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ORIGINAL ARTICLE

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Metabotropic glutamate receptors modulate serotonin release in the rat periaqueductal gray matter

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Abstract The role of metabotropic (mGluRs) and N-methyl-D-aspartate (NMDA) glutamate receptors on 5-hydroxytryptamine (5-HT) release has been studied in rat periaqueductal gray (PAG) matter by using in vivo microdialysis. (1S,3R)-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD; 0.5 or 1 mM], a group I/group II mGluRs agonist, increased the dialysate 5-HT concentration. (2S)-α-ethylglutamic acid (EGlu; 1 mM), an antagonist of group II mGluRs, but not (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA; 1 mM), an antagonist of group I mGluRs, antagonized the 1S,3R-ACPD-induced effect. (S)-3,5-dihydroxyphenylglycine (DHPG; 0.5 and 1 mM), an agonist of group I mGluRs, did not modify dialysate 5-HT. (2S, 3S, 4S)-α-(carboxycyclopropyl)-glycine (L-CCG-I; 0.5 and 1 mM), an agonist of group II mGluRs, increased extracellular 5-HT. This effect was antagonized by EGlu. Similarly, L-serine-O-phosphate (L-SOP; 1 and 10 mM), an agonist of group III mGluRs, increased extracellular 5-HT and this effect was antagonized by (RS)-α-methylserine O-phosphate (M-SOP; 1 mM), an antagonist of group III mGluRs. Out of the several N-methyl-D-aspartate concentrations used (NMDA; 10, 50, 100, 500 and 1000 μM) only the 50 μM infusion significantly decreased dialysate 5-HT. The $GABA_A$ receptor agonist, bicuculline (30 μM), increased 5-HT release on its own and antagonized the decrease caused by the opiate antagonist, naloxone (2 mM), as well as the increases caused by CCG-I or L-SOP. These data show that stimulation of PAG's group II/group III mGluRs increases 5-HT release, while stimulation of NMDA glutamate receptors may decrease it. We speculate that glutamate does not modulate 5-HT release in the PAG directly, but via activation of tonically active GABAergic interneurons.

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Introduction

There is growing evidence that L-glutamate may control 5-HT release (Mayer and Westbrook 1987; Becquet et al. 1990). Although several studies have suggested that NMDA and non-NMDA receptors stimulation led to changes in the concentration of extracellular 5-HT (Tao and Auerbach 1996; Whitton et al. 1994; Maione et al. 1997), to our knowledge little work has been done on the possible modulatory role of metabotropic glutamate receptors (mGluRs) on 5-HT release (McCormick and von Krosigk 1992). The role of mGluRs in the central nervous system (CNS) is raising considerable interest. This heterogeneous family of G-protein-coupled receptors is now known to include at least eight subtypes that can be placed into three groups according to sequence homology, intracellular transduction mechanisms and agonist-antagonist pharmacology (Nakanishi 1992; Watkins and Collingridge 1994; Pin and Duvoisin 1995). The periaqueductal gray (PAG) area is one of those brain structures featuring a high level of mGluRs (Catania et al. 1994) as well as serotonergic fibres originating from the dorsal raphe nucleus (DRN). Midbrain PAG matter may be considered the caudal pole of a longitudinally organized neural system which modulates behavioural and physiological changes. There is evidence that the PAG matter regulates emotional states such as fear and anxiety through the participation of 5-HT and glutamate (Handler and Depaulis 1988; Deakin and Graeff 1991; Guimar et al. 1991). In this regard we investigated the effect of direct infusion of mGluRs ligands or N-methyl-D-aspartate (NMDA) on 5-HT release in the PAG area using microdialysis in awake rats.

Materials and methods

Methods. Male Wistar rats (250–300 g, Charles River, Italy) were housed under controlled environmental conditions (21±1°C, 60% humidity, 12 h light-dark cycle, food and water ad libitum) for 5–7 days. Rats were anaesthetized with chloral hydrate (400 mg/kg i.p.) and concentric dialysis probes were implanted, with stereotaxic apparatus, in the PAG area (measured from the bregma AP: –7.8; L: 0.6; V: 5.0). Coordinates from the stereotaxis atlas of Paxinos and Watson (1986) were applied. Dialysis probes, with an active surface of 2 mm, were constructed as described by Hutson et al. (1985) with 25G (0.3 mm ID, 0.5 mm OD) stainless steel tubing (A-M systems, Everett, USA). Inlet and outlet cannulae (0.04 mm ID, 0.14 mm OD) consisted of fused silica tubing (Scientific Glass Engineering, Melbourne, Australia). Cuprophan hollow fibres (Enka AG, Wuppertal, Germany) were used for the dialysis membranes. The day after surgery, each implanted probe was perfused with artificial cerebrospinal fluid (ACSF: composition in mM: KCl, 2.5; NaCl, 125; $MgCl_2$, 1.18 and $CaCl_2$ 1.26) at a rate of 1 µl/min. After a 1 h period of equilibration, dialysate samples were collected every 30 min. Some consecutive samples (3–5) were collected before administering the drugs. All the drugs were dissolved in ACSF and infused into the PAG area via dialysis probes. In the case of pretreatment with an antagonist, this latter drug was infused through the same probe 30 min before the respective agonist. At the end of the experiment, all the rats were deeply anaesthetized and a 2% fast green solution was perfused through the dialysis probe to stain the surrounding tissue for histological inspection. 5-HT was determined using HPLC equipment fitted with an electrochemical detector as previously described by Hutson et al. (1985). The composition of the mobile phase was: 0.15 mM NaH_2PO_4, 0.01 mM octyl sodium sulphate, 0.5 mM EDTA (pH 3.8 adjusted with phosphoric acid) and 12.5% methanol and was delivered (flow rate: 1 ml/min) by a model 590 pump (Waters Associates, Milforal, USA) into an Ultrasphere 3 µm ODS column (4.6 mm×7.5 cm; Beckman Ltd, San Ramon, USA). The electrochemical detector was an ESA Coulochem mod. 5100A with a dual electrode analytical cell (mod. 5011). The conditioning cell was set at –0.05 V, electrode 1 at +0.10 V and electrode 2 at +0.25 V with respect to palladium reference electrodes. The limit of detection for 5-HT was found to be 2–3 fmol per sample injected with a signal-to-noise ratio of 2. The mean dialysate concentration of 5-HT in the first five samples represented the basal value and the results were expressed as percentage of the latter. In vitro recovery of the dialysis probe for 5-HT was 29–32%. For statistical analysis, treated and control animals were compared for significant differences at each stage using one and two-way ANOVA with repeated measurings followed by Newman-Keuls test for multiple comparison.

Drugs. The following drugs, dissolved in artificial cerebrospinal fluid, were used: (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R)-ACPD; (S)-3,5-dihydroxyphenylglycine [DHPG]; (RS)-1-aminoindan-1,5-dicarboxylic acid [AIDA]; (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), (2S)-α-ethylglutamic acid [(2S)-α-EGlu]; (RS)-α-methylserine-O-phosphate [M-SOP]; N-methyl-D-aspartic acid (NMDA) (Tocris Cookson Ltd, Bristol, UK); (–)bicuculline methochloride; L-serine-O-phosphate (L-SOP); chloral hydrate (Sigma Chemical Co., St. Louis, MO, USA) and naloxone hydrochloride (RBI, Natick, MA, USA).

Results

The mean basal value (not corrected for probe recovery) of extracellular 5-HT levels of the PAG area was 29±6 fmol/sample (mean ±SEM). Each animal was used once only and the reported value of basal 5-HT is the mean concentration from 14 randomly analyzed rats. Infusion of 500 µM 1S,3R-ACPD, an agonist of group I and II mGluRs (Pin and Duvoisin 1995) led to a significant increase (146±10% basal value) in extracellular 5-HT (Fig. 1A). This effect took at least 1.5–2 hours to appear from the start of the drug infusion. The highest dose of 1S,3R-ACPD (1 mM) caused a more pronounced increase in dialysate 5-HT (196±12% basal value) (Fig. 1A). Here again, the increase of extracellular 5-HT appeared delayed (0.5–1 hour) from the start of the drug infusion (Fig. 1A). The 1S,3R-ACPD-induced effect was not antagonized by pretreatment with AIDA (1 mM), a selective group I mGluRs antagonist (Moroni et al. 1997) (Fig. 1B). Pretreatment for 30 min with a group II mGluRs antagonist, EGlu (1 mM; Jane et al. 1996), blocked the effect of 1S,3R-ACPD (Fig. 1C). Intra-PAG infusion of

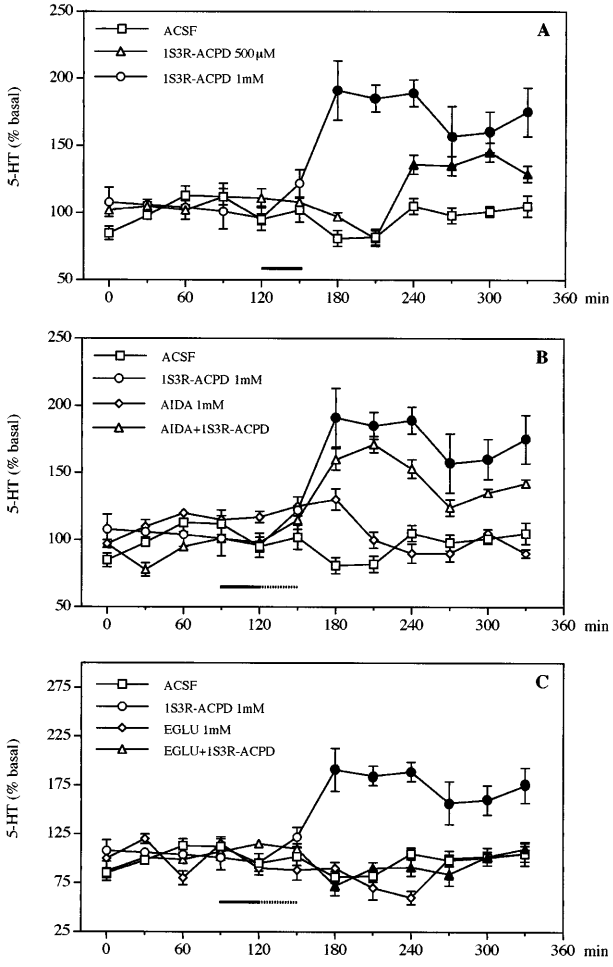

Fig. 1 Effect of artificial cerebrospinal fluid (ACSF) (A, B and C), (1S,3R)-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD; 0.5 or 1 mM) (A), 1S,3R-ACPD (1 mM) in combination with (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA, 1 mM) (B) and 1S,3R-ACPD (1 mM) in combination with (2S)-α-ethylglutamic acid (EGlu; 1 mM) (C) on periaqueductal grey dialysate 5-HT. Drugs were infused into the PAG through a concentric dialytic probe after collection of 4–5 basal samples. The unbroken bars represent the period of 1S,3R-ACPD (A), AIDA (B) or EGlu (C) infusion, while the dotted bars (B and C) indicate the period of 1S,3R-ACPD infusion. Data (7–10 rats for each group) are means ±SEM of 5-HT release in percent of the basal values. Filled symbols denote significant differences between drugs and the corresponding control group (ANOVA followed by Newman-Keuls test)

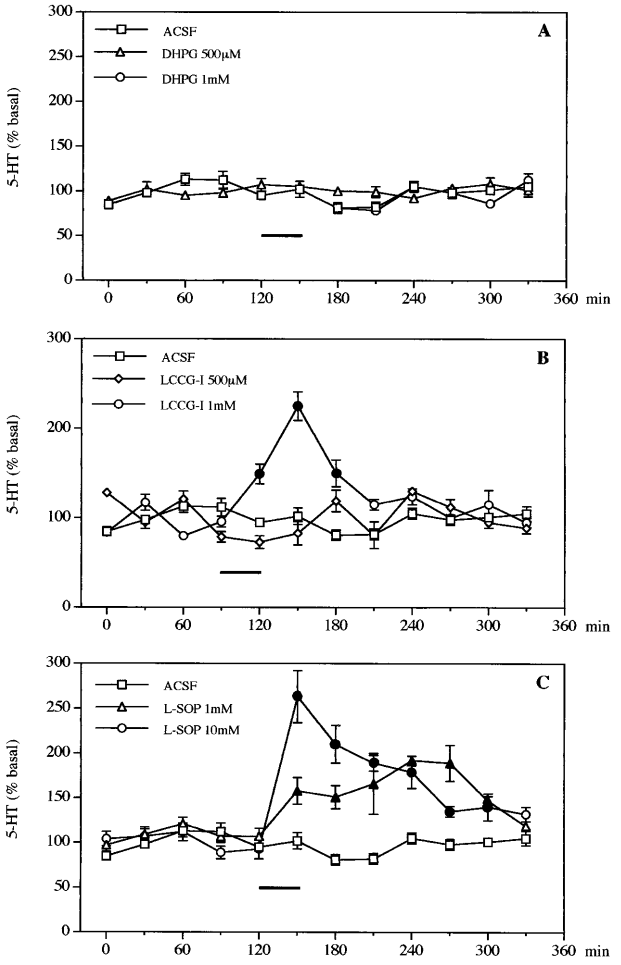

Fig. 2 Effects of artificial cerebrospinal fluid (ACSF) (A, B and C), (S)-3,5-dihydroxyphenylglycine (DHPG; 0.5 and 1 mM) (A), (2S,3S,4S)-α-carboxycyclopropylglycine (L-CCG-I; 0.5 and 1 mM) (B) and L-serine-O-phosphate (L-SOP; 1 and 10 mM) (C) on periaqueductal grey dialysate 5-HT. Drugs were infused into the PAG through a concentric dialytic probe after collection of 4–5 basal samples as indicated by the bar. Data (7–10 rats for each group) are means ±SEM of 5-HT release in percent of the basal values. Filled symbols denote significant differences between drugs and the corresponding control group (ANOVA followed by Newman-Keuls test)

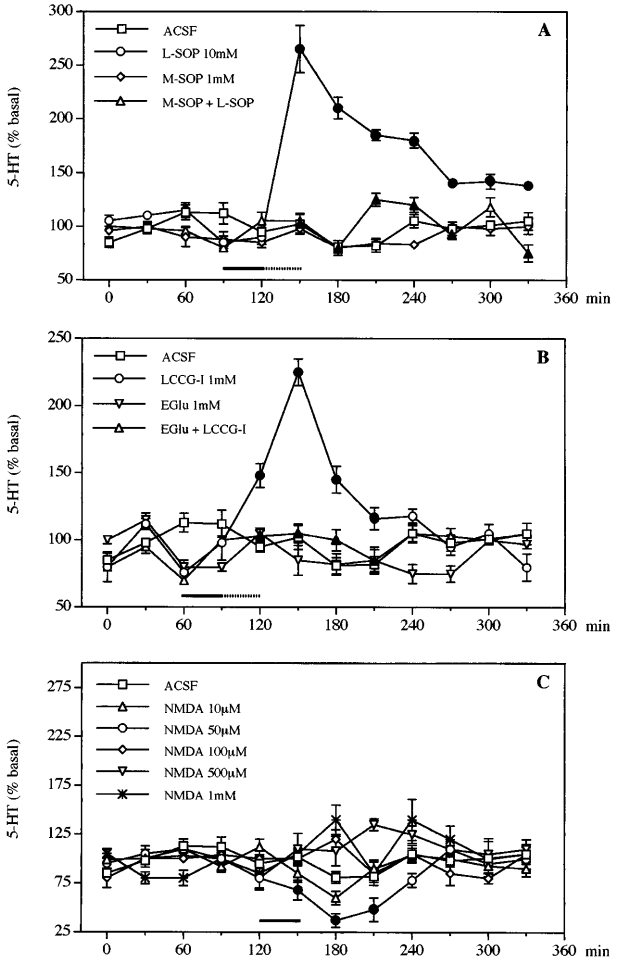

Fig. 3 Effects of artificial cerebrospinal fluid (ACSF) (A, B and C), L-serine-O-phosphate (L-SOP; 10 mM) alone or in combination with (RS)-α-methylserine-O-phosphate (M-SOP; 1 mM) (A), (2S,3S,4S)-α-carboxycyclopropylglycine (L-CCG-I; 1 mM) alone or in combination with (2S)-α-ethylglutamic acid (EGlu; 1 mM) (B) and N-methyl-D-aspartic acid (NMDA; 0.1–0.5–1 mM) (C) on periaqueductal grey dialysate 5-HT. Drugs were infused into the PAG through a concentric dialytic probe after collection of 3–5 basal samples. The unbroken bars represent the period of M-SOP (A), EGlu (B) or NMDA (C) infusion, while the dotted bars indicate the period of L-SOP (A) or L-CCG-I (B) infusion. Data (7–10 rats for each group) are means ±SEM of 5-HT release in percent of the basal values. Filled symbols denote significant differences between drugs and the corresponding control group (ANOVA followed by Newman-Keuls test).

the selective agonist of group I mGluRs (Sekiyama et al. 1996), DHPG (500 μM or 1 mM), did not modify dialysate 5-HT levels (Fig. 2A). L-CCG-I (500 μM), an agonist of group II mGluRs, did not significantly augment dialysate 5-HT. A sharp and significant increase (220±15% basal value) of extracellular 5-HT lasting approximately 2 h was observed with the dose of 1 mM (Fig. 2B). Similarly, L-SOP (1–10 mM), an agonist of group III mGluRs (Pin and Duvoisin 1995), induced a significant and dose-dependent increase of 5-HT dialysate of 195±14% and 265±16% with 1 and 10 mM respectively (Fig. 2C). M-SOP (1 mM), a selective antagonist of group III mGluRs (Thomas et al. 1996), completely blocked the effect induced by L-SOP 10 mM (Fig. 3A). EGlu 1 mM, infused 30 min before LCCG-1 1 mM, completely blocked the L-CCG-I-induced effect (Fig. 3B). NMDA (10–350–100–500–1000 μM) infusion, although only at the concentration of 50 μM, significantly decreased the dialysate 5-HT (Fig. 3C). However, a dramatic and concentration-dependent behavioural change (running, jumping and piloerection) was observed at all the NMDA concentrations tested (data not shown). Concentrations of NMDA below 10 μM (i.e. 1 μM, data not shown) as well as over 50 μM were unable to significantly modify the extracellular 5-HT concentration (Fig. 3C). Bicuculline (30 μM), a selective antagonist of $GABA_A$ receptors, when infused into the PAG induced an increase of extracellular 5-HT (Fig. 4A). On the contrary, intra-PAG infusion of naloxone (2 mM), an antagonist of the opiate receptors, generated a short, but significant, decrease of dialysate 5-HT

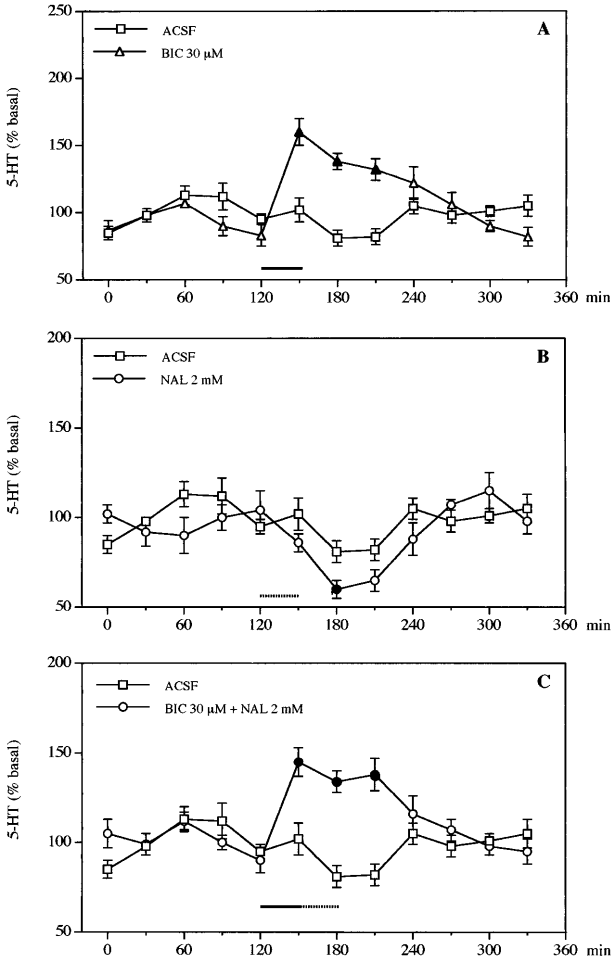

Fig. 4 Effects of artificial cerebrospinal fluid (ACSF) (A, B and C), bicuculline (BIC; 30 µM) (A), naloxone (NAL; 2 mM) (B) or bicuculline infused 30 min before naloxone (C) on periaqueductal grey dialysate 5-HT. Drugs were infused into the PAG through a concentric dialytic probe after collection of 5 basal samples. The unbroken bars represent the period of BIC infusion (A and C), while the dotted bars indicate the period of NAL infusion (B and C). Data (7–8 rats for each group) are means ±SEM of 5-HT release in percent of the basal values. Filled symbols denote significant differences between drugs and the corresponding control group (ANOVA followed by Newman-Keuls test)

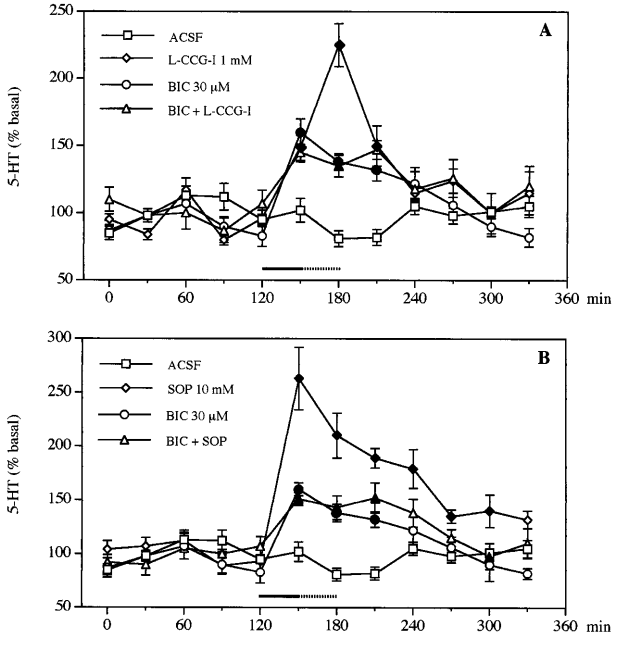

Fig. 5 Effects of artificial cerebrospinal fluid (ACSF) (A and B), bicuculline (BIC; 30 µM) alone (A and B) or in combination with (2S,3S,4S)-α-carboxycyclopropylglycine (L-CCG-I; 1 mM) (A) or L-serine-O-phosphate (L-SOP; 10 mM) (B) on periaqueductal grey dialysate 5-HT. Drugs were infused into the PAG through a concentric dialytic probe after collection of 5 basal samples. The unbroken bars represent the period of BIC infusion (A and B), while the dotted bars indicate the period of L-CCG-I or L-SOP infusion (A and B). Data (7–8 rats for each group) are means ±SEM of 5-HT release in percent of the basal values. Filled symbols denote significant differences between drugs and the corresponding control group (ANOVA followed by Newman-Keuls test)

(Fig. 4B). Bicuculline (30 µM), when infused 30 min before naloxone (1 mM), prevented the decrease of 5-HT expected by infusing naloxone (Fig. 4C). Similarly, bicuculline (30 µM), infused 30 min before L-CCG-I (1 mM) or L-SOP (10 mM), prevented the increase of 5-HT induced by L-CCG-I and L-SOP (Fig. 5).

Discussion

Since experimental evidence indicates that the PAG receives a serotonergic innervation (Mammounas et al. 1991), as well as a dense glutamatergic innervation (Beart et al. 1990), there is anatomical substrate for hinting at a participation of 5-HT and glutamate in the modulation of fear and anxiety at this level (Graeff 1990). Previous studies showed that both neurotransmitters are involved, although in opposite ways, in the regulation of experimentally induced aversive experiences. Schütz et al. (1985) showed that intra-PAG injections of 5-HT receptor agonists significantly reduce aversion after electrically-induced PAG stimulation. There is evidence that 5-HT reduces the aversive behaviour at the level of the PAG through the stimulation of GABAergic interneurons (Audi and Graeff 1987). Bandler and Depaulis (1988) demonstrated that activation of dorsal PAG ionotropic glutamate receptors elicits defensive rage behaviour with fear and panic. Moreover, the possibility of such a 5-HT/glutamate interaction at the PAG in modulating defensive behaviour, is supported by the evidence that 8-OH-DPAT, a selective agonist of 5-HT_{1A} subtype receptors, antagonizes the aversive effects induced by microinjection of amino acids into the PAG (Beckett et al. 1992).

In order to further evaluate the possible interaction between glutamate and 5-HT in the PAG, we attempted to assess the participation of mGluRs (as well as of NMDA glutamate receptors) on 5-HT release in this midbrain area.

We found that the PAG mean dialysate concentration of 5-HT was high (29±6 fmol/sample) in comparison to other 5-HT-rich areas such as the raphe nuclei, amygdala and hip-

pocampus. In these areas, very high 5-HT concentrations are detectable when a selective 5-HT reuptake inhibitor (i.e. citalopram) is co-infused (Whitton et al. 1994; Maione et al. 1997). Although we do not know why such a high 5-HT concentration was measurable in the PAG, we think that, at least in part, it could be due to: i) the very closeness of the PAG matter to the raphe nuclei and, therefore, to the huge 5-HT somatodendritic share, or ii) the different serotonergic pathway which innervates the PAG matter. The serotonergic axons projecting to the amygdala originate from the dorsal raphe-forebrain tract, while those directed to the PAG, as well as to the medial hypothalamus, run through the dorsal raphe-periventricular tract (Azmitia 1978).

In this in vivo study, we used high concentrations of both phenylglycine (i.e. 1S,3R-ACPD, DHPG, L-CCG-I) and non-phenylglycine (i.e. AIDA, L-SOP) derivatives because of their low potency. These new pharmacological tools, which exhibit greater selectivity for the mGluRs, are active in vitro at 200–500 micromolar concentrations (Roberts, 1995; Constantino and Pellicciari, 1996; Moroni et al. 1997; Doherty et al. 1997). Therefore, due to the efficiency of the probes, the recovery of which is about 30%, the actual drug concentrations reaching the cerebral tissue may resemble those used in the in vitro studies.

Infusion of 1S,3R-ACPD, a non-selective agonist of group I and group II mGluRs, induced a long lasting increase of extracellular 5-HT throughout the observation period, but it only became evident 1–2 hours from the start of the drug infusion. This apparently does not reflect a true pharmacological effect. One would expect the opposite: a rapid concentration-dependent increase of 5-HT, followed by a fading at the end of the experiment. However, the blockade of the 1S,3R-ACPD-induced effect obtained by pretreatment with a selective group II mGluRs antagonist (but not with a selective antagonist of group I mGluRs) nevertheless suggests that it is due to selective activation of group II mGluRs. The involvement of group II mGluRs was further confirmed by infusing L-CCG-I, a selective agonist of these mGluRs. An increase of extracellular 5-HT was also observed after stimulation of group III mGluRs with L-SOP, a selective agonist of this group of mGluRs. The increase of 5-HT induced by L-CCG-I and L-SOP was prevented by pretreatment with EGlu and MSOP, which are selective antagonists for group II and group III mGluRs respectively. Since these antagonists per se had no effect on PAG dialysate 5-HT at the concentrations used, it may well be that these receptors are not tonically modulating 5-HT release in the PAG matter.

In contrast to group II and group III mGluRs stimulation, the infusion of NMDA decreased extracellular 5-HT concentration, although this effect was evident only at the 50 μM dosage. This could mean that in response to a restricted range of NMDA concentrations (perhaps closer to a physiological situation), NMDA glutamate receptors exert a negatively modulatory role of the 5-HT release in this area. Although we cannot exclude other possibilities, it could be that over-stimulation of NMDA glutamate receptors after infusion of the highest NMDA concentrations, may generate unknown secondary neurochemical changes which prevented a further decrease of extracellular 5-HT. However, NMDA always generated a concentration-dependent behavioural change (i.e. teething, jumping, piloerection, etc.). Such a behavioural effect is in agreement with previous findings which showed that glutamate at this level exerts a phasic control of the aversive behaviour (Zhang et al. 1990).

Therefore, while stimulation of NMDA glutamate receptors decreased 5-HT release, this study showed that the activation of group II or group III mGluRs, which in other cerebral areas negatively modulate glutamate release (Lombardi et al. 1993; East et al. 1995; Shigemoto et al. 1996), increased extracellular 5-HT concentration in the PAG. In light of the current knowledge about NMDA glutamate receptors and mGluRs physiology, an opposite result would be expected. It was predictable that a depolarization induced by NMDA glutamate receptors (as well as by group I mGluRs) would increase extracellular 5-HT concentration, whereas a hyperpolarization or a decreased release of excitatory neurotransmitters induced by stimulation of group II or group III mGluRs (Pin and Duvoisin 1995; Shigemoto et al. 1996; Wigmore and Lacey 1998), would decrease it. This apparent contradiction may be due to the complex cytoarchitectonic organization in the PAG matter, where other neurotransmitters also participate in regulating 5-HT release. Evidence that endogenous opiates modulate 5-HT release in the PAG was first demonstrated by Johnson and Crowley (1984). A more recent study further confirmed the existence of synapses between opioidergic and serotonergic fibres in the PAG (Allen et al. 1993): damaging of serotonergic terminals induced a loss of mu and delta opiate receptors in this area. On the other hand, there is also evidence that glutamate affects 5-HT release in the PAG via GABAergic interneurons (Bequet et al. 1990). These latter cells, which project onto dorsal raphe serotonergic neurons (Jolas and Aghajanian 1997), are, in turn, under a negative opioidergic control (Maione et al. 1995).

There is, therefore, evidence that GABA and opioids may affect the glutamatergic control of 5-HT release within the PAG. In order to further evaluate how mGluRs operate on 5-HT release in the PAG and to assess whether or not GABA and opioids are involved in the glutamate-induced effect, we carried out experiments with bicuculline, a selective $GABA_A$ receptor antagonist, and naloxone, a non-selective opioid receptor antagonist, alone or in combination with L-CCG-I or L-SOP. Bicuculline per se increased extracellular 5-HT concentration and this suggested a tonic GABAergic control on 5-HT release. Opposite to bicuculline, naloxone generated a short, but significant, decrease of extracellular 5-HT. This effect was unexpected in consideration of the inhibitory nature of the opioids. However, bicuculline prevented the naloxone-induced effect and this suggested that GABAergic interneurons participate in the decrease of extracellular 5-HT that followed infusion of naloxone. Although synapses between opioid and serotonergic terminals have been demonstrated in the PAG (Allen et al. 1993), our data showed, in agreement with other studies (Bequet et al. 1990; Maione et al. 1995; Solas and Aghajanian 1997), that opioids may modulate 5-HT release in the PAG through inhibition of GABAergic interneurons which tonically and negatively modulate sero-

tonergic terminals. Further evidence excluding a direct glutamatergic control on 5-HT release, seems to be provided by NMDA infusion. NMDA glutamate receptor stimulation induced a decrease, rather than an increase, of extracellular 5-HT concentration, although such an effect was apparent only at a lower concentration of NMDA. It is, therefore, reasonable to speculate that GABAergic cells receive both opioidergic and glutamatergic inputs. A decrease of 5-HT release would also be expected after DHPG infusion. However, this was the case only with NMDA. It is difficult to say why stimulation of group I mGluRs did not modify 5-HT release. This could be due to a preferential role of the NMDA receptors on GABAergic neurons. However, further investigation is needed here.

Based on these data, we hypothesized that the stimulation of group II and group III mGluRs lead to inhibition of glutamate release in the PAG and this may, in turn, reduce the GABA-mediated inhibition of 5-HT release. Using this hypothesis as our starting point, we tested the effect of L-CCG-I and L-SOP after pretreatment with bicuculline. Although bicuculline increased the basal 5-HT release, we did not observe any further increase of 5-HT release during the blockade of $GABA_A$ receptors. This suggested that probably functioning GABAergic interneurons are necessary in order to generate the 5-HT increase observed after stimulation of group II and group III mGluRs.

In conclusion, this study provides evidence that PAG serotonergic terminals are probably not directly innervated by glutamatergic fibres. Since NMDA, an agonist of ionotropic glutamate receptors with a prevalent post-synaptic distribution, decreased extracellular 5-HT concentration, the existence of synapses between glutamatergic and serotonergic fibres seems unlikely. Moreover, the possible decrease of glutamate release induced by the activation of pre-synaptic group II and group III mGluRs, may generate a lower degree of PAG GABAergic tone and, therefore, elicit a rise of extracellular 5-HT. We therefore speculate that glutamate may modulate 5-HT release in the PAG not directly, but through the activation of tonically active GABAergic interneurons.

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