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European Journal of Pharmacology 483 (2004) 233–239
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# Evidence for participation of $GABA_A$ receptors in a rat model of secondary hyperalgesia

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Received 24 April 2003; received in revised form 6 October 2003; accepted 10 October 2003

**Abstract**

We investigated the involvement of endogenous γ-aminobutyric acid (GABA) in the modulation of secondary hyperalgesia induced by intraplantar (i.pl.) injection of 5% formalin in the rat tail-flick test. Intraplantar injection of gabamimetic drugs such as gabapentin (150–600 μg/site) or phenobarbital (20–80 μg/site) reversed secondary hyperalgesia, as measured by an increase in the tail-flick latency, thus displaying a peripheral antihyperalgesic effect. Central inhibition of the secondary hyperalgesia response by gabapentin was obtained following injection of either 200 μg intrathecally (i.t.) or 50 mg intraperitoneally (i.p.). The effects induced by gabamimetics were blocked locally or centrally by prior treatment with the specific $GABA_A$ receptor antagonist, bicuculline (80 ng/paw or 20 ng, i.t.). These data indicate the participation of endogenous GABA in the modulation of secondary hyperalgesia, through either a peripheral and/or a central action. They also indicate that $GABA_A$ receptors might be involved since a specific antagonist of these receptors (bicuculline) blocked this response.


*Keywords:* Hyperalgesia, secondary; $GABA_A$ receptor; Gabapentin; Phenobarbital

## 1. Introduction

A considerable body of evidence has accumulated demonstrating that neurotransmission involving the GABAergic system plays a key role in the modulation of pain pathways (Andrews and Johnston, 1979; Dickenson et al., 1997). In the mammalian spinal cord, the neurotransmitter γ-aminobutyric acid (GABA) is found in high levels in the superficial dorsal horn (laminae I–III) in inhibitory interneurones. These interneurones, functioning through inhibitory GABA transmission, are presynaptic to dendrites, second-order neurones, and nerve endings from Aδ and C fibre afferents located predominantly in laminae II and III (Todd and Mckenzie, 1989; Malcangio and Bowery, 1996). Distinct receptors, namely $GABA_A$ and $GABA_B$, are reported to be involved in this inhibitory modulation (Matsumoto, 1989; Malcangio and Bowery, 1996).

$GABA_A$ receptors consist of a matrix of a complex protein containing multiple recognition sites. They contain a GABA binding site coupled with chloride ($Cl^-$) channels that stabilise the transmembrane potential through an increase in $Cl^-$ conductance. Barbiturates, benzodiazepines, neurosteroids, and ethanol can modulate allosterically this receptor, facilitating GABA-mediated inhibition. $GABA_B$ receptors seem to be coupled to $Ca^{2+}$ and $K^+$ channels via G proteins and second messengers systems. They are activated by baclofen and are resistant to drugs that modulate $GABA_A$ receptors (Matsumoto, 1989; Dirig and Yaksh, 1995; Malcangio and Bowery, 1996).

Gabapentin is a well-tolerated anticonvulsant drug with an as yet unknown mechanism of action (Goa and Sorkin, 1993). Despite structural similarity to GABA, gabapentin does not exert actions at $GABA_A$ or $GABA_B$ receptors, or on either the uptake or degradation of GABA. It is also without effect on glycine, *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (Rock et al., 1993).

In recent years, accumulating evidence has shown that gabapentin is an effective antihyperalgesic agent. In controlled studies, it has been shown that gabapentin given

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0014-2999/$ - see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2003.10.015

locally at the periphery or spinally attenuates nociceptive or pain behaviour following peripheral nerve injury, in both animals and humans (Rosner et al., 1996; Singh et al., 1996; Xiao and Bennett, 1996; Shimoyama et al., 1997; Carlton and Zhou, 1998; Carlton et al., 1999). All these studies were related to inhibitory effects of gabapentin in a primary hyperalgesia model. In addition, gabapentin has been shown to exert anticonvulsant and antinociceptive effects by increasing GABA synthesis or release, which would culminate in GABA receptor activation (Gotz et al., 1993; Petroff et al., 1993).

Methods for measuring secondary hyperalgesia, defined as a pain reaction observed distant from the site where injury is generated, seemed to us a suitable way to assess pain modulation. In this context, the aims of the present work were (1) to assess whether secondary hyperalgesia can be detected in rats injected with intraplantar (i.pl.) formalin and measured by the tail-flick test, and (2) to use this model to investigate the participation of endogenous GABA via activation of $GABA_A$ receptors in the modulation of secondary hyperalgesia, at the spinal and the peripheral level. For this, drugs that potentiate GABA effects, such as gabapentin and phenobarbital, and a specific $GABA_A$ receptor antagonist (bicuculline) were used.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats weighing 160–200 g were used for the experiments. The animals were housed under controlled temperature (23 ± 1 °C) under a 12-h light/dark cycle (0600–1800 h) with food and water ad libitum. Tests were conducted during the light part of the cycle. Before the experiments, the animals were familiarised with the algesimetric apparatus in order to minimise stress. Procedures were in accordance with protocols approved by the local ethics committee (Federal University of Minas Gerais), which in essence follow the ethical guidelines from the International Association for the Study of Pain in conscious animals (Zimmerman, 1983).

### 2.2. Measurement of secondary hyperalgesia

Secondary hyperalgesia is described as a reduced latency, in seconds (s), for the animal to flick its tail in response to a nociceptive stimulation given by i.pl. administration of 50 μl of 5% formalin (Wiertelak et al., 1994). The tail-flick test used in the present study used a slight modification of the procedure described by D'Amour and Smith (1941). In brief, a radiant heat source was applied to the tail of the animals at 3 cm from the tip, and the time (s) taken for the animal to withdraw its tail from the heat source is described as the tail-flick latency. The intensity of the radiant heat was adjusted so that the baseline latencies were between 4 and 6 s. To avoid tissue damage, the cutoff time was established at 9 s. The baseline latency was obtained for each animal before drug administration, and it was determined from the average of three consecutive trials. Subsequent response latencies to formalin injection were measured at 5 and 10 min, and subsequently at 10-min intervals during 60 min of observation. In order to analyse data, a tail-flick index (TFI), as proposed earlier (Tatsuo et al., 1997; Yokoro et al., 2001), was calculated from the following formula:

$$\mathrm{TFI} = \frac{\mathrm{TF\ latency} - \mathrm{BL}}{9 - \mathrm{BL}}$$

where BL is the mean baseline latency (under no treatment), TF (tail-flick) latency is the mean latency at any time following formalin administration, and 9 indicates the cutoff time (maximal time for stimulus exposure).

### 2.3. Assessment of primary hyperalgesia

Primary hyperalgesia measurements were taken as described by Carlton and Zhou (1998) and were based on a behavioural method. For the test, 5% formalin was used for secondary hyperalgesia measurements and the number of spontaneous behaviours described as flinches was counted for 20 min, 10 min after injection, which corresponds to phase 2 in the test. Flinches were characterised by a rapid and repetitive withdrawal of the animal's paw. Control animals were injected with formalin vehicle and monitored as test animals.

### 2.4. Drugs, solutions, and routes of administration

Formalin (at 5%) was prepared from a dilution of formaldehyde (Labsynth, Brazil) with physiological saline (NaCl, 0.9%), and injected (50 μl) into the plantar surface of one of the rat hindpaws (right). Gabapentin (Neurotin®; Parke Davis, USA) and phenobarbital (Gardenal®; Rhodia, Brazil) were used as gabamimetic agents, and bicuculline (Sigma, St. Louis, MO, USA) was used as a specific $GABA_A$ receptor antagonist. The drugs (gabapentin and bicuculline) were diluted in isotonic saline, whereas phenobarbital was diluted in propylene glycol saline, (30:70, vol/vol; Vetec Química Fina, Brazil). The effect induced by the $GABA_A$ receptor antagonist, bicuculline, was assessed by either intraplantar or intrathecal (i.t.) routes of administration (see below) in a volume of 20 μl, administered 10 min before the gabamimetic drugs.

#### 2.4.1. Intraplantar route

A peripheral effect of gabamimetic drugs on secondary hyperalgesia was assessed by their administration in a volume of 20 μl, using a 30-gauge hypodermic needle, in

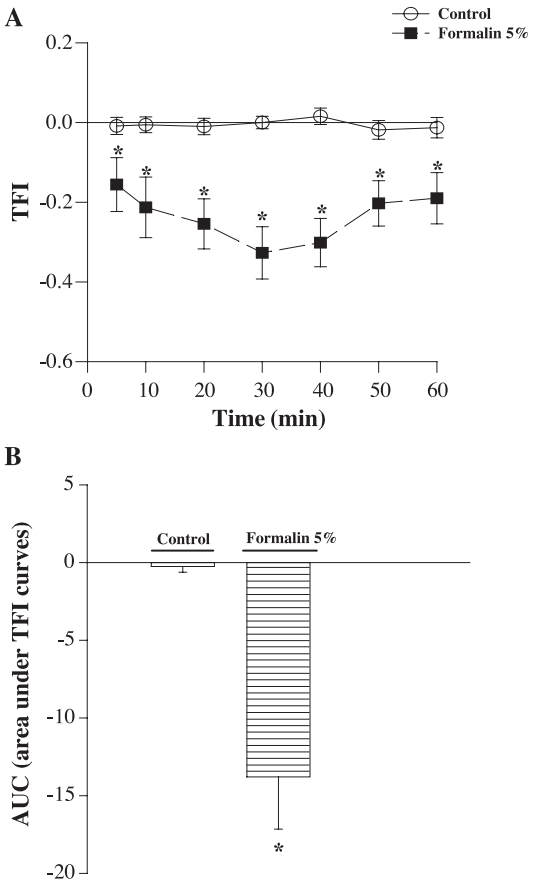


Fig. 1. Reduction by formalin of tail-flick latency, as measured by TFI, indicative of the development of secondary hyperalgesia. Rats were intraplantarly injected with formalin (50 μl of a 5% solution diluted in isotonic saline) at zero time. Control animals received the same saline volume by the same route ($n = 6$/group) at zero time (panel A). Mean TFI ± S.E.M. was obtained as described in Materials and Methods and calculated at the time points depicted. TFI data from formalin and control animals were transformed to AUC by a computer program and are shown in panel B. *Indicates a significant difference between formalin- and control-injected animals ($P < 0.05$, Mann–Whitney $U$ test).

the pad of either the ipsilateral or contralateral paw 10 min before or 5 min following the local injection of formalin.

*2.4.2. Intrathecal route*

To determine the central effect of gabapentin, 20 μl of the drug was administered 10 min before formalin. This injection was made according to Mestre et al. (1994), by holding the rat firmly in one hand by the pelvic girdle and inserting a 30-gauge hypodermic needle in tissues directly between $L_5$ and $L_6$. This method is rapid, reproducible, easy to employ, and unaccompanied by motor impairment, and is used for pharmacological studies of spinal mechanisms, particularly in the pain field.

*2.4.3. Intraperitoneal route*

A systemic effect of gabapentin was assessed using 0.1 ml solution/100 g of animal, which was administered 40 min before formalin.

*2.5. Statistical analysis*

Data are presented either as mean value or mean area under the curve (AUC) ± standard error of the mean (S.E.M). Negative and positive AUC values indicated a hyperalgesic and a hypoalgesic (antihyperalgesic) response, respectively. Statistical analysis was carried out by analysis of variance (ANOVA) using the Mann–Whitney $U$ test for comparison of two groups (control and treatment) and Kruskal–Wallis test followed by the Student–Newman–Keuls test for multiple comparisons for nonparametric data. The significance level was considered when the probability for type I error was less than 0.05 ($P < 0.05$), using Sigma Stat software (1.199 version).

## 3. Results

*3.1. Formalin-induced secondary hyperalgesia*

Injection of 5% formalin into the plantar surface of the rat paw produced a significant decrease in TFI, which expressed a reduction in the latency for the animals to flick their tails from a heat source in comparison with respective controls as early as 5 min following formalin administration ($P < 0.05$, Mann–Whitney $U$ test; Fig. 1, panel A). This reduction in TFI characterised what we here describe as secondary hyperalgesia. The TFI value was further decreased in the next 25 min, attained a maximal level 30 min after formalin injection, and remained significantly reduced for the whole period of observation (60 min). Clear-cut secondary hyperalgesia (negative column) was

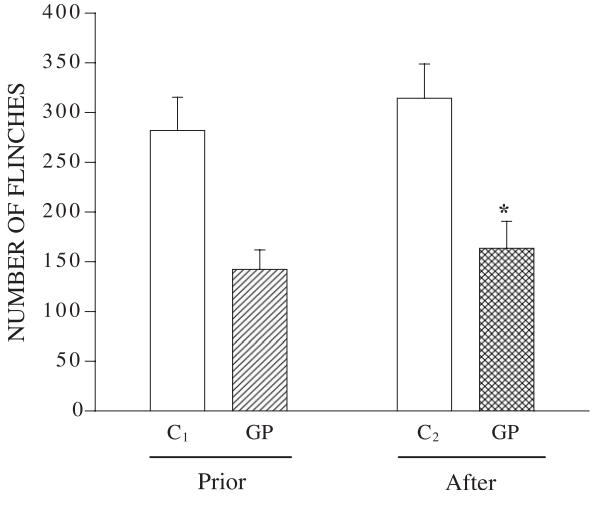


Fig. 2. Reduction of formalin-induced primary hyperalgesia by gabapentin. Data are presented as mean of flinches ± S.E.M. ($n = 6$/group), measured in phase 2 over a 20-min period of the formalin test. Gabapentin (GP, 600 μg/site) was administered intraplantarly 10 min (prior) or 5 min following formalin in the same paw. Control animals received 20 μl of saline. *Indicates a significant difference between formalin- and drug-treated animals ($P < 0.05$, ANOVA followed by Student–Newman–Keuls test).

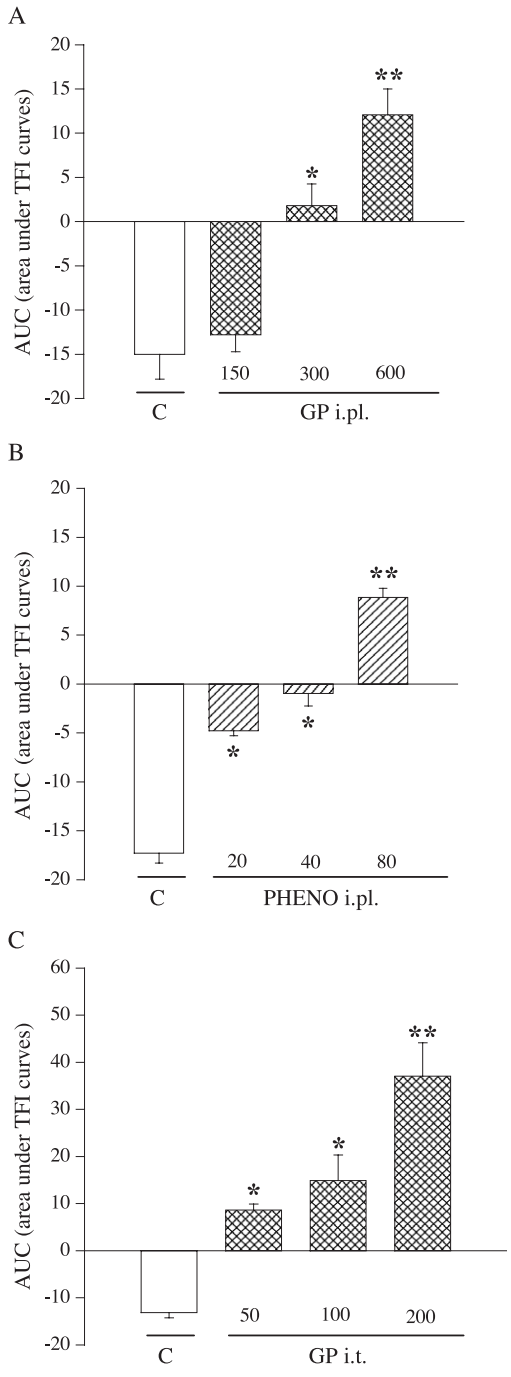


Fig. 3. Dose-dependent inhibition of formalin-induced secondary hyperalgesia by local (i.pl.) or central (i.t.) administration of gabapentin (GP) and phenobarbital (PHENO). Data are presented as AUC + S.E.M. ($n = 6$/group) obtained from TFI distribution over the observation period (60 min). Five percent formalin was prepared and administered as in Fig. 1 (controls, C). Gabapentin was administered in doses varying from 50 to 600 μg/site either intraplantarly (i.pl., panel A) or spinally (i.t.; panel C) 10 min before formalin. Phenobarbital (20–80 μg/site, panel B) was diluted in propylene glycol:saline (30:70, vol/vol) and injected intraplantarly according to the same therapeutic schedule used for gabapentin. *Indicates a significant difference between formalin and drug-treated animals. **Indicates a significant difference compared to other groups ($P < 0.05$, ANOVA followed by Student–Newman–Keuls test).

seen following transformation of TFI values versus time to AUC for formalin and control animals, as presented in panel B from Fig. 1. For this reason, all further data are presented in this format.

### 3.2. Formalin-induced primary hyperalgesia and gabapentin effects

Ten minutes after 5% formalin administration in rat paws, flinches (a behavioural component of formalin-induced primary hyperalgesia) were observed for the whole period of observation (next 20 min), as illustrated by $C_1$ and $C_2$ columns in Fig. 2. Local injection of gabapentin (GP, 600

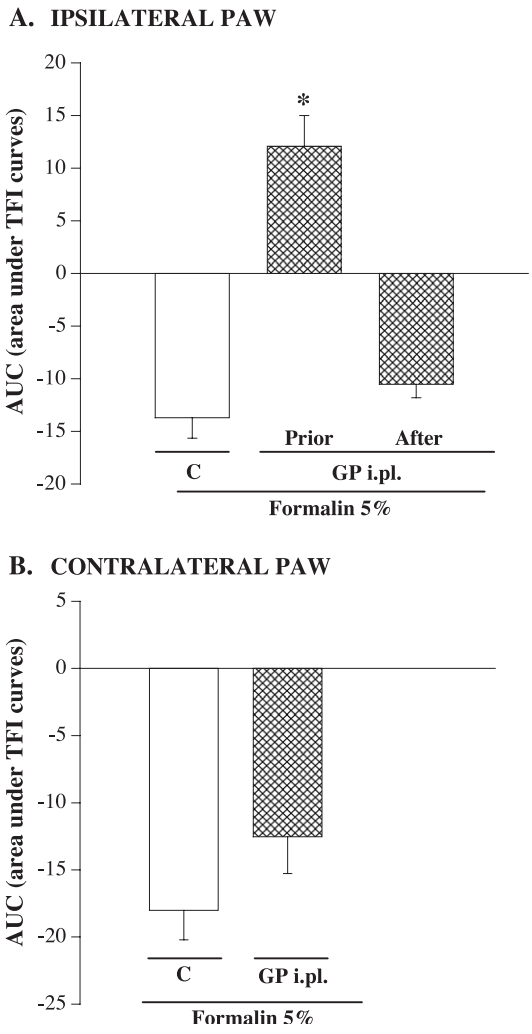


Fig. 4. Dependence of gabapentin effects on site and period of administration in the model of formalin-induced secondary hyperalgesia. Gabapentin was injected into ipsilateral or contralateral paws either before or after formalin (time zero). Gabapentin (GP, 600 μg/site; panel A) or vehicle in 20 μl was injected intraplantarly in the ipsilateral paw either 10 min before or 5 min following formalin administration. In panel B, gabapentin (GP, 600 μg/site) or vehicle was administered in the contralateral paw 10 min before formalin. Columns represent mean AUC ± S.E.M. ($n = 6$/group) for the TFI. *Indicates a significant difference between groups ($P < 0.05$, ANOVA followed by Student–Newman–Keuls test).

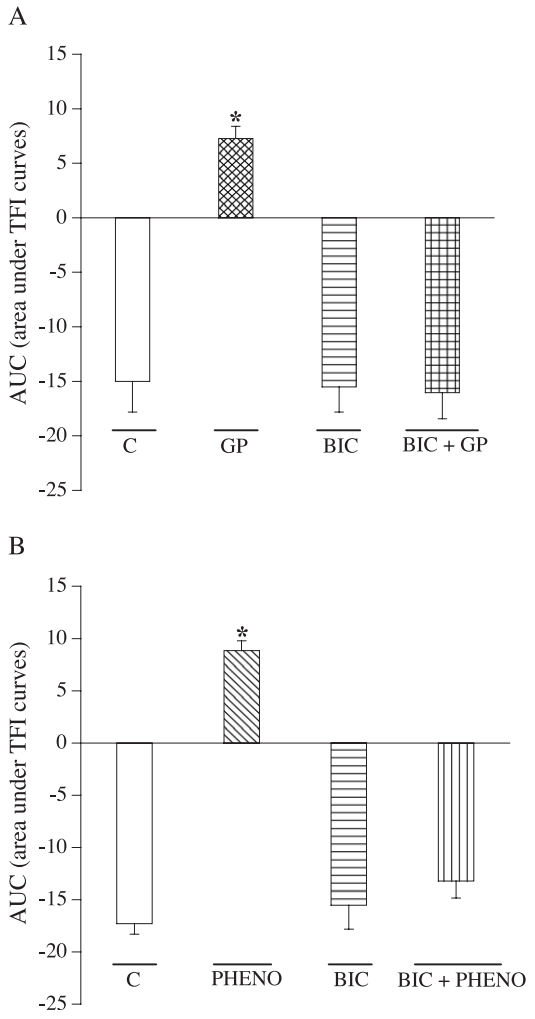


Fig. 5. Reversal by local bicuculline administration of the inhibitory effect shown by gabapentin and phenobarbital in formalin-induced secondary hyperalgesia. Bicuculline (BIC, 80 ng/site) or vehicle was administered in 20 μl intraplantarly (i.pl.) 10 min before gabapentin (panel A) or phenobarbital (panel B). Gabapentin (GP, 600 μg/site) or phenobarbital (PHENO, 80 μg/site) was also administered intraplantarly in a 20-μl volume 10 min before formalin administration. The results are expressed as area under the curve for the TFI and each column represents the mean AUC ± S.E.M ($n=6$). *Indicates a significant difference between control (formalin) and drug-treated groups ($P<0.05$, ANOVA followed by Student–Newman–Keuls test). It is noteworthy that bicuculline per se did not affect the formalin-reduced tail-flick latency, as measured by the AUC.

μg/site), 10 min prior to or 5 min following formalin, significantly reduced the number of flinches, as also shown in Fig. 2 ($P<0.05$, Mann–Whitney $U$ test).

### 3.3. Reversal by gabapentin and phenobarbital of formalin-induced secondary hyperalgesia

Intraplantar injection of gabapentin (150, 300, or 600 μg/site; Fig. 3, panel A) or phenobarbital (20, 40, or 80 μg/site; Fig. 3, panel B) 10 min before injection of formalin dose-dependently reduced negative AUC values or induced positive AUC values, which are characteristic of an anti-hyperalgesic or a hypoalgesic effect, respectively, at the higher doses used. In control animals (hyperalgesia absence), gabapentin did not affect the tail-flick response. In addition, previous intrathecal administration of increasing doses of gabapentin (50, 100, or 200 μg/site) induced progressively positive AUC values, also indicating the development of hypoalgesia in the animals (Fig. 3, panel C). However, in contrast to the effect observed with the prior injection, gabapentin at 600 μg/site administered intraplantarly 5 min after formalin administration did not inhibit secondary hyperalgesia (Fig. 4, panel A). In addition, a previous intraplantar dose of either gabapentin (600 μg) or phenobarbital (80 μg) was effective in preventing the development of secondary hyperalgesia only when administered into the formalin-injected paw, as injection into the contralateral paw did not modify formalin-induced secondary hyperalgesia (Fig. 4, panel B).

### 3.4. Bicuculline effects on the hypoalgesic effect induced by gabapentin and phenobarbital

Ipsilateral intraplantar administration of bicuculline (80 ng/site) 10 min prior to gabapentin (600 μg) or phenobarbital (80 μg) reversed the hypoalgesic effect induced by both drugs (Fig. 5A and B, respectively). Reversal of secondary hyperalgesia was also observed in formalin-injected animals in which intrathecal bicuculline (20 ng/site) administration was followed 30 min later by intraperitoneal injection of 50 mg/kg gabapentin (Fig. 6).

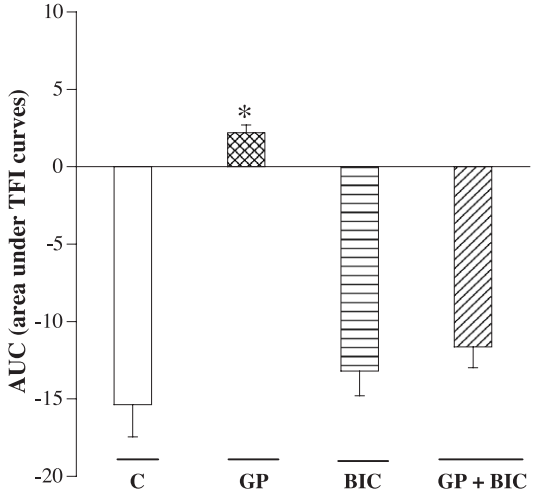


Fig. 6. Reversal by spinal bicuculline administration of the inhibitory effect of systemic injection of gabapentin in formalin-induced secondary hyperalgesia. Bicuculline (BIC, 20 ng/site) or vehicle was administered by the i.t. route in a volume of 20 μl, 10 min before formalin (C). Gabapentin (GP, 50 mg/kg) was diluted in physiological saline (0.1 ml/100 g animal) and administered intraperitoneally 30 min before formalin. Data are shown as AUC for TFI and columns represent the mean AUC ± S.E.M. ($n=6$). *Indicates a significant difference between control (formalin) and drug-treated groups ($P<0.05$, ANOVA followed by Student–Newman–Keuls test).

## 4. Discussion

Intraplantar injection of formalin in rats elicits a biphasic nociceptive behavioural response (Dubuisson and Dennis, 1977), with phase 1 (0–10 min of injection) being associated with activation of primary afferent C fibres, whereas phase 2 (15–30 min) is associated with sensitisation of dorsal horn neurones (Coderre et al., 1993). The present study gives support to the notion that formalin elicits a state of local sensitisation, known as primary hyperalgesia, that extends beyond the injured area (paw), such as that detected in the rat tail (secondary hyperalgesia). Secondary hyperalgesia represents a true hyperalgesic state as the TFI was reduced relative to that at baseline, in agreement with earlier observations of Wiertelak et al. (1994).

Central modulation of pain pathways by GABAergic neurotransmission has been extensively demonstrated (Matsumoto, 1989; Malcangio and Bowery, 1996). In the present study, we demonstrated that drugs that mimic GABA actions at $GABA_A$ receptors, such as gabapentin and phenobarbital, have an antihyperalgesic or a hypoalgesic effect in the model of formalin-induced secondary hyperalgesia. The blockade of secondary hyperalgesia by local administration of these drugs could only be observed if the drugs were administered before, but not after, formalin. These data are supported by those from Coderre et al. (1993), who reported that the initial phase of trauma is critical for the development of secondary hyperalgesia. In contrast, primary hyperalgesia was blocked by gabapentin treatment administered either prior or following formalin, as shown by the results of this study.

Gabapentin, a structural analogue of GABA, has been considered as an alternative treatment for neuropathic pain, as it was shown to reduce allodynia and hyperalgesia in animal models of neuropathic pain (Xiao and Bennett, 1996). The mechanisms underlying gabapentin-induced antinociceptive effects, however, remain unclear. Although gabapentin does not bind to GABA receptors or to any known neurotransmitter receptor (Taylor et al., 1998), some studies have suggested an involvement of gabapentin with the GABAergic system, through an increase in either GABA synthesis or release, which in turn culminates in GABA receptor activation (Gotz et al., 1993; Petroff et al., 1993). Alternatively, data from other studies suggested that gabapentin might bind to a specific site associated with a subunit of $Ca^{2+}$ channels (Gee et al., 1996). According to Gu and Huang (2001), gabapentin produces an increase in NMDA transmission, which increases activity in spinal excitatory and inhibitory neurones, the latter resulting in reduced nociceptive neurotransmission. There is evidence demonstrating that the effects of gabapentin depend on the previous condition shown by the animals, since inhibitory effects can only be detected following the development of inflammation (Field et al., 1997; Shimoyama et al., 1997). In support of this, Stanfa et al. (1997) showed that gabapentin facilitated the firing of convergent neurones in the deep dorsal horn in normal rats, but inhibited C fibre-evoked responses in carrageenan-treated animals.

We observed that administration of gabapentin into the ipsilateral paws, but not into the contralateral paws, dramatically attenuated the development of both formalin-induced primary and secondary hyperalgesia, confirming the findings of Carlton and Zhou (1998) for formalin-induced primary hyperalgesia. These authors demonstrated the potent blockade by gabapentin of sustained nociceptive responses elicited by inflammatory agents. Results similar to those for gabapentin were obtained with local administration of phenobarbital, which is known for its ability to bind to an as yet characterised recognition site in the $GABA_A$ receptor, facilitating GABA actions (Matsumoto, 1989).

As already discussed, several lines of evidence indicate that, in fact, gabapentin is an effective primary antihyperalgesic agent (Field et al., 1997; Ceseña and Calcutt, 1999). Our data extended this concept by showing that gabapentin, besides being an antihyperalgesic effect, also induced a hypoalgesic effect, characterised by the reversal of negative AUC values to positive values. Strikingly, this hypoalgesic effect was also seen when gabapentin was given in the periphery, an effect similar to that observed following specific COX-2 inhibitor (celecoxib, rofecoxib) administration in rat paws (Francischi et al., 2002). Because gabapentin is able to cross the blood–brain barrier (Goa and Sorkin, 1993), it was necessary to determine whether the latter effect was due to a peripheral or a central action. To this end, gabapentin was injected into the contralateral paw and, under such conditions, animals still presented with secondary hyperalgesia, clearly indicating a lack of an antihyperalgesic effect. In line with this, a similar result was obtained with phenobarbital, another gabamimetic agent active at $GABA_A$ receptors. Taken together, we can postulate for the first time the existence of peripheral $GABA_A$ receptors. However, a central modulatory role of $GABA_A$ receptors in secondary hyperalgesia could not be discarded, since gabapentin and phenobarbital also have antihyperalgesic and hypoalgesic effects following intrathecal administration. A corollary for this postulate is that peripheral administration of gabapentin or phenobarbital could constitute an alternative method for pain relief in inflammatory conditions (Stanfa et al., 1997; Ceseña and Calcutt, 1999).

Further support for a peripheral GABAergic modulatory mechanism in pain transmission came from the reversal of the gabapentin- or phenobarbital-induced hypoalgesic effect by local administration of bicuculline, a specific $GABA_A$ receptor antagonist. In addition, intrathecal administration of bicuculline also blocked the effect obtained after systemic administration of gabapentin. The latter results suggest that both peripheral and central $GABA_A$ receptors are involved in the modulation of formalin-induced secondary hyperalgesia.

## 5. Conclusion

The present study demonstrated, through the use of gabamimetics (gabapentin and phenobarbital) and a specific antagonist of $GABA_A$ receptors (bicuculline), the involvement of the GABAergic system in the modulation of secondary hyperalgesia, and in particular the participation of $GABA_A$ receptors. In addition, as gabamimetics and the specific antagonist also acted following administration at the site of injury, we postulate the presence of active $GABA_A$ receptors in the periphery. As the noxious agent used in the present study caused inflammation (formalin), we suggest that gabamimetic drugs may offer a novel therapeutic alternative for the local treatment of inflammatory pain.

## Acknowledgements

This research was supported by FAPEMIG.

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