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Pain Inhibits Pain: an Ascending-Descending Pain Modulation Pathway Linking Mesolimbic and Classical Descending Mechanisms

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Abstract

The ability to modulate pain perception is as critical to survival as pain itself. The most known pain modulation pathway is the PAG–RVM (periaqueductal gray–rostral ventromedial medulla) descending system. In this study, we hypothesized that it is functionally linked to the ascending nociceptive control, which is a form of pain-induced analgesia dependent on mesolimbic mechanisms. To test this hypothesis, we used a pharmacological approach, in which the antinociception induced by noxious stimulation (forepaw injection of capsaicin) was detected in a standard rat model of inflammatory pain (hindpaw injection of carrageenan). This antinociception was blocked by interventions known to block the ascending nociceptive control-mediated analgesia: the blockade of μ-opioid (Cys^2,Tyr^3,Orn^5,Pen^7amide (CTOP) 0.5 μg) or of dopamine (SCH23390 1.8 μg and raclopride 5 μg) receptors within the NAc (nucleus accumbens) and that of cholinergic nicotinic receptors (mecamylamine 0.6 μg) within the RVM. The antinociception was also blocked by standard interventions known to block mechanisms of descending inhibition within either the PAG or the RVM: local acute neuronal blockade (lidocaine 2%), blockade of μ-opioid receptors (CTOP 0.5 μg), or activation of $GABA_A$ receptors (muscimol 10 ng). Consistently, interventions that are known to block spinal mechanisms of descending inhibition also blocked antinociception: lesion of dorsolateral funiculus and the spinal blockade of serotonergic (WAY100135 46 μg or tropisetron 10 μg) or adrenergic (idazoxan, 50 μg) receptors. Neuronal activity indirectly estimated by c-Fos expression within the NAc, PAG, and RVM supports behavioral observations. Therefore, this study provides functional data indicating that noxious stimulation triggers an ascending–descending pain modulation pathway linking the mesolimbic system to the PAG–RVM descending system.

Keywords Pain modulation · Ascending nociceptive control · Periaqueductal gray-rostral ventromedial medulla descending system · Mesolimbic system · Nucleus accumbens · Rat

Introduction

Acute pain is essential for life; it signals potential danger and draws attention, requiring the individual to engage in recuperative behaviors to avoid further injury. Precisely because of this, the ability to suppress pain perception is essential to survival as well. For example, during life-threatening situations, such as prey–predator confrontations, pain perception should be suppressed in order to allow a threatened animal to engage in defensive responses instead of recuperative activities [1]. In order to achieve this goal, conserved neurobiological mechanisms of pain modulation are recruited leaving the individual free to express the most advantageous behavior. Understanding how the brain suppresses pain perception may contribute not only to understand the essence of adaptive behavior but also to develop drugs to control one of the greatest epidemics of mankind: chronic pain, a pathological kind of pain devoid of biological purpose. In fact, several drugs already in use for pain management activate pain modulation mechanisms to induce analgesia [2]. However, we are still far from efficiently treating persistent pain, and it still afflicts around 19% of the general population with an alarming socio-economic burden [3, 4].

Ever since Melzack and Wall published their remarkable gate control theory of pain [5], we have seen great advances in our knowledge about pain modulation mechanisms. In the 1970s and 1980s, a series of studies advanced our knowledge

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about the most well-known and probably powerful circuitry engaged in endogenous pain modulation: the periaqueductal gray–rostral ventromedial medulla (PAG–RVM) descending system (reviewed by [6, 7]). In this system, inputs from multiple forebrain regions are integrated within the periaqueductal gray (PAG), which projects to the rostral ventromedial medulla (RVM), from where descending pathways target the dorsal horn to control nociceptive transmission [6, 7]. More recently, from the 1990s, an ascending pain modulation pathway triggered by noxious stimuli has been described [8–10]. Named ascending nociceptive control [8], it mediates a form of pain-induced analgesia, in which potent and long-lasting antinociception is induced by opioid- and dopamine-dependent mechanisms within the nucleus accumbens (NAc), the target of the mesolimbic system in the ventral striatum.

Previous attempts have failed to link the ascending nociceptive control with the PAG–RVM descending system [8, 9]. In fact, to our knowledge there is no evidence linking either mesolimbic mechanisms of analgesia with the PAG–RVM descending system or the PAG–RVM descending system with pain-induced analgesia. However, from a perspective of adaptive mechanisms for behavior selection, it would be greatly advantageous if the ascending and descending systems were two components of the same neurobiological mechanism to suppress pain perception during noxious stimulation. Through the ascending component, the nociceptive information reaches the mesolimbic system, a key region for behavior selection [11]. Through the descending component, nociceptive transmission is suppressed at its entry, the spinal dorsal horn [6, 7]. Therefore, the aim of this study is to provide functional support to the hypothesis that noxious stimulation triggers an ascending-descending pain modulation pathway linking the mesolimbic system to the PAG–RVM descending system.

Material and Methods

Animals

The experiments were performed in 353 male Wistar rats (270–300 g), housed five per cages with free access to food and water. They were maintained in a room with controlled 12:12-h light/dark cycle and temperature (± 23 °C). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the Federal University of Parana (protocol no. 782) and followed the guidelines of the Ethics Standards of the International Association for the Study of Pain in animals. Each animal was subjected to a single trial.

Drugs

- Capsaicin (250 μg), an agonist of the transient receptor potential vanilloid 1 (TRPV1) [8]
- Carrageenan-λ (20 or 100 μg) [12], an inflammatory agent
- Morphine (5 mg/kg), an opioid receptor agonist [13]
- [D-Ala2,*N*-Me-Phe4,Gly5-ol]-enkephalin (DAMGO, 0.3 μg), a μ-opioid receptor agonist [14]
- Cys^2,Tyr^3,Orn^5,Pen^7amide (CTOP, 0.5 μg), a μ-opioid receptor antagonist [15]
- Mecamylamine (0.6 μg), a nicotinic acetylcholine receptor antagonist [10]
- Lidocaine *N*-ethyl bromide salt (lidocaine 2%), a quaternary derivative of lidocaine [16]
- Muscimol (10 ng), a $GABA_A$ receptor agonist [8]
- WAY100135 (46 μg), a selective serotonin 5-HT_{1A} receptor antagonist [17]
- Tropisetron (10 μg), a selective serotonin 5-HT_3 receptor antagonist [18]
- Idazoxan (50 μg), a selective α2-adrenoceptor antagonist [19]
- Raclopride (1 or 10 μg for intrathecal injection; or 5 μg for intra-NAc injection), a selective dopamine-D2 receptor antagonist [7, 20]
- SCH23390 (SCH, 1.8 μg), a selective dopamine-D1 receptor antagonist [21]

All drugs were dissolved in 0.9% NaCl except capsaicin that was initially dissolved in Tween-80 (50%) and ethanol (50%) to a concentration of 50 μg/μL and then diluted in 0.9% saline. All drugs were obtained from Sigma-Aldrich (St. Louis, MO) except WAY100135 obtained from Tocris Bioscience (Bristol, UK).

Pre-Experimental Procedures

Surgical procedures described below were performed in anesthetized rats (xylazine 10 mg/kg, i.p., and ketamine 60 mg/kg, i.p.). Dipyrone (30 mg/kg i.m.) and enrofloxacin (0.5 mg/kg s.c.) were administered post-surgically. Rats exhibiting any signal of motor impairment in the open-field test were excluded from additional testing.

Stereotaxic Surgery

The rats were placed in a stereotaxic instrument, the skull was exposed, and a small hole was made to introduce a 26-gauge guide cannula within the NAc core, ventrolateral PAG (vlPAG), or RVM. For the NAc core, bilateral cannula were inserted according the following coordinates from the bregma: 1.3 mm rostral, 7.2 mm dorsoventral, and ± 1.8 mm lateral with incisor bar at 3.3 mm [15]. For the vlPAG, a key region for the descending system [6], the coordinates were from the lambda: 0 mm anteroposterior, − 2 mm lateral, and 5.4 mm dorsoventral, with a 1.8° angle and the incisor bar at 2.5 mm [22]. For the RVM, a central cannula was vertically inserted

from the skull according the coordinates from the interaural line: 2.3 mm caudal and 0.2 mm ventral [15]—this coordinates from bregma are 11.3 mm rostral, 10.2 mm dorsoventral, and 0.0 mm lateral, with incisor bar at 3.3 mm. The cannula was then fixed to the skull with a screw and dental cement. Experiments were performed 7–9 days later. The depth in the coordinates represents the tip of the injection cannula.

Lesion of the Dorsolateral Funiculus

Lesion of the dorsolateral funiculus (DLF) was performed as previously described [22], ipsilateral to the carrageenan and capsaicin injection. Briefly, a laminectomy was performed at the T1–T3 level to expose the spinal cord using a bone drill under a surgical microscope. The lesion was performed by cutting a portion of the dorsolateral quadrant of the spinal cord with a 26-gauge curved needle. After hemostasis, the wounds were closed and the animals were allowed to recover for 28 days before the experiment. Sham DLF was performed by exposing the vertebrae without cutting any neuronal tissue.

Lesion location and extension were histologically assessed in each animal after experiments. The rats were transcardially perfused with saline followed by 4% formaldehyde. The spinal cords were removed, at the level of the lesions, and histologically processed (50 μm sections stained with 1% neutral red) to allow the microscopic verification of lesion extension and location.

Behavioral Model to Study Ascending Nociceptive Control-Mediated Analgesia

The ascending nociceptive control has been studied in anesthetized [9] and awake [8] animals by using different experimental strategies, always characterized by applying the noxious stimulus (to induce analgesia) in a body region far from where the test stimulus (to detect analgesia) is applied. This ensures that nociceptive testing is performed segmentally remote from the site of noxious stimulation, eliminating intrasegmental effects that might influence assays. For example, the noxious stimulus, commonly a capsaicin injection, can be applied into the hindpaw and analgesia detected in the orofacial formalin test [15].

In the present study, the antinociceptive effect induced by a forepaw injection of capsaicin was evaluated in the carrageenan model [23] of inflammatory pain (see Fig. 1 for experimental design). With this purpose, carrageenan (100 μg) or its vehicle was injected in the left hindpaw. Three hours later, when carrageenan-induced hyperalgesia is maximal [12], capsaicin (250 μg) or its vehicle was injected in the ipsilateral forepaw to activate the ascending nociceptive control [8]. The ability of the forepaw injection of capsaicin to decrease carrageenan-induced hyperalgesia in the hindpaw was tested over time until the antinociceptive effect wore off. Whenever brain microinjections or lumbar intrathecal injections were necessary (to block endogenous pain modulation mechanisms), they were performed immediately before the forepaw injection of capsaicin. There were two investigators, one who performed the injections (into the brain, paws, and spinal cord) and other who performed the behavioral tests and was blinded to which group the rats belonged. Noteworthy, capsaicin and carrageenan induce edema and redness, two unavoidable signs that compromise the blind design of the experiment. Capsaicin also induces a behavioral response characterized by licking and shaking the injected forepaw. This response lasts no longer than 5 min, and the first evaluation of capsaicin-induced antinociception in carrageenan-induced hyperalgesia was performed 30 min after capsaicin injection.

Drugs Injections

Paw Injections

Following the protocol detailed above, animals were gently held to receive a hindpaw injection (30 μL) of carrageenan or its vehicle, followed 3 h later by a forepaw injection (30 μL) of capsaicin or its vehicle. The injections were performed into the dorsal surface of the paws using a 26-gauge needle connected to a PE-50 cannula and also to a 50-μL Hamilton syringe.

Brain Microinjections

Microinjections were performed by inserting a 30-gauge stainless steel injection cannula through the guide cannulas stereotaxically implanted. The injection cannula was connected to a PE-10 polyethylene tube and also to a 2-μL Hamilton syringe. Injection volume was 0.3 μL carried out over a period of 60 s, after which injection cannula was left in place for 30 more seconds to minimize backflow along the cannula tract. Injection sites were histologically verified in each animal after experiments. Under anesthesia, Evans blue dye (1%, 0.5 μL) was microinjected and the rats were transcardially perfused with saline followed by 4% formaldehyde. Coronal sections (50 μm) were performed to allow the microscopic verification of dye location and spread [24]. Only animals in which the dye was restricted to the selected nucleus were included in the figures and data analysis.

Intrathecal Drug Administration

Intrathecal drug administration was performed as previously described [12, 25]. Briefly, rats were anesthetized by inhalation of isoflurane, and a small skin area overlying the lumbar region was shaved with an electric razor. For injection, rats were positioned in dorsal recumbency with the iliac region resting on a falcon tube. A 26-gauge needle

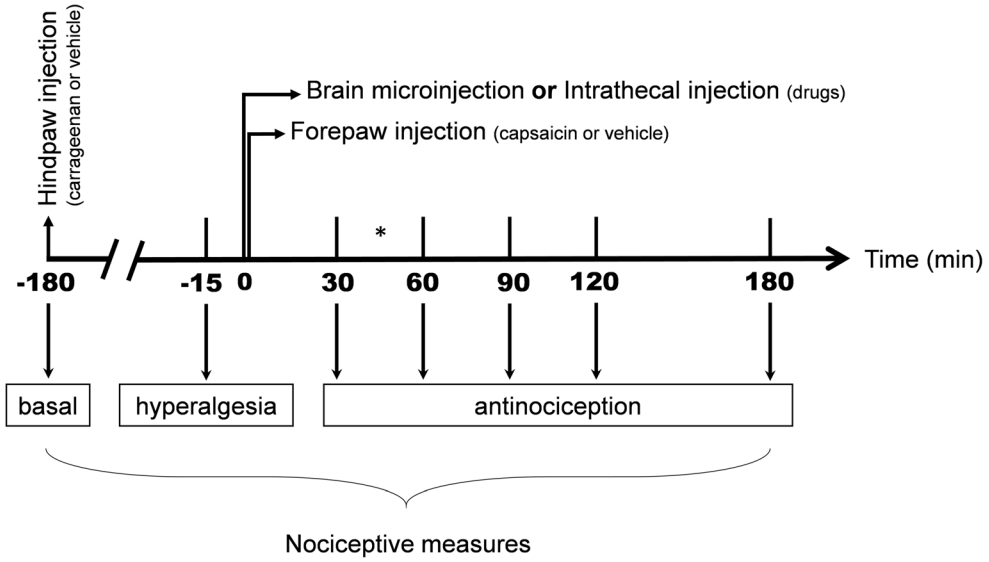


Fig. 1 Experimental design. The total duration of the experiments was 360 min (6 h). Negative values in the experimental timeline represent experimental interventions performed before forepaw injection. Positive values represent interventions performed after forepaw injection. Down arrows along the timelines indicate the sequence of nociceptive measurements. Up arrows indicate injection procedures. Asterisk indicates when the open field test was performed. The boxes at the bottom describe the expected effect of key interventions: carrageenan-induced hyperalgesia and capsaicin-induced antinociception

was inserted in the subarachnoid space on the midline between L5 and L6 vertebrae, and a flick of the tail was used as indicator of the precise positioning. The injections were performed at a volume of 5 μL (1 μL/s). The animals regained consciousness approximately 1 min after discontinuing the anesthetic.

Behavioral Testing

Behavioral testing was performed during the light phase (between 9:00 a.m. and 5:00 p.m.), in a quiet room maintained at 23 °C, and each animal was used once. The animals were habituated to the experimental conditions.

Mechanical Paw Withdrawal Test

The mechanical nociceptive threshold was assessed by the Randall–Selitto test [26] (Randall–Selitto apparatus, Insight, Ribeirão Preto, Brazil), and its decrease was used as a measure of hyperalgesia (nociception), while its increase as a measure of analgesia (antinociception). In this test, an increasing pressure (weight in grams) is applied to the dorsal surface of the rat's hindpaw until the animal withdrew its paw. The value in grams obtained from the mean of three readings (performed in intervals of 2 min) represents the mechanical nociceptive threshold. A cutoff pressure of 240 g was set to avoid tissue damage. Data were expressed in figures as variation (after–before) from the basal (pre-experiment) value over time following interventions.

Open-Field Test

The open-field test was used to provide an overall indication of locomotor activity, discarding the possibility that microinjections or surgery procedures affected animals' motor behavior and their response to the nociceptive tests. The open-field arena consists of a circular area (90 cm of diameter), divided into 12 squares, limited by a 50-cm-high wall. The exploratory behavior was quantified by the number of crossed squares during 1 min [16]. The test was performed between 30 and 60 min of the experimental time-course (see Fig. 1).

c-Fos Immunohistochemistry

The rats were transcardially perfused, under general anesthesia, with 0.9% NaCl followed by 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and subsequently immersed for 1 week in formaldehyde at 4%. The brains were then placed in 30% sucrose solution for 48 h before sectioning. Six sections of 30 μm per animal were taken between the bregma 1.44- and 1.20-mm coordinates to NAc, between bregma − 8.04- and − 8.28-mm coordinates to vlPAG, and between interaural − 2.16 and − 2.40-mm coordinates to RVM [24]. Tissue sections were incubated overnight at 4 °C with rabbit anti-c-Fos primary antibody (1:500 in phosphate buffer saline (PBS) plus 0.3% Triton X-100; Chemicon, USA). Sections were then incubated with a biotin-conjugated secondary antibody (1:500, Vector Laboratories, USA) for 2 h at room temperature. After several washes with PBS, the antibody complex was localized using the ABC system (Vectastain ABC Elite kit, Vector

Laboratories) followed by reaction with 3,3′-diaminobenzidine with nickel enhancement. The sections were then mounted onto gelatin-coated slides and coverslipped after dehydration by ascending concentrations of ethanol–xylene solutions [15]. The slides were digitized with a microscope scanner (Axio Imager Z2, Carl Zeiss, Jena, DE) coupled to an imaging system (Metasystems, Altlussheim, DE). Quantification of c-Fos immunoreactive (c-Fos-ir) cells was performed automatically by optical density in an area delimitated for NAc (×5 magnification), PAG (×2 magnification), and RVM (×5 magnification) using ImageJ 1.37c (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA—Public Domain) image analysis software.

Statistical Analysis

Data from mechanical nociceptive threshold were analyzed by repeated-measures analysis of variance (ANOVA) with one within-subjects factor (time) and one between-subjects factor (treatment). Data from c-Fos expression were analyzed by ANOVA with two (hindpaw and forepaw injections) between-subjects factors. Data from locomotion in the open field were analyzed by one-way ANOVA. If there were significant differences between groups, post hoc contrasts, using the Student–Newman–Keuls test, were performed to determine the basis of the significant difference in each case. The level for statistical significance was $p < 0.05$. Data are plotted in figures as mean ± S.E.M. SigmaPlot® software (Systat Software, San Jose, CA, USA) was used to perform data analysis and graphical representation.

Results

Activation of Ascending Nociceptive Control Blocks Carrageenan-Induced Inflammatory Hyperalgesia

The subcutaneous administration of carrageenan into the hindpaw significantly decreased the mechanical nociceptive threshold, which is referred as hyperalgesia (Fig. 2a, $p = 0.003$, shown in figures as the decrease in paw withdrawal threshold from the basal (pre-injection) value). The effect of noxious stimulation on carrageenan-induced hyperalgesia was tested by injecting capsaicin, at a dose known to activate the ascending nociceptive control, into the forepaw. Capsaicin not only reversed the hyperalgesia induced by carrageenan ($p < 0.001$) but also induced a potent antinociceptive effect, which can also be seen in animals receiving vehicle (0.9% NaCl, $p = 0.02$) instead of carrageenan into the hindpaw (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 2a: $F_{treatment}(3,20) = 17.397$, $p < 0.001$; $F_{treatment \times time}(12,80) = 4.463$, $p < 0.001$). The effect of capsaicin in carrageenan-induced hyperalgesia was similar in magnitude and time-course to that induced by a standard systemic dose of morphine (5 mg/kg, repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S1: $F_{treatment}(2,16) = 16.370$, $p < 0.001$; $F_{treatment \times time}(8,64) = 7.350$, $p < 0.001$).

In order to make sure that the antinociceptive effect of noxious stimulation with capsaicin depends on the ascending nociceptive control activation, we blocked its mechanisms within the NAc and the RVM. The administration of a μ-opioid receptor antagonist (CTOP) into the NAc, at a dose known to block the ascending nociceptive control [21], prevented ($p = 0.002$) the antinociceptive effect induced by capsaicin (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 2b: $F_{treatment}(2,18) = 13.715$, $p < 0.001$; $F_{treatment \times time}(8,66) = 14.728$, $p < 0.001$). Similarly, the co-administration of a dopamine D1 (SCH23390) and D2 (raclopride) receptor antagonists into the NAc prevented ($p = 0.013$) capsaicin-induced antinociception, while each antagonist alone had an intermediate effect (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 2c: $F_{treatment}(4,31) = 4.753$, $p < 0.004$; $F_{treatment \times time}(16,119) = 6.505$, $p < 0.001$).

Although the standard dose of carrageenan (100 μg) induces an intense hyperalgesic response, optimal to evaluate the efficacy of antinociceptive strategies in inflammatory pain, the decrease in paw withdrawal threshold is close to the test limit. For this reason, the effect of each drug by itself on carrageenan-induced hyperalgesia was evaluated using a lower dose of carrageenan (20 μg). This dose induces an intermediate decrease in paw withdrawal threshold, optimal to observe any tendency of drugs to further decrease (or increase) mechanical nociceptive threshold (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 A: $F_{treatment}(2,18) = 11.219$, $p < 0.001$; $F_{treatment \times time}(8,72) = 1.025$, $p = 0.426$). By themselves, neither CTOP nor the combination of raclopride and SCH23390 affected carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 B: $F_{treatment}(2,10) = 0.679$, $p = 0.529$; $F_{treatment \times time}(8,39) = 1.091$, $p = 0.390$).

The administration of a nicotinic acetylcholine receptor antagonist (mecamylamine) into the RVM, at a dose known to block the ascending nociceptive control [10], prevented ($p = 0.003$) capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 2d: $F_{treatment}(2,18) = 8.784$, $p = 0.002$; $F_{treatment \times time}(8,68) = 12.956$, $p < 0.001$). By itself, the administration of mecamylamine into the RVM, did not change carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 D: $F_{treatment}(4,15) = 0.067$, $p = 0.991$; $F_{treatment \times time}(12,45) = 1.069$, $p = 0.407$).

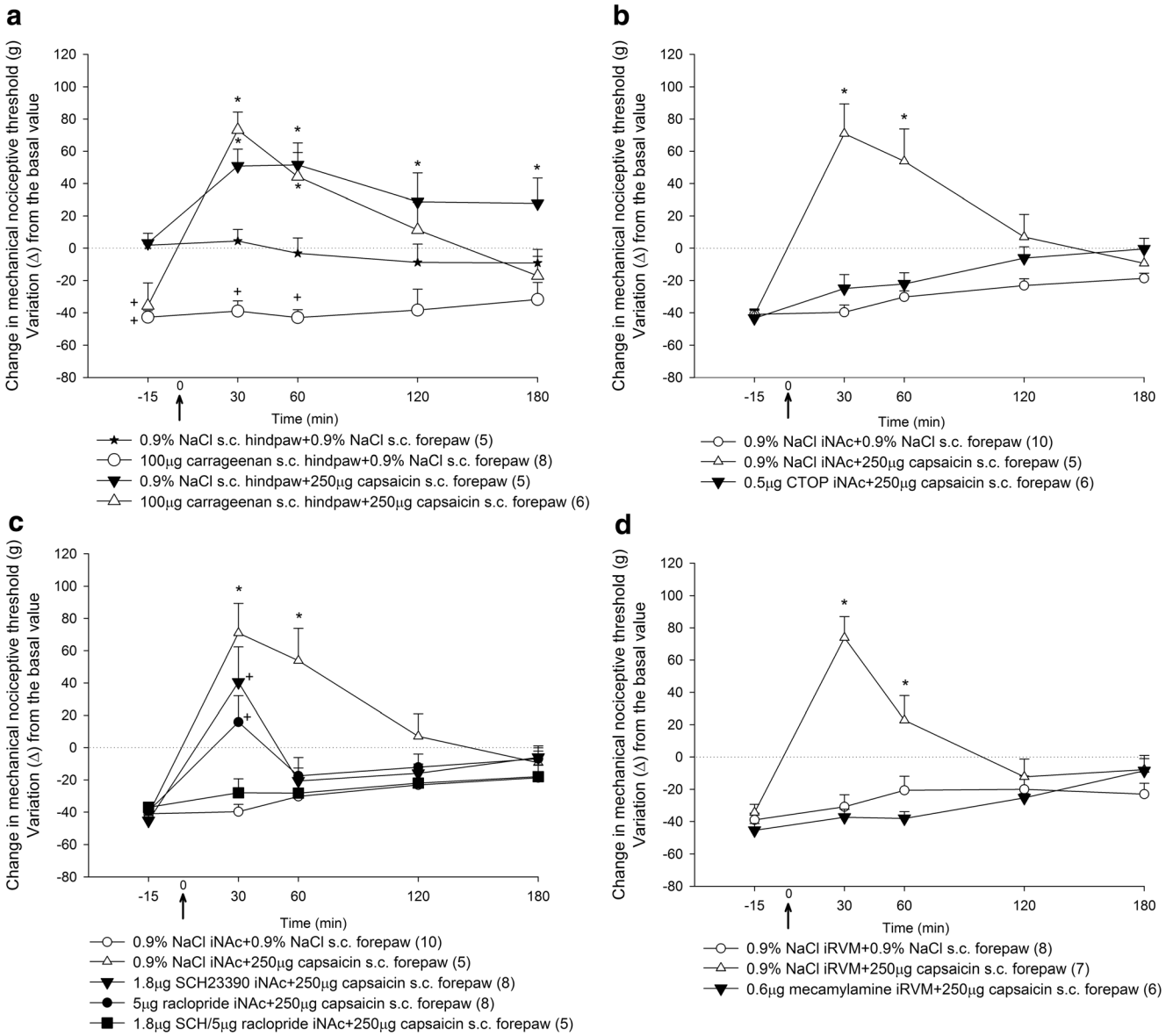


Fig. 2 Capsaicin-induced antinociception on carrageenan-induced hyperalgesia depends on μ-opioid and dopamine receptors within NAc and nicotinic acetylcholine receptors within RVM. **a** The hindpaw injection of carrageenan (100 μg) significantly decreased the mechanical nociceptive threshold (indicated by the plus sign). The forepaw injection of capsaicin (250 μg) significantly increased mechanical nociceptive threshold either in animals that have received carrageenan or saline into the hindpaw (indicated by asterisk). **b** Capsaicin-induced antinociception in animals that have received the hindpaw injection of carrageenan (indicated by asterisk) was prevented by the administration of CTOP (μ-opioid receptor antagonist) or by the **c** co-administration of SCH23390 and raclopride (D1 and D2 dopamine receptor antagonists, respectively; the plus sign in this panel indicates the intermediate effect of each antagonist alone) into the NAc as well as by **d** mecamylamine (nicotinic acetylcholine receptor antagonist) into the RVM (repeated-measures ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). See Supplementary Fig. S2 and "Results" section for details regarding the effect of the antagonists by themselves on carrageenan-induced hyperalgesia. In this and in subsequent figures, data represent the change in mechanical nociceptive threshold (g) as a variation from basal value (after–before). Numbers in parenthesis indicate the number of animals in each group. See "Material and Methods" for additional details regarding data presentation and analysis. iNAc intra-nucleus accumbens, iPAG intra-periaqueductal gray, iRVM intra-rostral ventromedial medulla

The Antinociceptive Effect Mediated by the Activation of Ascending Nociceptive Control Depends on the PAG and on the RVM

To evaluate the contribution of the vlPAG and RVM to the ascending nociceptive control-mediated antinociception, we used pharmacological approaches classically known to block the descending pain inhibitory system within either the PAG or the RVM level.

The blockade of vlPAG neural activity by the local administration of lidocaine prevented capsaicin-induced antinociception ($p < 0.001$), showing that the PAG is essential

to such effect. The selective pharmacological blockade of μ-opioid receptors (by CTOP, $p < 0.001$), as well as activation of $GABA_A$ receptors (by muscimol, $p < 0.001$) within the PAG, prevented capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 3a: $F_{treatment}(4,19) = 12.219$, $p < 0.001$; $F_{treatment \times time}(12,57) = 24.295$, $p < 0.001$). By themselves, neither lidocaine, nor CTOP or muscimol within the PAG changes carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 C: $F_{treatment}(3,12) = 0.273$, $p = 0.844$; $F_{treatment \times time}(9,36) = 0.926$, $p = 0.514$). Noteworthy, the administration of a μ-opioid receptor agonist (DAMGO) into the PAG of pain-free animals (hindpaw injection of saline instead of carrageenan) induced an antinociceptive effect similar in magnitude and time-course to that induced by capsaicin (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S3: $F_{treatment}(2,14) = 3.563$, $p < 0.050$; $F_{treatment \times time}(4,28) = 1.594$, $p = 0.204$). Since μ-opioid receptor agonists within the PAG are classically known to activate the descending inhibitory system, this finding demonstrates the similarity between the antinociceptive effect induced by capsaicin and by the descending system.

Consistently, the blockade of RVM neural activity by the local administration of lidocaine also prevented capsaicin-induced antinociception ($p < 0.001$), showing that the RVM is essential to such effect. The selective pharmacological blockade of μ-opioid receptors ($p < 0.001$), as well as activation of $GABA_A$ receptors ($p < 0.001$) within the RVM, prevented capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 3b: $F_{treatment}(4,24) = 31.970$, $p < 0.001$; $F_{treatment \times time}(12,68) = 11.021$, $p < 0.001$). By themselves, neither lidocaine, nor CTOP or muscimol within the RVM changes carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 D: $F_{treatment}(4,15) = 0.067$, $p = 0.991$; $F_{treatment \times time}(12,45) = 1.069$, $p = 0.407$). Since activation of μ-opioid receptors and inhibition of $GABA_A$ receptors within the PAG and RVM are key mechanisms underlying their pain inhibitory descending activity, these findings provide the first piece of evidence that the ascending nociceptive control-mediated analgesia depends on the descending pain modulation system.

The Antinociceptive Effect Mediated by the Activation of Ascending Nociceptive Control Depends on Descending Projections Through the Dorsolateral Funiculus

To evaluate whether descending projections through the DLF contribute to the ascending nociceptive control-mediated antinociception, we sectioned this funiculus at the thoracic level, a procedure known to abolish descending influences on spinal nociceptive transmission.

The DLF section prevented capsaicin-induced antinociception ($p < 0.001$), demonstrating that its integrity is essential to the ascending nociceptive control-mediated antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 4a: $F_{treatment}(2,17) = 70.168$, $p < 0.001$; $F_{treatment \times time}(6,51) = 30.438$, $p < 0.001$). By itself, DLF section did not change carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 E: $F_{treatment}(5,19) = 0.557$, $p = 0.732$; $F_{treatment \times time}(15,57) = 1.285$, $p = 0.242$). The DLF lesions were performed ipsilateral to the paw injections, and its extension was restricted to the DLF and the adjacent dorsal horn (Fig. 4b, c; Fig. 4d shows lesion reconstruction).

Since descending pathways from PAG and RVM reach the dorsal horn to modulate nociceptive transmission by traveling through the DFL, this finding provides the second piece of evidence that the ascending nociceptive control-mediated analgesia depends on the descending pain modulation system.

The Antinociceptive Effect Mediated by the Activation of Ascending Nociceptive Control Depends on Spinal Noradrenergic and Serotonergic Activity

To evaluate whether spinal noradrenergic, serotonergic, or dopaminergic activity contributes to the ascending nociceptive control-mediated antinociception, selective antagonists were spinally administered at doses known to block spinal pain modulation mechanisms.

The administration of either a serotonergic $5HT_{1A}$ receptor antagonist (WAY100135, $p < 0.001$) or a $5HT_3$ receptor antagonist (tropisetron, $p < 0.001$) prevented capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 5a: $F_{treatment}(3,17) = 45.833$, $p < 0.001$; $F_{treatment \times time}(9,51) = 13.218$, $p < 0.001$). Similarly, the administration of a selective α2-adrenoceptor antagonist (idazoxan, $p < 0.001$) also prevented capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 5b: $F_{treatment}(2,13) = 60.755$, $p < 0.001$; $F_{treatment \times time}(6,39) = 10.871$, $p < 0.001$). In contrast, the administration of a selective dopamine D2-like receptor antagonist (raclopride 1 or 10 μg, $p > 0.05$) did not significantly affect capsaicin-induced antinociception (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Fig. 5c: $F_{treatment}(3,19) = 24.652$, $p < 0.001$; $F_{treatment \times time}(9,57) = 10.089$, $p < 0.001$). By themselves, none of the antagonists changed carrageenan-induced hyperalgesia (repeated-measures ANOVA and Student–Newman–Keuls post hoc test; Supplementary Fig. S2 E: $F_{treatment}(5,19) = 0.557$, $p = 0.732$; $F_{treatment \times time}(15,57) = 1.285$, $p = 0.242$). Since the descending pathways involved in pain modulation are mostly

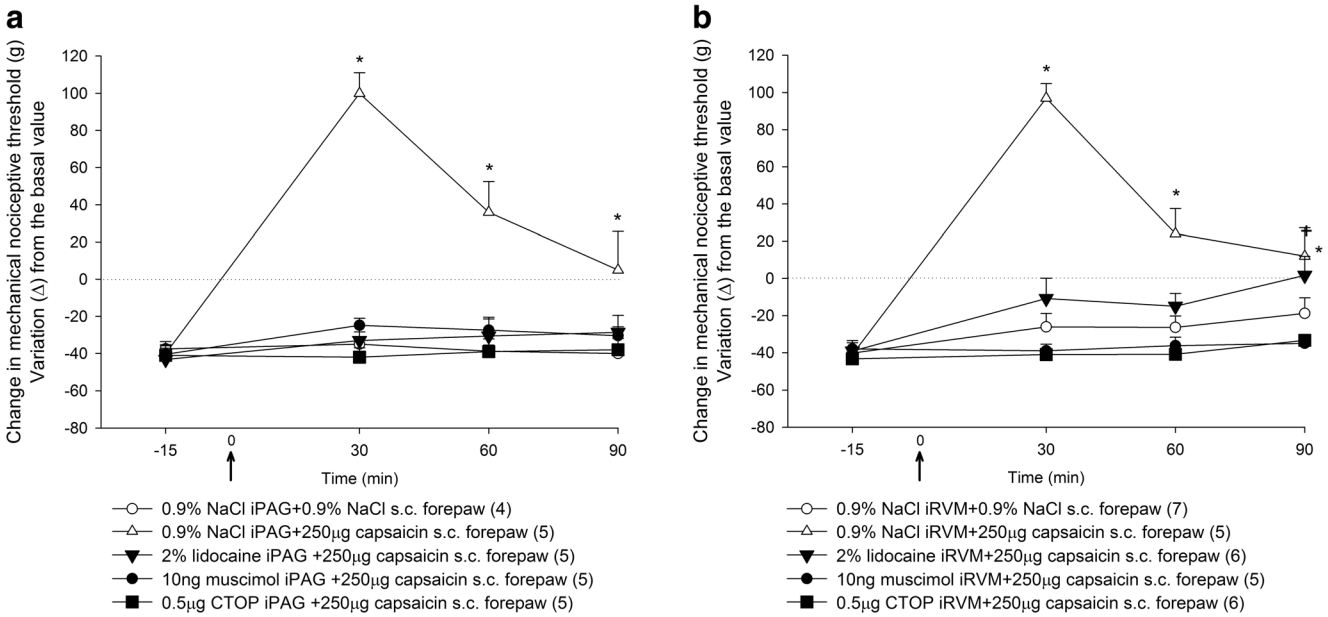


Fig. 3 Capsaicin-induced antinociception on carrageenan-induced hyperalgesia depends on activation of μ-opioid receptors and inhibition of $GABA_A$ receptors located in the PAG and RVM. The effect of capsaicin-induced antinociception in animals that have received the hindpaw injection of carrageenan (indicated by asrterisk) was prevented by the administration of lidocaine: CTOP (μ-opioid receptor antagonist) or muscimol ($GABA_A$ receptors agonist) either within the PAG (**a**) or the RVM (**b**) (repeated-measures ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). See Supplementary Fig. S2 and "Results" section for details regarding the effect of the drugs by themselves on carrageenan-induced hyperalgesia

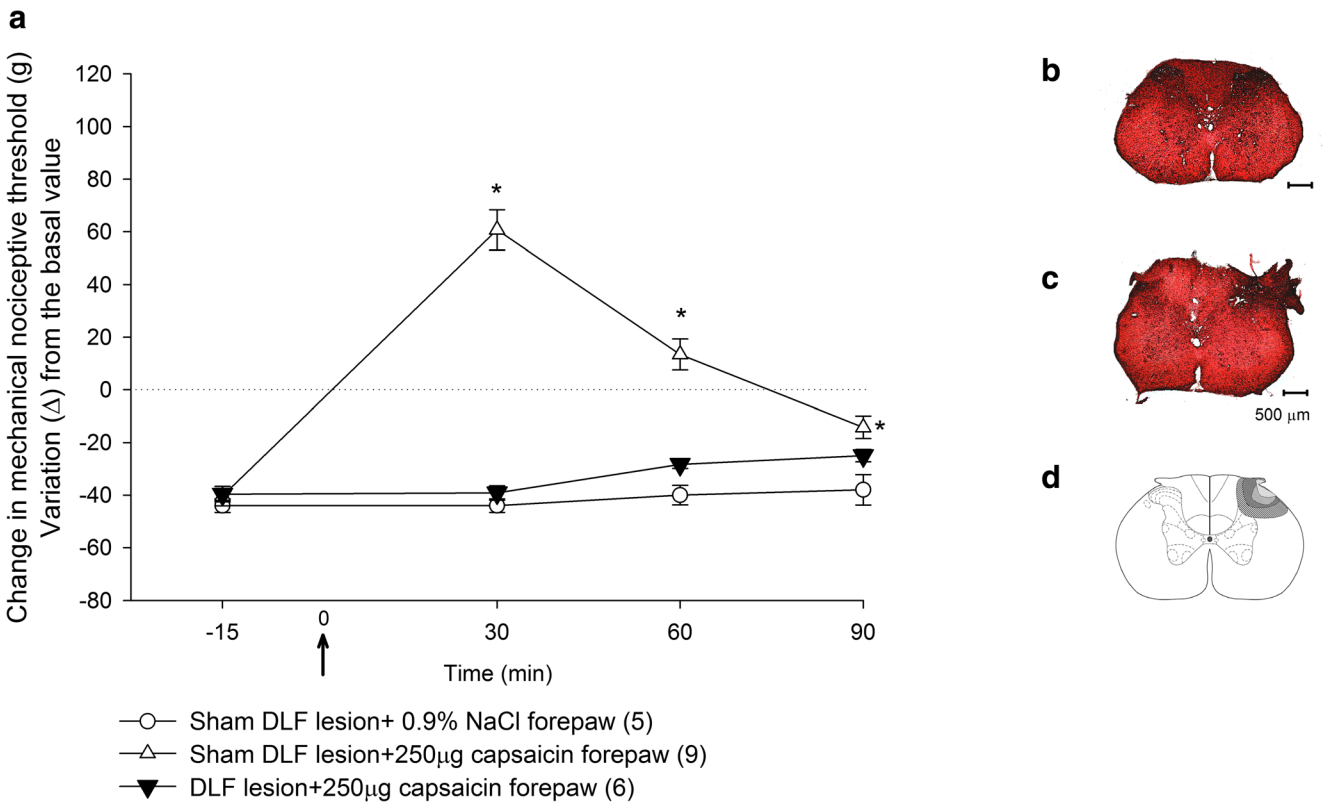
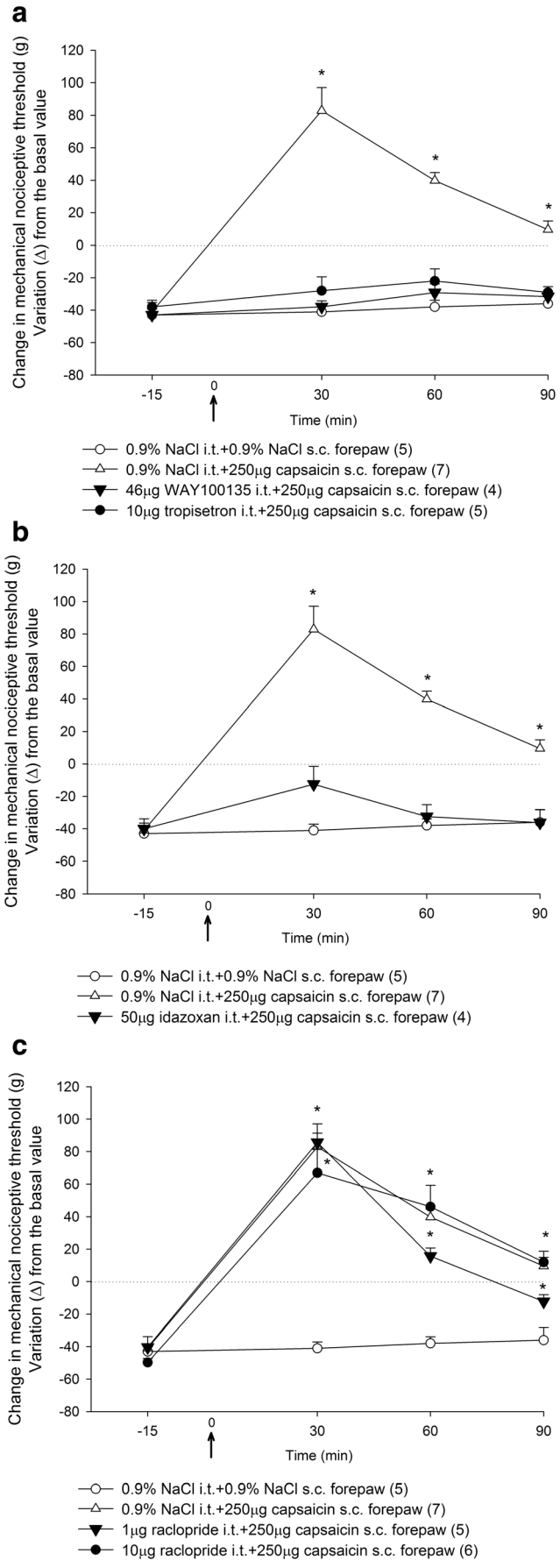


Fig. 4 Capsaicin-induced antinociception on carrageenan-induced hyperalgesia depends on the integrity of the dorsolateral funiculus. **a** Capsaicin-induced antinociception in animals that have received the hindpaw injection of carrageenan (indicated by asterisk) was prevented by the lesion of the DLF at thoracic level (repeated-measures ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). See Supplementary Fig. S2 and "Results" section for details regarding the effect of DLF lesion by itself on carrageenan-induced hyperalgesia. **b** Representative photographs from spinal cord sections (50 μm, neutral red) of the sham DLF-lesion and **c** DLF-lesion. **d** The extent of the DLF lesion is shown in the diagram adapted from the atlas of Paxinos and Watson. DLF dorsolateral funiculus



◀ **Fig. 5** Capsaicin-induced antinociception on carrageenan-induced hyperalgesia depends on spinal serotonergic and adrenergic but not dopamine receptors. **a** Capsaicin-induced antinociception in animals that have received the hindpaw injection of carrageenan (indicated by asterisk) was prevented by the intrathecal administration of WAY100135 (a $5HT_{1A}$ serotonin receptor antagonist), tropisetron (a $5HT_3$ serotonin receptor antagonist), or **b** idazoxan (a α2 adrenergic receptor antagonist) but not that of **c** raclopride (a D2 dopamine receptor antagonist) (repeated-measures ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). See Supplementary Fig. S2 and "Results" section for details regarding the effect of the antagonists by themselves on carrageenan-induced hyperalgesia. The groups 0.9% NaCl i.t. + 0.9% NaCl s.c. forepaw and 0.9% NaCl i.t. + 250 μg capsaicin s.c. forepaw in **b** and **c** were replotted from **a**

serotonergic and noradrenergic, these findings provide the third evidence that the ascending nociceptive control-mediated analgesia depends on the descending pain modulation system.

c-Fos Expression in NAc, PAG, and RVM

In order to indirectly estimate neural activity within the NAc, PAG, and RVM, we quantified local c-Fos protein expression, which rapidly and transiently increases in response to neuronal firing.

Within the NAc, c-Fos expression significantly increased with the hindpaw injection of carrageenan ($p = 0.006$). This effect was not observed when capsaicin was injected into the forepaw, no matter whether carrageenan ($p = 0.393$) or vehicle ($p = 0.168$) was injected into the hindpaw (two-way ANOVA and Student–Newman–Keuls post hoc test; Fig. 6a: $F_{\text{hindpaw} \times \text{forepaw}}(1,12) = 2.774$, $p = 0.122$).

Within the PAG, c-Fos expression significantly increased with the injection of either carrageenan ($p < 0.001$) or capsaicin ($p < 0.001$) and carrageenan plus capsaicin ($p < 0.001$) (two-way ANOVA and Student–Newman–Keuls post hoc test; Fig. 6c: $F_{\text{hindpaw} \times \text{forepaw}}(1,12) = 28.230$, $p < 0.001$).

Within the RVM, a non-significant tendency of c-Fos expression to increase with the different treatments was detected (two-way ANOVA and Student–Newman–Keuls post hoc test; Fig. 6d: $F_{\text{hindpaw} \times \text{forepaw}}(1,12) = 2.373$, $p = 0.149$).

Representative photomicrographs of c-Fos-ir cells within the NAc, PAG, and RVM are shown in Fig. 6b, d, f, respectively.

Locomotor Activity

Locomotion in the open field test did not significantly change with different treatments and experimental manipulations (Supplementary Table S1).

The anatomical reconstruction of the injection sites into the NAc core, vlPAG, RVM, and off sites is shown in supplementary material (Supplementary Fig. S4).

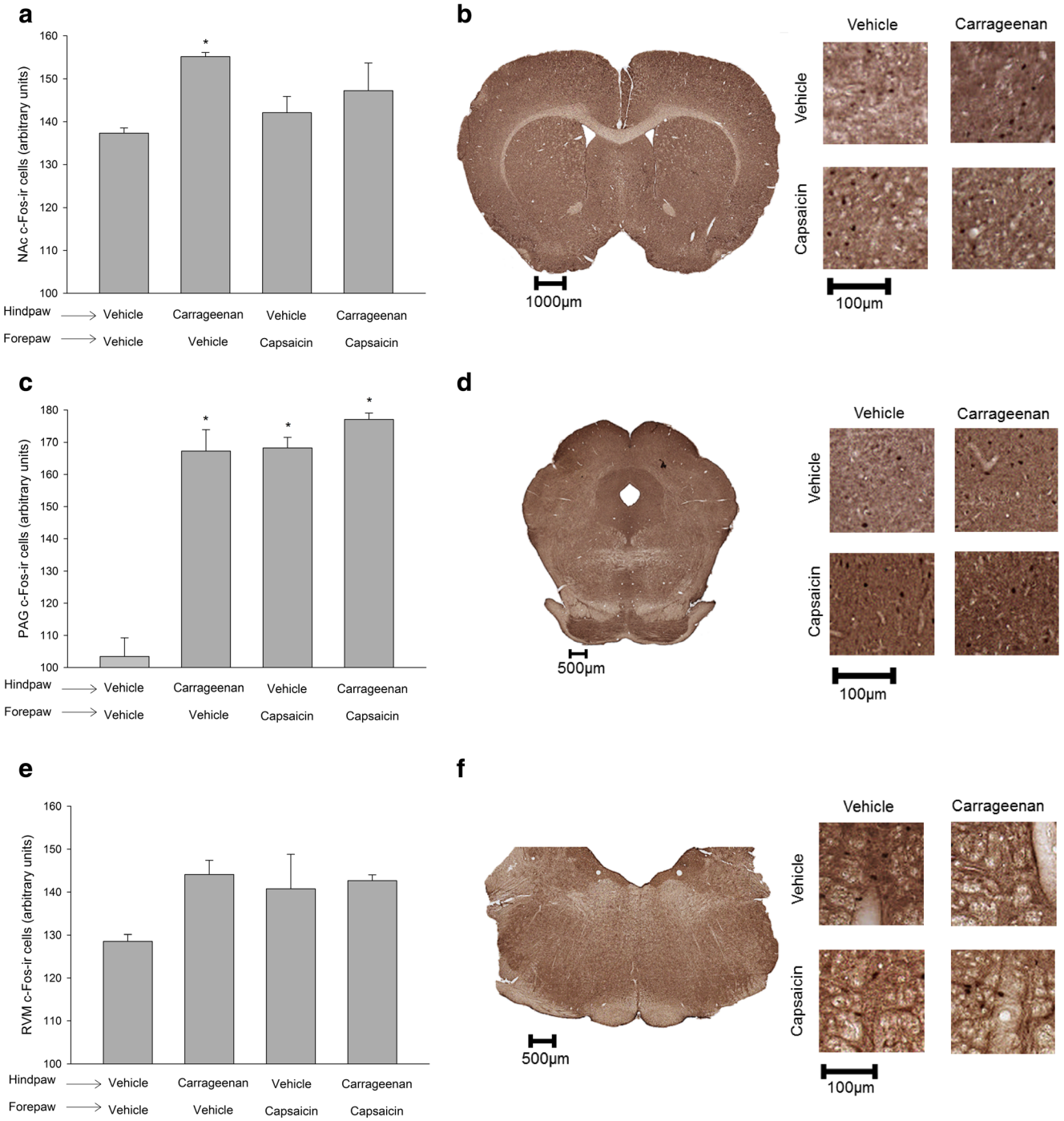


Fig. 6 c-Fos expression in the NAc, PAG, and RVM. **a** The hindpaw injection of carrageenan significantly increased c-Fos-immunoreactive (c-Fos-ir) optical density within the NAc (indicated by asterisk); the forepaw injection of capsaicin reversed this effect and did not affect c-Fos-ir optical density by itself (two-way ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). **c** All three experimental interventions significantly increased c-Fos-ir optical density within the PAG (two-way ANOVA and Student–Newman–Keuls post hoc test, $p < 0.05$). **e** c-Fos-ir optical density within the RVM was not significantly changed (two-way ANOVA and Student–Newman–Keuls post hoc test, $p > 0.05$). The brains were collected 30 min after forepaw injection. Representative photomicrographs of c-Fos-ir cells within the **b** NAc (magnification ×0.4 left; ×5 right), **d** PAG (magnification ×0.7 left; ×5 right), and **f** RVM (magnification ×0.7 left; ×5 right). Scale bar is represented in the figures

Discussion

This study describes an ascending–descending pain modulation pathway triggered by pain and dependent on the NAc and on the PAG–RVM descending system. The ascending component, named ascending nociceptive control, is triggered by noxious stimulation and induces potent and long-lasting remote antinociception dependent on opioidergic and dopaminergic mechanisms within the NAc [8, 21]. The descending component is the most well-known pain modulation mechanism, the PAG–RVM descending system [6, 7]. The functional link between the two components was demonstrated using a pharmacological approach, in which the antinociception induced by noxious stimulation was prevented either by all the already known supraspinal interventions that block the ascending component or by standard interventions known to block the descending one. To our knowledge, this is the first description of a spinal-mesolimbic-PAG-spinal pathway for pain modulation. Such mechanism may be the neurobiological basis to the proper behavior selection during life-threatening situations accompanied by intense pain.

The mechanisms underlying the antinociception induced by noxious stimulation in this study differ from other forms of pain-induced analgesia, such as diffuse noxious inhibitory control (DNIC) or conditioned pain modulation (CPM), the most used terms in animal and human studies, respectively [27]. DNIC-like effects are characteristically short-term and dependent on the maintenance of the noxious stimulation, disappearing soon after it has ceased (for review see [28]). In contrast, the ascending nociceptive control-mediated analgesia lasts more than an hour, and once initiated, it becomes independent on the eliciting stimulus [29]. In addition, DNIC-like effects are mediated by mechanisms restricted to the lower brainstem [2, 30]. Therefore, the ascending nociceptive control is the only known form of pain-induced analgesia dependent on forebrain mechanisms [8].

In fact, ascending nociceptive control-mediated analgesia has been shown to be dependent on opioidergic and dopaminergic mesolimbic mechanisms within the NAc [8, 21]. This was further demonstrated in the present study, since capsaicin-induced antinociception was prevented by the intra-accumbal administration of either a μ-opioid receptor antagonist (Fig. 2b) or a combination of dopamine D1 and D2 receptor antagonists (Fig. 2c). When administered alone, each dopaminergic antagonist induced an intermediate effect, suggesting that endogenous dopamine act on both D1 and D2 receptors to induce antinociception. This data extends previous findings that have demonstrated the ability of a non-selective dopamine receptor antagonist to prevent ascending nociceptive control-mediated antinociception [8]. Subsequent attempts to identify additional supraspinal nuclei involved in ascending nociceptive control-mediated analgesia have identified a cholinergic nicotinic mechanism within the RVM [10]. This was confirmed by our finding that capsaicin-induced antinociception was prevented by the intra-RVM administration of a nicotinic receptor antagonist (Fig. 2d).

The involvement of the PAG–RVM descending system in ascending nociceptive control-mediated antinociception has been already investigated and ruled out. This is because the administration of naloxone (a non-selective opioid receptor antagonist) within the PAG or the RVM did not affect antinociception, though the administration of muscimol within RVM has blocked it [8, 9]. These findings were interpreted as evidence that both systems are independent because opioid mechanisms within the PAG and the RVM are the neural basis of the descending system [6]. However, in this study, we have revisited this interpretation providing a bundle of data to argue that both systems are functionally linked. The initial evidence is that neuronal inactivation of either the PAG (Fig. 3a) or the RVM (Fig. 3b) by the local injection of lidocaine prevented capsaicin-induced antinociception. These findings demonstrate that both the PAG and the RVM are essential to the ascending nociceptive control-mediated antinociception. In addition, the blockade of μ-opioid receptors, as well as the activation of $GABA_A$ receptors within either the PAG (Fig. 3a) or the RVM (Fig. 3b), prevented capsaicin-induced antinociception. These are standard procedures to block descending inhibitory activity [31–34] because neurons responsible by its control are under tonic GABAergic inhibition that can be suppressed by μ-opioid mechanisms [6, 7]. Therefore, the blockade of mechanisms of descending inhibition within either the PAG or the RVM blocks ascending nociceptive control-mediated antinociception. In the next step, we have shown that either the DLF lesion (Fig. 4a) or the spinal administration of serotonergic ($5HT_{1A}$ and $5HT_3$, Fig. 5a) or noradrenergic (α2, Fig. 5b) but not dopaminergic (D2, Fig. 5c) receptor antagonists prevented capsaicin-induced antinociception. These strategies were used because the descending pathways reach the dorsal horn via the DLF [7] and release serotonin and norepinephrine. In contrast, dopaminergic descending projections originate mainly in the hypothalamic A11 region [7], which explains the lack of effect of the dopamine antagonist. Therefore, once again, but now at the spinal level, procedures classically known to block the antinociception mediated by the descending system also block that mediated by the ascending nociceptive control.

The inability of naloxone administered into the PAG or RVM to block ascending nociceptive control-mediated antinociception in previous studies [8, 9] may result from its non-selectivity. Naloxone could eventually block κ-, in addition to μ-opioid receptor-mediated effects, and this may be critical since opposite effects on nociception have been attributed to each receptor subtype expressed within these regions [35]. In fact, selective μ-opioid receptor antagonists are more effective than equipotent doses of non-selective antagonists in

blocking the antinociceptive effects mediated by the descending system [36, 37].

Neuronal activity indirectly estimated by c-Fos expression within the NAc, PAG, and RVM supports behavioral observations. Within the NAc, c-Fos expression increased with carrageenan-induced hyperalgesia, an effect not observed when capsaicin was injected in the forepaw of animals receiving either carrageenan or vehicle into the hindpaw (Fig. 6a). Since NAc activity is pronociceptive and the analgesia mediated by the ascending nociceptive control depends on its inhibition [38], increased c-Fos expression with carrageenan may reflect increased NAc activity during hyperalgesia. Complementarily, the decrease in c-Fos expression with capsaicin may reflect inhibition of NAc to induce antinociception. Within the PAG, c-Fos expression significantly increased in response to peripheral stimulation, no matter if it was with carrageenan, capsaicin, or both together (Fig. 6c). A non-significant tendency in the same direction was observed within the RVM (Fig. 6e). This may reflect increased PAG and RVM activity during either inflammatory hyperalgesia or endogenous analgesia, something expected considering their role on pain processing and modulation. In fact, previous studies have demonstrated increased c-Fos expression within the PAG and the RVM in response to either nociceptive activity [39, 40] or endogenous analgesia [41].

Nucleus accumbens is the critical target of the dopaminergic mesolimbic system which, along with its well-known role in reward and motivation [42], has been shown to have an important role in pain modulation (for excellent reviews see [11, 43]). However, in the current literature, the mesolimbic and the descending system appears to modulate pain through totally independent mechanisms, to such an extent that important revisions in each system barely mention the other one [11, 44]. In fact, although the PAG is anatomically connected with the ventral tegmental area and the NAc [45, 46] and evidence from the early 1990s suggest a bidirectional opioid loop between them [47–49], to our knowledge, this is the first evidence linking the PAG–RVM descending and mesolimbic systems in an endogenous pain modulation mechanism. Recently, Baliki and Apkarian have proposed that acute pain experience depends on peripheral nociceptive drive and on a threshold phenomenon, responsible to determine when nociception turns to pain perception [11]. This nociception-pain threshold is supposed to be dependent on mesolimbic activity; however, the underlying mechanistic basis is unclear. The findings from the present study fit well as a possible mechanism in this scenario, because the descending system has a powerful ability to decrease nociceptive transmission, which consequently increases the pain perception threshold. Evidently, further studies are needed to support this idea as well as to determine whether the mesolimbic system connects with the descending system through cortical or subcortical circuitry.

Although previous human brain image studies have indirectly correlated PAG activity with analgesia in response to some kinds of noxious stimulation [50–52], to our knowledge, this is the first functional evidence showing that the PAG–RVM descending system mediates pain-induced analgesia. This may be of adaptive importance, since during threatening situations, pain modulation ensures that the individual will be free for engaging in defensive responses. In fact, either the PAG–RVM descending system [53] or the ascending nociceptive control [54] is known to facilitate the expression of defensive behaviors during life-threatening situations. The first is activated, in such situations, in response to fear systems in the amygdala, in anticipation of an eventual noxious stimulation [55]. The second is by actual noxious stimulation that by itself signifies the presence of immediate threat. We propose that during intense noxious stimulation, the ascending nociceptive control is recruited to enhance the activation of the descending system, ensuring that this potent pain modulation mechanism will work in an optimal way when most necessary, during threatening situations accompanied by intense acute pain.

In summary, this study demonstrates that noxious stimulation triggers an ascending–descending pain modulation pathway linking the mesolimbic system to the PAG–RVM descending system. The mesolimbic system is believed to codify the value, salience, and expectation related to pain experience adjusting the pain perception threshold and selecting the most advantageous behavioral action [11]. Pain perception can disturb the expression of the selected behavioral action. An excellent way to prevent this from happening is to increase the pain perception threshold by activating powerful pain modulation mechanisms. The present study presents a possible mechanism to achieve this goal and adds important pieces to the endogenous pain modulation puzzle.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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