The management and search for mechanism-based therapies of neurodegenerative disorders such as Alzheimer’s or Parkinson’s disease (PD) is one of the greatest challenges of modern human societies. As of today PD is an incurable progressive illness, which is usually controlled by various interventions including psychotherapy, surgical treatment options and medication with various dopaminergic and non-dopaminergic drugs (Obeso et al. 2010; Schulz et al. 2011). PD is predominantly characterized by a degeneration of dopaminergic neurons in the substantia nigra caused by the progressive loss of dopamine-containing neurons. Consequently, PD is marked by the development of motor and non-motor symptoms which can severely impinge on patients’ quality of life and burden of caregivers. As of today PD is an incurable progressive illness, which is usually controlled by various interventions including psychotherapy, surgical treatment options and medication with various dopaminergic and non-dopaminergic drugs (Obeso et al. 2010; Schulz et al. 2011). PD is predominantly characterized by a degeneration of dopaminergic neurons in the substantia nigra caused by the progressive loss of dopamine-containing neurons. Consequently, PD is marked by the development of motor and non-motor symptoms which can severely impinge on patients’ quality of life and burden of caregivers.

9-Methyl-β-carboline-induced cognitive enhancement is associated with elevated hippocampal dopamine levels and dendritic and synaptic proliferation

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Abstract

β-Carbolines (BCs) belong to the heterogenous family of carbolines, which have been found exogenously, that is, in various fruits, meats, tobacco smoke, alcohol and coffee, but also endogenously, that is, blood, brain and CSF. These exogenous and endogenous BCs and some of their metabolites can exert neurotoxic effects, however, an unexpected stimulatory effect of 9-methyl-β-carboline (9-me-BC) on dopaminergic neurons in primary mesencephalic cultures was recently discovered. The aim of the present study was to extend our knowledge on the stimulatory effects of 9-me-BC and to test the hypothesis that 9-me-BC may act as a cognitive enhancer. We found that 10 days (but not 5 days) of pharmacological treatment with 9-me-BC (i) improves spatial learning in the radial maze, (ii) elevates dopamine levels in the hippocampal formation, and (iii) results after 10 days of treatment in elongated, more complex dendritic trees and higher spine numbers on granule neurons in the dentate gyrus of 9-me-BC-treated rats. Our results demonstrate that beyond its neuroprotective/neurorestorative and anti-inflammatory effects, 9-me-BC acts as a cognitive enhancer in a hippocampus-dependent task, and that the behavioral effects may be associated with a stimulatory impact on hippocampal dopamine levels and dendritic and synaptic proliferation.

Keywords: dendritic plasticity, HPLC, spatial learning, synaptic plasticity.


Read the Editorial Highlight for this article on page 841.

The management and search for mechanism-based therapies of neurodegenerative disorders such as Alzheimer’s or Parkinson’s disease (PD) is one of the greatest challenges of modern human societies. As of today PD is an incurable progressive illness, which is usually controlled by various interventions including psychotherapy, surgical treatment options and medication with various dopaminergic and non-dopaminergic drugs (Obeso et al. 2010; Schulz et al. 2011). PD is predominantly characterized by a degeneration of dopaminergic neurons in the substantia nigra caused by the progressive loss of dopamine-containing neurons. Consequently, PD is marked by the development of motor and non-motor symptoms which can severely impinge on patients’ quality of life and burden of caregivers.
presumably multi-factorial pathomechanisms. For instance, a dysfunction of the mitochondrial respiratory chain, an overshoot in the production of reactive oxygen species, and an involvement of neuroinflammatory processes including the activation of microglia were shown to be involved in the etiology of PD (Obeso et al. 2010; for a recent review see Shulman et al. 2011). Thus, the search for new, highly specific and effective drugs, which overcome the limitations and side effects of currently used medications, and which have neurostimulatory, neuroprotective/neurorestorative, and anti-inflammatory properties, is of major importance (Meissner et al. 2011).

β-Carbolines (BCs) belong to the heterogenous family of carbolines and have been found in various fruits, fish and beef, in tobacco smoke, alcohol and coffee, and can also be measured in the blood, brain and CSF (for a review see Polanski et al. 2011). Depending on the specific structural characteristics and/or dosage, these exogenous and endogenous BCs and some of their metabolites can exert neurotoxic effects (Neafsey et al. 1995; Collins et al. 1996; Matsubara et al. 1998; Hamann et al. 2006). For instance, the endogenously formed 2-methyl-β-carbolinium and 2,9-dimethyl-β-carbolinium ions are suspected to be involved in the pathogenesis of PD (Hamann et al. 2006; Lorenc-Koci et al. 2006). In contrast, an unexpected stimulatory effect of 9-methyl-β-carboline (9-me-BC) on dopaminergic neurons was recently discovered in cultures of primary mesencephalic neurons (Hamann et al. 2008; Polanski et al. 2010). This finding has now directed the focus of research on the stimulatory and protective action of 9-me-BC on the nigrostriatal dopaminergic system and its potential use as anti-Parkinson drug (see review by Polanski et al. 2011). In this context, one of the most interesting features of 9-me-BC is its uptake via dopamine (DA) transporters into dopaminergic neurons, where in vitro it can act as a neurostimulant by elevating tyrosine-hydroxylase, the rate-limiting enzyme of DA synthesis (Polanski et al. 2011). This, together with the inhibitory function of 9-me-BC on monoamine oxidases A and B (Ho 1972) may result in enhanced DA release in the target regions of dopaminergic fibers (Polanski et al. 2011). Furthermore, 9-me-BC promotes neurite outgrowth of dopaminergic cells in vitro, and there is evidence that this involves elevations of astrocyte-derived neurotrophic factors including the brain-derived neurotrophic factor (Polanski et al. 2011).

9-Methyl-β-carbolinium-induced cognitive enhancement

Materials and methods

Animals

Female Wistar rats (Han:Wist) from the breeding colony at the Friedrich Schiller University Jena, Germany, were used for all experiments. Animals were reared under normal animal facility conditions (temperature: 21 ± 2°C; humidity: 55 ± 5%; artificial 12:12-h light–dark cycle: light on at 06:00 AM) with ad libitum access to food (Altromin 1326®; Lage, Germany) and water. Six offspring were reared with their dam until postnatal day (P) 30. After weaning at P31 rats were housed in groups of four rats in translucent standard laboratory cages type IV (E. Becker & Co. GmbH, Castrop-Rauxel, Germany) until entering the experiments.

The experiments were conducted in accordance with the European Communities Council Directive of November 1986 (86/609/EEC) as well as the German guidelines for the care and the use of animals in laboratory research (§8, Abs.1, 25 May 1998). The ethics committee of the State of Thuringia (02-038/08) approved all experimental protocols which were intended to minimize animal suffering and the quantity of experimental animals.

9-me-BC treatment

9-me-BC was synthesized as described by Hamann et al. (2008). At the age of 7 weeks, rats were randomly assigned to one of the following experimental groups: non-injected animals (ni): remained without any pharmacological treatment until used in the experiments; vehicle-treated animals (veh): treated intraperitoneally (i.p.) once daily with 1 mL saline (Merck, Darmstadt, Germany)/100 g body weight (b.w.) for 10 days; animals treated with 9-me-BC for 5 days (9-me-BC-5d): treated i.p. with 2 pmol 9-me-BC/100 g b.w. dissolved in 1 mL saline/100 g b.w. for 5 days, followed by 5 days of vehicle injection; and animals treated with 9-me-BC for 10 days (9-me-BC-10d): treated i.p. with 2 pmol 9-me-BC/100 g b.w. dissolved in 1 mL saline/100 g b.w. for 10 days. The 9-me-BC dosage used in the present study was chosen because it was shown to decrease the number of working memory errors induced by scopolamine in the radial maze (Gille et al. 2011).

Spatial learning

The eight-arm radial maze (RAM) was used according to Olton and Samuelson (1976). Each arm (44 cm length, 30 cm height, 14 cm width) radiated from an octagonal platform that served as a starting point. A food cup (3 cm diameter) was located at the end of each arm. The entire arms and food cups were painted grey and placed in a dark and quiet room. During the RAM test, the experimenter stayed in the room where the RAM was located, but was not visible to the animal. The rat’s behavior was monitored in real time via computer-coupled camera recording. For quantification, animal behavior was videotaped, image analysis and pattern recognition.
were performed using the Video-Mot 2 software (video tracking, motion analysis and behavior recognition system) provided by TSE Systems (Bad Homburg, Germany). The computerized recording systems were located in the same room as the RAM.

**Adaptation to the RAM**

Adaptation procedure was started when rats were weaned from their dam with a handling period of 10 days, during which the rats were frequently exposed and habituated to the laboratory staff. The following 2 days rats were allowed to freely explore the RAM, where they received a food reward once a day for 10 min. All arms were baited with dustless precision rodent pellets (Bilaney Consultants Ltd, Sevenoaks, Kent, UK). After this adaptation procedure, food was restricted to reduce the rat’s body weight by 10% and then kept constant during the entire experiment.

**Learning trials**

At the age of 7 weeks, the first trial was conducted prior to the first injection (trial 0). The following trials (trials 1–11) were performed 3 h after the application of either vehicle or 9-me-BC. The rats were placed once daily in the centre of the RAM to visit all eight arms and eat all reward food baits. Each trial was performed until the rat has entered all eight arms and eaten all pellets or made 16 errors (re-entry into an arm that has been previously visited). The training was continued until the rats reached the criterion to enter all arms and scoring ≤ 1 error.

**Statistical analyses**

Each group of rats consisted of 15 animals. Arithmetic means ± SEM of the number of errors and total exploration time were calculated. ANOVA for repeated measures was done. Two-tailed Student’s t-test was used to assess significant differences between the control and 9-me-BC-treated groups (p < 0.05).

**Statistical analyses**

As no significant differences were found between left and right hemisphere, data were pooled and the comparison between the vehicle- and 9-me-BC-treated groups was carried out using the Student’s t-test. Significance levels were set to p < 0.05. Values were expressed as mean ± SEM.

**Results**

9-me-BC improves spatial learning

To determine the effect of 5 or 10 days of 9-me-BC treatment on spatial learning in the RAM, the number of errors was analyzed. Rats treated for 10 days with 9-me-BC...
reached the criterion to enter all eight arms with ≤ 1 error (Fig. 1) already after seven training trials. The ANOVA revealed a significant effect of 10 days of 9-me-BC treatment on test acquisition (F3,42 = 4.986, p = 0.032), which became significant in the t-test (p < 0.05) after 7 days of training. There was no difference between the 9-me-BC-5d and vehicle group.

9-me-BC increases hippocampal dopamine

The effect of 9-me-BC treatment on levels of DA and its related metabolites was analyzed in brain homogenates of hippocampal tissue. As there were no significant differences of these analytes between the left and right hippocampus (data not shown), data were collapsed for further statistical analysis. Statistical comparison of the experimental groups revealed a main effect of treatment conditions on DA levels (F3,45 = 3.174, p = 0.033; Fig. 2). Post hoc analysis revealed a significant increase of DA in the 9-me-BC-10d-treated, but not in the 9-me-BC-5d-treated rats compared with ni (p = 0.004) and veh-treated animals (p = 0.039), indicating that 9-me-BC enhances hippocampal DA after 10 days of treatment, i.e. the procedure which improved spatial learning.

The levels of DA metabolites DOPAC (F3,24 = 0.174, p = 0.913), and HVA (F3,45 = 0.447, p = 0.721) as well as the ratios of DO/DA (F3,24 = 0.505, p = 0.682), HVA/DA (F3,45 = 1.771, p = 0.166), and (DOPAC + HVA)/DA (F3,24 = 2.733, p = 0.066) did not show any significant difference between groups (Table 1).

9-me-BC stimulates dendritic and synaptic growth

Dendritic length

The length of dendrites was significantly increased in the animals treated with 9-me-BC (Fig. 3a). Significant elongation was observed at 60 µm (p = 0.044), 110 µm (p = 0.035) and 160 µm distance from the soma (p = 0.024).

Dendritic spines

The total number of dendritic spines per dendritic tree was significantly increased in the 9-me-BC-treated animals (Fig. 3a). Significant differences were detected for dendritic segments at 60 µm (p = 0.020), 110 µm (p = 0.028) and 160 µm distance from soma (p = 0.024).

Spine frequency

No statistical differences between vehicle- and 9-me-BC-treated animals were detectable for spine frequency (data not shown).

Dendritic complexity

The number of dendritic intersections was significantly increased in the animals treated with 9-me-BC (Fig. 3c). A significant difference was obtained at 110 µm distance from

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Non-injected</th>
<th>Vehicle</th>
<th>9-me-BC-5d</th>
<th>9-me-BC-10d</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPAC (ng/g wet weight)</td>
<td>1.21 ± 0.35</td>
<td>1.34 ± 0.32</td>
<td>1.31 ± 0.30</td>
<td>1.59 ± 0.53</td>
</tr>
<tr>
<td>HVA</td>
<td>2.29 ± 0.64</td>
<td>1.57 ± 0.22</td>
<td>1.99 ± 0.48</td>
<td>1.84 ± 0.34</td>
</tr>
<tr>
<td>DOPAC/DA</td>
<td>0.16 ± 0.04</td>
<td>0.10 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>HVA/DA</td>
<td>0.31 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>0.23 ± 0.05</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>DOPAC + HVA/DA</td>
<td>0.60 ± 0.15</td>
<td>0.26 ± 0.03</td>
<td>0.37 ± 0.07</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>+ HVA/DA</td>
<td></td>
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soma ($p = 0.014$), and a strong trend was seen at 160 μm distance from soma ($p = 0.058$).

**Discussion**

**9-me-BC increases hippocampal dopamine**

Our neurochemical results indicate that 9-me-BC stimulates the synthesis of DA in the hippocampus without affecting the overall metabolism of dopaminergic cells. The 9-me-BC-induced increase of DA, and of dendritic length/complexity and number of dendritic spines in the hippocampal formation (see below) extends the spectrum of the neurostimulatory action of this BC-derivative. Moreover, these findings translate results gained from *in vitro* approaches to the *in vivo* system and thereby expand our knowledge of 9-me-BC actions in the intact brain in several directions. Our study revealed direct evidence that 9-me-BC treatment does not only stimulate cultured DA neurons *in vitro* (Hamann *et al.* 2008; Polanski *et al.* 2010, 2011) but also DA neurons within the intact meso-cortico-limbic pathway *in vivo*. A recent study in an animal model of PD described that *in vivo* 9-me-BC is not only able to stimulate dopaminergic cells in drug-naïve animals (as shown in the present study), but also in 1-methyl-4-phenyl-pyridinium ion (MPP⁺)-pre-treated rats and thereby rescuing the PD-like phenotype indicating the neuroprotective properties of 9-me-BC (Wernicke *et al.* 2010). In addition, here we demonstrate that the effect of 9-me-BC is not restricted to the nigro-striatal dopaminergic pathway because we verified it also for meso-limbic pathways (with the hippocampus as one of the prominent projection fields), that is, it affects circuits that are critically involved in learning and memory formation.

Several studies revealed a close relationship between spatial learning and dopaminergic transmission (Korz and Frey 2007), which is not surprising considering the prom-
ient dopaminergic innervation particularly in the ventral portion of the hippocampus. For instance, lesions of the hippocampal dopaminergic innervation cause selective deficits in spatial working memory (Gasbarri et al. 1996), and especially the dopamine D2 receptor subtype in the ventral hippocampus was shown to be critically involved in spatial working memory. For instance, local application of quinpirole, a D2 receptor agonist, into the ventral hippocampus improved working memory performance, while raclopride, a D2 receptor antagonist, impaired performance in the radial-arm maze in a dose-related manner (Wilkerson and Levin 1999). Furthermore, dopamine D1 receptors play an important role in processing spatial information, it was demonstrated that D1 knockout mice show deficits in spatial learning and in learning new tasks (El-Ghundi et al. 1999). Dopamine D1 but not D3 receptors are critical for spatial learning and memory and act via activation of synaptic plasticity-related proteins, including the N-methyl-D-aspartic acid receptor, Ca2+/calmodulin-dependent protein kinase II, mitogen-activated protein kinases and cAMP-response element binding protein (Xing et al. 2010). On the cellular level there is evidence that dopamine D1/D2 receptors are involved in bidirectional synaptic plasticity at the Schaffer collaterals – it was suggested that long-term depression encodes novel spatial configurations (Lemon and Manahan-Vaughan 2006).

Regarding the time course of the effect of 9-me-BC it is important to note that the neurostimulatory action of 9-me-BC, that is, the elevation of hippocampal DA levels, becomes evident after 10 days, but not after 5 days of treatment. This parallels the findings in the pharmacobehavioral approach (see below) and strongly suggests a direct and most likely causal relationship of the enhanced DA levels and the improved spatial learning. In vitro the numerous effects induced by 9-me-BC were diminished after withdrawal of this drug (Hamann et al. 2008; Polanski et al. 2010), which seems to be true also for the in vivo situation, as we found that the amount of DA was back to control levels in animals, in which the 9-me-BC treatment was discontinued after five days. Even though in vitro an increase of tyrosine-hydroxylase expression as well as neurite outgrowth were detected as early as 48 h after 9-me-BC treatment, indicating the fast rate of these changes (Hamann et al. 2008; Polanski et al. 2010), the kinetics in vivo appear to be slower, and the underlying mechanisms might be much more complex.

In addition to the 9-me-BC-induced effects on hippocampal DA levels, we cannot completely rule out that other experimental conditions might also have contributed, such as food deprivation, which was used to motivate learning, as well as the food baits, which served as rewarding stimuli. Wilson et al. (1995) demonstrated that food-deprivation for about 20 h resulted in significantly enhanced DA concentrations in the nucleus accumbens. In addition, DA can increase significantly during consumption of food rewards (Wilson et al. 1995; reviewed by Wise 2004). However, as the rats of all of our experiments (i.e. the pharmacobehavioral and neurophysiological approaches) were food-deprived, it is likely to conclude that the increase of DA concentration in the hippocampus of 9-me-BC-10d rats was mainly caused by 9-me-BC administration.

9-me-BC improves spatial learning

Our results demonstrate that, beyond its neuroprotective/neurorestorative and anti-inflammatory effects, 9-me-BC acts as a cognitive enhancer in a hippocampus-dependent task. Treatment of rats with 9-me-BC for 10 days showed significantly accelerated acquisition compared with controls: while the 9-me-BC-10d group reached the criterion (≤ 1 error) from seventh day onwards, control rats needed 15 days to reach this criterion (Appenroth and Fleck 2010). This indicates that 10 days of treatment with 9-me-BC reduced the number of training trials by 50%.

The dopaminergic system plays an essential role in spatial learning (Korz and Frey 2007) and a stimulatory effect of 9-me-BC on DA was shown in vitro (Hamann et al. 2008; Polanski et al. 2010, 2011) and in vivo (Wernicke et al. 2010; own results presented here). Interestingly, the improved learning performance was observed after 10 days, but not after 5 days of 9-me-BC treatment, which indicates that 9-me-BC develops its effect over time. It is likely to speculate that 9-me-BC specifically interferes with the late phase of spatial learning (i.e. after a certain level of performance is reached), but this requires further evaluation. Furthermore, our neurochemical results (see above) revealed that a significant elevation of hippocampal DA levels requires longer (10 days) treatment with 9-me-BC, which most likely explains why only this treatment was efficient to accelerate spatial learning. In addition, our neuromorphological results demonstrate that long-term (10 days) 9-me-BC treatment induces structural changes in hippocampal neuronal networks, which most likely contribute to the improvement in spatial learning.

9-me-BC stimulates dendritic and synaptic growth

Our neuromorphological analysis revealed that 9-me-BC administration stimulates the growth of granule cells in the dentate gyrus of rats. Dendritic length as well as the number of dendritic intersections (an indicator for more complex dendritic ramifications) and the total number of dendritic spines were significantly increased in the 9-me-BC-treated animals compared with vehicle-injected controls. It is tempting to speculate that the 9-me-BC-induced increase in synaptic input on dentate granule cells prior to the learning tests might underlie the improved spatial learning of the 9-me-BC-treated rats. Increased synapse turnover and concomitant changes in connectivity patterns are assumed to be critical for the processing of information for long-term storage. There is evidence that pronounced, time-dependent
and partly transient synaptic changes occur following hippocampal-dependent learning. For instance, profound changes in both the size and density of perforated synapses have been reported in the dentate gyrus in response to spatial learning (Marrone 2007), and transient increases of axo-synaptic synapse density and synapse-to-neuron ratio were observed 9 h after the start of training, but not at earlier or later time points (Eyre et al. 2003). A significant learning-associated increase in spine number was observed in the dentate gyrus at 6 h post-training in the water maze, this increase was transient as spine number returned to control levels at 72 h post-training (O’Malley et al. 2000). Spatially trained adult rats, which showed faster spatial learning, displayed about 10% higher basal dendritic spine density (but no changes in dendritic length or complexity) on CA1 pyramidal neurons compared with controls, but it was not assessed whether the better learners had more synapses prior to the training (and therefore learned faster) or whether the training has induced more pronounced synaptic changes in the better learners (Andersen et al. 1996).

The 9-me-BC-induced dendritic and synaptic proliferation might involve dopaminergic mechanisms. Dopaminergic modulation of fiber and synaptic growth has been shown in a variety of in vivo and in vitro systems, even though data are controversial with respect to an inhibitory or stimulatory role of DA (Lauder 1993). Evidence for a stimulatory role of DA was shown by Critchlow et al. (2006) in primary dissociated hippocampal neurons, where haloperidol, an antipsychotic drug which blocks dopaminergic D2 receptors, reduces spinophilin, a protein which is located in dendritic spines (Ouimet et al. 2004). Treatment with chlorpromazine and risperidone, antipsychotic drugs which block D2 receptors, or with olanzapin, an atypical neurolepticum and D2-receptor antagonist, increases the gene expression of microtubule-associated protein 2, a major dendritic structural protein, in the dentate gyrus of rats (Law et al. 2004). DA depletion results in shorter dendrites and reduced spine densities in the nucleus accumbens of rats (Meredith et al. 1995), whereas treatment with drugs that elevate DA levels such as methylphenidate, cocaine and D-amphetamine has been shown to increase the pre-synaptic proteins GAP-43 and synaptophysin (Stroemer et al. 1998), stimulate dendritic growth (Levitt et al. 1997), and increase the density of dendritic spines (Lee et al. 2006; Zehle et al. 2007). A stimulatory effect of 9-me-BC was also demonstrated in vitro. 48 h treatment with 9-me-BC increased the number of dopaminergic neurons in a concentration-dependent manner and these neurons displayed an increased number and ramification of neurites (Hamann et al. 2008; Polanski et al. 2010).

In summary, our results demonstrate that beyond its neuroprotective/neurorestorative and anti-inflammatory effects, 9-me-BC acts as a cognitive enhancer in a hippocampus-dependent task, and that the behavioral effect may be associated with a stimulatory impact on DA levels and dendritic and synaptic growth.

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Disclosure/conflict of interest

The authors have no conflict of interest.

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