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ORIGINAL INVESTIGATION

M. Isabel Colado · Esther O'Shea · Blanca Esteban
A. Richard Green

Studies on the neuroprotective effect of the enantiomers of AR-A008055, a compound structurally related to clomethiazole, on MDMA ("ecstasy")-induced neurodegeneration in rat brain

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Abstract *Rationale:* 3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") administration produces neurotoxic degeneration of 5-HT nerve endings in several regions of rat brain. Administration of the GABAmimetic drug clomethiazole protects against this damage. *Objective:* We wished to see whether the enantiomers of AR-A008055 (1-4-methyl-5-thiazolyl-1-phenyl-methylamine), a compound structurally related to clomethiazole, were also neuroprotective against MDMA-induced degeneration. *Methods:* (*R*)-(+)-AR-A008055 or (*S*)-(–)-AR-A008055 (100 mg/kg IP) was injected 5 min prior to and 55 min after MDMA (15 mg/kg IP) administration to Dark Agouti rats. Rectal temperature was measured during this time and the concentration of 5-HT and 5-HIAA measured in hippocampus, cortex and striatum 7 days later. [^{3}H]-Paroxetine binding was also measured in cortex. *Results:* Both enantiomers abolished the acute MDMA-induced hyperthermia and attenuated the subsequent neurotoxic loss of 5-HT, 5-HIAA and [^{3}H]-paroxetine binding. When rats given the enantiomer plus MDMA were warmed to keep their rectal temperature elevated to near that of animals given only MDMA, the neuroprotective effect of (*S*)-(–)-AR-A008055 was still seen, while the effect of (*R*)-(+)-AR-A008055 was abolished. Protection was also seen when (*S*)-(–)-AR-A008055 (50 mg/kg) was given, a dose which produced only a modest attenuation of MDMA-induced hyperthermia. *Conclusions:* The current data suggest that a major proportion of the neuroprotective action of (*S*)-(–)-AR-A008055 did not involve an attenuating effect on MDMA-induced hyperthermia. The protection afforded by (*R*)-(+)-AR-A008055, which is not a GABA agonist, appears to be solely due to its action on body temperature, strengthening the contention that abolishing the acute MDMA-induced hypothermia can produce neuroprotection. Since (*S*)-(–)-AR-A008055 has a similar pharmacology to clomethiazole, these data suggest that drugs which increase $GABA_A$ receptor channel opening are neuroprotective against MDMA-induced damage.

Keywords 3,4-Methylenedioxymethamphetamine · GABA · Ecstasy · Neurodegeneration · 5-Hydroxytryptamine · Hyperthermia · Neuroprotection · Clomethiazole · AR-A008055

M.I. Colado · E. O'Shea · B. Esteban
Departamento de Farmacologia, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain

A.R. Green (✉)
AstraZeneca R&D Charnwood, Bakewell Road, Loughborough LE11 5RH, UK
e-mail: richard.green@astrazeneca.com

A.R. Green
Pharmacology Research Group, School of Pharmacy, De Montfort University, Leicester LE1 9BH, UK

Introduction

Administration of the recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") produces 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 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 nerve endings (e.g. Hewitt and Green 1994).

Clomethiazole, a drug with marked efficacy against ischaemia-induced neurodegeneration (Green 1998; Green et al. 2000a) has been shown to also protect against MDMA-induced damage (Colado et al. 1993; Hewitt and Green 1994). Subsequently, we demonstrated that clomethiazole had a neuroprotective action distinct from its effect of attenuating MDMA-induced hyperthermia (Colado et al. 1998). Pentobarbitone, like clomethiazole, also enhances GABA receptor function by interacting at a site associated with the chloride channel of the GABA receptor complex (Cross et al. 1989; Moody and

Skolnick 1989; Green et al. 1996) and was also initially shown to be neuroprotective against MDMA-induced degeneration. However, a subsequent investigation showed that this drug achieved this effect solely by its effect of preventing MDMA-induced hyperthermia (Colado et al. 1999). In an analogous way clomethiazole, but not pentobarbitone, is neuroprotective in animal models of acute ischaemic stroke (Sternau et al. 1989; Cross et al. 1991; Ito et al. 1999). This apparent dichotomy may be related to the fact that barbiturates and clomethiazole do not appear act at an identical binding site on the $GABA_A$ receptor (Green et al. 1996; Zhong and Simmonds 1997).

Recently it has been shown that clomethiazole is able to interact with the $GABA_A$ receptor to inhibit ischaemia induced glutamate release, apparently by opening the chloride channel in the absence of endogenous GABA, This property is not shared by pentobarbitone (Nelson et al. 2000a). It was suggested that this property might explain the neuroprotective activity of clomethiazole (Nelson et al. 2000a; Green et al. 2000b). Support for this proposal has been obtained with a study on (*S*)-(–)-AR-A008055, a novel, structurally related, derivative of clomethiazole which is also neuroprotective (Nelson et al. 2000b). This compound has very similar pharmacological properties to clomethiazole (Green et al. 2000a) and also appears to be able to open the chloride channel in the absence of endogenous GABA (Nelson et al. 2000b). In contrast, the other enantiomer, (*R*)-(+)-AR-A008055, does not possess the same pharmacological characteristics and does not have the ability to directly open the channel (Green et al. 2000a; Nelson et al. 2000b) although it can also increase GABAergic activity (Nelson et al. 2000b).

We have, therefore, now investigated whether these two enantiomers protect the brain of rats from MDMA-induced neurodegenerative damage in order to determine whether neuroprotection in this paradigm is associated with the ability of a compound to act as a $GABA_A$ agonist. In order to remove the influence of changes in body temperature in any neuroprotective effect, some experiments were conducted in which the temperature of the rats given MDMA and the AR-A008055 enantiomers was kept elevated to match that of rats given only MDMA by use of a homeothermic blanket. Seven days after MDMA administration, 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

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±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 either (*R*)-(+)-AR-A008055 or (*S*)-(–)-AR-A008055 (1-4-methyl-5-thiazolyl-1-phenyl-methylamine; synthesised at the former Astra Neuroscience Research Unit, London) or saline 5 min before and 55 min after MDMA (15 mg/kg). Drugs were dissolved in 0.9% w/v NaCl (saline) and administered IP. Rats were injected with a volume of 1 ml/kg MDMA and 5 ml/kg of the AR-A008055 enantiomers. Doses are quoted in terms of the base. Rectal temperature was measured by means of a digital readout thermocouple (Type K thermometer, Portec, UK) with a resolution of ±0.10°C and accuracy of 0.20°C. This was attached to a CAC-005 rodent sensor that 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.

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 (NHI publication No. 85-23, revised 1985).

Experiments with the homeothermic blanket

In two experiments, the temperature of the rats given MDMA plus AR-A008055 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-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 (GraphPad Prism, version 3). 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.

Results

Effect of administration of (*R*)-(+)- and (*S*)-(–)-AR-A008055 on neurotoxic damage following MDMA

MDMA (15 mg/kg IP) produced a major neurotoxic loss of 5-HT and 5-HIAA in the regions of the brain exam-

Table 1 Percent changes in 5-HT and 5-HIAA concentration in rat brain 7 days following administration of MDMA (15 mg/kg) and the effect of (*R*)-(+)-AR-A008055 and (*S*)-(–)-AR-A008055 (both given at a dose of 100 mg/kg 5 min prior and 55 min post-MDMA) on the changes

	Hippocampus		Striatum		Cortex	
	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA
Saline	100±4	100±1	100±4	100±4	100±2	100±3
(*R*)-(+)-AR-A	98±1	104±6	104±3	102±3	103±6	107±5
MDMA	47±3**	49±3**	67±3**	76±3*	60±4**	55±1**
MDMA+(*R*)-(+)-AR-A	68±3••	80±3•••	82±3•	87±4	77±3•	72±4••
(*S*)-(–)-AR-A	102±3	99±3	99±2	106±5	95±6	101±3
MDMA	44±2**	51±3**	70±6**	74±6**	62±3**	63±3**
MDMA+(*S*)-(–)-AR-A	67±6••	78±7••	87±3••	94±4•••	76±2•••	81±2•••

Results shown as mean±SEM of n=5–6. Different from saline treated: *P<0.01, **P<0.001. Different from MDMA-treated: •P<0.05, ••P<0.01, •••P<0.001. The values in saline-treated rats were: 5-HT: 329±13 ng/g and 5-HIAA: 344±4 ng/g in the hippocampus; 5-HT: 464±7 ng/g and 5-HIAA: 477±6 ng/g in the striatum and 5-HT: 267±11 ng/g and 5-HIAA: 162±4 ng/g in the cortex

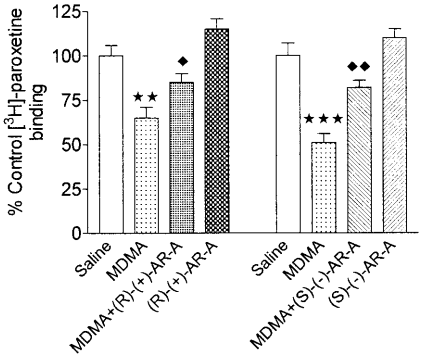

Fig. 1 Changes in the cortical [^{3}H]-paroxetine binding 7 days following administration of MDMA (15 mg/kg, IP) and the effect of (*R*)-(+)-AR-A008055 and (*S*)-(–)-AR-A008055 (both given IP at a dose of 100 mg/kg 5 min prior and 55 min post-MDMA) on the changes. Results shown as mean±SEM, n=6. Different from saline-treated: **P<0.01, ***P<0.001. Different from MDMA-treated: ❖ P<0.05, ❖❖ P<0.01

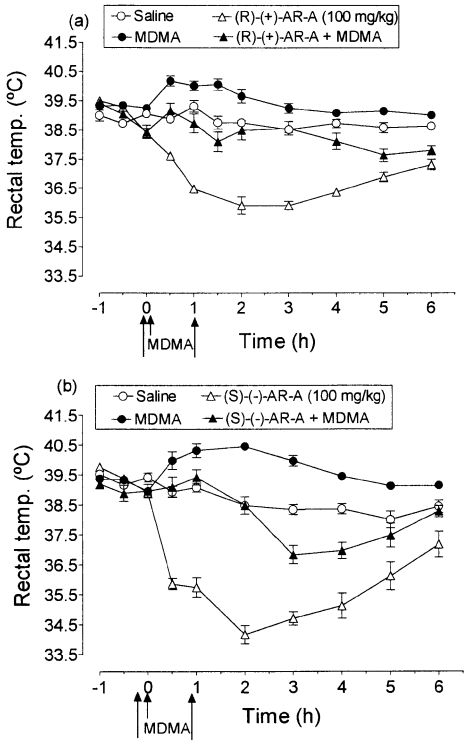

Fig. 2 Effect of **a** (*R*)-(+)-AR-A008055 and **b** (*S*)-(–)-AR-A008055 on MDMA-induced hyperthermia. Both enantiomers (100 mg/kg, IP) or saline were administered 5 min before and 55 min after MDMA (15 mg/kg, IP) or saline. Results shown as mean and vertical lines indicate SEM, n=5–6. **a** MDMA produced a significant rise in body temperature [F(1,9)=26.89, P<0.001] compared with the saline-injected group. (*R*)-(+)-AR-A008055 prevented the MDMA-induced hyperthermia [F(1,10)=28.80, P<0.001] and administered to saline-treated rats produced a significant hypothermia [F(1,9)=203.36, P<0.001]. **b** MDMA produced a significant rise in body temperature [F(1,9)=41.91, P<0.001] compared with the saline injected group. (*S*)-(–)-AR-A008055 prevented the MDMA-induced hyperthermia [F(1,10)=57.00, P<0.001] and administered to saline-treated rats produced a significant hypothermia [F(1,8)=86.66, P<0.001]

ined (Table 1). Administration of (*R*)-(+)-AR-A008055 or (*S*)-(–)-AR-A008055 produced a substantial attenuation of this MDMA-induced loss (Table 1). The prevention of damage to the 5-HT nerve endings was also indicated by the decrease in the size of the loss of [^{3}H]-paroxetine binding in cortical tissue (Fig. 1).

Both enantiomers when injected alone produced hypothermia and also effectively abolished the acute MDMA-induced rise in rectal temperature (Fig. 2).

Effect of using a homeothermic blanket on MDMA-induced neurotoxicity following AR-A008055 administration

Since earlier studies has demonstrated that preventing MDMA-induced hyperthermia attenuates or prevents the neurodegeneration that follows administration of this amphetamine derivative (see Discussion), we repeated the experiment above but kept the rats given MDMA plus AR-A008055 in a cage warmed with a homeother-

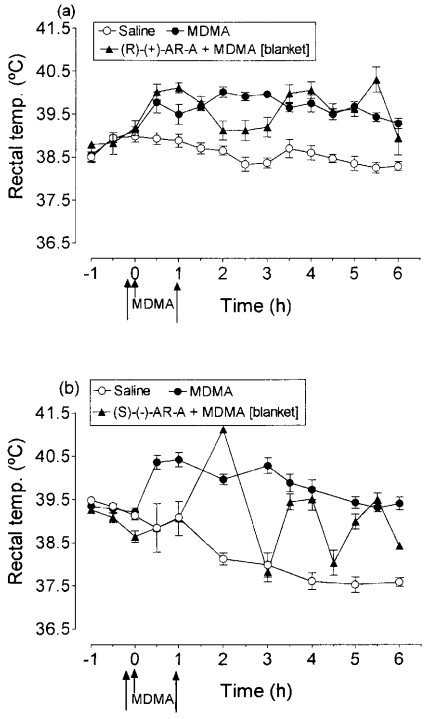

Fig. 3 Effect of **a** (*R*)-(+)-AR-A008055 and **b** (*S*)-(–)-AR-A008055 on MDMA-induced hyperthermia when groups injected with each enantiomer plus MDMA were kept at high ambient temperature. Both enantiomers (100 mg/kg, IP) or saline were administered 5 min before and 55 min after MDMA (15 mg/kg, IP) or saline. Using the homeothermic blanket, the rectal temperature of the rats given (*R*)-(+)-AR-A008055+MDMA or (*S*)-(–)-AR-A008055+MDMA was significantly higher [$F(1,9)=87.64$, $P<0.001$ and $F(1,8)=75.82$, $P<0.001$, respectively] than that of the saline group Results shown as mean and *vertical lines* indicate SEM, $n=5–6$

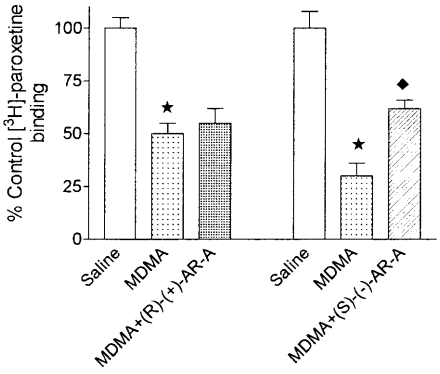

Fig. 4 Changes in the cortical [^{3}H]-paroxetine binding 7 days following administration of MDMA (15 mg/kg, IP) and the effect of (*R*)-(+)-AR-A008055 and (*S*)-(–)-AR-A008055 (both given IP at a dose of 100 mg/kg 5 min prior and 55 min post-MDMA) on the changes when the MDMA plus enantiomer groups were kept at high ambient temperature. Results shown as mean±SEM, $n=4–5$. Different from saline-treated: *$P<0.001$. Different from MDMA-treated: ❖ $P<0.05$

mic blanket (see Materials and methods). We thereby attempted to keep the rectal temperature of the animals to that of rats given MDMA alone.

The use of the blanket increased the rectal temperature of the rats injected with both MDMA and either (*R*)-(+)-AR-A008055 or (*S*)-(–)-AR-A008055 to a value near that of rats given MDMA alone (Fig. 3). No protection was observed in the (*R*)-(+)-AR-A008055 treated rats when the temperature was kept elevated, either as measured by the concentration of 5-HT and 5-HIAA in several brain regions (Table 2) or by the degree of [^{3}H]-paroxetine binding in the cortex (Fig. 4). In contrast, rats injected with (*S*)-(–)-AR-A008055 and MDMA and kept at elevated temperature still showed significant neuroprotection, as measured by the brain indole concentration (Table 2) or paroxetine binding in the cortex (Fig. 4).

Neuroprotective effect of a lower dose of (*S*)-(–)-AR-A008055 against MDMA-induced neurodegeneration

Since the previous experiment suggested that (*S*)-(–)-AR-A008055 possessed neuroprotective activity independent of any effect on body temperature we examined the effect of a lower dose of the compound. (*S*)-(–)-AR-A008055 (50 mg/kg) was also neuroprotective (Table 3), an effect being clearly seen on indole concentrations in both cortex and hippocampus and on [^{3}H]-paroxetine

Table 2 Percent changes in 5-HT and 5-HIAA concentration in rat brain 7 days following administration of MDMA (15 mg/kg) and the effect of (*R*)-(+)-AR-A008055 and (*S*)-(–)-AR-A008055 (both given at a dose of 100 mg/kg 5 min prior and 55 min post-MDMA) on the changes when the MDMA plus enantiomer group were kept at high ambient temperature

	Hippocampus		Striatum		Cortex	
	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA
Saline	100±5	100±5	100±3	100±3	100±8	100±7
MDMA	53±5**	60±2**	61±2**	76±2*	70±6*	69±2**
MDMA+(*R*)-(+)-AR-A	51±9	56±7	68±4	79±6	66±4	61±6
MDMA	37±5**	39±6**	56±5**	61±3**	46±7**	44±6**
MDMA+(*S*)-(–)-AR-A	61±3••	69±7••	80±3••	84±4••	69±4	68±4•

Results shown as mean±SEM of $n=4–6$. Different from saline-treated: *$P<0.01$, **$P<0.001$. Different from MDMA-treated: •$P<0.05$, ••$P<0.01$. The values in saline-treated rats were: 5-HT: 346±19 ng/g and 5-HIAA: 339±16 ng/g in the hippocampus; 5-HT: 441±16 ng/g and 5-HIAA: 464±14 ng/g in the striatum and 5-HT: 269±23 ng/g and 5-HIAA: 165±11 ng/g in the cortex

Table 3 Changes in 5-HT biochemistry in rat brain following MDMA and the effect of (*S*)-(–)-AR-A008055 (50 mg/kg) on these changes

	Saline	MDMA	MDMA+(*S*)-(–)-AR-A008055
Hippocampus			
5-HT	401±40 (5)	171±22 (5)***	247±19 (4)•
5-HIAA	381±45 (5)	186±24 (5)**	239±15 (5)•
Striatum			
5-HT	555±55 (5)	366±20 (5)*	475±35 (4)
5-HIAA	538±46 (5)	403±24 (5)	499±40 (5)
Cortex			
5-HT	269±14 (5)	140±8 (5)***	209±7 (4)•••
5-HIAA	151±7 (5)	83±4 (5)***	104±10 (5)
[^{3}H]-paroxetine binding	85±7 (5)	35±5 (5)***	54±4 (5)•

Results shown as mean±SEM of n=4–5. Different from saline-treated: *P<0.05, **P<0.01, ***P<0.001. Different from MDMA-treated: •P<0.05, •••P<0.001. The values in saline-treated rats were: 5-HT: 401±40 ng/g and 5-HIAA: 381±45 ng/g in the hippocampus; 5-HT: 555±55 ng/g and 5-HIAA: 538±46 ng/g in the striatum and 5-HT: 269±14 ng/g and 5-HIAA: 151±7 ng/g in the cortex. The [^{3}H]-paroxetine binding value in the cortex was 85±7 fmol/mg protein

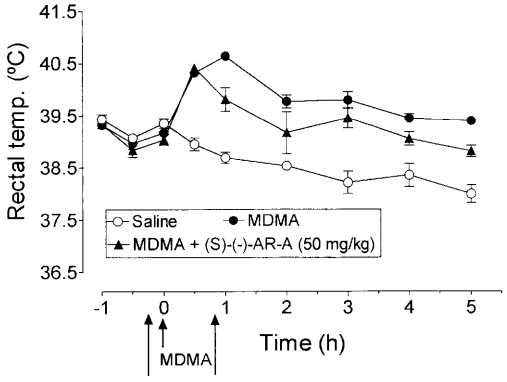

Fig. 5 Effect of a lower dose of (*S*)-(–)-AR-A008055 on MDMA-induced hyperthermia. (*S*)-(–)-AR-A008055 (50 mg/kg, IP) or saline was administered 5 min before and 55 min after MDMA (15 mg/kg, IP). MDMA produced a significant rise in body temperature [F(1,7=144.70, P<0.001] compared with the saline injected group. (*S*)-(–)-AR-A008055 slightly inhibited the MDMA-induced hyperthermic response [F(1,7)=12.06, P<0.05] but rats still showed a body temperature higher than that observed in the saline group [F(1,8)=47.49, P<0.001]. Results shown as mean and *vertical lines* indicate SEM, n=4–6

binding which increased by 42%. (*S*)-(–)-AR-A008055 administration had little effect on the rectal temperature of the rats also administered MDMA (Fig. 5).

Discussion

The current study has demonstrated that (*S*)-(–)-AR-A008055, a compound structurally related to clomethiazole is an effective neuroprotective agent against the neurotoxic degeneration that is seen after administration of MDMA. The degree of protection was similar to that seen after the same dose of clomethiazole.

The role of body temperature in neuroprotection has been demonstrated repeatedly in studies on experimental stroke where it has been shown that even modest hypothermia induces significant protection (Busto et al. 1987; Corbett et al. 1990; Nurse and Corbett 1996). It is clear in recent studies on MDMA-induced neurotoxicity that changes in body temperature can also play a major role in protecting against the long-term damage to 5-HT neurones. Evidence also suggests that it was not necessary to make the animals hypothermic, merely abolishing the acute hyperthermic response which follows MDMA administration had the effect of markedly attenuating or abolishing the neurotoxic degeneration (Colado et al. 1998; Farfel and Seiden 1995a, 1995b; Malberg et al. 1996). The current investigation examined the role of body temperature on the neuroprotective effect of (*S*)-(–)-AR-A008055 and demonstrated that it had a protective action that was probably mainly independent of any effect on body temperature.

This was demonstrated in two ways. In the first experiment using the homeothermic blanket, the rectal temperature was kept elevated for at least much of the time with the overall temperature increase in the (*S*)-(–)-AR-A008055+MDMA group being 58% of that seen in the MDMA alone group. In the second study when the lower dose (50 mg/kg) of (*S*)-(–)-AR-A008055 was used, the temperature of the (*S*)-(–)-AR-A008055+MDMA group was 70% of the MDMA alone group. Very substantial neuroprotection was still seen in this group, in contrast to the (*R*)-(+)-AR-A008055+MDMA group when no protection was seen even though their temperature was only 85% of the MDMA alone group. Therefore, while we acknowledge that we cannot fully eliminate a role of temperature in at least part of the protection seen, it seems reasonable to propose that much of the effect is by a mechanism independent of temperature. In this regard, therefore, the action of (*S*)-(–)-AR-A008055 appears to be similar to that of clomethiazole. Studies on clomethiazole demonstrated that a major part of its neuroprotective action against MDMA-induced damage was also by a mechanism that was primarily independent of body temperature (Colado et al. 1998).

While (*R*)-(+)-AR-A008055 was also demonstrated to protect against MDMA-induced damage, the study with the homeothermic blanket showed unequivocally that this effect was solely due to the action of the drug in lowering body temperature of the MDMA treated animals to that of the saline injected controls. In this regard, therefore, it has a neuroprotective action analogous to pentobarbitone (Colado et al. 1999).

While the pharmacological actions of pentobarbitone and clomethiazole are, in many ways, similar, biochemical studies indicate they do not interact with the $GABA_A$ receptor in an identical manner (Cross et al. 1989; Green et al. 1996; Zhong and Simmonds 1997). There are also difficulties in correlating the available electrophysiological and biochemical data on the compounds. While it has been demonstrated electrophysiologically that both pentobarbitone (Peters et al. 1988) and clomethiazole (Harrison and Simmonds 1983; Hales and Lambert 1992) can interact directly with the receptor complex to activate the chloride channel, such an interaction has never been shown using ligand binding techniques (Peters et al. 1988; Cross et al. 1989; Green et al. 1996). However, a recent in vitro study examining the effect of GABA, clomethiazole and pentobarbitone on glutamate release demonstrated that clomethiazole was able to enhance $GABA_A$ function in the absence of endogenous GABA, but that this property was not shared pentobarbitone (Nelson et al. 2000b).

(*S*)-(–)-AR-A008055 has a very similar pharmacological profile to clomethiazole and recent data indicated that it not only potentiated the action of endogenous GABA at the $GABA_A$ receptor (a property shared by pentobarbitone) but also acted as an agonist at this receptor, directly opening the channel (Nelson et al. 2000b). Clomethiazole, pentobarbitone and (*S*)-(–)-AR-A008055 all 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; Green et al. 2000a). Displacement of [^{35}S]-TBPS does not therefore reflect on the ability of compounds to act directly to open the channel or to act as neuroprotective agents.

While (*R*)-(+)-AR-A008055 can also increase GABAergic activity it does not do so by a action at the $GABA_A$ receptor (Nelson et al. 2000b). It is clear in the current investigation that it only protects against MDMA-induced damage by an effect on body temperature and not through a specific neurochemical mechanism, in this regard it is therefore similar to pentobarbitone (Colado et al. 1999).

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