Bile duct ligation increased dopamine levels in the cerebral cortex of rats partly due to induction of tyrosine hydroxylase

Running title: Increased DA of BDL rats due to induction of tyrosine hydroxylase

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Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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Author contributions

WK, LL and XL designed the research. WK, XS, SY, PL and XZ carried out the experiments. JZ, LZ, TJ, MJ and JG participated in part of the experiments. WK, XL and LL performed data analysis. WK drafted the manuscript. WK, XS, YY, JZ, PL, XL and LL revised the manuscript and supervised the project. All of the authors have read and approved the final manuscript.

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The authors have no conflicts of interest to declare.

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BULLET POINT SUMMARY

What is already known?

Liver failure, associated with psychiatric alterations, partly resulting from increased dopamine levels in brain

What this study adds?

BDL increased dopamine levels partly due to bilirubin-mediated TH induction via activating NF-κB signaling pathway.

Clinical significance?

Provide an explanation for increased DA levels and mental behavior abnormalities in patients with cholestasis.

Abstract

Background and Purpose

Liver failure is often associated with psychiatric alterations, partly resulting from the increased dopamine levels in brain. We aimed to investigate relationship between increased dopamine levels and mental abnormalities using bile duct ligation (BDL) rats and document mechanism that liver failure increased dopamine levels in SH-SY5Y cells.

Experimental Approach

Psychiatric alterations were operated following 14-day BDL. Dopamine and its metabolite levels in cortex, expressions of enzymes and transporters related to dopamine metabolism and transport in cortex and hippocampus were measured. SH-SY5Y cells were used to investigate whether NH_4Cl, bile acids and bilirubin affected expression of tyrosine hydroxylase (TH) or not. TH expression in SH-SY5Y cells co-incubated with bilirubin and signal pathway inhibitors was measured.

Key Results

Open-field test results showed a remarkable increase in exploratory behavior following BDL. BDL increased dopamine levels and expression of TH protein in cortex. MAO-A and Mb-COMT slightly but significantly decreased. Data from SH-SY5Y cells showed that increased bilirubin levels was a factor in inducing TH expression. Both inhibitor of NF-κB pathway BAY117082 and silencing p65 remarkably reversed bilirubin-induced upregulation of TH protein. NF-κB activator TNF-α increased expression of TH protein. Roles of bilirubin in TH expression and increases in dopamine levels were documented using hyperbilirubinemia rats. Significant increases in dopamine levels, expressions of TH, p65 and p-p65 protein were observed in hyperbilirubinemia rats.

Conclusion and Implications

BDL significantly increased dopamine levels in rat cortex partly due to bilirubin-

mediated TH induction. Increased bilirubin induced TH expression via activating NF-κB signaling pathway.

Keywords
Liver failure; tyrosine hydroxylase; dopamine; bilirubin; Hepatic encephalopathy

Abbreviations

HE: Hepatic encephalopathy; DA: dopamine; 5-HT: serotonin; BDL: bile duct ligation; MAO: monoamine oxidase; DOPAC: 3,4-dihydroxyphenylacelic acid; COMT: catechol-O-methyltransferase; HVA: homovanillic acid; TH: Tyrosine hydroxylase; Tyr: tyrosine; Phe: phenylalanine; UCB: bilirubin; ALT: alanine transaminase; AST: aspartate transaminase; AKP: alkaline phosphatase; CHX: Cycloheximide; TNF-α: human tumor necrosis factor alpha; BAY: BAY117082; CTR: control; PBS: phosphate buffered saline; HB: Hyperbilirubinemia; p-TH: phospho-tyrosine hydroxylase (Ser40); p65: NF-κB p65; p-p65: phosphorylated NF-κB p65; BAs: mix bile acids

1. INTRODUCTION

Hepatic encephalopathy (HE) is a complication of cirrhosis or other liver diseases, characterized by a wide spectrum of neurological or psychiatric abnormalities, ranging from subclinical alterations to coma (Ferenci, 2017; Nardone et al., 2016; Weissenborn, 2019). Various factors including brain accumulation of neurotoxins (such as hyperammonemia), dyshomeostasis of neurotransmitters, disorder of neurosteroids, infections and neuroinflammation are considered to contribute to HE, but none of above factors is generally accepted as the significant pathogenetic factor and measurement parameters in various types of HE (Ferenci, 2017; Nardone et al., 2016).

Several studies have demonstrated that HE is accompanied by the increased levels of dopamine (DA) in brain (Ding et al., 2014; Ding et al., 2013; Ding et al., 2016; Ding, Wang, Zhuge, Yang, & Zhuge, 2017; Rai & Dhiman, 2013; Zhuge et al., 2019). In

thioacetamide-induced acute liver failure rats, it was found that levels of 5-hydroxyindoleacetic acid and DA in frontal cortex and hippocampus as well as serotonin (5-HT) levels in frontal cortex were significantly higher than those in control rats (Yurdaydin et al., 1990). Significantly increased concentration of DA was also found in cerebrum and hippocampus of rats at 12 h following hepatectomy (Hadesman, Wiesner, Go, & Tyce, 1995). TAA-induced chronic liver failure also significantly elevated DA levels in rat hippocampus, accompanied by decreases in learning and memory ability, which were contributed to inhibition of glutamate-NO-cGMP pathway due to the elevated DA (Ding et al., 2014; Ding et al., 2013). In mice, liver failure by bile duct ligation (BDL) also led to abnormalities in the dopaminergic systems (Nasehi, Kafi, & Zarrindast, 2013; Nasehi, Mafi, Ebrahimi-Ghiri, & Zarrindast, 2016). Analysis of brain samples from liver cirrhosis patients with HE also demonstrated significantly higher DA levels compared with cirrhosis patients without HE (Cuilleret, Pomier-Layrargues, Pons, Cadilhac, & Michel, 1980). Similarly, analysis of CSF samples from patients with HE revealed remarkably higher concentrations of DA, 5-HT, histamine and gamma-aminobutyric acid than those of control. The increases were positively related to HE (Borg et al., 1982). However, real mechanisms for increasing levels of these neurotransmitters (such as DA and 5-HT) in brain were not fully understood.

DA is an important neurotransmitter in the brain, which is closely related to some neurological and psychiatric disorders (Zhuge et al., 2019). Homeostasis of DA levels in brain is highly controlled via synthesis and metabolism process. DA is mainly metabolized by monoamine oxidases (MAOs) to 3,4-dihydroxyphenylacelic acid (DOPAC) or to 3-methoxytyramine by catechol-O-methyltransferase (COMT). DOPAC and 3-MT are metabolized to homovanillic acid (HVA) by COMTs and MAOs, respectively (Enayah, Vanle, Fuortes, Doorn, & Ludewig, 2018; Miekus et al., 2020; Zhong et al., 2019). Tyrosine hydroxylase (TH) is a step-limiting enzyme of DA synthesis, catalyzing the conversion of tyrosine (Tyr) into L-dopa, which is further converted into DA under L-dopa decarboxylase (Enayah et al., 2018). Liver failure

may affect expressions of some enzymes mediated synthesis and metabolism of DA, although the reports are often contradictory. El Hiba et al. reported that TH immunoreactivity significantly decreased in both substantia nigra pars compacta and ventral tegmental area concomitantly with the cortical and the striatal outputs of BDL rats (El Hiba, Gamrani, Chatoui, & Ahboucha, 2013). Portacaval anastomosis remarkably lowered TH immunoreactivity in rat brain (Suarez, Bodega, Rubio, & Fernandez, 2017). Reduction of TH immunoreactivity was also found in a patient suffered from sick sinus syndrome and liver cirrhosis (Okoshi, Hayashi, Kanda, & Yamamoto, 2014). In contrast, Panayotacopoulou et al. reported that liver cirrhosis increased TH immunoreactivity in paraventricular and supraoptic Neurons (Panayotacopoulou et al., 2002). $CC1_4$-induce liver failure did not alter TH activity in the corpus striatum of rats (Takei et al., 1983). TH activity in post-mortem brains of human patients with hepatic coma was within normal limits (Jellinger, Riederer, Rausch, & Kothbauer, 1978). Liver failure also affects activity of MAOs in brain. Activity of MAOs in cortex and hippocampus of rats undergoing 4-week BDL was reported significant increases, the extent of increases in MAO-A was larger than those in MAO-B (Dhanda & Sandhir, 2015). Autopsied brain tissues from male cirrhotic patients with HE showed an increased MAO-A activity in frontocortical and cerebellar tissues, but MAO-B parameters were unaltered (Mousseau, Baker, & Butterworth, 1997). In portocaval shunt rats, it was also showed elevated MAO-B activity in the hypothalamus but not in other brain regions. MAO-A activity was unaltered (Fogel, Andrzejewski, & Sasiak, 1999). Frontal cortex and caudate nucleus of patient with hepatic coma also showed significant increases in activities of MAO-A and MAO-B (Rao, Giguere, Layrargues, & Butterworth, 1993). All these results indicated that alterations in expressions of enzymes related to DA metabolism and synthesis are special-brain rejoin and dependent on type and extent of liver failure too. The aim of the study was: 1) To determine associations of levels of DA in brain and the psychiatric alterations using BDL-induced chronic liver failure rats; 2) to measure expressions and functions of main enzymes related to DA metabolism and synthesis

such as MAOs, COMTs and TH in brain of BDL rats. Pre-experiment showed among the 3 tested enzymes, alteration in expression of TH was the strongest. Thus, we also screened and identified the main factors in serum of BDL rats affecting expression of TH as well as the real mechanism using SH-SY5Y cells as in vitro model. The in vitro data was confirmed by in vivo data. The results would partly highlight mechanisms that cholestasis increased brain DA levels.

2. METHODS

2.1 Materials

Dopamine hydrochloride (DA) and DOPAC were from Macklin Institute (Shanghai, China). L-Tyrosine (Tyr), L-phenylalanine (Phe), bilirubin (UCB) and ammonium chloride (NH_4Cl) were purchased from Aladdin Institute (Shanghai, China). Alanine transaminase (ALT), aspartate transaminase (AST), aspartate transaminas (AKP), total bile acids, and total bilirubin, direct bilirubin and blood ammonia assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MG-132 and U0126 were purchased from EFEBIO (Shanghai, China). Cycloheximide (CHX) was from Beyotime Institute of Biotechnology (Nanjing, China). Recombinant human tumor necrosis factor alpha (TNF-α) and BAY117082 (BAY) were obtained from MedChemExpress (Shanghai, China). Dulbecco's Modified Eagle Medium/ Ham‘s F12 nutrient medium (DMEM/F12) and fetal bovine serum (FBS) were purchased from Gibco (CA, USA). Penicillin and streptomycin were obtained by Sunshine Biotechnology Co., Ltd. (Nanjing, China). Radio Immunoprecipitation Assay (RIPA) lysis buffer, bicinchoninic acid kit and Coomassie brilliant blue G250 protein fast stain kit for protein content determination were supplied by Beyotime Institute of Biotechnology (Nanjing, China). All the other reagents and chemicals were of analytical grade.

2.2 Animals

Male Sprague-Dawley rats (weighing about 220g) from Super-B&K Laboratory

Animal Co., Ltd. (Shanghai, China) were housed in a relative humidity (50 ± 5%), temperature-controlled (24 ± 2 °C) and 12-h day/night cycles environment with water and food available free. The experimental protocols were carried out according to Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by China Pharmaceutical University Animal Ethics Committee.

2.3 Development of BDL rats

BDL rats were developed by bile duct ligation operation according to the previous method (Xu et al., 2016). Following 1-week acclimation, the rats were randomly divided into the sham-operation (Sham) group and BDL group. Sham rats were treated in the same manner without bile duct ligation only. On day 14 following surgical operation, the rats were sacrificed under ether anaesthesia and plasma, serum, liver, spleens and brain (cortex and hippocampus) were collected and stored rapidly at -80 °C for analysis. Part of the fresh cortex of rats was placed directly in the 4% paraformaldehyde fixative for immunohistochemical experiments.

2.4 Open field test

Another patch of BDL rats was developed for open field tests on Joint opening (open field) experimental video analysis system (Zhongshidichuang Institute, Beijing, China) according to the manufacturer's instruction. The open field was divided into inside circle and outer circle. The rats were placed in the open field for five minutes and repeated three times to record their behavior track. Software Any-maze Video Tracking System was used to record and process the experimental data. Before surgical operation, open field tests were carried out. Based on data of open field tests, the rats were divided into Sham rats and BDL rats, assuming that no significant differences for psychiatric behaviors between two group rats happened. Then, open field tests were again carried out on the 6th and 13th day following surgical operation, respectively.

2.5 Determination of DA and its metabolite in brain and plasma

About 50 μL plasma or 50 mg brain sample (weighing) was added in 2 mL Eppendorf tube and homogenized in 450 μL of precipitant containing 0.35 mol L^{-1} perchloric acid and 1.5 mmol L^{-1} cysteine. The supernatant was obtained by centrifuging at 18000 rpm. The contents of DA and DOPAC were determined by HPLC-fluorescence detector (ex/em, 290 nm/330 nm). The linear range of DA and DOPAC was 7.8 ng mL^{-1}-1000 ng mL^{-1} and 7.8 ng mL^{-1}-2000 ng mL^{-1}, respectively. Separation of DOPAC was performed on C18 column (YMC-Triart, 4.6×100 mm, 5 μm), whose mobile phase consisted of 8% methanol and 92% water (50 mmol L^{-1} KH_2PO_4 and 0.08 mmol L^{-1} EDTA). Flow rate was set as 1 mL min^{-1}. Separation of DA was operated on C18 bonds to pentafluorophenyl columns (Agilent, poroshell 120 PFP, 4.6*100 mm, 2.7 μm), whose mobile phase was 7% methanol and 93% water (0.05 mol L^{-1} NaH_2PO_4 and 0.02 mol L^{-1} citric acid). Flow rate was set as 0.7 mL min^{-1}.

The contents of Phe and Tyr in brain and plasma were detected by HPLC coupled with tandem mass spectrometry (LCMS-2020, Shimadzu, Japan). Tyr and Phe were extracted with n-butanol saturated with water and acidized using 0.01 mol L^{-1} HCl. After volatilization, the sample was redissolved with mobile phase (35% methanol and 65% water (0.01% acetic acid)). The internal standard was phenacetin. Flow rate was set as 0.2 mL min^{-1}. The linear range of both Phe and Tyr was 39 ng mL^{-1}-10000 ng mL^{-1}.

2.6 Quantitative real time-PCR (QT-PCR)

The mRNA levels of enzymes and transporters related to DA (OCT1/SLC22A1, OCT2/SLC22A2, OCT3/SLC22A3, dopamine transporter (DAT/SLC6A3), TH, COMT, MAO-A, MAO-B and p65/RELA) in rat brain (cortex and hippocampus) and in SH-SY5Y cells were detected by QT-PCR. Total RNA was extracted using TRIpure reagent (Takara, Shiga, Japan) and cDNA was synthesized using HiScript® III RT SuperMix (Vazyme, Nanjing, China) according to the manufacturer's protocol. Real-time PCR amplification was performed in lightcycler 96 real-time PCR system from

QuantStudio 3 Real-Time PCR Systems (Applied Biosystems) with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Primers of genes in rats and SH-SY5Y cells were listed in Table 1. The protocol was: pre-incubation for 300 s at 95 °C, 3-step amplification (10 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C), melting (10 s at 95 °C, 60 s at 65 °C, 15 s at 95 °C), then cooling 30 s at 37 °C. The comparative CT method was used to determine the relative mRNA expression using β-actin as reference.

2.7 Western blot analysis

Rat cortex, hippocampus and SH-SY5Y cells were prepared in RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride in appropriate proportion for measuring levels of OCT1-3, TH, Mb-COMT, S-COMT, MAO-A and MAO-B. Levels of phospho-tyrosine hydroxylase (Ser40) (p-TH) in SH-SY5Y cells were also measured in presence of phosphatase inhibitor. The extracted proteins were loaded in sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to PVDF/NC membranes. Membranes were incubated for 2 h with 5% non-fat milk-Tris-buffered saline containing 0.1% Tween 20 for blocking. The membranes were incubated with corresponding primary antibodies against β-actin (dilution 1:2500) (Proteintech, cat# 66009-1-Ig, RRID: AB_2687938), OCT1 (dilution 1:750) (Thermo Fisher Scientific, cat# PA5-86190, RRID: AB_2802983), OCT2 (dilution 1:5000) (Thermo Fisher Scientific, cat# PA5-104461, RRID: AB_2853762), OCT3 (dilution 1:1000) (Thermo Fisher Scientific, cat# PA5-96860, RRID: AB_2808662), MAO-A (dilution 1:3000) (Abcam, cat# ab126751, RRID: AB_11129867) and MAO-B (dilution 1:2500) (Santa Cruz Biotechnology Inc, cat# sc-515354, RRID: AB_2819030), COMT (dilution 1:500) (Sigma-Aldrich, cat# C6870, RRID: AB_1840764), TH (dilution 1:2500) (Santa Cruz Biotechnology Inc, cat# sc-25269, RRID: AB_628422), p-TH (dillution 1:500) (Cell Signaling Technology, cat# 2791S, RRID: AB_2201522), NF-κB p65 (p65, dillution 1:500) (Cell Signaling Technology, cat# 8242S, RRID: AB_10859369) and phosphorylated NF-κB p65 (p-p65, dillution 1:500) (Cell Signaling Technology,

cat# 3033S, RRID: AB_331284) at 4 °C overnight, respectively. Membranes were incubated for 1.5 h at room temperature with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:8000 dilution) (Bioworld Technology, cat# BS13278, RRID: AB_2773728) or anti-mouse antibody (1:5000 dilution) (Bioworld Technology, cat# BS12478, RRID: AB_2773727). The immunoreactivity was detected by a gel imaging system (Tanon 5200 Multi, Tanon Technology, Co., Ltd., Shanghai, China) using Super Signal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA) and intensity values were normalized to β-actin.

2.8 Measurement of MAO enzymes activity

MAOs (MAO-A and MAO-B) exist in mitochondria. Cortical mitochondria was roughly prepared by the method reported in the literature (Cai et al., 2016) and MAOs activity was measured by literature method (G. Huang et al., 2016). Benzylamine and 5-HT oxidation was respectively metabolized by MAO-B and MAO-A, these products were measured spectrophotometrically at 465 nm and 425 nm, and then corrected by protein concentration (Absorbance/mg protein).

2.9 Effect of abnormally altered compounds in serum of BDL rats on TH and p-TH protein expressions in SH-SY5Y cells

Cholestasis is often associated with increases in levels of bile acids, bilirubin (UCB) and ammonium in serum. SH-SY5Y cells were used to identify which compounds in serum of BDL rats affected expression of TH. In brief, SH-SY5Y cells, from Guangzhou Cellcook Biotech Co.,Ltd, (Guangzhou, China), were seeded in 12-well plates at 1.0×10^5 per well in DMEM/F12 which contains 10% FBS. The cells were given 10 μmol L^{-1} retinoic acid to differentiate for 3 days (Khwanraj, Phruksaniyom, Madlah, & Dharmasaroja, 2015; Kovalevich & Langford, 2013). The differentiated cells were treated with NH_4Cl (0, 0.1, 0.5, 5 mmol L^{-1}), mix bile acids (BAs) (20 μmol L^{-1} cholic acid, 10 μmol L^{-1} chenodeoxycholic acid, 5 μmol L^{-1} deoxycholic acid, 5

μmol L^{-1} hyodeoxycholic acid and 0.5 μmol L^{-1} lithocholic acid) and UCB (0, 0.01 μmol L^{-1}, 0.1 μmol L^{-1} and 0.5 μmol L^{-1}), respectively. The concentration of the mixed bile acids was designed according to concentration of BDL animals (Thakare, Alamoudi, Gautam, Rodrigues, & Alnouti, 2018). Following 72-h incubation, the cultured cells were collected for measuring protein expressions of corresponding target proteins.

2.10 Effect of proteasome ubiquitination system, ERK1/2 pathway inhibitor U0126 and NF-κB pathway inhibitor BAY117082 on TH expression induced by UCB

To investigate whether UCB induced expression of TH protein via affecting proteasome ubiquitination system, the differentiated SH-SY5Y cells, in the presence of 50 nmol L^{-1} CHX, were incubated with 0.1 μmol L^{-1} or 0.5 μmol L^{-1} UCB for 72 h. MG-132 (20 nmol L^{-1} and 50 nmol L^{-1}) served as positive inhibitor. The treated cells were collected for assessing expression of TH protein.

To investigate whether ERK1/2 signaling pathway or NF-κB signaling pathway was involved in TH expression induced by UCB, the differentiated SH-SY5Y cells were co-incubated with 0.1 μmol L^{-1} UCB and 1 μmol L^{-1} U0126 or BAY117082 (0.5 μmol L^{-1}) for 72 h. The cells were collected for assessing the expression of TH protein.

2.11 Effect of NF-κB signaling pathway on TH expression in SH-SY5Y cells

To investigate further roles of NF-κB signaling pathway in UCB-mediated expression of TH protein, SH-SY5Y cells were simultaneously incubated with NF-kB signaling pathway inhibitor BAY117082 and UCB (0.1 μmol L^{-1}) for 72 h. Treated with 0, 0.01, 0.1 and 0.5 μmol L^{-1} UCB, expressions of TH, p65 and p-p65 protein were simultaneously detected. Roles of NF-κB signaling pathway in UCB-mediated expression of TH were confirmed in cells treated with TNF-α (10 ng mL^{-1}) and silencing p65 using p65 siRNA.

SH-SY5Y cells were transfected with 90 nmol/L p65 siRNA (forward 5′-

CCUGAGCACCAUCAACUAU-3′ and reverse 5′-AUAGUUGAUGGUGCUCAGG-3′) or scrambled nagative control (forward 5′-UUCUCCGAACGUGUCACGU-3′ and reverse 5′-ACGUGACACGUUCGGAGAA-3′) by Lipofectamine 3000 (Invitrongen, America) according to manufacturer's directions. Following 12 h transfections, the transfected cells were cultured with culture medium containing 0.1 μmol L^{-1} UCB for another 72 h and expressions of TH and p65 protein were detected.

2.12 Effects of hyperbilirubinemia on DA levels and TH expression in rat brain

The rats, following 1-week acclimation, were randomly divided into control (CTR) and rats treated with bilirubin (Hyperbilirubinemia, HB). The HB rats i.p. 85.5 μmol kg^{-1} bilirubin daily for 14 days according to the previous report (Xu et al., 2016). On day 14 following bilirubin, the rats were sacrificed under ether anaesthesia. Plasma, serum, liver, spleens and brain (cortex and hippocampus) were collected and stored rapidly at -80 °C for analysis. Part of the fresh cortex of the 14-day group was placed directly in the 4% paraformaldehyde fixative for immunohistochemical analysis.

2.13 Immunofluorescence

Slides of paraffin-embedded tissue sections from the cortex of BDL and HB rats were prepared for immunofluorescence staining. Then deparaffinze and rehydrate the paraffin section. The sections were blocked with 3% BSA in phosphate buffered saline (PBS) for 1 h. TH (1:50 dilution), a mouse polyclonal antibody, was incubated overnight at 4 °C. After washing by PBS, the cells were incubated with a goat polyclonal secondary antibody to mouse IgG-H & L (Alexa Fluor® 594) (1:500 dilution) for 50 min and DAPI was used to stain the nuclei for 3 min. Immunofluorescence imaging was performed on the Imaging system (Nikon DS-U3).

2.14 Statistical analysis

All values were expressed as mean ± standard deviation (SD). Comparisons between

two groups were performed using t-tests. Comparisons among multiple groups were performed by one-way analysis of variance followed by Bonferroni's post hoc test. And statistical significance was assumed at $p < 0.05$.

3. RESULTS

3.1 Alterations in physiological and biochemical parameters in BDL rats

Physiological and biochemical parameters were measured (Table 2) at the second week following surgical operation. Then results demonstrated that BDL rats showed significantly increased liver and spleen weight, remarkably higher levels of AST, ALT, AKP, total bile acids, direct bilirubin, total bilirubin and blood ammonia. Compared with Sham rats, relative liver and spleen weight of BDL rats were increased. Levels of direct bilirubin and total bilirubin in serum of BDL rats were increased to 189.4-folds and 112.7-folds, respectively. All these results indicated that the model of BDL-induced liver injury was successfully constructed.

3.2 The alterations in psychiatric behaviors of BDL rats on open field test

On day 6 and day 13 following surgical operation, psychiatric behaviors of rats were measured (Fig. 1) using open field test, which were indexed as the percent of Sham rats. The results showed that compared with Sham rats, 13-day BDL rats showed longer travelled distance, outside distance and inside distance, which were significantly increased to 448%, 418% and 609% of Sham rats, respectively (Fig. 1B-D). it was also found that 13-day BDL also increased the travelled time of rats inside, which were obviously increased to 549% of Sham rats. 6-day BDL also showed a trend to alter psychiatric behaviors, but no significance was obtained (Fig. 1A).

3.3 Concentration of DA and its metabolite in brain and plasma

Concentration of DA and its metabolite in brain and plasma of BDL rats was measured (Fig. 2). The results showed that BDL significantly increased concentration of DA and DOPAC in cortex of rats, inducing increase by 26% and 84% of Sham rats, respectively, but concentration of DA and DOPAC in hippocampus was unaltered by

BDL (Fig. 2C-D). Significantly lower DOPAC levels were observed in plasma of BDL rats (Fig. 2D). It was also found that BDL also significantly increased Tyr and Phe levels in the cortex, hippocampus and plasma of rats (Fig. 2A-B).

3.4 The expressions of mRNA and proteins related to DA transport in brain of BDL rats

The expressions of genes related to DA transport in brain of BDL rats were measured (Fig. 3A-B). Data from QT-PCR demonstrated that BDL slightly but significantly decreased mRNA expressions of cortex OCT2 and hippocampus OCT1 (Fig. 3A-B). Western blotting analysis showed that BDL significantly decreased expression of OCT2 in cortex and hippocampus of rats, decreasing to 79% ($p < 0.05$) and 66% ($p < 0.01$), respectively (Fig. 3D). In contrast, BDL remarkably induced expression of hippocampus OCT1 (Fig. 3C). BDL little affected expressions of OCT3 mRNA, DAT mRNA and OCT3 proteins in cortex and hippocampus of rats (Fig. 3A-B, E).

3.5 The expressions of mRNA and proteins related to DA metabolism in brain of BDL rats

Expressions of enzymes to DA metabolism MAOs (MAO-A and MAO-B), COMTs (Mb-COMT and S-COMT) and TH in brain tissues of BDL rats were measured. The QT-PCR analysis showed that BDL slightly but significantly decreased expression of MAO-B mRNA in rat cortex (Fig. 4A). Western blotting analysis showed that BDL significantly decreased expression of MAO-A in cortex by 38% of Sham rats (Fig. 4E). The activities of MAO-A and MAO-B were measured. It was found that the activities of MAO-A and MAO-B were obviously decreased to 79% and 78% in the cortex of BDL rats, respectively (Fig. 4C). BDL also slightly but significantly down-regulated Mb-COMT protein in cortex and S-COMT protein in hippocampus of rats (Fig. 4G).

Expression of TH, a step-limiting enzyme of DA synthesis in cortex and hippocampus

of rats, was also measured. The results showed that expression of TH in cortex of rats was obviously higher than that in hippocampus. BDL remarkably increased TH protein expression in the cortex to 161% ($p < 0.05$) (Fig. 4D). BDL showed a trend to increase expression of TH in hippocampus, but no significance was obtained due to large variety. Consistence with the results of western blot, the significantly increased expression of TH in BDL rats was copied by immunofluorescence analysis (Fig. 4H).

3.6 Abnormal increases in bilirubin induced expression of TH protein in SH-SY5Y cells

Cholestasis is often associated with abnormal increases in levels of bile acids, bilirubin (UCB) and ammonia. Whether these abnormally altered compounds were factors inducing expression of TH protein was investigated using SH-SY5Y cells as an in vitro model. The results showed that among the tested agents, only bilirubin concentration-dependently induced expression of TH protein (Fig. 5A-C). Moreover, the tested agents did not affect expressions of other enzymes (MAO-A, MAO-B and S-COMT) (Fig. 5D). In line, QT-PCR analysis demonstrated that bilirubin induced expression of TH mRNA without affecting other enzymes except there was a decrease in mRNA levels of MAO-B at 0.5 μmol/L (Fig. 5F). Expression of phosphate TH (p-TH), activating TH, was also measured. It was also found that bilirubin concentration-dependently induced expression of p-TH protein (Fig. 5E).

3.7 Involvement of NF-κB pathway in UCB-induced expression of TH protein

To investigate whether bilirubin induced expression of TH protein via inhibiting proteasome degradation, MG-132 served as positive control (CTR). Similarly to bilirubin, MG-132 concentration-dependently increased levels of TH protein (Fig. 6A). However, following treatment with CHX, bilirubin and MG-132 no longer increased levels of TH protein in SH-SY5Y cells, indicating that bilirubin induced expression of TH protein via affecting the process of transcription and translation

(Fig. 6D-F).

ERK1/2 and NF-κB pathways were reported to be involved in UCB-induced inflammation in astrocytes (Fernandes et al., 2006). Whether ERK1/2 and NF-κB pathways were involved in UCB-induced expression of TH protein was also documented. The results showed that NF-κB pathway inhibitor BAY but not ERK1/2 pathway inhibitor U0126 significantly reversed the induction of TH protein by UCB (Fig. 6B-C). Further study showed that in line with induction of TH protein, UCB concentration-dependently increased levels of both p65 and p-p65 (Fig. 6F). These results indicated that UCB induced expression of TH protein via activating NF-κB pathway.

Roles of NF-κB pathway in UCB-induced expression of TH protein were further documented by silencing p65 using p65 siRNA and NF-κB pathway activator TNF-α. It was consistent with our expectation that transfection with p65 siRNA significantly decreased expressions of p65 and TH proteins to 65% and 51% of control cells, respectively (Fig. 6G). Moreover, silencing p65 completely abolished induction of TH proteins by UCB and the levels of TH proteins in p65-silenced cells combined with UCB were significantly lower than that of control cells (Fig. 6G). In contrast, treatment with TNF-α (10 ng mL^{-1}) significantly increased expression of TH. Expression of p65 protein also showed a trend to increase, but no significance was obtained (Fig. 6H). In vivo data also showed that in line with induction of TH protein, BDL remarkably increased expressions of p65 and p-p65 in BDL rats (Fig. 6I).

3.8 Effects of hyperbilirubinemia on concentration of DA and expression of TH protein in rat brain

Cholestasis is often associated with hyperbilirubinemia. Hyperbilirubinemia rats were developed to investigate effects of hyperbilirubinemia on concentration of DA and expression of TH protein in rat brain. The results showed following 14-day treatment with bilirubin, the hyperbilirubinemia rats were successfully established, whose concentration of direct bilirubin and total bilirubin was increased to 6.2 folds and 12.6

folds of control rats, respectively (Table 3).

The concentration of DA and expression of TH protein in cortex and hippocampus of rats were measured. The results showed that hyperbilirubinemia significantly increased levels of DA in cortex of rats to 215% of control rats, levels of DA in hippocampus of rats were little altered (Fig. 7C). Hyperbilirubinemia also remarkably increased levels of Tyr in cortex and hippocampus of rats (Fig. 7A).

Expression of TH protein was measured using western blot and immunofluorescence. Western blotting analysis showed hyperbilirubinemia significantly increased expression of TH protein in rat cortex, which was further confirmed using immunofluorescence (Fig. 7E-F). In line with expression of TH protein, hyperbilirubinemia induced expressions of cortex p-65 and p-p65, which were significantly increased to 232% ($p < 0.05$) and 155% ($p < 0.01$), respectively (Fig. 7G).

Raw data was listed in Supplementary Information.

4. DISCUSSION

Liver failure is often associated with HE. These patients often show various neurological or psychiatric abnormalities ranging from subclinical alterations to coma (Ferenci, 2017; Nardone et al., 2016; Weissenborn, 2019). HE was also reported to be a significant association between anxiety/depressive disorders (psychiatric comorbidity) (Eftekar, 2020). It was reported that depression and anxiety occurred in 4.5%–64% and 14%–45% of patients with cirrhosis, respectively. However, the specific pathogenesis of hepatic encephalopathy is still not fully understood (Eftekar, 2020). BDL rats is a common model of chronic liver failure, reflecting the association of liver failure with cirrhosis and portal hypertension (Nasehi et al., 2016). At first, BDL rats, as cholestasis models, were used to investigate psychiatric behaviors on open field test. It was in consistent with findings in BDL mice (Nasehi et al., 2013)

that BDL rats showed the increased exploratory behaviors (anxiety), evidenced by increases in spontaneous activity. Several reports have demonstrated the increased levels of DA in brain of AFL animals (Ding et al., 2014; Ding et al., 2013; Ding et al., 2016; Ding et al., 2017; Hadesman et al., 1995; Rai & Dhiman, 2013; Yurdaydin et al., 1990; Zhuge et al., 2019) and patients (Borg et al., 1982; Cuilleret et al., 1980). The present study also showed that BDL obviously increased DA levels in cortex but not hippocampus of rats. Some neurological and psychiatric disorders (such as anxiety) are considered to be related to alterations in DA turnover in brain (Taylor et al., 1982). These results indicated that the increased levels of DA cortex of rats at least partly contributed to psychiatric abnormalities under cholestasis status, which was supported by previous reports (Ding et al., 2013; Nasehi et al., 2013; Reza Zarrindast, Eslimi Esfahani, Oryan, Nasehi, & Torabi Nami, 2013; Zhuge et al., 2019).

Next, we designed a series of experiments to investigate the mechanism increased DA levels in cerebral cortex by cholestasis. DA is completely ionized at physiological pH values (Chemuturi & Donovan, 2007), its transport is mainly mediated by transporters such as OCT1, OCT2, OCT3 and DAT. OCT2 and OCT1 are located microvessel endothelial cells of brain, showing their roles in BBB, but OCT2 has a stronger affinity for DA than OCT1 (Chemuturi & Donovan, 2007; Gasser, 2019). OCT3 located in plasma membrane of glial cells and neurons. The OCT2 and OCT3 belong to uptake 2 monoamine transport system, functioning as a compensatory clearance system of aminergic neurotransmitters including DA in the brain (Liu, 2019). We investigated whether the increased DA in cortex of BDL rats was attributed to alterations of these transporters. The results showed that BDL slightly but significantly decreased mRNA expression of OCT1 in hippocampus and OCT2 in cortex without affecting DAT and OCT3 (Fig. 3A-B). Western blotting showed that BDL significantly lowered expression of OCT2 in rat cortex and hippocampus but obviously increased expression of OCT1 protein in hippocampus (Fig. 3C-D) without affecting expression of OCT3 protein (Fig. 3E). These results indicated that the increased DA levels in cerebral cortex by cholestasis possibly did not come from

alterations in expressions of OCT1-3 and DAT. Concentration of DA in plasma was below 7.8 ng/ml, which impossibly contributed to increased DA levels in brain.

Brain DA is highly controlled by DA synthesis and metabolism. DA was metabolized to DOPAC by MAOs and to 3-methoxytyramine by COMT (Enayah et al., 2018; Miekus et al., 2020; Zhong et al., 2019). Expressions and functions of MAOs were measured. The results showed that BDL significantly decreased expressions of MAO-B mRNA and MAO-A protein in cortex of rats (Fig. 4A-B, E-F). In line with protein expression, activity of MAO-A and MAO-B in the cortex of BDL rats was obviously decreased by about 20% (Fig. 4C). Although BDL decreased activity of MAOs in cortex of rats, the findings did not explain why the MAO-catalyzed-DOPAC levels in cortex of BDL rats were increased rather than decreased, inferring that increased DA levels in cortex of BDL rats possibly did not result from the decreased activity of MAOs, which needs further investigations. COMTs (Mb-COMT and S-COMT), another family of enzyme related to DA metabolism, were further measured. BDL slightly but significantly lowered expressions of Mb-COMT protein in the cortex and S-COMT protein in the hippocampus (Fig. 4G), which contributed to the increased DA levels in cortex of BDL to some extent. Expression of TH, a speed-limiting enzyme of DA synthesis (Enayah et al., 2018), was also simultaneously investigated. It was consistent with previous report that expression of TH in rat cortex was higher than that in hippocampus (Ramesh & Jadhav, 1998). BDL slightly decreased expression of TH mRNA, but remarkably increased expression of TH protein in cortex, without affecting expressions of TH mRNA and proteins in hippocampus of rats (Fig. 4A, B and D). The mechanisms whether transcript of TH was directly related to expression of TH protein were unclear. Immunofluorescence data further confirmed induction of TH in cortex of BDL rats (Fig. 4H). Among the tested 3 kinds of enzymes related DA turnover, alterations in TH expression by BDL were the most striking (Fig. 4D-G), indicated that increased DA levels in cortex were mainly attributed to TH induction. Other factors such as increased levels of Tyr, inhibition of MAOs and COMT, to some extent, may also contribute to the increased DA levels.

BDL did not induce expression of TH in hippocampus, which also explain that DA levels in hippocampus of BDL rats were unaltered.

Cholestasis is characterized by increases in ammonia, bilirubin and bile acids, which were confirmed by the study (Table 2)**.** SH-SY5Y cells were used to investigate whether the abnormally increased components induced expression of TH. The results that among the tested three components (NH_4Cl, mix bile acids and bilirubin), only UCB concentration-dependently increased expression of TH protein without affecting expressions of MAOs and COMT (Fig. 5A-D), demonstrating that UCB is one of the main factors inducing TH expression. In line with TH expression, UCB concentration-dependently increases phosphorylation of TH at ^{40}Ser (p-TH), which is the activated TH (Kawahata, Ohtaku, Tomioka, Ichinose, & Yamakuni, 2015) (Fig. 5E).

Bilirubin was reported to inhibit proteasome (H. Huang et al., 2017). MG-132 is a 26S proteasome inhibitor. Levels of TH protein were significantly increased by both MG-132 and UCB (Fig. 6A). It was in contrast to our expectation that in presence of 50nM CHX, both MG-132 and UCB no longer induced expression of TH (Fig. 6D-E), indicating that UCB and MG-132 increased expression of TH via involving de novo mRNA synthesis. In line with deduction, UCB induced expression of TH mRNA (Fig. 5F). Several reports have demonstrated that MG-132 significantly increased monocyte chemotactic protein-1 induced protein 1 expression (Skalniak, Koj, & Jura, 2013) and IL-6 expression (Shibata et al., 2002), both at mRNA and protein level.

ERK1/2 and NF-κB pathways were also reported to be involved in UCB-induced inflammation in astrocytes (Fernandes et al., 2006). Our results showed that the NF-κB pathway inhibitor BAY but not ERK1/2 pathway inhibitor U0126 reversed the increased expression of TH protein by UCB (Fig. 6B-C), In accordance, UCB increased expressions of p65 and p-p65 protein (Fig. 6F). NF-κB activator TNF-α also up-regulated the expression of TH (Fig. 6H). In contrast, silencing p65 down-regulated the expression of TH and remarkably attenuated the induction of TH expression by UCB too (Fig. 6G). Significant inductions of p65 and p-p65 were also observed in cortex of BDL rats. All these results gave a conclusion that UCB up-

regulated the expression of TH protein partly via activating NF-κB signaling pathway (Fig. 6G-H).

To further validate the effect of bilirubin on expression of TH in vivo, HB rats were developed. In agreement with BDL, significant increases in levels of DA and Tyr were observed in cortex of HB rats (Fig. 7C). HB also significantly induced expression of TH in cortex of rats, which were in parallel with increases in expressions of p65 and p-p65 protein (Fig. 7E-G). All these results confirmed that HB increased levels of DA in cortex of rats via inducing expression of TH, which may be involved in activation of NF-κB signaling pathway.

In conclusion, BDL increased levels of DA in cortex of rats, at least partly via inducing expression of TH. The increased levels of UCB by BDL were one of the main factors inducing TH expression through activating NF-κB signaling pathway. These findings provide an explanation for the increased brain DA levels and the mental behavior abnormalities in patients with cholestasis.

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Figure legands

Fig. 1 The results of open field test (n = 6). A-E: Track (A), relative travelled time (inside) (B) and relative travelled distance (total (C), inside (D) and outside (E)) of Sham rats on day 0, 6 and 13 in the open field (n = 6). *$p < 0.05$ vs Sham rats.

Fig. 2 The concentration of substances in the metabolic pathway of DA in brain and plasma of Sham and BDL rats (n = 6). A-D: The concentration of Phe (A), Tyr (B), DA (C) and DOPAC (D) in brain and plasma of Sham and BDL rats. *$p < 0.05$ or **$p < 0.01$ vs Sham rats.

Fig. 3 The mRNA and protein expressions of transporters (n=8). A-B: The mRNA levels of transporters in rat cortex (A) and hippocampus (hippo) (B) (n = 6). C-E: The protein expression levels of OCT1 (C), OCT2 (D) and OCT3 (E) in rat cortex and hippocampus, respectively. *$p < 0.05$ or **$p < 0.01$ vs Sham rats.

Fig. 4 The protein expressions and activity of DA metabolizing enzymes in rat brain (n = 6). A-B: The mRNA levels of enzymes in rat cortex (A) and hippocampus (hippo) (B). C: The activity of MAOs in rat cortex. D-G: The protein expressions of TH (D), MAO-A (E), MAO-B (F) and COMTs (G) in rat cortex and hippocampus (n = 8). H: The results of immunofluorescence of TH protein in Sham and BDL rat cortex. *$p < 0.05$ or **$p < 0.01$ vs Sham rats.

Fig. 5 Effects of main influencing factors on the protein and mRNA expressions of metabolic enzymes in SH-SY5Y cells (n = 6). A-C: The effect of NH_4Cl (A), UCB

(B) and BAs (C) on TH protein expression. D: The effect of 0.1 μmol/L UCB on MAO-A, MAO-B and S-COMT protein expressions. E: The p-TH protein expression in SH-SY5Y cells given different UCB concentrations (0, 0.01, 0.1 and 0.5 μmol L^{-1}). F: TH, MAO-A, MAO-B and COMT mRNA expressions in SH-SY5Y cells treated with different UCB concentrations (0, 0.01, 0.1 and 0.5 μmol L^{-1}) (n = 5). *$p < 0.05$ or **$p < 0.01$ vs CTR.

Fig. 6 Study on signaling pathway of increased expression of tyrosine hydroxylase induced by bilirubin (n = 6). A-C: The TH protein expressions of SH-SY5Y cells, after the cells were treated with bilirubin (0.1 μmol L^{-1}) and MG-132 (20 and 50 nmol L^{-1}) (A), U0126 (1 μmol L^{-1}) (B) or BAY (0.5 μmol L^{-1}) (C). D-E: The TH protein expressions of SH-SY5Y cells, after the cells were treated with CHX, MG-132 and bilirubin (50 nmol L^{-1} CHX, 50 nmol L^{-1} CHX and 50 nmol L^{-1} MG-132, 50 nmol L^{-1} CHX, 50 nmol L^{-1} MG-132 and 0.1 μmol L^{-1} UCB (D); 50 nmol L^{-1} CHX, 50 nmol L^{-1} CHX and 50 nmol L^{-1} MG-132, 50 nmol L^{-1} CHX and 0.1 μmol L^{-1} UCB, 50 nmol L^{-1} CHX and 0.5 μmol L^{-1} UCB (E)) for 72 h. F: The p-65 and p-p65 protein expressions in SH-SY5Y cells given different bilirubin concentrations (0, 0.01, 0.1 and 0.5 μmol L^{-1}). G: The p65 and TH protein expressions in cells transfected with p65 siRNA and treated with 0.1 μmol L^{-1} UCB only or together. H: The p65 and TH protein expressions after treated with 10 ng mL^{-1} TNF-α or not. I: The p-65 and p-p65 protein expressions in the cortex of Sham and BDL rats. *$p < 0.05$ or **$p < 0.01$ vs CTR, CHX or Sham. $^{\#\#}p < 0.01$ vs UCB.

Fig. 7 The concentration of substances in the metabolic pathway of DA and protein expressions of TH, p65 and p-pp65 in brain of HB rats (n=6). A-D: The concentration of Phe (A), Tyr (B), DA (C) and DOPAC (D) in brain and plasma of CTR and HB rats. E-F: The results of protein expression (E) and immunofluorescence (F) of TH in the control and HB rat cortex. G: The p-65 and p-p65 protein expressions in the cortex of control and HB rat. *$p < 0.05$ or **$p < 0.01$ vs CTR rat.