Dorsal striatum metabotropic glutamate receptor 8 affects nocifensive responses and rostral ventromedial medulla cell activity in neuropathic pain conditions

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Abbreviated title: Effect of DS mGluR8 on pain responses and RVM cell activity
Abstract

The current study has investigated the role of the metabotropic glutamate receptor subtype 8 (mGluR₈) in the dorsal striatum (DS) in modulating thermonociception and rostral ventromedial medulla (RVM) ON and OFF cell activities in conditions of neuropathic pain induced by the spare nerve injury (SNI) of the sciatic nerve in rats. The role of DS mGluR₈ on mechanical allodynia was also investigated. Intra-DS (S)-3,4-DCPG, a selective mGluR₈ agonist, did not modify the activity of the ON and OFF cells in sham rats. In SNI rats, which showed a reduction of the mechanical withdrawal threshold, intra-DS microinjection of (S)-3,4-DCPG inhibited the ongoing and tail flick-evoked activity of the ON cells while increased the activity of the OFF cells. AZ12216052, a selective mGluR8 positive allosteric modulator (PAM), behaves as (S)-3,4-DCPG in increasing tail flick latency, the OFF cell activity and decreasing the ON cell activity in SNI rats only, but was less potent. VU0155041, a selective mGluR4 PAM was ineffective in changing thermal nociception and ON and OFF cell activity in both shams and SNI rats. (S)-3,4-DCPG did not change mechanical withdrawal threshold in sham rats while increased it in SNI rats. Furthermore, a decreased level of mGluR₈ gene and immunoreactivity, expressed on GABAergic terminals, associated with a protein increase was found in the DS of SNI rats. These results suggest that the stimulation of mGluR₈ inhibits thermoceptive responses and mechanical allodynia. These effects were associated with an inhibition of the ON cells and the stimulation of OFF cells within RVM.

Keywords: mGluR₈, spare nerve injury, dorsal striatum, rostral ventromedial medulla, mechanical allodynia
Neuropathic pain, which is often resistant to conventional analgesics (Sindrup and Jensen 1999; Woolf and Mannion 1999), remains a significant clinical problem. Following peripheral or central nervous system injury, spinal and brain plastic changes lead to central sensitization and consequent thermal hyperalgesia and mechanical allodynia symptoms (Chudler and Dong 1995; Hagelberg et al. 2004; Neugebauer 2006; Jaggi and Singh 2011). Glutamate plays a key role in persistent activation of nociceptive neurons and hypersensitivity to painful stimuli. An increase in glutamate tone was also demonstrated in a rat model of neuropathic pain (Santangelo et al. 2012). The deleterious consequence of glutamate increase and associated over-activation of postsynaptic receptors is a well known event in several neuro-pathologies and can be reversed by inhibiting postsynaptic ionotropic glutamate receptors (iGluRs) or activating presynaptic glutamate autoreceptors (Niswender and Conn 2010). Indeed, group II and III metabotropic glutamate receptors (mGluRs) finely regulate glutamate release since they function as presynaptic autoreceptors (Scanziani et al. 1997; Cartmell and Schoepp 2000). These receptors are widely expressed at spinal, supraspinal and peripheral sites where they display different roles in nociception (Varney and Gereau 2002; Goudet et al. 2009; Chiechio and Nicoletti 2012). In this context, the role of individual group III mGluR subtypes in pain is only beginning to emerge concomitantly with the availability of subtype-selective agents.

In our previous studies the stimulation of mGluR subtype 8 (mGluR8) by the selective agonist (S)-3,4-dicarboxyphenylglycine, (S)-3,4-DCPG (Thomas et al. 2001), in the ventrolateral periaqueductal grey (VL PAG) and central nucleus of the amygdala (CeA) has proven to inhibit pain and associated changes in the rostral ventromedial medulla (RVM) neuron activity in models of neuropathic and inflammatory pain (Marabese et al. 2007a; Palazzo et al. 2008; Palazzo et al. 2011). RVM, a relay station in the pain descending system, contains different pain responding neurons: ON-cells which are activated, OFF-cells which are inhibited and neutral cells which are unaffected by nociceptive stimuli (Fields et al. 1983; Heinricher et al. 1989). Systemic or local
administration of opioids or cannabinoids, able to inhibit nociception, inhibit ON cells whilst
increase OFF cells (Heinricher and Tortorici 1994; Fields et al. 1995; Field et al. 2006; de Novellis
et al., 2005). Changes of ON and OFF cell activity are thus predictive of pain response modulation.

There is accumulating evidence indicating that the dorsal striatum, the main input of the basal
ganglia, might play a role in pain processing (Barcelo et al. 2012). In situ hybridization and
immuno-histochemistry studies have demonstrated the expression of mGluRs at different levels of
the dorsal striatum (DS) (Testa et al. 1994; Wang et al. 1997; Ferraguti and Shigemoto 2006). In
particular, group III mGluRs have been reported to be expressed in the striatum at presynaptic level
on excitatory corticostriatal terminals and GABAergic output fibers (Testa et al. 1994, 1998;
Kosinski et al. 1999; Corti et al. 2002). Moreover, previous studies demonstrated that RVM could
mediate the action of the striatum, indeed, electrolytic or kainic acid lesion of RVM blocked the
inhibition of the nocifensive reflex produced by striatal activation in a model of orofacial pain
(Barcelo et al. 2012).

In this study we have therefore investigated the contribution of DS mGluR8 on pain responses in
control and in condition of neuropathic pain induced by the spare nerve injury (SNI) of the sciatic
nerve in rats. A selective mGluR8 agonist, the (S)-3,4-DCPG, was therefore microinjected in the DS
and mechanical allodynia, latencies to tail flick and the activity of RVM ON and OFF cells in sham
and SNI rats have been investigated. The effects AZ12216052 and VU0155041, positive allosteric
modulators (PAMs) of mGluR8 and mGluR4 (Niswender et al., 2008; Duvoisin et al., 2010)
respectively, on thermoceptive responses and ON and OFF cell activity of the RVM were also
evaluated together with changes of expression of DS mGluR8 in SNI rats.
Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Italy) weighing 250-280 g were housed three per cage under controlled illumination (12 h light/12 h dark cycle; light on 06.00 h) and standard environmental conditions (ambient temperature 20-22°C, humidity 55-60 %) for at least 1 week before the commencement of experiments. Rat chow and tap water were available ad libitum. All surgery and experimental procedures were done during the light cycle and 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 EC (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to reduce both animal numbers and suffering during the experiments.

Neuropathic pain induction

The spared nerve injury model of neuropathic pain was induced according to the method used by Decosterd and Woolf (2000). Rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.). The sciatic nerve was exposed at the level of its trifurcation into sural, tibial and common peroneal nerves. The sural and common peroneal nerves were ligated tightly then transected just distal to the ligation, leaving the tibial nerve intact. Sham rats were anaesthetised, the sciatic nerve was exposed at the same level, but not ligated. Fourteen days after surgery sham and SNI rats were used for tail flick tests coupled with single unit extracellular recording or mechanical allodynia experiments or were sacrificed for immunohistochemistry, RT-PCR and western blot analysis.

Surgical preparation for intra-DS microinjection

In order to perform direct intra-DS microinjections sham and SNI rats were anaesthetised with pentobarbital (50 mg/kg, i.p.) and a 26-gauge, 12 mm-long stainless steel guide cannula was stereotaxically lowered until its tip was 0.8 mm above the DS by applying coordinates from the atlas of Paxinos and Watson (1986) (AP: 0.20 mm and L: 2.8 mm from bregma, V: 3.2 mm below the dura). 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-DS administration of drugs or respective vehicle (artificial cerebrospinal fluid, ACSF: composition in mm: 125 NaCl, 2.5 KCl, 1.18 MgCl₂, and 1.26 CaCl₂), was conducted with a stainless steel cannula connected by a polyethylene tube to a SGE 1-μl syringe, inserted through the guide cannula and extended 0.8 mm beyond the tip of the guide cannula to reach the DS (Fig. 1A). Volumes of 200 nl of drug solution, or vehicle, were injected into the DS over a period of 60 sec and the injection cannula gently removed 2 min later.

RVM extracellular recordings associated to tail flick

20-24 hrs after the guide cannula implantation (day 13), and at the day 14 after sciatic nerve surgery (day 0), anaesthesia was induced with pentobarbital (50 mg/kg, i.p.) and maintained with a continuous infusion of propofol (5-10 mg/kg/h, i.v.) 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 approximately 3-5 cm from the tip. The intensity of the radiant heat source was adjusted to 50 mW (corresponding to 50 mJ per sec) at the beginning of each experiment in order to elicit a constant tail flick latency. Tail flicks were elicited every 5 min for at least 15 min prior to microinjecting the drug or its vehicle into the DS. A glass-insulated tungsten filament electrode (3–5 MW) (FHC Frederick Haer & Co., ME, USA) was lowered into the RVM using the following stereotaxic coordinates: 2.8–3.3 mm caudal to lambda, 0.4–0.9 mm lateral and 8.9–10.7 mm depth from the surface of the brain (Paxinos and Watson 1986) (Fig 1B). RVM noxious stimuli-responding neurons were identified by the characteristic OFF cell pause and ON cell burst of activity immediately prior to tail flick response (Fields et al. 2006). Neutral cells, which did not show any significant change in activity associated with the tail withdrawal, where also encountered during cell searching procedure but were not recorded in this study. The recorded signals were amplified and displayed on both analogue and a digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were sampled by a CED 1401 interface (Cambridge Electronic Design Ltd., UK) and analyzed by Spike2
window software (CED, version 4) to create peristimulus rate histograms on-line and to store and analyse digital records of single-unit activity off-line. The configuration, shape, and height of the recorded action potentials were monitored and recorded continuously using Spike2 software for on-line and off-line analyses. 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 be clearly discriminated from the background activity throughout the entire experiment. By doing so, we were able to determine the activity of only one neuron. In each rat, the activity of only a single neuron was recorded before and after vehicle or drug administration. Ongoing and tail flick-related activity of the OFF cells was recorded before and after the DS microinjection of drugs or vehicle in shams and SNI rats 14 days after the surgical procedure for neuropathic pain induction. For each ON and OFF neuron the ongoing activity was obtained by averaging the firing rate (spikes/sec) for 50 sec before the tail flick trials (carried out every 5 min). The latency to the onset of the pause (time between the onset of heat application and the last action potential) and the duration of the tail flick-related pause (the time elapsing between the pause onset and the first action potential following tail flick) of the OFF cells were also quantified. Moreover, the tail flick-related burst (the peak height of burst in spikes/sec calculated by subtracting the ongoing activity to the burst activity) and the onset of the ON cell burst (the time elapsing between the onset of heat application and the increase in the frequency rate, which was at least twofold higher than its baseline) were quantified for the ON cells. At the end of the experiment, a volume of 200 nl of neutral red (0.1%) was injected into the DS 30 min before killing the rats with a lethal dose of pentobarbital. Rats were then perfused intracardially with 20 ml phosphate buffer solution (PBS) followed by 20 ml of 10% formalin solution in PBS. The brains were removed and immersed in a saturated formalin solution for 2 days. After fixation, the microinjection and recording sites were identified (Fig. 1A and B, respectively). The injection sites were ascertained using two consecutive sections (40 μm), one stained with cresyl violet to identify the DS, and the other unstained to determine dye spreading. The recording site was marked with a
20 μA DC current applied for 20 sec immediately prior to the end of the electrophysiological recordings. Only the data from microinjection and drug diffusion sites located within the DS and those from the recording sites in RVM neurons were included in the results.

Mechanical allodynia

Mechanical allodynia was evaluated 14 days after SNI or sham surgery using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Rats were allowed to move freely in one of the two compartments of the enclosure, positioned on the metal mesh surface and allowed to adapt to the testing environment before any measurement was taken. After 30 min of habituation period, the mechanical stimulus, a steel rod (2 mm) pushed with ascending force (0–30 g in 10 s), was delivered to the plantar surface of the hind paw of the rat from below the floor of the test chamber by an automated testing device. When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn and the force was recorded to the nearest 0.1 g. Mechanical withdrawal threshold (MWT) was measured in grams for 1 h before treatment and for 2 h after vehicle or drug administration by an experimenter blind to the treatments. A single trial at each time point (15 min) was performed on the ipsilateral hind paw to the sham or SNI surgery for each rat. Nociceptive responses for mechanical sensitivity were expressed as mean ± SEM in grams. Groups of 5 rats per treatment were used, with each animal being used for one treatment only.

Treatments

Single unit extracellular recordings in the RVM associated to tail flick have been carried out in anaesthetized SNI and sham rats. Mechanical allodynia was measured in awake SNI and sham rats. Both experiments have been carried out 14 days after sciatic nerve surgery. mGluR₈ agonist, (S)-3,4-DCPG, AZ12216052, a selective PAM for mGluR₈ and VU0155041, a selective PAM for mGluR₄ or respective vehicle were microinjected into the DS in sham and SNI rats in a volume of 0.2 μl. (S)-3,4-DCPG in combination with LY341495, a group II mGluR antagonist with a selectivity for mGluR₈ over mGluR₄/7 and LY341495 alone were microinjected only in SNI rats. In electrophysiology associated to tail flick experiments groups of 10 rats per each treatment have
been used in order to have at least 5 recordings for ON and OFF cells with each animal being used for a single cell recording. Rats received 14 days after surgery a single intra DS microinjection of drugs or vehicle and were divided as follows:

1) Sham and SNI rats receiving an intra-DS microinjection of vehicle,

2) Sham and SNI rats receiving intra DS-microinjection of (S)-3,4-DCPG (2 nmol for sham rats, 1 and 2 nmol for SNI rats).

3) SNI rats receiving intra DS-microinjection of (S)-3,4-DCPG (2 nmol) in combination with LY341495 (10 \( \mu \)mol)

4) SNI rats receiving intra DS-microinjection of LY341495 (10 \( \mu \)mol)

5) Sham and SNI rats receiving intra DS-microinjection of AZ12216052 (20 nmol)

6) Sham and SNI rats receiving intra DS-microinjection of VU0155041 (40 nmol)

7) In the awake rats tested for mechanical allodynia a group of SNI rats received intra-DS microinjection of (S)-3,4-DCPG (2 nmol) in combination with (RS)-\( \alpha \)-methylserine-O-phosphate, MSOP, a group III mGluRs selective antagonist.

Motor coordination

Motor coordination was tested using the accelerating rotarod (model 47700, Ugo Basile) in which rats were required to walk against the motion of a rotating drum with the speed accelerating from 4 to 40 rpm/min over 300 s. The time on the rod from the start of acceleration until the animal fell from the drum onto the counter-trip plate was recorded. A 300 s cut off was used. One training period per day was performed for 2 d before experiments in which ACSF or the (S)-3,4-DCPG (2 nmol) was microinjected into DS. On the day of testing, sham and SNI rats were tested before and 15-30-45-60 and 90 min after intra-DS ACSF (n = 5) or (S)-3,4-DCPG (n = 5). Time spent on the rod was taken as the mean ± SEM in seconds in the consecutive attempts by an experimenter who was blind to the treatment.

RNA extraction and RT-PCR
Total RNA was extracted from homogenized DS (contralateral and ipsilateral to the spared nerve injury) using an RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer’s protocol. The extracted RNA was subjected to DNase I treatment at 37° C for 30 min. The total RNA concentration was determined by UV spectrophotometer. The mRNA levels of the genes under analysis were measured by RT-PCR amplification, as previously reported (Galderisi et al., 1999). RT minus controls were carried out in order to check potential genomic DNA contamination. These RT minus controls were performed without using the reverse transcriptase enzyme in the reaction mix. Sequences for the mouse mRNAs from GeneBank (DNASTAR INC., Madison, WI) were used to design primer pairs for RT-PCRs (OLIGO 4.05 software, National Biosciences Inc., Plymouth, MN). Each RT-PCR was repeated at least four times so as to achieve optimal reproducibility data. A semi-quantitative analysis of mRNA levels was carried out using the “Gel Doc 2000 UV System” (Bio-Rad, Hercules, CA). The measured mRNA levels were normalised using three housekeeping genes: hypoxanthine-guanine phosphoribosyltransferase (HPRT), 18S ribosomal RNA (18S) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The HPRT, 18S and GAPDH housekeeping genes have been chosen since their gene expression did not change in different experimental conditions included SNI-induced neuropathic pain (Maione et al., 2002; Siniscalco et al., 2007, 2010; Bangaru et al., 2012; Piller et al., 2013). The gene expression values were expressed as arbitrary units ± SEM.

Amplification of gene of interest and HPRT, 18S and GAPDH were performed simultaneously.

**Western blotting**

For the protein extraction, the striatum (contra end ipsi of the spared nerve injury) was minced into small pieces with a blender, then was suspended in lysis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% blue-bromophenol, Tris-HCl, pH 6.8, containing 6 M urea, 50 μM Na₃VO₄, 50 μM PMSF (Sigma Chemical Co., St. Louis, MO). The total protein concentration was determined by the method described by Bradford (1976). Each sample was loaded, electrophoresed in a 8% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Primary antibodies
to detect mGluR-8 was used according to the manufacturer’s instruction at 1:500 dilution (Santa Cruz Biotecnology; Santa Cruz, CA). Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody and reacted with an ECL system (Amersham Pharmacia, Uppsala, Sweden). Protein levels were normalized with respect to the signal obtained with three housekeeping proteins: anti-β-actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO; 1:1000 dilution), β-tubulin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution) and GAPDH monoclonal antibody (Sigma Chemical Co., St. Louis, MO; 1:20000 dilution).

**Immunohistochemistry**

Sham and SNI rats where anaesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, post fixed for 3 hours in the perfusion fixative, cryoprotected for 72 hours in 10, 20 and 30% sucrose in 0.1 M phosphate buffer and frozen in O.C.T. embedding compound. Transverse sections (15 μm) were cut using a cryostat and those containing the whole striatum were thaw-mounted onto glass slides. Sections were subsequently incubated for 1 day at room temperature in a humid chamber with the respective polyclonal antibodies (all diluted in specific block solution). All sections were processed for goat anti-vesicular glutamate transporter-1 (VGluT1) (1: 100, Santa Cruz, USA), goat anti-vesicular GABA transporter (VGAT) (1:100, SySy, Germany), rabbit-anti mGluR8 (1:100, Santa Cruz, USA), rabbit-anti synaptophysin (1:200, SySy, Goettingen, Germany), mouse anti-parvalbumin (1:500, Millipore, Billerica, MA, USA). Following incubation, sections were washed and incubated for 3 hours with secondary antibody solution (donkey anti-goat, or donkey anti-rabbit IgG-conjugated AlexaFluorTM 488 and 568; 1:1000; Molecular Probes, USA). Slides were washed, cover-slipped with Vectashield mounting medium (Vector Laboratories, USA) and visualized under a Leica fluorescence microscope. Negative control by using secondary antibodies alone did not reveal any positive staining.

**Drugs**
(S)-3,4-DCPG, M-SOP, LY341495 and VU0155041 were purchased from Tocris Bioscience (Bristol, UK) and were dissolved in ACSF (vehicle) on the day of the experiment. AZ12216052 was purchased from AxonMedchem (Groningen, The Netherlands). The dose of (S)-3,4-DCPG, VU0155041, LY341495 and AZ12216052 has been chosen according to EC50/IC50 and to our and other in vivo studies using brain local microinjections which have shown to change pain behaviour (Chi et al., 2006; Marabese et al., 2007b; Niswender et al., 2008; Palazzo et al., 2008; Duvoisin et al., 2010; Palazzo et al., 2011; Dong et al., 2012; Liu et al., 2012).

Data analysis and statistics

All data are given as means ± SEM. For behavioural and electrophysiology experiments 2-way ANOVA for repeated measures followed by Newman-Keuls post hoc test have been used to analyze statistical differences between the different groups of rats. Comparisons between pre- and post-treatment ongoing and tail flick–related cell activity changes were performed by ANOVA for repeated measures. One-way ANOVA followed by t-test were used for biomolecular, protein analysis and immunohistochemistry. P values < 0.05 were considered statistically significant.

Results

Effect of intra-DS (S)-3,4-DCPG, AZ2216052 or VU01550412 on tail flick latencies in sham and SNI rats

Tail flicks were elicited every 5 min for 15 min prior to microinjecting drugs or respective vehicle into the DS. In sham rats tail flick latencies before any treatment were 5.2 ± 0.26 s, n=10. In SNI rats tail flick latencies were significantly lower compared to the shams (2.9 ± 0.7 s, \( P < 0.05 \), n=10). Intra-DS microinjection of vehicle did not change tail flick latency in sham and SNI rats (5.0 ± 0.23 s, n=10 and 3.1 ± 0.4 s, n=10, respectively) compared with pretreatment values. (S)-3,4-DCPG (2 nmol) significantly increased the tail flick latency to 5.88 ± 0.5 s (\( P < 0.05 \); n=10) in SNI but not in sham rats (Fig. 2A). This effect lasted for all the period of observation. Intra-DS microinjection of
the lowest dose of (S)-3,4-DCPG (1 nmol) significantly changed the nocifensive response in SNI rats at 15 min after drug microinjection (4.36 ± 0.6 s, P < 0.05; n=10) (Fig. 2A). The effect of (S)-3,4-DCPG (2 nmol) observed in SNI rats was antagonized by LY341495 (10 μmol), a group II mGluR antagonist with a selectivity for mGluR8 over mGluR4/7 (Fig. 2B). LY341495 (10 μmol) per se did not change the tail flick latency in SNI rats (not shown).

Intra-DS microinjection of AZ2216052 (20 nmol), a selective mGluR8 PAM, significantly increased the tail flick latency to 4.99 ± 0.4 s (P < 0.05; n=10) in SNI, but not in sham rats (Fig. 2C). Intra-DS microinjection of VU1550412 (40 nmol) did not change the nocifensive response in sham and SNI rats (not shown).

Effect of intra-DS (S)-3,4-DCPG on the ongoing activity of RVM ON and OFF cell in sham and SNI rats.

Neurons identified as ON cells by a burst of activity just before tail flick responses were spontaneously active in 33.3% of the cases and inactive in the remaining cases. ON cells with spontaneous activity were chosen to better characterize the activity of this ON cell subgroup and to consider postdrug changes in their spontaneous activity. In sham the population of spontaneous active ON cells had a mean frequency of 7.4 ± 0.9 spikes/s. In SNI rats the spontaneous activity of the ON cells was significantly increased (14.1 ± 1.2 spikes/s, P<0.05, n=5). Microinjection of vehicle did not change the spontaneous activity of the ON cells (7.6 ± 0.68 spikes/s, n=5 and 14.8 ± 1.3 spikes/s, n=8) in sham and SNI rats, respectively (Fig. 3A). The highest dose of (S)-3,4-DCPG (2 nmol) did not change the spontaneous activity of the ON cells in sham rats (Fig 3A). Intra-DS microinjection of (S)-3,4-DCPG (1 and 2 nmol) caused a decrease in the spontaneous firing activity of the ON cells (9.0 ± 0.6 spikes/s, P<0.05; n=5 and 0.9 ± 0.5 spikes/s, P<0.05; n=5, respectively) in SNI rats. The decrease of ON cell ongoing activity produced by (S)-3,4-DCPG (2 nmol) was observed after 15 min and remained significant for all the period of recording. This last effect was
agonized by LY341495 (10 μmol) (Fig. 3C). LY341495 (10 μmol) per se did not change the ON
cell spontaneous activity in SNI rats (Fig. 3C). The population of cells identified as OFF cells were
spontaneously active and they had a mean frequency of spontaneous activity of 8.3 ± 1.0, n=5 in
sham animals. In SNI rats the spontaneous activity was significantly reduced (4.6 ± 0.9 spikes/s,
P<0.05, n=5) compared to the shams. Microinjection of vehicle did not change the spontaneous
activity of the OFF cells (8.1 ± 0.8 spikes/s, n=5 and 4.0 ± 0.5 spikes/s, n=5) in sham and SNI rats,
respectively (Fig. 3B). The higher dose of (S)-3,4-DCPG (2 nmol) did not change the spontaneous
activity of the OFF cells in sham rats (Fig. 3B). Intra-DS microinjections of (S)-3,4-DCPG (1 and 2
nmol) caused an increase in the spontaneous firing activity of the OFF cells in SNI rats which was
already significant after 15 min and lasted until 60 min (9.4 ± 0.9 spikes/s, P<0.05; n=5 and 20.1 ±
1 spikes/s, P<0.05; n=5), respectively (Fig. 3B). The effect of (S)-3,4-DCPG (2 nmol) on the OFF
cell spontaneous activity in SNI rats was also antagonized by LY341495 (10 μmol) (Fig. 3D).
LY341495 (10 μmol) per se did not change the OFF cell spontaneous activity in SNI rats (Fig. 3D).

Effect of intra-DS AZ2216052 or VU01550412 on the ongoing activity of RVM ON and OFF cell in
sham and SNI rats.

Intra-DS microinjection of AZ2216052 (20 nmol) did not change the ON cell ongoing activity in
the sham rats (Fig. 4A). It, however, caused a decrease in the spontaneous firing of the ON cells
(8.6 ± 0.8 spikes/s, P<0.05; n=5) in SNI rats (Fig. 4A). AZ2216052 (20 nmol) also significantly
increased the OFF cell spontaneous activity in SNI rats (15.8 ± 1.2 spike/s, P<0.05, n=5) but it was
devoid of activity in the shams (Fig. 4A). Intra-DS microinjection of VU01550412 (40 nmol) did
not change the ON cell ongoing activity in the sham and SNI rats (Fig. 4B), nor it changed the OFF
cell spontaneous activity in sham and SNI rats (Fig 4C and D).
Effect of intra-DS (S)-3,4-DCPG on tail flick-related ON and OFF cell activity in sham and SNI rats

The ON cells had a tail flick-induced burst of firing of 6.1 ± 0.9 spikes/s and 8 ± 0.4 spikes/s in sham and SNI rats, respectively. The onset of the burst was 2650 ± 40 ms and 1680 ± 90 ms in sham and SNI rats, respectively. Thus, the mean of the frequency of the ON cell burst and the onset of burst in the sham rats did not differ from healthy rats (6.9 ± 1.0 spikes/s and 2200 ± 33 ms, n=5) whereas the mean frequency of the ON cell burst was significantly increased and the onset of the burst was decreased in SNI rats compared to both sham and healthy rats (P<0.05; n=5 and P<0.05; n=5). The OFF cells had a pause of 7.0 ± 1.5 s and 16.9 ± 2.5 s and an onset of pause of 2500 ± 45 ms and 1230 ± 80 ms in sham and SNI rats, respectively. The duration and the onset of the OFF cell pause in the sham rats did not differ from healthy rats (6.24 ± 2.4 s and 2370 ± 26 ms) whereas the pause of OFF cells was significantly increased and the onset of the pause was significantly decreased in SNI rats compared to both sham and healthy rats (P < 0.05; n=5 and P < 0.05; n=5, respectively). Microinjections of vehicle in sham and SNI rats did not change the tail flick-induced ON cell burst (5.6 ± 1.0 spikes/s, n=5 and 7.89 ± 0.3, spikes/s n=8, respectively) (Fig. 5A), the onset of ON cell burst (2240 ± 28 ms, n=5 and 1700 ± 200 ms, n=5, respectively) (Fig 5C), the OFF cell pause (6.48 ± 1.35 s, n=6 and 15.8 ± 2.5 s, n=5, respectively) (Fig. 5B) and the onset of the OFF cell pause (2520 ± 78 ms, n=6 and 1420 ± 100 ms, n=5, respectively) (Fig. 5D). Intra-DS microinjections of (S)-3,4-DCPG (1 and 2 nmol) caused a decrease in both ON cell burst (1.6 ± 1.0 spikes/s, P< 0.05; n=5 and 0.3 ± 0.6 spikes/s, P< 0.05; n=5, respectively) (Fig 5A) and OFF cell pause (8.8 ± 1.8 s, P< 0.05; n=5 and 3.95 ± 1.85 s, P< 0.05; n=5, respectively) (Fig 5B) and an increase in both the onset of the ON cell burst (2350 ± 180 ms, n=5 and 2400 ± 108 ms, n=5, respectively) (Fig. 5C) and the onset of the OFF cell pause (2400± 230 ms, n=5 and 2250 ± 140 ms, n=5, respectively (Fig 5D) in SNI rats. The higher dose of (S)-3,4-DCPG did not change the tail flick-related activity in sham rats (not shown). Figure 7 shows representative ratemeter records showing the ongoing and tail flick-related activity of ON (A, C and E) and OFF cells (B, D and F)
before and after (S)-3,4-DCPG in sham (A and B) and SNI (C, D and E) rats. The effect of (S)-3,4-
DCPG (2 nmol) on the burst of firing, onset of the burst of the ON cells, as well as, the pause
duration and onset of pause of OFF cells was antagonized by LY341495 (10 μmol) in SNI rats (Fig.
5).

Effect of intra-DS AZ2216052 or VU01550412 on tail flick-related ON and OFF cell activity in
sham and SNI rats

Intra-DS microinjections of AZ2216052 (20 nmol) caused a significant decrease in both ON cell
burst (3.5 ± 0.4 spikes/s, P< 0.05; n=5) and OFF cell pause (5 ± 0.3 s, P< 0.05; n=5) (Fig 6A and
B). Moreover AZ2216052 (20 nmol) significantly increased the onset of the ON cell burst and of
the OFF cell pause (2272 ± 189 ms, n=5 and 2329 ± 113 ms, n=5, respectively) and the onset of the
OFF cell pause (2296± 245 ms, n=5 and 2181 ± 129 ms, n=5, respectively) in SNI rats. Intra-DS
microinjections of VU01550412 (40 nmol) did not affect the tail flick-related of both ON and OFF
cells activity in sham or SNI rats (Fig 5C and D).

Effect of intra-DS (S)-3,4-DCPG on mechanical allodynia in sham and SNI rats

The mechanical withdrawal threshold in the shams rats was 29.1 ± 5 g before any
treatment. Microinjection of vehicle did not change the mechanical withdrawal threshold in the
shams (Fig. 8A). The intra-DS microinjection of (S)-3,4-DCPG (2 nmol) did not show significant
changes on mechanical withdrawal threshold in the shams (1 nmol not shown). SNI of the sciatic
nerve resulted in a significant decrease in mechanical withdrawal threshold (11 ± 7 g , P< 0.05,
n=8) in the ipsilateral paw of rats 14 days after surgery (Fig. 8A and B), while there were no
significant changes on the contralateral side (not shown). The intra-DS microinjection of (S)-3,4-
DCPG (1 and 2 nmol) induced a significant increased in the mechanical withdrawal threshold (26.2
± 0.8 g, P< 0.05, n=10 and 24.7 ± 2.0 g , P< 0.05, n=10 vs 12±0.7g and 28 ± 0.6, P< 0.05, n=10; 25
± 1.46 g, P< 0.05, n=8 vs 11±7 g, respectively) (Fig. 8A). M-SOP (100 nmol) was microinjected in combination with (S)-3,4-DCPG (2 nmol) antagonized the effects of this latter on mechanical withdrawal latency in the SNI rats (Fig. 8B). M-SOP alone did not change the mechanical withdrawal threshold in the SNI rats (not shown). No overt behavioural changes were observed in this study following intra-DS administration of the drugs used in freely moving un-anesthetized rats. Rats remained alert and generally active throughout the experiment.

Rota Rod

In the rotarod test, neither vehicle (228 ± 15 s) or (S)-3,4-DCPG (1 nmol) (238±10 s) affected motor performance compared to pre-treatment latency (240 ± 12 and 233 ± 10 s, respectively) when administered in DS in sham rats.

Expression and localization of mGluR8 receptor

The semiquantitative analysis of mRNA levels in the contralateral DS of SNI rats, measured by RT-PCR amplification, showed a significant decrease of the mGluR8 gene expression (1.9 ± 0.21, HPRT; 0.65 ± 0.20, 18S and 0.60 ± 0.22, GAPDH; mean ± SEM of arbitrary units, P<0.05, n=5) compared to the contralateral DS of the shams (3.00 ± 0.30, HPRT; 1.30 ± 0.11, 18S and 1.25 ± 0.10, GAPDH) (Fig. 9A and B). No changes of mGluR8 gene expression has been detected in the ipsilateral DS of SNI rats compared to ipsilateral DS of sham rats. Western blot analysis showed a significant increased of the mGluR8 protein level in contralateral Ds of SNI rats (0.51 ± 0.05 HPRT; 0.55 ± 0.03 18S; 1.12 ± 0.07 GAPDH) compared to the contralateral DS of sham rats (0.15 ± 0.03 HPRT; 0.15 ± 0.02 18S; 0.28 ± 0.05 GAPDH (Fig. 9 C and D). Moreover, we observed a significant increase of the mGluR8 protein level in the contralateral DS (0.51 ± 0.05 HPRT; 0.55 ± 0.03 18S; 1.12 ± 0.07 GAPDH) compared to ipsilateral DS in the SNI rats (0.14 ± 0.04, HPRT; 0.18 ± 0.04, 18S and 0.40 ± 0.04, GAPDH). A significant
decrease of the mGluR<sub>8</sub> protein level was also found in the ipsilateral DS of the SNI rat vs the ipsilateral DS of the sham rats (0.31±0.03 (HPRT); 0.33±0.02 (18S); 0.56±0.04, (GAPDH).

Finally, immunofluorescence showed the mGluR<sub>8</sub> localization in the DS on the VGAT expressing neurons, rather than VGLUT1. The localization mGluR<sub>8</sub> positive profile was counterstained with the presynaptic marker synaptophysin (Fig. 10A). Moreover, a double staining mGluR<sub>8</sub>/parvalbumin did not reveal significant expression of mGluR<sub>8</sub> on parvalbumin positive GABAergic interneurons (Fig. 10B).

Discussion

The first finding of the study was that intra-DS mGluR<sub>8</sub> stimulation by (S)-3,4-DCPG, a selective mGluR<sub>8</sub> agonist (Thomas et al. 2001), increased thermal threshold and the mechanical withdrawal threshold in the SNI rats. Intra-DS (S)-3,4-DCPG failed instead to change pain responses in sham rats.

The mGluR<sub>8</sub> stimulation was already evaluated in the VL PAG and CeA, where it reduced pain responses in normal and chronic pain conditions (Marabese et al. 2007a; 2007b; Palazzo et al., 2008; Palazzo et al., 2011) and proved to be associated with a GABA decrease and glutamate increase (Marabese et al. 2005; 2007a; Palazzo et al., 2011). Within the nucleus tractus solitarius mGluR<sub>8</sub> stimulation produced instead a pain facilitatory effect on cardiac nociception (Liu et al. 2012). These different pain responses may be related to the mGluR<sub>8</sub> location on different neuron terminals within supraspinal sites modulating differently pain perception. mGluR<sub>8</sub> proved to be expressed on presynaptic sites of asymmetrical and symmetrical synapses within the PAG (Marabese et al. 2005). Since GABAergic neural population constitutes ~ 50% of total neural elements (the majority are active tonic interneurons) of the PAG controlling its intrinsic antinociceptive activity (Reynolds, 1969; Reichling and Basbaum, 1991) mGluR<sub>8</sub> stimulation would induce an inhibition of GABAergic tone and consequent PAG disinhibition, which is consistent with the antinociceptive effect (Marabese et al. 2005; Marabese et al. 2007a; 2007b).
Within the CeA mGluRs proved to be expressed on GABAergic terminals and its stimulation reduced pain responses and GABA release (together with glutamate and serotonin increase) (Palazzo et al. 2008; 2011). In the NTS mGluRs stimulation activated the descending facilitatory pathway and enhanced the nociceptive transmission at the spinal cord level (Liu et al. 2012). Beside pain responses in the current study we have also investigated whether mGluRs receptor stimulation within the DS was able to affect the activity of the ON and OFF cells of RVM. Among the several forebrain or brainstem nuclei, the RVM is also under the influence of the DS. There are not direct projections from DS to the RVM. The convergence point among DS and the RVM seems to lie into the medullary dorsal reticular nucleus (DRt). DRt receives projections from globus pallidum and substantia nigra pars reticulata (which in turn receive projections from the DS) and sends inputs to the RVM (Leite-Almeida et al. 2006), as illustrated in the Fig. 11. Thus the DRt is strategically positioned among the extrapiramidal and pain descending systems indicating a possible involvement in the motor reaction to pain. It has been also recently reported that RVM mediates the analgesic action of the striatum: lesion of RVM blocked the inhibition control of striatum on orofacial pain (Barcelo et al. 2012). Evidence that stimulation of striatum leads to pain reflex inhibition (Belforte et al. 2001) through RVM has been however only scarcely investigated so far (Barcelo et al. 2012). Within the RVM, ON cells are activated by nociceptive stimuli and have a pain facilitatory effect (Fields et al. 1983), whereas OFF cells are inactivated and have an inhibitory effect on nociception (Fields et al., 1983, 2006; Heinricher and Tortorici, 1994; Neubert et al., 2004; Kincaid et al., 2006). We found in this study, as already show in others (Pertovaara 2000; Porreca et al. 2001; Porreca et al. 2002; Ossipov and Porreca 2006; Goncalves et al. 2007; Palazzo et al. 2011; Palazzo et al. 2012) that RVM cell activity changed in neuropathic pain conditions: the ongoing and tail flick evoked activity of the ON cells increased, whereas the ongoing activity of OFF cells decreased and the pause and onset of the pause of the OFF cells increased and decreased, respectively. Thus, it appears that ON and OFF cell activity in the RVM undergoes functional phenotypic changes after SNI which leads to ON cell hyperactivity and OFF cell hypoactivity. The
microinjection of (S)-3,4-DCPG into the DS increased the ongoing activity of the OFF cells and inhibited that one of the ON cells in rats with SNI. (S)-3,4-DCPG also reduced tail flick-induced ON cell burst and OFF cell pause and increased the onset of both ON cell burst and OFF cell pause in the same animals. A delayed onset of the ON cell burst, as well as the reduction of the burst of firing, proved to be critical events in the occurrence of antinociception (Heinricher et al. 1989; Foo and Mason, 2003; Jinks et al., 2004). Moreover, an inhibition of pain transmission correlates with an increase of spontaneous OFF cell activity and a reduction of its pause as well as a delay of the pause onset (Heinricher and Tortorici 1994). Thus, interestingly, mGlur8 stimulation into the DS modified the ongoing and tail flick-evoked activity of RVM ON and OFF cells, consistently with the antinociceptive effect produced by DS mGlur8 stimulation in the tail flick test and in mechanical allodynia.

(S)-3,4-DCPG has already shown to inhibit RVM ON cell and to enhance OFF cell activity consistently with mGlur8-induced antinociception in the SNI rats when locally microinjected into the VL PAG or CeA (Marabese et al. 2007b; Palazzo et al. 2011).

A critical finding of this study was however that S-3,4-DCPG into the DS was devoid of effect in sham rats. Indeed, the intra-DS administration of (S)-3,4-DCPG did not change thermal nociception, the mechanical withdrawal thresholds and the ongoing and tail flick-evoked ON and OFF cell activity in sham animals. Evidence that group III mGluR stimulation inhibits pain in different pathological pain states of various etiologies but not in normal conditions have been already reported (Chen and Pan, 2005; Goudet et al. 2008; Neugebauer, 2006; Palazzo et al., 2008; 2011; 2013; Zhang et al 2009). In particular, we have found that intra-CeA (S)-3,4-DCPG failed to change pain and affective behaviour, neurotransmitter release and RVM cell activity in physiological conditions (Palazzo et al. 2008; 2011). Accordingly, (S)-3,4-DCPG did not affect baseline synaptic transmission in hippocampal slices (Ayala et al. 2008) and failed to modify anxiety-like behavior when administered in the basolateral amygdala (Stachowicz et al. 2005). Therefore the analgesic effect of mGlur8 activation requires some neuroplasticity generated by...
pathological conditions, including chronic pain, which may change mGluR8 sensitivity. Indeed, in our previous study the expression of mGluR8 proved to be upregulated in the CeA after peripheral carrageenan administration (Palazzo et al. 2011). Apart from a mGluR8 change of expression other factors have to be taken into considerations to justify why mGluR8 stimulation reduces pain responses and RVM cell activity only in neuropathic conditions: i) the control of glutamate level, played by mGluR8, which is critical for maintaining normal sensory transmission, may be increased in pathological conditions associated with glutamatergic overactivity (Dougherty et al., 1992; Leem et al., 1996; Tolle et al., 1996; Santangelo et al., 2012); ii) the neuron terminals expressing mGluR8 may be normally inactive and be activated by nerve injury and iii) the downstream effectors associated with mGluR8 may be over-expressed in a way that even if a decrease of receptor expression is detected, an improved regulation of mGluR8 signal can result. The analysis of mGluR8 expression carried out in this study, showed that mGluR8 mRNA level was down-regulated in the DS of SNI rats, in contralateral, but not in the ipsilateral DS. Intriguingly, the protein levels showed an opposite trend in the contralateral DS of SNI. Indeed, while mGluR8 protein levels proved increased in the contralateral side, a reduction was observed in the ipsilateral DS, as also evidenced by immunofluorescence outcomes. These findings could explain, at least in part, why the (S)-3,4-DCPG exerts anti-nociceptive effect, also in term of the RVM ON and OFF cell activity in SNI but not sham animals. However, is still critical to explain the different mGluR8 mRNA and protein changes in neuropathic conditions which deserves further investigations.

When MSOP, a group III mGluRs antagonist, was administered alone did not changed mechanical allodynia, notwithstanding mGluR8 expression changes in SNI animals. Since MSOP blocks mGluR4, mGluR7 and mGluR8 when locally administered into the DS (a mGluR8 antagonist is not available) it can be suggested that the co-recruitment of the other mGluR subtypes such as mGluR7 (which plays an opposite functional action on nociception with respect to mGluR8, Marabese et al., 2007a,b; Palazzo et al., 2008; Palazzo et al., 2013) may lead to an annulment of the final effect.
Immunochemistry evidenced a preferential GABAergic expression of the mGluR8 in the DS and it is consistent to previous studies in the VL PAG and CeA (Marabese et al. 2005, Palazzo et al. 2011). In the DS mGluR8 expression on GABAergic terminals, as confirmed by counterstaining with the presynaptic marker synaptophysin, appears relevant since GABAergic population constitutes up to 90% of total neuron population. The lack of a double staining mGluR8/parvalbumin suggest that mGluR8 is not expressed on GABAergic interneurons. Thus, the mGluR8 stimulation leads to an GABAergic inhibition and consequent DS dis-inhibition, associated with analgesia (Belforte et al. 2001). Moreover, the DS is among the neural structures with highest concentrations of endogenous opiates and their receptors (Angulo and McEwen 1994; Hebert et al. 1990; McGeer and McGeer 1993), which could be crucial in the analgesia induction (Thorn-Gray and Levitt 1983; Kurumaji et al. 1988; Hebert et al. 1990). Another issue of the current study concerns the (S)-3,4-DCPG selectivity towards mGluR8. Indeed, this compound shows a dose-response curve overlapping on mGluR8 and mGluR4 (Thomas et al. 2001). Since a selective mGluR8 antagonist is not available, we performed further experiments by exploiting the selectivity of LY341495, a group II mGluR antagonist which shows a higher selectivity on mGluR8 over mGluR4 (K_i/IC50 = 173 over 22000, respectively). LY341495 antagonized the effect of (S)-3,4-DCPG on both thermal threshold increase and on RVM ON and OFF cell activity changes. Moreover, we carried out experiments with AZ12216052 and VU0155041, two selective mGluR8 and mGluR4 PAMs, respectively. While AZ12216052 was able to increase the tail flick latency and to decrease and increase the ON and OFF cell activity in SNI rats, as the (S)-3,4-DCPG did, the VU0155041 was not. These further experiments altogether underline the role of mGluR8 in modulating pain responses and RVM cell activity at the DS level. By contrast the mGluR4 does not seem be involved.

In conclusion mGluR8 stimulation into the DS of SNI rats: i) inhibited thermoceptive responses in the tail flick test in anaesthetized SNI rats, ii) changed RVM cell activity accordingly to behavioural analgesia and iii) reduced mechanical allodynia in a way that was antagonized by M-SOP, a group
III mGluR antagonist. mGluR8 stimulation did not affect motor coordination in the rota rod experiments, RVM activity and pain responses in sham rats. The effects of (S)-3,4-DCPG stimulation only in neuropathic pain condition, associated to mGluR8 gene decrease and protein increase, deserves further studies.

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Legends

**Figure 1** Schematic illustration of the location of dorsal striatum (DS) microinjection sites (A) and rostral ventromedial medulla RVM ON or OFF cell recording sites (B). Vehicle or drug microinjections were performed in the DS (filled circles). Cell recordings were performed by lowering a tungsten electrode into the RVM. ON cells (filled triangles) or OFF cells (open triangles) recording sites are shown in B. Distances (in mm) from the interaural line are indicated.

**Figure 2** Effect of intra-DS microinjections of vehicle (ACSF) or (S)-3,4-DCPG (1 and 2 nmol (A) or vehicle, (S)-3,4-DCPG (2 nmol) alone or in combination with LY341495 (10 μmol) (B) and ACSF or AZ12216052 (20 nmol; C) on tail flick latencies in sham and SNI rats 14 days after SNI. Vehicle or drugs were administered at the time 0. Each point represents the mean ± S.E.M of 10 rats per group. * indicates statistically significant difference versus sham/vehicle, º versus SNI/vehicle and # versus SNI/DCPG (2 nmol). P values < 0.05 were considered statistically significant.

**Figure 3.** Effects of intra-DS microinjections of vehicle (ACSF) or (S)-3,4-DCPG (1 and 2 nmol) (A) or vehicle, LY341495 (10 μmol), (S)-3,4-DCPG (2 nmol) alone or in combination with LY341495 (10 μmol) on the spontaneous firing of RVM ON (A and C) or OFF cells (B and D) in sham and SNI rats after 14 days after sciatic nerve surgery. Vehicle or drugs were administered at the time 0. Each point represents the mean ± S.E.M of 5-8 neurons. * indicates statistically significant difference versus sham/vehicle, º versus SNI/vehicle and # versus SNI/DCPG (2 nmol). P values < 0.05 were considered statistically significant.
Figure 4. Effects of intra-DS microinjections of vehicle or AZ2216052 (AZ, 20 nmol) (A and B) and vehicle or VU01550412 (VU, 40 nmol) (C and D) on the spontaneous firing of RVM ON or OFF cells in sham and SNI rats after 14 days after sciatic nerve surgery. Vehicle or drugs were administered at the time 0. Each point represents the mean ± S.E.M of 5 neurons. * indicates statistically significant difference versus sham/vehicle and º versus SNI/vehicle. P values < 0.05 were considered statistically significant.

Figure 5. Effects of intra-DS microinjections of vehicle or (S)-3,4-DCPG (1 and 2 nmol) on tail flick-evoked ON cell burst of firing (A) and onset of the burst (C) or tail flick-evoked OFF cell pause (B) and onset of the pause (D) in sham and SNI rats after 14 days after sciatic nerve surgery. Vehicle or drugs were administered at the time 0. Each point represents the mean ± S.E.M of 5-10 neurons per group. * indicates statistically significant difference versus sham/vehicle, º versus SNI/vehicle and # versus SNI/DCPG (2 nmol). P values < 0.05 were considered statistically significant.

Figure 6. Effects of intra-DS microinjections of vehicle or AZ2216052 (AZ, 20 nmol) on tail flick-evoked ON cell burst of firing (A and C) and OFF cell pause (B and D) in sham and SNI rats after 14 days after sciatic nerve surgery. Vehicle or drugs were administered at the time 0. Each point represents the mean ± S.E.M of 10 neurons per group. * indicates statistically significant difference versus sham/vehicle and º versus SNI/vehicle. P values < 0.05 were considered statistically significant.

Figure 7. Examples of ratemeter records which illustrate the effect of intra-DS microinjection of (S)-3,4-DCPG (1 and 2 nmol) on either the ongoing or tail flick-related burst of activity of identified RVM ON cells (A, C and E) and ongoing or tail flick-related pause of identified RVM OFF cells (B, D and F) in sham (A and B) and SNI (C, D, E and F) rats. Intra-DS microinjection of
S)-3,4-DCPG (2 nmol in sham and 1 and 2 nmol in SNI) reduced the ongoing activity and tail flick-related burst of the ON cells in SNI rats (C and E) compared to sham rats in which no significant change was observed (A). The same treatment increased the ongoing activity and reduced the tail flick-related pause of the OFF cells in SNI rats (D and F) compared to sham rats in which no significantly change was observed (B). Scales bars indicate 5 min for ratermater records. Small triangles indicate the tail flick stimulation. A time expanded scale illustrates pause duration changes: the grey arrows show the noxious stimulus application and the black one the tail flick reflex (scale bar = 5 sec)( B, D and F).

**Figure 8.** Effects of intra-DS microinjections of vehicle and (S)-3,4-DCPG (1 and 2 nmol) alone or (S)-3,4-DCPG (2 nmol) in combination with M-SOP (100 nmol) on mechanical withdrawal threshold (PWT) in sham (A) and SNI rats (B). The grey arrows show the vehicle or (S)-3,4-DCPG microinjection. Each point represents the mean ± S.E.M of 10 animals per group. (*) Indicates significant differences versus the sham/veh, (°) versus SNI/veh and (#) versus SNI/(S)-3,4-DCPG 2 nmol. P values <0.05 were considered statistically significant.

**Figure 9.** mGluR₈ mRNA and protein levels in the DS of sham and SNI rats. (A) and (B) show the mGluR₈ mRNA levels in the contralateral and ipsilateral DS in sham and SNI rats 14 days after surgery. Data represent mean ± SEM of 5 rats per group, normalized with respect to HPRT, 18S and GAPDH chosen as housekeeping genes. (C) and (D show the mGluR₈ protein levels in the contralateral and ipsilateral DS in sham and SNI rats 14 days after surgery. Data represent mean ± SEM of 5 rats per group, normalized with respect to β–actin, β–tubulin and GAPDH chosen as housekeeping proteins.

* indicates statistically significant differences vs sham contra DS, ° indicates statistically significant differences vs sham and SNI ipsi DS, # indicates statistically significant differences vs sham ipsi DS. P< 0.05 has been considered as value of significance.
(E) shows the immunostaining for the mGluR₈ in the DS of sham and SNI rats. mGluR₈ signal is reduced in DS of SNI rat. Double staining indicates the preferential counterstaining of mGluR₈ with vesicular GABA transporter (VGAT), rather than vesicular glutamate transporter (VGLUT).

**Figure 10.** Co-localization of mGluR8 (green) and the pre-synaptic marker synaptophysin (red). The panel on the left shows counterstaining of the mGluR8 positive profiles and synaptophysin, although several synaptophysin positive profiles were negative to mGluR8. The panel on the right shows that mGluR8 positive profiles do not colocalize with parvalbumin positive GABAergic interneurons (red). Some mGluR8 counterstained with the parvalbumin positive profiles (not shown in the pic). Scale bars= 100-50 µm

**Figure 11.** A simplified scheme illustrating the DS connections to the RVM via dorsal reticular nuclei area. Dorsal reticular nuclei receives projections from globus pallidus and substantia nigra pars reticulata (SNr) which in turn receive projection from DS. The effect of mGluR8 stimulation on RVM ON and OFF cell (which is part of the descending pain pathway which includes PAG) is far from being direct.
Cerebral Cortex → PAG → Globus Pallidus → SNr → Thalamus

DS → mGluR₈

Dorsal Reticular Nuclei

RVM
ON and OFF cells

Dorsal Horn