Effects of endogenous morphine deprivation on memory retention of passive avoidance learning in mice

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Abstract

Memory and the processes of learning in mammals are well known to be affected by opioid agonists such as morphine, which has been proven to interfere and cause amnesia. The presence of endogenous morphine has been demonstrated in various tissues from mammals to invertebrates. In this study, we have investigated the effects caused by in-vivo immunodepletion of endogenous morphine on working memory under different experimental conditions. When mice were submitted to fasting, a stress condition, acquisition and consolidation of memory were significantly impaired compared to controls. This was demonstrated by a decrease in entry latency into the dark room in the retention session of the passive avoidance test. This effect was significantly reversed to baseline values when endogenous morphine was depleted from the extracellular brain space. These findings support a role for endogenous morphine in weakening memory processes under stress conditions.

Introduction

Endogenous morphine (*eM*)

The presence of eM has been demonstrated in various tissues from mammals and lower animals using highly sensitive techniques such as immunological recognition, liquid and gas chromatographic retention times and mass spectrometry (Cardinale et al., 1987; Donnerer et al., 1986; Gintzler et al., 1976; Goldstein et al., 1985; Goumon and Stefano, 2000; Guarna et al., 1998; Stefano et al., 1993, 2000; Sonetti et al., 1999). eM corresponding HPLC fractions were analysed by gas chromatography/mass spectometry (GC/MS) and were found to be identical to synthetic morphine (Donnerer et al., 1986; Guarna et al., 1998). Major morphine precursors, i.e. reticuline, thebaine, codeine, etc. originally found in the poppy plant have now been identified in mammals (Donnerer et al., 1986; Epple et al., 1994; Kodaira et al., 1988; Weitz et al., 1987; Zhu et al., 2003). Furthermore, morphine-like immunoreactivity has been demonstrated in cell bodies, fibres and terminals of neurons in different brain areas of the rat (Bianchi et al., 1993), mouse (Gintzler et al., 1978) and man (Bianchi et al., 1994). We also have demonstrated that eM can be released in a chemically detectable form from rat brain slices in a Ca^{2+}- and K^+-dependent manner, suggesting a role for eM as a neurotransmitter or neuromodulator in the mammalian CNS (Guarna et al., 1998).

Working hypothesis

Memory and the process of learning in mammals are affected by opioid agonists, which have been proven to interfere with these processes and to produce amnesia. Several studies also have reported facilitation

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of memory retention by opiate antagonists, primarily naloxone, in normal animals in a variety of tasks, such as passive avoidance (Izquierdo, 1980). In mice and rats submitted to the passive avoidance test, the systemic administration of exogenous morphine impairs memory processes (Castellano, 1975; Izquierdo, 1980); therefore, a similar role might be hypothesized for eM.

Purpose of the study

In this study, fed and stressed animals were both submitted to the passive avoidance test; we used 12 h food deprivation (fasting) to induce a stress condition because it does not interfere with motor coordination, spontaneous motility and exploratory activity. We investigated this effect on the passive avoidance test by in-vivo depletion of eM in fasted mice. This method was previously used to demonstrate the effects of eM deprivation on acute thermonociception in mice (Guarna et al., 2002) and is based on immunoneutralization of eM from brain extracellular spaces through the intracerebroventricular (i.c.v.) administration of affinity-purified anti-morphine (anti-MF) IgG. Anti-MF IgG administration binds eM, inducing a decrease of free morphine present in extracellular brain spaces making eM unavailable to opioid receptors (Guarna et al., 2002).

Materials and methods

Animals

Sexually mature male Swiss albino mice (23–30 g) (Charles River, Como, Italy) were used. They were housed 3–4 per cage at 22 ± 1 °C with a 12 h light/dark cycle and free access to food and water for 1 wk before the experiments. The cages were placed in the experimental room 24 h before the test for acclimatization. Commercially bottled mineral water, all from 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 the guidelines of the European Community Council legislation on the use and care of laboratory animals.

GC/MS determination of eM in mouse brain

Brains from normally fed and fasted mice that may or may not have been administered with anti-MF IgG, were used for GC/MS detection of eM by a previously described method (Guarna et al., 1998). Briefly, the brains were homogenized using a blade blender and then combined with a saturated solution of sulphate ammonia and acidified with 1 ml of 3 M HCl at 121 °C

for 30 min (Felby et al., 1974). The sample was cooled and filtered through a Buchner funnel using Whatman No. 1 filter paper. The fraction obtained was set to pH 9 and then 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 addition 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 by selected ion monitoring (SIM) mode; derivatized morphine was identified by fragments 429, 414 and 236. Derivatized nalorphine was identified 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 did validate both the retention times and the characteristic ions for this substance.

I.c.v. injection technique

I.c.v. administration was performed under ether anaesthesia, according to the method of Haley and McCormick (1957). During anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter hypodermic needle, attached to a 10 μl syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl was then administered. The injection side was 1 mm to the right or left from the midpoint of a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricles, some mice (20%) were injected with 5 μl of 1:10 diluted India ink and their brains examined macroscopically after sectioning. The accuracy of injection technique evaluated as a percentage of correct injection was 95%.

Passive avoidance test

Passive avoidance learning and retention were assessed using a step-through paradigm modelled

according to Jarvik and Koop (1967). The apparatus consisted of two chambers connected by a guillotine door. One chamber, the start chamber, was lighted and the other chamber was dark. The behavioural tests were always performed between 08:00 and 15:00 hours during the light period, and an acclimatization period of 1 h to the conditions of the experimental room preceded all the experimental procedures. During training, passage into dark chamber was followed by the closing of the guillotine door and the mice falling into a cold-water bath (10 °C). The amount of time that the mice remained in the lighted chamber without crossing into the dark chamber was taken as the first latency for the mouse observed. Once finished, the animal was immediately taken away from the apparatus and dried. Mice that did not enter the dark chamber within 60 s were not used. Twenty-four hours later animals were tested for retention by placing each animal into the lighted chamber and the latency of the mouse entering the dark chamber (second entry) was recorded. This procedure was performed in the same manner as on day 1, with the exception that the cold-water bath was not available when the mouse entered the dark compartment. If mouse did not enter within 180 s, it was not used. The differences (delta) between the second- and the first-entry latencies were calculated. The increase in avoidance latency indicated an improved avoidance learning behaviour.

Drugs

A polyclonal antibody to morphine, raised in sheep, characterized for affinity and cross-reactivity, as previously described in Guarna (2002), was i.c.v.-injected into mice in a final volume of 5 μl. A dose of 5 μg anti-MF IgG was used; this dose was proven to be effective in inducing the maximum eM depletion from extracellular brain spaces (Table 1). Naloxone (1 mg/kg), morphine (1, 7, 10, 15 mg/kg) and D-amphetamine were purchased from Sigma (Milan, Italy) and dissolved in saline (NaCl 0.9%) immediately before i.p. administration. Five per cent glucose was provided in the drinking water throughout the fasting period to a separate group of mice.

Experimental design

The following phases in the learning/memory processes were evaluated:

(a) *Acquisition*. Four groups of mice were submitted to 12 h of fasting before training and injected with saline, naloxone, anti-MF IgG and normal serum 10 min before the training session; 4 groups of

Table 1. GC/MS determination of IgG-bound eM from fasted or fed mice which were prevísously administered anti-MF IgG

Anti-MF IgG dose (μg)	IgG-bound eM (pg/g) fed mice	IgG-bound eM (pg/g) fasting mice
2	190 $\pm$ 32	380 $\pm$ 42[a,b]
5	121 $\pm$ 19	812 $\pm$ 53[b]
7	107 $\pm$ 28	807 $\pm$ 36[b]

[a] 2 μg dose compared to other doses in fasting mice.
[b] Fasted compared to fed mice administered with the same dose, significance level $\alpha = 0.01$.

normally fed mice received the same treatment as above. Morphine, or saline, at different doses were administered 10 min before the training session to different groups of normally fed mice (saline was administered to fasted or fed mice).

(b) *Consolidation*. The same experiments as in (a) were performed except that treatments were administered to mice immediately after the training session.

Fasted mice from experiments (a) and (b) were fed again at the end of the training session.

(c) *Retrieval*. The same experiments as in (a) were performed except that mice were submitted to 12 h of fasting before the retention test and treatments were administered to mice 10 min before the retention test.

Five per cent glucose was provided in the drinking water throughout the fasting period to different groups of mice which had undergone passive avoidance test; these mice then endured 12 h of fasting before the training or retrieval tests.

Rota-rod test

Up to five mice were tested simultaneously on the rota-rod apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). Those mice that scored less than 3, and more than 6, falls in the pre-test were rejected. The performance time was measured before pre-test and 15, 30 and 45 min after i.p. administration of saline solution. Ten minutes before testing, animals were treated with saline, normal serum, anti-MF IgG, and morphine at different doses. A separate group of mice were submitted to 12 h of fasting before the rota-rod test. Twelve mice per group were tested.

Hole-board test

The hole-board test consisted of a 40-cm square plane with 16 flush mounted cylindrical holes distributed 4×4 in an equidistant grid-like manner. Mice were placed on the centre of the board one by one and allowed to move freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of the opposite side, thus dividing the plane into four equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. The test was performed 10 min after administration of saline, normal serum, anti-MF IgG, and morphine at different doses, or D-amphetamine (1 mg/kg, i.p.), which was used as reference drug. A separate group of mice was submitted to 12 h of fasting before the hole-board test.

GC/MS determination of IgG-bound eM in mice brains

In order to extract the IgG-bound eM from mice brains, the method modified by Takahashi and Das (1985) was used. Independent groups of mice ($n = 10$ each), previously submitted to 12 h of fasting or normally fed, were i.c.v.-administered with anti-MF IgG, or saline, at different doses (2–7 μg). The brains were extracted from mice under deep anaesthesia, 20 min after administration. The meninges were then peeled and the brains were minced into small pieces and homogenized at 4 °C with 10 vol. (w/v) of PBS (pH 7.2) in the presence of protease inhibitor (2 mM phenylmethylsulphonyl-fluoride, Sigma). 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, Spectrum Laboratories, USA). The IgGs were separated from the dyalized sample using a purification kit (Bio-Rad Econo-Pac, serum IgG purification kit; Bio-Rad, Milan, Italy). 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 analysed by GC/MS for morphine determination.

Statistical analysis

All experimental results are given as mean $\pm$ S.E.M. Analysis of variance by Fisher protected least significant difference (PLSD) procedure for post-hoc comparisons was used to verify significance between two means. Data were analysed with the Statview Software for the Macintosh (1992; SAS Institute, Cary, NC, USA).

Results

GC/MS determination

The brain content of eM quantified by GC/MS was 0.29 ± 0.01 ng/g (mean $\pm$ S.E.M., $n = 10$) in the fed mice (ad libitum) and 1.06 ± 0.13 ng/g (mean $\pm$ S.E.M., $n = 10$) after 12 h of fasting, demonstrating a significant increase of this opiate alkaloid. Morphine contamination was not found in the blank samples, food and water.

### Passive avoidance response in fed and fasted mice

The entry latency was significantly decreased in the fasted mice in the acquisition and consolidation tests (Figure 1a,b) whereas no significant difference was observed between fasted and fed mice in the retrieval test (Figure 1c). The entry-latency decrease in the fasted mice was fully reversed to the saline value by naloxone and anti-MF IgG administration in the acquisition and consolidation tests (Figure 1a,b). Naloxone administration to the fed mice did not induce any significant effects, with respect to the saline-administered fed mice. Fasted or fed mice injected with normal sheep IgG showed no significant entry-latency difference, with respect to the corresponding control group of saline-administered fasted or fed mice. Glucose administration did not modify passive avoidance response in the fasting mice (results not shown).

Dose–response curve of morphine in the mouse passive avoidance test

Morphine at higher doses (7–15 mg/kg) caused a significant decrease in entry latency in comparison to i.p. isotonic saline solution values when administered before, or shortly after, training (Figure 2a,b). The entry-latency decrease was linearly correlated to morphine doses. Pre-test administration of morphine did not cause any significant difference in entry latency in comparison to saline administration at the various doses (Figure 2c).

Rota-rod test and spontaneous activity meter

None of the treatments significantly increased the number of falls from the rotating rod in comparison with saline-treated mice; the number of falls in the rota-rod test progressively decreased as the mice learned how to balance on the road (Figure 3). The
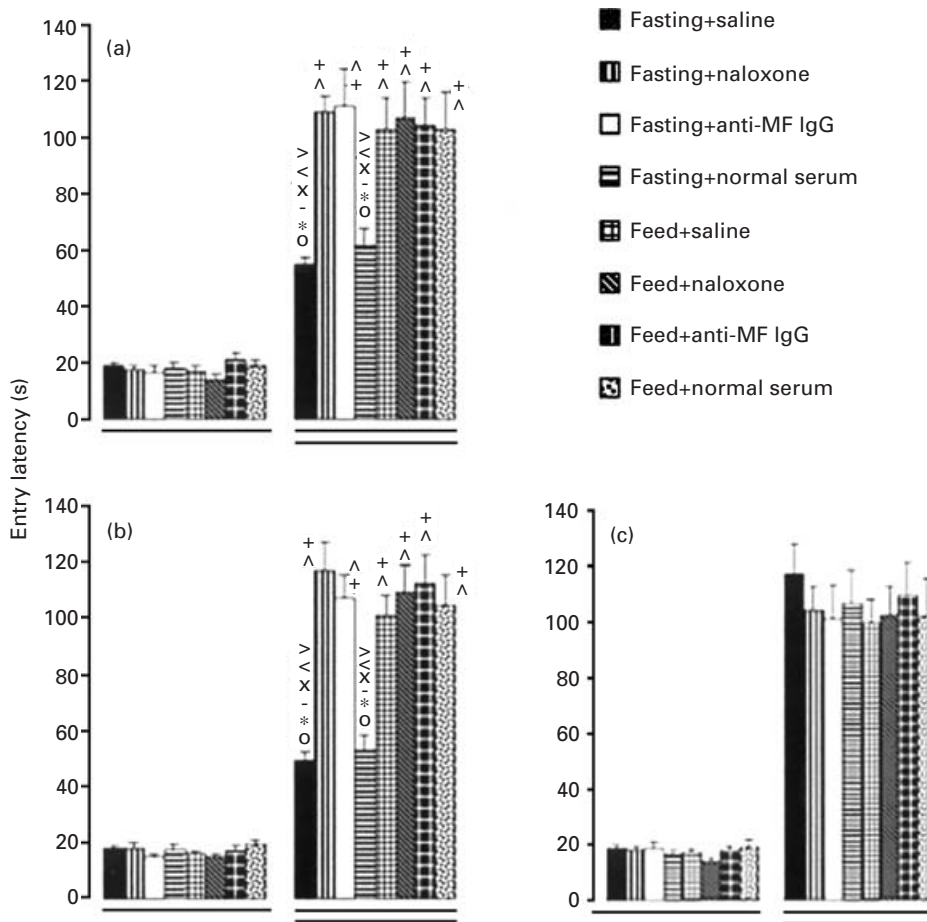
Figure 1. Effects of different pre-treatment on (a) memory learning, (b) consolidation and (c) retention in the mouse passive avoidance test. Entry latency (mean ± S.E.M.) into the dark compartment in the passive avoidance test is showm following saline, naloxone (1 mg/kg), anti-MF (5 μg) or normal sheep IgG (5 μg) administration to fed or fasted mice. The entry latency values (s) were measured in the training test (——) and after 24 h in the retention test (===). Vertical lines represent S.E.M. Between 19 and 25 mice were tested for each treatment. ∧=Other treatments compared to saline administration in fasted mice; ∗=other treatments compared to naloxone administration in fasted mice; ∘=other treatments compared to anti-MF IgG administration in fasted mice; +=other treatments compared to normal serum administration in fasted mice; −=other treatments compared to saline administration in fed mice; ×=other treatments compared to naloxone administration in fed mice; <=other treatments compared to anti-MF IgG administration in fed mice; >=other treatments compared to normal serum administration in fed mice; significance level α=0.05.

spontaneous motility and exploratory activity was not modified by treatment in comparison with saline administration, as demonstrated by the hole-board test (Figure 4a,b). In the same experimental conditions D-amphetamine (1 mg/kg, i.p.) used as the reference drug, increased both parameters evaluated in the hole-board and rota-rod tests.

GC/MS determination of IgG-bound eM in mice brains

IgG-bound eM was extracted from the mouse brain, separated from IgG and determined by GC/MS in mice that were subjected to 12 h of fasting and may, or may not, have been given anti-MF IgG. An eM value

of 812 ± 53 pg/g brain tissue was obtained in mice after 20 min of anti-MF IgG exposure at a dose of 5 μg; at a higher dose of anti-MF IgG, the quantity of eM bound to the antibody was constant (Table 1). Lower eM-bound quantities were obtained from normally fed mice (Table 1). No eM-bound IgG was obtained from mice that were administered with saline.

Discussion

eM in normally fed and fasted rat

The eM whole-brain concentrations found in the present study from fed and fasted mice were similar
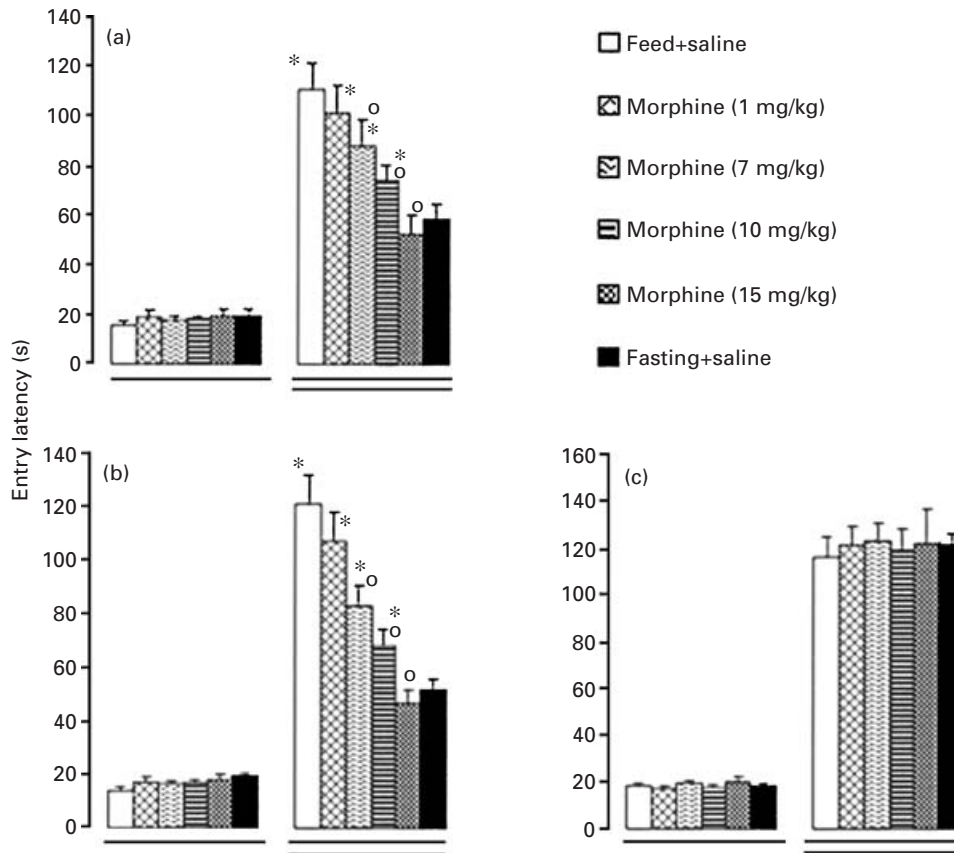
Figure 2. Dose–effect curve of morphine on (a) memory learning, (b) consolidation and (c) retention in the mouse passive avoidance test. Entry latency (mean±s.e.m.) into the dark compartment in the passive avoidance test are shown following saline and morphine (1 or 7 or 10 or 15 mg/kg) administration. Saline was administered to fed or fasted mice. The entry latency values (s) were measured in the training test (——) and after 24 h in the retention test (═══). Vertical lines represent s.e.m. Between 19 and 25 mice were tested for each treatment. ○=Treated compared to saline-treated mice in the retention test; *=treated compared to untreated fasting animals in the retention test; significance level $\alpha=0.01$.

to those reported by other laboratories (Guarna et al., 1998; Lee and Spector, 1991; Molina et al., 1995). A concentration of 0.29 ± 0.01 ng/g brain tissue was determined in fed mice. As expected, brain eM concentrations were significantly higher after fasting, increasing to a value of 1.06 ± 0.13 ng/g in fasting mice. Various stressors, including fasting, are known to increase synthesis, turnover, metabolism and release of neurotransmitters in nerve tissue (Pacak et al., 1996). In the present study, when mice were subjected to fasting, eM values in extracellular brain spaces increased 8-fold in comparison to controls, as determined by GC/MS values of anti-MF IgG-bound eM. The brain extracellular eM values obtained from normally fed mice were in agreement with the basal release value of eM (0.1–0.5 ng/g.min) by rodent nerve terminals (Guarna et al., 1998).

Effects of eM deprivation on memory

Fasting before the training session induced amnesia in the mouse passive avoidance test, whereas no memory impairment was seen in mice which were submitted to 12 h of fasting before the test session. A prolonged fasting condition has been proved to elevate eM values which slowly rise to maximum values and take several hours to return to basal values after the end of the fasting period (Lee and Spector, 1991). Thus, we cannot separate the effects of fasting on acquisition and consolidation of memory, which were both significantly impaired as shown by the significant decrease in entry latency into the dark room in the passive avoidance test. The amnesic effect obtained after 12 h of fasting was comparable to the one obtained by pre- or post-training administration of 15 mg/kg exogenous
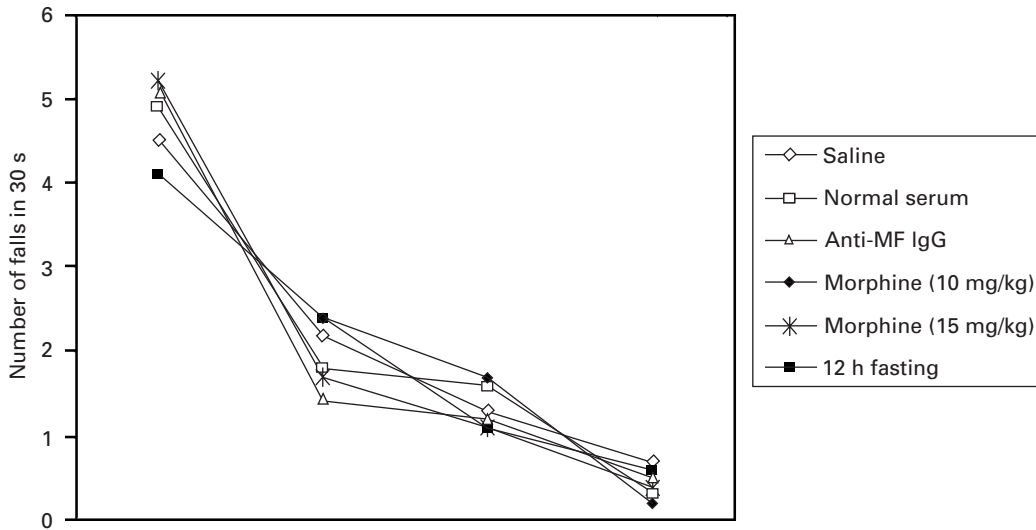
Figure 3. Lack of effect of pre-treatment with saline, normal serum, anti-MF IgG and morphine at different doses, or fasting, on motor coordination in the mouse rota-rod test. s.e.m. are so slight that they cannot be represented; 12 mice per group were tested.
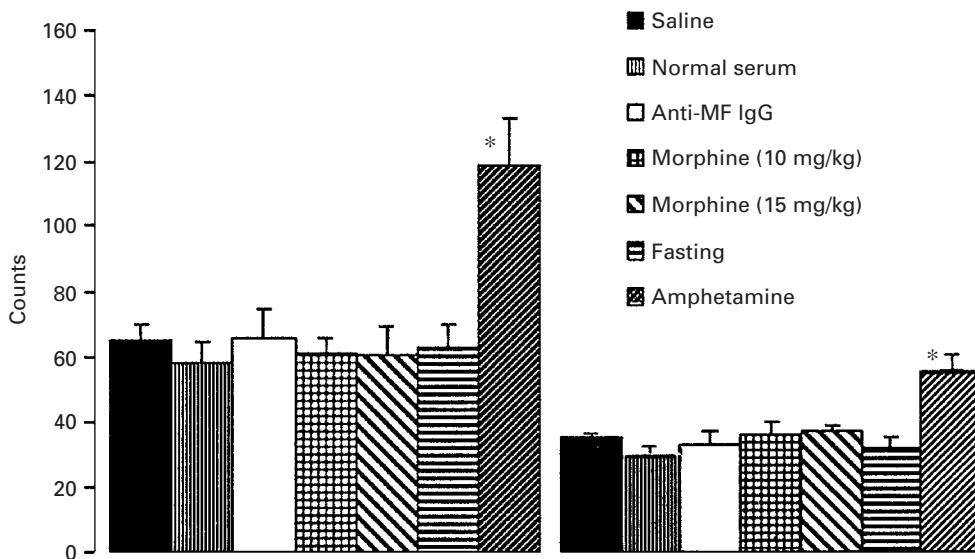
Figure 4. Lack of effect of pre-treatment with saline, normal serum, anti-MF IgG and morphine at different doses, or fasting, on (a) spontaneous motility and (b) inspection activity in the mouse hole-board test. Vertical lines represent s.e.m.; 12 mice per group were tested. *=Treated compared to saline; significance level $\alpha = 0.01$.

morphine, which was proven to prevent memory retention in passive avoidance tests (Castellano, 1975; Izquierdo, 1980). The decreased memory retention of passive avoidance learning obtained after fasting might be ascribed to increased levels of eM in mice. When eM was depleted in fasted mice by i.c.v. administration of affinity-purified anti-MF IgG, given before or shortly after training, the amnesic response values were the same as the values obtained after

saline administration. Thus, eM appears to contribute to the impairment of acquisition and consolidation of passive avoidance learning whereas, in our experiments, it had no effect on memory retrieval. The fasting-induced impairment of working-memory function could be antagonized by naloxone administered before or after training, confirming that this effect was mediated through opioid receptors. Glucose administration in fasting mice did not modify passive

avoidance response, excluding a significant effect of decreased glucose metabolism in the effect of fasting on working memory. In all the tested experimental conditions, no effect on memory was induced by eM deprivation in unstressed mice; the dose of naloxone used in this study did not have any effect, per se, on memory in normally fed mice. Therefore, eM does not appear to act on working memory in the absence of a stress condition.

Conclusions

A highly significant regression coefficient between analgesia and memory impairment was found after opiate administration (Braida et al., 1994). Further support for this close relationship was provided by other investigators who showed that highly morphine-tolerant rats display complete recovery of cognitive function if they are tested after analgesia (Sala et al., 1994). Fasting, which we used as the stress condition, was proven to be a physiological stimulus of vagus-mediated enhancement of nociception in the rat; food deprivation raised the activity in the subdiaphragmatic vagus inducing increased nociceptive behaviour and this effect might be ascribed to decreased gut distension (Randich and Gebhart, 1992). Different neurotransmitters have been identified for vagally mediated effects on nociception through noradrenergic and serotoninergic systems (Randich and Gebhart, 1992), which are known to be modulated by exogenous and endogenous opioids. Endogenous morphine was previously demonstrated to play a role in the modulation of thermal nociception (Guarna et al., 2002) and was localized in brainstem regions involved in the descending pain inhibitory pathway such as midbrain and periaqueductal grey matter, including nucleus raphe magnus (Gintzler et al., 1978). Morphinergic cells and fibres were also localized in hippocampal formation and other limbic areas (Bianchi et al., 1993), which play a major role in memory processes and mediate motivational, affective and autonomic responses often accompanying pain. Therefore, memory impairment could be related to nociceptive behaviour in a stress condition, i.e. fasting. Further investigations are warranted to reveal the mechanisms involved in this phenomenon. However, our results raise the intriguing possibility that eM plays a role in weakening the memory of a nociceptive experience.

Acknowledgements

Statement of Interest

None.

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