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ORIGINAL INVESTIGATION

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Studies on the neuroprotective effect of pentobarbitone on MDMA-induced neurodegeneration

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Abstract Administration of a dose of 15 mg/kg of the recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") to Dark Agouti rats resulted in an acute hyperthermic response which was followed 7 days later by a marked (≈45%) loss of 5-HT and its metabolite 5-HIAA in cortex, hippocampus and striatum and a similar loss of [^{3}H]-paroxetine binding in cortex. These losses reflect the MDMA-induced neurotoxic degeneration of 5-HT nerve endings. Administration of pentobarbitone (40 mg/kg) concurrently with MDMA produced a significant attenuation of the neurotoxic damage, but also acute hypothermia. When the temperature of the MDMA plus pentobarbitone-treated group was kept elevated to that of the MDMA-treated group by the use of a homeothermic blanket, the neuroprotective effect of pentobarbitone was lost. These data demonstrate that pentobarbitone appears to possess no intrinsic neuroprotective activity and the previously reported activity is due to a hypothermic action of the drug.

Key words 3,4-Methylenedioxymethamphetamine · GABA · "Ecstasy" · Neurodegeneration · Pentobarbitone · 5-Hydroxytryptamine · Hyperthermia · Neuroprotection

Introduction

Administration of the recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") has been shown to produce long term neurotoxic degeneration of 5-hydroxytryptamine (5-HT) nerve terminals in several regions of the brain of a variety of animal species (Green et al. 1995). The degeneration has been demonstrated both histologically (Molliver et al. 1990) and biochemically, the damage being reflected in a long term decrease in the concentration of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) and a decrease in the binding of [^{3}H]-paroxetine to the presynaptic 5-HT transporter on the nerve ending (e.g Hewitt and Green 1994).

It has previously been shown that dizocilpine, a compound that is an effective neuroprotective agent in several animal models of acute ischaemic stroke (Small and Buchan 1997), protects against MDMA-induced neurodegeneration (Farfel et al. 1992; Colado et al. 1993; Hewitt and Green 1994). However, whilst this observation was recently confirmed, evidence was also presented which suggested that dizocilpine only protected because in combination with MDMA it induced hypothermia (Farfel and Seiden 1995a). This proposal has recently received support from a study which examined the effect of another NDMA antagonist (AR-R15896AR) which found that this drug neither prevented the MDMA-induced hyperthermia nor protected against MDMA-induced damage (Colado et al. 1998).

Clomethiazole is another compound which also protects against neurodegeneration in a wide variety of animal models of stroke (Green 1998) and which has been found to produce protection against MDMA-induced damage (Colado et al. 1993; Hewitt and Green 1994). Recently, we showed that this agent provided significant protection against MDMA-induced neurodegeneration and did so by a mechanism that did not involve lowering of body temperature (Colado et al. 1998).

Since clomethiazole is known to enhance $GABA_A$ receptor function (see Cross et al. 1989; Green et al. 1996), it seemed possible that it was this property that

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was responsible for its ability to protect against MDMA-induced damage to the brain, particularly as we have previously shown that pentobarbitone, another compound which, like clomethiazole, can enhance GABA receptor function by interacting at a site associated with the chloride channel of the GABA receptor complex (see Cross et al. 1989; Moody and Skolnick 1989; Green et al. 1996), was also neuroprotective against MDMA-induced degeneration (Colado and Green 1994). However, the pentobarbitone study was conducted before it became apparent that changes in body temperature could markedly affect the protective effect of drugs on MDMA-induced neurotoxicity (Farfel and Seiden 1995a,b; Malberg et al. 1996; Colado et al. 1998). We have therefore now investigated whether the activity of pentobarbitone (PTB) as a neuroprotective agent against MDMA-induced damage is also independent of any effect on body temperature. In order to prevent hypothermia being induced by PTB, one experiment was conducted in which the temperature of the rats given MDMA and PTB was kept elevated to match that of rats given only MDMA with a homeothermic blanket. Seven days later, several markers of neurodegeneration of 5-HT nerve terminals, namely the concentration of 5-HT, 5-HIAA and [^{3}H]-paroxetine binding to the presynaptic 5-HT transporter, were quantified in the brain.

Materials and methods

Animals, drug administration and temperature measurement

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985). Adult male Dark Agouti rats (Interfauna, Barcelona) weighing 150–170 g were used in all experiments. They were housed in groups of five in conditions of constant temperature (21°C ± 2°C) and a 12-h light dark cycle (lights on: 0700 hours) and given free access to food and water. Rats were injected with pentobarbitone (PTB, 25 or 40 mg/kg, IP, Sigma Chemical Co., UK) or saline 5 min before and 55 min after MDMA (15 mg/kg, IP). PTB was dissolved in volumes of 12.5 and 20 mg/ml and rats injected with a volume of 2 ml/kg. Rectal temperature was measured for the next 6 h by means of a digital readout thermocouple (Type K thermometer, Portec, UK) with a resolution of ± 0.1°C and accuracy of 0.2°C attached to a CAC-005 Rodent Sensor which was inserted 2.5 cm into the rectum, the rat being lightly restrained by holding in the hand. A steady readout was obtained within 10 s of probe insertion.

Experiment with the homeothermic blanket

In one experiment the temperature of the rats given MDMA plus PTB was kept elevated to match that of rats given only MDMA using a homeothermic blanket. This was achieved by placing the rats in a cage containing a Harvard Homeothermic Blanket system (Model 50–7087).

Measurement of 5-HT, 5-HIAA and [^{3}H]-paroxetine binding

For measurement of 5-HT and 5-HIAA rats were killed by cervical dislocation and decapitation, the brains rapidly removed and cortex, hippocampus and striatum dissected out on ice. Tissue was homogenised and 5-HT and 5-HIAA measured by high performance liquid chromatography (HPLC) as described in detail by Colado et al. (1997). [^{3}H]-Paroxetine binding was measured in the cortex by the method described in detail by Hewitt and Green (1994). Briefly, tissue was homogenized in ice-cold TRIS-HCl (50 mM; pH 7.4) containing NaCl (120 mM) and KCl (5 mM) using an Ultra-Turrax. The homogenate was centrifuged at 30 000 *g* for 10 min at 4°C. The supernatant was discarded and the wash procedure repeated twice more. The pellet finally resuspended in the TRIS buffer at a concentration of 10 mg tissue/ml. The assay solution (1 ml) contained [^{3}H]-paroxetine (1 nM) and 800 µl tissue preparation with the addition of 5-HT (100 µM) for determination of non

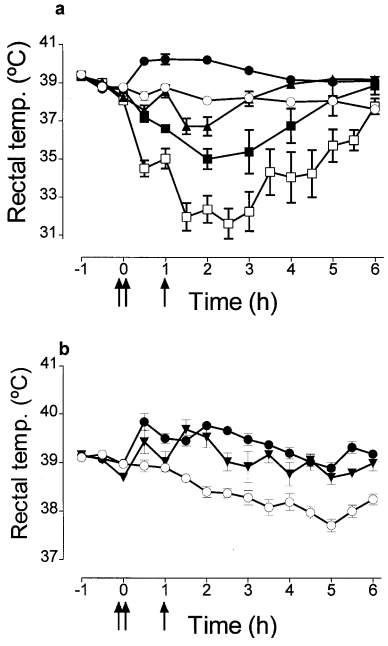

Fig. 1 a,b Effect of pentobarbitone (*PTB*) on MDMA-induced hyperthermia. **a** Rectal temperature of rats injected with saline or MDMA (15 mg/kg, IP) at time zero and of rats injected with PTB (25 or 40 mg/kg, IP) at −5 min and +55 min and treated with saline or MDMA at time zero. Temperature of rats given PTB (40 mg/kg) and saline is also shown. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature [$F(1,10) = 106.29$, $P < 0.001$] compared with the saline injected group. PTB prevented the MDMA-induced hyperthermia at both doses, 25 mg/kg [$F(1,10) = 30.83$, $P < 0.001$] and 40 mg/kg [$F(1,9) = 33.7$, $P < 0.001$] and rats given PTB 40 mg/kg and MDMA showed a marked hypothermia [$F(1,9) = 6.72$, $P < 0.05$] compared with the saline group. PTB administered to saline-treated rats also produced a significant hypothermia [$F(1,10) = 33.99$, $P < 0.001$]. **b** Effect of PTB (40 mg/kg) on MDMA-induced hyperthermia when the group injected with PTB and MDMA was kept at high ambient temperature to prevent the hypothermia resulting from this drug combination. There was no difference in basal temperature of the groups. Using the homeothermic blanket, the rectal temperature of the PTB + MDMA-treated rats was significantly higher than that of the saline group [$F(1,19) = 79$, $P < 0.001$]. Statistical analysis among groups was performed over the 6 h following MDMA administration. All results shown as mean ± SEM of 5–11 rats. ○ Saline, ● MDMA, ▲ PTB (25 mg/kg + MDMA), □ PTB (40 mg/kg), ■ PTB (40 mg/kg) + MDMA, ▼ MDMA + PTB (40 mg/kg) (homeothermic blanket)

specific binding. Incubation was for 60 min at room temperature. Assays were terminated by rapid filtration and counting of the radioactivity by scintillation spectrometry.

Data analysis

All neurochemical data were analysed by one-way ANOVA followed by Newman-Keuls test (Pharmacological Calculations, Tallarida). Analysis of the temperature data was performed by use of the statistical computer package BMDP/386 Dynamic (BDMP Statistical Solutions, Cork, Eire). Data were analysed by analysis of variance (ANOVA) with repeated measures (program 2V) or, where missing values occurred, an unbalanced repeated measure model (program 5V) was used. Both used treatment as the between subjects factor and time as the repeated measure. ANOVA was performed on both pre-treatment and post-treatment data.

Table 1 Changes in the concentration of 5-HT (ng/g tissue) and [^{3}H]-paroxetine binding (fmol/mg protein) in rat brain following MDMA (15 mg/kg, IP) and the lack of effect of pentobarbitone (PTB, 25 mg/kg, IP) on these changes. PTB was injected 5 min before and 55 min after MDMA. The rats were killed 7 days later. Results shown as mean ± SEM, $n = 5–6$

Measure	Saline	MDMA	MDMA/PTB
Cortex			
[^{3}H]-Paroxetine	80 ± 5	52 ± 5	58 ± 5
5-HT	255 ± 20	153 ± 11	183 ± 16
Hippocampus			
5-HT	276 ± 16	137 ± 6	170 ± 8
Striatum			
5-HT	440 ± 21	297 ± 15	345 ± 13

Results

Injection of MDMA (15 mg/kg, IP) resulted in a hyperthermic response lasting for more than 6 h (Fig. 1a). Administration of PTB (25 mg/kg, IP) 5 min prior and 55 min post-MDMA prevented the hyperthermic response and even produced a brief and modest hypothermia (Fig. 1a). Seven days later, rats injected with MDMA had an average 43% loss of 5-HT in hippocampus, striatum and cortex and this loss was not prevented by PTB (25 mg/kg) (Table 1).

When this experiment was repeated using a dose of PTB of 40 mg/kg, a clear and significant attenuation of the MDMA-induced monoamine loss and reduction in [^{3}H]-paroxetine binding was observed (Fig. 2). PTB (40 mg/kg) alone had no effect on the concentration of 5-HT, 5-HIAA or [^{3}H]-paroxetine binding 7 days later (Fig. 2).

However, this higher dose of PTB, in combination with MDMA resulted in a sustained hypothermia (Fig. 1a). This was perhaps not surprising, since administration of PTB (40 mg/kg) alone produced a profound hypothermia (Fig. 1a). It therefore appeared possible that the protection afforded by PTB was the result of the hypothermia observed in the MDMA plus PTB group. This experiment was therefore repeated but the rectal temperature of the MDMA plus PTB

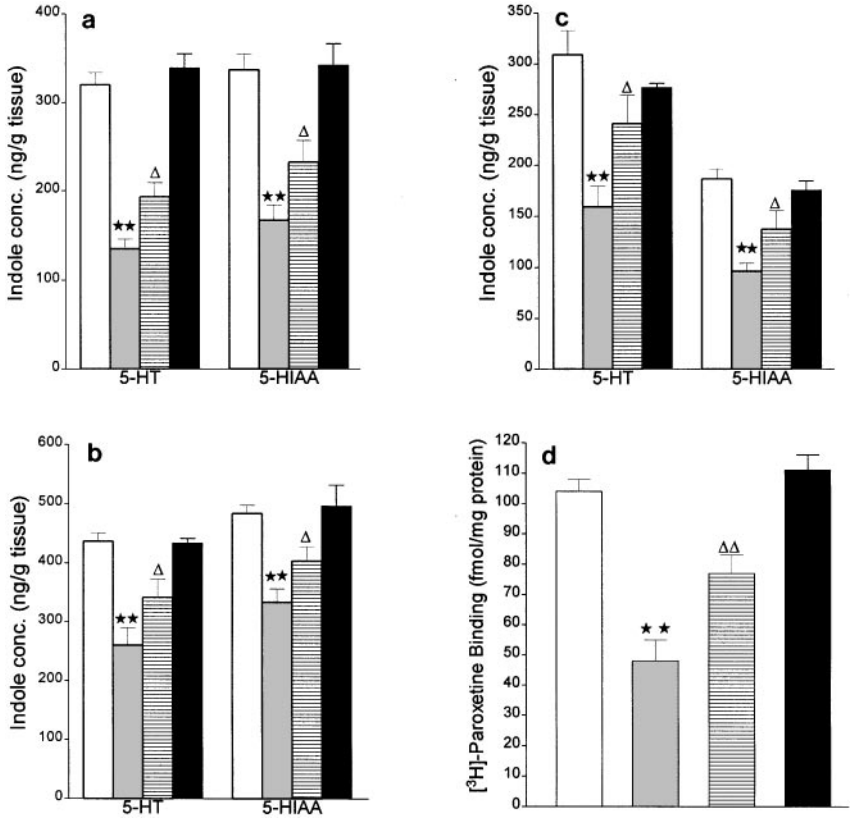

Fig. 2 a–d Indole concentrations in **a** hippocampus, **b** cortex and **c** striatum, and **d** [^{3}H]-paroxetine binding values in cortex 7 days following saline or pentobarbitone (*PTB*, 40 mg/kg, IP) 5 min before and 55 min after saline or MDMA (15 mg/kg, IP). Results shown as mean ± SEM, $n = 4–6$. Different from saline-treated: $^{**}P < 0.01$. Different from MDMA-treated: $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$. □ Saline, ▒ MDMA, ■ PTB, ▤ MDMA + PTB 40 mg/kg

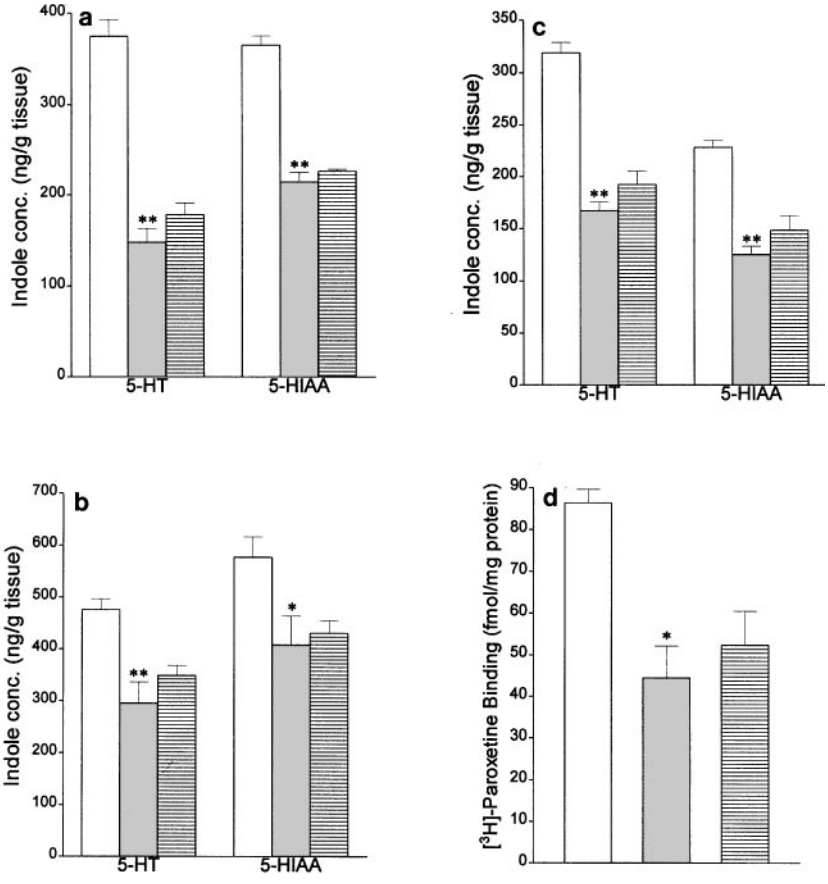

Fig. 3 a–d Indole concentrations in **a** hippocampus, **b** cortex and **c** striatum, and **d** [^{3}H]-paroxetine binding values in cortex 7 days following saline or pentobarbitone (*PTB*, 40 mg/kg, IP) 5 min before and 55 min after saline or MDMA (15 mg/kg). The MDMA + PTB group was kept at high ambient temperature during the 6 h after the first PTB injection. Results shown as mean ± SEM, $n = 4–6$. Different from saline-treated: $*P < 0.05$, $**P < 0.01$. ☐ Saline, ▩ MDMA, ▤ MDMA + PTB 40 mg/kg (homeothermic blanket)

(40 mg/kg) group was kept elevated to that seen in rats given MDMA alone by placing the rats in a cage containing a homeothermic blanket (Fig. 1b). Seven days later, brain 5-HT, 5-HIAA and [^{3}H]-paroxetine binding was measured and values found to be not statistically different from rats given MDMA alone (Fig. 3).

Discussion

The current study has confirmed our earlier report (Colado et al. 1993) that administration of pentobarbitone attenuates the neurotoxic degeneration that follows MDMA administration. However, the study also suggests strongly that this neuroprotective effect is due solely to the hypothermic action of the drug, since elevating the temperature of the PTB + MDMA-treated group to that of the MDMA alone group abolished the protection. This result therefore adds yet another compound to the increasing list of drugs which protect against MDMA-induced neurodegeneration by reducing body temperature (see Farfel and Seiden 1995a,b; Malberg et al. 1996; Colado et al. 1998). In contrast, both PBN (Colado and Green 1995; Colado et al. 1997) and clomethiazole (Colado et al. 1998) are able to protect against MDMA-induced damage through some specific neurochemical mechanism and not merely by decreasing body temperature.

These results in turn raise the question as to why pentobarbitone does not protect against MDMA-induced neurodegeneration, given that this compound, like clomethiazole, potentiates $GABA_A$ function (Cross et al. 1989; Green et al. 1996). Both compounds have been shown to displace the binding of [^{35}S]-butyl-bicyclophosphorothionate (TBPS) to the chloride channel of the $GABA_A$ receptor complex (Cross et al. 1989; Moody and Skolnick 1989; Green et al. 1996). However, there is evidence that barbiturates and clomethiazole do not act at an identical binding site (Green et al. 1996; Zhong and Simmonds 1997) and clomethiazole, unlike pentobarbitone, is also able to act as a GABA agonist (Hales and Lambert 1992; Anderson et al. 1993) The current data therefore do not rule out the involvement of GABA in the protective effect of clomethiazole.

Interestingly, the current results are compatible with studies on PTB and clomethiazole in models of global ischaemia where PTB, in contrast to clomethiazole, has minimal neuroprotective activity (Cross et al. 1991). The current results therefore do not deny a role for GABA in neuroprotection, but do illustrate further the major role that temperature can play in affording neuroprotection, not only in stroke (Busto et al. 1987;

Corbett et al. 1990; Nurse and Corbett 1996) but also in preventing neurotoxic degeneration.

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