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Neuronal Per Arnt Sim (PAS) Domain Protein 4 (NPAS4) Regulates Neurite Outgrowth and Phosphorylation of Synapsin I*

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Background: NPAS4 is involved in memory formation, but its roles in neuronal function remain to be fully elucidated.
Results: NPAS4 increased CDK5-dependent phosphorylation of synapsin I that was associated with neurite elongation.
Conclusion: NPAS4 induced CDK5-dependent phosphorylation of synapsin I to facilitate neurite outgrowth.
Significance: NPAS4 plays an important role in the structural and functional plasticity of neurons.

Neuronal Per Arnt Sim domain protein 4 (NPAS4), a brain-specific basic helix-loop-helix transcription factor, has recently been shown to regulate the development of the GABAergic inhibitory synapses and transcription program for contextual memory formation in the hippocampus. We previously reported that chronic social isolation or restriction stress in mice resulted in an impairment in memory and emotional behavior, which was associated with a decrease in *Npas4* mRNA levels. In this study, we investigated the role of NPAS4 in neuronal function *in vitro* and *in vivo*. Differentiation medium-induced neurite outgrowth was inhibited in *Npas4* knockdown Neuro2a cells, whereas overexpression of NPAS4 accelerated the neurite outgrowth in Neuro2a cells. Furthermore, depolarization-induced neurite outgrowth was abolished in *Npas4* KO hippocampal neurons. NPAS4 overexpression increased cyclin-dependent kinase 5 (CDK5)-dependent synapsin I phosphorylation in Neuro2a cells and primary cultured hippocampal neurons. A CDK5 inhibitor, roscovitine, inhibited the neurite outgrowth and the increase in phosphorylated synapsin I (p-SYN I) levels in *Npas4*-overexpressed Neuro2a cells. Interaction of NPAS4 with promoters of *Cdk5* and *NeuN* genes was demonstrated by a chromatin immunoprecipitation assay. In an *in vivo* study, pentylenetetrazole-induced convulsions in mice resulted in an increase in NPAS4 and p-SYN I levels in the prefrontal cortex of wild-type mice, although no changes in p-SYN I levels were observed in *Npas4* knock-out mice. These results suggest that NPAS4 plays an important role in the structural and functional plasticity of neurons.

The basic helix-loop-helix family has been studied for its important roles in various physiological and pathophysiological processes, including as transcription factors, in the regulation of neuronal differentiation and even in psychiatric disorders (1–6). Neuronal Per Arnt Sim domain protein (NPAS) is a member of the basic helix-loop-helix family, which is categorized as Npas1, Npas2, Npas3, and Npas4 (7–9). Npas3 has been identified as a candidate gene associated with schizophrenia, learning disabilities, and bipolar disorder (5, 10), and it is known to play a role in hippocampal neurogenesis (1, 11). NPAS4 is a neuron-specific transcription factor (12), and the *Npas4* expression level in the hippocampus is regulated by cerebral ischemic insults, the AMPA receptor agonist and kainic acid (13–15). Lin *et al.* (13) have reported that NPAS4 regulates the development of GABAergic inhibitory synapses in an activity-dependent manner and suggested its homeostatic role in the balance between excitatory and inhibitory neuronal activities in the brain. We previously reported that reduced *Npas4* mRNA levels may contribute to impairments in adult neurogenesis in the hippocampus, memory and emotional behaviors induced by social isolation or restriction stress (16, 17). Recent studies (18, 19) revealed that NPAS4 is important in memory formation and consolidation. Synaptic remodeling is considered to be a way for neurons to adapt cellular and neuronal circuits to environmental changes (20), and neurite arborization and rewiring may contribute to the neuronal plasticity in the brain (21–24). In this study, to investigate a possible role for NPAS4 in structural and functional plasticity of neurons, we examined the effect of knockdown or overexpression of NPAS4 on neurite outgrowth in Neuro2a cells. Neurite outgrowth in *Npas4* knock-out primary cultured hippocampal neurons was also investigated. Then, we investigated the underlying mechanism by which NPAS4 regulates neurite outgrowth. We focused on

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the phosphorylation of a synaptic vesicle-associated protein, Syn I,[3] via cyclin-dependent kinase 5 (CDK5). Finally, we conducted an *in vivo* study to see if the NPAS4-induced CDK5/SYN I pathway could be operated under physiological and pathophysiological conditions using a pentylenetetrazole (PTZ)-induced epilepsy model in mice.

EXPERIMENTAL PROCEDURES

Cell Culture—Neuro2a cells were kindly donated by Dr. Sigeru Yoshida (Taisho Pharmaceutical Co., Ltd., Saitama, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics/antimycotics (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2. For neurite development, the medium was replaced with differentiation medium (DM), DMEM containing lithium chloride (LiCl, 20 mM, Wako Chemicals, Osaka, Japan), and *myo*-inositol (10 mM, Wako Chemicals) with 2% FBS, and the cells were cultured for 12–48 h. The number of Neuro2a cells seeded was $1–5 \times 10^4$ cells/ml, 2 ml per well (6-well dishes), according to the experimental design.

Quantitative Real Time RT-PCR—Complementary DNA was synthesized from total isolated RNA using a SuperScriptIII first-strand synthesis system for RT-PCR (Invitrogen). Levels of mRNA expression were quantified by using a 7300 real time PCR System (Applied Biosystems, Foster City, CA). The quantitative real time RT-PCR was performed in a volume of 25 μl with 1 μg of cDNA and 500 nM primers in a Power SYBR Green Master Mix (Applied Biosystems). The primers used were as follows: 5′-AGCATTCCAGGCTCATCTGAA-3′ (forward) and 5′-GGCGAAGTAAGTCTTGGTAGGATT-3′ (reverse) for *Npas4*; 5′-TGTCAAGCTCATTTCCTGGTATGA-3′ (forward) and 5′-CTTACTCCTTGGAGGCCATGTAG-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase for GAPDH; and 5′-CGATGCCCTGAGGCTCTTT-3′ (forward) and 5′-TGGATGCCACAGGATTCCA-3′ (reverse) for *β-Actin* used as internal controls.

Immunocytochemistry—The cells were rinsed twice in phosphate-buffered saline (PBS) at room temperature. Fixation was performed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, and permeabilization was carried out with 0.2% Triton X-100 in PBS for 15 min at room temperature. The cells were incubated in 5% goat serum (Vector Laboratories Inc., Burlingame, CA) in PBS or Tris-buffered saline (TBS) for 1 h at room temperature. Polyclonal antibodies, NPAS4 antibodies 1 and 2, were raised against a peptide sequence of FHYTEKEQNEIDRL at the C terminus (for Neuro2a cells, rabbit, 1:300, Japan Bio Services Co., Ltd., Saitama, Japan) and a recombinant protein sequence (597–802, for hippocampal neurons, rabbit, 1:5,000, MBL, Nagano, Japan) of the NPAS4 protein, respectively. The cells were incubated with anti-Npas4, anti-Myc (mouse, 1:2,000), anti-Cdk5 (rabbit, 1:300, Santa Cruz Biotechnology), anti-Tau (mouse, 1:500, Santa Cruz Biotechnology), anti-GFP (rabbit, 1:2,000, MBL), anti-MAP2 (mouse, 1:500, Abcam, Cambridge, MA), anti-Tuj1 (mouse or rabbit, 1:500, Sigma), or anti-p-Syn I (goat, 1:300) antibodies at 4 °C overnight. The cells were rinsed in PBS three times for 10 min and incubated with anti-rabbit Alexa 594 (goat, 1:1,000, Invitrogen), anti-rabbit Alexa 488 (donkey, 1:1,000), anti-mouse Alexa 488 (goat, 1:1,000), anti-goat Alexa 546 (donkey, 1:1,000), anti-mouse Alexa 594 (donkey, 1:1,000), anti-mouse Alexa 405 (goat, 1:1,000), or anti-rabbit Alexa 405 (goat, 1:1,000) antibodies at room temperature for 2 h. Rinsed cells were mounted with coverslips and visualized under a microscope (Axio Imager, Zeiss). p-SYN I or CDK5 fluorescence intensity was measured with the "histogram" feature of ImageJ after selecting puncta with "freehand selections" (National Institutes of Health, rsbweb.nih.gov).

Npas4 siRNA Transfection and Neurite Length Measurement—The sequence of *Npas4* siRNA is 5′-GGTTGACCCTGATAATTTA-3′, and the scrambled siRNA sequence is 5′-GGTTCAGCGTCATAATTTA-3′ according to Lin *et al.* (13). Neuro2a cells (1×10^4 cells/ml) were cotransfected with *Npas4* siRNA (50 nM) or scrambled siRNA (50 nM) and pZsGreen1-N1 (100 ng, Clontech) vectors using Lipofectamine RNAiMAX (7.5 μl, Invitrogen). Seventy two hours later, the medium was replaced with DM, and the cells were cultured for 24 h. Six to seven areas were photographed randomly from three independent experiments each, and TUJ1-positive neurite length (25) was measured using a microscope and a visual image analysis system (Axio Imager, Zeiss, Jena, Germany).

NPAS4 Overexpression and Neurite Length Measurement—On the basis of the GenBank™ sequence, *Mus musculus Npas4* mRNA (BC129861) was chosen to construct the overexpression vector. Briefly, PCR was carried out using mouse cDNA with primers, which introduced a NotI site with deletion of the stop codon at the 3′ end. The PCR product was subcloned into the pCR®-XL-TOPO (Invitrogen) and sequenced. The cloned vector was digested using PstI and NotI endonuclease (Takara, Otsu, Shiga, Japan), and the fragment was inserted at the PstI and NotI site of pCMV/myc/nuc vector (Invitrogen). Neuro2a cells (2×10^4 cells/ml) were transfected with the Npas4/pCMV/myc/nuc vector (1 μg, *Npas4* vector) or pCMV/myc/nuc vector (mock vector) using FuGENE 6 (6 μl, Roche Diagnostics). Seventy two hours later, the medium was replaced with DM, and the cells were cultured for 12 h. Neurite length was measured as described above.

Immunoblot Analysis of NPAS4-overexpressed Neuro2a Cells—The NPAS4 overexpression vector was transfected to the Neuro2a cells (5×10^4 cells/ml) as mentioned above. Seventy two hours later, whole cell lysates were collected by general methods. For Tuj1 analysis, the medium was replaced with DM, and the cells were cultured for 12 h. The homogenates were subjected to SDS-PAGE (7.5%), and immunoblotting was performed. After blocking, membranes were incubated with anti-p-SYN I (goat, 1:300, Santa Cruz Biotechnology), anti-SYN I (goat, 1:300, Santa Cruz Biotechnology), anti-β-actin (goat, 1:300, Santa Cruz Biotechnology), anti-Npas4 (antibody 2, 1:1,000), anti-Tuj1 (mouse, 1:5,000, Sigma), anti-Myc (mouse, 1:1,000, Cell Signaling Technology, Beverly, MA), or anti-neuronal nuclei (NeuN) (mouse, 1:1,000, Chemicon International, Temecula, CA) antibodies at 4 °C overnight. Horseradish per-

[3] The abbreviations used are: Syn I, synapsin I; ANOVA, analysis of variance; DM, differentiation medium; p-Syn I, phosphorylated synapsin I; DIV, day *in vitro*; PTZ, pentylenetetrazole.

oxidase-conjugated anti-rabbit (1:1,500, Kirkegaard & Perry Laboratories), anti-mouse (1:2,000, Kirkegaard & Perry Laboratories), or anti-goat antibodies (1:1,000, R&D Systems, Minneapolis, MN) were treated for 1 h at room temperature, and the immunoreactivity was visualized with an ECL Plus detection system (GE Healthcare).

CDK5 Immunoblot and Activity Assay—After transfection of *Npas4* or mock expression vector in Neuro2a cells, the transfected cells were enriched by G418 (1.2 μg/ml, Calbiochem, Merck KGaA) selection. The whole cell lysates were collected, and an immunoblot assay was performed with anti-Cdk5 (rabbit, 1:1,000, Santa Cruz Biotechnology) antibody. CDK5 activity assay was performed with ADP-Glo^{TM} kinase assay kit according to manufacturer's instructions (Promega, Madison, WI).

Primary Hippocampal Neuron Culture and the Measurement of p-SYN I and Neurite Outgrowth—Hippocampal neurons were dissociated and prepared from E16 to E17 mouse embryos as described previously (26). To measure p-SYN I levels, ICR mice were killed on gestational day 16 or 17, and embryo hippocampi were digested with 0.25% trypsin and 0.01% DNase for 10 min at 37 °C and mechanically dissociated by gentle pipetting. Neurons were plated at 5×10^4 cells on poly-L-lysine-coated coverslips (12 mm, BD BioCoat, Bedford, MA) and cultured in DMEM (D6546, 10% fetal bovine serum, 2 mM L-glutamine, antibiotics/antimycotics) on day *in vitro* (DIV) 0. DMEM was replaced with glial conditioned medium (nerve cell and microglial cell culture system, SUMITOMO BAKELITE, Tokyo, Japan) with Ara-C (5 μM, Sigma) and antibiotics/antimycotics. The *Npas4* overexpression vector was transfected on DIV 4. Briefly, the medium was removed and kept at 37 °C in a humidified atmosphere with 5% CO_2. Npas4/pCMV/myc/nuc vector (1 μg) was transfected with Opti-MEM I (100 μl, Invitrogen) and Lipofectamine 2000 (5 μl, Invitrogen) in a new medium (500 μl, without antibiotics/antimycotics), and the transfection medium was replaced with old medium 4 h later. Immunocytochemistry was performed on DIV 5, and the p-SYN I fluorescence intensity was measured as described above. To measure neurite outgrowth, hippocampal neurons were dissociated and prepared from E16 to E17 mouse embryos of *Npas4* knock-out (KO) or wild-type (WT) littermates as described above. *Npas4* KO mice were kindly donated by Dr. Greenberg (13). The longest Tau-positive axonal length was measured at DIV 3. MAP2-positive dendrites length was measured at DIV 9, after treatment of KCl (16 mM) for 48 h (DIV 7–9) according to Wayman *et al.* (27). The measurement of neurite length and Sholl analysis were performed using a microscope and a visual image analysis system (Neurolucida, MBF Bioscience, Williston, VT).

Chromatin Immunoprecipitation Assay (ChIP Assay)—Neuro2a cells (3.9×10^7 cells, 10 cm dish) were seeded, and 96 h later the medium was replaced with DM, and the cells were cultured for 48 h. Primary cultured cortical neurons (4.0×10^7 cells, 10-cm dish) on DIV 4 were treated with tetrodotoxin and AP5 (an *N*-methyl-D-aspartate receptor antagonist) followed by KCl according to the protocol of Lin *et al.* (13). A ChIP assay was performed with a Simple Chip Enzymatic Chromatin IP kit (Cell Signaling Technology) following the manufacturer's manual. The following primers were used for quantitative PCR in the chromatin immunoprecipitation assay: 5′-CAGGATGACTCACACTGACAGTATTTTTAG-3′ (forward) and 5′-GTGGGAGAAGAGCTATTTATATCACCAG-3′ (reverse) for *Npas4* (13); 5′-GCTAGCTCTAGATTTCGTGT-3′ (forward) and 5′-GGAAAGTTGAGTTGGGAGA-3′ (reverse) *Cdk5*; 5′-CCGTGGTATGTGCCTCA-3′ (forward) and 5′-CTGATACACGTTGCTGGA-3′ (reverse) for *NeuN*; and 5′-GTTGGCCAGACAGAAGACAAGAGA-3′ (forward) and 5′-TATACACAGCCCTCCAAAGGCAGA-3′ (reverse) for *p35*.

Pentylenetetrazole-induced Convulsion Model of Epilepsy—*Npas4* KO mice or WT littermates at 6–8 weeks old were housed 4–5 per cage under standard conditions (23 $\pm$ 1 °C, 50 $\pm$ 5% humidity), with a 12-h light/dark cycle. Food and water were available *ad libitum*. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University. *Npas4* KO and WT mice were administered intraperitoneally with PTZ (Sigma) at a dose of 60 mg/kg, and the intensity of convulsions induced by PTZ was scored according to previous studies (28, 29). After seizure testing, mice were sacrificed. The prefrontal cortex was isolated, and an immunoblot assay was performed as described above (including p35 detection with p35 antibodies, rabbit, 1:1,000, Santa Cruz Biotechnology).

Statistical Analysis—Differences between the two groups were analyzed by Student's *t* test. One-way analysis of variance (ANOVA) followed by Dunnett's test or two- and three-way ANOVA followed by Bonferroni's test was used for multigroup comparisons. To compare neurite length, differences between two groups were analyzed by the Kolmogorov-Smirnov test, and differences among more than two groups were analyzed by one-way ANOVA followed by Dunnett's test and confirmed by the Kolmogorov-Smirnov test. The relationship between CDK5 and p-SYN I expression was analyzed by linear regression. The comparison of regression lines was performed by parallel line analysis.

RESULTS

Effect of Differentiation Medium on Neurite Outgrowth, NPAS4, and p-SYN I Expression in Neuro2a Cells—Neuronal differentiation of Neuro2a cells can be induced by treatment with DM leading to morphological changes with longer neurites. When Neuro2a cells were treated with DM for 48 h, neurite outgrowth in Neuro2a cells was accompanied by an increase in p-SYN I levels (Fig. 1, *A–C*) with no change in total SYN I levels. Next, we investigated whether neurite outgrowth of Neuro2a cells and the increase in p-SYN I levels induced by DM were associated with changes in *Npas4* mRNA levels. We measured *Npas4* mRNA levels in DM-treated Neuro2a cells using quantitative RT-PCR. DM significantly increased the mRNA levels of *Npas4* in Neuro2a cells (Fig. 1*D*).

Effect of Npas4 Knockdown by siRNA Treatment on Neurite Outgrowth in Neuro2a Cells—To study the causal relationship between the DM-induced increase in NPAS4 expression and the neurite outgrowth in Neuro2a cells, we next examined whether knockdown of *Npas4* expression by siRNA treatment suppressed neurite outgrowth induced by DM treatment. The siRNA or scrambled siRNA of *Npas4* with a pZsGreen1-N1 vector, which encodes green fluorescent protein (GFP), was

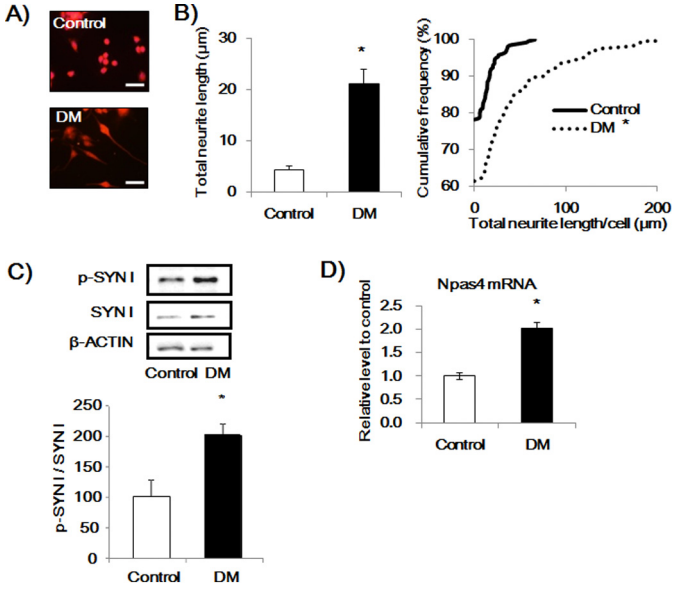

FIGURE 1. **Effect of DM treatment for 48 h on neurite outgrowth, NPAS4, and p-SYN I expression levels in Neuro2a cells.** *A,* representative photographs showing control (*top*) and DM-treated cells (*bottom*). *Scale bar,* 20 μm. *B,* quantitative analysis of neurite length. Values indicate the mean ± S.E. (*left*) and the cumulative frequency of neurite length (*right*). ($n = 261$ for control and $n = 251$ for DM.). *, $p < 0.001$ *versus* control (Student's *t* test, Kolmogorov-Smirnov test). *C,* immunoblot analysis of p-SYN I expression levels induced by DM. Values indicate the mean ± S.E. ($n = 8$). *, $p < 0.05$ *versus* control (Student's *t* test). *D,* quantitative RT-PCR analysis of the *Npas4* mRNA level. Values indicate the mean ± S.E. ($n = 3$). *, $p < 0.01$ *versus* control (Student's *t* test).

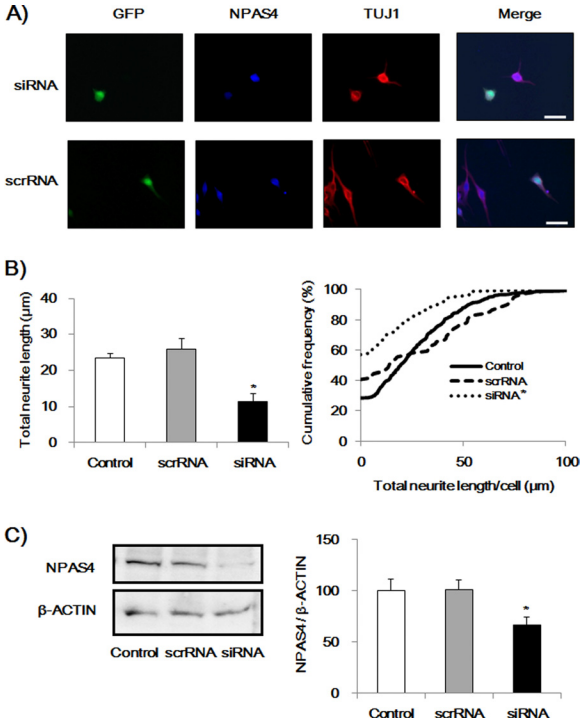

FIGURE 2. **Effect of NPAS4 knockdown on neurite outgrowth induced by DM in Neuro2a cells.** *A,* representative photographs showing immunocytochemical analysis of siRNA- or scrRNA-transfected cells (GFP-positive). *Scale bar,* 20 μm. *B,* quantitative analysis of neurite length. Values indicate the mean ± S.E. (*left*) and the cumulative frequency of neurite length (*right*). ($n = 79$ for *Npas4* siRNA transfection (siRNA), $n = 81$ for scrambled siRNA transfection (scrRNA), and $n = 463$ for nontransfection (control).). *, $p < 0.01$ *versus* scrRNA and control (one-way ANOVA followed by Dunnett's test, confirmed by the Kolmogorov-Smirnov test). *C,* immunoblot analysis of NPAS4 expression levels in control, *Npas4* siRNA, or scrRNA-transfected Neuro2a cells. Seventy two hours after transfection, the medium was replaced with DM, and the cells were cultured for 24 h. Values indicate the mean ± S.E. ($n = 10–11$). *, $p < 0.05$ *versus* control and scrRNA (one-way ANOVA followed by Dunnett's test).

transfected in Neuro2a cells. Seventy two hours after the transfection, cells were treated with DM for 24 h, and the TUJ1-positive neurite length (25) was quantified. GFP-positive cells were regarded as siRNA- or scrambled RNA-transfected cells, whereasGFP-negative cells were considered to be nontransfected cells. The siRNA transfection significantly suppressed the neurite outgrowth induced by DM, whereas scrambled siRNA did not affect the neurite length (Fig. 2).

Effect of NPAS4 Overexpression on Neurite Outgrowth in Neuro2a Cells—To investigate the role of NPAS4 in neurite outgrowth in Neuro2a cells further, we examined whether overexpression of NPAS4 facilitated neurite outgrowth induced by DM treatment. Seventy two hours after the transfection, cells were treated with DM for 12 h, and the neurite length was quantified. At this time point, neurite outgrowth induced by DM treatment in control cells was minimum (Fig. 3) compared with the length at 24 h (Fig. 2). Myc-positive cells were regarded as *Npas4* vector- or mock vector-transfected cells, whereas Myc-negative cells were considered to be nontransfected cells. Overexpression of NPAS4 significantly increased the neurite outgrowth induced by DM, whereas the mock vector had no effect on the neurite length in Neuro2a cells (Fig. 3).

Effect of NPAS4 Overexpression on NeuN, p-SYN I, and Tuj1 Expression in Neuro2a Cells—To clarify the role of NPAS4 in neuronal maturation, the expression levels of NeuN, p-SYN I, and TUJ1 were investigated in NPAS4-overexpressed Neuro2a cells. Seventy two hours after the transfection, cells were harvested, and immunoblot analysis was performed. Overexpression of NPAS4 increased NeuN and p-SYN I expression levels, whereas the mock vector had no effect in Neuro2a cells (Fig. 4, *A* and *B*). In addition, overexpression of NPAS4 significantly increased the neurite marker TUJ1 expression levels induced by DM (Fig. 4*C*).

Involvement of CDK5 in p-SYN I Expression in NPAS4-overexpressed Neuro2a Cells—The anti-p-Syn I antibodies used in this study recognize phosphorylated Ser^{553} of SYN I, which is known to be a substrate of CDK5 (30, 31). Therefore, we measured CDK5 expression levels and its role for p-SYN I expression levels in NPAS4-overexpressed cells. Immunocytochemical analysis was performed 24 and 72 h after transfection with the Npas4 overexpression vector in Neuro2a cells. Overexpression of NPAS4 significantly increased CDK5 and p-SYN I expression levels in Neuro2a cells, whereas mock transfection had no effect (Fig. 5, *A* and *B*). Regression lines (72 h) were analyzed. There was a positive correlation between p-SYN I and CDK5 expression levels in the control ($y = 0.691x + 0.308$, $R^2 = 0.288$, $p < 0.001$) and NPAS4-overexpressed Neuro2a cells ($y = 1.159x + 2.427$, $R^2 = 0.156$, $p < 0.001$), and the slope for NPAS4-overexpressed Neuro2a cells was significantly greater than that for the control ($p < 0.05$). However, no correlation was observed in mock-transfected Neuro2a cells ($y = -0.092x + 2.731$, $R^2 = 0.000$) (Fig. 5*C*).

Furthermore, we measured the CDK5 expression levels and kinase activity in NPAS4-overexpressed Neuro2a cells. Overex-

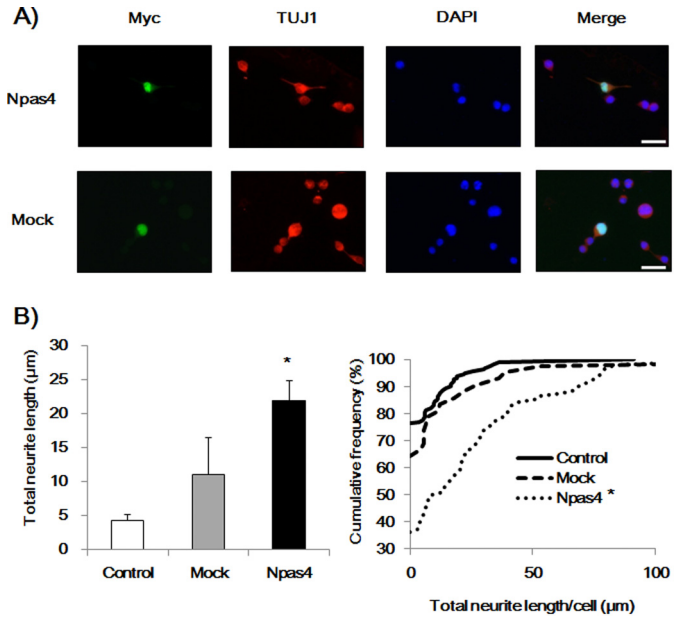

FIGURE 3. **Effect of NPAS4 overexpression on neurite outgrowth induced by DM in Neuro2a cells.** *A,* representative photographs showing immunocytochemical analysis of *Npas4*- or mock vector-transfected cells (Myc-positive). *Scale bar,* 20 μm. *B,* quantitative analysis of neurite length. Values indicate the mean ± S.E. (*left*) and the cumulative frequency of neurite length (*right*). ($n = 88$ for *Npas4* vector transfection (Npas4), $n = 42$ for mock vector transfection (*Mock*), and $n = 160$ for nontransfection (*Control*).) *, $p < 0.01$ *versus* mock and control (one-way ANOVA followed by Dunnett's test, confirmed by the Kolmogorov-Smirnov test).

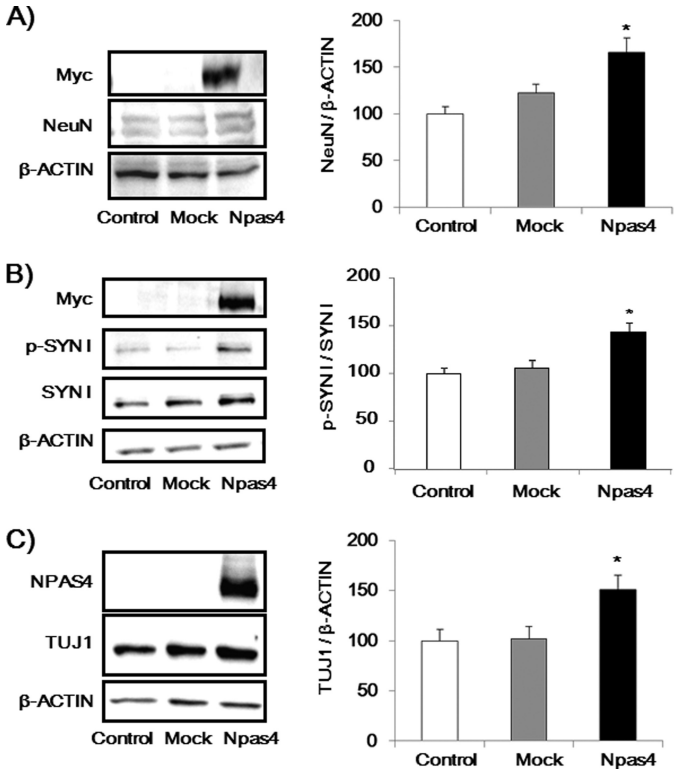

FIGURE 4. **NPAS4 overexpression increased NeuN, p-SYN I, and TUJ1 expression levels in Neuro2a cells.** *A,* immunoblot analysis of NeuN expression levels induced by NPAS4 overexpression ($n = 10–11$). *B,* immunoblot analysis of p-SYN I expression levels induced by NPAS4 overexpression ($n = 10–11$). *C,* immunoblot analysis of TUJ1 expression levels induced by NPAS4 overexpression with DM ($n = 6$). Values indicate the mean ± S.E. *, $p < 0.05$ *versus* mock and control (one-way ANOVA followed by Dunnett's test).

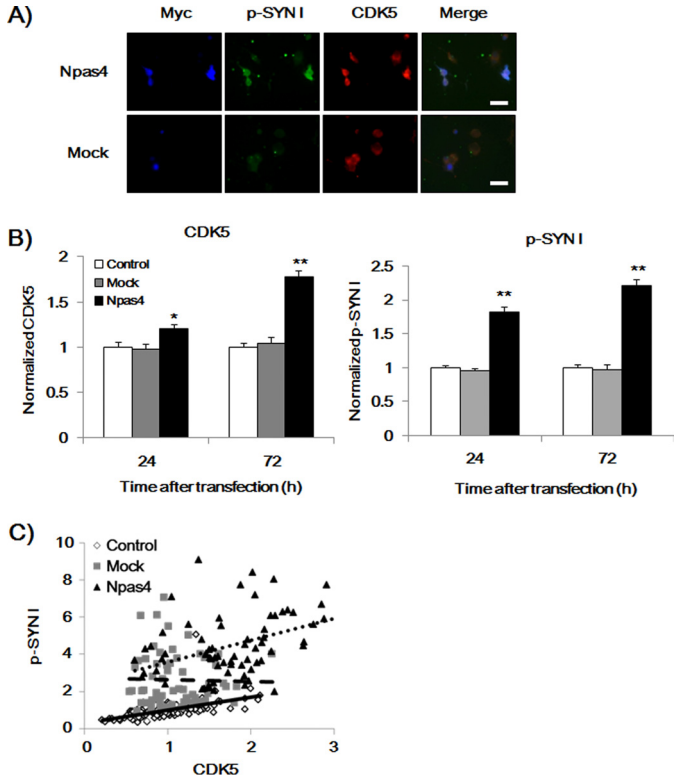

FIGURE 5. **NPAS4 overexpression increased CDK5 and p-SYN I expression levels and the relationship between CDK5 and p-SYN I expression in Neuro2a cells.** *A,* representative photographs showing immunocytochemical analysis of CDK5 and p-SYN I expression levels (72 h after transfection). *Scale bar,* 20 μm. *B,* quantitative analysis of CDK5 expression levels at 24 and 72 h after transfection (*left*). Values (the mean ± S.E.) indicate normalized CDK5 levels. ($n = 81$ for *Npas4* vector transfection (Npas4), $n = 32$ for mock vector transfection (Mock), and $n = 74$ for nontransfection (control), 24 h after transfection. $n = 103$ for *Npas4* vector transfection (Npas4), $n = 39$ for mock vector transfection (Mock), and $n = 154$ for nontransfection (control), 72 h after transfection.) *, $p < 0.01$; **, $p < 0.001$ *versus* control and mock (one-way ANOVA followed by Dunnett's test). Quantitative analysis of p-SYN I expression levels at 24 and 72 h after transfection (*right*). Values (the mean ± S.E.) indicate normalized p-SYN I levels. ($n = 62$ for Npas4 vector transfection (Npas4), $n = 32$ for mock vector transfection (mock), and $n = 109$ for nontransfection (control), 24 h after transfection. $n = 74$ for *Npas4* vector transfection (Npas4), $n = 36$ for mock vector transfection (mock), and $n = 43$ for nontransfection (control), 72 h after transfection.) **, $p < 0.001$ *versus* control and mock (one-way ANOVA followed by Dunnett's test). *C,* regression analysis of the relationship between CDK5 and p-Syn I expression levels at 72 h after transfection. ($n = 68$ for Npas4 vector transfection (Npas4), $n = 60$ for mock vector transfection (*Mock*), and $n = 117$ for nontransfection (*Control*).). The expression levels of p-SYN I were correlated with CDK5 expression levels in control and NPAS4-overexpressed cells. There are significant differences among the slopes ($p < 0.05$, parallel line analysis).

pression of NPAS4 increased CDK5 expression and activity, whereas the mock vector had no effect in Neuro2a cells (Fig. 6, *A* and *B*). To explore the causal relationship, NPAS4-overexpressed Neuro2a cells (72 h after the transfection) were treated with DM for 12 h, during which a CDK5 inhibitor, roscovitine (30 μM, Calbiochem), or DMSO was added to the culture medium (total 84 h), and p-SYN I expression levels and neurite length were measured. Roscovitine significantly reduced the p-SYN I expression (Fig. 6, *C* and *D*) and neurite outgrowth (Fig. 7) in Neuro2a cells induced by NPAS4 overexpression.

Phosphorylated SYN I Expression and Neurite Outgrowth in Hippocampal Neurons—To confirm NPAS4-induced phosphorylation of SYN I in hippocampal neurons, p-SYN I expression levels were investigated in NPAS4-overexpressed hip-

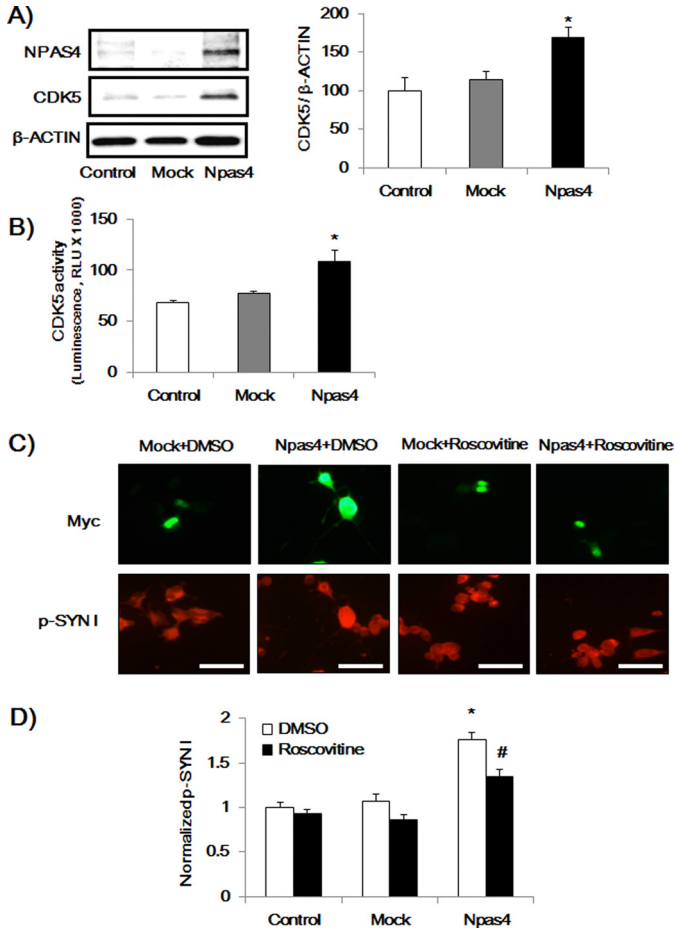

FIGURE 6. **NPAS4 overexpression increased CDK5 expression levels and kinase activity and roscovitine inhibited p-SYN I expression levels.** *A,* immunoblot analysis of CDK5 expression levels induced by NPAS4 overexpression ($n = 3$). Values indicate the mean ± S.E. *, $p < 0.05$ *versus* mock and control (one-way ANOVA followed by Dunnett's test). *B,* CDK5 activity assay in NPAS4 overexpressed Neuro2a cells ($n = 4$). Values indicate the mean ± S.E. *, $p < 0.05$ *versus* mock and control (one-way ANOVA followed by Dunnett's test). *RLU,* relative light units. *C,* representative photographs showing immunocytochemical analysis of p-SYN I expression in *Npas4* vector-transfected cells (Myc-positive) in the presence or absence of roscovitine treatment. *Scale bar,* 20 μm. *D,* quantitative analysis of p-SYN I expression levels. Values (mean ± S.E.) indicate normalized p-SYN I levels. ($n = 55$ for Npas4 + DMSO; $n = 33$ for Npas4 + roscovitine; $n = 41$ for mock + DMSO; $n = 28$ for mock + roscovitine; $n = 51$ for control + DMSO, and $n = 47$ for control + roscovitine.) *, $p < 0.05$ *versus* control + DMSO and mock + DMSO; #, $p < 0.05$ *versus* Npas4 + DMSO (one-way ANOVA followed by Dunnett's test).

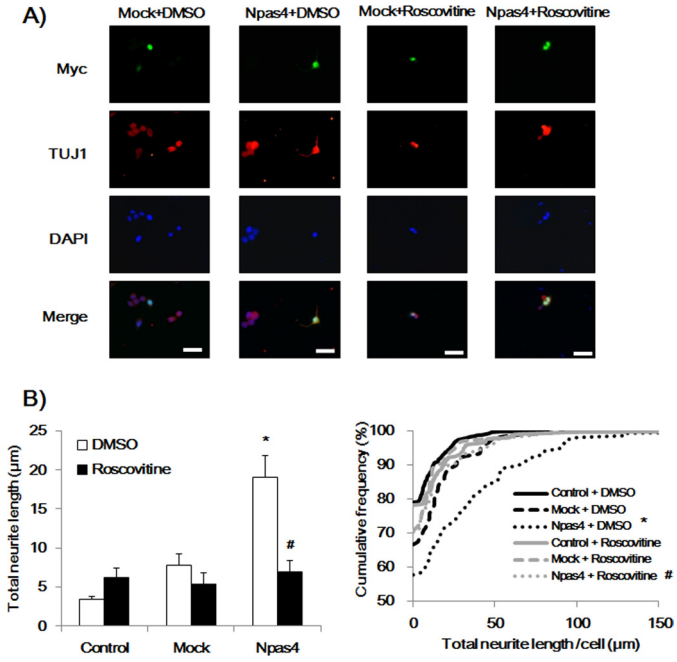

FIGURE 7. **Roscovitine inhibited neurite outgrowth in NPAS4-overexpressed Neuro2a cells.** *A,* representative photographs showing immunocytochemical analysis of neurite outgrowth in *Npas4* vector-transfected cells (Myc-positive) in the presence or absence of roscovitine treatment. *Scale bar,* 20 μm. *B,* quantitative analysis of neurite length. Values indicate the mean ± S.E. (*left*) and the cumulative frequency of neurite length (*right*). ($n = 89$ for Npas4 + DMSO; $n = 120$ for mock + DMSO; $n = 545$ for control + DMSO; $n = 148$ for Npas4 + roscovitine; $n = 65$ for mock + roscovitine, and $n = 308$ for control + roscovitine). *, $p < 0.001$ *versus* mock + DMSO and control + DMSO; #, $p < 0.001$ *versus* Npas4 + DMSO (one-way ANOVA followed by Dunnett's test, confirmed by the Kolmogorov-Smirnov test).

pocampal neurons. Twenty four hours after the transfection, immunocytochemical analysis was performed. Overexpression of NPAS4 increased p-SYN I levels in hippocampal neurons, whereas the mock vector had no effect (Fig. 8). We compared neurite length between WT and *Npas4* KO hippocampal neurons. *Npas4* KO hippocampal neurons showed an impaired MAP2-positive dendritic (Fig. 9, *A* and *B*) and Tau-positive axonal outgrowth (Fig. 9, *D* and *E*) and a reduction in the number of intersections of dendrites (Sholl analysis, Fig. 9*C*) compared with WT hippocampal neurons. Furthermore, KCl treatment increased dendritic length and complexity in WT hippocampal neurons but not in *Npas4* KO hippocampal neurons (Fig. 9, *A*–*C*).

Binding of NPAS4 to Promoter Regions of CDK5 and NeuN Genes in Neuro2a Cells and Primary Cultured Neurons—We used a ChIP assay to confirm the binding of NPAS4 to *Cdk5* and *NeuN* promoters. An *Npas4* promoter was used as a positive control (13). The ChIP assay revealed that NPAS4 binds to promoter regions of *Cdk5* and *NeuN* in DM-treated Neuro2a cells (Fig. 10*A*). In primary cultured neurons, a ChIP assay was conducted with or without KCl stimulation because the treatment was reported to induce NPAS4 in cultured neurons (13). As shown in Fig. 10*C*, we confirmed the increase in NPAS4 protein levels after KCl stimulation in primary hippocampal neurons. Only under such conditions was the binding of NPAS4 to the promoters of *Npas4* and *Cdk5* proven by the ChIP assay (Fig. 10*B*). Furthermore, NPAS4 bound to a promoter of p35, a coactivator of CDK5, in Neuro2a cells as well as in primary cultured neurons (Fig. 10).

Effect of PTZ Administration on NPAS4 and p-SYN I Expression in the Prefrontal Cortex of Npas4 KO and WT Mice—Finally, we investigated if p-SYN I expression was affected by *in vivo* PTZ treatment in WT and *Npas4* KO mice because it has been reported that PTZ-induced convulsions in mice lead to an increase in the *Npas4* mRNA levels in the hippocampus (32). First, we measured NPAS4, p-SYN I, SYN I, p35 and CDK5 expression levels 2, 6, 1, and 24 h after PTZ administration in the prefrontal cortex of WT mice (Fig. 11). PTZ-induced NPAS4 and p-SYN I were most highly expressed 2 h after PTZ administration. We next compared the effects of PTZ on NPAS4 and p-SYN I levels in WT and KO mice 2 h after treatment. PTZ administration increased Npas4 levels in the prefrontal cortex of WT mice but not in *Npas4* KO mice (Fig. 12). Similarly, p-SYN I levels were increased by PTZ administration

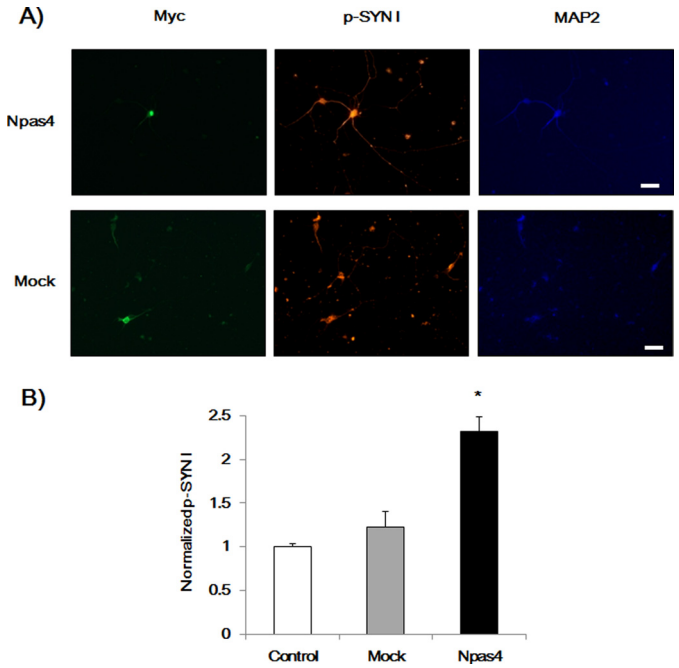

FIGURE 8. **NPAS4 overexpression increased p-SYN I expression levels in primary cultured hippocampal neurons.** *A,* representative photographs showing immunocytochemical analysis of p-SYN I expression in *Npas4* vector-transfected cells (Myc-positive). *Scale bar,* 20 μm. *B,* quantitative analysis of p-SYN I expression levels. Values (the mean ± S.E.) indicate normalized p-SYN I levels. ($n = 48$ for *Npas4* vector transfection (Npas4), $n = 34$ for mock vector transfection (*Mock*), and $n = 77$ for nontransfection (*Control*)). *, $p < 0.05$ *versus* control and mock (one-way ANOVA followed by Dunnett's test).

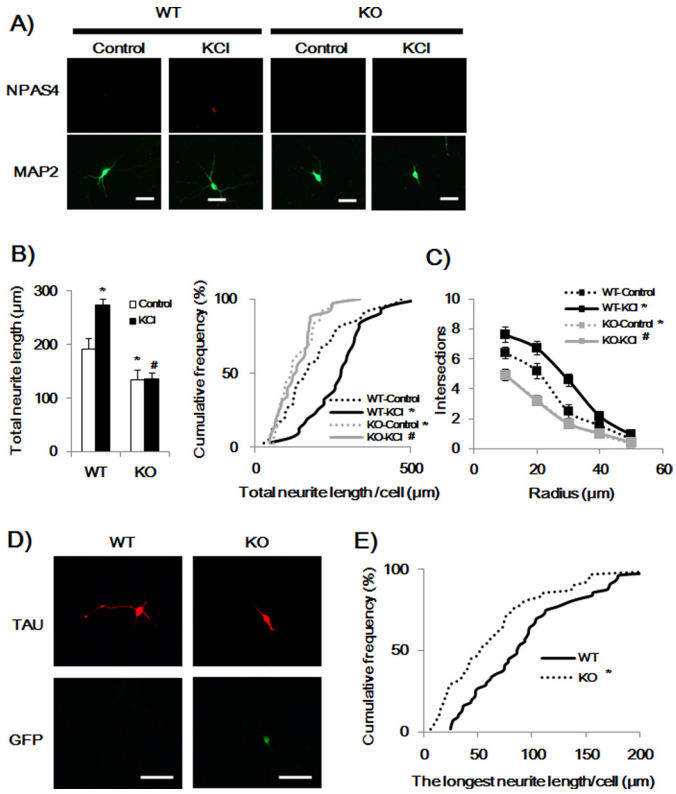

FIGURE 9. **Impairment of neurite outgrowth in primary cultured hippocampal neurons of *Npas4* KO mice.** *A,* representative photographs showing immunocytochemical analysis of dendritic outgrowth in hippocampal neurons of WT and KO mice in the presence or absence of KCl (16 mM, DIV 7–9) treatment. *Scale bar,* 20 μm. *B,* quantitative analysis of dendritic length. Values indicate the mean ± S.E. (*left*). *, $p < 0.01$ *versus WT-Control*; #, $p < 0.001$ *versus WT-KCl* (two-way ANOVA, Bonferroni's test), and the cumulative frequency of dendritic length (*right*). *, $p < 0.05$ *versus WT-Control*, #, $p < 0.001$ *versus WT-KCl* (Kolmogorov-Smirnov test). ($n = 34$ for WT-Control; $n = 31$ for *WT-KCl*; $n = 36$ for *KO-Control*; $n = 35$ for KO-KCl.) *C,* intersections. The concentric circle method of Sholl was used for dendritic quantification. *, $p < 0.001$ *versus WT-Control*; #, $p < 0.001$ *versus WT-KCl* (three-way ANOVA followed by Bonferroni's test). *D,* representative photographs showing immunocytochemical analysis of axonal outgrowth in hippocampal neurons of WT and KO mice (DIV 3). GFP signals indicate *Npas4* KO hippocampal neurons (13). *Scale bar,* 20 μm. *E,* quantitative analysis of axonal length. Values indicate the mean ± S.E. ($n = 31$) *, $p < 0.01$ *versus* WT (Kolmogorov-Smirnov test).

in WT mice, whereas the same treatment had no effect on p-SYN I levels in *Npas4* KO mice (Fig. 12). There was no difference in the intensity score of convulsions induced by PTZ treatment between *Npas4* KO mice (convulsion score 4.5 ± 0.5) and WT mice (3.9 ± 0.13), suggesting that the changes in p-SYN I levels after PTZ treatment in *Npas4* KO mice are not due to the reduced excitability of the mutant mouse brains.

DISCUSSION

NPAS4 is a brain-specific neuronal transcription factor that may respond to various excitatory stimuli and has a role in GABAergic neuronal synapse development (13–15). A recent study demonstrated that the activity-dependent expression of NPAS4 regulates a transcriptional program in the CA3 hippocampus required for contextual memory formation (18).

In this study, we showed that DM induced an increase in neurite outgrowth with an elevated *Npas4* mRNA level. DM-induced neurite outgrowth was inhibited in *Npas4* knockdown Neuro2a cells, whereas overexpression of NPAS4 accelerated the neurite outgrowth induced by DM. Furthermore, we demonstrated that axonal and dendritic outgrowths were impaired in *Npas4* KO hippocampal neurons. Although it was reported that NPAS4 was expressed after maturation in primary cultured neurons (13), our data indicate that this transcription factor is expressed even in immature neurons at DIV 3 (Fig. 10*C*). Under such culture conditions, an impaired axonal outgrowth was evident in *Npas4* KO neurons compared with WT neurons. Lin *et al.* (13) showed that *Npas4*-RNAi transfection at DIV 6 did not decrease dendritic complexity at a later stage (DIV 25–26). We compared the dendritic length of WT and conventional KO mice hippocampal neurons at DIV 9, and we found that the dendritic length of *Npas4* KO neurons was significantly shorter than that of WT neurons. Furthermore, KCl-induced dendritic outgrowth, which was evident in WT neurons, was abolished in *Npas4* KO hippocampal neurons. These results suggest that the role of NPAS4 in dendritic outgrowth varies depending on neuronal development stages in an activity-dependent manner.

Activity-dependent neurite outgrowth in cultured hippocampal neurons is reported to be mediated via activation by CaM-dependent protein kinase of the membrane-associated γ isoform of CaMKI (27), whereas the DM-induced neurite outgrowth in Neuro2a cells is associated with inhibition of GSK-3β (33). In our study, NPAS4 overexpression elevated CDK5 and p-SYN I expression levels and enhanced neurite outgrowth. The CDK5 inhibitor roscovitine reduced p-SYN I expression, as well as neurite outgrowth induced by NPAS4 overexpression. SYN I has been characterized as one of the major phosphoproteins in nerve terminals and is thought to be involved in the

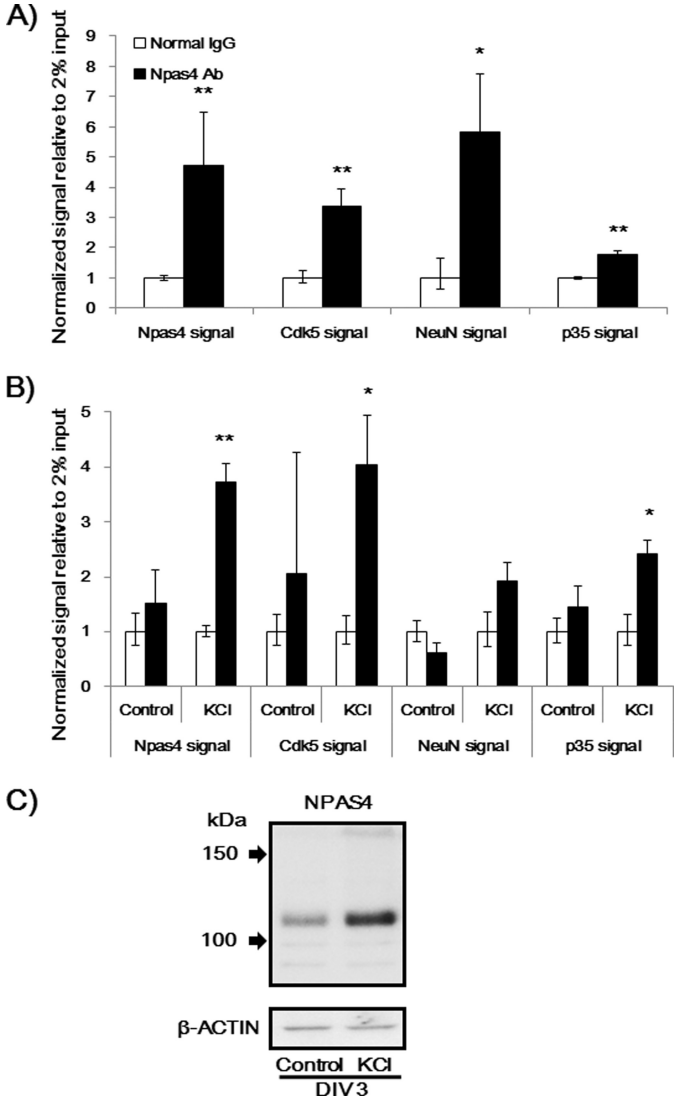

FIGURE 10. **NPAS4 binds to promoters of *Cdk5*, *Neu*, and *p35*.** *A,* ChIP assay in Neuro2a cells. Values (mean ± S.E.) indicate a normalized signal to 2% input. *, $p < 0.05$; **, $p < 0.01$ *versus* normal IgG (Student's *t* test, $n = 3$–5). *B,* ChIP assay in primary cultured neurons. Values (the mean ± S.E.) indicate a normalized signal to 2% input. *, $p < 0.05$; **, $p < 0.001$ *versus* normal IgG (Student's *t* test, $n = 3$). *C,* immunoblot analysis of NPAS4 expression in primary cultured hippocampal neurons on DIV 3 with or without KCl (50 mM) stimulation.

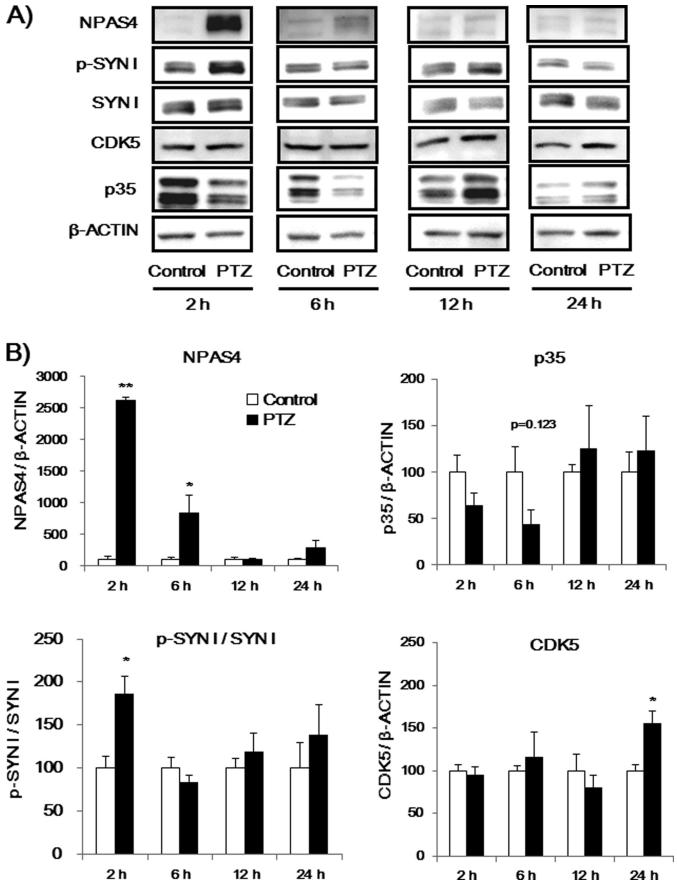

FIGURE 11. **PTZ-induced NPAS4, p-SYN I, SYN I, CDK5, and p35 expression in the prefrontal cortex of mice at different time points.** *A,* immunoblot analysis of NPAS4, p-SYN I, SYN I, CDK5, and p35 expression levels in the prefrontal cortex of mice at various time points after PTZ injection. *B,* quantitative analysis of immunoblots (*A*). Values indicate the mean ± S.E. ($n = 4$). *, $p < 0.05$; **, $p < 0.001$ *versus* control (Student's *t* test).

regulation of neurotransmitter release (34, 35). Both phosphorylated and nonphosphorylated Syn I regulate neuronal development (36–38). The anti-p-Syn I antibodies used in this study recognize phosphorylated Ser^{553} of SYN I, which is known to be phosphorylated by CDK5 (30, 31). Although previous DNA microarrays and ChIP-Seq studies did not reveal that CDK5 or CDK5-coactivator p35 is a target of NPAS4 (13, 46), CDK5 is essential for neurite outgrowth (39–41) and has roles in the physiological regulation of cytoskeletal components during neurotransmitter release via phosphorylation of SYN I (30). We demonstrated that NPAS4 overexpression increased CDK5 protein and the kinase activity in Neuro2a cells. In addition, NPAS4 bound to promoter regions of CDK5 as well as p35 in KCl-activated primary cultured neurons and DM-treated Neuro2a cells. Accordingly, it is suggested that NPAS4 may directly regulate transcription of CDK5 and consequently increase the protein expression with kinase activity, which is associated with neurite outgrowth.

We also demonstrated that PTZ-induced phosphorylation of SYN I *in vivo* was absent in NPAS4 KO mice, suggesting that NPAS4 has a crucial role in SYN I phosphorylation *in vivo*. However, there might be concern that the NPAS4/CDK5/SYN I signaling in neurite outgrowth described here may operate only *in vitro* cell culture systems because the *in vivo* study showed that phosphorylation of SYN I was induced by PTZ without an increase in the levels of CDK5 or p35. In fact, CDK5 expression was increased at 24 h, but not 2 h, after PTZ treatment, whereas an increase in p-SYN I and NPAS4 was observed at 2 h, but not 24 h, after PTZ treatment (Fig. 11). It is therefore likely that unidentified downstream signaling mechanisms other than CDK5 of NPAS4 may operate in the regulation of SYN I phosphorylation in neurons. To address this issue, further experiments with other animal models of epileptogenesis (42) are necessary in Npas4 KO mice.

A useful mature neuronal marker, NeuN, was induced in NPAS4-overexpressed Neuro2a cells, whereas NeuN was hardly detected in nontransfected control cells, as demonstrated in previous reports (43, 44). Although *NeuN* was not identified as a target of NPAS4 in previous studies (13, 45), NeuN has been identified as a transcriptional factor, FOX-3,

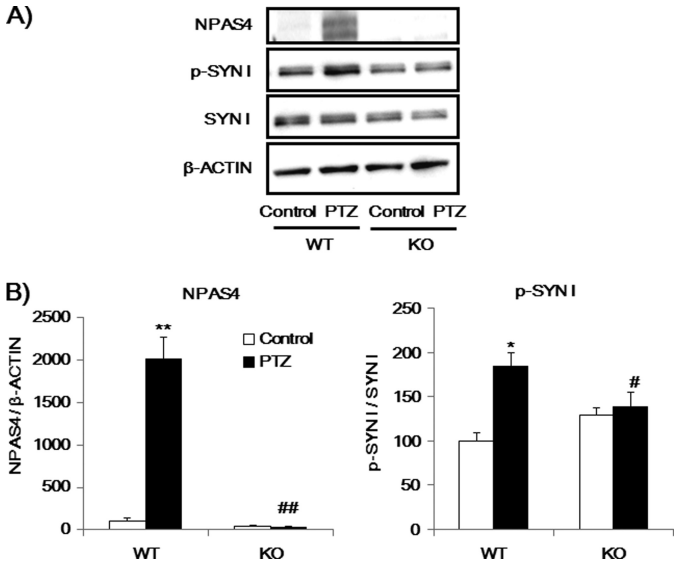

FIGURE 12. **NPAS4 is a key factor in PTZ-induced phosphorylation of SYN I.** *A,* immunoblot analysis of NPAS4, p-SYN I, and SYN I expression levels in the prefrontal cortex of mice 2 h after PTZ injection. *B,* quantitative analysis of immunoblots (*A*). Values indicate the mean ± S.E. ($n = 5–8$). *, $p < 0.05$; **, $p < 0.001$ *versus WT-Control*. #, $p < 0.05$; ##, $p < 0.001$ *versus* WT-PTZ (two-way ANOVA followed by Bonferroni's test).

and appears to be a nervous system-specific nuclear regulatory molecule and a splicing regulator (46, 47). In our study, a ChIP assay showed that Npas4 binds the promoter of NeuN in Neuro2a cells. Accordingly, NPAS4 may have indirect effects through the regulation of other transcriptional factors (18). Taking these findings together, it is suggested that NPAS4 contributes to neurite development and functional maturation of neurons through the transcriptional regulation of neurotransmission and neurodevelopment-associated molecules.

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REFERENCES

1. Erbel-Sieler, C., Dudley, C., Zhou, Y., Wu, X., Estill, S. J., Han, T., Diaz-Arrastia, R., Brunskill, E. W., Potter, S. S., and McKnight, S. L. (2004) Behavioral and regulatory abnormalities in mice deficient in the NPAS1 and NPAS3 transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 13648–13653
2. Havrda, M. C., Harris, B. T., Mantani, A., Ward, N. M., Paolella, B. R., Cuzon, V. C., Yeh, H. H., and Israel, M. A. (2008) Id2 is required for specification of dopaminergic neurons during adult olfactory neurogenesis. *J. Neurosci.* **28,** 14074–14086
3. Ide, M., Yamada, K., Toyota, T., Iwayama, Y., Ishitsuka, Y., Minabe, Y., Nakamura, K., Hattori, N., Asada, T., Mizuno, Y., Mori, N., and Yoshikawa, T. (2005) Genetic association analyses of PHOX2B and ASCL1 in neuropsychiatric disorders. Evidence for association of ASCL1 with Parkinson's disease. *Hum. Genet.* **117,** 520–527
4. Lee, S., Lee, B., Lee, J. W., and Lee, S. K. (2009) Retinoid signaling and neurogenin2 function are coupled for the specification of spinal motor neurons through a chromatin modifier CBP. *Neuron* **62,** 641–654
5. Pickard, B. S., Pieper, A. A., Porteous, D. J., Blackwood, D. H., and Muir, W. J. (2006) The NPAS3 gene–emerging evidence for a role in psychiatric illness. *Ann. Med.* **38,** 439–448
6. Shimizu, N., Watanabe, H., Kubota, J., Wu, J., Saito, R., Yokoi, T., Era, T., Iwatsubo, T., Watanabe, T., Nishina, S., Azuma, N., Katada, T., and Nishina, H. (2009) Pax6-5a promotes neuronal differentiation of murine embryonic stem cells. *Biol. Pharm. Bull.* **32,** 999–1003
7. Ooe, N., Saito, K., Mikami, N., Nakatuka, I., and Kaneko, H. (2004) Identification of a novel basic helix-loop-helix-PAS factor, NXF, reveals a Sim2 competitive, positive regulatory role in dendritic-cytoskeleton modulator *drebrin* gene expression. *Mol. Cell. Biol.* **24,** 608–616
8. Zhou, Y. D., Barnard, M., Tian, H., Li, X., Ring, H. Z., Francke, U., Shelton, J., Richardson, J., Russell, D. W., and McKnight, S. L. (1997) Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 713–718
9. Brunskill, E. W., Witte, D. P., Shreiner, A. B., and Potter, S. S. (1999) Characterization of *npas3*, a novel basic helix-loop-helix PAS gene expressed in the developing mouse nervous system. *Mech. Dev.* **88,** 237–241
10. Pickard, B. S., Christoforou, A., Thomson, P. A., Fawkes, A., Evans, K. L., Morris, S. W., Porteous, D. J., Blackwood, D. H., and Muir, W. J. (2009) Interacting haplotypes at the NPAS3 locus alter risk of schizophrenia and bipolar disorder. *Mol. Psychiatry* **14,** 874–884
11. Pieper, A. A., Wu, X., Han, T. W., Estill, S. J., Dang, Q., Wu, L. C., Reece-Fincanon, S., Dudley, C. A., Richardson, J. A., Brat, D. J., and McKnight, S. L. (2005) The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 14052–14057
12. Moser, M., Knoth, R., Bode, C., and Patterson, C. (2004) LE-PAS, a novel Arnt-dependent HLH-PAS protein, is expressed in limbic tissues and transactivates the CNS midline enhancer element. *Brain Res. Mol. Brain Res.* **128,** 141–149
13. Lin, Y., Bloodgood, B. L., Hauser, J. L., Lapan, A. D., Koon, A. C., Kim, T. K., Hu, L. S., Malik, A. N., and Greenberg, M. E. (2008) Activity-dependent regulation of inhibitory synapse development by NPAS4. *Nature* **455,** 1198–1204
14. Ooe, N., Motonaga, K., Kobayashi, K., Saito, K., and Kaneko, H. (2009) Functional characterization of basic helix-loop-helix-PAS type transcription factor NXF *in vivo.* Putative involvement in an "on demand" neuroprotection system. *J. Biol. Chem.* **284,** 1057–1063
15. Shamloo, M., Soriano, L., von Schack, D., Rickhag, M., Chin, D. J., Gonzalez-Zulueta, M., Gido, G., Urfer, R., Wieloch, T., and Nikolich, K. (2006) NPAS4, a novel helix-loop-helix PAS domain protein, is regulated in response to cerebral ischemia. *Eur. J. Neurosci.* **24,** 2705–2720
16. Ibi, D., Takuma, K., Koike, H., Mizoguchi, H., Tsuritani, K., Kuwahara, Y., Kamei, H., Nagai, T., Yoneda, Y., Nabeshima, T., and Yamada, K. (2008) Social isolation rearing-induced impairment of the hippocampal neurogenesis is associated with deficits in spatial memory and emotion-related behaviors in juvenile mice. *J. Neurochem.* **105,** 921–932
17. Yun, J., Koike, H., Ibi, D., Toth, E., Mizoguchi, H., Nitta, A., Yoneyama, M., Ogita, K., Yoneda, Y., Nabeshima, T., Nagai, T., and Yamada, K. (2010) Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: possible involvement of a brain-specific transcription factor NPAS4. *J. Neurochem.* **114,** 1840–1851
18. Ramamoorthi, K., Fropf, R., Belfort, G. M., Fitzmaurice, H. L., McKinney, R. M., Neve, R. L., Otto, T., and Lin, Y. (2011) NPAS4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science* **334,** 1669–1675
19. Ploski, J. E., Monsey, M. S., Nguyen, T., DiLeone, R. J., and Schafe, G. E. (2011) The neuronal PAS domain protein 4 (NPAS4) is required for new and reactivated fear memories. *PLoS ONE* **6,** e23760
20. Russo, S. J., Mazei-Robison, M. S., Ables, J. L., and Nestler, E. J. (2009) Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology* **56,** 73–82
21. Dickstein, D. L., Kabaso, D., Rocher, A. B., Luebke, J. I., Wearne, S. L., and Hof, P. R. (2007) Changes in the structural complexity of the aged brain. *Aging Cell* **6,** 275–284
22. Le Bé, J. V., and Markram, H. (2006) Spontaneous and evoked synaptic rewiring in the neonatal neocortex. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 13214–13219
23. Majewska, A. K., Newton, J. R., and Sur, M. (2006) Remodeling of synaptic structure in sensory cortical areas *in vivo*. *J. Neurosci.* **26,** 3021–3029
24. Parrish, J. Z., Emoto, K., Kim, M. D., and Jan, Y. N. (2007) Mechanisms that

regulate establishment, maintenance, and remodeling of dendritic fields. *Annu. Rev. Neurosci.* **30,** 399–423
25. Pautot, S., Wyart, C., and Isacoff, E. Y. (2008) Colloid-guided assembly of oriented 3D neuronal networks. *Nat. Methods* **5,** 735–740
26. Fath, T., Ke, Y. D., Gunning, P., Götz, J., and Ittner, L. M. (2009) Primary support cultures of hippocampal and substantia nigra neurons. *Nat. Protoc.* **4,** 78–85
27. Wayman, G. A., Impey, S., Marks, D., Saneyoshi, T., Grant, W. F., Derkach, V., and Soderling, T. R. (2006) Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron* **50,** 897–909
28. Schröder, H., Becker, A., and Lössner, B. (1993) Glutamate binding to brain membranes is increased in pentylenetetrazole-kindled rats. *J. Neurochem.* **60,** 1007–1011
29. Becker, A., and Grecksch, G. (1995) Flunarizine–its effect on pentylenetetrazole-kindled seizures and on related cognitive disturbances. *Pharmacol. Biochem. Behav.* **52,** 765–769
30. Matsubara, M., Kusubata, M., Ishiguro, K., Uchida, T., Titani, K., and Taniguchi, H. (1996) Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and CDK5 and its effects on physiological functions. *J. Biol. Chem.* **271,** 21108–21113
31. Levy, M., Faas, G. C., Saggau, P., Craigen, W. J., and Sweatt, J. D. (2003) Mitochondrial regulation of synaptic plasticity in the hippocampus. *J. Biol. Chem.* **278,** 17727–17734
32. Flood, W. D., Moyer, R. W., Tsykin, A., Sutherland, G. R., and Koblar, S. A. (2004) Nxf and Fbxo33. Novel seizure-responsive genes in mice. *Eur. J. Neurosci.* **20,** 1819–1826
33. García-Pérez, J., Avila, J., and Díaz-Nido, J. (1999) Lithium induces morphological differentiation of mouse neuroblastoma cells. *J. Neurosci. Res.* **57,** 261–270
34. Bykhovskaia, M. (2011) Synapsin regulation of vesicle organization and functional pools. *Semin. Cell Dev. Biol.* **22,** 387–392
35. Shupliakov, O., Haucke, V., and Pechstein, A. (2011) How synapsin I may cluster synaptic vesicles. *Semin. Cell Dev. Biol.* **22,** 393–399
36. Fornasiero, E. F., Bonanomi, D., Benfenati, F., and Valtorta, F. (2010) The role of synapsins in neuronal development. *Cell. Mol. Life Sci.* **67,** 1383–1396
37. Bonanomi, D., Menegon, A., Miccio, A., Ferrari, G., Corradi, A., Kao, H. T., Benfenati, F., and Valtorta, F. (2005) Phosphorylation of synapsin I by cAMP-dependent protein kinase controls synaptic vesicle dynamics in developing neurons. *J. Neurosci.* **25,** 7299–7308
38. Kao, H. T., Song, H. J., Porton, B., Ming, G. L., Hoh, J., Abraham, M., Czernik, A. J., Pieribone, V. A., Poo, M. M., and Greengard, P. (2002) A protein kinase A-dependent molecular switch in synapsins regulates neurite outgrowth. *Nat. Neurosci.* **5,** 431–437
39. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F., and Tsai, L. H. (1996) The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.* **10,** 816–825
40. Cheung, Z. H., Chin, W. H., Chen, Y., Ng, Y. P., and Ip, N. Y. (2007) CDK5 is involved in BDNF-stimulated dendritic growth in hippocampal neurons. *PLoS Biol.* **5,** e63
41. Cheung, Z. H., and Ip, N. Y. (2007) The roles of cyclin-dependent kinase 5 in dendrite and synapse development. *Biotechnol. J.* **2,** 949–957
42. Wilczynski, G. M., Konopacki, F. A., Wilczek, E., Lasiecka, Z., Gorlewicz, A., Michaluk, P., Wawrzyniak, M., Malinowska, M., Okulski, P., Kolodziej, L. R., Konopka, W., Duniec, K., Mioduszewska, B., Nikolaev, E., Walczak, A., Owczarek, D., Gorecki, D. C., Zuschratter, W., Ottersen, O. P., and Kaczmarek, L. (2008) Important role of matrix metalloproteinase 9 in epileptogenesis. *J. Cell Biol.* **180,** 1021–1035
43. Evangelopoulos, M. E., Weis, J., and Krüttgen, A. (2005) Signalling pathways leading to neuroblastoma differentiation after serum withdrawal. HDL blocks neuroblastoma differentiation by inhibition of EGFR. *Oncogene* **24,** 3309–3318
44. Evangelopoulos, M. E., Weis, J., and Krüttgen, A. (2009) Mevastatin-induced neurite outgrowth of neuroblastoma cells via activation of EGFR. *J. Neurosci. Res.* **87,** 2138–2144
45. Kim, T. K., Hemberg, M., Gray, J. M., Costa, A. M., Bear, D. M., Wu, J., Harmin, D. A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P. F., Kreiman, G., and Greenberg, M. E. (2010) Widespread transcription at neuronal activity-regulated enhancers. *Nature* **465,** 182–187
46. Soylemezoglu, F., Onder, S., Tezel, G. G., and Berker, M. (2003) Neuronal nuclear antigen (NeuN). A new tool in the diagnosis of central neurocytoma. *Pathol. Res. Pract.* **199,** 463–468
47. Kim, K. K., Adelstein, R. S., and Kawamoto, S. (2009) Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the *Fox-1* gene family of splicing factors. *J. Biol. Chem.* **284,** 31052–31061