[Frontiers in Bioscience E4, 2101-2109, January 1, 2012]

The contributions of unscheduled neuronal cell cycle events to the death of neurons in Alzheimer's disease

Karl Herrup[1]

[1]*Deptartment of Cell Biology and Neuroscience, Nelson Biological Laboratories, 604 Allison Road, Piscataway, NJ 0854*

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1. ABSTRACT

Alzheimer's disease is a neurodegenerative disorder that accounts for the majority of the dementia in individuals over the age of 65. While much has been learned about the biology and biochemistry of the tau tangles and beta-amyloid plaques, less is known about the cell biology of the neuronal cell death process. This review examines one feature of this process, the unexpected occurrence of unscheduled cell cycle events in mature and normally non-mitotic neurons in the at-risk neuronal populations. The correlation of neuronal cell cycling and cell death is not unique to Alzheimer's, but the evidence in both human Alzheimer's disease and its mouse models suggests that these events are early disease related processes, that they are driving forces of the disease rather than indirect symptoms. Defining the biochemistry behind cell cycle initiation holds promise as a fresh therapeutic approach in the battle against this devastating disease.

2. BACKGROUND

Alzheimer's disease (AD) is a late-onset neurodegenerative disease, with profound cell losses occurring in neuronal populations throughout the brain. The hippocampus and basal forebrain are perhaps the best known of these at-risk populations. As the most commonly mentioned feature of AD is the loss of a person's ability to form new memories it is fitting that these structures head the list of affected areas. But to end the list here misses a substantial portion of the neurobiological consequences of AD and channels thinking about its causes far too narrowly. In addition to hippocampus, there are significant neuronal losses in other regions of the limbic system such as the amygdala, entorhinal and cingulate cortex (Figure 1). Also included in the list of heavily affected populations are the adrenergic neurons of the locus coeruleus and the serotonergic neurons of the dorsal raphe as well as other regions of the neocortex. Even the cerebellar deep nuclei

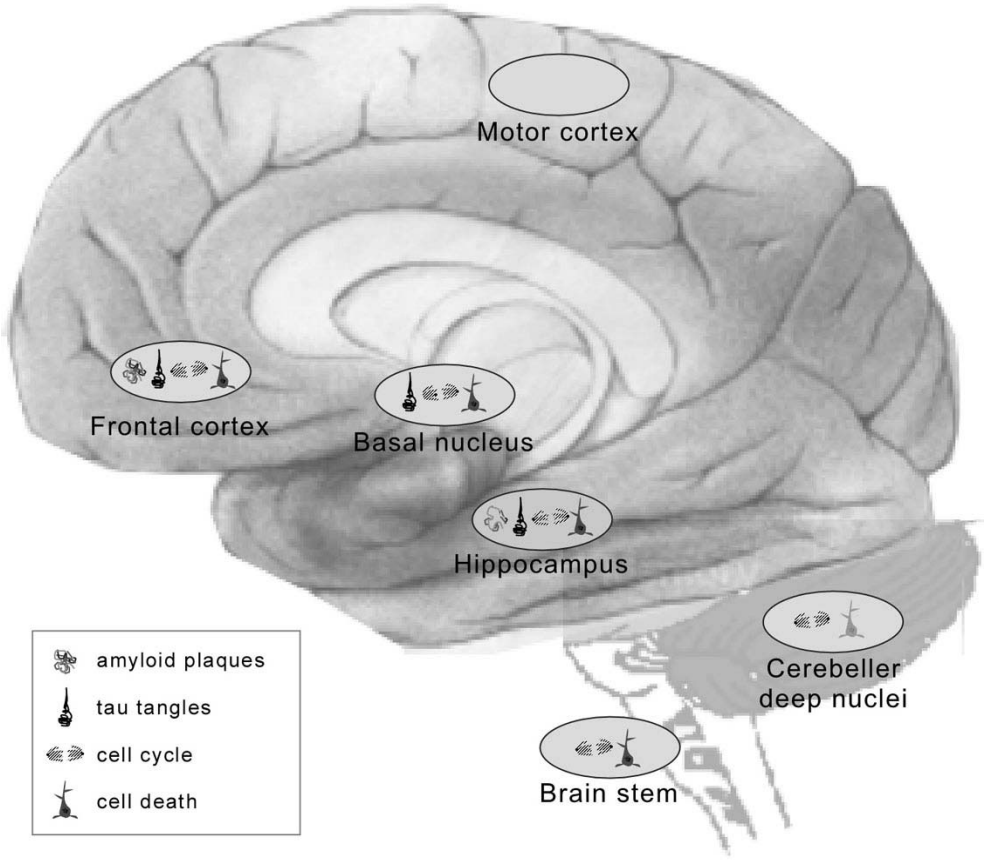

Figure 1. Concordence of pathological markers in various AD brain regions. The location of four types of histopatholgoical markers are shown by the presence or absence of their icons (see legend on the diagram) in the various brain regions illustrated. The motor cortex is listed as spared although significant evidence suggests that at later disease stages, all four types of pathology are found here as well.

have come under fresh scrutiny as a site of where neuronal dysfunction and loss may play an important part in the neurological and behavioral symptoms of the disease (1). These structures suffer the heaviest loss, but by end stages of AD the volume of the entire brain seems to shrink suggesting a wide-spread phenomenon that spares few populations of CNS neurons. A key problem facing current research into the neurobiology of AD is what are the precise cellular mechanisms that underlie this profound loss of neuronal cell numbers. Much has been learned in recent years, yet gaps remain in our understanding.

The vast majority of the literature on topic of cell death in AD is heavily focused on the role of the small Aβ peptide that is released from the larger Type I membrane protein, APP (amyloid precursor protein). Excellent reviews are written on this topic (2-6) as well as the microtubule associate protein tau (7, 8) and preclude the need to repeat the information here. Rather, the goal of this manuscript will be to examine what is known about other aspects of the cell death process in AD and their cell biological basis. The long term goal of the field is to integrate all of these concepts into a single unified disease model. For the moment this remains a laudable, but unattained, goal.

2.1. The strange linkage between cell death and cell cycle regulation

In an attempt to understand this widespread neuronal degeneration in AD, many labs have discovered that there is an unexpected involvement of processes normally associated with the cell cycle. The adult CNS neuron has a very unusual relationship to the cell cycle. Most brain structures are populated by nerve cells that underwent their last cell division during the embryonic time period. For these neurons, emigration from the ventricular zone means commitment to a permanent post-mitotic state. This is a conceptually simple statement, but its implementation *in vivo* turns out to be anything but simple. That is because, rather than adopt a rigorous set of traits that completely block cell cycle initiation, neurons have instead evolved a system where they can (and do) re-enter a cell cycle at any point during the life of the organism. This means that CNS neurons must constantly suppress their cell cycles in order to remain 'permanently' post-mitotic.

The evidence for this perspective comes from several sources. The evidence is most straightforward for immature neurons that have left the ventricular zone, but have yet to establish their full adult phenotype. If these

cells are forced to re-enter a cell cycle, either by ectopic expression of an oncogene (9, 10) or by genetic deletion of a tumor suppressor such as retinoblastoma (11-13), they rapidly enter S-phase and begin DNA replication. Rather than divide, however, these neurons die. While these deaths may be viewed as artificially induced, the association between cell cycle events and the process of programmed cell death is seen in more naturalistic settings as well. As part of their maturation process, many neuronal populations undergo a pruning process in which they match their numbers in precise quantitative fashion to the size of their synaptic targets (14, 15). These deaths are a normal part of the developmental process, yet they too are characterized by a cell cycle related neuronal degeneration (CRND). In cerebellar granule cells, where the timing of the process has been examined more closely (16), an injection of BrdU given during the cell death process results in a wave of incorporation of this DNA precursor into nuclei of the dying granule cells that peaks at 10 hours after injection. This rapid kinetics of this process is identical in both normal animals and those in which the target has been genetically eliminated (*Rora*^{sg}).

Neurons from rodent cerebellum or neocortex also behave as immature nerve cells when they are placed in tissue culture. If any of a number of different toxins (17-21) are applied to these cultures or if the cells are taken from animals that render them unable to suppress their cell cycle (22), these neurons will also undergo CRND. In all immature neurons that have been examined, the neurons that cycle are the neurons that die (16, 19, 23). Equally important, if the cell cycle is blocked, so is the cell death (24, 25).

2.2. Cell death in Alzheimer's disease – general considerations

This discussion of CRND in immature cells of the embryo and dissociated neurons in culture would seem a far cry from the question of how neurons die in Alzheimer's disease. Yet the CRND process would appear to be a major contributor to AD neurodegeneration. This suggestion arises because there is a consistent correlation between the anatomical regions where cell death is found in the AD brain and those regions in which neuronal cell cycle events, such as expression of cell cycle-related proteins, are found. Given the evidence cited above for the loss of cell cycle control acting as a major driving force in the induction of neuronal cell death, in immature neurons and in culture, one might assume that there was a simple and direct connection. But the process of CRND has proven to be more complex than the early studies suggested and thus there are questions that remain as to exactly how it applies to the neurodegeneration in AD. Before considering these questions, however, it is worthwhile to review what is know about the origins of the neuronal cell death process in Alzheimer's

There is substantial evidence that one of the first events in the Alzheimer's degenerative process is a major loss of synapses (5). This can be seen in the reduced density of staining for synaptic markers such as PSD-95, glutamate receptors and others. It can also be seen in the loss of neuronal dendritic complexity and a decreased density of synaptic spines. This synaptic loss is most prevalent in the areas where neurodegenerative changes are observed (26), and the comparison of neuropathological material from individuals who died at different stages of the disease strongly suggests that the synaptic dystrophy precedes the death of the cell body. In keeping with this neuronal locus of AD atrophy, the oft-reported incidence of tau abnormalities has been linked to the findings of defective axonal transport in AD (27). Such deficits would weaken a neuron and make it more susceptible to cell death (28-30).

There is also a growing body of evidence that defects in the endocytic pathway are an early and disease-specific event in the loss of neurons in Alzheimer's disease (31). The defects are most common in the sporadic rather than familial forms, although individuals with Down's syndrome manifest problems in the endocytic process at very early ages. The endocytic problems are consistent with the known involvement of endocytosis in the proper functioning of the ApoE lipoprotein in regulating the processing of the APP protein and b-amyloid, emphasizing the importance of this aspect of AD.

More recently, defects in the process of autophagy are being identified as a significant contributor to the neurodegeneration of AD (32, 33). This defect is a significant problem for nerve cells in a disease where, as in AD, macromolecular aggregates of misfolded proteins are common. Underscoring the importance of the linkage between autophagy and Alzheimer's is the recent finding that one of the important functions of the presenilin protein is to ensure the proper acidification of the lysosome, and that the mutant forms of PS1 do not function as well as wild type forms in this process. In the final steps autophagy, the vesicle known as the autophagosome fuses with a lysosome. The fusion allows for the efficient and complete digestion of the contents of the autophagosome; anything that interfered with this disposal process would be expected to have negative consequences for a nerve cell.

Endocytic and autophagic abnormalities and, as we will see below, CRND, are commonly found in neurons of the regions where cell death is found (Figure 1). Yet over the years, investigations into the classical forms of cell death have shown that they are less prevalent. For example, the search for a typical apoptotic process has suggested that this mechanism of programmed cell death is involved (34), but the numbers of such events is low in any one pathological specimen. Combined with the discussion of the other degenerative processes outline, the suggestion is that the final loss of the neuronal cell body may involve apoptosis, but that this final demise of the cell comes at the end of a long series of earlier degenerative events that likely have network and functional consequences for brain function.

2.3. Cell death in Alzheimer's disease – the involvement of cell cycle processes

The first suggestion that the death of neurons in the AD brain might involve problems with ectopic cell

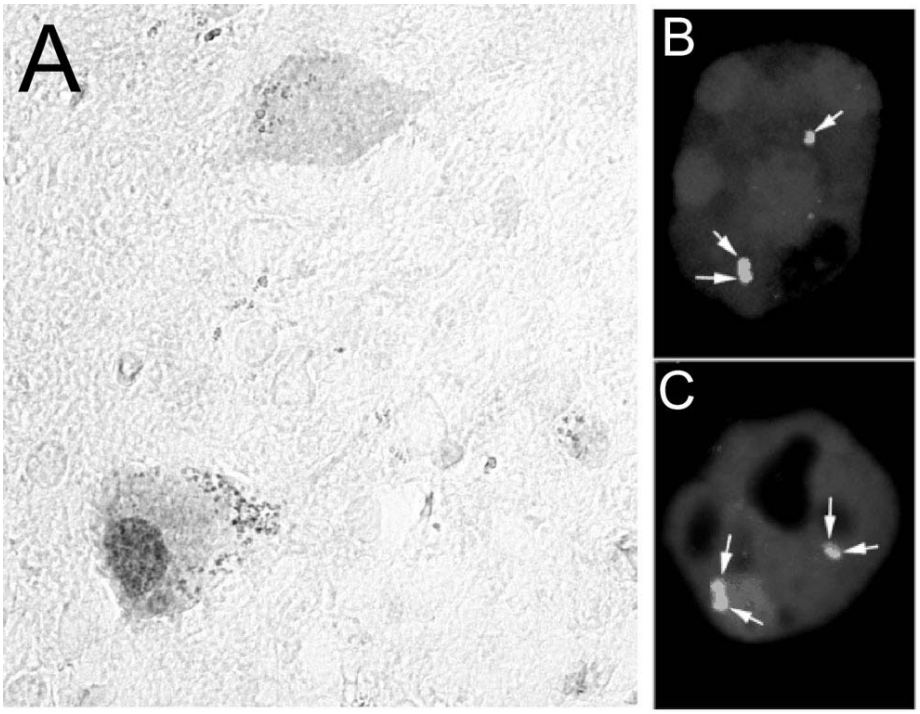

Figure 2. Cell cycle events in neurons of the Alzheimer's disease brain. Neurons in areas that are subject to cell death during the course of AD are found associated with ectopic cell cycle events – protein synthesis and DNA replication – in both the human disease and its mouse models. A) Cyclin A immunostaining of the the human cerebellar dentate nucleus of an individual who died with AD reveals ectopic expression of this cycle protein in neuronal nuclei. Such staining is significantly reduced in cognitively normal subjects (not shown). B, C) Two representative neuronal nuclei demonstrating that cell cycle activity is found in AD mouse models such as the R1.40 YAC transgenic model of Lamb *et al.* (70). This latter event can be detected by fluorescent *in situ* hybridization (FISH) with fluorescent probes to unique genomic sequences found in a single genomic loci. Normal adult neurons have two copies of each chromosome; thus two spots of hybridization should appear. The cells in B and C (from the frontal cortex of an R1.40 mouse) were identified as neurons based on the size and shape of their cell body, and their nucleus as well as on their location. The FISH signal indicates that both have replicated their genome. Three of these spots are shown in the confocal image of the cell in B; four are shown in the cell in C.

cycling in neurons came from studies involving the microtubule associated protein, tau. Tau levels in brain are high; the protein is found to decorate the microtubules of the neuronal axon where it provides structural support and serves to regulate the speed of axonal transport. The affinity of tau for the microtubule is regulated in large part by its levels of phosphorylation; the greater the levels of phosphorylation, the lower the microtubule binding affinity. In Alzheimer's disease, for reasons that are not completely understood, tau becomes hyperphosphorylated. In this state, microtubule affinity is reduced and the tau protein is more prone to aggregation. Various levels of aggregation have been described; they culminate in the paired helical filaments that can be seen in silver stained preparations as neurofibrillary tangles. Abnormal deposits of these tangles are a well known feature of the AD brain. Yet tau is found in other locations apart from the neuronal axon. In particular, it is a major component of the mitotic spindle and the same forms of hyperphosphorylated tau that are pathonmemonic of AD are also found to decorate the spindle of cells in mitosis. This led Vincent and Davies to suggest that there was a problem with cell cycle regulation in the neurons of the AD brain (35, 36). Coming shortly after the discovery that nerve cell death could be driven by forcing a mitosis in a mature neuron (9, 37), an important connection was made. At present, over a dozen labs around the world have repeated the observation and the correlation between ectopic cell cycle events and neuronal populations whose members are at risk for death has been repeatedly shown. The neuron in Figure 2A is an illustration of the robust presence of proteins such as cyclin A in neurons of the AD brain. The proteins that are ectopically expressed come from all phases of the cell cycle with the exception of M-phase. Cyclin proteins are the most commonly reported (36, 38, 39), but cyclin-dependent kinases (Cdks) are also found (39, 40) as are the Cdk inhibitors (40-42) and other proteins (43, 44).

The cell cycle process that occurs in the AD brain involves more than just protein expression or a reflection of other homeostatic processes. Repair of DNA damage is unquestionably more common in AD brain, and uses many of the same cell cycle proteins during the process. Consistent with the findings from experimental systems, however, much of the cell cycle protein expression found in the AD brain is there to assist in a

process of true DNA replication. In humans this cannot be shown by BrdU injection, but it has been conclusively demonstrated by fluorescent *in situ* hybridization (FISH) that chromosome copy numbers increase (45-49). An increase in chromosome copy number (ploidy) only occurs during true DNA replication (Figure 2). Another important finding, demonstrated by both cell cycle protein expression (1, 50) and FISH (45), is that the cell cycle events are not co-morbid events found only at end stages of the disease. Rather, they are present is significant numbers as soon as dementia is manifested clinically.

It is worth noting that there have been persistent reports challenging the DNA replication aspect of CRND in AD (51, 52), although the re-expression of cell cycle proteins is well accepted. It is fair to say that, due to the difficulties inherent in these types of studies, a detailed quantitative assessment of the involvement of DNA replication during the observed cell cycle events is not yet complete. One source of the reported discrepancies may be the nature of the material that is analyzed. For the most part, the studies that have examined sectioned pathological material find evidence of DNA replication (hyperploidy) in AD, while those that use isolated nuclei processed in solution find less evidence of this phenomenon. While both methodologies have their weaknesses, there is reason to suspect that the larger neuronal nuclei are lost during the solution based protocol unless specific steps are taken to preserve them (53). This is a critical question and until there is full agreement in the field, more work remains to be done.

Also significant is while the foregoing discussion links the appearance of ectopic cell cycle events in neurons with the degenerative process, it must always be remembered that correlation does not mean causality. The experimental findings from the many studies involving immature neurons make it tempting to draw direct connections to the adult situation. Such connections may yet prove valid, but caution is warranted. The chief reason for this is that the process of CRND is different in the mature adult neuron, and the difference is significant. In the adult, the linkage between cell cycle initiation and cell death is blocked. This is seen most simply in the percentages of cells in the AD brain that can be shown to be 'cycling'. The numbers are several hundred times higher than would be predicted of a rapid apoptotic process such as that seen *in vitro* or in developing systems *in vivo*. It has been suggested based on some simplifying assumptions that final neuronal death can be delayed follow cell cycle initiation by many months and possibly a year or more (39, 49, 54, 55). Further, there are key pieces of evidence that are missing in the demonstration of a direct homology between CRND in immature and mature neurons. The first of these is that it cannot be proven that in the AD brain it is the cycling neurons that die. This is highly likely, but in theory it is possible that the cycling neurons are in fact the protected ones (55). The second key piece of information that is missing is that we do not know that if we inhibit the cell cycle we will inhibit the cell death. Again, the strong analogy with the situation in developing brain makes this the most likely possibility, but as of this writing, formal proof is missing.

2.4. CRND in Alzheimer's disease mouse models

Even though there are still gaps in our knowledge about the process of cell cycle related neuronal death in AD, there are additional data that strongly validate its importance in the disease mechanism. The mouse models of Alzheimer's are perhaps the best example of this validation. Current murine models of familial AD have greatly stimulated research in the field, yet as a group they only weakly resemble the full phenotype of Alzheimer's disease. The behavioral symptoms are mild compared with the loss of function observed in advanced human AD. The neuropathological replication is also incomplete. While most develop amyloid plaque pathology, decreases in the density of synaptic proteins and early abnormalities in tau hyperphosphorylation, the classic neurofibrillary tangles do not appear and the extent of neuronal degeneration is quite modest, often zero. Yet several independent reports in different model systems reveal that cell cycle events are found, suggesting that the first stages of neurodegeneration are replicated faithfully (56-59). Indeed in every mouse model we have examined, ectopic neuronal cell cycle events are found although their distribution and timing are often quite different (Li, Cheung, *et al*, unpublished). This finding of cell cycle re-activation in our AD mouse models not only supports the concept that this re-activation is a central part of the disease process, it also offers us a new outcome measure in a variety of pre-clinical drug development paradigms.

Where it has been looked at most carefully, these cell cycle events in the mouse capture the AD disease process quite faithfully. The R1.40 model of Lamb (60) is the best studied model to date. In this model, the human APP gene carrying the Swedish fAD mutatation, has been integrated into the mouse genome by way of electroporation into ES cells, followed by passage through the germline of chimeras made from these modified cells. What is unique about this model is that it is a genomic model rather than a cDNA driven by a heterologous promoter. It carries the entire human gene including all introns and tens of kilobases of both 3' and 5' regulatory sequences. The human APP gene and all of its splice variants are expressed in a spatiotemporal sequence that matches that of the homologous gene in the human. In this animal, if cell cycle events are used as 'biomarkers' for impending neuronal degeneration, the fidelity of the mouse model is quite high (58). Cell cycle events appear early in frontal cortex and in brain stem (locus coeruleus and dorsal raphe); later they appear in the entorhinal cortex and the hippocampus. Within the cortex, the cells activate cell cycle processes in a fashion comparable to human – the upper layers (II and III) activate first followed months later by cell cycle activation in the deeper layers (V and VI) (61). These ectopic cell cycle events occur early in the process, just as they do in the human. And just as they do in the human, the cell cycle proteins are correlated with evidence for DNA replication (Figure 2B, C). The timing is informative as well. The first evidence of cell cycling is found at 6 months of age (on the C57BL/6 genetic background); by contrast, the first plaque deposits are not found until after 13 months. One potential discordance is the failure to observe cell cycle events in the cholinergic

neurons of the basal forebrain nucleus. These cells are lost in large numbers in human AD and neurons in the remaining cells are regularly positive for both expression of cell cycle proteins and hyperploidy as seen by FISH (49). This may be a technical problem that arises from trying to model a complex human disease in the simpler brain of the mouse. Or it may be that even though basal forebrain neurons undergo many changes early in the progression of the human disease (62, 63), they do not begin a death process until their hippocampal target neurons are actually lost. As the mouse never progresses very far along the pathogenic process, there is no cell loss in hippocampus and this may preclude the stress that normally activates cell cycling in the basal forebrain.

Beyond a close reproduction of the natural history of the disease, the cell cycle events in the mouse are beginning to prove their worth as outcome measures in pre-clinical experiments designed to examine different types of Alzheimer prevention and treatment paradigms. For example, evidence from human epidemiological studies shows a 50% reduction in the lifetime risk of AD in individuals who had been exposed to long periods of very high doses of certain non-steroidal anti-inflammatory drugs (NSAIDs) (64). The effect of two such drugs (ibuprofen and naproxen) on the cell cycle events in the R1.40 mouse model is completely consistent with this finding in the human. Transgenic mice allowed to eat NSAID-supplemented chow from 6-months to 9-months of age had no cell cycle events present in their brains while their transgenic littermates had developed them in several regions. The mouse data also extends the human epidemiological data in important ways. If the NSAID treatment of R1.40 animals was delayed until 9 months, well after the initiation of the cell cycle events had begun, then just as in the first experiment, no new cell cycle events appeared in any region. But those populations of neurons where neuronal cell cycle events had already appeared (layer II and III of frontal cortex, for example) were unaffected by the treatment and continued to express several cell cycle proteins. These provocative findings suggest that once the cells enter the state of hyperploidy, there is no going backwards. Removal of the stress that triggered their initial attempt at cell cycling (presumably an inflammatory pressure that could be prevented by NSAID treatment) is ineffective in causing them to down-regulate the expression of a variety of cell cycle proteins (65, 66). The failure of the ADAPT human trial of NSAID therapy (67) is unfortunately a predicable consequence of the continued cell cycle protein abnormalities in the mice.

2.5. The R1.40 model accurately predicts cerebellar involvement in AD

One of the important attributes of a good model system is that it not only validates information that was already known, it accurately predicts new and unexpected features of the system. This has been the case for the R1.40 AD mouse model. In assessing the distribution of cell cycle events in the AD mouse, we consistently noted the robust presence of neuronal cell cycling events in the cerebellar deep nuclear neurons. This was unexpected as the cerebellum is thought to be mostly spared in AD and is often used as a region against which functional imaging results in other parts of the human brain are calibrated. Nonetheless, a careful examination of a large number of AD cases and controls confirmed that in the cerebellar dentate nucleus, a disease-specific enhancement of cell cycle events can be found as well as higher levels of markers associated with DNA damage (1). Unlike the other brain regions we have examined, these cell cycle events do not appear during early stages of the disease, but rather appear to represent a later reflection of the growing atrophy and degeneration of the AD process. This may yet prove to be an important insight into Alzheimer's symptoms. Although the cerebellum is best known for its role in the control of fine motor skills, there is a substantial body of literature that suggests its involvement in executive functions, behavior and language (68) – all domains that are impinged upon by AD.

While cerebellar involvement in AD has been suggested previously (6-11,36), our findings linking cell cycle events in the cerebellar deep nuclei to AD argue that this lesser-studied brain region is worthy of closer attention as a contributor to the complex symptoms of AD. A curious finding that points the way to future studies was our discovery that cyclin A, a S-phase cell cycle marker, was significantly associated with disease state, but PCNA expression (a second S-phase marker of the cell cycle) was not disease correlated. We believe that since PCNA is involved in DNA damage repair in addition to cell cycle progression (30), our data suggest that the latter, but not the former is the true harbinger of neurodegeneration in AD.

3. CONCLUDING COMMENTS

There are many mysteries remaining to be solved before we can achieve a full understanding of the neurodegenerative process in Alzheimer's disease. Aggregations of fragments of the amyloid precursor protein and various isoforms of the tau protein clearly make major contributions to the loss of neurons, but recent findings have changed the simple relationships that once connected aggregates to disease. For both proteins the initial focus on macromolecular aggregates (plaques and tangles) has given way to a recognition that more nanomolecular aggregates known as oligomers may represent the truly toxic species. Indeed, models of AD have been proposed where these aggregates, though correlated, are neither necessary nor sufficient to trigger AD (69). Even with this shift in focus, the actual cell biological processes that connect the oligomers to the diverse examples of neuronal cell death in AD have yet to be worked out. The participation of other brain cell types – microglia, capillary endothelial cells, astrocytes, oligodendricytes – are all surely factors and play important contributing roles in the inflammation, cerebrovascular amyloidosis, axonal dystrophy and other events that help define AD pathogenesis. In this complicated cellular context, the involvement of cell cycle events represent a particularly attractive area for future research. These events are distinctly neuronal in nature. They are tightly correlated with disease state, both anatomically and temporally. Though not specific to AD, they mark the neurons at risk for death in AD as faithfully

as the presence of tau pathology and more reliably than amyloid-beta (in particular the plaques). Work in cultured neurons has shown that when the cell cycle events are stopped, so too are the cell death events. And the studies that have been done to date support their validity as pre-clinical biomarkers. In partnership with anti-aggregation therapies for tau and for amyloid, it seems likely that anti-cycling strategies might well be developed in the near future to stave off the worst effects of the devastation of Alzheimer's disease.

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Key Words: Alzheimer's disease, Neuron, Nerve, Central nervous system, Review

Send correspondence to: Karl Herrup, Department of Cell Biology and Neuroscience, Nelson Biological Laboratories, 604 Allison Road, Piscataway, NJ 0854, Tel: 732-445-3306, Fax: 732-445-1794, E-mail: herrup@biology.rutgers.edu

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