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Journal of Neurochemistry, 2002, **80**, 271–277

Endogenous morphine modulates acute thermonociception in mice

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Abstract

The endogenous synthesis of morphine has been clearly demonstrated throughout the phylogenesis of the nervous system of mammals and lower animals. Endogenous morphine, serving as either a neurotransmitter or neurohormone, has been demonstrated in the nervous system of both vertebrates and invertebrates. As one of the effects of exogenous morphine is the modulation of pain perception, we investigated the effects that the depletion of endogenous morphine had on nociceptive transmission. The immunoneutralization of endogenous morphine from brain extracellular spaces was obtained through the intracerebroventricular administration of affinity purified anti-morphine IgG to mice, which then underwent the hot plate test. Endogenous morphine immunoneutralization decreased thermal response latency and attenuated the anti-nociceptive effect of the mu selective agonist DAMGO in hot plate test suggesting that endogenous morphine is involved in pain modulation.

Keywords: endogenous morphine, gas chromatography-mass spectometry, hot plate test, thermal nociception.

J. Neurochem. (2002) **80,** 271–277.

The presence of endogenous morphine (eM) in various tissues from mammals and lower animals has been proven using highly sensitive techniques such as immunological recognition, liquid and gas chromatographic retention times and mass spectrometry (Gintzler *et al*. 1976; Goldstein *et al*. 1985; Cardinale *et al*. 1987; Stefano *et al*. 1993; Liu *et al*. 1997; Sonetti *et al*. 1999; Goumon and Stefano 2000; Stefano *et al*. 2000). The morphine corresponding HPLC fractions was analyzed by gas-chromatography/mass-spectrometry (GC/MS) and were found to be identical to synthetic morphine by different authors (Donnerer *et al*. 1986; Guarna *et al*. 1998). These studies conclusively proved that morphine alkaloid is endogenous to the mammalian brain.

The pathways of morphine biosynthesis have been established in the oppium poppy and animal tissue starting from L-tyrosine. Coclaurine, tetrahydropapaveroline, reticuline, salutaridine, thebaine and codeine are the main intermediates in the poppy plant, and similar pathways have been either demonstrated or presumed in mammals (Weitz *et al*. 1987). The conversion of salutaridine, thebaine and codeine into morphine has been demonstrated in several rat tissues, including the brain (Donnerer *et al.* 1986). Radiolabeled reticuline is transformed into salutaridine by rat liver microsomes *in vitro* (Weitz *et al.* 1987; Kodaira and Spector 1988) and a similar conversion has been observed *in vivo* (Weitz *et al.* 1987).

Morphine immunoreactivity has been demonstrated inside the cell body, fibers and terminals of neurons in different brain areas of the rat (Bianchi *et al*. 1993), mouse (Gintzler *et al*. 1978) and humans, and has been localized by electron microscopy in nerve terminals forming synaptic contacts (Bianchi *et al*. 1994). The immunohistochemical study of eM distribution in the mouse brain revealed the simultaneous presence of naturally occurring morphine in different parts

Received April 23, 2001; revised manuscript received October 9, 2001; accepted October 13, 2001.

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Abbreviations used: EKC, ethylketocyclazocine; eM, endogenous morphine; i.c.v., intracerebroventricular; GC/MS, gas chromatography/mass spectometry; PBS, phosphate-buffered saline; SIM, selected ion monitoring.

of the pain modulatory system. Endogenous morphine was localized in brainstem regions involved in the descending pain inhibitory pathway, such as midbrain periacqueductal gray matter including nucleus raphe magnus and their major caudal projection target, the rostral ventral medulla complex (Gintzler 1978). Neuron depolarization by high potassium concentrations caused release of eM from eel chromaffin cells (Epple *et al*. 1993) and rat brain slices (Guarna 1998). This effect was calcium dependent.

Although eM meets some of the criteria of mammalian neurotransmitters, at this time, the function of eM in mammalian nervous systems is largely unknown. Many studies examining opiate/opioid functions do not differentiate the effects of endogenous opioid peptides from eM or they examine opiate mediated processes by the application of exogenous morphine. It is therefore important to examine the events which may be modulated by eM, and determine if highly selective processes exist that exclusively involve eM signaling. In this regard, exogenous morphine application and particularly eM, may be involved in the regulation of certain kinds of pain (Donnerer *et al*. 1987). An effect of exogenous morphine is the modulation of pain perception with an increase in the threshold of noxious stimuli (Matthes *et al*. 1996; Elmer *et al*. 1998). In this study we have investigated the effects induced on hot plate test by the *in vivo* immunoneutralization of eM in mice. Affinity purified anti-morphine IgG was injected by intracerebroventricular (i.c.v.) administration into mice (Ohno *et al*. 1988; Kavaliers and Yang 1989; Obal *et al*. 1992; Opp and Krueger 1994; Callahan *et al*. 2000) to determine the effects of the immunoneutralization of eM on thermal nociception.

Materials and methods

Animals

Sexually mature albino male CD−1 mice (Charles River, Italy) weighing 30–40 g were used. They were housed 3–4 per cage at 22°C with a 12-h light/dark cycle and free access to food and water for one week before the experiments. Testing was routinely performed between 09.00 and 15.00 and independent groups of mice were used for each experimental condition. Commercially bottled mineral water, all of the same lot, was used throughout the experiments. Specimens of food and water were randomly sampled and analysed by GC/MS. The procedures used in this study were in strict accordance with legislation on the use and care of laboratory animals.

GC/MS determination of endogenous morphine in mouse brains

Mouse brains were used for GC/MS detection of eM by a previously described method (Guarna 1998). Briefly, the brains were homogenized using a blade blender and then added with a saturated solution of sulfate ammonia and acidified with 1 mL of 3M HCL at 121°C for 30 min (Felby *et al*. 1974). The sample was cooled, filtered through Buchner funnel using a Whatman n°1 filter paper. The obtained fraction was set to pH 9 and submitted to morphine extraction by solid phase on Bond Elut Certify columns (Varian, Harbor City, CA, USA) containing 300 mg of active silica. The eluate was collected in glass tubes to which an external standard consisting of nalorphine solution (250 ng/mL) in methanol was added. The samples were dried under a stream of warm air together with a series of standard solutions containing morphine from 10 ng/mL to 0. 5 ng/mL and nalorphine (25 ng). The dried samples were derivatized (Knapp 1979) by adding of a 20% solution of 50 μL of *N*-methyl-*N*-trimethyl-silyltrifluoroacetamide (Pierce, Rockford, IL, USA) in toluene; 1 μL of each derivatized sample was submitted to GC/MS analysis which was performed using a Hewlett Packard 5890 series II gas chromatograph coupled with a Hewlett Packard 5791 MS detector. The analysis was performed in selected ion monitoring (SIM) mode; derivatized morphine was identified by fragments 429, 414 and 236 and derivatized nalorphine by fragments 455, 440 and 414. The quantifier ion was 429 for morphine and 455 for nalorphine. Previous GC/MS analysis of standard solutions of derivatized morphine and nalorphine validated both the retention times and the characteristic ions for this substance.

Drugs

DAMGO, nalorphine HCl, codeine and DPDPE were purchased from Sigma Chemicals (St Louis, MO, USA). β-endorphin, met-enkephalin, endomorphin-1 and endomorphin-2 from the America Peptide Company (Sunnyvale, CA, USA). EKC was purchased from Sterling-Winthrop (Collegeville, PA, USA).

The purification of anti-morphine antibody

A polyclonal antibody to morphine-6-keyhole limpet hemocyanin was raised in sheep (Biogenesis). After the fractionation of IgGs from the serum with ammonium sulfate, anti-morphine IgG (3 mL) was applied to an affinity column previously prepared by the attachment of morphine 6-hemosuccinate (Simon *et al*. 1972) to CNBR activated 4B Sepharose (Pharmacia) according to the manufacturer's instructions. The column was then eluted with 0.5 M acetic acid, ph 2.7 (Spratt and Jones 1976). The IgGs were separated from normal sheep serum (Sigma) using a purification kit (Bio-Rad Econo-Pac, serum IgG purification kit). The dialyzed concentrated IgGs gave a final protein concentration of 1.5 μg/μL for anti-morphine IgG and 0.4 μg/μL for normal serum. The cross-reactivity of the antibody was characterized by the competitive inhibition of [^{3}H]morphine (35 Ci/mM; Amersham, Milan, Italy) by various unlabeled compounds with anti-morphine IgGs. The compounds assayed were codeine, β-endorphin, met-enkephalin, endomorphin-1, endomorphin-2 and DAMGO. Cross-reactivity was expressed as the quantity of cross-reactive compound that inhibited antibody binding by 50% divided by the quantity of morphine that inhibited antibody binding by 50%.

Anti-morphine antibody cross-reactivity

We generated an anti-morphine antibody for use in our *in vivo* experiments. To demonstrate the specificity of the anti-morphine antibody we measured the cross-reactivity of the affinity purified anti-morphine IgG with other compounds through the competitive inhibition of [^{3}H]-morphine binding. The cross-reactivity of the affinity purified anti-morphine IgG ($IC_{50,compound}/IC_{50\ MF}$) was: morphine (1), codeine (> 9000), DAMGO (> 10 0000), β-endorphin (> 10 0000), met-enkephalin (> 10 0000), endomorphin-1

(> 10 0000), endomorphin-2 (> 10 0000). This demonstrates that our antibody exhibits minimal cross-reactivity to codeine, DAMGO and endogenous opioid peptides.

Habituation procedure

All the animals were previously habituated to the laboratoty according to Abbott *et al*. 1986).

Intracerebroventricular administration

Immunoglobulins were injected in a final volume of 5 μL phosphate-buffered saline (PBS) and the mice were not anesthetized during these procedures. The i.c.v. injections were performed into the right and left cerebral ventricle according to the method described by Haley and McCormick (1957) injecting 2.5 μL in each side of the brain.

Hot-plate test

The test was based on the one described in Eddy and Leimbach (1953). A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mice on the hot plate, which was at a temperature of 50 ± 0.5°C. Here we have used lower temperatures in hot plate test (50°C instead of 54°C) to reveal possible subtle alterations that may occur in basal nociception. The licking latency was measured immediately prior i.c.v. injections, at starting time and at 15 min intervals for 90 min with a 30-s cut-off to prevent tissue damage. The endpoint for the licking response was the first paw lick whether it was lick of the front or rear paw. Anti-nociception was seen as increased latencies to the responses evaluated while increased nociception was seen by shorter latencies. Hot plate sessions were videotaped for later measurement by an observer blind to the treatment.

Motor coordination test

Rota-rod test

Up to five mice were tested simultaneously on the apparatus, with a rod rotating speed of 16 r.p.m. The integrity of motor co-ordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught *et al*. (1985). Their performance was measured before, and at 15, 30 and 45 min after the beginning of the experiments.

Spontaneous activity meter

IgG and PBS pre-treated mice were used. Locomotor activity was quantified using a type-S Animex activity meter (LKB, Farad, Sweden) set to maximum sensitivity. Mice were placed on the meter which transformed their movements into digital signals. Activity counts were made every 15 min for 45 min.

Experiment I – effects of eM immunoneutralization on thermonociception

The administration of exogenous morphine *in vivo* has been shown to induce thermal analgesia in the hot plate in mice (Matthes 1996). Therefore, its counterpart, eM, may be considered to induce an analogous effect. The effects of eM on thermal nociception have been studied *in vivo* by immunoneutralization of the endogenous alkaloid. This was obtained through the i.c.v. administration of affinity purified anti-morphine IgG to mice which then underwent hot plate test.

Independent grsoups of mice ($n = 10$ each) were i.c.v. injected with either PBS, affinity purified anti-morphine IgG or affinity purified normal sheep IgG. The IgGs (0.05 μg, 0.2 μg, 2 μg and 5 μg) were administered 5 min before testing in the hot plate.

Experiment II – GC/MS determination of IgG bound eM in mouse brains

In order to extract the IgG bound eM from the mice brains, the method modified by Takahashi was used (Takahashi and Das 1985). Independent groups of mice ($n = 10$ each) previously administered with anti-morphine IgG (5 μg, 2 μg, 0.2 μg, 0.05 μg, 0.005 μg) or PBS were submitted to hot plate test (experiment I). At the 45th min of the test run (experiment I), the brains were extracted from mice under deep anesthesia, the meninges peeled, the brains minced into small pieces and homogenized at 4°C with 10 volume (wt/vol) of PBS pH 7.2 in the presence of protease inhibitor (2 mM phenylmethylsulfonyl-fluoride; Sigma, Milan, Italy). The homogenates were centrifuged at 1000 *g* for 5 min and the pellets were washed 20 times with PBS, resuspended 1 : 20 in the same medium and dyalized against PBS (50-kDa molecular weight cut-off, Spectra/Por membranes; Spectrum, CA, USA). The IgGs were separated from the dyalized sample using a purification kit (Bio-Rad Econo-Pac, serum IgG purification kit). The eluted IgGs were boiled to separate the antibody from the antigen and centrifuged (30 000 *g*) at 4°C for 45 min. The supernatant was ultrafiltered, lyophilized and analyzed by GC/MS for morphine determination.

Experiment III – reversion of DAMGO, EKC or DPDPE induced analgesia by anti-morphine IgG

In order to study the effects of i.c.v. administration of anti-morphine IgG on the analgesia induced by the μ agonist DAMGO or the κ agonist ethylketocyclazocine (EKC) or the δ agonist [D-Pen2, D-Pen5] enkephalin (DPDPE), immediately before hot plate test, different doses of DAMGO (1–10 mg/kg i.v.), EKC (0.5–10 mg/kg, s.c.) or DPDPE (1–80 μg/mouse i.t.) were injected into PBS or anti-morphine (2 μg) independent groups of mice ($n = 10$ each) i.c.v. injected 5 min before starting time.

Statistics

The data were analyzed with a mixed design repeated measures analysis of variance and post hoc comparisons. A Dunnet multiple comparison test was used to determine the significance of differences in the hot plate response after different treatment with respect to controls.

Controls

The animals which underwent the hot-plate test were submitted to paw temperature measurement 24 h, 10 min and 1 min before testing. The temperature was measured with an infrared thermometer (Omega, Standford, CT).

At the conclusion of the experiments, the mice were anesthetized and their brains excised which were then cut coronally to check the visible path of i.c.v. injection. Only the data from mice in which the injection was correctly located within the ventricles was considered.

Levels of eM were determined in brains extracted from a different group of mice submitted to i.c.v. administration of PBS at the

following times: 15 min before (control), 5 and 30 min after i.c.v. injection.

Results

GC/MS determination

The brain content of eM quantified by GC/MS was 0.31 ± 0.012 ng/g (mean ± SE, $n = 10$; Fig. 1). These levels are similar to those previously reported for the rats (Guarna 1998). No morphine contamination was found in the blank samples, food and water.

Experiment I – effects of eM immunoneutralization on thermonociception

The effects of different *in vivo* pre-treatments on nociceptive threshold in the hot plate test are reported in Fig. 2. The i.c.v. injection of 5 and 2 μg anti-morphine IgG resulted in a highly significant decrease in licking latency with respect to the control injection of PBS. Maximum nociceptive effects were evident at 45 and 60 min after the starting time. Lower doses (< 0.2 μg) lacked a hyperalgesic effect. In contrast, pre-treatment with normal sheep IgGs at the same concentrations did not produce any appreciable change in licking latency compared to the controls (Fig. 3).

Rota-rod test

No significant differences were observed in the number of falls at different times between PBS and IgG pre-treated mice.

Spontaneous activity meter

No difference in the activity counts of IgG pre-treated and control mice (i.c.v. PBS) was detected. Therefore, no motor disturbances were observed in either test.

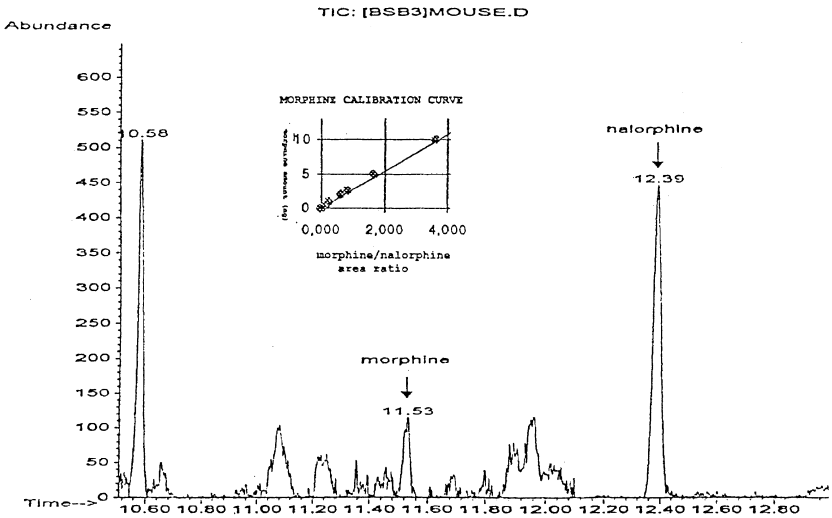

Fig. 1 Chromatographic plot of mouse brain morphine content [derivatized morphine retention time (RT) = 11.53; derivatized nalorphine RT = 12.39]. In the middle, a morphine calibration curve that was constructed by plotting the morphine/nalorphine peak area ratio (X axis) versus the quantity of morphine (Y axis).

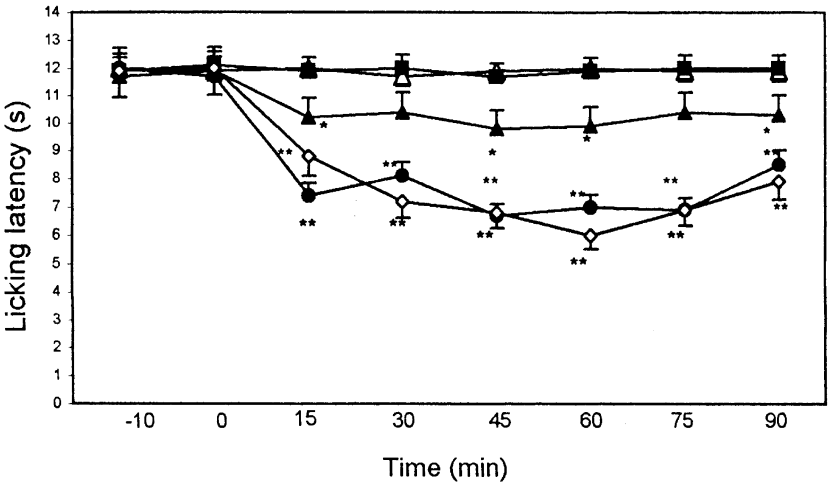

Fig. 2 Hot plate test-latencies for nociceptive responses in the hot plate test in mice pre-treated with either PBS, anti-morphine IgGs or or normal sheep IgGs. **Significant statistical difference at $p < 0.01$ versus PBS. *Significant statistical difference at $p < 0.05$ versus PBS. Vertical bars are the corresponding SEM. △, PBS; ■, anti-morphine IgG (0.05 μg); ▲, anti-morphine IgG (0.2 μg); ●, anti-morphine IgG (2 μg); ◇, anti-morphine IgG (5 μg).

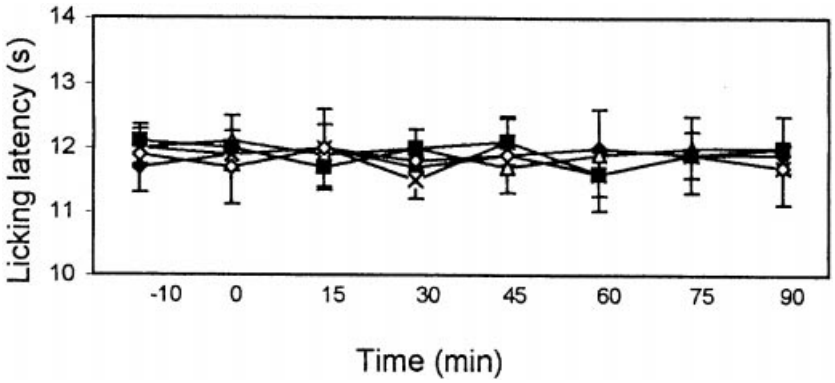

Fig. 3 Hot plate test-latencies for nociceptive responses in the hot plate test in mice pretreated with either PBS or normal sheep IgGs. Vertical bars are the corresponding SEM. ◆, PBS; △, normal sheep IgG (0.05 μg); X, normal sheep IgG (0.2 μg); ◇, normal sheep IgG (2 μg); ■, normal sheep IgG (5 μg).

Skin temperature

The skin temperature of the paws remained unchanged after microinjection of IgG and PBS ($\alpha < 0.05$ all conditions, data not shown).

Endogenous morphine levels in brain before and after PBS i.c.v. administration

The brain content of eM quantified by GC/MS at different times after PBS central injection (Table 1) did not show any significant differences with respect to controls (15 min before administration).

Table 1 GC/MS determination of eM levels in mouse brains before and after PBS central injection

15 min before (ng/g brain tissue)	5 min after (ng/g brain tissue)	30 min after (ng/g brain tissue)
0.29 ± 0.02	0.33 ± 0.04	0.30 ± 0.03

Each value of the columns represents the brain content of eM quantified by GC/MS and expressed as mean value ± SEM from groups of 10 mice each.

Table 2 GC/MS determination of IgG bound eM from mice which were administered previously with increasing doses of anti-morphine IgG and submitted to the hot plate test

IgG-bound eM (pg/g)	Licking latency (s)	Anti-morphine IgG dose (μg)
543	6.9 ± 0.6**	5
536	7.1 ± 0.8**	2
190	10.3 ± 1.1*	0.2
105	12.1 ± 1.2	0.05
nd	11.9 ± 0.9	0.005

Each value of the licking latency column represents the mean licking latency measured at 45 min which corresponds at the maximum nociceptive effect. At the end of the 45 min licking latency measurement, the brains were extracted from the mice in order to determine IgG bound morphine. nd, not detectable; **significance level of 0.01; *significance level of 0.05.

Experiment II – GC/MS determination of IgG bound em in mouse brains

Immunoglobulin bound eM was extracted and determined by GC/MS in the brains of mice which were subjected to the hot plate test after anti-morphine IgG administration. The results are reported in Table 2. In the brains (Table 2) IgG bound eM appeared to be correlated with licking latency. The hyperalgesic effect increases with the quantity of eM bound by anti-morphine IgG previously i.c.v. administered to mice. Higher doses of anti-morphine IgG (2–5 μg) reached the maximum hyperalgesic effect. At higher doses of anti-morphine IgG, the quantity of eM bound to anti-morphine IgG was constant; therefore it can be surmised that the unbound antibody does not modify the hyperalgesic effect. No eM bound IgGs were detected in the brains from the mice which had undergone the hot plate test after the i.c.v. administration of PBS. Therefore, it can be concluded that anti-morphine antibodies which behave as antagonists *in vivo* do not naturally occur in mouse brain.

Experiment III – reversion of DAMGO, EKC or DPDPE induced analgesia by anti-morphine IgG

Administration of anti-morphine IgG to mice induces a right shift of the DAMGO dose-effect curve in hot-plate test (Fig. 4a). The value of DAMGO ED_{50} significantly increases when anti-morphine IgG (2 μg) was previously administered to mice. No significant right shift of the dose-effect curve was obtained after EKC or DPDPE administration (Fig. 4b–c).

Discussion

The present study demonstrates the presence of naturally occurring morphine alkaloid in the mouse brain at concentrations similar to those found in the rat brain (Donnerer 1986; Guarna 1998). The morphine corresponding HPLC fractions were analyzed by GC/MS and found to be identical to synthetic morphine.

The i.c.v. administration of affinity-purified anti-morphine IgG which exhibited minimal cross-reactivity with codeine and opioid peptides, induced a hyperalgesic response in mice

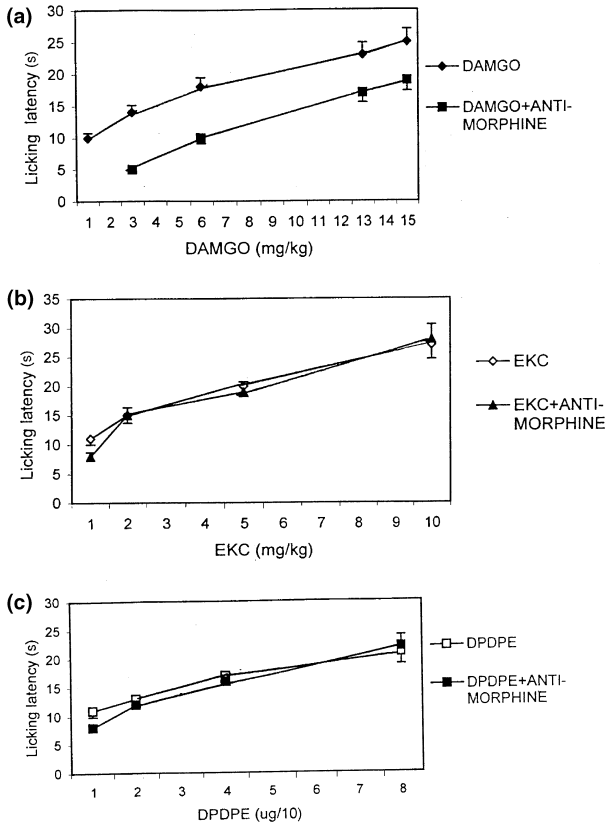

Fig. 4 Anti-nociceptive responses induced in hot plate test (paw lick latencies) in mice after administration of DAMGO and DAMGO plus anti-morphine IgG (a), EKC and EKC plus anti-morphine IgG (b), DPDPE and DPDPE plus anti-morphine IgG. (c) The paw licking latencies are expressed as mean value ± SEM from groups of 10 mice each and were measured at the time which corresponds to the maximum hyperalgesic effect, that is 30 min after DAMGO or EKC and 15 min after DPDPE administration. The estimated values of DAMGO ED_{50} were: 3.4 ± 0.6 mg/kg for DAMGO and 6.97 ± 1.2 for DAMGO plus anti-morphine IgG (Graph-Pad InPlot, Graph-Pad Software, San Diego, USA).

subjected to thermal nociception. As shown by the licking latency in the hot plate test, the nociceptive threshold in response to thermal stimulus was significantly lowered by anti-morphine IgG with respect to controls. The hyperalgesic effects obtained after anti-morphine IgG pre-treatment correlate with the quantity of eM bound to the anti-morphine IgG extracted from the brains of mice previously i.c.v. injected with different doses of anti-morphine IgGs. At the highest anti-morphine IgG doses tested, the maximum hyperalgesic effect was reached whereas the quantity of eM extracted from the immune complex did not change at these doses. The observed hyperalgesic effect increased with the quantity of eM bound to the antibody, whereas it did not appear to be modified by higher doses of anti-morphine IgGs. The levels of morphine endogenously present in the mouse brain might be considered comparable to a subcutaneous administration of 30 μg/kg exogenous morphine according to a 2–3% brain uptake of the intravenous morphine dose (Glare and Walsh 1991) and a 70% bioavailability (Miyamoto *et al*. 1993). This dose appeared to be equipotent in eliticing inhibitory/analgesic and exitatory/hyperalgesic effects (Crain and Shen 2001). As reported in the findings by Shen and Crain (2001), morphine injected at low doses, that is less than 30 μg/kg, induces a hyperalgesic response mediated by the activation of opioid receptor coupled G_s protein (Crain and Shen 1998). This effect appears to be dose dependent. Therefore, the hyperalgesic effect we observed in the hot plate test after eM immunodepletion might be ascribed to a decrease in eM levels present in the mouse brain extracellular spaces with respect to the basal morphine level which is maintained by the continuos release of eM (0.1–0.5 ng/g/min, Guarna 1998) by nerve terminals. No change was induced by non-immune sheep IgG pre-treatment at different doses thereby demonstrating that higher protein levels do not affect nociceptive response. No naturally occurring anti-morphine antibodies could be detected in the control mice when administered with PBS. Endogenous morphine immunoneutralization was found to significantly attenuate the anti-nociceptive effect of DAMGO, a μ selective agonist, as showed by the right shift of the DAMGO dose-effect curve. In contrast to DAMGO, no significant right shift of the dose-effect curve was obtained when different doses of the selective κ agonist EKC or of the selective δ agonist DPDPE were administered to mice previously treated with anti-morphine IgG. Therefore κ and δ receptors might not to be involved in the anti-morphine IgG effect.

Endogenous morphine is present (Gintzler 1978; Bianchi 1993, 1994) in the mammalian brain regions which are involved in the modulation of pain transmission (Willis and Westlund 1997) where opioids and opiates act by modulating the descending inhibitory pathway of nociceptive transmission (Fields *et al*. 1991). These findings, taken as a whole, suggest that eM may play a role in the modulation of thermal nociception.

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