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The role of gamma-aminobutyric acid/glycinergic synaptic transmission in mediating bilirubin-induced hyperexcitation in developing auditory neurons

Xin-Lu Yin[a,1], Min Liang[a,1], Hai-Bo Shi[a], Lu-Yang Wang[b], Chun-Yan Li[a,*], Shan-Kai Yin[a,*]

[a] Department of Otorhinolaryngology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Yishan Road 600, Shanghai 200233, PR China
[b] Program in Neurosciences & Mental Health, SickKids Research Institute and Department of Physiology, University of Toronto, Toronto, ON M5G 1X8, Canada

HIGHLIGHTS

- Bilirubin facilitates GABA/Glycinergic synaptic transmission in both bushy and stellate cells in VCN of P2-6 rats.
- Bilirubin-induced enhancement of GABA/Glycinergic transmission is Ca^{2+}-dependent.
- Excitatory action of GABA/Glycinergic transmissions in early development is engaged in bilirubin-induced hyperexcitation and potentially contribute to high vulnerability of developing neurons to bilirubin.

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ABSTRACT

Hyperbilirubinemia is a common clinical phenomenon observed in human newborns. A high level of bilirubin can result in severe jaundice and bilirubin encephalopathy. However, the cellular mechanisms underlying bilirubin excitotoxicity are unclear. Our previous studies showed the action of gamma-aminobutyric acid (GABA)/glycine switches from excitatory to inhibitory during development in the ventral cochlear nucleus (VCN), one of the most sensitive auditory nuclei to bilirubin toxicity. In the present study, we investigated the roles of $GABA_A$/glycine receptors in the induction of bilirubin hyperexcitation in early developing neurons. Using the patch clamp technique, $GABA_A$/glycine receptor-mediated spontaneous inhibitory synaptic currents (sIPSCs) were recorded from bushy and stellate cells in acute brainstem slices from young mice (postnatal day 2–6). Bilirubin significantly increased the frequency of sIPSCs, and this effect was prevented by pretreatments of slices with either fast or slow Ca^{2+} chelators BAPTA-AM and EGTA-AM suggesting that bilirubin can increase the release of GABA/glycine via Ca^{2+}-dependent mechanisms. Using cell-attached recording configuration, we found that antagonists of $GABA_A$ and glycine receptors strongly attenuated spontaneous spiking firings in P2-6 neurons but produced opposite effect in P15–19 neurons. Furthermore, these antagonists reversed bilirubin-evoked hyperexcitability in P2-6 neurons, indicating that excitatory action of GABA/glycinergic transmission specifically contribute to bilirubin-induced hyperexcitability in the early stage of development. Our results suggest that bilirubin-induced enhancement of presynaptic release GABA/Glycine via Ca^{2+}-dependent mechanisms may play a critical role in mediating neuronal hyperexcitation associated with jaundice, implicating potential new strategies for predicting, preventing, and treating bilirubin neurotoxicity.

1. Introduction

Jaundice is observed in up to 85% of newborns (Watchko and Tiribelli, 2013). Severe jaundice may lead to bilirubin encephalopathy of a subset of brain regions, with cortex, cerebellum and brainstem being most vulnerable (Ingelfinger et al., 2013). Clinical studies have shown that elevated bilirubin places infants at risk of

* Corresponding authors at: Department of Otorhinolaryngology,Shanghai Jiao Tong University Affiliated Sixth, People's Hospital, Yishan Road 600, Shanghai, 200233, PR China. Fax: +86 21 6483 4143.
E-mail addresses: licycrystal@sina.com (C.-Y. Li), yinshankai@china.com (S.-K. Yin).
[1] These authors contribute equally.

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cognitive, perceptual, motor, and sensory (particularly auditory) disorders (Connolly and Volpe, 1990; Hansen, 2002; Martinez-Cruz et al., 2014; Shapiro and Popelka, 2011). Excitotoxicity has been proposed as an important contributing factor in bilirubin-induced neuronal injury (Watchko, 2006). We recently demonstrated that excessive synaptic release of glutamate and consequent overstimulation of glutamate receptors directly result in bilirubin excitotoxicity (Li et al., 2011a). However, the cellular mechanisms underlying bilirubin excitotoxicity are not yet completely understood.

In addition to enhanced glutamate synaptic transmission, our work demonstrated that bilirubin can also facilitate gamma-aminobutyric acid (GABA)/glycinergic synaptic transmission in the ventral cochlear nucleus (VCN) (Li et al., 2011b), yet the potential roles of such a facilitation remain unknown. GABA and glycine are the primary inhibitory neurotransmitters in the mammalian central nervous system (CNS), and primarily exert a hyperpolarizing influence on adult neurons through the activation of chloride (Cl^-) receptor channels. However, GABA and glycine have also been shown to excite immature neurons during early postnatal development due to the high concentration of intracellular chloride (Gulledge and Stuart, 2003; Marty, 2003; Marzia martina and Pare, 2001). Activation of excitatory GABA and glycine receptors can produce membrane depolarization (Ben-Ari et al., 2007), which, in some cases, reaches the spike threshold to generate action potentials (Fujiwara-Tsukamoto et al., 2003; Voigt et al., 2001). In addition, GABA/glycine depolarization may also be sufficient to relieve the voltage-dependent magnesium block from *N*-methyl-D-aspartate (NMDA) receptors (Ben-Ari et al., 1997), leading to an influx of calcium and subsequent toxicity in neurons (Ben-Ari et al., 2012). Many reports have demonstrated the involvement of a depolarizing effect of GABA in excitotoxicity associated with seizure, autism, and Alzheimer's disease (Ben-Ari et al., 2012; Fujiwara-Tsukamoto et al., 2003; Khazipov et al., 2004a,b; Pitkänen, 2000; Pizzarelli and Cherubini, 2011). Other studies have indicated that GABA and glycine are protective in mature rats under hypoxia but toxic to immature rats under the same conditions (Zhao et al., 2005). Accordingly, we investigated whether GABA/glycinergic synaptic transmission exerts excitatory or inhibitory action in bilirubin-induced excitotoxicity during the early stages of development.

In the present study, we used whole-cell and cell-attached voltage-clamp techniques to probe the mechanisms underlying bilirubin-induced potentiation of presynaptic release of GABA/glycine and neuronal hyperexcitability in the VCN, an auditory nucleus that is one of the most sensitive nuclei to bilirubin toxicity. Our results demonstrated that bilirubin strongly potentiates the excitatory action of GABA/glycinergic synaptic transmission by elevating presynaptic Ca^{2+} and release probability during early development, dramatically promoting spontaneous postsynaptic spike firings. Such mechanisms are likely involved in bilirubin-induced neuronal hyperexcitability and vulnerability of auditory brainstem and other brain regions to bilirubin during early development.

2. Materials and methods

Experiments were performed in accordance with the Guiding Principles for the Care and Use of Animals. This study was approved by the Ethics Review Committee for Animal Experimentation at Shanghai Jiaotong University. All efforts were made to minimize possible pain and discomfort of animals during the experimental procedures.

2.1. Preparation of VCN brain slices

Sprague-Dawley rats, aged between postnatal day (P) 1 and P17, were anesthetized with sodium pentobarbital (55 mg/kg, intraperitoneal [i.p.]) and then decapitated. The brain was quickly removed and placed into oxygenated, ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose. Transverse slices were cut at a thickness of 300 um with a microslicer (VT-1000S; Leica, Germany). Slices containing the VCN were maintained in oxygenated (95% O_2 and 5% CO_2) ACSF at 37 °C for at least 1 h, and then transferred to a recording chamber at room temperature before use.

2.2. Reagents

Reagents used in the experiments included: free bilirubin, bicuculline, strychnine, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline-2,3-dione(NBQX), l-2-amino-5-phosphonovalericacid (APV), avidin, biocytin (all from Sigma, St. Louis, MO,USA), BAPTA-AM, and EGTA-AM (from Life Technologies, USA). Bilirubin was dissolved in 0.1 M NaOH at 1 mM as a stock solution, stored in single-use aliquots in the dark at −20 °C (for less than 48 h), and diluted to the final solution concentration prior to application. Because bilirubin is sensitive to light, the bilirubin solution was protected from light at all times. Bicuculline, NBQX, BAPTA-AM, and EGTA-AM were prepared as stock solutions using 100% dimethyl sulfoxide (DMSO; Sigma), and diluted to the required concentration in ACSF immediately prior to use, resulting in a maximal DMSO concentration of <0.1%. Other drugs were prepared as stock solutions in distilled water and diluted to the required concentration in ACSF immediately prior to use. All drugs were locally applied via square glass capillary (0.4 mm in width, Cat. #64-0121, Warner, USA) that was placed onto the slice as close as possible to the VCN region (Joshi and Wang, 2002). Developmental profiles of glutamate receptors and synaptic transmission at a single synapse in the mouse auditory brainstem.

2.3. Electrophysiological measurements and data analyses

Cell-attached recordings and whole-cell recordings were performed using a patch-clamp amplifier (EPC10; HEKA, Lambrecht/Pfalz, Germany). The electrode capacitance and liquid junction potential were compensated. Data were filtered at 1–3 kHz and sampled at 3–10 kHz using a Dell computer equipped with PatchMaster software (HEKA). Patch pipettes were pulled from borosilicate capillary glass through two stages by a vertical pipette puller (P-9; Narishige, Tokyo, Japan). The resistance of the electrode was 5–8 MΩ. Patch electrodes were filled with the following solutions (in mM) for cell-attached recordings: 97.5 K-gluconate, 32.5 KCl, 0.5 ethylene glycol tetraacetic acid (EGTA), 40 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 $MgCl_2$. For whole-cell recordings, the patched electrodes were filled with (in mM): 92CsCl, 50Cs-methanesulfonate, 5 tetraethylammonium (TEA)-Cl, 2 EGTA, 4 adenosine triphosphate (ATP)-Mg, and 10HEPES. All of the internal solutions were adjusted to a pH of 7.2 and 300 mM Osm. Only one recording was conducted per brain slice. All experiments were performed at room temperature (21–26 °C). Differences in the frequency of action potential currents and spontaneous inhibitory synaptic currents (sIPSCs) were examined using Wilcoxon signed-ranks tests for between-groups comparisons. Statistical analyses were performed with SPSS 17.0 software. Values of $P < 0.05$ were considered to be statistically significant. The data are presented as means ± standard errors (SEs).

2.4. Cell staining

VCN neurons were identified by injecting biocytin (1–2%, dissolved in the internal solution) through the recording electrode. To optimize diffusion of biocytin into the neuron, the injection was usually taken for 10–20 min. Then the slices were removed from the recording chamber, fixed with 4% paraformaldehyde overnight, and stored at 4 °C. The slices were thoroughly washed three times with phosphate buffered saline (PBS), and then incubated in 0.5% Triton X-100 (sigma, USA) and 5% bovine serum albumin (BSA; Life Technologies, USA) for 0.5–1 h. The recorded neurons were labeled using Alexa Fluor® 488-labeled avidin (1:2,000; Sigma) at room temperature, protected from light, for 2–3 h. The slices were further rinsed thoroughly with PBS three times, mounted onto slides, and cover-slipped. Pictures of biocytin-filled neurons were acquired using a laser scanning confocal microscope (LSM 710; Zeiss, Germany) and photographed.

3. Results

3.1. Properties of VCN neurons

Two major cell types have been described in the VCN: stellate and bushy cells (Song et al., 2012). In the present study, these cells were identified based on their morphological and electrophysiological properties. In general, stellate cells possessed a multipolar soma, with three or more dendrites extending from the cell body, and their somas were larger than those of bushy cells (Fig. 1B). Bushy cells had a round or oval soma, and their dendrites were mostly bipolar in opposing directions (Fig. 1A). However, morphological characteristics alone were not sufficient to determine the cell type; electrophysiological characteristics were also examined. Fig. 1C and D show different responses of two types of cells to depolarizing current pulses. Stellate cells fired a series of action potentials with relatively stable interspike intervals when they were injected with a depolarizing current. Increases in current strength correspondingly increased the number of action potentials (Fig. 1D). Bushy cells fired a single action potential at the beginning of the depolarizing current, independent of the increasing current (Fig. 1C).

3.2. Bilirubin facilitated sIPSC frequency of P2–P6 VCN neurons.

In the present study, we first recorded spontaneous inhibitory postsynaptic current (sIPSCs) in whole-cell configuration with high concentration of Cl^- in the Cs+/TEA-based pipette solution(Kotak et al., 1998). This intracellular solution help block other voltage-gated conductances and improve the quality of voltage-clamp, but

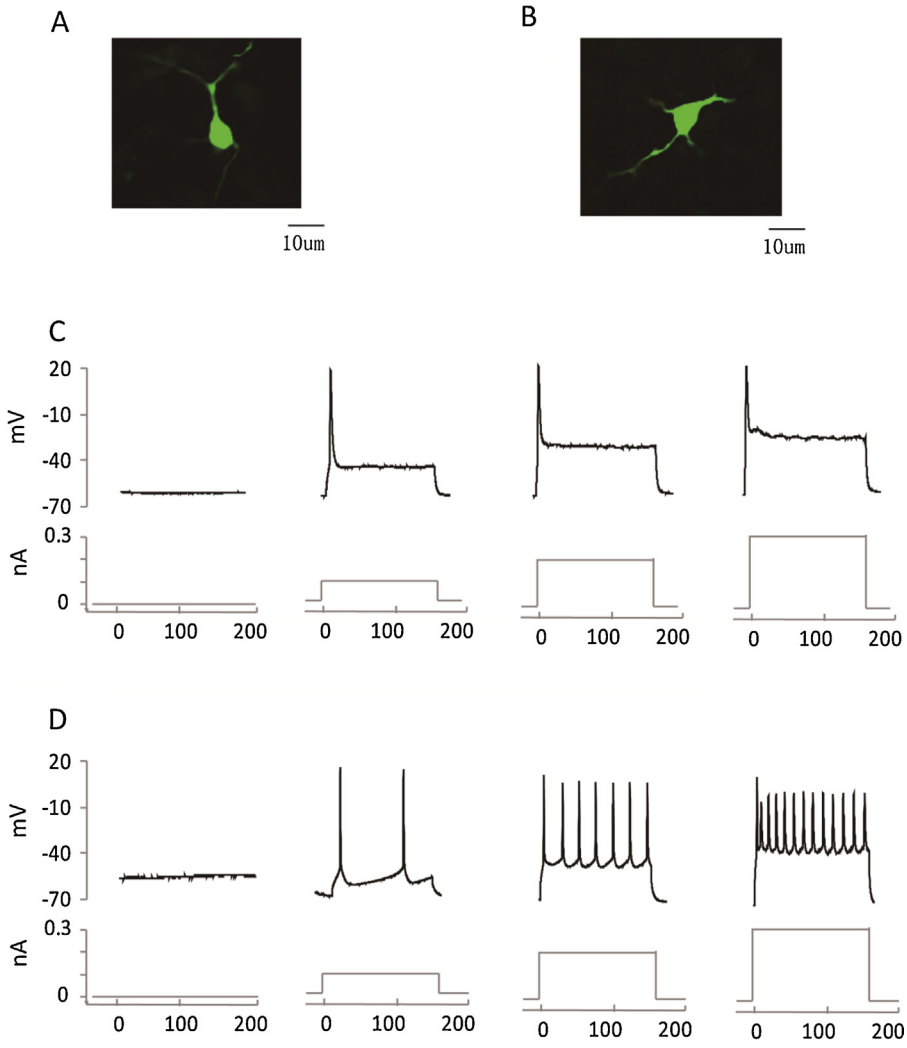

Fig. 1. Identification of bushy and stellate neurons based on their morphological and electrophysiological characteristics. (A) Morphology of a typical postnatal day (P) 7 bushy cell. (B) Morphology of a typical P13 stellate cell. (C) Bushy cell voltage responses (A) to current injections of 0 pA, 50 pA, 100 pA, and 150 pA (traces from left to right). (D) Stellate cell voltage responses (B) to current injections of 0 pA, 50 pA, 100 pA, and 150 pA (traces from left to right).

precluded us from positively differentiating two major cell types, and therefore data included in this study was unbiased towards either type of neurons. These inward currents could be completely blocked by antagonists of $GABA_A$ (50 μM bicuculline) and glycine (1 μM strychnine) receptors (data not shown), indicating that the currents were a mixture of GABAergic and glycinergic sIPSCs (Shi et al., 2006). Next, we investigated the effects of bilirubin (3 μM) on the above mentioned sIPSC during early development (P2–P6) (Fig. 2). To this end, we applied 2 μM NBQX and 50 μM APV to block glutamatergic transmission, and then applied bilirubin at a concentration of 3 μM. During the 9-min bilirubin application, the normalized sIPSC frequencies were increased to150 ± 12.3% of the control ($n = 6$, $P < 0.05$) (Fig. 2B). These results indicate that bilirubin can enhance the frequency of sIPSCs (i.e., facilitate GABA/glycinergic synaptic transmission) in early developing neurons.

3.3. Both the fast calcium chelator BAPTA-AM and the slow calcium chelator EGTA-AM prevent increased sIPSC activity induced by bilirubin

To investigate whether and how such Ca^{2+} rise by bilirubin is coupled to facilitation of GABA/glycine exocytosis, we pretreated brain slices for 30 min with the membrane permeable fast and slow calcium chelator BAPTA-AM and EGTA-AM (50 μM), respectively. These buffers have been widely used to probe the spatial coupling of Ca^{2+} influx to transmitter release. Although both chelators have similar equilibrium dissocation constants (K_D), the forward binding rate of BAPTA is about 100-fold faster than that of EGTA (Wang et al., 2009). Hence, if the source of Ca^{2+} is tightly coupled to its target (i.e. nanodomain <100 nm), EGTA will not be able to effectively intercept Ca^{2+} ions in transit, and in contrast BAPTA can effectively capture Ca^{2+} ions regardless of nanodomain or microdomain couplings (>100 nm).

After a 10 min incubation in Ca^{2+}-buffer free ACSF (in the presence of 2 μM NBQX and 50 μM APV), we recorded sIPSCs from VCN neurons in slices pretreated with EGTA-AM or BAPTA-AM. We found that BAPTA-AM pretreatment abolished the bilirubin induced increases in the frequency of sIPSCs (Fig. 3A). The frequency of spontaneous activity during bilirubin application was similar to the control (101.5 ± 5.6% of the control, $n = 4$, $P > 0.05$) (Fig. 3B). EGTA-AM had an effect similar to BAPTA-AM (Fig. 3C). After EGTA-AM pretreatment, the frequency of sIPSCs during bilirubin perfusion was similar to the control level (97.7 ± 6.2%, $n = 4$, $P > 0.05$) (Fig. 3D). These results indicate that bilirubin facilitates GABA/glycinergic synaptic transmission by enhancing Ca^{2+}-dependent of transmitter release, and that the source of Ca^{2+} ions (including Ca^{2+} release from internal stores) is spatially distant from the sensor of fusion.

3.4. Developmental switch of GABAergic responses from excitation to inhibition

To investigate functional consequence of enhanced GABA/glycinergic synaptic transmission, we made cell-attached voltage-clamp recordings of spontaneous spikes (in the form of compounded inward and outward currents) from developing VCN neurons. We discovered that after 3 min application of bicuculline (50 μM) in very immature neurons (P2–P6), the spontaneous discharge was significantly reduced to 68.1 ± 9.3% of the control ($n = 4$, $P < 0.05$). Co-application of strychnine (1 μM) further decreased the activity to 47.4 ± 14.4% of the control value ($n = 4$, $P < 0.05$), suggesting that enhanced presynaptic release of GABA and glycine by bilirubin synergistically depolarized postsynaptic neurons and increased their spontaneous firings (Fig. 4A and C). In contrast, in the P15–19 neurons which had much higher basal spontaneous discharge, we found that the spike firings of these neurons increased in response to bicuculline (180.7 ± 12.5% of the control, $n = 4$, $P < 0.05$) and co-application of strychnine (221.9 ± 13.2% of the control, $n = 4$, $P < 0.05$). These results collectively demonstrated that GABA/glycine receptors switch their action from excitatory to inhibitory transmission during development of the VCN (Fig. 4B and D).

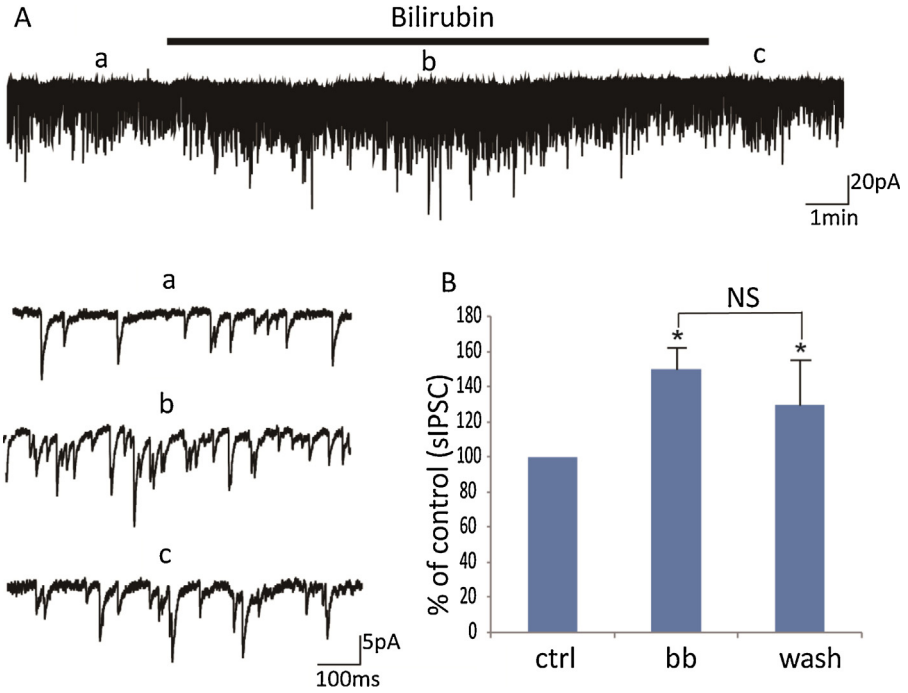

Fig. 2. Effects of bilirubin (3 μM) on the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in ventral cochlear nucleus (VCN) neurons. (A) Typical sIPSC recording before, during, and after 3 μM bilirubin perfusion. Bottom panel: sections of the traces shown at the top are presented at higher resolution (a, b, and c). (B) Histograms depicting normalized average sIPSC frequencies of four neurons before (3 min), during (9 min), and after (4 min) bilirubin application. Vertical error bars represent the standard error (SE). *$P < 0.05$; NS: not significant.

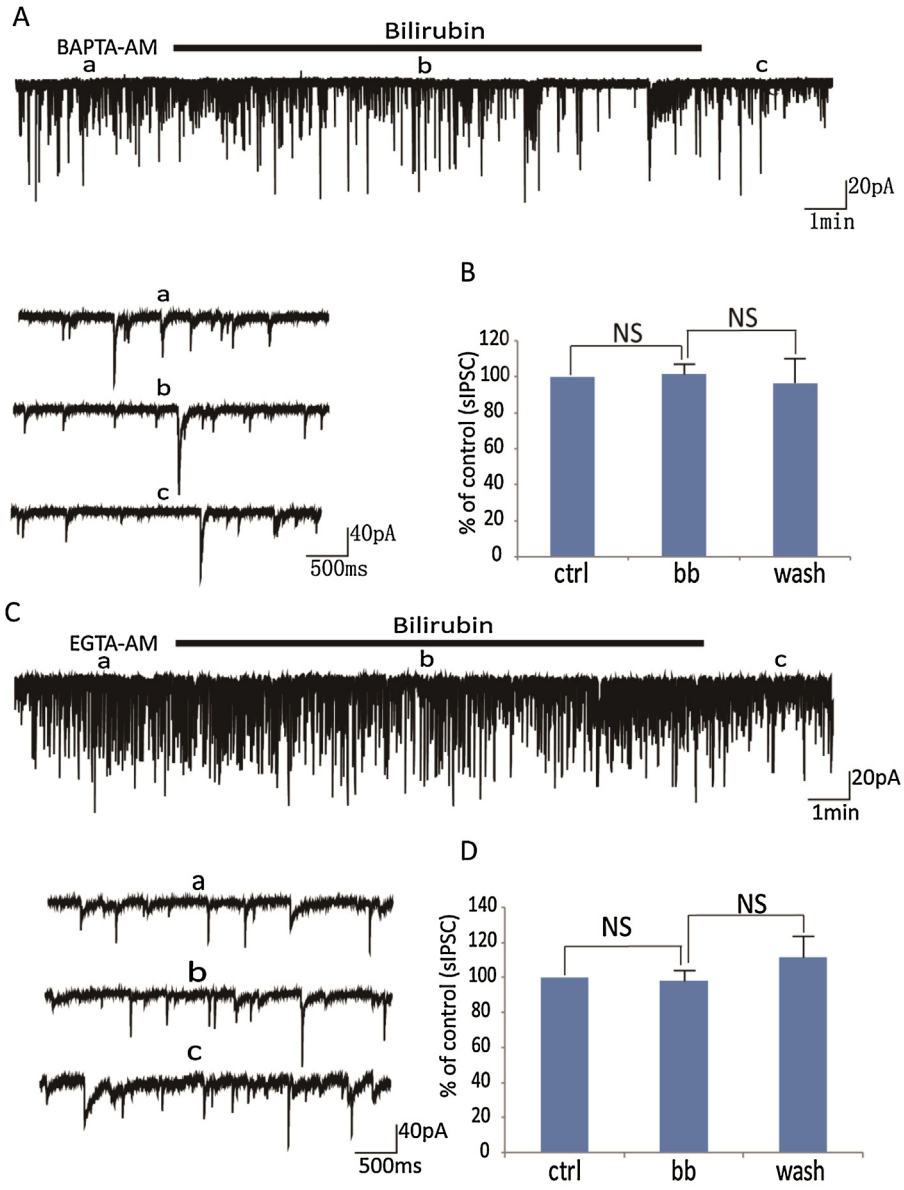

Fig. 3. Both the fast calcium chelator BAPTA-AM and the slow calcium chelator EGTA-AM prevented the increased sIPSC activity induced by bilirubin. (A) Top panel: typical sIPSCs recorded from a neuron after 30 min BAPTA-AM pretreatment in the control solution (a), 3 μM bilirubin application (b), and washout with the control solution (c). Bottom panel: sections of the traces shown at the top are presented at higher resolution (a, b, and c). (B) Comparison of normalized average sIPSC frequencies recorded from four neurons before, during (9 min), and after (3 min) bilirubin application. Vertical error bars represent the SE. NS: not significant. (C) Top panel: typical sIPSCs recorded from a neuron after 30 min pretreatment with EGTA-AM in the control solution (a), 3 μM bilirubin application (b), and washout (c). Bottom panel: sections of the traces shown in the top are presented at higher resolution (a, b, and c). (D) Comparison of normalized average sIPSC frequencies recorded from four neurons before, during (9 min), and after (3 min) bilirubin application. Vertical error bars represent SE. NS: not significant.

3.5. Role of GABA/glycinergic synaptic transmission in bilirubin-induced hyperexcitability

All of the above results demonstrated the presence of excitatory GABA/glycine action in very young neurons. Next we explored whether excitatory GABA/glycine receptors are also involved in the mechanism of bilirubin-induced hyperexcitability in early development (P2–6). After 3 min of baseline recording, we found that the perfusion of 3 μM bilirubin dramatically increased the spontaneous activity. The spontaneous firing rate increased by more than 1-fold (125% ± 10% above the basal frequency ($n = 8$, $P < 0.05$) (Fig. 5A and B). This increase in the firing rate was largely reversed to 102% ± 12.1% above control after co-application of bicuculline (50 μM) ($n = 8$, $P < 0.05$) (Fig. 5A and B). Co-application of bicuculline and strychnine (1 μM) further reduced the spontaneous firing rate to 78% ± 13.9% above the basal firing rate ($n = 8$, $P < 0.05$) (Fig. 5A and B). These results indicated that the excitatory action of GABA and glycine receptors contribute significantly to bilirubin-induced hyperexcitability in VCN neurons of early postnatal rats. More importantly, blocking excitatory GABA/glycine receptors in young neurons as demonstrated in P2-6 can at least partially attenuate the level of hyperexcitability caused by bilirubin.

4. Discussion

The VCN is the first relay of the central auditory system and plays a significant role in processing incoming acoustic information. It is one of the most sensitive nuclei to bilirubin toxicity in the CNS (Shapiro, 2010). By whole-cell and cell-attached patch-clamp

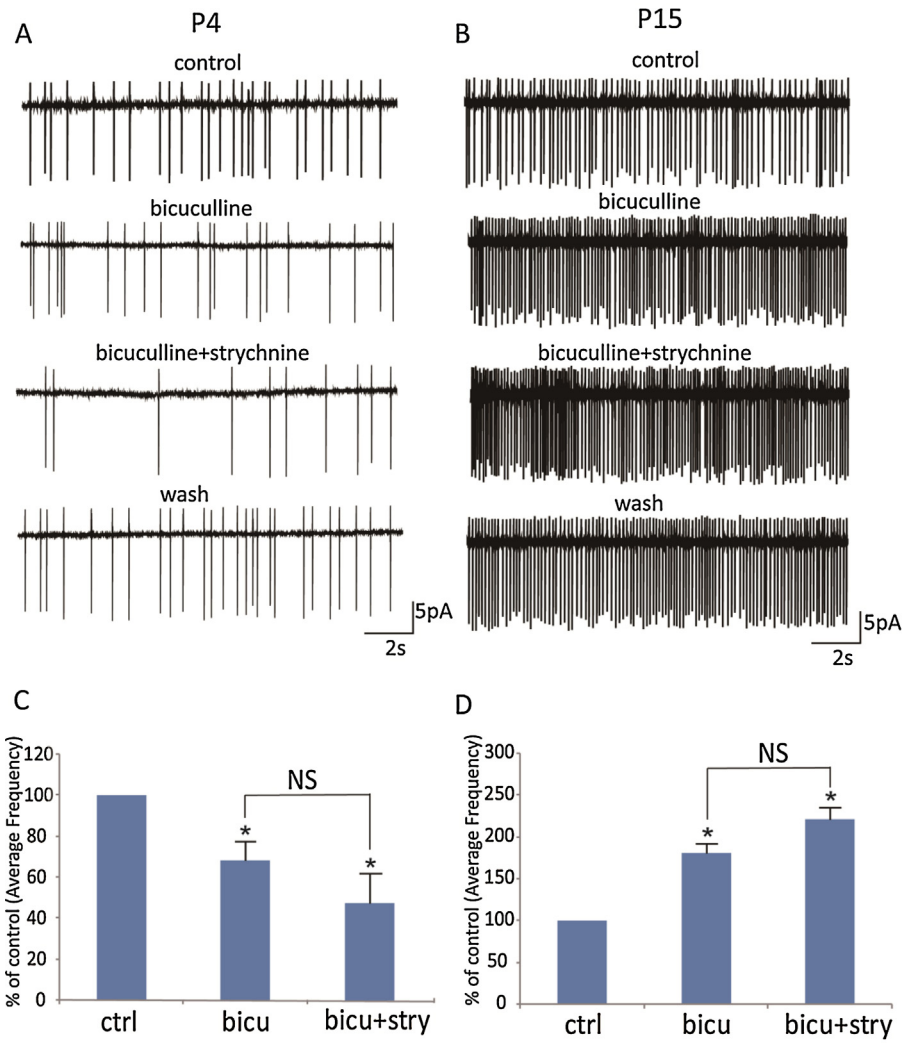

Fig. 4. Gamma-aminobutyric acid (GABA)/glycinergic synaptic transmission underwent a switch from excitatory to inhibitory action during development. (A) Spontaneous activity recorded from a P4 neuron under control, bicuculline, bicuculline and strychnine co-application, and washout conditions. (B) Spontaneous activity recorded from a P16 neuron under control, bicuculline, bicuculline and strychnine co-application, and washout conditions. (C) Summary data of normalized average spontaneous firing frequency from VCN neurons (P2–P6) in control solution, following bicuculline perfusion, and bicuculline and strychnine co-application conditions. (D) Summary data of the normalized average spontaneous firing frequency from VCN neurons (P15–P19) in control solution, bicuculline perfusion, and bicuculline and strychnine co-application conditions.

recordings from VCN neurons of newborn rats, we discovered that bilirubin significantly enhances the release of GABA/glycine via elevation of presynaptic Ca^{2+} level, and these transmitters exert their excitatory action on young neurons (<P6), underlying at least in part the dramatic enhancement of postsynaptic spike firings. We suggest such a bilirubin-dependent hyperexcitability may ultimately contribute to Ca^{2+} overload and neurotoxicity associated with jaundice and encephalopathy. Although our experimental paradigms prevented us from positively identifying the cell type of each recording, we consistently observed bilirubin-dependent enhancement of sIPSCs and spike discharge rate in our recordings, indicating that VCN neurons possess the similar sensitivity to bilirubin regardless of bushy or stellate subtype at the new born stage.

Our previous study showed that bilirubin increased presynaptic release of GABA/glycine in the VCN and lateral superior olive neurons in P12–15, likely via Ca^{2+} release from internal stores (Li et al., 2011b; Shi et al., 2006). Here, we observed bilirubin-induced potentiation of sIPSCs in VCN neurons in much younger animals (P2–6). Taking advantage of different forward binding rate of fast and slow Ca^{2+} chelators, BAPTA an d EGTA, we further probed whether the spatial coupling between the source of Ca^{2+} rise and the sensor of Ca^{2+} for exocytosis is in nanodomain or microdomain regime (Adler et al., 1991; Fedchyshyn and Wang, 2005; Wang et al., 2009). We found that both buffers were equally effective in attenuating bilirubin-dependent enhancement of GABA/Glycine release, indicating a global elevation in residual Ca^{2+} in nerve endings is sufficient to cause an increase in the release probability and results in an increase in the frequency of synaptic transmission (Goswami et al., 2012; Ivanov and Calabrese, 2003).

To investigate the functional consequence of enhanced GABAergic/glycinergic transmission in bilirubin-induced hyperexcitation, we recorded neuronal spontaneous activity (SA) using a cell-attached patch mode, which is noninvasive to homeostasis of the intracellular environment and preserves the endogenous spike activity most relevant to the physiological status of neurons (Minlebaev et al., 2013). Using this approach, we demonstrated that both bicuculline and strychnine could decrease SA frequency in neurons of P2–6 rats; on the contrary, bicuculline and strychnine increased the SA frequency in neurons of P15–19 rats. These results directly implicate the excitatory action of GABA/glycinergic neurotransmission in the generation of SA in early development,

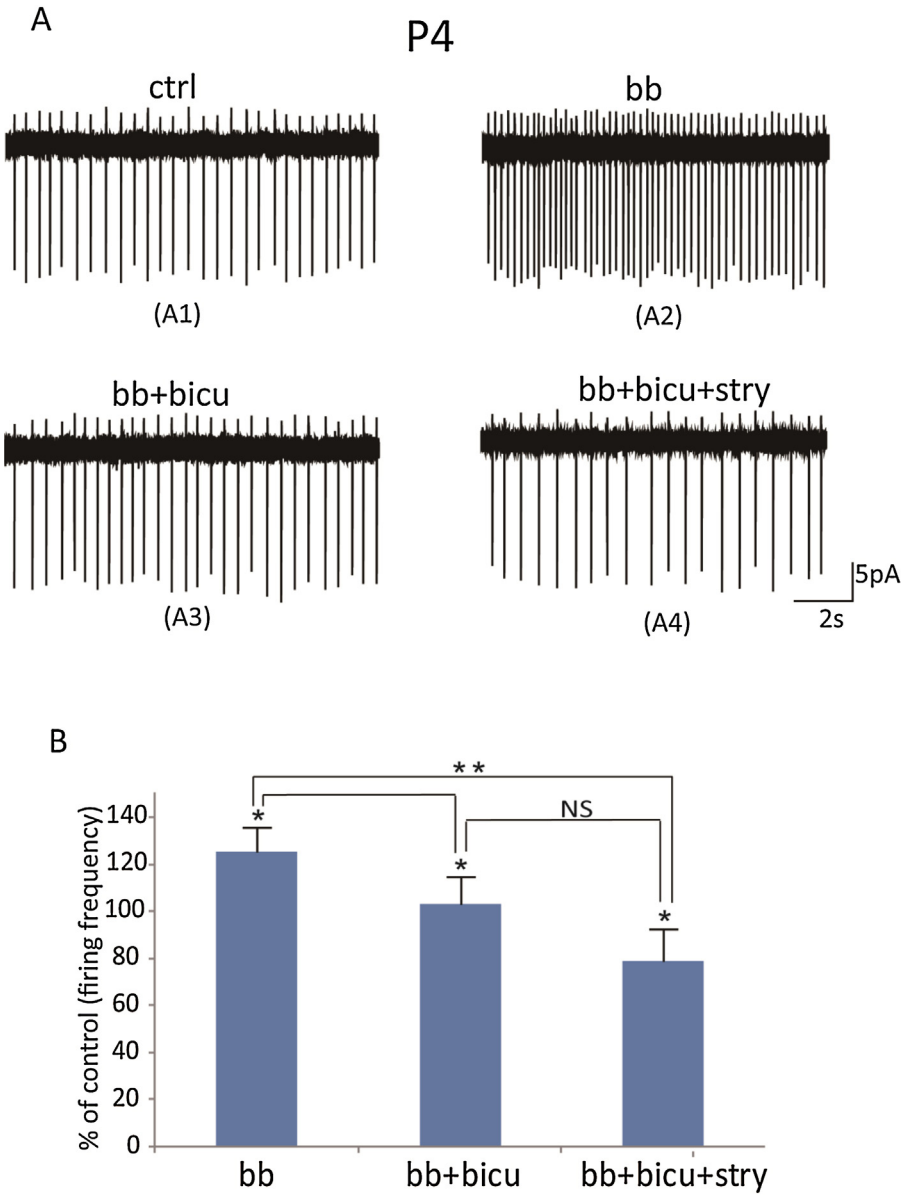

Fig. 5. The role of GABA/glycinergic synaptic transmission in bilirubin-induced hyperexcitability. (A) Top panel: spontaneous firing recorded from a P4 VCN neuron in control (A1) and bilirubin-containing solutions (A2). Bottom panel: additional application of bicuculline (A3) and strychnine (A4) in the presence of bilirubin. (B). Normalized spontaneous activity recorded from P2–P6 VCN neurons under bilirubin alone, bicuculline, and bilirubin co-application, and further application of strychnine conditions. Vertical error bars represent SE. $^{*}P < 0.05$; $^{**}P < 0.01$; NS: not significant.

in line with previous studies that demonstrated a shift from mainly depolarizing to hyperpolarizing action mediated by GABA/glycine receptors during the early postnatal period in many developing CNS nuclei (Jang et al., 2002; Kriegstein and Noctor, 2004; Ye et al., 2004). Our previous study in VCN neurons revealed GABA/Glycine receptors in bushy cells switch from predominantly depolarizing to hyperpolarizing in the first postnatal week and stellate cells make a similar switch at the end of the second postnatal week (Song et al., 2012). The results of the present study are also consistent with these previous results (i.e., GABA/glycine action in both types of VCN neurons is excitatory before P7). It should be noted that the increase in SA by bilirubin was only partially reversed by a combination of bicuculine and strychnine, implying that bilirubin may potentially increase SAs via other unidentified mechanisms.

In early development, activation of GABA/glycine receptors increases Cl^- conductance (Ben-Ari, 2002). Developmental changes in two cation–chloride co-transporters, chloride accumulating Na^+–K^+–$2Cl^-$ co-transporter (NKCC1) and chloride extruder potassium-chloride transporter member (KCC2), play a pivotal role in the developmental chloride concentration changes. NKCC1 uses the inwardly directed sodium gradient to raise chloride ion concentration and maintain it at a steady-state level. Expression levels of NKCC1 are reportedly high at birth and subsequently decline, which may also contribute to the shift in GABA/glycinergic responses (Achilles et al., 2007; Pfeffer et al., 2009). KCC2 is the principal transporter for Cl^- extrusion from neurons. KCC2 expression appears around birth, dramatically increases after the first week of postnatal life, and becomes abundant in the mature CNS. This developmental expression of KCC2 is pivotal for the development of hyperpolarizing GABA-mediated inhibition. For example, Lee et al. (2005) showed that early overexpression of KCC2 in immature cortical neurons, before the upregulation of KCC2, can produce a negative shift in GABA reversal potential and reduce GABA-elicited calcium responses. In

addition, GABA itself also reportedly promotes the developmental switch in neuronal GABAergic responses from excitation to inhibition (Ganguly et al., 2001; Leitch et al., 2005). It is yet to be tested whether bilirubin has additional effects on Cl^- transporters to promote the hyperexcitability of VCN neurons.

Many previous studies have implicated the excitatory action of GABA/Glycine transmission in mediating excitotoxicity in developing neurons (Lukasiuk and Pitkanen, 2000), or mature neurons under pathological conditions such hypoxia. Moreover, there are reports indicating that the excitatory action of GABA may be one reason for greater susceptibility of immature neurons to seizure (Nardou et al., 2013; Roustem Khazipov, 2004). Our results showed that blockers of $GABA_A$ and glycine receptors can decrease the frequency of enhanced spontaneous firing induced by bilirubin. This suggests that excitatory GABA/glycinergic synaptic transmission is directly involved in bilirubin induced neural hyperexcitability, potentially leading to Ca^{2+} overload and neurotoxicity. Auditory neurons at maturity express the highest level of endogenous Ca^{2+} binding proteins in brain and can effectively buffer Ca^{2+} overload, but such expression level takes several postnatal weeks to reach(Felmy and Schneggenburger, 2004; Lohmann and Friauf, 1996).

We therefore hypothesize that depolarizing GABA and glycine action may be an important factor to exacerbate the susceptibility of immature neurons to bilirubin-dependent hyperexcitability and Ca^{2+} overload, ultimately neurotoxicity. Furthermore, our previous work showed that bilirubin enhanced neuronal excitability by increasing glutamatergic transmission (Li et al., 2011a), consistent with those by McDonald (McDonald et al., 1998), Grojean (Grojean et al., 2000), and Brito et al. (Brito et al., 2010). However, Shapiro et al. (Shapiro et al., 2007) showed that the NMDA channel antagonist MK-801 did not protect against bilirubin-induced neurotoxicity in hippocampal neurons, raising the possibility that other pathways, such as the GABA/glycinergic synaptic transmission, may be engaged in bilirubin-dependent excitotoxicity, and present another set of potential targets to prevent or treat jaundice and encephalopathy.

In conclusion, we demonstrated that bilirubin facilitates GABA/glycinergic synaptic transmission in Ca^{2+}-dependent mechanism. This enhanced GABA/glycine neurotransmission promotes bilirubin-induced hyperexcitability and potentially excitotoxicity during early development of auditory neurons with limited endogenous Ca^{2+} buffering capacity. Our results also provide new insight into the mechanisms of bilirubin excitotoxicity and subsequent hearing impairment caused by bilirubin. A better understanding of the mechanism of bilirubin-induced hyperexcitation would be useful for the development of new strategies for predicting, preventing, and treating bilirubin neurotoxicity.

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