

ROLE OF PROTEIN KINASES IN NEURODEGENERATIVE DISEASE: CYCLIN-DEPENDENT KINASES IN ALZHEIMER'S DISEASE

Edward A. Monaco III and Mary Lou Vallano

Department of Neuroscience and Physiology, SUNY Upstate Medical University, Syracuse, New York, 13210, USA

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1. ABSTRACT

Cyclin-dependent kinases (Cdks) are serine/threonine kinases that regulate a number of cellular processes including the cell cycle and neuronal differentiation. Accumulating evidence indicates that two distinct Cdk pathways may have a role in the neuronal loss that is responsible for Alzheimer's disease. One pathway involves the aberrant reactivation of the cell cycle, a process believed to be incompatible with neuronal function. A second involves dysregulation of Cdk5, a member of this kinase family with no known cell cycle functions, but prominently expressed in postmitotic neurons. Reports supporting the involvement of both pathways are plentiful, but the story is not yet complete. In particular, difficulties incorporating the extended latency of AD into model approaches persist. Despite this, the theory that Cdks are involved in the pathogenesis of AD has generated considerable interest.

2. INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative affliction characterized histopathologically by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (1). It is believed that between 1 and 5 million people in the United States have the disease, with around 360,000 new cases diagnosed each year (2, 3). Considering the rapid rate at which the aged population is growing, it is clear that this tremendous public health problem is becoming worse. However, clinical therapies for the prevention of AD do not exist and current treatments for symptomatology are inadequate, due to our limited understanding of the cause and effect relationships that underlie the neuronal loss that is central to AD pathology.

Over the last two decades, a theme that has generated much study is that aberrant activation of protein kinases, in particular those associated with various forms of cell stress, is an early event leading to neurodegeneration in AD (4-12). The cyclin-dependent kinase (Cdk) family, has garnered intense interest and is the focus of this review.

There are 13 known Cdks encoded by the human genome, and they are well established regulators of the cell cycle (Cdks 1-4, 6, 7), but also have important roles in regulating transcription (Cdks 2, 7-9, 11), neuronal morphogenesis (Cdks 4, 5, 11), differentiation (Cdks 2, 5, 6, 9), and cell death (Cdks 1, 2, 4-6, 11). Cdk activity is normally tightly regulated by post-translational modification and the limiting availability of regulatory molecules. However, accumulating evidence indicates that cell cycle Cdks or Cdk5 can become dysregulated, the results of which are catastrophic, leading to neuronal demise. Herein, we discuss the data that implicate Cdk dysregulation as a causal element in the neuronal loss of AD.

3. DISCUSSION

3.1. Cyclin-dependent kinases and their regulation

Comprised of around 300 amino acids, Cdks are proline directed serine/threonine kinases that are activated on association with a regulatory partner, typically one of several cyclins, but also non-cyclin proteins (13, 14). Based on x-ray crystallographic studies, the general events surrounding Cdk activation have been elucidated (15-18). The binding of a regulatory partner initiates Cdk activation by inducing conformational changes, including movement of a segment of the protein, called the T-loop, away from a position blocking the catalytic domain. This also exposes a critical site (i.e. Thr160 of Cdk2) on the T-loop, and phosphorylation of this site by Cdk-activating kinase (CAK) is essential for full activation of the kinase as a result of further conformational changes. The CAK itself, is a Cdk/cyclin complex comprised of a Cdk7 and requires cyclin H as a binding partner (19).

Negative regulation of Cdks is accomplished in a number of ways, including alterations in the abundance of the regulatory cyclins/proteins. This process is important in shaping our understanding of Cdk dysregulation in neurodegenerative disease, and will be revisited later. Inhibitory phosphorylation of Cdks 1 and 2 (Thr14/Tyr 15) is

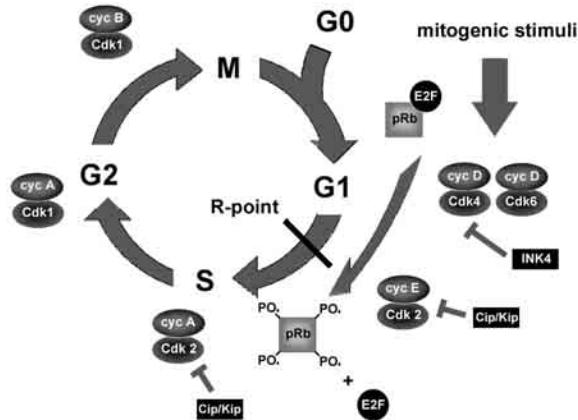


Figure 1. A simplified model depicting the cell cycle machinery. G₀, G₁, S, G₂, and M refer to quiescence, the first gap phase, DNA replication, the second gap phase, and mitosis. The restriction point (R-point) is shown as the process by which pRb becomes phosphorylated by G₁ phase Cdk activity. INK4 and Cip/Kip are endogenous Cdk inhibitors that regulate cell cycle Cdk activity.

achieved by Wee1/Myt1 kinases (20). In this way, Cdk1 activity is kept 'in check' until it is dephosphorylated by the dual specificity phosphatase Cdc25. Dephosphorylation of the T-loop threonine residues offers another level of regulation, though its physiological relevance remains to be determined. Endogenous peptide Cdk inhibitors are another important mode of negative regulation. Through direct physical interactions, these proteins suppress Cdk function (21, 22). Two classes of the peptide inhibitors are known: the INK4 (inhibitors of Cdk4), and the Cip/Kip (Cdk interacting protein/kinase inhibitory protein) families. Each family has a distinct specificity for Cdk, INK4 proteins for Cdk4/6 and Cip/Kip proteins for Cdk2/4. The INK4 family consists of four known proteins that share common amino acid sequence motifs: p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d} (23-26). Inhibition by INK4 proteins is the result of an allosteric interaction with a site distinct from the Cdk binding site. Specifically, INK4 protein binding induces a conformational change that distorts both the cyclin binding site and the ATP binding site (22). Three proteins comprise the Cip/Kip family: p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} (27-31). These Cip/Kip proteins interact with both the cyclin and Cdk, ultimately triggering conformational changes that prevent ATP binding and distort the catalytic domain (18, 21). Interestingly, Cdk4/cyclin D can sequester p21^{Cip1} and p27^{Kip1} without affecting the activity of the complex (32). When the D-type cyclins become less abundant due to reduced mitogenic stimuli, p21 and p27 are released to act on Cdk2 complexes.

Finally, compartmentalization serves to segregate Cdk activity from potential substrates (33). For example, while cyclin A can trigger DNA replication, centrosome duplication, and mitosis, cyclin B can only induce mitosis (34). Indeed, in interphase cells cyclins A and B are located in distinct cellular compartments, the nucleus and the cytosol, respectively (35). However, a chimeric cyclin B, containing the cyclin E nuclear localization sequence, can enter the nucleus and induce DNA replication (36). These findings suggest that rather

than merely conferring unique substrate specificities, the cellular cyclin library is degenerate with multiple layers of regulation to ensure that Cdk/cyclin complexes are active when and where they are needed (33). Although many of these regulatory mechanisms are conserved among the different Cdk/cyclin complexes, Cdk5 demonstrates noteworthy variations of these themes.

Unlike other Cdk, Cdk5 is activated through complex formation with one of several non-cyclin proteins: p35 and p39 (13, 14), or their proteolytic cleavage products p25 and p29 (37, 38), respectively. As with the cyclins, changes in the abundance of Cdk5 regulatory proteins, via increased or decreased expression and degradation, are an important mode of regulation (39-44). Although Cdk5 activating proteins share little amino acid sequence homology with cyclins, they exhibit cyclin-like tertiary structure (45, 46). However, whereas most Cdk require phosphorylation of the T-loop to become fully active, binding alone of Cdk5 to its activators results in an activated T-loop conformational change that is virtually indistinguishable from that of Thr160 phosphorylated Cdk2/cyclin A (46). In fact, although a similar phosphorylation event can occur at Ser159, it is not a requirement for full Cdk5 activation (47, 48) and may actually hinder association with p35 (46). Threonine 14 and Tyr15 are also regulated sites of phosphorylation on Cdk5, but not by the Wee1 kinase (48). Phosphorylation of Thr14 by an unknown kinase, purified from bovine thymus, is inhibitory (49), while phosphorylation of Tyr15 by the *c-Abl* kinase or the *src*-family member *Fyn* is stimulatory (50, 51). p35 and p39 possess consensus sequences for post-translational myristoylation of their N-termini (37), which confers upon these proteins a membrane localization. Indeed, both are enriched in cellular membrane fractions (52-54). In this way, Cdk5 activity is normally limited to substrates that are co-localized near the cell membrane.

Collectively, this multifaceted system of regulation highlights the importance of strictly controlling Cdk/cyclin complex activity to maintain cellular homeostasis. As such, it is not difficult to appreciate that dysregulation of these processes could be catastrophic. Although classically associated with cell cycle regulation, Cdk5 also have important roles in transcription, neuronal development, differentiation, and cell death. Aberrant reactivation of cell cycle Cdk5 (1-4, 6, 7) and dysregulation of the Cdk that functions in mature neurons (Cdk5) are associated with human neurodegenerative diseases like AD.

3.2. Cyclin-dependent kinases and the cell cycle

Cell division is a carefully orchestrated sequence of events that ensures the faithful replication and conduction of genetic material from parent to daughter cell. It is typically divided into four phases beginning with the first gap phase (G₁), proceeding to a DNA replication phase (S), a second gap phase (G₂), and ultimately mitosis (M) (55). Regulation of these events is a function of Cdk/cyclin complex activity (55, 56) and much work has contributed to the elucidation of a basic sequential framework by which they subserve this role since their initial discovery over two decades ago (57) (Figure 1). In general, during different phases of the cell cycle, unique

Cdk/cyclin complexes are formed and catalyze phosphorylation of a discrete subset of substrates (58-60). The temporal control of their subsequent degradation by the ubiquitin proteasome pathway (61, 62) is necessary to proceed through to the end of mitosis (63). Extrinsic mitogenic factors activate signaling cascades, inducing D-type cyclin expression (64-68). Transition from quiescence (G_0) into early G_1 is marked by the formation of complexes between these D-type cyclins and Cdks 4 or 6 (69). Their major function is to inactivate the retinoblastoma protein (pRb) by altering its phosphorylation state. In general, pRb can exist in two states: hypophosphorylated or hyperphosphorylated. When hypophosphorylated, pRb physically interacts with members of the E2F family of transcription factors and suppresses their transcriptional activity (70). Upon phosphorylation by activated Cdks 4 and 6, the affinity of pRb for E2F is reduced, E2F is released, and E2F-mediated gene expression required for advancement through later cell cycle phases occurs (71). This critical regulatory function of Cdk4-6/cyclin D complexes is confirmed by the observation that, in cells lacking pRb, this early G_1 phase Cdk activity is not required for cell cycle progression (72). These early pRb-related events constitute the 'restriction point', the point after which mitogenic stimuli are no longer required for completion of cell cycle events (73).

Later in G_1 , a second wave of Cdk activity ensues as cyclin E/Cdk2 complexes form (74, 75). By further phosphorylating pRb (76) and regulating centrosome duplication (77), these complexes contribute to the G_1/S transition. Near the beginning of S phase, cyclin E is degraded (78) and cyclin A replaces it in a complex with Cdk2. Cdk2/cyclin A phosphorylates a number of substrates that are critical for the regulation of DNA replication. For instance, Cdc6, a regulator of initiation of DNA replication, is phosphorylated by Cdk2/cyclin A (79). The result is that Cdc6 translocates from the nucleus to the cytoplasm, constraining DNA replication to one round. Near the end of G_2 , cyclin A is degraded and Cdk1/cyclin B complexes are generated to regulate the transition into M phase. Cyclin B must eventually be degraded to complete mitosis and enter into another round of replication or proceed to quiescence (G_0). Ultimately, successful production of daughter cells requires that the sequential activity of Cdk/cyclin complexes be tightly coordinated by checkpoints, such that initiation of subsequent steps requires completion of the previous.

3.3. Neuronal development and cyclin-dependent kinase 5

Based on its distinct binding partners, substrate specificity profile, and mode of regulation, it is clear that Cdk5 is a unique member of the Cdk family. Cdk5 was first identified from bovine brain (80) and by virtue of its ~60% homology to Cdk 1 (81). While several Cdks have prominent roles in cell division, Cdk5 is highly expressed and active in post-mitotic neurons (82-84). A crucial role for Cdk5 in the developing mammalian nervous system is evidenced by the disturbances that result in its absence. Cdk5 knockout mice demonstrate perinatal mortality associated with perturbed cortical laminar organization and cerebellar foliation, as well as abnormal neuronal migration

and neuronal pathology (85). p35 knockouts display a similar, albeit less severe phenotype (86), consistent with the functional redundancy of Cdk5 activation provided by p39. These observations indicate that neurons are dependent upon Cdk5 activity to establish normal nervous system patterning. However, much subsequent work demonstrates the multifunctional nature of this kinase, as it phosphorylates substrates that are critical for processes as diverse as synaptic neurotransmission and the maintenance of cellular architecture (Table 1). In light of this ever increasing list of potential substrates, it becomes important to consider the temporal and spatial patterns of Cdk5 activity within a cell. In other words, Cdk5 can be expected to regulate distinct processes at distinct times during neuronal maturation, and a unique subset of substrates will be targeted at particular developmental phases.

3.4. Cell cycle reactivation in Alzheimer's disease

It is widely accepted that the symptomatology of AD is the result of the extensive neuronal loss occurring in discrete brain regions over the course of many years, not merely the presence of senile plaques or NFTs. Elucidating the root causes of the death process inspires the efforts of many scientists. Neurons differ from many other cell types because their highly specialized functions require that their population be long-lived and relatively static. This enables them to subserve the complex task of controlling an organism's response to its environment. Not surprisingly then, one of the longest enduring dogmas of neuroscience is that neurons, once born, lose their proliferative capacity as they acquire highly specialized functions. Indeed, cancers of the CNS of neuronal origin are virtually unknown. Moreover, experimental work in which neuronal cell division was artificially induced by the addition of an oncogene (SV40 Large T antigen) or a mutant tumor suppressor gene (pRb) to transgenic mice shows that, unlike other tissues in which these genetic alterations would be expected to produce uncontrolled cellular proliferation, developing neuronal populations undergo substantial cell death (87-92). These observations led to the theory that neurons, once postmitotic, are incapable of sustaining cell cycle activity, and instead enter a default death pathway. On this basis, a little more than a decade ago, it was hypothesized that aberrant reactivation of the cell cycle is part of a death effector pathway in irreversibly postmitotic neurons (93). An abundance of experimental evidence, both *in vitro* and *in vivo*, supports this hypothesis.

Following NGF withdrawal in neuronally differentiated PC12 cells, an apoptotic stimulus, cyclin D1 expression is induced early in the process of cell death (94). The aberrant expression of cyclin D1 suggested that G_1 phase cell cycle events (i.e. phosphorylation of pRb by Cdks 4 and 6) could be occurring. Consistent with this, overexpression of the endogenous Cdk inhibitor p16^{INK4a} or dominant negative mutants of Cdks 4 and 6 are neuroprotective (95, 96), indicating that Cdk activity is critical for death. Likewise, KCl withdrawal in cerebellar granule neurons, another apoptotic stimulus, generates increases in the amounts of cyclins D and E, as well as their associated Cdk activities, prior to evidence of cell death (97). Moreover, this aberrant cell cycle activity corresponds

Table 1. Reported Cdk5 Substrates

Cdk5 Substrate	Putative Function
Tau	Regulation of microtubule stability (170, 171)
Microtubule associated protein 2	Regulation of microtubule stability (129)
Synapsin 1	Regulation of synaptic transmission (172)
Neurofilament	Maintenance of cytoskeletal structure (173, 174)
Microtubule associated protein 1b	Regulation of microtubule stability (39, 41)
Retinoblastoma protein	Neuronal differentiation and apoptosis (175)
PAK1	Regulation of PAK1 activity, actin dynamics (176, 177)
Munc18	Disrupts Munc18/syntaxin 1A interaction (178, 179)
p35/p39	Promotes ubiquitin-mediated degradation (40, 52)
c-Src	Regulation of cell adhesion, actin dynamics, integrin signaling (180)
DARPP-32	Regulation of dopaminergic-mediated PKA signaling (181)
NUDEL	Regulates dynein-mediated axonal transport (53, 182)
beta-amyloid precursor protein	Regulation of APP localization (145)
Pgamma	Regulation of retinal phosphodiesterase activity (183, 184)
Cables	Regulation of interaction with c-Abl (50)
beta-catenin	Regulation of Presenilin 1 binding (147)
ErbB	Regulation of signaling at the neuromuscular junction (54)
NMDA receptor subunit 2a	Up-regulates NMDA receptor activity (185, 186)
Protein phosphatase 1 inhibitor-1	Modulation of cAMP-mediated signaling (187)
Amphyphysin 1	Regulation of synaptic vesicle endocytosis (188)
Protein phosphatase 1 inhibitor-2	Activation of inhibitor-2 (189)
Disabled 1	Regulation of cell positioning (190)
Presenilin 1	Regulation of Presenilin 1 metabolism (146)
P/Q-type Ca ²⁺ channel	Downregulates channel activity (191)
c-Jun N-terminal kinase 3	Inhibition of JNK/c-Jun signaling (192)
Pctaire1	Enhances kinase activity ³⁵ (193)
MAP kinase kinase-1	Inhibition of kinase activity (194)
p53	Regulates stability and transcriptional activity (195)
Canoe	Regulation of Canoe function (196)
Myocyte enhancing factor 2	Inhibits prosurvival transcriptional activity (197)
Nestin	Modulation of nestin dynamics (198)
Cdk5/p35 regulated kinase	Inhibition of kinase activity (199)
Ezrin	Regulation of membrane-cytoskeletal signaling (200)
Focal adhesion kinase	Regulation of neuronal migration (201)
Dynamin I	Regulation of synaptic vesicle endocytosis (202)
Synaptojanin	Regulation of synaptic vesicle endocytosis (202)
Outer dense fibers	Regulation of sperm tail development (203)
Postsynaptic density 95	Regulation of PSD-95 clustering (204)

with increases in pRb phosphorylation (97), an event consistent with the transition from G₀ to G₁. A trophic withdrawal paradigm has also been modeled *in vivo*. In two mouse neurological mutants, *staggerer* and *lurcher*, the absence of Purkinje neurons in the cerebellar cortex results in massive granule neuron death (98-100). Further, the resulting target deprived granule neuron death is accompanied by cyclin D expression and evidence of cell cycle activity (101). These are but a few examples of the myriad death stimuli that yield evidence of neuronal cell cycle reactivation, including: DNA damage (102, 103), cerebral ischemia and excitotoxicity (104-107), and oxidative stress (108). For these examples to be relevant to the pathogenesis of AD, evidence of cell cycle reactivation must be detectable in AD tissue. Numerous investigators have established the presence of cell cycle markers in AD brains (Table 2). Indeed, several cyclins (109-111), Cdks (110-112), and endogenous Cdk inhibitors (112-114), as well as other markers of the cell cycle (111, 115, 116), are

significantly increased AD versus controls. These differences occur in brain regions known to be affected by AD, are predictive of neuropathology, and correspond to the presence NFTs. Moreover, in individuals with mild cognitive impairment, a putative prodromal stage of AD, evidence of lethal cell cycle changes is also evident (117). These cell cycle changes are not simply unrelated artifactual events, as they are sufficiently coordinated to allow for DNA replication (118). Thus, the preponderance of evidence supports a role for cell cycle dysregulation as a marker of AD, though causation has not been shown. For example, the events leading to abnormal cell cycle reactivation remain to be determined. Also, the data do not address how reactivation of cell cycle-elements mediates neuronal pathology. Several theories regarding these points are noteworthy.

Heintz proposed that abortive cell cycle reactivation in neurons is triggered in response to tumorigenic

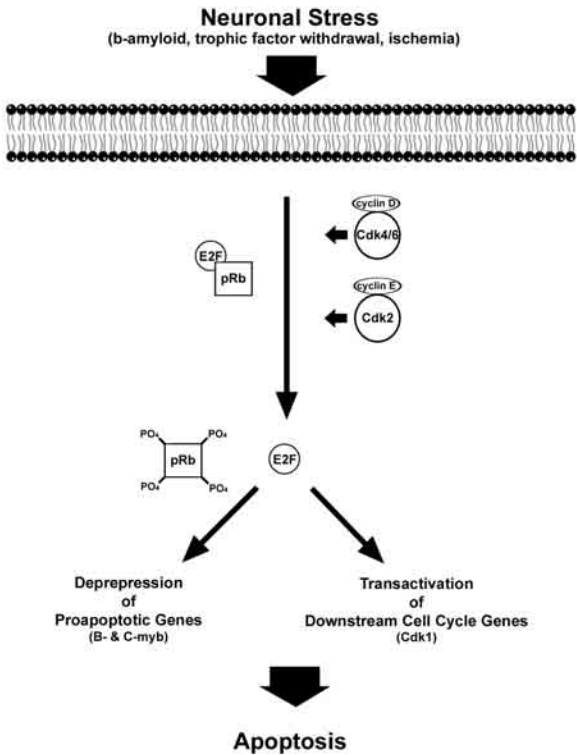


Figure 2. A simplified model depicting dysregulated cell cycle Cdk activity. Neuronal stress, evoked by various stimuli, triggers aberrant G₁ phase Cdk activity. pRb becomes phosphorylated, releasing E2F. E2F-regulated genes are both derepressed (i.e. B- and C-myb) and transactivated (i.e. Cdk1), resulting in further lethal cell cycle progression and the post-translational modification of pro-apoptotic proteins.

Table 2. Select Reports of Cell Cycle Dysregulation in AD

Re-expression of Cdks in AD (110-112, 205)	
	Cdk1
	Cdk4
	Cdk7
Re-expression of Cyclins in AD (109-111)	
	Cyclin B
	Cyclin D
	Cyclin E
Re-expression of Endogenous Cdk Inhibitors in AD (113, 114)	
	p15 ^{INK4b}
	p16 ^{INK4a}
	p18 ^{INK4c}
	p19 ^{INK4d}

stimuli that would cause proliferation in other tissues (93). In this context, he compared cancer and neurodegeneration. Both demonstrate remarkable cellular specificity, and just as distinct etiologic agents produce unique molecular lesions to yield cell-type specific transformation, similar insults could trigger abortive cell cycle in particularly vulnerable neuronal populations. Both cancer and neurodegeneration exhibit differential latencies, indicating that multiple insults may have to accumulate before disease

is demonstrable. Finally, both cancer and neurodegenerative diseases can occur in either a familial or spontaneous manner. Like cancer, spontaneous neurodegeneration may be derived from numerous somatic genetic lesions, while familial disease could result from the combination of a pre-existing germ-line mutation coupled with a second lesion. An alternative, proposed by Arendt and Herrup, posits that neurons in AD affected regions persist in the tenuous position between differentiation and plasticity (8, 119). Both states are necessary, not mutually exclusive, and likely result in a condition of incomplete differentiation. Differentiation allows for specialization of function and durability of critical synaptic connections. In contrast, plasticity allows for adaptation to successfully conduct the complex tasks of learning and forming new memories. To remain plastic, neurons must successfully integrate complex extrinsic signals from the environment. In other tissues, these signals would modulate positional and proliferative processes, but in neurons they regulate plasticity. Disturbing the delicate balance between these two states by any number of ill-defined insults could cause neurons to revert to an ineffective program of de-differentiation, and ultimately die. This notion is consistent with similarities in the factors and signaling systems governing both proliferation and plasticity in neuronal systems (8, 119). A particularly appealing feature of this model is the extended latency of the death process, which has not been successfully modeled in cell culture or animal models.

With regard to the final executioners of the death process, as indicated, pRb serves as the guardian of the restriction point by sequestering and suppressing the activity of E2F family transcription factors. Consistent with this, adenovirus mediated overexpression of E2F1 in cerebellar granule neurons is sufficient to trigger apoptotic death (120), whereas dominant negative interference of E2F activity attenuates cortical and sympathetic neuron death in response to DNA damaging stimuli. E2F can function both as a transcriptional activator and repressor. Evidence from experiments in which a mutant E2F1 construct, only possessing repressor functions, was tested in several cell culture paradigms indicates that it can promote death by derepressing the expression of pro-apoptotic genes (121, 122). Two genes in particular, which encode the transcription factors B- and C-myb, may be involved, as their overexpression is sufficient to induce neuronal apoptosis (121). On the other hand, release of E2F may regulate the death pathway via transactivation of additional cell cycle genes. For instance, KCl withdrawal in cerebellar granule neurons results in E2F1-mediated expression of Cdk1 (123). Using this same paradigm, it has been shown that Cdk1 phosphorylates BAD, a pro-apoptotic member of the Bcl-2 family of proteins, rendering it resistant to sequestration and degradation (124). Collectively, these observations suggest that aberrant cell cycle activity in neurons results in both altered patterns of gene expression and the post-translation modification of signaling molecules that are responsible for neuronal death (Figure 2).

3.5. Cyclin-dependent kinase 5 dysregulation in Alzheimer’s disease

One of the first indications that Cdk5 may have a

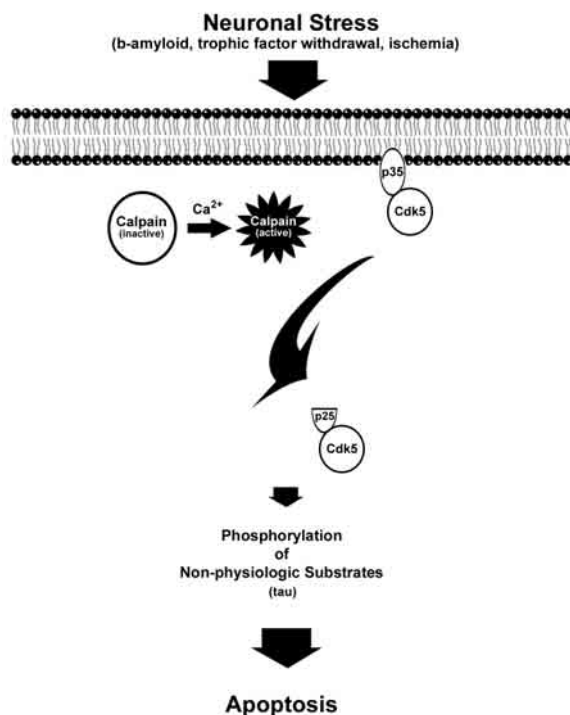


Figure 3. A simplified model depicting dysregulated Cdk5 activity. Following neuronal stress, calcium homeostasis is disturbed, resulting in activation of calpain, and calpain-mediated proteolysis of p35 to p25. This triggers increased and sustained Cdk5 activity that can access a wider array of previously unavailable substrates (i.e. tau). Altered regulation of these substrates results in apoptosis.

role in AD neuropathology was the identification of the microtubule associated protein tau as a substrate (125). *In vitro* analyses have extended these initial findings and identified several proline-directed Ser/Thr sites on tau that are targets of Cdk5 phosphorylation: Ser²⁰², Thr²⁰⁵, Thr²¹², Ser²³⁵, Ser³⁹⁶, and Ser⁴⁰⁴ (126-128). Moreover, many of the tau sites phosphorylated by Cdk5 *in vitro* are the same sites known to be phosphorylated in tau from paired helical filaments (PHFs) (129). Further support is provided by data indicating that increased Cdk5 and Cdk5-mediated phospho-tau immunoreactivity are found in neurons in the early stages of neurofibrillary tangle (NFT) formation (130, 131). These findings generated considerable enthusiasm for the theory that Cdk5 contributes to the early derangement of neurons in AD.

Interestingly, physiological Cdk5p/35 activity is unlikely to be substantially directed against tau. For example, COS-7 cells co-transfected with a human tau construct and Cdk5/p35, display little detectable AT8 (Ser²⁰², Ser²⁰⁵) or PHF-1 (Ser³⁹⁶, Ser⁴⁰⁴) phospho-tau immunoreactivity (37). Also, triple transgenic mice overexpressing p35, Cdk5, and human tau do not demonstrate increased tau phosphorylation (132). In marked contrast, Cdk5 efficiently phosphorylates these tau epitopes when complexed with p25 (37), indicating that p25 alters the regulation of Cdk5 activity. p25 is a 208

amino acid carboxy-terminal fragment of p35 derived from proteolysis by the calcium-dependent cysteine protease calpain (133-135). While p25 is sufficient to activate Cdk5, it lacks the N-terminal myristoylation that localizes Cdk5 to cellular membranes and likely restricts its potential substrate profile to those located in this subcellular region (37). A similar process can occur with p39, producing the truncated p29 (38). When complexed with p25, Cdk5 demonstrates a profoundly different cellular distribution. Instead of being located near the cellular periphery, Cdk5/p25 is diffusely distributed in the cytoplasm (37), thus allowing access to a wider array of potential substrates. The kinetics of Cdk5 catalysis may also be enhanced by association with p25 (127). *In vitro*, phosphorylation of human tau by Cdk5/p25 occurs at a faster rate when compared to Cdk5/p35 ($p25_{kcat/km} > p35_{kcat/km}$). Furthermore, Cdk5/p25 phosphorylates tau with greater stoichiometry than Cdk5/p35 (3.3 vs. 2.3 moles per mol tau). Collectively, these observations indicate that dysregulation of Cdk5 by p25 is the result of enhanced and sustained kinase activity that is mislocalized and directed against substrates that are not normally targeted, ultimately bringing about neuronal death. Consistent with this, expression of Cdk5/p25 complexes in transfected cells and primary neurons results in increased tau phosphorylation on AD specific phosphoepitopes (37). Furthermore, cortical neurons expressing these complexes demonstrate morphological and cytoskeletal disruptions, ultimately leading to apoptosis (37).

But what is the trigger for conversion of p35 to p25? As calpain mediates the pathogenic cleavage of p35, it is predictable that insults triggering Ca^{2+} dyshomeostasis would be involved. Indeed, neurotoxic injury by maitotoxin, ischemia, and glutamate excitotoxicity, all known to perturb Ca^{2+} regulation, results in calpain activation and the generation of p25 (133-135). It is important to note that these insults may not always lead to tau phosphorylation. Treatment of cultured hippocampal neurons with N-methyl-D-aspartate, glutamate, or a Ca^{2+} ionophore, ionomycin increases p25 levels and Cdk5 activity, but does not change the phosphorylation state of tau (136). β -amyloid, the primary component of senile plaques (137), also appears to affect p35 regulation. Application of fibrillogenic β -amyloid peptides to neuronal cultures results in neurotoxicity accompanied by calpain activation, p25 generation, and tau hyperphosphorylation (133, 138, 139). However, neuronal pathology in the aged brain likely precedes the detectable formation of β -amyloid plaques (140-143). Data regarding soluble β -amyloid, representing a pre-deposit paradigm, indicate that it too triggers p35 cleavage, increased Cdk5 activity, and tau hyperphosphorylation (144). Consistent with the involvement of a Ca^{2+} mediated process, disturbances of Cdk5 regulation are largely attenuated by the calcium channel blocker, verapamil, and calpain inhibitor I (144). Incidentally, Cdk5 recognizes substrate proteins involved in β -amyloid processing: β -amyloid precursor protein (β APP) (145), Presenilin-1 (146), and β -catenin (147) (Table 1), but it is not known whether these interactions are involved in the genesis of senile plaques. This collection of observations is summarized by the model depicted in Figure 3, linking neuronal stress, calpain activation, and cleavage of p35 to p25.

Table 3. Select Transgenic Mouse Models of Cdk5 AD Neuropathology

Transgenic Model	AD Associated Neuropathology
Human Cdk5/p35/tau(132)	None
βAPP Mutant (Tg2576)(149)	β-amyloid plaques, ↑ tau phosphorylation, ↑ p25, astrogliosis, neuronal loss
Double βAPP Mutant (TgCRND8)(150)	No changes in Cdk5, p35, p25 immunoreactivity
Human p25(151)	↑ tau & neurofilament phosphorylation, ↑ Cdk5 activity, cytoskeletal disruptions
Human p25(152)	↑ Cdk5 activity, axonal degeneration, ↑ tau phosphorylation, focal tau accumulation
Bovine p25(153)	None
Human p25 (p35 knockout background)(155)	None
Mouse p25(154)	None
Human p25/mutant (P301L) tau(156)	↑ Cdk5 activity, ↑ tau phosphorylation, NFTs, GSK3-tau co-localization
Inducible human p25(160)	↑ Cdk5 activity, ↑ tau phosphorylation, NFTs, cortical & hippocampal neuronal loss, forebrain atrophy, astrogliosis, caspase-3 activation

AD demonstrates extended latency, with disease manifestation typically apparent during the later decades of life. As such, in any theory of AD neuropathology latency must be incorporated. As indicated, one level of Cdk5 regulation involves the availability of p35. This is, in part, the function of Cdk5 itself, in the form of a negative feedback loop in which it phosphorylates p35, thereby targeting it for degradation by the ubiquitin proteasome pathway (40). The result is a protein with a half-life of ~30 minutes and limited Cdk5 activity. In contrast, p25 possesses a half-life 5-10 times longer (37). Curiously, the regulation of p35 phosphorylation, and thus its proteolysis, appear to be developmentally regulated. For example, p35 in fetal brain tissue is phosphorylated on two distinct sites (148). One site promotes proteasomal degradation, while the other confers resistance to calpain-mediated cleavage. In adult brain, p35 is not detectably phosphorylated (148). Thus, neurons in the adult may become susceptible to dysregulation as a result of an intrinsic maturation-dependent phenomenon.

A number of transgenic mouse models have been developed in an attempt to model the contribution of Cdk5/p25 to AD-like pathology, but results have been conflicting (Table 3). As mentioned previously, overexpression of p35 in transgenic mice does not induce tau hyperphosphorylation or brain pathology, despite increasing Cdk5 activity (132). Again, this supports the notion that p25 confers upon Cdk5 unique properties, including distinct substrate availabilities. Mice possessing a mutant transgene for βAPP (Tg2576) exhibit Cdk5-dependent tau phosphorylation and p25 accumulation that accompanies neuronal loss and astrogliosis (149), consistent with the existence of pathological β-amyloid/p25 signaling. On the other hand, no changes in Cdk5 regulation are detectable in another transgenic mouse strain which expresses a double mutant form of βAPP (TgCRND8) (150). Two different p25 overexpressing transgenic mice show increases in Cdk5 activity and tau phosphorylation, as well as cytoskeletal disturbances and axonal degeneration (151, 152). However, in both of these models, there is no evidence of NFTs, PHFs, or neuronal loss, indicating that while Cdk5 dysregulation may contribute to tau hyperphosphorylation, and related changes, it is, by itself, insufficient to generate NFTs. It is noteworthy that three additional and independently generated p25 transgenic mice fail to show significant tau hyperphosphorylation or neuropathology (153-155), but in

two of these cases this could be attributed to the relatively low levels of p25 expression produced (154, 155). A double transgenic mouse overexpressing p25 and mutant (P301L) human tau exhibits similar Cdk5-mediated tau changes, but these are accompanied by the presence of silver-stained NFTs (156). Interestingly, in this transgenic background, insoluble tau was also co-localized with glycogen synthase kinase-3 (GSK3), supporting the theory that phosphorylation by a number of distinct kinases might contribute to tau derangement (157, 158). This result is also consistent with evidence supporting a role for GSK3 in tau hyperphosphorylation and AD neuropathology (i.e. ref. (159)). More recently, a transgenic animal was developed utilizing a tetracycline-controlled transactivator system to generate inducible forebrain expression of p25 during the postnatal period. In this background, a more complete recapitulation of AD neuropathology is achieved including: increases in Cdk5 activity; accumulation of hyperphosphorylated tau; NFT formation; astrogliosis; and substantial brain atrophy including neuronal degeneration of the hippocampus and cerebral cortex (160).

If a Cdk5-mediated process is responsible for neurodegeneration in AD, then the most difficult studies to reconcile are those in which postmortem human tissue has been analyzed (Table 4). The first study to examine postmortem human tissues surveyed samples from 8 patients with AD, 1 with Huntington's disease, and 4 controls (37). In this study, p25 was found to be more abundant in AD tissue, and it was present in neurons with NFTs. Also, despite equivalent levels of Cdk5 protein in AD and control brains, immunoprecipitated Cdk5 kinase activity against histone H1 was increased in AD samples. This group also performed a larger study on samples from 28 AD patients and 25 age-matched controls (161). Again, Cdk5 dysregulation correlated with AD, evidence by increased mean ratios of p25 to p35 in AD versus controls across all brain regions investigated. These results lend support to previous *in vitro* analyses and transgenic mouse models, but have proven difficult to reproduce. For example, a second group independently examined 8 AD samples and 9 age-matched controls (162), but were unable to discern any differences in the level of p25 between the two groups. Four additional studies also reported no significant increases in p25 in AD (150, 153, 163, 164). One of these reports (150) used the same protocol, antibodies, and antisera as the original study (37). Thus, we are left to ponder the source of these conflicting data.

Table 4. Human Postmortem Studies of Cdk5 Dysregulation

#s AD vs. Ctrl	Mean PMI AD vs. Ctrl (hrs)	AD Mean Age (yrs)	Significant Cdk5 Dysregulation (+/-)
8:4(37)	~14 : ~21 ¹	71.5	+
8:9(162)	~3.25 : ~2.35 ¹	82.3	-
6:8(163)	26.83±22.32 : 37.38±20.51	61.33±9.42	-
5:5(153)	NR	78.8	-
10:10(164)	14.15±3.74 : 16.10±2.89	77.4±2.1	-
28:25(161)	2.66±1.18 : 2.83±0.92	83.8	+
22:11(150)	~11 : ~6.4 ¹	68.8±3.8	-

¹ Approximations calculated from available published data, NR-not reported

The time following death after which tissue is obtained is known as the postmortem interval (PMI). During this time, depending on its length, a number of changes occur within tissue that could confound analyses (i.e. proteolysis, pH alterations). Thus, it is likely that PMIs could affect evidence of Cdk5 dysregulation as determined by the abundance of p25 or p25/p35 ratios. Patrick and colleagues (37) suggest that after long PMIs (i.e. 24-hrs), once detectable alterations in the p25/p35 ratio between AD samples and controls can diminish, or disappear altogether. Indeed, after as little as one hour post mortem, and peaking around 14 hours, p35 is degraded to p25 by artifactual increases in calpain activity in postmortem tissue (162). However, in three of the studies that failed to detect changes in p25 abundance, PMIs were \leq ~14 hours (150, 162, 164). Another potential confound is the extent of AD progression. The bulk of the data suggest that the biochemical events responsible for neuronal degeneration in AD are likely to occur long before the disease is diagnosed. This is consistent with one finding in which tissue from a patient with late-stage AD (i.e. already extensive neuronal loss) was analyzed and found not to exhibit increases in p25 similar to other diseased samples (37). It is interesting to note, however, that tissue from individuals of diverse ages was utilized in all of these studies, averages ranging from 61.33 to 83.8. As the prevalence of AD increases with age from ~1% at age 60, to nearly 50% by age 80 (165), and the average survival from disease onset is ~8 years (166), it is unlikely that the majority of patients from these studies demonstrated late-stage disease.

4. CONCLUSIONS AND PROSPECTS

Cdks subserve numerous and diverse cellular processes. As we have discussed, mounting evidence indicates that when dysregulation of cell cycle Cdks occurs, neuronal survival is compromised. However, it still remains to be determined if investigators' attempts to recapitulate this process are adequately predictive of human neuropathology. Specifically, it is important to recognize that much of the experimental evidence of cell cycle dysregulation has been limited to the analysis of young, developing neurons both *in vivo* and *in vitro*. In these paradigms, cell cycle dysregulation results in rapid cell death, within hours (i.e. (94, 124)). In contrast, aberrant cell cycle expression and ensuing neuronal death in AD brain appears to occur over an extended duration, likely days to months to years, as evidenced by the relatively high percentages of neurons (4-9%) with detectable cell cycle changes (111, 118). The same concern can be expressed

with regard to the observations pertaining to Cdk5. Moreover, it is rather curious that unlike aberrant cell cycle reactivation, Cdk5 dysregulation has not been reproducibly detected in AD brain. The reasons for this have yet to be convincingly explained. What is clear, though, is that the extended latency of AD persists as a major experimental obstacle that must be overcome if we are to authentically model the disease and develop rational therapeutics for AD sufferers. Despite these difficulties, Cdks remain an attractive therapeutic target and a number of potent pharmacologic agents and molecular approaches have already been identified to inhibit their activity (167-169). For Cdk5 dysregulation in particular, it is unclear whether such approaches would be efficacious considering its multifunctionality and broad substrate specificity. Targeted disruption of Cdk5 would likely affect both normal and pathologic substrates and could yield undesirable consequences. Thus, we must acknowledge that additional study is necessary to develop a complete picture of how Cdks are involved in this devastating neurodegenerative illness.

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Send correspondence to: Mary Lou Vallano, Ph.D., Department of Neuroscience and Physiology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, New York 13210, USA, Tel: 315-464-7969, Fax: 315-464-7712, E-mail: vallanom@upstate.edu

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