

Formalin-induced spinal glutamate release in freely moving rats : comparison of two spinal microdialysis approaches

L. SHI (*, **), I. SMOLDERS (***), S. SARRE (***), Y. MICHOTTE (***), M. ZIZI (**) and F. CAMU (*)

Summary : Two different spinal microdialysis approaches using either a linear tissue probe (LM-3) or a loop probe were explored on freely-moving rats to investigate the basal and formalin-evoked release of glutamate (Glu) in the spinal dorsal horn or in the cerebrospinal fluid (CSF). Adult male Wistar rats were implanted either with a LM-3 probe transversely through the spinal dorsal horn or with a loop probe in the CSF. After 24 hours recovery, microdialysis was initiated with perfusion of modified Ringer's solution at a flow rate of 5 μ l/min and the basal Glu concentrations were sampled for 1 hour. The effects of altering the microdialysis flow rate and perfusion solution on basal Glu release were next investigated. Following the injection of 50 μ l of formalin 5% into the hind paw, 10-min samples were collected for 90 min. The baseline levels of Glu were $0.82 \pm 0.09 \mu$ M with LM-3 probes and $5.96 \pm 0.22 \mu$ M with the loop probes. Decreasing the flow rate from 5 to 2 μ l/min increased extracellular Glu concentrations by $222.7 \pm 7.3\%$, whereas perfusion with artificial CSF reduced baseline Glu by $61.5 \pm 9.5\%$ with LM-3 probes. Injection of formalin induced a short-lasting but significant increase of Glu with a similar profile and time course when using either of the microdialysis approaches. In conclusion, microdialysis in the dorsal horn or in the CSF are both effective techniques to assess the alterations in Glu release following peripheral nociceptive input. The loop probe technique in CSF is more reproducible for routine investigation of drug effects, whereas the microdialysis of the dorsal horn provides a useful tool to precisely locate where the release of the neurotransmitters occurs.

Key words : Spinal cord ; microdialysis ; neurotransmitters ; glutamate ; formalin.

The microdialysis sampling technique was originally developed in the neurosciences for use in brain tissue. Its use in pharmacokinetics research offered many benefits such as more frequent data points, clean samples, no loss of body fluid and consumption of fewer experimental animals per study (6). Spinal cord microdialysis in rat was recently introduced in pain research (3, 8, 10, 11, 14) because of the increasing interest in the trans-

mitter pharmacology of the spinal cord. It is regarded as an effective technique for measuring the release of excitatory amino acids as well as other neurotransmitters into the extracellular fluid of the dorsal horn. SKILLING and SMULLIN (12) successfully demonstrated for the first time the release of glutamate (Glu) and aspartate (Asp) in response to formalin-induced acute nociceptive stimulation. The results from VETTER *et al.* (18) and OKUDA (10) also provided evidence to support the hypothesis that Glu and Asp are dorsal horn neurotransmitters involved in nociception.

Different approaches of spinal cord microdialysis have been used. A transverse microdialysis system through the spinal cord dorsal horn (12, 13, 17) provided the ability to locate precisely the spinal terminals from which the neurotransmitter release occurs, but its utility is limited by the difficulty of the surgical preparation, the construction of the dialysis probe, and the performance in awake rats. Alternatively, spinal loop dialysis (7, 9) samples the neurotransmitters from the cerebrospinal fluid (CSF) and allows to perform the perfusion concurrently with behavioural assessment in the unanesthetized rat. Nevertheless, the latter method is unable to locate precisely the origin of the release of the neurotransmitters. The present study aimed to validate and compare two spinal microdialysis techniques in awake, freely moving rats. The basal release of the neurotransmitter glutamate was assessed with a linear tissue probe implanted in the dorsal horn of the spinal cord and with a loop probe

L. SHI, M.D. ; I. SMOLDERS, Ph.D ; S. SARRE, Ph.D ; Y. MICHOTTE, Ph.D ; M. ZIZI, M.D., Ph.D ; F. CAMU, M.D., Ph.D.

(*) Department of Anesthesiology, AZ-VUB, Laarbeeklaan 101, 1090 Brussels.

(**) Department of Physiology, Faculty of Medicine, Vrije Universiteit Brussel (VUB), 1090 Brussels.

(***) Department of Pharmaceutical Chemistry and Drug Analysis, Vrije Universiteit Brussel (VUB), 1090 Brussels.

Correspondence address : Prof. F. CAMU, Department of Anesthesiology, AZ-VUB, Laarbeeklaan 101, 1090 Brussels. E-mail : frederic.camu@az.vub.ac.be.

sampling CSF. The influences of changing flow rate and perfusion solution on the basal release of Glu were evaluated. Also, formalin-induced Glu release was investigated using the same experimental approaches.

METHODS

The experimental protocol was approved by the Bioethical Committee for Animal Experimentation of the Vrije Universiteit Brussel and was in accordance with the guidelines for animal experimentation of the International Association for the Study of Pain (IASP). Adult male Wistar rats (300-350 g; B & K Universal Limited; England) were housed in groups of four, with free access to food and water. The experiments were carried out between 8:00 and 15:00 hours to minimize animal-dependent variability in the signals.

Surgical preparation

Spinal dorsal horn microdialysis

The rats were anesthetized with pentobarbital (60 mg/kg, i.p.) for surgical preparation. The skin was incised above the vertebral column and muscle tissue cleared away from vertebrae T₁₂-L₁. Two small holes were drilled through the lateral surface of vertebra T₁₃ at the level of the dorsal horn. A dialysis linear probe (LM-3) with a 3 mm active length membrane (Fig. 1A; diameter 320 µm, molecular weight cut-off 32 kD; BAS, West Lafayette, Indiana) was placed transversely through the spinal dorsal cord as described previously (11, 12). The dialysis membrane was covered with epoxy glue, except for the part located in the spinal cord. The ends of the dialysis tube were connected to polyethylene tubing that was exteriorized and fastened in the neck. All rats received 50 µl of buprenorphine S.C. (0.3 mg/ml) for postoperative analgesia.

Lumbar CSF microdialysis

A spinal triple loop catheter (Fig. 1B; Marsil scientific, San Diego, USA) was introduced subarachnoidally via the atlanto-occipital membrane under similar anesthesia condition by using the method of MARSALA *et al.* (9). The loop of the catheter (9 cm length, including an active dialysis fiber of 4 cm with 200 µm inner diameter, 300 µm outer diameter, 11 KD cut-off) was placed at the rostral margin of the lumbar enlargement. The free ends of the catheter were externalized through the

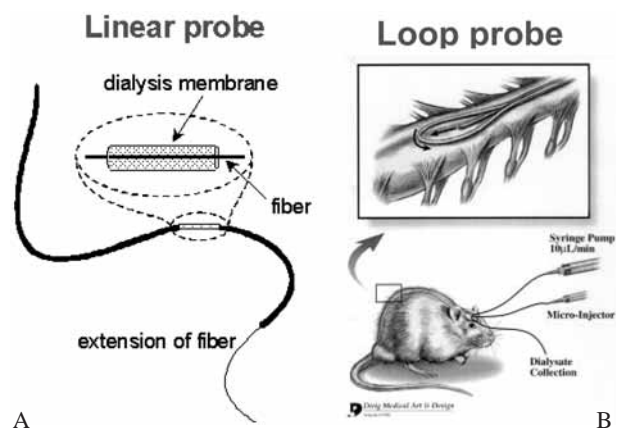


Fig. 1. — The diagram of the linear probe (A; BAS, West Lafayette, USA) and the triple loop probe (B; Marsil Scientific, San Diego, USA). The picture of the loop probe is adapted from the Marsil Scientific.

skin at the top of the skull. All rats received 50 µl of buprenorphine S.C. (0.3 mg/ml) for postoperative analgesia.

Experimental setting

Baseline glutamate sampling

The rats were placed in a large chamber (Freely Moving System, BAS/Microdialysis, West Lafayette, Indiana) and were allowed to recover from surgery overnight. The dialysis probes were connected to a microdialysis pump (CMA 100, CMA/Microdialysis, Stockholm, Sweden) and perfused with modified Ringer's solution (NaCl 147 mM, KCl 4 mM, CaCl₂ 2.3 mM) at a flow rate of 5 µl/min. After an equilibration phase of 1 hour, 6 baseline dialysates were collected every 10 min over 1 hour. For validating the microdialysis technique with the LM-3 in the spinal dorsal horn, two subgroups were set to analyze the influence of altering the perfusing solution and the flow rate on basal Glu release: 1) Artificial CSF (NaCl 124 mM, KCl 3.0 mM, CaCl₂ 1.35 mM, Na₂HPO₄ 0.242 mM, NaHCO₃ 20 mM, MgCl₂ 1.1 mM) at a flow rate 5 µl/min. 2) Modified Ringer's solution at flow rate of 2 µl/min.

Formalin-induced Glu release

After the six baseline microdialysate collections with modified Ringer's solution at the flow rate of 5 µl/min, 50 µl formalin 5% was subsequently injected into the plantar of the hind paw of the rat, and 10 min-samples were further collected for the additional 90 min. In the sham group, 50 µl of saline was injected after 60 min of baseline

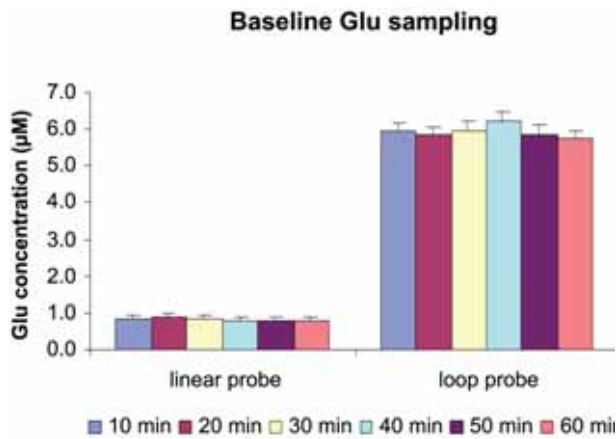


Fig. 2. — Basal glutamate (Glu) levels ($\mu\text{M} \pm \text{SEM}$) in the extracellular fluid of the spinal dorsal horn (left panel, $n = 7$) and in the CSF (right panel, $n = 6$) during 1 hour sampling. The basal concentrations of Glu were significantly different ($p < 0.01$) with two different probes.

sampling and dialysates collected for 90 min. All samples were stored at -70°C for subsequent analysis of glutamate. The concentrations of Glu were analyzed by microbore liquid chromatography with fluorescence detection after precolumn derivatisation with ortho-phthalaldehyde and β -mercaptoethanol, as described previously (15).

Verification of probe positioning

After each experiment, the animals were killed with an overdose of pentobarbital. The catheters were injected with methylene blue and the part of the spinal cord containing the microdialysis membrane was dissected and stored in 4% formaldehyde. The position of the microdialysis membrane in the dorsal horn was confirmed microscopically.

Data analysis

Data are reported as mean value \pm SEM and were analyzed with SPSS 11.5 for Windows. Statistical differences in Glu release in time within one group were assessed by one-way ANOVA for repeated measures. Statistical significance of differences was considered if $P < 0.05$.

RESULTS

Stable baseline levels of Glu ($0.82 \pm 0.09 \mu\text{M}$, $n = 7$) were found in the spinal dorsal horn with the linear probe perfused with modified Ringer's solution at a flow rate of $5 \mu\text{l}/\text{min}$ (Fig. 2, left panel).

Larger baseline concentrations of Glu ($5.96 \pm 0.22 \mu\text{M}$, $n = 6$) were found with the loop probes in the CSF of awake rats (Fig. 2, right panel).

As we applied the LM-3 probe for the first time to the spinal dorsal horn in conscious rats, we further investigated the influence of altering the perfusing fluid and the flow rate on basal Glu release. Decreasing the flow rate from $5 \mu\text{l}/\text{min}$ to $2 \mu\text{l}/\text{min}$ increased extracellular Glu concentrations by $222.7 \pm 27.3\%$. Since microdialysis is a kinetic dialysis process, lower flow rates will result in higher recovery of Glu across the dialysis membrane (Fig. 3, left panel). Perfusion with ACSF reduced baseline Glu levels to $61.5 \pm 9.5\%$, which is compatible with the lower $[\text{Ca}^{2+}]$ in the ACSF (Fig. 3, right panel).

Injection of 5% formalin $50 \mu\text{l}$ at the plantar of the hind paw of the rats resulted in a significant short-lasting increase of Glu ($P < 0.01$) in the spinal dorsal horn. Extracellular Glu levels increased by $154.1 \pm 10.7\%$ during the first 10 min-sample after the injection of formalin (Fig. 4, left panel). A similar profile and time course of increase of Glu release was observed in the CSF with the loop probe microdialysis ($174.7 \pm 10.2\%$ increase at the first 10-min interval, $P < 0.01$, Fig 4, right panel).

Correct probe positioning was obtained in 12 out of 16 operated rats with LM-3 probe in the dorsal horn, and in 11 out of 12 rats with the loop microdialysis probe in lumbar CSF. No significant inflammatory morphologic changes were found around the dialysed area of the spinal cord after 2-3 days following implantation of either probe. Fig. 5 shows the position of the probes.

DISCUSSION

Using two different methods of spinal microdialysis in awake and freely moving animals, stable baseline levels of Glu were found in the spinal dorsal horn and in the lumbar CSF during the 1 hour sampling period. Although the basal concentrations of Glu in the dialysates were significantly different, the profile, time course of the increase of Glu, and the relative change of Glu release induced by formalin remained similar.

The spinal cord microdialysis in the spinal dorsal horn is a useful tool to investigate changes of neurotransmitters release at their precise location (13, 17). However, its complicated surgical preparation and especially the construction of the probe limit its routine use. Each laboratory manufactures its own probes which may contribute to

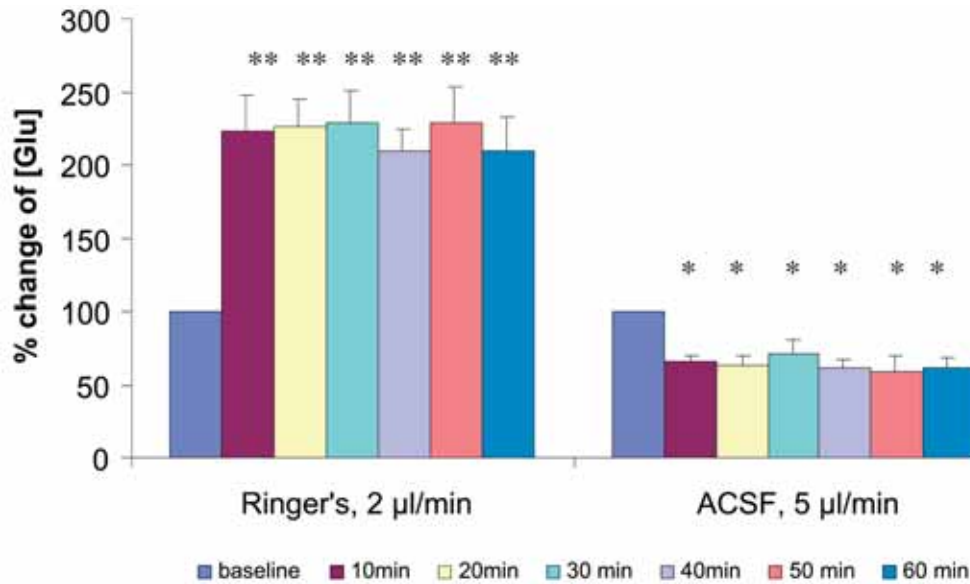


Fig 3. — Change of Glu levels with altering the flow rate (left panel) and composition of perfusion fluid (right panel). Y-axis represents the relative change of Glu concentration compared to the basal concentration (perfusion with modified Ringer's solution at a flow rate of 5 µl/min, first bar) that was set as 100% (n = 4 ; ** p < 0.01 or * p < 0.05 vs. basal value). Ringer's : modified Ringer's solution ; ACSF : artificial cerebrospinal fluid.

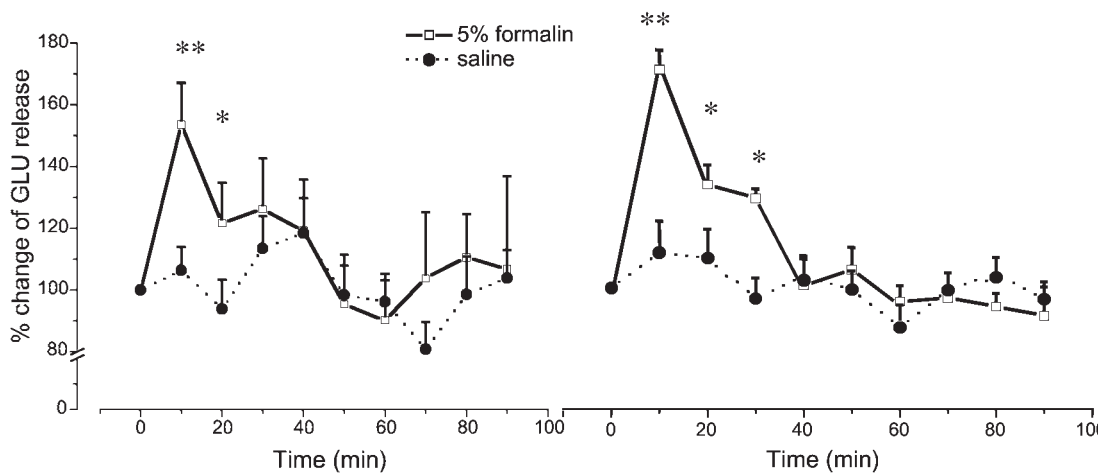


Fig. 4. — Formalin-induced increase of Glu release in the spinal dorsal horn (left panel), and in the lumbar cerebrospinal fluid (right panel). Baseline levels in both formalin and sham (saline) groups were set as 100%. Note the significant increase of Glu following the injection of formalin at time 0 min. **P < 0.01 or *P < 0.05 vs. baseline value (n = 6 in each group).

large inter-laboratory variations (12, 18-20). The linear tissue probe is designed for *in vivo* sampling from peripheral tissues such as the dermis and subcutaneous tissue, and from the liver and other organs (4). The probe consists of a short length of hollow dialysis fiber attached to narrow-bore inlet and outlet tubes. The 3 mm active length of the dialysis membrane allowed it to be located within the spinal dorsal cord. To the best of our knowledge, we are the first to validate this LM-3 linear probe to sample the extracellular fluid of the spinal dorsal horn in awake and freely moving rats. The basal Glu concentrations in most of the tested rats were

lower (< 1 µM) when compared to results from constructed probes (12, 14, 16). Differences in membrane recovery, flow rate, and perfusing solution may account for this. Because the dialysis is a kinetic process, decreasing the flow rate should result in increased extracellular Glu concentrations, which was indeed found in our experiments. Also, the Ca²⁺ concentration in the perfusion fluid is important as shown by the higher Glu release in presence of the modified Ringer's solution compared with artificial CSF. Taken together, reliable measurements of Glu release in the dorsal horn can be obtained with the LM-3 linear tissue microdia-



Fig. 5. — Verification of the probe positioning. A. Linear probe in the spinal dorsal horn ; B. Loop probe in the intrathecal space

lysis probe using the modified Ringer's solution at a flow rate of 5 μ l/min.

The microdialysis of the CSF with a loop probe using the modified method of MARSALA (9) was also set up in our laboratory for comparing the profile of the release of Glu in the CSF. The 4 cm active membrane of the loop probe offered a much larger recovery than the 3 mm one of the linear probe. The triple dialysis catheter permits simultaneous intrathecal drug delivery and dialysis by single catheterization. Because of the minimal surgical preparation and dissection, the recovery of the animal is accelerated, and this technique represents a less traumatic intervention. The basal as well as the evoked releases of Glu assessed with the loop probe are reproducible and consistent with the similar profile and time course of Glu release with the LM-3 probe following formalin injection. Loop probe microdialysis of the CSF provides an effective system to assess the effects of spinally delivered agents on the release of Glu and other neurotransmitters (5, 8). However, a specific limitation of the loop dialysis system is the inability to locate precisely the spinal terminals from which the Glu release occurs. Major advantages of this technique include preservation of spinal tissue integrity and the ability to perform the spinal perfusion concurrently with behavioral assessments in the awake rat.

As mentioned above, a marked increase in Glu concentrations was observed with both approaches at the early phase of the formalin injection but not in the later phase. It is known that paw injection of formalin causes a prolonged activation of primary afferent C-nociceptors, resulting in enhanced activity of nociceptive dorsal horn neurons (central sensitization) (1, 2). Following peripheral tissue injury glutamate as well as other

excitatory neurotransmitters are released from primary afferents and dorsal horn neurons (13, 18). Our results provide kinetic evidence of increased Glu release from the dorsal horn and in the CSF after paw injection of formalin in freely-moving animals.

In conclusion, the microdialysis of the dorsal horn or of the CSF are both effective systems to assess the alteration in Glu release following peripheral nociceptive input. The loop probe system in the CSF is more reproducible for routine investigation of drug effects, whereas the dialysis in dorsal horn provides a useful tool to precisely locate where the release of the neurotransmitters occurs.

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