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Evidence that GABAergic neurons in the spinal trigeminal nucleus are involved in the transmission of inflammatory pain in the rat: a microdialysis and pharmacological study

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Abstract

The aim of this experiment was to investigate the role of the γ-aminobutyric acid (GABA)-ergic transmission in the nociception within the spinal trigeminal nucleus.

The formalin test was used as an animal model of inflammatory pain. Two groups of six rats were used. The behavioural response to the labial injection of formaldehyde (50 μl of a 5% solution) (group 1) or saline (group 2) was evaluated by recording the time spent in facial grooming during a period of 8 min (one period before and seven consecutive periods after the injection). The extracellular concentration of GABA in the trigeminal caudalis nucleus was evaluated, during the formalin test, on samples of 30 μl each (one sample before and three samples after the labial injection) obtained by microdialysis and analysed by HPLC with electrochemical detection of the *o*-phtalaldeyde pre-column derivate. Subsequently, three more groups of six rats each were injected with saline, muscimol (GABAa receptor agonist), or bicuculline (GABAa receptor antagonist) in the trigeminal caudalis nucleus, before performing the formalin test.

The injection of formaldehyde induced a biphasic behavioural response and an increase of the GABA levels at 15–45 min. The injection of bicuculline, but not muscimol or saline, strongly decreased the behavioural response of the formalin test.

These findings suggest that GABAergic neurons in the trigeminal caudalis nucleus are involved in the transmission of nociceptive information.

Keywords: GABAergic neuron; Spinal trigeminal nucleus; Microdialysis

1. Introduction

The neurochemical pathways involved in pain transmission have been largely studied although many points are still unsettled (Conderre et al., 1993; Zieglgänsberger and Tolle, 1993; Mason, 1999). In particular, the major relay for orofacial pain is the subnucleus caudalis of the spinal trigeminal nucleus. The trigeminal caudalis nucleus differs from the other subnuclei of the sensory trigeminal complex, since it is characterized by a marginal layer and a substantia gelatinosa (laminae I and II) resembling the upper cervical dorsal horn with which it is continuous. Primary afferents to this nucleus are glutamatergic (Weinberg et al., 1987; Zieglgänsberger and Tolle, 1993) but the mechanisms for further integration and transmission to other superior nuclei is not well understood. It has been suggested that glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors are down-regulated after the formalin test (Florenzano and De Luca, 1999) and that extracellular glutamate increases during the test (Chiefari et al., 1996; for review, see Yeo, 2002). Another important neurotransmitter is probably γ-aminobutyric acid (GABA), as GABAergic interneurons have been described in the trigeminal caudalis nucleus. These have been suggested to modulate the primary afferent inputs through pre- and post-synaptic inhibition (Ginestal and Carlos, 1992; Almond et al., 1996). Furthermore, it has

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been reported that neurons projecting to the thalamus from the trigeminal caudalis nucleus receive GABAergic input via GABAa receptors (Kondo et al., 1995). Since the thalamus is the relay for cortical (conscient) transmission, the present experiments used the formalin test as an animal model of inflammatory pain, recording the time spent in facial grooming as an estimate of the perception of conscious pain. The formalin test is one of the most widespread models for studying pain. It consists of a subcutaneous injection of a formaldehyde solution which gives rise to an acute direct stimulation of nociceptors, followed by a quiescent period and a subsequent persistent pain due to tissue inflammation (Tjolsen et al., 1992).

The aim of the present study was to evaluate: (i) changes in the GABAergic transmission in the trigeminal caudalis nucleus during the formalin test, estimated by in vivo microdialysis; (ii) modifications of pain perception after injection of a $GABA_A$ receptor agonist (muscimol) or antagonist (bicuculline) into the trigeminal caudalis nucleus.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were, weighting 280–300 g, housed at 20 ± 1 °C and 70% humidity, with a 12:12-h light–dark cycle with lights on from 07:00 to 19:00. Standard laboratory food (Mil Morini, Italy) and water were available at all time. All protocols respected the guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Surgery

Rats were anaesthetized with i.p. penthobarbital sodium (45 mg/kg body weight) and a microdialysis guide cannula (CMA11 guide cannula, Carnegie Medicine) was stereotaxically implanted in the trigeminal caudalis nucleus (11 mm posterior to bregma, 2.6 mm lateral to midline, 6.4 mm deep from the teca; Pellegrino et al., 1979). Rats were given 7–10 days to recover from surgery as judged by recovery of the preoperative body weight. The same cannulas were used for both the microdialysis experiment and the drug injections. In the last case, we inserted a microdialysis probe (CMA/11) in which the tip was cut at half of the membrane length to make a simple injection.

2.3. Microdialysis

The day of the experiment, each rat was placed in a stand for freely moving microdialysis (Carnegie Medicine) and a microdialysis probe (CMA/11, 2 mm membrane length, 6 kDa cut-off) was inserted into the guide cannula. The probe was perfused with artificial cerebrospinal fluid (140 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) at a rate of 2 μl/min. The experiment started 2 h after the insertion of the probe to allow the preparation to stabilize.

2.4. GABA analysis

The dialysate was collected in samples of 30 μl (15 min) each. To each sample, 150 μl of an *o*-phtalaldeyde solution (8 mM in borate buffer pH 10 and 4 μl/ml *t*-butylmercaptan) was added and then injected into an hi-pressure liquid chromatography (HPLC) system (Jasco 880 PU, Japan) after 3 min. A reverse phase column (BAS MF-6213) was used with isocratic perfusion of a phosphate (48 mM, pH 4.9)/acetonitrile (23%) solution. Electrochemical detection (BAS LC-4b with a Uni-Jet cell) was obtained at +800 mV and recorded by a Chromjet Intergrator (Spectra-Physics). The in vivo concentration of GABA was estimated by evaluating the recovery from in vitro microdialysis of a standard GABA solution.

2.5. Experimental procedure

Twelve rats were divided in two groups of six rats for the microdialysis experiment. Microdialysis samples were collected every 15 min (30 μl). After the first sample was collected (basal), 50 μl of a 5% formaldehyde solution (group 1) or physiologic saline solution (group 2) were injected into the upper right lip (omolateral to the microdialysis side) and four more samples were collected.

Eighteen more rats were divided in three groups of six rats for the second experiment. Bicuculline metiodide (70 ng/0.7 μl) (group 1) or muscimol (28 ng/0.7 μl) (group 2) or 0.7 μl vehicle (NaCl 0.9%) (group 3) were injected into the trigeminal caudalis nucleus through the guide cannula. Within 1 min from the microinjection, 50 μl of formaldehyde solution were injected into the upper right lip as previously described. It has already been demonstrated that this dose of muscimol induces analgesia when injected intrathecally (Ueda et al., 1987), so it was expected to have even a stronger effect when injected in a nucleus. We have, in fact, already seen elsewhere that this dose produces effects when injected in vivo (Monda et al., 2000). We choose also a dose of bicuculline that is not convulsant and that induces analgesia when injected intracisternally (Ueda et al., 1987).

During all the experiments, an observer recorded the time spent in facial grooming through a custom software written in LabView (National Instruments) that cumulates the grooming events every 7 min with a resolution of 1 s.

Six more rats were injected into the trigeminal caudalis nucleus with saline (two rats) or muscimol (two rats) or bicuculline (two rats) as described in order to eventually reveal a direct effect of such treatments on grooming behavior.

2.6. Histology

At the end of each experiment, the animals were given a lethal dose of pentobarbital (150 mg/kg body weight). A 3% formaldehyde solution was perfused through the left ventricle and brains were removed and stored in the formaldehyde solution for 24 h. The brains were then equilibrated to 30% sucrose for 1 week to prevent tissue damage during subsequent freezing. Sagittal sections (50 μm) were cut and stained to check the correct position of the probes.

2.7. Statistical analysis

Results are presented as means ± standard error. Statistical significance was evaluated by analysis of variance (ANOVA) (Winer, 1971). Multiple comparisons were performed by the Newman–Keuls post hoc test.

3. Results

3.1. First experiment

The labial injection of formaldehyde caused a strong biphasic grooming response, as expected, after 0–10 and 16–45 min (Fig. 1). The saline injection caused only an initial and short grooming behaviour. ANOVA showed a significant effect for treatment ($F(1,6)=13.96$, $P<0.05$). Labial injection of formaldehyde caused an increase in the GABA concentration after 15–45 min (Fig. 2), while the saline injection had no effect. ANOVA showed a significant effect for treatment ($F(1,6)=6.02$, $P<0.05$). The histological control demonstrated that the trace left by the semipermeable portion of the microdialysis probe was adjacent to the trigeminal caudalis nucleus in all rats (Fig. 3). Fig. 4 illustrates two chromatograms for the analysis of GABA from the microdialysis samples, before and after the formaldehyde injection. The overall time course for each analysis was 10 min. The GABA peak was well resolved with an elution time of 4.25 min. Quantification was obtained by the peak area computed by the Integrator.

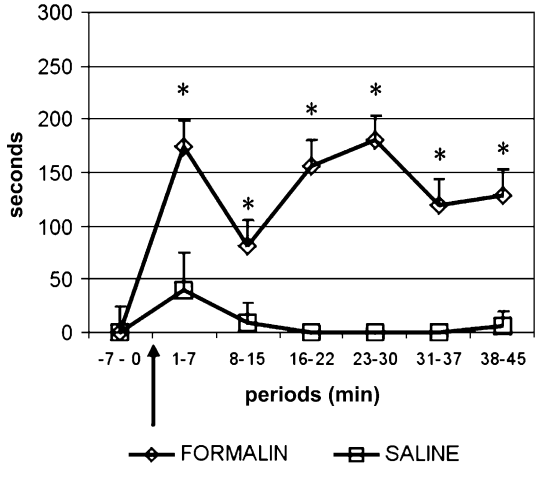

Fig. 1. Time spent in facial grooming before and after labial injection of formalin or saline during the microdialysis experiment. The injections were made at time 0 (arrow). Asterisks indicate statistical difference ($P<0.05$).

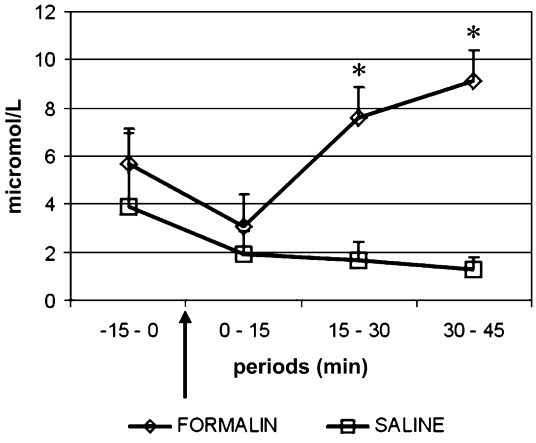

Fig. 2. Extracellular GABA concentrations before and after labial injection of formalin or saline. The injections were made at time 0 (arrow). Asterisks indicate statistical difference ($P<0.05$).

3.2. Second experiment

Fig. 5 shows the time spent in facial grooming during the second experiment (bicuculline vs. muscimol vs. vehicle injection). Bicuculline caused a decrease in the time spent in facial grooming during the entire formalin test while muscimol did not alter the behavioural parameter relative to vehicle injection. ANOVA showed a significant effect with treatments ($F(2,9)=4.37$, $P<0.05$). The post hoc test shows that the group treated with bicuculline differed from the others at any time after formaldehyde injection.

In the additional experiments, we found that neither bicuculline injection alone, nor muscimol nor saline into the trigeminal caudalis nucleus induced any unusual grooming behaviour; the grooming episodes were rare like the

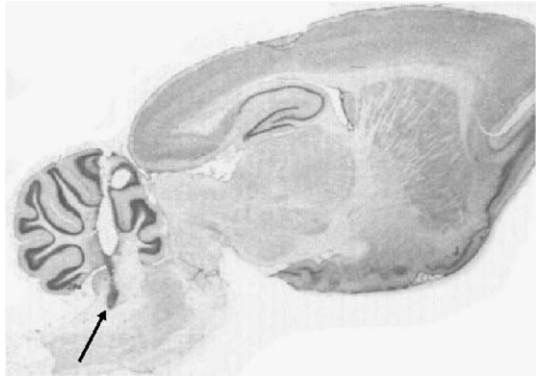

Fig. 3. Sagittal section showing the guide cannula rout reaching the trigeminal caudalis nucleus (arrow).

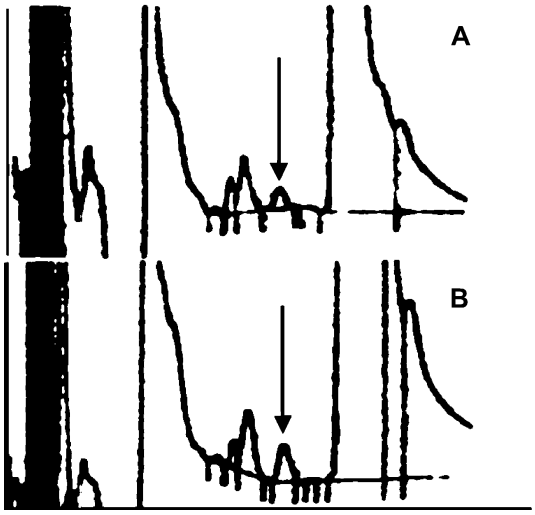

Fig. 4. Chromatograms of basal (panel A) and second sample (15–30 min, panel B) after formaldehyde injection. Eluting time for the GABA peak (arrows) was 4.25 min. The greater area under the peak in the second sample means a greater concentration of GABA after formaldehyde injection.

normal rat behavior during the light phase of the day (data not shown).

4. Discussion

To our knowledge, the findings of this experiment are the first to demonstrate with a direct evidence the increase in GABA in the trigeminal caudalis nucleus during a pain test. We have also demonstrated that the blockade of the $GABA_A$ receptor with bicuculline prevents the behavioural expression of the pain perception, suggesting that GABAergic transmission in the trigeminal caudalis nucleus is involved in signalling persistent painful stimuli to higher nervous centres.

The injection of muscimol, a $GABA_A$ receptor agonist, does not induce a behavioural response. This indicates that the stimulation of $GABA_A$ receptors alone in the trigeminal caudalis nucleus does not simulate the reaction due to the injection of formaldehyde in the labial zone. On the other hand, bicuculline is able to reduce the behavioural response to the formalin test. This suggests that the stimulation of $GABA_A$ receptors in trigeminal caudalis nucleus is necessary, but not sufficient to transmit to superior cerebral structures nociceptive signals from labial zone.

It is already known that treatment with large doses of muscimol suppresses the transmission of incoming signals from primary afferents (Sokal and Chapman, 2003). We used smaller doses of muscimol to verify how strong is the inhibitory effect the GABA receptors in the retransmission functions of the trigeminal caudalis nucleus. If the inhibition of the (re)transmission of pain signals is the unique role played by the GABAergic neurons, than an analgesic effect of muscimol would be expected even at the smallest active doses. Our results clearly demonstrate that the inhibition of the (re)transmission of pain signals is not the unique role played by the GABAergic neurons.

We cannot describe the exact mechanism by which GABA release is increased during nociception, but our results agree with previous anatomical and functional data. GABAergic neurons have been largely described in the trigeminal spinal tract (Ginestal and Carlos, 1992), with a major density in the trigeminal caudalis nucleus. These are interneurons possibly involved in pre- and post-synaptic inhibition of primary sensory afferent. A particular structure in which this function should take place, the synaptic glomerulus, has been described. This synaptic glomerulus contains the primary afferent terminal, axon and dendrites of intrinsic inhibitory interneurons, relay neurons, supraspinal neurons, all of which are partially enveloped within glial cells (Gobel and Hocfield, 1977). The marginal layer of the trigeminal caudalis nucleus projects to the ventro-posterior thalamic nucleus (Burton and Craig, 1979) and it has been demonstrated by immunohistochemistry that these fibers express $GABA_A$ receptors in the trigeminal caudalis nucleus (Kondo et al., 1995). From a functional point of view, it has been reported that: GABA level increases in the fourth ventricle during painful stimuli (Ge et al., 1988); intracisternal bicuculline administration produce potent analgesia (Ueda et al., 1987); GABA transporters expression increases after facial carrageenan injections (Ng and Ong, 2000).

Thus, we can hypothesize that nociceptive stimuli carried by primary afferents into the trigeminal caudalis nucleus can activate GABAergic neurons within the trigeminal caudalis nucleus which in turn can act on fibers expressing $GABA_A$ receptors and projecting to the ventro-posterior thalamic nucleus (Burton and Craig, 1979). The latter will projects to the somato-sensory cortex, thus

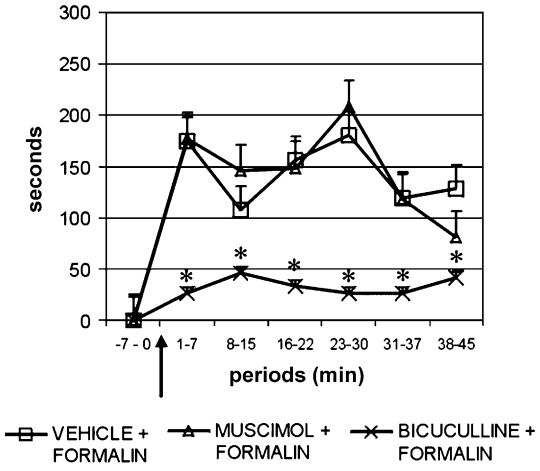

Fig. 5. Time spent in facial grooming before and after labial injection of formaldehyde in rats treated with vehicle or muscimol or bicuculline into the trigeminal caudalis nucleus. All injections were made at time 0 (arrow). Asterisks indicate statistical difference between the bicuculline treated group and other groups ($P < 0.05$).

making the stimulus conscious. According to this model, the blockade of the GABAergic transmission within the trigeminal caudalis nucleus results in an alteration of the retransmitting system that gives the conscious perception of the nociceptive stimulus. It is not possible to explain the lack of effect of the muscimol injection because it is not known, at present, what are the exact functions and the exact interplay between every type of neurons within the trigeminal caudalis nucleus.

The neuronal inhibition produced by GABA does not really preclude a possible role in mediating the ''presence of pain''-information. Such kind of neuronal coding is already know to exist, for instance, in the retina, where the ''presence of light''-information is coded by a decrease in the firing rate of cones and rods (Bear et al., 1996). Another possibility is that the transmission of pain stimuli requires the inhibition of other (perhaps antalgic) circuitries (opioid receptors?). It can be also noted that this counterintuitive ''pro-nociceptive'' role played by the $GABA_A$ receptors has already been seen within the rostral ventromedial medulla (Fields, 1991).

The above data and our findings strongly suggest that GABA is involved in the transmission of pain signals in the trigeminal caudalis nucleus. In perspective, it would be of interest to follow the trend of the GABA level during a longer time course after the formalin test, in order to see how long the increase in GABA is sustained. The sensitivity of the HPLC method used to quantify GABA did not allow to perform the analysis on samples smaller than 30 μl; so it was not possible to collect samples at time intervals smaller than 15 min. This implies that it is not possible to recognize shorter GABA release modification. The 15-min sampling would explain the lack of increase during the first phase of the test.

While the trigeminal caudalis nucleus is classically viewed as the anatomical equivalent of the spinal dorsal horn, it seems the GABAergic neurons play different roles in these regions, as pain transmission seems to be blocked in the spinal cord by GABAergic neurons. Kaneko and Hammond (1997), in fact, reported that intrathecal pre-treatment with bicuculline increases the number of flinches in the interphase and second phase of the formalin test, while intrathecal pre-treatment with muscimol significantly decreases the number of flinches in the first and second phase. On the other hand, Ueda et al. (1987) have also reported that intracisternal (but not intrathecal) pre-treatment with bicuculline has an analgesic effect in the tail pinch test. We can argue that GABAergic neurons could functionally belong to different neuronal populations, arranged in networks, which have different integrating and retransmitting roles. Anatomical and functional differences between the trigeminal caudalis nucleus and the spinal dorsal horn, in fact, have already been focused by other authors (Bereiter et al., 2000).

Actually, a behaviour observation cannot give univocal information about the conscious perception of painful stimuli. In addition, functional alterations of motor pathways, for example, could give rise to a different behaviour. However, no particular alterations in posture or movement were observed after the injection of bicuculine or muscimol. It must also be noted that that the bicuculline solution was injected in an essentially sensitive nervous centre.

Although modern pain therapy already comprises GABA receptor agonists, further studies are needed to better explore doses, types and sites of action of GABAergic drugs that could have antalgic effects.

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