Dysfunctional intracellular calcium homoeostasis: a central cause of neurodegeneration in Alzheimer’s disease

Cora O’Neill*, Richard F. Cowburn†, Willy L. Bonkale†, Thomas G. Ohm‡, Johan Fastbom†, Mark Carmody* and Mary Kelliher*

*Department of Biochemistry, University College, Lee Maltings, Prospect Row, Cork, Ireland, †Karolinska Institute, NEUROTEC, Section for Geriatric Medicine, NOVUM, KFC, S-141 86, Huddinge, Sweden, and ‡Institute fur Anatomie, University Klinikum Charité, D-10098 Berlin, Germany

Abstract

The clinical symptoms of all forms of Alzheimer’s disease (AD) result from a slowly progressive neurodegeneration that is associated with the excessive deposition of β-amyloid (Aβ) in plaques and in the cerebrovasculature, and the formation of intraneuronal neurofibrillary tangles, which are composed primarily of abnormally hyperphosphorylated tau protein. The sequence of cellular events that cause this pathology and neurodegeneration is unknown. It is, however, most probably linked to neuronal signal transduction systems that become misregulated in the brains of certain individuals, causing excessive Aβ to be formed and/or deposited, tau to become aggregated and hyperphosphorylated and neurons to degenerate. We hypothesize that a progressive alteration in the ability of neurons to regulate intracellular calcium, particularly at the level of the endoplasmic reticulum, is a crucial signal transduction event that is linked strongly to the initiation and development of AD pathology. In this chapter we will discuss the key findings that lend support to this hypothesis.

*To whom correspondence should be addressed.
Cellular mechanisms proposed to underlie the neurodegeneration of AD: where does calcium fit in?

Calcium (Ca$^{2+}$) has long been implicated as a key life and death signal for neurons [1] and many studies support a key role for deregulated Ca$^{2+}$ homoeostasis in the neurodegenerative processes of AD [2–4]. Although it is indisputable that Ca$^{2+}$ is involved in the neurodegeneration of AD, it has been difficult to evaluate whether dysfunctions in neuronal Ca$^{2+}$ metabolism are a proximal cause or an associated consequence of the disease pathology. For dysfunctional Ca$^{2+}$ homoeostasis to be a primary cause of the neurodegeneration of AD, it should occur early on in the disease and have the potential to cause progressive β-amyloid deposition, abnormal tau hyperphosphorylation and selective neuronal cell death. Evidence outlined in this chapter indicates that this could be the case. Findings discussed below also support the possibility that dysfunctional Ca$^{2+}$ homoeostasis is a driving force for AD pathology, secondary to increased Aβ, increased C-100 [the 100 C-terminal amino acids of the β-amyloid precursor protein (APP)] and/or reciprocal decreased sAPPα (a secreted product of APP processing) in the disease. Recent findings have also shown that mutations in presenilin 1 and presenilin 2, which cause familial forms of AD, deregulate intracellular Ca$^{2+}$ homoeostasis [5]. Together, this data suggests that altered Ca$^{2+}$ homoeostasis may be a common cellular instigator for neurodegeneration in both sporadic and familial forms of AD.

Neuronal Ca$^{2+}$ homoeostasis

Intracellular Ca$^{2+}$ ([Ca$^{2+}$]i) is a critical neuronal second messenger playing a vital role in synaptic transmission and the maintenance of neuronal function and survival [1,5]. Neurons possess elaborately controlled systems for regulating and responding to changes in [Ca$^{2+}$]i because of its crucial and powerful signalling role. (Figure 1 shows a simplified scheme of the mechanisms which control [Ca$^{2+}$]i homoeostasis.) Briefly, intraneuronal Ca$^{2+}$ is derived from sources both outside and inside the cell. Ca$^{2+}$ enters the cell through voltage- and ligand-operated ion channels and is also released within the cell from endoplasmic reticulum (ER) stores by activation of Ins(1,4,5)P$_3$ (IP$_3$) receptors (IP$_3$Rs) and ryanodine receptors (RyRs). In general IP$_3$Rs respond to IP$_3$ which is produced through agonist activation of the phosphatidylinositol signalling pathway, whereas RyRs are directly responsive to [Ca$^{2+}$]i, so-called Ca$^{2+}$-induced Ca$^{2+}$ release.

A variety of other proteins play a crucial role in maintaining [Ca$^{2+}$]i homoeostasis. These include: the SERCA (sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase) pump, which accumulates Ca$^{2+}$ into the ER; Ca$^{2+}$-ATPase and Na$^+/Ca^{2+}$ membrane exchangers in the plasma membrane, which move Ca$^{2+}$ out of the cell; and Ca$^{2+}$-binding proteins which sequester free Ca$^{2+}$. The mitochondria also sequester some of the Ca$^{2+}$ signal and Ca$^{2+}$ shuttles between the ER and mitochondria. The maintenance of [Ca$^{2+}$]i homoeostasis in neu-
APP metabolism and neuronal calcium homoeostasis

The influence of Ca\(^{2+}\) on APP processing

Altered APP processing to overproduce A\(\beta\)42/43 is believed to be a primary event in the pathological cascade that is AD [6]. Proteolytic degradation of APP, by the combined action of two proteases dubbed \(\beta\)-secretase and \(\gamma\)-secretase, produces the A\(\beta\) peptide [6]. \(\beta\)-secretase cleaves APP immediately N-terminal to the A\(\beta\) sequence yielding a large, soluble, secreted APP fragment, sAPP\(\beta\), and a C-100 fragment. C-100 is subsequently processed by \(\gamma\)-secretase to yield A\(\beta\) which is predominantly 42/43 and 40 amino acids long. These A\(\beta\) peptides are secreted but can also be found inside neurons. A\(\beta\)42 accounts for only 10% of the secreted A\(\beta\) but is the major fibrillogenic species found accumulated in senile plaques of AD. APP can also be endoproteolyti-
cally cleaved at the cell surface within the Aβ sequence by α-secretase generating sAPPα and the non-amyloidogenic 3 kDa (p3) Aβ peptide.

It has been widely shown that APP processing can be modulated by cell signal transduction pathways including Ca²⁺ ([7]; see also chapter 16). Of interest, firstly with respect to intracellular Ca²⁺ signalling, is the finding that Aβ42 localizes to the ER in neurons but does not appear to do so in other cell types [8,9]. This suggests that the ER may be an important site for generating Aβ42. In agreement with this studies have shown that activation of ER Ca²⁺ release from RyRs in HEK-293 cells increased total Aβ and p3 secretion four-fold [10]. This effect was potentiated by thapsigargin and cyclopiazonic acid agents which raise [Ca²⁺]i by preventing its reuptake into the ER [10]. Other studies have reported that thapsigargin could either increase or decrease Aβ release, depending on the concentration used, and that it could increase formation of soluble APP (sAPP) from CHO cells overexpressing APP751, following down-regulation of protein kinase C (PKC) [11]. In contrast to these findings, Nitsch and colleagues did not observe any changes in the levels of sAPP release in 3T3 cells following treatment with thapsigargin [12]. Treatment of cells with the Ca²⁺ ionophore A23187 has also been shown to up-regulate total Aβ production, this effect appeared to be dependent on extracellular Ca²⁺ [13]. The exact relationship between the release and uptake of Ca²⁺ and the processing of APP in the ER remains to be defined, particularly in neurons where APP processing differs from that detected in cell lines of peripheral origin [14]. The mechanism(s) underlying the effect of disrupted ER Ca²⁺ signalling on APP processing may involve alteration of the exit or export of APP or C-100 from the ER, and/or alteration in the regulation of Ca²⁺-dependent cofactors/proteins controlling the secretase enzymes, or indeed direct effects of Ca²⁺ on the secretase enzymes that cleave APP. In this respect, the regulation of ER Ca²⁺ release exerted by the presenilin proteins may influence their role in γ-secretase processing of APP (see section on presenilins).

The influence of Aβ, C-100 and sAPP on Ca²⁺ homoeostasis

A greater quantity of work has focused upon the effects of APP and its metabolites on Ca²⁺ homoeostasis than on the effect of Ca²⁺ homoeostasis on APP processing. The overall outcome of this work suggests that overproduction of Aβ and its precursor C-100 or diminished production of sAPPα has a primary effect on neuronal Ca²⁺ homoeostasis. This is proposed to initiate a pathological neuronal cell death cascade that could potentially be inhibited by blocking these detrimental Ca²⁺ responses. Work examining the effect of Aβ on Ca²⁺ homoeostasis has been performed in a variety of cell culture systems using a broad array of Aβ types, Aβ preparation methods and Aβ concentrations. Primarily, studies have used micromolar concentrations of Aβ40 and Aβ25–35 peptides with fewer studies using Aβ42. It is still unclear which species of Aβ is neurotoxic, e.g. protofibrils, oligomers or higher-molecular-mass species (see chapter 1), and whether the Aβ is only neurotoxic outside the neuron or can be toxic upon intracellular production. Results have shown that Aβ peptides can induce neurotoxicity by increasing [Ca²⁺]i [15] which can involve enhanced excitatory-amino-acid- and Ca²⁺-ionophore-induced Ca²⁺
flaxes [16–18]. In agreement with this, Aβ peptides have been shown to affect Ca\(^{2+}\) uptake into PC12 cells [19] and cortical and hippocampal neurons [20]. They have also been shown to increase the activity of Ca\(^{2+}\)-channel proteins, including the L-type voltage-operated Ca\(^{2+}\) channels [21,22], possibly through mitogen-activated protein (MAP) kinase phosphorylation of the channel [23], and N-type voltage-gated Ca\(^{2+}\) channels [24]. The IP\(_3\) Ca\(^{2+}\)-release channel has been shown to exhibit enhanced \(^{3}H\)IP\(_3\) binding in the presence of Aβ peptides [25] and also to mobilize Ca\(^{2+}\) in response to Aβ peptides [26]. The involvement of voltage-operated Ca\(^{2+}\) channels in Aβ-induced neurodegeneration is further confirmed by findings that Aβ-induced neurodegeneration can be blocked by nimodipine and Co\(^{2+}\) [21,22]. Aβ peptides have also been found to impair K\(^{-}\)-dependent Ca\(^{2+}\) flux [27,28] and to block Na\(^{+}/K\(^{-}\)ATPase activity, thus indirectly impairing the removal of excess [Ca\(^{2+}\)]\(_{i}\) by ion-exchange pumps [29,30].

The mechanism(s) by which Aβ affects such a range of the cellular Ca\(^{2+}\) signalling machinery and the relevance of the in vitro work to the situation that occurs in vivo is unknown. The observation that Aβ40 [31,32] and Aβ42 [33] can insert into synthetic lipid bilayers and reconstituted lipid vesicles as a Ca\(^{2+}\)-carrying ionophore may be important. In this respect the localization of Aβ42 to the ER in neurons and the identification of specific binding sites for Aβ in the ER [34] suggest the possibility that Aβ42 may disrupt ER Ca\(^{2+}\) homoeostasis by inserting into ER membranes. The exact sequence of signalling events by which Aβ-induced increases in [Ca\(^{2+}\)]\(_{i}\) cause neuronal cell death is not entirely clear but it has been proposed to trigger intracellular events that eventually cause cell dysfunction and death, including oxidative stress and activation of apoptotic cell death pathways.

C-100, cleaved from APP by β-secretase, which includes the Aβ sequence has also been shown to affect Ca\(^{2+}\) homoeostasis. Studies have shown that PC12 cells expressing C-100 show altered Ca\(^{2+}\) handling [35] and exhibit enhanced sensitivity to the Ca\(^{2+}\) ionophore A23187 [36], with this peptide also showing the ability to propagate intercellular Ca\(^{2+}\) waves [37]. Further studies have shown that this APP fragment inhibits the Na\(^{+}/\)Ca\(^{2+}\)-exchanger activity in SK-N-SH cells [38] and decreases the activity of the Mg\(^{2+}/\)Ca\(^{2+}\)-ATPase of the ER, suggesting that this peptide, which is only found inside neurons, can inhibit the ability of the ER to sequester Ca\(^{2+}\) in AD [39].

sAPP\(_{α}\) has also been found to modulate [Ca\(^{2+}\)]\(_{i}\), where it normalizes [Ca\(^{2+}\)]\(_{i}\) increases induced by cellular insults including Aβ. Increases in the second messenger cGMP and its responsive cGMP-dependent kinase [40], and activation of the transcription factor nuclear factor κB (NF-κB) [41], have been shown to be involved in the Ca\(^{2+}\)-normalizing effect of sAPP\(_{α}\). sAPP\(_{α}\) has also been found to activate K\(^{+}\) channels [42], an effect that was mimicked by cGMP and that reduced Ca\(^{2+}\) influx [42,43], suggesting that activation of K\(^{+}\) channels and membrane hyperpolarization may be the primary neuroprotective target of sAPP\(_{α}\). In summary it is believed that increased production of Aβ in association with decreased production of sAPP\(_{α}\) will have detrimental effects on the neuronal Ca\(^{2+}\) signalling machinery activating cell death pathways in the AD brain.
Presenilin modulation of Ca\(^{2+}\) homeostasis

Disrupted Ca\(^{2+}\) homeostasis may be a common cellular pathway for neurodegeneration both in sporadic and familial forms of AD. This evolves from findings showing that presenilin 1 (PS1) and presenilin 2 (PS2) mutations which cause familial forms of AD, cause disruptions in intracellular Ca\(^{2+}\) homeostasis which can cause neurodegeneration. The effect of presenilin mutations on Ca\(^{2+}\) homeostasis primarily targets the ER, affecting both IP\(_3\)-R- and RyR-induced Ca\(^{2+}\) release (see chapter 15). This fits in with the cellular localization of the presenilins, which are detected predominantly in the ER [44–46]. Results have shown that familial AD-causing PS1 mutations enhance Ca\(^{2+}\) release in a variety of systems including: cells transfected with PS1 mutants [47,48]; mutant PS1 knockin mice [49]; synaptosomes prepared from transgenic mice expressing mutant PS1 [50]; and fibroblasts from patients with familial AD [51,52]. Increased [Ca\(^{2+}\)]\(_i\) levels and resultant neurotoxic responses to A\(_\beta\) and other stressors induced by PS-1 mutations could be blocked by dantrolene, an inhibitor of RyR-induced Ca\(^{2+}\) release [47,48,50], suggesting that the neurodegenerative effect induced by PS1 mutations involves disruption of RyR-induced Ca\(^{2+}\) release from the ER. Recent results confirm this and have shown that PS2 interacts with the Ca\(^{2+}\)-binding protein sorcin [53], a known modulator of RyR activity [54], which influences the interaction of RyRs with L-type Ca\(^{2+}\) channels [55], and that RyR expression levels are enhanced by PS1 mutations (see chapter 15). PS1 mutations have also been found to affect the IP\(_3\) receptor, where IP\(_3\)-evoked Ca\(^{2+}\) signals were found to be potentiated significantly by PS1 mutants, in Xenopus oocytes [56]. Furthermore, fibroblasts from patients with PS1 mutations, and PC12 cells transfected with PS1 mutants, exhibit increased Ca\(^{2+}\) release from intracellular stores in response to agonists that activate the phosphatidylinositol signal transduction system [47,51,52].

The importance of ER Ca\(^{2+}\) release in the neurodegeneration of AD is shown by findings which demonstrate that blocking enhanced ER Ca\(^{2+}\) release, particularly with dantrolene which targets the RyR can protect against the neurodegeneration initiated by PS mutations and A\(_\beta\). The relationship between PS mutant-induced increases in A\(_\beta\)42 production and ER Ca\(^{2+}\) homeostasis remains to be determined. In this respect it is of importance to investigate whether increased A\(_\beta\)42 production is a cause or consequence of ER Ca\(^{2+}\)-release dysfunction.

Calcium signalling and tau hyperphosphorylation

Tau proteins are the predominant component of the paired helical filaments (PHFs) in intracellular neurofibrillary tangles (NFTs), neuritic threads and neuritic plaques of the AD brain. PHF-tau differs from normal tau by several post-translational modifications, including hyperphosphorylation, altered glycosylation and altered proteolysis. Tau hyperphosphorylation is regarded as the major driving force for PHF formation and severely affects tau function,
reducing its ability to bind to and stabilize microtubules, thus leading to disturbances in neuronal microtubular function in the AD brain [57,58].

Tau hyperphosphorylation must ensue through deregulation of one or several signal transduction cascades regulating kinases and/or phosphatases targeting tau [57]. However, the cellular mechanisms contributing to phosphorylation of tau to form PHFs in AD are still unclear and appear to be complex. Presently the consensus is that the proline-directed kinases, including: glycogen synthase kinase-3 (GSK-3); cyclin-dependent kinases, cdc(2) (cell division control) and CDK5 (p35); and MAP kinases, extracellular signal-related protein kinase, c-jun N-terminal kinase and p38 MAP kinase, are prominently involved in tau phosphorylation [57]. Of these, GSK-3β is believed to be a crucial physiological kinase for tau, and studies have shown that GSK-3 preferentially localizes to NFT-bearing neurons [59] and that the distribution of the active form of the enzyme parallels the spread of AD neurofibrillary changes [60]. Phosphorylation of tau by non-proline-directed kinases also occurs. These include protein kinase A (PKA), myristoylated alanine-rich C-kinase, calcium/calmodulin-dependent kinase II (CaM kinase II), casein kinase I and II and protein kinase C (PKC), some of which have been shown to act synergistically with the proline-directed kinases in the phosphorylation of tau [57]. The protein phosphatases PP2A, PP2B and PP1 also have important roles in the regulation of the phosphorylation state of tau and can dephosphorylate tau that has been phosphorylated by many of the above kinases, converting it to a normal form [61]. It has also been shown that PP2A and PP2B can catalyse tau dephosphorylation in PHFs prepared from AD brains [62].

Intracellular Ca²⁺ signals of varying amplitude and frequency are vital regulators of cellular kinase and phosphatases [63,64]. It is therefore possible that hyperphosphorylation of tau is an event downstream of a primary defect of [Ca²⁺][i] homoeostasis. In this respect it has been shown that Ca²⁺ can control many of the kinases and phosphatases implicated in tau phosphorylation including GSK-3β, the MAP kinase family, the cyclin-dependent kinases and many of the non-proline-directed kinases, particularly PKA, PKC and CaM kinase II, plus the Ca²⁺-dependent phosphatase PP2B or calcineurin. Transient increases in [Ca²⁺][i] have been shown to cause persistent tyrosine phosphorylation and activation of GSK-3β resulting in prolonged site-selective increases in tau phosphorylation [65]. It has also been shown that Ca²⁺ influx through the N-methyl-d-aspartate (NMDA) receptors dephosphorylates tau [67]. Impaired Ca²⁺ influx through the NMDA receptor may thus cause tau hyperphosphorylation possibly through down-regulation of the CaM kinase II–Akt-pathway [68] which would cause activation of GSK-3β.

Results have shown that calcineurin can dephosphorylate tau at select sites [62] and can revert tau that has been phosphorylated in vitro by CaM kinase II, PKA, MAP kinase and cdc(2) kinase to a normal-like state [61] that can bind to microtubules and promote tubulin polymerization. These results suggest that impaired calcineurin activity in the AD brain can lead to neurofibrillary pathology. Ca²⁺ has been shown to regulate other aspects of tau function other than the kinases and phosphatases that phosphorylate tau. These
include the ability to induce aggregates of PHF-tau but not normal human tau [69], and the proteolysis of tau by calpains [70].

Studies of the direct effect of altering \([\text{Ca}^{2+}]_i\) on the state of tau phosphorylation within cells have for the most part not investigated the response to changes in the amplitude and frequency of the \([\text{Ca}^{2+}]_i\) signal, which plays an important role in mediating phosphorylation-dependent cell function. Rather, these studies have simply totally up-regulated intracellular \([\text{Ca}^{2+}]_i\) using agents such as \([\text{Ca}^{2+}]_i\) ionophores. These studies have found that the treatment of primary neuronal cultures with \([\text{Ca}^{2+}]_i\) ionophores both increases [17,71] and decreases [72] tau phosphorylation. \([\text{Ca}^{2+}]_i\) ionophore treatment of human neuroblastoma cells was also shown to result in both increases [73] and decreases [74] in tau phosphorylation. Further studies have shown that increasing intracellular \([\text{Ca}^{2+}]_i\) by activation of NMDA receptors results in the dephosphorylation of tau in rat brain slices [75] and cortical neuronal cultures [72]. More in-depth studies are needed to define which aspects of \([\text{Ca}^{2+}]_i\) signalling control tau phosphorylation, and how this relates to the effects of \([\text{Ca}^{2+}]_i\) on APP metabolism.

**\([\text{Ca}^{2+}]_i\) signal transduction dysfunction in the AD brain**

Regulation of \([\text{Ca}^{2+}]_i\) homoeostasis is impaired in the post-mortem AD brain when compared to control brains, which correlates with the *in vitro* work outlined above. This occurs at a number of levels including alterations in \([\text{Ca}^{2+}]_i\)-channel proteins and the status of many \([\text{Ca}^{2+}]_i\)-activated enzymes, such as key neuronal proteases, kinases and phosphatases. Prominent among the \([\text{Ca}^{2+}]_i\)-channel defects are critical alterations in the IP\(_3\)R and RyR \([\text{Ca}^{2+}]_i\)-release channels of the ER, which are affected at very early stages of AD pathology and in the specific brain areas which are damaged in the disease. Work by our groups suggests that a primary, but slowly progressive impairment in the control of \([\text{Ca}^{2+}]_i\) release from the ER in AD brain, would have a downstream effect on an array of vital \([\text{Ca}^{2+}]_i\)-regulatable systems and on ER function in neurons, thus causing progressive neuronal damage and AD pathology and neuronal loss. Figure 2 shows the proposed model.

**IP\(_3\)Rs and RyRs in AD**

The activation of IP\(_3\)R and RyR ER \([\text{Ca}^{2+}]_i\)-release channels plays a crucial role in regulating diverse \([\text{Ca}^{2+}]_i\) signals and an array of neuronal functions such as excitability, release of transmitters, synaptic plasticity, gene expression and neuronal survival, being central to efficient receptor communication and coordination in brain [1]. Disruption of efficient ER \([\text{Ca}^{2+}]_i\) release would be expected to have a negative effect on all of the above neuronal functions, many of which are known to be impaired in the AD brain.

The first evidence suggesting that \([\text{Ca}^{2+}]_i\) release from the ER was impaired in the AD brain *post mortem* was provided by Young and colleagues [76] who reported significant reductions in the number of \([^3\text{H}]\text{IP}_3\) binding sites in
homogenates of AD parietal cortex and hippocampus, but not in the frontal, occipital or temporal cortices, when compared to control brains. This study was confirmed by Garlind and colleagues [77], who detected reduced [3H]IP3 binding in the same brain regions and also in AD temporal cortex, frontal cortex and cerebellum when compared to matched control brains. Subsequently it was reported that reduced IP3R levels in the AD temporal cortex, as determined by immunoblotting (using an antiserum that did not discriminate between IP3R isoforms), correlated with a semi-quantitative score for neuritic plaque and NFT accumulation [78]. In agreement with this it has been shown that IP3R loss in the entorhinal cortex, subiculum and CA1 pyramidal layer of the anterior hippocampus correlated with the staging for both neurofibrillary changes and amyloid deposition [79]. These latter results strongly suggest that progressive loss of IP3R and [3H]IP3 binding sites in AD is linked mechanistically to the development of disease pathology.

Historically RyRs have been little examined in human brain and not at all in neurodegenerative disorders. This has been an oversight as RyRs may be as or more important than IP3Rs when considering human brain Ca2+ homoeostasis in AD. This is owing to the presence of RyR, particularly RyR-2, in areas that are susceptible to degeneration in AD [80,81] and to the fact that the unit conductance for RyR is about 10 times that of IP3R. Our group was the first to examine the integrity of the RyR in the AD brain [82]. These studies

**Figure 2 Simplified model outlining mechanisms by which disrupted neuronal Ca2+ release from the ER may cause the neurodegeneration and pathology of AD.**
found that the $B_{\text{max}}$ (maximum number of binding sites) for $[^3H]$ryanodine binding was reduced significantly in areas of the brain affected by AD pathology (temporal cortex) but not in areas which are relatively unaffected pathologically (putamen, occipital cortex). This reduction in $B_{\text{max}}$ was due in part to loss of the RyR-2 isoform, which was the only RyR isoform detected in the human brain temporal cortex. Deficiencies in the ability of magnesium and Ruthenium Red to inhibit $[^3H]$ryanodine binding were also found, suggesting that the RyRs are impaired functionally in AD brain. Autoradiographical analysis of $[^3H]$ryanodine binding in sections of entorhinal cortex and anterior hippocampus staged for disease pathology, according to a protocol devised by Braak and Braak [83], found that $[^3H]$ryanodine binding was significantly and selectively increased in areas of the hippocampus (CA1, subiculum) which succumb to early (Stage I–II) neurofibrillary pathology, compared to brain sections from matched neurologically normal control cases. This up-regulation effect was selective for RyRs, as $[^3H]$IP$_3$ or $[^3H]$phorbol 12,13-dibutyrate binding was not increased in these same sections [79]. Following on from this, $[^3H]$ryanodine binding decreased progressively in a fashion that correlated with the advancement of neurofibrillary pathology in the hippocampus and also with Aβ build-up in some hippocampal regions. At the end-stage of the disease, loss of $[^3H]$ryanodine binding was extremely severe in the hippocampal sections (74–84%), suggesting that RyR Ca$^{2+}$-release function is radically impaired once neurofibrillary pathology becomes severe (Stage V–VI) [82]. This would have severe repercussions for efficient neuronal function. We believe that the initial up-regulation of $[^3H]$ryanodine binding and increased RyR Ca$^{2+}$ release may precede initial neuronal pathology in vulnerable brain regions.

Further studies in our group have shown that levels of cytosolic FKBP12 (FK506-binding protein MW 12 kDa), a key modulatory protein for both RyRs and IP$_3$Rs [84], is significantly and selectively reduced in brain regions affected by the pathology of AD [85]. This loss of FKBP12 correlates positively with the loss of RyR-2 and $[^3H]$ryanodine binding in the disease ([85] and M. Carmody, M. Kelliher and C. O’Neill, unpublished work) suggesting that loss of FKBP12 may be linked mechanistically to RyR-2 dysfunction in the disease. Loss of FKBP12 is known to have severe repercussions on RyR function, rendering these calcium channels ‘leaky’ and unable to exhibit full conductance states [84,86], a situation that may thus occur in the AD brain.

Such an alteration in both RyRs and IP$_3$Rs as detected in AD would be expected to put the ER under progressive and eventually substantial stress, a phenomenon that has been hypothesized to underlie neuronal degeneration [5] and which will influence vital ER functions including protein folding, secretion, aggregation and export [87]. This impairment of RyR and IP$_3$R responses would also severely compromise key neuronal signal transduction pathways that underlie cognitive and higher mental function. Inherent in this is impaired function of the vast array of enzymes and binding proteins that are controlled by temporal and spatial increases in [Ca$^{2+}$]i brought about by RyR and IP$_3$R which is discussed in the sections below.
**Ca\(^{2+}\)**-dependent enzymes in the AD brain

Many key Ca\(^{2+}\)-regulated enzymes have been found to be impaired in AD brain. These include: proteases, including the calpains; kinases, including PKC; CaM kinase II; the phosphatase calcineurin; and oxidative stress-linked enzymes including nitric oxide synthase (NOS) and phospholipase A\(_2\) (PLA\(_2\)). Other key Ca\(^{2+}\)-regulated signalling molecules such as adenylate cyclase and phospholipase C are also dysfunctional in AD brain and are discussed in chapter 16.

**Ca\(^{2+}\)**-dependent proteases in the AD brain

One of the most thoroughly studied groups of Ca\(^{2+}\)-dependent proteases are the calpains, which are Ca\(^{2+}\)-activated cysteine proteases. There are two isoenzymes, calpain I and calpain II, which require micromolar and millimolar levels of Ca\(^{2+}\) respectively for full activity in vitro. These proteases regulate protein function by limited proteolysis. Both isoforms have been found to be consistently up-regulated in the brains of patients with AD [88–90]. Grynspan and colleagues [88] have shown that active calpain II accumulates in NFTs and is an early-appearing pervasive component of the neurofibrillary pathology in the disease. Studies by Tsuji and colleagues [91] have detected significantly increased levels of calpain II in cytosolic fractions from AD brains post mortem when compared to control tissue. It is possible that increased Ca\(^{2+}\) release from RyRs is responsible for calpain II activation in the hippocampus at early stages of AD although this has not been investigated. Calpain II has been shown to be activated at very early stages of neurofibrillary pathology in the entorhinal cortex and hippocampus. This would be consistent with the up-regulation of \(^{[\text{3H}]\text{ryanodine}}\) binding sites observed by us in these regions at early stages in the development of neurofibrillary pathology. Calpain mobilization and its long-term and abnormal activation are believed to be a major contributor to proteolysis and removal of cytoskeletal fragments during NFT formation. It is also believed to play an important role in aggregation events, particularly of the cytoskeleton, while also cleaving and modulating kinases and phosphatases that modulate cytoskeletal proteins including tau.

Calpain activity is thought also to be necessary for apoptotic cell death in certain systems [92,93] and to proteolytically cleave enzymes and proteins involved in apoptosis. Amongst these are the caspase protease family which are believed to play a key role in apoptotic cell death pathways in the nervous system [92] and have been shown to be regulated by [Ca\(^{2+}\)]i and calpains [92,94,95]. Work is presently emerging to support a role for the caspase protease family in the neurodegeneration of AD [96,97]. An extensive study of the integrity of these cysteine proteases in the AD brain has not been performed. However, studies have detected a 50–100% elevation in caspase I or interleukin-1β-converting enzyme (ICE) in the hippocampus of AD brains compared to controls [98], with overexpression of ICE-α cDNA also reported in the AD brain [99]. Furthermore, transcription factors activated by caspase-1, such as NF-κB, can be induced by elevated [Ca\(^{2+}\)]i and drive important
responses to neuronal stress. Activated NF-κB p65 has been shown to be up-regulated in the AD brain [100] where it has been found to associate with the plaque pathology of the disease [101,102].

**Ca^{2+}-activated kinases in the AD brain**

Central and critical alterations in the PKC family of enzymes have been described in the brains of AD patients. PKC dysfunction has been the most consistent and reported alteration in the phosphoinositide signalling pathway in AD brain and is discussed in detail in chapter 16. Disrupted intracellular Ca^{2+} homoeostasis may be a driving force for PKC dysfunction in AD as the conventional PKC isoforms (α, βI, βII and γ) are regulated by Ca^{2+} and show reduced activity in AD brain compared to control tissue.

CaM kinase II is one of the most abundant kinases in the brain and has a key role in memory function and synaptic plasticity [103,104]. This Ca^{2+}-dependent kinase is widely expressed in neurons vulnerable to AD pathology and has been shown to be differentially modulated in AD, where it is shown to be associated with PHF [105,106]. The finding that GSK-3β is overactive in the brains of AD patients compared to controls, and that the active form of the enzyme co-localizes with NFTs in the disease, suggests that the enzyme is mis-regulated in AD. This altered regulation may include loss of the normal inhibitory input to GSK-β induced by growth factors, wingless (wnt)/β-catenin signalling and also by CaM kinase II activation, all of which can increase the kinase activity of Akt to phosphorylate and inactivate GSK-3β.

**Ca^{2+}-dependent phosphatases in the AD brain**

PP2B or calcineurin is expressed at very high levels in the brain and can dephosphorylate PHF-tau in vitro as mentioned above. Calcineurin has a variety of roles in the nervous system including: the regulation of Ca^{2+} release from IP_{3}R and RyR, neurotransmitter release, synaptic plasticity and neuronal survival/apoptosis. Studies examining calcineurin in the brain post mortem detected a co-localization of calcineurin with NFT and a selective reduction of calcineurin expression in neurons containing NFTs [59]. Furthermore, knock-out mice lacking the gene for the calcineurin catalytic subunit show accumulation of phosphorylated tau in the hippocampus [107]. Initial immunoblot analysis did not detect any difference in total levels of calcineurin when co-mapping homogenates prepared from AD and matched control brains [108,109]. However, more detailed studies by Ladner and colleagues [110] detected reduced calcineurin activity in the AD brain, which correlated inversely with NFT and neuritic plaque density and which was selective to areas pathologically affected by the disorder.
The integrity of Ca\(^{2+}\)-activated enzymes involved in Ca\(^{2+}\)-induced oxidative stress pathways in the brain, including the neuronal and endothelial isoforms of NOS (nNOS and eNOS) and PLA\(_2\), has been examined in AD brains. NOS has received the most attention, owing to the key role that NO is believed to play in neurodegenerative pathways. Results from investigations examining the integrity of NOS in AD brain have been somewhat contradictory. Thus some investigations have shown nNOS-containing neurons to be susceptible to neurodegeneration, with decreased levels of nNOS detected in the entorhinal cortex and hippocampus of AD brains [111,112] compared to controls. Whereas other investigations have reported nNOS-containing neurons to be resistant to the neurodegeneration of AD [113]. The eNOS enzyme has been reported to be aberrantly expressed in the AD brain compared to control brain tissue [114]. Studies performed in our group have detected a selective impairment of the nNOS isoform in areas pathologically affected by AD with highly significant reductions in nNOS levels in the presence of unaltered nNOS activity, suggesting a loss of control of this enzyme in AD ([115] and M. Kenny, R. Ravid and C. O’Neill, unpublished work).

PLA\(_2\) activates the arachidonic acid cascade leading to the generation of multiple eicosanoids, many of which are believed to play a role in the inflammatory process. PLA\(_2\) is activated by very low levels of Ca\(^{2+}\) and thought to control receptor-mediated eicosanoid production. Ross and colleagues have detected significant reductions in PLA\(_2\) activity, both in the presence and absence of Ca\(^{2+}\) in the parietal and temporal cortex of AD brains compared to control brains [116]. On the other hand, Stephenson and colleagues, showed elevations in cytosolic PLA\(_2\) immunoreactivity in AD brains suggestive of the presence of an increased inflammatory response mechanism through this pathway in the disease [117].

Ca\(^{2+}\)-binding proteins in AD

Ca\(^{2+}\)-binding proteins buffer intracellular Ca\(^{2+}\) levels and regulate many Ca\(^{2+}\)-dependent processes playing a vital role in maintaining efficient Ca\(^{2+}\) homoeostasis in the brain. In general, many of the Ca\(^{2+}\)-binding proteins investigated so far in the AD brain are expressed to much higher levels in interneurons than in pyramidal neurons, the latter being the major neuronal type to degenerate in AD. These Ca\(^{2+}\)-binding proteins include: parvalbumin, calretinin, and calbindin 28K. The general consensus from work performed is that neurons containing these binding proteins are resistant to the neurodegeneration of AD [118–121]. Some investigations have detected a selective loss of calbindin 28K in specific cortical layers of the AD brain, when compared to control brains [122,123], with more recent results also detecting subfield- and layer-specific changes in parvalbumin and calretinin in the entorhinal cortex in AD [124]. A more specific study of the functional integrity and profile of the Ca\(^{2+}\)-binding proteins of pyramidal neurons would be beneficial in elucidating...
whether changes in the function or expression of these proteins are linked to the Ca\textsuperscript{2+}-induced neurodegenerative pathways of AD.

**Summary and future directions**

Disrupted Ca\textsuperscript{2+} release from the ER, particularly from the RyR, is one of the earliest changes described in neuronal Ca\textsuperscript{2+} homoeostasis in the AD brain. We believe that slowly progressive impairments in both the RyR and IP\textsubscript{3}R cause the progressive deregulation of a number of critical Ca\textsuperscript{2+}-dependent enzymes to induce cellular stress leading to the neurodegeneration and pathology of AD. This raises the exciting possibility that targeting these ER Ca\textsuperscript{2+} channels to negate their malfunction may protect against the neurodegeneration of AD. In the future, experiments examining the role of the RyR, IP\textsubscript{3}R and ER functions in neurodegeneration will be important. In this respect, development of neuronal expression systems, both cell lines and transgenic animals, for normal and genetically modified RyRs and IP\textsubscript{3}Rs will provide valuable information about the role of these receptors in brain function and neurodegeneration.

We are grateful to the Health Research Board of Ireland, the Alzheimer Society of Ireland and Enterprise Ireland for the financial support of our research work.

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