



9-Methyl- β -carboline has restorative effects in an animal model of Parkinson's disease

Catrin Wernicke¹, Julian Hellmann², Barbara Zięba³, Katarzyna Kuter⁴, Krystyna Ossowska⁴, Monika Frenzel⁵, Norbert A. Dencher⁵, Hans Rommelspacher^{1, 2}

¹Department of Psychiatry, CCM, Charité-University Medicine Berlin, Dorotheenstr. 94, 10117 Berlin, Germany

²Section of Clinical Neurobiology, Department of Psychiatry, CBF, Charité-University Medicine Berlin, Akazienallee 36, 14050 Berlin, Germany

³Department of Neurobiology, ⁴Department of Neuro-Psychopharmacology, Institute of Pharmacology, Polish Academy of Sciences, Smetna 12, PL 31-343 Kraków, Poland

⁵Physical Biochemistry, Department of Chemistry, Technische Universität, Petersenstrasse 22, 64287 Darmstadt, Germany

Correspondence: Catrin Wernicke, e-mail: catrin.wernicke@charite.de

Abstract:

In a previous study, a primary culture of midbrain cells was exposed to 9-methyl- β -carboline for 48 h, which caused an increase in the number of tyrosine hydroxylase-positive cells. Quantitative RT-PCR revealed increased transcription of genes participating in the maturation of dopaminergic neurons. These *in vitro* findings prompted us to investigate the restorative actions of 9-methyl- β -carboline *in vivo*. The compound was delivered for 14 days into the left cerebral ventricle of rats pretreated with the neurotoxin 1-methyl-4-phenyl-pyridinium ion (MPP⁺) for 28 days applying a dose which lowered dopamine by approximately 50%. Interestingly, 9-methyl- β -carboline reversed the dopamine-lowering effect of the neurotoxin in the left striatum. Stereological counts of tyrosine hydroxylase-immunoreactive cells in the substantia nigra revealed that the neurotoxin caused a decrease in the number of those cells. However, when treated subsequently with 9-methyl- β -carboline, the number reached normal values. In search of an explanation for the restorative activity, we analyzed the complexes that compose the respiratory chain in striatal mitochondria by 2-dimensional gel electrophoresis followed by MALDI-TOF peptide mass fingerprinting. We found no changes in the overall composition of the complexes. However, the activity of complex I was increased by approximately 80% in mitochondria from rats treated with MPP⁺ and 9-methyl- β -carboline compared to MPP⁺ and saline and to sham-operated rats, as determined by measurements of nicotinamide adenine dinucleotide dehydrogenase activity. Microarray technology and single RT-PCR revealed the induction of neurotrophins: brain-derived neurotrophic factor, conserved dopamine neurotrophic factor, cerebellin 1 precursor protein, and ciliary neurotrophic factor. Selected western blots yielded consistent results. The findings demonstrate restorative effects of 9-methyl- β -carboline in an animal model of Parkinson's disease that improve the effectiveness of the respiratory chain and promote the transcription and expression of neurotrophin-related genes.

Key words:

neuronal regeneration, neurotrophins, respiratory chain, dopamine, Parkinson's disease, rat

Abbreviations: Armet11 – arginine-rich, mutated in early stage tumor-like 1, synonymous with CDNF – conserved dopamine neurotrophic factor, ATP – adenosine triphosphate, BC – β -carboline, BDNF – brain-derived neurotrophic factor, BMP2 – bone morphogenetic protein 2, BN-PAGE – blue native-polyacrylamide gel electrophoresis, Cbln1 – cerebellin 1 precursor protein, DA – dopamine, DAT – dopamine transporter, DRD1 – dopamine receptor 1, DRD2l – long variant of the DRD2 receptor, FRET – fluorescence resonance energy transfer, GDNF – glial cell line-derived neurotrophic factor, hDAT – human dopamine transporter, HEK-293 – human embryonic kidney cell-293, Hmbs – hydroxymethylbilane synthase, HPLC – high performance liquid chromatography, HSP 60 – 60 kDa heat shock protein, LDH – lactate dehydrogenase, LSD – Fisher's least significant difference *post-hoc* test, MALDI-TOF-MS – matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 9-me-BC – 9-methyl- β -carboline, MPP⁺ – 1-methyl-4-phenyl-pyridinium ion, NADH – nicotinamide adenine dinucleotide dehydrogenase, NGF – nerve growth factor, NPY – neuropeptide Y, Nurr 1 – nuclear receptor regulated 1 protein, OXPHOS – oxidative phosphorylation, PD – Parkinson's disease, PTX – paired homeodomain transcription factor, Ret – rearranged during transfection, RKIP – Raf-1 kinase inhibitor protein, RT-PCR – reverse transcriptase polymerase chain reaction, Sirt – silent information regulator, SLC – left striatum, sham-operated control, SLM – left striatum after administration of MPP⁺ and then saline, SLM+BC – left striatum after administration of MPP⁺ and then 9-me-BC, SNc – substantia nigra pars compacta, SRC – right striatum, sham-operated control, SRM – right striatum after administration of MPP⁺ and then saline, SRM+BC – right striatum after administration of MPP⁺ and then 9-me-BC, TH – tyrosine hydroxylase, THir – tyrosine hydroxylase immunoreactivity, TNF – tumor necrosis factor, VTA – ventral tegmental area

Introduction

β -Carbolines (BC) are produced in mammals from tryptophan and tryptophan-derived indoleamines [80]. Several BCs have been detected in mammalian brain and other organs as well as in human cerebrospinal fluid and plasma [6, 17, 28, 33, 45]. Animal studies revealed the preference of BCs for dopaminergic neurons. The unsubstituted BC, trivial name: norharman, induced a release of dopamine (DA) in the nucleus accumbens of rats (*in vivo* microdialysis, intraperitoneal application [67]). Furthermore, 1-methyl-BC (1-me-BC, trivial name: harman) facilitated L-DOPA-induced stereotypy in mice, suggesting a pro-dopamine effect [62]. Intraperitoneal application of 1-me-BC (2.27 μ mol/kg) induced a release of DA in the nucleus accumbens of the freely moving rat, whereas higher doses (41 μ mol/kg and 82 μ mol/kg) also induced the

release of 5-hydroxytryptamine [66]. Nanomolar concentrations of 1-me-BC inhibited monoamine oxidase subtype A *in vitro* [25, 46, 47] and *in vivo* [65]. In the brain, the unsubstituted BC is converted by certain S-adenosyl-methionine-dependent N-methyltransferases to the 2-methyl- β -carbolinium ion and subsequently to the 2,9-dimethyl- β -carbolinium ion (2,9-dime-BC⁺) [9, 19, 43–45]. There is strong evidence that 2,9-dime-BC⁺ is neurotoxic and involved in the pathogenesis of Parkinson's disease [10, 11, 39, 45].

There are numerous examples of compounds that exert dose-dependent effects, which may even oppose each other (e.g., sedation-behavioral activation). Furthermore, the specific effect depends on the substituents involved. Thus, we rationalized that by modifying substituents, BCs with neuroprotective actions might be detected. We synthesized a large number of BCs and screened them for neuroprotective properties using HEK-293 cells transfected with the human DA transporter (hDAT) [87] and primary dopaminergic cultures of the midbrain from embryonic mice, as models with relevance to PD [22]. We found evidence that 9-methyl- β -carboline (9-me-BC) is a compound with neuroprotective properties. Previously, Matsubara and co-workers reported that 9-me-BC systemically administered to C57BL/6 mice for 7 days (250 μ mol/kg twice per day) decreased levels of DA and serotonin in several brain regions [42]. In our experiments, the mesencephalic culture was exposed to 9-me-BC for 48 h (25 to 100 μ M; higher concentrations were toxic). This procedure promoted an increase in tyrosine hydroxylase (TH)-positive cells as represented by a roughly 20% increase in DA levels and high-affinity [³H]DA uptake. The total number of cells in culture did not change, whereas the number of neurons appeared to increase slightly, and that of dopaminergic neurons rose significantly. Thus, dopaminergic neurons were more susceptible to the actions of 9-me-BC than other neurons. Further experiments suggested neuroprotective properties of 9-me-BC: a reduction in levels of lactate dehydrogenase (LDH) in the medium and in the number of necrotic cells. Furthermore, ATP levels increased.

Quantitative real-time RT-PCR revealed increased transcription of genes participating in the maturation of dopaminergic neurons and of several genes involved in neuronal differentiation. In contrast, genes engaged in inflammation and apoptosis were down-regulated [22]. These results indicate that 9-me-BC is neuroprotective.

The *in vitro* effects of 9-me-BC, including pro-dopamine actions and increased numbers of tyrosine hydroxylase-positive neurons, prompted us to investigate possible restorative actions *in vivo* in an animal model of PD. The rat model used has been reported by Yazdani and coworkers [89]. It is based on the damage to dopaminergic neurons due to chronic delivery of the neurotoxin 1-methyl-4-phenyl-pyridinium ion (MPP⁺) into the left cerebral ventricle. MPP⁺ delivery to rats over a four-week period followed by a two-week period of vehicle infusion provides a chronic model of mitochondrial dysfunction without mortality and low inter-animal variability with regard to the degree of neuropathology. Furthermore, we sought to determine the basic mechanisms of the restorative actions of 9-me-BC. Previous studies have reported the rescuing effects of neurotrophins in Parkinson's disease. Therefore, we investigated the transcription and expression of neurotrophins by array technology, single real time-PCR and western blot. Furthermore, it has been reported that some BCs bind to complex I of the respiratory chain in mitochondria. These findings suggested that 9-me-BC might affect the respiratory chain as well. Since our *in vitro* experiments revealed that low concentrations promoted favorable effects in a neuronal culture while higher doses were toxic, a low dose was used in the present study.

Materials and Methods

Materials

9-Methyl- β -carboline-HCl was synthesized by Yvonne Schott, Institute of Pharmacy (head: Prof. J. Lehmann), Friedrich-Schiller University, Jena, Germany, as described previously [22]; MPP⁺ was obtained from Sigma Aldrich, Taufkirchen, Germany. The following compounds were obtained as indicated in parentheses: protease inhibitor cocktail (P8340, Sigma, Taufkirchen, Germany), mouse monoclonal anti-TH antibody (Chemicon Int., Temecula, CA, USA), ABC-peroxidase kit (Vector Laboratories, Burlingame, CA, USA). The RNeasy Lipid Tissue Mini-Kit was from Qiagen (Hilden, Germany), the RT² PCR Array First Strand Kit, the SYBR Green/Rox PCR Master Mix, and the Neurotrophin and Receptors RT² ProfilerTM PCR Array

were from Super Array (Frederick, MD, USA). We also used a 1st strand cDNA synthesis kit for single real time RT-PCR (Roche Applied Science, Mannheim, Germany) and a LightCycler FastStart DNA Master Hybridization Probes kit (Roche Applied Science). Appropriate primers and probes were designed and synthesized by Tib Molbiol (Berlin, Germany).

The following devices were obtained as indicated: Alzet[®] osmotic pump (Alzet, Cupertino, CA, USA, model 2ML4), centrifugal filter device (Durapore, 0.22 μ m, Millipore, Bedford, MA, USA), biofuge (Heraeus, Osterode, Germany), Nova-Pak HPLC-column, Waters (Milford, MA, USA), coulochemical detector (ESA Coulochem, Bedford, MA, USA). We also used an HPLC pump (Knauer, Berlin, Germany), a microscope (Leica, DMLB; Leica, Ballerup, Denmark) equipped with a projecting camera and a microscope stage connected to an xyz stepper (PRIOR ProScan, Prior Scientific, Cambridge, UK) and controlled by a computer using CAST2 software (Olympus, Ballerup, Denmark), a densitometer (GS-800, BioRad, Munich, Germany), the Stratagene Instrument Mx3005p (Cedar Creek, TX, USA), and a LightCycler system (Roche Applied Science).

Animals

Male Wistar rats (initial body weight 280–300 g; breeder: Charles River, Sulzfeld, Germany) were housed 4 animals per cage in an artificial 12-h light-dark cycle with food and water available *ad libitum*. The rats were kept under these conditions for at least 7 days until surgery. All experiments were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals (Publication no. 85–23, revised in 1985) and were approved by the animal care and use committee of the Senate of Berlin (Registration no. G-0129/04).

Surgery and chronic delivery of test compounds

Rats received MPP⁺-iodide dissolved in saline infused into the anterior part of the left cerebral ventricle *via* an Alzet[®] osmotic mini-pump implanted subcutaneously on the back. Cannula placement was performed stereotactically using the following coordinates proposed by Paxinos and Watson [59]: anterior –0.3 mm from the bregma, lateral left +1.4 mm, depth –3.5 mm. Cannula and pump implantations were per-

formed under anesthesia: ketamine (80 mg/kg) and xylazine (2.5 mg/kg), both delivered *ip*. In pilot experiments, several doses of MPP⁺ were administered (0.142 mg/kg/day (as reported by Yazdani et al. [89], 0.213 mg/kg/day, 0.284 mg/kg/day, 0.426 mg/kg/day) for 28 days with a drug delivery rate of 2.5 µl/h followed by replacement of the osmotic pump by a second pump that administered vehicle (0.9% sodium chloride, pH 7.4) for 14 days. A dose of 0.284 MPP⁺ mg/kg/day was chosen to lower the level of DA by approximately 50% in the left striatum by 14 days later, after delivery of vehicle (saline) for 14 days. In experiments with 9-me-BC·HCl, half the amount of MPP⁺ on an equimolar basis was chosen, i.e., 0.105 mg 9-me-BC /kg/day (0.48 µmol/kg/day). Three groups of rats with six to eight animals each were treated. The first group received MPP⁺ (0.284 mg/kg/day) for 28 days and subsequently 9-me-BC (0.105 mg/kg/day) for 14 days. The second group received MPP⁺ for 28 days and subsequently saline for 14 days. The third group was sham-operated twice with a 28-day interval. Animals were killed after discontinuation of the 42-day infusions. Their brains were dissected on ice and divided in two parts at the level of the caudal hypothalamus: the anterior part containing the striatum for biochemical studies, and the posterior part for TH immunohistochemistry and stereology in the substantia nigra pars compacta (SNc).

Preparation of tissue

Striatal tissue dissected from the brain was immediately frozen (left and right striatum separately) in liquid nitrogen and stored at -80°C. Total RNA was isolated using the RNeasy Lipid Tissue Mini-Kit. The kit is suited to process a maximum of 100 mg of brain tissue. Frozen tissue was weighed and immediately transferred into a 1.5-ml reaction tube and homogenized in 0.5 ml QIAzol Lysis Reagent (containing phenol) using a homogenizer that fit the reaction tube. A drill on ice was used to gently rotate the samples. The homogenizer was washed with 0.5 ml QIAzol Lysis reagent, and the solution was combined with the homogenate. After incubation at room temperature for 5 min, 200 µl of chloroform was added. After vigorous shaking and further incubation at room temperature for 3 min, the tube was centrifuged at 12,000 × *g* and +4°C. The upper phase was transferred to a collection tube and after adding the same volume of 70% ethanol and thorough mixing, samples were applied to

a column which had been placed in a 2-ml collection tube. The tube was centrifuged at 8,000 × *g* for 15 s at room temperature. The eluent was transferred into a separate tube and used to measure DA levels (see below). The solid portion retained on the filter consisted of total RNA and was further processed as suggested by the manufacturer, which included DNase digestion and several washing steps. RNA was eluted with 30 µl RNase-free water. A second elution step with the first eluate was performed to concentrate the RNA. RNA concentration was determined at 260 nm. RNA quality was determined at 260/280 nm using a BioPhotometer from Eppendorf (Hamburg, Germany).

Measurement of DA levels in the striatum

DA levels were measured in left and right striatal homogenates separately using HPLC with coulometric detection as described previously [39]. The eluent mentioned in the previous section was transferred to a centrifugal filter device and then centrifuged (13,000 × *g*, 10 min, +4°C) using a Biofuge. The resulting eluent was immediately applied to the HPLC apparatus. DA was separated using a Nova-Pak C-18 column. The detection limit for DA was approximately 15 fmol.

Tyrosine hydroxylase immunohistochemistry in the substantia nigra

The analyses were performed following a protocol described previously [39] with slight modifications. Caudal parts of the brains (see "Surgery and chronic delivery of test compounds" subchapter) were fixed in cold buffered 4% paraformaldehyde for a week, then cryoprotected in 20% sucrose solution in phosphate-buffered saline (PBS) for at least 5 days. The brains were cut on a freezing microtome into 30-µm frontal sections containing the substantia nigra and ventral tegmental area (VTA) at levels from the bregma -4.80 to -6.3 mm, according to Paxinos and Watson [59].

Stereology

THir neurons in the substantia nigra and VTA were counted stereologically as described previously [39, 56, 57]. Systemic, uniform sampling was used to choose the sections. The first sample was taken at random from the level A = -4.6 mm from the bregma

[60] and all following samples were taken at a fixed distance from the previous one. At least 8–10 sections throughout the entire lengths of both structures were sampled. All stereological counts were performed using a microscope equipped with a projecting camera and a microscope stage connected to an xyz stepper controlled by a computer using CAST2 software.

The regions of the SNc and VTA were outlined under low magnification (5 \times). The total volume of the structure $V(ref)$ was estimated using Cavalieri's principle [21] according to:

$$V(ref) = t \times a(p) \times \Sigma P,$$

where t is the known distance between sections, $a(p)$ is the area associated with each point on a grid and ΣP is the total number of points counted throughout all sections from one rat.

The total number of THir neurons was estimated without bias using a three-dimensional probe under high magnification (63 \times). The simple three-dimensional probe was the dissector [79]. The sampling volume, $v(dis)$, was the volume of the test frame. An unbiased estimation of the total number of THir neurons in the above-mentioned structures was obtained from:

$$N = \Sigma Q \times V(ref) / v(dis) \times \Sigma P,$$

where ΣQ was the total number of THir neurons in the sections uniformly sampled with dissectors and meander sampling, $v(dis)$ was the total volume of the dissector and ΣP was the total number of all dissector points.

Analysis of the respiratory chain including complex V in striatal mitochondria

Isolation of mitochondria

Gentle isolation of a crude mitochondrial fraction from rat striatum was performed on ice immediately upon dissection of the tissue. The brain tissue was minced and homogenized by a motor-driven, micro-pistill homogenizer (9 strokes, 600 rpm) in 4 vol (1 g tissue/4 ml buffer) of homogenization buffer (5 mol/l HEPES/NaOH, pH 7.4, 320 mmol/l sucrose, 1 mmol/l Na^+ /EDTA, 0.5% protease inhibitor cocktail (Sigma P8340) and 2% (v/v) SCAVEGR (mixture of antioxi-

dants [7]. After centrifugation at $1,300 \times g$ (3 min, 4°C) the mitochondria in the supernatant were collected. To increase the yield, the pellet was washed twice with homogenization buffer ($1,500 \times g$, 4°C, 3 min). To collect the mitochondria, the merged supernatants were centrifuged at $17,000 \times g$ (10 min, 4°C). The pellet was suspended in homogenization buffer (containing 0.5 mmol/l pefabloc SC, a serine protease inhibitor). Aliquots of the mitochondrial fraction were shock-frozen in liquid nitrogen and stored at -80°C .

2D Blue-Native/SDS-PAGE

Solubilization of striatal mitochondria was performed at 4°C for 30 min using an 8 g digitonin/g protein ratio at a final detergent concentration of 1% (w/v), according to Reifschneider et al. [64].

Linear 4–13% gradient gels overlaid with a 3.5% stacking gel were used for Blue-Native (BN)-PAGE. Lanes from the first dimension BN-PAGE were excised and analyzed by two-dimensional SDS-PAGE (5% stacking gel, 13% separation gel) [32, 52, 64]. One-dimensional gels were stained with Rotiblock and 2D-SDS gels were stained with silver.

Mitochondrial proteins were identified by their specific migration behavior in the first native-PAGE dimension as well as by the characteristic subunit pattern in the 2D SDS-PAGE gel, using a recent protein profiling of rat brain mitochondria by MALDI-TOF-MS peptide mass fingerprinting as a reference [64].

In-gel complex I activity assay

The NADH dehydrogenase activity of complex I was probed by in-gel formazan precipitation using a modified protocol described by Kuonen et al. [34] and Grandier-Vazille and Guerin [20] and modified by Schaefer et al. [69]. The in-gel activity tests were performed immediately after BN-PAGE without fixation of proteins. The gels were incubated for up to 1.5 h at room temperature in 100 mmol/L Tris, 768 mmol/l glycine, 0.04% (w/v) 4-nitro blue tetrazolium chloride, and 0.1 mmol/l β -NADH, pH 7.4. Subsequently, the gels were fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for 10 min. For quantitative evaluation of protein abundance and enzyme activity a calibrated densitometer for gel-scanning and Quantity One 1-D analysis software (units of signal intensity are optical density, O.D.) were employed.

Real-time RT-PCR

Array analysis

The rat Neurotrophin and Receptors RT² Profiler™ PCR Array was used to analyze transcription changes for several genes related to neuronal differentiation, regeneration, and survival. Four animals from each group were included; the left and right striatum of each animal were investigated separately. As advised by the manufacturer, 0.5 µg RNA of each probe was pretreated with the genomic DNA elimination buffer supplied before synthesizing cDNA using the RT² PCR Array First Strand Kit. In accordance with the procedure in the user manual, cDNA was diluted with water and RT Real-Time™ SYBR Green/Rox PCR Master Mix and applied to the 96-well plate provided, which already contained the appropriate primers. Cycling was performed on a Stratagene Instrument Mx3005p following the advised Super Array procedure for this instrument: after 10 min denaturation at 95°C, samples were subjected to 40 cycles of 15 s at 95°C and 60 s at 60°C. The procedure ended with a melting curve program for quality control, as described by the manufacturer (Super Array). Additionally, quality was controlled by agarose gel electrophoresis. Analyses of the data were conducted as advised by the provider using the Excel sheet provided on the Super Array website. The array contains five different housekeeping genes, controls for genomic DNA contamination, reverse transcription, and positive PCR.

Single RT-PCR

For each isolated probe, 1 µg RNA was reverse-transcribed, using random primers and the 1st strand cDNA synthesis kit for RT-PCR in a final volume of 40 µl. Real-time RT-PCR was performed using the fluorescence resonance energy transfer (FRET)-probe format of the LightCycler system with the LightCycler FastStart DNA Master hybridization probes kit. The hydroxymethylbilane synthase gene (Hmbs) was chosen as a housekeeping gene for relative quantification. Appropriate primers and probes were designed and synthesized by Tib Molbiol (Tab. 1). Primers or probes were designed to span exon boundaries in order to exclude possible DNA fragments. Most of the primers and probes were specific for cDNA of both rat and human origin. The optimal annealing tempera-

ture and amount of MgCl₂ were assessed; these values are compiled in Table 1. To each reaction, 25 ng of the cDNAs were added. Cycling conditions were: denaturation at 94°C for 5 min, 55 cycles of denaturation at 94°C for 7 s, primer and probe annealing at the appropriate temperature for 10 s, and elongation at 72°C for 10 s. The run ended with a melting curve analysis. The amount of LC-Red-specific fluorescence was measured in each cycle after probe annealing and analyzed with LightCycler software. The relative amount of target was calculated as amount of target for each probe compared to the amount of the housekeeping gene for the same probe. The quantification was performed by the 2^{-ΔΔCt} method [38].

Protein isolation and western blot analysis from the same striatal tissue as used for RT-PCR and the determination of DA levels

The lower phase after tissue disruption and centrifugation (see mRNA isolation) was processed as described by the kit manufacturer (Qiagen). At the end, protein was dissolved in PBS containing 10 mM N-ethylmaleimide, 2 µg/ml pefabloc and aprotinin each, 1 µg/ml leupeptin and pepstatin each (all from Roche, Mannheim, Germany), and 0.2% digitonin, using a B-12 sonifier (Branson Sonic Power Company, Danbury, CT, USA), and incubated for 1 h at 4°C. Probes were centrifuged for 10 min at 2,000 × g and 4°C. The supernatant was used for further analyses. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Each probe was diluted in Laemmli buffer and the proteins were denatured for 10 min at 95°C. Twenty-five µg per lane were loaded on 4–20% Precise Protein Gels (Pierce, Rockford, IL, USA). The Dual Color Precision Plus Protein™ Standards (Bio-Rad Laboratories, Munich, Germany) was used as a molecular weight reference.

After running the gel, the protein was blotted onto polyvinylidene fluoride microporous membrane (Millipore, Schwalbach, Germany) for 1 h at 150 mV/cm². Ponceau-S red staining of the blot was performed to assess even blotting. After destaining, detection was conducted using the Amersham ECL Advanced Western Blotting Detection Kit and the appropriate primary antibodies (all developed in rabbit): anti-Armet11 (Pro Science, Poway, CA, USA), anti-cerebellin (Life Span, Seattle, WA, USA), anti-CNTF,

Tab. 1. Sequences of primers and FRET-probes, and the respective annealing temperature, and concentration of MgCl₂ used for quantitative real-time RT-PCR

Gene name	Primer	FRET-probes
ARMETL1 56°C, 2.5 mM	AAAgATTCTTAAACCGATTCTACA CAWCTTCTTCAgCTTCTCACAAAT	TCCTAgATAATAgCACAggCggTTTTTC-FL LC640-TTTCCTTgTgTCCRMgCAAAARCT-PH
BDNF 58°C, 2 mM	TTgTACACKTCCMgggTgATg CTCRCTAATACTgTCACACAgCT	AggTAATTTTTgTATTCCTCCAgCagAAAg-FL LC640-AgAggAggCTCCAAAggCACTTgA-PH
BMP2 56°C, 2.5 mM	gTgCCCCCTAYATgCT ggWWgTTTTCCCACTCRTTTC	ACCACAggYTggAgAgggC-FL LC640-gCCAgCCgMgCCAACACyGT-PH
BMP4 60°C, 2 mM	CTTgTTTTCTgTCAAgACACCATgATT gCAgAAgTgTgCgCCTCgAAg	CCCgTCTCAggTATCAAACAgCATggCTC-FL LC640-CgCCTCCTAgCAggACTTggCA-PH
Cbln1 58°C, 2 mM	CAAgTgCCTggTggTgT gTTCACAgTACCTggTgCgAAgTAg	gCgATTACTCATCTCggACggCTC-FL LC640-TggTTggTgCTCCTgATggCag-PH
CNTF 58°C, 2 mM	gACAgTTgAKTTARgggATgg TgATgYTTYACATAgATTCRTAAg	AgAggTCCCgRCggTgARgg-FL LC640-TCAgMggTgWWTgCTCTgYgAAAgCC-PH
CNTFR 56°C, 2.5 mM	CCTgYTTCCACAgTgACTC gCTgggTCCTTCTCACAgAC	CgCCKCgggARCCyTgTCTCA-FL LC640-CTgCCgYTCCAACACTTACCCCAAggg-PH
Fas 60°C, 2 mM	CCCggACCCAgAATACC CTTgCATTTggTgTgCTg	TgCTTCTCTCTgTgACCACTgTTATCAC-FL LC640-gCACCTCgTgTggACTTgAagACA-PH
GAL 56°C, 2.5 mM	gAggCTggACCCTgAACA AgggCMCCggCCTCTTT	ATgCCRTTgRCAACCACAgRTCATTYAgCg-FL LC640-CAAgMATggCCTCACMRgCAAgMgggAg-PH
GALR1 54°C, 2 mM	gCACCACCAACCTgTTCAT AggTgggCagYgCgTAC	ACCTggCCTACCTgCTCTTCTgC-FL LC640-TCCCYTTCAAggCCACCG-PH
GALR2 62°C, 3 mM	gTCTCCCTggACAggTATC ggTAgCTRAAgACgAAggTg	ggCMgCCATCgggCTCATCTgg-FL LC640-ggCTRKCRCTgCTCTTCTCCgggCC-PH
GDNF 56°C, 3 mM	CCAgAgAATTCCAgAggRAAAg TgCAAACATgCCTgSCCTAC	TAgCCARACCCAAgTCAgTgACA-FL LC640-AARTgTATTgAgTAAgACRCAMCCCCg-PH
HCRT 56°C, 2.5 mM	CCTTCYACAAAggTYCCTggg gCKCCgTgCARCagYTCgT	CagCCYCTgCCCgACTgCTg-FL LC640-CgYCARAAgACKTgYTCYgCCgYCTCTACg-PH
HCRT1 62°C, 3 mM	TgggCAACACKCTggTCTg CCTgKAgATAgggATgACCTg	gCCAgggACAggTTgACAATgAA-FL LC640-TAgTTggTgACTgTCCTCATgTggTggT-PH
HCRT2 56°C, 2.5 mM	CCSTTTTAAACCCACMgACT gCCACRCARACCAgAgCgTT	TgCggTACCTgTggAgggAATACC-FL LC640-gCACCCgAAAgAATAgAgTgggTCCT-PH
Hmbs 54°C, 3 mM	gATgAAggATgggCAACTgTA CAgTgATTCCAACCACTgTg	ggCCACCATCCAggTCCCT-FL LC640-TTCAgCAAgAagATggTCCAgAggATgA-PH
NGF 61°C, 2 mM	TTTggCCWgTggTgTgTg gTCAAgggAATgCTgAAGTTTAgT	CTgAggTgCATAgCgTAATgTCCATgT-FL LC640-gTTCTACACTCTgATCACAgCKTTTYgATCg-PH
NPY 56°C, 2.5 mM	CCAgCCCWgAgACACTgATT gARATRTgggSKgAAAMTAggAAAAg	CCCgAACWMggCTTgAagACCCTKCMATg-FL LC640-ggTgATgggAAATgARACTTgCTCTCYKgMC-PH
Nurr1 61°C, 2 mM	AAAggYTTCTTTAAgCgCAC gAgggYARACgACCTCTCCg	CTgYCGRTTTCagAagTgCCTggC-FL LC640-gTTgggATggTYAAAgAagTggTTCgCA-PH
PITX3 63°C, 2 mM	gggSCAggAgCACAgYgACTC CgTgCTCATgTgCggggTA	CgCACTTCACCAgCCAgCAgC-FL LC640-CAggAgCTRgAggCSACCTTCCAgAggAA-PH
Sirt1 54°C, 2 mM	ATgACACTgTggCagATTgT AAggCgAgCATAAAATACCAT	TgCTgTgAARTTACTRCAAgAgTgCAAAA-FL LC640-ATAgTTCTRACTggAgCTgggTKTCTgT-PH
Th 54°C, 3 mM	CCCgTTCTCCCAgACAT gCTggATACgAgAggCATAgT	AgCCTTTgACCCAgACACAgCA-FL LC640-CTgTgCagCCCTACCAAgATCAAACC-PH
TNF 56°C, 2.5 mM	CAYgTYgTAgCAAACCMYCAAg gCTgAYggTgTgggTgA	CTKARAgAYAACCARCTggTggTRCCAKCA-FL LC640-AKggSCTgTACCTYATCTACTCCAggT-PH

anti-GDNF, anti-NGF, and anti-TH (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA, all diluted 1:400) were applied. After incubation with the primary antibody overnight at 4°C, blots were rinsed three times with 0.05 M Tris, 0.9% NaCl containing 0.05% Tween-20 (T-TBS) and 0.05 M Tris, 0.9% NaCl (TBS) for 5 min at room temperature and incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibody directed against rabbit-IgG (Chemicon, Temecula, CA, USA) and diluted in PBS at 1:5,000. After washing the blots with T-TBS and TBS, blots were incubated with the substrate from the detection kit for 5 min. Chemiluminescence signals were detected and analyzed using the LAS 3000 imaging system and Aida image analysis software, version 4.1 (Raytest, Straubenhardt, Germany). Afterwards, the blots were stripped by washing for 5 min in water, then 0.2 M NaOH, then water. The blots were re-probed with horseradish peroxidase-conjugated anti- β -actin antibody (1:10,000; Sigma-Aldrich, Taufkirchen, Germany). After washing the blots with T-TBS and TBS, blots were incubated with the substrate of the LumiLight^{PLUS} kit from Roche to produce a chemiluminescence signal.

The two-way ANOVA from GraphPad Prism software (GraphPad Prism software, San Diego, CA, USA) was used to analyze the differences.

Statistics

The statistical significance of all biochemical differences was estimated using Student's *t*-test. Stereological analysis was carried out using repeated measures

ANOVA followed by LSD *post-hoc* tests with Statistica 8.0 software (StatSoft, Inc., Tulsa, OK, USA). Array analyses were performed using the Excel spreadsheet provided on the Super Array website. The two-tailed *t*-test on the Excel program was employed to analyze the data from single RT-PCR.

Results

DA in the left and right striata

We found in pilot experiments that the dose used by Yazdani et al. [89] was not high enough to lower the DA levels to approximately 50% in our rats. To investigate the restorative potential of a particular compound, the neurons studied had to have been damaged by the neurotoxin but not destroyed. This condition may be fulfilled if the level of the index substance, that is DA in the striatum, is lowered by approximately 50% in the left striatum, i.e., the side of the infusion. As shown in Figure 1, delivery of 0.284 mg/kg/day MPP⁺ for 28 days followed by delivery of saline for 14 days caused an approximately 50% reduction in DA levels in the left striatum ($p < 0.001$ compared with both the sham-operated rats and with the right side), while the levels in the right striatum were in the range of sham-operated rats (5.1 ± 0.8 ng/mg, $n = 6$). Presented are the means \pm SD with values plotted separately for the left and right striata. A second group of rats was treated with 0.284 mg/kg/day MPP⁺ for 28 days and subsequently 0.105 mg/kg/day 9-me-

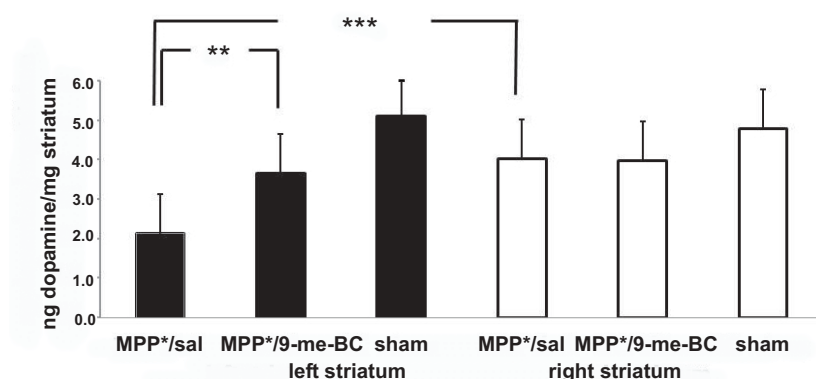


Fig. 1. Concentrations of dopamine (DA) in the left (black columns) and right (light columns) striata of rats. 1-Methyl-4-phenyl-pyridinium ion (MPP⁺; 0.284 mg/kg/day) had been delivered continuously for 28 days, followed by saline (sal) and 9-methyl- β -carboline (9-me-BC; 0.105 mg/kg/day), respectively, for 14 days into the cranial part of the left ventricle of the rat brain by means of an osmotic mini-pump. DA levels were determined at the end of the 42-day period. The concentration of DA in the left striatum of MPP⁺+ sal-treated rats was different from that in the left striatum from MPP⁺+ 9-me-BC ($p < 0.01$) and from sham-operated control rats ($p < 0.001$) and from the right side of the MPP⁺+ sal-treated rats ($p < 0.001$). The values are the means \pm SD. Each treatment group consisted of 6 rats. Not all significant differences are plotted in the drawing

BC for 14 days. The levels on the left side normalized in rats that underwent the combined treatment (Fig. 1).

TH immunohistochemistry in the substantia nigra and stereology

Histological examination and stereological counting of immunoreactive (THir) cells were performed in the SNc and in the VTA. The number of THir cells decreased by 15.4% in the left SNc as compared with the right side in rats infused with MPP⁺ and saline (left $6,736 \pm 238$ vs. right $7,985 \pm 464$ cells, $p < 0.05$, $n = 8$; Fig. 2). Delivery of 9-me-BC instead of saline normalized the number of THir-positive cells (left $7,480 \pm 478$ vs. right $8,199 \pm 498$ cells, $n = 8$).

The density of THir-positive cells as assessed by the number of THir-cells per mm³ decreased by 12.3% in the left SNc of rats treated with MPP⁺ and saline (left $5,182 \pm 257$ vs. right $5,912 \pm 249$ cells/mm³, $p = 0.05$) but not in those treated with MPP⁺ and 9-me-BC (left $5,699 \pm 333$ vs. right $5,891 \pm 376$ cells/mm³, Fig. 2).

The VTA was less affected than the SNc. The density of THir-cells increased by 9% in the left VTA after combined treatment (left $5,337 \pm 293$ vs. right $4,854 \pm 163$ cells/mm³, $p < 0.05$, Fig. 3).

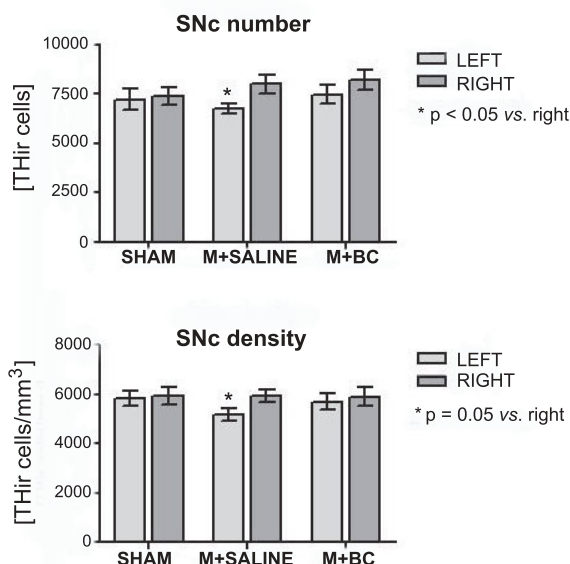


Fig. 2. Stereological analysis of the number and density of tyrosine hydroxylase immunoreactive (THir) neurons in the substantia nigra pars compacta (SNc). The numbers of animals were: SHAM $n = 5$, MPP⁺ + saline (M+Saline) $n = 7$, MPP⁺ + 9-me-BC (M+BC) $n = 7$. Results are shown as the mean \pm SEM and statistical significance is marked when $p \leq 0.05$.

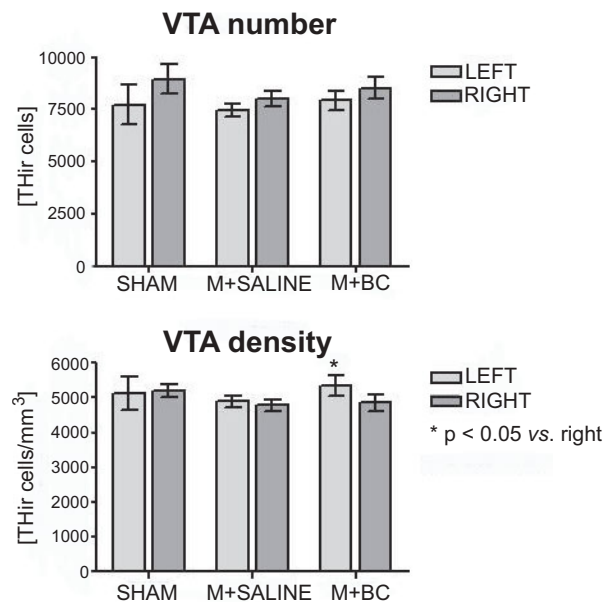


Fig. 3. Stereological analysis of the number and density of the tyrosine hydroxylase-immunoreactive (THir) neurons of the ventral tegmental area (VTA). The numbers of animals were: SHAM $n = 5$, MPP⁺ + saline (M + Saline) $n = 7$, MPP⁺ + 9-me-BC (M + BC) $n = 7$. Results are shown as the mean \pm SEM and statistical significance is marked when $p \leq 0.05$.

Analysis of respiratory chain complexes including complex V in striatal mitochondria

The rat model employed produces selective, progressive loss of nigrostriatal dopaminergic cells through perturbation of mitochondrial function [89]. Considering that the dose of MPP⁺ used in our experiments caused a 50% reduction in DA levels, our model should correspond to an early stage in PD. Thus, we were interested in exploring possible deficits of mitochondrial function, which would allow us to evaluate the key mechanism of the neurotoxic process. Therefore, we studied mitochondria isolated from striatal tissue. The mitochondrial proteome was investigated with emphasis on the composition, abundance, structure, and activity of membrane proteins and their modulation by consecutive treatment with MPP⁺ and saline and with MPP⁺ and 9-me-BC, respectively. In particular, we addressed the question of the occurrence, architecture, and function of oxidative phosphorylation (OXPHOS) in mitochondrial complexes as well as supercomplexes, i.e., the natural assembly

of respiratory complexes I, III, and IV into supercomplexes, as well as ATP-synthase (complex V) oligomers [13]. By application of BN-PAGE, separating proteins in their native, active state and preserving all functional relevant protein-protein interactions, we were able to identify distinct protein bands containing either the individual complexes or the supercomplexes with a defined complex stoichiometry (Fig. 4). Presented are the findings from mitochondria of the left and right striata from single rats, either sham-operated twice, treated with MPP⁺ and saline, or treated with MPP⁺ and 9-me-BC.

Inspection of Figure 4 reveals that the composition and abundance of the building blocks of the respiratory chain were comparable in all 6 samples. There

were no pronounced differences in the relative proportion of specific supercomplexes and individual complexes or in the monomeric versus oligomeric (dimers, trimers, tetramers) arrangement of the proton-ATP synthase. This is in line with a previous study that analyzed the substantiae nigrae, tegmenta, and cerebella of PD patients [70]. Moreover, the abundance of mitochondrial HSP60, a stress response protein that acts as an important chaperone, was not affected by the various treatments.

The activity of complex I as an individual complex and in supercomplexes was similar in all striata except in the striatum treated with 9-me-BC (Fig. 5A). The activity in the left striatum from the rat treated with MPP⁺ and 9-me-BC was approximately 80% higher than that from the sham-operated rat and 75% higher than in the rat treated with MPP⁺ and saline. In this respect it is worth mentioning that about 90% of all complex I is found in various supercomplexes; only the remaining 10% are present as individual complexes. Therefore, we measured the complex I activity of the different supercomplexes. The specific

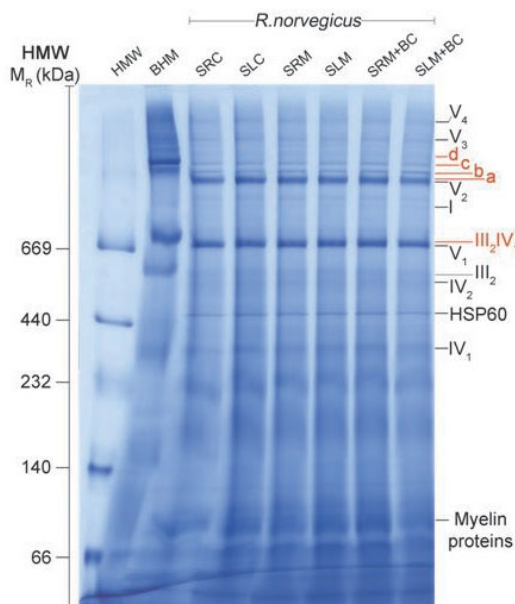


Fig. 4. Analysis of the abundance and the supramolecular organization of mitochondrial proteins prepared from rat striatum. Rats were treated with MPP⁺ (28 days) and then saline and 9-me-BC, respectively (14 days), delivered into the cranial section of the left ventricle. BN-PAGE (linear 4–13% gradient gel with a 3.5% stacking gel, stained with Rotiblock; Roth, Karlsruhe, Germany) of digitonin-solubilized rat mitochondria. For mass calibration, high molecular weight (HMW) standard and digitonin-solubilized bovine heart mitochondria (BHM) were used: individual complexes I–IV (130–1,000 kDa) and supercomplexes a–d (I1III2IV0–3, 1,500–2,100 kDa). The membranes were solubilized with 8 g digitonin/g protein at a final detergent concentration of 1%. The characteristic bands of the individual OXPHOS complexes I, III₂, IV and V and their preserved supercomplexes are recognizable. In addition, heat shock protein HSP60 and myelin proteins [Myelin basic protein isoform 5, Lipophilin (proteolipid protein)] are indicated. Abbreviations: striatum left sham-operated control (SLC); striatum right, sham-operated control (SRC); striatum left, MPP⁺ and subsequently saline (SLM); striatum right, MPP⁺ and subsequently saline (SRM); striatum left, MPP⁺ and subsequently 9-me-BC (SLM+BC); striatum right, MPP⁺ and subsequently 9-me-BC (SRM+BC)

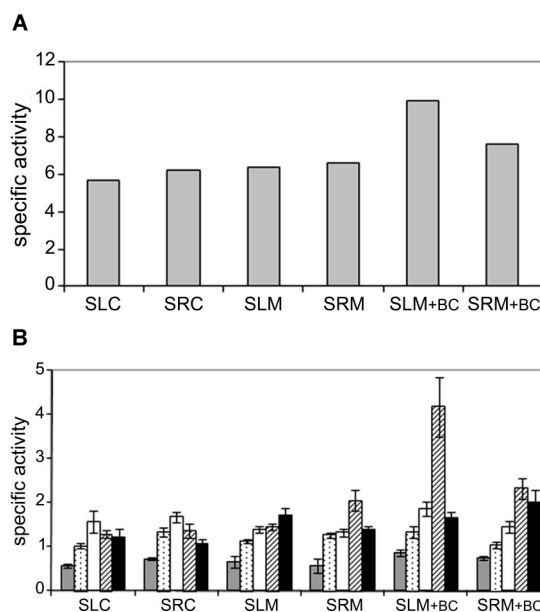


Fig. 5. Specific NADH dehydrogenase activity (in relative units) of complex I in striatal mitochondria. **(A)** Enzymatic activity of all protein bands containing complex I, i.e., individual complex I solely or the various supercomplexes composed of complex I and the dimer of complex III with 0–3 copies of complex IV, were probed by in-gel formazan precipitation. About 90% of all complex I is assembled in the various supercomplex species. **(B)** NADH dehydrogenase activity of individual complex I (gray), supercomplex I₁III₂ (dotted), I₁III₂IV₁ (open column), I₁III₂IV₂ (hatched), and I₁III₂IV₃ (black). Abbreviations: see legend to Figure 4

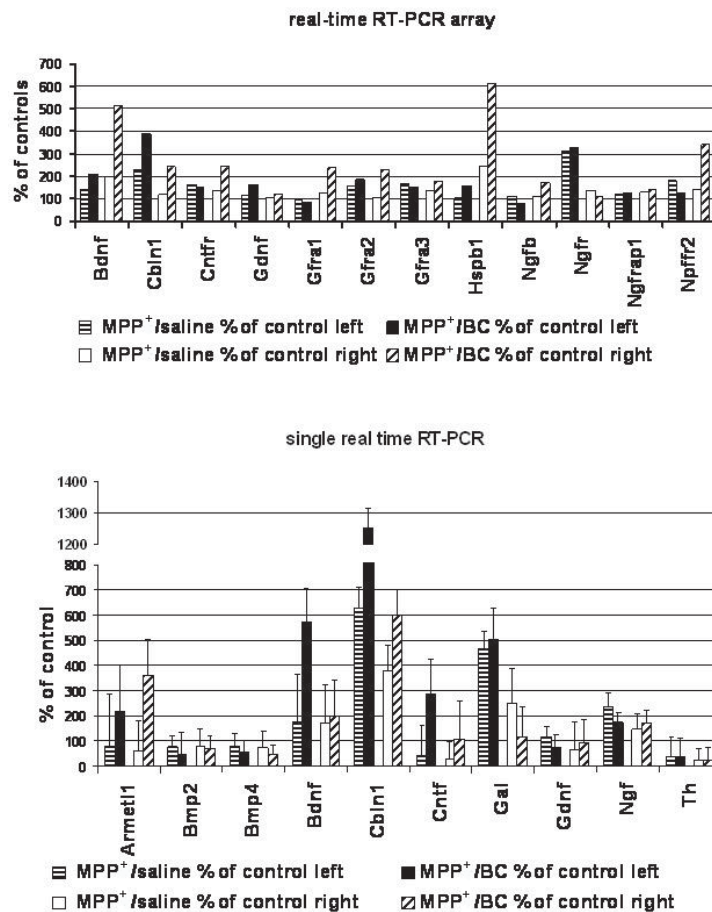


Fig. 6. Selected results of the array analysis (upper part), and results of single real time RT-PCR (lower part). Fold difference in transcript amount for the respective genes. The analyses were conducted separately in isolates of the left and the right striata from rats treated with either MPP⁺ and then saline or MPP⁺ and then 9-me-BC delivered into the cranial part of the left cerebral ventricle. The findings were compared with those of sham-operated controls (each group, *n* = 4). Abbreviations: see abbreviation list text. The values are the means from 4 animals

activity of supercomplex I₁III₂IV₂ was approximately three times higher in the left striatum of the rat with the combined treatment as compared to both the sham-operated rat and the rat treated with MPP⁺ and saline (Fig. 5B).

The amount of mitochondrial HSP60 was determined as a control protein. The heat shock protein provides differential protection against intracellular dysfunction and cell death by maintaining mitochondrial oxidative phosphorylation [84]. Levels in the striata did not differ between the various treatment conditions. It is noteworthy that levels in striata from the MPP⁺ + vehicle-treated rats did not differ.

Effects of the various treatment regimes on gene transcription in the left and right striata

Based on our findings of antiproliferative and differentiation-inducing actions of 9-me-BC in primary culture and in human neuroblastoma SH-SY5Y cells [22], we hypothesized that possible neuron-rescuing

actions of 9-me-BC result from stimulation of neurotrophin gene expression and improvement of energy homeostasis in mitochondria. Therefore, we investigated the impact of 9-me-BC on the transcription of neurotrophins, their receptors, and some factors involved in the differentiation of DA neurons.

The analysis was conducted separately in homogenates of the left and right striata from rats treated with either MPP⁺ and then saline or MPP⁺ and then 9-me-BC delivered into the cranial part of the left cerebral ventricle. These samples were compared with those obtained from sham-operated controls (each group *n* = 4). The homogenate was the same as used to measure DA levels.

First, we conducted real time RT-PCR using the RT Profiler PCR Array for rat neurotrophins and receptors supplied by Super Array. The array profiles the expression of 84 genes involved in the normal functions of neurons including neuronal cell growth, differentiation, regeneration and survival (for the whole list see: www.sabiosciences.com). Selected results are shown in Figure 6 (upper part).

In order to confirm and extend the findings obtained with the array, an additional gene transcription analysis was conducted by applying single quantitative real time RT-PCR. Additional genes reported to be important for differentiation and survival of dopaminergic neurons were included in this investigation (for details see below). Since we used FRET probes to quantify the PCR products, the results obtained with single RT-PCR were more specific. The animals were the same as in the array analysis. Results were normalized to the housekeeping gene hydroxymethylbilane synthase (Hmbs). Again, only expression differences ≥ 1.5 -fold compared to control rats are presented in Figure 6 (lower part). The following transcripts were analyzed but omitted from Figure 6 because only minor changes were found: Bone morphogenetic protein 2 (BMP2), BMP4, Ciliary neurotrophic factor receptor (CNTFR), Fas (death receptor), galanin receptor 1 (GalR1), Hypocretin (orexin) (Hcrt), Hcrt1, Hcrt2, neuropeptide Y (NPY), nuclear receptor regulated 1 protein (Nurr1), paired homeodomain transcription factor 3 (Ptx3), Sirtuin (Sirt), tumor necrosis factor (TNF).

Recently, a conserved DA neurotrophic factor (CDNF, also denoted arginine-rich, mutated in early stage tumors-like 1, Armet1 1) has been reported. A single injection of CDFN before the delivery of the neurotoxin 6-hydroxydopamine into the striatum almost completely rescued dopaminergic Th-positive cells [37]. We found a minor reduction in levels of the Armet1 1 transcript after MPP⁺ and saline treatment. The amount was doubled by 9-me-BC in the left striatum and the combination induced a greater than three-fold increase in the right striatum (it should be mentioned that Armet1-1 was not provided with the array).

The amount of BDNF mRNA was almost doubled after MPP⁺ administration in both the left and right striata; 9-me-BC induced a six-fold (left) and two-fold (right) increase, respectively. The array analysis yielded differing results, which will be discussed later.

The strongest effects were observed for cerebellin 1 precursor protein (Cbln1), which was concordant with both methods, though only qualitatively. MPP⁺ induced a roughly three-fold increase in the left striatum and a six-fold increase in the right striatum. The combination caused a twelve-fold increase in the left striatum and a six-fold increase in the right striatum. The values from single RT-PCR were consistently higher than those determined with the array method.

There were differences between both methods in the amount of up-regulation observed for BDNF and Cbln gene transcripts. The specificity of both methods depends on the specificity of primers and optimal conditions for primer annealing, leading to amplification of the desired cDNA fragment. If the primers or conditions are not specific enough, additional amplifications may be generated. The array works with the DNA-specific dye Sybrgreen, which cannot distinguish between different fragments. Only agarose gel electrophoresis can uncover possible artifacts. Notably, for BDNF as well as Cbln, two bands were visible after gel electrophoresis, with the amount varying from sample to sample. For single RT-PCR we used FRET-probes, which bind to specific sequences on the amplified products, thus excluding potentially incorrect amplification products from detection. Thus we consider the results from single RT-PCR for these two genes as more representative than those obtained with array analysis.

Single RT-PCR revealed that ciliary neurotrophic factor (CNTF) decreased in both sides after MPP⁺. This effect was reversed in the right striatum. In the left striatum, 9-me-BC induced a three-fold increase. The array revealed that levels of the specific receptor CNTFR α were slightly elevated in the right striatum.

Galanin transcript levels (not provided by the array) were affected by MPP⁺, which caused a five-fold increase in the left striatum. This change was unaffected by 9-me-BC and an almost three-fold increase in the right striatum after MPP⁺ which was completely reversed by 9-me-BC.

The glial cell line-derived neurotrophic factor (GDNF) was not affected with either method. The amount of nerve growth factor ([NGF], beta subunit, which is solely responsible for nerve growth-stimulating activity of NGF) was nearly doubled in the right striatum after the combined treatment in the array analysis. After single RT-PCR, NGF levels were roughly doubled in all homogenates.

We also investigated the human homologue of silent information regulator (Sirt), an NAD⁺-dependent protein deacetylase that is involved in the regulation of energy homeostasis in mitochondria [16]. The levels of Sirt were not affected by the treatments.

The transcription of other genes was apparently not affected, e.g. nuclear receptor subfamily 4, group A, member 2 (Nurr1), Ptx3, BMP2 and BMP4, which were increased in primary cultures of cells derived from embryonic mice exposed to 9-me-BC [22].

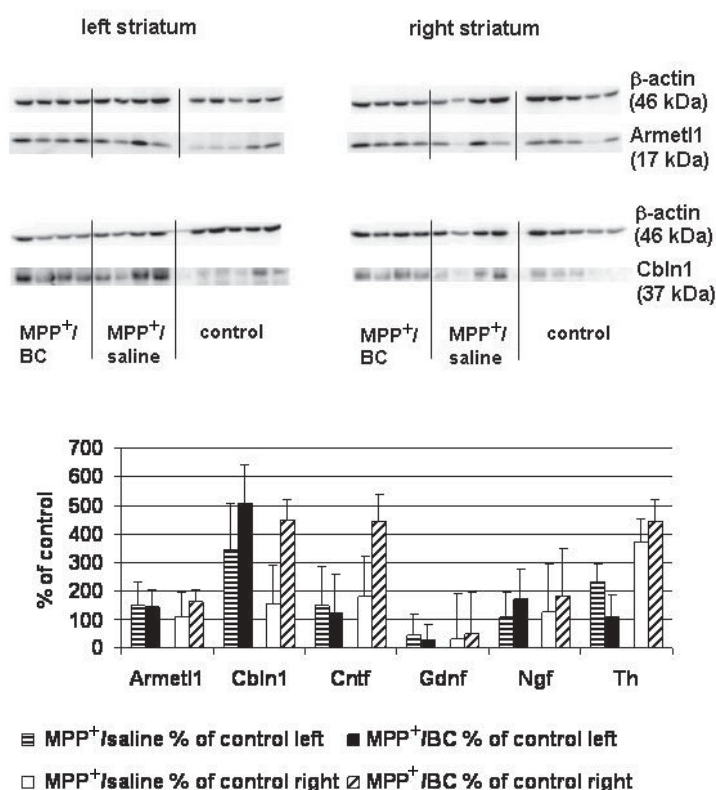


Fig. 7. Western blot analyses. The upper part shows representative western blots for Armet1 and Cbln1 and the respective β -actin counterstaining. Each group is represented by at least four independent animals. The lower part shows the mean and standard deviation of the investigated proteins normalized to β -actin and related to the control group for each treatment group for the left and right side of the striatum

The array comprised further transcripts in addition to those identified by single RT-PCR. Corticotropin-releasing hormone (CRH) and CRH-binding protein (CRHBP) were nearly doubled in all homogenates; 9-me-BC seemed to enhance this effect. CRH receptors 1 and 2 were unchanged. Levels of nerve growth factor receptor (TNFR superfamily, member 16) were upregulated three-fold by MPP⁺ and not affected by 9-me-BC. Among the other factors investigated, signal transducers and transcription activators (Stat) 1 to 4 were differentially affected. The strongest changes were observed for Stat 4, levels of which were doubled by MPP⁺ and increased three-fold by MPP⁺ + 9-me-BC.

Western blot analysis

Western blots were prepared for selected proteins (Fig. 7). Armet1 was slightly increased by MPP⁺ and by 9-me-BC compared with sham-operated rats (100%). Changes in the levels of Cbln1 and CNTF correlated with the results of single RT-PCR with the exception of the left striatum and CNTF. It is noteworthy that 9-me-BC induced a four- to five-fold increase. The levels of GDNF were unchanged, which

is consistent with findings at the mRNA level. NGF was slightly increased by 9-me-BC. Interestingly, tyrosine hydroxylase levels were increased by MPP⁺ in the left striatum and by both treatment regimes in the right striatum. These changes contrast with the results of the single RT-PCR experiments.

Discussion

The main findings of this study are the observations that 9-me-BC has restorative effects in an animal model of Parkinson's disease. This has never been reported before and has been reproduced recently by experiments with primary dopaminergic neurons from embryonic mice [63]. In search of an explanation of the underlying mechanisms we found that 9-me-BC improved the effectiveness of the respiratory chain and promoted the gene transcription of neurotrophins.

A single injection of MPP⁺ into the rat striatum induced retrograde damage to dopaminergic neurons in the SN, along with extensive oxidative stress and microglia activation [48]. However, only continuous ad-

ministration of the neurotoxin produced progressive behavioral changes and triggered the formation of neuronal inclusions characteristic of a Parkinsonian syndrome [18]. Therefore, we infused MPP⁺ continuously into the anterior part of the left cerebral ventricles of rats over a four-week period. Yazdani et al. [89] observed a selective reduction in striatal DA, without affecting 5-HT, GABA or glutamate at the dose applied. Therefore, the findings differing from those in sham-operated control rats should be attributed to changes in dopaminergic neurons. We found a significant reduction of THir cells in the ipsilateral SNc, which was not observed in the VTA. However, the reduction was much less than that observed by Yazdani et al., who found a 65% reduction in the number of THir SNc neurons. We know from pilot experiments that our rats were less sensitive to MPP⁺ than the rats used by Yazdani et al. Therefore, we had to administer twice as much MPP⁺ to reduce the levels of DA in the striatum by approximately 50%. Another explanation may be that the anatomy differed and that we infused the toxin more rostrally than did the other group. Furthermore, we investigated Wistar rats whereas Yazdani et al. used Sprague-Dawley rats. There are profound differences among strains of rats in response to MPP⁺. Wistar rats were protected from MPP⁺ neurotoxicity by coadministration of N-methyl-D-aspartate antagonists, a phenomenon which has not been confirmed for Sprague-Dawley rats [77, 82].

In rats treated with MPP⁺ and subsequently 9-me-BC, the number of THir-cells normalized in the SNc. These findings indicate that 9-me-BC rescues damaged dopaminergic neurons and that dopaminergic neurons in the VTA are less sensitive to MPP⁺ than those in the SNc [51, 73].

There are no *in vivo* studies reporting effects of 9-me-BC in drug naive animals. It should be mentioned that we conducted behavior experiments using the chimney test and the tilted plane. We did not observe differences between the three groups of rats, with regard to broad behavior, locomotion, behavior in the chimney test or the tilted plane. This is not unexpected because drug-naive patients with Parkinson's disease in Hoehn and Yahr stages I and II exhibited a 65% reduction in dopaminergic neurons in the putamen. The patients were examined by imaging methods using [¹²³I]β-CIT SPECT, which labels dopamine transporters *in vivo* [81]. Therefore at an early stage of the disease when more than 50% of dopaminergic neurons are destroyed, the first motor changes can be

observed. We did not measure the expression of DA transporters in our rats. It should be considered that DA levels of 50% do not necessarily imply complete disappearance of half of the dopaminergic neurons.

Recently, the restoring properties of 9-me-BC were confirmed in an *in vitro* model of PD. Primary dopaminergic neurons from mesencephalon of embryonic mice were exposed to the neurotoxin rotenone (1 nM) for 6 days. This caused a 40% reduction in THir neurons. After withdrawal for another 8 days, the number of THir neurons decreased further (by ~50%). Exposure of the neurons to 9-me-BC (50 μM) during the withdrawal period normalized the number of THir neurons [63]. These findings are consistent with the results reported in the present study.

MPP⁺ produces a loss of nigrostriatal dopaminergic cells through perturbation of mitochondrial function induced by the inhibition of complex I [53]. Therefore, the degree of perturbation might be reflected in changes in the amount of complex expressed, the abundance of supercomplexes, and activity level. However, the polypeptide patterns of SN from control individuals and patients with PD did not differ [24, 71, 72]. Others reported that in mitochondria from frontal cortex of PD patients the 8 kDa subunit of complex I was decreased by 34% and the proteins comprising the catalytically active core of complex I were oxidatively damaged [30]. In our study, the complexes and the supercomplexes could already be detected in the first-dimensional native gel (Fig. 4). This determination was performed by analysis of the subunit pattern of denatured complexes and supercomplexes in the second dimension. We did not find changes of the abundance of the complexes in the striatum of the rats treated with MPP⁺ (Fig. 4, quantification not shown). The amount and composition of supercomplexes were not changed either, a finding not investigated by others. Reduced catalytic activity of complex I in frontal cortex and SN, respectively, was reported for PD patients [30, 72] which was not confirmed by others [70]. We did not observe changes in the catalytic activity of isolated complex I and of supercomplexes in striatal homogenate from rats treated with MPP⁺. The in-gel measurement of nicotinamide adenine dinucleotide dehydrogenase (NADH) activity suggested that 9-me-BC stimulated the enzyme activity of complex I in rats pre-treated with MPP⁺ (+80%; Fig. 5A). This increase was mainly caused by a specific supercomplex (I₁III₂IV₂), which was approximately three times more active (Fig. 5B).

The abundance of complex IV did not vary among the treatment groups (data not shown). With respect to the pronounced and specific enhancement of the NADH activity of supercomplex I₁III₂IV₂ by 9-me-BC, it is remarkable that in the presence of complex IV (I₁III₂IV₁) the activity of complex I was nearly 2.5-fold higher than in its absence (I₁III₂) [69]. It is tempting to speculate that 9-me-BC specifically interacts with the dimer of IV in I₁III₂IV₂.

There is evidence that neurodegeneration is linked to a lack of trophic support in brain areas associated with PD [86]. Thus far, results of clinical trials using neurotrophins in PD have been disappointing [31, 35]. One option to attenuate neurodegeneration would be administering drugs that selectively modulate and enhance endogenous neurotrophin expression. Therefore, we investigated whether the restorative effects of 9-me-BC can be explained by activation of the transcription of neurotrophic factors known to affect dopaminergic neurons. The CDNF also denoted Armet11; [61] was recently described as a trophic factor for dopaminergic neurons [37]. We found reduced levels of Armet11 in MPP⁺-treated rats; this effect was suspended by treatment with 9-me-BC. In contrast, an almost four-fold increase was observed in the contralateral striatum after 9-me-BC administration. Another neurotrophic factor utilized by dopaminergic neurons is GDNF. GDNF has been shown to exert neurotrophic effects at the level of the cell bodies in the SNc and of the axon terminals in the striatum [4]. Others demonstrated that GDNF expression in the mouse striatum prevents the MPTP-induced loss of dopaminergic neurons [8]. We found only minor changes in GDNF levels in striatal tissue induced by MPP⁺ and 9-me-BC (single RT-PCR), whereas the transcription of GDNF receptor α and β was elevated by 9-me-BC (array technology).

Next, we investigated neurotrophic factors that exert less specific actions. BDNF is the most prevalent growth factor in the brain and regulates diverse aspects of neuronal function. We found an increase in both the ipsilateral and the contralateral striatum after MPP⁺ treatment. In the ipsilateral striatum, delivery of 9-me-BC in MPP⁺-pretreated rats induced a six-fold stronger effect than in controls; this effect was half as large in the contralateral striatum. This is an interesting finding because BDNF is important for the peri-wound sprouting response associated with striatal injury and prevents nigrostriatal degeneration induced by glycoprotein 120 [2, 55]. The strongest ef-

fect of both MPP⁺ and 9-me-BC concerned the Cbln1. The levels of Cbln1 mRNA closely parallel synapse formation between granule cells and Purkinje cells [76, 83]. Immunohistochemical studies in adult mice revealed Cbln1-like immunoreactivity in other brain regions in addition to the cerebellum [85]. Cbln1-null mice failed to eliminate supernumerary climbing fibers to yield a one-to-one relationship with Purkinje cells [26]. It is tempting to speculate that Cbln1 regulates the precise formation of new synaptic connections during the restitution of damaged neurons in the striatum. This could explain the high gene expression after MPP⁺ and MPP⁺ + 9-me-BC.

Unilateral dopaminergic denervation of the striatum by 6-hydroxydopamine reduces CNTF mRNA expression in adult mice [88], which is consistent with our findings of reduced CNTF mRNA in both the ipsilateral and contralateral striatum after MPP⁺ treatment. The dopaminergic pathway normally promotes CNTF expression by astrocytes of the striatum, a process mediated by D2 receptors [88]. Furthermore, CNTF promotes neuronal survival [29, 54] and stimulates neurite growth and axon regeneration in the developing and mature nervous system in several *in vitro* and *in vivo* experimental paradigms [12, 23, 74]. Therefore, the present findings of increased CNTF expression in MPP⁺ + 9-me-BC-treated rats are consistent with the observed restitution of the DA deficit in the striatum.

Levels of the neuropeptide galanin are markedly increased in the central and peripheral nervous system following injury. Galanin plays a survival role in hippocampal models of apoptosis and excitotoxicity, mediated by galanin-receptor 2 (GalR2); [27]). GalR2 plays a key role in neurite outgrowth from adult sensory neurons [40] and protects the hippocampus from neuronal damage [1, 15]. We observed strongly increased galanin gene expression in striata from rats treated with MPP⁺ in single RT-PCR experiments without modulation by 9-me-BC, which is consistent with an injury-associated rise in galanin levels. On the other hand, 9-me-BC induced an increase in receptor levels of both GalR1 and GalR2, possibly contributing to the repair of damaged dopaminergic neurons.

The NAD⁺-dependent deacetylase Sirt1 was originally described as a factor regulating longevity, apoptosis, and DNA repair [5, 75]. Sirt1 expression was stimulated by MPP⁺ in neuroblastoma B65 cells. No significant changes were observed in samples from human brain from PD (phase IV) patients or patients

with dementia with Lewy bodies [58]. Sirt1 activates PPAR γ coactivator-1 α (PGC-1 α) which coactivates the expression of many subunits of the respiratory chain [68]. Moreover, PGC- α positively regulates the expression of several ROS-detoxifying enzymes, such as the superoxide dismutase SOD2 and glutathione peroxidase GPx1 [78]. Increased Sirt1 gene expression in striata from both sides of rats treated with MPP⁺ fits well with these observations. Furthermore, animals treated with the combination had normal expression levels of Sirt1, supporting the notion of a neuroprotective effect of 9-me-BC.

Finally, we want to highlight the unexpected observation of changes in gene expression in the contralateral striatum with respect to the infusion site. Yazdani et al. [89] reported on the appearance of activated microglia cells in both the left and the right SNc and both striata of the rats after chronic infusion of MPP⁺ into the anterior left ventricle. The microglia might activate gene transcription. Furthermore, dopaminergic neurons projecting from the left SNc to the right striatum might compensate for neurotoxic processes in the left striatum. This crossed projection is involved in reciprocal control of the uncrossed nigrostriatal dopaminergic pathway [36, 41]. Unilateral intra-striatal 6-hydroxydopamine injections caused a reduction in the number of DA uptake sites within the contralateral VTA and SNc [3]. Thus, a few dopaminergic neurons cross to the contralateral side [14, 49, 50] and exert compensatory activity in unilaterally lesioned animals [14, 49, 90].

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