

Inhibitory effect of group II mGluR agonist 2R, 4R-APDC on cell proliferation in dentate gyrus in rats with epileptic seizure

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Abstract. – OBJECTIVE: Epileptic seizure can increase the cell proliferation in dentate gyrus in brain, but the mechanism remains unclear.

MATERIALS AND METHODS: In this study, using systemic bromodeoxyuridine (BrdU) to label the dividing cells, the inhibitory effect of group II metabotropic glutamate receptor (mGluR) agonist 2R, 4R-4-aminopyrrolidine-2, 4-dicarboxylate (2R, 4R-APDC) on cell proliferation in dentate gyrus in rats after pilocarpine-induced status epilepticus (SE) was investigated.

RESULTS: Results found that, 2R, 4R-APDC could significantly inhibit the behavioral seizure and block the seizure-induced increase of BrdU-positive cells in dentate gyrus, especially in hilus. Double-label immunofluorescence staining showed that, 2R, 4R-APDC did not affect the ability of newborn cells to differentiate into neurons or astrocytes.

CONCLUSIONS: 2R, 4R-APDC not only has anticonvulsant effect on adult rats with pilocarpine-induced SE, but also has neuroprotective effect by reducing the abnormal regeneration of nerves.

Key Words:

mGluR, 2R, 4R-APDC, Epileptic seizure, Cell proliferation, Neuroprotective.

adult neurogenesis are achieved through growth factors and several neurotransmitter systems³. The neurotransmitter glutamate controlled neurogenesis in the SVZ, DG, or both in previous studies⁴.

Several lines of evidence implicate newly generated neurons in structural and functional network abnormalities in the epileptic hippocampal formation of adult rodents⁵. Some findings indicated that seizure-induced abnormalities of neuroblast migration lead to abnormal integration of newborn dentate granule cells (DGC) in the epileptic adult hippocampus. This abnormal integration causes aberrant neurogenesis in the development or progression of recurrent seizures⁶.

Recently, metabotropic glutamate receptors have been considered as potential targets for neuroprotective and/or anticonvulsant drugs⁷⁻⁸. (2R, 4R)-4-aminopyrrolidine-2, 4-dicarboxylate (2R, 4R-APDC), a highly selective and potent group II mGluR (metabotropic glutamate receptor) agonist, retards the development of amygdaloid kindling in rats and increases the generalized threshold in fully kindled animals⁹. In the present study, we investigate neurogenesis of the hippocampus following status epilepticus (SE) in adult rats and the effect of 2R, 4R-APDC on neurogenesis and the potential anticonvulsant effect of 2R, 4R-APDC on pilocarpine-induced SE.

Introduction

Data accumulated over the past four decades have led to the widespread recognition that neurogenesis, the growth of new neurons, persists in the hippocampal dentate gyrus (DG) and rostral forebrain subventricular zone (SVZ) of the adult mammalian brain¹. Experimental seizures may stimulate the proliferation of precursor cells and differentiation into dentate granule cells in the subgranular zone². Regulation of proliferation and survival of these neural precursor cells in

Materials and Methods

Adult male Sprague-Dawley (SD) rats were anesthetized for intracerebroventricular (ICV) infusion with 2R, 4R-APDC (10 nmol in 10 ml, dissolved in 0.9% NaCl, n=16) or saline (Sham-treated, n=16)⁹⁻¹⁰.

After 1hr of preconditioning with 2R, 4R-APDC or saline, pilocarpine hydrochloride (350

mg/kg) was injected intraperitoneally (i.p.) to induce seizures. Animals were monitored throughout SE induction, and seizure severity was assessed according to the Racine scale¹¹. The control animals received vehicle injections (0.9% NaCl; $n=6$). After initial experiments to assess the effect of seizure severity on newborn granule cells, only rats that had multiple level III-V seizures within 3h of pilocarpine injection (status epilepticus) were used for the further studies. Animals were then observed periodically in the vivarium for the occurrence of SE. The effect of 2R, 4R-APDC on pilocarpine-induced seizures was assessed on the basis of the SE appearance, percentage, and latency of SE. The number of animals filling the inclusion criteria was: Saline+PILO ($n=7$), APDC+PILO ($n=6$) and Saline+Saline ($n=6$).

The thymidine analog 5-bromodeoxyuridine (BrdU) was administered i.p. (200 mg/kg per injection) twice on the 2nd day, 12h apart¹². All rats were killed 24h after the last BrdU administration, and the brains were removed for immunohistochemistry or immunofluorescence. BrdU immunohistochemistry was performed according to a previously reported protocol¹³. Double-label immunofluorescence experiments were utilized to examine the neuronal or glial differentiation of adult DGC progenitors, DNA denaturation. Immunofluorescent images were obtained using a confocal laser scanning microscope.

Quantitation of BrdU-positive cells in tissue sections were carried out using a modified stereology protocol previously described¹⁴. Quantitation was performed blindly with coded sections to eliminate bias.

Statistical Analysis

Differences of the average number of BrdU-positive cells were determined by one-way ANOVA and percent colocalization of BrdU-positive cells with NeuN or GFAP among three groups were determined by two-way ANOVA followed by Bonferroni post hoc test using SPSS 13.0 software. p values < 0.05 were considered statistically significant. Values are expressed as mean $\pm$ SE.

Results

Behavior Observation

Following pilocarpine injection, peripheral cholinergic stimulation signs (piloerection, salivation, and diuresis) were observed, along with

tremor and limbic automatism (scratching and chewing). Some rats exhibited head nodding, rearing and forelimb clonus. Sequentially, animals fell down on their side or back with generalized clonic seizures. SE continued unabated for hours. The percentage of SE of APDC+PILO treated rats was 37.5% and Saline+PILO treated rats was 87.5%. Pretreatment with 2R, 4R-APDC significantly decreased the percentage of SE in comparison to Saline+PILO group ($p < 0.05$). The average latency of SE onset was significantly longer in the APDC+PILO rats (46.33 ± 8.89 min) as compared to the Saline+PILO rats (32.29 ± 1.67 min) ($p < 0.05$). None of the Saline+Saline group showed seizure activity.

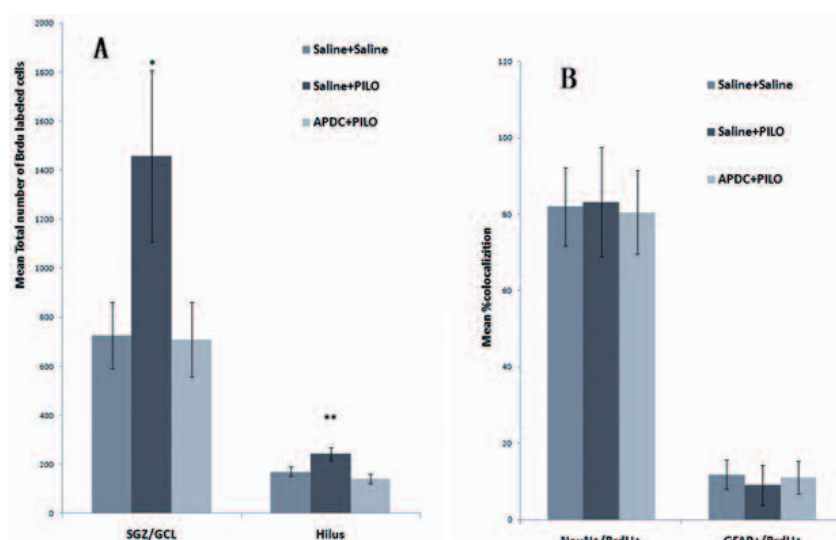
The Pilocarpine Increased Neurogenesis after Pilocarpine-Induced Seizures

After BrdU labeling, pilocarpine-induced seizures caused a marked increase in the number of BrdU-positive cells in the hippocampal dentate gyrus in contrast to the Saline+Saline rats (Figure 1). In the DG of all rat brains, the highest density of BrdU labeling was seen in the SGZ (subgranular zone) and the majority of GCL (granule cell layer) labeled cells were in close proximity to SGZ (Figure 2 B). Newly generated DGCs present following SE also appeared in ectopic locations in the hilus and inner molecular layer of the DG (Figure 1 A, Fig 2 B-C). The nucleus of BrdU+ and GFAP+ (glial fibrillary acidic protein) labeled cells in the DG were smaller in size ($< 5 \mu\text{m}$ in diameter) than granule neurons and their progenitors (5-10 μm). Most astrocytes were located within the hilus and not along the molecular layer border (Figure 3 B-C).

The Effect of APDC on Neurogenesis in the DG after SE

The effects of SE and 2R, 4R-APDC pretreatment on the proliferation of adult dentate granule cell progenitors were assessed using the mitotic marker BrdU to label dividing progenitors. The number of BrdU-positive cells within the SGZ/GCL and hilus exhibited a significant increase in SE animals. Moreover, 2R, 4R-APDC pretreatment significantly reduced the number of BrdU labeled cells in the SGZ/GCL ($p < 0.05$), most evident in the hilus ($p < 0.01$), 3 days after seizures (Figure 1 A). In addition, there was no significant difference in the number of BrdU-labeled cells between control rats without pilocarpine treatment and the rats receiving 2R, 4R-APDC and pilocarpine ($p > 0.05$). These results indicate that 2R,

Figure 1. Quantification of BrdU-positive cells in the SGZ/GCL and hilus of Saline+Saline, Saline+PILO and APDC+PILO rats (A). Bars represent mean $\pm$ S.E.M. The asterisk indicates a significant difference from controls using one-way ANOVA with Bonferroni post hoc test ($*p < 0.05$, $**p < 0.01$). The percentages of BrdU-labeled cells that colocalized NeuN or GFAP were compared among three groups (B), using two-way ANOVA with Bonferroni post hoc test ($p > 0.05$). The results are expressed as the mean $\pm$ SEM of percent colocalization of BrdU-positive cells with NeuN or GFAP in the dentate gyrus. SGZ: subgranular zone, GCL: granule cell layer.



4R-APDC pretreatment completely blocked the enhancing effects of pilocarpine-induced seizures on neural precursor cell proliferation in the DG.

The Effects of 2R, 4R-APDC on Neuronal Differentiation after SE

We used double-label immunofluorescence experiments to examine the neuronal or glial differentiation of adult DGC progenitors. Cells that colocalized with BrdU and NeuN (neuron-specific nuclear protein) were classified as neurons and the vast majority of these mitotically active cells differentiated into neurons in the granule cell layer

(Figure 1 B). BrdU-positive cells that co-localized with GFAP were classified as GFAP-positive glia. However, the astrocytic marker GFAP rarely co-localized with BrdU-immunostained nuclei, indicating that a population of newly dividing cells also included astrocytes (Figure 3 F). In addition, we found that there was no difference among Saline+PILO, APDC+PILO and Saline+Saline groups of BrdU colabeled with NeuN or GFAP positive cells ($p > 0.05$ for both comparison) (Figure 1 B). This suggested that APDC treatment did not affect the ability of new cells to differentiate into neurons or astrocytes.

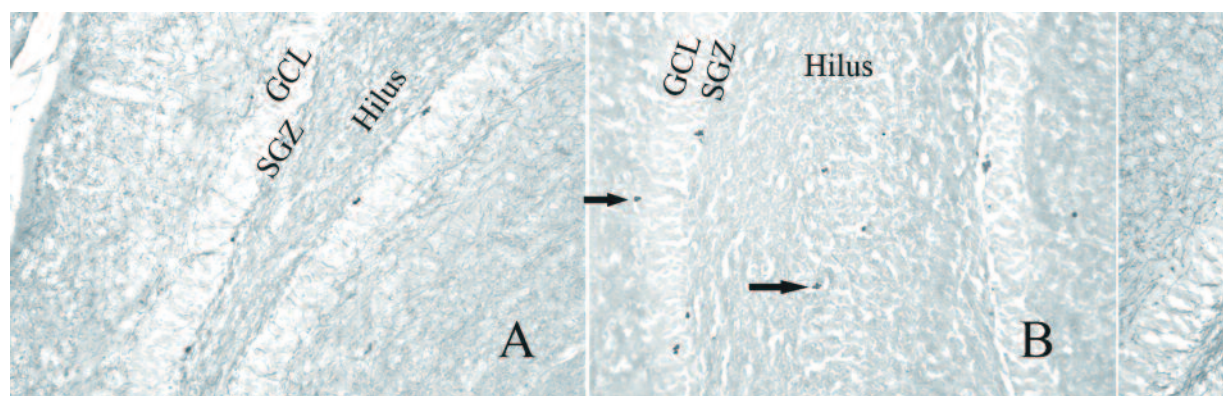


Figure 2. The number of BrdU positive cells in the dentate gyrus was significantly increased following pilocarpine administration. 2R, 4R-APDC-treated SE individuals exhibited a significant reduction in the number of SGZ/GCL and hilus BrdU-positive cells as compared to Saline+PILO rats. Some newly generated dentate granule cells after SE also appeared in ectopic locations in the hilus and inner ML of the dentate gyrus (arrows). Shown are representative differential interference contrast photomicrographs of BrdU-positive cells from Saline+Saline (A) Saline+PILO (B) and APDC+PILO rats (C). SGZ: subgranular zone, GCL: granule cell layer, ML: molecular layer.

Discussion

Recent studies approved that some antiepileptic drugs (AEDs), valproate, levetiracetam and lamotrigine, significantly suppress seizure-induced increases in neuronal proliferation and ectopic migration of newborn neurons into the dentate hilus¹⁵⁻¹⁷. This suggested that AEDs may exert antiepileptogenic effects through the suppression of seizure-induced neurogenesis. We also observed that the highly selective and potent AED group II mGluR agonist, 2R, 4R-APDC, prominently suppressed pilocarpine-induced SE. These results provide the first evidence that 2R, 4R-APDC pretreatments could prevent seizure-induced increases in the proliferation of precursor cells. This study further showed that neither pilocarpine-induced SE nor 2R, 4R-APDC pretreatment could affect the differentiation of neurons or astrocytes.

Consequences of Neurogenesis after Pilocarpine-Induced SE

Similar to previous reports^{6,18}, we observed that pilocarpine-induced SE caused a marked increase in cell proliferation in the dentate SGZ. The vast majority of these mitotically active cells differentiated into neurons. Several lines of evidence implicate newly generated neurons in structural and functional network abnormalities in the epileptic

hippocampal formation of adult rodents. These abnormalities include aberrant mossy fiber reorganization, persistence of immature DGC structure (e.g. basal dendrites), and the abnormal migration of newborn neurons to ectopic sites in the DG^{15,19}. Questions concerning the effects of seizure-induced neurogenesis in the epileptic brain, in terms of either a pathological or reparative role, have been discussed and emerging data suggests that altered neurogenesis in the epileptic dentate gyrus may be pathological and promotes abnormal hyperexcitability¹⁹. Some of the newly generated cells that arise after seizures appear to incorrectly migrate deep within the hilar region. Scharfman et al²⁰ found that unlike mature granule neurons in the GCL, the ectopic hilar granule cells exhibited hyperexcitability in the form of abnormal burst firing that occurred synchronously with CA3 pyramidal cells. They also determined that newly generated granule cells are actively involved in hippocampal integration and participate in the spontaneous seizures of pilocarpine-treated rats. Based on intracellular recordings from ectopic hilar granule cells, they confirmed that newly born granule cells (i.e., generated after pilocarpine-induced status epilepticus) receive excitatory synapses from the perforant path with major input to the dentate gyrus.

Structural differences between hilar ectopic following SE and normal DGCs are present. Rib-

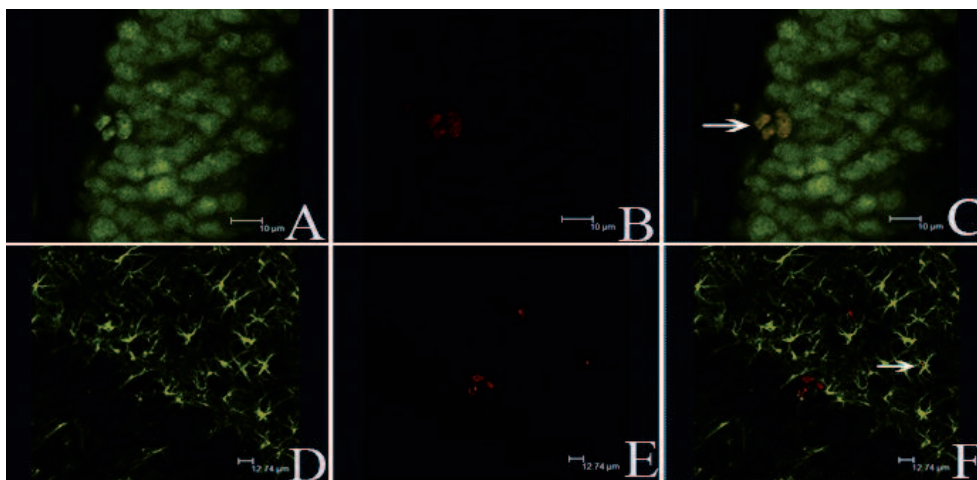


Figure 3. Immunofluorescent identification of newborn neurons in the dentate gyrus. Double immunofluorescent labeling for BrdU (red) and the cell-specific markers NeuN (green) or GFAP (green) were analyzed by laser scanning confocal microscopy. **A**, NeuN linked to FITC (fluorescein isothiocyanate) shows uniform, bright fluorescence of granule cells. **B**, Along the hilar border BrdU linked to TRITC (tetramethyl rhodamine iso-thiocyanate). **C**, Superposition of images of BrdU and NeuN (yellow cells) show most cells colocalize (arrows). **D**, GFAP fluorescence shows most astrocytes are located within the hilus and not along the border of the granule/hilar layers. **E**, BrdU linked to TRITC. **F**, Superposition of images of BrdU and GFAP labeling show few cells coexpress both proteins (arrows).

ak et al²¹ reported that the number of excitatory synapses on the dendrites of hilar ectopic DGCs in epileptic rats was substantially increased compared with granule neurons in the GCL. This morphological abnormality may result from a failure of the normal developmental pruning of processes and thereby lead to seizure generation²². Taken together, these findings suggest a pro-epileptogenic role of seizure-induced neurogenesis in the epileptic hippocampal formation.

Effects of group II mGluR Agonist Pretreatment on Neurogenesis within the Dentate Gyrus after Pilocarpine-Induced SE

Augmented release of glutamate and subsequent activation of iGluRs (ionotropic glutamate receptors) play a key role in seizures and seizure-induced neurogenesis^{12,23}. More recently, the presynaptic mechanisms involved in glutamate release have become a primary focus of research with the possibility that failure in the control of this synaptic release by presynaptic receptors might lead to a rise in extracellular glutamate levels leading to seizure activity. Also, some AEDs may exert antiepileptogenic effects through the suppression of seizure-induced neurogenesis^{15,17}. 2R, 4R-APDC, a highly selective and potent group II mGluR agonist, may decrease the extracellular glutamate level and cell proliferation in the DG after seizure by presynaptic mechanism as our previous study demonstrated¹⁰; therefore, they exert an antiepileptic effect.

Several studies have indicated that group II mGluR agonists have a protective effect in several models, such as global ischemia²⁴, traumatic brain injury²⁵, and homocysteic acid-induced neuronal damage⁹. Thus 2R, 4R-APDC has potential neuroprotection via inhibiting the aberrant neurogenesis induced by diffuse brain injury²⁶. In the present study, our results confirmed that 2R, 4R-APDC pretreatment could inhibit seizure-induced proliferation of precursor cells in the DG, especially in hilus. This result suggests that the protection against hyperexcitability of 2R, 4R-APDC could be explained by the mechanism that group II mGluR agonist decreases the number of newborn granule cells, especially hilar ectopic DGCs. The present work is the first to show that the group II mGluR agonist, 2R, 4R-APDC, can serve a neuroprotective role via inhibiting precursor cell proliferation in the DG in the model of seizures induced in adult rats by pilocarpine.

Potential Mechanisms Underlying the Anticonvulsant and/or Neuroprotective Effect of 2R, 4R-APDC

Depression of excitatory synaptic transmission may occur since group II mGluR agonists inhibit presynaptic glutamate and aspartate release in many brain regions and attenuate excitatory postsynaptic potentials²⁷. Activation of group II mGluR was also shown to inhibit G-protein activation of adenylate cyclase and decrease the formation of cyclic adenosine monophosphate (cAMP). This can result in the inhibition of voltage-operated Ca^{2+} channels that limit NMDA (n-Methyl-D-Aspartate) toxicity⁸. Other authors claim that activation of group II mGluR could inhibit voltage activated (HVA) Ca^{2+} channels. Inhibition of HVA Ca^{2+} currents could limit both the presynaptic release of glutamate as well as the response of postsynaptic cells to glutamate receptor activation.

The agonists for group II mGluRs exert neuroprotective effects indirectly via astrocyte production and release of transforming growth factor- β (TGF- β), evoked by activation of mGlu3 subtype receptors localized on glial cells²⁹. Activation of mGluRs protect against cell injury by regulating the production of reactive oxygen species and oxidative stress in nerve cells³⁰.

Conclusions

We showed that 2R, 4R-APDC exerts a neuroprotective effect against hyperexcitability by decreasing the number of ectopic newborn DGCs, which may contribute to the aberrant network reorganization in the adult rat hippocampus.

Conflict of interest

The Authors declare that they have no conflict of interests.

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