Alpha-1B adrenergic receptor knockout mice are protected against methamphetamine toxicity

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Abstract
The psychostimulant methamphetamine (MA) is toxic to nigrostriatal dopaminergic terminals in both experimental animals and humans. In mice, three consecutive injections of MA (5 mg/kg, i.p. with 2 h of interval) induced a massive degeneration of the nigro-striatal pathway, as reflected by a 50% reduction in the striatal levels of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC), by a substantial reduction in striatal tyrosine hydroxylase and high-affinity DA transporter immunostaining, and by the development of reactive gliosis. MA-induced nigro-striatal degeneration was largely attenuated in mice lacking α1b-adrenergic receptors (ARs). MA-stimulated striatal DA release (measured by microdialysis in freely moving animals) and locomotor activity were also reduced in α1b-AR knockout mice. Pharmacological blockade of α-adrenergic receptors with prazosin also protected wild-type mice against MA toxicity. These results suggest that α1b-ARs may play a role in the toxicity of MA on nigro-striatal DA neurons.

Keywords: α1-adrenergic receptor antagonists, methamphetamine neurotoxicity, Parkinson’s disease.


Neurotoxicity by psychostimulants is a major medical concern for the widespread use of these drugs in Western countries. Amphetamines are potentially harmful to central dopaminergic pathways (Ricurte and McCann 1992) and, among these drugs, methamphetamine (MA) is the most harmful because of its high potency and slow elimination kinetics (Cho et al. 2001). MA abusers show a reduction in the brain levels of the high-affinity dopamine transporter and other biochemical markers of dopaminergic terminals (Wilson et al. 1996; McCann et al. 1998; Volkow et al. 2001a,b). Brain dopamine neurotoxicity is also shown in baboons treated with doses of MA comparable with those recreationally abused by humans (Villemagne et al. 1998). The recent availability of the free base of MA (‘ice or crank’) will likely increase the incidence of neurodegeneration in MA abusers. Finding new strategies that would limit the occurrence of neurological complications in these individuals is a primary need in the management of MA addiction. MA treatment in mice induces the degeneration of striatal dopaminergic terminals, and is therefore considered as one of the experimental animal models of parkinsonism (Gerlach and Riederer 1996). Consistently, we have recently found that MA-treated mice show nigral intraneuronal inclusions reminiscent of Lewy bodies observed in idiopathic Parkinson’s disease (PD) and other neurodegenerative disorders (Fornai et al. unpublished data). We decided to examine the involvement of α1-adrenergic receptors (α1-ARs) in the...
pathophysiology of MA-induced nigro-striatal damage for the following reasons: (i) chemical lesions of central noradrenergic pathways is known to affect the onset and progression of experimental parkinsonism in rodents and monkeys (Mavridis et al. 1991a; Marien et al. 1993; Bing et al. 1994; Fornai et al. 1995; Gesi et al. 2000); (ii) 1b-ARs are critically involved in the trans-synaptic effects controlling the activity of dopaminergic neurons in response to d-amphetamine (Paladini et al. 2001); (iii) overexpression of 1b-ARs in transgenic mice results in a complex pattern of neurodegeneration resembling the multiple system atrophy (Zusick et al. 2000). Using knockout mice and prazosin, an 1b-AR antagonist, we now report that the lack or the pharmacological blockade of 1b-AR confers substantial protection against nigro-striatal damage induced by MA.

**Experimental procedures**

**Animals**

The generation 1b-AR knockout mice (kindly provided by S. Cotecchia, Université de Lausanne, Switzerland) has been described previously (Cavalli et al. 1997). Briefly, 129/C57BL is the common genetic background for knockout mice and their wild-type controls, which derive from the same germline of microinjected clones. For each strain, mice from different litters were randomly intercrossed to obtain wild-type and knockout progenies and never intercrossed with other strains or mated with those from the same litters; thus, their genetic background should not be too different from that of the animals described in the previous study. Since 1997, at least 40 intercrosses have been made. Wild-type mice were derived from the initial intercrosses between the heterozygous mice and bred in parallel with the knockout progeny. Mice were kept at constant temperature (21 ± 1°C) with food and water ad libitum. As MA toxicity depends on the aggregation of the animals (Wagner et al. 1981), male mice, 10 weeks old, weighing 22–24 g, were housed 10 per cage in small cages (38 × 22 cm wide and 15 cm high). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health.

**MA toxicity**

Wild-type or knockout mice were treated with (+)-MA at a dose of 5 mg/kg, i.p., injected three times, with a 2-h interval. Control groups were injected with saline. In some experiments, wild-type mice were injected with prazosin (0.5 mg/kg) 30 min prior to each (+)-MA injection. Five days after the administration of saline or (+)-MA, mice were killed by decapitation, and the brains were dissected for the determination of striatal monoamine levels.

**MPTP toxicity**

Wild-type or knockout mice were treated with MPTP hydrochloride, at a dose of 36 mg/kg, i.p.). Control groups were injected with saline. Five days after the administration of MPTP or saline, mice were killed by decapitation, and the brains were dissected for the determination of striatal monoamine levels.

**Monoamines assay**

The striatum was sonicated in 0.6 mL of ice-cold 0.1 m perchloric acid. Fifty microlitres of the homogenates were used for protein determination (Lowry et al. 1951). The remaining aliquots were centrifuged at 8000 g for 10 min, and 20 μL of the supernatants were injected into an HPLC equipped with an autosampler module 507, a programmable solvent module 126, an analytical C18 reverse-phase column kept at 30°C, Ultrosphere ODS 5 μm, 80 Å pore, 250 × 4.6 mm (Beckman Instruments, Fullerton, CA, USA), and a Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA, USA). The holding potentials were set at ±350 and ±350 mV for the detection of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The mobile phase consisted of 80 mM sodium phosphate, 40 mM citric acid, 0.4 mM EDTA, 3 mM 1-heptansulfonic acid and 12.5% methanol, brought to pH 2.75 with phosphoric acid (run under isocratic conditions, at 1 mL/min).

**Measurement of body temperature**

To test whether 1b-AR knockout mice showed an altered regulation of body temperature, we measured body temperature in wild-type or 1b-AR knockout mice. Mice were treated with (+)-MA (5 mg/kg × 3, 2 h apart). Body temperature was measured at 60, 180 and 300 min after saline or (+)-MA injection, i.e. 60 min after each injection of (+)-MA or saline. Measurements were carried out at constant daytime intervals starting at 09.00 h to avoid the influence of circadian variations. Core temperature was measured by inserting a lubricated thermometer probe in the rectum of mice (Battaglia et al. 2002). In order to prevent sudden movements, animals were handled with a wool glove while their tail was moved to allow the probe insertion. This was done in order to prevent the effects of restraint stress on body temperature. When the attempt to insert the probe was not successful (i.e. sudden movements of the animal or the need of restraining the mouse) the animal was excluded from the groups.
Assessment of locomotor activity
To measure the effects on motor activity induced by systemic administration of (+)-MA, wild-type or α1b-AR knockout mice were treated with (+)-MA at the dose of 5 mg/kg, i.p., injected three times, with a 2-h interval. Animals were weighed and injected with either saline or (+)-MA (5 mg/kg), and returned to their home cage(s) with their normal cagemates. Only one mouse at a time was injected per experimental session and placed in a plastic apparatus. Starting 5 min after injection, activity was assessed for 2 min as the number of quadrant (10 × 10 cm) crossings in the square open field (each time an injected mouse entered a different square of the cage, 1 activity count was scored).

Microdialysis in freely moving mice
Male wild-type or α1b-AR knockout mice, 10 weeks old, were used to measure the release of DA in the striatum of freely moving mice by microdialysis. Mice weighing 24–26 g were implanted with microdialysis intracerebral guides (CMA/7 Guide Cannula, CMA/Microdialysis, Stockholm, Sweden), under ketamine (100 mg/kg) + xylazine (10 mg/kg), i.p., anaesthesia, in a Kopf stereotaxic frame. The site of implantation was the left striatum (coordinates: 0.6 mm anterior to the bregma, 1.7 mm lateral to the midline, 3.5–5.5 mm ventral from the surface of skull, according to the atlas of Franklin and Paxinos 1997). After surgery, mice were housed in separate cages in a temperature-controlled environment on a 12-h light/dark cycle, with free access to water and food, and allowed to recover for 4 days before the experiment. On the evening before the experiment, a probe was inserted into the intracerebral guide, after removing a dummy, and mice were transferred to a plastic bowl cage with a moving arm (CMA/120 System for Freely Moving Animals, CMA/Microdialysis) with free access to water and food. Concentric vertical microdialysis probes 2-mm long and 0.24 mm in outer diameter, having a cuprophane membrane with a molecular cut-off of 6000 Da (CMA/7 Microdialysis Probe, CMA/Microdialysis), were used. The probes were perfused continuously with artificial cerebro-spinal fluid (ACSF), at a flow rate of 1.5 μL/min, using a microinjection pump (Bioanalytical System Inc., West Lafayette, IN, USA). The ACSF contained: 150 mM NaCl, 3 mM KCl, 1.7 mM CaCl₂, 0.9 mM MgCl₂. This solution was not buffered, and the pH was typically 6.5. The following morning, 30 μL (20 min) of consecutive perfuse sample fractions were continuously collected by a fraction collector (CMA/142 Microfraction Collector, CMA/Microdialysis). After four sample fractions, used to determine the basal levels of monoamines, wild-type or knockout mice received three injections of (+)-MA (5 mg/kg, i.p.), 2 h apart, and sample fractions were collected for the following 2 h, after each (+)-MA injection. Analysis of DA was performed by HPLC with electrochemical detection, as described above.

Measurements of (+)-MA levels
To exclude that the absence of α1b-ARs could have induced changes in the central bioavailability of (+)-MA, we measured striatal levels of (+)-MA in wild-type or α1b-AR knockout mice. Mice were treated with (+)-MA (5 mg/kg) and (+)-MA levels were measured at 60, 120 and 240 min after (+)-MA injection. The striatum was sonicated in 0.6 mL of ice-cold 0.1 M perchloric acid. Fifty microliters of the homogenates were used for protein determination (Lowry et al. 1951). The remaining aliquots were centrifuged at 8000 g for 10 min, and 100 μL of the supernatants were assayed for (+)-MA, using a fluorescence polarization immunoassay (Adx, Abbott Park, IL, USA). To exclude any cross-reactivity within the immunoassay, we used the corpus striatum of saline-injected mice as controls. The specificity of the MA assay has been validated performing a mass spectrum of the molecule in the homogenate: gas chromatography mass-spectrometry was carried out both in the scan mode and ion monitoring (Fornai et al. 1999) to distinguish between MA and amphetamine.

Results
Lack of MA toxicity in α1b-AR knockout mice
In wild-type mice, MA treatment (5 mg/kg, i.p., injected three times with 2 h of interval) led to about 50% reduction in the striatal DA and DOPAC levels and a smaller reduction in HVA levels 5 days after the treatment (Fig. 1). MA did not cause any drop of DA and its metabolites in α1b-AR knockout mice, although the basal DA levels did not differ between knockout and wild-type mice (Fig. 1). The resistance of α1b-AR knockout mice to the neurotoxic action of MA was confirmed by the immunodetection of TH, DAT and GFAP. In wild-type mice, MA treatment also led to a substantial reduction in striatal TH and DAT immunostaining associated with an increase GFAP immunostaining, which is indicative of reactive gliosis (Eisch et al. 1992; Pu and Vorhees 1993; Battaglia et al. 2002) (Fig. 2). No significant changes in TH or DAT immunoreactivity were detected in the substantia nigra, at least 5 days after MA treatment (not shown). MA-induced changes in striatal TH, DAT and GFAP immunoreactivity were substantially attenuated in α1b-AR knockout mice (Fig. 2). As hypothermia has been implicated in the protective effect of some drugs against MA neurotoxicity (Ali et al. 1994; Bowyer et al. 1994; Miller and O’Callaghan 1994; Albers and Sonsalla 1995), we measured core body temperature in both wild-type and α1b-AR knockout mice treated with MA (5 mg/kg, i.p., injected three times with 2 h of interval). Body temperature was measured three times, 60 min after each single injection of MA. Each injection of MA increased body temperature by about 1.5–2°C in both wild-type and knockout mice with no difference between the two groups of animals (Table 1). To exclude that the resistance of α1b-AR knockout mice was due to changes in the central bioavailability of MA, we measured striatal MA levels at 1, 2 or 4 h after a single MA injection (5 mg/kg, i.p.). We did not observe any difference in striatal MA levels between wild-type or knockout mice (Table 2).

MA-stimulated DA release and locomotor activity in α1b-AR knockout mice
We examined whether the primary mechanism of action of MA required the presence of α1b-ARs by measuring the
stimulation of DA release in the striatum of freely moving mice. MA treatment in wild-type mice induced a rapid rise in extracellular DA, which was higher and more prolonged after the second and third injections of the drug. Interestingly, it was the response to the second and third injections of MA that was specifically reduced in α1b-AR knockout mice (Fig. 3). We obtained the same results by measuring locomotor activity. MA treatment in wild-type mice induced an increase in locomotor activity, which was slight after the first injection, but became remarkable after the second and third injections. Behavioural sensitization to MA was completely abolished in α1b-AR knockout mice, which did not respond to the second and the third injections of MA (Table 3). Taken together, these results suggest that α1b-ARs are involved in mechanisms contributing to the progressive amplification of MA response.

Pharmacological blockade of α-adrenergic receptors protects against MA toxicity
MA toxicity (5 mg/kg, i.p., three injections with a 2-h interdose interval) was tested in wild-type mice treated with the α1-AR antagonist, prazosin (0.5 mg/kg, i.p., injected 30 min prior to each injection of MA). Prazosin treatment prevented the 50% reduction of striatal DA and DOPAC levels induced by MA (Fig. 4). Neuroprotection was confirmed by the immunohistochemical analysis of striatal TH, DAT and GFAP (Fig. 5).

MPTP toxicity in α1b-AR knockout mice
We extended the study to mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to assess whether the presence of α1b-ARs could contribute to nigrostriatal degeneration in a second (and more-established) experimental model of parkinsonism. A single injection of MPTP (30 mg/kg, i.p.) in wild-type mice led to a substantial reduction in striatal DA levels (35.2 ± 6.5 ng/mg protein vs. 64.9 ± 7.1 in mice treated with saline) and DOPAC levels (6.3 ± 1.3 ng/mg protein vs. 9.5 ± 1.4 in mice treated with saline), when assessed 5 days later. The same extent of reduction was observed in α1b-AR knockout mice treated with MPTP (DA levels: 32.5 ± 8.2 ng/mg protein vs. 53.4 ± 10.3 ng/mg protein in mice treated with saline; DOPAC levels: 6.1 ± 2.1 ng/mg protein vs. 9.0 ± 1.6 ng/mg protein in mice treated with saline). This indicated that neuroprotection induced by the lack of α1b-ARs was specific for MA toxicity and could not be extended to MPTP toxicity.

Discussion
Recent evidence suggests that α1-ARs are involved in the response of central dopaminergic neurons to amphetamine. Activation of α1b-ARs by the DA released in response to amphetamines desensitizes the metabotropic glutamate receptor-mediated hyperpolarization of mid-brain dopaminergic neurons, a mechanism that may sustain the rewarding properties of amphetamine (Paladini et al. 2001). In addition, α-AMPH fails to increase extracellular dopamine levels in α1b-AR knockout mice (Auclair et al. 2002). The rewarding properties of cocaine and morphine are also reduced in mice lacking α1b-ARs (Drouin et al. 2002), although animals which go through development without the receptor may be deficient in complex ways. A prolonged use of amphetamine derivatives, like MA, causes nigro-striatal damage in both experimental animals and humans (Ricaurte et al. 1982, 1984; Woolverton et al. 1989; Ricaurte and McCann 1992; McCann et al. 1998; Villemagne et al. 1998; Volkow et al. 2001a,b; Battaglia et al. 2002). It is well established that low doses of MA (5 mg/kg × 3, i.p.) damage DA nerve terminals (see Davidson et al. 2001 for a review) without affecting DA cell bodies in substantia nigra, although
high doses (10 mg/kg × 4, i.p.) may cause neuronal cell body loss in this region (Sonsalla et al. 1996). A role for α1b-ARs in neurodegeneration is supported by the finding that transgenic mice overexpressing either wild-type or constitutively active α1b-ARs show nigro-striatal damage associated with serious locomotor dysfunction, tremor at rest, epileptic seizures and autonomic dysfunctions (Zuscik et al. 2000). Present data show that α1b-ARs are directly involved in the mechanism of MA-induced neurodegeneration. MA is transported into dopaminergic terminals, where it displaces DA from synaptic vesicles, causing large increases in DA release (O’Dell et al. 1991; Cubells et al. 1994). α1b-ARs

Table 1 Core body temperature in wild-type and α1b-AR knockout mice treated with MA

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Body temperature (°C)</th>
<th>60 min</th>
<th>180 min</th>
<th>300 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>37.2 ± 0.11</td>
<td>37.3 ± 0.15</td>
<td>37.4 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>38.7 ± 0.32*</td>
<td>38.9 ± 0.35*</td>
<td>39.0 ± 0.40*</td>
<td></td>
</tr>
<tr>
<td>α1b-AR knockout mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>37.4 ± 0.08</td>
<td>37.2 ± 0.11</td>
<td>37.3 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>38.5 ± 0.29*</td>
<td>38.6 ± 0.26*</td>
<td>38.7 ± 0.21*</td>
<td></td>
</tr>
</tbody>
</table>

Body temperature was measured 1 hour after each injection of methamphetamine (5 mg/kg, i.p., repeated three times with 2 h of interval). Values are mean ± SEM of four individual determinations. *p < 0.01 (student’s t-test) versus the respective values in mice injected with saline.

Table 2 Striatal MA levels in wild-type and α1b-AR knockout mice

<table>
<thead>
<tr>
<th>Treatments</th>
<th>MA (µg/mg protein)</th>
<th>60 min</th>
<th>120 min</th>
<th>240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type mice</td>
<td>0.065 ± 0.003</td>
<td>0.026 + 0.001</td>
<td>0.010 + 0.001</td>
<td></td>
</tr>
<tr>
<td>α1b-AR knockout mice</td>
<td>0.064 ± 0.003</td>
<td>0.029 + 0.001</td>
<td>0.011 + 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Striatal MA levels were measured 60, 120 and 240 min after injection of MA (5 mg/kg, i.p.). Values are mean ± SEM of four individual determinations.

Fig. 2 Immunohistochemical analysis of TH (\(\text{a}^1\)), DAT (\(\text{b}^1\)), and GFAP (\(\text{c}^1\)) in the corpus striatum of wild-type mice injected with saline (a) or MA (b), and of α1b-AR knockout mice injected with saline (c) or MA (d). Densitometric analysis of striatal TH (e) and DAT (f) immuno-

staining has been performed on comparable sections from five to six mice per group. Scale bars = 50 µm *p < 0.05 (one-way ANOVA + Fisher’s PLSD) versus saline-treated wild-type mice. **p < 0.05 (one-way ANOVA + Fisher’s PLSD) versus MA-treated wild-type mice.
are key regulators of the trans-synaptic mechanisms that sustain the release of DA in response to MA (Paladini et al. 2001). Accordingly, the progressive increase in DA release and locomotor activity induced by MA was substantially attenuated in our mice lacking α1b-ARs. Similar results were obtained in mice pre-treated with the α1-AR antagonist, prazosin, although this drug is not subtype-selective. Although the mechanism by which α1b-ARs (or other α1-AR subtypes) amplify the response to MA is unknown, these findings suggest that pharmacological inhibition of these receptors may limit the potential damage to nigrostriatal DA neurons induced by MA. Knowing that in mice, MA induces nigral inclusions reminiscent of the Lewy’s bodies of Parkinson’s disease (Fornai et al. unpublished data), it was reasonable to expect that the lack of α1b-ARs could be protective in a more established model of parkinsonism, such as the MPTP model. However, this was not the case because a substantial drop in striatal DA and DOPAC levels was equally seen in wild-type and α1b-AR knockout mice treated with MPTP. Although MPP+ (the toxic metabolite of MPTP) and MA are both transported into dopaminergic terminals by the high-affinity DA uptake system, the mechanism underlying the neurotoxic effect of these drugs is different. While MA kills dopaminergic...
terminals by generating reactive oxygen species as result of DA oxidation (and perhaps through other mechanisms – Battaglia et al. 2002), MPP+ inhibits complex I of the mitochondrial respiratory chain (Cubells et al. 1994; Przedborski and Jackson-Lewis 1998). This latter mechanism may be sufficient to the induction of nigro-striatal degeneration independent of any additional contribution provided by α1b-ARs. Thus, we conclude that endogenous activation of α1b-ARs specifically contributes to nigro-striatal degeneration induced by MA, and perhaps other amphetamine derivatives, whereas a role for these receptors in the pathophysiology of neuronal damage in Parkinson’s disease remains unclear. In addition, it is noteworthy that α1-AR antagonists may worsen motor symptoms in Parkinson’s disease, as suggested by a stimulating effect of α1-ARs on dopamine release (Mavridis et al. 1991b; Andersson et al. 1994; Sommermeyer et al. 1995; Bedard et al. 1998; Auclair et al. 2002).

Acknowledgements

We thank Dr S. Cotecchia (Université de Lausanne, Switzerland) for kindly providing α1b-AR knockout mice. We thank Fabrizio Pontarelli for technical assistance.

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