3,4-Methylenedioxymethamphetamine (MDMA), but not morphine, alters APP processing in the rat brain

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Abstract

The abuse of drugs such as opioids and 3,4-methylenedioxymethamphetamine (MDMA or ‘ecstasy’) can have detrimental effects on the cognitive functions, but the exact molecular mechanism whereby these drugs promote neurodegeneration remains to be elucidated. The major purpose of the present pilot study was to determine whether the chronic in-vivo administration of morphine (10 mg/kg) or MDMA (1 mg/kg) to rats can alter the expression and processing of amyloid precursor protein (APP), the central molecule in the proposed pathomechanism of Alzheimer’s disease. MDMA treatment significantly decreased the production of APP in the cytosolic fraction of the brain cortex. A concomitant 25% increase was found both in the β-secretase (BACE) and APP mRNA levels (108%). In contrast, in the applied single dosage chronic morphine treatment did not influence either the APP and BACE protein levels or the APP mRNA production. These results indicate that the chronic use of ‘ecstasy’, but not morphine, may be harmful via a novel mode of action, i.e. by altering the APP expression and processing in the brain.

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Introduction

The amyloid hypothesis of Alzheimer’s disease (AD) is based on the observation that β-amyloid protein (βAP), the major component of the senile plaques, is proteolytically cleaved from a larger membrane-associated amyloid precursor protein (APP) (Selkoe, 1999). In certain genetical forms of AD, the over-expression of APP may result in aberrant processing, leading to amyloidogenic fragments (Citron et al., 1998). In both the familial and sporadic forms of AD, a subtle balance exists between the normal, α-secretase-mediated cleavage of APP, resulting in soluble, secreted and neuroprotective αAPPs, and the abnormal, β- and γ-secretase-mediated proteolysis, which leads to neurotoxic βAP production and deposition (Scheuner et al., 1996). APP gene transcription and processing are regulated by a number of cellular mediators and messengers, but no information is yet available as to how abused drugs such as morphine and 3,4-methylenedioxymethamphetamine (MDMA) interfere with this process.

A derivative of amphetamine, MDMA or ‘ecstasy’, is a popular recreational drug with potent and complex effects on the central nervous system (Green et al., 2003). It releases mainly serotonin (5-HT) from the nerve terminals, but the neurotransmission in other biogenic amine-containing neurons, e.g. dopamine (DA) and noradrenaline is also enhanced (Gough et al., 1991). Besides its effect at a synaptic level, the induction of immediate, early genes, and increases of
lipid peroxidation and free radical formation have also been proposed to be involved in the MDMA-induced specific neurotoxic damage to serotonergic nerve terminals in the forebrain (Colado et al., 1997; Hashimoto et al., 1997). The MDMA-induced neurodegeneration lasts for months or even years, depending on the species (Malpass et al., 1999) and has been found to be associated with impairment of memory functions such as verbal and delayed visual functions and prospective memory (Fox et al., 2002). Similar cognitive deficits have been found as early signs of AD.

The very addictive opiates and synthetic analogues with specific effects on the brain reward circuitry are also commonly abused. The most typical opiate, morphine, has a common characteristic with MDMA: they both facilitate DA release (Law et al., 2004). Morphine acts on its specific opiate receptors linked to $G_\text{o}$ proteins and related second-messenger systems such as adenylate cyclase, K+ and voltage-gated Ca2+ channels (Hack and Christie, 2003). $G_\text{o}$ is highly expressed in neurons and mediates the effects of opioid receptors (Harlan et al., 2004). On the other hand, $G_\text{o}$ is also activated by APP in vitro (Mahlapuu et al., 2003). Moreover, morphine, like $\beta$-AP, can activate Ca2+/calmodulin-dependent protein kinase II and augments $\beta$-AP-induced neuronal apoptosis (Lin et al., 2004). The opiate receptor antagonist naloxone is able to inhibit $\beta$-AP-induced degeneration in vitro (Liu et al., 2002).

The major purpose of the present study was, therefore, to determine whether the chronic in-vivo administration of the addictive drugs morphine and MDMA, with different targets and modes of molecular action, can alter the expression and processing of APP, the central molecule in the proposed pathomechanism of AD. Drug abusers use the racemic form of MDMA, which is also applied in most of animal experiments. However, the dextrarotary form, (+)-MDMA, is considered to be more active in releasing both 5-HT and DA (Schmidt, 1987) and only (+)-MDMA causes DA release in the striatum (Hiramatsu and Cho, 1990). The (+)-enantiomer has more marked stimulatory activity on biogenic amine release and this form is responsible for the neurotoxic effect of MDMA (Gyarmati et al., 2002; Schmidt et al., 1987). Accordingly, the (+)-MDMA enantiomer was used in this study.

The present paper provides experimental evidence that in chronic low doses MDMA treatment increases APP mRNA and $\beta$-amyloid-cleaving enzyme (BACE) protein levels and decreases the amount of soluble APP in the rat brain, while in the applied dosage and time morphine treatment is unlikely to exert an effect on APP processing.

**Methods**

**Animals**

Regardless of their oestrous cycle, a total of 21 female Wistar rats (seven in each group) weighing 200–250 g were maintained under standard laboratory conditions. The animals were injected s.c. daily with 10 mg/kg morphine or $1 \text{mg/kg}$ (+)-3,4-methylenedioxymethamphetamine [(+)-MDMA] for 42 d. Control rats received physiological saline. After the treatment period, the rats were decapitated under anaesthesia, cerebral cortices from the brain were removed on ice and the samples were stored at $-80^\circ \text{C}$ until required for measurement. Experiments were performed in accordance with a protocol approved by the University Ethics Committee on laboratory animals.

**Semi-quantitative reverse transcription–PCR (RT–PCR)**

Total cellular RNA was extracted from the brain cortex with acid guanidium-thiocyanate phenol/chloroform (Chomczynski and Sacchi, 1987). Five micrograms of total RNA was transcribed into cDNA with oligo(dt) primers (RevertAidTM First Strand cDNA Synthesis kit, Fermentas, St Leon-Rot, Germany). cDNA was amplified by PCR with oligonucleotides for $\beta$-actin and APP. For $\beta$-actin, the oligonucleotide primers were sense, 5'-GGCTGTGGTGGTCCCCGTAT-3', 2203–2221 bp; and antisense, 5'-CCGGCTCTTTGCCATAGTG-3', 2554–2536 bp (Genbank accession no. J00691) (Shi et al., 1997). The numbers of nucleotide bases were according to the rat APP genomic sequence numeration from Genbank (accession no. X074648). The APP oligonucleotides were: sense, 5'-GATCCGGAGTTCCGGACATG-3', 1788–1807 bp; antisense, 5'-GTCTGCATCTGCTCAAAG-3', 2085–2067 bp sense primers, which includes the transmembrane domain. PCR was performed in a final volume of 25 $\mu$l, containing 2.5 $\mu$l of 10 $\times$ PCR buffer, 2 $\mu$l of 25 mm MgCl2, 1 $\mu$l of 5 mm dNTP, 6 pmol $\beta$-actin, 25 pmol APP, 2 $\mu$l of the cDNA described above and 0.25 $\mu$l of Taq DNA polymerase (5 units) (Fermentas). Thirty cycles consisted of 1 min at 94 °C, followed by 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. After 30 cycles, extension was performed at 72 °C for 5 min. PCR products were separated by 1.3% agarose gel electrophoresis. The ratio of APP/$\beta$-actin mRNA was calculated.
Sample preparation for Western blotting

For APP immunoblotting, six samples from each group were homogenized in 50 mM Tris buffer (pH 7.5), containing 0.15 M NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 2 µg/ml leupeptin and 1 µg/ml pepstatin by using a glass-Teflon potter (1500 rpm, 1 min). The homogenates were centrifuged at 10 000 g for 30 min at 4 °C. The supernatant represented the soluble fraction, while the pellet was resuspended in the same volume of buffer containing 1% sodium dodecyl sulphate (SDS) and centrifuged as before. The new supernatant represented the membrane fraction.

For BACE measurement, the soluble and membrane-bound fractions were not separated. Cortices were homogenized in 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl, 2 mM PMSF, 2 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin and 1% SDS. The homogenates were centrifuged at 10 000 g for 30 min at 4 °C. The supernatants were used for protein assay (Hess et al., 1978).

Electrophoresis and Western blotting

Twenty-microgram samples of protein from cytosolic and membranous fractions in SDS sample buffer were loaded onto 9% SDS–polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes by using the Bio-Rad Mini Electrophoresis and Western blotting apparatus. The nitrocellulose was washed again with TBST three times, incubated with the rabbit IgG-HRP (1:5000; Jackson Immunoresearch) for 1 h. The nitrocellulose was washed with TBST again with TBST three times, incubated with the monoclonal 22C11 antibody (5 µg/ml; against residues 68–81 of APP, Chemicon International Inc., USA) or Alz-90 monoclonal antibody (10 µg/ml; against residues 511–608, Chemicon International Inc.) was applied. In independent experiments, BACE polyclonal antibody (1:1000; Chemicon International Inc.) was used to detect the enzyme cleaving APP to produce β-amyloid. After three washes with TBST, goat anti-mouse IgG-HRP (1:500; Jackson Immunoresearch, West Grove, PA, USA) or goat anti-rabbit IgG-HRP (1:500; Jackson Immunoresearch) was added for 1 h. The nitrocellulose was washed again with TBST three times, incubated with the Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to Kodak autography film.

Densitometric analysis and statistics

In the case of the semi-quantitative RT–PCR experiments, the ratios of APP mRNA/actin mRNA were calculated. The ratio for the control animals, taken as 100%, was compared with the ratios for the treatment groups. Western blot optical densities of immunoreactive bands were quantified by means of the NIH Image Analyzer System. The levels of APP and BACE in the control group were taken as 100% and changes were calculated in respect to them. The results were assessed statistically with ANOVA and a LSD post-hoc probe was used for individual comparisons. A probability level of p < 0.05 was considered significant.

Results

Semi-quantitative RT–PCR

In order to detect changes in APP transcription after the chronic morphine and (+)MDMA treatment, semi-quantitative RT–PCR was performed. APP (297 bp) and β-actin mRNA (351 bp) could be detected in the control-, morphine- and (+)MDMA-treated rat brain cortices. The same APP mRNA band, corresponding to that including the Kunitz protease inhibitor domain, was similarly present in all examined groups.

The APP/β-actin mRNA ratios were calculated and expressed as percentages of the control values (94% ± 0.026) in the soluble fraction (ANOVA: df = 3.6) and 108% ± 0.129 (ANOVA: df = 2.9) in the membrane-bound fraction (ANOVA: df = 2.7) in the morphine- and (+)MDMA-treated groups respectively. A significant increase in the APP/β-actin mRNA ratio was observed after the 10 mg/kg (+)MDMA treatment (ANOVA: df = 16, F = 8.886, p = 0.003; LSD post-hoc probe p = 0.026) while morphine administration had no significant affect (LSD post-hoc probe p = 0.059).

Western immunoblotting

The APP signal of lysates from samples obtained from untreated or treated rats was resolved at ~105–110 kDa (Figure 1a, b). Membrane-bound APP levels were not significantly altered either by the morphine or the (+)MDMA treatments (ANOVA: df = 19, F = 1.324, p = 0.292; LSD post-hoc probes p = 0.129 and p = 0.561 respectively).

Treatment of the animals with (+)MDMA caused a significant decrease (ANOVA: df = 19, F = 16.302, p = 0.001; LSD post-hoc probe p = 0.026) in the soluble APP level (55% of the control value), while it remained unchanged after the morphine treatment (LSD post-hoc probe p = 0.165) (Figure 1c).

The 22C11 antibody used to analyse APPs cross-reacts with both APP and amyloid-like precursor protein 2 (Wolozin et al., 1996). For this reason, we have investigated the effects of morphine or MDMA on APP levels using Alz-90, an antibody specific to
APP. Very similar changes in APP levels were observed with both antibodies (data not presented).

To investigate regulatory factors participating in the APP processing, BACE levels were monitored by immunoblotting after treatment with morphine or (+)MDMA. The BACE antibody detected a single band at 75 kDa (Figure 2). Morphine treatment did not cause any significant change (ANOVA: df = 11, \( F = 3.574, p = 0.072 \); LSD post-hoc probe \( p = 0.165 \)) in BACE levels (Figure 2a, b), while a consistent, significant (25%) increase (LSD post-hoc probe \( p = 0.026 \)) was observed after (+)MDMA treatment (Figure 2a, b).

**Discussion**

No information was previously available on the effects of MDMA and morphine treatment on APP processing. The present study demonstrates that chronic (+)MDMA treatment results in a 25% increase in BACE levels. BACE activity is expected to play a fundamental role in the control of \( \beta \)AP production and thus in the aetiopathogenesis of AD. Increased BACE-1 protein expression (Holsinger et al., 2002), levels (Fukumoto et al., 2002) and \( \beta \)-secretase activity (Yang et al., 2003) have been found in the brain of AD patients. Since the amount of \( \beta \)AP produced by the neurons is related to the BACE expression level (Bigl et al., 2000), we conclude that chronic (+)MDMA treatment might increase the possibility of \( \beta \)-site-specific cleavage of APP.

An explanation of our finding that chronic (+)MDMA treatment can increase the BACE protein levels is still missing. The chronic, neurotoxic effect of MDMA in the brain is very complex, including the long-term depletion of biogenic amines, mostly 5-HT, and the induction of early, immediate genes, inflammation and free radicals, leading to a final neurodegenerative process (Green et al., 2003). MDMA-evoked increased free-radical production may be one possible mechanism whereby BACE can be induced in the rat brain.

In-vitro experiments show that oxidative stress induces increased BACE protein levels and activity (Tamagno et al., 2002). It is pertinent that similar findings of increased BACE production have recently been reported after experimental transient cerebral ischaemia (Wen et al., 2004) and traumatic brain injury (Blasko et al., 2004), conditions where oxidative damage has likewise been proposed to be involved in
the pathomechanism. On the other hand, the chronic (+)-MDMA-induced increased production of BACE could also provoke and contribute to cell death and neuronal degeneration (Wen et al., 2004), both of which are observed in MDMA-treated (O’Shea et al., 1998) and AD brains (Hugon et al., 2000).

The polyclonal antibody used in our experiment to recognize BACE is not able to distinguish between BACE1 and BACE2. Since BACE1 is the more predominant form in the brain and colocalizes with intracellular sites of βAPP production, while BACE2, a homologue of BACE1, is less abundant (Farzan et al., 2000), we presumed that most of the increase in BACE protein levels we observed after MDMA treatment was due to changes in the BACE1 level.

Our observation, of a 45% decrease in the amount of soluble APP unfortunately does not clearly indicate whether chronic (+)-MDMA treatment is likely to shift the alternative cleavage of APP to the BACE-mediated process or not, since the applied antibody (22C11) is not able to distinguish between the different soluble APP isoforms, such as soluble APP, soluble βAPP and soluble βAP. On the other hand, soluble αAPP is regarded as a non-amyloidogenic product of α-secretase with neuroprotective and trophic effects (Milward et al., 1992; Mucke et al., 1995). The fact that chronic (+)-MDMA treatment might be able to decrease the amount of this product provides further evidence that this type of abusive drug can be detrimental to the brain. It has been shown earlier that 5-HT is able to stimulate the secretion of APPs in a PKC- and PLA2-mediated process (Nitsch et al., 1996). Since one of the most robust effects of MDMA is the release and depletion of biogenic amines, mainly 5-HT, from nerve terminals, leading to specific neurotoxic damage and neurodegeneration of 5-HT neurons, it is tempting to speculate that our observation of the 45% decrease in the amount of APPs reflects the MDMA treatment-induced neurodegenerative process.

Our finding is that the decreased amount of soluble APP may be explained by reduced translation or increased degradation of the APP molecule. Since the regulation of APP processing involves many factors, further in-vivo and in-vitro investigations are required to contribute to an understanding of the exact mechanism of the changes in APP processing caused by MDMA.

The slight but significant increase in the production of APP mRNA as a consequence of the low doses chronic (+)-MDMA treatment might be associated with the reported neuroprotective effect of the APP molecule (Kogel et al., 2003) since even multiple low doses of MDMA could induce long-term serotonergic depletion and neurotoxicity and the increased production of this molecule could be a compensatory stress response. The underlying mechanism of action responsible for the increased cortical APP mRNA production may either be related to the (+)-MDMA-associated release of neurotransmitters and signalling, or the induction of immediate early genes since several recent studies have provided evidences that the stimulation of these multiple pathways could trigger increased APP mRNA production (Trejo et al., 1994).

Although it has long been known that the chronic use of MDMA is detrimental to learning and memory functions, there is no epidemiological or experimental evidence indicating a link or association between the use of substituted amphetamines or morphine and AD.

Surprisingly, the other addictive compound we tested, morphine, had only partial effect on the transcriptional and translational regulation of APP in our experimental system. Since only one dosage was tested we can not exclude the possibility that chronic morphine treatment, or the stimulation of μ-type opioid receptors with other compounds or higher morphine doses could be more effective. Further experiments are necessary in order to answer these questions. It is pertinent to mention here that treatment with the weak partial agonist naloxone is able to prevent βAP-induced peroxidative damage, but its effect is not related to the opiate receptor-binding capacity of this molecule (Liu et al., 2002).

It is important to distinguish the short-term changes that result from a single (acute) exposure to MDMA or opioids from the chronic effects of these drugs. The latter develop gradually over time in response to repeated treatments resulting in tolerance, addictive state and dependence in the case of morphine, while on the other hand resulting in sensitization and neurotoxicity in the case of MDMA. These states are all associated with responses in cellular and subcellular level within the CNS and persist for a long time after the cessation of the exposure (Shalev et al., 2002). Since AD is considered as a chronic neurodegenerative disorder where the development of specific amyloid related neuropathology starts decades before the onset of the clinical symptoms the acute abuse or single doses of MDMA and morphine are, therefore, not likely to provoke long-lasting alterations in the APP metabolism. This was the reason why we wanted to focus only on the chronic effect of these addictive drugs in our experimental design and why acute treatment experiments with multiple doses were not performed.
Regarding the selection of the treatment doses, big strain dependent differences have been reported for both compounds tested in our experiments. A direct neurotoxic effect of MDMA has been found on Wistar rats with doses >1 mg/kg (Shankaran and Gudelsky, 1999). Furthermore recent studies demonstrated MDMA neurotoxicity in rats following doses that are fraction of those published earlier (O’Shea et al., 1998). A multiple low dose (1 mg/kg) of (+)MDMA was therefore chosen, which does not induce sensitization or neurotoxicity on the serotonergic system. Higher doses, e.g. 2.5 mg/kg MDMA given for 6 d, have been shown to produce sensitization both to hyperthermic and hyperkinetic responses (Dafters, 1995). Moreover, chronic administration of even higher doses (4 mg/kg) induce long-term serotonergic depletion in hippocampal and striatal areas, therefore, a dose of 1 mg/kg was applied in our study. This dose of (+)MDMA roughly corresponds to that used by non-addict human ecstasy users (Bolla et al., 1998). However, the validity of the inter-species dose scaling in relation to MDMA has been also questioned (Vollenweider et al., 2001).

Furthermore, it should be considered that the dose of morphine given once a day in our experiments is too low to induce marked tolerance or dependency in rats. In other experimental designs where chronic morphine-induced adaptive changes resulting in tolerance and dependence were examined, much higher doses and a different route of administration and time were used than those applied here (Fábian et al., 2002; Timár et al., 2005).

In the case of morphine there are no in vivo data indicating the dose of the neurotoxic effect except one recent paper where the dose and the time-frame was similar to our experimental design (Atici et al., 2004). According to the review of Tegeder and Geisslinger (2004) the applied doses were also in the range of 1–20 mg/kg when growth-promoting or inhibiting effects of opioids were tested in vivo on non-neuronal tissues in chronic experiments, and the length of the treatment period was also similar to our experiments.

Unfortunately we can not exclude the possibility that acute treatments with the same or even higher doses than those applied here would have a similar effect on the APP metabolism as found in the present investigation. Beside the dilemma of the applied treatment doses, another possible limitation of our study that was that only female rats were used. We can not answer the question, therefore, whether the gender, sexual cycle and sexual hormone levels might have an impact on MDMA- and morphine-induced changes in APP metabolism in our experiments. A further methodological problem is that the use of a housekeeping gene as an internal standard was omitted in our Western-blot experiments. Careful interpretation of these type of results is therefore necessary.

In conclusion, we found in vivo evidence that chronic (+)MDMA treatment increases APP mRNA and BACE protein levels and decreases soluble APP levels in the rat brain. These results point to new routes whereby abuse of this recreational drug can cause degeneration in neuronal cells. Further studies are necessary on humans with MDMA or morphine abuse and addiction to answer the question of whether the APP metabolism is similarly affected as we observed here.

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Statement of Interest

None.

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