Neurobiology of Disease 31 (2008) 230–241

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Cyclin D1 in excitatory neurons of the adult brain enhances kainate-induced neurotoxicity

Hajira B. Koeller [a,b], M. Elizabeth Ross [a,b], Sara B. Glickstein [b,*]

[a] Graduate Program in Neuroscience, Weill Medical College of Cornell University, 1300 York Avenue, Box 239, New York NY 10065, USA
[b] Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York NY 10065, USA

ARTICLE INFO

Article history:
Received 20 February 2008
Revised 22 April 2008
Accepted 25 April 2008
Available online 10 May 2008

Keywords:
Cyclin D1
Kainate
Pentylenetetrazole
CA1
Excitotoxicity
Seizure
Hippocampus
Cell cycle
Apoptosis
Excitatory neurons

ABSTRACT

G1-phase cyclin D1 (cD1) expression has been documented in post-mitotic neurons undergoing apoptosis, leading others to propose that attempted cell cycle re-entry may induce cell death. Here, cD1 immunoreactivity was found in a subpopulation of healthy excitatory neurons throughout the brain. Most striking was the selective cD1 expression in hippocampal pyramidal neurons, an especially vulnerable cell group. Seizure threshold, cD1 induction and CA1 neuron death were examined following application of kainate (KA) or pentylenetetrazole (PTZ) in cD1 heterozygous (+/−) and wildtype mice to determine whether baseline cD1 correlates with pathology. cD1+/− mice displayed resistance to KA, but not PTZ-induced seizures and had reduced or equivalent cytotoxicity respectively, compared with wildtype. KA administration, but not PTZ, induced cD1 expression. These findings suggest that basal cD1 expression may render hippocampal circuits more susceptible to particular epileptogenic agents and excitotoxic cell death, though cD1 is not a direct precipitant in apoptosis.

Introduction

D-type cyclins (cD1, cD2, cD3) are positive regulators of the mid-G1-transition — the only point in the cycle when a cell can commit to another round of division or exit the cell cycle (Sherr and Roberts, 2004). The cyclins D activate binding partners cyclin-dependent kinases 4 or 6 that catalyze the initial inactivating phosphorylation of the retinoblastoma protein (pRb) and resultant de-repression of proliferation-associated E2F target genes and also, sequester cdk inhibiting proteins, p57and p27, from the cyclin E/cdk2 holoenzyme complex, which completely inactivates pRb and allows for cell-cycle progression. Disruption of tightly regulated G1/S phase cyclin expression results in cell-cycle arrest. Non-redundant functions of D-type cyclin subtypes are demonstrated by distinct impairments observed in mice deficient in one D-type (Sicinski et al., 1995, 1996; Huard et al., 1999). These distinctions are likely accounted for by tissue specific expression requirements and by unique properties of each protein (Ciemerych et al., 2002; Carthon et al., 2005), some of which may be independent of cdk activity regulation (Landis et al., 2006).

Mice lacking cD1 are runted and die prematurely due to starvation (Sicinski et al., 1995). In addition to other physical defects, these mice are neurologically impaired, though no distinct neuroanatomical abnormality has been identified (Sicinski et al., 1995; Chen et al., 2005). In the forebrain, the expression of cD1 is restricted to the embryonic ventricular zone, but curiously, in adult mice, is present in post-mitotic neurons (Tamaru et al., 1994; Glickstein et al., 2007). Several studies have detailed de-novo expression of cell-cycle proteins in apoptotic, post-mitotic neurons in various neurodegenerative mouse models and in pathological samples from patients having neurodegenerative conditions including Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, Parkinson's disease and stroke (Freeman et al., 1994; Jordan-Sciutto et al., 2003; Ranganathan and Bowser, 2003; Yang and Herrup, 2005; Burns et al., 2007; Herrup and Yang, 2007). Re-activation of the cell cycle in terminally differentiated neurons is hypothesized to lead to death rather than cell-cycle progression — a theory drawing support from neuroprotection afforded by cell-cycle blocking in models of neuronal apoptosis (Kranenburg et al., 1996; Park et al., 1996, 1997; Padmanabhan et al., 1999). Several papers have examined cell cycle re-activation in apoptosis, but the unequivocal observation of cD1 in healthy post-mitotic neurons that are not destined for imminent apoptosis has not been addressed (Park et al., 2000; Di Giovanni et al., 2005). Despite the plethora of research articles on the pathogenic consequences of neuronal cD1 expression, we have no understanding of the function served by cD1 in healthy neurons, nor the neuronal cell types and brain regions demonstrating robust, invariable expression.

* Corresponding author.
E-mail address: sbglick@med.cornell.edu (S.B. Glickstein).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.04.010

The present study shows that cD1 expression is predominantly nuclear and restricted to a subset of excitatory neurons. Its widespread localization indicates that neuronal cD1 expression is not a harbinger of imminent apoptosis. Analysis of hippocampal cell death following seizures induced in wildtype and cD1 heterozygous littermates supports that basal cD1 expression marks a vulnerable population of excitatory neurons.

Materials and methods

Animals

Mice were housed in climate-controlled Thoren units with 12-h light-dark cycle. Inactivation of the cyclin D2 (cD2) gene was accomplished by disruption of exons I and II (Sicinski et al., 1996). The cD1 gene was inactivated by the deletion of exons I, II, and III (Sicinski et al., 1995). All mice were subsequently maintained on a C57BL/6J background and were backcrossed more than 12 times. Heterozygous cD1+/− and cD2+/− mice were bred to produce WT, null and heterozygous littermates for all studies (Sicinski et al., 1995, 1996). Male mice aged 8–12 weeks were used for all analyses unless otherwise indicated. Intergenotype comparisons were always performed on littermate cohorts. Brain tissues from adult male Sprague Dawley rats ($n=3$) weighing 300–350 gm were used. Rats were fed lab chow *ad libitum* and maintained in a thermally controlled (27 °C), 12 h light/dark cycle environment. All procedures involving animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Weill Medical College of Cornell University.

Human tissue

Normal human cerebral cortical tissue stored in formalin was received from the NICHD Brain and Tissue Bank for Developmental Disorders under contracts N01-HD-4-3368 and N01-HD-4-3383. One sample was obtained from a 42-year old Caucasian female after a post-mortem interval of 4 h and was stored for 2 years. The second was acquired from an African-American 34-year old female after a post-mortem interval of 2 h and tissue was stored for 4 years. Death of both subjects was attributed to natural causes unrelated to brain damage. Tissue was paraffinized and sectioned at 10 μm thickness and processed using standard protocols described below.

Genotyping

Genomic DNA extraction from tail snips was performed using standard protocols, followed by PCR-based genotyping using primers for WT and null alleles as previously published (Sicinski et al., 1995, 1996). All genotyping of animals was validated by using a second post-mortem DNA sample.

Immunohistochemistry

Animals were anesthetized with ketamine/xylazine and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were blocked and post-fixed overnight at 4 °C before processing (Tissue Tech 2000, 11 h protocol for adult brain) for paraffin embedding in the coronal plane. Brains were sectioned at 10 μm and mounted on adhesive-coated capillary gap slides (Fisher Scientific, Pittsburgh, PA) and were immunostained on a TechMate 500 semi-automated stainer after antigen retrieval by steam in Reveal buffer (pH 5.9; Biocare Medical, Concord, CA) for 30 min and cooling for 10 min. Sections were treated with 3% H_2O_2 for 10 min and blocked in Sniper (Biocare Medical, Concord, CA) for 30 min and then incubated with primary antibody at the specified concentrations overnight at +4 °C. The next day sections were sequentially incubated in appropriate secondary antibody and peroxidase conjugated tertiary (Signet Rabbit or Murine kit (Covance, N.J) or Vectastain Elite Kit (Vector Laboratories, Burlingame, CA) for antibodies raised in other species), for 30 min each, followed by reaction with 3, 3-diaminobenzidine (DAB) (Covance, NJ). For free floating immunohistochemistry, brains were removed following transcardial perfusion (4% PFA in PB) and post-fixed in 4% PFA for 1 h. Brains were then cryoprotected overnight in 30% sucrose (in PB) and sectioned on a sliding microtome (40 μm). To test the effect of fixatives, some mice were both perfused and post-fixed in 10% neutral buffered formalin (1–1.5% methanol) until paraffinization and processing with the standard protocol for immunostaining. For GABA IHC, tissue from animals perfused with 0.5% glutaraldehyde (Sigma), 3% PFA in 0.1 M phosphate buffer was used. When comparing between experimental groups or across genotypes, slides were processed together thereby minimizing variability secondary to immunohistochemical staining. To fully characterize the expression of cD1 immunoreactivity in the normal adult C57BL/6J mouse brain, one 10 μm paraffin section every 100 μms was systematically sampled along the rostrocaudal axis.

Primary antibodies for immunohistochemistry included: rabbit anti-cyclin D1 (1:500, LabVision #RM9104, Freemont, CA), mouse anti-cyclin D2 (1:1000, LabVision, #MS-221), rat anti-somatostatin (1:100, Chemicon MAD35R), goat anti-calretinin (1:10,000, Chemicon AB1550), mouse anti-parvalbumin (1:50,000, Swant 35), mouse anti-glial fibrillary acidic protein (GFAP) (1:1000, Sigma), rabbit anti-gamma-a-butyric acid (GABA) (1:5000, Sigma), rabbit anti-c-Fos (1:10,000, Ab-5, Calbiochem). The specificity of the cD1 antibody used for this study has been previously established by western blot analysis and by negative staining in cD1 null tissues (Glicktein et al., 2007).

Dual immunolabeling

Following standard IHC with DAB, sections were blocked in Sniper and incubated with primary antibodies at +4 °C overnight. Sections were washed in phosphate buffered saline and incubated in Alexa Fluor-conjugated secondary antibodies (488 or 594 λ excitation; 1:500, Invitrogen, CA) for 1 h, washed and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA) and fluorescence was examined and photographed using an upright compound microscope (Nikon, Japan).

Western blot analysis

Following decapitation and brain retrieval, hemi-cortices and hippocampi were dissected out and quick frozen on dry ice. Tissue was stored at −80 °C until further processing. Tissue was homogenized in lysis buffer (Mammalian cell-lytic buffer, Sigma, MO) supplemented with protease inhibitors (Sigma, MO) and phosphatase inhibitors (Calbiochem, CA). Lysate was cleared by centrifugation at 10,000 rpm for 10 min at +4 °C. Cleared lysates were used for BCA assay (Pierce, IL) for protein quantification. Approximately 75 μg of total protein in duplicate was loaded per lane in 4–12% polyacrylamide Bis-Tris gels (NuPage, Invitrogen) and electrophoresed. Protein was transferred to nitrocellulose membrane and immunoblotted using rabbit anti-cD1 (1:500, Labvision) and mouse α-tubulin (1:10,000, Sigma) in 1% milk in Tris-buffered saline. Protein was detected using chemiluminescent detection (Pierce, IL). Blots were scanned and ImageJ (NIH, http://rsb.info.nih.gov/ij/) was used for densitometry. The absence of cD1 protein in cD1 nulls has been verified (Glickstein et al., 2007).

Seizure behavior and CA-field pyknosis

Mice were weighed and housed individually in fresh cages 1 h prior to seizure testing. Cages were coded so that observers were blind