Functional interaction between TRPV1 and μ-opioid receptors in descending antinociceptive pathway activates glutamate transmission and induces analgesia

Sabatino Maione¹#, Katarzyna Starowicz²,³#, Luigia Cristino⁴, Francesca Guida¹, Enza Palazzo¹, Livio Luongo¹ Francesca Rossi⁵, Ida Marabese¹, Vito de Novellis¹ and Vincenzo Di Marzo²,³*

¹Endocannabinoid Research Group, Second University of Naples, Department of Experimental Medicine, via Costantinopoly 16, Naples, Italy.


³Department of Pain Pharmacology, Institute of Pharmacology PAS, ul. Smetna 12, 31-343 Cracow, Poland.

⁴Endocannabinoid Research Group, Institute of Cybernetics, CNR, Via dei Campi Flegrei 34, 80078 Pozzuoli, Italy.

⁵Department of Pediatrics, Second University of Naples, via De Crecchio 4, Naples, Italy.

#These authors contributed equally to this work

*Correspondence to: Sabatino Maione, Endocannabinoid Research Group, Second University of Naples, Department of Experimental Medicine, via Costantinopoly 16, Naples, Italy, Tel.: +39-081-5667650. E-mail: sabatino.maione@unina2.it

or Vincenzo Di Marzo - Endocannabinoid Research Group, Institute of Biomolecular Chemistry, C.N.R., Via Campi Flegrei 34, 80078 Pozzuoli (Naples), Italy. Tel.: +39-081-8675093. Fax.: +39-081-8041770. E-mail. vdimarzo@icmib.na.cnr.it
Abstract

The transient receptor potential vanilloid-1 (TRPV1) receptor is involved in peripheral and spinal nociceptive processing and is a therapeutic target for pain. We have shown previously that TRPV1 in the ventrolateral periaqueductal grey (VL-PAG) tonically contributes to brainstem descending antinociception by stimulating glutamate release into the rostral ventromedial medulla and OFF neuron activity. Since both opioid and vanilloid systems integrate and transduce pain sensation in these pathways, we investigated here the potential interaction between TRPV1 and µ-opioid receptors in the VL-PAG-RVM system. We found that the TRPV1 agonist, capsaicin, and the µ-receptor agonist DAMGO, when co-administered into the ventrolateral-PAG ad doses non-analgesic per se, produce: 1) antinociception in tests of thermal nociception; 2) stimulation of glutamate release into the RVM; 3) inhibition of ON neuron activity in the RVM. These effects were all antagonized by the TRPV1 and opioid receptor antagonists 5'-iodo-resiniferatoxin and naloxone, respectively, thus suggesting the existence of TRPV1-µ opioid interaction in the VL-PAG/RVM system. By using double immunofluorescence techniques, we found that TRPV1- and µ-opioid receptors are co-expressed in several neurons of the VL-PAG. These findings suggest that µ-receptors activation not only acts on inhibitory neurons to disinhibit PAG output neurons, but also interacts with TRPV1 activation at increasing glutamate release into the RVM, possibly by acting directly on PAG output neurons projecting to the RVM.

Keywords: vanilloid, opioid, pain, µ receptors, endovanilloid, cannabinoid

Running title: TRPV1 and µ receptors in the periaqueductal grey
Introduction

Opioids produce analgesic effects at both spinal and supraspinal levels largely via µ opioid receptors (MORs). The study of the descending control of pain dates back to 1962, when the gate hypothesis of pain was postulated with the periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) being the key players in the brainstem and described as “the origin of the descending control of pain” (Millan 2002; Melzack and Wall, 1962). Since 1969, when it was demonstrated that electrical stimulation of the PAG enhances descending inhibition and produces antinociception (Reynolds, 1969), many studies have shown that this effect is the result of complex PAG-mediated processes. It was demonstrated that microinjection of morphine into the ventrolateral (VL) PAG inhibits pain perception in animals (Yaksh et al., 1976; Tsou and Jang 1964). Morphine is believed to indirectly excite PAG output neurons projecting to the RVM, resulting in activation of RVM neurons projecting to the spinal dorsal horn (Sandkühler and Gebhart; 1984; Gebhart et al., 1983; Behbehani and Fields, 1979), which in turn inhibit nociceptive transmission in the spinal cord. Three main populations of nociception-modulating RVM neurons, i.e. neutral, ON and OFF cells, are considered (Fields and Heinricher 1985). ON cells are characterized by a burst of activity associated with nocifensor withdrawal reflexes. Their sustained activation produces hyperalgesia, whereas reduction in the threshold of the ON cell burst is associated with a decrease in reflex latency (Fields et al. 1995; Heinricher and Neubert 2004; Neubert et al. 2004; McGaraughty et al. 2003). OFF cells are defined by a pause in firing associated with withdrawal reflexes and are generally thought to exert antinociception (Neubert et al. 2004; Heinricher and Tortorici 1994; Heinricher et al. 1994). ON and OFF cells respond in opposite ways to stimulation of MORs: systemic or local injections of MOR agonists at doses inhibiting nociceptive reflexes will inhibit ON-cell and increase OFF-cell activities (Heinricher and Tortorici 1994; Fields et al. 1983).
The recent identification of transient receptor potential vanilloid type-1 (TRPV1) channels in the brain (Cristino et al., 2006, 2008; Starowicz et al., 2008 for review; Mezey et al., 1999) suggested for these proteins, which act as polymodal nociceptors in sensory neurons, an additional way through which they might contribute to pain transmission. We showed that microinjections of a TRPV1 agonist, capsaicin, into the VL-PAG produces analgesia, whereas the selective TRPV1 antagonist 5’-iodo-resiniferatoxin (I-RTX) evokes hyperalgesia (Starowicz et al., 2007; Maione et al., 2006; Palazzo et al., 2002). These effects are accompanied by concomitant increase or decrease, respectively, of glutamate release into the RVM (Starowicz et al., 2007). We proposed that a set of VL-PAG neurons respond to TRPV1 stimulation by releasing glutamate into the RVM, thereby activating OFF cells and producing analgesia.

In the present study we investigated the potential interaction between μ-opioid and TRPV1 receptors in the PAG–RVM system. To this end we studied in rats the effect of simultaneous intra-VL-PAG administration of sub-threshold doses of a TRPV1 and a MOR agonist on glutamatergic and GABAergic signaling and ON and OFF cell activity in the RVM, and concomitantly, on the animal response to noxious heat in the tail flick or plantar tests. We also characterized by means of immunohistochemistry the possible co-localization of μ-opioid and TRPV1 in glutamatergic or GABAergic PAG and RVM neurons.

Materials and methods

Animals

Male Wistar rats (250-300 g) were housed 3 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 at least 1 week before the start of the 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 EU (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Surgical preparation for intra-PAG microinjections

In order to perform direct intra-VL PAG administrations of drugs or respective vehicle, 10% dimethyl sulfoxide in artificial cerebrospinal fluid (ACSF, composition in mM: KCl 2.5; NaCl 125; MgCl2 1.18; CaCl2 1.26), rats were anaesthetised with pentobarbital (60 mg/kg, i.p.) and a 31-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 (VL-PAG) was considered in this study, since previous studies have shown in this area the presence of excitatory output neurons projecting to RVM neurons (Sandkulher and Gebhart 1984; Moreau and Fields 1986). 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, USA) with the animal positioned on a homeothermic temperature control blanket (Harvard Apparatus Limited, Edenbridge, Kent). Direct intra-VL PAG administration of drugs, or respective vehicle, was conducted with a stainless steel cannula connected by a polyethylene tube to a SGE 1-microlitre syringe, inserted through the guide cannula and extended 1.5 mm beyond the tip of the guide cannula to reach the VL-PAG. Vehicle or drug solutions were given into the VL-PAG in a final volume of 200 nl, both when capsaicin and DAMGO were injected alone and when capsaicin and DAMGO were co-injected. The microinjection was performed over a period of 60 sec and the injection cannula 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 VL-PAG 30-40 min before killing the rat. Rats were then perfused intracardially with 20 ml phosphate buffer solution (PBS) followed by 200 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 2 consecutive sections (40 μm), one stained with cresyl violet to identify nuclei and the other unstained to determine dye spreading (Fig. 1A). Only those rats whose microinjected site was located within the VL-PAG were used for data computation as already described and demonstrated in Starowicz et al. (2007).

**Thermal withdrawal latency**

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 (see below), was placed in a plastic cage (22cm x 17cm x 14cm; length x width x height) with a glass floor. After a 60 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 photoelectric 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 sec; the cut-off time was 20 sec in order to prevent tissue damage. Vehicle or drugs were microinjected into the PAG after the collection of four basal microdialysis samples and the simultaneous recording of basal thermal withdrawal latencies every 15 min. The results were expressed as thermal withdrawal latency in sec. Nociceptive responses were measured every 15 min for a period of 2 hrs.

**In vivo electrophysiology**

After implantation of the guide cannula into the VL-PAG, a tungsten microelectrode was stereotaxically lowered through a small craniotomy into the RVM (AP: 11.5; L: +0.3; V: 9.9-10.9) (Paxinos and Watson 1986) to record the activity of ON and OFF cells. These neurons
were identified by the characteristic OFF cell pause and ON cell burst of activity just before
tail flick responses (Fields et al. 1983; Xu et al., 2007). Anaesthesia was maintained with a
constant, continuous infusion of propofol (5-10 mg/kg/h, i.v.). Anaesthesia was adjusted so
that tail flicks were elicited with a constant latency of 4-5 sec. A thermal stimulus was elicited
by a radiant heat source of a tail flick unit (Ugo Basile, Varese, Italy) focused on the rat tail
approximately 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 in order to elicit a constant tail flick latency. Tail flicks were elicited every 4-5 min for at least 15-20 min prior to microinjecting
drugs, the respective vehicle, or their combination into the VL-PAG.

Extracellular single-unit recordings were made in the RVM with glass insulated
tungsten filament electrodes (3-5 MΩ) (FHC Frederick Haer & Co., ME, USA). The recorded
signals were amplified and displayed on digital storage oscilloscope to ensure that the unit
under study was unambiguously discriminated throughout the experiment. Signals were
processed by a CED 1401 interface (Cambridge Electronic Design Ltd., UK) connected to a
Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate
histograms on-line and to store and analyse 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-line and off-
line analysis. Once an ON or OFF cell was identified from its background activity, we
optimised spike size before all treatments. This study only included neurons whose spike
configuration 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 and, at the
end of the experiment, recording sites were marked with an electrolytic lesion and all rats
euthanized with an overdose of pentobarbital and perfused intracardially with physiological
saline followed by 10% formalin. Consistently with previous studies from this laboratory
(Marabese et al., 2007; Starowicz et al., 2007) recording sites were found to be distributed in the RVM (Fig. 1C).

**RVM microdialysis**

In vivo microdialysis experiments were performed in awake and freely moving rats. In brief, rats already implanted with guide cannula (as described above) and still anaesthetized with pentobarbital (60 mg/kg, i.p.) were stereotaxically implanted with concentric microdialysis probes, which were constructed as previously described (Biggs et al., 1992), into the RVM using coordinates: AP: -11.5 mm and L: +0.3 mm from bregma, V: 10.5 mm below the dura (Paxinos and Watson 1986). Following a post-operative recovery period of approximately 18 h dialysis was commenced by perfusing ACSF at a rate of 0.8 μl/min using a Harvard Apparatus infusion pump (mod. 22). On the day of the experiment each animal was placed in a Plexiglas cage and allowed to move freely. Following an initial 60 min equilibration period, 12 consecutive 15 min dialysate samples were collected. On completion of each experiment, rats were anaesthetized with pentobarbital and their brains perfused-fixed via the left cardiac ventricle with 20 ml phosphate buffer solution (PBS) followed by 200 ml 10% formalin solution in PBS. Brains were removed after fixation, and coronal sections cut in order to verify probe placements (Fig. 1B). Dialysates were analysed for amino acid content using an HPLC method. The system comprised two Gilson pumps (mod. 303), a C18 reverse-phase column and a Gilson fluorimetric detector (mod. 121). Dialysates were pre-column derivatised with o-phtaldialdehyde (OPA) (10 μl dialysate + 10 μl OPA) and amino acid conjugates resolved using a gradient separation. The detection limit of GABA and glutamate in 10-μl samples was about 0.5-1 and 2-3 pmol, respectively. The mobile phase consisted of two components: (A) 50 mM sodium dihydrogen orthophosphate, pH 5.5, with 20% methanol and (B) 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 was collected using a Dell Corporation PC system 310 interfaced to the detector via a Drew data collection unit.

**Drugs**

Capsaicin, I-RTX (5’-Iodo-resiniferatoxin) and DAMGO were purchased from Tocris Bioscience, Bristol, UK. Naloxone was purchased from Sigma. Drugs were dissolved in 10% dimethyl sulfoxide (DMSO) in ACSF with final pH = 7.2 for intra-VL PAG microinjections. Since recent evidence suggests that high concentration of DMSO can have direct effects on plasma membrane and receptor functioning (Fossum et al., 2008; Gustovenko and Anwar 2007), additional experiments of microdialysis combined with plantar test were performed with capsaicin and DAMGO dissolved in 0.5% DMSO in ACSF in order to exclude any interference of a higher concentration of DMSO.

**Treatments**

Groups of 8-10 animals per treatment were used with each animal being used for one treatment only. Rats (both those for microdialysis/plantar test and those electrophysiology/tail flick test combined experiments) received intra-VL-PAG microinjections of vehicle (10% DMSO in ACSF), capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) alone, or a co-administration of capsaicin and DMGO in a single 200 nl microinjection at the same doses. Naloxone (5 nmol/rat), an opioid receptor antagonist, or I-RTX (0.5 nmol/rat), a TRPV1 receptor antagonist, were microinjected 5 min before capsaicin and DAMGO.

A further group of rats for microdialysis/plantar test combined experiments (n=9) received intra-VL-PAG microinjections of vehicle (0.5 % DMSO in ACSF), capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) alone, or a co-administration of capsaicin and DMGO in a single 200 nl microinjection in this novel vehicle in order to exclude any interference due to a higher (10%)
DMSO concentration.

The doses of drugs to be administered into the VL-PAG were chosen based on our and others’ previous in vivo studies in the rat (Maione et al., 1995; Maione et al., 2000; McGaraughty et al., 2003; Starowicz et al., 2007). We also performed preliminary experiments with several doses of drugs used in this study in order to find minimal doses able to change RVM cell activities or thermoceptive thresholds.

Statistics

Single-unit extracellular recording (action potentials) was analysed off-line from peristimulus rate histograms using Spike2 software (CED, version 4). The neuron responses, before and after intra-VL-PAG vehicle or drug microinjections, were measured and expressed as spikes/sec (Hz). Baseline activities of neurons were measured between tail flicks. In particular, basal values were obtained by averaging the activities recorded 30-50 sec before the application of 3-4 thermal stimulations (each stimulation trial was performed every 3-4 min). Data are presented as mean ± standard error (S.E.) either of changes in withdrawal latencies (tail flick test) or changes in neuron responses (extracellular recordings). The mean ± standard error (S.E.) values of the RVM background cell activity was obtained by averaging ongoing cell firing recorded every 3 min from the commencement of the experiments and subtracting the tail-flick-induced changes when they occasionally coincided with the tail flick testing. The tail-flick-induced firing of the ON cell was calculated after subtraction of its ongoing activity immediately preceding the tail-flick application.

In order to analyse tail flick-related ON cell activities (before and after drug treatment), the ongoing activity (spikes/sec) was determined 30-50 sec before tail flick application, and then the peak of ON cell activity related to the tail flick (peak firing) was quantified. 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. 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.

We also performed analysis of tail flick related OFF cell activities before and after drug treatments. The ongoing activity (spikes/sec, 40-50 sec before radial heat application), the latency to onset of the OFF cell pause (the interval between the onset of thermal stimulus and the last spike), and the duration of the cell pause (the interval between the pause onset and the first spike after the tail flick) were determined. The interval between the onset of applied noxious radiant heat and the beginning of the tail flick related cell pause (pause latency), was also calculated. Comparisons between pre- and post-treatment ongoing activity, tail flick-related cell burst and pause were performed by applying the non-parametric Wilcoxon matched-pairs signed rank test. Burst latency and the latency to the onset of the cell pause were analysed using two-way ANOVA for repeated measures followed by the Tukey-Kramer test for post-hoc comparisons to determine the statistical significance between different treated groups of rats.

Microdialysis, tail flick or plantar test data are represented as means ± standard error of the mean (S.E.M), and statistical analysis of these data was 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 $P < 0.05$.

**Immunohistochemistry**

Animals (n=4) were deeply anaesthetized with pentobarbital and perfused transcardially with saline followed by ice-cold 4 % paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains were removed, post-fixed for 2 h and then washed. Tissues to be cut at cryostat were cryoprotected overnight in PB containing 30% (w/v) sucrose at 4°C until they sank. Serial cryostat sections were cut at 14 μm and mounted onto gelatine-coated slides (Mezel,
Germany). For double immunofluorescence serial sections were incubated for 1 h in 10% normal donkey serum (NDS, Jackson Immunoresearch Laboratories, West Grove, PA) in PB containing 0.3% Triton X-100 (block solution). Subsequently the sections were incubated for 2 days at 4°C in a humid chamber with the respective polyclonal antibodies (all diluted in block solution). All sections were processed for anti-TRPV1 receptor immunoreaction (1:250 guinea pig anti-TRPV1, Abcam, Cambridge, UK) coupled to rabbit anti-μ-opioid receptor (MOP) (1:1000, Abcam, Cambridge, UK). After three washes in PB, double immunofluorescence was revealed by incubation for 2 h in the appropriate fluorochrome conjugated secondary antibody: Alexa Fluor488 anti guinea pig for TRPV1 (Molecular Probes Inc. Eugene, OR); Alexa Fluor546 anti rabbit for MOR (Molecular Probes Inc. Eugene, OR) diluted 1:250 in NDS block solution. Thereafter, sections were washed with PB and coverslipped with Aquatex mounting medium (Merck, Darmstadt, Germany).

Controls included: (1) preabsorption of diluted antibodies with their respective immunizing peptides; and (2) omission of either the primary antisera or the secondary antibodies. These control experiments did not show staining. The sections processed for immunofluorescence were studied with an epifluorescence microscope (Leica DM IRB); settings for excitation of fluorescein isothiocyanate (488 nm) and Texas Red (543 nm) were identical throughout the analysis. Images were acquired using the digital camera Leica DFC 320 connected to the microscope and the image analysis software Leica IM500, which allows both single and merged pictures acquisitions. Digital images were processed in Adobe Photoshop, with brightness and contrast being the only adjustments made.

Quantification of immunoreactivity

Quantification of the mean percentage value of the number of neurons double labeled for TRPV1/MOR was performed by an observer blinded to the experimental protocols, on the total of VL-PAG and RVM neurons identified with respect to each adjacent section labeled with cresyl violet, with nuclei, unstained or lightly stained, in the focal plane. The level of
section evaluated for immunohistochemistry covered the entire extension of the VL-PAG or RVM regions, approximately for 1.0 mm rostrally from bregma AP:-8.0 mm, L:0.5 mm and AP:-12.0 mm, L:+0.3 mm respectively. For each region we counted nine sections per animal, using 3 animals per group.

Results

Effect of intra-VL-PAG capsaicin and DAMGO in 10% or 0.5% DMSO in ACSF vehicle on thermal nociception

Microinjections of vehicle, capsaicin (3 nmol/rat) or DAMGO (6 pmol/rat), into the VL-PAG did not change the latency of the thermoceptive reaction (Fig. 2A). Co-injection of capsaicin (3 nmol/rat) in combination with DAMGO (6 pmol/rat) produced a significant (P< 0.05) increase in the latency of the nociceptive reaction (17.1± 0.8 sec) with respect to the vehicle (10.9±0.4 sec) (Fig. 2). The antinociception induced by this treatment was significantly prevented by naloxone (5 nmol/rat, 200 nl) or I-RTX (0.1 nmol, 200 nl) (P< 0.05) (Fig. 2B). The antagonists alone (I-RTX and naloxone) did not change per se the latency of the nociceptive reaction (not shown). Microinjections of a vehicle with lower DMSO concentration (0.5 %), capsaicin (3 nmol/rat) or DAMGO (6 pmol/rat) dissolved in 0.5 % DMSO vehicle, into the VL-PAG did not change the latency of the thermoceptive reaction (Fig. 2C). Co-injection of capsaicin (3 nmol/rat) in combination with DAMGO (6 pmol/rat) in 0.5 % DMSO vehicle produced a significant (P< 0.05) increase in the latency of the nociceptive reaction (16.2± 0.7 sec) with respect to the vehicle (10.6±0.3 sec) (Fig. 2C). In spite of the evidence that the PAG is a midbrain area integrating aversive behaviors, we did not observe any overt behavioral change (i.e. jumping, running, freezing, etc.) after drug microinjections.
Effect of intra-VL-PAG vehicle, capsaicin and DAMGO on OFF and ON cell spontaneous activity in the RVM

The results are based on RVM neurons (group size = 8-10; one cell recorded from each animal per treatment) at a depth 9,405-10,740 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. A total of 187 cells were recorded. We selected for recording only neurons that showed spontaneous activity (mean frequency of 6.6±0.7 spikes/sec for ON cells and 8.6±0.4 spikes/sec for the OFF cells). These neurons were identified by the characteristic OFF cell pause and ON cell burst of activity just before tail flick responses. Microinjections of vehicle, capsaicin (3 nmol/rat) or DAMGO (6 pmol/rat) did not cause any significant effect, whereas the co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) caused a decrease in the firing activity of the ON cells, which was significant between 3 and 21 min, and maximal between 6 and 15 min after administration (Fig. 3A). The same treatment did not change the OFF cell ongoing activity (Fig. 3B). The effects of co-administration of capsaicin and DAMGO were prevented by pre-treatment with I-RTX (0.5 nmol/rat) or naloxone (5 nmol/rat) (Fig 3C and D) at doses that did not significantly change per se the RVM ON and OFF cell ongoing activity (data not shown).

Spontaneous activities of RVM neutral neurons (n=6) as identified by their non-responsiveness to tail flick were also analysed before and after intra-VL-PAG microinjections of drugs, which failed to cause any change in their spontaneous activity (data not shown).

Effect of intra-VL-PAG vehicle, capsaicin and DAMGO on tail flick-related RVM ON and OFF cell activities

Capsaicin (3 nmol/rat) did not significantly change the ON cell onset of burst (1.8±0.2 vs 2.2±0.2 sec), or OFF cell pause (2.3±0.3 vs 2.1±0.4 sec). Similarly, DAMGO (6 pmol/rat) did not significantly change the ON cell onset of burst (1.6±0.3 vs 1.9±0.4 sec), or OFF cell pause
(1.8±0.4 vs 2.0±0.4 sec). Consistently, capsaicin did not change tail flick-induced ON cell peak firing (from 12.6±3.2 to 11.2±2.5 spikes/sec), or the OFF cell pause duration (from 5.3±0.5 to 6.2±0.4 sec) (Fig. 4A and C). Similarly, DAMGO did not change tail flick-induced ON cell peak firing (from 10.6±3.5 to 9.5±2.8 spikes/sec), or the OFF cell pause duration (from 4.6±0.4 to 4.3±0.3 sec) (Fig. 4B and D). However, co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) decreased the ON cell onset of burst (1.7±0.2 vs 3.6±0.3 sec), and increased the onset of the OFF cell pause (1.6±0.5 vs 3.3±0.5 sec) (p<0.05). Consistently, co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) decreased the background firing frequency of the ON cell (from 12.7±3.1 to 5.2±1.7 spikes/sec) (Fig. 5A), and the OFF cell pause duration (from 5.7±0.6 to 2.4±0.4 sec) (Fig. 5B).

**Effect of intra-VL-PAG vehicle, capsaicin and DAMGO on tail-flick latencies from RVM recording studies.**

Tail flicks were elicited every 3-4 min for at least 20 min prior to microinjecting drugs, or respective vehicles, into the VL-PAG. Data related to pre-treatment interval were considered as basal tail flick latencies (4.9±0.4 sec). Intra-VL-PAG microinjection of vehicle, capsaicin (3 nmol/rat ) or DAMGO (6 pmol/rat) did not change the tail flick latency as compared to basal values (4.7±0.5 sec) (Fig. 6). Tail flick latency was increased to 7.8±0.5 sec by capsaicin in combination with DAMGO (p<0.05) (Fig. 6). The effect of co-administration of capsaicin and DAMGO was prevented by pre-treatment with I-RTX (0.5 nmol/rat) or naloxone (5 nmol/rat) (Fig. 6).

**Effect of intra-VL-PAG vehicle, capsaicin and DAMGO on glutamate and GABA release in the RVM**

The mean basal extracellular GABA and glutamate levels in the RVM (not corrected for probe recovery of 21 ± 6% and 26 ± 5% for GABA and glutamate, respectively) were 5.8 ±
0.6 and 19.5 ± 6.2 pmol/10 μl of dialysate (mean ± S.E.M.), respectively. These values are concordant with those obtained in our previous studies (Starowicz et al., 2007). Each animal was used only once and the reported basal values of GABA and glutamate are the mean concentrations obtained from all experiments pooled as controls. Intra-VL-PAG administration of the TRPV1 agonist capsaicin (3 nmol/rat), did not significantly change the extracellular levels of glutamate or GABA in the RVM. Intra-VL-PAG microinjection of the μ-opioid receptor (MOR) agonist DAMGO (6 pmol/rat), induced a delayed decrease of glutamate levels (48±5% of basal value, 90 min after injection), but very rapidly decreased the GABA levels (58±10% of basal value) (Fig.2 A and B). Importantly, intra VL-PAG co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) induced an immediate and significant (p <0.05) elevation of the extracellular levels of glutamate (50±8% of basal value), but not GABA in the RVM (Fig.7A and B). The effect on glutamate release was antagonised by pre-treatment with naloxone (5 nmol/rat, 200 nl), an opioid receptor antagonist, or I-RTX (0.1 nmol/rat, 200nl), a selective TRPV1 receptor antagonist (Fig. 7C and D). The two antagonists administered per se at these doses did not affect glutamate or GABA release (data not shown).

Effect of intra-VL-PAG vehicle, capsaicin and DAMGO with 0.5 % DMSO concentration on RVM glutamate and GABA release and thermal nociception

In order to exclude that the observed effects were due to a 10% DMSO concentration in the vehicle, select control microdialysis/plantar test combined experiments were carried out using a lower DMSO concentration. A 0.5 % DMSO vehicle solution or capsaicin (3 nmol/rat) dissolved in 0.5% DMSO did not produce any effect per se. Intra-VL-PAG administration of DAMGO (6 pmol/rat) dissolved in 0.5% vehicle significantly reduced the extracellular levels of glutamate and GABA in the RVM (Fig 8A and B) Importantly, intra VL-PAG co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) dissolved in 0.5% vehicle
induced an immediate and significant (p < 0.05) elevation of the extracellular levels of glutamate but not GABA in the RVM (Fig. 8A and B). Briefly, when capsaicin, DAMGO or the capsaicin/DAMGO combination were dissolved in 0.5 % DMSO did not produce effects different from those obtained using a 10% DMSO (Fig. 7).

Localization of MOR and TRPV1 in the rat VL-PAG and RVM

The immunohistochemical localization of MOR and TRPV1 receptors in the rat PAG (Fig. 9a-c) and RVM (Fig. 9d-f) was determined by double immunofluorescence. We found TRPV1-positive neurons in the VL-PAG area, where a density of 82.5%±7.2% MOR/TRPV1 positive profiles was observed within the ventrolateral sub-region with respect to 10.4%±2.7% of neurons MOR-immunopositive but TRPV1-immunonegative (Fig. 9a-c, arrows). Generally the TRPV1-ir signal was mostly found in the cell bodies whereas MOR-ir was prevalently on the somatic cellular membrane and fibers (see also Fig. 9 lower left panels a1–c1).

In the RVM the MOR immunoreactivity signal was prevalently somatic but less coinciding with TRPV1-ir with respect to the VL-PAG since the percentage of MOR/TRPV1 neurons was 54.7%±5.6% and many neurons were MOR-immunopositive but TRPV1-immunonegative (20.3±3.2%) or MOR-immunonegative but TRPV1-immunopositive (26.4±2.8%, Fig. 9d-f arrows, Fig. 9 lower right panels d1–f1).
**Discussion**

We have presented here the results of the first study ever performed to investigate a possible functional cross-talk *in vivo* of the endovanilloid and opioid systems within the framework of supraspinal nociceptive transmission. Recent studies have revealed that negative interactions occur between TRPV1 and MOR in heterologous cell expression systems and isolated sensory neurons. Both Vetter et al. (2006) and Endres-Becker et al. (2007) observed that MOR activation counteract TRPV1 activation by the inhibiting cAMP-mediated sensitization of this channel (De Petrocellis et al., 2001). Conversely, Chen et al. (2008) found that TRPV1 activation leads to morphine tolerance, although chronic administration of morphine causes up-regulation of TRPV1 receptors in DRG neurons. Therefore, the results presented here represent the first example of a positive interaction between MOR and TRPV1, since we have shown that, in the rat VL-PAG, MOR ligands, which are known to act at inhibitory neurons to disinhibit PAG output neurons (Vaughan et al., 1997), also interact with a TRPV1 ligand at inducing an early glutamate release into the RVM, thereby inhibiting ON cell activity (via RVM inhibitory interneurons, see Rea et al. 2007 for review), decreasing the tail-flick-related OFF cell pause, and reducing nociception. In fact, by correlating the antinoceptive activity in the plantar test with the *in vivo* microdialysis data obtained in freely moving rats and the electrophysiology data obtained in anaesthetized rats, we have observed that co-administration of non-analgesic doses of capsaicin and DAMGO produces significant effects on ongoing RVM ON cell activity, in a way antagonized by both naloxone and I-RTX.

The doses of the two agonists used here were selected as the highest inactive doses on analgesia, based on previous studies that had shown anti-nociceptive effects of intra-PAG DAMGO (Sykes et al., 2007; Rossi et al., 1994) or capsaicin (Starowicz et al., 2007) *per se*. In a previous study, however, Palazzo et al. (2002) demonstrated that the dose of capsaicin (3 nmol/rat) used here as inactive proved antinociceptive when injected into the dorsolateral PAG of rat. Methodological and technical differences (i.e. the presence of a microdialysis
probe into the RVM in the same animal, as well as a different microinjected PAG subregion) may have contributed to these different outcomes. In fact, both morphine and capsaicin are known to produce antinociception via µ-opioid and TRPV1 receptors, respectively, located in different neuronal populations of the PAG, RVM and spinal cord, and all known to be important for descending pain modulation. Excitatory cells in the VL-PAG receive glutamatergic fibers from several forebrain structures and MOR-expressing GABAergic fibers, and in turn project to neurons in the RVM, which send their axons to the dorsal horn of the spinal cord (for reviews see Rea et al., 2007 and Palazzo et al., 2008). As a result of the activation of these excitatory PAG output neurons, which might result from their disinhibition following activation of MOR on GABAergic fibers, or from their depolarisation induced by TRPV1, RVM OFF cell activity can be enhanced, thereby suppressing the activity of ascending nociceptive projection neurons in the dorsal horn and increasing the nociceptive threshold (Fields et al., 1991; Basbaum and Fields, 1984). Importantly, we found that co-injection of the two agonists produced effects that are also qualitatively different from those previously observed with higher intra-VL-PAG doses of the two compounds. In fact: 1) high doses of DAMGO cause antinociception accompanied by enhanced glutamatergic signalling in the RVM and inhibition and stimulation of both spontaneous and tail-flick related ON and OFF cell activity, respectively (Tortorici and Morgan, 2002; Spinella et al., 1996; Fang et al., 1989), whereas the low dose exerted in this study an early and prolonged inhibition of GABA release and a delayed inhibition of glutamate release in the RVM, two effects that were not accompanied by any effect on nociception or ON/OFF cell activity; 2) a high dose of intra-VL-PAG capsaicin causes antinociception accompanied by increased glutamate and decreased GABA release in the RVM, and inhibition and stimulation of both spontaneous and tail-flick related ON and OFF cell activity (Starowicz et al., 2007), whereas the low dose used here had no effect on both amino acids release and spontaneous and tail-flick related ON and OFF cell activity; 3) co-injection of the low doses of the two drugs resulted in antinociception
accompanied by enhanced glutamate release in the RVM and inhibition of ON cell activity, whilst enhancing OFF cell activity only in the presence a nociceptive stimulus, which is different from what observed previously with single administration of high doses of either MOR agonists or capsaicin. These observations suggest that the interaction between the two receptors produced by their co-stimulation with low doses of agonists results in a different outcome in the neuronal pathways mediating descending antinociception.

We recently showed that several VL-PAG output neurons receiving both GABAergic and glutamatergic inputs express TRPV1 receptors (Starowicz et al., 2007). Here, in agreement with the hypothesis of a cross-talk between TRPV1 and MOR, we have found that several of these VL-PAG TRPV1-ir neurons are surrounded by MOR-expressing neurons and also express MOR. A similar degree of fiber and somatic co-localization was also recently shown to occur in rat DRG neurons (Chen et al., 2008). Therefore, both pre-/post-synaptic and post-/post-synaptic interactions might occur between MORs and TRPV1, respectively, in the VL-PAG, and underlie the functional interaction revealed in the present study. These data are compatible with what has already been published by others using confocal microscopy, namely that MOR-ir is present in both cells and small processes within the VL-PAG (Kalyuzhny et al., 1996), whereas it is restricted to cell membranes and cytoplasm in the RVM (Kalyuzhny et al., 1996). Anatomical studies show that MORs are frequently located on the somata or dendrites of GABAergic PAG neurons (Commons et al., 2000; Kalyuzhny and Wessendorf, 1998) and that about 50% of the MOR-ir in axon terminals also contain GABA (Commons et al., 2000). Out of these terminals, some are double labelled for MOR/GABA and make contacts with both unlabeled and GABA-ir dendrites (Commons et al., 2000). However, an ultrastructural study also showed that MOR is strongly co-localized with post-synaptic NMDA receptors in asymmetric (i.e. excitatory) neurons of the VL-PAG (Commons et al., 1999). In summary, the immunohistochemical results of these previous studies together with ours support both the model in which MOR ligands act by inhibiting GABA release from
GABAergic neurons, and the possibility that MORs are also present on excitatory/glutamatergic neurons, where TRPV1 receptors are most likely located (Starowicz et al., 2008; 2007). Performing experiments aimed at directly investigating the molecular mechanism of action of the MOR-TRPV1 interaction observed here was outside the aims of this study. However, we can speculate, based on the above immunohistochemical data, that whereas pre-synaptic MORs on GABAergic neurons and post-synaptic TRPV1 might interact by disinhibiting and exciting VL-PAG output neurons, respectively, the two receptors, when co-localised in post-synaptic neurons, might instead produce together an extracellular Ca\(^{2+}\)-induced intracellular Ca\(^{2+}\) mobilization from intracellular stores, an event that has been described to be coupled to active doses of both MORs and TRPV1 channels in DRG neurons (Tang et al., 1996; De Petrocellis et al., 2001).

We cannot exclude the possibility, however, that the antinociceptive effect observed here might occur as a consequence of the stimulation of TRPV1 channels and MORs located on distant neurons. For example, MORs on pre-synaptic glutamatergic terminals innervating GABAergic interneurons might cause the same effect of MORs located on the axons of latter neurons, i.e. indirectly disinhibit the antinociceptive VL-PAG output neurons. In fact, presynaptic MORs would also influence glutamatergic terminals in the same way as they do GABAergic terminals, i.e. by inhibiting neurotransmitter release. Indeed, some authors have shown that opioid receptors are not exclusively located in inhibitory synapses (Chiou and Huang 1999; Vaughan and Christie, 1997). Thus, the inhibitory effect of DAMGO on the levels of GABA in the RVM might be also the result of MOR activation on excitatory synapses in the PAG, where a population of VL-PAG neurons projecting to a specific RVM nuclei receive direct opioidergic inputs (Kalyuzhny et al., 1996; Osborne et al., 1996; Williams and Beitz, 1990). If such output neurons were GABAergic, their inhibition by MOR activation would cause a reduction on GABA release in the RVM, consistently with the DAMGO-induced effect. Inhibition of RVM GABA release tone would, in turn: 1) disinhibit
GABAergic interneurons in the RVM, which in turn causes ON cell inhibition, and 2) directly disinhibit ON cells (see Palazzo et al., 2008 and Rea et al., 2007 for reviews), thus causing a net null effect on ON cell activity. We speculate that co-activation of TRPV1 “shifts” the effects of low dose of DAMGO from PAG output inhibitory to excitatory neurons. Interestingly, however, there was no change in GABA concentration when capsaicin and DAMGO were co-injected in the presence of I-RTX, i.e. when only the effect of DAMGO should be present, suggesting that TRPV1 blockade does not simply affect TRPV1 receptors, and underlying the complexity of TRPV1-MOR interactions. Ad hoc studies should be designed to address specifically the nature of these interactions and to fully investigate the reasons of the little inconsistencies observed here.

In conclusion, we found that MOR- and TRPV1-receptors are strongly co-expressed in brainstem descending antinociceptive circuits. The results of our pharmacological, electrophysiology and microdialysis experiments indicate the occurrence of a functional interaction between the two receptors in the VL-PAG, which result in the excitation of excitatory VL-PAG output neurons to the RVM. The resulting ON cell inhibition, together with increased onset and decreased duration of OFF cell pause, which are both crucial for the stimulation of the descending inhibitory circuitry to the spinal cord, produce antinociception. In view of our present findings, the action of opioid analgesics in the brainstem will have to be revisited also in view of their possible interactions with TRPV1 receptors. Future studies will have to investigate the molecular and cellular mechanism of MOR-TRPV1 cross-talk in the VL-PAG.
Acknowledgements

This study was supported by MIUR, Italy (PRIN 2005). KS acknowledges the support of the Foundation for Polish Science and Iceland, Liechtenstein and Norway through the EEA Financial Mechanism.
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Legends to Figures

Figure 1. Schematic illustration of the location of PAG microinjection sites (A), RVM microdialysis probes location (B) and RVM ON- or OFF-cell recording sites (C). Vehicle or drug microinjections were performed into the VL-PAG (filled circles) (A). The site of microdialysis probe (B) was also histologically confirmed and marked as correct (black bars) when the probe tip fell within the RVM areas studied. Moreover, the cell recording was performed by lowering a tungsten electrode into the RVM and filled triangles represent ON-cells and open triangles the OFF-cell sites (C). Many sites are not shown because of symbol overlapping. Scale bar is 100 micron. Distances from the interaural line are indicated.

Figure 2. Effect of a co-injection of capsaicin and DAMGO into the VL-PAG on thermal withdrawal latency in sec. Thermal nociception was evaluated by using the Plantar test and expressed as thermal withdrawal latency in sec. “A” shows the effect of VL-PAG microinjection of vehicle (10% DMSO in ACSF), capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) alone or the co-injection of capsaicin (3 nmol/rat) and DAMGO (6 nmol/rat). “B” shows the effect of I-RTX (0.1 nmol/rat) or naloxone (5 nmol/rat) when they were microinjected in combination with capsaicin and DAMGO. The curves of vehicle and capsaicin (3 nmol/rat) in combination with DAMGO (6 nmol/rat) have been duplicated in B for clarity. Nociceptive responses were measured every 15 min for a period of 2 hrs. “C” shows the effect of a vehicle with lower DMSO concentration (0.5 %), capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) or co-injection of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) all dissolved in 0.5 % DMSO vehicle on thermoceptive reaction. Each point represents the mean ± S.E.M of 8-10 animals per group. (*) Indicates significant differences vs. vehicle (A, B,C) and (°) significant differences vs. capsaicin (3 nmol/rat) + DAMGO (6 pmol/rat) (B). P values <0.05 were considered statistically significant.
**Figure 3.** Effect of capsaicin or DAMGO alone or co-administered on the spontaneous firing of RVM ON (A and C) or OFF (B and D) cells. “A” and “B” show the effect of intra-VL-PAG administration of vehicle, capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) alone or co-administered. “C” and “D” show the effect of intra-VL-PAG administration of vehicle, capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat), alone or in combination, with and without naloxone (5 nmol/rat) or I-RTX (0.5 nmol/rat). The black arrows show the time of microinjections. Each point represents the mean ± standard error of the mean (S.E.M) of 8-10 neurons. * indicates significant differences vs. vehicle and ° significant differences vs. capsaicin (3 nmol/rat) + DAMGO (6 pmol/rat). P values < 0.05 were considered statistically significant.

**Figure 4.** Examples of ratemeter records which illustrate the effects of intra-PAG microinjections of capsaicin (CPS, 3 nmol/rat) (A and C), DAMGO (6 pmol/rat) (B and D) on either the ongoing or tail flick-related discharges of identified RVM ON (A, B) and OFF (C, D) cells. Traces report overall firing before and after drug injections into the VL-PAG. Filled triangles indicate tail flick trials, 1sec bins. Open arrows show the time of microinjections within the VL-PAG. Scale bar 4 min.

**Figure 5.** Examples of ratemeter records which illustrate the effects of intra-PAG microinjections of DAMGO (6 pmol/rat) in combination with capsaicin (CPS, 3 nmol/rat) on either the ongoing or tail flick-related discharges of identified RVM ON (A) and OFF (B) cells. Traces report overall firing before and after drug injections into the VL-PAG. Filled triangles indicate tail flick trials, 1sec bins. Single oscilloscope traces indicated by the long black arrows show the effect from tail flick stimulation (small white arrows) to tail flick.
reflex (small black arrows). Open arrows show the time of microinjections within the VL-PAG. Scale bar 4 min.

Figure 6. Tail flick latencies before and after microinjections into the VL-PAG of vehicle, capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) alone or co-administered (A), and in combination with naloxone (5 nmol/rat) or I-RTX (0.5 nmol/rat) (B). Each point represents the mean ± standard error of the mean (S.E.M) of 8-10 observations. * indicates significant differences vs. vehicle and ° significant differences vs. capsaicin (3 nmol/rat) + DAMGO (6 pmol/rat). P values < 0.05 were considered statistically significant.

Figure 7. Effects of intra-VL-PAG injection of vehicle, capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) or co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) alone (A,B) or in combination with I-RTX (0.1 nmol/rat) or naloxone (5 nmol/rat) (C,D) on RVM dialysate levels of glutamate (A,C) and GABA (B,D). Each point represents the mean ± SEM of amino acid extracellular concentrations as a percentage of the basal values (8-10 rats per group). * indicates significant difference vs. vehicle and ° vs. capsaicin (3 nmol/rat) + DAMGO (6 pmol/rat). P values <0.05 were considered statistically significant.

Figure 8. Effects of intra-VL-PAG injection of vehicle (0.5 % DMSO in ACSF), capsaicin (3 nmol/rat), DAMGO (6 pmol/rat) or co-administration of capsaicin (3 nmol/rat) and DAMGO (6 pmol/rat) all dissolved in 0.5% DMSO in ACSF on RVM dialysate levels of glutamate (A) and GABA (B). Each point represents the mean ± SEM of amino acid extracellular concentrations as a percentage of the basal values (9 rats per group). * indicates significant difference vs. vehicle. P values <0.05 were considered statistically significant.
Figure 9. Immunohistochemical localization of MOR and TRPV1 receptors in the rat ventrolateral PAG (a-c) and RVM (d-f) as determined by double immunofluorescence. a-c: VL-PAG immunoreactivity (ir) of MOR (a), TRPV1 (b) and MOR/TRPV1 (c, merged image). Note the dense MOR-ir, prevalently in cellular membranes and processes, and the TRPV1-ir in cytoplasm. Some neurons MOR-immunopositive and TRPV1-immunonegative are also seen in the VL-PAG (arrows). a1–c1, High magnification of the respective boxed areas of a–c. d-f: RVM immunoreactivity distribution of MOR (d), TRPV1 (e) and MOR/TRPV1 (f, merged image). Note the cytoplasmatic features of both MOR and TRPV1 receptor labeling in the majority of neurons, whereas some neurons are TRPV1-ir only (arrows). d1–f1, High magnification of the respective boxed areas of d–f. 3v: lumen of the aqueduct; VL: ventrolateral PAG. Scale bar a-c= 80μm; d-f= 120μm. a1–f1 = 20μm Images are representative of those obtained in 9 sections for animal (n=4).
A

- vehicle 10% DMSO
- capsaicin 3 nmol
- DAMGO 6 pmol
- capsaicin+DAMGO

Thermal withdrawal latency (s)

min

B

- vehicle
- capsaicin 3 nmol+DAMGO
- IRTX 0.1 nmol + capsaicin + DAMGO
- nalox 5 nmol+DAMGO+capsaicin

Thermal withdrawal latency (s)

min

C

- vehicle 0.5% DMSO
- capsaicin 3 nmol
- DAMGO 6 pmol
- capsaicin+DAMGO
**A**

- **vehicle**
- **capsaicin 3 nmol**
- **DAMGO 6 pmol**
- **capsaicin+ DAMGO**

**B**

- **vehicle**
- **capsaicin+ DAMGO**
- **Nalox 5 nmol+DAMGO+capsaicin**
- **IRTX 0.5 nmol+capsaicin+DAMGO**

**Tail Flick Latency (s)**

- **pre-drug**
- **post-drug**
Figure A shows the effects of various treatments on % basal glutamate levels over 120 minutes. Treatments include vehicle 0.5% DMSO, capsaicin 3 nmol, DAMGO 6 pmol, and capsaicin + DAMGO. Asterisks indicate significant differences from controls.

Figure B displays the % basal GABA levels over the same time period, comparing the same treatments. Significant differences are also indicated by asterisks.

Graphs depict time (0-120 min) on the x-axis and % basal neurotransmitter levels on the y-axis.