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ORIGINAL ARTICLE

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.

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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

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Abbreviations used: 9-me-BC, 9-methyl-β-carboline; b.w., body weight; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; i.p., intraperitoneally; P, postnatal day; RAM, eight-arm radial maze.

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-carbolinum and 2,9-dimethyl-β-carbolinum 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 nigro-striatal 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).

Until now, the studies on 9-me-BC effects were focused on the nigro-striatal pathways. The aim of the present study was to extend this to other dopaminergic functional pathways, such as the meso-cortico-limbic dopaminergic pathway, and to investigate whether 9-me-BC might exert a broader stimulatory effect. In a pharmaco-behavioural approach, we tested the hypothesis that 9-me-BC improves spatial learning in a radial maze (Olton and Samuelson 1976), a hippocampus-dependent task modulated by the meso-limbic dopaminergic pathway (McGurk *et al.* 1992; Wilkerson and Levin 1999; Tinsley *et al.* 2001). To unveil the underlying mechanisms of the predicted 9-me-BC-induced cognitive enhancement we assessed the neurochemical and neuromorphological effects of 9-me-BC in the hippocampus.

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 μmol 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 μmol 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 ($p < 0.05$).

Neurochemical analysis

Preparation of tissue

Three hours after the last application of vehicle or 9-me-BC (non-injected animals were used in a time-matched manner), animals naive to the spatial learning procedure were decapitated. Both hippocampi were prepared on an ice-cold dissection plate, immediately frozen (left and right hippocampus separately) using liquid nitrogen, and stored at −80°C until assayed by HPLC.

HPLC procedure

To quantify levels of DA, as well as its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), either the left or right hippocampus (the remaining hippocampus was used in a different experimental approach) of non-injected ($n = 13$), vehicle- ($n = 12$), 9-me-Bc-5d- ($n = 12$), or 9-me-BC-10d-treated animals ($n = 12$) was weighted (i.e. wet weight) and processed as described previously (Marques Pereira *et al.* 2008). Briefly, samples were homogenized by ultrasonic disruption (Labsonic©M, Sartorius, Germany), centrifuged (20 800 *g* for 15 min at 4°C), filtered (0.2 µm pore size; Whatman, Clifton, NJ, USA), and injected onto a HPLC system. DA, DOPAC and HVA were separated by reversed phase HPLC (column: MD-150 × 3.2 mm; mobile phase: MD-TM 70-1332; ESA Biosciences Inc., Chelmsford, MA, USA), electrochemically detected, and quantified using external standard substances. The final amounts are expressed as nanogram per gram wet weight (ng/g w.wt.).

Statistical analyses

Using SPSS (version 15.0; SPSS Inc., Chicago, IL, USA) the data were analyzed with the General Linear Model for univariate analysis of variance followed by, if applicable, a LSD *post hoc* test. For all analyses, Bonferroni confidence interval adjustment for multiple comparisons was used. For comparison of two experimental groups, a Student's *t*-test was used. All tests were two-tailed, and the level of significance was set to $p < 0.05$. Data are presented as mean ± SEM.

Neuroanatomical analysis

Three hours after the last pharmacological application of 9-me-BC-10d or vehicle (each group n = 6 animals), the animals naive to the spatial learning procedure were decapitated, the brains were removed and incubated in 50 ml of Golgi-Cox solution for two weeks. Transverse sections of 150 µm thickness were further processed using a modified Golgi-Cox technique as described in (Bock and Braun 1999). For each animal two granule neurons/hemisphere were analyzed in the dentate gyrus, and mean values were generated for each individual animal. The quantitative analysis was conducted on selected neurons, which were impregnated in their entirety, and which were not obscured by neighboring neurons, glia or blood vessels. Reconstruction was made at 1000× using a computer-based neuron tracing system (NEUROLUCIDA®, MicroBrightField, Williston, VT, USA), which allows the quantitative three-dimensional analysis of complete dendritic trees. The following parameters were quantified: (i) mean dendritic length, (ii) spine numbers, i.e. the total number of spines per dendritic tree, (iii) spine frequency, i.e. the number of spines per µm dendritic length, and (iv) dendritic complexity, i.e. the total number of dendritic intersections along the entire length of the dendrite. The length of the dendritic trees was measured by tracing the entire dendrite while counting dendritic spines. All protrusions - thin, stubby or mushroom type - were counted as spines if they were in direct continuity with the dendritic shaft. For analyzing dendritic complexity a Sholl analysis (Sholl 1953) with concentric rings of 50 µm distance (starting 10 µm from a reference point in the centre of the soma) from each other was conducted. All measurements were performed by an experimenter who was unaware of the experimental conditions of the animals.

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

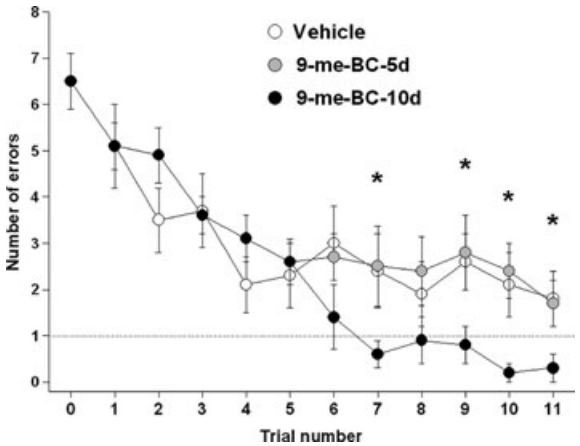

Fig. 1 9-me-BC treatment improves learning in the radial maze. The number of working memory errors was registered first before (trial 0) the administration of 9-me-BC (trial 1–11). Values are given as mean ± SEM. *Statistically significant differences compared with controls (*t*-test, $p < 0.05$).

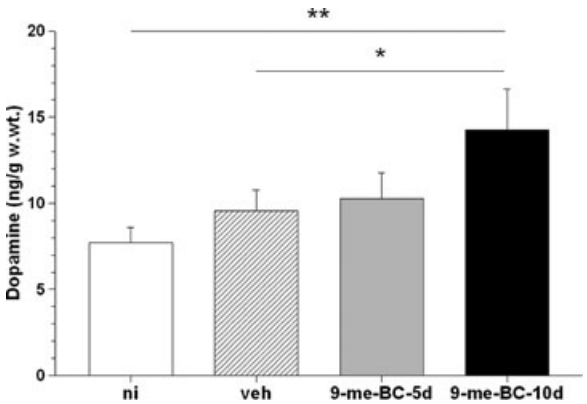

Fig. 2 9-me-BC (2 μmol/100 g body weight) increases the hippocampal levels of dopamine after 10 days (9-me-BC-10d; black bar), but not 5 days of treatment (9-me-BC-5d; grey bar) in comparison with non-injected (ni; white bar) and vehicle-treated animals (veh; hatched bar). Values are given as mean ± SEM. **$p < 0.01$, *$p < 0.05$ (LSD *post hoc* test).

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 ($F_{3,42} = 4.986$, $p = 0.032$), 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 ($F_{3,45} = 3.174$, $p = 0.033$; 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 ($F_{3,24} = 0.174$, $p = 0.913$), and HVA ($F_{3,45} = 0.447$, $p = 0.721$) as well as the ratios of DO/DA ($F_{3,24} = 0.505$, $p = 0.682$), HVA/DA ($F_{3,45} = 1.771$, $p = 0.166$), and (DOPAC + HVA)/DA ($F_{3,24} = 2.733$, $p = 0.066$) 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 Hippocampal levels of DOPAC and HVA (ng/g wet weight), as well as ratios of DOPAC/DA, HVA/DA, and DOPAC + HVA/DA (mean ± SEM)

	Non-injected	Vehicle	9-me-BC-5d	9-me-BC-10d
DOPAC	1.21 ± 0.35	1.34 ± 0.32	1.31 ± 0.30	1.59 ± 0.53
HVA	2.29 ± 0.64	1.57 ± 0.22	1.99 ± 0.48	1.84 ± 0.34
DOPAC/DA	0.16 ± 0.04	0.10 ± 0.02	0.13 ± 0.02	0.13 ± 0.05
HVA/DA	0.31 ± 0.09	0.19 ± 0.03	0.23 ± 0.05	0.14 ± 0.02
DOPAC + HVA/DA	0.60 ± 0.15	0.26 ± 0.03	0.37 ± 0.07	0.25 ± 0.05

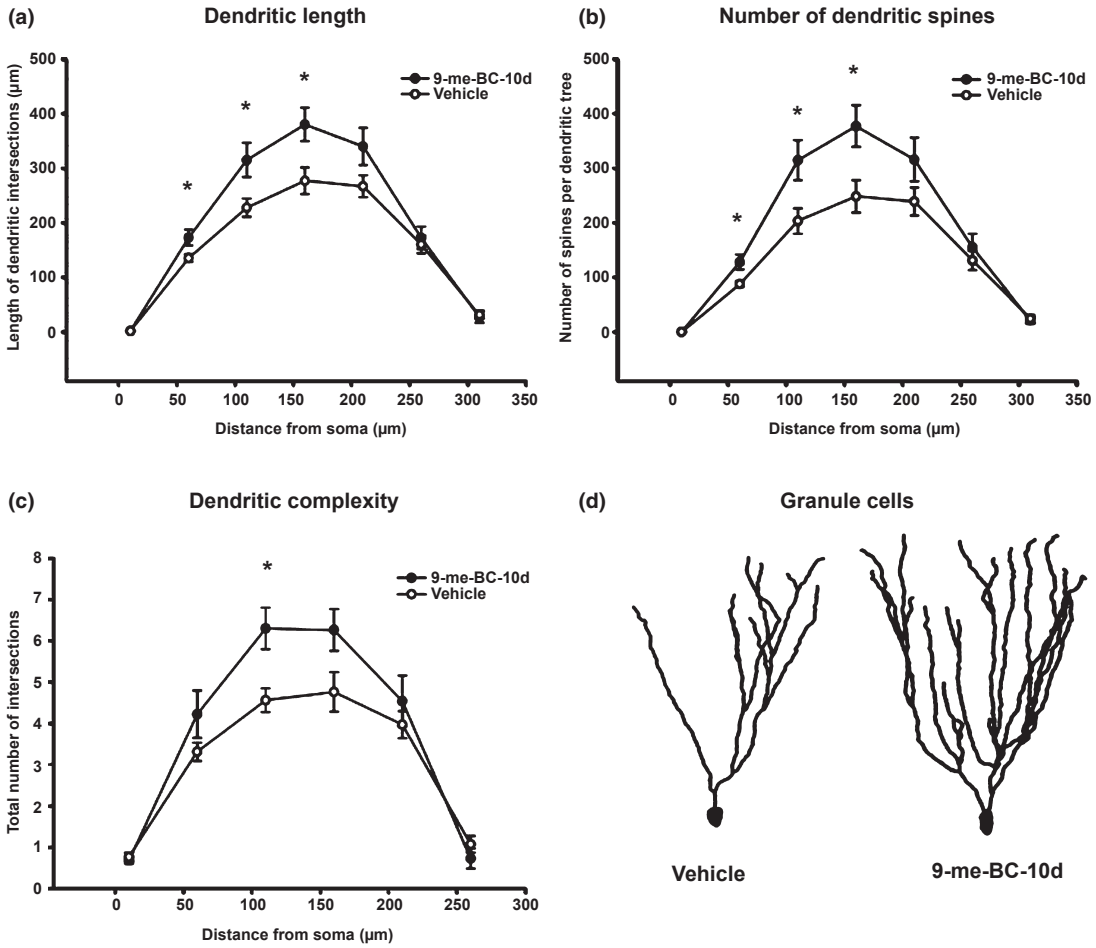

Fig. 3 Treatment of male rats with 9-me-BC for 10 days increased the length of dendrites (a), the number of dendritic spines (b), and the dendritic complexity (c) in several concentric rings of granule cells from the dentate gyrus. A representative reconstruction of granule cells of the dentate gyrus from rats treated with vehicle or 9-me-BC is given in panel (d). Values are given as mean ± SEM. *$p < 0.05$ (*t*-test).

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-naive 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-

inent 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 D_2 receptor subtype in the ventral hippocampus was shown to be critically involved in spatial working memory. For instance, local application of quinpirole, a D_2 receptor agonist, into the ventral hippocampus improved working memory performance, while raclopride, a D_2 receptor antagonist, impaired performance in the radial-arm maze in a dose-related manner (Wilkerson and Levin 1999). Furthermore, dopamine D_1 receptors play an important role in processing spatial information, it was demonstrated that D_1 knockout mice show deficits in spatial learning and in learning new tasks (El-Ghundi *et al.* 1999). Dopamine D_1 but not D_3 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, Ca^{2+}/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 D_1/D_5 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 pharmaco-behavioral 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 pharmaco-behavioral 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-spinous 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 controverse 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 D_2 receptors, reduces spinophilin, a protein which is located in dendritic spines (Ouimet *et al.* 2004). Treatment with chlorpromazine and risperidone, antipsychotic drugs which block D_2 receptors, or with olanzapin, an atypical neurolepticum and D_4-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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