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

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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

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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).