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NFATs and Alzheimer's Disease

Hafiz Mohammad Abdul^{1,*}, Jennifer L. Furman^{2,*}, Michelle A. Sama^{2,#}, Diana M. Mathis³, and Christopher M. Norris^{1,2}

¹Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky

²Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, Kentucky

³Graduate Center for Gerontology, University of Kentucky, Lexington, Kentucky

Abstract

Nuclear factor of activated T cells (NFAT) is a transcription factor that translocates from cytosol to nucleus following dephosphorylation by the Ca^{2+} /calmodulin dependent protein phosphatase calcineurin (CN). In nervous tissue, aberrant CN signaling is increasingly linked to a variety of pathologic features associated with Alzheimer's disease (AD), including synaptic dysfunction, glial activation, and neuronal death. Consistent with this linkage, our recent work on postmortem human hippocampal tissue discovered increased nuclear accumulation of select NFAT isoforms at different stages of AD. Some of these changes occurred at the early stages of the disease process and/or paralleled diminishing cognitive status. In addition, inhibition of astrocytic NFAT activity in primary cultures of neurons and glia dampened glutamate levels and alleviated neuronal death in response to pathogenic amyloid- β peptides. In this article, we discuss our recent findings and expand upon the possible isoform specific contributions of NFATs to the progression of AD. We also consider the possible benefits of using NFAT inhibitors to treat AD and other neurodegenerative disorders, as well.

Keywords

NFATs; Alzheimer's disease; Calcineurin; Neurodegenerative disorders

Introduction

Alzheimer's disease (AD) is an aging-dependent, progressive neurodegenerative disease, characterized by β -amyloid (A β) plaques, neurofibrillary tangles, gliosis, oxidative stress, neuronal degeneration, and dementia. More than two decades ago, it was proposed that the dysregulation of cellular Ca^{2+} across the lifespan leads to impaired cognition and increased susceptibility to AD and other neurodegenerative conditions (1–4). Numerous studies on human AD tissue, and especially on multiple animal models of AD, have largely confirmed the importance of Ca^{2+} dysregulation during the disease process (5,6). Recently, our group and several other labs have compiled strong evidence to suggest that the Ca^{2+} -dependent protein phosphatase calcineurin (CN) provides an important link among the multiple

Correspondence: Christopher M. Norris, Ph.D., University of Kentucky, 131 Sanders-Brown Building, 800 South Limestone St, Lexington, KY 40536, USA. Tel. 859-257-1412, Fax. 859-323-2866. cnorr2@uky.edu.

*These authors contributed equally to this work.

#Current affiliation: Department of Physiology and Neurobiology, Dartmouth Medical School, Lebanon, New Hampshire.

Conflicts of Interest

No potential conflicts of interest to disclose.

pathologic hallmarks that emerge during AD. This article will discuss some of the recent findings concerning CN signaling and AD, with particular emphasis on our new research on the CN-dependent transcription factor, nuclear factor of activated T cells (NFAT) (7).

Calcineurin

CN signaling properties and regulation have been described in several excellent reviews, *e.g.* (8), and therefore will not be described in detail here. Briefly, the CN holoenzyme consists of an ~60 kDa catalytic subunit (CN A) and an ~19 kDa Ca^{2+} binding regulatory subunit (CN B). Multiple isoforms of each subunit (*e.g.* CN A α , CN A β and CN B α , and CN B β) have been identified and are expressed in brain. CN is strongly regulated by cooperative interactions between Ca^{2+} , CN B, and Ca^{2+} /calmodulin. Maximal catalytic activity is achieved when Ca^{2+} /calmodulin is bound to the CN A subunit. Several unique regulatory, expressional, and functional properties of CN make it a molecule of interest in AD and other neurodegenerative disorders. First, while most enzyme classes (*e.g.* protein kinases and proteases) consist of multiple Ca^{2+} sensitive family members, CN is the only Ca^{2+} -activated protein phosphatase in mammalian cells. Moreover, of the many Ca^{2+} dependent enzymes, CN is among the most sensitive to fluctuating intracellular Ca^{2+} levels and is therefore especially vulnerable to the Ca^{2+} dysregulation that arises with AD. Second, CN is one of the most abundantly expressed proteins in the brain, with particularly high levels found in structures (*e.g.* the hippocampus) showing the earliest and most extensive signs of AD pathology (9). Third, elevated CN signaling is strongly associated with numerous deleterious processes linked to AD including, synaptic dysfunction and spine loss (10,11), neuritic atrophy and neuronal death (12), astrocyte activation (13), β -amyloid (A β) production (14), and impaired cognition (15,16). Fourth, pathogenic molecules in AD brain, especially oligomeric A β peptides, robustly activate CN activity in neurons (17,18) and astrocytes (7). Moreover, numerous deleterious effects of oligomeric A β including synaptic depression (19), NMDA receptor endocytosis and synaptic spine retraction (20,21), dendritic atrophy (22), neuronal apoptosis (18), and glutamate dyshomeostasis are blocked or ameliorated by inhibition of CN signaling pathways. Finally, several recent studies have shown that neural CN activity/expression is increased in mouse models of AD, as well as postmortem AD tissue (7,13,23,24).

NFATs are CN dependent transcription factors

NFATs are the most thoroughly characterized CN substrate and are the subject of several excellent reviews, *e.g.* (25). These transcription factors tend to reside in the cytosol in a highly phosphorylated state when intracellular Ca^{2+} levels are low, but are bound tightly by activated CN and dephosphorylated when Ca^{2+} levels rise. CN-mediated dephosphorylation of NFATs reveals a nuclear import sequence which permits transport into the nucleus. Conversely, a nuclear export sequence is exposed and NFATs are shuttled back to the cytosol upon re-phosphorylation by a variety of protein kinases (*e.g.* glycogen synthase kinase 3 β). Elevated CN activity is therefore generally required to maintain the nuclear localization of NFATs. Once in the nucleus, NFATs interact with specific DNA binding elements to regulate gene expression in conjunction with other transcription factors (*e.g.* AP1, MEFs, and NF κ B). NFATs play an important role in immune/inflammatory signaling and are best known for coordinating T cell activation through the regulation of cytokine genes. Nonetheless, NFATs have been found in most other mammalian tissues, as well, where they regulate numerous and diverse cellular processes. In brain, CN-mediated effects on Ca^{2+} homeostasis, neuronal viability, A β production, and neuroinflammation have been shown to depend, in part, on NFAT activation (14,26–30), suggesting a possible role for these transcription factors in AD. Based on these observations, we set out to determine

whether AD and cognitive decline were associated with changes in NFAT signaling properties.

Isoform specific changes in NFAT signaling properties with AD

In our study (7), postmortem human hippocampal and cerebellar tissue was collected from non-demented control cases and confirmed AD cases. Subjects diagnosed with mild cognitive impairment (MCI) were also studied, as these patients appear to be at a transition between normal age-related cognitive decline and dementia associated with AD. Hippocampus and cerebellum were chosen because of their different vulnerabilities to AD pathology (hippocampus = high vulnerability; cerebellum = low vulnerability). Tissue from each subject was separated into cytosolic and nuclear fractions using differential centrifugation and protein levels were measured in each fraction. At least four CN-dependent NFAT isoforms have been identified (NFATs1-4), all of which are expressed in brain (31). In our study, we measured levels for NFATs 1–3 (due to the lack of a satisfactory antibody, NFAT4 was not investigated). Levels for multiple CN A isoforms and glycogen synthase kinase 3 β (GSK3 β) were also measured. The proportion of NFAT protein associated with the nuclear fraction (nucNFAT) was obtained by dividing nuclear protein levels by total (nuclear + cytosolic) protein levels. Table 1 summarizes changes in the nuclear localization of NFATs 1, 2, and 3, CN A α and CN A β , and GSK3- β during MCI and AD, as reported in Abdul et al., 2009 (7).

Because NFATs tend to accumulate in the nucleus when active, we predicted that AD and/or MCI would be associated with elevated nucNFAT levels in the hippocampus. Our results both supported and refuted this prediction. Of the three NFAT isoforms studied, only the NFAT2 isoform remained unchanged in the hippocampus across subject categories (see Table 1). Moreover, changes in NFATs1 and 3 occurred during different stages of disease progression. Hippocampal nucNFAT1 levels were increased during MCI, but then fell precipitously as pathology and cognitive status worsened. In contrast, hippocampal nucNFAT3 levels dipped slightly in MCI but were markedly increased with intermediate to severe AD. In contrast to the hippocampus, cerebellar nucNFAT levels were very similar across subject groups, suggesting that changes in NFAT signaling with AD may be relatively restricted to the most vulnerable structures.

To determine whether isoform specific changes in NFAT localization were attributable to different expression patterns across neuron and glial populations, we double-labeled postmortem hippocampal sections from each of the subject groups with anti-NFAT antibodies and an antibody to glial fibrillary acidic protein (GFAP), an intermediate filament highly enriched in astrocytes. Using confocal microscopy, we showed that NFATs1 and 3 were expressed in both neurons (dentate granule cells) and astrocytes in the hippocampus, regardless of clinical and pathological symptoms. However, relative to controls and AD cases, NFAT1 tended to show greater localization to astrocyte nuclei in the MCI group, whereas nuclear localization of NFAT3 in astrocytes was more readily apparent in the AD group. Within dentate granule neurons of all subject groups, NFATs1 and 3 each showed 'donut-like' expression patterns, consistent with cytosolic localization. These expression changes are generally consistent with our Western blot results and suggest that alterations in NFAT signaling during AD progression may occur to a greater extent in astrocytes. Caution is needed with this interpretation, however, as NFAT expression in CA pyramidal neurons were not examined closely.

Isoform-specific contributions of NFATs to AD and dementia?

If multiple NFAT isoforms are expressed within the same cell populations, why would one isoform respond differently than the others during the progression of cognitive decline in

AD? One possibility is that NFAT isoforms exhibit different sensitivities to CN, or to NFAT kinases. In fact, we found that nucNFAT 1 and 3 levels in human hippocampus were preferentially associated with different CN A isoforms (Table 1), but not with GSK-3 β . NucNFAT1 levels were moderately, but positively, associated with the nuclear localization of both CN A α and CN A β isoforms, whereas nucNFAT3 exhibited a much stronger association with CN A α , and did not correlate with CN A β . Isoform differences in NFAT activation may also depend on the presence of other transcription factors and/or on the temporal and spatial properties of the Ca²⁺ signal, among other things. Regardless of the mechanism, our results demonstrate that NFATs 1 and 3 are excessively activated at different stages of cognitive decline and AD, and therefore may contribute to entirely different cellular processes.

Whether in peripheral tissues, or in brain, NFATs appear to play an important role in regulating immune/inflammatory signaling, which is an important component of most all neurodegenerative diseases, including AD. To determine the extent to which NFATs 1, 2, and 3 are associated with changes in inflammatory mediators in AD brain, we measured activated protein levels for several cytokines, including IL-1 β , tumor necrosis factor α (TNF α), and granulocyte macrophage colony stimulating factor (GM-CSF) in the same postmortem samples used in the Abdul et al study (7), and compared these measures to nucNFAT levels (32). The results revealed an increase in the expression of all three cytokines with either MCI or AD. Moreover, each cytokine exhibited a significant positive correlation with nucNFAT1, as assessed within subjects. GM-CSF showed the strongest association ($r = 0.51$, $p < 0.01$), especially when evaluated across normal control cases, MCI cases, and so-called pre-clinical AD cases (*i.e.* individuals with prominent AD pathology but little to no clinical symptoms of cognitive decline). Thus, levels for several cytokine species increase as NFAT1 levels accumulate in the nucleus, consistent with a role for NFAT1 in neuroinflammation during the early stages of AD-related cognitive decline. Interestingly, none of the cytokines examined correlated significantly with NFAT3, suggesting that this isoform may have a limited role in neuroinflammation with AD.

In contrast to NFAT1, NFAT3 may have more of an impact on neurodegenerative processes, including cell death. In animal models of acute injury, NFAT3 translocates to nuclear compartments of dentate granule cells where it interacts with responsive elements within the promoter region of the pro-apoptotic Fas ligand (FasL) (30). Similar nuclear translocation of NFAT3 and FasL upregulation were observed in apoptotic cochlear neurons of mice following deafferentation (28). In both of these studies, blockade of CN/NFAT activity was neuroprotective. The link between NFAT3 and FasL is interesting as FasL protein levels are increased in the brain during end stage AD (33), similar to nucNFAT3. Moreover, recent work identified NFAT3 as a critical component of A β -mediated neuronal apoptosis (34). While these results suggest that elevated nucNFAT3 promotes or drives cell death cascades during AD, other reports have described neuroprotective properties of NFAT3. Benedito et al., (2005) found that NFAT3 mediated the pro-survival properties of serum in primary cerebellar neurons (35). Similarly, Vashishta et al., (2009) revealed anti-apoptotic effects of NFAT3 in cortical neuronal cultures following an NMDA insult (36). These results are generally consistent with earlier studies showing robust activation of NFAT3 in response to a variety of neuroprotective factors, including BDNF (37). Thus, as with other transcription factors, NFAT3 activation may promote, or prevent cell death. Additional work will be necessary to define the contextual variables that turn NFAT3 from killer to protector.

Positive feedback interactions between CN/NFAT signaling and A β

A β appears to be a primary factor for triggering aberrant CN/NFAT activity during the progression of AD. As discussed, A β robustly stimulates CN/NFAT activity in both neurons

and astrocytes. Moreover, we observed a relatively strong positive correlation ($r = 0.54$, $p < 0.01$) between nucNFAT levels (particularly nucNFAT3) and soluble A β (1–42) in the Abdul et al., 2009 study (7). However, in addition to reacting to A β , CN/NFAT activity may also help drive A β production through the transcriptional regulation of the β -site APP-cleaving enzyme 1 (BACE-1). BACE1 is the rate-limiting protease involved in the conversion of APP to pathogenic A β peptides and is upregulated in AD brain (38). In both cell cultures and intact AD model mice, NFAT1 interacts with the BACE1 promoter and stimulates BACE1 protein levels (14,39). Increased NFAT1 activation in the early stages of cognitive decline, as shown by our work (7) may therefore help potentiate amyloidosis through a BACE1 regulatory mechanism, leading to a precipitous loss of neuronal function and viability.

Working model for the role of CN/NFAT signaling in AD

From these results, we constructed a working model for the role of CN/NFAT signaling in the progression of AD (Figure 1). This model suggests that aging related changes in Ca²⁺ homeostasis, glial activation, and oxidative stress lead to an increase in A β production, aberrantly high CN activity, or both. A β and CN engage in a positive feedback cycle that exacerbates the dysregulation of both molecules. In the early stages of cognitive decline, A β -CN interactions result in synaptic dysfunction/deterioration and glial activation, which also progress in a cyclical manner (*i.e.* synaptic deterioration $\rightarrow$ glial activation $\rightarrow$ synaptic deterioration). We propose that NFAT1 is especially critical at this stage as it stimulates neuroinflammatory responses and drives further production of A β through upregulation of BACE1. As these feedback interactions progress, critical mechanisms for maintaining neuronal viability are lost, such as astrocytic glutamate uptake (see below), leading to further Ca²⁺ dysregulation and oxidative stress in neurons. During this state, NFAT3 activation is selectively increased leading to further neurodegeneration, cell death, and ultimately, dementia.

Possible use of NFAT inhibitors for treating AD?

According to this model, blockade of CN/NFAT activity at each stage should help disrupt these positive feedback cycles and slow neurodegeneration and cognitive decline. In fact, CN inhibitors (such as tacrolimus) have been shown to improve cognition (23,40), prevent degeneration of neuronal processes (22), and extend the lifespan (41) of transgenic mice expressing amyloid or tau pathology. However, the use of CN inhibitors for treating AD in humans may be limited because of the numerous serious, and possibly deadly, adverse effects associated with these drugs (42), which may be even more pronounced in elderly subjects (43). In the last 10 years, new immunosuppressive agents, such as the peptide, VIVIT, have been developed as possible alternatives to CN inhibitors. VIVIT mimics a CN docking site expressed in all CN-dependent NFATs (44), and possibly in a few other CN substrates, as well. When present, VIVIT prevents CN from binding to and activating NFATs, without suppressing CN activity. In animal models, VIVIT exerts powerful immunosuppressant/anti-inflammatory properties, and appears to be less toxic than CN inhibitors (45). Recently, we observed neuroprotective effects of VIVIT in primary cultures of neurons and astrocytes (7,29). Specifically, delivery of VIVIT to primary astrocytes prevented the downregulation of critical astrocytic glutamate transporter levels during chronic exposure to IL-1 β or A β . Effects of VIVIT were especially robust for the type II excitatory amino acid transporter (EAAT2), which contains multiple NFAT binding sites in its promoter region. Selective delivery of VIVIT to astrocytes in a mixed (neurons/glia) primary culture model limited IL- and A β -dependent elevations in extracellular glutamate and ameliorated neurotoxicity.

Based on these findings, we initiated pilot studies to investigate the effects of VIVIT in a mouse model of AD. We used Alzet miniosmotic pumps to unilaterally deliver VIVIT peptide or a scrambled control peptide, IVEET, to the cerebral ventricles of 14-month-old APP/PS1 mice (VIVIT $n = 3$, IVEET $n = 2$) over a period of 14 days (~60 $\mu\text{g/day}$). Peptides were synthesized by Synbiosci (Livermore, CA) and contained a polyarginine tag at the N terminus (*i.e.* 11R-VIVIT, 11R-IVEET) to facilitate transport across cell membranes, as previously described (45). Mice were homozygous double knock-in mutants (APP^{NLh/NLh} X PS-1^{P264L/P264L}) that express humanized A β and the P264L familial Alzheimer's disease presenilin-1 mutation. As described elsewhere (46,47), these mice show extensive amyloid pathology and/or gliosis well before 14 months-of-age. At the end of the treatment period, mice were transcardially perfused and brains fixed, and cut into 60 μm sections for immunohistochemical labeling of astrocytes, using anti-GFAP primary antibody (Cell Signaling) and HRP-coupled secondary antibody. All mice were treated in accordance with institutional animal usage guidelines.

Figure 2 shows representative photomicrographs taken of the ipsilateral and contralateral hippocampus of a mouse treated with VIVIT peptide. Note that images in Figure 2A and 2B are from the same coronal section. Relative to the contralateral hemisphere, the ipsilateral VIVIT-perfused hemisphere shows far fewer and less ramified GFAP positive cells (compare Figs 2A1 and 2A2 with Figs 2B1 and 2B2). Similar inter-hemispheric differences were observed in the remaining two VIVIT-treated mice. Note also that the ipsilateral hippocampus of VIVIT-treated mice shows fewer GFAP positive cells relative to the ipsilateral hemisphere of IVEET-treated mice (compare Figs 1A1 and A2 with Figs 2C1 and 2C2). Though preliminary, these data suggest that VIVIT treatment blunts or limits astrocyte activation in APP/PS1 mice and may therefore be useful in treating synaptic dysfunction, amyloid pathology, and cognitive decline in this, and other animal models of AD.

Of course, it is possible that inhibition of CN/NFAT activity and blockade of astrocyte activation in intact animals could have detrimental rather than beneficial effects. Indeed, several studies have shown that glial activation helps clear amyloid peptides from the interstitial space (48,49). In addition, Fernandez et al. (2007) found that transgenic mice overexpressing an activated form of CN in astrocytes were less vulnerable to neurotoxicity following an acute trauma (26). Anti-inflammatory effects of VIVIT, as shown in Figure 2, may therefore worsen amyloid pathology and hasten cognitive decline in animal models. On the other hand, chronic neuroinflammation observed in AD (or amyloidogenic animals) and inflammation arising from acute neural injury may have fundamentally different effects on neuronal viability. Ongoing work in our lab is therefore testing the effects of VIVIT in multiple animal models of injury and amyloidosis.

Conclusions and future directions

Mounting evidence from our laboratory and from several other groups suggest that aberrant CN/NFAT signaling significantly contributes to the pathologic and clinical features of AD. Interestingly, different CN/NFAT signaling pathways appear to be activated at different stages of AD: NFAT1 is selectively activated early in cognitive decline, while NFAT3 is recruited later in dementia. The precise isoform-specific functions of NFATs in nervous tissue, and their respective roles in AD, will require extensive investigation. Finally, CN inhibitors show promising anti-inflammatory, neuroprotective, and/or cognitive enhancing properties in a variety of experimental models of AD. Whether the beneficial properties of these drugs offset their numerous adverse effects remains to be seen. In addition to CN inhibitors, reagents with greater selectivity to NFAT activation (*e.g.* VIVIT) also hold promise for treating neurodegeneration. Studies are presently underway in our lab to test the

neuroprotective and nootropic properties of VIVIT in multiple experimental models of injury and disease.

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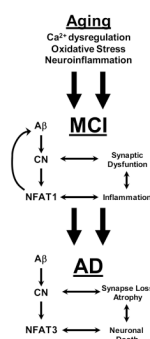


Figure 1. Working model for the role of CN/NFAT signaling in AD

During aging, gradual dysregulation of Ca^{2+} , oxidative stress, and/or neuroinflammation lead to increased production of $\text{A}\beta$, increased activation of CN or both. In turn, aberrant CN activity leads to synaptic dysfunction and neuroinflammation, in part through increased stimulation of NFAT1. This chain of events leads to mild cognitive impairment (MCI). Note that cyclical interactions between neuroinflammatory processes (*i.e.* glial activation) and synaptic dysfunction) can maintain the CN/NFAT1 pathway in an activated state, which helps perpetuate deleterious neuronal and glial activities. NFAT1 also helps exacerbate amyloid toxicity through upregulation of BACE1 (see text). Eventually, these interactions progress to a degenerative state (*i.e.* Alzheimer's disease) characterized by severe amyloidosis and severe Ca^{2+} dyshomeostasis leading to greater CN activity. CN triggers neuritic atrophy and cell death cascades through preferential activation of NFAT3.

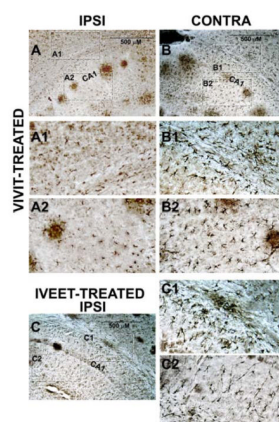


Figure 2. The NFAT inhibitor, VIVIT, reduces astrocyte activation in AD mice

A and B, photomicrographs of a GFAP-labeled coronal section from a 14-month-old APP/PS1 mouse treated intraventricularly for two weeks with 11R-VIVIT. Dashed boxes indicate regions of higher magnification shown in A1 and A2 (ipsilateral, VIVIT-treated hemisphere—Ipsi) and B1 and B2 (contralateral hemisphere—contra). C, GFAP-labeled coronal section (Ipsilateral hemisphere) from a 14-month-old APP/PS1 mouse treated for two weeks with 11R-IVEET control peptide. Dashed boxes indicate regions of higher magnification shown in C1 and C2. IVEET-treated mice and the contralateral hemisphere of VIVIT-treated mice appear to express more numerous and more ramified astrocytes relative to the ipsilateral hemisphere of VIVIT-treated mice. CA1 = cornu ammonis 1 pyramidal cell layer.

Changes in nuclear protein levels for each NFAT isoform (nucNFATs) in patients with mild cognitive impairment (MCI) and Alzheimer’s disease (AD) and their relationships with other nuclear-localized proteins: CN A α , CN A β , and GSK3- β , as reported in Abdul et al., 2009

Table 1

	MCI	AD	Correlation with nucCNA α	Correlation with nucCNA β	Correlation with nucGSK3 β
nucNFAT1	Up	Down Slightly	Yes, Positive	Yes, Positive	No
nucNFAT2	No Δ	No Δ	No	No	No
nucNFAT3	Down Slightly	Up	Yes, positive	No	No