

## Dysfunction of M-channel enhances propagation of neuronal excitability in rat hippocampus monitored by multielectrode dish and microdialysis systems

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### Abstract

To explore the pathogenesis of benign familial neonatal convulsions (BFNC), we determined effects of KCNQ-related M-channels (KCNQ-channels) on hippocampal glutamate (Glu) and  $\gamma$ -aminobutyric acid (GABA) releases using microdialysis, and propagation of evoked field-potentials (FP) using multielectrode (64-ch)-dish system as two-dimensional monitoring. KCNQ-channel inhibitor, Dup996, enhanced hippocampal  $K^+$ -evoked Glu and GABA releases without affecting basal releases of them. Dup996 unaffected FP-amplitude, but enhanced FP-propagation. The GABA<sub>A</sub>-receptor antagonist, bicuculline, enhanced the stimulatory effects of Dup996 on FP-propagation, however, this stimulatory effects of Dup996 were abolished by the  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/glutamate-receptor antagonist, DNQX. These results suggest that the occurrence of BFNC cannot be produced by KCNQ-channel dysfunction alone, but by reciprocal action between impaired KCNQ-channel and other unknown elements (possibly dysfunction of inhibitory neurotransmission system). © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Epilepsy; Dup996; Benign familial neonatal convulsions; MED64; Microdialysis; KCNQ

Mutations in either KCNQ2 or KCNQ3 were recently identified as a cause of benign familial neonatal convulsions (BFNC) [1–3,17], a dominantly inherited epilepsy that occurs during the neonatal period [14]. KCNQ3 interacts with KCNQ2 and KCNQ5 are co-expressed almost exclusively in central nervous system (CNS) [7,15,18,19], suggesting that they may form heteromeric  $K^+$  channels. Indeed, when KCNQ2/KCNQ3 and KCNQ3/KCNQ5 were co-expressed in *Xenopus* oocytes, the currents of these heterometric channels were much larger than those obtained from KCNQ2, KCNQ3 or KCNQ5 alone [7,15,19]. The pharmacological profile (voltage-dependence and kinetics) of these KCNQ-related M-channels (KCNQ-channels) suggest that these KCNQ-channels contribute to a formation of native M-currents, which play an important inhibitory regulator of neuronal excitability [8], in central nervous system [7,15,19].

Several previous studies regarding the mutant KCNQ-

channels have investigated the intraneuronal transmission mechanisms of BFNC [1,3,15,18]. These studies suggested that none of the BFNC mutations had been identified exerted dominant negative effects, and consequently the reduction of M-currents in patients was predicted to be small [1,3,15,18]. However, the interneuronal transmission mechanisms of BFNC have not been fully clarified yet. Hence, to clarify the possible pathogenesis of BFNC, the present study determined the effects of reduction of KCNQ-channels activity using the selective inhibitor, Dup996, which inhibits both KCNQ2/KCNQ3- and KCNQ3/KCNQ5-heterometric channels without affecting Erg-related M-current [7,15,16], on hippocampal Glu and  $\gamma$ -aminobutyric acid (GABA) releases by microdialysis in freely moving rat. Additionally, we determined the propagation of neuronal excitability by a new technique for monitoring evoked field-potential (FP), using the multielectrode(64-ch)-dish (MED64) system in hippocampal slice.

All experiments described in this report were performed in accordance with the specifications of the Ethical committee of Hirosaki University and met the guidelines

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of responsible governmental agency. Male Wistar rat (Clea, Japan), weighing 250–300 g, was placed in a stereotaxic frame and kept under halothane anesthesia (1.5% mixture of halothane and O<sub>2</sub> with N<sub>2</sub>O). A concentric I-type dialysis probe (0.22 mm diameter; 3 mm exposed membrane; Eicom, Japan) was implanted in the hippocampus (A = -5.8 mm, L = 4.8 mm, V = -4.0 mm relative to bregma) and the perfusion experiments were started 18 h after the rats had recovered from anesthesia [10]. The perfusion rate was 1  $\mu$ l/min, using modified Ringer's solution (MRS) composed of (in mM): 145 Na<sup>+</sup>, 2.7 K<sup>+</sup>, 1.2 Ca<sup>2+</sup>, 1.0 Mg<sup>2+</sup> and buffered with 1.25 mM phosphate buffer and 22 mM carbonate buffer to adjust the pH to 7.4. To study the effects of an increase in the extracellular K<sup>+</sup> level on the hippocampal extracellular Glu and GABA levels, MRS containing 50 mM K<sup>+</sup> (HKMRS) was perfused for 20 min (K<sup>+</sup>-evoked stimulation) [10]. The ionic composition and isotonicity was maintained by an equimolar decrease of Na<sup>+</sup> [10]. The extracellular Glu and GABA levels were determined by high performance liquid chromatography (HPLC) with fluorescence-detection [6]. The analytical column (100  $\times$  3 mm internal diameter) was packed with mightysil RP-18 (gift from Kanto Chemicals; particle size 3  $\mu$ m) by Masis (Hirosaki, Japan). Excitation and emission wave-lengths of fluorescence detector were set at 340 and 445 nm, respectively. Determination of diffusion rate of Dup996 from dialysis probe to hippocampal tissue was performed by the methods of our previous study [5] and Pieniaszek et al. [13].

To study the effects of KCNQ-channels inhibition on propagation of fiber-volley (FV), field-excitatory postsynaptic potential (fEPSP) and population-spikes (PS) in hippocampal CA1 area, 2-week old male Wistar rat was sacrificed by decapitation after halothane anesthesia, and transverse slices of hippocampus (350  $\mu$ m thickness) were prepared with a vibrating tissue slicer (DTK-1000, Dosaka, Japan). The procedures for preparation of rat hippocampal slices and MED64 system (Panasonic, Japan) were prepared mainly according to the methods of Oka et al. [9]. During monitoring of FP, the MED64 probe (MED-P5305, 8  $\times$  8 array, inter-polar distance 300  $\mu$ m, Panasonic) was superfused with artificial-cerebrospinal-fluid composed of (in mM): 150 Na<sup>+</sup>, 2.5 K<sup>+</sup>, 2.0 Ca<sup>2+</sup>, 1.0 Mg<sup>2+</sup> and buffered with 1.25 mM phosphate buffer and 25 mM carbonate buffer to adjust pH to 7.35 at 35°C, and maintained at a 2 ml/min flow rate [9]. A single pair of planar microelectrodes with bipolar constant current pulses (10–100  $\mu$ A, 0.1 ms) was used for stimulation of the hippocampal slice. The stimulation site was selected at the Schaffer collateral pathway in hippocampal CA1 region, and FP were recorded at all 64 planar microelectrodes which were covered with hippocampal slice.

The chemical agents used in this study were KCNQ-channel inhibitor, Dup996 (Research Biochemicals, USA), GABA<sub>A</sub>-receptor antagonist, bicuculline (Biomedicals, USA) and  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propio-

nic acid (AMPA)/glutamate-receptor antagonist, DNQX (Research Biochemicals).

The basal Glu and GABA releases were insensitive and partially sensitive (40%) to tetrodotoxine (TTX) and Ca<sup>2+</sup>-free medium perfusion, respectively, [11]. Both K<sup>+</sup>-evoked release of Glu and GABA were TTX-sensitive and Ca<sup>2+</sup>-dependent [11]. The basal extracellular Glu and GABA levels were 17.1  $\pm$  5.1 and 1.0  $\pm$  0.3 pmol/sample per 20  $\mu$ l, respectively. The recovery (from outside to inside) rates of probes for Glu and GABA were 12.8  $\pm$  3.2 and 10.4  $\pm$  4.5%, respectively. Thus, the estimated basal hippocampal extracellular Glu and GABA levels were 6.7  $\pm$  2.0 and 0.5  $\pm$  0.2  $\mu$ M, respectively. The K<sup>+</sup>-evoked stimulation for 20 min increased extracellular Glu and GABA levels to 203.8  $\pm$  30.6 and 520.9  $\pm$  64.9% (Fig. 1), respectively. The diffusion rate of Dup996 was ranged 8–11%. Dup996 (100 and 1000  $\mu$ M: estimated concentration in hippocampal brain tissue was 8–11 and 80–110  $\mu$ M, respectively), did not affect basal releases (Fig. 1), but did enhance K<sup>+</sup>-evoked

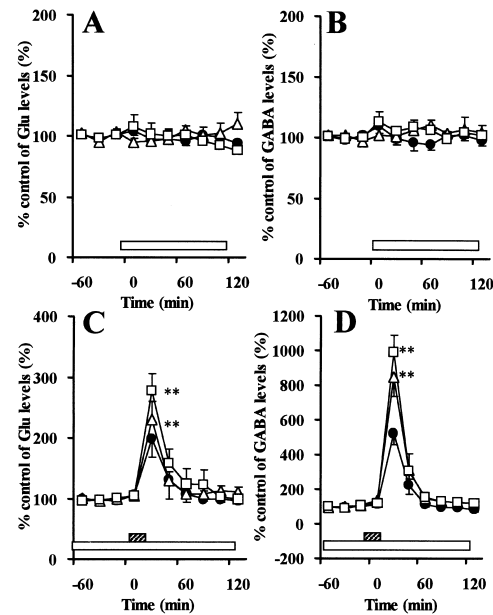


Fig. 1. Effects of Dup996 on basal and K<sup>+</sup>-evoked hippocampal Glu and GABA releases. The effects of Dup996 on basal extracellular Glu and GABA levels were expressed in (A,B), respectively. After confirming stabilization, the perfusion medium was changed from MRS to MRS containing free (●), 100  $\mu$ M ( $\Delta$ ) or 1000  $\mu$ M ( $\square$ ) Dup996. The effects of Dup996 on K<sup>+</sup>-evoked Glu and GABA releases were expressed in (C,D), respectively. After confirming stabilization, the perfusion medium was changed from MRS containing free (●), 100  $\mu$ M ( $\Delta$ ) or 1000  $\mu$ M ( $\square$ ) Dup996 to HKMRS containing the same agent. Ordinate indicates the hippocampal extracellular levels of Glu and GABA (% control) and abscissa shows the time in minutes. The data was expressed as percentage (mean  $\pm$  SEM) of control value. The opened columns indicate the perfusion with Dup996, and striped columns indicate the K<sup>+</sup>-evoked stimulation. Comparisons were made between mean values obtained by perfusion without and with Dup996 contained in MRS (\* $P$  < 0.05; \*\* $P$  < 0.01) using repeated measurement ANOVA with Tukey's multiple comparison.

releases of Glu ( $F = 6.02$ ,  $P < 0.01$ ,  $n = 18$ ), and GABA ( $F = 6.63$ ,  $P < 0.01$ ,  $n = 18$ ) (Fig. 1).

To determine the effects of impaired KCNQ-channels on the propagation of neuronal excitability, the present study monitored the effects of Dup996 on both amplitude and propagation of FP using MED64 system in hippocampal CA1 regions. In this study, MED64 system could detect FV in the CA1 cell body layer (Fig. 2B). However, our previous study, using glass extracellular recording electrode, could not determine the FV response in the same regions [4]. These controversial results may be caused by the different recording electrodes, because the recording electrode of MED64 system was  $60 \times 60 \mu\text{m}$  platinum (impedance:  $6 \text{ k}\Omega$ ) [9], and the impedance of glass electrode were more than  $1 \text{ M}\Omega$  [4]. In other words, the MED64 may

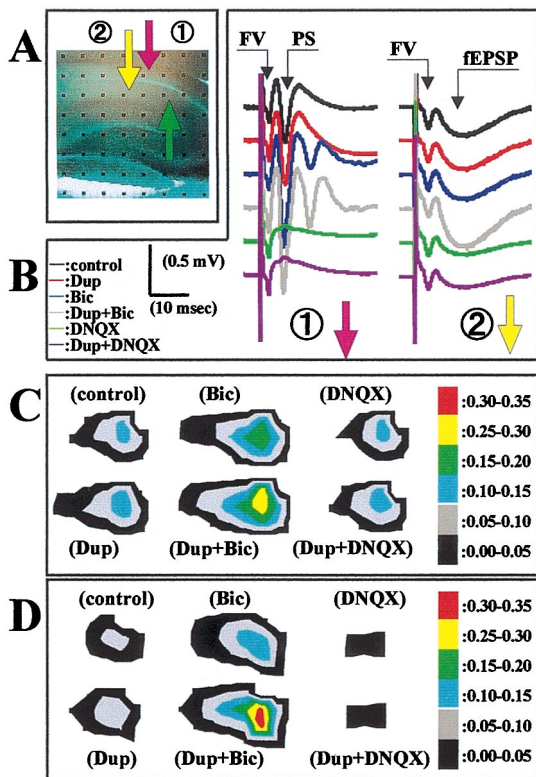


Fig. 2. Interaction among Dup996, bicuculline and DNQX on amplitude and propagation of FP in hippocampal CA1 regions expressed as two-dimensional monitoring. (A) Shows the phase-contrast microphotographs of rat hippocampal slice as positioned on the MED64 probe, and the stimulation (green arrow) and recording ((1) red and (2) yellow arrows) configuration used to elicit Schaffer collateral CA1 pyramidal cells responses. (B) Indicates the example of Schaffer collateral-evoked field potentials in electrode (1) (red arrow) and (2) (yellow arrow) during superfusion with Dup996 ( $10 \mu\text{M}$ : Dup), bicuculline ( $10 \mu\text{M}$ : Bic) or DNQX ( $40 \mu\text{M}$ ). (C,D) Show the interaction among Dup996 (Dup), bicuculline (Bic) and DNQX on Schaffer collateral-evoked FV- and fEPSP-responses in hippocampal slice as two-dimensional monitoring, respectively. Data expressed as the FV- or fEPSP-amplitude in each electrode. The evoked response amplitude of FV and fEPSP is represented in the right side panels.

be able to detect the evoked presynaptic response (FV) beside CA1 cell body layer. To clarify this problem, in the next study we are going to use smaller electrode in MED64.

GABA<sub>A</sub>-receptor antagonist, bicuculline ( $10 \mu\text{M}$ ), stimulated both amplitude and propagation of FV, fEPSP and PS, whereas AMPA/glutamate-receptor antagonist, DNQX ( $40 \mu\text{M}$ ) reduced amplitude of fEPSP and PS, propagation of FV, fEPSP and PS without affecting FV-amplitude (Figs. 2 and 3). Dup996 ( $10 \mu\text{M}$ ) unaffected amplitude of FV, fEPSP and PS, and PS-propagation ( $n = 6$ ), but enhanced propagation of FV and fEPSP ( $P < 0.05$ ,  $n = 6$ ) (Figs. 2 and 3). Under the conditions of inhibition of GABA<sub>A</sub>-receptor by bicuculline ( $10 \mu\text{M}$ ), Dup996 enhanced amplitude of fEPSP and PS, and propagation of FV, fEPSP and PS ( $P < 0.05$ ,  $n = 6$ ), but unaffected FV-amplitude (Figs. 2 and 3). Under the conditions of inhibition of AMPA/glutamate-receptor by DNQX ( $40 \mu\text{M}$ ), the stimulatory effects of Dup996 on FP were abolished (Figs. 2 and 3).

BFNC typically suffers from frequent brief seizures that occur within the first days of life and disappear spontaneously after weeks to months. BFNC is characterized by clusters of generalized seizures, however, it has been well known BFNC is often accompanied by partial seizures [3,14]. KCNQ2, KCNQ3 and KCNQ5 are expressed in hippocampus [7,15,18]. Additionally, the neurological examination, interictal electroencephalogram (EEG), and development of BFNC children are usually normal [14]. Therefore, the dysfunction of KCNQ-channels activities does not play an important role in the early neurodevelopmental stage.

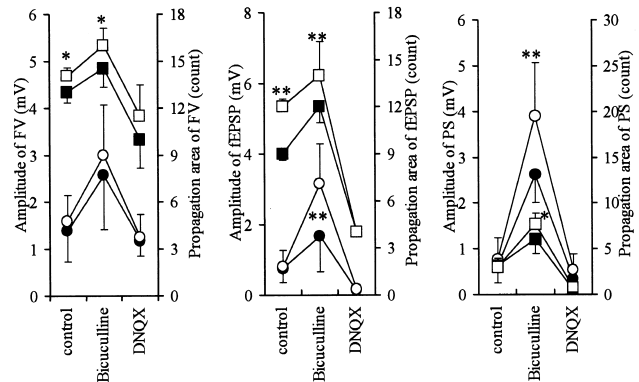


Fig. 3. Interaction among Dup996, bicuculline and DNQX on amplitude and propagation of FV, fEPSP and PS. The interaction among  $10 \mu\text{M}$  Dup996,  $10 \mu\text{M}$  bicuculline and  $40 \mu\text{M}$  DNQX on amplitude and propagation of Schaffer collateral-evoked FV (A), fEPSP (B) and PS (C) in rat hippocampal CA1 region are represented. Left side ordinates indicate the amplitude (mV) of FV, fEPSP and PS, and right side ordinates indicate the propagation areas (counts of responsible electrodes) of FV, fEPSP and PS. The differences between amplitude (circles) or propagation (squares) values, during superfusion with (opened marks) or without (closed marks)  $10 \mu\text{M}$  Dup996, were analyzed by repeated measurement of AVOVA with Tukey's multiple comparison or Friedman's test with Tukey's multiple comparison, respectively ( $*P < 0.05$ ;  $**P < 0.01$ ).

The enhancement of excitatory neurotransmission and/or reduction of inhibitory neurotransmission, produced seizure activity. In addition, the mechanism of epileptic seizure has been recognized to be the result of a disturbance of balance between the excitatory and inhibitory neurotransmission systems [3]. KCNQ-channels control membrane excitability by being the only sustained current in the range of action potential initiation [8]. Dup996 inhibits KCNQ2/KCNQ3 and KCNQ3/KCNQ5 heterometric channels selectively without affecting Erg-related M-currents. This study demonstrated that inhibition of KCNQ-channels stimulated neuronal excitability. Dup996 enhanced propagation of presynaptic response of FV and postsynaptic response of fEPSP, without affecting amplitude of them, in CA1 regions. Furthermore, Dup996 enhanced  $K^+$ -evoked neurotransmitter release, but unaffected fEPSP-amplitude. This contradiction suggests that inhibition of KCNQ-channels increase the gross count of responsible synapses which can transmit Glu without affecting the volume of neurotransmitter release per synapse, resulting in the enhancement of level of Glu release monitored by microdialysis.

The EEG showed no differences between wild-type and heterogenous KCNQ2 knock-out mice, whereas the heterogenous KCNQ2 knock-out mice were more sensitive to a proconvulsant, pentylenetetrazole [20]. Taken together with this evidence, the present results, bicuculline enhanced the stimulatory effects of Dup996 on propagation of FV, fEPSP and PS, suggest that slight reduction of KCNQ-channels alone cannot produce seizure activity, even though it can facilitate seizure activity under conditions of unbalanced neurotransmission. In other words, under the normal balance of neurotransmission, dysfunction of KCNQ-channels cannot yield seizure activity. On the other hand, it has been demonstrated that the immature neuron could be readily depolarized by GABA [12]. Therefore, the present study suggests that mechanism for spontaneous improvement of BFNC may be produced by the normalization of imbalances that occur in neurotransmission during the period of neurodevelopment, and the mechanisms of BFNC occurrence may be the result of cooperative interaction between dysfunction of the KCNQ-channels and imbalances of neurotransmission that occur in immature CNS. Our laboratory is in the process of conducting further investigation to clarify this hypothesis.

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