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RESEARCH ARTICLE

Hepatic encephalopathy induces site-specific changes in gene expression of GluN1 subunit of NMDA receptor in rat brain

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Abstract We investigate changes in gene expression of GluN1 subunit of N-Methyl-D-Aspartate (NMDA) receptor in the prefrontal cortex (PFC), hippocampus and striatum in a rat model of hepatic encephalopathy (HE). We used male Wistar rats in which HE was induced after a common bile duct ligation (BDL). The animals were divided into three sets, and each set included three groups of control, sham operated and BDL. In the first set of animals, blood samples collected for biochemical analysis on day 21 of BDL. In the second set, changes in nociception threshold was assessed on day 21 of BDL using a hotplate test. In the third set, whole brain extracted, and the PFC, the hippocampus and the striatum in each rat were immediately dissected. We used a semi-quantitative RT-PCR method for evaluating the *GluN1* gene expression. The biochemical analyses showed that plasma levels of ammonia and bilirubin in BDL rats were significantly increased compared to the sham control group on day 21 of BDL ($P<0.01$). Nociception threshold was also increased in rats with BDL compared to sham group ($P<0.001$). The results revealed that the *GluN1* gene expression at mRNA levels in BDL group was decreased by 19 % in the PFC ($P<0.05$) but increased by 82 % in the hippocampus ($P<0.01$) compared to the sham control group; however, no significant change was observed in the striatum. It can be concluded that HE affects the *GluN1* gene expression in rat brain with a site-specific pattern, and the PFC and hippocampus are more sensitive areas than striatum.

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Introduction

Hepatic encephalopathy (HE) is a term that is used to describe the alterations in cerebral function that result from liver failures (Butterworth 2008). There are different reports that HE is primarily induced by hyperammonemia followed by liver failure and subsequently causes morphological and physiological changes in astrocytes (Butterworth 2008; Rodrigo et al. 2010). In addition, it has been reported that HE affects neurons and neurotransmitter systems including gamma-aminobutyric acid (GABA) and glutamate, which may play main roles in the pathogenesis of the neurological alterations in HE (Cauli et al. 2009b; Felipo 2013; Llansola et al. 2013). Glutamate is the main excitatory neurotransmitter in the central nervous system that involved in many cerebral and neurological functions (Paoletti and Neyton 2007; Rousseaux 2008). It has been shown that blocking of *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors improves alterations induced by HE in rats and delays their death (Cauli et al. 2014; Felipo 2013; Vogels et al. 1997).

NMDA receptors play key roles in excitatory synaptic transmission and are also thought to be potential targets for the treatment of pathological changes of HE (Felipo 2013). According to molecular data, functional NMDA receptors are tetramers composed of different subunits namely GluN1, GluN2A-D, GluN3A-B, which were formerly known as NR1, NR2A-D and NR3 A-B in the old nomenclatures (Glasgow et al. 2015; Paoletti 2011). Different assemblies of NMDA receptor subunits have been reported in different sites of brain but GluN1 is an obligatory subunit of all endogenous NMDA receptors (Sanz-Clemente et al. 2013). It has been

shown that changes in expression of the GluN1 subunit modulate NMDA receptor function, which subsequently may set the tone of the nervous system's response to noxious stimuli and tissue injury (Da Silva et al. 2010).

Rats with common bile duct ligation (BDL) has been accepted and used as a model of chronic liver failure and HE (Butterworth et al. 2009; Rodrigo et al. 2010). In case of chronic liver failure and the subsequent HE, concentration of ammonia in circulation increases and it has been proposed that the reserve mechanisms of detoxification of ammonia in astrocytes may turn on (Felipo 2013; Rodrigo et al. 2010). Astrocytes turn ammonia into glutamine, which in turn it is transported to neurons where glutaminase deaminate glutamine into glutamate (Olde Damink et al. 2009). Therefore, it is possible that an increase in glutamate and excessive activation of NMDA receptors subsequently could also contribute to changes by which HE affects neural functions (Cauli et al. 2009b).

NMDA receptors are highly expressed in the forebrain structures including the PFC, hippocampus and striatum (Paoletti 2011). Considering the importance of GluN1 as an obligatory subunit of NMDA receptors, the aim of this study was to investigate changes in the *GluN1* gene expression in the PFC, hippocampus and striatum in a rat model of HE to correlate changes in gene expression of NMDA receptors within these areas with HE. We also aimed to propose a shared link between possible changes in expression of NMDA receptors in the above areas of rat brain with nociception processing.

Materials and methods

Subjects

In this study, we used 60 male Wistar rats weighing 300–350 g. The animals were kept in an animal house at a constant temperature (22±2 °C) under a 12 h light/dark cycle (light beginning at 7:00 a.m.). They had free access to food and water except for during the experiments. Experimental groups consisted of either eight rats in biochemical analysis of the serum and hotplate test or four rats in the gene expression studies, respectively. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (2011), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

Surgical laparotomy

Three groups of control, sham and rats with BDL were used in each part of the study. Sham and BDL groups were undergone laparotomy under anesthesia induced by intraperitoneal (i.p.) injection of a mixture of ketamine/xylazine (50 and 5 mg/kg, respectively). Sham operation consisted of laparotomy, bile duct identification and manipulation but without ligation and resection. In BDL rats, common bile duct was exposed, ligated at two points with approximately 5 mm apart, and then transected at the midpoint between the ligatures. Each rat received injection of 1 ml normal saline (i.p.) after closure of the abdominal wound and moved to a clean box until complete recovery. Mortality due to operation was about six percent during and/or after laparotomies.

Experimental design

Three sets of animals were used to complete this study. In the first set, a total number of 24 rats in three groups of control, sham and BDL (n=8, in each group) were used to examine plasma levels of ammonia and bilirubin. In the second set, three groups of control, sham and BDL (n=8, in each group) were used to study nociception threshold using a hotplate test. In the third set, three groups of control, sham and BDL (n=4, in each group) were used to examine the *GluN1* gene expression in the PFC, hippocampus and striatum. These animals were independent of those that we used in our previous study (Ahmadi et al. 2015).

Biochemical analysis

Twenty one days of bile duct ligation (BDL) rats of the first set of animals were anaesthetized and blood samples collected from heart for biochemical analysis. Plasma levels of ammonia was determined using a Biorex Ammonia Assay Kit (Biorex Diagnostics, Muckamore, Antrim, UK). In this assay, ammonia combines with α-ketoglutarate and NADPH in the presence of glutamate dehydrogenase to produce glutamate and NADP. This reaction results in a decrease in NADPH absorbance measured at 340 nm which is proportional to the concentration of ammonia in plasma. Total and direct bilirubin levels in plasma were also measured using standard kits (Darmankav, Esfahan, Iran). These assays are based on the reaction of bilirubin with diazotized sulphanilic acid in acidic medium to form the pink colored azobilirubin. The intensity of absorbance of the colored produced at 578 nm for total bilirubin and at 546 nm for direct bilirubin are directly proportional to their concentration present in the sample. Indirect bilirubin was indirectly calculated from difference of total and direct bilirubin.

Measurement of nociception

We used the second set of animals including control, sham and BDL groups to assess pain behavior using a hotplate test according to the method that we have earlier reported (Ahmadi et al. 2015). In brief, time elapse between placement of each animal on the hotplate (52±0.1 °C) and licking one of the hind

paws or first jumping was measured as an index of pain reaction latency. First, baseline latency was measured 1 day before surgical laparotomies. Second, different experimental groups (control, sham and BDL) on day 21 of BDL were tested to measure a test latency on the hotplate apparatus. A cutoff time of 80 s was set to prevent tissue damage. Finally, the two measured latencies were converted to percentage maximum possible effect (%MPE) using the following formula: %MPE = [(test latency − baseline latency)/(cut−off time − baseline latency)]×100 (Keil and Delander 1995; Ossipov et al. 1990). An increase in %MPE means an elevation in nociception threshold.

Dissection of the PFC, hippocampus and striatum from rat brains to examine the *GluN1* gene expression on day 21 of BDL

Three groups of control, sham and BDL rats (n=4, in each group) were used to examine the *GluN1* gene expression in the PFC, hippocampus and striatum. Twenty-one days of BDL, each rat was sacrificed, whole brain was quickly removed from the skull and the PFC, the hippocampus and the striatum were immediately dissected on an ice-chilled sterile surface according to previous reported method (Chiu et al. 2007). Then, each tissue was immediately moved into a tube in which it was submerged in RNAlater RNA Stabilization Reagent (QIAGEN, USA) and incubated overnight at 4 °C. Then, the RNAlater solution was drained after 24 h and the tubes containing the tissues were stored at −80 °C until further analysis.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 70 mg of each tissue using a TRIZOL method according to our previous study (Ahmadi et al. 2015). In brief, each tissue sample homogenized for 60 s in a tube containing 1 ml lysis buffer (RNX+ reagent, Cinagen, Tehran, Iran), with a high speed homogenizer (Silent Crusher S., Heidolph, Germany), then was subjected to a total RNA extraction according to manufacturer's manual (Cinagen, Tehran, Iran). Quality of the extracted total RNA was assessed by electrophoresis on 1 % agarose gel to visualize 28 s and 18 s ribosomal RNA. The quantities of total RNAs were also measured spectrophotometerically (Specord210, Analytic Jena, Germany). Synthesis of cDNA from the total RNAs was performed using a Viva 2-step RT-PCR Kit according to manufacturer's protocol (Vivantis Technologies, Selangor Darul Ehsan, Malaysia).

Polymerase chain reaction (PCR)

We used a semi-quantitative RT-PCR method to evaluate the *GluN1* gene expression in the brain areas (Marone et al. 2001). Related cDNAs of the *β-actin* (as control) and the *GluN1* genes were amplified using thermal cycling (C1000 Thermal Cycler, BIO-RAD, USA). Primers for both genes were designed at exon-exon junctions to prevent amplifications of genomic DNA fragments of the genes. The accession numbers were NM-031144 for *β-actin* gene and NM-017010 for the *GluN1* subunit of NMDA receptor gene. For primer designing, we have selected a sequence of the *GluN1* gene that was shared between its different isoforms. Primers had the following sequences: the *β-actin* forward primer, 5′-CTGGGTATGGAATCCTGTGGC-3′; the *β-actin* reverse primer, 5′-AGGAGGAGCAATGATCTTGATC-3′; the *GluN1* forward primer, 5′-TGGCATCATCGGACTTCAG-3′; the *GluN1* reverse primer, 5′- TCTGGTGGACATCTGGTATC-3′.

PCR was carried out in a reaction volume of 20 μl consisting of 10 μl of PCR Master Mix (Thermo Scientific), 2 μl of cDNA, 3 μl of upstream and downstream mix of the *GluN1* primers (10 μM), 2 μl of upstream and downstream mix of the *β-actin* primers (10 μM), and nuclease free water up to 20 μl. According to a pilot PCR optimization process, thermal cycling was initiated with a first denaturation step of 95 °C for 3 min, followed by 27 cycles of thermal cycling of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s, and finally followed by 10 min of a final extension step at 72 °C and was terminated at 4 °C for 5 min. Finally, the PCR products were subsequently analyzed on 2 % agarose gel electrophoresis and the bands were quantified with densitometry using Image J software.

Statistical analysis

Plasma levels of ammonia and bilirubin data failed to pass normality and/or equal variance tests, so these data were analyzed by Kruskal-Wallis one-way non-parametric ANOVA. Then, pairwise comparisons were done with Mann-Whitney's *U*-test and Holmes-Bonferroni's correction test was used after multiple comparisons. The obtained data for hotplate test and the quantified values for the *GluN1* gene expression passed normality and equal variance tests and they were analyzed with one-way ANOVA. Then, after a significant F value, Holm-Sidak's test was used for pairwise comparisons. The quantified value for the *GluN1* gene expression in each sample were normalized as percentage of the *GluN1* to *β-actin* ratio and then was set at 100 % as the relative the *GluN1* gene expression in the control group. $P<0.05$ was considered statistically significant level.

Results

Plasma levels of ammonia and all types of bilirubin were increased in BDL rats

Kruskal–Wallis one-way ANOVA revealed that plasma levels of ammonia was significantly altered between experimental

Table 1 Plasma levels of ammonia and all types of bilirubin in control, sham and BDL groups on day 21 of BDL

	Control	Sham	BDL
Ammonia (μg/dl)	50.7 (44.1–58.3)	57.1 (46.4–63-4)	290.2 (257.2–308.2)**
Direct Bilirubin (mg/dl)	0.19 (0.18–0.2)	0.2 (0.19–0.21)	2.83 (2.8–2.9)**
Indirect Bilirubin (mg/dl)	0.45 (0.43–0.49)	0.45 (0.43–0.47)	3.22 (3.1–3.3)**
Total Bilirubin (mg/dl)	0.64 (0.63–0.68)	0.65 (0.62–0.67)	6.05 (5.86–6.12)**

Data are median and interquartile range; **$P<0.01$ compared to the respective data of the sham control group

groups on day 21 of BDL [H (2)=15.68, $P<0.001$]. In addition, all types of bilirubin were significantly altered between experimental groups on day 21 of BDL [direct bilirubin, H (2)=16.23, $P<0.001$; indirect bilirubin, H (2)=15.4, $P<0.001$; and total bilirubin H (2)=15.39, $P<0.001$]. Pairwise comparisons with Mann-Whitney's U-test showed a significant increase ($P<0.01$) in plasma levels of ammonia and all types of bilirubin in the group with BDL compared to the sham control group (Table 1).

Nociception threshold was increased in rats with BDL

One-way ANOVA revealed that nociception threshold in experimental groups was significantly altered on day 21 of BDL [F (2, 21)=59.18, $P<0.001$]. The post hoc test revealed that nociception threshold in rats with BDL compared to the sham group was significantly increased (Fig. 1).

The *GluN1* gene expressions at mRNA levels were significantly decreased in the PFC, increased in the hippocampus and remained without significant change in the striatum

One-way ANOVA showed that the *GluN1* gene expression in the PFC was altered between the experimental groups [F (2, 9)=7.87, $P<0.05$], and in the hippocampus [F (2, 9)=11.36, $P<0.01$]. However, no significant change in the *GluN1* gene expression was observed between experimental groups in the striatum [F (2, 9)=0.72, $P>0.05$]. Post hoc test with Holm-Sidak's method revealed a significant decrease of the *GluN1* gene expression by 19 % in the PFC (Fig. 2a) but a significant increase by 82 % in the hippocampus (Fig. 2b) compared to the sham control group after 21 days of BDL. However, post hoc test revealed no significant change for the *GluN1* gene expression in the striatum (Fig. 2c).

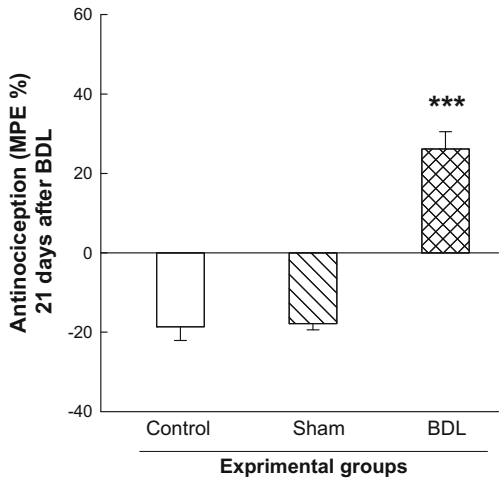

Fig. 1 Nociception was altered in rats with BDL. Three groups of control, sham and BDL rats (n=8) were submitted to the hotplate test on day 21 of BDL. Each bar represents mean ± S.E.M. of MPE% in each group. ***$P<0.001$ compared to the sham control group

Discussion

The results of biochemical analyses in this study showed that plasma levels of ammonia and all types of bilirubin including direct, indirect and total bilirubin were significantly increased in rats with BDL. An increase in plasma bilirubin levels is a common result of BDL that has been widely reported across literatures (Adler et al. 1977; Maillette de Buy Wenniger and Beuers 2010; Rodriguez-Garay 2003). The increased levels of bilirubin in plasma confirmed obstruction of common bile duct during the surgical laparotomy, which in turn might cause chronic liver failure (Butterworth et al. 2009; Magen et al. 2009; Wright et al. 2010). In addition, chronic liver failure may induce hyperammonemia and HE, which has been previously reported by other investigators (Cauli et al. 2009a; Rodrigo et al. 2010).

According to the previous researches, accumulations of ammonia and bilirubin and other toxic substances in the liver and plasma could affect functions of not only the liver but also the brain (Benedetti et al. 1997; Garcia-Ayllon et al. 2008; Huang et al. 2004). It has been also reported that liver failure alters brain functions via affecting neurotransmitter systems (Garcia-Ayllon et al. 2008; Lozeva et al. 2004). During hyperammonemia in HE, astrocytes detoxify ammonia into glutamine. Then, glutamine is transported to neurons and is converted into glutamate by glutaminase (Olde Damink et al. 2009). Therefore, it is possible that an increase in glutamate levels in neurons during HE may lead to imbalances in neural circuits that may finally affect neural functions.

The result of the hotplate test in the present study revealed that nociception threshold was increased in rats with HE on day 21 of BDL. This may reflect slowing of sensory

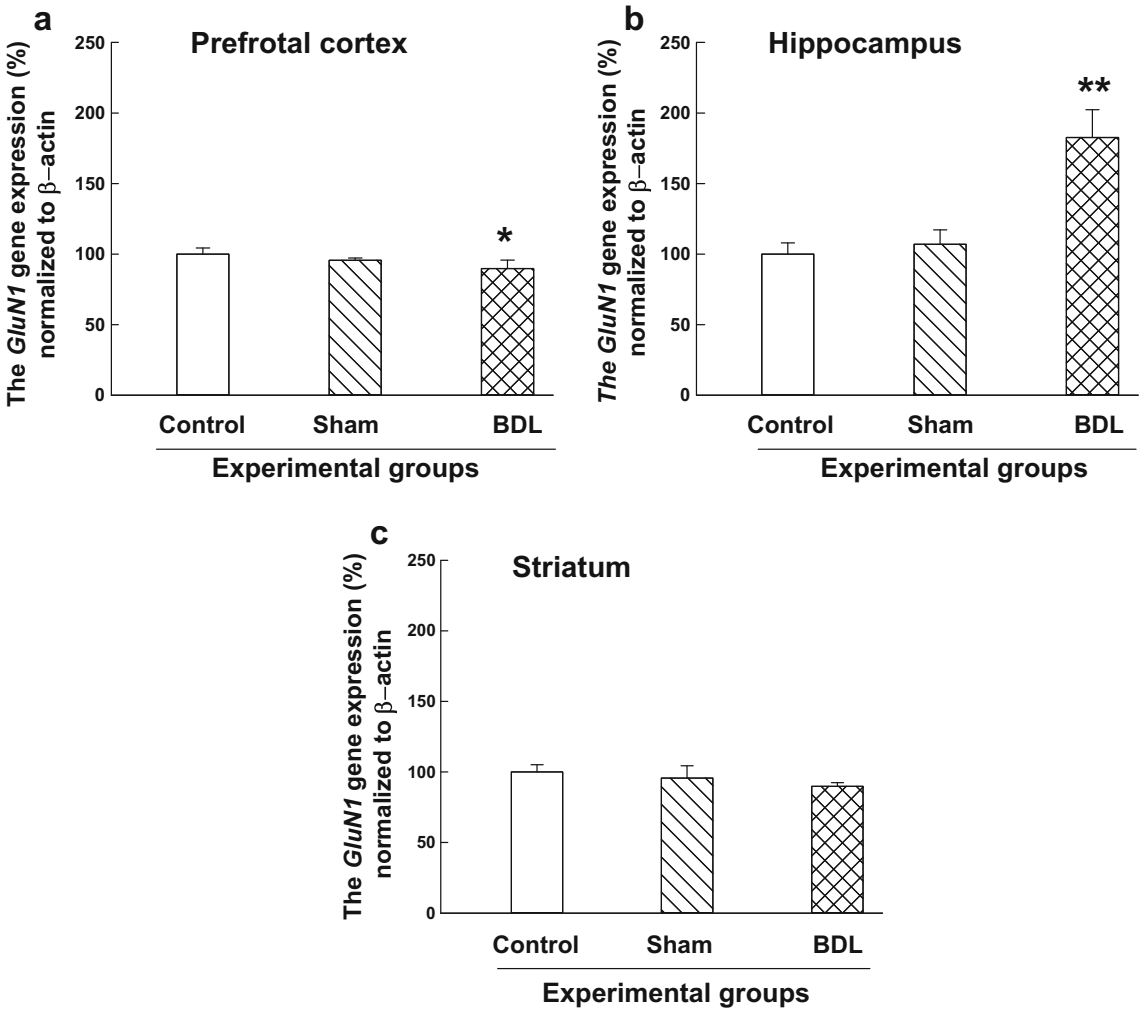

Fig. 2 The *GluN1* gene expression on day 21 of BDL in the prefrontal cortex (**a**), in the hippocampus (**b**) and in the striatum (**c**) of control, sham and BDL groups (n=4). Each *bar* represents mean ± S.E.M of the *GluN1* gene expression normalized to *β-actin* as control gene. *P<0.05 and **P<0.01 compared to the *GluN1* gene expression in the respective sham control group

processing in HE. It has been shown that plasma total opioid levels in cholestatic liver disease is increased, which may underlie changes including pruritus and analgesia after BDL (Alemi et al. 2013; Bergasa et al. 1995; Nicoll et al. 2005). In support of the involvement of a opioid system in the antinociception induced by BDL, we have recently reported that it was almost completely prevented by injection of a mu-opioid receptor antagonist, naloxone, on day 21 of BDL (Ahmadi et al. 2015). In addition, NMDA receptor pathway has been shown to be involved in modulation of cholestasis-induced antinociception in rats (Hasanein et al. 2007). According to previous researches, NMDA receptors play a key role not only in pain signal transmission in the spinal cord but also in pain perception at higher brain centers (Piovesan et al. 2008; Rodriguez-Munoz et al. 2012; Zhou et al. 2011). Therefore, we propose possible changes in NMDA receptor system may account for, at least partly, alteration in nociception threshold in the BDL model of HE.

To test changes in NMDA receptor expression in HE, we examined changes in gene expression of *GluN1* as an obligatory subunit of NMDA receptors in some forebrain areas including the PFC, hippocampus and striatum. The results revealed that the *GluN1* gene expression was significantly decreased in the PFC but was increased in the hippocampus; however, it was remained without significant changes in the striatum. It has been reported that alterations in certain neurotransmitters including GABA and glutamate play a main role in the pathogenesis of the neurological alterations in HE (Cauli et al. 2009b). According to previous reports, the process of neurotransmission can be altered in HE at different steps (Wen et al. 2013). We propose that hyperammonemia in HE may alter expression of NMDA receptors at mRNA levels. It has been reported that for many genes, transcript and protein levels do not correlate well (Tian et al. 2004). However, there are some reports that transcript levels of the GluN1 subunit are correlate with its protein levels (Jayanthi

et al. 2014; Priya et al. 2013). In addition, it has been recently reported that complete deletion of the obligatory GluN1 subunit of the NMDA receptors in hippocampal slice cultures completely eliminates NMDA receptors (Incontro et al. 2014). Therefore, it can be proposed that changes in the *GluN1* gene expression in the present study may importantly affect the amount of NMDA receptors in neurons of the examined brain areas, which subsequently may affect neural functions in HE.

Furthermore, according to the present results, it is possible that changes in the *GluN1* gene expression in a specific brain area in rats with HE cannot be extrapolated to other areas or to the whole brain. These results may be result from special pattern of afferents and interneurons in each area of the brain. In support of the different changes of the *GluN1* expression in different brain areas, Cauli et al. (2009a) have also reported that GABAergic tone is increased in cerebellum of rats with chronic hyperammonemia but reduced in cerebral cortex of the same animal (Cauli et al. 2009a). One may propose that hyperammonemia increases glutamate in the PFC, which subsequently induces a decrease in the *GluN1* gene expression. On the other hand, an increase in the *GluN1* gene expression in the hippocampus may result from imbalances in neurotransmitter systems that in turn may cause cognitive dysfunctions in HE (Rodrigo et al. 2010). However, the results of the *GluN1* gene expression in the striatum further indicate that cellular adaptation in different brain areas in HE may be a site-specific process.

The results of a recent research from our laboratory revealed that the mu-opioid receptor 1 *(MOR1)* gene expression was also influenced by BDL in the PFC, hippocampus and hypothalamus but not in the striatum (Ahmadi et al. 2015). We proposed that the observed changes in the *MOR1* gene expression might directly or indirectly result from an increase in endogenous opioids. Alternatively, one may propose that changes in opioid systems due to cholestasis may also influence neural circuits affecting NMDA receptor systems. Therefore, alteration in the *GluN1* gene expression could also be a consequence of changes in opioid systems and their subsequent resulting changes in rats with HE. However, more complementary experiments needs to be done to reveal the exact mechanism of these changes.

Conclusion

In summary, our results showed that hyperammonemia in a rat model of HE decreased the *GluN1* gene expression in the PFC but increased it in the hippocampus, and remained without significant changes in the striatum. These results may propose that changes in the *GluN1* gene expression in rat brain with HE have a site-specific pattern and consequence of these changes may underlie slowness of brain functions including sensory perception (for example nociception) and cognitive dysfunctions in HE.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed."

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