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Role of PAG in the antinociception evoked from the medial or central amygdala in rats

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ABSTRACT: The effects of stimulating the periaqueductal gray (PAG) against the rat tail flick reflex (TFR) was not changed significantly by the microinjection of lidocaine (5%/0.5 μl) into the medial (ME) or central (CE) nuclei of the amygdala. In contrast, lidocaine into the PAG blocked the effects from the ME or CE. The microinjection of naloxone (1 μg), β-funaltrexamine (2 μg), propranolol (1μg), or methysergide (1μg), but not atropine (1 μg) or mecamylamine (1 μg) into the PAG significantly reduced the effects from the CE. The effect from the ME was not altered significantly by microinjecting naloxone into the PAG. Therefore, the ME or CE are unlikely to be intermediary stations for depression of the TFR evoked by stimulating the PAG, but the PAG may be a relay station for the effects of stimulating the ME or CE. The circuitry activated from the CE, but not the ME, utilises opioid mediation in the PAG. The effect from the CE depends at least on μ-opioid, serotonergic, and probably β-adrenergic mediation in the PAG. © 2001 Elsevier Science Inc.

KEY WORDS: Antinociception, Amygdala, Periaqueductal gray, Stimulation-produced antinociception, Medial nucleus of the amygdala, Central nucleus of the amygdala.

INTRODUCTION

The literature has provided evidence for the participation of amygdaloid nuclei in endogenous antinociceptive mechanisms. Electrical stimulation at the medial or central nuclei of the amygdala in rats reduces the tonic phase of the formalin test, elevates the threshold for vocalisation during or after tail stimulation, and increases the tail flick latency to noxious heat [36]. Electrical stimulation of the central nucleus of the amygdala inhibits the unit discharges of thalamic neurones in response to the stimulation of the splanchnic nerve [55]. Lesions of the amygdala, mainly at its basolateral aspect, the central or medial nuclei attenuate several forms of environmentally induced antinociception [18,21,22,58, 59]. Bilateral lesion of the central nucleus of the amygdala abolishes the antinociceptive effects of low doses of systemic morphine in both the rat tail flick [32] and formalin [33] tests. Many types of agonists, including morphine, carbachol, serotonin, and neurotensin, evoke antinociception in several pain models following injection into the medial or central nuclei of the amygdala [2,17,26,27,41,43,50,58,61]. We have more recently confirmed that brief electrical stimulation applied to the medial or central nuclei of the amygdala evokes antinociception in the rat tail flick test [42]. The effectiveness of stimulating the medial or central nuclei of the amygdala on the latency for the tail flick reflex is indicative that descending pathways from those nuclei can somehow depress spinal reflexes, and the inclusion of these nuclei into current models of descending pain control mechanisms has already been proposed [32].

Few reports are available regarding direct projections from the amygdala to the spinal cord. A sparse population of central nucleus neurons in monkeys [37] and cats [52] projects to the cervical spinal cord. Alternatively, anatomical studies have demonstrated direct reciprocal projections between the amygdala and the mesencephalic periaqueductal gray [7,10,15,30,47, 57]. The stimulation of the periaqueductal gray, which is widely known to be a key structure in descending pain control mechanisms, also suppresses spinally mediated nociceptive inputs (see [8]). The central nucleus of the amygdala have direct and indirect (via hypothalamus) ipsilateral connections with the dorsomedial, lateral and ventrolateral portions of the periaqueductal gray [25,47]. Small group of axons from the medial nucleus of the amygdala courses ipsilaterally through the hypothalamus into the lateral periaqueductal gray and some of them provide a sparse input to the dorsal raphe nucleus [11,15, 47]. On the other hand, the amygdala receives direct and indirect projections from the periaqueductal gray [10,15], mainly from cells found rostrally in its dorsolateral portion and caudally in its ventrolateral portion [47]. More recently, a neural circuit that includes the amygdala, periaqueductal gray and rostral ventromedial medulla has been proposed to play a role in the expression of several forms of hypoalgesia [24].

The present study was therefore undertaken to examine whether the periaqueductal gray participates as a relay station in the antinociceptive mechanisms activated from the medial or central nuclei of the amygdala. The possibility that these amygdaloid nuclei act as alternative stations in the antinociceptive effects of stimulating the periaqueductal gray was also investigated. It is shown that stimulation-induced antinociception from the amygdaloid medial or central nuclei depends on the activation of distinct pathways in the periaqueductal gray.

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MATERIALS AND METHODS

Subjects and Surgery

The experiments were conducted on male Wistar rats (140–160 g) housed two to a cage with free access to food and water and maintained at an average ambient temperature of 24°C with a 12-h light-dark cycle before and after surgery. The proposals of the Committee for Research and Ethical Issue of IASP [62] were followed throughout the experiments. A group of 10 animals was prepared for each experimental sequence, but frequently less than 10 rats had both electrode tract and microinjection site found in the correct position during histological examination (see below). Each animal was used in only one experimental sequence. Each animal was anesthetized with sodium thiopentone (50 mg/kg, intraperitoneal). A teflon-insulated monopolar electrode (o.d. = 0.007″) and a 12-mm long stainless-steel guide cannula (gauge 23) were stereotaxically implanted into the skull to lie in the medial or central nuclei of the amygdala, or ventrolateral periaqueductal gray. The coordinates (in mm) used for implanting the electrode were: AP = 0.6; L = 0.4; H = −5.5, for the periaqueductal gray, AP = +5.8; L = 3.5; H = −3.2, for the medial nucleus of the amygdala, and AP = +5.8; L = 4.4; H = −5.5, for the central nucleus of the amygdala, as proposed elsewhere [28]. These coordinates were also used for guide cannula implantation but the ventral coordinates were changed to allow the guide cannula tip to lay 3 mm above the target structure. The electrode and guide cannula were then fixed to the skull with two steel screws and dental cement. One of these screws was used as the indifferent electrode. After receiving penicillin (50 mg/kg, intramuscular) the animal was allowed to recover for at least one week before the experiments.

Tail Flick Test

The animal was introduced in a ventilated glass tube for period of up to 20 s, with the tail laid across a small wire that was at room temperature (23 ± 2°C). The coil temperature was then raised by the passage of an electric current which was previously adjusted to ensure a tail withdrawal reflex within 2.5–3.5 s. A cut-off time of 6 s was established to minimize the probability of skin damage. Tail flick latencies were measured at 10-min intervals until a stable baseline (BL) was obtained over three consecutive trials. Only rats showing a stable BL after six trials were used in each experiment. To allow easier comparison with former studies [27,41,42], each tail flick latency (TL) was normalized by an "Index of Antinociception" (IA) using the formula: IA = (TL − average BL)/(6 − average BL).

Microinjection Procedures

After baseline tail flick latency determination, drugs or saline were microinjected intracerebrally using a glass needle (70–90 μm, o.d.) protected by a system of telescoping steel tubes as described elsewhere [4]. The assembly was inserted into the guide cannula immediately before the microinjection and the needle advanced to protrude 3 mm beyond the guide cannula tip. The volume of microinjection was 0.5 μl delivered at a constant rate over a period of 3 min, and the needle was removed 20 s after completion of this procedure. The tail flick latency was then determined at 5-min intervals over a period of 10 or 15 min.

Stimulation Procedures

Ten or 15 min after the intracerebral microinjection the animal was placed inside a glass-walled box (20 × 30 × 35 cm). Electrical stimulation (AC, 60 Hz) at the intensity of 35 μA root mean square was applied to the electrode of freely moving rats during 15 (periaqueductal gray) or 30 s (medial or central nuclei of the amygdala) and the tail flick latency determined within 10 s and later repeated at 5-min intervals over a period of 30 min. These current intensities and duration of the stimulation periods for periaqueductal gray [45] or amygdaloid nuclei [42] have been determined previously. During the stimulation period the drop in voltage across a 1-kΩ resistor in series with the electrode was continuously monitored on an oscilloscope. No attempt was made to test for the presence of antinociception during the stimulation.

In some experiments lidocaine (5%/0.5 μl) was microinjected intracerebrally to produce neural block of the periaqueductal gray, medial or central nuclei of the amygdala. Once the purpose of such procedure was to produce neural block of each structure, concentration-effect correlation for the effects of lidocaine was not carried out. Soon after the period of observation, a monopolar electrode was inserted into the guide cannula to reach the site in which lidocaine was previously microinjected (about 45 min after the local anesthetic). Electrical stimulation was then applied to the electrode as described above. Only rats showing no change in the tail flick latency soon after this procedure were considered for further analysis.

Histology

At the end of the experiment Fast green (0.5 μl) dye was microinjected to label the site of injection, the animal was sacrificed with an overdose of sodium thiopentone and perfused through the heart with formalin. Dye spots and electrode tracks were localized on 50-μm serial coronal sections stained with Neutral Red, and identified on diagrams from the atlas of König and Klippel [28]. Rats showing sites in the medial or central nuclei of the amygdala, or ventrolateral portions of the periaqueductal gray were considered for further analysis.

Drugs

A range of drugs was microinjected intracerebrally: lidocaine chloride (5%) was purchased from Merril-Lepetit (Brazil). Atropine sulfate, β-funaltrexamine hydrochloride, mecamylamine hydrochloride, methysergide maleate, naloxone hydrochloride, and propranolol hydrochloride were from Research Biochemicals International (Natick, MA, USA). Except for lidocaine, which was not diluted, the remaining drugs were diluted in saline.

Statistical Analysis

The effects of different treatments on IA were analyzed statistically by multivariate analysis of variance (MANOVA) with repeated measures to compare the groups over all times. The factors analyzed were treatments, time and treatment x time interaction. In the case of significant Treatment × Time interaction a one-way ANOVA followed by the Duncan or Student *t*-tests was performed for each time. The analysis was performed using the statistical software package SPSS/PC+, version 3.0, and the level of significance was set at $p < 0.05$.

RESULTS

Stimulation-produced Antinociception from the Periaqueductal Gray: Effects of Microinjecting Lidocaine Into the Amygdaloid Medial or Central Nuclei

The electrical stimulation of the periaqueductal gray produced a short lasting increase in the tail flick latency (Figs. 1,B). Vocalization, jumping, piloerection, and freezing were occasionally seen during periaqueductal gray stimulation in rats pretreated with saline in the amygdaloid medial or central

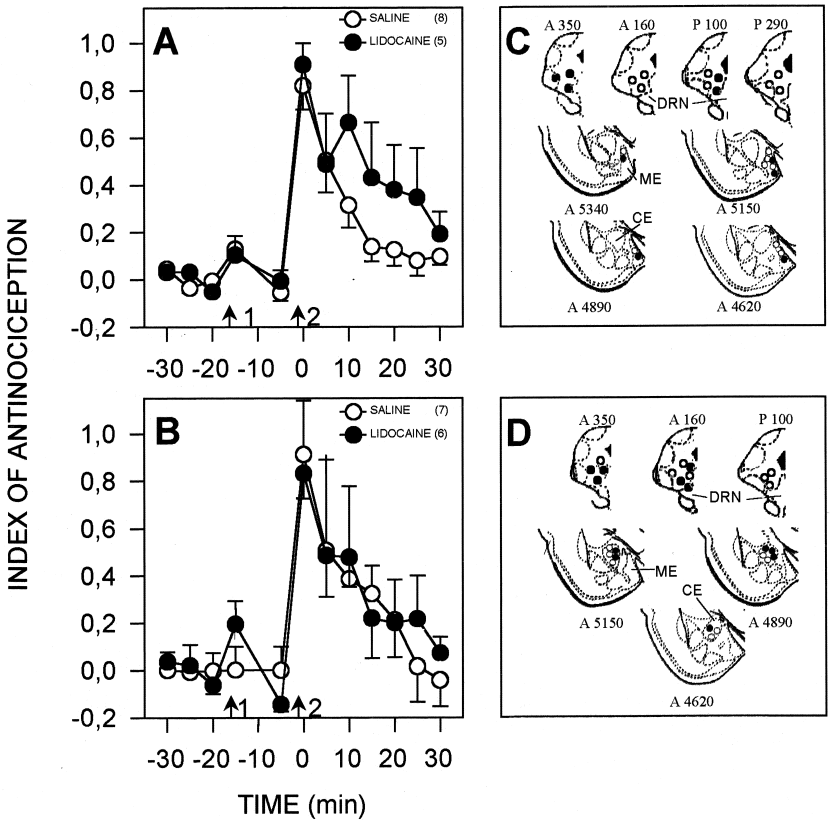

FIG. 1. Effects of the microinjection (arrow 1) of saline (0.5 μl) or lidocaine (5%/0.5 μl) into the medial (A) or central (B) nuclei of the amygdala on the antinociception induced by the electrical stimulation (arrow 2) of the periaqueductal gray (PAG) of rats submitted to the tail flick test. Cross sections taken from the atlas of König and Klippel [28], at the indicated AP levels, show the location of stimulation sites in the ventrolateral PAG and the microinjection sites in the medial (C) or central (D) amygdaloid nuclei. Abbreviations: DRN, dorsal raphe nucleus; ME, medial nucleus of the amygdala; CE, central nucleus of the amygdala. The number of rats is given in parentheses. Data are reported as mean ± SEM for each group of rats.

nuclei. The administration of lidocaine (5%/0.5 μl) into the medial (Fig. 1A) or central (Fig. 1B) nuclei of the amygdala did not change significantly the baseline tail flick latencies or the antinociceptive effect of stimulating the periaqueductal gray. MANOVA indicated that the curves in Fig. 1A did not differ regarding treatments [$F(1,11) = 1.89$; $p = 0.19$] or had significant Treatment × Time interaction [$F(11,121) = 1.37$; $p = 0.19$]. The curves in Fig. 1B also did not differ regarding treatments [$F(1,11) = 0.06$; $p = 0.81$] or had significant Treatment × Time interaction [$F(11,121) = 0.92$; $p = 0.52$]. Although not quantified, the frequency of behavioral manifestations during periaqueductal gray stimulation remained apparently unchanged in rats pretreated with lidocaine in amygdaloid nuclei. Electrical stimulation at the amygdaloid medial or central nuclei conducted 5 min after the period of observation (i.e., 45 min after lidocaine) did not produce latencies significantly different from baseline tail flick latencies (not shown in figures). These results are indicative that the inhibitory effect of microinjecting lidocaine into the amygdaloid nuclei lasts at least 45 min.

The location of the stimulation sites in the periaqueductal gray and microinjection sites in the amygdaloid nuclei is shown in Figs. 1 C,D, respectively.

Stimulation-Produced Antinociception from the Amygdaloid Medial or Central Nuclei: Effects of Microinjecting Lidocaine or Antagonists Into the Periaqueductal Gray

The electrical stimulation of the medial nucleus of the amygdala produced a short-lasting antinociceptive effect in a group of rats in which saline was previously microinjected into the periaqueductal gray (Figs. 2A,B). This effect was completely avoided by the previous administration of lidocaine (5%/0.5 μl) (Fig. 2A), but not naloxone (1 μg/0.5 μl) (Fig. 2B) into the PAG. The data from Figs. 2A,B were analyzed together because only one control (saline) group of rats was used for these experiments. The curves in Fig. 2 did not differ regarding the different treatments [$F(2,21) = 0.72$; $p = 0.50$] but showed a significant Treatment × Time interaction [$F(22,231) = 2.7$; $p < 0.001$]. ANOVA followed by the Duncan test detected a significant difference between saline- and lidocaine-treated rats at time $t = 0$, only [$F(2,24) = 4.25$; $p = 0.02$]. The location of the stimulation (medial nucleus of the amygdala) and microinjection (periaqueductal gray) sites for the experiments with lidocaine and naloxone is indicated in Figs. 2C,D, respectively.

Electrical stimulation of the central nucleus of the amygdala evoked a stronger and longer-lasting effect in a group of rats in

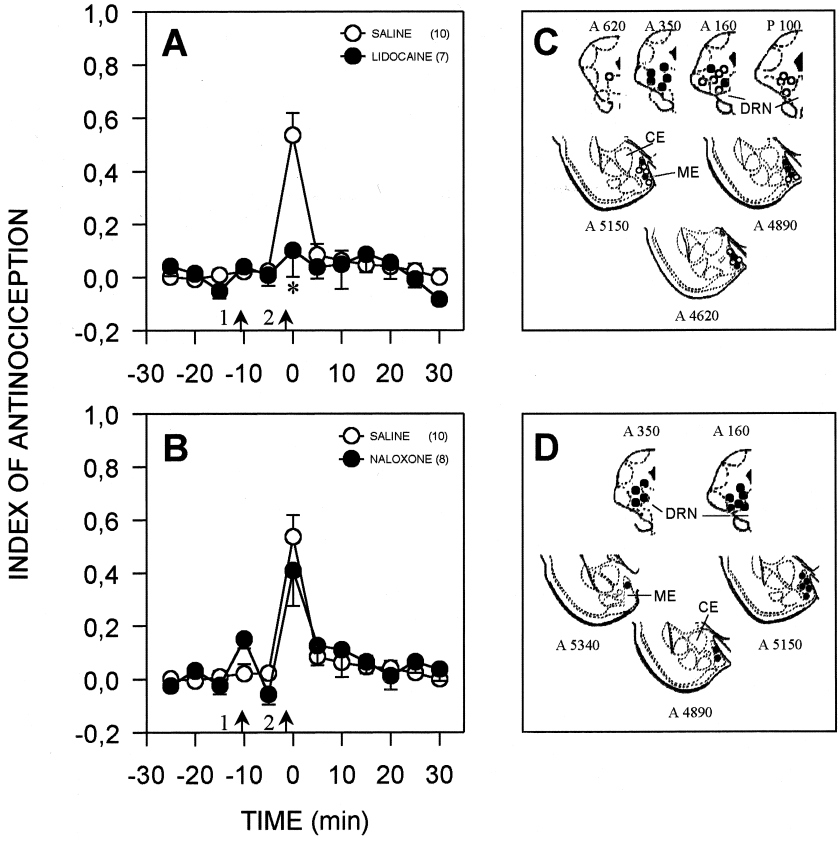

FIG. 2. Effects of the microinjection (arrow 1) of lidocaine (5%/0.5 μl) (A), naloxone (1 μg/0.5 μl) (B) or saline (0.5 μl) into the ventrolateral periaqueductal gray on the antinociception induced by the electrical stimulation of the medial nucleus of the amygdala (arrow 2) of rats submitted to the tail flick test. Cross sections taken from the atlas of König and Klippel [28], at the indicated AP levels, show the location of stimulation sites in the medial nucleus and the sites of microinjection of lidocaine (C) or naloxone (D) in the periaqueductal gray. Abbreviations: DRN, dorsal raphe nucleus; ME, medial nucleus of the amygdala; CE, central nucleus of the amygdala. Control curve (saline) of (A) is the same as on (B) and the location of the corresponding sites in the periaqueductal gray is shown on (C). The number of rats is given in parentheses. Data are reported as mean ± SEM for each group of rats. *$p < 0.05$ vs. control (Student's *t*-test).

which saline was previously microinjected into the periaqueductal gray (Fig. 3). The effect was inhibited by the previous administration of lidocaine (5%/0.5 μl) (Fig. 3A), naloxone (1 μg/0.5 μl) (Fig. 3B), propranolol (1 μg/0.5 μl) (Fig. 3C), or methysergide (1 μg/0.5 μl) (Fig. 3D), but not by atropine (1 μg/0.5 μl) (Fig. 3E), or mecamylamine (1 μg/0.5 μl) (Fig. 3F) into the periaqueductal gray. The data from Fig. 3 were analyzed altogether since only one control group of rats was used for these experiments. The curves in Fig. 3 were significantly different regarding treatments [$F(6,46) = 9.12$; $p < 0.001$] and had significant Treatment × Time interaction [$F(66,506) = 2.25$; $p < 0.001$]. Figure 4 shows the location of the stimulation sites in the central nucleus of the amygdala and the saline or antagonists microinjection sites in the periaqueductal gray. Some rats stimulated in the central nucleus of the amygdala displayed masticatory movements as reported elsewhere [3].

The microinjection of β-funaltrexamine (2 μg/0.5 μl) into the periaqueductal gray was also effective against the antinociception evoked by stimulating the central nucleus of the amygdala 24 h later (Fig. 5 A). The curves in Fig. 5A differ significantly regarding treatments [$F(1,13) = 15.55$; $p = 0.002$] and had significant Treatment × Time interactions [$F(9,117) = 5.76$; $p < 0.001$]. The ANOVA followed by the Student t test detected significant difference between saline and β-funaltrexamine-treated rats at times $t =$ 0 to 20 min. Microinjection of lidocaine or antagonists alone into the periaqueductal gray did not change significantly the tail flick latency. The location of the stimulating (central nucleus of the amygdala) and β-funaltrexamine microinjection (periaqueductal gray) sites is indicated in Fig. 5B.

Electrical stimulation at the periaqueductal gray conducted 5 min after the period of observation (i.e., 45 min after lidocaine) did not produce latencies significantly different from baseline tail flick latencies (not shown in figures). The result is indicative that the inhibitory effect of microinjecting lidocaine into the periaqueductal gray lasts at least 45 min.

Some rats stimulated in the amygdaloid medial or central nuclei had lidocaine microinjected into structures close to but not inside the periaqueductal gray. The data obtained from these cases were then pooled to show that the stimulation of the amygdaloid nuclei was still effective to reduce the tail flick latency (Fig. 6).

After the end of stimulation at the amygdaloid medial or central

nuclei, no gross motor disturbance was detected. The animals

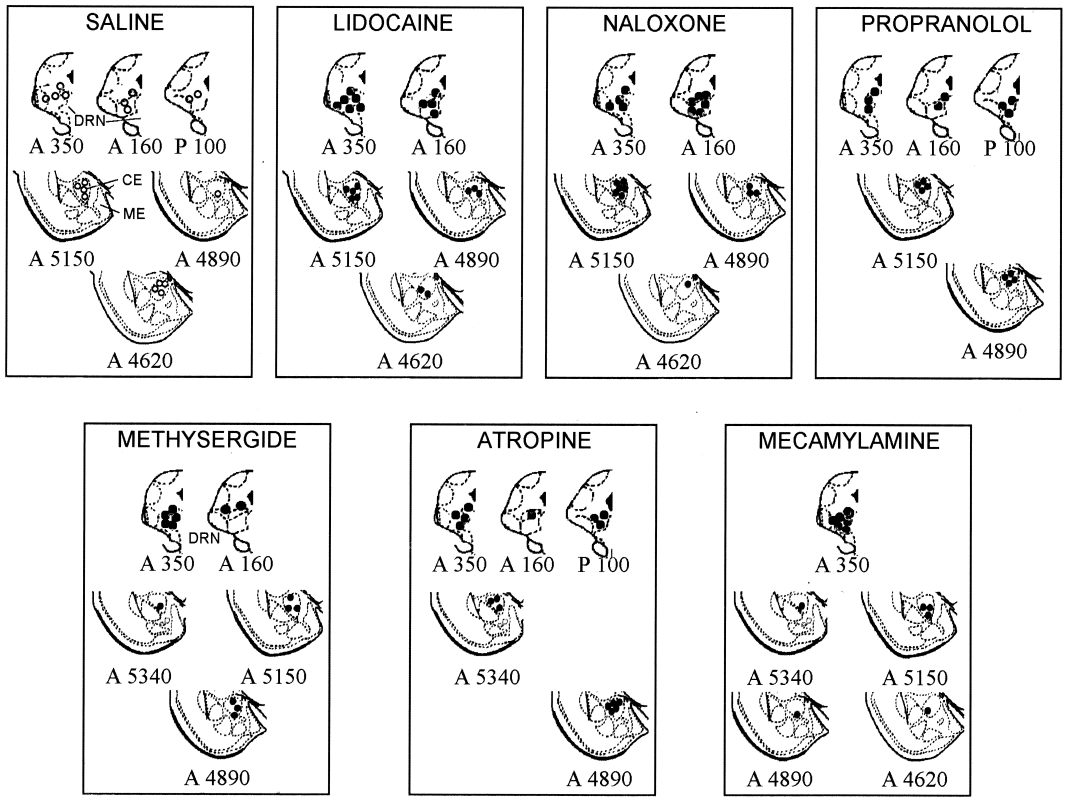

FIG. 4. The location of stimulation sites in the central nucleus and the sites of microinjection of saline, lidocaine or antagonists in the ventrolateral periaqueductal gray. Cross-sections taken from the atlas of König and Klippel [28], at the indicated AP levels. Abbreviations: DRN, dorsal raphe nucleus; ME, medial nucleus of the amygdala; CE, central nucleus of the amygdala.

0.5 mm [51]. In our experiments, neural block produced by the local anesthetic was confirmed in all cases. The antinociceptive efficacy of stimulating the periaqueductal gray during neural block of the medial or central nuclei of the amygdala is therefore indicative that connections from the periaqueductal gray to these nuclei are unlikely to participate in the modulation of the depressive effects of stimulating the periaqueductal gray against the tail flick reflex.

We have also shown that the antinociceptive effects of stimulating the amygdaloid medial or central nuclei was blocked by the previous microinjection of lidocaine into the periaqueductal gray but was unaffected when lidocaine was administered into control misplacements neighbouring the periaqueductal gray, thus indicating that the effects from the amygdaloid nuclei may depend on their connections with the periaqueductal gray. Neuroanatomical studies have shown direct connections from the amygdaloid medial [11] or central [7] nuclei to the periaqueductal gray. Efferents from the medial nucleus sparsely reach the ventral periaqueductal gray in its whole rostrocaudal extension [11]. The main connection from the medial nucleus to the periaqueductal gray, however, is indirect and utilises the medial hypothalamus as an intermediary station [12,31,54]. Inputs from the central nucleus are found in the entire rostrocaudal extension of the periaqueductal gray. They terminate more rostrally in the dorsal, dorsolateral, and ventrolateral portions of the periaqueductal gray, but they are predominantly in the caudal ventral periaqueductal gray [47]. The inhibition of the antinociception evoked from the amygdaloid medial or central nuclei by microinjecting lidocaine into the periaqueductal gray, however, does not allow us to conclude for a direct or indirect connection between the amygdaloid nuclei and the periaqueductal gray.

The antinociceptive effect of stimulating the central, but not the medial nucleus of the amygdala, was significantly inhibited by the previous microinjection of naloxone, a non-selective opioid antagonist, into the periaqueductal gray. Thus, the effect from the central, but not the medial nucleus of the amygdala, seems to depend on the activation of a pathway that utilises opioid modulation in the periaqueductal gray. Moreover, the results also indicate that the antinociception evoked from the amygdaloid medial nucleus is unlikely to be due to current spreading to the central nucleus or vice versa. The microinjection of β-funaltrexamine, a non-reversible μ-opioid antagonist, into the periaqueductal gray also prevented the antinociception evoked from the amygdaloid central nucleus, thus indicating that μ-opioid modulation may be regulating in the periaqueductal gray the stimulation-produced antinociception from the amygdaloid central nucleus. These conclusions agree with an earlier electrophysiological study indicating that inputs from the central nucleus of the amygdala to the periaqueductal gray utilises opioid mechanism [13]. The necessity of periaqueductal gray opioid receptors (δ_2-type, and to a lesser degree, μ-type) for the expression of opioid analgesia elicited from the central nucleus of the amygdala have also been proposed elsewhere [43].

Several other studies support the idea of an involvement of periaqueductal gray opioid mechanisms in the antinociception evoked from the central nucleus of the amygdala. The amygdaloid

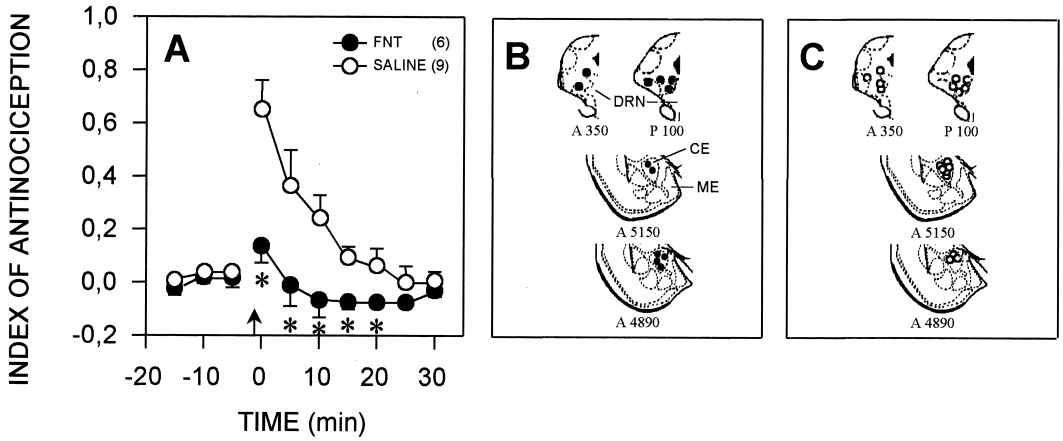

FIG. 5. Effects of the microinjection (arrow 1) of β-funaltrexamine (FNT, 2 μg/0.5 μl) or saline (0.5 μl) into the ventrolateral periaqueductal gray on the antinociception induced by electrical stimulation of the central nucleus of the amygdala (arrow 2) of rats submitted to the tail flick test (A). Cross sections taken from the atlas of König and Klippel [28], at the indicated AP levels, show the location of stimulation sites in the central nucleus and the sites of microinjection of β-funaltrexamine (B) or saline (C) in the periaqueductal gray. Abbreviations: DRN, dorsal raphe nucleus; ME, medial nucleus of the amygdala; CE, central nucleus of the amygdala. The number of rats is given in parentheses. Data are reported as mean ± SEM for each group of rats. *$p < 0.05$ vs. control (Student's *t*-test).

central nucleus directly innervates opioid-sensitive neurones of the ventrolateral periaqueductal gray involved with antinociception [23]. At least three types of opioid receptors (μ- and κ-, and few δ-opioid receptors) were found in the periaqueductal gray [19,34] and the μ receptors seem to be involved with the opioid analgesic function of the periaqueductal gray [16]. Moderate expression of mRNA for μ receptors was predominantly found in the ventrolateral portion of the periaqueductal gray, but the expression may be higher in the caudal periaqueductal gray [34]. Enkephalinergic cells were demonstrated in the amygdaloid central nucleus [38,48] and a direct enkephalinergic connection between the amygdaloid central nucleus and the periaqueductal gray was not discarded [13,56].

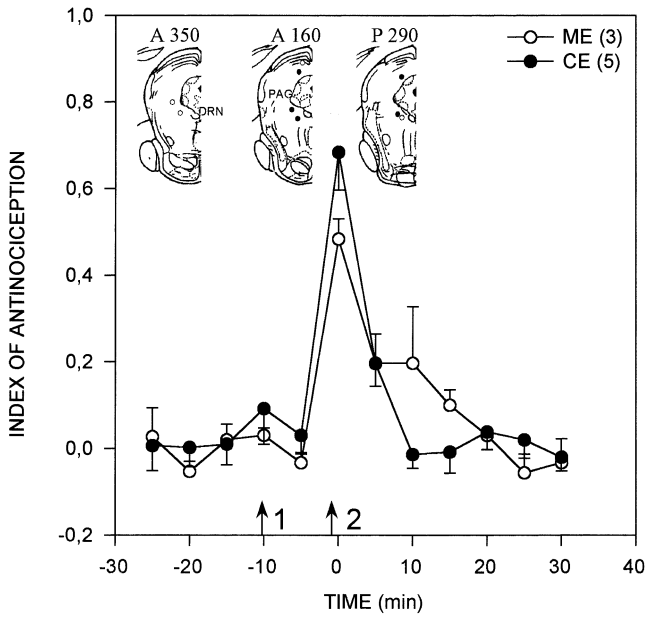

FIG. 6. Effects of the microinjection (arrow 1) of lidocaine (5%/0.5 μl) into sites close to but not inside the periaqueductal gray (PAG) on the antinociception induced by the electrical stimulation of the medial (ME) or central (CE) nuclei of the amygdala (arrow 2) of rats submitted to the tail flick test. Cross sections taken from the atlas of König and Klippel [28], at the indicated AP levels, show the location of microinjection sites. The number of rats is given in parentheses. Data are reported as mean ± SEM for each group of rats.

The effect of stimulating the amygdaloid central nucleus was also inhibited by the previous administration of propranolol or methysergide into the periaqueductal gray but resisted to atropine or mecamylamine used at doses earlier found to be fully effective against carbachol-induced antinociception from the amygdala [41] or periaqueductal gray [20]. Methysergide is a non-selective serotonin antagonist, whereas propranolol, in addition to a non-selective β-adrenergic antagonistic property, also has affinity for a range of serotonergic receptors (see [53]). Therefore, the stimulation-produced antinociception from the amygdaloid central nucleus also depends on a pathway that utilises serotonin, but not acetylcholine, as a modulator in the periaqueductal gray. The results obtained with propranolol, however, do not allow us to rule out that an β-adrenergic mechanism is also involved in the same mechanism. High density of noradrenergic terminals has been demonstrated in the ventrolateral periaqueductal gray [29], and α- and β-adrenergic receptors have already been found in the periaqueductal gray (see [5]). An eventual local anaesthetic effect of propranolol is unlikely to explain its inhibitory effect because this property depends on very high concentration of this drug [44].

In summary, the presented results are indicative that the periaqueductal gray may act as a relay station for the descending pathways activated from the amygdaloid medial or central nuclei. The effectiveness of microinjecting naloxone into the periaqueductal gray against the effect of stimulating the amygdaloid central, but not the medial nucleus, is suggestive that these amygdaloid nuclei connect to the periaqueductal gray via distinct pathways. This conclusion is somewhat reinforced by the recent demonstration that the antinociceptive effects of stimulating the amygdaloid medial nucleus is significantly reduced by the previ-

ous intraperitoneal administration of naloxone, methysergide, atropine, phenoxybenzamine, or propranolol, but not by mecamylamine, whereas the previous systemic administration of naloxone, atropine, or propranolol, but not methysergide, phenoxybenzamine, or mecamylamine, is effective against the antinociceptive effects of stimulating the amygdaloid central nucleus [42].

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