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Effect of agmatine on locus coeruleus neuron activity: possible involvement of nitric oxide

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1 To investigate whether agmatine (the proposed endogenous ligand for imidazoline receptors) controls locus coeruleus neuron activity and to elucidate its mechanism of action, we used single-unit extracellular recording techniques in anaesthetized rats.

2 Agmatine (10, 20 and 40 μg, i.c.v.) increased in a dose-related manner the firing rate of locus coeruleus neurons (maximal increase: $95 \pm 13\%$ at 40 μg).

3 I_1-imidazoline receptor ligands stimulate locus coeruleus neuron activity through an indirect mechanism originated in the paragigantocellularis nucleus *via* excitatory amino acids. However, neither electrolytic lesions of the paragigantocellularis nucleus nor pretreatment with the excitatory amino acid antagonist kynurenic acid (1 μmol, i.c.v.) modified agmatine effect (10 μg, i.c.v.).

4 After agmatine administration (20 μg, i.c.v.), dose-response curves for the effect of clonidine ($0.625-10$ μg kg^{-1} i.v.) or morphine ($0.3-4.8$ mg kg^{-1} i.v.) on locus coeruleus neurons were not different from those obtained in the control groups.

5 Pretreatment with the nitric oxide synthase inhibitors N^{ω}-nitro-L-arginine (10 μg, i.c.v.) or N^{ω}-nitro-L-arginine methyl ester (100 μg, i.c.v.) but not with the less active stereoisomer N^{ω}-nitro-D-arginine methyl ester (100 μg, i.c.v.) completely blocked agmatine effect (10 and 40 μg, i.c.v.).

6 Similarly, when agmatine (20 pmoles) was applied into the locus coeruleus there was an increase that was blocked by N^{ω}-nitro-L-arginine methyl ester (100 μg, i.c.v.) in the firing rate of the locus coeruleus neurons (maximal increase $53 \pm 11\%$ and $14 \pm 10\%$ before and after nitric oxide synthase inhibition, respectively).

7 This study demonstrates that agmatine stimulates the firing rate of locus coeruleus neurons *via* a nitric oxide synthase-dependent mechanism located in this nucleus.
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Keywords: single-unit extracellular recordings; excitatory amino acids; imidazoline receptors; *in vivo*; α_2-adrenoceptor; μ-opioid receptor

Abbreviations: ANOVA, analysis of variance; D-NAME, N^{ω}-nitro-D-arginine methyl ester; LC, locus coeruleus; L-NA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester

Introduction

Agmatine (1-amino-4-guanidinobutane) is a primary amine, derived from the decarboxylation of arginine by arginine decarboxylase and metabolized into putrescine by agmatinase, which has been isolated from the brain and other mammalian organs (see review, Reis & Regunathan, 2000). The biosynthetic and the metabolic enzymes of agmatine have been found to be expressed in the mammalian brain (Li *et al*., 1994; Sastre *et al*., 1996). Agmatine is stored in vesicles within neurons (Reis *et al*., 1998) and is released from synaptosomes by depolarization in a Ca^{24}-dependent manner (Sastre *et al*., 1997). Moreover, it can be biologically inactivated by uptake into synaptosomes in the mammalian brain (Sastre *et al*., 1997). Agmatine-like immunoreactivity identified by light microscopy, has been visualized in the cytoplasm of neuronal perikarya in several brain areas, including the locus coeruleus nucleus (LC) (Otake *et al*., 1998). Agmatine is a biologically active substance that produces several central and peripheral effects. Thus, in the central nervous system, agmatine potentiates morphine analgesia, blocks opiate tolerance development (Kolesnikov *et al*., 1996; Aricioglu-Kartal & Uzbay, 1997) and stimulates pituitary hormone release (Kalra *et al*., 1995).

On the whole, these findings suggest that agmatine may play a role as a neurotransmitter/neuromodulator. However, the mode and sites of its actions have not been completely defined. It is well known that agmatine binds reversibly and with relatively high affinity to α_2-adrenoceptors and imidazoline receptors (Li *et al*., 1994; Piletz *et al*., 1995). Moreover, this compound is thought to be the endogenous ligand for imidazoline receptors (see review, Raasch *et al*., 2001). Accordingly, the central distribution of agmatine correlates with the regional distribution of imidazoline receptors (Feng *et al*., 1997).

On the basis of distinct pharmacological profiles and tissue and subcellular distribution, imidazoline receptors were initially classified as I_1 and I_2 imidazoline receptors and later, a third non-I_1/I_2 imidazoline receptor type (also called I_3) was included (see review, Eglen *et al*., 1998). In this context, we have shown that neurons of the LC, the main noradrenergic nucleus in the brain, respond to certain

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imidazoline drugs by increasing in their firing rate (Pineda *et al*., 1993; Ugedo *et al*., 1998). This effect involves two different types of imidazoline receptors (Pineda *et al*., 1993; Ugedo *et al*., 1998).

The present study was therefore designed to assess if agmatine modulates LC neuron activity *in vivo* and to establish whether imidazoline or other receptors (such as α_2-adrenoceptors or μ-opioid receptors) could be implicated in the effect of agmatine. To this end, we used single-unit extracellular recordings of LC neurons in anaesthetized rats.

Methods

Animal preparation

Male, albino Sprague-Dawley rats weighing 250–320 g were housed under controlled environmental conditions (22°C and a 12-h light/dark cycle) with free access to food and water. Rats were anaesthetized with chloral hydrate (400 mg kg^{-1} i.p.), a tracheal cannula was inserted and the right jugular vein was cannulated for additional injections of anaesthetic and other drugs. Animal body temperature was maintained at ~37°C for the entire experiment by means of a heating pad connected to a rectal probe. The rat was placed in a stereotaxic frame, with the head oriented at 15° to the horizontal plane (nose down). The skull was exposed and a 3 mm bur hole was drilled 3.7 mm posterior to the lamboid fontanel and 1.1 mm lateral to the midline (Paxinos & Watson, 1986). Lesions of the lateral paragigantocellularis nucleus (PGi) were performed as described (Ruiz-Ortega & Ugedo, 1997). The head was oriented at 24° to the horizontal plane (nose down), the neck tissue at the caudal skull margin was drawn back and the occipital bone over the caudal cerebellum was removed to reveal the obex (caudal apex of the IVth ventricle). Briefly, a recording electrode was placed 1.9–2.1 mm lateral to the midline and 2.0–2.3 mm rostral to the edge of the obex and was lowered through the cerebellum into the medulla; a group of neurons exhibiting prominent discharge with respiration was encountered within 1–1.5 mm dorsal to the ventral brain surface, the latter being revealed by a sharp increase in noise in unfiltered pipette records. The recording electrode was removed and an electrode, consisting of a twisted pair of wires (250 μm diameter) was implanted at the same coordinates, except for being 500–700 μm dorsal to the ventral brain surface. Electrical lesions of the PGi were performed ipsilaterally to the recording site in the LC, by passing direct current pulses of 1 mA for 15 s through the electrode from a square-wave stimulator and a constant-current stimulus insulation unit (custom-made). The location and extension of the lesioned area is shown in Figure 2. This protocol for PGi destruction has been useful to demonstrate that electrical lesion of the PGi greatly attenuates the non α_2-adrenoceptor effect of clonidine (Ruiz-Ortega & Ugedo, 1997). For intracerebroventricular administrations, a 23-gauge steel catheter was inserted into the left lateral ventricle, 1.0 mm caudally and 1.3 mm laterally to bregma, at a depth of 4–5 mm from the skull surface, and fixed, with dental cement. The intraventricular position of the catheter was controlled by inspection of the level of an air bubble in a plastic tube connected to the cannula.

Electrophysiological procedures

Single-unit extracellular recordings of LC neurons were performed as described previously (Pineda *et al*., 1993). The recording electrode, consisting of an Omegadot single-barrel, glass micropipette was filled with 2% solution of Pontamine Sky Blue in 0.5% sodium acetate. This was broken back to a tip diameter of 1–2.5 μm (*in vitro* impedance 2–6 MΩ) and was situated 1.1 mm lateral, 3.7 mm caudal, and 5.5–6.5 mm ventral to the cortical surface. The extracellular signal from the electrode was amplified with a high-input impedance amplifier, and then monitored on an oscilloscope and on an audio monitor. Individual neuronal spikes were discriminated by means of a custom-made unit, fed into an electronic rate counter and finally analysed using a custom-made computer programme. LC neurons were identified by standard criteria (Cedarbaum & Aghajanian, 1976) which included: (1) spontaneous activity displaying a regular rhythm and a firing rate between 0.5–5 Hz; (2) characteristic spikes with a long-lasting, positive–negative waveform; and (3) biphasic excitation–inhibition response to pressure applied to the contralateral hind paw (paw pinch). Additional clues to facilitate the location of the LC included a zone of relative electrical silence, just dorsal to the LC (corresponding to the IVth ventricle) and the presence, just lateral to the LC, of the mesencephalic nucleus of the Vth nerve, revealed by cells, which were activated by the displacement of the mandible.

Pressure microinjection into the LC

A thick-walled pipette with a calibrated narrow inner diameter was broken at 2 μm from the tip and was glued adjacent to a recording micropipette (Ruiz-Ortega & Ugedo, 1997). The calibrated pipettes were filled with Dulbecco's buffered saline solution containing (in mM): NaCl 136.9, KCl 2.7, NaH_2PO_4 8.1, KH_2PO_4 1.5, $MgCl_2$ 0.5 and $CaCl_2$ 0.9 (pH of 7.40), with or without 10 mM of 2-BFI. Drug ejection was performed by applying pressure pulses (50–150 ms) using a solenoid-controlled pneumatic pressure device (Picospritzer™II, General Valve Corp.) driven by synthetic air. The injected volume was measured by monitoring the meniscus movement in the calibrated pipette. We have previously shown that Dulbecco's pressure microinjection into LC does not affect neuron-firing rate (Ruiz-Ortega & Ugedo, 1997).

Histological verifications

At the end of the experiment, a 5 μA cathodal current was passed through the recording electrode, leaving a blue spot at the recording site. The brain was then fixed by transcardial perfusion with 10% formaldehyde solution. Brains were removed, cut in sections of 50 μM and stained with neutral red. The recording site and the electrically lesioned area were examined microscopically. All cells recorded outside the LC were excluded from this study.

Analysis of data

To study the effect of agmatine on the inhibition induced by morphine or clonidine, cumulative dose-effect curves were analysed by Prism programme (GraphPad, v. 3.0). Experimental data were normalized to the initial basal firing rate for

each cell (the firing rate at the beginning of the experiment was taken as 100%), and then analysed for the best simple non-linear fit to the three-parameter logistic equation $E = E_{max} / \{1 + (ED_{50}/A)^n\}$, where E is the effect induced by a certain dose of the agonist (A), E_{max} is the maximal effect, ED_{50} is the dose of agonist needed to elicit 50% of E_{max}, and n is the slope factor of the dose-effect curve (Parker & Waud, 1971). ED_{50} and n were estimated by this analysis (E_{max} was constrained to 100% since maximal inhibition was always complete).

Statistics

Statistical significance was assessed by means of two-way repeated-measures analysis of variance (ANOVA; agmatine effect, pretreatment and agmatine effect x pretreatment interaction), the Student *t*-test for comparisons of two independent groups or the paired Student *t*-test for comparisons of the firing rate before and after drug application. The level of significance was chosen as $P = 0.05$. Data are given as mean ± s.e.mean.

Drugs

Agmatine sulphate, clonidine HCl, kynurenic acid and N^{ω}-nitro-L-arginine (L-NA) were obtained from Sigma (Madrid, Spain); Morphine HCl from Alcaliber, S.A. (Madrid, Spain); N^{ω}-nitro-D-arginine methyl ester (D-NAME) HCl and N^{ω}-nitro-L-arginine methyl ester (L-NAME) HCl from Research Biochemical International (Madrid, Spain). Drugs were dissolved in 0.9% NaCl for intravenous administrations, and in Dulbecco's buffered saline solution containing (in mM): NaCl, 136.9; KCl, 2.7; NaH_2PO_4, 8.1; KH_2PO_4, 1.5; $MgCl_2$, 0.5 and $CaCl_2$, 0.9 (pH 7.4) for intracerebroventricular applications. Drugs were always administered at the same volume (10 μl).

Results

Agmatine increases the firing rate of LC neurons in vivo

In an initial series of experiments, the putative modulation of LC neuron activity by agmatine was studied. Intracerebroventricular administration of agmatine (40 μg) increased the firing rate in all cells studied. The maximal effect was reached after 4–5 min, and the effect lasted at least 15–20 min after agmatine administration ($n = 5$) (Figure 1A). This effect was specifically due to agmatine, since similar intracerebroventricular applications of 10 μl Dulbecco's solution (the vehicle in which agmatine was dissolved) did not modify the firing rate of LC neurons ($n = 5$) (Figure 1B). In addition, as shown in Figure 1C, the stimulatory effect of agmatine was dose-related (maximal increases in firing rate after 10, 20 and 40 μg, i.c.v. of agmatine were 34 ± 4%, $n = 5$, 69 ± 10%, $n = 11$ and 95 ± 13%, $n = 5$, respectively; ANOVA; $P < 0.05$).

I_1 imidazoline receptors are not involved in the effect of agmatine

It has been reported that clonidine stimulates LC neurons through I_1-imidazoline receptors located in the PGi, and that this response is mediated by excitatory amino acids (Ruiz-Ortega & Ugedo, 1997). To study whether the stimulatory effect of agmatine on LC neurons was due to an interaction with I_1-imidazoline receptors, electrolytic lesions of the PGi (Figure 2A) were performed, and in another series of experiments, the excitatory amino acid receptor antagonist, kynurenic acid (1 μmol, i.c.v.) was administered 60 and 10 min, respectively, before agmatine (10 μg, i.c.v.). As has been previously described (Ruiz-Ortega & Ugedo, 1997), lesioning of the PGi did not cause any change in the firing rate of LC neurons (mean firing rate was 1.4 ± 0.2 Hz; $n = 5$ and 1.4 ± 0.4 Hz; $n = 5$ in the control and lesioned groups, respectively). In contrast, kynurenic acid blocked response of LC cells to paw pinch and slightly reduced the basal firing rate of LC neurons (1.2 ± 0.2 Hz; $n = 8$). Two-way repeated-measures ANOVA showed that there were no significant differences when the agmatine (10 μg, i.c.v.)-induced effect in control animals was compared with that in lesioned rats, or with that in kynurenic acid pretreated rats (Figure 2B). Consequently, it can be concluded that the I_1-imidazoline receptor does not mediate the effect of agmatine on LC neurons.

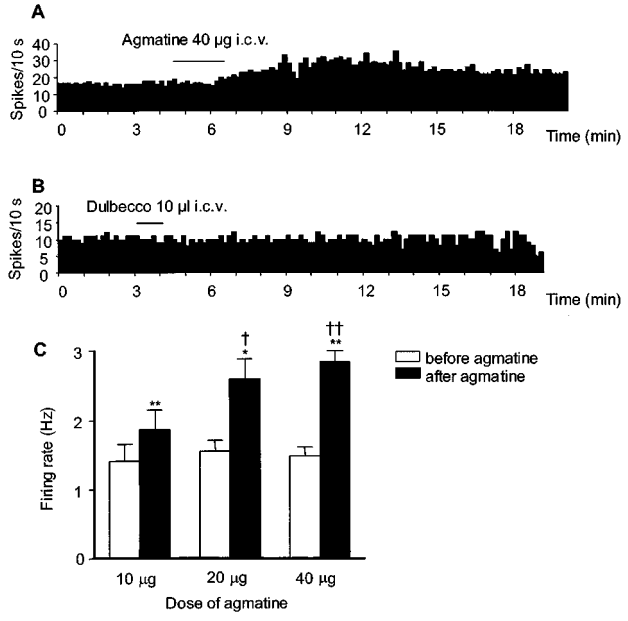

Figure 1 Representative recordings showing (A) the stimulatory effect of agmatine (40 μg, i.c.v.) and (B) the lack of effect of the vehicle of agmatine (Dulbecco's solution 10 μl, i.c.v.) on the firing of LC neurons. Vertical lines represent the firing rates extracellularly recorded and displayed on a chart recorder as integrated time histograms (spikes per 10 s). (C) Bar histograms showing the dose-dependence of agmatine for the three doses studied (10, 20 and 40 μg, i.c.v.). Bars are the mean ± s.e.mean for each dose before and after agmatine administration. For all doses studied, agmatine increased the firing rate of LC neurons (*$P < 0.05$, **$P < 0.001$ 2-tailed, paired Student *t*-test), when the basal firing rate of LC neurons was compared with the maximal effect observed after agmatine administration. †$P < 0.05$, ††$P < 0.001$ two-way repeated-measures ANOVA, when the agmatine (10 μg i.c.v.) induced effect was compared with that observed after other doses of agmatine (20 and 40 μg, i.c.v.).

Agmatine does not interact with α_2-adrenoceptors or μ opioid receptors

Some studies have demonstrated that agmatine has an affinity for α_2-adrenoceptors and that it can interact with the opioid system, potentiating the analgesic effects of morphine (see Introduction). Therefore, we investigated if the stimulatory effect of agmatine could be mediated by an interaction with α_2-adrenoceptors or μ-opioid receptors. In order to do this, we studied the inhibitory effect of increasing doses of clonidine (0.625–10 μg kg^{-1} i.v., 2×, every minute) and morphine (0.3–4.8 mg kg^{-1} i.v., 2×, every minute), 4–5 min after agmatine administration (20 μg, i.c.v.). Under these circumstances, the dose-effect curves for clonidine and morphine were not different from those obtained in control animals (Figure 3A,B). The ED_{50} parameters were also unaltered (ED_{50} for clonidine in the control group: 2.74±0.32 μg kg^{-1}, $n=5$; after agmatine 2.66±0.40 μg kg^{-1}, $n=5$ and ED_{50} for morphine in the control group: 0.72±0.13 mg kg^{-1}, $n=5$; after agmatine 0.91±0.13 mg kg^{-1}, $n=5$). These results indicate that agmatine stimulates the LC *via* a mechanism which seems to be independent of both α_2-adrenoceptors and μ opioid receptors.

The agmatine effect is mediated through a nitric oxide-dependent mechanism

Some studies have pointed out that agmatine may affect nitric oxide synthesis. To determine if the stimulatory effect of agmatine is dependent on a nitric oxide pathway, the nitric oxide synthase inhibitors L-NA and L-NAME, and the less active stereoisomer D-NAME, were administered 20 min prior to application of agmatine (10 and 40 μg, i.c.v.). Administration of L-NAME (100 μg, i.c.v.) and L-NA (10 μg, i.c.v.) produced a slight and short-lived decrease in the LC neuron firing rate (data not shown) as reported by others (Torrecilla *et al*., 1999). However, although the mean firing rate immediately before agmatine administration was not statistically different from that obtained in the control group

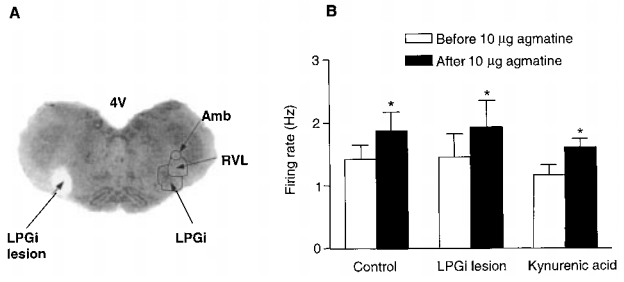

Figure 2 (A) Representative example of a rat brain tissue slice showing the electrolytic lesion of the PGi nucleus. (B) Bar histograms showing the effect of agmatine (10 μg, i.c.v.) in control rats, in PGi lesioned rats and in rats pretreated with kynurenic acid (1 μmol, i.c.v.). Bars are the mean±s.e.mean of five neurons for each dose before and after agmatine administration. In all groups studied, agmatine increased the firing rate of LC neurons (*$P<0.05$, 2-tailed, paired Student *t*-test), when the basal firing rate of LC neurons was compared with the maximal effect after agmatine administration. No statistical differences were found when the agmatine (10 μg, i.c.v.) induced effect was compared with that for the other groups. Amb, ambiguous nucleus; RVL, rostroventral lateral nucleus; 4A, fourth ventricle.

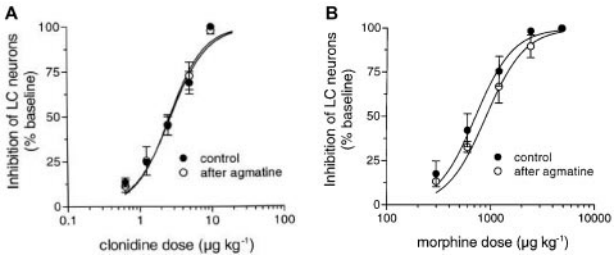

Figure 3 Dose-effect curves for the inhibitory effect of (A) clonidine or (B) morphine on the firing rate of LC neurons, showing the lack of effect of pretreatment with agmatine (20 μg, i.c.v.) on the dose-effect curve parameters. Symbols are mean±s.e.mean of the percentage reduction from the basal firing rate of *n* rats. In all groups the number of rats was $n=5$. The horizontal axis represents the cumulative doses of clonidine or morphine, administered i.v. at 1-min intervals. The lines through the data are the theoretical curves in each group constructed from the mean of the dose-effect curve parameters, as estimated individually by non-linear regressions (see Methods for details).

(firing rate after L-NAME: 1.3±0.3 Hz, $n=5$, after L-NA: 1.4±0.3 Hz, $n=5$ and after D-NAME: 2.5±0.3 Hz, $n=5$), the stimulatory effect of agmatine (10 μg, i.c.v.) was completely blocked by L-NA ($n=5$; $P<0.05$) and L-NAME ($n=5$; $P<0.05$), but it was not changed by D-NAME ($n=5$) (Figure 4A). Furthermore, the effect of the highest dose of agmatine (40 μg, i.c.v.) was also completely blocked L-NA ($n=5$; $P<0.05$) and L-NAME ($n=5$; $P<0.05$), but it was not changed by D-NAME ($n=5$) (Figure 4B). The present data indicate that agmatine elicits its stimulatory effect on LC neurons through a nitric oxide synthase-dependent mechanism.

The agmatine effect is mediated by a mechanism located in the LC

To evaluate whether the stimulatory effect of agmatine could be caused by a mechanism located within the LC area and related to NOS activity, we studied the effect of local application of agmatine (20 pmoles) in 4–5 cells and then in other 4–5 cells after L-NAME (100 μg, i.c.v.) application in the same rat. As it is shown in Figure 5, in five rats, pressure microinjection of agmatine increased the firing rate of all LC neurons studied, but this stimulatory effect of agmatine was prevented by L-NAME (maximal increase caused by agmatine before and after NOS inhibition 53±12%, $n=22$, 14±10%, $n=21$ respectively).

Discussion

The present study demonstrates that i.c.v. administration of agmatine, a proposed imidazoline receptor endogenous ligand, elicits a dose-related stimulatory effect on the activity of LC neurons. This effect was not modified by electrolytic lesioning of the PGi (an area enriched with I_1-imidazoline receptors) or by excitatory amino acid receptor antagonism. No interaction between agmatine and α_2-adrenoceptor or opiate receptor agonists was found, but the agmatine effect was abolished by nitric oxide synthase inhibitors.

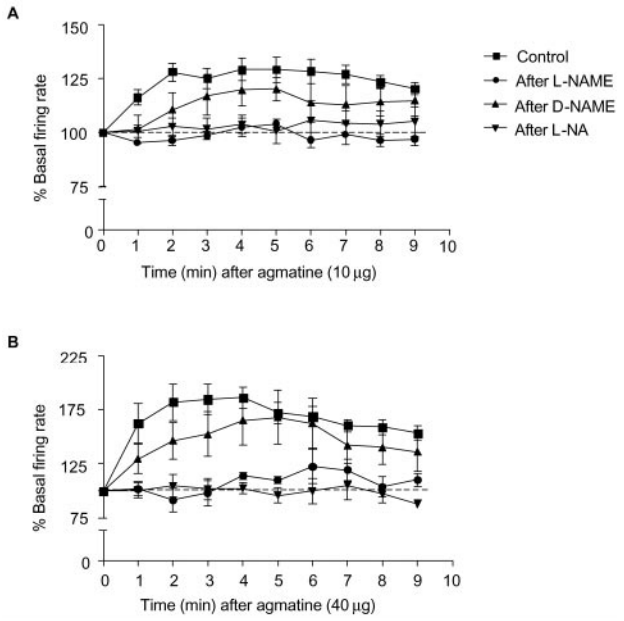

Figure 4 Time course of the effect of agmatine (A, 10 μg, and B, 40 μg, i.c.v.) on LC neuron firing rate in control rats and after administration of nitric oxide synthase inhibitors, showing the effect of agmatine after pretreatment with the nitric oxide synthase inhibitors, N^{ω}-nitro-L-arginine (L-NA) (10 μg, i.c.v.) and N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (100 μg, i.c.v.) and the less active stereoisomer, N^{ω}-nitro-D-arginine methyl ester hydrochloride (D-NAME) (100 μg, i.c.v.). The nitric oxide synthase inhibitors were administered 20 min before agmatine injection. The stimulatory effect of agmatine (10 and 40 μg, i.c.v.) was completely blocked after L-NA ($P<0.05$) ($n=5$) or L-NAME ($P<0.05$) ($n=5$) but not after D-NAME ($n=5$) administration (Two-way repeated-measures ANOVA). Note: statistical analysis was performed with firing rate data and the time course of the agmatine effect for each group was presented as a percentage of stimulation.

LC neuron activity is under the control of vegetative changes such as alterations in blood pressure (Elam *et al*., 1984). In the present study, previous bilateral vagotomy did not modify the agmatine effect on LC neurons (data not shown). Therefore, the agmatine stimulatory effect we describe here can be interpreted as an effect mediated by a centrally-located mechanism, which could be due to the interaction of agmatine with several types of receptors. In favour of this hypothesis, local application of agmatine into the LC also stimulated cell activity of this nucleus. The fact that the stimulation caused by local application did not reach the same magnitude as that caused by intracerebroventricular administration could be due to methodological reasons since it is not possible to apply locally more volume of solution or a more concentrated solution of agmatine. Alternatively, agmatine could act upon receptors not only located in the LC but also located outside the LC.

The first hypothesis tested was that imidazoline receptors participated in mediating the effect of agmatine, since agmatine binds to imidazoline receptors and is thought to be an endogenous ligand of these receptors (see Introduction). Thus, our previous studies have demonstrated that the activation of imidazoline receptors by clonidine and other imidazoline compounds elicits a stimulatory effect on LC neurons (Pineda *et al*., 1993) *via* the activation of I_1-imidazoline receptors located in the PGi and mediated by excitatory amino acids (Ruiz-Ortega *et al*., 1995; Ruiz-Ortega & Ugedo, 1997). However, in the present study, neither electrolytic lesions of the PGi nor pretreatment with kynurenic acid, modified the effect of agmatine on the LC, which is in conflict with the hypothesis that the effect of agmatine is due to an interaction with I_1-imidazoline receptors. On the other hand, agmatine does not seem to interact with non-I_1/I_2-imidazoline receptors since it does not cause any effect on the LC when it is applied *in vitro* (Pineda *et al*., 1996b).

It has been shown that agmatine interacts with α_2-adrenoceptors (Molderings *et al*., 2000) and also with the opioid system (Kolesnikov *et al*., 1996; Sánchez-Blázquez *et al*., 2000). However, in the current study, agmatine did not modify the dose-response curve for clonidine and morphine, which excludes any functional interaction.

Equivocal results have been obtained regarding the possible interaction between agmatine and nitric oxide synthase. Thus, it has been shown that agmatine activates nitric oxide synthase in endothelial cells (Morrissey & Klahr, 1997) and kidney (Schwartz *et al*., 1997) but agmatine also inhibits selectively the inducible form of the nitric oxide synthase (Auguet *et al*., 1995; Galea *et al*., 1996) and the neuronal nitric oxide synthase *in vitro* (Demady *et al*., 2001). In the present study, the stimulatory effect of agmatine on LC neurons was completely blocked by administration of the nitric oxide synthase inhibitors L-NAME and L-NA, but not by the less active stereoisomer D-NAME. L-NAME and L-NA are two substituted analogues of L-arginine, which inhibit constitutive isoforms of nitric oxide synthase both *in vivo* and *in vitro* (Moore & Handy, 1997). Therefore, our data suggest that nitric oxide mediates the stimulatory effect of agmatine on LC cell activity. The fact that L-NAME blocked the effect of locally applied agmatine indicates that nitric oxide may be formed in the LC area. This hypothesis is consistent with the role proposed for the nitric oxide/cyclic GMP pathway in the LC (Pineda *et al*., 1996a). Thus, nitric oxide has been reported to stimulate directly LC neurons *via* a cyclic GMP-dependent mechanism (Pineda *et al*., 1996a). Moreover, nitric oxide synthase immunostaining has been described in the vicinity of the LC (Xu *et al*., 1994), and LC neurons are endowed with high levels of guanylate cyclase (Matsuoka *et al*., 1992) and type II cyclic GMP-dependent protein kinase (El-Husseini *et al*., 1995).

An increase in nitric oxide formation after administration of agmatine could be explained either by the formation of an intermediate product, N^{ω}-hydroxyagmatine (Ishikawa *et al*., 1995), or by cross-talk between the enzymatic pathways producing agmatine (arginine decarboxylase) and nitric oxide (nitric oxide synthase), both of which use L-arginine as a common substrate (Blantz *et al*., 2000). The failure of agmatine to affect LC neurons *in vitro* (Pineda *et al*., 1996b) suggests that some elements required for the activity of agmatine or nitric oxide synthesis itself, are lost after severing the LC inputs during brain slice preparation. Likewise, nitric oxide synthase inhibitors are able to alter tonic activity of LC cells *in vivo* (Torrecilla *et al*., 1999), but not *in vitro* (Pineda *et al*., 1996a).

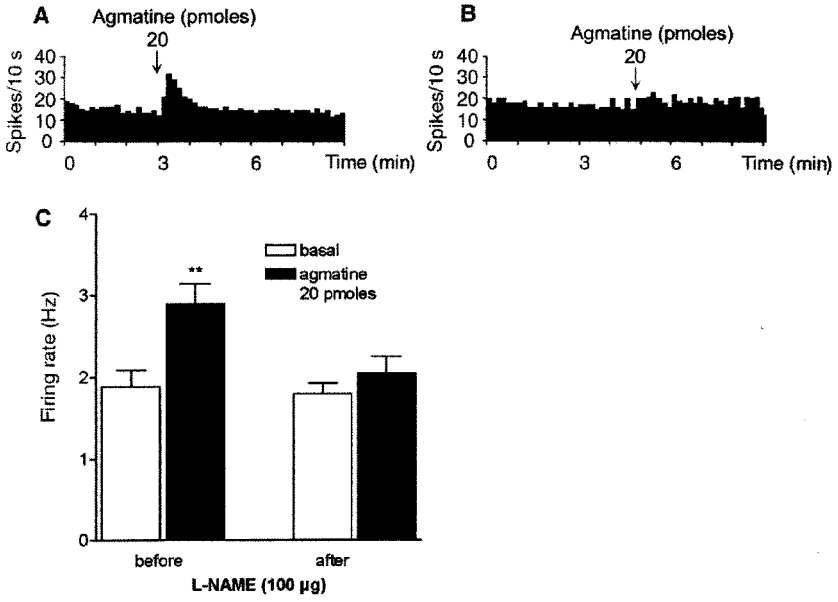

Figure 5 Representative recordings showing (A) the stimulatory effect of local administration of agmatine (20 pmoles) and (B) the lack of effect of local administration of agmatine after application of L-NAME (100 μg, i.c.v.) on the firing of LC neurons. Vertical lines represent the firing rates extracellularly recorded and displayed on a chart recorder as integrated time histograms (spikes per 10 s). (C) Bar histograms showing the effect of local administration of agmatine (20 pmoles) before and after application of L-NAME (100 μg, i.c.v.) on the firing of LC neurons. Bars are the mean $\pm$ s.e.mean of five neurons before and after agmatine administration. Before L-NAME application, agmatine increased the firing rate of LC neurons (**$P<0.005$, paired Student t-test), when the basal firing rate of LC neurons was compared with the maximal effect after agmatine administration.

In summary, the results of the present study demonstrate that agmatine stimulates neuronal activity of the LC and suggest that imidazoline receptors and α_2-adrenoceptors do not directly mediate this effect. However, while no interaction between agmatine and the opioid system was found, agmatine does interact with the nitric oxide pathway in the LC and may function as an activator of nitric oxide formation in the LC or in its vicinity, thereby stimulating the LC. This study not only supports the hypothesis that agmatine has a role as a neurotransmitter/neuromodulator but also contributes to our knowledge of its mechanism of action.

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