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Cyclin D1 in Excitatory Neurons of the Adult Brain Enhances Kainate-induced Neurotoxicity

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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 sub-population 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 pentylentetrazole (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.

Keywords

Cyclin D1; Kainate; Pentylentetrazole; CA1; Excitotoxicity; Seizure; Hippocampus; Cell Cycle; Apoptosis; Excitatory Neurons

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 catalyzes the initial inactivating phosphorylation of the retinoblastoma protein (pRb) and resultant derepression of proliferation-associated E2F target genes and also, sequesters cdk inhibiting proteins, p57 and 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

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in one D-type (Sicinski et al., 1995; Sicinski et al., 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; Park et al., 1997; Padmanabhan et al., 1999). Several papers have examined cell cycle reactivation in apoptosis, but the unequivocal observation of cD1 in healthy postmitotic 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.

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; Sicinski et al., 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 hr 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 4h and was stored for 2 years. The second was acquired from an African-American 34-year old female after a post-mortem interval of 2h 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 10µm 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; Sicinski et al., 1996). All genotyping of animals was validated with using a second post-mortem DNA sample.

Immunohistochemistry

Animals were anesthetized with ketamine/xylazine and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). Brains were blocked and postfixed overnight at 4°C before processing (Tissue Tech 2000, 11h 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% H₂O₂ 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 hour. 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 0.1M 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 (1:10000, 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 (Glickstein 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 1h, 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 10,000rpm for 10 min at +4°C. Cleared lysates were used for BCA assay (Pierce, IL) for protein quantification. Approximately 75 μ g 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 to genotype. Immediately following the intraperitoneal injection of kainic acid (KA), pentylenetetrazol (PTZ) or saline, mice were observed for 2h and behaviors were scored as described below. All mice were re-housed following testing and sacrificed 48h later to examine hippocampal pathology.

KA (Ascent Scientific, UK) was prepared in saline and injected at 25 mg/kg. For sub-threshold acute administration, 20 mg/kg dose was used. Seizure behavior was scored at 10 min intervals using a previously described scale (McLin and Steward, 2006). Briefly, Stage 1: unmoving and crouched; Stage 2: body stretched out, tail straight and rigid; Stage 3: repetitive head bobbing, rearing into sitting position with forepaws resting on belly and Stage 4: rearing and subsequent tonic-clonic seizures interrupted by total stillness, jumping or running clonus.

PTZ (Sigma) was prepared in phosphate buffered solution and injected intraperitoneally at 30 mg/kg. For sub-threshold acute testing, 25 mg/kg dose was used. Behavior was scored at 30 second intervals for 30 minutes. PTZ was reinjected at 30 minute intervals until a tonic clonic seizure occurred, usually after the second dose. The following behavioral scale was used: Stage 1: normal behavior; Stage 2a-b: freezing or generalized shaking; Stage 3a-b: myoclonic jerking or head, neck spasms and tail flexion; Stage 4a-b: rearing or falling on side; Stage 5a-b: tonic-clonic seizure involving all limbs (Golarai et al., 2001).

In some studies, histological samples were obtained from mice sacrificed 6h, 14h, 24h and 48h following KA, PTZ or saline injection. For evaluation of pyknotic nuclei in the hippocampus, tissue sections were stained with hemotoxylin and eosin (Biocare Medical, Concord, CA) and coverslipped. Pyknotic nuclei with dense and shrunken appearance were scored in a 0.1mm² region of the CA1 or CA3 fields in 4 sections per animal and reported as a fraction of total number of healthy cells (McLin and Steward, 2006). Free-floating immunohistochemistry was used to survey c-Fos activation 1h following administration of a subthreshold dose of KA (20 mg/Kg), PTZ (25 mg/Kg) or Saline.

Image Preparation and Statistics

All images were taken using an upright compound microscope (Nikon, Japan); images were acquired with a digital camera and Spot Insight Mosaic 3.2 (Diagnostic Instruments, USA) and processed using Adobe Photoshop. For double-labeled samples, bright field images were inverted and superimposed on fluorescent images. Images were not modified in any way, except for adjustments of brightness and contrast. All quantitative data was compiled in MS Excel and used the following tests: Chi-squared distribution test and Student's t-test.

RESULTS

Previously, we have described robust cD1 and cD2 immunoreactivity in proliferative zones of the embryonic brain that subsequently decreased towards the end of the embryonic neurogenic period (Glickstein et al., 2007). Notably, we observed a resurgence of cD1 expression, but not cD2, in post-mitotic neurons of the cortex and hippocampus postnatally (Glickstein et al., 2007). Toward understanding the function of this cyclin in terminally differentiated neurons, in this study we fully characterize the regional and cellular expression of cD1 in the adult brain, examine the ability of excitotoxins to alter cD1 expression and, in cD1^{+/-} mice, assess the consequences for cell viability of reduced cD1 protein levels in two models of pharmacologically induced seizures.

cD1 immunolabels neuronal nuclei in all segments of the brain and spinal cord

cD1 immunoreactivity was predominantly nuclear and observed in all cortical regions, but was more robust in rostral than in caudal neocortex (not shown). In the somatosensory cortex, cD1 immunoreactivity was in all layers but sparsest in layer IV (Fig. 1A, 1B). cD1 labeling was observed in all layers of the motor cortex, including large Betz cells in layer V–VI (Fig. 1D–D') and also was robust in the basolateral amygdala and hippocampal formation (Fig. 1CE). Remarkably, cD1 was highly expressed in most of the pyramidal neuronal nuclei in the CA1 fields (Fig. 1F), but paler and sparser in the CA3 field neurons (Fig. 1G). In the dentate gyrus, cD1 immunoreactivity was present in the molecular and hilar regions, occasionally in the subgranular zone and rarely in the granule layer (Fig. 1H). Co-labeling of cD1 nuclei with a proliferative cell marker in the subgranular zone indicates that some of the labeled nuclei in this neurogenic niche belong to proliferative cells (Glickstein et al., 2007). No region of the neocortex or archicortex was completely devoid of cD1 immunostaining, although the density and morphology of labeled cells varied.

Immunoreactivity for cD1 was observed throughout the brain, retina and spinal cord. In the cerebellum, cD1 immunoreactivity was present in the white matter, the granule and molecular layers, and all three deep cerebellar nuclei, but was absent in Purkinje cells (Suppl. Fig. 1A–B). In the brainstem, cD1 immunoreactivity was relatively sparse; although, several prominently labeled cells with nuclear and cytoplasmic labeling were observed in the facial motor nucleus (Suppl. Fig. 1C). The spinal cord displayed scattered cD1 labeling of small cells in the dorsal horn and large motor neurons in the ventral horn, which had both nuclear and cytoplasmic labeling (Suppl. Fig. 1D', 2D'). In the diencephalon, immunolabeled cells were scattered in most nuclei and concentrated in the thalamic anterior-dorsal and hypothalamic paraventricular nuclei (Suppl. Fig. 2). cD1 labeling in the retina, a tissue exhibiting retinopathy in cD1 homozygous null mice (Fantl et al., 1995), was confined to the inner nuclear layer and occasionally the ganglion cell layer (Suppl. Fig. 3A–C). Retinal labeling did not overlap with the proliferative marker Ki67, supporting that post-mitotic neurons were labeled. The regional extent of basal cD1 immunolabeling is summarized in Table 1.

We also detected neuronal cD1 immunopositive neuronal nuclei in the rat cortex (Suppl. Fig. 4A–B), but cD1 labeling was absent in the rat CA1 fields (Suppl. Fig. 4B). Nuclear labeling

in other brain regions, including the ventrolateral thalamus, was unequivocally neuronal (Suppl. Fig. 4C). In comparison to both mouse and rat, no labeling was observed in the human neocortex (Suppl. Fig. 4D). Labeling observed in proliferative zones of human fetal cortex (Glickstein, unpublished observation) supports that cD1 immunoreactivity, if present, should be detected with our antibodies in human tissues. Normal cD1 immunolabeling in mouse brain tissue following prolonged (8 weeks) formalin-fixation of mouse brain tissue (Suppl. Fig. 4E, F) supports that lack of staining in human tissues is not a fixation artifact.

cD1 is expressed in a subpopulation of excitatory neurons

Based on their nuclear morphology and location, a significant proportion of these neurons were clearly neuronal and excitatory (Vaughn, 1984; Peters et al., 1991). A thorough survey of neocortex and hippocampus in sections double-immunolabeled for cD1 and GABA did not reveal any overlap (Fig. 2A–C), indicating that cD1 is not expressed in inhibitory neurons. Moreover, no double-labeling for cD1 and the inhibitory interneuron subtype markers parvalbumin or somatostatin was observed (Fig. 2D–I). While cD1 and calretinin dual-labeled cells were absent in most of the neocortex and hippocampus (Fig. 2J–L), there were rare co-labeled cells in the entorhinal cortex (not shown), a region in which some calretinin neurons are excitatory (Wouterlood et al., 2000). We also did not observe overlap of cD1 and GABA immunoreactivities in the retina or cerebellum (Suppl. Fig. 3). Moreover, cD1 immunoreactivity was not present in readily identifiable GABAergic neurons, including Purkinje cells or in striatal medium spiny neurons. Whether cD1 immunolabeling was a general characteristic of large-nucleated neurons was explored; however, cD1 labeling did not coincide with tyrosine hydroxylase immunoreactivity in the substantia nigra (Suppl. Fig. 3F–H). Altogether, cD1 was identified in a subset of excitatory neurons regardless of their size.

We observed no colocalization of cD1 immunoreactivity with the glial-cell marker GFAP in the neocortex (not shown), the rostral migratory stream (Suppl. Fig. 5A–C) corpus callosum (Suppl. Fig. 5D–F), or dentate gyrus (Suppl. Fig. 5G–I). Higher power magnification examination of Nissl and cD1 co-staining in several brain regions (Suppl. Fig. 5J–L) revealed that some cD1 immunopositive neurons had small dense nuclei characteristic of microglia (Vaughn, 1984; Peters et al., 1991).

cD1 expression was found in significant numbers of post-mitotic excitatory neurons and potentially proliferating microglia (Table 2). Because labeling was not observed in all glutamatergic cells (e.g., CA3, thalamic neurons), only a subset of excitatory neurons expresses cD1. Several studies have suggested that neuronal damage elicits expression of cell cycle proteins that lead to cell death (Herrup and Yang, 2007). To understand the role of cD1 in the context of neurotoxicity, we examined CA1 field pyramidal neurons, a highly vulnerable neuronal population.

WT-like cD1 expression is not essential for the structural integrity of hippocampal CA field neurons

Whether cD1 is essential for the healthy morphology of CA1 neurons in wildtype, cD1^{+/-} and ^{-/-} littermates was explored after first examining cD1 expression in cD1^{+/-} mice. Reductions of cD1 expression were confirmed in cD1^{+/-} animals compared to wildtype by western blot analysis (Fig. 3A–B). cD1 protein was reduced by 40% in the cortex and 32% in the hippocampus ($p < 0.05$; $n = 3$) (Fig. 3B). There were no gross cytoarchitectural abnormalities in the hippocampus of cD1^{-/-} or cD1^{+/-} mice, as previously reported (Sicinski et al., 1995), nor obvious defects in nuclear appearance of CA1 field neurons in cD1^{-/-} or ^{+/-} mice compared to wildtype (Fig. 3C–E). cD1 immunoreactivity was absent in cD1^{-/-} mice and generally paler in an equivalent number of CA1 nuclei in cD1^{+/-} compared with wildtype (Fig. 3F–H), supporting that expression of the remaining allele is not increased. Labeling for cD2 was not

increased in a compensatory manner in the cD1^{+/-} and null mice (Fig. 3I–K). The absence of immunolabeling for activated (cleaved)-caspase-3 established that reduction in or absence of cD1 did not elicit apoptosis (Fig. 3L–N). Immunostaining for a synaptic protein, synaptophysin, also displayed no obvious difference across genotypes (Fig. 3O–Q). These data suggested that deficiency of cD1 did not impact the overall structural organization or mortality of CA1 neurons.

cD1 expression in CA1 is reduced by the excitotoxin kainic acid

In mice, systemic application of the excitotoxin and convulsant kainic acid (KA) induces cD1 in all CA subfields, but particularly in CA3, a region that appears more susceptible to KA induced-cell death and that, in our analysis, has very low basal cD1 levels (Hu et al., 1998; Park et al., 2000). For this reason, we examined KA-induced cD1 levels in CA1 compared with CA3 to explore whether neurons that basally express cD1 have a more rapid or likely induction of cD1 expression.

Wildtype animals were given single injections KA that produced brief tonic-clonic seizures, and then sacrificed 6h, 14h, 24h or 48h later to examine cD1 expression in the CA fields. A modest number of pyknotic nuclei were observed from 6h onwards (not shown; typically 1–2 pyknotic cells per field of view at 6h and 14h and an increased number at 24h and 48h). cD1 immunoreactivity vanished in CA1 6h following KA (Fig. 4A–E). At 14h, cytoplasmic levels of cD1 increased and formed a halo around an unlabeled-nucleus and thereafter, nuclear labeling increased and returned to baseline levels by 48h (Fig. 4A–E). Following KA, cD1 expression did increase in small nuclei outside of CA1 that potentially are part of a gliotic response. In contrast to CA1, cD1 immunoreactivity modestly increased in CA3 neurons 6–14h following KA and returned to normally low basal levels by 48h (Fig. 4K–O). There were a small number of pyknotic nuclei in CA3 that were comparable to that observed in CA1 (not shown). However, as in CA1, the largest increases in cD1 immunoreactivity were apparent in small nuclei scattered outside of CA3. We examined the expression of the proto-oncogene c-Fos, an activity marker and an early death signal induced by KA (Planas et al., 1994; Kasof et al., 1995). Immunolabeling in adjacent sections indicated that, in contrast to cD1, c-Fos immunoreactivity in both CA1 and CA3 was elevated at 6- and 14h following KA and returned to baseline by 24- and 48h (Fig. 4F–J, P–T). This finding implies that the initial drop in cD1 immunoreactivity is distinct from other proteins associated with cellular death pathways and if basal cD1 in CA1 is involved in cellular death in response to KA, it exerts its effect immediately or in those cells dying at later times following KA.

To understand whether the KA-induced alterations in cD1 levels were secondary to seizure and unrelated to excitotoxicity, cD1 expression was examined in both CA1 and CA3 regions following seizures induced by pentylenetetrazol (PTZ), which elicits minimal CA neuron death (Kasof et al., 1995; Liu et al., 1999) and also elicits c-Fos expression (Yount et al., 1994). CA1 neurons appeared non-pyknotic (not shown) and had no significant changes in cD1 immunoreactivity despite increased c-Fos expression (Supp. Fig. 6). In CA3, there was similarly no alteration of cD1 immunoreactivity despite moderate c-Fos induction 6–14h after PTZ treatment and the presence of a small number of pyknotic nuclei (Suppl. Fig 6). These data, summarized in Fig. 4U, suggest that reduced cD1 levels in CA1, and increased neuronal cD1 in CA3 were elicited by KA and were not a general feature associated with seizures.

Elevated seizure threshold and reduced cell death following KA, but not PTZ in cD1 heterozygous mice

Pro-convulsant and pro-cell death signal roles for cD1 were explored through the examination of seizure threshold and cytotoxicity in KA and PTZ-treated wildtype and cD1^{+/-} littermates. KA application elicited stage 4 seizures within an average of 35 min. While there was no

significant difference in the latency to stage 4 seizure (WT vs. cD1+/-, 33±8min vs. 42±8min; $p=0.46$), in cD1+/- mice, time spent in this seizure stage was significantly reduced by 67%, while time spent in stages 1–2 and 3 was increased by 75% and 39%, respectively, compared with wildtype (Fig. 5A). This suggests that cD1+/- mice have an elevated threshold to KA induced seizures. In contrast, no difference in the duration of PTZ-induced tonic-clonic seizures was observed amongst genotypes (Fig 5B). There were also no differences in the latency to seize following PTZ (WT vs. cD1 +/-, 38 ± 2min vs. 41 ± 2; $p = 0.55$). These findings suggest that cD1 is not a general pro-convulsant molecule.

The proportion of pyknotic nuclei 48h following KA was significantly reduced, by ~85%, in both CA1 and CA3 fields of cD1+/- mice (Fig. 5C, E–H). Additional wildtype mice receiving reduced KA dose were prepared to compare cell death between genotypes having similar stage 4 seizure duration. CA1 field cell death in cD1+/- was significantly reduced, by ~80%, compared with wildtype mice. After PTZ treatment, where similar behavior was observed between genotypes, only rare pyknotic nuclei were observed equivalently in the CA1 fields of both genotypes (Fig. 5D, I–L). These findings support that cD1 haploinsufficiency is neuroprotective in response to KA, but that nuclear cD1 expression alone does not commit a cell to death following KA.

To explore the degree of involvement of hippocampal and cortical neurons in the generation of seizure by KA and PTZ, and to determine whether cD1 heterozygous mice respond to KA similarly to WT, we examined c-Fos immunoreactivity in CA1, CA3 and neocortex 1h following KA, PTZ or saline. We employed sub-threshold doses of these drugs to focus on drug-specific neuronal activity and not secondary effects of seizure. One hour after saline injection, c-Fos labeling was increased in both the cortex and CA1 of both genotypes above baseline levels in non-injected mice (Fig. 6A–D), but c-Fos immunolabeling was modestly elevated in cD1+/- animals in the cortex and CA1 fields suggesting a likely increased response to stress (Fig. 6E–H). Numbers and intensity of c-Fos labeled nuclei were equivalently increased by KA in both the cortex and CA1 above that following saline in both genotypes (Fig. 6I–L). Cortical immunolabeling for c-Fos following PTZ was similar to that observed following KA and did not differ between genotypes (Fig. 6M–N). In contrast, PTZ appeared to attenuate c-Fos induction in CA1 of both genotypes (Fig. 6O–P). Taken together these data suggest that KA has a more pronounced impact on the CA fields than PTZ and that both genotypes respond to KA in a similar manner at the cellular level. The contribution of cD1 to seizure threshold and pathology may thus be downstream of KA-elicited CA neuron activation.

DISCUSSION

This is the first study to characterize the regions and cell types exhibiting basal cD1 expression in the mouse brain. We observed neuronal cD1 throughout the brain and spinal cord, primarily in large neurons, which are the targets of many neurodegenerative conditions, and in microglia, which participate in the inflammatory response to neuronal injury. cD1 immunoreactivity was predominantly nuclear, though cytoplasmic labeling was particularly apparent in the largest motor neurons of the brainstem and spinal cord. cD1 immunoreactivity was not detected in astrocytes. While neuronal cD1 expression is not likely to result in cell cycle progression (negative immunostaining for Ki-67 and pRb, not shown), it is probable that microglial cD1 immunolabeling is consistent with on-going proliferation. Neuronal cD1 immunolabeling was robust, but was not present in GABAergic neurons as shown by the lack of dual-labeling and absence of cD1 in many known and easily identifiable GABAergic neurons (i.e., Purkinje cells & medium spiny striatal neurons). While numerous labeled nuclei were unequivocally identifiable as belonging to glutamatergic cells (i.e., CA1 field neurons, lamina V pyramidal neurons), not all glutamatergic neurons had cD1 labeling (e.g., CA3 fields, many thalamic neurons). cD1 labeling did not appear to be simply restricted to larger nucleated cells. Although

we did not observe basal cD1 in the human brain, our observations of basal cD1 expression in the mouse are therefore highly relevant to those studies that seek to examine re-activation of the cell cycle in apoptotic cell death using rodent models. Some neurons having basal cD1 expression (i.e., CA fields) are known to be especially susceptible to delayed cell death following excitotoxic and ischemic insults (Kirino, 1982). While we observed no gross morphological alterations in hippocampal CA1 neurons in cD1 heterozygous and nullizygous mice, having reduced or no basal cD1, respectively, we did observe specific reductions in both the seizure severity and CA-field cell death in cD1 heterozygotes following KA, but not PTZ. These findings, in addition to rapid changes in cD1 immunoreactivity in the CA fields following KA, support a role for cD1 in the toxic response of CA neurons to KA. They further indicate that basal cD1 immunolabeling characterizes a subset of vulnerable excitatory neurons in the mouse brain. However, cD1 expression is not necessarily a sign of imminent apoptosis.

Restricted basal expression of cD1 in excitatory neurons

The mechanisms that restrict expression of cD1 in neurons are not known. Postnatal cD1 expression develops during the second week of life (Glickstein et al., 2007), at a time when neuronal growth and later stages of neuronal differentiation occur. While basal cD1 has not been found in healthy human brain tissues (Yang et al., 2003), others observed neuronal, nuclear cD1 immunoreactivity in hemimegalencephalic brain specimens (Yang et al., 2003; Aronica et al., 2007). In that study, neuronal cD1 was unrelated to seizure and was only observed within abnormally large neurons characteristic of this brain malformation, which is consistent with a role for neuronal cD1 in the modulation of cell size secondary to activation of the PTEN pathway (Backman et al., 2001). It is possible that normal CA1 somal sizes in mice lacking cD1 observed in this study results from developmental compensation by cyclin E or the remaining D-type cyclins (Chen et al., 2005). cD1 has been shown to have other noncell cycle functions that include the modulation of transcription factor activity (Lamb et al., 2003), the promotion of DNA repair (Fishel et al., 2007; Schwartz et al., 2007) and the generation of short-term and long-term plasticity in the hippocampal circuits (Li et al., 2007). It is therefore possible that cD1 plays a non-cell cycle role not directly related to any pathogenic process. It remains to be elucidated whether this expression in excitatory neurons is a normal step in their differentiation or whether it is induced by regular physiological processes.

Pro-apoptotic functions of cD1 are the best characterized among the atypical roles identified for this protein (Herrup and Yang, 2007). The proposed cell death mechanism involves a cdk4-dependent de-repression of E2F4 leading to the expression of the transcription factors B- and C-myc. These transcription factors bind to the promoter for the pro-apoptotic BH3-only protein Bim, a protein that harnesses the apoptotic cascade by activating caspases (Greene et al., 2007). Numerous studies have reported nuclear expression of cD1 in damaged neurons (Freeman et al., 1994; Herrup and Yang, 2007). It is therefore possible that neurons expressing cD1 are under a physiological stress that is sufficient to yield cD1 expression, but that is not significant enough to trigger the latter steps of the apoptotic cascade. Cellular stress may result from enhanced cellular metabolism, which is characteristic of CA1 compared with CA3 neurons (Davolio and Greenamyre, 1995), or reduced calcium buffering, a feature of excitatory compared with inhibitory neurons (Lee et al., 2000). Furthermore, alterations in cellular metabolism in mice compared with rats (Davson, 1964) could underlie the species differences in cD1 staining pattern. In the relatively young, healthy human brain we observed no basal cD1; however it is worth examining whether basal cD1 is observed in aged human brain tissues that have an increased lifetime accumulation of stresses. cD1 expression in the large neurons associated with hemimegalencephaly may also be secondary to enhanced cellular metabolism (Aronica et al., 2007). That there was no gross CA1 pathology in mice with reduced expression of cD1 indicates that this cyclin is not essential for cellular health.

Kainate-induced cyclin D1, seizures and cell death

Systemic kainate application results in seizures and death of CA field neurons. KA-induced synchronized activation of CA3 neurons, by way of their recurrent collaterals, is central to seizure production. CA3 neurons are directly excited by stimulation of their KA receptors and indirectly, by increased glutamate efflux secondary to KA stimulation of mossy fibers (Ben-Ari and Cossart, 2000; Schmitz et al., 2001). Seizure activity itself, as a result of KA or occurring in response to other agents, elevates mossy fiber glutamate efflux, which also leads to CA3 synchronization. CA3 synchronization produces spreading epileptiform activity that extends to CA1 and other limbic structures to produce seizure-related behaviors and also cell death in the CA fields. In mice, the CA3 region displays increased cell death compared with CA1 following KA, converse to that observed in the rat hippocampus (Weiss et al., 1996; Hu et al., 1998; Park et al., 2000). We compared KA and PTZ-elicited cD1 induction to understand changes in cD1 induction secondary to seizure compared with those resulting from excitotoxic cell death. While both agents evoked seizures, only KA resulted in significant CA field pathology. cD1 expression changed rapidly in the CA fields following KA, but not PTZ treatment. Thus, cD1 is not induced (CA3) or degraded (CA1) as a general feature of the activation of hippocampal networks during a seizure. Nuclear expression of cD1 had a different time-course in CA1 compared with CA3 subfields. Our results partially conflict with those reported in another mouse strain (Park et al., 2000) and in rats (Timsit et al., 1999) in that both of these studies observed significant increases in cD1 in CA3 following kainate and neither detected significant basal cD1 in the hippocampus. It is possible that CA3 induction represents a strain or species dependent response to KA. It is also possible that any cD1-mediated engagement of the apoptotic machinery occurs at different times in CA1 compared with CA3 or occurs by different mechanisms. Differences in c-Fos activation in CA1 compared with CA3 in the KA model suggest that neuronal activation alone does not induce cD1 expression. However, c-Fos expression following acute, sub-threshold doses of KA or PTZ support that these agents differentially affect CA1 neurons. Reduced cellular death in response to KA in cD1^{+/-} mice, even when controlling for seizure behavior, fits with the current pro-apoptotic view of cD1 function in post-mitotic neurons and that shown in rats following KA treatment (Ino and Chiba, 2001). However, widespread nuclear cD1 expression, seemingly uncoupled from a death signaling cascade, and diminished seizure threshold observed in cD1 heterozygous mice are novel findings and suggest a role for cD1 in the neuronal network response to KA or the development of relevant circuits.

In summary, cD1 is basally expressed in a subset of post-mitotic neurons. Our findings of cD1 suppression and cell death following KA compared to PTZ treatments suggests that basal cD1 may enhance neuronal activation or cell death processes. This study supports the literature showing the cD1 is a cell death signal, but it places cD1 rather far upstream of the final apoptotic events. It is possible that cD1 accumulation with aging or sub-threshold insults produces a basal cD1 expression profile observed in mice that results in differential cellular vulnerability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of cD1 in post-mitotic neurons in the neocortex and archicortex

A, B, In the somatosensory cortex, cD1 immunolabeling predominantly marks layers II–III and VVI of the normal adult mouse cortex. cD1 immunoreactivity is dense in layer II, V and VI and sparse in layer IV as shown by co-staining for Nissl (A) and cD1 only (B) in S1 cortex. cD1 staining is clearly nuclear (higher magnification in B'). **C,** cD1 labeling in the amygdala also has distinct nuclear staining. White matter tracts along the basolateral nucleus are indicated with the dotted line. **D–D',** cD1 labeling pattern is conserved in other cortical regions such as the primary motor cortex (D) and in the nuclei of large Betz pyramidal neurons of the deep layers V–VI (D'). **E,** cD1 is dispersed throughout the hippocampal formation and overlying white matter as shown by Nissl co-staining. **F–G,** At higher magnification, cD1 is clearly present in the CA1 field pyramidal neurons (F) and sparse in the CA3 fields (G). In both regions, cD1 positive cells with smaller nuclei are present that could be microglia (arrows). **H:** **Several** cD1 immunoreactive cells are in the neurogenic SGZ (arrows) and the SG of dentate gyrus. Abbreviations: S1, primary somatosensory cortex; M1, primary motor cortex; Amyg, amygdala; wm, white matter; pir, piriform cortex; rf, rhinal fissure; CeC, central amygdaloid nu capsule; BLA, basolateral amygdala CA1, cornus ammonus 1; CA3, cornus ammonus 3; dg, dentate gyrus; ml, molecular layer; sgz, sub granular zone; sg, stratum granulosum; hil, hilus.

Figure 2. cD1 immunolabeled neurons are not GABAergic

A–C, cD1 expression (A and inverted image A') does not overlay with GABA immunoreactivity (B, C). **D–F**, cD1 immunolabeling (D, D') is absent in parvalbumin labeled neurons (E, F). Somatostatin positive cells (H) do not overlay with cD1 immunostaining (G, G', I). **J–L**, cD1 expression in SS1 (J, J') does not coincide with calretinin labeling (K, L). Abbreviations: SS1, primary somatosensory cortex; PV, parvalbumin; SSN, somatostatin; Calr, calretinin.

Figure 3. Loss of cD1 expression does not affect CA1 neuronal morphology or survival

A, Representative immunoblot for cD1 (top panel) in cortical and hippocampal lysates from adult cD1 wildtype and heterozygous (cD1 +/-) animals were normalized to α -tubulin (bottom panel). **B**, Optical densitometry (OD) reveals significant reductions in cD1 protein in cD1 +/- cortex and hippocampus (n=3). **C–O**, Comparison of various morphological and immunohistochemical markers in 10 μ m adjacent sections in cD1 wildtype, heterozygous and null littermate P21 mice. **C–E**, Nissl staining of 10 μ m sections of level matched CA1 fields shows normal morphology in cD1 heterozygous (D) and cD1 nulls (E) compared to wildtype (F). **F–H**, cD1 immunolabeling is present in the nuclei of CA1 pyramidal neurons on WT (F), reduced in heterozygous (G) animals and is absent in the cD1 null animals (H). **I–K**, Cyclin

D2 (cD2) expression is absent in the CA1 neurons of WT (I) and cD1 heterozygous (J) animals. CA1 fields of cD1 null animals (K) do not have a compensatory expression of cD2 in the CA1 neurons. **L–N**, Immunostaining for the apoptotic marker cleaved caspase-3 (casp3) in adjacent sections reveals that the CA1 field neurons are non-apoptotic and stable in WT (L) heterozygous (M) and null (N) animals. **O–Q**, The synaptic inputs of the CA1 fields are similar in cD1 WT (O), heterozygous (P) or null (Q) mice as visualized by immunostaining for synaptophysin (synP). Abbreviations: WT, wildtype; CA1, cornus ammonus 1; Or, stratum oriens; Py, stratum pyramidale; Rad, stratum radiatum; α -tub, α -tubulin.

Figure 4. cD1 falls and recovers in CA1 while cD1 increases in primarily non-neural cells in CA3 following systemic application of kainate (KA) in wildtype mice

A–E, cD1 immunostaining at baseline (A) is dramatically reduced at 6h (B) and 14h (C) after KA treatment. At 14h (C) cD1 immunolabeling appears non-nuclear and densest at the nuclear membrane in many cells (arrows). cD1 levels begin to recover at 24h (D) and are at baseline levels by 48h (E). **F–J,** Baseline c-Fos immunoreactivity (F) is minimal in the CA1 fields and numbers of c-Fos immunolabeled neurons are robustly increased at 6h (G) and 14h (H) post KA treatment. By 24h (I) and 48h (J), c-Fos immunolabeling is minimal. **K–O,** cD1 immunolabeling in CA3 fields is sparse at baseline (K). At 6h post KA administration (L), cD1 levels are unchanged, while they are slightly elevated at 14h (M). Note the increased cD1

immunoreactivity in presumably non-neuronal cells in the CA3 region. At 24h (N) and 48h (O) after KA treatment, cD1 levels are similar to baseline. **P–T**, c-Fos labeling is absent at baseline (P). c-Fos expression is highly induced at 6h (Q) and 14h (R) following KA and, by 24h(S) and 48h (T), c-Fos immunostaining is minimal. **U**, Plot summarizing the effect of KA and PTZ over time on cD1 and c-Fos expression in CA1 and CA3 regions. Abbreviations: KA, kainic acid; PTZ, pentylentetrazol; CA1, cornus ammonus 1; CA3, cornus ammonus 3.

Figure 5. cD1^{+/-} mice have an elevated seizure threshold to kainate but not PTZ compared with WT littermates

A–B, cD1^{+/-} animals are resistant to KA (25 mg/kg) and spend significantly more time in pre-seizure stage 3 and significantly less time in seizure stage 4 compared to WT littermates (A). There is no behavioral difference between cD1 WT and heterozygous littermates upon PTZ (30 mg/kg) administration (B). **C–D**, Cell damage in CA1 and CA3 fields. WT animals have significantly more pyknotic cells in the CA1 and CA3 fields compared with cD1^{+/-} littermates 48 hr after treatment with same dose of KA (C). When comparing animals with similar duration of KA induced seizure behavior, pyknosis is reduced in both CA subregions, in WT compared with cD1 heterozygotes. No pyknotic nuclei were apparent in CA1 fields of all animals tested

with PTZ (not graphed), and there was no difference in pyknotic cells in the CA3 fields between cD1 WT and heterozygous animals (D). **E–H**, Representative images of H&E stained sections in cD1 WT (E, G) and cD1 heterozygous animals (F, H) in CA1 (E, F) and CA3 fields (G, H) 48h following KA. **I–L**, Representative images of H&E stained sections in cD1 WT (I, K) and cD1 heterozygous animals (J, L) in CA1 (I, J) and CA3 fields (K, L) 48h following PTZ. Abbreviations: WT, wildtype; KA, kainic acid; PTZ, pentylenetetrazol. H&E, hematoxylin and eosin.

Figure 6. Robust activation of CA1 neurons acutely following kainate but not PTZ application
A–D, At baseline, c-Fos labeling in the cortex (A, B) and the CA1 neurons (C, D) is low in both WT (A, C) and cD1 heterozygous animals (B, D). **E–H**, c-Fos immunostaining is elevated 1h after saline injection in cortex (E, F) and CA1 neurons (G, H) of both WT (E, G) and cD1 heterozygous (F, H). **I–L**, Acute treatment with kainate (20 mg/kg) induces comparable levels of c-Fos immunolabeling in both WT (I, K) and cD1 heterozygous animals (J, L) in the cortex (I, J) and CA1 neurons (K, L). **M–P**, Conversely, c-Fos immunoreactivity is weakly induced by PTZ administration (25 mg/kg) after 1h in the CA1 fields (O, P) in either WT (O) or cD1 heterozygous animals (P), while high immunolabeling is observed in both genotypes in the

cortex (M, N). Abbreviations: LTPA, lateral parietal association cortex; KA, kainic acid; PTZ, pentylenetetrazol.

Table 1

Summary of cD1 immunolabeling in the CNS.

Region	cD1 Immunolabeling
Overall	dispersed in the entire brain, brain stem and spinal cord
Cortex	rostral > caudal gradient; layer II-III, V-VI; S1, M1 (large Betz cells, layer V-VI), amygdala
Hippocampus	CA1 fields, sparse in CA3 and SG in the dentate gyrus
Cerebellum	scattered in ml, gcl, wm; no difference in cD2 nulls
Retina	inner nuclear layer
Thalamus	anterior thalamic nuclei, posterior hypothalamic nuclei
Brain Stem	Dispersed in brainstem, particularly notable in facial nucleus
Spinal Cord	dorsal, ventral horn motor neurons

Table 2

Summary of cD1 co-expression with neuronal and glial subtype markers.

Criteria	Regions	Dual Label
<i>Excitatory Markers</i>		
Glutamate (morphology, location)	Cortex (S1, M1), Hippocampus	Yes
<i>Interneuron Markers</i>		
Parvalbumin	Cortex (S1), Hippocampus	No
Somatostatin	Cortex (S1), Hippocampus	No
Calretinin	Cortex (S1), Hippocampus	No
GABA	Entorhinal cortex	Some
<i>Glial Markers</i>		
GFAP	Cortex (S1), Hippocampus	No
Microglia Morphology	Olfactory bulb, Corpus Callosum, Hilus, hippocampus	No
<i>Other Neuronal cell types</i>		
Tyrosine Hydroxylase	Cortex, Hippocampus	Yes
	Substantia Nigra	No