

Melatonin prevented spatial deficits and increases in brain asymmetric dimethylarginine in young bile duct ligation rats

Mei-Hsin Hsu^a, Yu-Chieh Chen^a, Jiunn-Ming Sheen^a, Shih-Wen Li^a
and Li-Tung Huang^{a,b}

Bile duct ligation (BDL) in young rats can cause impaired liver function and cognition deficits. Nitric oxide is implicated in hepatic encephalopathy and is also involved in cognition. In this study, we examined the role of brain asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, in young BDL rats with spatial deficits. Young male Sprague-Dawley rats aged 17 days were assigned to four groups: laparotomy (SHAM), laparotomy plus 5 mg melatonin delivered through a pellet (SHAMM) for 4 weeks, BDL for 4 weeks, and BDL plus 5 mg melatonin delivered through a pellet (BDLM) for 4 weeks. Their spatial memory was assessed using a Morris water-maze task. Plasma and brains were collected for biochemical and ADMA analyses. We found that the BDL group had significantly elevated levels of ADMA in the plasma, the prefrontal cortex, and the dorsal hippocampus, and worse spatial performance than that of the control groups. Melatonin administration prevented an increase in the ADMA levels in the plasma, prefrontal cortex, and dorsal hippocampus, and prevented spatial deficits in BDL rats.

Introduction

Hepatic encephalopathy (HE) is a serious neuropsychiatric complication of acute liver failure or cirrhosis [1]. As per the International Society for Hepatic Encephalopathy and Nitrogen Metabolism, the rodent bile duct ligation (BDL) model is an animal model of chronic liver failure associated with HE [2]. BDL in rats can cause brain dysfunction, including increased oxidative/nitrosative stress [3] and cognition impairment [4,5].

Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid that can inhibit nitric oxide (NO) synthase and consequently decrease the synthesis of NO [6]. NO, which is inhibited by ADMA, plays an important role in spatial memory [7]. The glutamate–NO–cyclic GMP pathway is compromised in the brain of individuals with HE [8]. ADMA is primarily metabolized by the liver. BDL in rats can cause increased circulating and

In addition, melatonin maintained brain-derived neurotrophic factor in the dorsal hippocampus at a level comparable with controls. We concluded that melatonin is effective in preventing spatial deficits and decreasing ADMA levels in the plasma, prefrontal cortex, and dorsal hippocampus in young BDL rats. Brain ADMA levels might play a role in BDL-induced spatial deficits. *NeuroReport* 29:541–546 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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^aDepartment of Pediatrics, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung and ^bDepartment of Traditional Medicine, Chang Gung University, Linkow, Taiwan

Correspondence to Li-Tung Huang, MD, Department of Pediatrics, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, 123 Dabi Road, Niao Sung, Kaohsiung 833, Taiwan
Tel: +886 975 056 169; fax: +886 7 7338009; e-mail: litung.huang@gmail.com

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brain ADMA concentrations [6]. However, the role of ADMA in HE remains largely unknown.

Melatonin can decrease kidney and liver ADMA levels in the context of BDL [9]. Melatonin (*N*-acetyl-5-methoxytryptamine), an effective free radical scavenger and antioxidant, is mainly secreted as a neurohormone by the pineal gland [10]. Melatonin can pass the blood–brain barrier and is found to be protective after experimental traumatic brain injury [11], hypoxic brain damage [12], and HE [4].

Cognitive dysfunction is one of the most challenging complications of HE, but no specific treatment is currently available. In this study, we evaluated the effect of melatonin released through a pellet on plasma and brain ADMA levels and impaired spatial memory in young BDL rats.

Materials and methods

Animals

This experiment was conducted under the Guidelines for Animal Experiments of Chang Gung Memorial Hospital. All experiments were conducted using Sprague-Dawley rats, and day of birth was designated as postnatal day 0

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(PND 0). Only male rats were used to avoid the sex effects on spatial memory and oxidative stress. At PND 17, an age equivalent to human early childhood, male Sprague-Dawley rats with an average weight about 50 ± 5 g were operated as we reported previously [5]. Attempts were made to minimize the numbers of animals used. All animals were housed in a room maintained at 24°C with 12-h light/dark cycles. All animals had free access to standard chow and tap water.

Treatment of animals and grouping

All surgical procedures were performed under ketamine (50 mg/kg) and xylazine (23 mg/kg) anesthesia with clean surgical techniques at PND 17 as described previously [4]. The young BDL rats were induced by opening a midline incision and the common bile duct was ligated and divided with double ligatures of the proximal duct (BDL group, $n = 10$). In addition, rats that received sham ligation of the bile duct were designated as the sham-control group (SHAM group, $n = 10$). The third group included sham-operated rats that received melatonin pellet treatment ($n = 10$, SHAMM group). Melatonin-treated rats received melatonin between PND 17 and 45 through a slow-release melatonin pellet (5 mg; Innovative Research, Sarasota, Florida, USA) implanted into the peritoneum. In addition, BDL rats that received melatonin pellets were designated as the BDLM group ($n = 10$).

Morris water maze: spatial memory

The Morris water-maze test was performed to assess spatial learning and memory between PND 37 and 42 [5,13]. The water-maze was a circular pool (180 cm diameter, 50 cm high) filled with opaque water ($24 \pm 1^\circ\text{C}$) to a depth of 25 cm. The invisible platform was 12 cm in diameter and made of white Plexiglas and placed 1.5 cm below the water level and equidistant from the sidewall and middle of the pool. The pool was divided into cardinal points, and the hidden platform location and spatial cues remained constant throughout experimentation. A video camera was set up above the center of the pool and connected to a video traction system (Noldus, Ethovision, The Netherlands). On the first day, PND 37, each rat was placed in the pool for 120 s without the platform to acclimatize them to the training environment. One day after acclimatization, rats were trained for six trials per day to locate and escape onto the submerged platform (PND 38–41). If a rat failed to escape within 120 s, it was placed manually on the platform. Latencies to reach the platform, the distance traveled, and the average swimming speed were recorded. Latency to reach the platform during each trial was used as a measure of acquisition. Retention of memory was evaluated on PND 42 in the absence of the platform from the pool. The percentage of time spent in the quadrant where the platform was located previously was used as a measure of retention of memory.

Measurement of plasma biochemistry parameters

After receiving BDL for 4 weeks, blood samples were collected by cardiocentesis in all four experimental groups. Rats ($n = 10/\text{group}$) were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct bilirubin, and total bilirubin according to the methods that we have published previously [4].

Tissue collection

The rats were euthanized on PND 45, and the prefrontal cortex and dorsal hippocampus were immediately collected.

Enzyme-linked immunosorbent assay

Plasma (Immunodiagnostic AG, Bensheim, Germany), prefrontal cortex, and hippocampus ADMA (Bluegene, Shanghai, China) and hippocampus brain-derived neurotrophic factor (BDNF; Bluegene) were examined using enzyme-linked immunosorbent assay kits according to the manufacturers' instructions as we reported previously [5].

Immunohistochemistry assay

For immunohistochemical (IHC) staining, rat brain tissues were fixed in formaldehyde and dehydrated in 70% ethanol for 30 min, in 95% ethanol for 30 min, and finally in 100% ethanol for 30 min. The tissues were embedded in paraffin at 58°C, and then cut at 4–15- μm thick section using a rotary microtome. Before staining, the sections were floated in a 56°C waterbath and the sections were mounted onto slides. The slides with paraffin-embedded sections of rat brain tissue were dewaxed in xylene two times for 5 min, rehydrated in 100% ethanol for 1 min, in 90% ethanol for 1 min, and finally in 80% ethanol for 1 min. Slides with rat cortex tissues were incubated in 3% H_2O_2 for 1 min to remove endogenous superoxidase activity, washed with PBS, and heated with 100°C EDTA (pH 9.0) for 20 min to induce antigen retrieval. Subsequently, slides were blocked with a protein block solution and incubated with rabbit anti-ADMA antibodies for 30 min. After washing with PBS, slides were reacted with Polymer-horseradish peroxidase for 20 min, developed using the 3,3'-diaminobenzidine substrate, and counterstained with hematoxylin.

Statistical analysis

Morphologic parameters and biochemical parameters were analyzed by one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test. Results from the Morris water-maze acquisition memory were evaluated using a two-way ANOVA (with group as the between-subjects factor) with repeated measures (day). The major result of the probe test (e.g. dwell time in the platform quadrant vs. all other quadrants) was compared using one-way ANOVA, followed by Bonferroni post-hoc tests. All analyses were carried out using the SPSS version 15

(IBM, Armonk, New York, USA) in a PC-compatible computer. Values were expressed as mean±SEM and significance was defined as *P* value less than 0.05 for all tests.

Results

Morris water maze

The water-maze tests indicated that all rats learnt how to find the platform and that there was no significant difference in swim velocity between the different treatment groups at any time (*P*>0.1).

Two-way ANOVA indicated that escape latencies improved over time in all four groups as shown by a significant effect of day [*F*(3,34)=7.984, *P*=0.001], indicating that learning occurred (Fig. 1a). There were significant differences among the groups for the number of trial blocks that were needed to learn to escape by swimming using visual cues [*F*(3,34)=7.699, *P*=0.001; SHAM vs. BDL, *P*<0.01; BDL vs. BDLM,

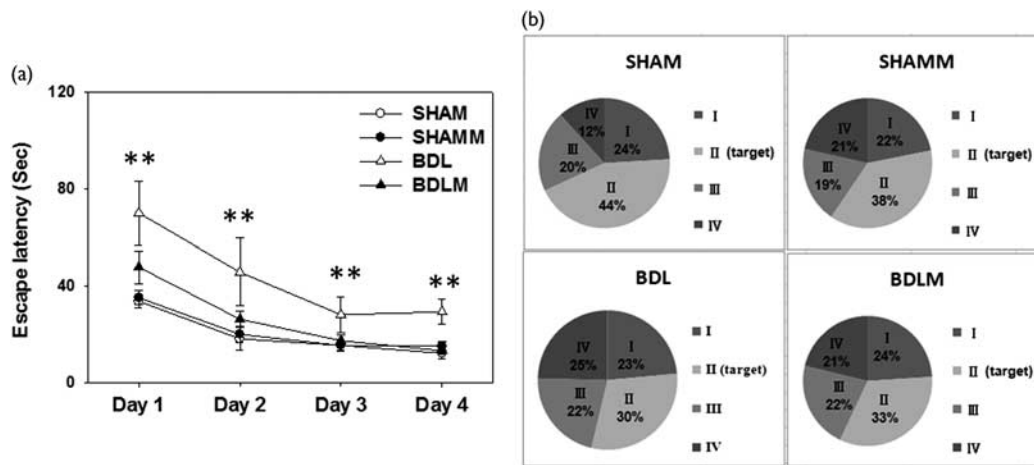
P<0.01] (Fig. 1a). These results show that BDL caused acquisition memory deficits that melatonin could prevent.

There were quadrant differences among the SHAM, SHAMM, and BDLM groups [SHAM, *F*(3,39)=10.445, *P*=0.001; SHAMM, *F*(3,51)=4.623, *P*=0.006; BDLM, *F*(3,23)=3.202, *P*=0.045] on retention in PND 41. However, there was no significant difference of retention in the target quadrant in the BDL group [*F*(3,23)=1.695, *P*=0.2]. These results showed that BDL caused retention memory deficits that were prevented by melatonin.

Plasma biochemistry parameters

Plasma levels of direct and total bilirubin, AST, and ALT were higher in the BDL group than in the SHAM group (all *P*<0.05) (Table 1). However, the BDLM group had normal AST, ALT, and direct/total bilirubin levels (Table 1).

Fig. 1



Spatial learning and memory tested by a Morris water maze. (a) Escape latencies to the platform in the Morris water maze (mean±SEM). Rats in the BDL group swam for a longer period to locate the submerged platform on all four acquisition days compared with rats from the other three groups (all *P*<0.001). There was no significant difference between BDLM and SHAM or SHAMM groups. (b) There were quadrant preferences in SHAM, SHAMM, and BDLM groups (all *P*<0.05). In contrast, there was no quadrant preference in the BDL group, indicating poor retention memory. ***P*<0.01 versus the SHAM group. BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; SHAM, sham control; SHAMM, sham control treated with melatonin.

Table 1 Clinical parameters

	SHAM (N=10)	SHAMM (N=10)	BDL (N=10)	BDLM (N=10)
AST (IU/l)	114.1±6.9	103.5±6.4	456.0±32.4*	295.8±23.3*#
ALT (IU/l)	48.2±2.4	53.8±2.7	139.0±16.1*	100.8±7.6*#
Direct bilirubin (mg/dl)	0.1±0.0	0.2±0.0	6.1±0.6*	4.3±0.5*#
Total bilirubin (mg/dl)	0.3±0.0	0.5±0.1	8.8±0.9*	6.06±0.7*#
Body weight (g)	195.9±8.5	182.4±6.7	115.4±7.7*	105.8±8.38

Values are represented as mean±SEM.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; SHAM, sham control; SHAMM, sham control treated with melatonin.

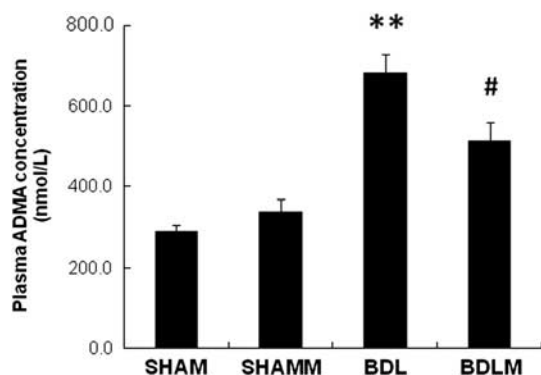
**P*<0.05 versus SHAM.

#*P*<0.05 versus BDL.

Asymmetric dimethylarginine enzyme-linked immunosorbent assay levels in the plasma, prefrontal cortex, and dorsal hippocampus

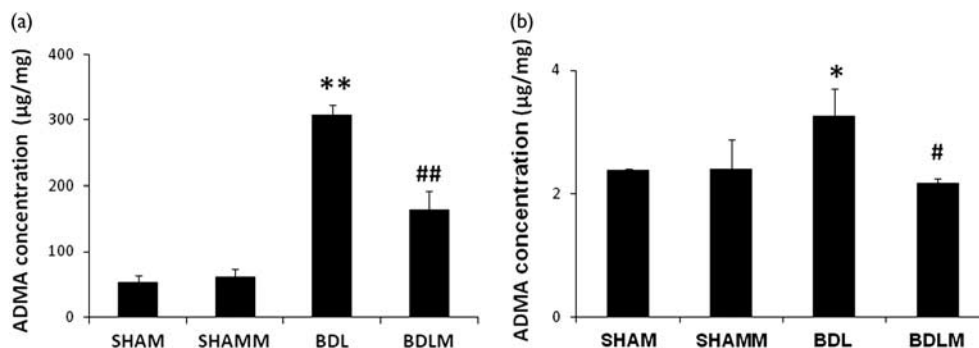
Plasma ADMA was significantly higher in the BDL group, but not in the BDLM group [$F(3,33)=17.975$, $P<0.001$; BDL vs. SHAM, $P<0.01$; BDL vs. BDLM, $P<0.05$] (Fig. 2). We detected higher ADMA in the prefrontal cortex of the BDL group, but not in the BDLM group [$F(3,40)=15.588$, $P<0.01$; BDL vs. SHAM, $P<0.01$; BDLM vs. BDL, $P<0.01$] (Fig. 3a). We also detected higher ADMA in the dorsal hippocampus in the BDL group, but not the BDLM group [$F(3,26)=4.229$, $P=0.017$; BDL vs. SHAM, $P<0.05$; BDLM vs. BDL, $P<0.05$] (Fig. 3b).

Fig. 2



Plasma ADMA level. The BDL group had a higher plasma ADMA level than the control group. The BDLM group had lower ADMA levels compared with the BDL group, indicating the effectiveness of melatonin. ** $P<0.01$ versus the SHAM group, # $P<0.05$ versus the BDL group. ADMA, asymmetric dimethylarginine; BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; SHAM, sham control; SHAMM, sham control treated with melatonin.

Fig. 3



ADMA levels in the rat prefrontal cortex and dorsal hippocampus. The BDL group had higher prefrontal cortex (a) and dorsal hippocampus (b) ADMA levels than the control groups. The BDLM group had lower prefrontal cortex and dorsal hippocampus ADMA than the BDL group, indicating the effectiveness of melatonin. * $P<0.05$, ** $P<0.01$ versus SHAM group; # $P<0.05$, ## $P<0.01$ versus BDL. ADMA, asymmetric dimethylarginine; BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; SHAM, sham control; SHAMM, sham control treated with melatonin.

Brain-derived neurotrophic factor enzyme-linked immunosorbent assay levels in the dorsal hippocampus

Previous studies showed that BDNF supports synaptic plasticity and that it is critically involved in memory processes. We detected lower levels of BDNF in the dorsal hippocampus in the BDL group, but not the BDLM group [$F(3,24)=8.796$, $P=0.021$; BDL vs. SHAM, $P<0.05$; BDLM vs. BDL, $P<0.05$] (Fig. 4).

Immunohistochemical staining of asymmetric dimethylarginine in the brain

IHC staining showed that the ADMA levels were higher in the BDL group than in the SHAM group. Moreover, melatonin treatment restored brain ADMA in the BDLM group [Fig. 5, $F(3,12)=3.451$, $P=0.01$; BDL ~1.4-fold of SHAM; BDL vs. SHAM, $P<0.01$; BDLM vs. BDL, $P<0.05$].

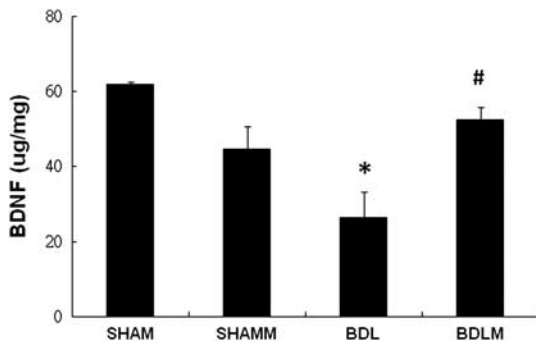
Discussion

The main findings of this study were as follows: (i) melatonin effectively prevented increases of ADMA in the plasma, prefrontal cortex, and dorsal hippocampus in young BDL rats; (ii) melatonin effectively upregulated the levels of BDNF in the dorsal hippocampus in young BDL rats; and (iii) melatonin effectively prevented a decline in spatial acquisition and retention memory deficits in young BDL rats.

A previous study showed an association between ADMA levels in the cerebrospinal fluid and cerebral vasospasm in a primate model of subarachnoid hemorrhage [14]. Conceivably, ADMA may contribute toward cerebral vasospasm and affect cognition [15]. Interestingly, epidemiological studies support a potential link between ADMA and cerebrovascular disease and cognitive impairment [16]. Therefore, agents that can lower ADMA are targets of current research.

Balasubramanian *et al.* [17] reported that brain ADMA levels were significantly higher in adult rats 4 weeks after BDL and their ADMA levels were reduced after treatment with ornithine phenylacetate. Similarly, Bajaj *et al.* [18] reported that patients with liver cirrhosis showed cognition dysfunction and higher serum ADMA levels. We have previously shown that rats with BDL for 2 weeks had increased plasma ADMA levels as well as cognitive deficits [19]. Here, we replicate our previous findings, showing increased ADMA levels in the prefrontal cortex in rats 4 weeks after BDL [5].

Fig. 4



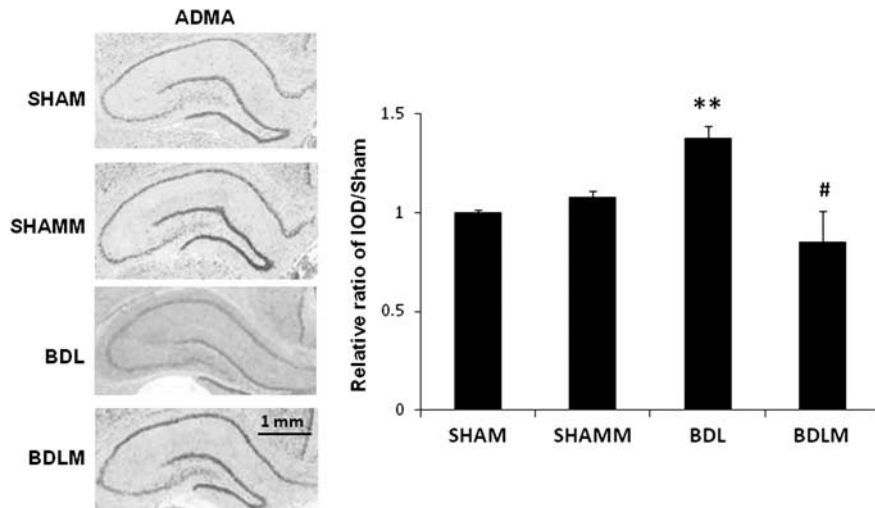
BDNF level in the rat dorsal hippocampus. The BDL group had a lower dorsal hippocampus BDNF level than the control groups. The BDLM group had higher dorsal hippocampus BDNF than the control groups, indicating the effectiveness of melatonin. * $P < 0.05$ versus the SHAM group; # $P < 0.05$ versus BDL. BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; BDNF, brain-derived neurotrophic factor; SHAM, sham control; SHAMM, sham control treated with melatonin.

The prefrontal cortex plays a role in memory, attentiveness, and perceptual awareness. The dorsal hippocampus and ventral hippocampus have distinct neuroanatomical profiles, whereas the dorsal hippocampus is primarily involved in cognition [20]. Spatial memory needs a wider network of inter-connected brain regions including the prefrontal cortex and the hippocampus. Rats with lesions of the prefrontal cortex or the hippocampus show spatial deficits [21]. In our previous study, the entire hippocampus did not show a significant difference in ADMA levels between controls and 4-week BDL rats [5]. Here, we found increased ADMA concentration in the dorsal hippocampus in 4-week BDL rats, that is, the BDL group, and melatonin infusion therapy prevented the increase in ADMA. In addition, IHC staining showed a protective effect of melatonin against increased hippocampal ADMA expression. To the best of our knowledge, this is the first report on the protective effect of melatonin against increases of ADMA levels in the dorsal hippocampus in a brain disorder.

BDNF supports synaptic plasticity and is critically involved in memory processing. Previous reports have shown decreased hippocampal *BDNF* mRNA in adult BDL rats [22] and decreased prefrontal cortex *BDNF* mRNA in young BDL rats [13]. Here, we showed a decrease in *BDNF* expression in BDL rats' dorsal hippocampus that was prevented by melatonin, suggesting a role of BDNF in cognition deficits in BDL rats.

Increased oxidative stress can interfere with BDNF expression. Oxidative stress may decrease the DNA-binding activities of activator protein-1 and cAMP

Fig. 5



Immunohistochemical staining of ADMA in rat brain. The ADMA level was decreased in the BDL rats. However, melatonin treatment prevented the decrease in ADMA levels (the BDLM group). The IOD in the positive areas of the rat cortex was measured using Image-Pro Plus 6.0 software. The IOD/area values of ADMA expression are shown in the histogram. ** $P < 0.001$ versus the SHAM group; # $P < 0.05$ versus the BDL group. $N = 3$ in each group. Scale bar = 100 μm . ADMA, asymmetric dimethylarginine; BDL, bile duct ligation; BDLM, bile duct ligation treated with melatonin; IOD, integrated optical density; SHAM, sham control; SHAMM, sham control treated with melatonin.

response element binding protein, and thus decrease *BDNF* gene expression [23].

ADMA is an oxidant and could therefore possibly interfere with *BDNF* synthesis. Interestingly, a 4-week ADMA infusion in healthy rats was shown to induce a marked reduction in serum *BDNF* levels [24]. This study thus provides supportive evidence that ADMA may affect cognition through downregulation of *BDNF*.

We have previously shown that melatonin prevented spatial deficits in young rats with BDL for 2 weeks [4]. We also found alterations in NADPH oxidase expression and blood–brain barrier in the prefrontal cortex and hippocampus in young BDL rats [25]. Here, we showed that melatonin released through a pellet could prevent spatial deficits in BDL rats by downregulation of circulating and brain ADMA and upregulation of dorsal hippocampus *BDNF*. In addition, melatonin prevented increases in plasma AST, ALT, and direct/total bilirubin levels in these rats.

Conclusion

We found that BDL in young rats cause spatial deficits and increased ADMA in the plasma, prefrontal cortex, and dorsal hippocampus. Melatonin released slowly through a pellet prevented changes in circulating and brain ADMA and prevented the development of any spatial deficit. These results could improve our understanding of the molecular mechanisms associated with HE and open new avenues for its treatment.

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Conflicts of interest

There are no conflicts of interest.

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