Neuroscience Letters 478 (2010) 56–60

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Elevated BDNF protein level in cortex but not in hippocampus of MDMA-treated Dark Agouti rats: A potential link to the long-term recovery of serotonergic axons

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ARTICLE INFO

Article history:
Received 11 March 2010
Accepted 26 April 2010

Keywords:
3,4-Methylenedioxymethamphetamine (ecstasy)
Serotonin
Brain derived neurotrophic factor (BDNF)
Tryptophan-hydroxylase (TpH)
Paroxetine-binding
ELISA

ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a widely used recreational drug known to cause selective long-term serotonergic damage. In this study, we examined the pattern of BDNF protein expression 1 day, 3, 8, 12 and 24 weeks after a single 15 mg/kg i.p. dose of MDMA to adolescent Dark Agouti rats. In parallel, we measured either tryptophan-hydroxylase immunoreactive (TpH IR) axon density, or [^{3}H]-paroxetine-binding in parietal cortex and hippocampus, two brain areas known to have different recovery capacity after MDMA, to test whether BDNF-levels were associated with the long-term recovery of serotonergic fibers after a neurotoxic dose of MDMA. Both TpH IR axon density and [^{3}H]-paroxetine-binding were significantly decreased 3 weeks after the treatment in both brain areas but while normalization in both parameters was found in parietal cortex 24 weeks after treatment, significant decreases remained evident in the hippocampus. In the parietal cortex, a significant reduction in BDNF protein levels was found in the acute phase after treatment (1 day), which was followed by a robust increase 8 weeks later and a return to control levels by 12 weeks. In contrast, no significant alteration of BDNF protein level was found in the hippocampus at any time points. This absence of any significant increase in BDNF protein levels in the hippocampus, and the persistence in this region of decreases in TpH IR axon density and [^{3}H]-paroxetine-binding, raises the possibility that BDNF has an important role in the long-term recovery of serotonergic axons after MDMA treatment.

The synthetic ring-substituted amphetamine derivate 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") has become widely used as a recreational party drug [10]. The positively reinforcing acute behavioral effects caused by MDMA are associated with a release of monoamines from nerve endings in the brain [6], but subsequently a long-term decrease of several serotonergic parameters has been described in the forebrain of rats, guinea pigs and primates [6,21].

Brain derived neurotrophic factor (BDNF) belongs to the family of neurotrophins [15]. It supports the maintenance of monoaminergic and cholinergic neurons [15] with many studies demonstrating a relationship between central serotonergic system and BDNF [16,18].

BDNF protein levels have already been examined acutely after the administration of various serotonergic drugs, including MDMA [3,5,13,17,19,31]. However, the long-term expression pattern of BDNF after MDMA treatment has only been reported in one recent study, in which neonatal Wistar rats were treated and BDNF protein level was followed up to 65 days after the treatment [25]. In the present study, we have analyzed long-term alterations in BDNF protein levels over a much longer time frame, up to 24 weeks after a single dose of MDMA administered to adolescent Dark Agouti rats, and, in parallel, have conducted a the long-term biochemical–anatomical study of serotonergic fibers in two areas of the brain (parietal cortex and hippocampus) known to have different recovery capacity after MDMA. By comparing temporal patterns of alterations in BDNF protein levels in these brain regions with both TpH immunoreactive (IR) axon density and [^{3}H]-paroxetine-

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doi:10.1016/j.neulet.2010.04.061

binding, we have been able to test the possibility that increased expression of BDNF is associated with the processes of recovery in serotonergic fibers after a neurotoxic dose of MDMA.

All animal experiments and housing conditions were carried out in accordance with the European Community Council Directive on 24 November 1986 (86/609/EEC). Adolescent (43- to 47-day-old, 130–145 g) male Dark Agouti rats (Harlan, Olac Ltd., Shaw's Farm, Blackthorn, Bicester, Oxon, UK, 5 weeks old upon arrival) were used in the experiments.

The animals (4 per cage) were kept under controlled environmental conditions (temperature at 21 ± 1 °C, and a 12-h light–dark cycle starting at 10.00). Standard food and water were freely available.

(±)3,4-Methylenedioxymethamphetamine hydrochloride (MDMA, certified reference compound, purity >99.5%) was provided by Sanofi-Synthelabo-Chinoin, Hungary. The drug was dissolved in 0.9% NaCl at a dose equivalent to 15 mg/kg free base and was injected intraperitoneally in a volume of 1 ml/kg. Control animals received an injection of 0.9% NaCl (SAL) in a volume of 1 ml/kg.

For immunohistochemistry, 3, 12 or 24 weeks after the single-dose MDMA treatment, rats (n = 5–7) were anesthetized with pentobarbital sodium (35 mg/kg, i.p.), thoracotomized and perfused transcardially with Zamboni-fixative [1]. The brains were embedded into paraffin. Coronal sections were cut at the levels of the striatum and hippocampus according to a conventional rat brain atlas [24]. Mouse monoclonal TpH antibody (PharMingen, USA; Cat. No.: 556310, IgG1, clone PH8) was used at a dilution of 1:3000. Immunostaining was developed using a peroxidase/DAB kit (EnVision™, DAKO, Glostrup, Denmark). The immunostaining procedure was as previously described in detail from this laboratory [1].

Morphometrical analysis of parietal cortex (layers II–V) and stratum radiatum of the CA1 region of the hippocampus was conducted with reference to the rat brain atlas of Paxinos [24]. TpH immunostaining was quantified by densitometric analysis with reference to counterstain-free sections, as described previously [1].

For paroxetine-binding measurements, at each of the same predetermined time points used for TpH immunohistochemistry, rats (n = 5–7) were sacrificed by decapitation, brains were immediately dissected out intact and rapidly frozen in pre-cooled 2-methylbutane (−45 °C). Frozen brains were sectioned in a cryostat (20 μm) for autoradiographic radioligand binding analysis of 5-HT uptake sites using [^{3}H]-paroxetine according to the protocol described by [7] and used by us previously [28].

For ELISA, animals (n = 5–7) were decapitated 24 h after the single-dose MDMA treatment, or after 3, 8, 12 and 24 weeks, and their brains were quickly removed and frozen on dry ice. Animals were sacrificed at the same hours of day (17:00–20:00) in all groups. Tissue was dissected from parietal cortex or hippocampus with reference to the atlas of [24]. Brain samples were homogenized in lysis buffer in the volume of 1 ml × 100 mg^{-1} wet tissue in Eppendorf tubes with a mechanical homogenizer. The lysis buffer contained 100 mM PIPES, 500 mM NaCl, 0.2% TritonX 100, 0.1 M Na-azide, 2% BSA, 2 mM EDTA, 200 μm PMSF, 10 μm leupeptin, 0.3 μM approtinin, 1 μm pepstatin, pH = 7.00. Homogenized samples were kept on ice for 30 min before being centrifuged for 20 min at 14,000 rpm at 4 °C. Following centrifugation, the supernatants were carefully removed into clean Eppendorf tubes which were stored at −70 °C, pending analysis.

A commercially available enzyme immunoassay kit (BDNF E_{max}® ImmunoAssay System, Promega, USA) was used to determine of BDNF protein level (free mature form) according to the manufacturer's suggested protocol, as published previously [3]. All hippocampal and cortical samples from any given time point were analyzed in one assay.

Table 1
TpH immunoreactive axon densities and [^{3}H]-paroxetine-binding in parietal cortex and hippocampus at variant time points after a single 15 mg/kg MDMA to Dark Agouti rats.

Brain region	Time after the treatment	SALINE	MDMA
TpH immunostaining density (arbitrary units)			
	3,12,24 weeks	7.20 ± 0.46	5.06 ± 0.53*
Parietal cortex		8.76 ± 0.93	6.05 ± 0.37*
		6.64 ± 0.21	7.46 ± 0.40
	3,12,24 weeks	6.32 ± 0.24	3.34 ± 0.42***
Hippocampus CA1		7.58 ± 0.58	4.56 ± 0.41**
		6.84 ± 0.73	4.06 ± 0.32**
Paroxetine-binding (fmol/mg tissue)			
	3,12,24 weeks	92 ± 8	31 ± 10***
Parietal cortex		88 ± 7	55 ± 7**
		86 ± 8	77 ± 9
	3,12,24 weeks	79 ± 8	28 ± 12***
Hippocampus CA1		82 ± 5	45 ± 6***
		80 ± 9	57 ± 7*

Note that TpH IR axon density and [^{3}H]-paroxetine-binding exhibit significant decrease 3 weeks after the treatment in both brain areas. Parietal cortex shows a complete restoration by 24 weeks in both parameters. In contrast, no recovery can be detected in the hippocampus in the TpH IR axon density and only partial recovery is noted in [^{3}H]-paroxetine-binding for 6 months. Statistics: two-way ANOVA, (variables: region, treatment) and Tukey *post hoc* analysis. Data are presented as mean ± SEM, n = 5–7.

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

BDNF protein levels in tissue samples were determined from the regression line of the BDNF standard and were reported in pg BDNF × g^{-1} wet weight.

Data obtained from densitometry analysis, ELISA and paroxetine-binding assays were evaluated using two-way analysis of variance (ANOVA) (variables: treatment, brain region) or three-way ANOVA (variables: time, treatment, brain region). Tukey-test was used for *post hoc* comparisons. All statistical data were evaluated with acceptable levels of significance set at $P < 0.05$ using Statistica® 7 Software.

Densitometric analysis of tryptophan-hydroxylase immunoreactive (TpH IR) fibers in the parietal cortex and the CA1 region of the hippocampus revealed that the MDMA treatment caused a significant decrease in TpH IR axon density with a distinct temporal and spatial pattern (treatment effect: $F_{(1,52)} = 57.01$, $P < 0.001$; time × treatment interaction: $F_{(2,52)} = 3.65$, $P < 0.05$; region × treatment interaction: $F_{(1,52)} = 6.98$, $P < 0.05$, time × treatment × region interaction: $F_{(2,52)} = 2.69$, $P = 0.07$; 24-week time point region × treatment interaction: $F_{(1,16)} = 13.35$, $P < 0.01$). In the parietal cortex, a significant reduction in TpH IR axon density of approximately 30% was found in MDMA-treated rats at both 3 weeks and 12 weeks after the treatment, however no significant effect was detectable by 24 weeks (Table 1, Fig. 1). In contrast, a significant MDMA-induced reduction in TpH IR axon density was found in hippocampus, which remained at similar levels throughout and ranged from −47% at 3 weeks to −40% at 24 weeks (Table 1, Fig. 1).

The pattern of [^{3}H]-paroxetine-binding to 5-HT transporter sites in response to MDMA treatment, and the changes that were found over time, were in general agreement with the quantified TpH IR fiber density data described above, with similar temporal and spatial patterns (treatment effect: $F_{(1,52)} = 28.21$, $P < 0.001$; time × treatment interaction: $F_{(2,52)} = 3.24$, $P < 0.05$; region × treatment interaction: $F_{(1,52)} = 4.04$, $P < 0.05$, time × treatment × region interaction: $F_{(2,52)} = 2.01$, n.s.; 24-week time point region × treatment interaction: $F_{(1,16)} = 4.83$, $P < 0.05$). Specifically, in the parietal cortex a statistically significant reduction of [^{3}H]-paroxetine-binding (−67%) was detected in MDMA-treated rats after 3 weeks, which was partially restored by 12 weeks (−38%) and returned to a level not significantly different from the control one by 24 weeks

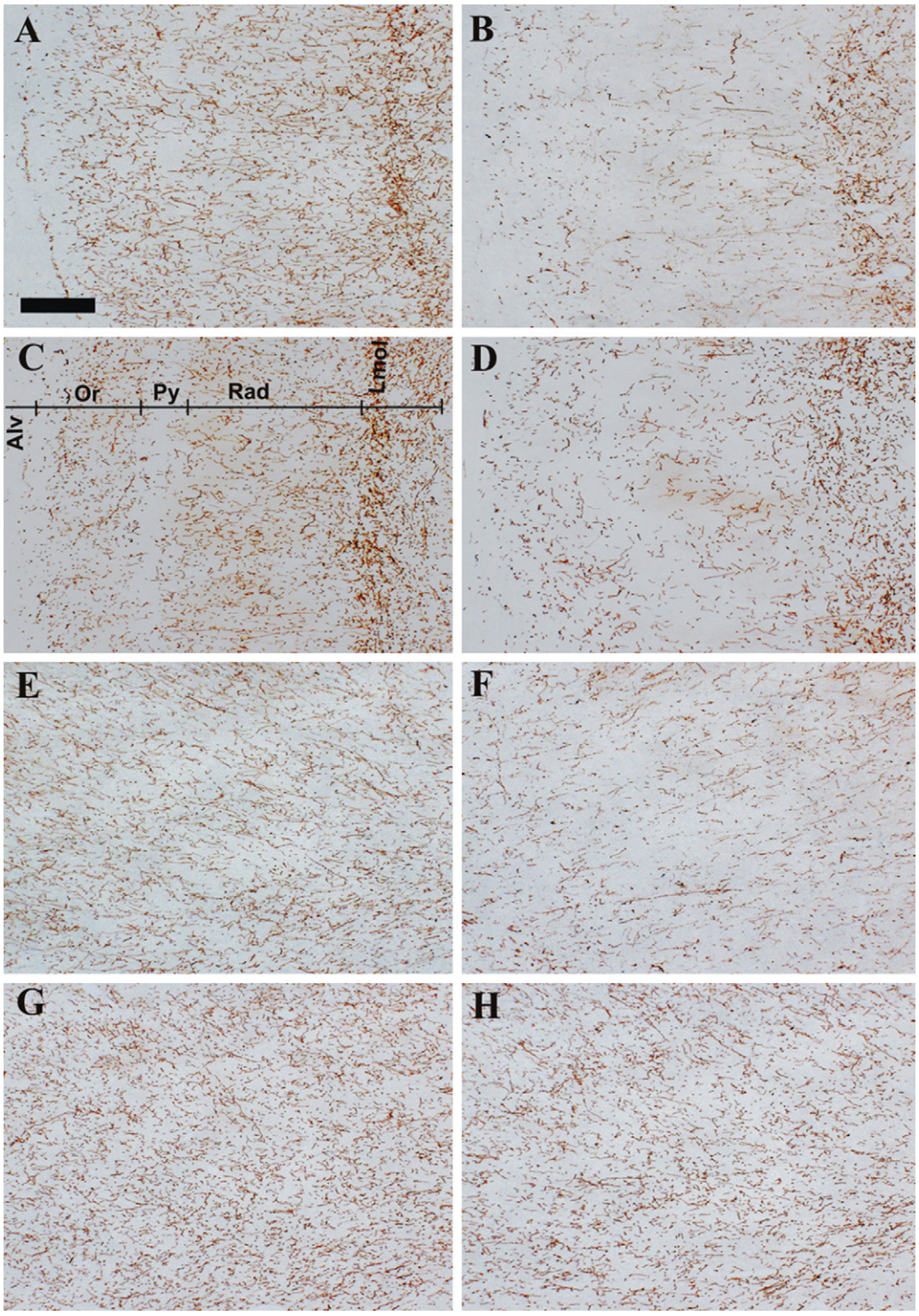

Fig. 1. TpH immunoreactive (TpH IR) axon densities in the hippocampus (A–D) and in the parietal cortex (E–H) in control (saline-treated) (A, C, E, G) or 15 mg/kg MDMA-treated (B, D, F, H) Dark Agouti rats 3 weeks (A, B, E, F) or 24 weeks (C, D, G, H) after the treatments. Note that TpH IR axon density exhibits a significant decrease 3 weeks after the treatment in both brain areas (A vs. B, E vs. F, hippocampus CA1 and parietal cortex, respectively). 24 weeks after the treatment, TpH IR axon density in the parietal cortex is similar to control (G vs. H). In contrast, there is no significant recovery in the hippocampus CA1 24 weeks after the treatment (C vs. D). Alv: alveus; Or: hippocampus CA1 stratum oriens; Py: stratum pyramidale; Rad: stratum radiatum; Lmol: stratum lacunosum-moleculare. Scale bar: 100 μm in panel A applies to all panels.

(Table 1). In contrast, in the hippocampus the effect of MDMA was still evident after 24 weeks (−29%), albeit at a lower level than at earlier time points (−65% at 3 weeks) (Table 1).

The protein level of brain derived neurotrophic factor (BDNF) (pg BDNF × g^{-1} tissue wet weight) was determined by ELISA in the parietal cortex and hippocampus. Significant treatment effect ($F_{(1,95)} = 6.16$, $P < 0.05$), time × treatment interaction ($F_{(4,95)} = 20.71$, $P < 0.001$) and region × time × treatment interaction ($F_{(4,95)} = 12.32$, $P < 0.001$) were found. In *post hoc* comparisons, a significant reduction in the BDNF protein levels was found 1 day after treatment (−25%) and a similar trend was noticed 3 weeks later (−22%, n.s.) in the parietal cortex. However, 8 weeks after treatment, BDNF protein levels were markedly elevated (+172%) and returned to levels close to control by 12 weeks in this brain region (Fig. 2). In con-

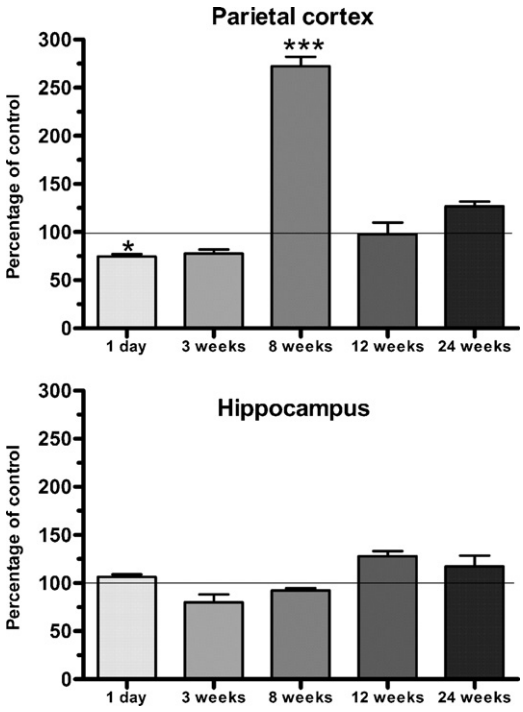

Fig. 2. BDNF protein levels in parietal cortex and hippocampus at variant time points after a single 15 mg/kg MDMA to Dark Agouti rats. In the parietal cortex, BDNF protein levels show a significant acute reduction 24 h after the treatment and a robust elevation 8 weeks later that returns to levels close control by 12 weeks. In contrast, no significant alterations can be found in the hippocampus. Statistics: two-way ANOVA, (variables: region, treatment) and Tukey *post hoc* analysis; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are presented as percentage of controls ± SEM, $n = 5–7$.

trast, no significant effect of MDMA treatment was found in the hippocampus at any of the time points although some small non-significant trends, namely tendency towards decrease at 3 weeks and tendency towards increase at 12 weeks were observed (Fig. 2).

In this study, we report MDMA-induced changes in the pattern of BDNF protein expression. These changes may be indicative of a potential role for BDNF in the processes of recovery following the serotonergic terminal depletion induced by a single dose of MDMA. The single 12.5–15 mg/kg dose of MDMA in Dark Agouti rats has been found to be neurotoxic in a number of studies [1,4,23] and based on interspecies scaling represents a dose similar to that used for recreational purposes by humans [22].

Densitometric analysis of TpH immunoreactive axons and paroxetine-binding measurements from parallel groups of similarly treated animals were used as markers for long-term depletion of serotonergic axons and terminals following MDMA exposure. Paroxetine-binding reflects the density of 5-HT uptake sites, and has frequently been used as a quantitative marker of serotonergic impairment following administration of MDMA or amphetamine derivatives [2,23,27]. Similarly, TpH immunohistochemistry is a well-established method for the visualization of serotonergic cell bodies and projections in the rat brain [1,30] at a level of resolution greater than that of radioligand autoradiography. These two approaches (paroxetine-binding and TpH IHC) complement each other to provide a comprehensive overview of the damage to serotonergic terminals produced by MDMA.

In our study, both TpH IR axon density and paroxetine-binding exhibited significant decreases in both the hippocampus and the parietal cortex 3 weeks after MDMA treatment. However, while the parietal cortex showed complete restoration in both parameters by 24 weeks, no or only a partial recovery could be detected in the hippocampal CA1 field using TpH IR and paroxetine-binding as markers, respectively. These results are in agreement with previous studies on the long-term consequences of MDMA treatment based on 5-HTT IHC [12] serotonin (5-HT) and 5-hydoxyindoleacetic acid (5-HIAA) measurements or radioligand binding to 5-HTT [8,14,26,27].

Brain derived neurotrophic factor (BDNF) has a prominent role in the maintenance of serotonergic fibers in the adult brain [9,16]. Moreover, the reverse interaction between 5-HT and BDNF has also been reported, since activation of 5-HT receptors can stimulate BDNF gene expression [18].

Only a limited number of papers have addressed the effects of serotonergic drugs upon the long-term patterns of BDNF expression. Thus, increased levels of BDNF mRNA were found in parietal cortex and in hippocampus 14 days after treatment with the potent serotonergic neurotoxin PCA [31] and in a recent paper [25] neonatal rats (at P1–4) treated with MDMA were found to show elevated BDNF protein levels in the occipital cortex only, and a clear dissociation between BDNF and 5-HTT levels 26 days after the last treatment (at P30). According to speculations of these authors, if 5-HT-specific neurotoxins do cause upregulation of BDNF as a trophic response to stimulate serotonergic fiber recovery, the effect might be characterized by delayed onset and a prolonged time course.

The present study is the first to describe the long-term time course of BDNF protein expression after a single neurotoxic dose of MDMA in Dark Agouti rat as measured by a well-characterized ELISA method. In the parietal cortex, BDNF protein levels showed a significant acute reduction 24 h after the treatment, a robust elevation 8 weeks later and a return to control levels by 12 weeks. In contrast, no significant alteration could be found in the hippocampus where only a non-significant tendency towards decreases at 3 weeks and a similarly non-significant tendency towards increase at 12 weeks may be noted. These results indicate a completely different BDNF protein expression response between parietal cortex and hippocampus following MDMA treatment, which is in agreement with the previous studies [5,17,29,31].

The mechanism by which levels of BDNF decrease acutely 24 h after MDMA in the cortex, but not in the hippocampus, has yet to be fully characterized, particularly as level of the mRNA encoding BDNF actually increases in the frontal cortex and decreases in the hippocampus at the same time point [17]. However, the dose of MDMA used in that study was lower than that we used, and the strain of rat was different (Wistar vs. Dark Agouti). It is possible that this acute effect on BDNF protein level could be associated with the acute depletion of 5-HT from terminals caused by MDMA [6]. Whatever the mechanism underlying these acute effects, and the cause of discrepancies in the results, the elevation of BDNF at 8 weeks after the treatment, described in the present study, is most likely involved in the neuroregenerative response to the long-term neurotoxic effects of the drug [25,31].

Considering the particular relationship between the serotonergic system and BDNF detailed above, our results raise the possibility that the robust elevation of BDNF in parietal cortex 8 weeks after the treatment might be necessary to support the recovery of serotonergic fibers in this brain region. This hypothesis is strengthened by the observed lack of any significant BDNF response in the hippocampus where significant serotonergic depletion persists right up to 24 weeks (and possibly beyond). It is worth noting that the BDNF response in the parietal cortex exhibits a delayed onset (sometime between 3 and 8 weeks) and diminishes months before the complete recovery of serotonergic parameters: that is, restoration of paroxetine-binding can first be detected by 12 weeks and TpH immunostaining density recovers between 12 and 24 weeks after the treatment. These results are comparable with Piper's study where significant elevation of BDNF protein level was detected only a month after MDMA treatment [25] and, albeit in a different experimental model, BDNF expression reached maximal levels at approximately 4 weeks after post-section of lesioned peripheral

nerves, a response that is much slower than NGF [20]. Moreover, the ability of cortically infused BDNF to elicit 5-HT axon sprouting after PCA-lesion in rat is not restricted to a narrow "critical period". In this case BDNF markedly stimulates the regrowth of axons at least 5 weeks after terminating exogenous BDNF delivery [16].

Further studies are needed to clarify the potential functional role of BDNF in the long-term recovery of serotonergic axons after MDMA, and the role of BDNF metabolism, turnover, receptor patterns or signaling pathways could usefully be examined in this context. In addition, other factors, such as the expression of S100B neurotrophic factor should perhaps be considered [11].

In conclusion, an increase of BDNF protein level in the parietal cortex but not in the hippocampus precedes the recovery of serotonergic fibers selectively in that cortical area observed at 6 months and raises the possibility that BDNF plays a significant role in the long-term recovery of serotonergic axons after MDMA treatment.

Acknowledgements

This study was supported by the 6th Framework Program of the European Community, LSHM-CT-2004-503474, the Hungarian Research Fund Grant T020500 and the Ministry of Health Research Grant 460/2006. We are grateful to Agnes Druskone, Zsuzsanna Anhauer, Ildiko Csontos and Mariann Lenkey for their excellent technical assistance.

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