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# Effect of agmatine on spinal nociceptive reflexes: Lack of interaction with $\alpha_2$-adrenoceptor or $\mu$-opioid receptor mechanisms

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**Abstract**

Agmatine has been tested i.v. in $\alpha$-chloralose anaesthetised rats for its effects on spinal nociceptive reflexes evoked by mechanical and electrical stimuli. Agmatine did not affect reflexes until very high doses (200 mg/kg, i.v.) which also caused complex cardiovascular disturbances. In spinally intact rats agmatine reduced reflexes; it was slightly less potent when there was carrageenan-induced hind paw inflammation. The $\alpha_2$-adrenoceptor antagonist atipamezole (80 $\mu$g/kg) did not significantly affect these reductions. In spinalised animals, agmatine caused a generalised increase in background firing which in animals with a non-inflamed paw was significantly reduced after atipamezole. There was no significant change in evoked responses once corrected for background activity. In all groups of animals agmatine, when administered at various doses and times prior to the $\mu$-opioid receptor agonist fentanyl, had no effect on the $ID_{50}$ of fentanyl. © 1997 Elsevier Science B.V.

*Keywords:* Agmatine; $\alpha_2$-Adrenoceptor; $\mu$-Opioid receptor; Analgesia; Fentanyl; Spinal cord

## 1. Introduction

Agmatine has recently been isolated from cow and rat brain as a putative 'clonidine-displacing substance' and has been shown to bind with reasonable affinity to both $\alpha_2$-adrenoceptors and imidazoline receptors (Li et al., 1994). It is widely distributed throughout the body (Raasch et al., 1995) including astrocytes (Regunathan et al., 1995) and its biosynthetic enzyme arginine decarboxylase (Li et al., 1995) and degradative enzyme, agmatinase (Sastre et al., 1996) are both present in rat brain. This, together with the similar concentration to that of other neurotransmitters in brain (Li et al., 1994) suggests a possible endogenous role for agmatine.

Agmatine is synthesised from L-arginine, which is a precursor of several other biologically active molecules including nitric oxide (NO), polyamines and endogenous opioids. Agmatine (Galea et al., 1996) and spermidine/spermine (Das and Khan, 1995) are both inhibitors of nitric oxide synthase and it has been suggested that there may be differential regulation of NO and agmatine synthesis (Regunathan et al., 1995); agmatine does not, however, serve as a substrate for nitric oxide synthase (Gilad et al., 1996). Agmatine has also been shown to potentiate morphine analgesia in behavioural tests although alone it is without effect in the mouse tailflick assay (Kolesnikov et al., 1996).

We have tested agmatine's effects intravenously on spinal nociceptive reflexes in anaesthetised rats. The use of spinalised and spinally intact rats allows the relative spinal and supraspinal effects of a compound to be evaluated. Comparison of effects in animals with normal and carageenan-inflamed paws allowed the compound to be evaluated under conditions of normal vs. increased excitability within the central nervous system (CNS). Comparison of mechanical stimulation of the peripheral receptive field and electrical stimulation proximal to the receptive field allowed separation of possible central and peripheral effects. We have also used this model to examine the effect that agmatine has on the potency of the $\mu$-opioid receptor agonist fentanyl. Preliminary data have been published in abstract form (Bradley and Headley, 1996).

## 2. Materials and methods

Details of methods have been described previously (Hartell and Headley, 1991; Herrero and Headley, 1996).

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36 male Wistar rats (240–350 g) were anaesthetised with halothane (1.5–2% in $O_2$) and in 15 of them 1 mg of the pro-inflammatory agent carrageenan (Sigma, in 0.1 ml distilled water) was administered by intraplantar injection into the right hind paw. The trachea, carotid artery and both jugular veins were cannulated. A laminectomy was subsequently performed, using topical local anaesthesia (Xylocaine 2%, Astra Pharmaceuticals), at the level of thoracic segments 9–10. The dura was opened; in 22 rats the spinal cord was transected (spinalised) and in the remainder it was left intact (sham spinalised). After surgery halothane was discontinued and anaesthesia was maintained with $\alpha$-chloralose (50 mg/kg i.v. bolus dose followed by 20 mg/kg per h i.v. infusion). Core temperature was maintained at $37 \pm 0.5$°C. Systolic blood pressure never fell below 100 mmHg except transiently following i.v. drug injections.

Animals were left for a minimum of 1 h after surgery, and 3 h after carrageenan injection, before any drug was tested. The degree of inflammation produced by carrageenan was assessed at the end of the experiment as the volume of water displaced by the paw relative to the volume at the start of the experiment; paw volume increased from $1.7 \pm 0.1$ to $2.8 \pm 0.1$ ml.

Single motor unit activity was recorded from hind limb flexor muscles using bipolar Teflon-coated tungsten electrodes. Responses were elicited in 3 min cycles each of 3 stimuli: noxious pinch (15 s, 1.0–2.6 N over 19 $mm^2$) and 2 trains of electrical stimuli (1 ms pulse width and 10 times threshold for eliciting a reflex). These trains were delivered via 2 needles inserted percutaneously at the proximal edge of the receptive field at either lower frequency electrical stimulation (0.2 Hz, 4 stimuli) or higher frequency electrical stimulation (1 Hz, 16 stimuli), during which frequency-dependent facilitation usually occurred ('wind-up', Mendell, 1966).

Drugs used were agmatine (Sigma), the $\mu$-opioid receptor agonist, fentanyl citrate (Manorpark Pharmaceuticals) and the $\alpha_2$-adrenoceptor antagonist, atipamezole (Antisedan, Norden Laboratories). All drugs were injected intravenously in a volume of 0.3 ml; fentanyl and agmatine were given in a cumulative dose-doubling regime at 6 min intervals. Evoked responses were recorded until recovery, or, if no effects were seen following the top dose, for a minimum of 30 min following the last dose of drug, in case of any slow onset actions or delayed penetration into the CNS.

Animals were divided into 4 groups: spinalised or sham spinalised, and with or without peripheral inflammation. In each group agmatine was tested alone, to a cumulative total of up to 200 mg/kg. At least 30 min after full recovery ($\geq$ 60 min after the last dose of agmatine) the same protocol was repeated after a single dose of atipamezole (80 $\mu$g/kg). In parallel studies in this laboratory, this dose has been shown to reverse fully the effects of systemically-administered medetomidine (5 $\mu$g/kg i.v.), a selective $\alpha_2$-adrenoceptor agonist (C.A. Capner, personal communication).

In separate experiments fentanyl was tested in a cumulative regime, until responses were reduced to $< 30\%$ control (4–16 $\mu$g/kg). At least 20 min after full recovery ($\geq$ 45 min after the last dose of fentanyl) agmatine (doses between 10 and 200 mg/kg) was given followed by a repeat cumulative dose regime of fentanyl starting between 6 and 60 min after agmatine. Previous experiments in this laboratory, and control experiments in the present series, have shown that a second cumulative dose/response run with fentanyl at intervals of 35–80 min did not show any sign of tolerance ($ID_{50}$ on pinch response were $6.5 \pm 2.6$ and $6.4 \pm 2.4$ $\mu$g/kg, $n = 3$).

Data are presented as percentages of control values ($\pm$ S.E.M.) where the control is the mean of the responses recorded over the last 3 cycles prior to administration of a drug; $n \geq 6$ unless otherwise stated. 'Wind-up' was calculated as the sum of the responses to higher frequency electrical stimulation (16 shocks) minus 4 times the response to lower frequency electrical stimulation (i.e., $4 \times 4$ shocks). For fentanyl, data are presented as the dose of fentanyl that reduced control responses by 50% ($ID_{50}$, estimated by Grafit, Erithaca Software). Statistical significance was assessed by non-parametric analysis of original spike count data and $ID_{50}$ values. Statistical analysis was performed using the Wilcoxon pairs test (for paired data) or the Friedman test with Dunn's post tests (Instat, Graphpad Software).

## 3. Results

### 3.1. Effects of agmatine alone on spinal nociceptive reflexes

Preliminary experiments ($n = 6$) using cumulative doses of agmatine up to 25 mg/kg i.v. showed no significant change in spike counts evoked by any of the stimuli (data not shown). In subsequent experiments cumulative doses of agmatine between 25 and 200 mg/kg i.v. were given.

In spinally intact animals with non-inflamed paws, agmatine caused a reduction of all responses at the highest dose. An example of a test performed on a single motor unit is shown in Fig. 1 and the pooled data for this group are in Fig. 2. Agmatine significantly reduced all evoked responses (pinch $p < 0.01$, lower and higher frequency electrical stimulation $p < 0.001$, Friedman test). For individual doses (Dunn's post test) the reduction reached significance at 100 mg/kg for responses to lower frequency electrical stimulation ($72 \pm 5\%$ control, $p < 0.05$) and at 200 mg/kg for responses to pinch (Fig. 2a and Table 1, $p < 0.01$), higher frequency electrical stimulation ($31 \pm 7\%$ control, $p < 0.001$) and the 'wind-up' component of the response to higher frequency electrical stimulation ($36 \pm 13\%$ control, $p < 0.01$). The $ID_{50}$ for agmatine on the pinch response was $166 \pm 69$ mg/kg.

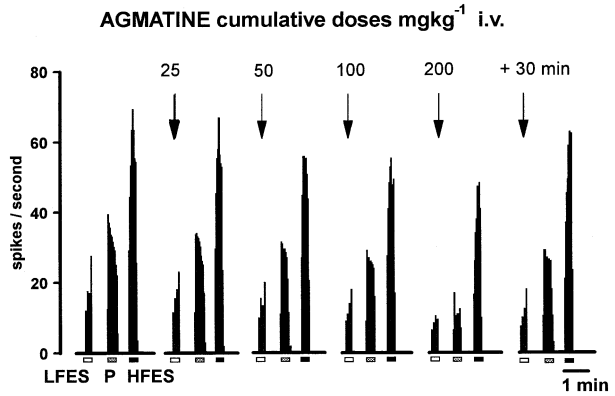


Fig. 1. The effects of cumulative doses of agmatine on nociceptive reflex responses of a single motor unit in a hindlimb flexor muscle of a spinally intact rat with a normal (non-inflamed) paw. Agmatine reduced responses to all stimuli at the highest doses. One control cycle and one 3 min cycle immediately following each dose of drug is illustrated. Drug doses were administered at 6 min intervals; data for the second cycle after each drug dose are not illustrated. Responses recovered to control levels 30 min after the last dose. LFES = lower frequency electrical stimulation (0.2 Hz, 4 stimuli, 1 ms pulse width, 10× threshold for eliciting a reflex), P = pinch (15 s, 1.8 N over 19 $mm^2$) and HFES = higher frequency electrical stimulation (1 Hz, 16 stimuli).

In spinally intact animals with an inflamed paw, agmatine appeared to be slightly less potent, although the top dose of 200 mg/kg was tested on only a small number of animals. The only significant reduction was in responses to higher frequency electrical stimulation at 100 mg/kg agmatine ($69 \pm 9\%$ control, $n = 7$, $p < 0.05$, Dunn's post test vs. control). In 3 animals a dose of 200 mg/kg reduced responses to pinch to $67 \pm 7\%$ control, to lower frequency electrical stimulation to $51 \pm 3\%$ control, and to higher frequency electrical stimulation to $48 \pm 17\%$ control.

In animals with a transected spinal cord there was no consistent effect of agmatine at doses of up to 100 mg/kg. At the top dose of 200 mg/kg it caused a generalised excitation, increasing background firing rate from zero to $5.7 \pm 1.4$ spikes/s in animals with non-inflamed paws ($n = 13$), and to $10.9 \pm 2.7$ spikes/s in carrageenan-inflamed animals ($n = 9$). After subtraction of the background firing, there was no significant change in evoked responses to pinch in either group (Table 1).

### 3.2. Effects of agmatine on blood pressure

In all animals agmatine ($\geq 25$ mg/kg) decreased mean blood pressure. The maximal effect was seen with the top dose (200 mg/kg) which, in animals with non-inflamed paws, caused a drop from $105 \pm 6$ to $67 \pm 7$ mmHg in spinalised animals ($n = 13$) and from $131 \pm 3$ to $69 \pm 4$ mmHg in intact animals ($n = 9$); blood pressure returned to pre-drug levels after $14 \pm 2$ min ($n = 22$). Similar decreases and durations of effect were seen in animals with inflamed paws. In 12 animals (10 spinalised) an initial decrease in blood pressure at 200 mmHg was followed by a secondary hypertensive response with mean blood pressure rising transiently to $133 \pm 8$ mmHg.

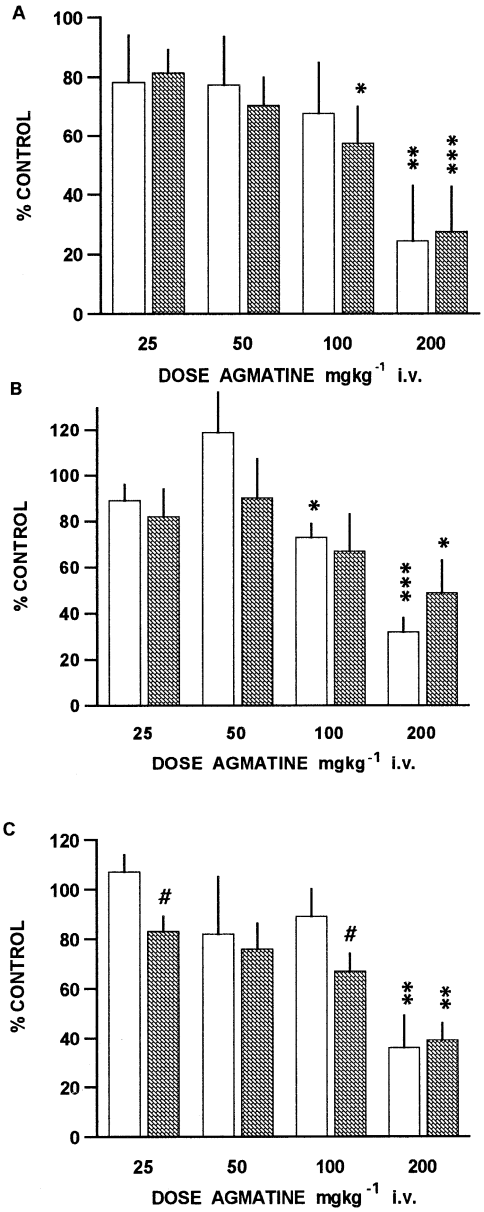


Fig. 2. Pooled data showing the effects of cumulative doses of agmatine on nociceptive reflex responses in spinally intact animals with non-inflamed paws when tested before (white bars) and after (filled bars) atipamezole (80 μg/kg i.v.). Data are limited to units with which atipamezole was tested and are expressed as % pre-agmatine control values. (A) Responses to pinch (1.0–2.6 N over 19 $mm^2$), (B) Responses to lower frequency electrical stimulation (4 stimuli at 0.2 Hz, 1 ms pulse width, 10× threshold for eliciting a reflex), (C) 'Wind-up' component of the responses to higher frequency electrical stimulation (16 stimuli at 1 Hz; see Section 2). Statistical symbols: * value is significantly different from control (Friedman test with Dunn's post test), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, # value is significantly different pre- vs. post-atipamezole (Wilcoxon test, $p < 0.05$). $n = 6$ in all cases. Bars represent S.E.M.

Table 1
Pooled data for reflex responses to noxious pinch, showing the effects of agmatine alone and after atipamezole (an $\alpha_2$-adrenoceptor antagonist), and the influence of pretreatment with agmatine on $ID_{50}$ values of the $\mu$-opioid receptor agonist fentanyl

| | | Spinally intact | | Spinalised [a] | |
|---|---|---|---|---|---|
| | | normal paw | inflamed paw | normal paw | inflamed paw |
| Dose of agmatine (mg/kg) | 25 | 78 ± 16 (6) | 83 ± 12 (5) | 90 ± 8 (13) | 84 ± 11 (9) |
| | 50 | 83 ± 15 (7) | 78 ± 18 (6) | 87 ± 10 (13) | 84 ± 7 (9) |
| | 100 | 71 ± 14 (8) | 74 ± 16 (6) | 84 ± 10 (13) | 120 ± 13 (9) |
| | 200 | 34 ± 16 (8) [c] | 67 ± 7 (3) | 95 ± 13 (13) | 118 ± 14 (9) |
| Dose of agmatine (after 80 $\mu$g kg$^{-1}$ atipamezole) | 25 | 81 ± 7 (6) | 112 (2) | 98 ± 5 (6) | 100 ± 3 (6) |
| | 50 | 71 ± 9 (6) | 113 (2) | 103 ± 6 (6) | 93 ± 3 (6) |
| | 100 | 57 ± 13 (6) [b] | 103 (2) | 101 ± 10 (6) | 99 ± 4 (6) |
| | 200 | 28 ± 15 (6) [d] | 81 (2) | 87 ± 25 (6) | 90 ± 14 (6) |
| $ID_{50}$ fentanyl ($\mu$g kg$^{-1}$) | pre-agmatine | 6.4 ± 2.5 (7) | 4.8 ± 0.9 (8) | 8.2 ± 2.3 (6) | 7.9 ± 1.1 (7) |
| | post-agmatine | 7.3 ± 2.2 (7) | 4.6 ± 0.9 (8) | 5.9 ± 2.0 (6) | 8.3 ± 1.7 (7) |

Data are shown as % of pre-drug control values ± S.E.M. (n), and $ID_{50}$ ($\mu$g/kg) ± S.E.M. ($n$).
[a] Values after subtraction of background firing rates.
[b] $p < 0.05$; [c] $p < 0.01$; [d] $p < 0.0001$: Values are significantly different from control by Friedman test with Dunn's post test vs. Control.

### 3.3. Influence of the $\alpha_2$-adrenoceptor antagonist atipamezole on the effects of agmatine

Atipamezole alone (80 $\mu$g/kg) had no effect on evoked responses to pinch or electrical stimulation in any group; for example, responses to pinch following atipamezole were 100 ± 4% control ($n = 20$). In spinally intact rats with normal paws agmatine caused similar reductions in responses when tested after atipamezole as when tested before. Responses to pinch were reduced to 28 ± 15% control (Table 1), and those to lower frequency electrical stimulation and the 'wind-up' component of higher frequency electrical stimulation were reduced to 49 ± 13% and 39 ± 7% control ($n = 6$), respectively, at the 200 mg/kg dose.

Following atipamezole in spinalised animals, agmatine (at 200 mg/kg) caused less increase in background firing. In rats with normal paws the excitation was significantly ($p < 0.05$) less, increasing to only 2.5 ± 1.0 spikes/s, compared with 8.0 ± 1.6 spikes/s in the same units before atipamezole ($n = 8$). In animals with an inflamed paw there was again a lower increase in background firing after atipamezole (to 5.4 ± 2.0 spikes/s, $n = 7$) but this was not significantly different to the rise prior to atipamezole (to 10.0 ± 2.5 spikes/s in this sample).

### 3.4. Effect of agmatine on $\mu$-opioid receptor-mediated antinociception

As expected, fentanyl dose-dependently reduced responses to all stimuli; the $ID_{50}$ values for fentanyl on responses to pinch are shown in Table 1. Fentanyl was somewhat more potent in spinally intact than in spinalised animals, and in inflamed animals compared to those with a normal paw, although these differences were not significant. Recovery from fentanyl was complete in all cases by 30 min after the last dose, but at least a further 30 min was allowed to elapse before another test with fentanyl was initiated.

Agmatine had no consistent effect on the $ID_{50}$ values for fentanyl. Because of this lack of consistency agmatine was tested over a wide dose range (10–200 mg/kg) and fentanyl was subsequently administered between 6 and 60 min after agmatine. No pattern of effect relating to either the dose of agmatine or to the agmatine–fentanyl interval emerged from these tests. The data were therefore pooled and are shown in Table 1. There was no significant change in the $ID_{50}$ values for fentanyl following agmatine in any of the four groups (±spinalisation, ±paw inflammation). A similar analysis was performed for data on responses to electrical stimulation and yielded similar lack of effects (data not shown).

### 3.5. Time-course of effects

The time-course of the changes in cardiovascular responses was marginally shorter than that for the changes in evoked reflex responses. In spinally intact animals the time to half recovery for the evoked responses to pinch was 22 ± 7 min ($n = 8$), for those to lower frequency electrical stimulation 30 ± 8 min ($n = 6$) and for those to higher frequency electrical stimulation 27 ± 5 min ($n = 7$). Blood pressure, in comparison, returned fully to pre-drug control levels in 17 ± 3 min ($n = 8$).

In spinalised animals the excitation seen persisted as long as the cardiovascular changes; the time to half recovery for background firing rates was 11 ± 3 min whereas blood pressure returned fully to pre-drug control levels in 11 ± 2 min ($n = 13$).

## 4. Discussion

These experiments show that in this model only at very high doses did agmatine affect reflex responses evoked by peripheral noxious stimuli. Doses under 25 mg/kg did not affect nociceptive reflexes, which is consistent with a previous study showing that doses of agmatine up to 10 mg/kg had no effect on the tailflick latency in mice (Kolesnikov et al., 1996). Depression of responses was only seen in spinally intact rats suggesting that this action is mediated at a supraspinal level. The small differences seen in the effectiveness of agmatine in normal animals vs. those with inflamed paws may be related to differences in baseline excitability of the cord under different conditions. In both groups there was a parallel reduction of responses to pinch and those to electrical stimulation proximal to the receptive field. Since any peripheral action of a drug would be expected to be mediated at the sensory endings (i.e., within the receptive field), the response to electrical stimulation of afferent nerve fibres proximal to the receptive field should not have been affected directly by any peripheral actions of agmatine. The result is therefore suggestive of a central rather than peripheral site of action. However, the extreme dose required to produce any effect, together with the accompanying major cardiovascular disturbances, casts doubt on the physiological or pharmacological relevance of these effects.

At all doses above 25 mg/kg in both spinalised and spinally intact animals, agmatine caused a depression of blood pressure. Similar results were found in a recent study, also in the rat, when agmatine (60 μmol, approximately 24 mg/kg) was administered intravenously; however, when given i.c.v. it had the opposite effect, causing a sympathoexcitation which lasted up to an hour (Sun et al., 1995). The effects seen with systemic administration could be due to blockade of sympathetic ganglia, as agmatine has been shown to block nicotinic receptors (Loring, 1990), or due to agonist activity at prejunctional $\alpha_2$-adrenoceptors causing an inhibition of noradrenaline release (González et al., 1996). Another possibility is an action via imidazoline $I_2$ receptors which have been shown to be present both centrally and peripherally, including on vascular smooth muscle and endothelium (Regunathan et al., 1996), although agmatine shows only a low affinity for these sites (Lione et al., 1996).

The excitation seen at the highest dose in spinalised rats was a consistent finding, occurring in all but two of the spinalised animals. The mechanism behind this excitation is unclear. It was significantly less, although not abolished, following atipamezole, which is a relatively selective antagonist of $\alpha_2$-adrenoceptors (Sjöholm et al., 1992). However, depression rather than excitation is the normal effect of spinal $\alpha_2$-adrenoceptor activation. Atipamezole did not influence the depressions caused by high doses of agmatine, suggesting that these depressions are not mediated by $\alpha_2$-adrenoceptors. This is supported by other studies which have shown that although agmatine can bind to $\alpha_2$-adrenoceptors, it does not activate them (Pinthong et al., 1995b), that agmatine does not have activity at $\alpha_2$-adrenoceptors modulating the firing rate of locus coeruleus neurones (Pineda et al., 1996) and that agmatine alone is unlikely to account for all of the biological activity of 'clonidine-displacing substance' (Piletz et al., 1995; Pinthong et al., 1995a,b). However agmatine has been shown to have agonist activity at prejunctional $\alpha_2$-adrenoceptors in the rat tail artery (González et al., 1996) and multiple effects on sympathetic neurotransmission in rat vas deferens (Jurkiewicz et al., 1996).

Agmatine had little effect on the $ID_{50}$ of fentanyl, which showed no significant change following agmatine in any group. These results contrast with those of a recent study investigating the interaction of agmatine and opioids in the mouse tailflick assay, in which agmatine given s.c. was shown to potentiate both morphine (given s.c., i.t. and i.c.v.) and the δ-opioid receptor agonist [D-Pen2, D-Pen5] enkephalin (DPDPE, given i.t.; Kolesnikov et al., 1996). The conflict in results is unlikely to be due to differences in protocol, since in our initial experiments we used similar doses and time intervals to the previous study and agmatine was administered systemically in both cases. The potency of fentanyl is similar for both mechanical and thermal nociceptive responses (Parsons et al., 1989), therefore the choice of noxious stimulus should not influence the results. Opioid receptor differences are also unlikely to account for the discrepancy in results as both fentanyl and morphine are μ-opioid receptor agonists (see Parsons et al., 1989). Some differences may have been due to the different preparations employed, since behavioural tests do not allow the measurement of other parameters such as altered blood pressure which can influence the response to a heat stimulus (Duggan et al., 1978, Hole and Tjølsen, 1993).

Any proposed spinal or supraspinal actions elicited by agmatine in our model are dependent on it being able to cross the blood brain barrier, which has not yet been proven. Systemic arginine, the precursor of agmatine, is able to reverse the central effects of NO synthase inhibitors (Meller and Gebhart, 1993), and must therefore be acting centrally. Agmatine s.c. enhanced the potency of morphine i.t. to a greater degree than morphine s.c. (Kolesnikov et al., 1996), suggesting that agmatine was acting centrally in this model. However agmatine i.c.v. had longer lasting and opposing effects on blood pressure to that of agmatine i.v. (Sun et al., 1995) suggesting that with systemic administration the peripheral effects outweigh the central effects; this leaves in doubt the ability of agmatine to penetrate the CNS.

In conclusion, our results showed that agmatine depressed reflex responses to noxious stimuli only at very high doses and only in spinally intact animals, by mechanisms independent of $\alpha_2$-adrenoceptors. At high doses in spinalised animals it caused a generalised excitation. The

receptor mechanisms of both of these actions are unclear. Our results did not demonstrate any enhancement by agmatine of the potency of the $\mu$-opioid receptor agonist, fentanyl.

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