NEURAL REGENERATION RESEARCH
April 2015, Volume 10, Issue 4
www.nrronline.org

RESEARCH ARTICLE

Short-term use of antiepileptic drugs is neurotoxic to the immature brain

Yu Liu[#], Xue-ying Wang, Dan Li, Lin Yang[*,#], Shao-ping Huang

Department of Pediatrics, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China

***Correspondence to:**
Lin Yang, M.D., yr_1217@163.com.

 # These authors contributed equally to this work.

***doi:**10.4103/1673-5374.155434*
***http:**//www.nrronline.org/*

***Accepted:** 2015-03-21*

Abstract

Previous studies have shown that the long-term use of antiepileptic drugs can cause nervous system damage. However, short-term antiepileptic drug treatment is frequently given to infants, especially neonates, to control seizure. Whether the short-term use of antiepileptic drugs is neurotoxic remains unclear. In the present study, immature rats, 3–21 days of age, were intraperitoneally injected with phenobarbital and/or topiramate for 3 consecutive days. Hematoxylin-eosin and immunohistochemical staining revealed that phenobarbital and topiramate, individually or in combination, were cytotoxic to hippocampal CA1 neurons and inhibited the expression of GluR1 and NR2B, excitatory glutamate receptor subunits. Furthermore, the combination of the two drugs caused greater damage than either drug alone. The results demonstrate that the short-term use of antiepileptic drugs damages neurons in the immature brain and that the combined use of antiepileptic drugs exacerbates damage. Our findings suggest that clinicians should consider the potential neurotoxic risk associated with the combined use of antiepileptic drugs in the treatment of seizure.

***Key Words:** nerve regeneration; seizure; antiepileptic drugs; immature brain; hippocampus; synaptic plasticity; glutamate receptor; NSFC grant; neural regeneration*

***Funding:** This study was financially supported by grants from the National Natural Science Foundation of China, No. 30973224; Talent Cultivation Funds of the Second Affiliated Hospital of Xi'an Jiaotong University of China, No. RC(XM)200908; a grant from the Key Science and Technology Project of Shaanxi Province of China, No. 2006k15-G1(2).*

Liu Y, Wang XY, Li D, Yang L, Huang SP (2015) Short-term use of antiepileptic drugs is neurotoxic to the immature brain. Neural Regen Res 10(4):599-604.

Introduction

A seizure is a sudden and transient cerebral dysfunction caused by abnormally excessive or synchronous neuronal activity (Clancy, 2006; Kiviranta et al., 2013; Liu et al., 2013). Clinical and laboratory studies have shown that convulsions, which are the physiological manifestations of seizures, can cause severe brain damage and long-term neurological sequelae (Lynch et al., 2000; Ferro et al., 2014; Reilly et al., 2014). At present, antiepileptic drugs (AEDs) are the preferred treatment choice for the prevention and control of seizures. Phenobarbital is still the first choice (Bartha et al., 2007; Blume et al., 2009; Kiviranta et al., 2013). A new antiepileptic drug, topiramate, is widely used for the control of convulsions in newborns and infants (Silverstein and Ferriero, 2008; Demir et al., 2013).

Although AEDs inhibit seizures, they also inhibit neuronal excitability, which may affect the normal functioning of the nervous system (Meador, 2003). Additionally, some AEDs are neurotoxic, and administration of AEDs to embryos, neonates and infants can cause cognitive impairment, microcephaly and birth defects (Sulzbacher et al., 1999; Jevtovic-Todorovic et al., 2003; Kaindl et al., 2006; Viinikainen et al., 2006; Verrotti et al., 2014). While it remains unclear how AEDs elicit neurotoxicity, short-term AED treatment is frequently given to infants, especially neonates, to control seizure. AEDs are given for a few days to months.

It is unknown whether the short-term use of AEDs in infants can impact the expression of glutamate receptors. There are three major glutamate receptors in the brain: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptors (Whitlock et al., 2006; Benarroch, 2008). GluR1 and NR2B are key subunits of AMPA and NMDA receptors, respectively (Omkumar et al., 1996; Cull-Candy et al., 2001; Lee et al., 2003; Nakagawa et al., 2005; Huganir and Nicoll, 2013). Whitlock et al. (2006) showed that the expression of the hippocampal AMPA receptor subunit GluR1/2 increased in rats during inhibitory avoidance training. Tang et al. (1999) demonstrated that the expression levels of NR2B in the hippocampus and cortex of NR2B-overexpressing transgenic rats were twice the normal levels. In the present study, immature rats were intraperitoneally injected with AEDs, and we examined cytohistological changes, as well as changes in the expression of GluR1 and NR2B, in the hippocampus, in an effort to better understand the effects of the short-term use of AEDs on the developing brain.

Materials and Methods

Animals

A total of 192 immature Sprague-Dawley rats, regardless of gender, of specific pathogen-free grade, were obtained from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, Shaanxi Province, China; license No. SCXK (Shaan) 2007-001). All rats were housed in a quiet, dry and airy environment prior to experimentation, and were allowed *ad libitum* access to food and water. The experimental procedures using animals were in accordance with the guidelines and standards formulated by the Animal Ethics Committee of Xi'an Jiaotong University, China. Sprague-Dawley rats were randomly and equally divided into four groups: phenobarbital (PB) group, topiramate (TPM) group, TPM + PB group and control group, with 48 rats in each group.

AED treatment in immature rats

At 3, 7, 14 and 21 days after birth, 12 rats from each group were randomly selected. The body surface area was used to calculate the dosage of drugs administered to each rat, as previously described (Manent et al., 2007; Aydin-Abidin et al., 2012; Forcelli et al., 2012). Phenobarbital was purchased from Fujian Mindong Rejuvenation Pharmaceutical Co., Ltd. (Fuzhou, Fujian Province, China; 100 mg phenobarbital dissolved in 32 mL saline). Topiramate was purchased from Xi'an Janssen Pharmaceutical Ltd. (Xi'an, Shaanxi Province, China; 25 mg topiramate dissolved in 12.5 mL saline). Rats in the PB group were injected intraperitoneally with 62.5 mg/kg phenobarbital per day (equivalent to a clinical dose of 10 mg/kg per day). Rats in the TPM group were injected intraperitoneally with 40 mg/kg topiramate per day (equivalent to a clinical dose of 7 mg/kg per day). Rats in the TPM + PB group were injected intraperitoneally with 40 mg/kg topiramate + 62.5 mg/kg phenobarbital per day. Rats in the control group were injected intraperitoneally with 20 mL/kg normal saline per day. All drugs were administered for 3 consecutive days. All rats were breastfed up to 21 days and then fed with normal diet until 6 weeks after birth.

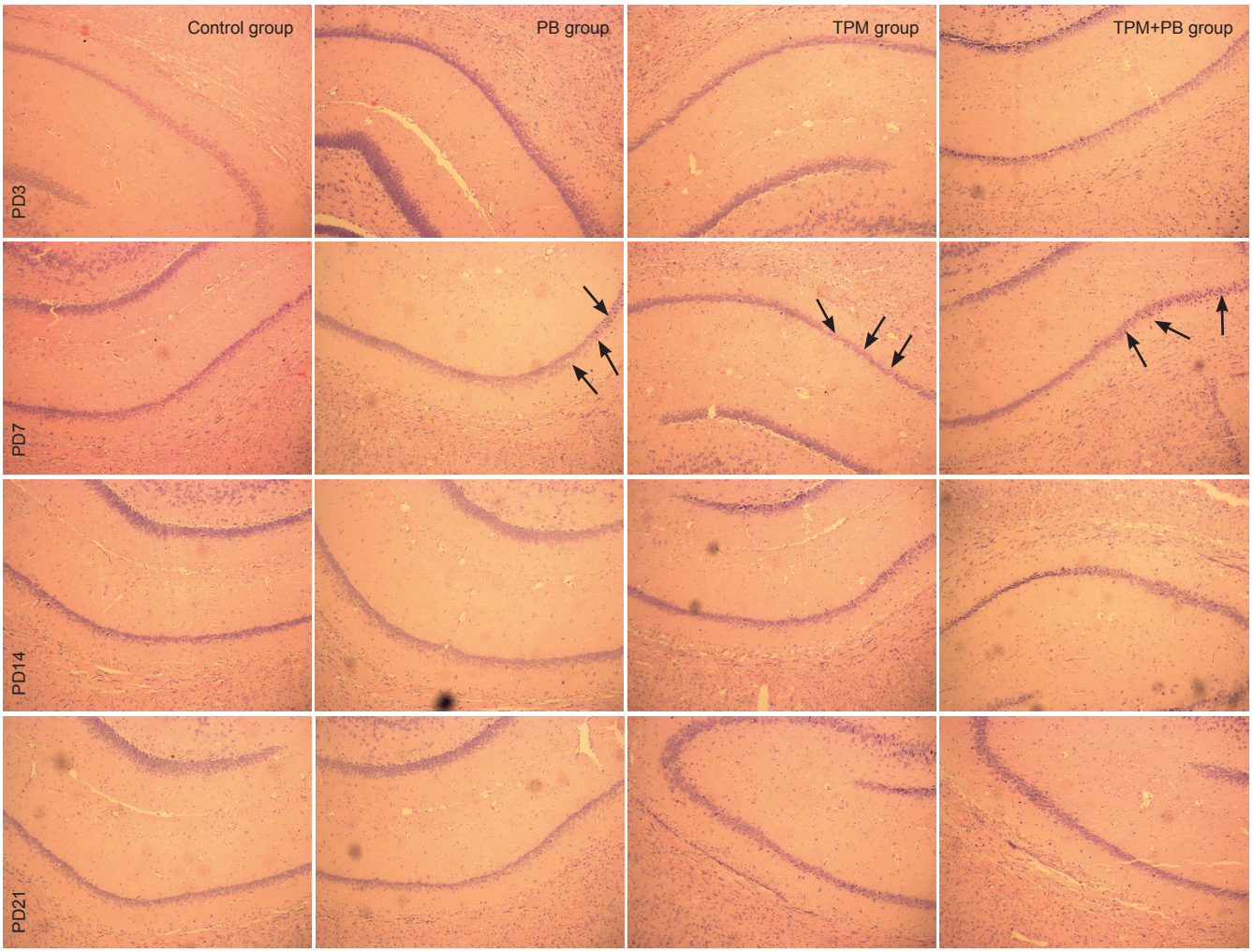

Figure 1 Effects of phenobarbital (PB) and topiramate (TPM) on the morphology of neurons in the hippocampal CA1 region in immature rats (hematoxylin-eosin staining, × 100).

In the control group, hippocampal neurons were distributed in a regular and uniform manner. In the PB, TPM and TPM + PB groups, hippocampal neurons were irregularly arranged. Arrows indicate hippocampal neurons. PD3, 7, 14, 21: Postnatal days 3, 7, 14, 21 (indicating when the rat was injected with drug).

Hematoxylin-eosin staining

After rats were anesthetized with 10% chloral hydrate through intraperitoneal injection, killed, and the brains were harvested. The brains were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3-μm-thick continuous coronal sections. For observation under an optical microscope (Olympus, Tokyo, Japan), the slices were dewaxed and stained with hematoxylin and eosin; neutral balsam was used for mounting (Huang et al., 2010).

Immunohistochemical staining

The slices were dewaxed, dehydrated in a graded ethanol series, and treated with freshly prepared 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidase and 0.01 M citrate buffer for antigen retrieval. Slices were blocked with normal goat serum for 10 minutes and incubated with rabbit anti-rat GluR1 monoclonal antibody (1:1,000; Millipore Corporation, Billerica, MA, USA) or mouse anti-NR2B polyclonal antibody (1:500; LifeSpan BioSciences, Seattle, WA, USA) at 4°C overnight. Subsequently, slices were incubated with 50 μg biotinylated goat anti-rabbit/mouse IgG (1:2,000; Amyjet Scientific Inc, Wuhan, Hubei Province, China) at 37°C for 30 minutes, followed by incubation in ABC complex solution for 30 minutes. Finally, the sections were developed with diaminobenzidine for 5 minutes. An optical microscope was used to observe the immunoreactivities of GluR1 and NR2B in the CA1 region of the hippocampus (Zhang et al., 2012). Three slices selected at random were tested for each specimen, and the average gray value was computed using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

All measurement data were expressed as the mean ± SD. Two-way analysis of variance was used for statistical comparison of the means. Significant results were analyzed with Tukey's *post hoc* test using SPSS 13.0 software (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered statistically significant.

Results

Effects of phenobarbital and topiramate on the morphology of neurons in the hippocampal CA1 region in immature rats

Hematoxylin-eosin staining showed that, in the control group, hippocampal neurons in immature rats were distributed in a regular and uniform manner, and had a normal morphology, without signs of degeneration or necrosis. The cell membrane was clearly visible and the cytoplasm was weakly eosinophilic. The nuclei were large, with a regular (oval or circular) shape, and were weakly eosinophilic. The nucleolus was clear and uniform, and no nuclear pyknosis was noted. Neurons were neatly aligned. In the PB, TPM and TPM + PB groups, hippocampal neurons were irregularly arranged, the proportion of glial cells was increased, and neurons showed signs of degeneration. Cell volumes were higher than in the control group, and the cytoplasm was strongly eosinophilic and its volume was increased. Furthermore, nuclear shrinkage was noted. The neurons were not well aligned. The younger the rat, the worse the alignment (**Figure 1**).

Effects of phenobarbital and topiramate on GluR1 and NR2B immunoreactivities in the hippocampal CA1 region in immature rats

Immunohistochemical staining showed that the immunoreactivities of GluR1 and NR2B in the hippocampal CA1 region in rats in the PB, TPM and TPM + PB groups at different ages was lower than in the control group ($P < 0.05$). Furthermore, the immunoreactivities of GluR1 and NR2B in the hippocampal CA1 region was lower in the TPM + PB group than in the PB or TPM group ($P < 0.05$, except GluR1 immunoreactivity at 21 days after birth). The immunoreactivities of GluR1 and NR2B in the hippocampal CA1 region in the PB, TPM and TPM + PB groups increased with age ($P < 0.05$; **Figures 2**, **3**).

Discussion

Increasing evidence shows that phenobarbital, valproate, lamotrigine and other AEDs are neurotoxic to immature brains (Viinikainen et al., 2006; Halbsgut et al., 2013; Morte et al., 2013). However, very few studies have examined the effects of AEDs on the immature brain at different ages after birth. In the present study, immature rats received intraperitoneal injection of AEDs at 3, 7, 14 or 21 days after birth. We found that the expression levels of GluR1 and NR2B receptors were all reduced by the AEDs. Furthermore, the AEDs had neurotoxic effects. In the immature brain, the earlier the AED treatment, the greater the injury to the brain. However, AEDs are widely used to control seizures in infants and young children. Our findings suggest that clinicians should consider the neurotoxic risk associated with the use of AEDs in this population.

Accumulating evidence indicates that the long-term use of AEDs in fetuses and infants causes cognitive impairment, intellectual deficits, microcephaly, and birth defects (Kaindl et al., 2006; Manent et al., 2007). Previous studies have shown that AEDs increase the activation of GABA receptors and induce rapid changes in the composition and function of AMPA receptor GluR1 subunits in the immature brain (Auberson et al., 2001; Manent et al., 2007).

Published studies have primarily examined the neurotoxicity of AEDs after long-term treatment. However, the neurotoxic effects of short-term treatment remained unclear. Therefore, in the present study, we aimed to determine whether short-term AED treatment can cause injury to the immature brain. In rats treated with AEDs (phenobarbital, topiramate or phenobarbital + topiramate), the expression levels of GluR1 and NR2B in each treatment group were lower than in the control group. This indicates that even short-term AED treatment can result in brain damage. Therefore, we recommend that clinicians take into consideration this potential risk before using AEDs for the treatment of seizure.

Phenobarbital, a traditional AED, has been widely demonstrated to have neurotoxic effects (Sulzbacher et al., 1999; Bittigau et al., 2002; Jevtovic-Todorovic et al., 2003; Viinikainen

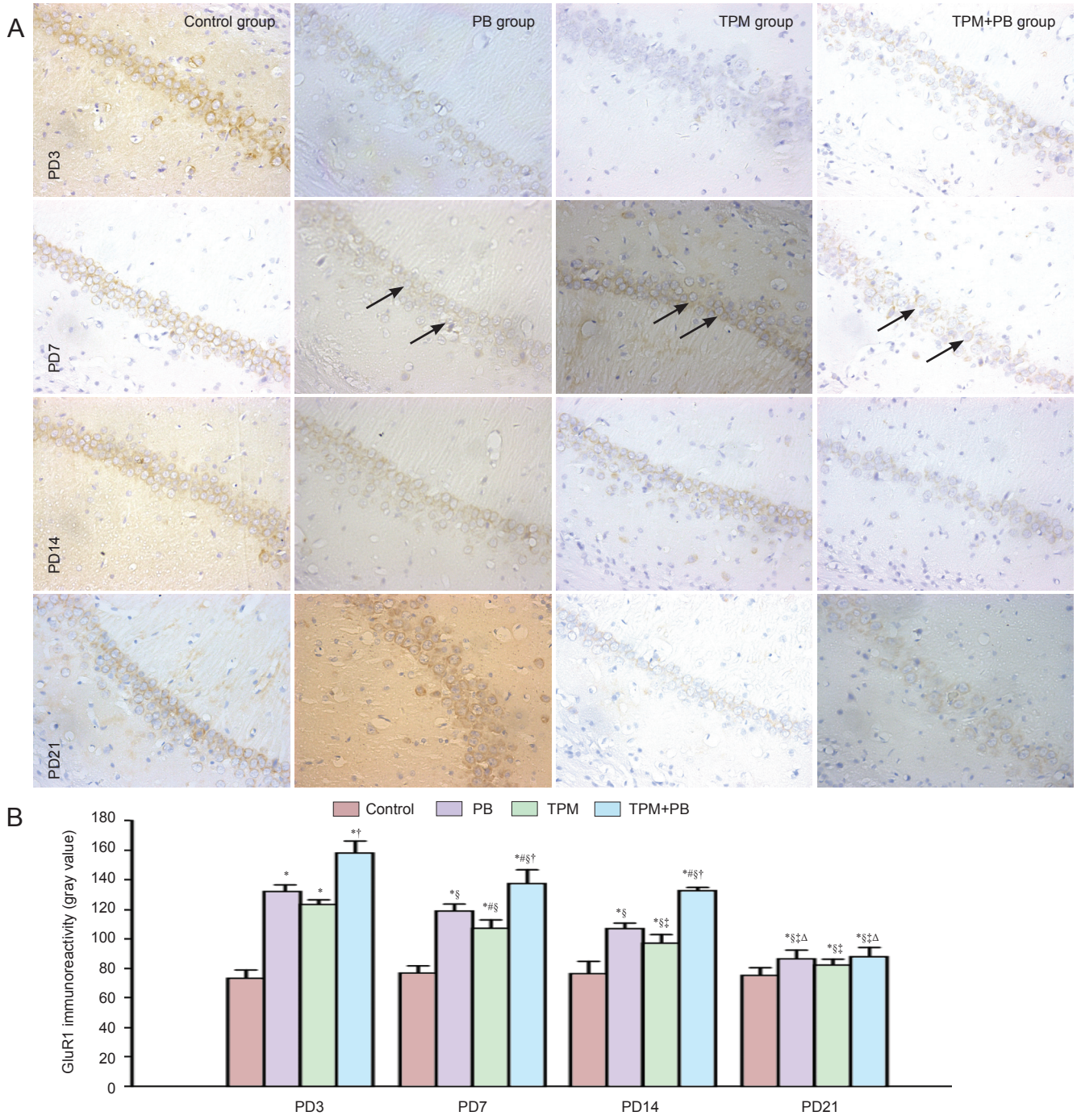

Figure 2 Effects of phenobarbital (PB) and topiramate (TPM) on AMPA receptor GluR1 immunoreactivity in the hippocampal CA1 region in immature rats.

(A) GluR1 immunoreactivity in the hippocampal CA1 region in immature rats (immunohistochemical staining, × 400). The brown color represents receptors on the cell membrane (arrows). (B) Quantification of GluR1-immunoreactive cells. All data are expressed as the mean ± SD. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* PB group; †$P < 0.05$, *vs.* TPM group; §$P < 0.05$, *vs.* PD3; ‡$P < 0.05$, *vs.* PD7; Δ$P < 0.05$, *vs.* PD14 (two-way analysis of variance and Tukey's *post hoc* test). PD3, 7, 14, 21: Postnatal days 3, 7, 14, 21 (indicating when the rat was injected with drug); AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid.

et al., 2006; Manent et al., 2007). Topiramate, a newer AED, may also have neurotoxic effects, although its neurotoxicity is unclear (Shannon et al., 2005; Filippi et al., 2012; Sommer et al., 2013). Topiramate can inhibit voltage-gated Na^+ channels and activate GABA receptors (Kwan and Brodie, 2004), similar to phenobarbital. Thus, we speculate that topiramate is also neurotoxic.

We found in this study that the expression of GluR1 and NR2B in all the groups was decreased compared with the control group. However, there was no difference between the PB group and the TPM group. In the combined treatment (PB + TPM) group, the expression levels of GluR1 and NR2B decreased more significantly compared with the PB and TPM groups. In addition, the combination of phenobarbital and

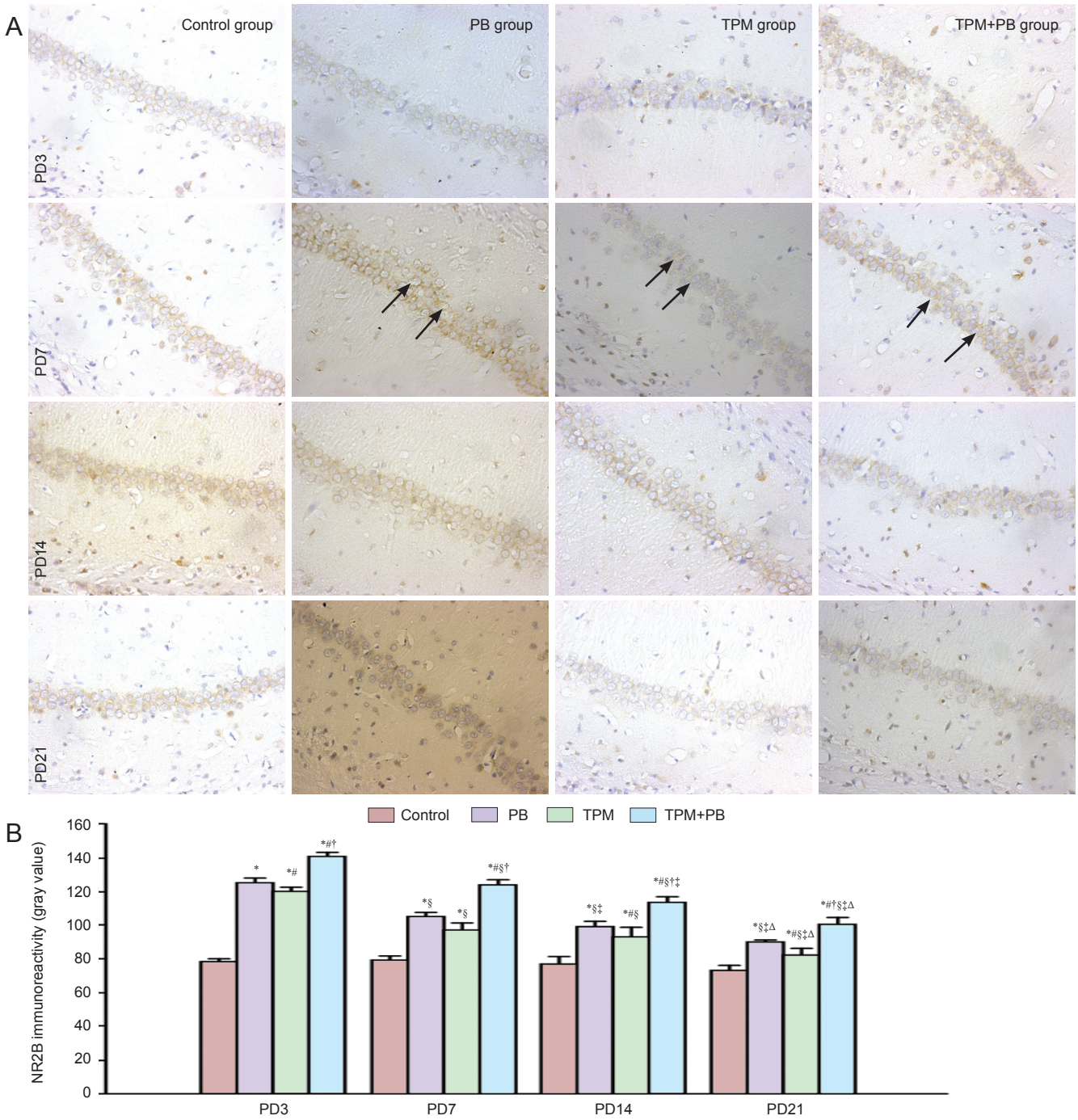

Figure 3 Effects of phenobarbital (PB) and topiramate (TPM) on NMDA receptor NR2B immunoreactivity in the hippocampal CA1 region in immature rats.

(A) NR2B immunoreactivity in the hippocampal CA1 region in immature rats (immunohistochemical staining, × 400). The brown color represents receptors on the cell membrane (arrows). (B) Quantification of NR2B-immunoreactive cells. All data are expressed as the mean ± SD. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* PB group; †$P < 0.05$, *vs.* TPM group; §$P < 0.05$, *vs.* PD3; ‡$P < 0.05$, *vs.* PD7; Δ$P < 0.05$, *vs.* PD14 (two-way analysis of variance and Tukey's *post hoc* test). PD3, 7, 14, 21: Postnatal days 3, 7, 14, 21 (indicating when the rat was injected with drug); NMDA: N-methyl-D-aspartate.

topiramate caused greater damage to the immature brain than either drug alone. Therefore, the immature brain will likely be injured by short-term treatment with these anticonvulsants (Tebb and Tobias., 2006).

In summary, we demonstrate that short-term AED treatment is neurotoxic, and that the neurotoxicity is greater in younger rats. Therefore, even short-term treatment with phenobarbital, topiramate or other AEDs will likely cause damage to the immature brain. The combination of phenobarbital and topiramate caused greater damage than either drug alone. Therefore, this neurotoxic risk should be considered before using AEDs for the treatment of seizure. We anticipate that our findings should encourage the development of AEDs with lower neurotoxicity.

Author contributions: *YL wrote the manuscript. All authors were responsible for designing the experiment, implementing the experiments, evaluating the study and approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

References

Auberson YP (2001) Compitive AMPA antagonism: a novel mechanism for antiepileptic drugs? Drugs Fut 26:463-471.
Aydin-Abidin S, Yildirim M, Abidin İ, Cansu A (2012) Chronic application of topiramate and carbamazepine differentially affects the EEG and penicillin-induced epileptiform activity in rats. Neurol Res 34:246-251.
Bartha AI, Shen J, Katz KH, Mischel RE, Yap KR, Ivacko JA, Andrews EM, Ferriero DM, Ment LR, Silverstein FS (2007) Neonatal seizures: multicenter variability in current treatment practices. Pediatr Neurol 37:85-90.
Benarroch EE (2008) Metabotropic glutamate receptors: synaptic modulators and therapeutic targets for neurologic disease. Neurology 70:964-968.
Bittigau P, Sifringer M, Genz K, Reith E, Pospischil D, Govindarajalu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW, Ikonomidou C (2002) Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. Proc Natl Acad Sci U S A 99:15089-15094.
Blume HK, Garrison MM, Christakis DA (2009) Neonatal seizures: treatment and treatment variability in 31 United States pediatric hospitals. J Child Neurol 24:148-154.
Clancy RR (2006) Summary proceedings from the neurology group on neonatal seizures. Pediatrics 117:S23-27.
Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327-335.
Demir CF, Ozdemir HH, Müngen B (2013) Efficacy of topiramate as add-on therapy in two different types of progressive myoclonic epilepsy. Acta Medica (Hradec Kralove) 56:36-38.
Ferro MA, Chin RFM, Camfield CS, Wiebe S, Levin SD, Speechley KN (2014) Convulsive status epilepticus and health-related quality of life in children with epilepsy. Neurology 83:752-757.
Filippi L, Fiorini P, Daniotti M, Catarzi S, Savelli S, Fonda C, Bartalena L, Boldrini A, Giampietri M, Scaramuzzo R, Papoff P, Del Balzo F, Spalice A, la Marca G, Malvagia S, Della Bona ML, Donzelli G, Tinelli F, Cioni G, Pisano T, et al. (2012) Safety and efficacy of topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia (NeoNATI). BMC Pediatr 12:144-144.
Forcelli PA, Janssen MJ, Vicini S, Gale K (2012) Neonatal exposure to antiepileptic drugs disrupts striatal synaptic development. Ann Neurol 72:363-372.
Halbsgut LR, Fahim E, Kapoor K, Hong H, Friedman LK (2013) Certain secondary antiepileptic drugs can rescue hippocampal injury following a critical growth period despite poor anticonvulsant activity and cognitive deficits. Epilepsy Behav 29:466-477.
Huang P, Sun YP, Gou WL, Zhang R (2010) Effect of behavioral training on learning and memory capacity and changes of hippocampal NR2B and GluR1 expressions in FGR offspring rats. Nan Fang Yi Ke Da Xue Xue Bao 30:708-711.
Huganir RL, Nicoll RA (2013) AMPARs and synaptic plasticity: the last 25 years. Neuron 80:704-717.
Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF (2003) Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 23:876-882.
Kaindl AM, Asimiadou S, Manthey D, Hagen Mvd, Turski L, Ikonomidou C (2006) Antiepileptic drugs and the developing brain. Cell Mol Life Sci 63:399-413.
Kiviranta AM, Laitinen-Vapaavuori O, Hielm-Björkman A, Jokinen T (2013) Topiramate as an add-on antiepileptic drug in treating refractory canine idiopathic epilepsy. J Small Anim Pract 54:512-520.
Kwan P, Brodie MJ (2004) Phenobarbital for the treatment of epilepsy in the 21st century: a critical review. Epilepsia 45:1141-1149.
Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 112:631-643.
Liu J, Xu Z, Pang AL, Meng BL, Wang XF, Ren H (2013) Abnormal expression of srGAP2 in the brain of rats with refractory epilepsy. Zhongguo Zuzhi Gongcheng Yanjiu 17:1207-1211.
Lynch M, Sayin Ü, Bownds J, Janumpalli S, Sutula T (2000) Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. Eur J Neurosci 12:2252-2264.
Manent JB, Jorquera I, Mazzucchelli I, Depaulis A, Perucca E, Ben-Ari Y, Represa A (2007) Fetal exposure to GABA-acting antiepileptic drugs generates hippocampal and cortical dysplasias. Epilepsia 48:684-693.
Meador KJ (2003) Newer anticonvulsants: dosing strategies and cognition in treating patients with mood disorders and epilepsy. J Clin Psychiatry 64 Suppl 8:30-34.
Morte MI, Carreira BP, Falcão MJ, Ambrósio AF, Soares-da-Silva P, Araújo IM, Carvalho CM (2013) Evaluation of neurotoxic and neuroprotective pathways affected by antiepileptic drugs in cultured hippocampal neurons. Toxicol In Vitro 27:2193-2202.
Nakagawa T, Cheng Y, Ramm E, Sheng M, Walz T (2005) Structure and different conformational states of native AMPA receptor complexes. Nature 433:545-549.
Omkumar RV, Kiely MJ, Rosenstein AJ, Min KT, Kennedy MB (1996) Identification of a phosphorylation site for calcium/calmodulin-dependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem 271:31670-31678.
Reilly C, Atkinson P, Das KB, Chin RFMC, Aylett SE, Burch V, Gillberg C, Scott RC, Neville BGR (2014) Neurobehavioral comorbidities in children with active epilepsy: a population-based study. Pediatrics 133:e1586-1593.
Shannon HE, Eberle EL, Peters SC (2005) Comparison of the effects of anticonvulsant drugs with diverse mechanisms of action in the formalin test in rats. Neuropharmacology 48:1012-1020.
Silverstein FS, Ferriero DM (2008) Off-label use of antiepileptic drugs for the treatment of neonatal seizures. Pediatr Neurol 39:77-79.
Sommer BR, Mitchell EL, Wroolie TE (2013) Topiramate: Effects on cognition in patients with epilepsy, migraine headache and obesity. Ther Adv Neurol Disord 6:211-227.
Sulzbacher S, Farwell JR, Temkin N, Lu AS, Hirtz DG (1999) Late cognitive effects of early treatment with phenobarbital. Clin Pediatr (Phila) 38:387-394.
Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G, Tsien JZ (1999) Genetic enhancement of learning and memory in mice. Nature 401:63-69.
Tebb Z, Tobias. JD (2006) New anticonvulsants--new adverse effects. Southern Med J 99:375-379.
Verrotti A, Scaparrotta A, Cofini M, Chiarelli F, Tiboni GM (2014) Developmental neurotoxicity and anticonvulsant drugs: A possible link. Reprod Toxicol 48:72-80.
Viinikainen K, Eriksson K, Mönkkönen A, Äikiä M, Nieminen P, Heinonen S, Kälviäinen R (2006) The effects of valproate exposure in utero on behavior and the need for educational support in school-aged children. Epilepsy Behav 9:636-640.
Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. Science 313:1093-1097.
Zhang J, Tao DQ, Zhao H, Yin ZY (2012) Expression of Hsp70 and Caspase-3 in rabbits after severe traumatic brain injury. Chin J Traumatol 15:338-341.

Copyedited by Patel B, Norman C, Yu J, Yang Y, Li CH, Song LP, Zhao M