Neuroscience Research xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Suppression of nucleocytoplasmic p27^{Kip1} export attenuates CDK4-mediated neuronal death induced by status epilepticus

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ARTICLE INFO

Article history:
Received 3 August 2017
Received in revised form 2 October 2017
Accepted 6 October 2017
Available online xxx

Keywords:
CDK5
Epilepsy
Flavopiridol
Leptomycin B
Seizure

ABSTRACT

Aberrant cell cycle re-entry promotes neuronal death in various neurological diseases. Thus, cyclin-dependent kinases (CDKs) seem to be one of potential therapeutic targets to prevent neuronal loss. In the present study, we investigated the involvements of CDK4, CDK5 and p27^{Kip1} (an endogenous CDK inhibitor) in status epilepticus (SE)-induced neuronal death. Following SE, CDK4 expression was increased in CA1 neurons, while CDK5 was decreased. Most of TUNEL-positive neurons showed CDK4 expression, but less CDK5 expression. Flavopiridol (a CDK4 inhibitor) attenuated TUNEL signal and CDK4 expression in CA1 neurons following SE. CDK5 inhibitors did not affect these phenomena. Both flavopiridol and leptomycin B (an inhibitor of chromosome region maintenance 1) mitigated SE-induced neuronal death by inhibiting nucleocytoplasmic p27^{Kip1} translocation. These findings suggest that SE may lead to nucleocytoplasmic p27^{Kip1} export that initiates CDK4, not CDK5, induction, which an abortive and fatal cell cycle re-entry progress in CA1 neurons.

1. Introduction

Aberrant re-entry of cell cycle evokes the degeneration of post-mitotic neurons (Herrup, 2013). Since cell cycle progression is regulated by the cyclin-dependent kinases (CDKs), inhibition of some CDKs seems to be one of potential strategies to prevent neuronal loss induced by various neurological diseases (Nguyen et al., 2003; Greene et al., 2004; Kim et al., 2014a,b; Hyun et al., 2017). Indeed, excessive glutamate stimulus triggers CDK5 activation that leads to CDK4-mediated neuronal apoptosis in rat cortical primary neurons via p27^{Kip1} (an endogenous CDK inhibitor) deletion (Veas-Pérez de Tudela et al., 2015). However, the binding of p27^{Kip1} with CDK5 plays a neuroprotective role in response to β-amyloid toxicity by inhibiting nucleocytoplasmic CDK5 transport (Zhang et al., 2010). Furthermore, we have recently reported that CDK4 involves programmed neuronal necrosis, not apoptosis, following status epilepticus (SE, a prolonged seizure activity), while CDK5 does not (Kim et al., 2014a,b; Hyun et al., 2017). Therefore, the roles of CDKs and p27^{Kip1} in neuronal death are still controversial. In the present study, we evaluated the roles of CDK4 and CDK5 in SE-induced neuronal death. Furthermore, to explore the link between CDKs and p27^{Kip1} in neuronal damage, we applied leptomycin B (LMB, an inhibitor of chromosome region maintenance 1, CRM1), and then determined the role of nucleocytoplasmic p27^{Kip1} export in CA1 neuronal death induced by SE.

2. Materials and methods

This study utilized male Sprague-Dawley rats (7 weeks old) obtained from Experimental Animal Center, Hallym University, Chunchon, Republic of Korea. The animals were provided with a commercial diet and water ad libitum under controlled temperature, humidity and lighting conditions (22 ± 2 °C, 55 ± 5% and a 12 : 12 light/dark cycle). Animal protocols were approved by the Institutional Animal Care and Use Committee of Hallym University (Chunchon, Republic of Korea). The number of animals used and their suffering was minimized in all cases. All reagents were obtained from Sigma-Aldrich, except as noted.

Rats were anesthetized with Isoflurane anesthesia (3% induction, 1.5–2% for surgery and 1.5% maintenance in a 65:35 mixture of N_2O:O_2) and placed in a stereotaxic frame. A brain infusion kit 1 (Alzet, USA) was implanted into the right lateral ventricle (1 mm posterior; 1.5 mm lateral; 3.5 mm depth), and connected to an osmotic pump (1007D, Alzet, USA) containing: (1) vehicle; (2) flavopiridol (50 μM); (3) olomoucine (100 μM); or (4) roscovitine (100 μM). The pump was placed in a subcutaneous pocket in the interscapular region. The dosage of each compound did not affect

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https://doi.org/10.1016/j.neures.2017.10.001
0168-0102/

seizure threshold in response to pilocarpine in our previous studies (Kim et al., 2014a,b; Hyun et al., 2017).

Three days after surgery, rats were treated with pilocarpine (380 mg/kg, i.p.) 20 min after methylscopolamine (5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of SE and repeated as needed. As controls, age-matched normal rats were treated with saline instead of pilocarpine. Three days after SE, rats were subjected to transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed immediately following infusion and kept in a solution of 4% paraformaldehyde for 48 h. Next, brains were transferred to a solution of 30% sucrose in phosphate-buffered saline (PBS, pH = 7.4) at 4 °C. After 72 h in the sucrose solution, brains were sliced in coronal sections of 30 μm using a cryostat.

Brain sections were incubated overnight at room temperature in a mixture of guinea pig anti-neuronal nuclear antigen (NeuN, 1:1000, Millipore, USA)/mouse anti-CDK4 (1:200, Abbiotec, USA), NeuN/rabbit anti-CDK5 (1:500, Millipore, USA) or NeuN/rabbit anti-p27^{Kip1} (1:100, Abcam, UK) antisera in PBS containing 0.3% Triton X-100. Next, sections were placed in the solution containing a mixture of FITC- and Cy3-conjugated IgG (Amersham, NJ, USA). Negative controls were obtained by pre-immune serum instead of primary antibody to verify the specificity of the antibodies. As the results, negative controls showed the absence of any immunoreactivity (data not shown). TUNEL staining was also performed with the TUNEL apoptosis detection kit (Upstate, USA) according to the manufacturer's protocol (http://www.upstate.com). After TUNEL reaction, CDK4 or CDK5 immunofluorescence staining was applied (Kim et al., 2014a,b; Hyun et al., 2017). Image acquisition and cell count were performed using an Axiocam HRc camera and AxioVision Rel. 4.8 software (Carl Zeiss, Germany).

For qRT-PCR, we rapidly removed the hippocampus and dissected CA1 region. The dissected CA1 tissues were homogenized and total RNA was extracted using Trizol Reagents, according to the manufacturer's protocol (Ambion, USA). Total RNA was reverse transcribed into first-strand cDNA using a PrimerScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). Quantification of mRNA expression was performed in triplicate using a SYBR Green

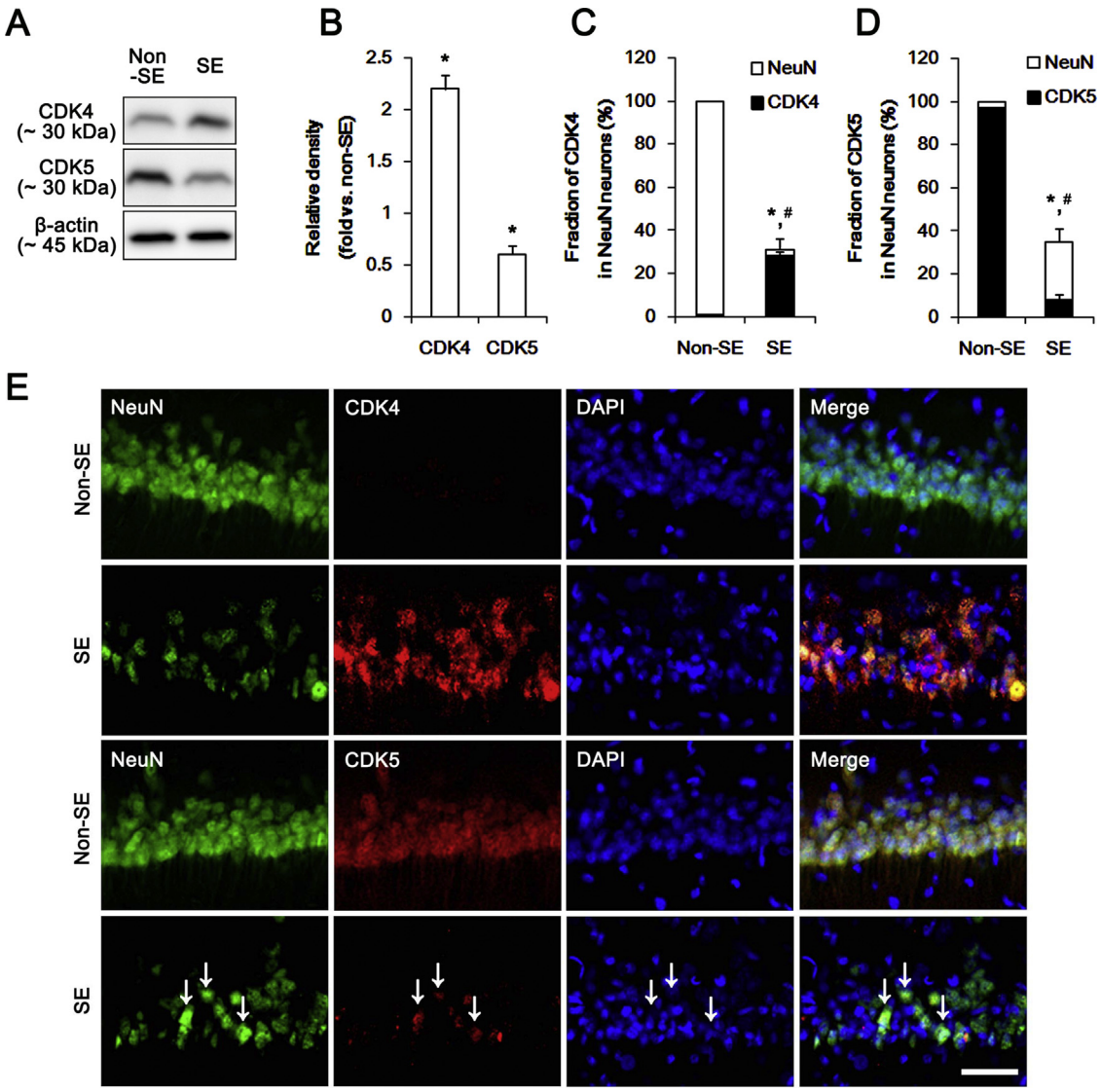

Fig. 1. Neuronal death and alterations in CDK4 and CDK5 expression in CA1 neurons following SE. **(A)** Representative western blot for CDK4 and CDK5 expression following SE. CDK4 expression is elevated, but CDK5 expression is decreased in CA1 neurons 3 days after SE. **(B)** Quantification of protein expressions based on the western blots. Error bars indicate SEM ($^{*}p < 0.05$ vs. non-SE animals, respectively; n = 7, respectively). **(C, D)** Quantification of the fractions of CDK4 and CDK5 positive cells in NeuN positive neurons. Error bars indicate SEM ($^{*,\#}p < 0.05$ vs. non-SE animals, * for NeuN and # for CDK4 or CDK5, respectively; n = 7, respectively). **(E)** Representative photos demonstrating CDK4 and CDK5 expressions in CA1 neurons. Arrows indicate NeuN positive cells showing CDK5 expression. Bar = 25 μm.

Please cite this article in press as: Kim, J.-E., Kang, T.-C., Suppression of nucleocytoplasmic p27^{Kip1} export attenuates CDK4-mediated neuronal death induced by status epilepticus. Neurosci. Res. (2017), https://doi.org/10.1016/j.neures.2017.10.001