Increase in brain corticosterone concentration and recognition memory impairment following morphine withdrawal in mice

MOHAMMED RABBANI¹, VALIOLLAH HAJHASHEMI¹, & AZADEH MESRIPOUR²

¹Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran, and ²School of Pharmacy and Pharmaceutical Sciences, Isfahan Pharmaceutical Sciences Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran

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Abstract
Glucocorticoid hormones evidently affect memory. Morphine withdrawal causes a cognitive deficit and an increase in corticosterone secretion. In the present study brain corticosterone concentrations were determined after morphine withdrawal. Male mice were made dependent by increasing doses of morphine over 3 days. Blood and brain samples were collected following withdrawal induced by injection of naloxone (0.1 mg/kg) or spontaneously after 4 and 14 h. Brain corticosterone was extracted and measured by enzyme immunoassay. Short-term memory was determined in the novel object recognition task, using a 20 min interval between the learning trial and the test trial. In this memory trial, the difference in exploration between a previously seen object and a novel object is taken as an index of memory performance (recognition index, RI). RI in morphine dependent mice undergoing withdrawal was decreased compared to their control group. Brain corticosterone concentrations after naloxone withdrawal or 4 h after spontaneous withdrawal were respectively 22 and 34% greater than control values. Corticosterone concentration was normalized 14 h after the last dose of morphine. The results indicate that increase in brain corticosterone concentration may play an important role in short-term memory impairment following morphine withdrawal.

Keywords: Corticosterone, memory, morphine withdrawal, naloxone, novel object recognition

Introduction
Modulation of learning and memory processes by morphine and other opioidergic agents has been demonstrated in many studies (Canli et al. 1990; Bodnar and Klein 2005). Reports have indicated that acute administration of opioids impairs learning and memory processing (Castellano and Pavone 1985; Itoh et al. 1994); additionally, chronic exposure to opiates can result in an impaired performance on a memory task in rats (Spain and Newsom 1991; Sala et al. 1994). Using the object recognition task we have observed recognition memory impairment in dependent mice following morphine withdrawal (Mesripour et al. 2007). However, memory recovered to control levels following the administration of metyrapone (a glucocorticoid synthesis inhibitor) and mifepristone (a glucocorticoid receptor antagonist; Mesripour et al. 2007).

It has long been recognized that prolonged exposure to stress or excess glucocorticoids impairs memory function in both animal and human subjects (Roozendaal 2002). It is now also known that glucocorticoids have acute influences on memory (Het et al. 2005). The consequences of activation of glucocorticoid production on memory depend largely on the different memory phases investigated, i.e., memory consolidation is enhanced by acute stress or acute glucocorticoid treatment, while retrieval is impaired (Roozendaal 2002).

Morphine withdrawal is associated with activation of the hypothalamus–pituitary adrenal axis (Morley 1981). Moreover, repeated short periods of drug withdrawal (24 or 72 h) in morphine-dependent mice represent a mild stress load (Zelena et al. 2005) and animals undergoing acute (12 h) morphine withdrawal display a potentiated and prolonged...
corticosterone response to restraint (Houshyar et al. 2004).

The relationship between corticosterone concentrations in the brain and memory impairment following morphine withdrawal is not yet clear. Therefore, in the present study memory was assessed following naloxone-precipitated morphine withdrawal and spontaneous withdrawal in morphine-dependent mice. Blood and brain corticosterone concentrations were determined in parallel groups of morphine dependent mice following withdrawal.

**Materials and methods**

**Animals**

Male NMRI mice (Pasteur Institute, Tehran, Iran) weighing 25–30 g were housed in cages of six at 21 ± 2°C in a 12 h:12 h light–dark cycle with the lights on during day time from 06.00–18.00 h. Tap water and standard food pellets were available ad libitum. Tests were performed only after the mice had acclimated to the above environment for at least 2 days. In order to minimize circadian rhythm influence, all experiments were conducted between 08:00 and 13:00 h, in a separate noise-free room with controlled illumination. All procedures were approved by the Ethical Committee of the Isfahan University of Medical Sciences, and conducted in accordance with the ‘Principles of Laboratory Animal Care’ (National Institutes of Health publication no. 86-23, revised 1985).

**Memory test**

Memory was evaluated by the novel object recognition task as originally developed by Ennaceur and Delacour (1988), and it is based on the natural tendency of rodents to explore a novel object more than a familiar one. The object recognition task was performed as described by Bertaina-Anglade et al. (2006) after habituation to the memory apparatus the day before starting the experiment. On the day of experiment, mice were submitted to two trials spaced by an intertrial interval of 20 min. During the first trial (acquisition trial, T1), mice were placed in the arena containing two identical objects for an amount of time necessary to explore the objects for 20 s. Any mouse not exploring the objects for 20 s within the 12-min period was excluded from the experiments. Exploration was defined as the mouse directing the nose within 2 cm of the object while looking at, sniffing, or touching it. The duration of T1 was measured as the time required to explore the object for 20 s in T1. For the second trial (test trial, T2), one of the objects presented in the first trial was replaced by a new object, the mice were returned to the arena for 5 min and the total time spent in exploration of the familiar object (F) and the new object (N) was determined. Behavior of the mice was recorded via a web camera mounted above the experimental apparatus and recordings were analyzed later. Recognition memory was evaluated using a recognition index (RI) calculated for each mouse using the formula: \( \frac{N - F}{N + F} \times 100 \), corresponding to the difference between the time exploring the novel and the familiar object, corrected for total time exploring both objects (Bertaina-Anglade et al. 2006). Positive values indicate a good discrimination performance, while negative values or those around zero indicate poor discrimination capacity.

**Drug treatments**

Mice were made dependent by injections of increasing doses of morphine sulfate (Temade co., Tehran, Iran), given subcutaneously twice daily at 12 h intervals; 30 and 45 mg/kg on the first day, 60 and 90 mg/kg on the second day, and 90 mg/kg on the third (last) day (Hajhashemi et al. 2004). Withdrawal was elicited by injection of 0.1 mg/kg naloxone (Tolid Daru Co., Tehran, Iran) intraperitoneally 3 h after the last morphine injection (Zelena et al. 2005; Mesripour et al. 2007). For mice that were candidates for memory evaluation, naloxone was injected after T1 (in the morphine + naloxone and naloxone alone groups). Twenty minutes after naloxone injection mice were either tested in the memory apparatus or they were decapitated. For the study of spontaneous morphine withdrawal different groups of mice were either tested or decapitated 4 and 14 h after they received the last dose of morphine. Control groups were treated only with saline twice daily for 3 days. In addition a group of control (non-dependent) mice was given naloxone in order to determine the effect of naloxone on memory performance. The doses were adjusted such that each mouse received an injection volume of 10 ml/kg.

**Tissue collection**

Mice were lightly anesthetized with diethyl ether inhalation and rapidly decapitated; trunk blood samples were collected. The time elapsed between the start of ether exposure and decapitation was 3–4 min (Głowa 1993). Following centrifugation of blood samples, serum was transferred to small-capped vials and stored frozen (−20°C) until analyzed. The whole brain was removed, weighed and homogenized in assay buffer. Corticosterone was extracted by ethyl acetate and the extract dried under nitrogen gas (the whole procedure was carried out on ice). Dried samples were kept frozen at −20°C until assayed.

**Corticosterone assay**

Corticosterone was assayed in duplicate samples using a corticosterone enzyme immunoassay procedure.
Corticosterone and morphine withdrawal

Sensitivity. The sensitivity of assays, defined as two standard deviations from the signal given by the zero blank, was 26.99 pg/ml.

Variation. The intra- and interassay coefficients of variation of biological samples and the recovery of three different amounts of corticosterone added to plasma were within the manufacturer’s acceptable range.

Statistical analysis

The corticosterone concentrations in serum and brain were analyzed by Student’s t-test. Memory performance (RI) after naloxone and spontaneous morphine withdrawal were analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple comparison tests. One-sample t-tests were used to determine whether the RI was different from zero. P values less than 0.05 were considered significant. Results are expressed as the group mean ± SEM. Sigmastat software was used for all the statistical analyses.

Results

Memory performance in the novel object recognition task

About 3 h after the last dose of morphine, mice were exposed to the T1, before they were given naloxone. These morphine-dependent mice took significantly more time for object exploration in T1 (t14 = -2.312, P = 0.03) compared to their control groups (saline only, with or without subsequent naloxone; Table I). However, 4 and 14 h after the last dose of morphine the duration of T1 did not differ between the morphine-dependent and control groups (Table I).

Data from evaluation of memory performance by the novel object recognition task (T2) are shown in Figure 1 and Table I. After the naloxone injection, before T2, in the morphine-dependent mice, some somatic signs of withdrawal such as penile grooming, digging, occasional jumps and teeth chattering were seen. In morphine-dependent mice undergoing naloxone-induced withdrawal, RI was significantly less than in the control groups (saline only and naloxone alone groups, f(2/18) = 27.182, P < 0.001;

Figure 1. Effect of naloxone-precipitated morphine withdrawal (A) and spontaneous morphine withdrawal (B) on memory performance (expressed as recognition index, RI = (N – FN + F) × 100) in morphine dependent mice in the novel object recognition task. Values are group mean ± SEM. *P < 0.05 compared with the saline naloxone group, #P < 0.05 compared with the saline group (Student’s t-tests or by ANOVA, followed by Duncan’s multiple comparison tests). In (A) in the saline only and naloxone alone groups, n = 6 mice; in the morphine + naloxone group, n = 9. In (B), in the saline only group, n = 9; in the spontaneously withdrawn groups (+4 h and +14 h), n = 6.

Table I. Duration of T1 (the time required to achieve 20 s of object exploration on the first trial), and time required in T2 to recognize the familiar (F) and new (N) objects.

<table>
<thead>
<tr>
<th></th>
<th>Duration of T1 (min)</th>
<th>F (s)</th>
<th>N (s)</th>
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</thead>
<tbody>
<tr>
<td>Naloxone-induced withdrawal</td>
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<tr>
<td>Control, saline only (n = 7)</td>
<td>3.3 ± 0.5</td>
<td>4.6 ± 1.1</td>
<td>10.0 ± 1.5</td>
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<tr>
<td>Naloxone alone (n = 6)#</td>
<td>2.1 ± 0.1</td>
<td>5.0 ± 1.0</td>
<td>13.5 ± 3.2*</td>
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<tr>
<td>Morphine + Naloxone (n = 9)#</td>
<td>9.1 ± 0.8*</td>
<td>5.9 ± 0.9</td>
<td>4.5 ± 0.7</td>
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<tr>
<td>Spontaneous withdrawal</td>
<td></td>
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<tr>
<td>Control, saline only (n = 9)</td>
<td>4.3 ± 0.6</td>
<td>5.6 ± 1.4</td>
<td>13.4 ± 2.4</td>
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<tr>
<td>Morphine + 4 h (n = 6)</td>
<td>5.5 ± 0.6</td>
<td>6.7 ± 0.9</td>
<td>5.5 ± 0.9*</td>
</tr>
<tr>
<td>Morphine + 14 h (n = 6)</td>
<td>3.3 ± 0.4</td>
<td>6.8 ± 1.0</td>
<td>6.1 ± 1.1*</td>
</tr>
</tbody>
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Results are expressed as mean ± SEM. *P < 0.05 compared with control group. Control groups received saline. #Naloxone alone controls and morphine-dependent rats received naloxone after T1, 3 h after the last morphine/saline injection; T2 was 20 min after naloxone/saline injection.
Figure 1(A)), which indicated that the mice withdrawn by naloxone did not remember the familiar object, and spent less time exploring the new object (Table I). Similar results were obtained from morphine dependent mice 4 and 14 h after the last dose of morphine (spontaneous withdrawal); thus, RI was significantly less in the 4 and 14 h groups than in the control group ($f(2/18) = 12.808, P < 0.05$; Figure 1(B)). One sample t-tests, used to examine whether the RI was different from zero (chance level), showed that in contrast to the control groups, which exhibited a significant exploration preference for the novel object ($P < 0.001$), naloxone-withdrawn or spontaneously (4 or 14 h) withdrawn mice did not show such a preference ($P > 0.05$).

**Serum and brain corticosterone concentrations**

Serum and brain samples were collected 20 min after naloxone injection. In the dependent mice brain corticosterone concentration was 22% greater than in the controls, receiving daily saline ($t_{10} = -2.813; P = 0.01$; Figure 2(A)). Brain corticosterone concentration in morphine dependent mice 4 h after the last dose of morphine was increased by 34% vs. controls receiving daily saline ($t_{15} = -4.757; P < 0.001$). Fourteen hours after the last dose of morphine brain corticosterone concentration was not significantly different from control values ($t_{10} = -0.0991; P = 0.9$).

The serum corticosterone concentrations after naloxone-precipitated morphine withdrawal and 4 h after the last dose of morphine were respectively 15.3 and 20% greater than in the controls receiving daily saline ($P < 0.001$; Figure 2(B)). As in the brain, 14 h after the last dose of morphine the serum corticosterone concentration did not differ from the control values ($t_{10} = -0.351, P = 0.733$).

**Discussion**

In order to determine if memory impairment after morphine withdrawal in the novel object recognition task is at least in part due to increased corticosterone concentration, the present study evaluated memory performance and blood and brain corticosterone concentrations following morphine withdrawal in mice.

The object recognition task allows a rapid evaluation of memory performance in mice. In contrast to studies of memory in human subjects, animal experiments generally use emotionally arousing learning tasks. But in this method no rewarding or aversive stimulation is used during training, so the learning occurs under conditions of relatively low stress or arousal (Ennaceur and Delacour 1988).

In agreement with previous studies, serum corticosterone concentration was increased following naloxone-precipitated morphine withdrawal, and 4 h after the last dose of morphine in morphine-dependent mice (Houshyar et al. 2004; Zelena et al. 2005). This increase in corticosterone concentration was not expected to be influenced by the anaesthetic used, since the time elapsed between ether exposure and decapitation was less than 5 min (Glowa 1993). Serum corticosterone concentration returned to control values 14 h after the last dose of morphine.

As presented in Table I, morphine impaired learning performance by increasing duration of $T1$, and this measure returned to control values after 4 h. The effect of morphine on the duration of $T1$ in dependent mice could be because of the behavioral sensitization and enhanced locomotor activity in morphine dependent mice (Robinson and Berridge 2000). As shown in Figure 1(A) and (B), recognition memory performance was impaired following morphine withdrawal, since the RI was low compared to control mice. In our initial experiments we observed that memory performance was impaired in the novel...
object recognition task 20 min after a single dose of morphine (60 and 90 mg/kg, sc) in naïve mice (data not shown). Previous experiments have shown that in the case of the intraperitoneal route, the biological half life of morphine (elimination phase) was estimated to be 49 ± 6 min ( Ishikawa et al. 1982). Therefore, memory impairment seen 4 h after the last dose of morphine is likely to be a result of morphine withdrawal. As shown in Figure 1(A) naloxone alone did not affect memory in control mice, hence memory impairment seen after naloxone injection in dependent mice is a result of induction of morphine withdrawal.

Memory impairments observed were associated with increased brain corticosterone concentrations. Since RI in control mice receiving naloxone did not differ from control values corticosterone determinations were not performed in this group. For mice undergoing 14 h morphine cessation there was memory impairment, although blood and brain corticosterone concentrations had returned to control values. Glucocorticoids exert their actions principally via intracellular receptors, which belong to the nuclear receptor superfamily and regulate the transcription of target genes. The biological actions of the steroids on tissue are thus generally slow in onset and persistent ( Buckingham 2006). However, in addition to genomic action, recent evidence indicates that glucocorticoids also induce rapid neural effects through non-genomic mechanisms involving membrane-associated receptors ( Makara and Haller 2001). Moreover, emotionally arousing stimuli activate noradrenergic mechanisms, which are critically involved in modulating memory processes ( McGaugh and Roozendaal 2002). Our finding that 14 h after morphine administration memory is impaired, while corticosterone concentrations have returned to control levels, may be explained by at least some part of glucocorticoid effects on memory after withdrawal being gene related, and outlasting increased corticosterone levels. By 24 h after spontaneous morphine withdrawal in dependent mice memory performance has returned to normal values ( Mesripour et al. 2007).

A previous study revealed that in rats that were not habituated to the object recognition apparatus, corticosterone impaired short-term (1 h) memory performance while enhancing 24 h retention performance ( Okuda et al. 2004). That study showed that the effects of glucocorticoid administration on memory retrieval depend on the level of emotional arousal associated with initial encoding. Hence, on the basis of previous studies morphine withdrawal may affect memory in the novel object recognition task by inducing a level of emotional arousal, which is an interesting aspect for further research.

The changes in brain corticosterone concentrations were concordant with blood level changes. Following 4 h-spontaneous withdrawal metyrapone (25 mg/kg, sc 90 min before T2), mifepristone (50 mg/kg, sc 40 min before T2) ( Mesripour et al. 2008) and spironolactone (50 mg/kg, sc 40 min before T2) ( Mesripour et al. 2007) improved RI by 34.8, 25.4 and 18% respectively, in morphine dependent mice. Since these anti-glucocorticoid manipulations improved memory performance after morphine withdrawal in the object recognition task, it can be concluded that increased brain corticosterone concentration plays an important role in the memory deficit that follows morphine withdrawal. The hippocampus serves a pivotal role in memory formation ( Roozendaal et al. 2003). In addition the basolateral amygdala ( BLA ) seems to be a key structure in a memory-modulatory system that regulates stress and glucocorticoid effects on memory. By activating the BLA, stress allows for strong consolidation of the current event, but simultaneously compromises memory retrieval ( Roozendaal 2003). Evidently, noradrenergic activity of the BLA is critical for this effect ( Roozendaal 2003). Synergistic actions of glucocorticoids and noradrenergic activation of the BLA constitute a neural mechanism by which glucocorticoids may selectively enhance memory consolidation for emotionally arousing experiences in the object recognition task ( Roozendaal et al. 2006).

Consistent with the role of the hippocampus in memory processing ( Roozendaal et al. 2003; Broadbent et al. 2004) and also the presence of a high density of glucocorticoid receptors in this structure ( Roozendaal 2002), it is likely that the increased corticosterone concentrations in the present study affected memory, at least in part, through actions in the hippocampus. This explanation for memory impairment during withdrawal in morphine dependence requires further investigation.

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References


