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Neuronal Cell Cycle Regulation of Cdk5 in Alzheimer's Disease

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Abstract

Neuronal cell cycle dysregulation is closely related with the neuronal death in Alzheimer's disease (AD), but the detailed mechanism linking the two processes is unclear. Cyclin-dependent kinase 5 (Cdk5) is described as an atypical Cdk, which has been shown to have no cell cycle promoting activity. Yet while Cdk5 may not promote the cycle, we have found that Cdk5 may play a role in maintaining the quiescent stage of post-mitotic neuron. In this chapter, we review recent findings concerning the cell cycle suppression activity of Cdk5, and relate this function to the initiation and progression of neurodegenerative diseases, in particular AD. Our data suggest that nuclear Cdk5 can block the cell cycle. When the post-mitotic neuron is subjected to β-amyloid stress, Cdk5 is translocated from nucleus to cytoplasm. Deprived of its nuclear Cdk5, the post-mitotic neuron will re-enter into cell cycle, ultimately leading the cycling neuron to die rather than divide. Our work has identified the molecular basis of the cell cycle suppression effect of Cdk5. Taken together, our data reveal that Cdk5 does indeed regulate cell cycle activity. These finding may provide new pharmaco-therapeutic approach to treating brain disorders such as AD.

Keywords: (Cyclin-dependent kinase 5) Cdk5, Cell Cycle; Neuronal Death; Alzheimer's disease

Cell Cycle Regulation

The cell cycle is a highly conserved mechanism that controls the cells decision to proliferate and regulate the process once it starts. Typically, the cell cycle is divided into four phases, namely G1 (first gap), S (DNA synthesis), G2 (second gap), and M (mitosis). The cell cycle process is regulated by the sequential expression, activation, and inhibition of Cyclin-dependent kinases (CDKs) associated with activating subunits, the cyclins, as well as two families of Cyclin-dependent kinase inhibitors (CKIs)–the Kip/Cip family of proteins (p21, p27 and p57) and the INK4 family (p16, p15, p19 and p18) [1-3]. There are ten Cdks and nine cyclins in mammal tissues that have been described to date. After mitogenic initiation, synthesized D-type cyclins bind to and activate Cdk4 and Cdk6. These Cdks target several proteins, chief among them the tumor suppressor protein, retinoblastoma (RB). Phosphorylated RB releases the E2F1 transcription factor which binds to DNA and allows cells to enter G1. The cyclin E/Cdk2 complex is required for transition from G1 to S phase. Later, in M phase, Cyclin B/Cdk1 activation is triggered allowing the cell to proceed through cytokinesis. During all four stages of the cell cycle, the activity of both Cdks and CKIs are tightly controlled by transcription, translation, and ubiquitin-mediated proteolysis [4-7].

Cdk5: A typical Cyclin Dependent Kinase

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the Cdk family, despite the fact that its cloning was based on its sequence homology to other Cdks [8]. Cdk5 expression can't drive the cell cycle forward, nor is it necessary for normal cell cycle progression. Yet, as described in more detail below, Cdk5 has a potent cell cycle activity that is inhibitory in nature. It is a strong force in mature cells to hold the cell cycle in check. As a proline directed serine/threonine kinase, Cdk5 is structurally most similar to CDC2 (Cdk1), but it functionally differs from traditional Cdks [8]. Cdk5 is abundant in nerve cells, but evidence for its existence in other cells is well established [9,10]. Several of the traditional cyclins can bind with Cdk5, but none can activate it. Instead, Cdk5 is primarily activated by p35 and p39, which are highly expressed in the nervous system [11-14]. The p35 and p39 share no homology to cyclins at the amino acid level but have remarkable structural similarity all the same [8]. The association of Cdk5 with p35 or p39 is required in processes such as neurite outgrowth, axonal migration, and cortical lamination, control of cell adhesion, axonal transport and synaptic activity [8]. Consequently, the loss of Cdk5 give rise to a failure of cell cycle suppression and neuronal cell death, both *in vivo* and *in vitro* [15,16].

Neuronal Cell Cycle Dysregulation in Alzheimer's Disease

In the adult mature brain, once the neurons of the central nervous system leave the ventricular, they will be permanently post mitotic, and never complete a full cell cycle again. But despite this non-mitotic state, a neuron is still capable of initiating a cell cycle. This leads to the provocative question of what would happen if a neuron loses its control of the cell cycle and attempted to divide? It has been shown in numerous studies that neurons that re-enter the cell cycle are fated to die rather than divide. For example, in areas where neurons are likely to die in neurodegenerative diseases such as Parkinson's or Alzheimer's disease (AD) there is substantial evidence from many labs that nerve cells are expressing cell cycle proteins and have completed at least one round of DNA replication. The induction and activation of CDC2 is found in degeneration neuron in AD brain, as is Cyclin B [17-19]. Other cell cycle proteins such as Cyclin D, Cdk4, Cdk6 and Ki-67 (a DNA binding protein that is found only in dividing cells) are also found in the neurons of the AD brain [20-22]. Prior to their death,

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genomic DNA replication has been demonstrated by fluorescent in situ hybridization (FISH) [23]. These experiments offer direct evidence that AD neurons probably complete enough of the cell cycle to enter G2 phase after completing G1 and S phase. The assignment of specific cell cycle phases to these neurons is probably not completely correct. For example, several cell cycle proteins are found in unnatural locations in the 'cycling' neurons. PCNA and Cyclin B, both normally nuclear proteins during the cell cycle are found in aberrant cytoplasmic locations. Yet despite these anomalies, numerous laboratories studying this abortive neuronal cell cycle re-entry have found a tight connection between evidence of cell cycle events in neurons and sites of apoptosis in AD and other neurodegenerative diseases.

Does Cdk5 Play a Role in Cell Cycle Regulation?

Data from our lab supports the suggestion that Cdk5 functions actively in cell cycle regulation. Unlike other Cdks, however, its role appears to be to suppress the cell cycle, both in post-mitotic neurons and neuronal cell lines. We have proposed that to perform this cell cycle suppression function Cdk5 must be located in the nucleus. Here it plays an active role in allowing neurons to remain post mitotic as they mature. Consistent with this hypothesis, we have found that loss of nuclear Cdk5 leads to cell cycle re-entry, even if the levels of cytoplasmic Cdk5 remain significant. The shift in sub-cellular location and cell cycle re-entry is accompanied by neuronal death. Significantly, we have found evidence for this phenomenon in non-neuronal cell as well. By synchronizing NIH 3T3 cells and neuronal N2a cells, we find low levels of nuclear Cdk5 in proliferating fibroblasts, as well as neuroblastoma cells. Here again, in cells that are actively proliferating, the nuclear/cytoplasmic ratio is low during S-phase of the cell cycle [16].

Historically, the first evidence to hint at the cell cycle regulatory effect of Cdk5 was the discovery that neurons in the *Cdk5*$^{-/-}$ mouse brain cortex re-express cell cycle proteins, such as cyclin D and incorporate BrdU before apoptotic death [15]. Experiments *in vitro* showed that the nuclear location of Cdk5 is the key to cell cycle arrest rather than its total amount [16]. Even in the AD brain, neurons that express cell cycle proteins (in the regions where neuronal death is prevalent) also lose their nuclear fraction of Cdk5, attesting to the generalizability of this phenomenon. Indeed, results in both human AD and its mouse models further prove that Cdk5 localization and cell cycle re-entry are intimately linked. In both the R1.40 AD mouse and human AD brain, neurons in vulnerable regions re-enter a cell cycle and lose their nuclear Cdk5. We suggest that without nuclear Cdk5, the neuronal cell cycle is released followed by an ultimately lethal series of subsequent events.

The consistency of the cell cycle suppression role of Cdk5 in a variety of cell types, in stressful situations both *in vivo* and *in vitro*, proves that the nuclear localization of Cdk5 that plays an unexpected but widespread role in neuronal cell cycle suppression. The types of stress that can induce a cell cycle related neuronal death are wide ranging and include oxidation (H_2O_2), DNA damage (camptothecin, etoposide), as well as the Alzheimer's peptide (Aβ).

How does Cdk5 Suppress Neuronal Cell Cycle?

In exploring the mechanism by which Cdk5 suppress neuronal cell cycle, our attention was drawn to the transcriptional factor E2F1. E2F1 is a well-known transcription factor that is involved in the regulation of cell proliferation, differentiation, and apoptosis through transcriptional regulation [24-26]. Just as Cdk5 needs its cyclin-like p35 protein to be fully active, E2F1 requires a co-factor, DP1, to bind DNA appropriately and drive cell cycle protein expression. Without DP1, E2F1-dependent transcriptional activation reduces to a large

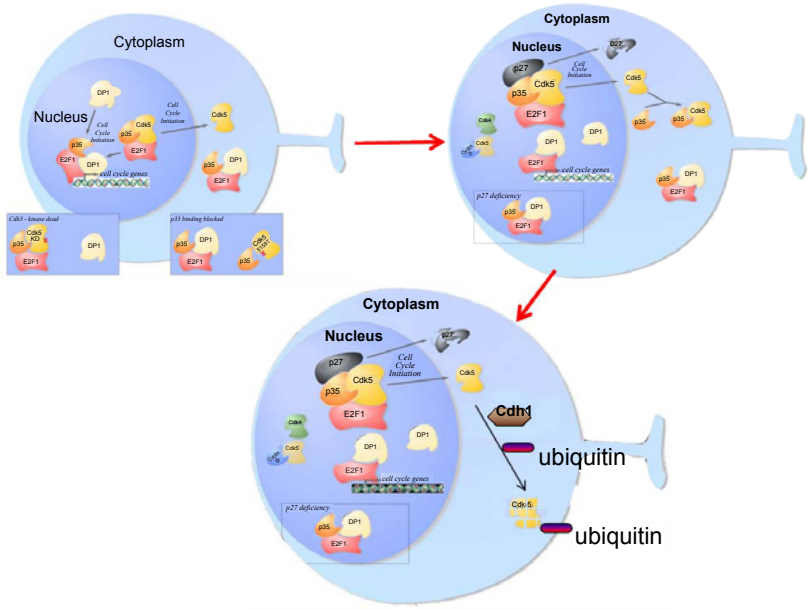

Figure 1: Neuronal cell cycle regulation of Cdk5.
Panel A: In post-mitotic neurons, Cdk5 was located both in nuclear and cytoplasm. Nuclear Cdk5 will disrupt the interaction between E2F1 with DP1 in the present of p35. Without DP1 binding, the cell cycle driving capacity of E2F1 was limited and the neurons were kept in post-mitotic stage.
Panel B: Nuclear Cdk5 localization is dependent on its binding with p27. Mitotic signals appear to dissociate the interaction between Cdk5 with p27 and trigger Cdk5 to shuttle from nucleus to cytoplasm by NES-CRM1 pathway.
Panel C: As soon as nuclear Cdk5 was transported into cytoplasm in S phase, the cytoplasmic Cdk5 will be ubiquitinated by the E3 ligase APC-CDH1. Ubiquitinated Cdk5 is degraded then by the proteasome.

extent [27]. We first explored the interactions among Cdk5, E2F1, DP1, and p35 at a physical level. We found that Cdk5 performs as a cell cycle suppressor by way of its participation in a multi-protein complex that contains E2F1. Electromobility shift assays proved that the DNA binding ability of E2F1 was significantly attenuated by overexpression nuclear Cdk5. The association of Cdk5 with E2F1 and its cofactor, DP1, provides evidence for this inhibition [28,29]. After binding with E2F1 and preventing the interaction of DP1 with E2F1, Cdk5 suppress cell cycle by decreasing the occupancy of the E2F1 promoter element. Unexpectedly, a kinase-dead Cdk5 produced the same results. This suggested that Cdk5 does not need its normal kinase activity to suppress the neuronal cell cycle [28,29]. But Cdk5 does need the binding of its activator p35 to inhibit the cell cycle. This p35-dependency is supported by lack of effect on E2F1 promoter occupancy in the Cdk5 (S159T) mutant that lacks the ability to bind p35, and by the observation that this mutant cannot function as a cell cycle suppressor. The p35 protein contains a myristoylation signal motif, which has been speculated to give it the ability to be anchored to the plasma membrane. Yet, cell fractionation and immunocytochemistry reveal that in addition to the membrane-bound form of p35, substantial amounts of the protein also exist in the nucleus of cultured neurons and other cells [30,31]. Here it is well positioned to participate in the E2F1/Cdk5/p35 complex. We also directly visualized the interaction between Cdk5 and p35 in the nucleus using BiFC (bimolecular fluorescence complementation).

It is the location in the nucleus or the cytoplasm that is the critical determinant of the function of the Cdk5-E2F1complexes. Our data suggested that each protein of complex has a potential binding site for each of the other three proteins. They can bind each other as heterodimers in all combinations. If all of them gather at once, Cdk5 and DP1 apparently compete to bind a p35–E2F1 complex. However, the DP1–p35–E2F1 trimer is most often observed in the cytoplasm while the Cdk5–p35–E2F1 trimer is more often seen in the nucleus. The nuclear Cdk5–p35–E2F1 complex keeps DP1 from binding with E2F1, which will dramatically decrease the cell cycle driving ability of E2F1. The direct consequence will be inhibiting the cell cycle (Figure 1).

The Nucleocytoplasmic Trafficking of Cdk5

As mentioned above, Cdk5 is normally located in both nucleus and cytoplasm, yet it is only nuclear Cdk5 that is effective in serving as a cell cycle suppressor. It is not surprising, therefore that in cultured post-mitotic neurons, the export of Cdk5 from the nucleus to the cytoplasm is pivotal for cell cycle re-entry, a first step on a pathway that leads to neuronal cell death. This places a premium on understanding the forces that regulate its location and in particular its nuclear retention. In pursuing this question, we have determined that the nuclear localization Cdk5 is dependent on its binding to the Cdk inhibitor, p27. When this binding is lost, Cdk5 adopts a more cytoplasmic localization that is mediated by NES-CRM-1 pathway [32,33].

The levels of Cdk5 in nucleus and cytoplasm are changed by cell cycle or cell death stimulation. Nuclear export serves as both a positive regulator of the cell cycle and an initiator of the cell death process. Regulation of Cdk5 transport is therefore critical for normal neuronal homeostasis. For most nucleocytoplasmic proteins contain both NLS (nuclear localization signal) and NES (nuclear export signal) sequences are found that serve to regulate the shuttling process. In a typical nuclear import process, the cargo containing the NLS is bound by an importin family member and then shuttled to the nucleus [34]. Despite extensive searching of the Cdk5 protein sequence and testing in functional assay, however, no functional NLS signal could be found. Instead, we found that the nuclear import of Cdk5 depends on its physical interaction with p27. The 17 amino acids of the Cdk5 N-terminus are vital for this interaction. Further, as predicted by our earlier studies, p27 is not only important for nuclear localization of Cdk5, but is also required for the cell cycle blocking activity of Cdk5. When we drove Cdk5 expression in p27 deficient NIH 3T3 P27 (D51) cell, Cdk5 retained none of its cell cycle blocking capacity, even when an exogenous NLS was added to the protein sequence that forced the protein to the nuclear compartment. This demonstrates a dual function of p27 in the regulation of Cdk5. It is required both for its nuclear localization and for its ability to from the E2F1/Cdk5/p35 complex that regulates the cell cycle.

The control of Cdk5 localization also depends on nuclear export processes. Unlike the lack of NLS sequences in Cdk5 itself, we have been able to show that endogenous NES signals direct the export of Cdk5 [32,33]. Similar to other nucleocytoplasmic proteins, nuclear export of Cdk5 is dependent on its ability to bind to CRM1 [35-37]. Truncation mutation studies allowed us to show that the export of Cdk5 relies on two atypical NES motifs; one is located between amino acids 64 and 83 and the other is between amino acids 128 and 147. We have just begun to explore this export process, yet already we have discovered several intriguing results. The most unexpected of these is that when Cdk5 is exported to the cytoplasm in post-mitotic neuron under stress, we find that the extra cytoplasmic Cdk5 shows every sign of being neuroprotective. The extra cytoplasmic Cdk5 attenuates the caspase-3 cleavage and degradation of BCL-2. Even though the mechanism behind this neuroprotection is unclear, the distinct nuclear and cytoplasmic function of Cdk5 points to the critical importance of the factors that regulate Cdk5 location. In this complex picture, we see several opportunities to develop pharmaceutical targets that would allow drugs to enhance the normal capacity of nuclear Cdk5 to block the cell cycle and thus block the first step to neuronal death in neurodegenerative diseases such as Alzheimer's disease.

Cdk5 is Degraded in Cycling Cells

The timely degradation of cyclin proteins is important for the progression of the normal cell cycle process. Cyclin degradation is usually mediated by a highly specific ubiquitin-dependent proteolysis [38]. In contrast to the cyclins, the levels of the Cdk proteins themselves are normally maintained unchanged during the cell cycle. Our recent data show that Cdk5 is a relatively an unstable protein, in neuronal cell lines and, surprisingly, its levels oscillate during the neuronal cell cycle [39]. This oscillation is not due to transcriptional regulation as mRNA levels remain relatively level. Rather, we have shown that Cdk5 is significantly degraded when cells enter S phase.

The mechanism behind this cells cycle stage-specific degradation is the UPS (ubiquitin-proteosome system). Cdk5 is ubiquitinated by the E3 ligase APC-Cdh1 after it is transported to the cytoplasm. The ubiquitin tag targets the protein for proteasomal degradation in a relatively short time. We used truncation mutations to identify the region containing the ubiquitination site on Cdk5, and found it to be in close proximity to the protein pocket that forms the binding site for p35. We validated the importance of this location by showing that the ubiquitination of Cdk5 was blocked when high levels of p35 were present. And when ubiquitination was blocked, the degradation of Cdk5 in S phase was also attenuated. This S-phase specific degradation of a Cdk once again marks Cdk5 as an atypical Cdk. In this behaviour, it is more similar to a cyclin than a true Cdk. As we know that the substrate recognition of ubiquitin-ligases relies on the E3 component [40], we identified that the Cdh1 is the E3 ligase to mediate the degradation of Cdk5 [39]. Three

major ubiquitin E3 ligases, APC-CDC20, APC-CDH1, and SCF (skp2), are responsible for cell cycle protein degradation. During a typical cell cycle, the multi-protein APC/C complex is activated only through early G1 [41]. Yet it has been shown that the complex can switch between two major activator proteins: CDC20 and CDH1, depending on the cell cycle phase [42-44]. Significantly, CDH1 has been proved to play major role in regulating neuron maturation. The identification of CDH1 serves as the E3 ligase for Cdk5 degradation fits well with this aspect of CDH1 function and suggests that understanding the factors that regulate the levels and location of the Cdk5 kinase will move us closer to a better understanding of how Cdk5 regulate the neuronal migration, differentiation and survival.

Conclusions

In many severe neuronal degeneration diseases, neuronal cell cycle re-activation is the earliest dysfunction found. These include Parkinson's and Alzheimer's disease as well as amyotrophic lateral sclerosis, HIV induced encephalitis, stroke, ataxia-telangiectasia and others. The primary causes of this wide range of afflictions vary enormously, but in each the process of neuronal cell death is tightly correlated with neuronal re-entrance into a cell cycle. The implication is that restoring cell cycle control to the affected neurons has significant potential as therapeutic strategy with wide-ranging applicability. As one of the key neuronal proteins whose normal function is to suppress the cell cycle, this places the proper regulation of Cdk5 levels and activity as an important aspect of the fight against these diverse set of diseases.

In the past several years, we have addressed several questions associated with the role of Cdk5 in cell cycle regulation:

1. How does Cdk5 suppress the neuronal cell cycle?
2. What is the nuclear-cytoplasm trafficking mechanism of Cdk5?
3. How are the levels of Cdk5 regulated in both nuclear and cytoplasmic compartments?

In a typical healthy post-mitotic neuron, the interaction between nuclear Cdk5 and E2F1 prevents the binding between DP1 and E2F1, which significantly blocks the ability of E2F1 to bind its usual sites on the genome and hence suppresses its transcription activity. Thus, in the presence of nuclear Cdk5, neurons are maintained in a non-mitotic state. In this situation, Cdk5 does not need its kinase activity but it must bind with its cyclin-like partner, p35 to form a stable complex with E2F1 (and exclude its binding to DP1). It is the first report on the non-kinase-related function of Cdk5. Next we identified p27 is vital for nuclear localization of Cdk5. In abnormal neurons like those found in AD patient's brain, it occurs as if those mitotic signals appear to trigger Cdk5 to shuttle from nucleus to cytoplasm by NES-CRM1 pathway (Figure 1), librating its interaction with p27 or E2F1, which eliminate the cell cycle suppression. The p27 is normally high expressed in nucleus in quiescent cell and post-mitotic neurons which facilitate the nuclear localization of Cdk5. What's more, P27 will be transported into cytoplasm if cell re-enter cell cycle, without nuclear p27, Cdk5 will also be lost its nuclear localized foundation. As soon as nuclear Cdk5 was transported into cytoplasm in S phase, the cytoplasmic Cdk5 will be ubiquitinated by the E3 ligase APC-CDH1. Ubiquitinated Cdk5 is degraded then by the proteasome (Figure 1).

Cdk5 was identified as an atypical member of Cdks and has been explored for more than 2 decades. Almost all Cdk5 research has documented about its kinase activity while its characteristics as a protein, its localization or its transport are partially studied. We focused on the localization and transportation of Cdk5 to investigate its neuronal cell cycle blocking ability for the first time to provide evidence that Cdk5 may perform neuronal cell cycle suppressor in post-mitotic neurons. This new finding will contribute to better understanding the involvement of Cdk5 in brain disorders including Alzheimer's disease.

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