, high striatal levels of BDNF protein as generated here produce motor abnormalities. In contrast to previously described neuroprotective effects of BDNF, the anterogradely delivered, high BDNF levels aggravated the ischaemic damage in the striatum, probably by enhancing glutamate-evoked excitotoxicity. In the same animals, BDNF promoted the initial phase of stroke-induced striatal neurogenesis. It is conceivable that anterograde delivery of BDNF via nigral afferents will be a useful tool to explore further the role of BDNF for the balance between neuronal death and neurogenesis in the striatum after brain insults. Our data show that the actions of BDNF on neuronal survival and neurogenesis are complex, and underscore the notion that many aspects of BDNF function have to be taken into account when considering potential therapeutic applications of this neurotrophin.

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Abbreviations

AMPT, 3-methyl-para-tyrosine; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2′-deoxyuridine-5′-monophosphate; BS, Block & Sample; ChAT, choline acetyltransferase; DCX, doublecortin; EEC, electrophoeresis chromatogram; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; i.p., intraperitoneally; KPBSD, potassium phosphate-buffered saline; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NeuN, neuronal-specific antigen; NMDA, N-methyl-D-aspartate; NPY, neuropeptide Y; PB, phosphate buffer; pCBA, plasmid cytomegalovirus promoter with chicken ß-actin intervening, sequence; rAAV, recombinant adeno-associated virus; SVZ, subventricular zone.

References


