

Involvement of over-expressed BMP4 in pentylenetetrazol kindling-induced cell proliferation in the dentate gyrus of adult rats

Jinbo Yin ^{a,1}, Yuxin Ma ^{a,1}, Qing Yin ^a, Haiwei Xu ^{b,2}, Ning An ^a, Shiyong Liu ^a, Xiaotang Fan ^c, Hui Yang ^{a,*}

^a Department of Neurosurgery, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, PR China

^b Department of Physiology, Third Military Medical University, Chongqing 400038, PR China

^c Department of Neurobiology, Third Military Medical University, Chongqing 400038, PR China

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Abstract

The dentate gyrus (DG) of the hippocampus is one of a few regions in the adult mammalian brain characterized by ongoing neurogenesis. Proliferation of neural precursors in the granule cell layer of the DG has been identified in pentylenetetrazol (PTZ) kindling epilepsy model, however, little is known about the molecular mechanism. We previously reported that the expression pattern of bone morphogenetic proteins-4 (BMP4) mRNA in the hippocampus was developmentally regulated and mainly localized in the DG of the adult. To explore the role of BMP4 in epileptic activity, we detected BMP4 expression in the DG during PTZ kindling process and explore its correlation with cell proliferation combined with bromodeoxyuridine (BrdU) labeling technique. We found that dynamic changes in BMP4 level and BrdU labeled cells dependent on the kindling stage of PTZ induced seizure-prone state. The number of BMP4 mRNA-positive cells and BrdU labeled cells reached the top level 1 day after PTZ kindled, then declined to base level 2 months later. Furthermore, there was a significant correlation between increased BMP4 mRNA expression and increased number of BrdU labeled cells. After effectively blocked expression of BMP4 with antisense oligodeoxynucleotides(ASODN), the BrdU labeled cells in the dentate gyrus subgranular zone(DG-SGZ) and hilus were significantly decreased 16d after the first PTZ injection and 1, 3, 7, 14d after kindled respectively. These findings suggest that increased proliferation in the DG of the hippocampus resulted from kindling epilepsy elicited by PTZ maybe be modulated by BMP4 over-expression.

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In the adult, neurogenesis occurs primarily in three locations: the subgranular zone (SGZ) of the dentate gyrus (DG), the olfactory bulb, and the subventricular zone (SVZa) lining the ventricular walls. Neurons are born in the underlying subgranular layer of the hippocampus and move into the granule cell layer (GCL) to become mature granule neurons [1,2]. Evidences have demonstrated that

adult hippocampus neurogenesis was increased dramatically after seizures [3–6]. Is enhanced neurogenesis in the DG beneficial, or might it actually exacerbate seizures? Recent results supported the hypothesis that newly-born granule cells might not necessarily act to ameliorate seizure, and might even contribute to seizures [7]. It has been reported that pentylenetetrazol (PTZ) kindling models of epilepsy stimulated DG cell neurogenesis [8], while the molecular mechanism underlying has not been made clear.

Precise control of bone morphogenetic protein-4 (BMP4) signaling in the extracellular space appears to play a critical role in multiple events during development, including neural induction [9], tissue patterning [10], epithelial–mesenchymal

* Corresponding author. Fax: +86 23 65218204.

E-mail addresses: haiweixu2001@yahoo.com.cn (H. Xu), huiyang64@yahoo.com (H. Yang).

¹ These authors contributed equally to this work.

² Co-corresponding author.

interactions underlying organogenesis [11], lineage selection [12] and in the creation of stem cell “niches” in developing and adult organs [13,14]. Bone morphogenetic proteins (BMPs) exert their biological functions by interacting with membrane bound receptors belonging to the serine/threonine kinase family including bone morphogenetic protein receptor type I (BMPRI) and type II (BMPRII) [15]. It has been demonstrated that both BMPs and their receptors were expressed in adult hippocampus [16]. A moderate increase in BMPRII mRNA expression was observed in granule cells of the DG after both mild head trauma and cerebral ischemia [17], which suggested that BMPs might modulate hippocampal plasticity.

Our previous results demonstrated that temporal and spatial expression patterns of BMP4 mRNA in embryonic and postnatal hippocampus were developmentally regulated, and BMP4 might play an important role during hippocampus development [18]. It has been generally accepted that the hippocampus undergoes extensive structural and functional changes, including dramatically increased dentate granule cell neurogenesis during PTZ kindling [8,19,20]. However, little is known about PTZ kindling-induced changes in expression of BMP4 and its correlation with cell proliferation during development of epilepsy.

The present study was designed to investigate the time-dependent alternation in BMP4 level during PTZ-kindling process using *in situ* hybridization and immunohistochemistry, and its correlation with cell proliferation was also explored by blocking the expression of BMP4 with antisense oligodeoxynucleotide (ASODN) to BMP4.

Materials and methods

Rats model of PTZ-kindled seizures. Adult male Sprague–Dawley rats (200–250 g, 3- to 4-month-old) (Animal Breeding Center, Third Military Medical University, Chongqing, China) were housed in temperature-controlled conditions under a 12-h–12-h light–dark cycle (lights on at 8:00 a.m.) with food and water supplied *ad libitum*. All experiments were performed in accordance with approved principles of laboratory animal care. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Rats received PTZ injections every other day (35 mg/kg, Sigma–Aldrich, dissolved in 0.9% saline, injected intraperitoneally (i.p.), 13 injections total). After each injection, the convulsive behavior was recorded for 20 min and behavioral changes were graded according to the criterion described by Racine [21]: 0—no behavioral changes; 1—facial movements, ear and whisker twitching; 2—myoclonic convulsions without rearing; 3—myoclonic convulsions with rearing; 4—clonic convulsion with loss of posture; 5—generalized clonicotonic seizures. Meanwhile, control animals were injected with 0.9% saline solution (13 injections as in the PTZ-treated group).

Intracerebroventricular (i.c.v.) injection of antisense oligodeoxynucleotides to BMP4. The sequences of antisense and sense BMP4 oligodeoxynucleotides (ODN) were 5-ACATCTGTAGAAGTGTCCG-3 and 5-GGCGACACTTCTACAGATGT-3 respectively, which were chosen in regions presenting little homology with sequences of related mRNAs, and were checked against the GenBank database. Phosphothioate antisense and sense ODN to BMP4 was synthesized at Sangon Biological Engineering Technology (Shanghai, China) and dissolved in sterile pyrogen-free 0.9% saline.

Adult Sprague–Dawley rats were anesthetized and a stainless steel cannula, stereotactically implanted into the left lateral ventricle: AP, –0.8 mm; ML, 1.4 mm; DV, –3.5 mm from bregma 1 week before PTZ

injection. At least 5 days before bromodeoxyuridine injection, antisense or sense ODN (20 µg/day) or sterile saline vehicle was administered *i.c.v.* twice a day for 3 consecutive days using a 10 µl Hamilton syringe ($n = 5$ per time point). The needle was left in place for 5 min and then slowly withdrawn to minimize cerebrospinal fluid leakage. At the end of experiment, cannula placement was verified by injection of 10 µl dye (0.05% cresyl violet) through the *i.c.v.* cannula and subsequent examination of the brain.

BrdU injections and immunohistochemistry. The thymidine analog 5-bromodeoxyuridine (BrdU, Sigma–Aldrich) was dissolved in 0.9% saline in all experiments and administered *i.p.* at a dose of 100 mg/kg per injection. 2 h after the corresponding PTZ injection, the rats received two injections of BrdU with a 2 h interval, starting at different time points (4d, 10d, 16d after the first injection showed as I4, I10 and I16; 1d, 3d, 7d, 14d, 30d, 60d after kindled showed as K1, K3, K7, K14, K30 and K60, respectively) ($n = 5$ per time point) or 0.9% saline treatment ($n = 8$), and were sacrificed 24 h after the last BrdU administration.

At the end of the experiment, animals were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*), then perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, post-fixed for 6 h in 4% PFA-PB, and placed in 30% sucrose until they sank. Coronal sections through the entire hippocampus were cut at 35 µm on a freezing microtome and sections were collected in phosphate-buffered saline (PBS, pH 7.4).

Every sixth section containing the DG of the hippocampal formation (for a total of 10 sections per rat) was used for each immunohistochemical or *in situ* hybridization procedure. Immunohistochemistry was performed according to our previous protocols [22]. Sections were incubated with 0.1% trypsin in Tris buffer (pH 7.4) for 10 min, rinsed twice in phosphate buffer saline (PBS, pH 7.4), incubated 30 min in 2 M HCl, rinsed three times in PBS (pH 6.0), incubated for 20 min in 3% normal horse serum in PBS (pH 7.4), and incubated in anti-BrdU antibody (mouse monoclonal, 1:1000; Sigma–Aldrich) or anti-BMP4 antibody (mouse monoclonal, 1:500; Chemicon), respectively, in PBS with 0.5% Tween-20 overnight at 4 °C. On the following day, sections were rinsed 3 times in PBS and then incubated in secondary biotinylated antisera (Vectastain Elite ABC, Vector Labs.) with normal serum in PBS for 90 min, and then reacted in 3,3'-diaminobenzidine and H₂O₂ for 2–10 min.

***In situ* hybridization of BMP4.** *In situ* hybridization for BMP4 mRNA was performed according to procedures we described previously [23]. Briefly, 35 µm frozen sections were collected into 4× standard saline citrate (SSC), and incubated in hybridization buffer (50 µl 4× SSC, 20% dextran sulphate, 0.5× Denhardt's, 50% deionized formamide, 0.1 M DTT, 0.25 mg/ml poly(A), 0.25 mg/ml tRNA, 0.25 mg/ml ssDNA) containing 2.5 ng/µl digoxigenin (DIG)-labeled BMP4 antisense oligonucleotide probe at 42 °C overnight. Immunological detection of DIG was carried out by incubation of the sections with anti-DIG antibody fragment conjugated with alkaline phosphatase (Roch, Germany, 1:1000), and hybridization signal was developed in nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NTB/BCIP)-containing buffers (Roch, Germany).

Cell counting and statistical analyses. For cell counting we employed a BX60 microscope (Olympus, Japan, objective with 40-fold magnification) equipped with a magnifier digital camera and a computer-assisted image analysis system (Spot Insight, cts, USA). The methods of quantitative analysis of BrdU-labeled cells were according to that of Parent [24] and Gray [25], 10 mounted sections containing the DG of the hippocampal formation spaced at 150 µm intervals were examined for each brain. A two-cell-body-wide zone along the border of the granule cell layer and the hilus as defined by Parent et al. [24] was analyzed. The numbers of BrdU-labeled cells were counted on the digital images by an experimenter who was not informed about group assignment, and the data were expressed as the number of positive cells per section.

Results were expressed as the average number (means ± SEM) of BrdU-labeled cells, BMP4 mRNA positive cells and BMP4 immunoreactive (IR) cells. Differences between groups were determined by one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant different (PLSD) post hoc test of SPSS 10.0 software. P values <0.05 was considered statistically significant.

Results

Progression of PTZ-kindled seizures

No significant behavioral changes were observed in rats received saline injection. A single injection of PTZ at a sub-convulsive dose resulted in short-term freezing, face autom-

atism of the rats, corresponding to the stage 1 of the Racine scale. Among the 30 rats assessed for seizure scores, repeated injections of PTZ at a sub-convulsive dose caused 23 rats generalized tonic–clonic seizures, which had at least 3 consecutive stage 5 seizures, 2 rats died after seizure, and the remaining 5 rats did not meet the criterion of 3 consecutive days of tonic–clonic seizures.

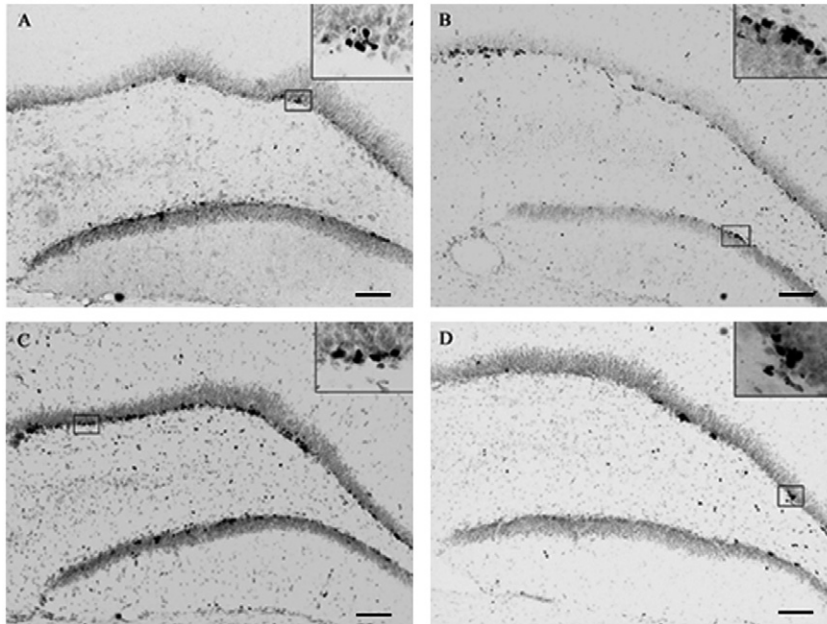


Fig. 1. BrdU-labeled cells in the DG of saline-treated rats and PTZ-treated rats. (A) BrdU labeled cells in DG of the saline injected rats. (B) Four days after the first PTZ injection, the number of BrdU labeled cells was increased significantly. (C) One day after PTZ-kindled seizures, the number of BrdU labeled cells reached its peak. (D) Sixty days after PTZ-kindled seizures, the number of BrdU labeled cells declined to base level. Scale bar = 50 μ m.

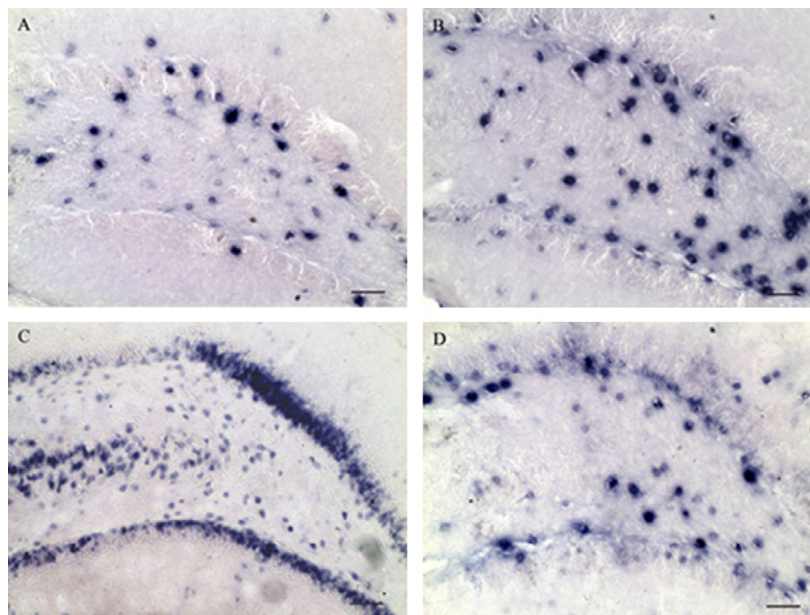


Fig. 2. Changes in the expression of BMP4 mRNA in the DG of the hippocampus during the PTZ kindling process. (A) Sporadic BMP4 mRNA positive cells localized in the DG of the rats received 0.9% saline injection. (B) The number of BMP4 mRNA positive cells was increased 4 days after the first PTZ injection. (C) The expression of BMP4 mRNA reached its peak 1 day after PTZ kindled seizures. (D) The number of BMP4 mRNA positive cells was declined to base level 60 days after PTZ-Kindled seizures. Scale bar = 50 μ m.

There was a basal level of BrdU incorporation into cells in the SGZ, which was an area where neurogenesis normally persisted in adult animals, this was consistent with previous reports [8]. The BrdU labeled cells appeared darkly and were irregularly stained and frequently in clusters of two or more, and were localized within the SGZ and throughout the hilus (Fig. 1A). This pattern is characteristic of DG precursor cells. After treated with PTZ at a sub-convulsive dosage, the number of BrdU labeled cells in the dentate GCL and hilus was increased significantly compared with

that in the saline injection rats. The number of BrdU labeled cells in the hippocampus was increased significantly 4 days after the first PTZ injection (Fig. 1B) and reached the peak 1 day after PTZ-Kindled seizure (Fig. 1C). It was decreased gradually after the PTZ kindled, and declined to base level 60 days after PTZ-Kindled seizure (Fig. 1D).

There were sporadic BMP4 mRNA positive cells localized in the DG of the rats received 0.9% saline injection (Fig. 2A). Four days after the first PTZ injection, the number of BMP4 mRNA positive cells in the DG was increased

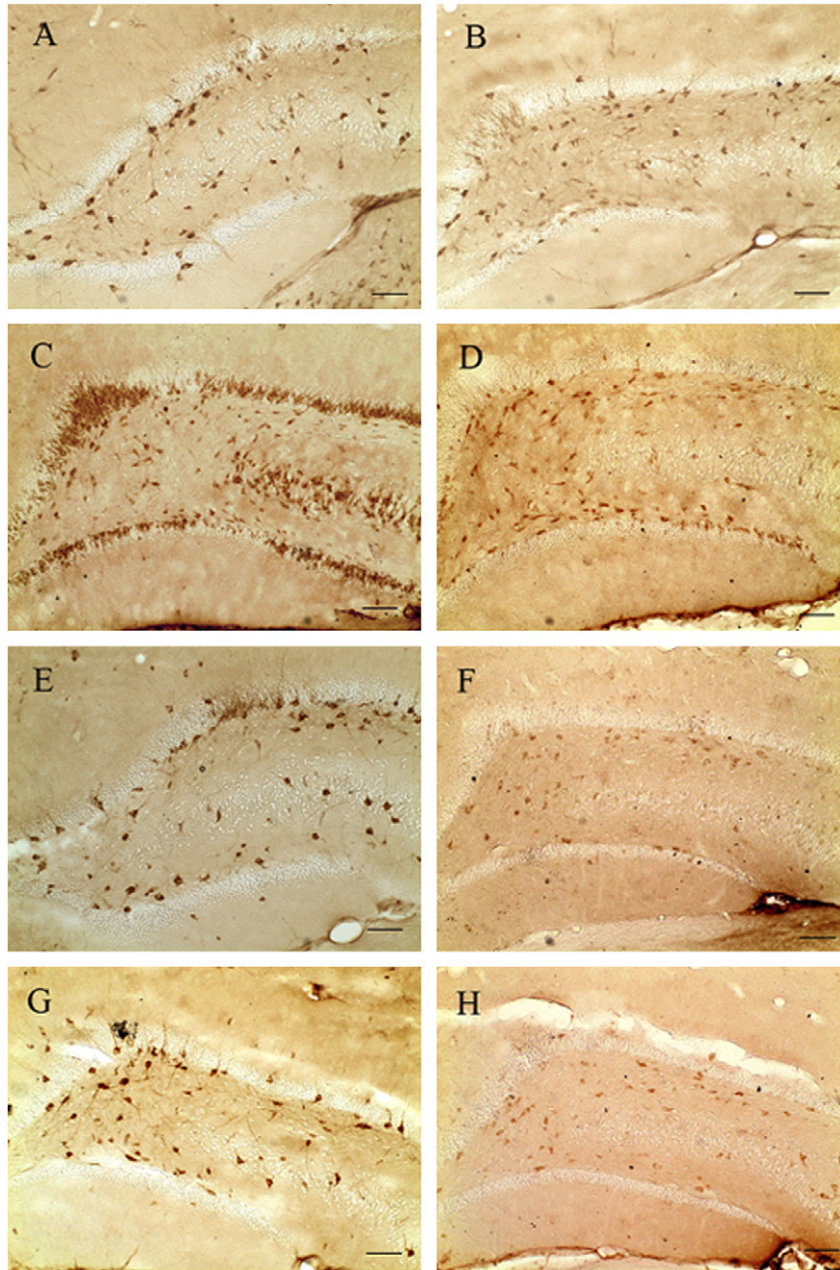


Fig. 3. Effect of ASODN on the number of BMP4-immunoreactive (IR) cells in the hippocampus during PTZ kindling process. BMP4 IR cells mainly localized in the DG of the rats 16 days after the first PTZ-injection (A), the number of BMP4 IR cells was decreased after i.c.v. ASODN to BMP4 (B). One day after PTZ-kindled, the number of BMP4 IR cells in the DG of the rats reached its peak during PTZ-kindling (C), it decreased significantly when ASODN to BMP4 was injected (D); 7 days after PTZ-kindled (E), the number of BMP4 IR cells was markedly decreased by ASODN (F); 14 days after PTZ-kindled (G), the number of BMP4 IR cells was significantly decreased by ASODN (H). Scale bar = 50 μ m.

significantly, the strong signals were also observed in CA3 region and moderate signals localized in both CA1 and CA2 (2B). One day after PTZ-kindled, the number of BMP4 mRNA positive cells in the DG reached the peak, densely and deeply stained BMP4 mRNA positive cells were concentrated in the granular cell layer of the DG (2C). The expression of BMP4 mRNA was decreased gradually after the PTZ kindled, but remained at high level 14 days after PTZ kindled, moderate hybridization signal was detected in the DG and CA3 region. The expression of BMP4 declined to base level 60 days after PTZ-kindled (2D).

Relation between BMP4 expression and cell proliferation in the DG of the hippocampus

A correlation analysis between the number of BMP4 mRNA-positive cells and the number of BrdU labeled cells in the DG of the rats during PTZ kindling process was performed. It showed that the increase of the numbers of BrdU labeled cells was strongly correlate to the increase

of the numbers of BMP4 mRNA positive cells (Fig. 4A). The P value was less than 0.01, and the r value was 0.962.

To examine more closely the relationship between BrdU-labeled cells and BMP4 expression, ASODN was injected (i.c.v.) to block expression of BMP4. The number of BMP4 mRNA positive cells in the DG of the hippocampus was decreased markedly by ASODN to BMP4 in comparing with PTZ injected rats in the group of 16d after the first PTZ injection and 1, 3, 7, 14d after kindled, respectively, while sense ODN did not decrease the number of BMP4 mRNA positive cells (Fig. 4B). To verify the effectiveness of ASODN on reducing the protein level of BMP4, the BMP4 IR cells in the DG was observed in I16, K1, K7 and K14 group, it showed that the number of BMP4-IR cells in the DG was decreased significantly when the rats received ASODN by i.c.v. (Fig. 3A–H). The number of BrdU labeled cells in the DG-SGZ and hilus was significantly decreased after ASODN to BMP4 treatment in comparison with group of 16d after the first PTZ injection and 1, 3, 7, 14d after kindled respectively (Fig. 4C). Treatment with BMP4 sense ODN had no effects on the number of BrdU-labeled cells in the hippocampus.

Discussion

New neurons normally develop from progenitor cells located in many areas of the immature nervous system during development. In the adult, it occurs primarily in three locations: the SGZ of the DG, the olfactory bulb, and the SVZa lining the ventricular walls [26]. In the DG, the SGZ is a thin layer that lies just beneath the GCL. Ordinarily, cells are born there at a modest rate throughout life, although this rate gradually decreases with age [27,28]. Remarkably, seizures appear to increase the rate of neurogenesis. This appears to occur after brief seizures, kindling and after status epilepticus induced by chemoconvulsants such as pilocarpine, pentylentetrazol and kainic acid or status epilepticus induced by electrical stimulation [3,25,29]. However, the mechanism underlying the neurogenesis induced by seizures remained unclear.

Although BMPs were initially identified for their osteo-inductive property, they have been demonstrated to play multiple roles in brain development and adult neurogenesis [13,30,31]. Evidences have showed that under pathological condition including brain trauma and cerebral ischemia, BMPRII mRNA was moderately increased in granule cells of the DG [17]. From neurogenesis to the maturation of the vertebrate nervous systems, BMPs mediate a diverse array of developmental processes including cellular survival, proliferation, morphogenesis, lineage commitment, inhibition of alternate lineages, differentiation, and apoptosis [12,32,33]. We demonstrated that 1 day after PTZ kindled, BMP4 was strongly expressed in the DG. The expression of BMP4 was decreased gradually after the PTZ kindled, but remained at high level 14 days after PTZ kindled, and declined to base level 60 days after PTZ-kindled. It suggests

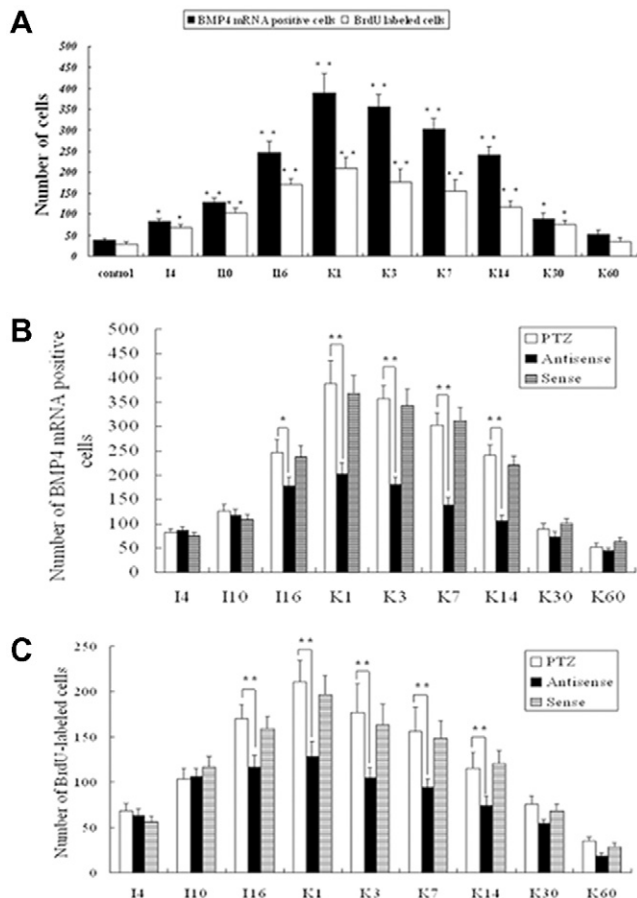


Fig. 4. The number of BMP4 mRNA-positive cells and BrdU-labeled cells in the DG during the PTZ kindling process (A, * P < 0.05 vs. control, ** P < 0.01 vs. control) and effect of BMP4 ASODN on the number of BMP4 mRNA positive cells (B) and BrdU-labeled cells (C) in the dentate gyrus during the PTZ kindling process (B,C, * P < 0.05, ** P < 0.01 vs. PTZ group).

that BMP4 is involved in regulating hippocampal plasticity during epileptogenesis.

Combined with BrdU labeled technique, we found that BrdU labeled cells were increased in the GCL and hilus during epileptogenesis which was consistent with previous reports [8]. Furthermore, we noticed that the increase of BrdU labeled cells was strongly correlate to the increase of BMP4 mRNA positive cells, when the over-expression of BMP4 was blocked, the increased cell proliferation in the DG-SGZ was normalized, it infers that BMP4 maybe affect hippocampal function through modulating hippocampal cell proliferation during epileptogenesis.

BMPs promote or inhibit the proliferation of neural stem cells (NSCs) depending on their concentration. Our previous study demonstrated that low concentrations (1–5 ng/ml) of BMP4 promoted the proliferation of SVZa NSCs, but high concentrations (10–100 ng/ml) of BMP4 inhibited this proliferation. However, the effect of BMP4 on neuronal commitment is time-dependent instead of dose-dependent, the neuronal differentiation in response to BMP4 at different concentrations (1–100 ng/ml) was gradually increased and reached a plateau level on day 2 and reduced on day 7, which indicated that BMP4 promoted the exit from the cell cycle and triggered the differentiation of neuron progenitors [34]. Recently, a BMP-based induction–termination mechanism has been proposed to describe the shift from proliferation to differentiation: BMPs cause proliferation through BMPRIA, and BMPRIA signaling simultaneously induces expression of BMPRII, and activation of BMPRII drives mitotic arrest via the CDK inhibitor p21^{cip1} and results in differentiation [35]. The results presented here seem to support that BMP4 promotes proliferation of NSCs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2007.01.107](https://doi.org/10.1016/j.bbrc.2007.01.107).

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