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Valproic acid downregulates Cdk5 activity via the transcription of the p35 mRNA

Miyuki Takahashi [a], Manami Ishida [a], Taro Saito [a], Toshio Ohshima [b], Shin-ichi Hisanaga [a,*]

[a] Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan
[b] Department of Life Science and Medical Bio-Science, Waseda University, Shinjuku, Tokyo 162-8480, Japan

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ABSTRACT

The cyclin-dependent kinase 5 (Cdk5) is a neuron-specific Ser/Thr kinase that is activated by the regulatory subunit p35. Overactivation of Cdk5, which is induced by the cleavage of p35 by calpain, is implicated in neuronal death in various neurodegenerative diseases. In contrast, depletion of the Cdk5 activity renders neurons vulnerable to stresses. Recent reports suggest the involvement of Cdk5 in mental disorders. We hypothesized that perturbation of Cdk5 activity is related to mental conditions. To verify this hypothesis, we investigated the effect of valproic acid (VPA), which is a drug of choice for psychiatric disorders, on Cdk5 activity. VPA decreased the expression of p35 at both the protein and mRNA levels in cultured neurons, resulting in a decrease of Cdk5 activity. VPA decreased the p35 mRNA via histone deacetylase inhibition. The chronic administration of VPA also downregulated p35 in mouse brains. These results indicate that VPA regulates Cdk5 activity in neurons via p35 transcription mediated by HDAC inhibition.

1. Introduction

Valproic acid (VPA) is widely used for the treatment of epilepsy, seizure, migraine, and bipolar disorder [1]. In contrast, prenatal VPA exposure causes malformation of the brain and behavioral abnormalities that are similar to those of autism spectrum syndrome (ASD) [2,3]. However, the mechanisms that underlie these observations are not understood currently. It is important to determine how VPA exerts its therapeutic action and its effect on the developing brain. VPA increases the levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [4]. Furthermore, VPA inhibits histone deacetylase (HDAC) activity [5,6]. HDAC inhibition leads to an increase in the acetylation levels of histones, which induces changes in chromatin structure and, subsequently, in gene expression. However, because the expression of many genes is affected by HDAC inhibition [7,8], the target proteins that are responsible for the effects of VPA have not been identified clearly.

The cyclin-dependent kinase 5 (Cdk5) is a unique member of the Cdk serine/threonine kinase family that is expressed mainly in postmitotic neurons [9,10]. The activity of Cdk5 is mainly determined by the available amount of its activators p35 and p39, with p35 being the major activator [11]. p35 is a short-life protein that is degraded by the proteasome [12–14], and the synthesis of p35 is stimulated by the Egr1 transcription factor via the MEK-ERK1/2 signaling pathway [15,16]. However, the mechanism that is involved in the regulation of the synthesis and degradation of p35 is not well understood.

Cdk5-p35 plays a role in synaptic activity, in addition to having developmental functions, such as neuronal migration and neurite outgrowth [17,18]. The downregulation of Cdk5 activity enhances memory formation and extinction [19,20]. Cdk5 regulates synaptic plasticity structurally by phosphorylating the TrkB BDNF receptor [21], or through the cell-surface expression of the NR2B glutamate receptor via its phosphorylation [22]. We reported that long-term potentiation is induced easily in p35-lacking brain slices and that inhibition of Cdk5 induces easier activation of CaMKII [23,24]. Based on these findings, we hypothesized that Cdk5 determines the threshold of synaptic excitation. Recent reports suggest the involvement of Cdk5 in mental disorders, such as attention deficit/hyperactivity disorder (ADHD) [25], bipolar disorder [26], and schizophrenia [27]. We noticed some similarities in phenotypes and behaviors between VPA-treated and Cdk5-deficient mice

Abbreviations: Cdk5, cyclin-dependent kinase 5; GABA, γ-aminobutyric acid; HDAC, histone deacetylase; MG132, benzyloxycarnonyl-leucyl-leucyl-leucinal; TSA, trichostatin A; VPA, valproic acid; VPM, valpromide.

* Corresponding author. Address: Department of Biological Sciences, Tokyo Metropolitan University, Minami-osawa, Hachioji, Tokyo 192-0397, Japan. Fax: +81 42 677 2559.
E-mail address: hisanaga-shinichi@tmu.ac.jp (S.-i. Hisanaga).

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[2,3,17,18,25–27], and hypothesized that the effect of VPA treatment is mediated by the downregulation of Cdk5 activity. Subsequently, we investigated the effect of VPA on Cdk5-p35 activity in cultured neurons and mice.

2. Materials and methods

2.1. Chemicals and antibodies

VPA and Dulbecco's Modified Eagle's Medium (DMEM)-Ham F-12 nutrient mixtures were purchased from Sigma (St. Louis, MO). Trichostatin A (TSA) and valpromide (VPM) were obtained from Wako (Osaka, Japan). Benzyloxycarnonyl-leucyl-leucyl-leucinal (MG132) was purchased from the Peptide Institute (Osaka, Japan). Antibodies directed against p35 (C-19), Cdk5 (DC-17 and C-8), Egr1, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-p39 antibody (C14) has been described previously [28]. Protein A Sepharose was from GE Healthcare (Buckinghamshire, U.K.). [γ-^{32}P]ATP was obtained from PerkinElmer Japan (Kanagawa, Japan).

2.2. Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from cultured neurons or mouse brains using the Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan). Single-stranded cDNAs were generated from 1 μg of total RNA using the SuperScript First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA). cDNAs were amplified by quantitative PCR using the following primers: 5′-AACAGGATTCCAGCTTTGCTGAC-3′ and 5′-ACACGGGCTTCACAATGGAAC-3′ for p35, 5′-TGTCCAAGGT GCCAATGATG-3′ and 5′-CAGCGCTTTATAGTTCGCTCCA-3′ for p39, 5′-AGCTACAACATAATTGGTGAACGTC-3′ and 5′-ATGCGCTGCACA GGGTTACA-3′ for Cdk5, 5′-CCCTTCCAGGGTCTGGAGAACCGT-3′ and 5′-GGGGTACTTGCGCATGCGGCTGGG-3′ for Egr1, and 5′-TGTGT CCGTCGTGGATCTGA-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ for GAPDH. Amplification was carried out in the presence of SYBR Premix Ex Taq (Takara, Kyoto, Japan) containing 1 μl of each primer and 10 ng of cDNA on a Thermal Cycler Dice (Takara).

2.3. Cell culture, immunoblotting, immunoprecipitation, and kinase assay

Brain cortical neurons were prepared from fetal ICR mice at embryonic day 16 and cultured at a density of 0.3×10^6 cells/cm^2 in DMEM and Ham's F-12 (1:1) containing 5% fetal bovine serum and 5% horse serum [29]. The protein concentration of cell lysates or brain extracts was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as the standard. Equal amounts of each sample were applied to a 10% polyacrylamide gel for SDS–PAGE. Immunoblotting and immunodetection were carried out as described previously [14] using an ECL system (GE Healthcare) or the Millipore Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Bedford, MA). Immunoprecipitation and kinase assay of Cdk5 were performed as described previously [29].

2.4. Animals and treatments

ICR mice were obtained from the Sankyo Laboratory Service (Tokyo, Japan) and C57BL/6J mice were purchased from CREA Japan (Tokyo, Japan). All animals used in this study were housed in a controlled environment (22–25 °C, 50% humidity) under a 12 h light/12 h dark cycle, with standard diet and distilled water available *ad libitum*. All experiments were performed in compliance with relevant laws and institutional guidance of the Tokyo Metropolitan University. C57BL/6J mice at the age of 7 weeks were assigned randomly to the vehicle control group and the VPA treatment group (n = 6 each group). Mice in the VPA group were injected intraperitoneally (i.p.) with VPA (400 mg/kg, dissolved in phosphate-buffered saline (PBS)) once a day for 2 weeks. Control mice were treated with the same volume of PBS. Brains were homogenized in 20 mM HEPES, pH 7.5, 0.1 mM EDTA, 2 mM $MgCl_2$, 5 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.2 mM Pefablock, and 1 μg/mL leupeptin, and the extracts were prepared as described previously [29].

2.5. Statistical analysis

Immunoreaction was captured as a digital image using a Chemidoc XRS apparatus (Bio-Rad Laboratory, Tokyo, Japan) or by scanning an X-ray film. The band intensities were measured using the Image J software. All experiments were performed a minimum of three times, with similar results; representative results are shown in the figures. The statistical significance of the data was analyzed using Student's t test.

3. Results

3.1. VPA downregulates Cdk5 activity by decreasing p35 in cultured cortical neurons

We examined whether VPA affects the expression of Cdk5 or p35 in mouse cortical neurons. Cortical neurons at 5 DIV were treated with various concentrations of VPA for 12 h. VPA decreased the levels of p35, but not Cdk5, in a dose-dependent manner in the range of 1–20 mM (Fig. 1A). Quantification indicated a decrease in p35 level by approximately 20% relative to the control after the administration of 20 mM VPA. The time course of this experiment is shown in Fig. 1B. p35 began to decrease at 1 h and continued up to 12 h after treatment (Fig. 1B). Neurons appeared to be healthy during VPA treatment (visual inspection), and p35 returned to control levels 24 h after the treatment (data not shown). In contrast to what was observed for p35, the levels of p39, which is another activator of Cdk5, did not change after VPA treatment (Fig. 1B). The effect of VPA on the activity of Cdk5 was also examined. Cdk5-p35 was immunoprecipitated from the neuron extracts with an anti-Cdk5 antibody using buffers that isolate Cdk5-p35 but not Cdk5-p39 [28]. VPA treatment decreased Cdk5-p35 activity (Fig. 1C). These results indicate that VPA downregulates Cdk5 activity by decreasing p35 in cultured neurons.

3.2. Degradation of p35 is not a target for VPA

p35 has a short half-life and is degraded by the ubiquitin–proteasome system. We assessed the effect of VPA on the degradation of p35. VPA treatment decreased p35 (Supplemental Fig. S1, VPA), whereas the proteasome inhibitor MG132 increased p35 (Supplemental Fig. S1, MG132), as expected. If the proteasomal degradation of p35 is a target for VPA, the effect of VPA should be canceled by MG132, and the amount of p35 should increase, as was observed in the presence of MG132. However, VPA suppressed the MG132-dependent increase in the levels of p35 (Supplemental Fig. S1, VPA + MG132). These results suggest that proteasomal degradation is not a main target of VPA.

3.3. VPA downregulates the levels of the p35 mRNA

VPA modulates the transcription of many genes. Therefore, we tested whether VPA decreases the transcription of p35. The levels

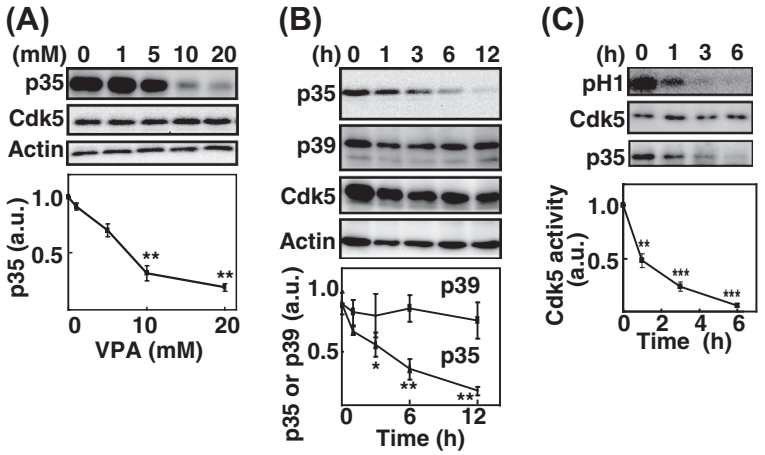

Fig. 1. VPA decreases the levels of p35 and Cdk5 activity in primary neurons. (A) Effect of VPA on p35 in cultured cortical neurons. Mouse cortical neurons at 5 DIV were treated with VPA at the indicated concentrations for 12 h. p35 and Cdk5 were detected by immunoblotting with anti-p35 and anti-Cdk5 antibodies, respectively. Actin was the loading control. The lower panel depicts the quantification (means ± sem, $n = 3$, $^{**}P < 0.01$, Student's t test). (B) Time course of the effect of VPA on p35 expression. Mouse cortical neurons were treated with 20 mM VPA for the indicated times. p35, p39, and Cdk5 were detected by immunoblotting with anti-p35, anti-p39, and anti-Cdk5 antibodies, respectively. Actin was the loading control. The lower panel depicts the quantification (means ± sem, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t test). (C) Effect of VPA on the kinase activity of Cdk5-p35. Cortical neurons were treated with VPA, and p35 and Cdk5 were detected in the cell extracts by immunoblotting, as described above. The kinase assay was performed with immunoprecipitated Cdk5-p35 using histone H1 as a substrate (pH1). Quantification is shown in the lower panel (mean ± sem, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.005$, Student's t test).

of the p35 transcript were measured by quantitative PCR. Treatment with VPA for 6 and 12 h decreased the p35 mRNA by about 50% relative to the control. We also measured the levels of the p39 and Cdk5 mRNAs in parallel. Consistent with the results regarding protein levels, the p39 and Cdk5 mRNAs did not decrease; rather, they increased slightly after VPA treatment (Fig. 2). These results suggest that VPA decreases Cdk5 activity by suppressing p35 transcription.

3.4. VPA decreases p35 transcription in an Egr1-independent manner

It is known that the transcription of p35 is regulated by Egr1, downstream of ERK1/2, in response to nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) [15]. We tested whether VPA modulates the transcription of p35 in an Egr1-dependent manner. We measured the protein and mRNA levels of Egr1 in neurons treated with 20 mM VPA for 3 or 6 h. In contrast to p35, the Egr1 protein was increased about twofold relative to the control neurons (Supplemental Fig. S2A). Egr1 mRNA levels were also increased fourfold in neurons treated with 20 mM VPA for 6 h compared with control neurons (Supplemental Fig. S2B). These results indicate that VPA downregulates p35 independent of the ERK1/2-Egr1 pathway.

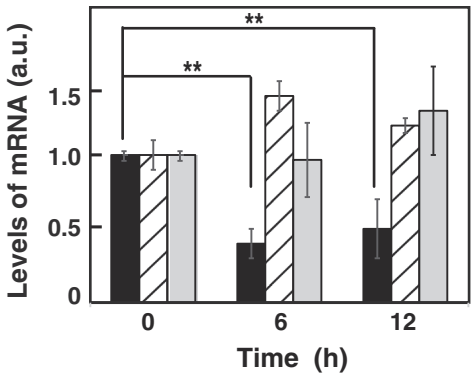

Fig. 2. VPA decreases the p35 mRNA in primary neurons. The mRNA levels of p35, p39, and Cdk5 were measured by quantitative PCR. Mouse cortical neurons were treated with 20 mM VPA for 6 or 12 h, and the mRNA levels of p35, p39, or Cdk5 were estimated using the primers described in the "Section 2". The levels of the p35 (black), p39 (diagonal), and Cdk5 (gray) mRNAs were estimated using the ΔΔCt method and are shown as the ratio against that of control neurons (means ± sem, $n = 5$, $^{**}P < 0.01$, Student's t test).

3.5. VPA-induced p35 downregulation is mediated by HDAC inhibition

VPA is an HDAC inhibitor [5,6]. As the acetylation of histones generally increases transcription [30], we wondered whether VPA decreases p35 transcription by inhibiting HDAC activity. To examine this possibility, we used valpromide (VPM), which is a derivative of VPA that has no HDAC inhibitory activity. VPM did not change p35 levels in cortical neurons (Fig. 3A). Next, we tested another HDAC inhibitor, trichostatin A (TSA), which is a structurally dissimilar HDAC inhibitor compared with VPA. TSA treatment downregulated p35 in a dose-dependent manner, from 0.25 to 1 mM (Fig. 3B). TSA, but not VPM, reduced the expression of the p35 mRNA (Fig. 3C). These results indicate clearly that VPA downregulates p35 via the inhibition of HDACs.

3.6. Chronic administration of VPA decreases the protein and mRNA levels of p35 in vivo in mouse brains

VPA is used to treat epilepsy, migraine, and bipolar depression disorders [1]. Therefore, it is important to identify the effect of VPA on p35 in vivo. VPA was administered intraperitoneally to 7-week-old mice at 400 mg/kg of body weight. The effect of VPA was confirmed by the observation of a suppressive behavior in the open-field test 30 min after the administration of the drug (data not shown). The cerebral cortex was dissected and subjected to immunoblotting for p35; however, the levels of the protein did not change, at least up to 120 min after VPA administration (Fig. 4A).

Next, we administered VPA to mice chronically for 14 days. The effect of VPA on the anxiety behavior was confirmed by a forced swimming test. Non-moving time was increased about 2.5-fold after the administration of the drug for 2 weeks (data not shown). p35 began to decrease at 3 days and reached 20% of the control at 14 days (Fig. 4B). The effect of VPA on p35 mRNA levels was also

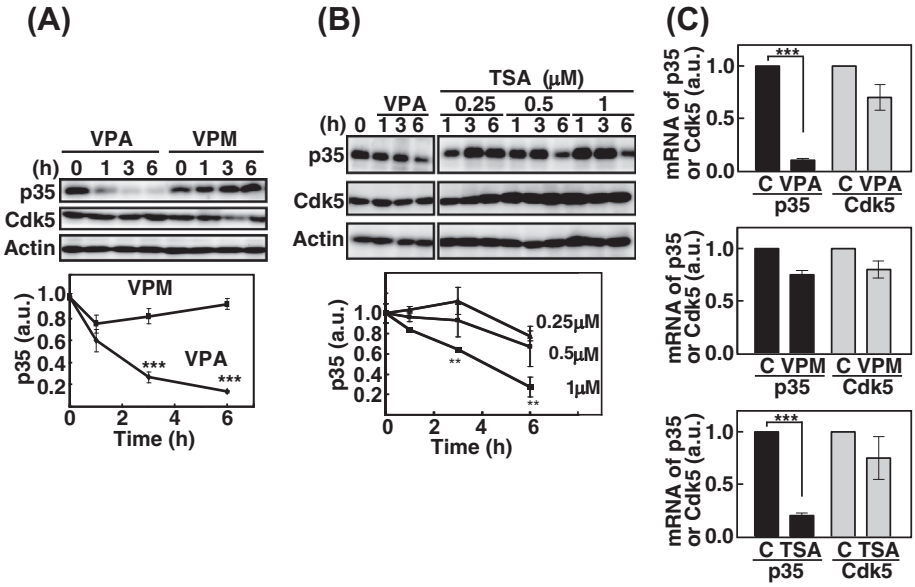

Fig. 3. Downregulation of p35 is caused by HDAC inhibition by VPA. (A) Effect of valpromide (VPM) on p35 expression. Cortical neurons at 5 DIV were treated with 20 mM VPA or 20 mM VPM for the indicated times. p35 and Cdk5 were detected by immunoblotting with anti-p35 and anti-Cdk5 antibodies, respectively. Actin was the loading control. Quantification is shown in the lower panel (means ± sem, $n = 3$, $^{***}P < 0.005$, Student's t test). (B) Effect of TSA on p35 expression. Cortical neurons were treated with 20 mM VPA or TSA at the indicated concentrations for the indicated times. p35 and Cdk5 were detected as described above. Actin was the loading control. Quantification is shown in the lower panel (means ± sem, $n = 3$, $^{**}P < 0.01$, Student's t test). (C) Effect of VPA, VPM, or TSA on the mRNA levels of p35 or Cdk5. Cortical neurons were treated with 20 mM VPA, 20 mM VPM, or 1 mM TSA for 6 h. The mRNA levels of p35 (black) or Cdk5 (gray) were measured by quantitative PCR (means ± sem, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.005$, Student's t test).

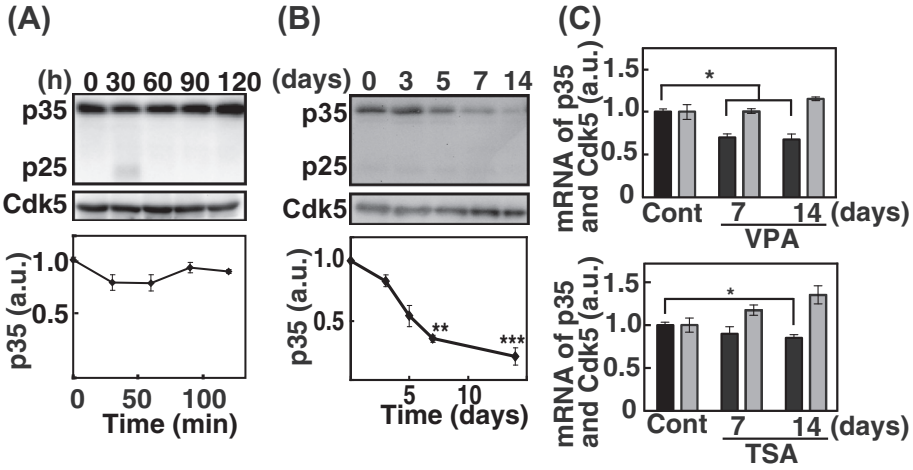

Fig. 4. Effect of acute or chronic VPA treatment on the protein and mRNA levels of p35 in mouse brains. (A) Effect of one-shot VPA administration on p35 expression in mouse brains. Mice at 7 weeks of age were administered VPA intraperitoneally at 400 mg/kg body weight. Control mice were injected with PBS. p35 and Cdk5 in the cerebral cortex were detected by immunoblotting with anti-p35 and anti-Cdk5 antibodies (upper panel). The position of p25 is indicated in a blot of p35. The lower panel is the quantification of p35 (means ± sem, $n = 3$, Student's t test). (B) Effect of chronic VPA treatment on the protein levels of p35 and Cdk5 in mouse brains. Mice were administered VPA daily (400 mg/kg) for the indicated days. Brains were excised, and p35 and Cdk5 were detected by immunoblotting. Quantification is shown in the lower panel (means ± sem, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.005$, Student's t test). (C) Effects of chronic VPA or TSA treatment on the mRNA levels of p35 and Cdk5 in mouse brains. Mice were administered with VPA or TSA as described above for 7 or 14 days. Control mice received PBS. mRNAs were measured by quantitative PCR (means ± sem, $n = 3$, $^{*}P < 0.05$, Student's t test).

examined by quantitative PCR. The p35 mRNA, but not the Cdk5 mRNA, was decreased significantly by 70% and 65% of the control mice after exposure to the drug for 1 and 2 weeks, respectively (Fig. 4C). Similarly, TSA treatment decreased p35 mRNA levels (Fig. 4C).

4. Discussion

Here, we found that VPA downregulates Cdk5 activity by suppressing p35 transcription via HDAC inhibition, whereas VPA is reported to suppress Cdk5 activity by reducing phospho-Cdk5 and the ratio of p25/p35 [31]. VPA is a chemical with a variety of biological activities, such as teratogenicity, modulation of GABAergic neurons, and antidepressant effects [2,3]. However, it is not well understood how VPA induces these biological actions. It is very important to determine the molecular mechanism underlying these actions, because VPA is a widely used medication [1]. Its antimanic efficacy may be mediated by GABA transaminase, the inhibition of which increases the concentration of GABA, resulting in suppression of synaptic activity [4]. However, the effect on GABA metabolism does not explain the other pharmacological activities of VPA. HDACs are another well-known target of this drug [5,6].

VPA inhibits HDACs, resulting in changes in chromatin structure that induce the expression of many genes. However, as it affects many genes, it is very difficult to correlate the teratogenic or psychiatric action of VPA with its specific target. We showed here that VPA downregulated Cdk5 activity by inhibiting p35 expression. There are several similarities between the biological actions of VPA and Cdk5-deficient phenotypes. For example, both induce brain developmental abnormalities and affect synaptic activity [2,3,17,18,25–27]. VPA may execute some of its neuronal functions via the downregulation of Cdk5 activity, which is an interesting hypothesis that should be investigated in greater detail in the future.

Cdk5-p35 is a multifunctional protein kinase, the activity of which is controlled by the level of p35 via its synthesis and degradation [11]. Although it has been shown that the degradation of p35 is catalyzed by the proteasome [12–14], it is not clear how the synthesis of p35 is regulated. The synthesis of p35 is affected by the extracellular matrix or NGF/BDNF [15,32]. We found that VPA decreased the expression of p35 at the level of transcription. Egr1 is a transcriptional factor that acts downstream of the NGF/BDNF-ERK1/2 pathway and regulates p35 synthesis [15,16]. If Egr1 was involved in decreasing p35 transcription, Egr1 activity should have been reduced. However, VPA increased Egr1 expression. Instead, we found that the effect of VPA on p35 expression was mediated by HDAC inhibition. Although a large number of genes are stimulated by HDAC inhibition, some genes are suppressed by it [5,6,33]. p35 is among the latter, which was consistent with the results of a comprehensive analysis of mRNA expression in VPA-treated cultured neurons [34]. Those authors detected p35 as a gene, the mRNA of which was reduced by VPA treatment. VPA would loosen the chromatin structure of the promoter region of the p35 gene, but negative transcription factors may bind to this region in the case of p35, to inhibit transcription. Only a few studies have addressed the transcriptional regulation of p35 [35].

Although VPA decreased p35 expression at 6 h after treatment in cultured neurons, one-shot exposure did not change the levels of p35 in mouse brains. Chronic treatment (for 14 days) was required to decrease p35 to levels that were comparable with those observed in cultured neurons. This difference may be, at least in part, caused by the difference in gene expression between culture embryonic neurons and in vivo neurons in adult mouse brains. In the case of therapeutic treatment, the effect of VPA becomes apparent after more than 10 days of medication [33]. This may be an indication that links Cdk5 activity and the usage of VPA as an antidepressant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.072.

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