Periaqueductal Gray Metabotropic Glutamate Receptor Subtype 7 and 8 Mediate Opposite Effects on Amino Acid Release, Rostral Ventromedial Medulla Cell Activities, and Thermal Nociception

Ida Marabese,1,* Francesca Rossi,1* Enza Palazzo,1 Vito de Novellis,1 Katarzyna Starowicz,2 Luigia Cristino,3 Daniela Vita,1 Luisa Gatta,1 Francesca Guida,1 Vincenzo Di Marzo,2 Francesco Rossi,1 and Sabatino Maione1,2

1Department of Experimental Medicine, Section of Pharmacology “L. Donatelli,” Faculty of Medicine and Surgery, Second University of Naples, Naples; 2Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy; and 3Endocannabinoid Research Group, Institute of Cybernetics “E. Caianiello”, Consiglio Nazionale delle Ricerche, Pozzuoli (Naples), Italy

Submitted 29 March 2007; accepted in final form 8 May 2007

INTRODUCTION

It has been shown that metabotropic glutamate subtype 7 and 8 receptors (mGluR7 and mGluR8), similarly to mGluR4 and mGluR6, the other group III mGluR subtypes, function as presynaptic receptors that modulate glutamate and GABA releases (Cartmell and Schoepp 2000; Schaffhauser et al. 1998; Schoepp 2001). Presynaptic modulation of the release of these amino acids at spinal and periaqueductal gray (PAG) levels may be effective in alleviating pain (Marabese et al. 2006; Thomas et al. 2001). Indeed, the PAG is an important processing center for the descending control of nociception (Harris 1996), and glutamate and GABA play a critical role in processing pain at this level (Behbehani and Fields 1979; Harris and Hendrickson 1987; Millan et al. 1987; Moreau and Fields 1986). We have previously shown that mGluRs modulate glutamate, GABA, and glycine releases within the PAG (de Novellis et al. 2002, 2003), contributing to the tonic modulation of nociception (Berrino et al. 2001; Maione et al. 2000). More recently, we have found that presynaptic mGluR8s are expressed within the PAG on both GABAergic and glutamatergic neurons, and their stimulation leads to a facilitation of glutamate and an inhibition of GABA release in rats (Marabese et al. 2005). Because GABAergic interneurons tonically inhibit the PAG antinociceptive pathway (Moreau and Fields 1986), the mGluR8 stimulation-induced increase in glutamate and reduction in GABA at that level may be crucial to produce analgesia. Indeed, stimulation of mGluR8 within the PAG has an important antinociceptive effect in inflammatory and neuropathic pain in mice (Marabese et al. 2006). Conversely, PAG mGluR7, unlike mGluR8, subtype facilitates pain transmission in mice (Marabese et al. 2006). It is reasonable to suppose that the opposite effects induced by PAG mGluR4 and mGluR7 on pain might be due to the preferential location of the mGluR7 on glutamatergic synapses (Bradley et al. 1996; Shigemoto et al. 1997). Thus their possible main autoreceptor role on glutamate terminals may justify an inhibition of the excitatory output in the PAG antinociceptive pathway and consequently the appearance of the mGluR7-induced hyperalgesia. In this study, we decided to further verify our hypothesis (the opposite effect of mGluR7 and mGluR8 on pain modulation) by performing in vivo microdialysis, behavioral, and electrophysiological studies. Thus the effects of PAG mGluR7 stimulation on glutamate and GABA release and of mGluR7 and mGluR8 on rostral ventromedial medulla (RVM) cell activities and tail flick-related behavioral and electrophysiological responses have...
been evaluated in the current study. Three neuronal classes with distinct physiology and pharmacology are found in the RVM (Fields et al. 1991). Cells of one class, “neutral cells,” show no modification in spontaneous activity associated with nociceptive stimulation. On the other hand, there is evidence that as regards the other two classes of cells, the “ON cells” and the “OFF cells,” have specific roles in nociceptive modulation. ON cells show a burst of activity just prior to withdrawal reflexes, and OFF cells are inhibited just prior to withdrawal reflexes. These cells respond in the opposite way to pharmacological stimulation with opioid receptor agonists: systemic or local injections of opioid receptor agonists sufficient to inhibit nociceptive reflexes inhibit ON-cell and increase OFF-cell activities (Fields et al. 1983; Heinricher and Tortorici 1994). A greater understanding of the role of mGluR$_{5}$ and mGluR$_{8}$ within the PAG-RVM circuitry in the modulation of two functionally counteracting neurotransmitters, such as glutamate and GABA, and in the electrophysiological and behavioral pain responses might provide further insight into the pathophysiology of pain and new approaches to its pharmacological control.

**METHODS**

**Animals**

Male Wistar rats (250–300 g) were housed three per cage under controlled illumination (12:12 h light:dark cycle; light on 06.00 h) and environmental conditions (ambient temperature: 20–22°C, humidity: 55–60%) for ≥1 wk before the commencement of experiments. Rat chow and tap water were available ad libitum. The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with Italian (D.L. 116/92) and European Economic Committee (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Surgical preparation for intra-PAG microinjections**

To perform direct intra-ventrolateral PAG administrations of drugs or respective vehicle, artificial cerebrospinal fluid [ACSF, composition (in mM): 2.5 KCl, 125 NaCl, 1.18 MgCl$_{2}$, and 1.26 CaCl$_{2}$] rats were anesthetized with pentobarbital (60 mg/kg ip), and a 23-gauge, 12 mm-long stainless steel guide cannula was stereotaxically lowered until its tip was 1.5 mm above the ventrolateral PAG by applying coordinates from the atlas of Paxinos and Watson (1986) (A: ~7.8 mm and L: 0.5 mm from bregma; V: 4.3 mm below the dura). Ventrolateral PAG was considered in this study because we have performed previous studies in the same area (Maione et al. 2006; Marabese et al. 2006). The cannula was anchored with dental cement to a stainless steel screw in the skull. We used a David Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the animal positioned on a homeothermic temperature control blanket (Harvard Apparatus Limited, Edenbridge, Kent, UK). The guide cannula for intra-PAG microinjection was implanted on the same day as the electrophysiological and tail flick latency recordings. Direct intra-ventrolateral PAG administration of drugs or respective vehicle was conducted with a stainless steel cannula connected by a polyethylene tube to a SGE 1-μl 26-gauge syringe, inserted through the guide cannula and extended 1.5 mm beyond the tip of the guide cannula to reach the ventrolateral PAG. Volumes of 200 nl drug solutions, or vehicle, were injected into the ventrolateral PAG over a period of 60 s, and the injection cannula was gently removed 2 min later. At the end of the experiment, a volume of 200 nl of neutral red (0.1%) was also injected in the ventrolateral PAG 30–40 min before killing the rat. Rats were then perfused intracardially with 20 ml phosphate buffer solution (PBS) followed by 20 ml 10% formalin solution in PBS. The brains were removed and immersed in a saturated formalin solution for 2 days. The injection site was ascertained by using two consecutive sections (40 μm), one stained with cresyl violet to identify nuclei and the other unstained to determine dye spreading. Only those rats the microinjected site of which was located within the ventrolateral PAG were used for data computation.

**RVM extracellular recording and tail flick test**

After implantation of the guide cannula into the ventrolateral PAG, a tungsten microelectrode was stereotaxically (Paxinos and Watson 1986) lowered through a small craniotomy into the RVM to record the activity of neutral, ON, and OFF cells. As first described by Fields et al. (1983), these neurons were identified by their responses to thermal noxious stimulus. OFF cells were inhibited, ON cells excited, and neutral cells unaffected by application of a thermoceptive stimulation of the tail able to evoke a withdrawal (tail flick responses) (Fields et al. 1983; Leung and Mason 1999; Mason 2005). ON and OFF cells also responded (a sudden increase or a decrease in the firing rate, respectively) to noxious stimulation at the hind paws (press/pinch) or to light touch of the corneal surface. In few cases, once RVM neutral, ON, and OFF cells were identified, we also performed intra-PAG morphine microinjections (2 μg/rat, 200 nl) to further verify their physiological characteristics (data not included in this study). Neutral cells did not modify their spontaneous activity after application of drugs or different nociceptive stimuli.

Anesthesia was maintained with a constant, continuous infusion of propofol (5–10 mg · kg$^{-1}$ · h$^{-1}$ iv). Anesthesia was adjusted so that tail flicks were elicited with a constant latency of 4–5 s. A thermal stimulus was elicited by a radiant heat source of a tail flick unit (Ugo Basile, Varese, Italy) focused on the rat tail ~3–5 cm from the tip. From 35°C, the temperature increased linearly to 53°C and was adjusted at the beginning of each experiment to elicit a constant tail flick latency. Tail flicks were elicited every 3–4 min for ≥15–20 min prior to microinjecting drugs, or respective vehicle, into the PAG.

Extracellular single-unit recordings were made in the RVM with glass-insulated tungsten filament electrodes (3–5 MΩ; FHC Frederick Haer) using the following stereotaxic coordinates: 2.8–3.3 mm caudal to lambda, 0.04–0.9 mm lateral, and 8.9–10.9 mm depth from the surface of the brain (Paxinos and Watson 1986). The recorded signals were amplified and displayed on analog and digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also fed into a window discriminator the output of which was processed by an interface (CED 1401; Cambridge Electronic Design) connected to a Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms on-line and to store and analyze digital records of single-unit activity off-line. Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously using a window discriminator and Spike2 software for on- and off-line analysis. Once cells were identified from their background activities, we optimized spike size before all treatments. This study only included neurons the spike configuration of which remained constant and could clearly be discriminated from activity in the background throughout the experiment, indicating that the activity from one neuron only and from that same neuron was measured. Only one neuron was recorded in each rat.

**Microdialysis procedure**

Intra-PAG microdialysis experiments were performed in awake and freely moving rats. In brief, rats were anesthetized with pentobarbital (60 mg/kg ip) and stereotaxically implanted with concentric microdialysis probes, which were constructed as previously described (Biggs...
et al. 1992), into the ventrolateral PAG using coordinates: A: −7.5 mm and L: +0.5 mm from bregma; V: 6.5 mm below the dura (Paxinos and Watson 1986). After a postoperative recovery period of ~18 h, dialysis was commenced by perfusing ACSF at a rate of 0.8 µl/min using a Harvard Apparatus infusion pump (mod. 22). The Ca\(^{2+}\)-free ACSF was produced from normal ACSF by omitting Ca\(^{2+}\) (composition, in mM: 2.5 KCl, 125 NaCl, 1.18 MgCl\(_2\)). On the day of the experiment, each animal was placed in a Plexiglas cage and allowed to move freely. After an initial 60-min equilibration period, 12 consecutive 30-min dialysate samples were collected. Rats received Ca\(^{2+}\)-free ACSF, TTX, or other drugs through the microdialysis probe during a period of 30 min (from 120 to 150 min from the commencement of the microdialysis sample collection). On completion of each experiment, rats were anesthetized with pentobarbital, and their brains were perfused-fixed via the left cardiac ventricle with heparinized paraformaldehyde saline (4%). Brains were removed 120 min after fixation, and coronal sections cut to verify probe placements. Dialysates were analyzed for amino acid content using an HPLC method. The system comprised two Gilson pumps (mod. 112), a C18 reverse-phase column, a Gilson refrigerated autoinjector (model No. 231), and a Gilson fluorometric detector (model No. 121). Dialysates were precolumn derivatized with o-phthaldialdehyde (OPA) (10 µl dialysate + 10 µl OPA), and amino acid conjugates were resolved using a gradient separation. The detection limit of GABA and glutamate in 10-µl samples was ~0.5–1 and 2–3 pmol, respectively. The mobile phase consisted of two components: 1) 50 mM sodium dihydrogen orthophosphate, pH 5.5, with 20% methanol and 2) 100% methanol. Gradient composition was determined with an Apple microcomputer installed with Gilson gradient management software. The mobile phase flow rate was maintained at 1.0 ml/min. Data were collected using a Dell Corporation PC system 310 interfaced to the detector via a Drew data-collection unit.

**Thermal withdrawal latency during microdialysis**

Thermal nociception was evaluated by using Plantar Test Apparatus (Ugo Basile, Varese, Italy). On the day of the experiment, each animal, which had been previously implanted with a microdialysis probe, was placed in a plastic cage (22 × 17 × 14 cm; length × width × height) with a glass floor. After a 30-min habituation period, the plantar surface of the hind paw was exposed to a beam of radiant heat through the glass floor within the time interval between dialysate sample collection. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W). A photodiode cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. Paw withdrawal latency was automatically displayed to the nearest 0.1 s; the cut-off time was 20 s to prevent tissue damage. Vehicle or drug perfusion by reverse microdialysis was performed after the collection of five basal microdialysis samples and the simultaneous recording of five basal thermal withdrawal latencies every 30 min. Thermoreceptive responses were expressed as paw withdrawal latency in seconds (means ± SE). Nociceptive responses were measured every 30 min for a period of 5 h and 30 min.

**Immunohistochemistry**

Animals were killed (pentobarbital, 60 mg/kg ip) and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed and paraffin embedded. Micrometre sections were cut at a thickness of 8 µm, collected on slides in three series each consisting of pairs of serial sections mounted onto gelatin-coated slides (Mezel). For mGluR, and mGluR\(_8\) antigen immunohistochemistry, the sections were dewaxed and rehydrated, immersed in diluted Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA), and then proceeded for ABC immunohistochemistry technique. The sections were reacted for 10 min in 0.3% H\(_2\)O\(_2\), to inactivate endogenous peroxidase activity and incubated for 1 h at room temperature in 10% normal goat serum (NGS, Vector Laboratories) in 0.1 M Tris-HCl-buffered saline, pH 7.3 (TBS), containing 0.3% Triton X-100 and 0.05% sodium azide (Sigma-Aldrich). The sections were then incubated for 24 h at 4°C with rabbit polyclonal mGluR\(_2\) antibody (Abcam, Cambridge, UK) diluted at 1:200 in NGS or guinea pig polyclonal mGluR\(_8\) antibody (Chemicon International). After three rinses, the sections were incubated for 2 h in biotinylated goat anti-rabbit diluted 1:100 in NGS or biotinylated goat anti-guinea pig IgGs diluted 1:500 in NGS (both Vector Laboratories), followed by incubation for 1 h at room temperature in the avidin-biotin-peroxidase solution (ABC Kit; Vectastain, TBS), and then in 0.05% 3’-3’-diaminobenzidine (DAB Sigma Fast, Sigma-Aldrich) in 0.01 M TBS. Then the brain sections were washed in water, and all sections were dehydrated in alcohol, cleared in xylene, and mounted in dibutylphthalate polysytrene xylene (DPX; Merck). Controls included: preabsorption of diluted antibody with respective immunizing peptide (synthesized on custom request by Custom Peptide Synthesis, Cambridge, MA), omission of either the primary antisera or the secondary antibodies. These control experiments did not show staining. The sections were investigated under bright-filed illumination (Leica DM IRB microscope). Images were acquired using the digital camera Leica DFC 320 connected to the microscope and the image analysis software Leica IM500. Digital images were processed in Adobe Photoshop with brightness and contrast being the only adjustments made.

**Treatments**

Groups of 8–10 animals per treatment were used with each animal being used for one treatment only.

For in vivo extracellular recording and tail-flick test, rats receiving intra-ventrolateral PAG administration of vehicle or different doses of [(S)-3,4-dicarboxyphenylglycine [(S)-3,4-DCPG] or N,N\(^2\)-dibenzhydryl-ethane-1,2-diamin dihydrochloride (AMN082), alone or in combination with (RS)-α-methylserine-α-phosphate (MSOP), were grouped as follows. 1) A group of rats was implanted with guide cannulae and received an intra-ventrolateral PAG microinjection of 50 nl of ACSF and served as a control of the intra-ventrolateral PAG drug microinjection. 2) Groups of rats received intra-ventrolateral PAG administration of (S)-3,4-DCPG (2 and 4 nmol/rat) and MSOP (100 nmol/rat) alone or (S)-3,4-DCPG (4 nmol/rat) in combination with MSOP (100 nmol/rat). When (S)-3,4-DCPG was administered in combination with MSOP, the latter was centrally delivered 3 min before the administration of (S)-3,4-DCPG. 3) Groups of rats received intra-ventrolateral PAG administration of AMN082 (1 and 2 nmol/rat) alone or in combination with intra-ventrolateral PAG MSOP (100 nmol/rat). When AMN082 (2 nmol/rat) was administered in combination with MSOP, the latter was centrally delivered 3 min before the administration of AMN082.

For the combined plantar test and microdialysis experiments, rats receiving intra-ventrolateral PAG perfusion with vehicle or different concentrations of AMN082, alone or in combination with MSOP, were grouped as follows. 1) Groups of rats implanted with a concentric microdialysis probe into the ventrolateral PAG were perfused for 30 min with ACSF or Ca\(^{2+}\)-free ACSF or TTX (1 µM) and served as a control of the intra-ventrolateral PAG drug perfusion, or to establish the origin of GABA and glutamate releases. 2) Groups of rats received intra-ventrolateral PAG perfusion with AMN082 (10 and 25 µM), MSOP (0.5 mM), or AMN082 (25 µM) in combination with MSOP (0.5 mM).

In agreement with our previous microdialysis experiments, we used higher agonist concentrations compared with their in vitro EC\(_{50}\) because of the relatively low probe recovery (~20%), the more efficient uptake/metabolism by glial and neural cells in vivo, and the drug diffusion from the probe site. Nevertheless, we have previously found (Marabese et al. 2006) and confirmed here that the antagonist...
MSOP was effective already at 500 μM, a fairly low concentration as compared with its IC$_{50}$ in vitro (Thomas et al. 1996), in erasing the effect of AMN082 or (S)-3,4-DCPG. Indeed this may be the consequence of some limitations of this technique in vivo, as there is no way to make any definitive assumption on the final concentration of drug reaching a specific target (de Lange et al. 1997).

**Drugs**

(S)-3,4-DCPG, AMN082, and (RS)-α-methylserine-o-phosphate, MSOP, were purchased from Tocris Cookson, Bristol, UK. Tetrodotoxin, TTX, was purchased from Sigma. TTX, (S)-3,4-DCPG, MSOP, and AMN082 were dissolved in ACSF with final pH = 7.2 for intra-PAG microinjection or perfusion.

**Statistics**

Single-unit extracellular recording (action potentials) was analyzed off-line from perstimulus rate histograms using Spike2 software (CED, version 4). The neuron responses, before and after intraventrolateral PAG vehicle or drug microinjections, were measured and expressed as spike/second (Hz). Baseline activities of neurons were measured between tail flicks. In particular, basal values were obtained by averaging the activities recorded 30–50 s before the application of three to four thermal stimulations (each stimulation trial was performed every 3–4 min). Data are presented as means ± SE either of changes in time latencies (tail flick test) or changes in neuron responses (extracellular recordings). Statistical comparisons of values from different treated groups of rats were made using the two-way ANOVA for repeated measures followed by the Tukey-Kramer test for post hoc comparisons.

To analyze tail flick-related ON cell activities (before and after drug treatment), the ongoing activity (spike/s) was determined 40–50 s before tail flick application, and then the peak of ON cell activity related to the tail flick (peak firing) was quantified. Interspike interval was used to distinguish between the beginning of an ON cell burst and ongoing activity. Tail flick-related ON cell firing was calculated as the number of spikes in the 2-s interval beginning 0.5 s before the tail flick. Comparisons between pre- and posttreatment ongoing activity and tail flick-related cell burst were performed by applying the nonparametric Wilcoxon matched-pairs signed-rank test. Furthermore, we calculated the ON cell burst latency; that is, the interval between the onset of the applied noxious radiant heat and the beginning of the tail flick-related cell burst. Burst latency was analyzed using a two-way ANOVA for repeated measures followed by the Tukey-Kramer test for post hoc comparisons.

We also performed analysis of tail flick related OFF cell activities before and after drug treatments. The ongoing activity (spike/s, 40–50 s before radial heat application), the latency to onset of the OFF cell pause (time between the onset of heat application and the last action potential prior to the tail flick), and the duration of the cell pause (the interval between the pause onset and the 1st spike after the tail flick) were determined. Comparisons between pre- and post-treatment ongoing activity and cell pause related to tail flick were performed by applying the nonparametric Wilcoxon matched-pairs signed-rank test. The latency to the onset of the cell pause was analyzed with a two-way ANOVA for repeated measures with the Tukey-Kramer test for post hoc comparisons.

Microdialysis, tail flick or plantar test data are represented as means ± SE, and statistical analysis of these data were performed by two-way ANOVA for repeated measures followed by the Student-Newman-Keuls multiple comparisons test to determine the statistical significance between different treated groups of rats. Differences were considered significant at $P < 0.05$.

**RESULTS**

**Effect of (S)-3,4-DCPG or AMN082 on the ongoing activities of RVM neutral, on, and off cells**

The results are based on RVM neurons (group size = 8–10; 1 cell recorded from each animal per treatment) at a depth of 8, 905–10,910 μm from the surface of the brain, the estimated location of the neurons being in nucleus raphe magnus, nucleus reticularis gigantocellularis pars α, and nucleus reticularis paragigantocellularis. All recorded neurons were spontaneously active and discharged with a mean frequency of 7.7 ± 0.4 (neutral cells), 6.7 ± 0.5 (ON cells) and 8.0 ± 0.4 (OFF cells) spike/s. These neurons were identified by the characteristic OFF cell pause and ON cell burst of activity just before tail flick responses. Neutral cells did not modify their spontaneous activity during or after application of thermoceptive stimulation of the tail. Microinjections of (S)-3,4-DCPG (2 and 4 nmol/rat) into the ventrolateral PAG caused a dose-dependent decrease in the firing activity of the ON cells, which was significant between 3 and 15 min, and maximal 12 min after administration of the highest dose (Figs. 1A and 2A). The same treatment produced a very rapid increase in the firing activity of the OFF cells, which was already significant after 3 min and maximal after 9 min from administration of the highest dose (Figs. 1B and 2B). Unlike (S)-3,4-DCPG, microinjections of AMN082 (1 and 2 nmol/rat) into the ventrolateral PAG caused a dose-dependent increase in the firing activity of the ON cells, which was significant between 3 and 24 min and maximal 12 min after administration (Figs. 1C and 2C), and a very rapid decrease in the firing activity of the OFF cells, already significant after 3 min and maximal 9 min after administration of the highest dose (Figs. 1D and 2D). The effects of (S)-3,4-DCPG or AMN082 were prevented by pretreatment with MSOP (100 nmol/rat). MSOP (100 nmol/rat) did not significantly change per se the RVM ON and OFF cell ongoing activities (Fig. 1). Spontaneous activities of RVM neutral neurons ($n = 5$) as identified by their nonresponsiveness to tail flick were also analyzed before and after microinjections of (S)-3,4-DCPG (4 nmol/rat) or AMN082 (2 nmol/rat) into the ventrolateral PAG. Both drug treatments failed to cause any change in their spontaneous activities (Fig. 2, E and F).

**Effect of (S)-3,4-DCPG and AMN082 on tail flick-related changes on RVM cell activities**

The highest doses of (S)-3,4-DCPG (4 nmol/rat) or AMN082 (2 nmol/rat) modified tail flick-related OFF cell activity. (S)-3,4-DCPG (4 nmol/rat) did not significantly change the ON cell onset of burst (4.5 ± 0.7 vs. 6.7 ± 0.9 s), whereas it decreased the duration of the OFF cell pause (from 10.5 ± 3.2 to 4.2 ± 2.2 s; $P < 0.05$; Figs. 2, A and B, and 3A). (S)-3,4-DCPG did not significantly affect tail flick-induced ON-cell peak firing (from 16.5 ± 3.7 to 11.2 ± 4.2 spike/s), whereas it delayed the onset of OFF cell pause (from 4.4 ± 0.4 to 9.3 ± 0.3 s; $P < 0.05$; Figs. 2, A and B, and 3A). AMN082 (2 nmol/rat) did not significantly change the ON cell onset of burst (4.5 ± 0.7 vs. 3.8 ± 0.6 s), but it increased the duration of the OFF cell pause (from 10.5 ± 3.4 to 18.4 ± 3.2 s; $P < 0.05$; Figs. 2, C and D, and 3B). AMN082 did not significantly affect tail flick-induced ON cell peak firing (from 17.5 ± 3.4 to
19.7 ± 5.2 spike/s) either, but it did shorten the onset of off cell pause (from 4.5 ± 0.5 to 2.2 ± 0.3 s; P < 0.05; Figs. 2, C and D, and 3B). Unlike RVM on and off cells, the administration of (S)-3,4-DCPG or AMN082 did not affect the activity of neutral cells before and after thermocceptive stimulation (Fig. 2, E and F).

**Effect of (S)-3,4-DCPG and AMN082 on tail-flick latencies**

Tail flicks were elicited every 3–4 min for ≥20 min prior to microinjecting drugs, or respective vehicle, into the ventrolateral PAG. Data related to pretreatment interval were considered as basal tail flick latencies (4.5 ± 0.3 s). Intra-ventrolateral PAG microinjection of vehicle did not change the tail flick latency as compared with basal values (4.6 ± 0.5 s; Fig. 4). Tail flick latency increased to 7.3 ± 0.6 and 10.2 ± 0.8 s or decreased to 2.9 ± 0.3 and 1.7 ± 0.6 s by intra-ventrolateral PAG microinjections of (S)-3,4-DCPG (2 and 4 nmol/rat) or AMN082 (1 and 2 nmol/rat), respectively (P < 0.05; Fig. 4, A and B). The effects of the higher dose of (S)-3,4-DCPG or AMN082 were prevented by pretreatment with MSOP (100 nmol/rat; Fig. 4, A and B). MSOP (100 nmol/rat) did not significantly change per se the tail flick latencies (Fig. 4).

**Effect of TTX and Ca²⁺-free ACSF on PAG glutamate and GABA extracellular values**

The mean basal extracellular GABA, glutamate and glutamine levels in the PAG (not corrected for probe recovery of 24 ± 8, 30 ± 6, and 35 ± 7% for GABA, glutamate and glutamine, respectively) were 6.9 ± 0.5, 25 ± 5, and 458 ± 29 pmol/10 μl of dialysate (means ± SE), respectively. These values are concordant with those obtained in our previous studies and with those of other laboratories (Marabese et al. 2005; Maione et al. 1999, 2000; Renno et al. 1992). Each animal was used only once, and the reported basal values of glutamate, GABA, and glutamine are the mean concentrations obtained from all experiments pooled as controls. Intra-PAG perfusion with TTX (1 μM) decreased the extracellular levels of glutamate and GABA (58 ± 5 and 51 ± 7% of basal value, respectively; Fig. 5, A and B). Similarly, Ca²⁺ free ACSF decreased the extracellular levels of glutamate and GABA (45 ± 5 and 47 ± 6% of basal value, respectively; Fig. 5, A and B).

**Effect of AMN082 on PAG glutamate and GABA extracellular values**

Intra-ventrolateral PAG perfusion with the selective mGluR₇ agonist, AMN082 (10 and 25 μM), decreased the extracellular...
FIG. 2. Examples of ratemeter records that illustrate the effects of intra-PAG microinjections of (S)-3,4-DCPG (4 nmol/rat; A and B) or AMN082 (2 nmol/rat; C and D) on either the ongoing or tail flick-related discharges of identified RVM ON (A and C) and OFF (B and D) cells. Spontaneous activity of RVM neutral cells were also analyzed before and after (S)-3,4-DCPG (4 nmol/rat; E) or AMN082 (2 nmol/rat; F) intra-PAG microinjections. Traces report overall firing before and after drug injections into the ventrolateral PAG. ▲ tail flick trials, 1-s bins. ↑, time of microinjections within the ventrolateral PAG. Scale bar 4 min.

FIG. 3. Examples of ratemeter records with expanded time scale which illustrate the effects of intra-PAG microinjections of (S)-3,4-DCPG (4 nmol/rat) (A) or AMN082 (2 nmol/rat) (B) on either the ongoing or tail flick-related discharge pauses of identified RVM OFF cells. Traces (all 3 traces from the same cell) report overall firing before and 10 min after drug injections into the ventrolateral PAG. ▲ tail flick trials, 1-s bins. Scale bar 0.5 min.

FIG. 4. Tail flick latencies before and after microinjections into the ventrolateral PAG of ACSF, (S)-3,4-DCPG (2 and 4 nmol/rat), MSOP (100 nmol/rat) alone, or (S)-3,4-DCPG (4 nmol/rat) in combination with MSOP (100 nmol/rat; A) and ACSF, AMN082 (1 and 2 nmol/rat), MSOP (100 nmol/rat) alone, or AMN082 (2 nmol/rat) in combination with MSOP (100 nmol/rat; B). Each point represents the mean ± SE of 8–10 observations. *, significant differences vs. ACSF; °, significant differences vs. (S)-3,4-DCPG (4 nmol/rat) or AMN082 (2 nmol/rat). P values <0.05 were considered statistically significant.
levels of glutamate in a concentration-dependent manner (60 ± 6 and 21 ± 9% of basal value, respectively; Fig. 6A). AMN082 (10 µM) did not change dialysate GABA concentration (69 ± 5% of basal value; Fig. 6B). The higher dose of AMN082 (25 µM) produced a significant delayed effect on GABA extracellular values (56 ± 6% of basal value; Fig. 6B). MSOP (0.5 mM, a group III mGlu receptor antagonist, perfused in combination with AMN082 (25 µM), antagonized the effect induced by AMN082 on extracellular glutamate and GABA releases (Fig. 6, A and B).

**Effect of AMN082 on thermal withdrawal latency (Plantar test)**

Because we have recently already evaluated the effect of (S)-3,4-DCPG on nocifensive behavior (Marabese et al. 2006), we decided to focus on the current study on the effect of AMN082 when administered into the PAG matter. Intra-PAG perfusion with AMN082 (10–25 µM), by reverse microdialysis, induced a dose-dependent decrease in thermal withdrawal latency (7.5 ± 0.5 and 5.6 ± 0.4 s vs. 10.2 ± 0.4 s; P < 0.05). When MSOP (0.5 mM) was perfused in combination with AMN082 (25 µM), it fully antagonized the pronociceptive effect of AMN082 but did not change per se thermal withdrawal latencies (Fig. 7). No overt behavioral changes were observed in this study after intra-PAG administration of all the drugs used in freely moving not unanesthetized rats. Rats remained alert and generally active throughout the experiment (not shown).

**Immunohistochemistry**

Immunohistochemical localization of mGluR7 and mGluR8 in rat PAG was determined by ABC immunohistochemistry technique. We found mGluR7 and mGluR8 positive neurons in all the PAG area. However, a higher density of mGlu7 positive profiles with strong immunoreactivity (ir) was observed within the ventrolateral sub-region (Fig. 8A). Numerous neurons display also strong mGluR8 immunoreactivity (Fig. 8D). Higher magnification of these profiles revealed mGlu7-ir and mGlu8-ir in cytoplasm on cell bodies and neuronal processes (Fig. 8, B and E).

**DISCUSSION**

The involvement of group III mGluRs in modulating pain responses has not yet been fully established (Fisher and
Our previous studies reported some important discrepancies in the action of group III receptor ligands in modulating pain. In particular, blockade of these receptors at PAG level, a major component of the descending pain modulatory system, either decreased or increased thermoceptive responses in the mouse or rat, respectively (Maione et al. 1998, 2000; Palazzo et al. 2001). More recently, however, we have found that selective stimulation of the mGluR8 decreases nociceptive behavior in different pain models in the mouse (Marabese et al. 2006).

Unlike mGluR4/8, PAG mGluR7 stimulation may be responsible for generating hyperalgesia. Indeed, a preliminary study showed that intra-PAG microinjection of AMN082, a selective mGluR7 agonist, induced dose-dependent thermal hyperalgesia (Marabese et al. 2006). One reason for the opposing effects obtained from mGluR4/8 and mGluR7 in pain control at the midbrain PAG could be due to the localization of the mGluR7 on glutamatergic synapses. A possible main auto-receptor role for these subtype receptors on glutamate terminals might justify the decrease in the excitatory output of the PAG antinociceptive pathway. However, in this study we have only had the possibility to see several profiles with strong mGluR7 immunoreactivity especially within ventrolateral PAG. From these data, unfortunately it is not possible to distinguish whether these cells correspond to PAG output excitatory neurons impinging on the RVM OFF cell population. Further ultra-structural studies would clarify such a possibility. Regarding mGluR8-ir, as compared with mGluR7, it appeared to be more diffusely expressed in many profiles within the PAG matter, and this finding seems to confirm our previous data that mGluR8 are present on both symmetrical and asymmetrical synapses (Marabese et al. 2005).

We performed in vivo microdialysis experiments to shed more light on the possibility that mGluR7 in the PAG may inhibit mainly the release of glutamate. Group III mGluRs are “classically” associated with a reduction in both glutamate and GABA (Cartmell and Schoepp 2000; Schoepp 2001). Nevertheless, intra-PAG perfusion with (S)-3,4-DCPG by reverse microdialysis increased glutamate, whereas it reduced GABA release (Marabese et al. 2005). The opposite (facilitatory or inhibitory) effects of mGluR8 stimulation on glutamate and GABA release could be due to the fact that group III mGluR stimulation, by decreasing GABA, increases glutamate release. Thus even if such a possibility is not detectable by microdialysis, we speculate that the effects on glutamate and GABA releases are not simultaneous but rather that one is the consequence of the other. Nevertheless, these neurochemical changes are consistent with evidence that mGluR8 are ex-
pressed on both symmetrical and asymmetrical synapses at that level (Marabese et al. 2005) and with the activation of an endogenous pain inhibitory system and consequent PAG-mediated analgesia (Marabese et al. 2006).

Further weight is given to this possibility here because AMN082 induced dose-dependent thermal hyperalgesia in the plantar test and, simultaneously, a massive decrease in PAG glutamate in the current study. As far as GABA is concerned, AMN082 produced a slight and delayed decrease in the same rats. This latter finding further suggests that the main effect of AMN082 may be consequence of a direct action of this drug on the excitatory glutamatergic cells. The possible synaptic nature of glutamate and GABA in the PAG dialysates seems in part confirmed by the fact that either TTX or Ca²⁺-free ACSF perfusions almost halved their extracellular concentrations. This finding suggests that almost 45–55% of extracellular glutamate and GABA we measured in the PAG may function as neurotransmitters. Nevertheless, one has to be cautious when using in vivo microdialysis as these experiments do not provide definitive results on synaptic release of either glutamate or GABA. Indeed there is evidence that glutamate receptors are expressed on both neural synaptic and glial processes (Gallo and Ghiani 2000), and there is no way in this study of distinguishing between glial and neural dialysate amino acids. Nevertheless, the changes in the PAG extracellular glutamate or GABA levels may deeply affect nociceptive perception (Gebhart et al. 1984).

An important issue at this point regards how we have chosen the doses of these drugs, and in particular of AMN082. The doses of AMN082 to be administered into the PAG were chosen based on our previous in vivo study in the mouse (Marabese et al. 2006). Indeed, because to our knowledge no other study has been published describing the effects of AMN082 by using a similar administration route, we performed extensive preliminary experiments with several doses of AMN082, as well as of (S)-3,4-DCPG, to find minimal doses able to change RVM cell activities. In contrast to the in vitro data showing that (S)-3,4-DCPG is expected to be more potent than AMN082 (Mitsukawa et al. 2005; Thomas et al. 2001), we found in this study that when injected into the PAG, 2 nmol/rat of AMN082 was as effective as 4 nmol/rat of (S)-3,4-DCPG in changing both RVM cell activities and thermocceptive thresholds. Such a discrepancy may be due to different receptor binding sites (allosteric or orthosteric) of these two ligands that may differ greatly in inducing receptor internalization/desensitization and, therefore, the functional responses (Ferguson 2001; Pelkey et al. 2007); the possible different pharmacokinetics (i.e., uptake and metabolism) related to these mGluR ligands by glial and neural cells in the whole animal. Nevertheless, it is also intriguing that MSOP was effective at 100 nmol/rat in antagonizing the AMN082- or the (S)-3,4-DCPG-induced effects, quite a low concentration as compared with its in vitro IC₅₀ (Thomas et al. 1996) that would call for a dose 10-fold higher that used in the current study. Indeed we cannot ignore the fact that in the whole brain glial, endothelial, and neural processes represent functional or physical factors, which affect diffusion in the extracellular space of drugs and also of neurotransmitters. Thus the actual drug concentrations in the extrasympathetic compartment will be the result of many factors contributing to the complex framework of the extracellular environment and make it so different from the in vitro preparations.

Together with the PAG, the nuclei of the RVM constitute part of the endogenous antinociceptive pathway. Two classes of neurons (i.e., ON and OFF cells) in the RVM control pain conversely (Fields and Basbaum 1999). When microinjected into the PAG, (S)-3,4-DCPG decreased the ongoing activity of the pro-nociceptive ON cells and increased the ongoing activity of the anti-nociceptive OFF cells in the RVM, unlike AMN082, which increased the ongoing activity of the pro-nociceptive ON cells and decreased the ongoing activity of the anti-nociceptive OFF cells. Unlike ON and OFF cells, spontaneous activity of neutral cells was not affected by the intra-ventrolateral PAG microinjection of (S)-3,4-DCPG or AMN082.

Thus selective stimulation of PAG mGluR₉ or mGluR₇ may have modified the activity of PAG output neurons and either increased or decreased the input required to modulate the ON and the OFF cell spontaneous activities. Because the ON cells may facilitate nociception (Fields et al. 1991), a delayed or shortened onset of the ON cell burst with either mGluR₉ or mGluR₇ might be expected to be a critical event to the occurrence of analgesia or hyperalgesia. However, such a possibility does not seem consistent with the current study or with another previous study showing that the ON cell burst can even be completely inhibited without any consequence to tail flick latency (Heinricher and McGaraughty 1998). It is possible that ON cell firing provides a critical regulatory pro-nociceptive output in persistent pain states (Wiertelak et al. 1997) or in other conditions such as opioid withdrawal. On cells but not OFF cells increased their spontaneous activity in naloxone-precipitated hyperalgesia (Bederson et al. 1990). Based on these considerations, spontaneous activity patterns of these cells are critical in determining nociceptive thresholds to any given noxious stimulus. Consistent with the idea that ongoing activity of both ON and OFF cells modulate nociceptive responsiveness, the latency of the nociceptive tail-flick reflex is shorter during periods of increased ON cell activity (Foo and Mason 2005; Heinricher et al. 1989; Jinks et al. 2004). Thus the gain in pain transmission is constantly changing, increasing during periods of ON cell activity and decreasing when OFF cells are active (Mason 2005; Ramirez and Vanegas 1989).

Stimulation of PAG mGluR₉ or mGluR₇, respectively, delayed or shortened the onset of the OFF cell pause and, respectively, decreased or increased the duration of the pause of these cells. These effects seem to be critical in generating RVM-mediated analgesia or hyperalgesia (de Novellis et al. 2005; Heinricher and Tortorici 1994; McGaraughty and Heinricher 2002).

This study provides initial evidence that a mGluR₉ agonist in the PAG, similarly to opioids or cannabinoids, is able to inhibit GABAergic tone, which, in turn, is responsible for the disinhibition of PAG antinociceptive output neurons impinging on OFF neurons in the RVM (Behbehani and Fields 1979; de Novellis et al. 2005; Maione et al. 2006; Ritchieimpr and Behbehani 1991). Because many GABAergic interneurons are tonically active in the PAG (Moreau and Fields 1986), the mGluR₉-induced decrease in the GABAergic tone may be responsible for the PAG output excitatory neuron disinhibition and therefore for the antinociception. Moreover, another important finding of this study is that the mGluR₇ in the PAG...
matter appears to be a critical presynaptic receptor specifically involved in the fine tuning of glutamate extracellular release.

In conclusion, this study shows that, although with different mechanisms, mGluR7 and mGluR6 stimulation may modulate the RVM on- and off-cell activities within the PAG. Thus if on the one hand PAG mGluR6 stimulation can play a pivotal role in inducing analgesia (likely by inhibiting GABAergic interneurons), the stimulation of mGluR7 may, on the other hand, reduce downstream the tonic excitatory control of glutamate on the endogenous antinociceptive pathways originating from the PAG. This study underlines the importance of focusing further efforts on the investigation of mGluR7 agonist analgesic potential and on the development of mGluR7 antagonists, which could be promising new pain-relief agents.

REFERENCES


Thomas NK, Wright RA, Howson PA, Kingston AE, Schoepp DD, Jane DE. (S)-3,4-DCPG, a potent and selective mGlu8a receptor agonist, activates metabotropic glutamate receptors on primary afferent terminals in the neonatal rat spinal cord. Neurpharmacology 40: 311–318, 2001.
