Riluzole is a Promising Pharmacological Inhibitor of Bilirubin-Induced Excitotoxicity in the Ventral Cochlear Nucleus

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Keywords

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SUMMARY

Background and purpose: Bilirubin encephalopathy as a result of hyperbilirubinemia is a devastating neurological disorder that occurs mostly in the neonatal period. To date, no effective drug treatment is available. Glutamate-mediated excitotoxicity is likely an important factor causing bilirubin encephalopathy. Thus, drugs suppressing the overrelease of glutamate may protect the brain against bilirubin excitotoxicity. Riluzole is a prescription drug known for its antigitamatergic function. This study was conducted in the rat's ventral cochlear nucleus, a structure highly sensitive to bilirubin toxicity, to find whether riluzole can be used to inhibit bilirubin toxicity. **Experimental approach:** Electrophysiology changes were detected by perforated patch clamp technique. Calcium imaging using Rhod-2-AM as an indicator was used to study the intracellular calcium. Cell apoptosis and necrosis were measured by PI/Hoechst staining. **Key results:** In the absence of bilirubin, riluzole effectively decreased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and suppressed neuronal firing but did not change the amplitude of sEPSC and glutamate-activated currents (I_{Glu}). Moreover, riluzole inhibited bilirubin-induced increases in the frequency of sEPSC and neuronal firing. Riluzole could prevent the bilirubin-induced increase in intracellular calcium, mediated by AMPA and NMDA receptors. Furthermore, riluzole significantly reduced bilirubin-induced cell death. **Conclusions and implications:** These data suggest that riluzole can protect neurons in the ventral cochlear nucleus from bilirubin-induced hyperexcitation and excitotoxicity through reducing presynaptic glutamate release.

Introduction

Severe hyperbilirubinemia can result in neurological disease, that is, bilirubin encephalopathy (BE), in newborns [1]. Bilirubin-induced toxicity affects the central nervous system by damaging neurons such as those in the basal ganglia and brainstem nuclei. The ventral cochlear nucleus (VCN), an auditory structure, is among the brainstem nuclei most sensitive to bilirubin toxicity [2]. It has been suggested that impairment of auditory function is the most consistent abnormality related to BE [1].

All healthy newborns are at potential risk of BE if their jaundice is unmonitored and/or managed inappropriately [3]. A timely and effective treatment to newborn jaundice can help reduce the risk of developing irreversible BE-related brain damage [1,4]. Currently, phototherapy and exchange transfusion remain the major treatments for infants with hyperbilirubinemia and BE. However, phototherapy has several known disadvantages such as the risk of skin cancer and bronze baby syndrome; exchange transfusion is invasive and may lead to significant mortality [5–7]. Thus, attention has been drawn to the development of novel pharmacological interventions that target specific neural mechanisms underlying the neurotoxicity of hyperbilirubinemia.

It has been proposed that excitotoxicity is an important factor causing BE [8]. This excitotoxicity is likely related to glutamate, the primary excitatory neurotransmitter in the central nervous system [9,10]. Recently, we demonstrated that excessive synaptic release of glutamate and consequent overstimulation of glutamate receptors contributed to bilirubin-induced hyperexcitation [11]. This evidence suggests that drugs targeting the overrelease of glutamate may serve as potential neuroprotectants against bilirubin-induced excitotoxicity.

Riluzole is currently the only drug approved for treating the fatal neurodegenerative disease amyotrophic lateral sclerosis [12–14]. The drug also has therapeutic potentials for treating other diseases and conditions, such as convulsions, anxiety, motor neuron disease, and ischemia [15–17]. The therapeutic potential of riluzole is believed to be related primarily to its well-known antigitamatergic actions. Riluzole has been defined as a glutamate release inhibitor and an antagonist of glutamate neurotransmission [17,18]. The molecular mechanisms underlying the antigitamatergic effect of riluzole are likely to be multifaceted, involving suppression of both presynaptic autoreceptors and voltage-dependent calcium (Ca^{2+}) channels [19,20]. Riluzole also inhibits the persistent sodium current (I_{NaP}), which underlies intrinsic spiking and

rhythm generation in motor neurons [21–23]. It has yet to be determined whether riluzole has a protective effect against bilirubin-induced excitotoxicity.

This study was conducted in the VCN to find whether bilirubin-induced hyperexcitation and excitotoxicity could be prevented by riluzole through blockade of presynaptic glutamate release. An answer to this question is important for developing a new pharmacological method for treating BE in newborns.

Materials and Methods

All experiments were carried out according to the guiding principles for the care and use of animals. The study was approved by the Ethics Review Committee for Animal Experimentation at Shanghai Jiaotong University (Shanghai, China).

VCN Neuron Preparation

The general procedures for preparing isolated VCN neurons were similar to those described previously [24]. VCN neurons were obtained from Sprague Dawley (SD) rats (12–15 days old). Briefly, after decapitation under anesthesia with sodium pentobarbital (55 mg/kg, i.p.), the brain was removed quickly from the skull and placed in oxygenated and ice-cold incubation solution, containing (in mM) 124 NaCl, 5 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, 24 NaHCO₃, and 10 glucose, saturated with 95% O₂/5% CO₂. Brain slices (300 μ m) containing the VCN were cut using a microslicer (VT-1000S, Leica Microsystems, Nussloch, Germany). After recovery at room temperature (21–26°C) for 20–30 min, the slices were transferred to Petri dishes (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) filled with oxygenated standard solution containing (in mM) 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES). The pH of the solution was adjusted to 7.4 with Tris-OH. The VCN region was identified visually using a dissecting microscope (XTL-2400; SOIEC, Shanghai, China). Mechanical dissociation of VCN neurons with attached, functional presynaptic terminals was accomplished using a custom-built vibration device with a fire-polished glass pipette oscillating at 50 Hz over a distance of 0.1–0.2 mm on the surface of the VCN region for 4 min. The isolated neurons then adhered to the bottom of the dish over 15–20 min after the vibration and could be readily identified visually with an inverted microscope (TE-2000U; Nikon, Tokyo, Japan).

Electrophysiological Measurements

To keep the neurons in as normal and physiological conditions as possible during recordings, we used the gramicidin-perforated patch-clamp technique as before [11,24]. Patch electrodes were prepared from borosilicate capillary glass pipettes using a vertical pipette puller (P-9; Narishige, Tokyo, Japan). The pipette was filled with internal solution containing (in mM) 150 KCl and 10 HEPES, with the pH adjusted to 7.2 with Tris-OH. The resistance of the pipettes was 6–8 M Ω . Gramicidin was first dissolved in methanol to prepare a stock solution of 100 mg/mL and then diluted to a final concentration of 10 mg/mL for the internal solution. To record spontaneous excitatory postsynaptic currents (sEP-

SCs), neurons were voltage-clamped at a holding potential (V_H) of –60 mV. The sEPSCs were pharmacologically isolated using bicuculline (50 μ M) and strychnine (1 μ M) to inhibit the activation of GABA_A and glycine receptors. The membrane potential was measured in current-clamp mode with no injection of current. Electrode capacitance and liquid junction potential were compensated. Recordings were conducted using an EPC-10 amplifier (HEKA, Lambrecht, Germany). The input resistance and series resistance were monitored continuously throughout the recording. If the series resistance (usually 25–55 M Ω) changed by more than 20%, the recording was terminated immediately. Recordings were conducted from only one neuron per dish. All experiments were performed at room temperature (21–26°C).

Cytosolic Ca²⁺ Measurements Using Rhod-2-AM

Dissociated neurons were washed three times with Hanks' balanced salt solution (HBSS), followed by incubation at 37°C in the dark for 30 min in HBSS-containing Rhod-2-AM (5 μ M). Neurons were then washed with HBSS three times and subsequently placed on a laser scanning confocal microscope (Lsm-710, Zeiss, Jena, Germany) for observation. The illumination wavelength was set to 543 nm. Digital images were acquired every minute using a 20 $\times$ objective with a 5–7 μ s pixel acquisition time. The average fluorescence intensity of all relevant regions was recorded and stored for subsequent analyses. The images were analyzed using ZEN software (Zeiss).

Cell Culture and Assessment of Cell Viability

Ventral cochlear nucleus neuron cultures were prepared in neurobasal/B27 medium, as described in a previous publication [25]. Briefly, postnatal day 3–5 rats were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and decapitated. After the brain was extracted and placed in HBSS, the VCN was dissected from the brainstem under a dissecting microscope using a microdissecting knife. The VCN was then placed in Dulbecco's modified Eagle's medium with high-concentration glucose (DMEM/HIGH) and cut into small pieces with Vannas scissors. Following a 30-min treatment with papain (20 mM) and recombinant DNase I (2.5 mM), the digested VCN was mixed with an equivalent amount of DMEM/HIGH containing 10% fetal bovine serum (FBS) to terminate the enzyme reaction. After centrifugation at 91 $\times g$ for 8 min, the cell suspension was transferred into cell culture plates precoated with poly-L-lysine at a density of 5–10 $\times 10^6$ cells/well in 20-well plates. The cultures were then placed in a 37°C incubator (5% CO₂/20% O₂). Three to four hours later, the culture medium was changed to neurobasal medium with B27, penicillin–streptomycin (100 U/mL penicillin, 100 μ g/mL streptomycin), and 2 mM L-glutamine. Half of the medium was refreshed every other day. To keep bilirubin in solution, before the experiment, unconjugated bilirubin (UCB) was dissolved in 0.1 M NaOH and then added to a bovine serum albumin (BSA) solution to obtain UCB/BSA, with a molar ratio (MR) of 1.5 [26]. After 9–10 days *in vitro*, VCN neurons were then exposed to bilirubin, riluzole, or riluzole followed by bilirubin, and afterward the cultures were washed with complete medium. Neurons exposed to NaOH and BSA at concentrations equivalent to those treated with

25 μM UCB served as controls. The degree of cell death was determined by nuclear staining with Hoechst 33,423 (10 $\mu\text{g}/\text{mL}$) and propidium iodide (PI) (10 $\mu\text{g}/\text{mL}$) for 20 min at 4°C in the dark. Apoptotic and necrotic cells were counted under a laser scanning confocal microscope (Lsm-710; Zeiss).

Reagents

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA). A bilirubin solution was made before an experiment by adding a bilirubin stock solution (bilirubin dissolved in 0.1 M NaOH) into a bovine serum albumin (BSA) solution at a 1.5 UCB/BSA molar ratio. Bilirubin stock solution was stored in single-use aliquots in the dark at -20°C and diluted to the final solution prior to use in electrophysiological recordings. Bilirubin solution was protected from light at all times. Riluzole was dissolved in dimethylsulfoxide (DMSO) at room temperature. The riluzole solution was kept for no more than 2 days before use. The reagents were applied to isolated neurons using a Y-tube system [27] that allowed for a complete exchange of the perfusion solution within 20 ms.

Data Analyses

The numbers of sEPSCs and action potentials were counted and analyzed using the MiniAnalysis software (Synaptosoft, Fort Lee, NJ, USA). Amplitudes were used as major criteria for identifying sEPSCs (≥ 6 pA) and action potentials (≥ 40 mV). In most cases, the average value of the sEPSC frequencies and amplitudes during the control period were scaled to 1.0. When comparing the data

between application of bilirubin and riluzole combined with bilirubin, the average value of the sEPSC frequencies during the bilirubin application period was scaled to 1.0. The data were evaluated to determine whether they followed a normal distribution using the Shapiro–Wilk test first. Statistical analysis was performed using a paired or unpaired Student's *t*-test for comparisons between two groups. Multiple comparisons were analyzed by one-way ANOVA for independent and/or repeated measures followed by Bonferroni test. Statistical analyses were performed using the SAS software (version 8.0; Cary, NC, USA). Values of $P < 0.05$ were considered to indicate statistical significance. Data are presented as means $\pm$ standard deviation.

Results

Riluzole Decreased sEPSC Frequency

The effects of riluzole on sEPSCs were examined in isolated VCN neurons on which the nerve terminals remain attached. Considering that the plasma therapeutic concentration of riluzole in the clinic is approximately 5 μM [17,28], we used 5, 10, and 15 μM riluzole to assess the concentration-dependency of its effects. As described previously, sEPSCs recorded in the present mode were mediated by ionotropic glutamate receptors, both α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors as the sEPSCs were totally suppressed by the specific receptor antagonists, D,L-2-amino-5-phosphonovaleric acid (APV) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (data not shown) [11]. The sEPSC frequency (before drug: 0.43–4.04 Hz) decreased

Figure 1 Riluzole decreased the frequency but did not affect the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) in VCN neurons. **A**, Top three traces: Riluzole reversibly decreased the incidence of sEPSCs in a VCN neuron. Bottom trace: averaged sEPSCs from the neuron shown in A before, during and after riluzole application. The rise time (2.25 ms) and decay time (13.6 ms) did not show obvious changes during riluzole application (rise time: 2.05 ms; decay time: 12.9 ms) and wash time (rise time: 2.1 ms; decay time: 12.6 ms).

B, Cumulative probability of interevent intervals and amplitudes of sEPSCs recorded from the same neuron shown in A before, during, and after riluzole application. **C**, A bar graph showing the mean sEPSC frequency and amplitude of five neurons during (6 min) and after (4 min) application of riluzole at 5 μM . **D**, Group results ($n = 5$) show sEPSC frequency during riluzole treatment at 5, 10, and 15 μM . Error bars indicate standard deviation; * $P < 0.05$; ** $P < 0.01$; ns: not significant.

persistently over the entire 6 min riluzole (5 μ M) application time, shifting the cumulative distribution curve of interevent intervals to the right (Figure 1B, left). The sEPSC frequency was reduced to 0.14–0.79 Hz, and the average was $52.4 \pm 6\%$ of the control ($n = 5$, $P < 0.01$; Figure 1C). At 10 μ M of riluzole, the sEPSC frequency (before drug: 0.24–4.27 Hz) was reduced to 0.23–1.09 Hz and was $50.2 \pm 4.6\%$ of the control ($n = 5$, $P < 0.05$; Figure 1D). There was no significant difference in the frequency reduction between 5 μ M and 10 μ M riluzole applications ($P > 0.05$). However, at 15 μ M, the action of riluzole was greatly increased and the sEPSC frequency (before drug: 1.55–5.61 Hz) decreased to only $10.8 \pm 2.8\%$ of the control and was 0.22–0.43 Hz ($n = 5$, $P < 0.01$). The drug effect on the sEPSC frequency at 15 μ M was significantly different from those at 5 and 10 μ M ($P < 0.01$; Figure 1D). The inhibition of sEPSC frequency by riluzole (5 μ M) was reversible. The average sEPSC frequency during the 4-min wash period was $102 \pm 15.1\%$ of the control ($n = 5$, $P > 0.05$; Figure 1C). In contrast, sEPSC amplitude (65 ± 25 pA) was little affected by riluzole (5 μ M, $103 \pm 10.9\%$ of the control, $n = 5$, $P > 0.05$; Figure 1B, right, and C). Riluzole also did not influence kinetics of the sEPSCs (Figure 1A).

Riluzole Suppressed sEPSC Potentiation Induced by Bilirubin

We next investigated whether riluzole could influence bilirubin-induced potentiation of glutamate release by examining the effect of riluzole following bilirubin application. Consistent with our previous studies, bilirubin (3 μ M) markedly increased the frequency of sEPSC over a 6-min application time from 0.42–2.1 Hz to 2.39–9.24 Hz ($692 \pm 110\%$ of the control, $n = 5$, $P < 0.01$), and with 2–5 min of bilirubin application, action potential currents (APc) which were proved to be tetrodotoxin sensitive appeared (Figure 2A, b). Further addition of riluzole (5 μ M) significantly decreased the sEPSC frequency to 0.51–0.92 Hz ($12.9\% \pm 7\%$ of the bilirubin alone condition, $P < 0.01$; $84 \pm 35\%$ of the control, $P > 0.05$; $n = 5$, 6 min) and suppressed the APc (Figure 2A, B). The effect of riluzole was reversible. After riluzole was removed, the sEPSC frequency rebounded to 3.41–6.45 Hz, a level similar to that evoked by bilirubin alone ($557 \pm 187\%$ of the control in 4-min bilirubin wash, $n = 5$, $P < 0.01$) and the APc reemerged (Figure 2A, d, B). Riluzole at 10 and 15 μ M decreased the frequency of sEPSCs to $10.8 \pm 8.7\%$ (0.36–1.36 Hz, $n = 5$, $P < 0.05$) and $9.1 \pm 4\%$ (0.02–0.63 Hz, $n = 5$, $P < 0.01$), respectively, compared to the bilirubin-alone condition. The protective action of riluzole against bilirubin was not significantly different among 5, 10, and 15 μ M concentrations (Figure 2C). The sEPSC amplitude was not significantly affected during the entire period of recording, and the average amplitude was 93 ± 6.8 pA, 71.5 ± 9.2 pA, and 72.9 ± 2.3 pA, respectively, in bilirubin, bilirubin plus riluzole (5 μ M), and bilirubin (wash) conditions (i.e., $125 \pm 25.3\%$, $95.2 \pm 14.7\%$, and $98.6 \pm 21.1\%$, respectively, compared to the control ($n = 5$, $P > 0.05$) (Figure 2B).

Postsynaptic currents mediated by glutamatergic receptors (I_{Glu}) were elicited by bath application of exogenous glutamate (500 μ M) through a “Y tube” perfusion device. In the presence of TTX, the mean amplitude of I_{Glu} over the 6-min bilirubin (3 μ M) application and 10-min combined application of riluzole (5 μ M)

and bilirubin (3 μ M) was $95 \pm 3\%$ (172 ± 23 pA) and $94 \pm 9\%$ (169 ± 26 pA), respectively, of that obtained under glutamate alone (Figure 2D, E). Thus, the mean amplitude of I_{Glu} was neither significantly affected by bilirubin nor by a combination of bilirubin and riluzole ($n = 4$, $P > 0.05$).

Pretreating with Riluzole also Prevented the Bilirubin-Induced Potentiation of sEPSCs

To investigate whether riluzole could prevent the potentiation of synaptic activity induced by bilirubin in advance, we pretreated neurons with riluzole (5 μ M) for 30 min before bilirubin (3 μ M) application. In the presence of riluzole, further addition of bilirubin (10 min) had no significant influence on either the frequency (0.53–3.39 Hz to 0.80–2.89 Hz) or amplitude (59.1 ± 9.5 pA to 59 ± 5.8 pA) of the sEPSCs ($113 \pm 25.6\%$ and $102 \pm 18\%$ of the control, respectively, $n = 5$, $P > 0.05$) (Figure 3).

Riluzole Suppressed Neuronal Hyperexcitation Induced by Bilirubin

Here, we investigated the action of riluzole on spontaneous firing and bilirubin-induced hyperexcitation in VCN neurons. Most neurons recorded displayed spontaneous action potentials. Under 5 μ M riluzole, spontaneous firing of action potentials was completely inhibited and only subthreshold spontaneous synaptic potentials remained ($n = 4$, Figure 4A). Consistent with our previous findings, bilirubin (3 μ M) dramatically increased the rate of the neuronal firing which was almost completely blocked by coapplication of NBQX (10 μ M) and APV (50 μ M) (Figure 4B) [11,29]. After application of bilirubin for 6 min (3 μ M), coapplication with riluzole could also suppress neuronal firing, although subthreshold synaptic events could still be detected. The inhibitory effect of riluzole was reversible as a high level of spontaneous firing reemerged after riluzole was removed from the perfusion solution (Figure 4C). During the recording and drug application, the resting potential of these neurons showed no significant change (control: -65 ± 8 mV, riluzole: -61 ± 6 mV, bilirubin: -64 ± 4 mV, riluzole plus bilirubin: -65 ± 6 mV).

Inhibitory Effects of Riluzole on Bilirubin-Induced Ca^{2+} Overload

To assess the potential role of intracellular Ca^{2+} homeostasis in the protective effects of riluzole against bilirubin neurotoxicity, we monitored the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using the fluorescent calcium indicator Rhod-2-AM. As shown by an example in Figure 5A, 20 min after the initiation of bilirubin application (3 μ M), intracellular Rhod-2-AM fluorescence was enhanced markedly, indicating an increased intracellular Ca^{2+} level. For a population of 10 neurons, the level of $[\text{Ca}^{2+}]_i$ during bilirubin was increased to $191 \pm 46\%$ of that under the control condition ($P < 0.05$; Figure 5A, left column and 5B). The action of bilirubin could be completely blocked by the ionotropic glutamate receptor (AMPA and NMDA receptors) antagonists, NBQX (10 μ M), and APV (50 μ M) ($101 \pm 23\%$ of the control, $n = 10$, $P > 0.05$; Figure 5A, middle column and 5B), indicating that bilirubin-induced intracellular calcium increase is mediated by

Figure 2 Riluzole suppressed the bilirubin-induced increase in the frequency of sEPSC and action potential currents, but did not change the amplitude of sEPSC. **A**, Top panel: A typical sEPSC trace recorded from a neuron in control solution (a), 3 μ M bilirubin (b), 3 μ M bilirubin plus 5 μ M riluzole (c), and 3 μ M bilirubin again (d). Bottom panel: Portions of the trace are also shown at higher resolution (a, b, c, and d). **B**, A bar graph shows mean sEPSC frequency and amplitude of five neurons during application of 3 μ M bilirubin, 3 μ M bilirubin plus 5 μ M riluzole, and 3 μ M bilirubin again. **C**, Histograms depict average sEPSC frequency for five neurons during the bilirubin plus riluzole (5, 10, 15 μ M) application period relative to the bilirubin alone condition. **D**, In the presence of TTX, typical IGLu traces recorded from a VCN neuron before, during bilirubin, and during combined application of bilirubin and riluzole. **E**, Amplitudes of the IGLu recorded in TTX and bilirubin, and TTX combined with bilirubin and riluzole were normalized against that obtained under the control condition (i.e., TTX alone) ($n = 4$). Error bars represent standard deviation; * $P < 0.05$; ** $P < 0.01$; ns: not significant.

activating AMPA and NMDA receptors. Riluzole (30 min, 5 μ M) alone had no significant influence on $[Ca^{2+}]_i$ ($92 \pm 3\%$ of the control, $n = 10$, $P > 0.05$). However, pretreating neurons with riluzole (30 min) significantly attenuated the potentiating action of bilirubin (20 min, $88 \pm 21\%$ of the control, $n = 10$, $P > 0.05$; Figure 5A, right column and 5B).

Neuroprotective Effects of Riluzole Against Bilirubin-Induced Cell Death

Previous studies showed that bilirubin-induced cell death was mediated by glutamate receptors. In this study, we have demonstrated that riluzole can effectively inhibit bilirubin-induced overactivation of glutamatergic synaptic transmission. Therefore, we further investigated the effect of riluzole on bilirubin-induced neuronal death. As shown in Figure 6B, treatment with 3 μ M and

6 μ M UCB (12 h, MR 1.5) did not significantly affect neuronal viability. However, UCB at concentrations of 12.5 and 25 μ M (12 h, MR 1.5) produced significant neuronal toxicity, as evidenced by staining nucleus of the neuron for apoptosis (bright blue) and/or necrosis (bright red) (Figure 6A, left bottom panel). Neurons treated with riluzole (5 μ M, 24 h) alone did not demonstrate any damage (Figure 6A, right top panel and B). Pretreating neurons with riluzole (5 μ M) for 2 h prior to UCB (12.5 or 25 μ M, 12 h, MR 1.5) markedly reduced the number of dead cells in the culture (Figure 6A, right bottom panel and B).

Discussion and Conclusions

Riluzole is a prescription drug well-known for its ant glutamatergic effect [17,30]. Recently, there has been increasing therapeutic use of riluzole in treating neurodegenerative diseases [15,17].

Figure 3 Pretreatment with riluzole suppressed bilirubin-induced potentiation in sEPSC frequency in VCN neurons. **A**, Typical sEPSC traces observed before, during and after bilirubin ($3 \mu\text{M}$) application in the presence of riluzole. **B**, Summary data of sEPSC frequency and amplitude during and after bilirubin application (10 min) with riluzole pretreatment (30 min). Error bars indicate standard deviations; ns: not significant.

Figure 4 Effects of riluzole ($5 \mu\text{M}$) on action potentials and bilirubin-induced synaptic potentiation. **A**, Spontaneous action potentials were suppressed by riluzole. **B**, left and Middle: Spontaneous action potentials were recorded from a neuron before and after bilirubin application. Right: The firing potentiated by bilirubin was blocked by NBQX and APV. **C**, Top panel: Bilirubin ($3 \mu\text{M}$) increased the spontaneous action potentials, while riluzole completely inhibited the potentiating action of bilirubin. Bottom panel: Portions of the trace are also shown at higher resolution (a, b, and c).

However, few studies have focused on its protective action in BE to date. Our results showed that riluzole acts to suppress presynaptic glutamate release and neuronal firing in VCN neurons. Furthermore, the drug inhibits bilirubin-induced potentiation of excitatory physiological activities. Riluzole was also shown to inhibit bilirubin-induced Ca^{2+} overload and neuronal death resulted from overactivation of glutamate receptors. This report provides novel information regarding using riluzole to treat BE in the future.

Riluzole Affects Presynaptic, but not Postsynaptic Glutamate Receptors

Results from the present study revealed that riluzole reduced the frequency of sEPSCs and spontaneous action potentials in VCN neurons without influencing the amplitude of sEPSCs and exogenous glutamate-activated postsynaptic currents even in the presence of TTX. These results suggest that riluzole inhibits presynaptic glutamate release, but does not influence the sensitivity

Figure 5 Effects of riluzole on bilirubin-induced Ca^{2+} overload as revealed by the intensity of Rhod-2-AM fluorescence in confocal microscopic images. **A** Left column: Images taken 30 min after a neuron was loaded with Rhod-2-AM (control, top panel) and 20 min after the initiation of application of bilirubin (bottom panel). Middle column: Images taken 30 min after a neuron was loaded with Rhod-2-AM (control, top panel) and 10 min after NBQX and APV were added to the perfusion solution (middle panel), and 20 min after bilirubin was added to the solution containing NBQX and APV (bottom panel). Right column: Images taken 30 min after a neuron was loaded with Rhod-2-AM (control, top panel) and 30 min after riluzole was added to the perfusion solution (middle panel), and 20 min after bilirubin was added to the solution containing riluzole (bottom panel). Color bar indicates low (left) and high (right) fluorescence intensities. **B**: A bar graph shows mean fluorescence intensities of 10 neurons under the four different drug conditions. Error bars indicate standard deviations; * $P < 0.05$; ns: not significant.

of postsynaptic glutamate receptors. This conclusion is consistent with findings using other techniques and methods [31–33]. It is noted that some studies indicated that for motor neurons and striatal spiny neurons riluzole could reduce the amplitude of currents evoked by direct activation of postsynaptic ionotropic glutamate receptors as well as the amplitude of sEPSCs [34,35]. This difference indicates that structural dependence may exist in the synaptic mechanisms that the drug targets.

Figure 6 Neuroprotective effects of riluzole against bilirubin-induced excitotoxicity. **A**, Top panel: riluzole (5 μ M) did not influence neuronal activity. Bottom panel: bilirubin (12.5 μ M) induced neuronal apoptosis and necrosis, and riluzole (5 μ M) effectively protected the neurons from bilirubin-induced cell death. **B**, The histogram shows the quantification of cell death with riluzole (5 μ M), bilirubin at four different concentrations, and in the bilirubin at four concentrations combined with riluzole (5 μ M) conditions. Vertical bars indicate SDs. ** $P < 0.01$, *** $P < 0.001$. ns: not significant.

The presynaptic mechanisms through which riluzole suppresses glutamate release can be multifaceted. Riluzole has been reported to depress presynaptic Ca^{2+} influx [20,36], block autoreceptors controlling transmitter release, and activate a class of background potassium (K^+) channels and G-protein-dependent signal transduction [37, 38]. The specific molecular mechanism(s) contributing to the effects observed in the present study has yet to be determined.

Riluzole Inhibits Bilirubin-Induced Potentiation of Presynaptic Glutamate Release and Hyperexcitation

Recently, we showed that bilirubin enhanced neuronal excitability by increasing presynaptic glutamate release [11,29]. In the present study, the results demonstrated that riluzole can suppress the potentiation of bilirubin on both sEPSCs and spike firing. This suppressive effect of riluzole was observed no

matter whether the drug was applied before or during bilirubin treatment. These results suggest that riluzole can be used both to prevent and to treat bilirubin-induced hyperexcitation. The effects of the drug are likely dependent on the suppression of presynaptic glutamate release. The lack of concentration-dependent within the range between 5 and 15 μM suggests that the dosage of riluzole used for treating amyotrophic lateral sclerosis is sufficient for preventing hyperexcitation in VCN neurons.

Postsynaptic Consequences of Bilirubin-Induced Enhancement of Glutamate Release and the Effect of Riluzole on these Consequences

Continuous activation of a large number of ionotropic glutamate receptors can lead to increases in intracellular Ca^{2+} loads and activation of catabolic enzymes, further triggering a cascade of events, eventually leading to apoptosis or necrosis [39–41]. In fact, Ca^{2+} overload is recognized as a major molecular mechanism of bilirubin-induced excitotoxicity [8,42]. Previous studies have also shown that bilirubin-induced neuronal cell death was decreased by glutamate receptor antagonists [9,10], indicating that glutamatergic synaptic transmission is indeed an important target of bilirubin toxicity.

Because riluzole can effectively inhibit bilirubin-induced potentiation of glutamate release and the subsequent overactivation of glutamate receptors, we suggest that riluzole may inhibit bilirubin-induced Ca^{2+} overload and neuronal death. Consistent with this hypothesis, our study illustrates that preincubating neurons with riluzole at therapeutic concentrations can effectively inhibit bilirubin-induced increase in the intracellular concentration of Ca^{2+} and the number of cell death. Our study provides direct evidence for a protective effect of riluzole on bilirubin-induced excitotoxicity. We also suggest the mechanisms of protective action of riluzole, that is, the inhibitory effects of riluzole are the consequence of decrease in glutamatergic transmission, likely due to reduction of presynaptic glutamate release.

Other Possible Mechanisms Involved in the Neuroprotective Effects of Riluzole

In addition to the inhibition of glutamatergic synaptic transmission, riluzole may protect neurons from bilirubin-induced cell death through other mechanisms. Riluzole is known to be an inhibitor of the persistent sodium current underlying sustained spike firing and excessive Ca^{2+} influx during spiking [18,19,23]. This inhibitory effect may also contribute to the neuroprotective effect of riluzole. A recent study showed that the level and activity of astrocyte glutamate transporters, which are responsible for the removal of glutamate from the synaptic cleft, were upregulated by riluzole [43]. Furthermore, there are studies showing that riluzole potentiated the inhibitory function of GABA_A-receptors and inhibited voltage-gated Ca^{2+} currents [20,44]. These actions of riluzole may also lead to an antiexcitotoxicity effect of the drug. Thus, several molecular mechanisms may contribute, possibly synergistically, to inhibiting excitotoxicity.

Riluzole as a Potential Neuroprotective Drug Against Hyperbilirubinemia-Related Excitotoxicity

It is believed that excitotoxicity is an important contributor to the pathogenesis of BE [8,11]. Thus, drugs reducing neuronal hyperexcitation may serve as effective neuroprotectants against bilirubin toxicity. In an early investigation, we studied the effects of some proposed drugs, such as minocycline, on bilirubin-induced hyperexcitation, but the results were disappointing. The failure of minocycline was apparently due to its inability to depress bilirubin-induced potentiation of presynaptic glutamate release [29].

Hearing loss is one of the most common symptoms in BE infants, and in some cases, can become permanent. Hearing aids and cochlear implants are the only available methods in such cases but with limited efficacy [1,45]. To date, exchange transfusions and phototherapy remain common interventions for newborns with BE, despite of the known adverse effects [4,46,47]. Because of the unsatisfactory clinical outcomes of current available interventions, there is a continuing need to develop new pharmacological interventions to prevent and treat BE in these infants.

Our study showed that riluzole can inhibit bilirubin-induced hyperexcitation and excitotoxicity in VCN neurons, regardless of whether riluzole is applied before or after bilirubin treatment. Riluzole not only prevents the occurrence of bilirubin-induced excitotoxicity but also alleviates the excitotoxicity after excitotoxic insults. The VCN is the most involved nucleus for hearing abnormalities in BE infants. The protective effect of riluzole on VCN neurons rises hope that this prescription drug may be useful for treating and/or preventing hearing loss in BE infants. Except VCN, other nuclei, such as Purkinje cells of the cerebellar cortex and neurons in the later superior olive which also suffer from excitotoxicity induced by bilirubin, may benefit from riluzole as well. Further studies in these neural structures will help us determine whether riluzole can be used clinically for dealing with bilirubin toxicity. Using riluzole as a prophylactic measure in high-risk infants recognized by a risk-based assessment may reduce the development of irreversible brain damage. Using this drug as a treatment for BE may have fewer side effects than existing treatments including exchange transfusions and phototherapy. A recent report indicated that a few patients with spinal cord injury had a mild elevated bilirubin level on admission. During riluzole administration, slightly more patients showed mild-to-moderate elevated bilirubin level, but no patients had abnormal level of bilirubin on the last day (day 14) of riluzole administration [48]. Moreover, there is no evidence that riluzole can increase bilirubin levels directly. Overall, the results in the present study suggest an additional advantage of the clinical use of riluzole as a pharmacological intervention for BE.

In conclusion, our study demonstrated that riluzole can effectively suppress the potentiation of presynaptic glutamate release and neuronal hyperexcitation caused by bilirubin in neurons in the VCN. This suppressive effect may subsequently prevent Ca^{2+} overload and cell death in these neurons. These effects make riluzole a promising drug for treating and preventing bilirubin-induced excitotoxicity and hearing loss in BE infants.

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Conflict of Interest

The authors declare no conflict of interest.

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