

# EFFECT OF CONNEXIN 36 BLOCKERS ON THE NEURONAL CYTOSKELETON AND SYNAPTIC PLASTICITY IN KAINIC ACID-KINDLED RATS

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## Abstract

In this study we investigated the potential anti-epileptogenic effect of neuronal connexin Cx36 gap junction blockage *via* inhibition of microtubule-associated protein 2 (MAP-2) and synaptophysin (SYP) overexpression. Thirty adult male Wistar rats were divided into five groups (six animals per group): control, sham, carbenoxolone (CBX), quinine (QN), and quinidine (QND). An epilepsy model was produced by injecting kainic acid (KA) into the rat amygdala. Broad-spectrum and selective blockers of the Cx36 channel (CBX, QN, and QND) were administered *via* intraperitoneal injection. Expression of MAP-2 and SYP was assessed by immunofluorescent and immunohistochemical examination. Expression of MAP-2 and SYP was significantly increased after KA administration in the sham group compared with the control group. Expression of MAP-2 and SYP was significantly decreased in the CBX, QN, and QND groups compared with the sham group. The results provide new evidence regarding the key role of MAP-2 and SYP overexpression in three important mechanisms: the modulation of neuronal plasticity, hyperexcitability of the hippocampal neuronal network, and persistent seizure discharge. Furthermore, the reversal of MAP-2 and SYP overexpression following administration of Cx36 channel blockers indicates a potential role for Cx36 channel blockers in anti-epileptogenic treatment and in doing so, highlights a critical need for further investigation of these compounds.

## Keywords

Epilepsy • microtubule-associated protein-2 (MAP-2) • synaptophysin (SYP) • Connexin 36

## Introduction

Neurons are characterized by a complex polar morphology that is essential for their structural and functional integrity. Microtubule-associated protein-2 (MAP-2) refers to an abundant group of cytoskeletal components that are predominantly expressed in neuronal cells. MAP-2 promotes the assembly of microtubules, while phosphorylation controls its association with the cytoskeleton [1]. Additionally, MAP-2 is an excellent substrate for various protein kinases, such as cyclic adenosine monophosphate (cAMP) dependent protein kinase, calcium/calmodulin-dependent protein kinase II, and protein kinase C. MAP-2 also plays an important role in the outgrowth of neuronal axons and dendrites, synaptic plasticity, and neuronal cell death [2]. Furthermore, alterations in neuronal morphology caused by neuronal cytoskeletal changes are responsible


for the initiation and stabilization of new fibers and synaptic contacts [3, 4].

Synaptophysin (SYP) is an axonal presynaptic vesicle protein that is responsible for synapse formation [5]. Its activity is tightly regulated by several protein kinases and phosphatases that modulate the interaction of SYP with synaptic vesicles and other synaptic proteins. SYP overexpression is associated with axonal sprouting and an increase in synaptic vesicles [6]. The most well characterized function of SYP is the modulation of neurotransmitter release at the presynaptic terminal. Synaptic plasticity is a fundamental element of neuronal plasticity. Thus, changes in SYP may lead to neuronal hyperexcitability [7, 8].

MAP-2 and SYP represent the building blocks of neuronal micro devices that are important for synaptic plasticity [4, 9]. Alterations in synaptic plasticity are associated with the sustained stimulation of limbic pathways

and development of generalized convulsions [7, 10]. Gap junctions provide morphological support for the direct diffusion of ions and low-molecular-weight molecules between adjacently coupled cells [11]. Enhanced interneuronal gap junction communication is associated with the modulation of MAP-2 function *via* interference with the phosphorylation process. Neuronal connexin Cx36 blockers may have an inhibitory effect on the propagation and synchronization of epileptiform activity *in vivo* [12-16] and *in vitro* [17]. To the best of our knowledge, the contribution of MAP-2 and SYP in the anti-epileptic process at the neuronal Cx36 gap junction has not been previously reported. Importantly, confocal microscopy imaging can be used to delineate the structure and distribution of the cytoskeleton, and the data obtained can be subjected to quantitative analysis.

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In this study, kainic acid (KA) was injected into the rat amygdala at a micro dose to produce an epilepsy model [18]. Carbenoxolone (CBX), quinine (QN), and quinidine (QND), which are broad-spectrum and selective blockers of the Cx36 channel [11], were administered to rats *via* intraperitoneal (i.p.) injection in order to disrupt the epileptic process. The aim of this study was to examine the changes in expression of MAP-2 and SYP using immunofluorescent and immunohistochemical methods. We hypothesized that alterations in MAP-2 and SYP expression may underlie the anti-epileptic mechanisms of Cx36 channel blockers.

## Materials

Adult male Wistar rats (200-250 g in weight) were obtained from the experimental animal center of the Bethune Medical Department. All rats had *ad libitum* access to food and water and were housed at  $25 \pm 1^\circ\text{C}$  with an alternating 12 h light/dark cycle. All animal experiments were approved by relevant local authorities and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering.

The rats were divided into five groups (six animals per group): control, sham (KA + Tween80), CBX, QN, and QND group. Kainic acid monohydrate, CBX, QN, and QND were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal anti-MAP-2 and polyclonal anti-SYP antibodies were obtained from Santa Cruz (Dallas, TX, USA). Ten percent chloral hydrate was obtained from the First Hospital of Jilin University. Kainic acid monohydrate was dissolved in phosphate-buffered saline (PBS). CBX, QN, and QND were dissolved in 0.8% (v/v) Tween80 and administered *via* i.p. injection. Pretreatment with CBX (20 mg/kg), QN (50 mg/kg), and QND (20 mg/kg) was initiated 30 min prior to KA application. The sham group was administered 0.8% (v/v) Tween80. Ten percent chloral hydrate was administered *via* i.p. injection at a dose of 3.5 ml/kg.

## Methods

### Surgery

The animals were first anesthetized with 10% chloral hydrate (3.5 ml/kg) and secured in a

stereotaxic frame with the incisor bar positioned at -3.3 mm. The position of the basolateral nuclear group in the right amygdala was 3.5 mm posterior to bregma, 4.5 mm lateral to the midline, and 8.0 mm below the skull surface. One  $\mu\text{l}$  KA (1 mg/ml) was injected into the amygdala using a 5  $\mu\text{l}$  syringe (0.2  $\mu\text{l}/\text{min}$ ) in all groups, with the exception of the control group.

### Tissue preparation

All animals were decapitated under deep ether anesthesia six hours after the administration of KA, followed by the immediate removal of the brain. The brains were placed in 10% formalin prior to paraffin embedding and cut into 10  $\mu\text{m}$  slices on a microtome. The slices were subsequently mounted on glass slides and prepared for immunochemical and immunofluorescent examination. The stained slices were qualitatively analyzed using a stereoscopic microscope (Olympus, Osaka, Japan).

### Immunohistochemistry

Paraffin sections were stained using MAP-2 and SYP rabbit polyclonal antibodies (Santa Cruz, Dallas, TX, USA). All reagents were obtained from Dako A/S (Glostrup, Denmark) and used according to the manufacturer's instructions. The paraffin sections were deparaffinized, and the endogenous peroxidase activity was quenched *via* immersion in 1.5% hydrogen peroxide, followed by heat-induced epitope retrieval in ethylene diamine tetraacetic acid (EDTA) buffer. The sections were subsequently incubated for 60 min with the MAP-2 and SYP antibodies (1:400) separately. The antigen-antibody complex was detected using anti-rabbit IgG, followed by its visualization using 3,3'-diaminobenzidine (DAB) as the chromogen. Images of the tissue sections were captured with an Olympus (Tokyo, Japan) microscope.

A quantitative analysis was conducted by measuring the positive area in the stratum lucidum with Image-Pro Plus (v. 6.0, Media Cybernetics, Silver Spring, MD, USA). The positive area was measured in at least six high-power fields per section and at least four sections were scored for each animal. The values of the positive areas versus the total image area per microscopic field were measured.

### Immunofluorescence staining

The paraffin-embedded sections were maintained at  $60^\circ\text{C}$  for 15 min and then sequentially transferred into 100% ethanol (EtOH), 95% EtOH, 70% EtOH, and 50% EtOH for 4 min at room temperature. The sections were rinsed in deionized water and stored in PBS. MAP-2 and SYP in hippocampal neuronal cells were examined using immunofluorescence with the rabbit polyclonal antibody to rat MAP-2 and SYP (Santa Cruz, Dallas, TX, USA). The blocking solution comprised 10% normal goat serum in PBS, and the antibodies were diluted in 1% goat serum. The sections were incubated for 60 min with the MAP-2 antibody (1:100). The affixed antibody was detected with tetramethylrhodamine isothiocyanate (TRITC) goat anti-rabbit IgG. The sections were also incubated for 60 min with the SYP antibody (1:200). The affixed antibody was detected with fluorescein isothiocyanate (FITC) goat anti-rabbit IgG. The coverslips were mounted using a mounting medium that contained 4,6-diamidino-2-phenylindole (DAPI, VWR International Aps, Glostrup, Denmark) for the identification of nuclei. The expression was visualized using confocal microscopy (Olympus, Osaka, Japan, FV-1000). The quantitative analysis was conducted as previously described.

### Statistical analysis

Statistical analysis was performed using the SPSS statistical software package, Version 14.0. (SPSS Inc., Chicago, IL, USA). The values are expressed as the mean  $\pm$  standard deviation (SD). The data was analyzed using analysis of variance (ANOVA). A value of  $P < 0.05$  was considered statistically significant.

## Results

### MAP-2 expression in the CA3 region of the hippocampus Immunohistochemical examination of MAP-2

MAP-2 was predominantly identified in the hippocampus of normal brain tissue, with a wide distribution in the membrane and dendrites of the neuronal cells in the hippocampal CA3 region. A faint staining was identified in the control group cells, whereas astrocytes, in

general, were negative for MAP-2 (Fig. 1A). In the sham group, the staining was thick and dense in the neurons of the KA kindled rats (Fig. 1B). In the CBX (Fig. 1C), QN (Fig. 1D), and QND (Fig. 1E) groups, there was comparatively less intense staining compared with the sham group. The expression of MAP-2 was significantly decreased, as assessed by quantitative analysis of the area of positive puncta per square  $\mu\text{m}$ , in the CA3 region of the rat hippocampus in the CBX, QN, and QND groups compared with the sham group ( $P < 0.05$ ). Furthermore, there was no other significant difference between the CBX, QN, and QND groups (Fig. 1F).

#### Immunofluorescence examination of MAP-2

To further confirm the changes in MAP-2 expression in neuronal cells, immunofluorescence staining was performed. MAP-2 expression was investigated in the hippocampus and is illustrated as a red plexiform or plaque-like staining (Fig. 2A). The sham group exhibited increased MAP-2 expression compared with the control group

(Fig. 2B). The MAP-2 expression was lower in the CBX, QN, and QND groups compared with the sham group (Fig. 2C, D, E, F).

#### SYP expression in the CA3 region of the hippocampus SYP immunohistochemistry

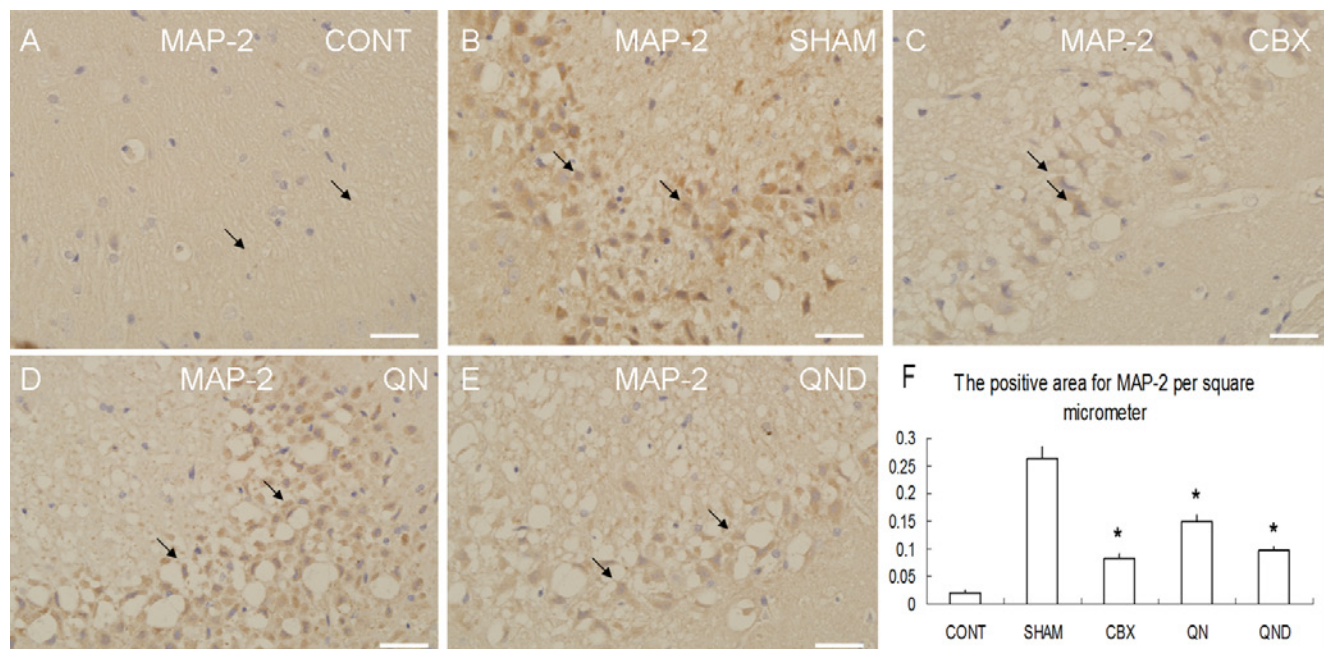
Synaptic plasticity was found to be abundant in the presynaptic vesicle membranes in the CA3 region of normal hippocampal tissue, and a wide distribution was identified in the neural terminals. A light and sparse staining was identified in the neural cells of the control group. In the sham group, the staining was thick and dense in the neurons of the KA kindled rats (Fig. 3A). There was a significant decrease in the SYP expression, as assessed by quantitative analysis of the area of positive puncta per square  $\mu\text{m}$ , in the hippocampal CA3 region in the CBX, QN, and QND groups (Fig. 3C, D, E) compared with the sham group ( $P < 0.05$ ). The SYP expression in the sham group was increased compared with the control group (Fig. 3B). In contrast, there was no significant difference among the CBX, QN, and QND groups (Fig. 3F).

#### SYP immunofluorescence staining

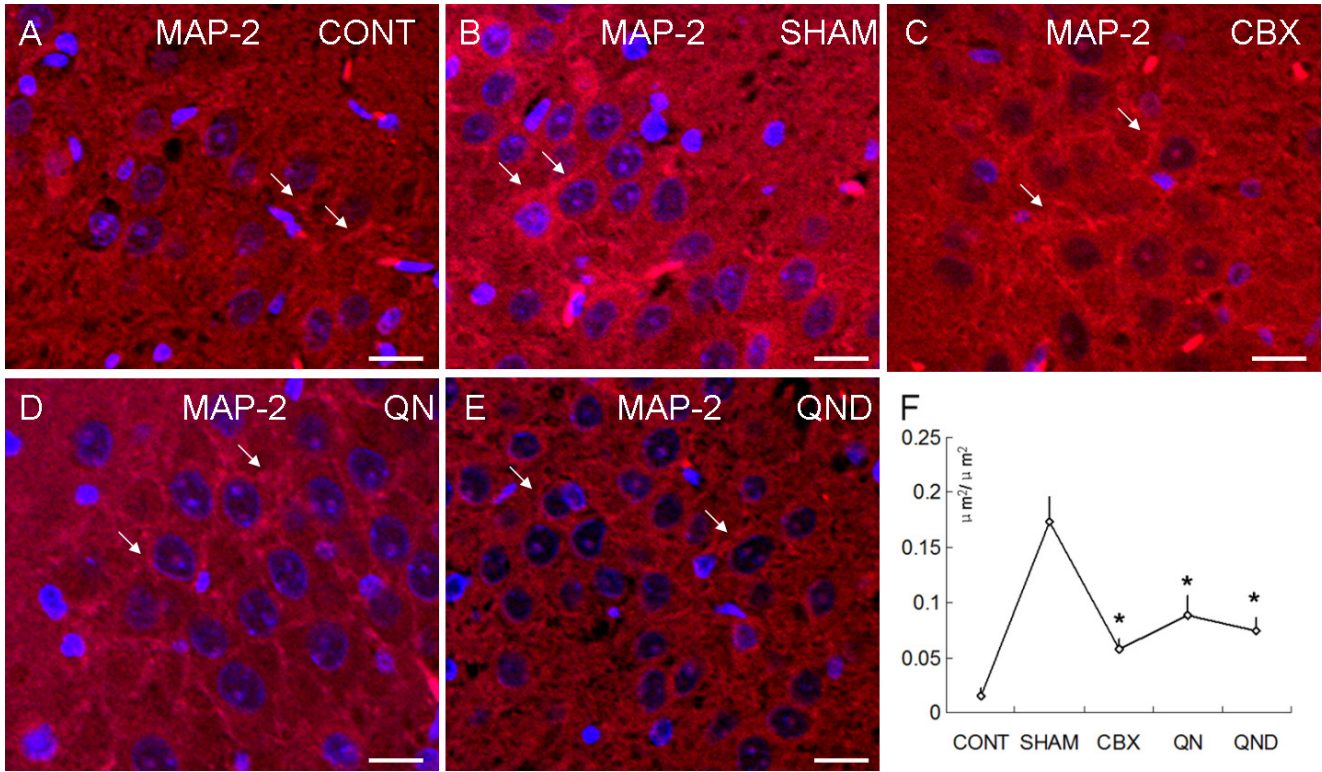
Change in SYP expression in neuronal cells was also assessed by immunofluorescent staining. The SYP expression was investigated in the hippocampus and is illustrated as green particles or with a lamellar staining (Fig. 4A). Similar results were identified for SYP expression with immunofluorescence staining as with SYP immunohistochemistry. The SYP in the sham group exhibited increased expression compared with the control group (Fig. 4B, F). In contrast, the SYP expression was decreased in the CBX (Fig. 4C), QN (Fig. 4D), and QND (Fig. 4E) groups compared with the control group (Fig. 4F).

#### Discussion

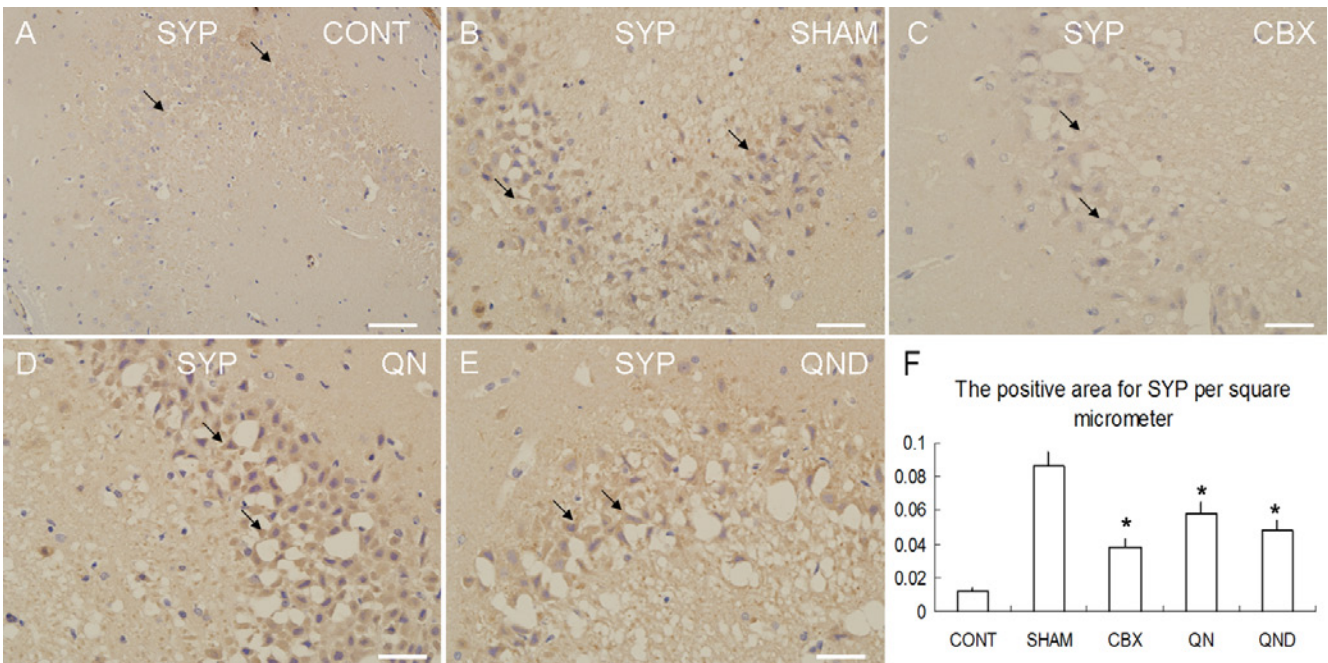
This study demonstrated a significant increase in the MAP-2 expression level in the sham group as compared with the control group. Previous studies have demonstrated an increase in MAP-2 expression during kindled seizures in rats, which thus suggests an association of MAP-2 with hippocampal plasticity in epilepsy [19]. Furthermore, a significant decrease of dendritic



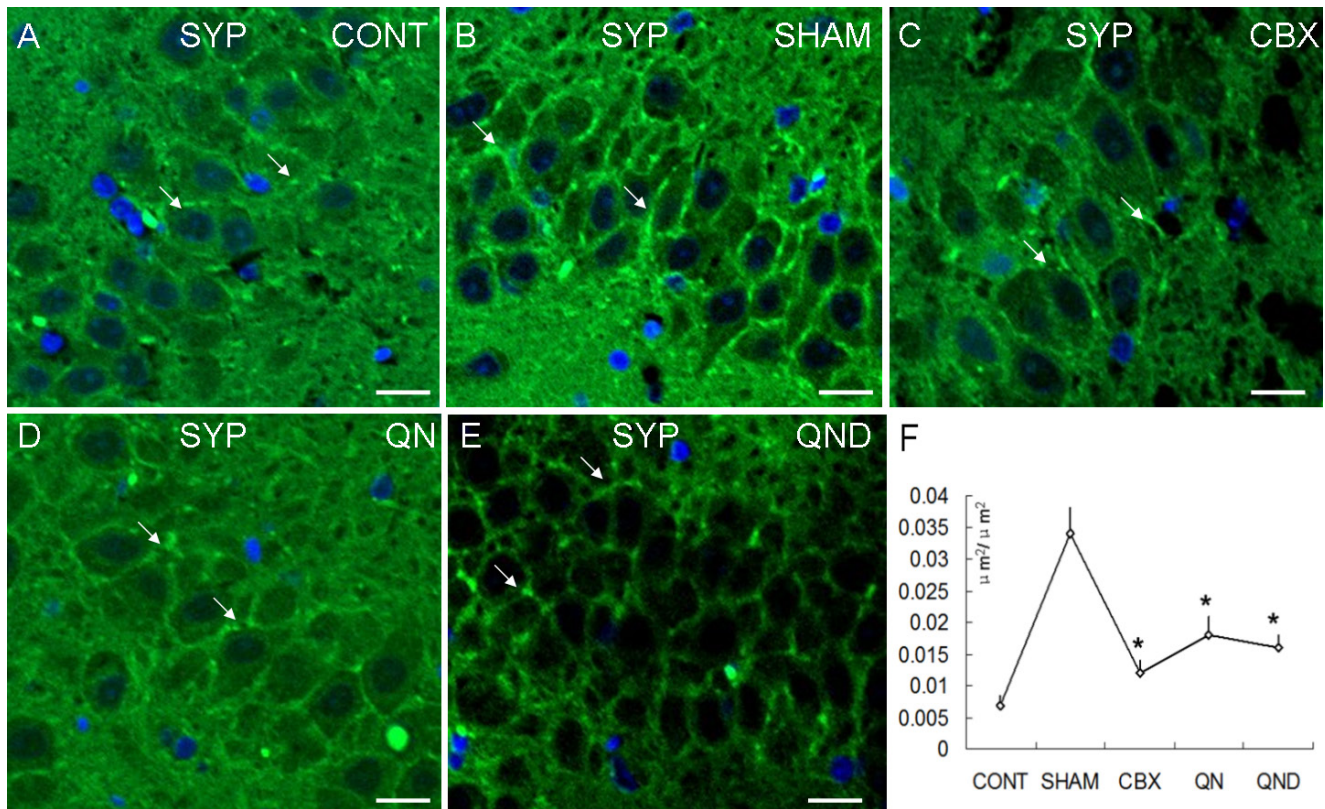
**Fig. 1.** Immunohistochemical analysis of microtubule-associated protein 2 (MAP-2) expression in the CA3 region of the hippocampus. MAP-2 was expressed in the hippocampal CA3 region in all groups (arrows). A: In the control group, the rats moved freely without drug administration. B: In the sham group, 0.8% (v/v) Tween80 was administered via an intraperitoneal (i.p.) injection 30 min prior to kainate acid (KA) administration. C, D and E: In the carbenoxolone (CBX), quinidine (QN), and quinidine (QND) groups, the drugs were administered via i.p. injection 30 min prior to the application of KA. F: Compared with the control group (A), MAP-2 appeared more intense in the hippocampus of the sham group (B). The decrease in MAP-2 was significant in the CBX, QN, and QND groups compared with the sham group ( $P < 0.05$ ). Scale bars: 50  $\mu\text{m}$ .



**Fig. 2.** Microtubule-associated protein 2 (MAP-2) expression in the hippocampal CA3 region. Effects of carbenoxolone (CBX), quinine (QN), and quinidine (QND) on MAP-2 expression (arrows) in the hippocampal CA3 region, as assessed *via* immunofluorescence examination. A: Control rats; B: 0.8% (v/v) Tween80 + KA; C: CB + KA; D: QN + KA; E: QND + KA. F: CBX, QN, and QND groups with significantly reduced MAP-2 expression compared with the sham group ( $P < 0.05$ ). Scale bars: 25 µm.



**Fig. 3.** Immunohistochemical examination of synaptophysin (SYP) expression in the hippocampal CA3 region. The SYP expression was immunohistochemically investigated in the control (A) and treatment groups (B, C, D, E). The sham group exhibited increased SYP expression (B). The SYP expression in the carbenoxolone (CBX; C), quinine (QN; D), and quinidine (QND; E) groups was significantly lower compared with the sham group ( $F, P < 0.05$ ). Scale bars: 50 µm.



**Fig. 4.** Immunofluorescence examination of synaptophysin (SYP) protein expression. A: Immunofluorescence staining indicated neuronal SYP protein (arrows) expression in the CA3 region of the hippocampus in the control group. B: SYP proteins (arrows) were highly expressed in the hippocampal CA3 region in the sham group. C, D, E, F: The SYP protein expression (arrows) was significantly lower in the carbenoxolone (CBX), quinine (QN), and quinidine (QND) groups compared with the sham group ( $P < 0.05$ ). Scale bars: 25µm.

MAP-2 expression has been demonstrated after epilepsy, whereas a significant increase has been documented during the reinnervation and synaptic reconstruction process after experimental traumatic brain injury [20]. Several reports have demonstrated that KA-mediated excitotoxicity induces neurodegeneration [21], and the MAP-2 level is reduced in seizure-related brain damage in adult rats [22]. However, synaptic plasticity occurred after injury, which led to an increase, rather than a decrease, in MAP-2. Another study demonstrated significant mossy fiber sprouting associated with altered MAP-2 expression [23]. Furthermore, studies have also linked MAP-2 with axonal and dendrite outgrowth, which reflects its involvement in neuronal budding [24, 25]. MAP-2 also contributes to the generation of new axons and synaptic plasticity in the reinnervation phase after epilepsy [26]. The overexpression of MAP-2 is an indirect indicator of mossy fiber sprouting and synaptic plasticity. We hypothesize that the mechanism of neuronal

network hyperexcitability caused by MAP-2 overexpression in the KA-induced models of epilepsy in this study may be as follows: 1) A substantial number of damaged neuronal cells may contribute to MAP-2 overexpression and provide the building blocks for mossy fiber sprouting after epileptic seizures, 2) The abnormal discharge of neurons leads to the abnormal activation of NMDA glutamate receptors. Calpain activation, as a result of calcium influx, may subsequently induce MAP-2 dephosphorylation. MAP-2 dephosphorylation may result in damage to the cytoskeleton that induces neuronal death [27], and 3) Repeated synaptic stimulation can induce phosphorylation by either the stimulation of protein kinases or inhibition of protein phosphatases. The phosphorylation of MAP-2 decreases its ability to promote microtubule assembly and contributes to an increased number of synaptic connections, whereas the dephosphorylation of MAP-2 reverses this effect that results in the stabilization of novel synaptic

connections through protein phosphatases activation and protein kinases inhibition [28]. It has also been demonstrated that 3, but not 6, hours of KA treatment is associated with an increased MAP-2 level in young rats [29]. However, these differential findings may be the result of variation in KA concentration or administration methods.

This study demonstrated MAP-2 overexpression in the rat hippocampus following induced seizures. Increased MAP-2 is the building block for axonal sprouting and the development of new synaptic connections with dendrites, which promote neuronal dendrite reinnervation. Abnormal neuronal network hyperexcitability that results from new synaptic connections in the hippocampus is most likely to be responsible for the maintenance of status epilepticus.

Carbenoxolone is a broad-spectrum blocker of neuronal gap junctions, whereas QN and QND are specific Cx36 channel blockers [11]. The administration of these three compounds

significantly decreased the overexpression of MAP-2 in the hippocampus in this study. This result may have been caused by the effect of these drugs on the transfer of calcium ions and chemical signals between nerve cells, thereby altering the phosphorylation and dephosphorylation status of MAP-2, leading to decreases in MAP-2 expression. As a result, the hyperexcitability present in the hippocampal neuronal network decreased. Additionally, neuronal gap junction blockers reduce neuronal cell damage *via* the inhibition of electrical coupling between cells. These changes can also potentially contribute to the reversal of MAP-2 overexpression.

In this study, SYP was significantly increased in the KA group compared with the control and sham groups. One previous study has also demonstrated increased SYP in the hippocampal CA3 region in electrically kindled animal models of epilepsy [30]. These findings suggest that the mossy fiber sprouting that occurs after seizures and in new synaptic connections is associated with the maintenance and propagation of epilepsy. These findings are consistent with results obtained in a study of the temporal lobe in epilepsy patients [31]. SYP is a marker of the presynaptic terminal and is widely distributed on presynaptic membrane vesicles. Furthermore, SYP is associated with synaptic plasticity [32]. Neuronal axon sprouting is responsible for SYP overexpression, and the overexpression of SYP is involved in the up-regulation of presynaptic terminals and synaptic vesicles [33]. As a result, the number of synaptic connections is increased. It has been reported that the synaptic connections between hippocampal sprouted mossy fibers and neuronal dendrites in the molecular layer of the dentate gyrus represent non-symmetric and excitatory synapses [8, 9]. Therefore, the

new synaptic contacts are likely associated with the maintenance and propagation of epilepsy [10]. Additionally, SYP could also have a potential modulatory role in  $Ca^{2+}$ -dependent release of neurotransmitters, e.g., acetylcholine and glutamate. This mechanism appears to be consistent with a previous report that the regulation of endogenous glutamate release in synaptic vesicles is the mechanism by which SYP exerts its influence on synaptic plasticity [34].

The Cx36 blocking compounds caused a significant reversal of hippocampal SYP overexpression. This finding could potentially be explained by reduced axonal sprouting resulting from a decrease in MAP-2 overexpression. The reduction in SYP overexpression and the subsequent decrease in the number of new synaptic connections between sprouted mossy fibers and granule cell dendrites could have a potential application in the development of antiepileptic treatments.

Gap junctions play an important role in the pathophysiology of epilepsy. To the best of our knowledge, the potential role of the neuronal Cx36 gap junction in epilepsy, by virtue of its influence on MAP-2 and SYP expression, has not been investigated previously. Thus, it is critical to clarify the mechanism underlying these effects. In this study, Cx36 channel blockers caused a significant reversal of the overexpression of MAP-2 and SYP in the hippocampus. The potential therapeutic role of Cx36 channel blockers in the inhibition of propagation of seizure activity requires confirmation.

Several limitations should be considered in the interpretation of these findings. First, the sample size is relatively small. Second, the use of anesthesia may have affected the kindling of seizure activity caused by KA. However, to minimize this potential confounding influence, KA was administered 30 min after

the anesthesia. Finally, the current preliminary study maintained a narrow focus with limited techniques as an initial step to begin to address our hypothesis. Thus, these preliminary findings require confirmation *via* larger, more robust studies using multiple techniques, such as an *in vivo* knock-out approach, to further confirm the mechanism of these effects and comprehensively evaluate the potential role of Cx36 channel blockers in future anti-epileptic therapies.

In conclusion, MAP-2, a marker of neurite growth, plays an important role in dynamic neuronal sprouting. SYP, a marker of synaptic terminals, is closely associated with synaptic plasticity. An increase in the number of new synaptic connections is known to be a fundamental mechanism in the repeated discharge of seizure activity. Therefore, the overexpression of MAP-2 and SYP is likely involved in the causation of neuronal plasticity, hyperexcitability of the hippocampal neuronal network, and persistent seizure discharge. In this study, CBX (a broad-spectrum neuronal gap junction blocker), as well as QN and QND (selective Cx36 channel blockers) caused a significant reversal of MAP-2 and SYP overexpression in the rat hippocampus because the blockers inhibit the intercellular transfer of ions and secondary messengers, which thereby effectively closes the gap junction channels. Our preliminary, yet novel findings, suggest a possible role for Cx36 channel blockers as potential anti-epileptogenic agents necessitating further research.

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*Conflict of interest statement:* The authors declare that they have no competing interests.

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