The discovery of analgesic agents in preclinical models of pain very poorly translates into successful clinical developments. In an effort to identify neurochemical markers of nociceptive processing that could be correlated to behavioral antinociceptive efficacy of analgesic drugs, an in vivo microdialysis method in rats has been developed to study neurochemical events in the dorsal horn of the spinal cord, a crucial region in pain transmission and control. Interestingly, the method was applied to conscious animals in which microdialysis sampling and behavioral nociception were simultaneously assessed. The method also allowed the differentiation between ipsi- and contralateral sides, which is relevant in most animal models of pain to focus on ipsilateral pain-related changes compared to control contralateral ones.

Three different methods for spinal microdialysis have been described based on the location of the fiber: transversal, intrathecal, and concentric (Fig. 1A). We used the last approach because it allows intradorsal horn microdialysis and it is the only one enabling the sole evaluation of the ipsilateral side. Noteworthy, some studies are available in anesthetized animals but the concentric microdialysis approach has never been used in conscious animals in pain research. In fact, only a few microdialysis studies on locomotor activity have employed concentric probes in awake animals (1–3). This is a key issue as anesthesia modifies pain perception and probably interferes with the neurochemical modulation of pain as well as the effect of analgesics. In this way, previous data have shown that certain anesthetic agents modulate glutamate (Glu) central release (4).

Glu is a neurotransmitter critical for spinal excitatory synaptic transmission and for the generation and maintenance of spinal states of sensitization and pain-related hypersensitivity (5). Understanding the regulation of spinal Glu release in the context of pain and analgesia is thus important to explore novel mechanisms and innovative therapeutic strategies for pain treatment. Following peripheral tissue injury or chemical sensitization by subcutaneous administration of formalin, Glu is released in the dorsal horn, leading to increased sensitivity of dorsal horn neurons (i.e., central sensitization) (6–8). These events occur mainly in the dorsal horn of the spinal cord ipsilateral to the site of the injury and thus the concentric microdialysis approach, in contrast to the transversal (dialysate comes from both sides) or the intrathecal ones, seems more appropriate to detect changes in neurotransmitter release in this region.

In the present study, the nociceptive response and changes in Glu levels in the ipsilateral spinal dorsal horn...
and their modulation by systemic morphine administration were investigated after intraplantar formalin injection using the concentric microdialysis approach in conscious and unrestrained rats (Fig. 1B).

Experiments were performed on male Wistar rats (Charles River, Lyon, France) weighing 260 – 320 g. Animals were housed 4 per cage in a 12-h light–dark cycle at room temperature (22°C) with food and water available ad libitum. Animal care and all experimental protocols were performed according to European Union regulations (O.J. of E.C. L 358/1 18/12/1986) and received approval by the local Ethical Committee.

Rats were anesthetized with chloral hydrate (440 mg/kg, i.p.) and an incision was made along the dorsal midline. Using a David Kopf stereotaxic frame (Düsseldorf, Germany), the Th13 was then immobilized on the horizontal plane by using a transverse process clamp and a burr hole created in the dorsal surface. The exposed dura mater was carefully opened and a microdialysis probe of concentric design (CMA/11) was inserted into the spinal cord at an angle of 45° from the vertical. The microdialysis probes (exposed tip, 2.0 mm × 0.24 mm) were implanted into the medial dorsal horn of the L4 lumbar region of the spinal cord. The probe was fixed by application of superglue-3 gel (Loctite®) and dental cement around the probe and a stainless steel anchorage screw located in the Th13 vertebra. The skin was then closed and rats were allowed to recover overnight, one per cage, with food and water ad libitum.

Only implanted rats showing normal behavior and neurological functions after the recovery period (no walking dysfunction, normal weakened extension withdrawal reflex of the hind limb, no reduced toe spread, normal food and water intake, no piloerection or apparent stress signals) were used in the study.

Around 20 h after probe implantation, rats were placed in a system for freely moving animals. The dialysis probes were connected to a CMA Microdialysis System (Solna, Sweden) and then perfused with CNS perfusion fluid (147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, and 0.85 mM MgCl₂, pH 7.4; supplied by CMA Microdialysis) at 2 μl/min flow rate. After 1 h for stabilization of baseline Glu release, consecutive samples were collected every 10 min into vials. Immediately after collection, dialysate samples were assayed by HPLC coupled to fluorescence detection (Waters, Cerdanyola del Vallès, Spain) after derivatization AQCh Fluor™ Reagent Kit (Waters). Separation was carried out using a 4-μm AccQ-Tag C18 column (150 mm × 3.9 mm I.D.; from Waters). Eluent A was aqueous acetate phosphate buffer prepared by diluting AccQ-Tag eluent A concentrate with water to a ratio of 1:10, and eluent B was 60% HPLC-grade acetonitrile. The AccQ-Tag column was heated at 37°C and operated at a flow rate of 1 ml/min. Detection was performed by fluorescence with excitation

Fig. 1. Spinal microdialysis in conscious and unrestrained rats. A) Drawings showing three different methods for spinal microdialysis based on the location of the fiber: transversal, intrathecal, and concentric. B) Photomicrograph showing the typical flinching behavior induced following hind paw intraplantar formalin (5%) injection in a conscious and unrestrained rat submitted to spinal cord microdialysis in the dorsal horn. C) Effect of local infusion of K⁺ at 100 mM in the dorsal horn of the spinal cord on extracellular concentration of Glu. Note that Glu released in the dorsal horn was stimulated by K⁺ (100 mM) in CSF. Points are means ± S.E.M. values from 7 animals and are expressed as percentages of their respective basal values. *P < 0.05, **P < 0.01, compared to the basal values (one-way ANOVA with post-hoc Bonferroni’s test).
Intradorsal Horn Microdialysis in Rats

Morphine hydrochloride was obtained from Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) (Madrid, Spain), dissolved in sterile physiological saline and administered in a volume of 2 ml/kg of body weight through the subcutaneous (s.c.) route. Twenty minutes after treatment with morphine or vehicle, 50 μl of 5% formalin solution was intraplantarly injected in the right hind paw and the occurrence of spontaneous flinching of the injected paw was quantified. An initial acute pain response (phase I, during the first 1 – 5 min after formalin injection) was followed by a relatively short quiescent period and then by a prolonged tonic phase (phase II, 10 – 60 min after formalin injection) (10). The number of paw flinches was counted every min for the first 5 min after the formalin injection. Thereafter, starting at 6 min after the formalin injection and up to 60 min, flinching was quantified at 5-min intervals. At the end of the experiment, animals were killed by CO₂ inhalation and spinal cords were dissected out for histological examination to verify that microdialysis probes were correctly implanted.

The mean value of two dialysate samples obtained before saline or morphine administration was considered as the 100% basal value. Extracellular Glu concentration of dialysate samples collected during an experiment was normalized as percentage of basal values. Values were not corrected for in vitro recovery through the dialysis probe. Behavioral data were expressed as means ± S.E.M. of the number of flinches and compared by using a two-way ANOVA (treatment × time). The effect of K+ infusion was analyzed by a one-way ANOVA. The level of significance was set at \( P < 0.05 \). Data were analyzed using the Prism version 4.0 computer program (GraphPad Software, San Diego, CA, USA).

In order to study the Glu responses to local depolarization, a high K+ solution (100 mM) was perfused through the probe at the end of experiments. K+ (100 mM) CSF infusion significantly increased Glu release (\( F[2,30] = 20.96, P < 0.001 \)) reaching a maximum effect of 297% ± 65% (Fig. 1C). Thus, neuronal release appears to be the source for Glu increases detected in the dorsal horn of the spinal cord.

The injection of 50 μl of 5% formalin solution into the right hind paw evoked significantly less flinching behavior in implanted (n = 9) when compared with the naïve group (n = 7) (\( F[1,210] = 13.84, P < 0.01 \)). However, the implanted animals kept the typical biphasic response on flinching behavior (Fig. 2). Systemic administration of morphine 3 mg/kg (s.c.) significantly prevented this nociceptive behavior in both naïve (n = 6) and implanted animals (n = 6) when compared with their control, saline-

![Fig. 2.](image)

**Fig. 2.** Effect on flinching behavior of formalin (5%) injected in naïve or implanted rats. Saline or morphine was s.c. administered 20 min before formalin injection. Note that formalin evoked a significantly reduced flinching behavior in implanted rats (n = 9) compared with naïve rats (n = 7). Morphine (3 mg/kg) significantly prevented flinching behavior in both naïve (n = 6) and implanted (n = 6) animals. Points are means ± S.E.M. of the number of flinches from 6 – 9 separate experiments. **\( P < 0.01 \), ***\( P < 0.001 \), compared to the saline naïve group or saline formalin group (two-way ANOVA).**

![Fig. 3.](image)

**Fig. 3.** Effect on extracellular concentration of Glu in the dorsal horn of the spinal cord of formalin (5%) in implanted rats. Saline or morphine was s.c. administered 20 min before formalin or saline injection. Note that in the saline + formalin–treated group (n = 8), formalin induced an increase of Glu released compared to the group receiving saline injection (n = 7). Morphine (3 mg/kg) (n = 5) significantly prevented this effect. Points are means ± S.E.M. and are expressed as percentages of the respective basal values. *\( P < 0.05 \), compared with the appropriate control group (two-way ANOVA).
treated groups (naïve animals: $F[1,165] = 28.43$, $P < 0.0001$; implanted animals: $F[1,195] = 12.92$, $P < 0.01$) (Fig. 2).

The intraplantar injection of formalin induced a significant increase in Glu ($F[1,117] = 8.19$; $P < 0.05$) compared to saline injection, reaching a maximum effect of 294% over basal values (Fig. 3). In contrast, intraplantar injection of saline did not modify Glu levels in the ipsilateral dorsal horn, as previously described (7). Administration of morphine at 3 mg/kg (s.c.) 20 min before formalin injection prevented the increase of extracellular Glu concentration in the ipsilateral side of the dorsal horn ($F[1,99] = 5.36$; $P < 0.05$) (Fig. 3). These findings agree with a previous study reporting suppression by morphine of formalin-induced flinching behavior and spinal glutamate release in rats, where a significant correlation between antinociception and inhibitory effect on glutamate release of morphine was observed (11).

Malmberg and Yaksh (8) using the intrathecal microdialysis approach in the rat showed an increase of 92% of Glu after formalin administration (using the same injection volume and dose as in this study). The increased Glu levels found in the present study suggest that concentric microdialysis is a more sensitive approach for studying neurochemical changes induced by pain at the spinal level.

Our results demonstrate that concentric microdialysis is a sensitive technique for studying the neurochemical modulation induced by pain in awake, freely-moving rats. In addition, measurement of Glu in the dorsal horn of the spinal cord provides relevant information on pain signaling and could provide insight about the mechanism of action of analgesic drugs.

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References