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Original Article

Inhibition of connexin 36 decreases seizure activity in the Kainic acid-induced rat model of epilepsy

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Abstract: Aim: To investigate the effect of connexin 36-associated (Cx36) channel blockers on the process of epilepsy and its mechanisms. Methods: We examined the anticonvulsant properties of carbenoxolone, quinine, and quinidine in the Kainic Acid-induced (KA-induced) model of epilepsy in rats, and we investigated the effects of these blockers on epileptic discharge and the expression of Cx36. Results: 30 min after KA injection, spike frequency and amplitude were noticeably and reversibly reduced in Cx36 blocker pretreated animals. At the 90 min time point, carbenoxolone, quinine, and quinidine significantly decreased the spike amplitude but not frequency. Cx36 protein level significantly increased in Cx36 blocker pretreated animals relative to control. There were no significant differences among the three drug treatment groups in spike frequency, spike amplitude, or level of Cx36 expression. Conclusion: Therefore, specific gap junction blockers may be efficacious for the treatment of epilepsy by preventing the propagation and synchronization of epileptiform activity.

Keywords: Epilepsy, gap junction, connexin 36, carbenoxolone, quinine

Introduction

Epilepsy represents a group of neurological disorders of varying severity that are characterized by recurring seizures and neurobiological, cognitive, psychological, and social ramifications [1]. Approximately 70% of patients with epilepsy are diagnosed with chronic and intractable temporal lobe epilepsy. Epilepsy is one of the most common neurologic disorders worldwide. In spite of the rapid development of anti-epileptic drugs, 20% to 30% of patients with epilepsy remain refractory to drug treatment [2]. Surgical resection is a not viable option for 50% of these patients because of the lack of a definite or suitable surgical location [2]. Pharmacological therapy remains the treatment of choice for temporal lobe epilepsy. Therefore, development of new anti-epileptic drugs remains a priority for epilepsy research.

A major factor driving neuronal synchrony during seizures is enhanced gap junction communication between neurons. The connexin Cx36, a gap junction protein, is predominantly expressed in mammalian neuronal cells [3], especially in CA3 of hippocampus. Given the role of gap junctions in epilepsy, it follows that pharmacological inhibition of their function using Cx36 blockers would be an efficacious approach for disrupting neuronal synchronization associated with seizure activity [4-6].

Based on specificity, Cx36 blockers can be divided into two main types: 1) non-specific gap junction blockers (ex. carbenoxolone), and 2) specific Cx36 blockers, (ex. the antimalarial drugs quinine and its stereoisomer quinidine [7]. The effect of quinine is dose-dependent and reversible for Cx36, but it also blocks voltage-dependent K+ channels at higher concentrations. Although several studies have demonstrated Cx36 blocker associated effects on seizure activity, there is no information available regarding the time course of possible alterations in the expression of Cx36 expression during the course of epilepsy [4-6]. Additionally, there are no reports on the use of quinidine in vivo in the control of epilepsy.

Although the relationship between gap junctions and epilepsy are well established, the specific details of their function and how this can be modulated for therapeutic purposes remain to be elucidated [4-6]. To date, there are no anticonvulsant drugs approved for clinical use that block gap junctions. Understanding the role of Cx36 and the effects of its blockers on seizure activity may contribute to the development of novel therapeutic strategies for the treatment of epilepsy. Here, we examined the anticonvulsant properties of carbenoxolone, quinine, and quinidine in the KA-induced model of epilepsy in rats, and we investigated the effects of these blockers on epileptic discharge and the expression of Cx36.

Materials

In total, 72 adult male Wistar rats (200~250 g, Experimental Animal Center of Bethune Medical Department) were used in this study. Animals were housed at 25 ± 1°C on an alternating 12 hr light/dark cycle with food and water available *ad libitum*. All animal experiments were carried out with the permission of local authorities and in accordance with the China law for animal protection in order to minimize suffering and the number of animals used.

Rats were divided into the following four groups (n = 18 in each group): control group, carbenoxolone group, quinine group, and quinidine group. Animals were divided accordingly: electroencephalogram (EEG) recording (n = 6), immunoblotting (n = 6), and immunohistochemistry (n = 6). KA monohydrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline and carbenoxolone (Sigma-Aldrich), quinine (Sigma-Aldrich), and quinidine (Sigma-Aldrich) were dissolved in 0.8% (v/v) Tween80. KA, carbenoxolone, quinine and quinidine were delivered intraperitoneally (i.p.). The control group received 0.8% (v/v) Tween80. Animals were anesthetized with an i.p. injection of 10% chloral hydrate (3.5 ml/kg, obtained from First Hospital of Jilin University). Carbenoxolone (200 mg/100 ml, 20 mg/kg), quinine (500 mg/100 ml, 50 mg/kg), and quinidine (200 mg/100 ml, 20 mg/kg) were administered 30 min prior to the injection with KA.

Methods

Surgery

After administration of anesthesia, animals were secured in a stereotaxic frame with the incisor bar positioned at -3.3 mm. The coordinates used for the basolateral nucleus in the right nucleus amygdala were AP = -3.5 mm, L = 4.5 mm lateral, and V = 8.0 mm from bregma.

Electroencephalogram (EEG) recording

One surface Ag-AgCl non-polarizable electrode was affixed on the parietal cortex to record the cortical EEG (coordinates: AP = -4.0 mm, L = 3.0 mm, and V = 1.5 mm from bregma) with dental cement. In addition, ground and indifferent electrodes were affixed on the tip of the nose and neck muscles, respectively. The electrode wires were attached to a socket connector, and animals were connected to a computerized EEG recording system (Power Lab, AD Instruments, Dunedin, New Zealand). EEG activity was recorded with a power filter with the low pass and high pass filters set at 30 Hz and 1 Hz, respectively. The data were acquired and stored on a computer hard disk. EEG recordings started immediately after a 30 min pretreatment and continued for 120 min after the administration of KA.

Tissue preparation

All animals were sacrificed by decapitation under deep ether anesthesia, and their brains were promptly removed six hours after the administration of KA. A portion of the hippocampus was quickly dissected, frozen immediately in liquid nitrogen, and stored at -80°C. The rest of the brain was placed in 10% formalin for paraffin embedding. Brains were cut into 10 μm thick sections on a microtome, mounted on glass slides, and prepared for histopathological and immunohistochemical analyses. The stained sections were qualitatively analyzed using a stereoscopic microscope (Olympus, Shinjuku, Japan).

Immunoblotting

Isolated hippocampus tissue was homogenized in RIPA lysis buffer containing: 150 mM NaCl, 0.25% sodium deoxycholate, 0.25% sodium

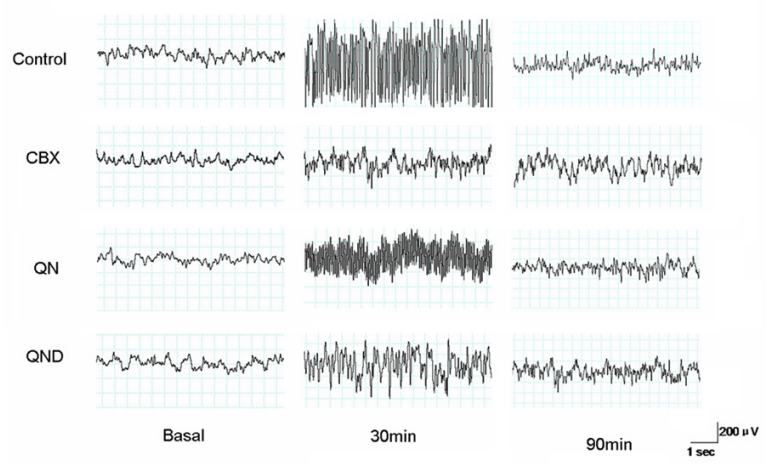


Figure 1. Effects of global or selective Cx36 channel blockers on electroencephalographic activities. The electroencephalographic sample traces show the effects of carbenoxolone (CBX), quinine (QN), and quinidine (QND) on the basic and epileptiform activities 30 and 90 min after KA injection. EEG samples on the left represent brain activity prior to KA application. Note that CBX, QN, and QND do not change baseline brain activity. Those traces in the middle and right show brain activity 30 and 90 min after KA administration. Injection of KA induces an epileptiform EEG activity characterized by biphasic spikes and spike-wave complexes. The administration of CBX, QN, and QND decrease the frequency and amplitude of epileptiform activity.

dodecylsulfate, 1 mM ethylenedia-minetetraacetic acid, 50 mM Tris-HCl, pH 7.4 with PMSF. Protein concentration of lysates was determined by the Bradford method (Bio-Rad, Hercules, CA, USA), and 30 g of sample was loaded per lane, resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analysis. Blots were probed with the primary antibodies anti-Cx36 (1:200, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-β-Actin (1:1000, Bioss, Woburn, MA, USA). After rinsing, blots were incubated in horseradish peroxidase conjugated goat anti-rabbit antibody (1:5000, Bioss). Highly sensitive enhanced chemiluminescence (ECL) reagent was used to visualize the bands, and band density was quantitated using Image J software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence immunohistochemistry

Paraffin-embedded sections were maintained at 60°C for 15 min, incubated in xylene at room temperature (RT) for 15 min, and transferred sequentially into 100% EtOH, 95% EtOH, 70% EtOH, and 50% EtOH for 4 min at RT. Sections were rinsed in deionized water and stored in phosphate buffered saline (PBS). Double-label immunofluorescence was performed with rabbit anti-rat Cx36 and mouse anti-rat microtubule associated protein-2 (MAP-2, Santa Cruz) to visualize localization of Cx36 in hippocampal neurons. Sections were blocked in 10% normal goat serum in PBS, and antibodies were diluted in 1% goat serum. The sections were incubated for 60 min with the Cx36 antibody (1:100), rinsed, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. After rinsing and additional blocking, sections were incubated with MAP-2 antibody (1:100), rinsed, and incubated with tetramethylrhodamine isothiocyanante (TRITC)-conjugated goat anti-mouse IgG. The coverslips were mounted with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (VWR International Aps, Radnor, PA, USA) to stain the nuclei. Co-expression was visualized using confocal microscopy (Olympus, Center Valley, PA, USA).

Statistical analysis

All statistical procedures were performed using SPSS (14.0) statistical software (Chicago, IL, USA). For immunoblot experiments, proteins levels in the different groups were compared using analysis of variance followed by Fisher LSD post hoc tests. All data are expressed as means ± SD., and the data were analyzed using analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Electroencephalographic activities

Injection of KA induced a status epilepticus. The seizures typically began with repetitive biphasic spikes of relatively low, but gradually increasing, voltage and frequency. These were represented in the EEG as either a spike-wave complex or a fast single spike of high amplitude

Table 1. Effects of global or selective Cx36 channel blockers on seizure discharge 30 min and 90 min after KA injection

	Latency s	Frequency 1 Hz	Frequency 2 Hz	Amplitude 1 μV	Amplitude 2 μV
Control	79.25 ± 19.43	13.26 ± 2.19	7.95 ± 1.25	168.48 ± 11.37	136.9 ± 5.51
CBX	128.3 ± 15.04	9.8 ± 0.93**	8.44 ± 0.73	74.8 ± 8.80**	115.2 ± 5.18**
QN	86.67 ± 7.77	11.11 ± 0.78**	9.09 ± 0.68	105.2 ± 15.75**	107.4 ± 8.87**
QND	69.5 ± 2.12	11.35 ± 1.22**	8.14 ± 0.84	123.4 ± 32.8*	112.1 ± 15.04*

The data illustrate the effects of carbenoxolone, quinine, and quinidine on different parameters of epileptiform activity. Carbenoxolone (CBX) group, quinine (QN) group, and quinidine (QND) group are each compared to the control group. Data are expressed as mean ± SD. Significance criterion: *P < 0.05, **P < 0.01.

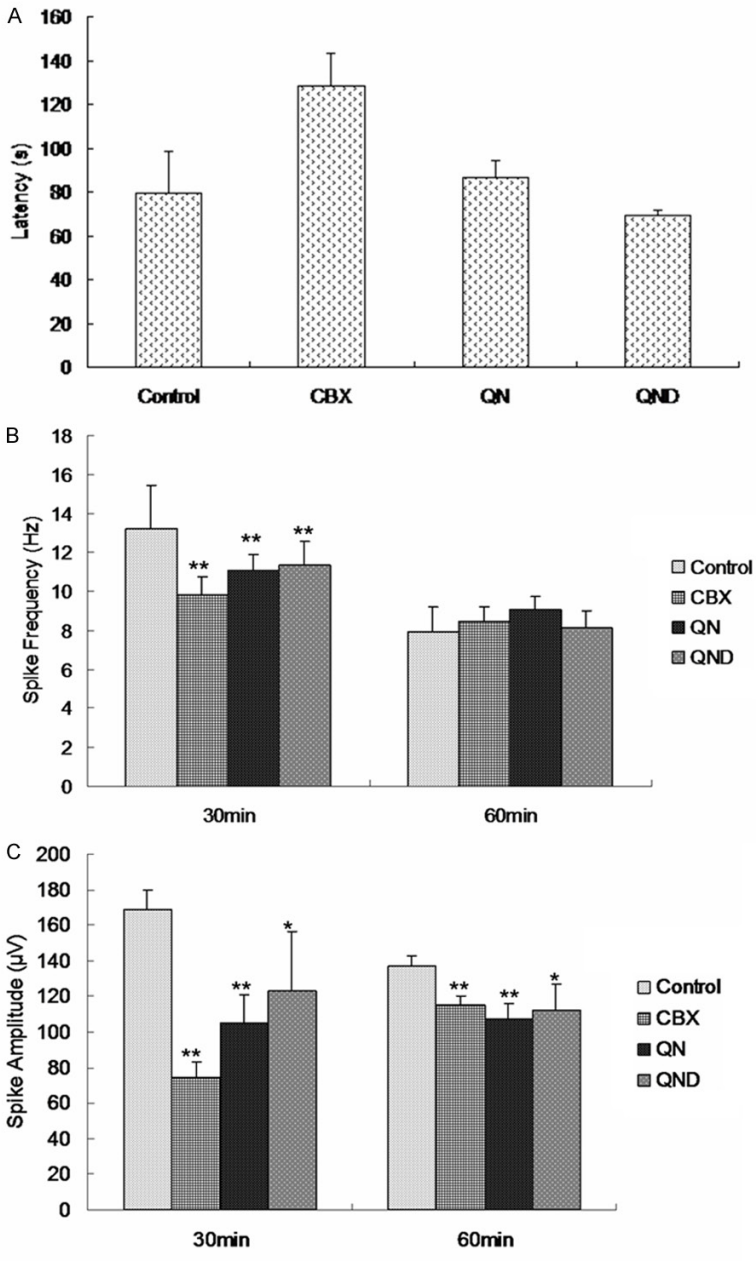


Figure 2. The statistical analysis of the changes on electroencephalographic activities. Effects of CBX, QN, and QND on latency (A), spike frequency (B), and amplitude (C) of seizure discharges 30 and 60 min after KA injection. Significance criterion: *P < 0.05, **P < 0.01, compared to the control group.

that was accompanied by a high frequency burst (**Figure 1**).

Injection of carbenoxolone, quinine, or quinidine did not significantly alter the frequency or amplitude of EEG activity. Although the average latency of the seizure discharges was shorter, it was not significantly different in the carbenoxolone pretreated animals (128.3 ± 15.04 s) relative to the control group (79.25 ± 19.43 s). Quinine and quinidine pretreatment for 30 min did not noticeably influence the latency of seizure discharges.

At 30 min after KA injection, the mean spike frequency was 13.3 ± 2.2 Hz, and the mean spike amplitude was 168.5 ± 11.4 μV in the control group. In the carbenoxolone and quinine groups, the spike frequencies and amplitudes were significantly and reversibly reduced (P < 0.01) (**Table 1**). Quinidine also significantly decreased the frequency (14.4%, P < 0.01) and amplitude (27.0%, P < 0.05) of seizure discharges relative to the control group (**Figure 2**).

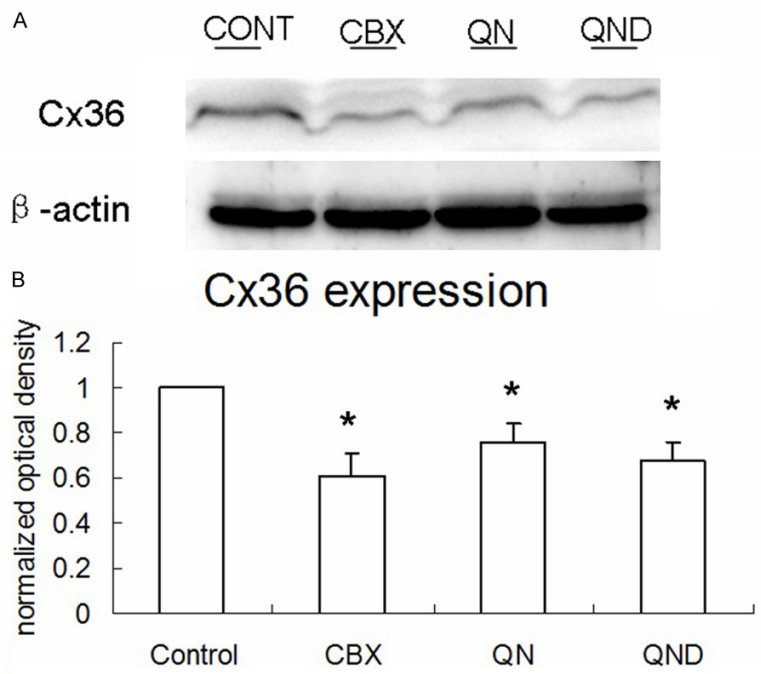


Figure 3. The immunoblot analysis changes of the Cx36 protein level. The effects of CBX, QN, and QND on the expression levels of Cx36 protein in the hippocampus of KA-induced seizure rats (A, B). Connexin 36 levels were normalized to that of β-actin protein (A). Data are expressed as means ± SD. *: $P < 0.05$, compared to the control group.

At the 90 min time point, administration of carbenoxolone, quinine, or quinidine decreased the spike amplitude to 115.2 ± 5.2 ($P < 0.01$), 107.4 ± 8.9 ($P < 0.01$), and 112.1 ± 15.0 ($P < 0.05$), respectively (**Figures 1**, **2**). There was no statistically significant difference in spike frequency among the groups.

Immunoblot analysis

In KA treated animals, the level of Cx36 protein expression in hippocampus increased significantly relative to control ($P < 0.05$). In rats treated with carbenoxolone, quinine, or quinidine 30 min prior to injection of KA, the expression of Cx36 protein was significantly reduced relative to KA treatment alone (**Figure 3**).

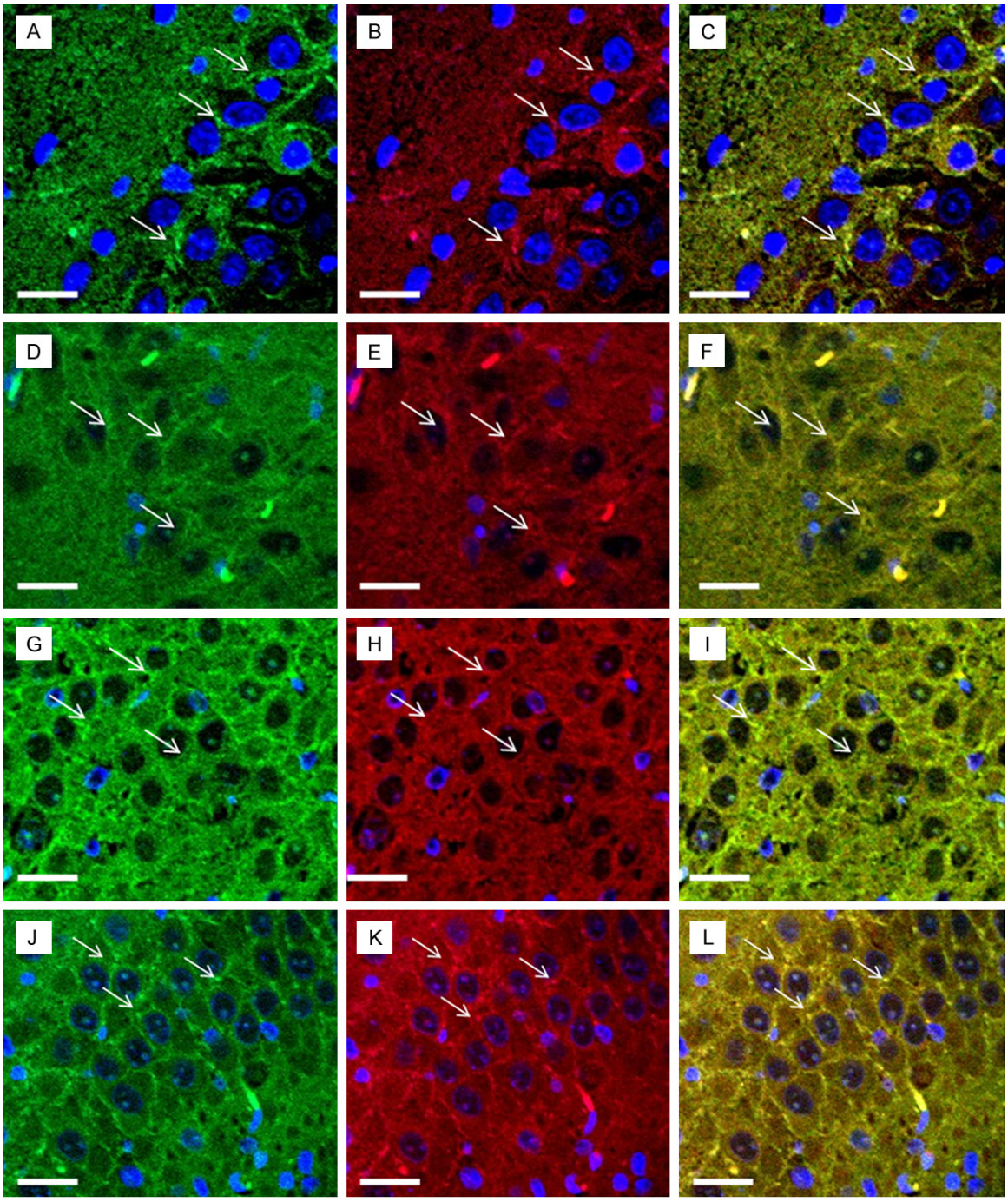
Confocal immunofluorescence analysis

Cx36 protein was localized to the membrane of neurons (**Figure 4A-L**). The immunofluorescence intensity value for Cx36 was significantly less in the carbenoxolone group (**Figure 4D-F**) compared with the control group (**Figure 4A-C**) (259.84 ± 51.89 and 974.04 ± 103.57, respectively, $P < 0.01$). The immunofluorescence intensity value for Cx36 protein was significantly less in the quinine (**Figure 4G-I**) and quinidine (**Figure 4J-L**) groups as well (491.52 ± 35.94 and 424.115 ± 55.55, respectively, $P < 0.01$).

Discussion

In this *in vivo* study, we examined the anticonvulsant properties of both broad (carbenoxolone) and selective (quinine and quinidine) pharmacologic blockers of the gap junction protein Cx36 in the KA-induced rat model of epilepsy. These drugs can diffuse into the brain and are metabolized over time. According to our findings, pretreatment with carbenoxolone, quinine, or quinidine significantly reduced the frequency and amplitude of the epileptic discharge at 30 min post KA injection. At the 90 min time point, carbenoxolone, quinine, or quinidine significantly decreased the spike amplitude but not spike frequency. This reduction in seizure activity was also associated with a decrease in Cx36 protein expression.

In a penicillin-induced rat model of epilepsy, quinine was shown to decrease the amplitude and frequency of epileptiform spikes and to attenuate epileptiform behavior [4]. In the 4-aminopyridine (4-AP) model of epilepsy, treatment with carbenoxolone and quinine at the already active epileptic focus had an anticonvulsive effect by modifying the manifestation of seizure discharges between 1 and 18 Hz [5]. This indicated that gap junction communication via Cx36 channels altered network synchronization that generated discharges with different frequencies. In addition, injection of quinine (35 pmol) into the entorhinal cortex of 4-AP-induced seizure animals decreased the amplitude and frequency of the discharge trains in the EC and CA1 of hippocampus [6]. Taken together, these studies indicate that blockade of Cx36 affects seizure activity, but they provided no information regarding possi-



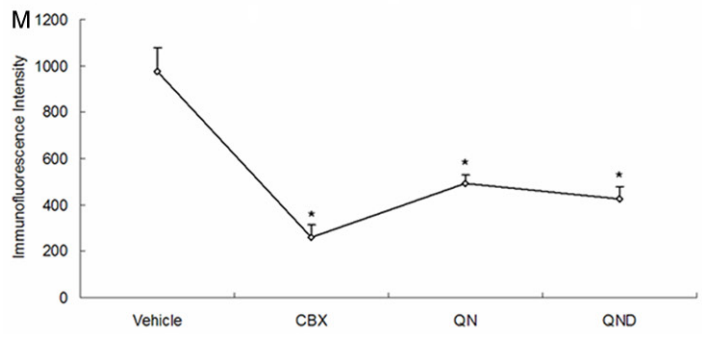


Figure 4. Effects of global or selective Cx36 channel blockers on the Cx36 protein level. Double immuno uorescence staining with Cx36 and MAP-2. Scale bars: 25 μm. Expression of Cx36 in neuron cells (neuronal marker MAP-2) was investigated using double immuno uorescence. Co-localization of Cx36 and MAP-2 was identi ed as a yellow dot-like staining. The Cx36 protein level was signi cantly reduced after pretreatment with CBX (D-F), QN (G-I), and QND (J-L) compared to the control group (A-C, M). *: $P < 0.01$, compared to the control group.

ble alterations in Cx36 expression during the course of epilepsy.

Quantitative immunoblot and confocal immunofluorescence analysis showed that Cx36 protein level was significantly reduced after pretreatment with all Cx36 channel blockers tested. Despite the differences of the drugs, there was no significant variation among the three groups regarding changes in Cx36 expression. This suggests that it is neuronal connexin and not glial connexin that drives the cooperation between neuronal networks during seizure. By antagonizing epileptic synchronization, without depressing neuronal excitability, gap junction blockade may prevent excitable network transmission and enhance synchronization by rebalancing excitation prior to seizure explosion.

Although previous studies have found alterations in baseline EEG activity with gap junction channels blockers, we found no significant effect of carbenoxolone, quinine, and quinidine on this parameter. This may be because the concentrations of the blockers were below a critical concentration threshold that did not influence basic electrophysiological parameters and neuronal excitability. Our observations are consistent with a study by Bostanci et al. [4] who found that quinine significantly decreased spike frequency, spike amplitude, and epileptic behavioral score while not altering baseline EEG activity.

An ideal antiepileptic drug should improve the abnormal pathophysiology of epileptogenesis but not interfere with physiological neurotransmission. Gap junction blockers fulfill these requirements. They inhibit the low-pass properties of gap junctions that can transmit spikes that contribute to the propagation and synchronization at low frequencies [8], and they do not affect basal EEG activity at low doses. Because pretreatment with carbenoxolone, quinine, or quinidine did not prevent the induction of seizures or influence the latency of seizure discharges, the neurons involved with epileptogenesis were unlikely interconnected by gap junction channels. In fact, interneurons have been shown to play a crucial role in epileptogenesis in several seizure types and models [9, 10].

Quinine dose dependently blocks Cx36 containing gap junctions, while at relatively high concentrations it non-specifically affects a number of other channels [11]. In vivo, a critical concentration threshold was identified where quinine does not influence the basic electrophysiological parameters and excitability of neurons [4]. We chose a relatively low concentration of quinine where quinine is selective for Cx36 with few or no effects on nonjunctional proteins. Overall, the reduction in the amplitude and frequency of discharges indicated that blockade of Cx36 channels decreased the synchronicity of cell firing involved in the propagation of seizure discharges.

In CA3 of KA treated animals, histopathology included neuronal cell death, disarray of the remaining neurons, cell body shrinkage, nuclear condensation, varying degrees of glial cell proliferation, and capillary changes [12]. In the carbenoxolone, quinine, and quinidine pretreatment groups, the changes were less severe than the control group. Possible mechanisms underlying neuronal death associated with the discharge include calcium overload, zinc toxicity, and apoptosis [13, 14].

Conclusions

This *in vivo* study showed that pretreatment with carbenoxolone, quinine, and quinidine significantly downregulated Cx36 protein expression and reduced the frequency and amplitude of the epileptic discharge without altering baseline EEG activity. The use of broad-spectrum and specific gap junction blockers in the treatment of epilepsy may prevent the propagation and synchronization of epileptiform activity, and use of these types of drugs may be a novel therapeutic strategy for epilepsy. Further research should focus on the development and refinement of new, more specific pharmacological therapies.

Disclosure of conflict of interest

None.

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