Involvement of opioidergic and nitrergic systems in memory acquisition and exploratory behaviors in cholestatic mice
Mohammad Nasehi\textsuperscript{a}, Morteza Piri\textsuperscript{b}, Kobra Abbolhasani\textsuperscript{c} and Mohammad R. Zarrindast\textsuperscript{d,e,f,g,h}

Bile duct ligation (BDL) is an animal model used in cholestatic disease research. Both opioidergic and nitrergic systems are known to be involved in cholestasis. The aim of this study was to investigate the possible interaction between these two systems in BDL-induced memory formation and exploratory behaviors in mice. Male mice weighing 25–30 g were divided into nonoperated controls, sham-operated, and BDL groups. One-trial step-down and hole-board paradigms were used to assess memory acquisition and exploratory behaviors, respectively. Cholestasis did not alter memory acquisition while increasing exploratory behaviors 7 days after BDL. A pretraining intraperitoneal injection of L-arginine (50, 100, and 200 mg/kg), L-NG-nitroarginine methyl ester (L-NAME) (5, 10, 20, and 40 mg/kg), or naloxone (0.125, 0.25, and 0.5 mg/kg) did not alter memory acquisition or exploratory behaviors, whereas morphine (5 and 7.5 mg/kg) decreased memory acquisition in sham-operated animals. Moreover, although injection of L-NAME and naloxone exerted no effect on memory acquisition in the 7 days post-BDL mice, L-arginine (100 and 200 mg/kg) and morphine (2.5, 5, and 7.5 mg/kg) injection reduced it. In contrast, L-NAME and naloxone, but not morphine or L-arginine, reduced the BDL-induced exploratory behaviors. Coadministration of subthreshold doses of morphine (1.25 mg/kg) and L-arginine (50 mg/kg) caused a memory deficit in 7 days post-BDL mice. However, the memory deficit induced by the effective doses of morphine (2.5 mg/kg) or L-arginine (200 mg/kg) in these mice was restored by the administration of either naloxone (0.5 mg/kg) or L-NAME (40 mg/kg). In addition, naloxone and L-NAME reduced the exploratory behaviors in L-arginine-pretreated mice but not in morphine-pretreated mice. We conclude that there appears to be a synergistic effect between opioidergic and nitrergic systems on memory acquisition and exploratory behaviors in cholestatic mice. \textit{Behavioural Pharmacology} 24:180–194 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: L-arginine, cholestasis, hole-board task, memory, morphine, mouse, naloxone, L-NAME

Introduction
Cholestasis is clinically characterized by stagnation of bile flow, resulting in elevated plasma concentrations of biliary constituents, jaundice, and liver damage (Garcia-Moreno \textit{et al.}, 2005). The primary and secondary etiologic factors, their respective adaptive processes, and the consequences that may result in cholestasis should be well differentiated (Oude Elferink \textit{et al.}, 2011). Cognitive functions such as memory formation (Huang \textit{et al.}, 2004, 2009, 2010; Zarrindast \textit{et al.}, 2012) and anxiety-like behaviors (Eslimi \textit{et al.}, 2011) are impaired in patients with liver disease and in animal models of chronic liver failure (Erceg \textit{et al.}, 2005; Mendez \textit{et al.}, 2008). Alterations in neurotransmitter systems such as opioidergic, nitrergic, glutamatergic, GABAergic, cholinergic, and serotoninergic systems have been implicated in these cognitive dysfunctions (Marrachelli \textit{et al.}, 2006; Cauli \textit{et al.}, 2009; Palomero-Gallagher \textit{et al.}, 2009; Magen \textit{et al.}, 2010; Marquez-Aguirre \textit{et al.}, 2010). An increased opioid neuromodulation, leading to elevated activity and plasma level of opioid peptides, has been evident both in animal models and humans subjected to cholestasis, supporting an association between activation of the opioid system and the pathophysiology of cholestatic liver disease (Talaenko \textit{et al.}, 2005; Ebrahimkhani \textit{et al.}, 2006). Opioid peptides induce their effects through three classes of receptors known as μ, κ, and δ (Herz, 1983). Furthermore, both acute and chronic activation of opioid receptors in animals have been reported to cause liver damage through increased oxidative stress and enhanced plasma liver enzyme activities (James \textit{et al.}, 1982; Zhang \textit{et al.}, 2004). In addition, liver failure may negatively affect glutamatergic neurotransmission, glutamate–nitric oxide (NO)–cGMP interaction, and hence long-term potentiation (Felipo, 2006). Previous studies have also indicated that memory formation in the passive avoidance task is impaired in adult bile duct ligation (BDL) mice (Zarrindast \textit{et al.}, 2012). NO has also been suggested...
recently to play a critical role in the BDL-induced spatial memory deficit in rodents (Huang et al., 2010). NO retains its critical role as a prominent secondary messenger in both the central and the peripheral nervous systems (Nasehi et al., 2010a, 2010b). It also participates in certain forms of long-term potentiation (Hawkins et al., 1998; Prast and Philippu, 2001).

The rodent BDL model has been widely used to evaluate the consequences of cholestasis (Zarrindast et al., 2010; Eslimi et al., 2011). The question addressed in the present study is how the opioidergic and nitrergic systems interact in cholestasis-induced memory acquisition and exploratory behaviors. Some studies have shown that at long intervals after BDL, mice show decreases in both memory formation (García-Moreno et al., 2005; Huang et al., 2010) and locomotor activity (Magen et al., 2009). However, we designed our experiment to assess the interactive effects of opioidergic and nitrergic systems in 7 days post-BDL (cholestatic) mice, which were expected to show memory impairment without alteration of locomotor activity.

**Methods**

**Subjects**

Male NMRI mice (24–28 g; Institute for Cognitive Science Studies, Tehran, Iran) were used. Animals were housed five per cage with food (22% protein, 53% carbohydrate, and 4.5% lipid; Behparvar Co., Tehran, Iran) and water freely available. Cages were kept in a room with a constant temperature (22±2°C), a 12-h light/dark cycle, and a relative humidity of 45–55%, except during the limited periods of the experiments. Behavioral experiments were conducted during the light phase of the light/dark cycle. Each animal was handled before and during the course of the experiments for the purposes of gentling and body weight measurements. Each group included 10 animals and each animal was used once only.

All experimental procedures involving animals were performed in accordance with the NIH Guideline for the Care and Use of Laboratory Animals as well as the ethical standards of the Research and Ethics Committee of the Faculty of Science, Tehran University of Medical Sciences.

**Bile duct ligation surgery and induced cholestasis**

There were three experimental groups of mice: non-operated, sham operated, and bile duct ligated. BDL surgery was performed under intraperitoneal (i.p.) anesthesia using a mixture of ketamine hydrochloride (50 mg/kg) plus xylazine (5 mg/kg). After laparotomy, the common bile duct was double ligated with silk threads and excised in between the ligatures to prevent regeneration. In the sham-operated controls, the bile duct was identified, manipulated, and left in situ (Bergasa et al., 1994; Zarrindast et al., 2012). A sterile 0.9% NaCl solution (1 ml/mouse) was injected i.p. immediately after the surgery. All surgeries were conducted using an aseptic technique. Immediately after the operation, each animal was placed in a cage by itself to prevent wound dehiscence and was moved to its original cage 4 h later (Rastegar et al., 2002). Operative mortality was less than 5%.

**Memory apparatus and procedure**

Mice were trained in a single trial, step-down inhibitory avoidance paradigm in accordance with previous studies (Zarrindast et al., 2008, 2009; Nasehi et al., 2010a, 2010b, 2012a, 2012b). The inhibitory avoidance training apparatus consisted of a wooden box (30 × 30 × 40 cm) with a floor of parallel stainless-steel rods (0.3 cm in diameter, spaced 1 cm apart). A wooden platform (4 × 4 cm) was set at the center of the grid floor. Electric shocks (1 Hz, 0.5 s, and 50 V DC) were delivered to the grid floor using an isolated stimulator (Grass S44; Grass Instruments, Quincy, Massachusetts, USA). During training, each mouse was gently placed on the wooden platform. On the training day, once the mouse stepped down from the platform and placed its four paws on the grid floor, it received foot shocks for 15 s and was then immediately removed from the training box. After 24 h, on the test day, each mouse was placed on the platform again and the step-down latency was measured using a stopwatch, and taken as an index of passive avoidance behavior. The trial was stopped if the mouse had not stepped down after a 300 s cutoff. All behavioral testing was carried out between 09:00 and 14:00 h.

**Exploratory behavior apparatus and procedure**

We used a hole-board apparatus (BorjSanat Co., Tehran, Iran) consisting of gray Perspex panels (40 × 40 cm, 2.2 cm thick) with 16 equidistant holes, 3 cm in diameter, in the floor. The dimensions were as described in previous reports (Vinade et al., 2003) and the board was positioned at a height of 15 cm on a table. This apparatus was first introduced by Boissier and Simon (1962) and has been used widely to evaluate exploratory behaviors, emotionality, anxiety, and/or response to stress in experimental animals (Rodriguez Echandia et al., 1987; Nasehi et al., 2012a, 2012b; Zarrindast et al., 2012). The different behaviors that can be observed and measured in this test enable a comprehensive description of the animal’s behavior. Anxiety testing was carried out 5 min after the memory test. Animals were placed singly in the center of the board facing away from the observer. The number of head dips was automatically recorded through photocells arranged below the holes during a 5 min period of free exploration. An increase or a decrease in the number of head dips indicated anxiolytic-like or anxiogenic-like behaviors, respectively. Locomotor activity and other indices were measured during the testing phase by an observer blinded to the treatments. The hole-board arena was divided into four equal-sized squares and locomotor activity was measured as the number of crossings from one square to another. Other behavioral indices such as...
latency to the first head dip, rearing, grooming, and defecation were recorded manually by the experimenter.

Drug treatment
Ten animals were used in each experimental group. In experiments where animals received two injections, control groups were injected with saline. The protocol is summarized in Table 1.

Experiment 1: The effects of cholestasis on memory formation and exploratory behaviors
Three groups of animals were used in this experiment, in which memory retrieval and exploratory behaviors were tested 7 days after BDL. The data obtained from cholestatic animals were compared with those of non-operated (intact) and sham-operated animals.

Experiments 2, 3, 4, and 5: The effects of morphine, naloxone, L-arginine, and L-NAME on memory acquisition and exploratory behaviors in the presence or absence of cholestasis
Sham-operated and BDL animals received saline (1 ml/kg, i.p.) or morphine (1.25, 2.5, 5, and 7.5 mg/kg, i.p., in experiment 2), naloxone (0.125, 0.25, and 0.5 mg/kg, i.p., in experiment 3), L-arginine (50, 100, and 200 mg/kg, i.p., in experiment 4), or L-NG-nitroarginine methyl ester (L-NAME) (5, 10, 20, and 40 mg/kg, i.p., in experiment 5) 15 min before the training session. The exploratory behaviors of animals were recorded in the hole-board task 5 min after the memory test.

Experiment 6: The effects of coadministration of subthreshold doses of morphine and L-arginine on memory acquisition and exploratory behaviors in BDL mice
In this experiment, four groups of animals were used. Animals received saline (1 ml/kg, i.p.), morphine (1.25 mg/kg, i.p.), L-arginine (50 mg/kg, i.p.), or coadministration of the subthreshold doses of morphine (1.25 mg/kg, i.p.) and L-arginine (50 mg/kg, i.p.) 15 min before training. The exploratory behaviors of animals were recorded in the hole-board task 5 min after the memory test.

Experiments 7 and 8: The effects of naloxone or L-NAME on impairment of memory acquisition induced by L-arginine or morphine in BDL mice
In these experiments, sham-operated and BDL animals received saline (1 ml/kg, i.p.) or different doses of naloxone (0.125, 0.5, and 0.5 mg/kg, i.p., in experiment 7) or L-NAME (10, 20, and 40 mg/kg, i.p., in experiment 8) 15 min before the pretraining injection of saline or an effective dose of L-arginine (200 mg/kg, i.p.) or morphine (2.5 mg/kg, i.p.). The exploratory behaviors of the animals were recorded in the hole-board task 5 min after the memory test.

Drugs
The drugs used in the present study were L-arginine and L-NAME (Sigma, St Louis, Missouri, USA), and naloxone and morphine sulfate (Temed, Tehran, Iran). The doses of drugs were chosen as per the published data in the scientific literature. All drugs were dissolved in 0.9% physiological saline just before the experiments and injected i.p. Control animals received saline.

Statistical analysis
As step-down latency did not follow a normal distribution, these data were analyzed using the Kruskal–Wallis nonparametric one-way analysis of variance (ANOVA), followed by a two-tailed Mann–Whitney’s U-test. The analysis compared treatment groups with their respective (sham vs. BDL) control groups. Holm’s sequential Bonferroni correction test was used for paired comparisons as appropriate. The step-down latencies for 10 animals in each experimental group are presented as medians and interquartile ranges.

The hole-board apparatus data are presented as mean ± SEM. One-way repeated-measures ANOVA, followed by post-hoc t-tests was used for the statistical evaluation.

In addition, independent t-tests were used to compare treatment groups with their respective (sham vs. BDL) control groups. In all statistical evaluations, a P value less than 0.05 was considered as the criterion for statistical significance.

Results
Induction of cholestasis
Five days after BDL, animals started to show signs of cholestasis (jaundice, dark urine, and steatorrhea), which have been tested qualitatively and reported clearly by other investigators.

The effect of cholestasis on memory acquisition and exploratory behaviors
Cholestasis did not significantly alter memory formation 7 days after BDL [Kruskal–Wallis ANOVA, H(2) = 3.27, NS, Fig. 1a]. However, one-way ANOVA showed that cholestasis increased head-dip counts [F(2,27) = 5.32, P < 0.01, Fig. 1b] while not affecting latency to the first head dip [F(2,27) = 0.13, NS, Fig. 1c], locomotor activity [F(2,27) = 0.57, NS, Fig. 1d], rearing [F(2,27) = 1.13, NS], grooming [F(2,27) = 1.16, NS], or defecation [F(2,27) = 0.5, P > 0.05] 7 days after BDL. These results show that BDL did not appear to alter memory formation but did induce an anxiolytic-like effect 7 days after BDL surgery.

The effects of morphine on memory formation and exploratory behaviors in the presence or absence of cholestasis
Kruskal–Wallis ANOVA and Mann–Whitney’s U-tests indicated that morphine injection at 5 and 7.5 mg/kg 15 min before training decreased memory acquisition...
**Table 1 Summary of the experimental design**

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<td>Sham 10</td>
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<td>8</td>
<td>BDL 10</td>
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BDL, bile duct ligation; L-NAME, L-NG-nitroarginine methyl ester.

Each figure shows four sets of behavioral data in different panels: avoidance responses, head dips, latency to first dip, and locomotor activity.

\[H(4) = 31.16, \ P < 0.001\] – (Fig. 2a, left panel). Figure 2b–d (left panels) show the effects of morphine on exploratory behaviors. One-way ANOVA and post-hoc analysis showed that, whereas morphine at 5 mg/kg decreased the latency to the first head dip \[F(4, 45) = 3.09, \ P < 0.05, \text{Fig. 2c}\], it did not alter head-dip counts \[F(4, 45) = 2.22, \text{NS, Fig. 2b}\], locomotor activity \[F(4, 45) = 0.33, \text{NS, Fig. 2d}\], rearing \[F(4, 45) = 0.93, \text{NS}\], grooming \[F(4, 45) = 0.46, \ P > 0.05\], or defecation \[F(4, 45) = 0.48, \text{NS}\]. To conclude, morphine appeared to decrease memory acquisition at 5 and 7.5 mg/kg.

Moreover, as shown in Fig. 2a (right panel), pretaining infusion of morphine reduced memory formation in cholestatic mice 7 days after BDL [Kruskal–Wallis ANOVA, \(H(4) = 38.32, \ P < 0.001\)]. Mann–Whitney’s U-tests showed that 2.5, 5, or 7.5 mg/kg of morphine reduced memory acquisition 7 days after BDL. Figure 2b–d (right panels) summarize the effects of morphine on exploratory behaviors in cholestatic mice. One-way ANOVA indicated that morphine did not alter the number of head-dip counts \[F(4, 45) = 2.38, \text{NS, Fig. 2b}\], latency to the first head dip \[F(4, 45) = 0.91, \text{NS, Fig. 2c}\], locomotor activity \[F(4, 45) = 2.1, \text{NS, Fig. 2d}\], rearing \[F(4, 45) = 0.66, \text{NS}\], grooming \[F(4, 45) = 0.97, \text{NS}\], or defecation \[F(4, 45) = 0.830, \text{NS}\] induced by cholestasis in mice.

In addition, Mann–Whitney’s U-tests indicated that morphine at the doses of 2.5 and 5 mg/kg increased the impairment of memory acquisition in the BDL mice as compared with the effects of these doses in the sham-BDL mice. In conclusion, 2.5 and 5 mg/kg of morphine appeared to decrease memory acquisition 7 days after BDL.

**The effects of naloxone on memory formation and exploratory behaviors in the presence or absence of cholestasis**

Kruskal–Wallis ANOVA showed that administration of naloxone 15 min before training did not alter memory acquisition in sham-BDL animals \[H(3) = 0.2, \text{NS}\] (Fig. 3a, left panel). In addition, one-way ANOVA showed that naloxone had no effect on head-dip counts \[F(3, 36) = 0.34, \text{NS}\] (Fig. 3b, left panel), latency to the first head dip \[F(3, 36) = 0.29, \text{NS}\] (Fig. 3c, left panel), locomotor activity \[F(3, 36) = 0.87, \text{NS}\] (Fig. 3d, left panel), rearing \[F(3, 36) = 0.24, \text{NS}\], grooming \[F(3, 36) = 1.5, \text{NS}\], or defecation \[F(3, 36) = 0.41, \text{NS}\]. It is inferred from these data that naloxone by itself does not alter memory acquisition.

Moreover, Kruskal–Wallis and one-way ANOVA analyses showed that, in sham-BDL animals, L-arginine did not alter memory acquisition and exploratory behaviors. In conclusion, 2.5 and 5 mg/kg of morphine appeared to decrease memory acquisition 7 days after BDL.

**The effects of L-arginine on memory formation and exploratory behaviors in the presence or absence of cholestasis**

Kruskal–Wallis and one-way ANOVA analyses showed that the same dose of L-arginine decreased head-dip counts \[F(3, 36) = 3.08, \ P < 0.05\] (Fig. 3b, right panel) in BDL mice, while not altering memory acquisition \[H(3) = 2.26, \text{NS}\] (Fig. 3a, right panel), latency to the first head dip \[F(3, 36) = 0.89, \text{NS}\] (Fig. 3c, right panel), locomotor activity \[F(3, 36) = 0.64, \text{NS}\] (Fig. 3d, right panel), rearing \[F(3, 36) = 0.74, \text{NS}\], grooming \[F(3, 36) = 0.31, \text{NS}\], or defecation \[F(3, 36) = 0.47, \text{NS}\]. This suggests that L-arginine may decrease BDL-induced anxiolytic-like behaviors.

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The effects of cholestasis on memory formation and exploratory behaviors. Three groups of animals were used. Inhibitory avoidance memory, head-dip counts, latency to the first head dip, and locomotor activity induced by cholestasis were tested 7 days after bile duct ligation (BDL). The data were compared with the normal (nonoperated) group. The bars that represent step-down latencies are expressed as median and quartile, whereas other bars represent mean±SEM. **P<0.01 when compared with the normal control group.

The effects of pretraining administration of morphine on memory formation and exploratory behaviors in the presence or absence of bile duct ligation (BDL). Different doses of morphine or saline were injected 15 min before training in sham-operated or BDL animals (left or right panels), respectively. On the test day, inhibitory avoidance memory (a), head-dip counts (b), latency to the first head dip (c), and locomotor activity (d) were evaluated. The bars that represent step-down latencies are expressed as median and quartile, whereas other bars represent mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 compared with the saline/sham-BDL group. +++P<0.001 compared with the saline/BDL group. +P<0.05 and +++P<0.001 compared with the respective control groups.
acquisition \( [H(3) = 1.1, \text{NS}] \) (Fig. 4a, left panel), head-dip counts \( [F(3, 36) = 0.28, \text{NS}] \) (Fig. 4b, left panel), latency to the first head dip \( [F(3, 36) = 0.51, \text{NS}] \) (Fig. 4c, left panel), locomotor activity \( [F(3, 36) = 0.5, \text{NS}] \) (fig. 4d, left panel), rearing \( [F(3, 36) = 0.4, \text{NS}] \), grooming \( [F(3, 36) = 1.1, \text{NS}] \), or defecation \( [F(3, 36) = 1.7, \text{NS}] \). From these results, \( \text{L-arginine, by itself, did not appear to alter memory acquisition.} \)

However, as shown in Fig. 4a (right panel), similar pretraining infusion of \( \text{L-arginine} \) 7 days after BDL decreased memory acquisition \( \text{[Kruskal–Wallis ANOVA,} H(3) = 32.96, P < 0.001\text{]. Analysis using Mann–Whitney’s} U\text{-tests showed that} \text{L-arginine at 100 and 200 mg/kg reduced memory acquisition as compared with the sham-BDL groups. Figure 4b–d (right panels) summarize the effects of} \text{L-arginine on exploratory behaviors in cholestatic mice. L-Arginine did not alter the head-dip count} \ [F(3, 36) = 0.14, \text{NS, Fig. 4b}], latency to the first head dip \ [F(3, 36) = 0.32, \text{NS, Fig. 4c}], locomotor activity \ [F(3, 36) = 0.77, \text{NS, Fig. 4d}], rearing \ [F(3, 36) = 0.96, \text{NS}], grooming \ [F(3, 36) = 0.78, \text{NS}], or defecation \ [F(3, 36) = 0.96, \text{NS}] \text{in cholestatic mice.} \)

The effects of \( \text{L-NAME on memory formation and exploratory behaviors in the presence or absence of cholestasis} \)

Kruskal–Wallis and one-way ANOVA analysis confirmed that \( \text{L-NAME did not alter memory acquisition in sham-BDL animals} \ [H(4) = 3.1, \text{NS}] \) (Fig. 5a, left panel). \( \text{L-NAME also exerted no effect on head-dip counts} \ [F(4, 45) = 0.91, \text{NS}] \) (Fig. 5b, left panel), latency to the first head dip \ [F(4, 45) = 0.89, \text{NS}] \ (Fig. 5c, left panel), locomotor activity \ [F(4, 45) = 1.31, \text{NS}] \ (Fig. 5d, left panel), rearing \ [F(4, 45) = 0.62, \text{NS}], grooming \ [F(4, 45) = 0.38, \text{NS}], or defecation \ [F(4, 45) = 0.64, \text{NS}] \). Figure 5a (right panel) shows that similar pretraining administration of the \( \text{L-NAME did not alter memory acquisition in BDL mice} \ [\text{Kruskal–Wallis ANOVA,} H(4) = 2.98, \text{NS}] \). However, one-way ANOVA and post-hoc analysis showed that \( \text{L-NAME (10 and 20 mg/kg) decreased head-dip counts in BDL mice} \ [F(4, 45) = 2.15, P < 0.05] \) (Fig. 5b, right panel) while not altering other exploratory behaviors such as latency to the first head dip \ [F(4, 45) = 0.95, \text{NS}] \ (Fig. 5c, right panel), locomotor activity \ [F(4, 45) = 0.57, \text{NS}] \ (Fig. 5d, right panel), rearing \ [F(4, 45) = 1.43, \text{NS}], grooming \ [F(4, 45) = 1.54, \text{NS}], or defecation \ [F(4, 45) = 0.51, \text{NS}] \). These results suggest that \( \text{L-NAME decreased the cholestasis-induced anxiolytic-like behaviors.} \)

The effects of coadministration of subthreshold doses of morphine and \( \text{L-arginine on memory formation and exploratory behaviors in cholestatic mice} \)

Kruskal–Wallis ANOVA and Mann–Whitney’s \( U\)-test analysis indicated that coadministration of subthreshold doses of morphine (1.25 mg/kg) and \( \text{L-arginine (50 mg/kg) 15 min} \)
The effects of pretraining administration of L-arginine on inhibitory avoidance memory and exploratory behaviors in the presence or absence of bile duct ligation (BDL). Different doses of L-arginine or saline were injected 15 min before training in sham-operated or BDL animals as shown in the left or right panels, respectively. On the test day, inhibitory avoidance memory (a), head-dip counts (b), latency to the first head dip (c), and locomotor activity (d) were examined. The bars that represent step-down latencies are expressed as median and quartile, whereas other bars represent mean±SEM. *P<0.05 and ***P<0.001 compared with the saline/BDL group.

The effects of pretraining administration of L-NG-nitroarginine methyl ester (L-NAME) on inhibitory avoidance memory and exploratory behaviors in the presence or absence of bile duct ligation (BDL). Different doses of L-NAME or saline were injected 15 min before training in sham-operated or BDL animals as shown in the left or right panels, respectively. On the test day, inhibitory avoidance memory (a), head-dip counts (b), latency to the first head dip (c), and locomotor activity (d) were evaluated. The bars that represent step-down latencies are expressed as median and quartile, whereas the other bars represent mean±SEM. *P<0.05 compared with the saline/sham-BDL group. +P<0.05 compared with the saline/BDL group.
before training decreased memory acquisition in BDL mice \( [H(3) = 22.22, P < 0.001] \) (Fig. 6a). However, Fig. 6b–d shows that coadministration of these drugs did not significantly alter head-dip counts \( [F(3, 36) = 2.8, \text{NS}, \text{Fig. } 6b] \), latency to the first head dip \( [F(3, 36) = 2.02, \text{NS}, \text{Fig. } 6c] \), locomotor activity \( [F(3, 36) = 2.19, \text{NS}, \text{Fig. } 6d] \), rearing \( [F(3, 36) = 0.33, \text{NS}] \), grooming \( [F(3, 36) = 2.2, \text{NS}] \), or defecations \( [F(3, 36) = 0.14, \text{NS}] \). These results suggest that morphine and L-arginine exert a synergistic amnesic effect in 7 days post-BDL mice without affecting anxiety behaviors.

The effects of naloxone on impairment of memory acquisition induced by L-arginine or morphine in cholestatic mice

Kruskal–Wallis and one-way ANOVA analysis showed that, in the sham-operated animals, i.p. pretraining injection of different doses of naloxone 15 min before saline injection did not alter memory acquisition \( [H(3) = 1.8, \text{NS}] \) (Fig. 7a, left panel), head-dip counts \( [F(3, 36) = 1.6, \text{NS}] \) (Fig. 7b, left panel), latency to the first head dip \( [F(3, 36) = 0.1, \text{NS}] \) (Fig. 7c, left panel), locomotor activity \( [F(3, 36) = 0.42, \text{NS}] \) (Fig. 7d, left panel), rearing \( [F(3, 36) = 0.13, \text{NS}] \), grooming \( [F(3, 36) = 0.41, \text{NS}] \), or defecation \( [F(3, 36) = 1.8, \text{NS}] \). However, in cholestatic mice, pretraining administration of naloxone (0.25 and 0.5 mg/kg) 15 min before injection of the effective dose of L-arginine (200 mg/kg) restored the L-arginine-induced memory impairment \( [Kruskal–Wallis ANOVA, H(3) = 25.12, P < 0.001] \) (Fig. 7a, middle panel) and decreased head-dip counts \( [F(3, 36) = 2.32, P < 0.05] \) (Fig. 7b, middle panel), while not altering other exploratory behaviors such as latency to the first head dip \( [F(3, 36) = 0.85, \text{NS}] \) (Fig. 7c, middle panel), locomotor activity \( [F(3, 36) = 0.91, \text{NS}] \) (Fig. 7d, middle panel), rearing \( [F(3, 36) = 0.5, \text{NS}] \), grooming \( [F(3, 36) = 1.42, \text{NS}] \), or defecation \( [F(3, 36) = 0.58, \text{NS}] \). This suggests that naloxone potentially restores the L-arginine-induced amnesia in BDL mice.

Similarly, pretraining administration of naloxone (0.25 and 0.5 mg/kg) 15 min before the effective dose of morphine (2.5 mg/kg) restored the morphine-induced memory impairment in cholestatic mice \( [Kruskal–Wallis ANOVA, H(3) = 19.6, P < 0.001] \) (Fig. 7a, right panel). Naloxone did not, however, alter exploratory behaviors such as head-dip counts \( [F(3, 36) = 1.23, \text{NS}] \) (Fig. 7b, right panel), latency to the first head dip \( [F(3, 36) = 0.76, \text{NS}] \) (Fig. 7c, right panel), locomotor activity \( [F(3, 36) = 0.56, \text{NS}] \) (Fig. 7d, right panel), rearing \( [F(3, 36) = 1.01, \text{NS}] \), grooming \( [F(3, 36) = 0.78, \text{NS}] \), or defecation \( [F(3, 36) = 1.22, P > 0.05] \). Therefore, naloxone seemed to restore morphine-induced amnesia but not anxiety-like behavior in BDL mice.

The effects of L-NAME on l-arginine-induced or morphine-induced impairment of memory acquisition in cholestatic mice

Kruskal–Wallis and one-way ANOVA analysis showed that, in sham-operated animals, i.p. pretraining injection of...
The effects of naloxone on L-arginine-induced or morphine-induced memory impairment in cholestatic mice. Bile duct ligation (BDL) animals received saline or naloxone 15 min before pretraining injection of saline (left panels), L-arginine (middle panels), or morphine (right panels). On the test day, inhibitory avoidance memory (a), head-dip counts (b), latency to the first head dip (c), and locomotor activity (d) were evaluated. The bars that represent step-down latencies are expressed as median and quartile, whereas other bars represent mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 compared with the respective control groups. +P<0.05 and +++P<0.001 compared with the saline, L-arginine/BDL group. ψψψP<0.001 compared with the saline, morphine/BDL group.
However, in cholestatic mice, pretraining administration of L-NAME 15 min before saline injection did not alter memory acquisition \( H(3) = 0.59, \text{NS} \) (Fig. 8a, left panel), head-dip counts \( F(3, 36) = 0.44, \text{NS} \) (Fig. 8b, left panel), latency to the first head dip \( F(3, 36) = 2.2, \text{NS} \) (Fig. 8c, left panel), locomotor activity \( F(3, 36) = 1.44, \text{NS} \) (Fig. 8d, left panel), rearing \( F(3, 36) = 0.74, \text{NS} \), grooming \( F(3, 36) = 0.88, \text{NS} \), or defecation \( F(3, 36) = 1.74, \text{NS} \).

Furthermore, pretraining administration of L-NAME (40 mg/kg) 15 min before the effective dose of L-arginine (200 mg/kg) restored the L-arginine-induced memory impairment \( H(3) = 24.66, P < 0.001 \) (Fig. 8a, middle panel). L-arginine also decreased the head-dip counts \( F(3, 36) = 3.01, P < 0.05 \) (Fig. 8b, middle panel) but did not alter other exploratory behaviors such as latency to the first head dip \( F(3, 36) = 1.13, \text{NS} \) (Fig. 8c, middle panel), locomotor activity \( F(3, 36) = 1.93, \text{NS} \) (Fig. 8d, middle panel), rearing \( F(3, 36) = 0.83, \text{NS} \), grooming \( F(3, 36) = 0.34, \text{NS} \), or defecation \( F(3, 36) = 2.1, \text{NS} \). It is inferred from these results that L-NAME restores L-arginine-induced amnesia and anxiety in BDL mice.

The present data indicate that, 7 days after BDL, whereas cholestasis does not alter memory formation, it increases exploratory behaviors in mice. In contrast, our recent study postulated that at 24 but not 12 days after BDL, cholestasis impaired and did not alter aversive memory and exploratory behaviors, respectively (Zarrindast et al., 2012). The presence of cholestasis-induced anxiolytic-like behaviors 7 days after BDL suggests the concept of the time dependency of BDL-induced cognitive deficits (Nasehi et al., 2012a, 2012b). Georgiev et al. (2008) showed that cholestasis induces dynamic changes in the liver of BDL mice, inasmuch as cholestasis induces acute injury with consequent reparative reactions 7 days after BDL, followed by a chronic injury phase resulting in liver fibrosis. In general, hepatic encephalopathy (HE) has been classified into three types: type A includes acute liver failure, type B is associated with portosystemic bypass without intrinsic hepatocellular disease, and type C corresponds to liver cirrhosis (Ferenci et al., 2002). Biliary cirrhosis, which refers to the type C-HE, has been observed 4–6 weeks after BDL (Kountouras et al., 1984; Garcia-Moreno et al., 2005; Geerts et al., 2008). However, there are few reports on the effect of cholestasis on passive avoidance or classical conditioning. Some studies have shown an impaired ability to discriminate between novel and previously encountered sample objects (Garcia-Moreno et al., 2005) as well as impaired spatial memory acquisition in the Morris water maze in BDL rodents (Huang et al., 2010). There are other reports suggesting deficits in visuospatial abilities (Tarter et al., 1987; Weissenborn et al., 2003) and working memory (Mendez et al., 2009) following HE, which might be because of changes in the hippocampal formation (Mechoulam et al., 2007). It should be noted that these two tasks (object recognition and Morris water maze) are completely different from the test used here, which hence assessed different behaviors and learning processes.

In those without clinical symptoms of HE, liver cirrhosis may cause mild cognitive impairment (Romero-Gomez et al., 2007). However, patients with signs of HE may present a full-blown state of attention deficit plus deterioration in memory and cognitive function. They may have some alterations in motor functions including psychomotor slowing, bradykinesia or hypokinesia and asterixis, which generally seem to be of central rather than peripheral origin (Burak et al., 2002). HE is characterized by deficits in several neurotransmitter systems in the brain (Butterworth, 1996; Lozeva et al., 2004) including opioidergic, dopaminergic, cholinergic, adrenergic, glutamatergic, GABAergic, and serotoninergic systems (Celik et al., 2005). Other studies have suggested that deficits in the release of corticotrophin-releasing hormone (Swain et al., 1993; Burak et al., 2002) and an imbalance in manganese homeostasis in the brain (Forton et al., 2004) are involved in cholestasis-induced behaviors.
The effects of morphine and naloxone on memory formation (7 days after BDL)

The present results showed that a pretraining injection of morphine, a μ-opioid receptor agonist, impairs memory acquisition while not altering exploratory behaviors. Moreover, the leftward shift in the morphine dose–effect curve in BDL mice and the effectiveness of a subthreshold dose of morphine in BDL mice suggest that BDL potentiates morphine-induced amnesia. However, pretraining injection of naloxone, a μ-opioid receptor
antagonist, in sham-operated or BDL mice did not alter memory acquisition, but reduced the anxiety-like behaviors. Different physiological and behavioral functions are under the influence of the opioidergic system and notable alterations in this system have been reported in patients with liver disease (Talaenko et al., 2005; Ebrahimkhani et al., 2006). Thus, several interactions between the cholestasis and the opioidergic system have been described. These include (a) the precipitation of an opioid-like syndrome in patients with cholestasis as well as in the mouse model of cholestasis upon administration of an opioid antagonist (Ghafourifar et al., 1997; Dehpour et al., 1998), (b) downregulation of μ-opioid receptors in the brain of BDL rats (Bergasa et al., 1992), (c) reversal of the antinociceptive effect of cholestasis by naloxone in rats, and (d) increased opioid activity in BDL rats as compared with controls (Bergasa et al., 1994; Dehpour et al., 2000). Although alterations in endogenous opioid peptides under impaired bile secretion have been shown (Bergasa et al., 1994; Dehpour et al., 2000), the role of the opioid system in the regulation of hepatothropic functions is not yet clear. There are reports indicating that cholestasis is accompanied by an increase in central opioidergic neurotransmission/neuromodulation tonus (Bergasa et al., 1994, 1995; Thornton and Losowsky, 1988).

HE has been shown to induce downregulation of opioid receptors and especially μ receptors in different experimental models (Tao et al., 1987; Bergasa et al., 1992). However, the actual mechanism of this downregulation has not been shown completely. One plausible mechanism is the internalization of these receptors because of their increased ligand levels (Bergasa et al., 1992). The increased level of opioidergic tonus is also considered a contributing mechanism in HE (Bergasa et al., 1992).

The effects of L-arginine and L-NAME on memory formation (7 days after BDL)

According to our data, pretraining administration of L-arginine, an NO precursor, did not alter memory acquisition or exploratory behaviors in sham animals. However, there was a notable leftward shift of the dose–effect curves of L-arginine in BDL mice. Pretraining injection of L-NAME, a nonselective nitric oxide synthesis (NOS) inhibitor, in either sham-operated or BDL mice, did not alter memory acquisition while decreasing head-dip counts.

NO, as a biological messenger, exerts a variety of different actions in the cardiovascular, immune, and nervous systems. This messenger is produced by the enzyme NOS (Heinzen and Pollack, 2003; Nasehi et al., 2010a, 2010b). Three isoforms of NOS have been identified including neuronal (type I), inducible (type II), and endothelial (type III) (Alderton et al., 2001). Although all NOS isoforms are present in the brain, neuronal NOS is the predominant isoform (Alderton et al., 2001); thus, the effects of NO on neurotransmission are mainly attributed to NO produced by neuronal NOS (Itzhak et al., 2000; Kiss, 2000). Moreover, a study showed that types I and III of NOS isoforms were located in pyramidal and interneurons of the hippocampus, respectively (Doyle and Slater, 1997).

HE-induced NO overproduction has been discussed previously (Rao, 2002); however, the actual mechanism of this phenomenon has not been completely understood. It has been proposed that there is a close relationship between NO and ammonium overproduction following HE, inasmuch as hyperammonemia induces the activation of NMDA receptors through opening of the calcium channels that are associated with NMDA receptors (Hermenegildo et al., 1998, 2000). NMN induces its response through the type I isoform of NOS, which is highly expressed in hippocampal interneurons. This response is therefore blocked by L-NAME, which is a NOS enzyme inhibitor (Segieth et al., 2000). These findings are in agreement with previous reports supporting the involvement of the NO system in the modulation of learning and memory (Holscher and Rose, 1992; Bohme et al., 1993; Baratti and Kopf, 1996) through the common glutamate–NO–cGMP pathway (Felipo, 2006). An earlier study indicated that the learning and memory deficit observed in the animal model of HE is because of impairment of the glutamate–NO–cGMP pathway (Felipo, 2006). This has also been suggested to contribute to bradycardia (Nahavandi et al., 2001a, 2001b; Mani et al., 2002), hypotension (Hajrasouliha et al., 2004), hyporesponsiveness of isolated atria (Mani et al., 2002; Hajrasouliha et al., 2004), and vascular beds (Namiranian et al., 2001) adrenoceptor stimulation in cholestatic liver disease.

It has been reported that L-NAME can block the naltrexone-induced restorative effect on cholestasis-induced amnesia in rats 28 but not 21 days after BDL in the spatial recognition memory task (Javadi-Paydar et al., 2013). This re-emphasizes the importance of the time frame (days after BDL) when investigating BDL-induced behaviors. The overproduction of both opioids and NO as well as opioid receptor downregulation following HE have been well described (Tao et al., 1987; Bergasa et al., 1992). Moreover, it has been accepted that NO signaling is required for the formation of long-term memory and disruption of NO signaling impairs this phenomenon (Schafe et al., 2005; Juch et al., 2009; Limon et al., 2009; Tanda et al., 2009; Zhou and Zhu, 2009). However, it has also been proposed that excess NO may be involved in disorders of neural circuitry such as schizophrenia (Boultdakis and Pitsikas, 2010). In general, excessive NO concentrations have been shown to lead to mitochondrial dysfunction (Das et al., 1998) and neuronal damage (Dawson et al., 1991). Nevertheless, the actual action of low doses of NOS mechanism(s) on learning and memory is not yet completely clear. It seems that mild and transient use of NOS inhibitors induces
The synergistic effects of opioidergic and nitrergic systems on memory formation (7 days after BDL)
The pretraining coadministration of subthreshold doses of morphine and L-arginine impaired memory acquisition in BDL mice. Moreover, the pretraining administration of subthreshold doses of naloxone or L-NAMe blocked the morphine-induced and L-arginine-induced impairment of memory acquisition 7 days after BDL. Moreover, exploratory naloxone and L-NAMe reduced exploratory behaviors only in L-arginine-treated, but not in morphine-treated, mice.

The results of some studies have postulated that opioid–NO interactions play a critical role in cholestasis-induced neural pathology. However, further experiments are required to elucidate the molecular basis of this interaction (Dehpour et al., 1998; Nahavandi et al., 2001a, 2001b). The interaction has been shown in different biological models such as morphine-induced amnesia (Rezayof et al., 2006; Zarrindast et al., 2006). In agreement with this notion, Fimiani et al. (1999) have shown that release of NO may be mediated through μ-opioid receptors (Zhu et al., 2004). In contrast, the activation of μ-opioid receptors stimulates the release of NO in various organ systems. Given these findings, the current data strongly suggest a synergistic interaction between opioidergic and nitrergic systems upon cholestasis-induced memory acquisition impairments in mice.

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Conflicts of interest
There are no conflicts of interest.

References
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