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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-5′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 intracerebroventricularly 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).

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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 nigrostriatal 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 intrastriatal BDNF levels, obtained through anterograde transport from substantia nigra, can influence neuronal survival and neurogenesis after stroke.

Materials and methods

Vector production

The rAAV2 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 pDG (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 anaesthetized 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 treatment, 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 anaesthezia 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 $N_2O : O_2$ (70 : 30), and then intubated and ventilated artificially with inhalation of 1–1.5% halothane during the rest of 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, PO_2, PCO_2, 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 (Koizumi *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

Physiological parameters	Animal group		
	GFP–rAAV–sham	GFP–rAAV–MCAO	BDNF–rAAV–MCAO
Glucose concentration (mM)	3.6 ± 0.9	4.0 ± 0.8	$7.3 \pm 1.8^{*\dagger}$
pH	7.40 ± 0.02	7.41 ± 0.02	$7.44 \pm 0.03^{*}$
pCO_2 (mmHg)	37.4 ± 1.6	36.0 ± 1.9	34.4 ± 3.6
pO_2 (mmHg)	107.7 ± 16.8	102.9 ± 11.8	103.0 ± 10.5
Body temperature (°C)			
Start of occlusion	37.0 ± 0.4	37.2 ± 0.3	37.2 ± 0.2
Start of reperfusion	37.0 ± 0.5	37.4 ± 0.6	37.2 ± 0.5
After 1 h of reperfusion	36.3 ± 0.6	$37.4 \pm 0.5^{*}$	$38.3 \pm 0.6^{*\dagger}$
After 24 h of reperfusion	38.1 ± 0.2	38.2 ± 0.2	38.8 ± 0.7
Mean arterial blood pressure (mmHg)	108 ± 12	111 ± 13	99 ± 10

Values are means ± SD. *Significantly different from GFP–rAAV–sham. †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 ischaemia, 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 ischaemic 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. Forty-micrometre-thick coronal sections were then cut in 10 series using a freezing microtome and stored in cryoprotectant 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), GFP (1 : 1000; rabbit polyclonal, Clontech, Palo Alto, CA, USA), neuropeptide Y (NPY, 1 : 1500; rabbit polyclonal, Sigma), parvalbumin (1 : 2000; mouse monoclonal, Sigma), BDNF (1 : 1000; rabbit polyclonal, a gift from Dr Q. Yan), BrdU (1 : 100; rat monoclonal, Harlan Sera-Laboratory, Loughborough, UK), Hu (1 : 500; mouse monoclonal, Chemicon), doublecortin (DCX, 1 : 250; rabbit polyclonal, a gift from Dr J. G. Gleeson and Dr C. A. Walsh) and Meis2 (1 : 1000; rabbit polyclonal, a gift from Dr A. M. Buchberg).

For single staining, sections were rinsed and endogenous peroxidase activity was blocked by 3% hydrogen peroxide and 10% methanol. After rinses, sections were incubated with the primary antibody in 2% appropriate serum in 0.25% Triton X-100 overnight at 4 °C. On the next day, sections were rinsed and incubated with the appropriate biotinylated secondary antibody: horse antimouse (1 : 200 for NeuN and parvalbumin, Vector Laboratories, Burlingame, CA, USA) and biotinylated goat antirabbit (1 : 200 for ChAT, NPY, BDNF and GFP, 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 diaminobenzidine.

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 BrdU 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 antirat (1 : 400 for BrdU, 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 E_{max} 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*.,

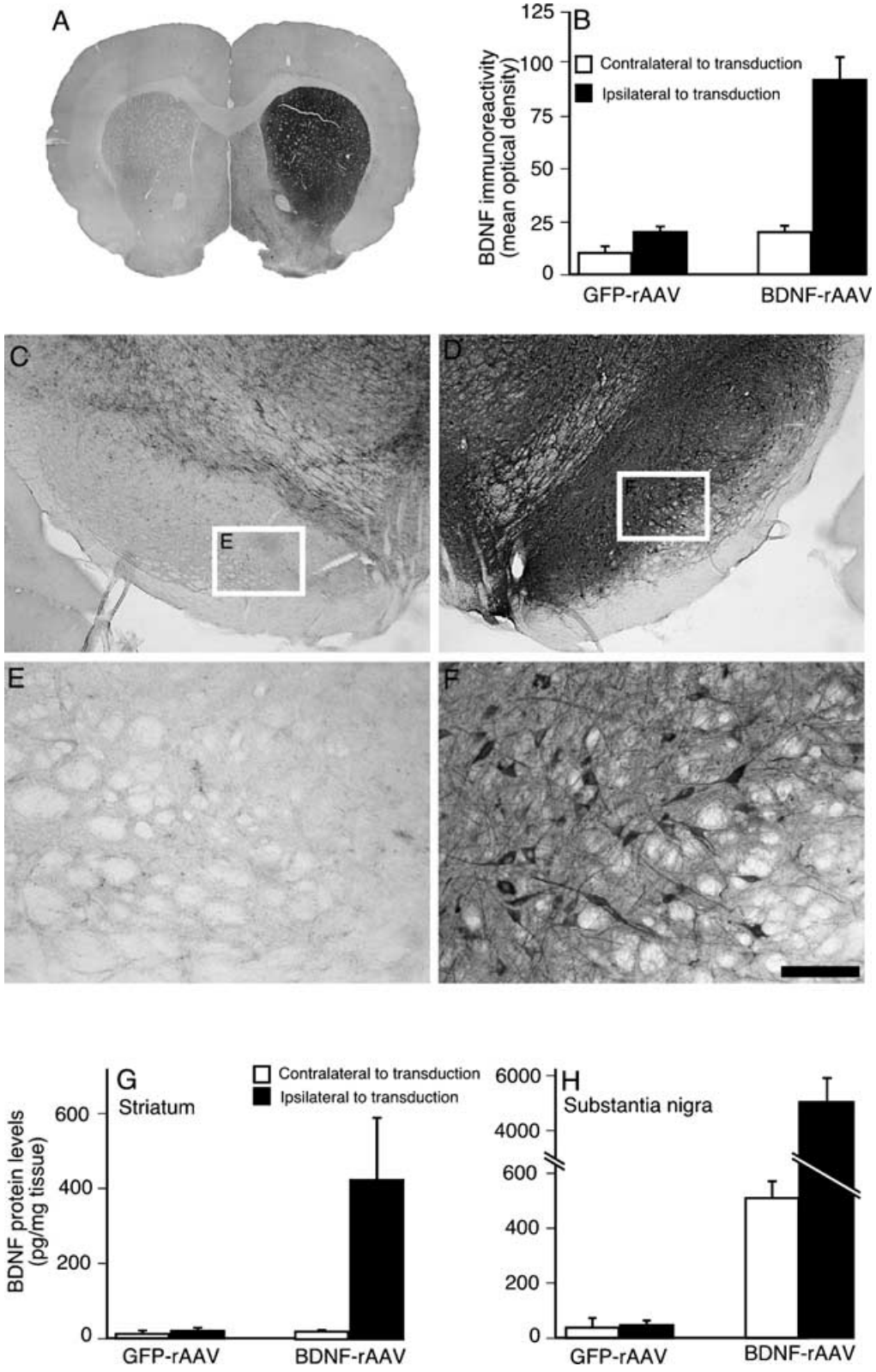

FIG. 1. (A) Photomicrograph showing brain-derived neurotrophic factor (BDNF)-immunoreactivity in a coronal section from a rat subjected to BDNF–recombinant adeno-associated viral (rAAV) transduction unilaterally in the substantia nigra. Note the high BDNF immunoreactivity distributed equally throughout 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 contra- (C, E) and ipsilateral (D, F) to 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).

1997), available in the C.A.S.T.-GRID software (Olympus). Between 50 and 75 NPY-positive cells per striatum were analysed in every 20th section through the striatum.

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 animals with or without MCAO, respectively (Fig. 2).

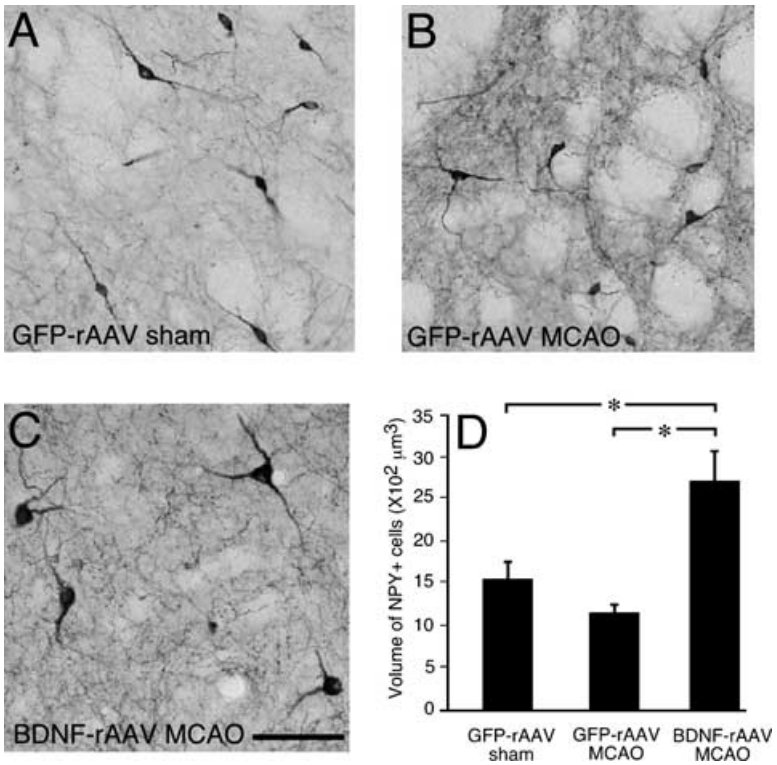

FIG. 2. (A–C) Photomicrographs of NPY-immunoreactive striatal interneurons in animals transduced with (A, B) GFP–recombinant adeno-associated virus (rAAV) or (C) brain-derived neurotrophic factor (BDNF)–rAAV and subjected to (A) sham treatment or (B and C) middle cerebral artery occlusion (MCAO). (C) 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-

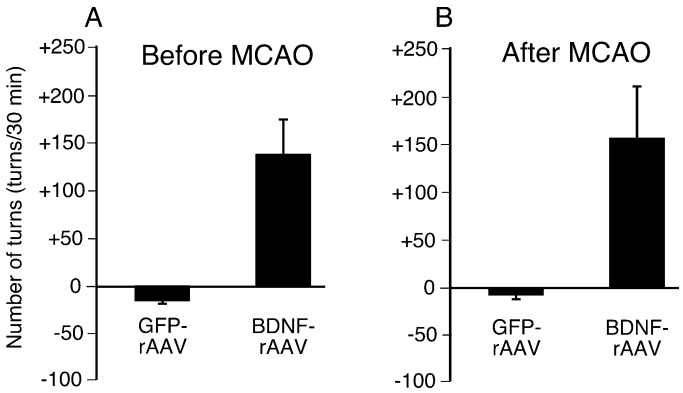

FIG. 3. Spontaneous rotation as assessed at 4 weeks after intranigral injection of GFP–recombinant adeno-associated viral (rAAV, $n = 8$) and brain-derived neurotrophic factor (BDNF)–rAAV ($n = 7$) vectors [A, before middle cerebral artery occlusion (MCAO)] and at 1 week after MCAO (B). '–' means rotation 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.

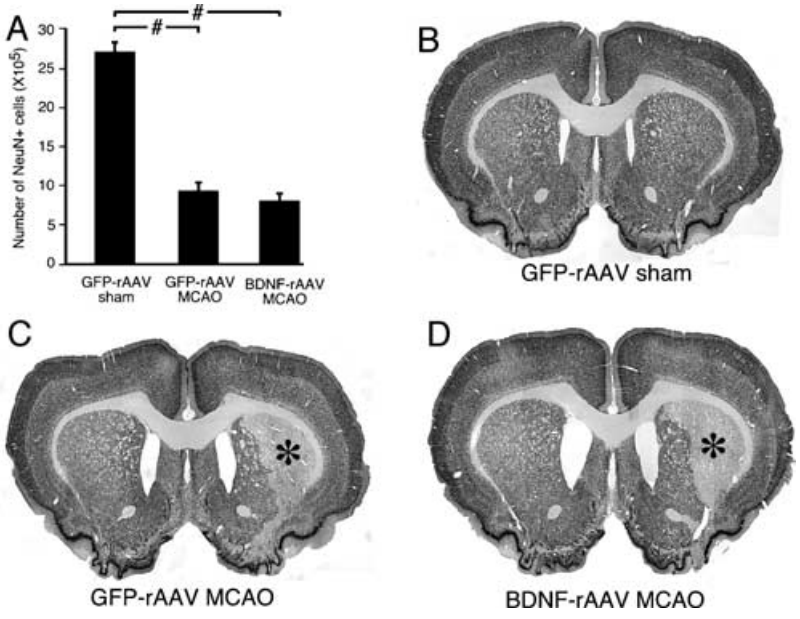

FIG. 4. (A) Total number of NeuN-stained cells in the striatum quantified using stereological procedures in animals transduced with GFP–recombinant adeno-associated virus (rAAV, $n = 13$) or brain-derived neurotrophic factor (BDNF)–rAAV ($n = 6$) and subjected to sham treatment ($n = 6$) or 30 min of 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.

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).

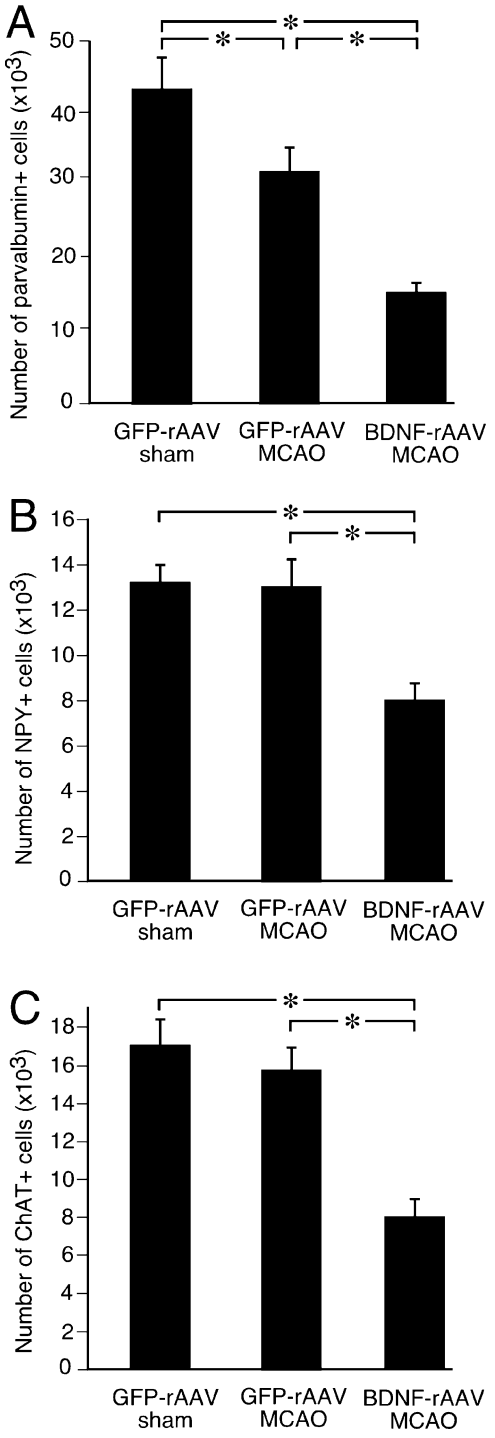

FIG. 5. Number of (A) parvalbumin-, (B) neuropeptide Y (NPY)- and (C) choline acetyltransferase (ChAT)- immunoreactive neurons in the striatum ipsilateral to sham surgery ($n = 6$) or 30 min of middle cerebral artery occlusion (MCAO, $n = 13$) in animals subjected to intranigral GFP–recombinant adeno-associated viral (rAAV, $n = 13$) or BDNF–rAAV ($n = 6$) transduction. Values are mean ± SEM. $^*P < 0.05$, one-way ANOVA, followed by Bonferroni–Dunn post-hoc test.

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 (Marusich *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 commisure. 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,

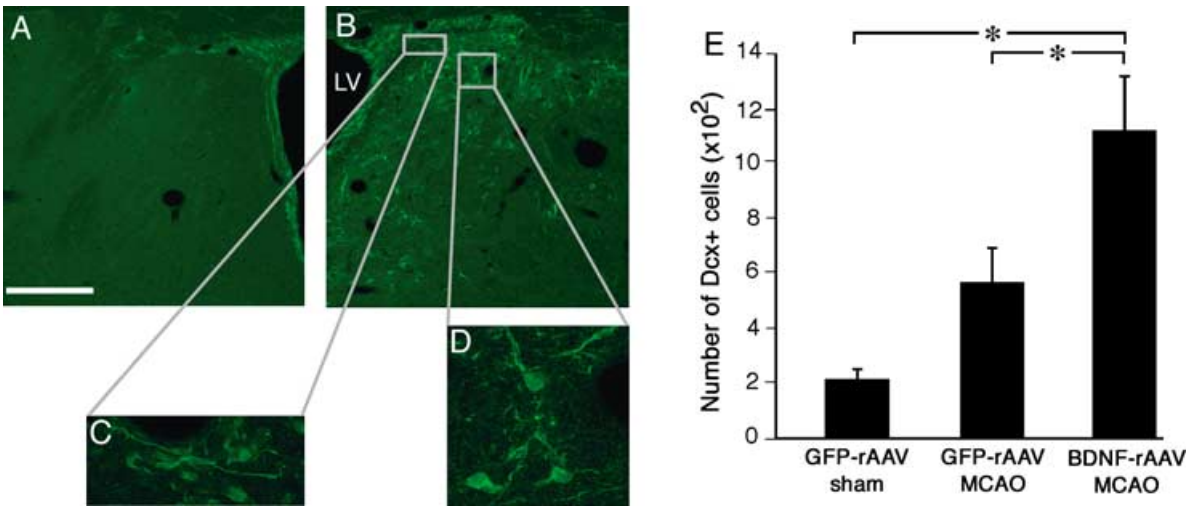
A
B
LV
C
D
E
Number of Dcx+ cells (x10^2)
0
2
4
6
8
10
12
14
*
*
GFP-rAAV sham
GFP-rAAV MCAO
BDNF-rAAV MCAO

FIG. 6.

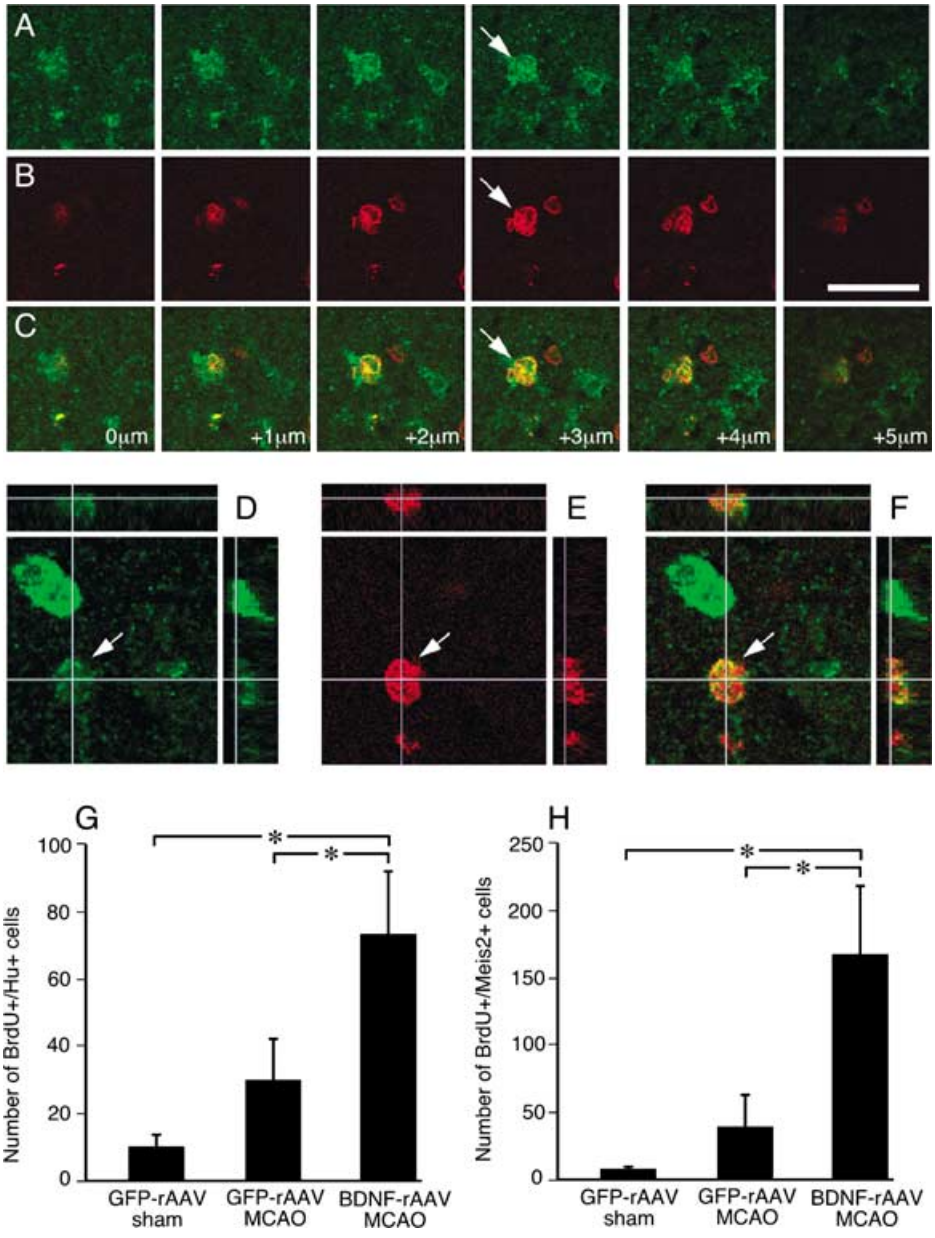
A
B
C
0μm
+1μm
+2μm
+3μm
+4μm
+5μm
D
E
F
G
Number of BrdU+/Hu+ cells
0
20
40
60
80
100
*
*
GFP-rAAV sham
GFP-rAAV MCAO
BDNF-rAAV MCAO
H
Number of BrdU+/Meis2+ cells
0
50
100
150
200
250
*
*
GFP-rAAV sham
GFP-rAAV MCAO
BDNF-rAAV MCAO

FIG. 7.

increased levels of intrastriatal 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 nigrostriatal 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 (Scharfman *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, intrastriatal 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 exogeneous BDNF counteracts MCAO-induced damage, predominantly in the cerebral cortex (Schäbitz *et al*., 1997; Yamashita *et al*., 1997; Schäbitz *et al*., 2000; Yanamoto *et al*., 2000; Zhang & Pardridge, 2001). BDNF also protects striatal neurons from excitotoxic lesions (Martínez-Serrano & Björklund, 1996; Bemelmans *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 intrastriatal 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 (Larsson *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 and route of 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 intrastriatal 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 corticostriatal 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, homogenously 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 (Arvidsson *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) Confocal 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 (G) 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–recombinant adeno-associated virus (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, α-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; EEG, electroencephalogram; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; i.p., intraperitoneally; KPBS, 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 R-actin intervening, sequence; rAAV, recombinant adeno-associated virus; SVZ, subventricular zone.

References

Ahmed, S., Reynolds, B.A. & Weiss, S. (1995) BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. *J. Neurosci.*, **15**, 5765–5778.

Akaneya, Y., Tsumoto, T., Kinoshita, S. & Hatanaka, H. (1997) Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J. Neurosci.*, **17**, 6707–6716.

Altar, C.A., Boylan, C.B., Jackson, C., Hershenson, S., Miller, J., Wiegand, S.J., Lindsay, R.M. & Hyman, C. (1992) Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. *Proc. Natl. Acad. Sci. USA*, **89**, 11,347–11,351.

Altar, C.A., Cai, N., Bliven, T., Juhasz, M., Conner, J.M., Acheson, A.L., Lindsay, R.M. & Wiegand, S.J. (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature*, **389**, 856–860.

Andsberg, G., Kokaia, Z., Björklund, A., Lindvall, O. & Martínez-Serrano, A. (1998) Amelioration of ischaemia-induced neuronal death in the rat striatum by NGF-secreting neural stem cells. *Eur. J. Neurosci.*, **10**, 2026–2036.

Andsberg, G., Kokaia, Z., Klein, R.L., Muzyczka, N., Lindvall, O. & Mandel, R.J. (2002) Neuropathological and behavioral consequences of adeno-associated viral vector-mediated continuous intrastriatal neurotrophin delivery in a focal ischemia model in rats. *Neurobiol. Dis.*, **9**, 187–204.

Arai, S., Kinouchi, H., Akabane, A., Owada, Y., Kamii, H., Kawase, M. & Yoshimoto, T. (1996) Induction of brain-derived neurotrophic factor (BDNF) and the receptor trk B mRNA following middle cerebral artery occlusion in rat. *Neurosci. Lett.*, **211**, 57–60.

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. & Lindvall, O. (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nature Med.*, **8**, 963–970.

von Bartheld, C.S., Byers, M.R., Williams, R. & Bothwell, M. (1996) Anterograde transport of neurotrophins and axodendritic transfer in the developing visual system. *Nature*, **379**, 830–833.

Bates, B., Hirt, L., Thomas, S.S., Akbarian, S., Le, D., Amin-Hanjani, S., Whalen, M., Jaenisch, R. & Moskowitz, M.A. (2002) Neurotrophin-3 promotes cell death induced in cerebral ischemia, oxygen-glucose deprivation, and oxidative stress: possible involvement of oxygen free radicals. *Neurobiol. Dis.*, **9**, 24–37.

Beck, T., Lindholm, D., Castren, E. & Wree, A. (1994) Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. *J. Cereb. Blood Flow Metab.*, **14**, 689–692.

Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R.L. & Bartkowski, H. (1986) Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*, **17**, 472–476.

Bemelmans, A.P., Horellou, P., Pradier, L., Brunet, I., Colin, P. & Mallet, J. (1999) Brain-derived neurotrophic factor-mediated protection of striatal neurons in an excitotoxic rat model of Huntington's disease, as demonstrated by adenoviral gene transfer. *Hum. Gene Ther.*, **10**, 2987–2997.

Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D. & Goldman, S.A. (2001) Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci.*, **21**, 6718–6731.

Carmignoto, G., Pizzorusso, T., Tia, S. & Vicini, S. (1997) Brain-derived neurotrophic factor and nerve growth factor potentiate excitatory synaptic transmission in the rat visual cortex. *J. Physiol. (Lond.)*, **498**, 153–164.

Cheng, B. & Mattson, M.P. (1994) NT-3 and BDNF protect CNS neurons against metabolic/excitotoxic insults. *Brain Res.*, **640**, 56–67.

Chesselet, M.F., Gonzales, C., Lin, C.S., Polsky, K. & Jin, B.K. (1990) Ischemic damage in the striatum of adult gerbils: relative sparing of somatostatinergic and cholinergic interneurons contrasts with loss of efferent neurons. *Exp. Neurol.*, **110**, 209–218.

Croll, S.D., Wiegand, S.J., Anderson, K.D., Lindsay, R.M. & Nawa, H. (1994) Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. *Eur. J. Neurosci.*, **6**, 1343–1353.

Davies, A.M. (1994) The role of neurotrophins in the developing nervous system. *J. Neurobiol.*, **25**, 1334–1348.

Ekdahl, C.T., Mohapel, P., Elmer, E. & Lindvall, O. (2001) Caspase inhibitors increase short-term survival of progenitor-cell progeny in the adult rat dentate gyrus following status epilepticus. *Eur. J. Neurosci.*, **14**, 937–945.

Endres, M., Fan, G., Hirt, L., Fujii, M., Matsushita, K., Liu, X., Jaenisch, R. & Moskowitz, M.A. (2000) Ischemic brain damage in mice after selectively modifying BDNF or NT4 gene expression. *J. Cereb. Blood Flow Metab.*, **20**, 139–144.

Ferrer, I., Ballabriga, J., Marti, E., Perez, E., Alberch, J. & Arenas, E. (1998) BDNF up-regulates TrkB protein and prevents the death of CA1 neurons following transient forebrain ischemia. *Brain Pathol.*, **8**, 253–261.

Gagliardi, R.J. (2000) Neuroprotection, excitotoxicity and NMDA antagonists. *Arq. Neuropsiquiatr.*, **58**, 583–588.
Goodman, Y. & Mattson, M.P. (1994) Staurosporine and K-252 compounds protect hippocampal neurons against amyloid beta-peptide toxicity and oxidative injury. *Brain Res.*, **650**, 170–174.
Gratacos, E., Perez-Navarro, E., Tolosa, E., Arenas, E. & Alberch, J. (2001) Neuroprotection of striatal neurons against kainate excitotoxicity by neurotrophins and GDNF family members. *J. Neurochem.*, **78**, 1287–1296.
Grimm, D., Kern, A., Rittner, K. & Kleinschmidt, J.A. (1998) Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum. Gene Ther.*, **9**, 2745–2760.
Gwag, B.J., Koh, J.Y., Chen, M.M., Dugan, L.L., Behrens, M.M., Lobner, D. & Choi, D.W. (1995) BDNF or IGF-I potentiates free radical-mediated injury in cortical cell cultures. *Neuroreport*, **7**, 93–96.
Han, B.H., D'Costa, A., Back, S.A., Parsadanian, M., Patel, S., Shah, A.R., Gidday, J.M., Srinivasan, A., Deshmukh, M. & Holtzman, D.M. (2000) BDNF blocks caspase-3 activation in neonatal hypoxia-ischemia. *Neurobiol. Dis.*, **7**, 38–53.
Han, B.H. & Holtzman, D.M. (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *J. Neurosci.*, **20**, 5775–5781.
Hauswirth, W.W., Lewin, A.S., Zolotukhin, S. & Muzyczka, N. (2000) Production and purification of recombinant adeno-associated virus. *Meth. Enzymol.*, **316**, 743–761.
Kawaguchi, Y., Wilson, C.J., Augood, S.J. & Emson, P.C. (1995) Striatal interneurones: chemical, physiological and morphological characterization. *Trends Neurosci.*, **18**, 527–535.
Kirik, D., Rosenblad, C., Björklund, A. & Mandel, R.J. (2000) Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J. Neurosci.*, **20**, 4686–4700.
Koh, J.Y., Gwag, B.J., Lobner, D. & Choi, D.W. (1995) Potentiated necrosis of cultured cortical neurons by neurotrophins. *Science*, **268**, 573–575.
Koizumi, J., Yoshida, Y., Nakazawa, T. & Ooneda, G. (1986) Experimental studies of ischemic brain edema. 1. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J. Stroke*, **8**, 1–8.
Kokaia, Z., Andsberg, G., Martínez-Serrano, A. & Lindvall, O. (1998a) Focal cerebral ischemia in rats induces expression of p75 neurotrophin receptor in resistant striatal cholinergic neurons. *Neuroscience*, **84**, 1113–1125.
Kokaia, Z., Andsberg, G., Yan, Q. & Lindvall, O. (1998b) Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: evidence for increased synthesis and anterograde axonal transport. *Exp. Neurol.*, **154**, 289–301.
Kokaia, Z., Nawa, H., Uchino, H., Elmer, E., Kokaia, M., Carnahan, J., Smith, M.L., Siesjö, B.K. & Lindvall, O. (1996) Regional brain-derived neurotrophic factor mRNA and protein levels following transient forebrain ischemia in the rat. *Mol. Brain Res.*, **38**, 139–144.
Kokaia, Z., Zhao, Q., Kokaia, M., Elm,R.E., Metsis, M., Smith, M.-L., Siesjö, B.K. & Lindvall, O. (1995) Regulation of brain-derived neurotrophic factor gene expression after transient middle cerebral artery occlusion with and without brain damage. *Exp. Neurol.*, **136**, 73–88.
Lapchak, P.A. & Hefti, F. (1992) BDNF and NGF treatment in lesioned rats: effects on cholinergic function and weight gain. *Neuroreport*, **3**, 405–408.
Larsson, E., Lindvall, O. & Kokaia, Z. (2001) Stereological assessment of vulnerability of immunocytochemically identified striatal and hippocampal neurons after global cerebral ischemia in rats. *Brain Res.*, **913**, 117–132.
Larsson, E., Mandel, R.J., Klein, R.L., Muzyczka, N., Lindvall, O. & Kokaia, Z. (2002) Suppression of insult-induced neurogenesis in adult rat brain by brain-derived neurotrophic factor. *Exp. Neurol.*, **177**, 1–8.
Larsson, E., Nanobashvili, A., Kokaia, Z. & Lindvall, O. (1999) Evidence for neuroprotective effects of endogenous brain-derived neurotrophic factor after global forebrain ischemia in rats. *J. Cereb. Blood Flow Metab.*, **19**, 1220–1228.
Lessmann, V. (1998) Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. *General Pharmacol.*, **31**, 667–674.
Li, P.-A., Shamloo, M., Katsura, K., Smith, M.-L. & Siesjö, B.K. (1995) Critical values of plasma glucose in aggravating ischemic brain damage: correlation to extracellular pH. *Neurobiol. Dis.*, **2**, 97–108.
Li, P.-A., Shamloo, M., Smith, M.-L., Katsura, K. & Siesjö, B.K. (1994) The influence of plasma glucose concentrations on ischemic brain damage is a threshold function. *Neurosci. Lett.*, **177**, 63–65.
Lindvall, O., Ernfors, P., Bengzon, J., Kokaia, Z., Smith, M.L., Siesjö, B.K. & Persson, H. (1992) Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. *Proc. Natl Acad. Sci. USA*, **89**, 648–652.
Lindvall, O., Kokaia, Z., Bengzon, J., Elmer, E. & Kokaia, M. (1994) Neurotrophins and brain insults. *Trends Neurosci.*, **17**, 490–496.
Linnarsson, S., Willson, C.A. & Ernfors, P. (2000) Cell death in regenerating populations of neurons in BDNF mutant mice. *Mol. Brain Res.*, **75**, 61–69.
Martínez-Serrano, A. & Björklund, A. (1996) Protection of the neostriatum against excitotoxic damage by neurotrophin-producing, genetically modified neural stem cells. *J. Neurosci.*, **16**, 4604–4616.
Martin-Iverson, M.T., Todd, K.G. & Altar, C.A. (1994) Brain-derived neurotrophic factor and neurotrophin-3 activate striatal dopamine and serotonin metabolism and related behaviors: interactions with amphetamine. *J. Neurosci.*, **14**, 1262–1270.
Marusich, M.F., Furneaux, H.M., Henion, P.D. & Weston, J.A. (1994) Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.*, **25**, 143–155.
Memezawa, H., Smith, M.-L. & Siesjö, B.K. (1992) Penumbral tissues salvaged by reperfusion following middle cerebral artery occlusion in rats. *Stroke*, **23**, 552–559.
Nacher, J., Crespo, C. & McEwen, B.S. (2001) Doublecortin expression in the adult rat telencephalon. *Eur. J. Neurosci.*, **14**, 629–644.
Numakawa, T., Takei, N., Yamagishi, S., Sakai, N. & Hatanaka, H. (1999) Neurotrophin-elicited short-term glutamate release from cultured cerebellar granule neurons. *Brain Res.*, **842**, 431–438.
Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N. & Ferriero, D.M. (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann. Neurol.*, **52**, 802–813.
Pascual, M., Climent, E. & Guerri, C. (2001) BDNF induces glutamate release in cerebrocortical nerve terminals and in cortical astrocytes. *Neuroreport*, **12**, 2673–2677.
Paxinos, G. & Watson, C. (1997) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego.
Pelleymounter, M.A., Cullen, M.J. & Wellman, C.L. (1995) Characteristics of BDNF-induced weight loss. *Exp. Neurol.*, **131**, 229–238.
Pencea, V., Bingaman, K.D., Wiegand, S.J. & Luskin, M.B. (2001) Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J. Neurosci.*, **21**, 6706–6717.
Perez-Navarro, E., Alberch, J., Neveu, I. & Arenas, E. (1999) Brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 differentially regulate the phenotype and prevent degenerative changes in striatal projection neurons after excitotoxicity in vivo. *Neuroscience*, **91**, 1257–1264.
Perez-Navarro, E., Canudas, A.M., Akerund, P., Alberch, J. & Arenas, E. (2000) Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 prevent the death of striatal projection neurons in a rodent model of Huntington's disease. *J. Neurochem.*, **75**, 2190–2199.
Rudge, J.S., Mather, P.E., Pasnikowski, E.M., Cai, N., Corcoran, T., Acheson, A., Anderson, K., Lindsay, R.M. & Wiegand, S.J. (1998) Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective. *Exp. Neurol.*, **149**, 398–410.
Sattler, R. & Tymianski, M. (2001) Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol. Neurobiol.*, **24**, 107–129.
Sauer, H., Fischer, W., Nikkhah, G., Wiegand, S.J., Brundin, P., Lindsay, R.M. & Bjorklund, A. (1993) Brain-derived neurotrophic factor enhances function rather than survival of intrastriatal dopamine cell-rich grafts. *Brain Res.*, **626**, 37–44.
Schäbitz, W.R., Schwab, S., Spranger, M. & Hacke, W. (1997) Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. *J. Cereb. Blood Flow Metab.*, **17**, 500–506.
Schäbitz, W.R., Sommer, C., Zoder, W., Kiessling, M., Schwaninger, M. & Schwab, S. (2000) Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. *Stroke*, **31**, 2212–2217.
Scharfman, H.E., Goodman, J.H., Sollas, A.L. & Croll, S.D. (2002) Spontaneous limbic seizures after intrahippocampal infusion of brain-derived neurotrophic factor. *Exp. Neurol.*, **174**, 201–214.
Siuciak, J.A., Boylan, C., Fritsche, M., Altar, C.A. & Lindsay, R.M. (1996) BDNF increases monoaminergic activity in rat brain following intracerebroventricular or intraparenchymal administration. *Brain Res.*, **710**, 11–20.
Smith, M.A., Zhang, L.X., Lyons, W.E. & Mamounas, L.A. (1997) Anterograde transport of endogenous brain-derived neurotrophic factor in hippocampal mossy fibers. *Neuroreport*, **8**, 1829–1834.

Takeda, A., Onodera, H., Sugimoto, A., Kogure, K., Obinata, M. & Shibahara, S. (1993) Coordinated expression of messenger RNAs for nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in the rat hippocampus following transient forebrain ischemia. *Neuroscience*, **55**, 23–31.

Takei, N., Sasaoka, K., Inoue, K., Takahashi, M., Endo, Y. & Hatanaka, H. (1997) Brain-derived neurotrophic factor increases the stimulation-evoked release of glutamate and the levels of exocytosis-associated proteins in cultured cortical neurons from embryonic rats. *J. Neurochem.*, **68**, 370–375.

Toresson, H., Parmar, M. & Campbell, K. (2000) Expression of Meis and Pbx genes and their protein products in the developing telencephalon: implications for regional differentiation. *Mech. Dev.*, **94**, 183–187.

Toth, E. & Lajtha, A. (1989) Motor effects of intracaudate injection of excitatory amino acids. *Pharmacol. Biochem. Behav.*, **33**, 175–179.

Uemura, Y., Kowall, N.W. & Beal, M.F. (1990) Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. *Ann. Neurol.*, **27**, 620–625.

Ungerstedt, U. & Arbuthnott, G.W. (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.*, **24**, 485–493.

Vecsei, L. & Beal, M.F. (1991) Comparative behavioral and neurochemical studies with striatal kainic acid- or quinolinic acid-lesioned rats. *Pharmacol. Biochem. Behav.*, **39**, 473–478.

Weber, U.J., Bock, T., Buschard, K. & Pakkenberg, B. (1997) Total number and size distribution of motor neurons in the spinal cord of normal and EMC-virus infected mice – a stereological study. *J. Anat.*, **191**, 347–353.

West, M.J. (1999) Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends Neurosci.*, **22**, 51–61.

West, M.J., Slomianka, L. & Gundersen, H.J. (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.*, **231**, 482–497.

Yamashita, K., Wiessner, C., Lindholm, D., Thoenen, H. & Hossmann, K.A. (1997) Post-occlusion treatment with BDNF reduces infarct size in a model of permanent occlusion of the middle cerebral artery in rat. *Metab. Brain Dis.*, **12**, 271–280.

Yanamoto, H., Nagata, I., Sakata, M., Zhang, Z., Tohnai, N., Sakai, H. & Kikuchi, H. (2000) Infarct tolerance induced by intra-cerebral infusion of recombinant brain-derived neurotrophic factor. *Brain Res.*, **859**, 240–248.

Zhang, Y. & Pardridge, W.M. (2001) Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood–brain barrier drug targeting system. *Stroke*, **32**, 1378–1384.

Zhao, Q., Smith, M.-L. & Siesjö, B.K. (1994) The w-conopeptide SNX-111, an N-type calcium channel blocker, dramatically ameliorates brain damage due to transient focal ischaemia. *Acta Physiol. Scand.*, **150**, 459–461.

Zigova, T., Pencea, V., Wiegand, S.J. & Luskin, M.B. (1998) Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. *Mol. Cell. Neurosci.*, **11**, 234–245.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J. & Muzyczka, N. (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.*, **6**, 973–985.