Anti-epileptic effect of ifenprodil on neocortical pyramidal neurons in patients with malformations of cortical development

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Abstract. Ifenprodil has been demonstrated to reduce spontaneous action potentials observed by local field potential in animal models. To investigate whether ifenprodil exerts an anti-epileptic effect on neuronal levels in humans, whole-cell patch clamp recordings were used to study the electrophysiological membrane properties of neocortical pyramidal neurons in human brain tissues. Electrophysiological membrane properties and spontaneous spikes of neocortical pyramidal neurons were investigated by using whole-cell patch clamp recordings, prior to and following the application of ifenprodil. In the present study, ifenprodil significantly decreased the membrane input resistance ($P<0.01$), membrane time constant ($P<0.01$), action potential amplitude ($P<0.01$), action potential rising rate ($P<0.05$) and falling rate ($P<0.05$) on neocortical pyramidal neurons in patients with epilepsy caused by malformations of cortical development (MCD). These results suggested that ifenprodil decreased neuronal excitability of neocortical pyramidal neurons in patients with epilepsy and MCD and demonstrated that ifenprodil may be a potentially specific treatment for refractory epilepsy caused by MCD.

Introduction

Epilepsy is a neurological disorder characterized by the chronic tendency of recurring, unprovoked seizures and ~30% of patients have refractory epilepsy (1). For the majority of patients, refractory epilepsy is caused by malformations of cortical development (MCD), which is a heterogenous group of disorders that involves focal cortical dysplasia, tuberous sclerosis and heteropia (2). Furthermore, epileptogenetic focus resection is not suitable for all patients, and previous studies have demonstrated that inflammatory pathways may be targeted as an effective therapeutic strategy in refractory epilepsy (3,4).

Previous studies have supported the promotion of inflammatory and immune processes in epileptogenesis (5), and the majority of them have been described in patients with MCD (6,7). Previous evidence has indicated that the high mobility group box 1 (HMGB1)-Toll-like receptor 4 (TLR4) axis was a proconvulsant pathway in animal models of temporal lobe epilepsy (TLE) (8). Another study showed that, in epileptic human tissues, TLR4 was expressed in astrocytes and dysplastic neurons and HMGB1 was expressed in the glial cytoplasm, which suggested that they may have an indispensable role in human epileptogenesis associated with MCD (6). In addition, HMGB1 has been shown to generate focal seizures by increasing the mean frequency of spontaneous discharges and lowering the ictal event threshold in animal models of TLE (9).

Ifenprodil, which is a selective antagonist of *N*-methyl D-aspartate receptor subtype 2B (NR2B), which contains N-methyl-D-aspartate (NMDA) receptors (10), is able to abrogate the epileptogenetic effect of HMGB1 (8). Despite these findings, the exact effect of ifenprodil on neuronal levels and epileptic network activity remains to be fully elucidated, particularly regarding human neocortical tissues. In the present study, it was investigated whether ifenprodil affected electrophysiological properties and spontaneous spikes in neocortical pyramidal cells (PCs), in addition to the epileptic network activity.

Materials and methods

Patients. Informed written consent for surgical resection of human tissues for research was obtained from patients or their

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relatives prior to surgery. The handling and use of human brain tissue was approved by the Ethics Committee of Sanbo Brain Hospital, Capital Medical University (Beijing, China). The study included a total of 6 patients enrolled between October 2014 and December 2015 (males, n=5; females, n=1; age, 11.6±9.6). These cases consisted of 5 patients with refractory epilepsy with the following inclusion criteria: i) Confirmed diagnosis of refractory epilepsy; and ii) pathological diagnosis of malformations of cortical development. Patients who had undergone radiotherapy or chemotherapy were excluded from the study. There was 1 case of glioma without epilepsy also included. Each patient received cortical focal resection. For each case, at least 2 PCs were recorded. The diagnosis of each patient was made by a multidisciplinary team according to the International League Against Epilepsy classification system (11).

Clinical information. The standardized information obtained from patients included clinical history evaluation, routine neurological examinations, such as motor, sensor, vision, language and auditory sense examinations and scalp electro-encephalogram (EEG) video-recordings, magnetic resonance imaging (MRI) and postoperative computed tomography. Clinical history was obtained from medical records and included the following: Age at seizure onset, age at surgery, clinical manifestation, type of surgery (multi-lobar or lobar/focal), side of operation, sex, seizure frequency, history of antiepileptic drugs (AEDs) and pathology examination described previously (12), as indicated in Table I. In particular, the region of brains sampled for *in vitro* electrophysiological studies were recorded.

Electrophysiological methods. Human brain tissues, chosen as epileptic onset zone according to preoperative evaluation (2/6 with high frequency oscillations in stereotactic electroencephalogram [SEEG]), were excised to investigate the electrophysiological character of cortical neurons.

Tissues were removed microsurgically and directly placed in ice-cold slicing solution containing 26 mM $NaHCO_3$, 2.5 mM KCl, 5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 10 mM dextrose, 213 mM sucrose, 1.0 mM $CaCl_2$ and 1.0 mM $MgSO_4$ (pH 7.2-7.4). Within 1-2 h, slices 350 μm thick were cut using a Leica slicer and incubated in regular artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM Dextrose, 2 mM $MgSO_4$ and 2 mM $CaCl_2$ (pH 7.2-7.4) for 45 min at 36˚C and then at room temperature until use.

During recordings, slices, without the cover slip, from the original and ifenprodil groups were perfused at 35-36˚C at 3 ml/min for 2 h in Mg^{2+}-free modified ACSF composed of 126 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM dextrose and 2 mM $CaCl_2$ (pH 7.2-7.4) to mimic epileptic activity (13,14). Whole-cell recordings were achieved using glass pipettes (3-5 MΩ) filled with 140 mM K-Gluconate, 3 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 2 mM Na_2ATP and 0.2% biocytin (pH 7.25-7.30; osmolality, 280-290 mOsm) for voltage and current clamp recordings. Spontaneous spikes lasting for at least 2 min, action potential induced by depolarizing currents (40 pA current increment; 500 msec) and membrane potential

(Vm) responses to hyperpolarized current (-100 pA; 500 msec) or a series of current steps (500 msec in duration) were recorded, respectively, to compare differences of intrinsic electrophysiological properties prior to and after the application of ifenprodil (5 μM; I2892; Sigma-Aldrich; Merk KGaA, Darmstadt, Germany).

The five patients with epilepsy underwent epileptogenic zone resections and the patient without epilepsy underwent tumor resection. Subsequently, patients with epilepsy were split into the following groups: i) According to video EEG recordings, patients were divided into a high frequency oscillations (HFOs) group (n=3 patients) and a non-HFOs (n=2 patients) group; and according to pathological results, ii) patients with focal cortical dysplasia type II and tuberous sclerosis (TS) were classed as the TS complex (TSC)-related group (n=3 patients) and iii) patients with focal cortical dysplasia type I and type IIId were classed as the idiopathic group (n=2 patients). The patient without epilepsy was classed as the non-epilepsy group.

For all brain tissue obtained from the 6 patients enrolled in the current study, whole-cell recordings on pyramidal-shaped neurons were performed and interneuron-shaped neurons under the DIC (differential interference contrast) microscope (x400) were avoided due the focus of the study being on excitatory modulation. According to our previous study, PCs and inhibitory interneurons were identified by their firing patterns (15). Depolarizing currents were applied (40 pA current increment; 500 msec) to induce action potentials, and all cells were recorded at resting membrane potential. Once the recordings were established, the type of neuron was re-confirmed using staining techniques, after having identified them by firing patterns.

Hemtoxylin and eosin staining. Tissue samples from all 6 patients were fixed in 10% formalin at room temperature for 24 h and embedded in paraffin prior to being cut into sections 4 μm thick. Sections were then mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Braunschweig, Germany). Sections of all specimens were stained with hematoxylin and eosin at room temperature for 4 h, and images were visualized using a Leica microscope, captured using an Optronics DEI-750 three-chip camera equipped with a BQ 8000 sVGA frame grabber (x400; Optronics, Goleta, CA, USA) and analyzed using Bioquant software 2011 (Bioquant Image Analysis Corporation, Nashville, TN, USA).

Drug treatment. For all slices obtained from patients enrolled in the current study, including the epilepsy and non-epilepsy groups, Vm responses to the hyperpolarized current, spontaneous spiking and Vm responses to serials of hyperpolarized current steps were recorded. Following original recordings for 20 min, brain tissue sections from all 6 patients were immersed in 5 μM ifenprodil for 5 min in 36˚C and the above-mentioned parameters were subsequently examined again. Slices were then fixed in 4% paraformaldehyde for 12 h at room temperature for subsequent avidin staining.

Histological procedures. Alexa Fluor 488 (200 μM) and 0.2% biocytin were added to internal solution (K-Gluconate 140 mM, KCl 3 mM, MgCl2·6H2O 2 mM, HEPES 10 mM, EGTA 10 mM, Na2ATP 2 mM, 285 mOsm, pH 7.3) as

Table I. Clinical information of patients with drug-resistant epilepsy caused by malformations of cortical development (P1-5) or without epilepsy (P6).

Patient	Sex	Age at seizure onset (years)	Age at surgery (years)	Clinical manifestation	Type of surgery	Side of surgery	Seizure frequency	Situation of AEDs	Pathology	Neurological examinations	EEG video-recordings	MRI	HFOs	Samples taken
P1	Male	4	8	Contralateral elementary motor signs; lower limbs clonic signs; generalized tonic clonic seizure	Focal	Left	6/month	None for 1 year	Tuberous sclerosis	Negative	Ictal type in right cerebral hemisphere	Bilateral multiple abnormal signal, considering tuberous sclerosis	Around tubers	Left frontal lobe, surrounding tissue of tubers
P2	Male	7	17	Impairment of consciousness; contralateral versive signs; facial expression; integrated gestural motor behavior	Focal	Right	7-9/month	Oxcarbazepine 600 mg bid; Valproate 500 mg bid	FCD IIId	Negative	Ictal type	Abnormal signals in bilateral occipital lobe, considering ischemic change	None	Right superior parietal lobe
P3	Male	8	13	Impairment of consciousness; both eyes contralateral turning with ipsilateral head leading; contralateral upper limb tonic posture; integrated gestural motor behavior with urinary incontinence	Focal	Left	1/day	Oxcarbazepine 450 mg bid; Levetiracetam 750 mg bid	FCD IIId	Negative	Ictal type in bilateral parietooccipital lobe, especially left side	Right occipital encephalomalacia	None	Left occipital lobe
P4	Male	20	25	Behavioral arrest; contralateral proximal stereotypes; autonomic signs; fixed facial expression	Focal	Right	2/week	Oxcarbazepine 300 mg once daily	FCD Ib	Negative	Diffuse ictal type	Abnormal signals in right mesial temporal lobe	Right inferior temporal gyrus+Right fusiform gyrus	Right inferior temporal gyrus
P5	Male	4	14	Autonomic aura; integrated gestural motor behavior	Focal	Left	>10/day	Oxcarbazepine 900 mg 750 mg; Phenobarbital 30 mg bid	FCD IIb	Negative	Diffuse ictal type, especially left side	Abnormal signals in left superior frontal gyrus, considering focal cortical dysplasia	None	Left superior frontal gyrus
P6	Female	No seizures	35	Headache for 1 year; exacerbated in 1 month	Focal	Right	N/A	N/A	Low grade glioma, WHO II	Negative	N/A	Focal mass in left frontotemporoinsula lobe, considering low grade glioma	N/A	Right frontal lobe, surrounding tissue of tumor

EEG, electroencephalogram; MRI, magnetic resonance imaging; N/A, not applicable; WHO, World Health Association; FCD, focal cortical dysplasia; AEDs, anti-epileptic drugs; HFOs, high frequency oscillations.

described previously (15) in order to study and record labeled cells. Following the completion of recordings by patch clamp, 3 human brain slices from the TSC, idiopathic and non-epilepsy groups were fixed in 8% PFA at room temperature and 8% sucrose (w/v) in 0.1 Mmol PBS (pH 7.4) for 1 h. Slices were rinsed in 0.01 Mmol PBS 3 times, and subsequently incubated in 0.5% Triton X-100 for 0.5 h at room temperature and 10% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as a blocking solution at room temperature for 1 h. Once slices were washed in PBS 3 times for 10 min, they were incubated in strept-avidin (1:1,000; cat no. SA-5000, Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h at room temperature. Confocal images were taken with a laser scanning confocal microscope (Nikon A1; Nikon Corporation; Tokyo, Japan) with a x40 objective. Z-stack images of labeled cells were attained with an interval of 0.75 μm.

Statistical analysis. All of the electrophysiological values were presented as mean ± standard error and all experiments were repeated in triplicate. For two independent observations, a two-sample Student's t-test was used for normally-distributed data, whereas non-normal data were compared using Wilcoxon rank-sum test. For multiple observations a one-way ANOVA followed by a Student-Newman-Keuls test was used to examine the significance between groups with a normal distribution, whereas when data did not have a normal distribution a Kruskal-Wallis H test was used. P<0.05 was considered to indicate a statistically significant difference. Passive parameters were derived from Vm responses to current steps.

Input resistance was calculated using the following formula: Steady state Vm changes/current amplitudes. Action potential (AP) amplitude was considered the voltage value between the threshold and peak. AP threshold was the voltage value at the time when the AP rising slope reached 20 V/sec. AP half-width was determined as the AP duration at half amplitude. AP rising rate was the highest value of voltage-time slope in the AP rising phase. To detect the AP falling rate, the lowest value of the voltage-time slope in the AP falling phase was used. Frequency-current intensity curve (F-I curve) indicated the association between serials of hyperpolarized currents and the number of spikes they evoked.

Every random spontaneous excitatory input was identified, including subthreshold depolarizations and spontaneous spikes, and then considered all-or-none random spontaneous bursts into an event. In the statistical analysis, excitatory inputs with an amplitude >15 mV were included. For each cell, the induced spontaneous spikes and events within the selected 120 sec were measured.

All analysis was performed using Matlab (R2014a, The Math Works; Natick, MA, USA), SPSS 21 for Mac (IBM Corp., Armonk, NY, USA) and Spike2 (7.11a; Cambridge electronic design; Cambridge, UK).

Results

Patient clinical information. Clinical information is presented in Table I. Focal lesions were localized by clinical manifestation, preoperative MRI (Fig. 1A) and SEEG (data not shown). Following the combination of postoperative CT and
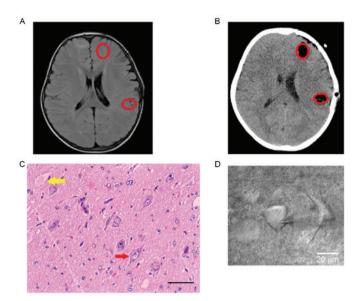

Figure 1. Example of human brain tissue. (A) P1 was an 8 year old male patient diagnosed with refractory epilepsy caused by tuberous sclerosis 4 years prior to the start of the current study. Fluid-attenuated inversion recovery weighted magnetic resonance imaging revealed tubers in left frontal lobe (red circles). (B) Postoperative computed tomography revealed the exact location of the human tissue for study (red circles). (C) Pathological examination of HE staining showed dysmorphic neuron (red arrow) and balloon cells (yellow arrow). Scale bar, 50 μm. (D) Example of recording pyramidal neuron using a differential interference contrast microscope. Magnification, x400.

pathological results, the exact locations of samples were studied and dysmorphic neurons and balloon cells were noted in Fig. 1B and C). The morphology of a pyramidal neuron is presented in Fig. 1D.

Investigation of electrophysiological properties of neocortical PCs in patients with MCD. To identify the correlation between clinical manifestations and PC electrophysiological properties, patients with focal cortical dysplasia type II and tuberous sclerosis (TS) were categorized as the TS complex (TSC)-related group, and patients with focal cortical dysplasia type I and type IIId were classed as the idiopathic group. According to video EEG recordings, patients were divided into the HFOs group (n=3) and non-HFOs (n=2) group. In addition, one patient without epilepsy was selected as the non-epilepsy group.

Electrophysiological properties of neocortical PCs in patients with MCD were determined (Fig. 2). The morphology of recorded pyramidal neurons, which were similar to those under physiological condition, were indicated in Fig. 2A. The rising and falling rate of AP in the TSC-related group was significantly faster than those in the idiopathic and non-epilepsy groups (Fig. 2C and D, respectively; P<0.01). The half-width of APs in the TSC-related group was significantly decreased compared with those in idiopathic and non-epilepsy groups (Fig. 2B; P<0.01), whereas there was no significant difference in the membrane input resistance, membrane capacitance, time constant, amplitude of AP, and threshold potential among the 3 groups (data not shown).

The characteristics determined by EEG indicated there was no significant difference in the membrane input resistance, membrane capacitance, time constant, AP rising rate, threshold potential, amplitude of AP, half-width of AP and AP
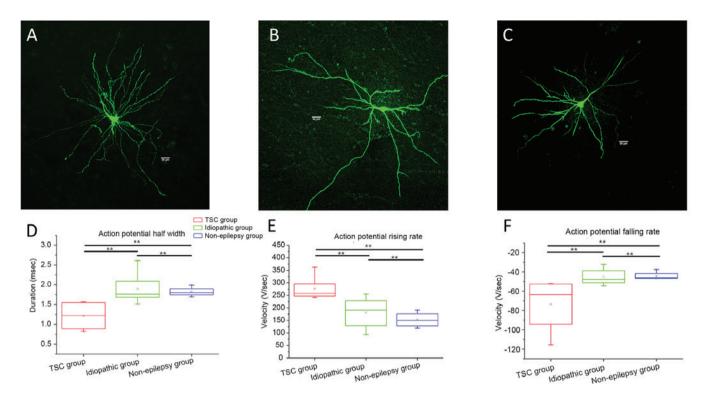
Figure 2. Investigation of electrophysiological properties of neocortical pyramidal cells in patients with malformations of cortical development. (A-C) Neocortical pyramidal cells, from the TSC group (A), idiopathic group (B) and non-epilepsy group (C), were stained with avidin staining. Scale bar, 30 μm. (D) Action potential half-width in the TSC-related group (n=6 cells) was decreased compared with that in idiopathic group (n=8 cells) and non-epilepsy group (n=4 cells) (Student-Newman-Keuls test; **P<0.01). (E and F) In aspect of pathology, action potential rising and falling rates in the TSC group (n=6 cells) were significantly faster compared with those in the idiopathic (n=8 cells) and non-epilepsy groups (n=4 cells) (Student-Newman-Keuls test, **P<0.01). Box-and-whisker plots present the range, interquartile range and median; 'o' indicates the mean value. TSC, tuberous sclerosis complex.

falling rate between the HFOs group and non-HFOs group (data not shown).

Modulation of electrophysiological properties in PCs by ifenprodil. As HMGB1 modulates the excitability of PCs by activating NR2B-containing NMDA receptors (16), the present study examined the exact electrophysiological changes in PCs. Changes in whole-cell membrane electrophysiological properties were obtained by injecting hyperpolarizing and depolarizing currents steps, respectively. Hyperpolarizing currents (-100 pA; 500 msec) were applied to the recording cell to examine passive membrane properties. Subsequently, depolarizing currents (40 pA current increment; 500 msec) were applied to induce action potentials. Cells were recorded at resting membrane potential.

Electrophysiological membrane properties for each cell were compared, including input resistance, time constant, capacitance, AP amplitude, AP half-width, AP threshold, AP rising rate and falling rate (Table II). Among patients with epilepsy, ifenprodil significantly decreased the input resistance and time constant compared with the original group (Fig. 3A; P<0.01), whereas no significant difference was indicated in capacitance (Table II; P=0.94). Furthermore, ifenprodil treatment significantly decreased AP amplitude compared with the original group (P<0.01; Table II; Fig. 3B), but no significant alteration in AP half-width was observed (Table II; Fig. 3B), which likely indicated decreased Ca^{2+} entry following application of ifenprodil (17,18). Additionally, AP rising and falling rates were significantly decreased following

ifenprodil treatment compared with the original group, respectively (Table II; Fig. 3B; P<0.05). However, the AP threshold showed no significant difference prior to and after application of ifenprodil. In patients without epilepsy, no significant differences were noted in the membrane properties prior to and after application of ifenprodil (Table II). In addition, no significant difference in the membrane properties between patients with and without epilepsy were exhibited, with one exception; significantly increased membrane capacitance was detected in epilepsy patients (Table II; P<0.05).

To investigate the modulation of neuronal excitability of PCs by ifenprodil, a series of depolarizing current steps were applied to generate an F-I curve. Fig. 4A indicates an example of the voltage response of recording PCs under the current stimulus with the same intensity prior to and after application of ifenprodil. Results suggested that in the epilepsy group, ifenprodil decreased the mean number of spikes compared with the original group; however, this was not a statistically significant difference (Fig. 4B). Conversely, the mean number of spikes was increased following the application of ifenprodil in non-epilepsy patients, although this was not a statistically significant difference (Fig. 4B). These results indicate that ifenprodil may decrease neuronal excitability of PCs in patients with epilepsy.

Modulation of epileptic network activity by ifenprodil. To mimic epileptic activity, the brain tissues were perfused in Mg^{2+}-free ACSF instead of normal ACSF. A total of 85.7% of slices (12/14) generated epileptiform activity in cortical tissue
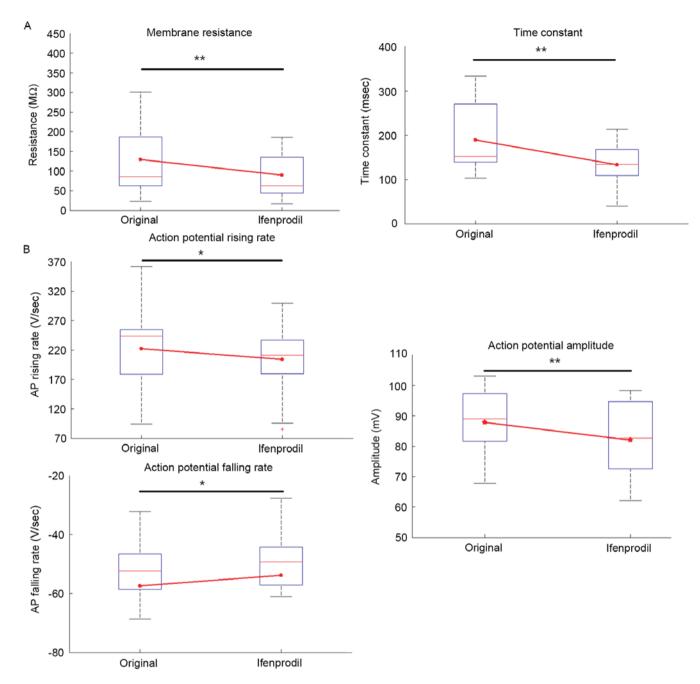
Figure 3. Modulation of electrophysiological membrane properties in neocortical pyramidal cells. (A) Membrane resistance and time constant were significantly decreased compared with the original group following the application of 5 μM ifenprodil. (Wilcoxon matched-pairs signed-rank test; [**]P<0.01; n=14 cells) (B) Ifenprodil significantly decreased AP amplitude (Wilcoxon matched-pairs signed-rank test; [**]P<0.01; n=14 cells), AP rising rate (two-tailed paired t-test; [*]P<0.05; n=14 cells) and AP falling rate (Wilcoxon matched-pairs signed-rank test; [*]P<0.05; n=14 cells) compared with the original group, respectively. Box-and-whisker plots present the range, interquartile range and median; red points indicate the mean value. AP, action potential.

obtained from epilepsy patients. Once a stable epileptic network activity was achieved in 5 slices for ≥25 min, ifenprodil was applied and the generation of epileptiform activity was decreased after applying ifenprodil (Fig. 5). The number of spikes for each depolarized event was decreased in the Ifenprodil group compared with the original group (Fig. 5A). As defined above, depolarizing potentials with amplitude >15 mV and duration >300 msec were considered as an event. Bath application of 5 μM ifenprodil significantly decreased the number of depolarizing events compared with the original group (Fig. 5D; P<0.05) and significantly increased the inter-burst-interval (Fig. 5C; P<0.05). Furthermore, the frequency distribution plot indicated

that ifenprodil significantly reduced the quantity of depolarizing events in every 10-Hz step within the selected 120 sec by (Fig. 5B; P<0.05).

Discussion

Immune mediators are functional in the immune response to infection; however, recent studies have demonstrated that they also have alternative roles (19,20). Increasing experimental evidence has demonstrated that immune mediators are associated with neuromodulation (21). In the last two decades, findings have suggested that immune mediators, including

Table II. Modulation of electrophysiological properties of pyramidal neurons.

| | Epilepsy | | | | | Non-epilepsy | | | | |
| | Original | | Ifenprodil | | | Original | | Ifenprodil | | |
Property	Mean ± SE	n	Mean ± SE	n	P-value	Mean ± SE	n	Mean ± SE	n	P-value
Passive membrane property										
Resistance (MΩ)	140.665±29.768	14	102.683±21.365	14	<0.01	191.297±22.947	4	218.875±15.168	4	0.144
Tau (msec)	18.923±2.091	14	13.295±1.208	14	<0.01	19.553±SE 3.943	4	21.752±3.236	4	0.465
Capacitance (pf)	189.267±36.891	14	188.007±31.819	14	0.778	101.774±12.895	4	98.112±10.180	4	0.715
AP amplitude (mV)	87.999±3.332	14	83.043±3.777	14	<0.01	78.699±3.881	4	76.352±4.354	4	0.715
AP half width (msec)	1.605±0.134	14	1.615±0.152	14	0.221	1.8223±0.0600	4	1.871±0.124	4	0.715
Active membrane property										
AP threshold (mV)	-43.549±3.100	14	-44.327±2.594	14	0.778	-36.885±1.1450	4	-36.030±2.829	4	0.465
AP rising rate (V/sec)	222.037±19.260	14	203.949±16.438	14	<0.05	152.413±15.599	4	144.990±13.998	4	0.715
AP falling rate (V/sec)	-57.454±6.013	14	-53.819±5.414	14	<0.05	-44.219±2.205	4	-43.360±3.507	4	0.715

Statistical significance of differences was evaluated via Wilcoxon test, with the exception of AP rising rate, which was tested using a paired two sample t-test. AP; action potential; SE, standard error.
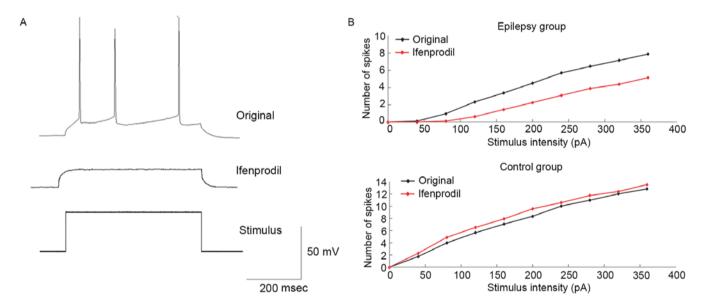

Figure 4. Modulation of neural excitability. (A) Voltage response of a neocortical pyramidal cell recording under current stimulus with the same intensity prior to and after application of 5 μM ifenprodil. (B) For patients with epilepsy, the frequency-current intensity curve indicated that ifenprodil reduced the mean number of spikes; however, this was not statistically significant. Conversely, the mean number of spikes was increased following the application of 5 μM ifenprodil in patients without epilepsy, although this was not statistically significant. P=0.098 and P=0.534, respectively; n=14 cells.

pathogen associated molecular patterns, danger associated molecular patterns (DAMPs) and extracellular matrix components, modulate voltage-dependent ion channels, receptor-dependent ion channel and synaptic strength (22). HMGB1, which is a type of DAMP, was expressed in the nucleus in both neurons and glial cells under normal physiological conditions, and is produced from cell necrosis or active release following stimulation (23). In an animal model, the HMGB1/TLR4 axis increased Ca^{2+} influx through phosphorylation of the NMDA-NR2B receptor (16). The present study revealed that AP amplitude in PC was significantly decreased following the application of ifenprodil in the epilepsy group, which indicated a reduction in Ca^{2+} influx (17). However, Ca^{2+} channels are categorized in three families and mediate different types of currents (24); therefore, further investigation is required to identify which family was associated with this effect. As Ca^{2+} influx is necessary to activate the release of HMGB1 (25), it was postulated that ifenprodil likely decreased the release of HMGB1 in PCs. HMGB1 also provokes astrocytes to release immune mediators (26), and application of ifenprodil potentially decreases the release of a variety of immune mediators.

As HMGB1 and NMDA receptors are upregulated in the nidus of the neuron among patients with MCD (27), it was
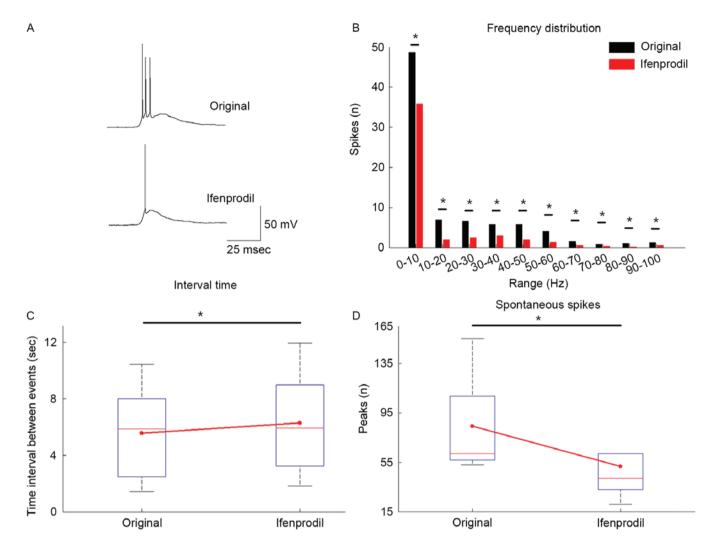
Figure 5. Modulation of spontaneous spikes. (A) Spontaneous spikes of a neocortical pyramidal cell recording prior to and after application of 5 μM ifenprodil. (B) Frequency distribution determined the application of ifenprodil reduced the quantity of depolarizing events in every 10-Hz step within the selected 120 sec. *P<0.05. (C and D) Application of 5 μM ifenprodil significantly decreased the quantity of depolarizing events (Wilcoxon matched-pairs signed-rank test; *P<0.05; n=5 slices) and significantly the increased inter-burst-interval (two-tailed paired t-test; *P<0.05; n=5 slices) compared with the original group within the selected 120 sec.

expected that application ifenprodil would exhibit pathological-specific modulation of neuronal excitability in PCs. In the present study, the application of ifenprodil reduced neuronal excitability of PCs in patients with epilepsy, but marginally enhanced PC neuronal excitability in patients without epilepsy. Coincidentally, application of ifenprodil significantly decreased membrane input resistance in patients with epilepsy. Previous studies showed that NR2B was predominantly expressed in the extrasynaptic region (28) and extrasynaptic neurotransmitter receptor activation appeared to modulated membrane potential (29). These findings suggest that NR2B blockage by application of ifenprodil may hyperpolarize membrane potential, which results in an increase in membrane conductance by HCN channel activation (30).

Neuronal excitability was determined by AP characteristics and spiking pattern, thereby neuronal excitability should be described by both AP shape and F-I curve alteration. The present results indicated that application of ifenprodil altered the AP characteristics in the epilepsy group by decreasing either AP amplitude, AP rising rate or AP falling rate. These findings

suggested that the application of ifenprodil likely decreased Ca^{2+} influx during the generation of APs, and decreased the conductance of Ca^{2+}-dependent K^+ channel in AP falling phase (31). Using calcium imaging in an animal model, a previous study demonstrated that HMGB1 increased spontaneous ictal-like discharges, which were blocked by tetrodotoxin, which is a voltage-gated sodium channel blocker (9). The present study showed that application of ifenprodil decreased the total number of spontaneous spikes and increased inter-burst-interval time among PCs in patients with epilepsy.

With the exception of HMGB1, experimental evidence has revealed interactions between interleukin-1β and NR2B subunits in hippocampal neurons (32). The interaction between HMGB1 and IL1β also exhibited amplification of the inflammatory response in osteoarthritis (33). We subsequently speculated that the application of ifenprodil likely had a potent anti-epileptic effect via the direct blockage of HMGB1/TLR4 axis (8).

In clinical practice, antiepileptic agents have non-specific effects on preventing epileptic activity by decreasing

excitation or increasing the inhibition in every neuron within the cortex (34,35). In patients with epilepsy and MCD, the expression of HMGB1 was upregulated in dysmorphic neurons and translocated from the nucleus to the cytoplasm in glial cells in a previous study (27). No similar findings in patients without epilepsy were identified in the present study. In addition, animal experiments have revealed that the activation of HMGB1/TLR4 axis also has long term effects on epileptogenesis by reducing the epileptogenetic threshold (36).

Ifenprodil has been proven to be safe for humans in the treatment of ischemic brain injury (37,38) as a non-competitive and highly selective antagonist of NMDA receptors containing the NR2B subunit (10), and an effective neuroprotectant for modulating NR2B receptor activity by changing the binding rate of the receptor (39). In the present study, ifenprodil showed specific antiepileptic effects by reducing PC neural excitability in patients with epilepsy and marginally increasing neural excitability in the patient without epilepsy. Furthermore, ifenprodil inhibited the neural network activity in brain tissue slices transformed from patients with MCD.

The limitations of the study are as follows: A small cohort of patients was used, which makes it challenging to avoid the bias brought by variables and in terms of the effect of ifenprodil, interneurons have not been involved in the current study. Therefore, further investigation for the expression, regulation, function of ifenprodil on interneurons is required.

In conclusion, although further studies are necessary to investigate whether ifenprodil exerts an effect on inhibitory interneurons and glial cells, the present findings suggest that ifenprodil may be utilized as a treatment method for epilepsy caused by MCD.

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