Antiepileptic effects of levetiracetam related to the regulation of cell cycle reentry in the parietal cortex of EL mouse brain

Yoshiya L. Murashima¹, Mitsunobu Yoshii¹

¹Division of Frontier Health Science, Tokyo Metropolitan University, Graduate School of Human Health Science, Tokyo, Japan

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Abstract

We have reported that cyclins and the corresponding cyclin-dependent kinase (CDK) family are related to cell proliferation during development as well as epileptogenesis. In the present study, we used EL mice to examine how levetiracetam (Lev) controls the altered expressions of cyclins and the CDK family during development, and further, the epileptogenesis as well in the parietal cortex, the seizure initiation site of EL. Developmental changes in the expression of cyclin and the corresponding CDK families (cyclin D/CDK-4, cyclin E/CDK-2, cyclin A/CDK-2, cyclin A/CDK-1, and cyclin B/CDK-1) in the parietal cortex of EL mice and the control DDY mice were examined by Western blotting. At different ages, one group of mice (n = 6) were administered a single dose of Lev 160 mg/kg Lev p.o. and a treatment naïve group (n = 6) was administered vehicle, three days before sacrifice. Compared with the control DDY mice, treatment naïve EL mice showed upregulations of cell cycle-specific cyclins/CDK during the early developmental stages, suggesting that reentry into cell cycle is promoted prior to the beginning of seizures. Lev abolished these effects and Lev-treated EL mice showed no seizures at all. These results
suggest that cyclins/CDK may be activated during early stages of development before exhibiting seizures, suggesting that reentry into cell cycle in the parietal cortex is a candidate mechanism for the seizure predisposition of EL mice. The antiepileptic effects of Lev may be related to regulation of cell cycle reentry.

Introduction

The EL mouse is an animal model of epilepsy. In this model, DNA fragmentation in the hippocampus CA1 and the parietal cortex [1, 2] has been demonstrated using in situ terminal transferase-mediated dUTP Nick labeling [1, 3]. However, in the hippocampus and the parietal cortex of EL mice, neuronal cell loss is not found despite occurrence of frequent seizures during development [4, 5]. In addition, various cell death-related proteins are overexpressed during early stages of development. Pro-apoptotic Bax is overexpressed from 8 to 19 weeks of age, which covers the period of epileptogenesis [4, 5, 6]. In parallel, anti-apoptotic Bcl-2 is also overexpressed from 8 to 19 weeks of age. Expression of anti-apoptotic Bcl-XL peaks at 5 and 24 weeks of age, corresponding to early period of ictogenesis and late period of epileptogenesis, respectively [4, 5, 6]. There is an altered equilibrium between pro-apoptotic Bax and anti-apoptotic Bcl-2, which are associated with increased DNA fragmentation without cell loss [6].

More recently, we have reported that various neurotrophic factors are also overexpressed during the development of EL mice. Brain-derived neurotrophic factor (BDNF) shows biphasic overexpression during 8 to 12 weeks as well as around 20 weeks of age, which cover the period of epileptogenesis [4, 5]. Neurotrophic factor-3 (NT-3) shows a peak expression from 8 to 12 weeks of age, which covers the early period of epileptogenesis. In addition, fibroblast growth factor-2 (FGF-2) shows a linear increase in expression with age and reaches a maximum level at 24 weeks, which covers the late period of epileptogenesis [4, 5, 6]. On the other hand, seizure activity also induces expressions of neurotrophic factor mRNA and protein [7, 8] and causes cell loss [9]. However, the level of NT-3 and BDNF in the hippocampus of EL increase significantly in earlier developmental periods before exhibiting frequent seizures [8]. The abundance of trophic factors may also facilitate the induction of cell division-related processes and promote epileptogenesis [10, 11, 12, 13].

In proliferating cells, the cell cycle consists of four phases. Gap1 (G1) is the interval between mitosis and DNA replication that is characterized by cell growth. The transition that occurs at the restriction point (R) in G1 commits the cell to the proliferative cell cycle. If the conditions that signal this transition are not present, the cells stop the cell cycle and enter G0, a non-proliferative phase during which growth, differentiation and apoptosis occur [14, 15]. Replication of DNA occurs during the synthesis (S) phase, which is followed by a second gap phase (G2) during which growth and preparation for cell division occurs [14, 15]. Mitosis and the production of two daughter cells occur in M phase (M) [16,
17]. A family of cyclins acting as regulatory subunits for cyclin-dependent kinase (CDK) regulates passage through the four phases of the cycle. The activities of the various cyclin/CDK complexes regulate the progression through G1/S/G2/M phases of the cell cycle [18] (Figures 1 and 2).

Levetiracetam (Lev) is a newly developed antiepileptic drug. This drug binds to SV2A, and regulates the synaptic membrane excitability [19]; however, other mechanisms of anti-epileptogenesis are suggested [20]. In the present study, we used EL mice to examine how Lev controls the altered cyclin and CDK

**Fig. 1.** Schematic representation of checkpoints of the cell cycle. Three negative feedback mechanisms control cell circle reentry.

**Fig. 2.** Schematic representation of the roles of cyclins and cyclin-dependent kinases in cell cycle reentry. The activities of various cyclin/CDK complexes regulate the progression through G1/S/G2/M phases of the cell cycle.
families during development, and further, the epileptogenesis as well in the parietal cortex, the seizure initiation site of EL.

Methods

EL mouse

The EL mouse is an inbred mutant strain used as an animal model of secondarily generalized seizures [21, 22]. The mode of inheritance is autosomal dominant and the penetration rate is 100%. Mice show the first seizures after 10 weeks of age, followed by frequent seizures after 20 weeks [21]. Several lines of evidence indicate that seizure discharges are initiated in the parietal cortex, and then spread through the hippocampus [23]. These findings have been substantiated by histochemical and biochemical analyses of glucose utilization [24] and study of inhibitory neurotransmission mediated by γ-aminobutyric acid (GABA) [25]. The developmental formation of the “focus complex”, the epileptogenic zone, which mainly involves the parietal cortex and the hippocampus, has been hypothesized to be the key to epileptogenesis in EL mice [21].

Treatment protocol

EL mice were administered p.o. with a single dose of Lev at 160 mg/kg, which is the double dosage of ED50 for seizure suppression in EL mice (Lev-treated group; n = 6). It was difficult to administer Lev p.o. without inducing seizures during administration. We found that a single dose was the maximum to induce CCR change without inducing seizures when administering the drug. Lev was given three days before sacrifice. None of the EL mice manifested seizures during the three days. Treatment-naïve EL mice were administered the same amount of vehicle three days before sacrifice (naïve group; n = 6). EL mice at the ages of 3, 5, 7, 10, 12, 15, 20 and 25 weeks were studied. As controls, DDY/TIP mice (mother strain of EL) at 10 and 20 weeks of age were subjected to the same procedures as EL mice (n = 6).

Preparation of tissues for immunoblotting analysis

For immunoblotting analysis of the cyclin and CDK families (cyclin A, B, D, E and CDK-1, -2, -4), EL mice at 3, 5, 7, 10, 12, 15, 20 and 25 weeks of age were used (n = 6). DDY/TIP mice (mother strain of EL) at 10 and 20 weeks were used as controls (n = 6). After decapitation, the brains were removed and the hippocampi was dissected out. Approximately 10% homogenates were prepared in 20 mM Tris-HCl buffer (pH 8.0). Just before use, homogenates with dye for 15% SDS-PAGE gels were boiled for 3 min and then centrifuged for 5 min at 2000 ×g.

Primary antibodies and their specificities

Rabbit anti-cyclin A polyclonal antibodies were purchased from Abcam (Cambridge, UK). The epitope cyclin A is a synthetic peptide corresponding to the C-terminal amino acids of the mouse cyclin A sequence. Rabbit anti-cyclin B polyclonal antibodies were purchased from Sigma (Saint Louis, Missouri, USA). The immunogen of the cyclin B antibody is a recombinant hamster cyclin B, and anti-cyclin B recognizes cyclin B in mouse, hamster and human cells. Rabbit anti-cyclin D
Polyclonal antibodies were purchased from Upstate (Charlottesville, VA, USA). The immunogen of cyclin D is a synthetic peptide corresponding to the 11 C-terminal amino acids of human cyclin D, and anti-cyclin D recognizes cyclin D in cells of human, mouse, and rat origin. Rabbit anti-cyclin E polyclonal antibodies were purchased from Lab Vision (Westinghouse Dr, Fremont, CA, USA). The immunogen of cyclin E is a synthetic peptide derived from the C-terminal amino acids of human cyclin E, and anti-cyclin E recognizes cyclin E in cells of human and mouse origin. Rabbit anti-CDK-1 polyclonal antibodies were purchased from Oncogene Research Products (San Diego, CA, USA). The immunogen of CDK-1 is a synthetic peptide corresponding to residues 220-227 in murine CDK-1, and anti-CDK-1 recognizes CDK-1 in cells of mouse, rat, and human origin. Rabbit anti-CDK-2 polyclonal antibodies were purchased from Covance Research Products (Denver, PA, USA). The immunogen of CDK-2 is a synthetic peptide corresponding to the C-terminal sequences of human CDK-2, and anti-CDK-2 recognizes CDK-2 in cells of rodents and human origin. Rabbit anti-CDK-4 polyclonal antibodies were purchased from Abcam (Cambridge, UK). The immunogen of CDK-4 is a human synthetic peptide, and anti-CDK-4 recognizes CDK-4 in cells of mouse and human origin.

Immunoblotting procedures and data analysis

Immunoblotting studies were carried out according to partially modified procedures of Arima et al. [26, 27]. For the immunoblot analysis, 10% mouse brain homogenates (total protein: 30.0–31.0 µg) were separated by 15% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), blocked and then incubated with either anti-cyclin A (1:200), anti-cyclin B (1:200), anti-cyclin D (1:1,000), or anti-cyclin E (1:200); or anti-CDK-1 (1:250), anti-CDK-2 (1:250) or anti-CDK-4 (1:1,000) antibody. After washing, the membrane was incubated with goat anti-rabbit conjugated with horseradish peroxidase (1:10,000) (Sigma, St Louis, Missouri, USA), followed by the Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA, USA). The luminescence of the membrane was detected using Hyperfilm-ECL (Amersham, Little Chalfont, UK). The data were semiquantitatively analyzed using the NIH Image software.

Results

Expression of cyclin/CDK playing major role at CCR checkpoint in the parietal cortex

During G1-S phase, cyclin D/CDK-4 and cyclin E/CDK-2 play major regulatory roles; especially cyclin D/CDK-4 regulates CCR from G0 to G1. In naïve EL mice, cyclin D expression increased to a peak at 15 weeks of age, and then gradually decreased. The corresponding CDK-4 expression showed a peak at 20 weeks of age.

Both cyclin D and CDK-4 showed a parallel expression pattern and reached a maximum level at 20 weeks of age and then decreased.
Lev did not markedly influence the upregulation of the first step of CCR (Figures 3 and 4).

**Expression of cyclins/CDKs playing major roles during G1, G2 and spindle assembly checkpoints in the parietal cortex**

In naïve EL, cyclin E expression increase, peaking at 5 weeks of age and, then gradually decreased. The corresponding expression of CDK-2 reached a peak at 8 weeks of age. Cyclin E and CDK-2 showed a parallel expression pattern during the early development. Lev upregulated the expression of cyclin E and CDK-2 only at 3 weeks of age and abolished the upregulation after that. (Figures 5 and 6)

Cyclin A and CDK-2 are major regulators of S phase. In naïve EL mice, cyclin A expression showed a peak expression at 5-10 weeks of age. The corresponding CDK-2 expression was maximum at 8 weeks of age. Lev upregulated the expression of cyclin A and CDK-2 only at 3 weeks of age and abolished the upregulation after that (Figures 6 and 7).

Cyclin A, B and CDK-1 play major roles during G2-M phase. In naïve EL mice, cyclin A showed a peak expression at 5-10 weeks of age. Cyclin B showed a peak at 20 weeks of age. The corresponding CDK-1 common for cyclin A and cyclin B showed a peak at 10 weeks of age. Lev upregulated the expression of cyclins A, B and CDK-1 only at 3 weeks of age and abolished the upregulation after that (Figures 7, 8 and 9).

**Figure 3.** Effects of levetiracetam on the expression of cyclin D in the parietal cortex of EL mice during the course of development. Lev did not influence the upregulation of the first step of CCR
**Figure 4.** Effects of Levetiracetm on the expression of CDK4 in the parietal cortex of EL mice during the course of development. Both cyclin D and CDK4 showed a parallel expression pattern and reached a maximum level at 20 weeks of age and then decreased.

**Figure 5.** Effects of levetiracetam on the expression of cyclin E in the parietal cortex of EL mice during the course of development. Lev upregulated the expression of cyclin E only at 3 weeks of age and abolished the upregulation after that.
**Figure 6.** Effects of levetiracetam on the expression of CDK2 in the parietal cortex of EL mice during the course of development. Cyclin E and CDK-2 showed a parallel expression pattern during the early development.

**Figure 7.** Effects of levetiracetam on the expression of cyclin A in the parietal cortex of EL mice during the course of development. Lev upregulated the expression of cyclin A only at 3 weeks of age and abolished the upregulation after that.
**Figure 8.** Effects of levetiracetam on the expression of Cyclin B in the parietal cortex of EL mice during the course of development. Lev upregulated the expression of cyclin B only at 3 weeks of age and abolished the upregulation after that.

**Figure 9.** Effects of levetiracetam on the expression of CDK1 in the parietal cortex of EL mice during the course of development. The expression of CDK-1 common for cyclin A and cyclin B showed a peak at 10 weeks of age.
Discussion

Both the apoptotic process and cell proliferation observed in the EL mouse brain appeared to be puzzling. However, in the last decade, the concepts of neural cell loss, cell proliferation, and cell migration have been changed dramatically [28-31].

With respect to cell death-related proteins and neurotrophic factors in the brain, anti-apoptotic Bcl-2 and pro-apoptotic Bax levels are increased during the periods of epileptogenesis in EL mice [6]. The neurotrophic factors NT-3 and BDNF increase significantly as Bcl-2 and Bax are elevated during epileptogenesis [6]. These lines of evidence indicate that in EL mice, the susceptibility of hippocampal neurons to “DNA fragmentation without cell loss” increases after experiencing repetitive seizures during development, probably due to a change in balance between protective mechanism and inactivation of pro-apoptotic pathway [6]. Together with the Bcl-2 family, neurotrophic factors may play a role in epileptogenesis by promoting abnormal synaptic plasticity [11, 12, 32].

Post-seizure “short-term” expression of FGF-2 may be one of the most specific effects of seizures themselves [11], whereas post-seizure “long-term” expression of FGF-2 may indicate neuron survival, neuroprotective effects or long-term synaptic changes following seizures. Similar evidence has been reported in chronic electro-convulsive shock (ECS) [8], amygdala kindled seizures [7], and EL mice [6]. The expression of BDNF after prolonged seizure may indicate adaptive changes during epileptogenesis, as reported in kindled seizures [8], chronic ECS [10], and EL mice [8]. However, the expression of BDNF before exhibiting seizures may trigger ictogenesis and subsequent epileptogenesis in EL mice [6]. NT-3 is known to trigger sprouting but inhibit epileptogenesis [33]. In EL mice, NT-3 expression is very low in the hippocampus of early developmental stage during epileptogenesis [8].

In the present study, cell cycle regulatory proteins were investigated with respect to epileptogenesis and the regulation effects of Lev for CCR. Passage through the four phases of the cell cycle is regulated by a family of cyclins that acts as regulatory subunits for CDK’s [16-18]. The activities of various cyclin/CDK complexes regulate the progression through G1/S/G2/M phases of the cell cycle [16]. In the parietal cortex, EL mice showed upregulation of all the cell cycle-specific cyclins and CDK during early developmental stage when compared with the control DDY mice, suggesting that reentry into cell cycle is promoted prior to the beginning of seizures, possibly due to the abundance of neurotrophic factors i.e. BDNF and NT3 [6]. Administration of Lev had no effects on the expression of cyclin D and CDK4, which suggests that Lev does not inhibit the CCR from G0 to G1. On the other hand, Lev inhibited the expressions of cyclins and CDKs related to the G1, G2 and spindle assembly checkpoints, except at the age under three weeks. In EL mice, the central nervous system development is delayed at the first three weeks and then catches up with normal development thereafter. EL mice show no neurological anomaly except manifesting sei-
zures, and they survive as long as the mother strain DDY [21]. Lev inhibited the abnormal upregulation of CCR after 5 weeks of age, but did not potentiate the delayed normal CCR at 3 weeks, which may compensate the abnormal plastic phenomena and contribute to the antiepileptic effects of Lev.

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