Neurochemical Research (2019) 44:994–1004
https://doi.org/10.1007/s11064-019-02734-3

ORIGINAL PAPER

2-Deoxy-D-Glucose Exhibits Anti-seizure Effects by Mediating the Netrin-G1-KATP Signaling Pathway in Epilepsy

Yuming Long[1] · Kai Zhuang[2] · Zhonghai Ji[1] · Yaru Han[1] · Yanqing Fei[1] · Wen Zheng[1] · Zhi Song[1] · Heng Yang[1]

Received: 3 June 2018 / Revised: 16 January 2019 / Accepted: 16 January 2019 / Published online: 25 February 2019

Abstract

Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures. The glycolytic inhibitor 2-deoxy-D-glucose (2-DG) has been reported to exert antiepileptic effects by upregulating KATP subunits (kir6.1 and kir6.2). We evaluated whether 2-DG exhibits anti-seizure effect by mediating the netrin-G1-KATP signaling pathway in epilepsy. In a mouse epilepsy model induced by lithium chloride-pilocarpine, 2-DG intervention increased the mRNA and protein expression levels of kir6.1 and kir6.2, and these increases were significantly reversed after knocking down netrin-G1 expression. Similarly, in cultured neurons with a magnesium-free medium, we found that the frequency of spontaneous postsynaptic potentials (SP) was increased, and in the meanwhile, expression levels of kir6.1 and kir6.2 were increased after pretreatment with 2DG. These effects were remarkably reversed after knocking down netrin-G1. Thus, our findings show that 2DG exhibits anti-seizure effects through the netrin-G1-KATP signaling pathway.

Keywords Epilepsy · Netrin-G1 · KATP · 2DG

Introduction

Epilepsy is a disorder of the brain characterized by an enduring predisposition to generating epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition. The diagnosis of epilepsy needs to meet one of the two criteria First, at least two unprovoked (or reflex) seizures occurring > 24 h apart. Second, one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the next 10 years [1]. Epilepsy is a global health issue affecting more than 50 million people [2].Thus, new targets of antiepileptic drugs are needed. The construction of abnormal neural networks is an important pathological source of intractable epilepsy [3, 4]. During the development of the nervous system, neurons and axons are guided to the right locations to form correct neural networks through various axon guidance molecules. Therefore, axon guidance molecule families play an essential role in the neural network construction of epilepsy patients.

Netrin-G1 is an axon guidance molecule involved in synaptic plasticity. It belongs to the netrin family which includes netrin1, netrin 3, netrin 4 and netrin-G2 [5, 6]. Netrin-G1 is located at chromosome 1p13 and encodes a protein of 539 amino acid residues with a molecular weight at 61 kDa. Netrin-G1 is highly expressed in the brain and kidney, and is weakly expressed in the spleen, liver and lungs [7]. In the brain, netrin-G1 is mainly expressed in the occipital pole, frontal and temporal lobes, putamen, hippocampus, and thalamus [8]. It consists of at least six isoforms, five of which are anchored to the presynaptic membrane via glycosyl phosphatidyl-inositol linkages [8]. After binding to its specific ligand (NGL-1), NetrinG1 promotes axon growth, regulates synapse formation, and maintains the balance between excitatory versus inhibitory neurotransmitter [9–12]. And NGL-1 is a transmembrane protein containing a C-terminal intracellular postsynaptic binding motif,

Yuming Long and Kai Zhuang contributed equally to this research.

✉ Zhi Song
docsong2009@126.com

✉ Heng Yang
johnnelyang@hotmail.com

[1] Department of Neurology, The Third Xiangya Hospital of Central South University, Changsha 410013, Hunan, People's Republic of China

[2] Department of Neurosurgery, The Third Xiangya Hospital of Central South University, Changsha 410013, Hunan, People's Republic of China

which interacts with scaffolding proteins such as PSD-95 family members [10, 13].Many studies have found that gene polymorphisms of netrin-G1 are associated with synaptic plasticity-related diseases, such as schizophrenia and bipolar disorder [14–18]. Epilepsy is a type of synaptic plasticity-related disease and synaptic recombination accompanied by axon sprouting can lead to the construction of abnormal network, which can promote the induction of epilepsy. Thus, it could be inferred that netrin-G1 is associated with epilepsy.

The KATP channel is a special non-voltage-dependent potassium channel which links cell metabolism with electrical activity [19]. In the brain, KATP channel is widely expressed in substantia nigra, striatum, and hippocampal neurons [20]. Structurally, KATP channel is octamultimeric complexes of four Kir6.1/ Kir6.2 subunits and four associated SUR subunits. Recent studies show that KATP channel plays an anti-seizure effect [21]. In our previous study, we had found that glycolysis inhibitor 2-DG plays an anti-seizure role by upregulating the mRNA and protein expressions of kir6.1 and kir6.2 [22]. However, the mechanism underlying 2-DG induced regulation of KATP channels is unclear. It is possible that netrin-G1 might act as an upstream regulator of the KATP channel. We hypothesize that 2-DG may exert its anti-seizure effect by regulating netrin G1- KATP signaling pathways.

Materials and Methods

All experimental protocols were approved by the Medical Experimental Center and Ethics Committee of the Third Xiangya Hospital of Central South University. All experiments were carried out in accordance with the approved guidelines.

Mice

Male C57BL/6 mice (5–6 weeks, 19–23 g, n = 150) were provided by the Third Xiangya Hospital experimental animal center and were maintained under a controlled standard condition, which included normal room light (12 h regular light/dark cycle) and temperature (22 ± 1 °C). Six mice were chosen for each group.

Establishment of the Epileptic Model

Mice were randomly and equally divided into 5 groups, including a control group (control); EP group: a lithium-chloride (LiCl) pilocarpine kindling epileptic model; 2DG group: 2-DG (250 mg/kg) (sigma, USA) was administered 30 min before LiCl-pilocaprine induced status epilepticus (SE). EPSI group: mice were transfected with si-netrin-G1 (50 nmol) (ribo.CHN) before LiCl-pilocarpine treatment. siRNA was transfected using intracerebroventricular microinjection. DGSI group: mice were transfected with si-netrin-G1 before 2-DG and LiCl-pilocarpine intervention.Drugs and siRNA were administered between 8 a.m. to 10 a.m. Status epilepticus was terminated by diazepam (10 mg/kg) after 30 min of the onset. The mice that were kindled by pilocaprine after 120 min were eliminated from the groups.

Behavior Measurement and EEG Recording

Behavior measurement was recorded by three aspects, including seizure score, seizure latency and seizure duration. Seizure score was classified according to the Racine scale [23, 24]. Mice with racine scores greater than four was considered as having seizure. Mice with scores less than four were excluded from the study. Electrographic activity after LiCl-pilocarpine treatment was assessed by EEG recordings in the hippocampus. 10% chloral hydrate was intraperitoneally injected into mice for anesthesia. After anesthesia, a recording electrode was placed in a burr hole 3.0 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.0 mm ventral to the surface of the skull. The other burr hole was for the ground screw, which was placed on the left side 4 mm anterior to bregma.

Real Time-PCR

Cervical dislocation was used for scarifying mice, and the procedure was performed out of sight of the other mice. Death was verified by sudden cardiac cessation and respiratory arrest. Only the mice that reach the criterion in this experiment were used for this study. Total RNA was extracted from the hippocampus using TRIzol reagent (life, USA) in accordance with the manufacturer's instructions. The concentration of total RNA was determined and measured with an absorbance ratio between 1.7 and 2.0 at 260/280 nm. Then, total RNA was transcribed into cDNA using the Reverse First Strand cDNA Synthase kit (TAKARA, Japan). Quantitative real-time polymerase chain reactions were performed using SYBR Green PCR premix (TAKARA, Japan). Sequences of mRNA primers were as follows:

GAPDH-F: GCAGTGGCAAAGTGGAGATT,
GAPDH-R: CGTTCCTGGAAGATGGTGAT,
Kir6.1-F:TCTTCACCACCTTGGTAGACCT,
Kir6.1-R: ACCTGACATTGGTCACACAGAC,
Kir6.2-F:AACACCATTAAAGTGCCCACAC,
Kir6.2-R:AGAGATGCTAAACTTGGGCTTG.
NetrinG1-F:TGCTAAACACAGTCATTTGCGT,
NetrinG1-R:GCACACATTCTCATCGTCCAG.

The thermal cycler conditions included 40 cycles at 95 °C for 5 s and then 60 °C for 30 s. The expressions of microRNA and mRNA were analyzed using the $2^{-\Delta\Delta CT}$ method [25].

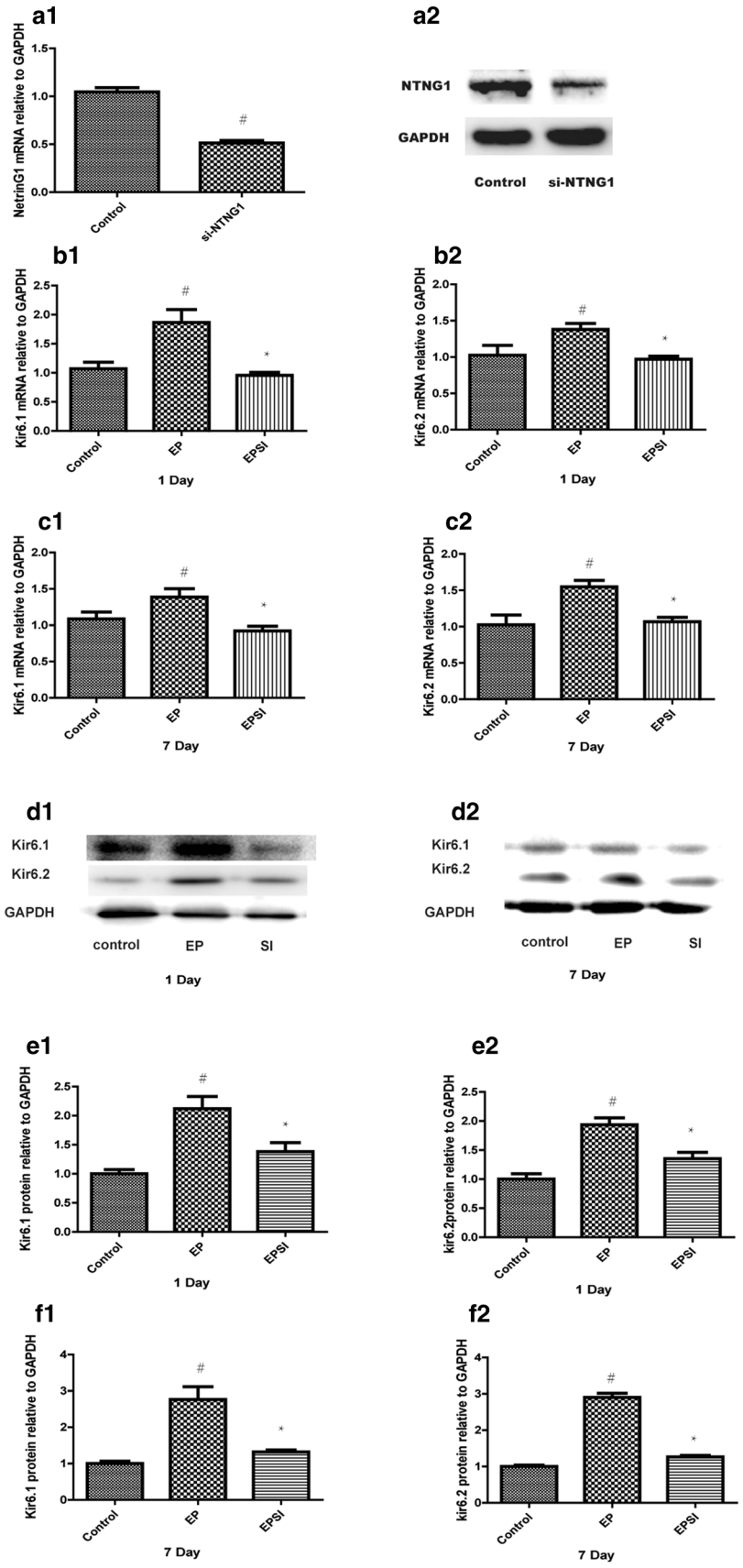


Fig. 1 Netrin-G1 can up-regulate the expressions of kir6.1 and kir6.2 in vivo model. Transfected with si- Netrin-G1 (50 nmol) before licl-pilocarpine treated mice named EPSI group. The effect of siRNA is proved by RT-PCR and Western blot (shown in **a**1 and **a**2). Compared with control group (n = 8), the expression of kir6.1 and kir6.2 mRNA increased in EP group at day1 (n = 6) and day7 (n = 6) (shown in figure **b** and figure **c**). Compared with EP group, the expression of kir6.1 and kir6.2 mRNA decreased in EPSI group at day1 (n = 6) and day7 (n = 6) (shown in figure **b** to figure **c**). As to protein, compared with control group, the expression of kir6.1 and kir6.2 protein increased in EP group (shown in **d** to **f**). Compared with EP group, the expression of kir6.1 and kir6.2 protein decreased in EPSI group at day1 and day 7 (shown in **d** to **f**). # represents compared with control group, $p < 0.05$.*Represents compared with EP group, $p < 0.05$

Western Blotting

Protein was extracted from the hippocampus using RIPA and PMSF. 20 μg of total protein were separated by 10% SDS–PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane (Minipoll, USA). After blocking with 5% no-fat milk in TBST, the membrane was incubated overnight with rabbit antimouse kir6.1 and kir6.2 (Sigma; 1:1000) overnight. Immunoreactive bands were visualized by electrochemiluminescence (Minipoll) after incubation with a horseradish peroxidase (HRP)-conjugated antirabbit or antigoat antibody. The ratio of the targeted protein of interest to GAPDH was used for statistical analyses.

Cultured Neurons

1 to 2 days Neonatal mice were provided by the Third Xiangya Hospital experimental animal center. Hippocampal tissues were removed from mice and digested by using papainase. Neurons were incubated with neurobasal medium (Gibco). Approximately half of the culture medium was changed every 2 days. The siRNA transfection reagents were used at day 5 and the cultured neurons were used for the subsequent experiment at day 8.

Patch-Clamp Recording

The membrane potentials of neurons were measured by whole-cell patch-clamp recording using a patch amplifier detector. A cell dish was placed on an inverted microscope (Olympus, Japan). Patch pipettes were filled with an intracellular solution containing 140 mM KCl, 0.5 mM EGTA, 5 mM HEPES and 3 mM Mg-ATP. The pH was adjusted to 7.3 with KOH, and the osmolarity was adjusted to 315 mOsm with dextrose. The experiments were performed at room temperature (20 °C). The pipette resistance intracellularly was 2–4 MΩ. The pipette resistance and capacitance were calculated electronically after the establishment of a gigaseal. Data were recorded only when the series resistance was < 20 MΩ. Cultured neurons with small dendritic arborizations, long axons and pyramidal somas with diameters of 20–26 μm were selected for the electrophysiological recordings. Whole-cell recordings were performed using an EPC-10 amplifier (HEKA, Germany) in the current-clamp mode. Data were collected and analyzed using Clamp-fit software.

Statistical Analysis

Data were presented as the mean ± standard error of the mean (SEM). One-way ANOVA with Tukey post hoc test was used for statistical analysis. Chi square test was used for the Racine scale. $P < 0.05$ was considered to indicate a significant difference.

Fig. 2 Netrin-G1 can up-regulate the expressions of kir6.1 and kir6.2 and activate KATP channel in vitro model. In cultured neurons, the patch–clamp recordings were divided into three groups (shown in figure **a**). Cultured neurons treated with none magnesium-free medium (non-MGF medium) named control group (a1) (n = 6), while MGF medium-treated group named EP group(a2) (n = 6). MGF epilepsy was built successfully (figure **b**). Cultured neurons transfected with si-Netrin-G1 reagent (50 nmol) was named SI group (a3) (n = 6). The effect of siRNA is proved by RT-PCR in figure c. Compared with EP group, AP frequency increased in SI group (figure **b**). Compared with control group, the expression of kir6.1 and kir6.2 mRNA increased in EP group (figure d1 and d2). Compared with EP group, the expression of kir 6.1 and kir 6.2 mRNA decreased in SI group(figure d1 and d2). As to protein, compared with control group, the expression of kir6.1 and kir6.2 protein increased in EP group (figure f1 and f2). Compared with EP group, the expression of kir6.1 and kir6.2 protein decreased in SI group (figure f1 and f2). # represents compared with control group, $p < 0.05$.* represents compared with EP group, $p < 0.05$

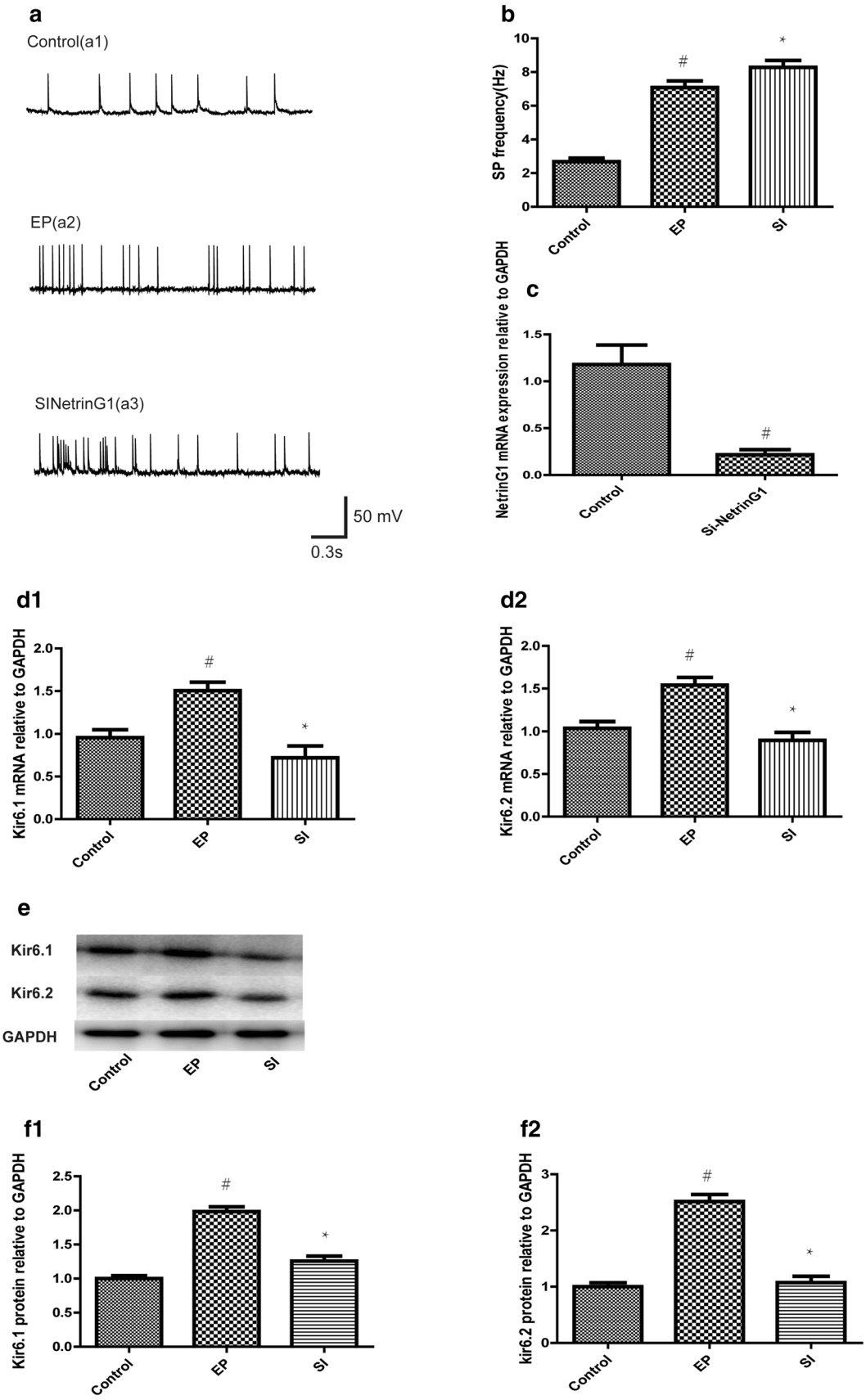
Results

Netrin-G1 Upregulated kir6.1 and kir6.2 Expressions In Vivo

The EPSI group included mice were transfected with si-netrin-G1 (50 nmol) before LiCl-pilocarpine treatment. The efficiency of siRNA was determined by RT-PCR ($p < 0.01$) and Western blotting (respectively shown in Fig. 1a1 and a2). Compared with the control group, 2DG intervention significantly increased the expressions of kir6.1 and kir6.2 both at mRNA and protein levels at day 1 and day 7 in lithium chloride-pilocarpine-induced epilepsy model (kir6.1 mRNA, $p = 0.041$, $p = 0.049$; kir6.1 protein, both $p < 0.01$; kir6.2 mRNA, $p = 0.047$, $p = 0.013$; kir6.2 protein, both $p < 0.01$) (shown in Fig. 1b, c, e, f). While knockdown of Netrin-G1 partially reversed these effects caused by 2DG reflected by decreased expressions of kir6.1 and kir6.2 (kir6.1 mRNA, $p = 0.025$, $p = 0.014$; kir6.1 protein, $p = 0.016$, $p < 0.01$; kir6.2 mRNA, $p = 0.037$, $p = 0.046$; kir6.2 protein, both $p < 0.01$) (shown in Fig. 1b, c, e, f).

Netrin-G1 Upregulated kir6.1 and kir6.2 Expressions and Activated KATP Channel In Vitro

In cultured neurons, the patch–clamp recordings were divided into three groups (shown in Fig. 2a). Cultured neurons without magnesium-free medium (non-MGF medium) served as the control group (Fig. 2a1), while MGF medium-treated group was named EP group (Fig. 2a2). According to other research articles, behavioral seizures (the defining feature of clinical epilepsy) cannot occur in cultured hippocampal neurons. Here, we recorded SP and used the terminologies outlined in comprehensive literature reviews, which were as follows: brief (typically < 100 msec), interictal-like


a
Control(a1)
EP(a2)
SINetrinG1(a3)
50 mV
0.3s
b
SP frequency(Hz)
Control
EP
SI
c
NetrinG1 mRNA expression relative to GAPDH
Control
Si-NetrinG1
d1
Kir6.1 mRNA relative to GAPDH
d2
Kir6.2 mRNA relative to GAPDH
e
Kir6.1
Kir6.2
GAPDH
f1
Kir6.1 protein relative to GAPDH
f2
kir6.2 protein relative to GAPDH

discharges as epileptiforms, epileptiform discharges, or epileptiform bursts, and longer (several sec, presumably ictal) seizure-like discharges as electrographic seizures [26–31]. Cultured neurons transfected with si-Netrin-G1 reagent (50 nmol) were categorized into SI group (Fig. 2a3). The effect of siRNA was detected by RT-PCR, as shown in Fig. 2c ($p < 0.01$). Compared with the control group, 2DG administration significantly increased the expressions of kir6.1 and kir6.2 at both mRNA and protein levels and decreased SP frequency in magnesium-free medium-induced epilepsy model (all $p < 0.01$) (shown in Fig. 2d, f), while knockdown of Netrin-G1 partially reversed these effects caused by 2DG (frequency, $p = 0.031$; mRNA and protein, all $p < 0.01$) (shown in Fig. 2b, d, f).

2DG Played an Anti-seizure Effect by Mediating NetrinG-1 to Regulate kir6.1,kir6.2 Expression In Vivo

LiCl-pilocarpine- and 2-DG (250 mg/kg)-treated mice were regarded as the 2DG group. The DGSI group is the mice treated with si-netrinG1 (50 mmol) before LiCl-pilocarpine and 2DG intervention. Compared with the EP group, 2DG administration significantly increased seizure latency time and decreased seizure duration($p < 0.01$, $p = 0.031$, $p = 0.014$) (Fig. 3b, c), while knockdown of Netrin-G1 partially reversed these effects caused by 2DG (EPSI group $p = 0.01$, $p = 0.378$, $p = 0.011$ DGSI group all $p < 0.01$). Figure 3a1 to a5 show the EEG recordings of the control, EP, 2DG, EPSI and DGSI groups. Compared with the EP group, treatment of mice with 2DG significantly increased the expressions of kir6.1 and kir6.2 both at mRNA and protein levels at day 1 and day 7 in lithium chloride-pilocarpine-induced epilepsy model. (kri6.1 mRNA, $p = 0.03$, $p = 0.041$; kir6.1 protein, $p = 0.01$, $p = 0.027$; kir6.2 mRNA, $p = 0.038$, $p = 0.044$; kir6.2 protein, $p = 0.048$, $p < 0.01$) (shown in Fig. 3d, e, g, h). While knockdown of Netrin-G1 partially reversed these effects caused by 2DG (DGSI group all, $p < 0.01$) (shown in Fig. 3d, e, g, h).

2DG Played Anti-Seizure Effects by Mediating Netrin-G1 to Regulate kir6.1, kir6.2 Expression and Activate KATP Channel In Vitro

Cultured hippocampal neurons were separately transfected with si-netrinG1 (50 nmol) and control reagent (50 nmol) at day 5 and treated with MGF medium at day 8. SP frequency was tested after MGF medium treatment for 3 h. The frequency of MGF group was increased by threefolds compared to the non-MGF medium group, while the frequency found in the MGF medium group was larger than 3 Hz. Compared with EP group, 2DG administration significantly decreased SP frequency ($p < 0.01$) (Fig. 4b), while knockdown of Netrin-G1 partially reversed the decreased SP frequency induced by 2DG ($p < 0.01$). In comparison with SI group, SP frequency of SI + DIA decreased and increased in SI + Gli, although this finding was not significant (SI + DIA group $p = 0.152$,SI + Gli group $p = 0.090$). Compared with the EP group, 2DG administration significantly increased the expressions of kir6.1 and kir6.2 both at mRNA and protein levels in magnesium-free medium-induced epilepsy model (kir6.1, $p = 0.044$, $p < 0.01$; kir6.2, $p = 0.012$, $p < 0.01$) (Fig. 4c, d), while knockdown of Netrin-G1 partially reversed these effects caused by 2DG (kir6.1, both $p < 0.01$; kir6.2, both $p < 0.01$).

Discussion

Epilepsy is a disorder of the brain characterized by an enduring predisposition to generating epileptic seizures. The glycolysis inhibitor 2-DG plays an anti-seizure effect in different models of seizure [32]. In a previous study, we found that 2-DG exerts anti-seizure effects by upregulating the expression of KATP subunits (kir6.1 and kir6.2) [22].Given that epilepsy is a plasticity-related disease and Netrin-G1 participates in the pathogenesis of synaptic plasticity-related diseases [14–18, 33], netrin-G1 might be also involved in the pathogenesis of epilepsy. In this experiment, our results first found that netrin-G1 has anti-seizure effects in a lithium chloride-pilocarpine-induced epilepsy model (in vivo epilepsy model) and magnesium-free medium-induced epilepsy model (in vitro epilepsy model), which was consistent with our hypothesis.

How did netrin-G1 have an anti-seizure effect both in vivo and vitro epilepsy models? We speculate that netrin-G1 may have anti-seizure effects by promoting axonal growth, regulating the synaptic formation, and inhibiting synaptic recombination and axon sprouting. The KATP channel may be the downstream effector of netrin-G1, and netrin-G1 may have an anti-seizure effect by mediating KATP channel. Our results show that the SP frequency was increased, while expressions of kir6.1 and kir6.2 were increased in vivo and vitro epilepsy models. These increases were significantly reversed after knocking down netrin-G1 expression, suggesting that netrin-G1 is an upstream regulator of KATP channel and has an anti-seizure effect by upregulating the expressions of kir6.1 and kir6.2 while activating KATP channels in in vivo and in vitro epilepsy models.

While our results in the in vivo epilepsy model are consistent with previous reports [22], they are inconsistent with the findings reported by Jiang et al [22, 34, 35]. The difference may be attributed to the difference in epilepsy models between the two studies. The characteristic of picrotoxin (PTX)-induced rats is that the seizure duration

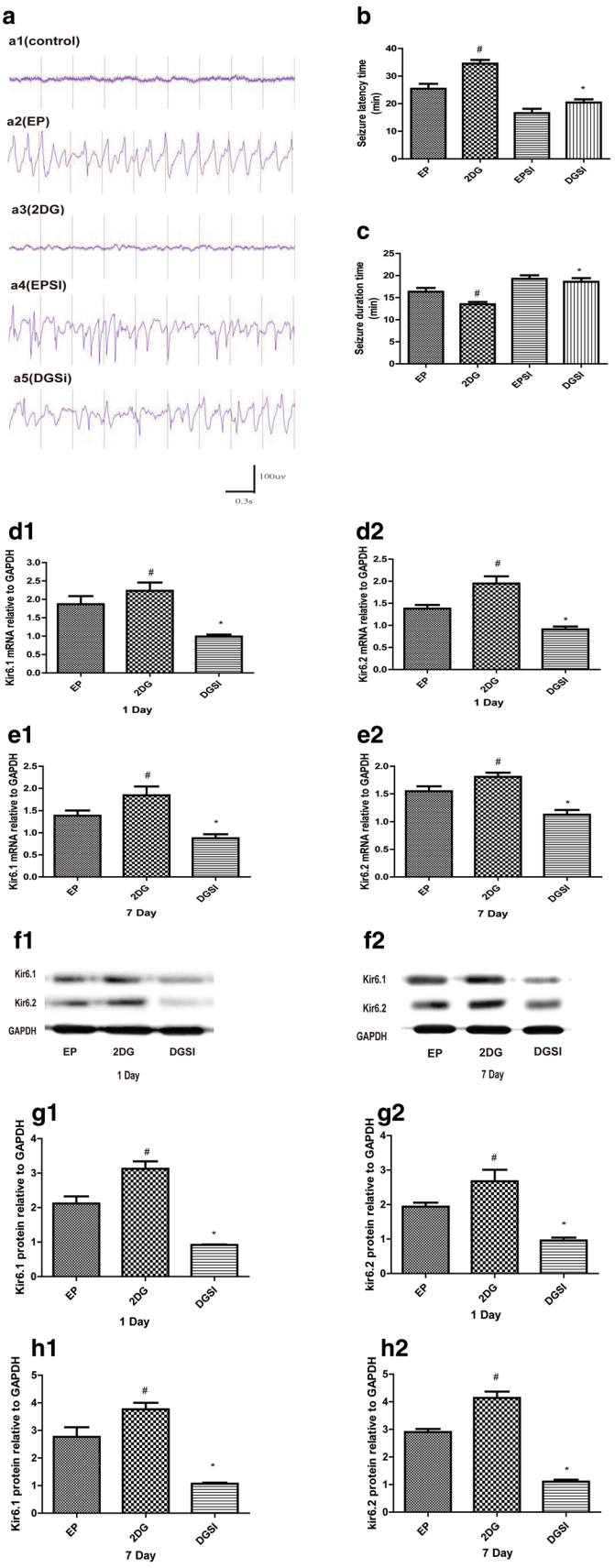
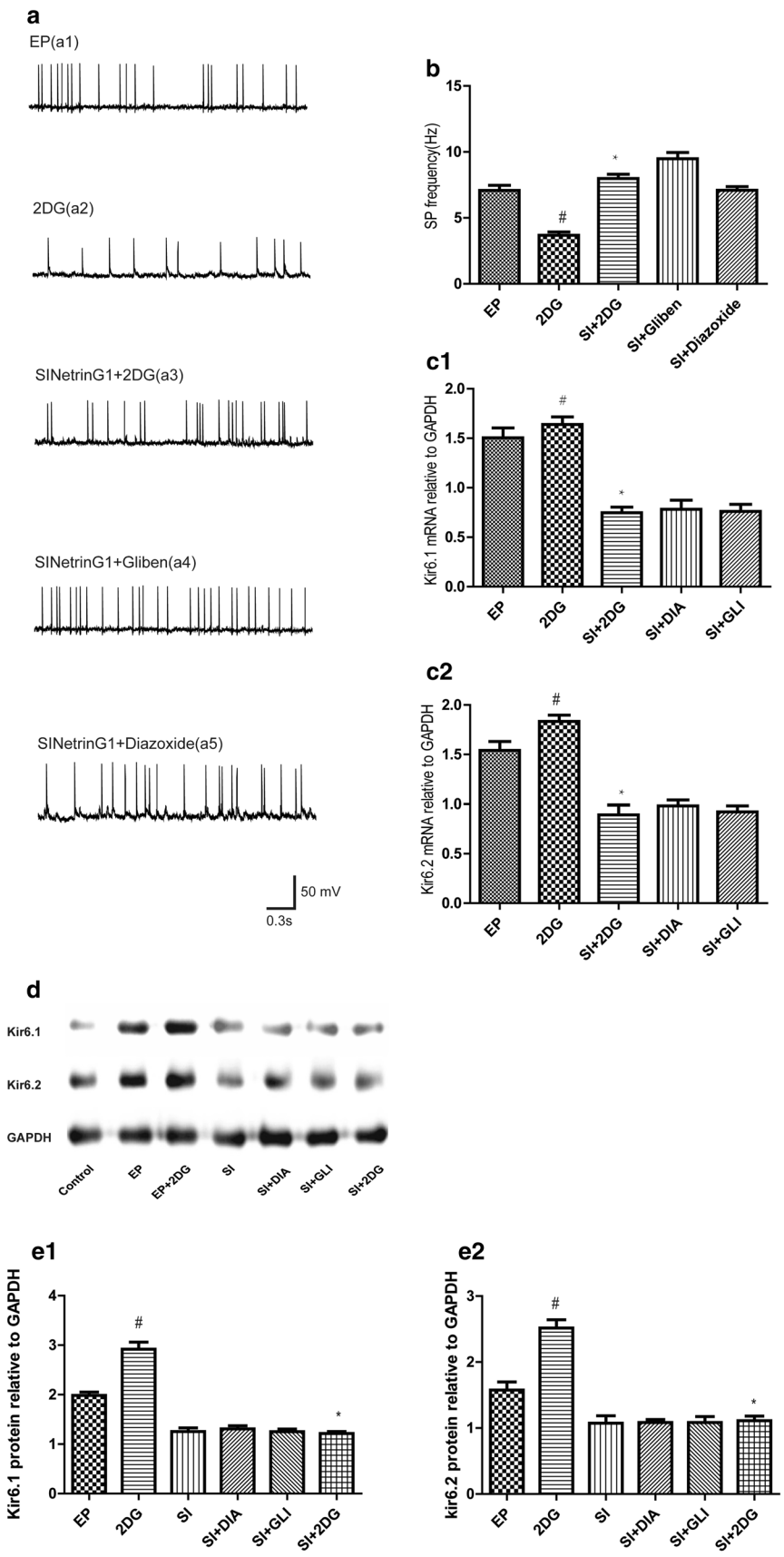


Fig. 3 2-DG plays anti-seizure effect by mediating Netrin-G1 to regulate kir6.1 and kir6.2 in vivo model. Figure a1 to a5 is the EEG recording of control, EP, 2DG, EPSI and DGSI groups. Compared with EP group (n = 6), seizure latency time increased and seizure duration decreased in 2DG group (n = 6) (figure **b** and **c**). Compared with EP group, seizure latency time decreased and seizure duration increased in EPSI group (n = 6). Compared with 2DG group, seizure latency time decreased and seizure duration increased in DGSI group (n = 6). Compared with EP group, the expression of kir6.1 mRNA and protein increased in 2DG group at day1 and day 7 (shown in figure d1, e1, g1 and h1). Compared with EP group, the expression of kir6.2 mRNA and protein increased in 2DG group at day1 and day7 (shown in figure d2, e2, g2 and h2). Compared with 2DG group, the expression of kir6.1 mRNA and protein decreased in DGSI group at day 1 and day 7 (shown in figure d1, e1, g1 and h1). Compared with 2DG group, the expression of kir6.2 mRNA and protein decreased in DGSI group at day 1 and day 7 (shown in figure d2, e2, g2 and h2). # represents compared with EP group, $p < 0.05$. *Represents compared with 2DG group, $p < 0.05$


a
EP(a1)
2DG(a2)
SINetrinG1+2DG(a3)
SINetrinG1+Gliben(a4)
SINetrinG1+Diazoxide(a5)
50 mV
0.3s
b
SP frequency(Hz)
EP
2DG
SI+2DG
SI+Gliben
SI+Diazoxide
c1
Kir6.1 mRNA relative to GAPDH
SI+DIA
SI+GLI
c2
Kir6.2 mRNA relative to GAPDH
d
Kir6.1
Kir6.2
GAPDH
Control
EP
EP+2DG
SI
SI+DIA
SI+GLI
SI+2DG
e1
Kir6.1 protein relative to GAPDH
e2
kir6.2 protein relative to GAPDH

◂**Fig. 4** 2-DG plays anti-seizure effect by mediating Netrin-G1 to regulate kir6.1 and kir6.2 and activate KATP channel in vitro model. Cultured hippocampal neurons were separated transfected with si-netrin-G1 (50 nmol) and control reagent (50 nmol) at DIV 5 and treated with MGF medium at DIV 8. SP frequency was tested after MGF medium treated 3 h. MGF epilepsy was built successfully for the frequency three times increased than non-MGF medium group, while the frequency of MGF medium group is larger than 3 Hz. (a1) represents recordings of MGF medium treated neurons (EP), which is identical to the same group in Fig. 2a2. (a2) represents recordings of neurons treated 2-DG 30 min before MGF medium treating (2DG). (a3) represents recordings of neurons transfected si-netrin G1 and treated 2-DG 30 min before MGF medium treating (SI + 2DG). (a4) represents recordings of neurons transfected si-netrin G1 and treated Gliben 30 min before MGF medium treating (SI + GLI). (a5) represents recordings of neurons transfected si-netrin G1 and treated Diazoxide 30 min before MGF medium treating (SI + DIA). While compared with EP group, SP frequency of 2DG group decreased (figure **b**). Compared with SI group, SP frequency of SI + DIA decreased and SI + GLI increased although no significance. Compared with 2DG group, SP frequency of DGSI group decreased. Compared with EP group, the expression of kir6.1 and kir6.2 mRNA increased in 2DG group (n = 6) (shown in figure c1 and c2). Compared with EP group, the expression of kir6.1 and kir6.2 mRNA decreased in SI group (n = 6). Compared with 2DG group, the expression of kir6.1 and kir6.2 mRNA decreased in SI + 2DG group (n = 6). The Western blot results shown in figure d. Compared with EP group, the expression of kir6.1 and kir6.2 protein increased in 2DG group (shown in figure e1 and e2). Compared with EP group, the expression of kir6.1 and kir6.2 protein decreased in SI group. Compared with 2DG group, the expression of kir6.1 and kir6.2 protein decreased in SI + 2DG group. #Represents compared with EP group, $p < 0.05$. *Represents compared with 2DG group, $p < 0.05$

is short, while repeated application of subconvulsive stimuli is needed to induce progressive seizure activity, which culminates in tonic–clonic convulsions. This might explain why mRNA expressions of kir6.1 and kir6.2 were decreased during the seizure-free interval in the hippocampus of picrotoxin (PTX)-induced model. Lithium chloride-pilocarpine induced epilepsy model exhibits more rapid acute status epilepticus followed by appearance of spontaneous recurrent seizures among several latent periods, and our team has demonstrated the expressions of kir6.1 and kir6.2 were increased in this model [22]. Our results are consistent with the findings of Alfredo Giménez-Cassina et al, who showed that KATP channel has an antiseizure effect [36].

A study by He XP et al showed that PLCγ1 is activated in the pilocarpine epilepsy model. Protein kinase C is an identified downstream protein of DAG, which act as a secondary messenger in the PLCγ signal pathway. More importantly, studies have shown that PLCγ activates protein kinase C [37]. There are many studies demonstrated that KATP channel is inhibited by protein kinase C [38–40] and netrin-G1 shows a specific association with PKC signaling [41]. Thus, netrin-G1 may exert an anti-seizure effect by mediating PLCγ-PKC-KATP channel signaling pathway. However, the details of this mechanism need to be further explored.

In our experiment, we investigated whether 2DG exerts its anti-seizure effect through netrin-G1-KATP signaling pathway. Our findings show that 2DG pretreatment increased the mRNA and protein levels of kir6.1 and kir6.2, as well as decreased SP frequency both in vivo and vitro epilepsy models. Furthermore, these effects were significantly reversed after knocking down netrin-G1 expression. The results show that 2DG exerts an anti-seizure effect through netrin-G1-KATP signaling pathway in in vivo and vitro epilepsy models. The results of behavioral and EEG recordings are consistent with our previous reports [22]. In addition, Forte's findings are similar to our results, which showed that 2DG has antiseizure effects by potentiating the extrasynaptic tonic GABAergic current in hippocampal slices [42].

It is currently believed that the mechanism of the anti-seizure effect of 2-DG is based on decreased expression of brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (trkB) [43]. No study has explored the possible association of2-DG's anti-seizure effect with netrin-G1 mediated PLCγ-PKC-KATP channel signaling pathway.

Currently, there is inadequate control over recurrent seizures in the majority of patients, despite the use of available anti-seizure drugs. Our study shows that 2DG may be used in seizure prevention through its interactions with netrin-G1 and activation of KATP channels. While there are still many unclear mechanisms that need to be explored, no study has elucidated the details of the interactions between netrin-G1 and KATP channels thus far. Our study provides a novel insight into the interaction between netrin-G1 and activation of KATP channels in epilepsy. Further studies exploring the antiepileptic mechanisms underlying the role of netrin-G1-KATP signal pathway are wanted.

Acknowledgements The work was supported by the National Natural Science Foundation of China (81501128) and National Natural Science Foundation of China (81671296). The Jiyizhupao of Third Xiangya Hospital (JY201520). Thanks to the Experimental Animal Center of the Third Xiangya Hospital of Central South University for affording animals.

Author Contributions Yuming Long and Kai Zhuang were both the first author to this article. Heng Yang and Zhi Song were both the corresponding author to this article. Yuming Long and Kai Zhuang conducted all the experiments and drafed the main manuscript text. Zhonghai Ji, Yaru Han, Yanqing Fei and Wen Zheng made statistical analysis and prepared all the figures in this article. Heng Yang and Zhi Song designed the whole experiment procedure and supervised the research.

Compliance with Ethical Standards

Conflict of interest The authors have declared that no competing interests exist.

References

1. Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE et al (2014) ILAE official report: a practical clinical definition of epilepsy. EPILEPSIA 55:475–482
2. Tanywe A, Matchawe C, Fernandez R (2016) The experiences of people living with epilepsy in developing countries: a systematic review of qualitative evidence. JBI Database System Rev Implement Rep 14:136–192
3. Nadler JV (2003) The recurrent mossy fiber pathway of the epileptic brain. Neurochem Res 28:1649–1658
4. Pan Y, Liu G, Fang M, Shen L, Wang L, Han Y et al (2010) Abnormal expression of netrin-G2 in temporal lobe epilepsy neurons in humans and a rat model. Exp Neurol 224:340–346
5. Yin Y, Miner JH, Sanes JR (2002) Laminets: laminin- and netrin-related genes expressed in distinct neuronal subsets. Mol Cell Neurosci 19:344–358
6. Moore SW, Tessier-Lavigne M, Kennedy TE (2007) Netrins and their receptors. Adv Exp Med Biol 621:17–31
7. Meerabux JM, Ohba H, Fukasawa M, Suto Y, Aoki-Suzuki M, Nakashiba T et al (2005) Human netrin-G1 isoforms show evidence of differential expression. GENOMICS 86:112–116
8. Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, Culotti JG et al (2000) Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. J Neurosci 20:6540–6550
9. Lin JC, Ho WH, Gurney A, Rosenthal A (2003) The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. Nat Neurosci 6:1270–1276
10. Kim S, Burette A, Chung HS, Kwon SK, Woo J, Lee HW et al (2006) NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. Nat Neurosci 9:1294–1301
11. Aoki-Suzuki M, Yamada K, Meerabux J, Iwayama-Shigeno Y, Ohba H, Iwamoto K et al (2005) A family-based association study and gene expression analyses of netrin-G1 and -G2 genes in schizophrenia. Biol Psychiatry 57:382–393
12. Medina-Ceja L, Pardo-Pena K, Ventura-Mejia C (2014) Evaluation of behavioral parameters and mortality in a model of temporal lobe epilepsy induced by intracerebroventricular pilocarpine administration. Neuroreport. https://doi.org/10.1097/WNR.0000000000000207
13. Woo J, Kwon SK, Kim E (2009) The NGL family of leucine-rich repeat-containing synaptic adhesion molecules. Mol Cell Neurosci 42:1–10
14. Ohtsuki T, Horiuchi Y, Koga M, Ishiguro H, Inada T, Iwata N et al (2008) Association of polymorphisms in the haplotype block spanning the alternatively spliced exons of the NTNG1 gene at 1p13.3 with schizophrenia in Japanese populations. Neurosci Lett 435:194–197
15. Eastwood SL, Harrison PJ (2008) Decreased mRNA expression of netrin-G1 and netrin-G2 in the temporal lobe in schizophrenia and bipolar disorder. Neuropsychopharmacol 33:933–945
16. Zakharyan R, Boyajyan A, Arakelyan A, Gevorgyan A, Mrazek F, Petrek M (2011) Functional variants of the genes involved in neurodevelopment and susceptibility to schizophrenia in an Armenian population. Hum Immunol 72:746–748
17. Zhu Y, Yang H, Bi Y, Zhang Y, Zhen C, Xie S et al (2011) Positive association between NTNG1 and schizophrenia in Chinese Han population. J Genet 90:499–502
18. Stepanyan A, Zakharyan R, Boyajyan A (2013) The netrin G1 gene rs628117 polymorphism is associated with ischemic stroke. Neurosci Lett 549:74–77
19. Foster MN, Coetzee WA (2016) KATP channels in the cardiovascular system. Physiol Rev 96:177–252
20. Zhou M, Tanaka O, Suzuki M, Sekiguchi M, Takata K, Kawahara K et al (2002) Localization of pore-forming subunit of the ATP-sensitive K(+)-channel, Kir6.2, in rat brain neurons and glial cells. Brain Res Mol Brain Res 101:23–32
21. Wickenden AD (2002) Potassium channels as anti-epileptic drug targets. Neuropharmacology 43:1055–1060
22. Yang H, Guo R, Wu J, Peng Y, Xie D, Zheng W et al (2013) The antiepileptic effect of the glycolytic inhibitor 2-deoxy-D-glucose is mediated by upregulation of K(ATP) channel subunits Kir6.1 and Kir6.2. Neurochem Res 38:677–685
23. McIntyre DC, Poulter MO, Gilby K (2002) Kindling: some old and some new. Epilepsy Res 50:79–92
24. Raedt R, Van Dycke A, Van Melkebeke D, De Smedt T, Claeys P, Wyckhuys T et al (2009) Seizures in the intrahippocampal kainic acid epilepsy model: characterization using long-term video-EEG monitoring in the rat. Acta Neurol Scand 119:293–303
25. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
26. Rutecki PA, Lebeda FJ, Johnston D (1985) Epileptiform activity induced by changes in extracellular potassium in hippocampus. J Neurophysiol 54:1363–1374
27. Traynelis SF, Dingledine R (1988) Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. J Neurophysiol 59:259–276
28. Traynelis SF, Dingledine R, McNamara JO, Butler L, Rigsbee L (1989) Effect of kindling on potassium-induced electrographic seizures in vitro. Neurosci Lett 105:326–332
29. Tancredi V, Hwa GG, Zona C, Brancati A, Avoli M (1990) Low magnesium epileptogenesis in the rat hippocampal slice: electrophysiological and pharmacological features. Brain Res 511:280–290
30. Sombati S, Delorenzo RJ (1995) Recurrent spontaneous seizure activity in hippocampal neuronal networks in culture. J Neurophysiol 73:1706–1711
31. Clark S, Wilson WA (1999) Mechanisms of epileptogenesis. Adv Neurol 79:607–630
32. Gasior M, Yankura J, Hartman AL, French A, Rogawski MA (2010) Anticonvulsant and proconvulsant actions of 2-deoxy-d-glucose. Epilepsia 51:1385–1394
33. Matsukawa H, Akiyoshi-Nishimura S, Zhang Q, Lujan R, Yamaguchi K, Goto H et al (2014) Netrin-G/NGL complexes encode functional synaptic diversification. J Neurosci 34:15779–15792
34. Jiang K, Shui Q, Xia Z, Yu Z (2004) Changes in the gene and protein expression of K(ATP) channel subunits in the hippocampus of rats subjected to picrotoxin-induced kindling. Brain Res Mol Brain Res 128:83–89
35. Jiang KW, Gao F, Shui QX, Yu ZS, Xia ZZ (2004) Effect of diazoxide on regulation of vesicular and plasma membrane GABA transporter genes and proteins in hippocampus of rats subjected to picrotoxin-induced kindling. Neurosci Res 50:319–329
36. Gimenez-Cassina A, Martinez-Francois JR, Fisher JK, Szlyk B, Polak K, Wiwczar J et al (2012) BAD-dependent regulation of fuel metabolism and K(ATP) channel activity confers resistance to epileptic seizures. Neuron 74:719–730
37. He XP, Pan E, Sciarretta C, Minichiello L, McNamara JO (2010) Disruption of TrkB-mediated phospholipase Cgamma signaling inhibits limbic epileptogenesis. J Neurosci 30:6188–6196
38. Aziz Q, Thomas AM, Khambra T, Tinker A (2012) Regulation of the ATP-sensitive potassium channel subunit, Kir6.2, by a Ca2+-dependent protein kinase C. J Biol Chem 287:6196–6207
39. Shi Y, Cui N, Shi W, Jiang C (2008) A short motif in Kir6.1 consisting of four phosphorylation repeats underlies the vascular KATP channel inhibition by protein kinase C. J Biol Chem 283:2488–2494
40. Park WS, Ko EA, Han J, Kim N, Earm YE (2005) Endothelin-1 acts via protein kinase C to block KATP channels in rabbit

coronary and pulmonary arterial smooth muscle cells. J Cardiovasc Pharmacol 45:99–108
41. Wierda KD, Toonen RF, de Wit H, Brussaard AB, Verhage M (2007) Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. Neuron 54:275–290
42. Forte N, Medrihan L, Cappetti B, Baldelli P, Benfenati F. 2-Deoxy-d-glucose enhances tonic inhibition through the neurosteroid-mediated activation of extrasynaptic GABAA receptors. Epilepsia 2016
43. Garriga-Canut M, Schoenike B, Qazi R, Bergendahl K, Daley TJ, Pfender RM et al (2006) 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. Nat Neurosci 9:1382–1387