

## Original Article

# Bilirubin in cerebrospinal fluid reduces nociceptive sensation through inhibition of synaptic activity of neurons in spinal cord

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**Abstract:** Objectives: To determinate the effect of bilirubin on nociceptive sensory in the spinal cord of bile duct ligation (BDL) rats. Methods: Mechanical pain thresholds were measured in rats on days 3, 5, and 7 after BDL surgery. Pain thresholds were also measured 0.5, 1, 1.5 and 2 hours after intrathecal administration of different concentrations of bilirubin. Spontaneous excitatory postsynaptic current (sEPSC) and spontaneous inhibitory postsynaptic current (sIPSC) of substantiagelatinosa neurons were recorded in normal condition and the changes in sEPSC and sIPSC were measured during a 2-minute perfusion of 10  $\mu$ M bilirubin using the whole-cell patch clamp technique. Results: We found that: (1) the mechanical pain thresholds were significantly increased in BDL rats compared with the sham group ( $P < 0.05$ ); (2) the mechanical pain thresholds were also significantly increased 0.5, 1, 1.5, and 2 hours after the intrathecal administration of 1 mM bilirubin; similarly, the 100  $\mu$ M bilirubin group also showed an increase in pain thresholds after 1, 1.5, and 2 hours compared with the NS group ( $P < 0.05$ ); (3) the sEPSC amplitude recorded by whole-cell patch clamp was significantly decreased (-47%,  $P < 0.01$ ), whereas the sIPSC frequency was significantly enhanced (+57%,  $P < 0.01$ ) after bilirubin administration. Conclusion: The increased bilirubin concentration in the cerebrospinal fluid (CSF) of obstructive jaundice (OJ) rats may reduce the nociceptive sensation by inhibiting the synaptic activity of neurons in the spinal cord.

**Keywords:** Bilirubin, mechanical pain threshold, substantiagelatinosa neurons

## Introduction

Elevated pain threshold is a special change in obstructive jaundice (OJ) caused by gallstones or malignant tumors [1]. This pain threshold change leads to a relative lower opioid consumption during the perioperative period. However, if the anesthesiologists give the conventional dosage of drugs without recognition of the change, it may result in a relative over-dosage of opioid drugs in OJ patients, which increases complications such as delayed recovery and respiratory depression. However, the underlying mechanism causing this elevated pain threshold remains unclear. In our previous work, the up-regulation of enkephalin [2] and the increase in  $\beta$ -endorphin were believed to be involved in this special pathophysiological

change in OJ. However, the pathogenesis of this elevated pain in OJ has not been identified yet.

Bile acids have been considered as candidates for the pain change observed in OJ. An increased circulating level of bile acids has been found in OJ by Alemi et al. [3], who estimated that this was induced by analgesia through the activation of TGR5 receptors, a G protein-coupled plasma membrane receptor for bile acids. Bilirubin was also considered to be a candidate for this pain change, since cholestatic patients showed increased bilirubin concentration in the circulation, which suggested that bilirubin could be an alternative potential mediator. The understanding of bilirubin neurotoxicity originates from the concern for neonatal bilirubin encephalopathy 200 years ago. The effect of bilirubin

on the nervous system leads to auditory impairment, mental retardation, other serious neurological sequelae, and even death in children [4, 5]. In addition, some studies have shown that bilirubin neurotoxicity could play an important role in secondary brain injury after cerebral hemorrhage [6]. However, little attention was paid to the effect of bilirubin on the spinal cord, especially regarding its role in pain sensation.

The spinal cord is the first 'transfer station' of the somatosensory pathway and also an important central part in pain modulation [7, 8]. It transmits the sensory information to the central nervous system by integrating the potential changes on the postsynaptic membrane. In our previous research, we confirmed that minimum alveolar concentration (MAC) of desflurane in OJ patients was significantly lower than in Non-OJ patients [9]. As the critical region affecting MAC [10], the spinal dorsal horn can be inhibited by general anesthetics. This results in the loss of nociceptive sensory input received by upstream systems such as motor neurons and sensory neurons, and then leads to the loss of body movement and consciousness [11]. The reduced MAC in OJ indicates that patients with jaundice are probably less sensitive to nociceptive stimulation. Whether the presence of bilirubin in the cerebrospinal fluid affects the spinal dorsal horn that closely relates to the nociceptive sensory transmission needs further research.

Whole-cell patch clamp, as an advanced technique allowing to study the synaptic function of the central and peripheral nervous systems, is a suitable tool for exploring synaptic activity [12-14]. In the present study, the effect of bilirubin on spinal dorsal horn neurons was observed using the whole-cell patch clamp technique, to explore the possible roles of bilirubin in pain transmission. Mechanical pain thresholds were measured in bile duct ligation (BDL) rats and in rats receiving an intrathecal bilirubin administration in order to explore the role of bilirubin in nociceptive sensation.

### Materials and methods

#### *Animals*

The experimental protocol was in accordance with the International Association for the Study of Pain guidelines for the use of animals in

research and was approved by the Second Military Medical University Animal Care and Use Committee. Adult male Sprague-Dawley (SD) rats were provided by the Shanghai Experimental Animal Center, Chinese Academy of Sciences (CAS). These SD rats (weight: 200-250 g, age: 10-12 weeks) were housed in a specific pathogen-free environment at room temperature (21-23°C) and 50% humidity, with a 12/12 hour light/dark cycle, and water and food available *ad libitum*. Every effort was made to minimize the number of animals used and reduce their suffering.

#### *Establishment of the bile duct ligation model*

Rats were randomly allocated into a bile duct ligation (BDL) group (n=12) or a sham operation group (n=12). Rats were anesthetized with chloral hydrate (300 mg/kg) by intraperitoneal administration after weighing. In the BDL group, the bile duct was ligated at a point proximal to the hilus and a point immediately distal to the entry of the bile duct, into the duodenum, after opening the abdominal cavity. The bile duct was then severed between the ligatures. In the sham group, the operation was carried out in a similar manner without ligating and severing the bile duct. Penicillin was administered intramuscularly (single dose of 80,000 units) to prevent infection after closing the abdominal cavity [15]. On the day of BDL surgery and on the 3rd, 5th, 7th, 14th days after surgery, concentrations of bilirubin, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and  $\gamma$ -Glutamyl Transferase ( $\gamma$ -GT) in the plasma were measured and pain threshold was assessed using an electronic von Frey apparatus.

#### *Intrathecal catheter implantation*

A polyethylene catheter PE-10 (Becton, Dickinson and Company, U.S.A) was sterilized prior to surgery. Rats were then anesthetized with chloral hydrate, before being placed on the operation table in prone position. A dorsal midline incision of 0.8-1.0 cm centered on the L5-6 vertebral segment was made for the lumbar intrathecal catheterization and a hole of 4-5 mm was drilled in the left lateral aspect of the L4 or L5 vertebra to expose the dura mater. The sterilized catheter was then inserted into the intrathecal space and rostrally advanced by 1.5-2 cm. Confirmation of successful implanta-

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**Table 1.** Liver function of rats

	Sham	BDL <sub>3d</sub>	BDL <sub>5d</sub>	BDL <sub>7d</sub>	BDL <sub>14d</sub>
Total bilirubin (μmol/L)	2.25±1.75	126.51±30.64*	122.34±42.81*	116.23±48.50*	94.41±30.05*
ALT (U/L)	62.25±5.75	463.41±46.83*	256.91±42.09*	66.38±14.58*	71.57±14.51*
AST (U/L)	173.4±42.11	1395.82±48.34*	1309.54±52.79*	1207.37±46.34*	441.94±80.68*
Γ-GT (U/L)	1.00±0.50	25.32±5.31*	38.32±7.21*	32.21±6.12*	35.45±20.07*

\*: significantly different from the sham group at P<0.05. On the 3rd, 5th, 7th, 14th days after BDL surgery, concentrations of total bilirubin, ALT, AST and γ-GT in the plasma were significantly increased in the BDL group as compared with that in the sham group (\*P<0.05, BDL group vs. sham group, n=12). Bile duct ligation is indicated as BDL; Rats receiving a similar operation without ligating the bile duct are indicated as sham.

**Table 2.** Bilirubin concentration in the CSF of rats

	SHAM	BDL <sub>3d</sub>	BDL <sub>5d</sub>	BDL <sub>7d</sub>	BDL <sub>14d</sub>
Total Bilirubin (μmol/L)	0	0.25±0.05*	0.60±0.10*	2.43±1.09*	2.53±1.38*
Indirect bilirubin (μmol/L)	0	0.1±0.00*	0.27±0.15*	1.76±0.57*	1.15±0.44*

\*: significantly different from the sham group at P<0.05. The total bilirubin and indirect bilirubin concentrations in the CSF were increased on the 3rd, 5th, 7th, 14th days after BDL surgery (\*P<0.05, BDL group vs. sham group, n=12). Bile duct ligation is indicated as BDL; Rats receiving a similar operation without ligating the bile duct are indicated as sham.

tion was determined by the presence of a cerebrospinal fluid flow from the tip of the catheter following the tail-flick reflex. The catheter was tightly fixed and the opposite end was protected at the cervical region by a subcutaneous tunnel. All wounds were closed and a 80,000-unit dose of penicillin was administered intramuscularly for the prevention of infection. After surgery, rats showing severe motor weakness and non-responsiveness to lidocaine were excluded from the study [16, 17]. Sixty rats with intrathecal catheter implantation were randomly divided into five groups (n=12): a NS group, a 1-μM bilirubin group, a 10-μM bilirubin group, a 100-μM bilirubin group, and a 1-mM bilirubin group. In the bilirubin groups, 25 μl of unconjugated bilirubin was injected with different concentrations. In the NS group, the same volume of normal saline was injected. The pain threshold was measured immediately (hour 0) and at hours 0.5, 1, 1.5, and 2 after the intrathecal bilirubin administration.

### Behavioral test for mechanical thresholds

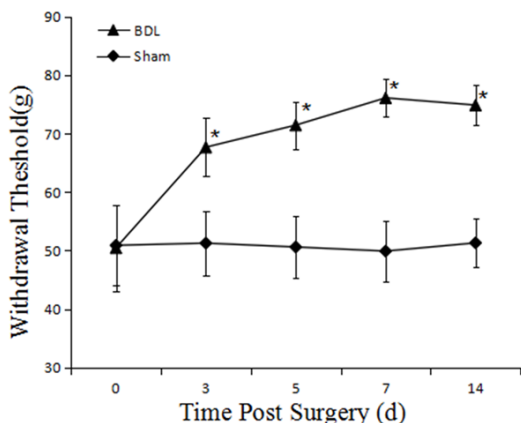
The pain threshold was measured using an electronic von Frey apparatus (IITC Life Science, Woodland Hills, CA, USA). Rats were acclimated to the environment for 1 hour after being placed on a tawny suspended 22×22×30 cm wire grid. The electronic von Frey rigid filaments were applied to the hind paw surface, and the intensity was gradually enhanced until a paw with-

draw reaction occurred. The maximal recorded value during the procedure was considered as the mechanical threshold. The tests were repeated three times on both paws at a 5-minute interval to avoid sensitization.

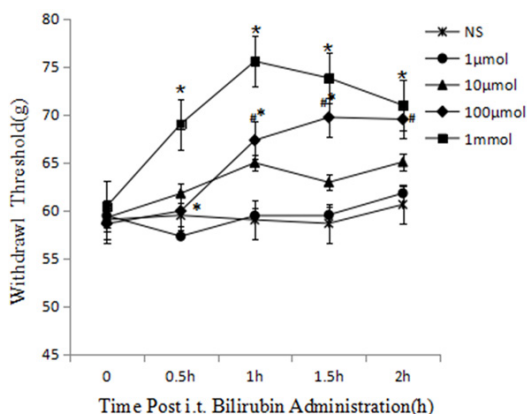
### Electrophysiological test

**Solutions:** An unconjugated bilirubin (Sigma, USA) stock solution (10 mM) was prepared in a 0.1 M NaOH solution immediately before use. The pH of the incubation medium was restored to 7.4 by the addition of an equal amount of 0.1 M of HCl. Then, the solution could be diluted to a required concentration using artificial cerebral spinal fluid (ACSF). All the experiments with unconjugated bilirubin were performed under light protection to avoid photodegradation. The ionic composition of the ACSF, which exerted an important role in the perfusion of spinal cord slices, was (in mM): NaCl 117, KCl 3.6, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, and Glucose 11 with 95% O<sub>2</sub> and 5% CO<sub>2</sub> saturated. The composition of the pipette solution (intracellular fluid) used in this study to record the spontaneous excitatory postsynaptic currents (sEPSC) and the spontaneous inhibitory postsynaptic current (sIPSC) was as follows (in mM): K-gluconate 135, KCl 5, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2, EGTA 5, HEPES 5, ATP-Mg 5 and Cs<sub>2</sub>SO<sub>4</sub> 110, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2, EGTA 5, HEPES 5, TEA 5, ATP-Mg 5, respectively.

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**Figure 1.** Mechanical pain threshold after bile duct ligation surgery. On the 3rd, 5th, 7th, and 14th days after BDL surgery, pain threshold significantly increased in the BDL group compared with the sham group (\* $P < 0.05$ , BDL group vs. sham group,  $n = 12$ ), suggesting a reduction in nociceptive sensation in jaundiced rats. Bile duct ligation is indicated as BDL; Rats receiving a similar operation without ligating the bile duct are indicated as sham.



**Figure 2.** Mechanical pain threshold after intrathecal injection of bilirubin. Pain thresholds were tested immediately (hour 0) and at hours 0.5, 1, 1.5, and 2 after intrathecal bilirubin injection. Compared with the NS group, pain threshold significantly increased in the 100- $\mu\text{M}$  group at hours 1, 1.5, and 2 (\* $P < 0.05$ , 100- $\mu\text{M}$  group vs. NS group,  $n = 12$ ) and in the 1-mM group at hours 0.5, 1, 1.5, and 2 (\* $P < 0.05$ , 1-mM group vs. NS group,  $n = 12$ ). Furthermore, pain thresholds were significantly different between the 100- $\mu\text{M}$  group and 1-mM group at hours 0.5, 1, and 1.5 (# $P < 0.05$ , 100- $\mu\text{M}$  group vs. 1-mM group,  $n = 12$ ).

### Preparation of spinal cord slices

Under  $\text{CO}_2$  anesthesia, the lumbar enlargement of 12-14-day-old Sprague-Dawley rats was quickly removed and transferred into cold ACSF. After removing endorhachis and cleaning the

nerve root under a microscope, the spinal cord was then cut transversely in the agar groove with a 500- $\mu\text{m}$  thickness for whole-cell recording using a vibratome at a 0.14-mm/s speed and 3500-rpm vibration frequency. The slices were incubated for one hour in a Gibb groove designed by Dr. Alasadair J Gibbat at a constant temperature of 33~35°C and then perfused with ACSF with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  saturated.

### Electrophysiological recording

The incubated slice was transferred to the recording chamber on the stage of a microscope and perfused with ACSF at room temperature. Substantiagelatinosa neurons, which are semitransparent, were identified under the microscopy and then visualized using infrared differential interference contrast optics [18]. The patch electrode filled with pipette solution was advanced onto the surface of the neuron until an increase in electrode resistance appeared, and then sucked the cytomembrane. A well-sealed neuron was confirmed by an action potential induced by a current input generated by pClamp10.2 software. Then, sEPSCs and sIPSCs were recorded in a voltage-clamp mode using MultiClamp 700B amplifier and pClamp10.2 data acquisition software under holding potentials of -60 mV and 0 mV, respectively. All drugs were administered through the Drug Delivery System, and a 2-minute perfusion of ACSF was essential before and after the 10  $\mu\text{M}$  bilirubin injection.

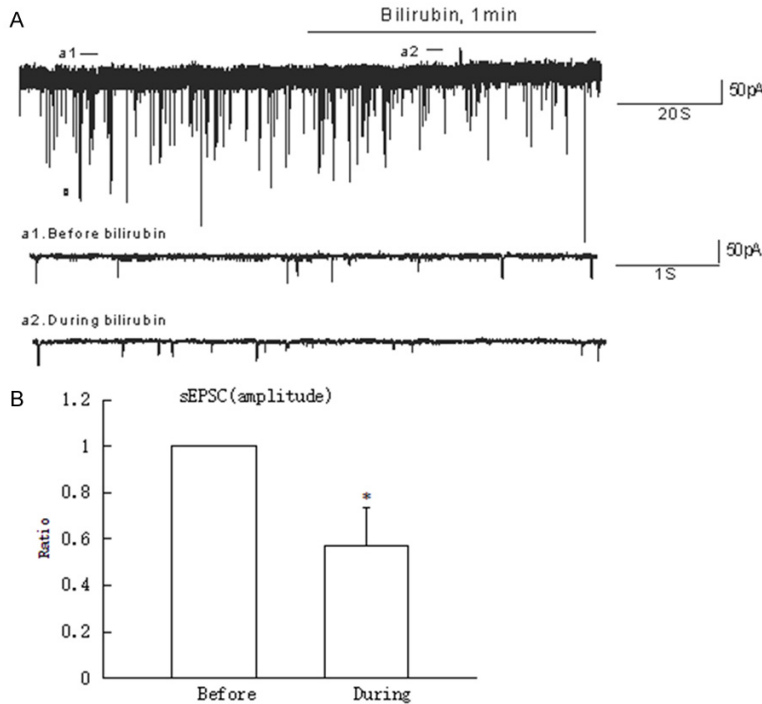
### Data analysis

All numerical data were expressed as mean  $\pm$  standard deviation and analyzed using SPSS 17.0 software. Data on mechanical pain thresholds were analyzed by two-way and multiple levels factorial analysis in order to fully compare the factors of time and drug administration. Statistical significance level was set at  $P < 0.05$ . Electrophysiological data were analyzed using pClamp10 software. Neurons with more than 20% change in current intensity were regarded as responding to drugs.

## Results

### Changes in bilirubin concentration and in the liver function

On the 3rd, 5th, 7th, 14th days after surgery, concentrations of bilirubin, ALT, AST and  $\gamma\text{-GT}$  in



**Figure 3.** sEPSC of substantiagelatinosa neurons recorded by whole-cell patch clamp. A. The amplitude of sEPSC. sEPSC amplitude was significantly inhibited during a 2-minute perfusion of 10 μM bilirubin. a1, an enlarged image of sEPSC before perfusion. a2, an enlarged image of sEPSC during perfusion. B. The inhibition ratio of sEPSC amplitude was 47% in neurons receiving bilirubin perfusion as compared with the neurons that did not receive bilirubin perfusion (\* $P < 0.01$ , during bilirubin perfusion vs. before bilirubin perfusion). The spontaneous excitatory postsynaptic current is indicated as sEPSC.

the plasma was significantly increased in the BDL group as compared to those in the sham group (Table 1,  $P < 0.05$ ). This suggested that the BDL model was successfully constructed and that the liver function was damaged. As compared to the sham group, the total bilirubin and indirect bilirubin concentrations in the CSF were increased in the BDL group as well (Table 2,  $P < 0.05$ ). This indicated that the increased level of bilirubin entered into the CSF through the blood brain barrier.

#### *BDL surgery increased mechanical pain threshold*

As shown in Figure 1, the mechanical pain threshold was measured after BDL surgery. On the 3rd, 5th, 7th, and 14th day, pain threshold increased significantly in the BDL group compared with the sham group ( $P < 0.05$ ). The up-regulation of pain threshold after BDL surgery

indicated a reduction in the nociceptive sensation of jaundiced rats.

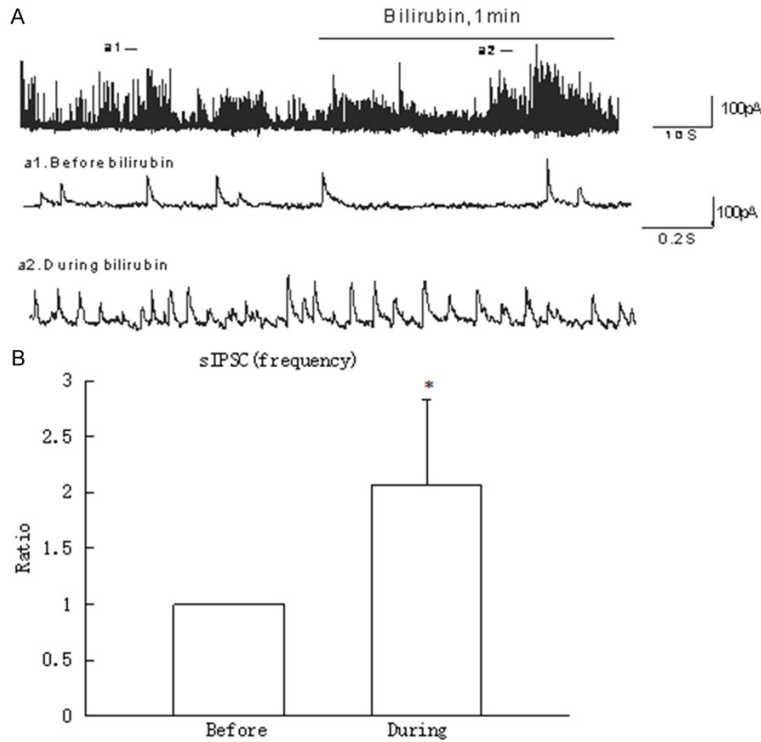
#### *Intrathecal bilirubin administration increased pain threshold*

To test the relationship between the increased pain threshold and bilirubin concentration, bilirubin with different concentration levels were administered intrathecally. Pain thresholds were tested immediately (hour 0) and at hours 0.5, 1, 1.5, and 2 after the bilirubin administration. As shown in Figure 2, compared with the NS group, a significant difference existed in the 100-μM bilirubin group at hours 1, 1.5, and 2 ( $P < 0.05$ ) and in the 1-mM bilirubin group at hours 0.5, 1, 1.5, and 2 ( $P < 0.05$ ). Furthermore, pain thresholds were significantly different between the 100-μM and the 1-mM bilirubin groups at hours 0.5, 1, and 1.5 ( $P < 0.05$ ). The results indicated that the concentrations of bilirubin administered

intrathecally were strongly related to pain threshold and time interval in a concentration-dependent manner.

#### *Effect of bilirubin on synaptic transmission in spinal cord*

We recorded the sEPSC of substantiagelatinosa neurons by whole-cell patch clamp. As shown in Figure 3A, sEPSC amplitude was significantly inhibited during bilirubin perfusion. The inhibition ratio of sEPSC amplitude was 47% during the perfusion of 10 μM bilirubin (Figure 3B, ratio = 0.53 during bilirubin perfusion vs. ratio = 1 before bilirubin perfusion,  $P < 0.01$ ). This result indicated that bilirubin had an inhibitory effect on postsynaptic receptors of excitatory neurons. Similarly, sIPSC of substantiagelatinosa neurons was also recorded by whole-cell patch clamp. During perfusion of 10 μM bilirubin, the frequency of sIPSC was significantly enhanced (Figure 4A). The enhancing



**Figure 4.** sIPSC of substantiagelatinosa neurons recorded by whole-cell patch clamp. A. The frequency of sIPSC. The frequency of sIPSC was significantly enhanced during a 2-minute perfusion of 10  $\mu$ M bilirubin, a1, an enlarged image of sIPSC before perfusion. a2, an enlarged image of sIPSC during perfusion. B. The enhancing ratio of sIPSC amplitude was 57% in neurons receiving bilirubin perfusion as compared with the neurons that did not receive bilirubin perfusion (\* $P < 0.01$ , during bilirubin perfusion vs. before bilirubin perfusion). The spontaneous inhibitory postsynaptic current was indicated as sIPSC.

ratio of sIPSC amplitude was 57% in neurons receiving bilirubin perfusion as compared to those that did not receive bilirubin perfusion (Figure 4B,  $P < 0.01$ ). The results indicated that bilirubin facilitated the presynaptic release of inhibitory neurotransmitter.

### Discussion

In the present study, we found that pain thresholds were elevated in BDL rats along with an increased concentration of bilirubin in the plasma and the CSF. To exclude other factors involved in pain changing, different concentrations of bilirubin were injected intrathecally and we found that the pain thresholds were up-regulated in a bilirubin concentration dependent manner. The electrophysiological recordings showed that the sEPSC amplitude was significantly inhibited and the sIPSC frequency was markedly enhanced by bilirubin, which suggest-

ed that bilirubin inhibited postsynaptic receptors of excitatory neurons and facilitated the presynaptic release of inhibitory neurotransmitters. In the present study, the reduction in nociceptive sensation in BDL rats may probably be due to the inhibitory effect of bilirubin on the synaptic activity of neurons in the dorsal horn of the spinal cord.

In a previous clinical study, we found that analgesic drug consumption was relatively lower in jaundice patients, which suggests that OJ patients could be less sensitive to nociceptive stimulation. Similar results were found in the present study since pain thresholds were elevated in BDL rats, which tends to confirm our hypothesis. A previous study reported that this elevated pain threshold observed in OJ may probably be due to an up-regulation of  $\beta$ -endorphin in the peripheral plasma and that the adhesion of leukocytes was involved in this up-regulation of  $\beta$ -end-

orphin. However, besides this peripheral mechanism, future studies should investigate the changes that may occur in the CNS because of the up-regulation of bilirubin in the CSF. Indeed, unconjugated bilirubin has a cytotoxic effect by crossing various biological membranes, especially the blood brain barrier. In the present study, rats with intrathecal injection of bilirubin showed less sensitivity to nociceptive stimulation in a dose-dependent manner than the NS group, which suggested that bilirubin may relate to the elevated pain threshold. An *in vitro* study by Tao et al. [2] showed that  $\beta$ -endorphin in the supernatant from non-jaundiced patients was up-regulated after leukocytes were treated with bilirubin, which supports the effect of bilirubin in the regulation of pain. However, whether bilirubin directly affects the CNS neurons in the CSF needs further research.

The substantiagelatinosa (SG) neurons of the spinal dorsal horn play an important role in regulating nociceptive transmission from the periphery [19, 20]. The inhibitory interneurons exert an inhibitory effect on the signal transmission process from the primary afferent fibers to the substantiagelatinosa neurons, and thus in the regulation of the amount of pain signal delivered to the brain [21, 22]. It has been reported that the sEPSC of SG neurons was significantly enhanced by a treatment with capsaicin [23] and was weakened by a treatment with opioids [24]. Regarding the neuropathic pain models proposed after a peripheral nerve injury, data shows that the primary afferent-evoked IPSCs are substantially reduced in incidence, magnitude, and duration [25]. In a specific pathophysiological state, both excitatory and inhibitory signals are received by neurons in the nervous system. Whether and which kind of action potential is induced depends on how the current is integrated. In OJ state, sEPSC amplitude was reduced and sIPSC frequency was increased by bilirubin, which suggests that the inhibitory signal was mainly received by interneurons. The insensitive response to nociceptive stimulation in OJ may probably be due to the inhibition of synaptic activity in the SG neurons by bilirubin.

Unconjugated bilirubin (not linked to albumin, also referred as indirect bilirubin) has a neural cytotoxic effect after crossing the blood brain barrier. The unconjugated bilirubin within the cerebrospinal fluid significantly increases in hyperbilirubinemia patients, some of which may even reach a bilirubin concentration up to 1  $\mu\text{M}$ . In our study, the bilirubin concentration increased up to  $126.51 \pm 30.64 \mu\text{M}$  in the plasma and  $2.53 \pm 1.38 \mu\text{M}$  in CSF of BDL rats. After the intrathecal administration of 1mM bilirubin, the final concentration in the cerebrospinal fluid was up to 18.75  $\mu\text{M}$ . The increased bilirubin in the CSF may affect neural activity and change the pain threshold. The *in vitro* experiments proved that a bilirubin concentration in the range of 71-770 nM caused neurotoxicity according to Ostrow et al.'s meta-analysis [26]. A study by Warr et al. showed that a bilirubin concentration of 10  $\mu\text{M}$  inhibited the current of NMDA receptors within 10-15 seconds [27]. The 10  $\mu\text{M}$  bilirubin concentration produced a significant reduction in the postsynaptic potential of the hippocampus, along with an inhibi-

tion of the long-term potentiation of the hippocampus and alterations in learning and memory [28]. In Shi et al.'s study, bilirubin induced a reversible increase in the spontaneous inhibitory synaptic transmission in P13-15 lateral superior olive neurons and caused bilirubin-related hearing impairment [29]. In most *in vitro* studies, neurons were directly exposed to a 10- $\mu\text{M}$  bilirubin concentration, which is consistent with the concentration used in our whole-cell patch clamp study since SG neurons were incubated with a 10  $\mu\text{M}$  bilirubin concentration. This bilirubin concentration directly inhibited the synaptic activity of SG neurons and changed pain threshold.

In conclusion, this study indicated that an increased bilirubin concentration can inhibit the excitability of the spinal dorsal horn neurons, this resulting in a reduction of the nociceptive sensation in OJ.

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### Disclosure of conflict of interest

None.

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