

Roles of Sodium-Calcium Exchanger Isoform-3 toward Calcium Ion Regulation in Alzheimers Disease

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Abstract

Alzheimer's disease (AD) is a late-onset progressive neurodegenerative disorder that leads to cognitive, memory and behavioural impairments. Substantial evidence indicates that disrupted neuronal calcium homeostasis is an early event in AD that could mediate synaptic dysfunction and neuronal toxicity. Sodium calcium exchangers (NCXs) play important roles in regulating intracellular calcium, and accumulated data suggests that reduced NCX function, following aberrant proteolytic cleavage of these exchangers, may contribute to neurodegeneration. This review, characterizes the expression and activity of NCX as a prominent feature of AD brain, identifies the molecular mechanisms underlying the effects of NCX isoforms, and pinpoints the molecular determinants responsible for the effects of NCX. Our findings suggest that calpain mediates cleavage of NCX3 in AD brain and therefore that reduced NCX3 activity contributes to the sustained increases in intraneuronal calcium concentrations that are associated with caspase-12 activation and neuronal death in AD.

Keywords: Alzheimers disease; Sodium; Calcium; Calpain; Caspase-12; Sodium calcium exchanger

Background of the Study

Alzheimers disease

AD is a late-onset progressive neurodegenerative disorder that results in the irreversible loss of cholinergic cortical neurons, particularly in the associative neo-cortex and hippocampus. The principal risk factor for AD is age, with the main onset time observed in people aged over 60, in particular between the age of 70 and 80 [1,2]. With the increasing longevity of our population, AD is already approaching epidemic proportions with no cure or preventative therapy available [3,4].

Currently, it is estimated that one in 10 persons over 65 age and more than a third of all people over 80 have AD. According to United Nations population projections, it is estimated that 370 million people will be older than 80 years by 2050 [5]. The aging of the world's population, therefore, will potentially pose an immense social and economic burden on future societies as this susceptible cohort continues to rapidly expand. Thus, a better understanding of the clinical and molecular events underlying AD will no doubt prove invaluable for combating this affliction.

Clinically, AD is characterized by the progressive impairment of higher cognitive function, loss of memory and altered behaviour that follows a gradual progression [6]. Pathological hallmarks of AD characterised at autopsy include: the presence of senile plaques composed of extracellular amyloid-beta (A β) protein aggregates, intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated forms of the microtubule associated tau (τ) protein deposits, the shrinkage of the cerebral cortex due to extensive neuronal loss and neuronal death [7,8]. These histopathological lesions do not occur diffusely throughout the brain, but are restricted to selective regions, particularly the hippocampus and neo-cortex. The pathogenetic mechanism that causes AD is unknown, but the discovery that mutations in presenilins (which cleave APP) and in APP itself (which is a substrate of the presenilin containing γ -secretase complex) in FAD patients gave rise to the most dominant, and commonly

accepted, hypothesis to explain pathogenicity in both FAD and SAD, namely the "amyloid cascade hypothesis" [9].

Protein abnormalities in Alzheimer's disease: A β and Tau protein:

The pathological AD hallmarks are the presence of cerebral senile plaques composed of extracellular amyloid-beta (A β) protein aggregates, intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau (τ) protein deposits and the shrinkage of the cerebral cortex due to extensive neuronal loss. Although the precise cause of AD remains elusive, it has been suggested that neuronal loss in AD is attributed to the accumulation of toxic protein β -amyloid (A β) [10]. The β -site amyloid precursor protein-cleaving enzyme 1 (BACE1 or β -secretase), the principal actor in amyloid precursor protein (APP) processing in AD [11], is a stress-response protein involved in several neurologic diseases including stroke [12], amyloid angiopathy, inflammation, and oxidative damage.

A β peptides are natural products of metabolism consisting of 39 to 43 amino acids, and it mainly exists in two isoforms: soluble A β 1-40 (~80-90%) and insoluble A β 1-42 (~5-10%) [13,14]. Monomers of A β 40 are much more prevalent than the aggregation-prone and damaging A β 42 species. A β peptides originate from proteolysis of the APP by the sequential enzymatic actions of BACE-1 and γ -secretase, a protein complex with presenilin-1 at its catalytic core [3,15] (Figure 1). Its primacy has been manifested in the 'amyloid cascade hypothesis', which posits that the aggregation and accumulation of A β (resulting

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from overproduction, altered processing or imbalance between production and clearance) is the initiating molecular event that triggers neurodegeneration in sporadic and familial AD [10].

This process, called “amyloid cascade hypothesis”, remains the main pathogenetic model, as suggested by familial AD, mainly associated with mutation in APP and presenilin genes [9]. A β peptides spontaneously self-aggregate into multiple coexisting forms. One form consists of oligomers (2 to 6 peptides), which link into intermediate assemblies [16] (Figure 1). A β peptides can also grow into fibrils, which arrange themselves into β -pleated sheets to form the insoluble fibers of advanced amyloid plaques. Soluble oligomers and intermediate amyloids are the most neurotoxic forms of A β [17]. The severity of the cognitive defect in AD correlates with levels of oligomers in the brain, not the total A β [18].

As for NFTs, it has been found that their major constituent is the protein tau. Experimental evidence indicates that A β accumulation precedes and drives tau aggregation [19]. Tau is normally an abundant soluble protein in axons that promotes assembly and stability of microtubules and vesicle transport. Hyperphosphorylated tau is instead insoluble, lacks affinity for microtubules and self-associates into paired helical filament structures. Several factors might be involved in tau hyperphosphorylation, including A β -mediated caspases activation, A β -mediated oxidative stress, chronic oxidative stress, and reduced insulin-like growth factor 1-mediated oxidative stress [20]. Like A β oligomers, the aggregates of abnormal tau molecules (neurofibrillary tangles) are cytotoxic and impair cognition. These filamentous inclusions are sited in pyramidal neurons and their number is a pathologic marker of the severity of AD.

Increased oxidative stress, the impaired protein-folding function of the endoplasmic reticulum (ER) and deficient proteasome-mediated and autophagic-mediated clearance of damaged proteins - all of which are also associated with aging - accelerate the accumulation of amyloid and tau proteins in AD [21].

APP processing and A β generation: A β is generated by the sequential cleavage of APP, a type I integral membrane protein anchored to the plasma membrane and internal membranes of the ER, Golgi and trans-Golgi apparatus [22,23]. A β is generated in very small quantities in normal healthy individuals and does not typically build up to very high levels [24]. However, in individuals afflicted with AD, differential processing of APP or the failure to degrade A β leads to its excessive accumulation.

Endoproteolysis of parental APP is achieved by the sequential cleavage of APP by groups of enzymes or enzyme complexes termed α -, β - and γ -secretases (Figure 1). For α -secretase there are currently three members of the ADAM family (a disintegrin- and metalloproteinase-family enzyme): ADAM-9, ADAM-10 and ADAM-17 [25]. ADAM-17 is also referred to as tumour necrosis factor α converting enzyme (or TACE) [26]. Several groups have identified the β -secretase (β -site APP cleaving enzyme or BACE) as a type I integral membrane protein belonging to the pepsin family of aspartyl proteases [27-29]. The identity of the γ -secretase has been identified as a complex of enzymes composed of presenilin-1 or -2, (PS1 and PS2), nicastrin, anterior pharynx defective (aph-1) and presenilin enhancer 2 (pen-2) [30].

The cleavage and processing of APP can be divided into a “non-amyloidogenic pathway” and an “amyloidogenic pathway” (Figure 1).

In the prevalent non-amyloidogenic pathway, a large portion of

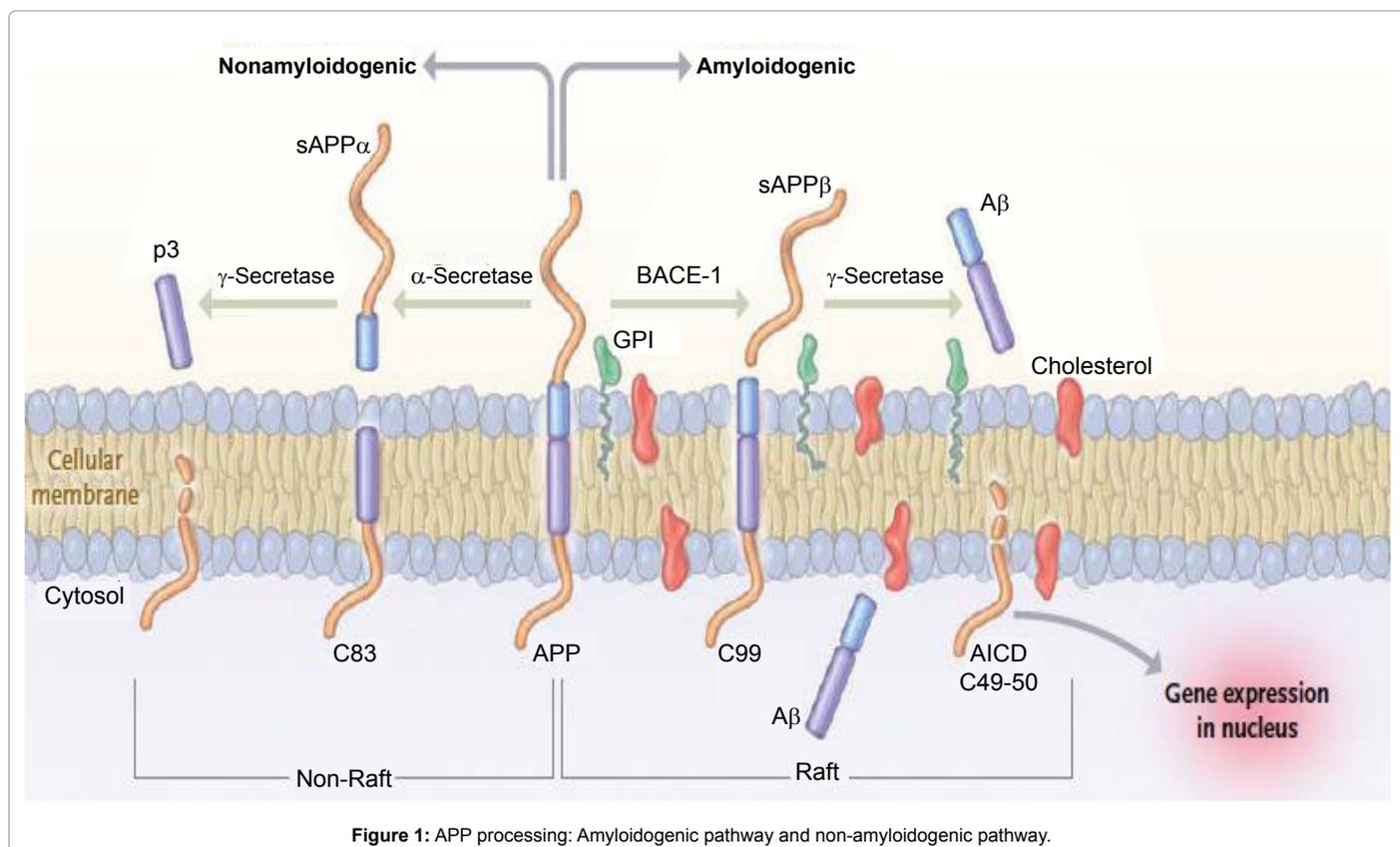


Figure 1: APP processing: Amyloidogenic pathway and non-amyloidogenic pathway.

APP is cleaved by the α -secretase at a position 83 amino acids from the carboxyl (C) terminus, producing a large amino (N)-terminal ectodomain (sAPP α) which is secreted into the extracellular medium [3,15]. The resulting 83-amino-acid C-terminal fragment (C83) in the membrane is retained within cells and subsequently cleaved by the γ -secretase, producing a short fragment termed p3. Importantly, cleavage by the α -secretase occurs within the A β region, thereby precluding formation of A β [31].

The amyloidogenic pathway is an alternative cleavage pathway for APP which leads to A β generation, under physiological conditions. The initial proteolysis is mediated by the β -secretase at a position located 99 amino acids from the C-terminus. This cut results in the release of a slightly smaller form of APP (sAPP β) into the extracellular space, and leaves the slightly larger 99-amino-acid C-terminal fragment (known as C99) within the membrane, with the newly generated N-terminus corresponding to the first amino acid of A β [5]. Subsequent cleavage of this fragment (between residues 38 and 43) by the γ -secretase liberates an intact A β peptide. Most of the full-length A β peptide produced is 40 residues in length (A β 1-40), whereas a small proportion (approximately 10%) is the 42 residue variant (A β 1-42). The A β 1-42 variant is more hydrophobic and more prone to fibril formation than A β 1-40; it is also the predominant isoform found in cerebral plaques [32]. A β can exist in a variety of forms, including monomers, oligomers, and fibrils [33].

Genetic mutations and A β production: Most cases of AD are not caused by a specific genetic defect but are sporadic in nature and are typically characterized by a later age of onset. However, there are a significant number of cases that are inherited in an autosomal dominant manner and generally these forms manifest at an earlier age of onset [34].

Mutations in three genes -APP, PS-1 and PS-2 - are known to cause autosomal dominant AD, which generally manifests with an early-onset pathogenesis [34]. All these mutations affect the metabolism or stability of A β . Most of the mutations occur within the APP gene and cluster around the various secretase sites [5]. Surprisingly, APP mutations account for a small percentage of FAD cases. Mutations in the genes encoding PS-1 and PS-2, found on chromosomes 14 and 1, respectively, serve as the major loci for FAD [35,36]. Whilst over 135 individual mutations have been linked with PS-1, only 10 have thus far been linked with PS-2 [37]. PS-1 mutations cause an aggressive form of FAD with a particularly early age of onset, whilst PS-2 mutations result in a form of FAD that is more akin to sporadic AD, bearing a later age of onset [37].

A key feature of these identified mutations is that, all causes elevated production of A β 1-42. Hence, these genetic mutations have been used to generate transgenic mouse models of the AD. One common mutation in APP is known as the Swedish mutation (APPSwe), in which a double amino acid change leads to increased cleavage of APP by the β -secretase [38]. Other mutations, such as the Arctic mutation (APPArc), increase the aggregation of A β , leading to early onset, aggressive forms of the disease [39]. Mutations in the presenilins, such as the PS1M146V mutation, increase levels of A β 1-42 [40], which aggregates more readily than A β 1-40. Increased dosage of the APP gene also results in AD [41].

Calcium

Calcium can be considered a ubiquitous intracellular messenger within cells acting as a regulator in multiple physiological functions. As a divalent cation, calcium can bind to several proteins, receptors

and ion channels. All of these properties are of great importance within neurons, where continuous firing of action potentials leads to calcium cycling and it implies an influx through the calcium channels at the plasma membrane level, intracellular buffering and an efflux through the calcium plasma membrane transporters.

Loss of calcium (Ca²⁺) regulation is common to several neurodegenerative disorders and studies indicate that Ca²⁺ dyshomeostasis is one of the earliest molecular defects in the pathogenesis of AD [42]. In AD, elevated concentrations of cytosolic calcium ([Ca²⁺]_i) stimulate A β aggregation and amyloidogenesis [43]. The presenilins modulate Ca²⁺ balance. Presenilin mutations might disrupt Ca²⁺ homeostasis in ER [44]. However, the main effect of the mutations is to increase A β 42 levels, which in turn increases Ca²⁺ stores in the ER and the release of Ca²⁺ into the cytoplasm [6]. The relevance of these mechanisms to sporadic AD is delineated. A chronic state of excitatory amino acid (glutamatergic) receptor activation is thought to aggravate neuronal damage in late-stage AD [45]. Glutamate increases [Ca²⁺]_i, which in turn stimulates calcium-release channels in the ER. A β forms voltage-independent, cation channels in lipid membranes [46], resulting in Ca²⁺ uptake and degeneration of neuritis [47]. Indirectly, glutamate activates voltage-gated calcium channels. The L-type voltage-gated calcium-channel blocker, MEM 1003, is in a phase 3 trial and an ionotropic glutamate receptor blocker, N-methyl-D-aspartate (NMDA) receptor blocker, is approved by the Food and Drug Administration.

Calcium regulation of A β production and linkage to AD: By screening genes located in known AD linkage regions, Marambaud et al. [48] discovered a novel calcium-conducting channel, with polymorphisms associated with increased risk for the development of Sporadic AD (SAD) [49].

They called this novel calcium channel Calcium Homeostasis Modulator 1 (CALHM1). It is a three-transmembrane domain containing glycoprotein. Expression of CALHM1 was found in all brain regions and cells of neuronal lineage. CALHM1 localized predominantly to the ER but also exists at the plasma membrane where it mediates a novel Ca²⁺ influx to the cytosol, which is unaffected by specific blockers of store-operated Ca²⁺ influx or voltage-gated calcium channels but inhibited by nonspecific cation channel blockers such as cobalt (Figure 2). CALHM1 appears to exist as multimeric complexes, forming a functional ion channel, and has structural similarities with the NMDA receptor within the ion selectivity region.

Critically, Ca²⁺ influx through CALHM1 decreases A β production and is accompanied by increases in soluble amyloid precursor protein alpha (sAPP α) [50]. The mechanism underlying this effect has not been elucidated but presumably involves a calcium-dependent effect on α -secretase, which are enzymes that are known to cleave APP 83 amino acids from the carboxyl terminus and can thereby prevent A β formation. Conversely, increases in A β occur after siRNA knockdown of endogenous CALHM1 in cells when combined with calcium influx.

Curiously, this observation is contradictory to the vast majority of studies published on cytosolic Ca²⁺ entry and A β production, which indicate that increasing Ca²⁺ influx into the cytosol, either from the extracellular media or from ER stores, increases A β production [51]. An unexplored possibility could be that CALHM1 and the polymorphism P86L variant (that decrease Ca²⁺ permeability and also increases A β) exert their effects on A β processing via their location in the ER rather than the smaller pool found on the plasma membrane, given that the vast majority of CALHM1 was localized to the ER. It is unknown

whether CALHM1 forms a functional cation-conducting pore within the ER, which could facilitate Ca^{2+} influx or efflux from the stores [52].

As the channel appears to be constitutively open (as membrane depolarization was not required for Ca^{2+} influx), it may exist as a potential leak channel at the ER, which would increase $[Ca^{2+}]_i$ and would be diminished by the P86L variant.

This finding would then be in agreement with previous studies showing how ER Ca^{2+} regulation modulates $A\beta$ production. Basal Ca^{2+} levels are tightly regulated by a number of calcium pumps and binding proteins, which sequester free cytosolic Ca^{2+} so that it cannot affect local enzymes and signaling cascades.

Calcium enters into the cytosol from the extracellular space through ionotropic receptor-operated (ligand-gated) channels (ROCs), voltage-operated Ca^{2+} channels (VOCCs), and also through store-operated calcium-entry channels. ROCs permeable to Ca^{2+} include the N-methyl-D-aspartate receptors (NMDARs), some a-amino-3-hydroxy-5-methylisoxazole-4-propionate acid receptors (AMPA). Calcium can also enter into the cytosol from intracellular stores such as the ER via the inositol 1, 4, 5-trisphosphate receptor (IP3R) [53] and the ryanodine receptors (RyR), as well as from the mitochondria [54,55]. When $[Ca^{2+}]_i$ increases are large, mitochondria become rapidly-sequestering Ca^{2+} buffers, ensuring protection against

excess of Ca^{2+} [56,57]. Indeed, slower Ca^{2+} clearance is mediated by Ca^{2+} pumps and exchangers located at plasma membrane level. Ca^{2+} ions are pumped out against a concentration gradient of four orders of magnitude by a plasma membrane Ca^{2+} ATPase (PMCA). Ca^{2+} is also removed from the cytoplasm by Na^+/Ca^{2+} exchanger (NCX) located in the cell membrane; NCX has low affinity but high capacity for Ca^{2+} compared with PMCA [58] for this it is perfectly suited to extruding large amounts of this ion.

Conversely, $[Ca^{2+}]_i$ is reduced via the presence of calcium-binding proteins, such as calbindin, acting as buffers, and also through the extrusion of Ca^{2+} either into intracellular stores, such as the ER via the sarco-endoplasmic reticulum calcium ATPase (SERCA) [59] or out across the plasma membrane via plasmalemmal calcium pumps and exchangers (Figure 2).

Presenilins and calcium homeostasis: In addition to direct effects on $A\beta$ formation, presenilin mutations have profound effects on cellular calcium homeostasis [60]. Familial Alzheimer's Disease (FAD)-associated mutations in the presenilins were found to enhance IP3-mediated Ca^{2+} release from the ER stores [61]. The presenilins were identified in 1995 as multi-transmembrane proteins, which predominantly localized to the ER, and were postulated to form a novel ion channel. Their involvement in the AD pathogenesis was cemented

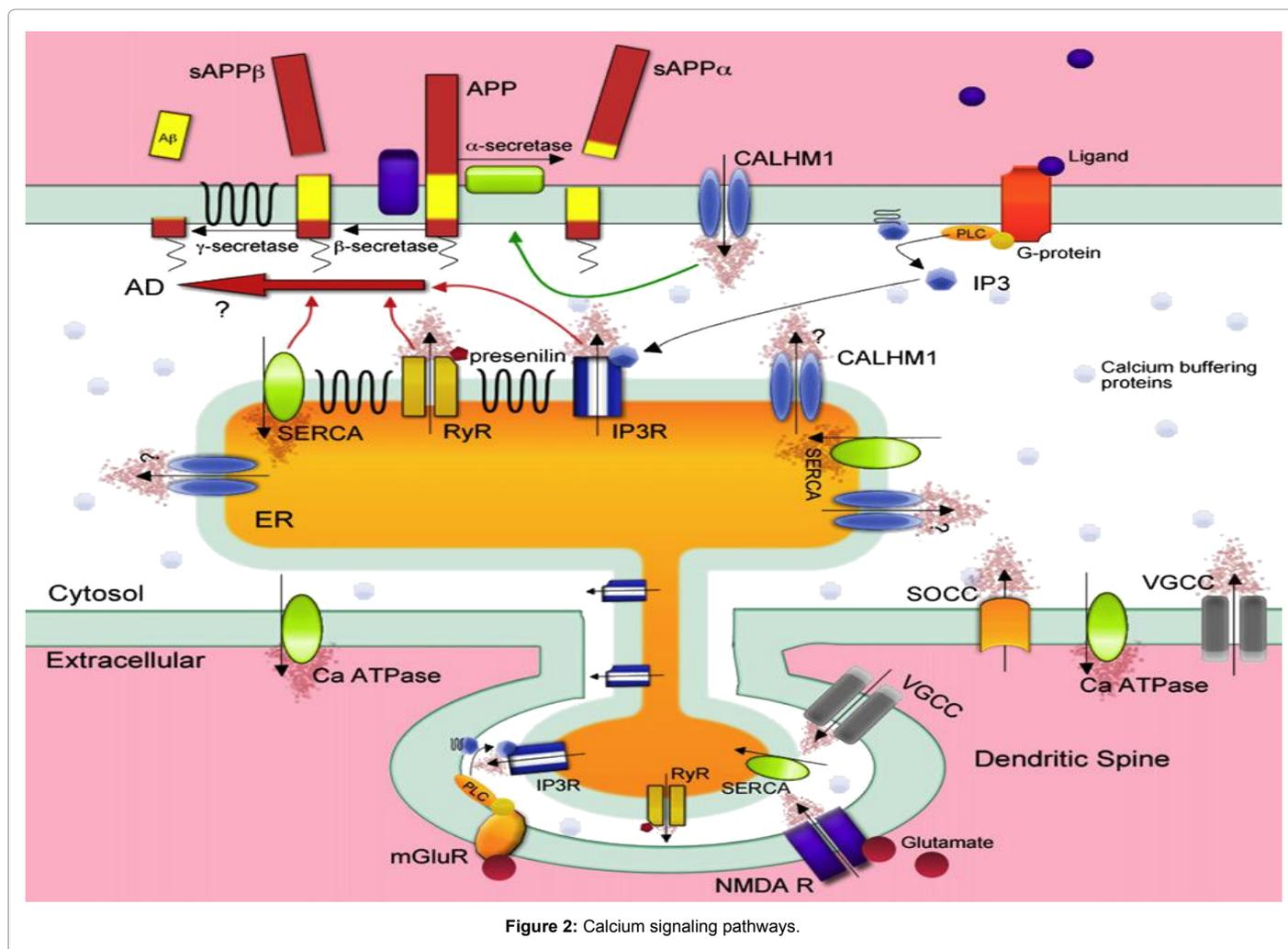


Figure 2: Calcium signaling pathways.

with the discovery that the presenilins formed the catalytic core of the γ -secretase complex, which liberates A β from the membrane fragment C99 (Figure 1). These FAD mutations lead to the formation of the more predominantly 42 amino acid long version A β , which aggregates more readily.

The effects of FAD presenilin mutations on Ca²⁺ are very significant given that FAD presenilin mutations enhance Ca²⁺ release from the ER via the IP3 receptor [53], the ryanodine receptor via caffeine [54,55] and through endogenous calcium leak channels [62], it was thought that these results could all be explained by an increase in ER Ca²⁺ load. However, the same FAD-linked mutations have also shown a reduction in ER Ca²⁺ load and ER release with SERCA inhibition [59]. Thus, it is unclear whether all mutations increase ER Ca²⁺ or not.

Foskett et al. [63] performed direct IP3 channel recordings via single channel patch-clamp electrophysiology of the ER membrane, expressing presenilin or FAD-linked mutants. Overexpression of mutant presenilin 1 or 2 directly increased IP3 channel activity by prolonging the channel open time [64]. Presenilin mutants appear to modulate the IP3 receptors directly, as they were found to physically interact and are known to co-localize to the ER membrane [65]. Presenilin have been shown to interact with the ryanodine receptor, via its N terminus and to increase the open channel probability and mean current [66], similar to that described with the IP3 receptor.

Furthermore to these “gain-of-function” interactions with native ER calcium receptors, FAD-linked presenilin mutations have also been shown to have a “loss-of-function” effect on ER Ca²⁺ dynamics by reducing endogenous Ca²⁺ leak from the ER [65]. Overexpression of wild-type presenilins also accelerates the sequestration of cytosolic Ca²⁺, an effect that can be blocked by pharmacological inhibition of SERCA, suggesting that presenilins modulate SERCA function and that SERCA pumping is impaired in the absence of both presenilins. Taken together, presenilins appear to interact and modulate Ca²⁺ influx into the ER via SERCA and Ca²⁺ extrusion from the ER via interactions with the ryanodine and IP3 receptors. ER Ca²⁺ regulation results to be a critical determinant for the production of A β , in addition to plasma membrane influx pathways such as with CALHM1.

The sodium/calcium exchanger

The Na⁺/Ca²⁺ exchanger (NCX) is a plasma membrane transporter that plays a major role in the maintenance of Ca²⁺ and Na⁺ homeostasis in various excitable and non-excitabile cells [67]. NCX belongs to the Ca²⁺ cation antiporter superfamily (CaCA) and catalyzes the exchange of Na⁺ and Ca²⁺ across the plasma membrane with a 3:1 stoichiometry [67]. Depending on the electrochemical gradient across the plasma membrane NCX can either extrude intracellular Ca²⁺ in its forward mode or take up extracellular Ca²⁺ in its reverse mode.

The regulation of Ca²⁺ and Na⁺ homeostasis is a crucial physiological phenomenon in excitable cells. In fact, Ca²⁺ ions play a key role as a second messenger in the cytosol and in the nucleus [68], while the Na⁺ ion regulates the cellular osmolarity, induces action potentials [69] and it is involved in the signal translation [30]. The control of this regulation is delegated to ionic channels selective for Ca²⁺ and Na⁺, to Na⁺ pumps, Ca²⁺ ATP-dependent and to NCX [70].

Molecular biology of NCX: NCX belongs to the superfamily of membrane proteins comprising the following members:

1) The NCX family, which exchanges three Na⁺ ions for one Ca²⁺ ion or four Na⁺ ions for one Ca²⁺ ion depending on [Na⁺]_i and [Ca²⁺]_i [67];

2) The Na⁺/Ca²⁺ exchanger K⁺-dependent family, which exchanges four Na⁺ ions for one Ca²⁺ plus one K⁺ ion [69];

3) The bacterial family which probably promotes Ca²⁺/H⁺ exchange [71];

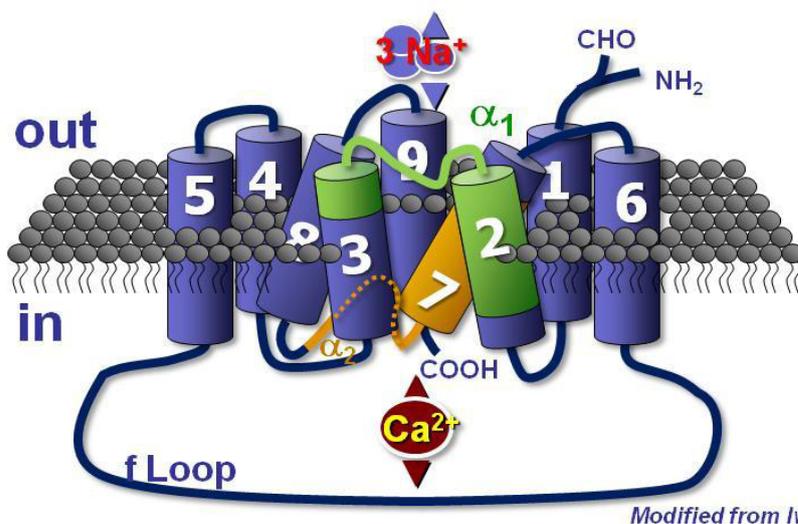
4) The nonbacterial Ca²⁺/H⁺ exchange family, which is also the Ca²⁺ exchanger of yeast vacuoles;

5) The Mg²⁺/H⁺ exchanger, an electrogenic exchanger of protons with Mg²⁺ and Zn²⁺ ions [72].

These membrane proteins are all peculiarly characterized by the presence of α -repeats, the regions involved in ion translocation. In mammals, the NCX family consists of three separate isoforms: NCX1, NCX2 and NCX3 [73]. NCX1 is predominantly expressed in heart, kidney and brain [74], NCX2 is most abundantly expressed in brain [75] and NCX3 is expressed in excitable tissues such as brain and skeletal muscle [76]. These three genes appear to be dispersed, since NCX1, NCX2 and NCX3 have been mapped in mouse chromosomes 17, 7 and 12, respectively [76]. At the post-transcriptional level, at least 12 NCX1 and 3 NCX3 proteins are generated through alternative splicing of the primary nuclear transcripts. These variants arise from a region of the large intracellular f-loop, are encoded by six small exons defined A to F and are used in different combinations in a tissue-specific manner. To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive [75].

NCX1 is composed of 938 amino acids in the canine heart and has a molecular mass of 120 kDa and contains nine transmembrane segments (TMS). NCX1 amino terminus (N-terminal) is located in the extracellular space, whereas the carboxyl terminus (C-terminal) is located intracellularly (Figure 3). The nine transmembrane segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1–5) and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1–5) TMS are separated from the last four (6–9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f-loop [77]. Although the f-loop is not implicated in Na⁺ and Ca²⁺ translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as Ca²⁺ and Na⁺ ions, NO, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA), phosphoarginine (PA) and ATP. In the center of the f-loop, a region of approximately 130 amino acids in length (371–508 amino acids) has been reported to exert a Ca²⁺ regulatory function. This region is characterized by a pair of three aspartyl residues and by a group of four cysteines [77]. At the N terminal end of the f-loop near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues and consisting of a 20-aminoacid sequence (219–238), named exchange inhibitory peptide (XIP) [78], has been identified. The f-loop is also characterized by alternative splicing sites named α 1-repeat and α 2-repeat. The NCX protein amino acid sequence found between TMS2 and TMS3 is called α -1 repeat, whereas the one found between TMS7 and TMS8 is named α -2 repeat [79]. With electrophoretic gels and under non reducing conditions, NCX1 migrates as a 120 and a 70 kDa band. The 120-kDa band represents the native protein, and the 70-kDa protein is a proteolytic fragment, which includes a large part of the f-loop and retains an NCX activity.

Interestingly, NCX2 and NCX3 have been found only in the brain and in the skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and



Modified from Iwamoto et al., Mol Pharmacol, 2004

Figure 3: Molecular pharmacology of NCX.

105 KDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 [76]. All three NCX gene products share the same membrane topology.

NCX can facilitate both Ca^{2+} and Na^+ flow in a bidirectional way through the plasma membrane [67] with a stoichiometry of 3 Na^+ ions versus 1 Ca^{2+} ion. Depending on the intracellular levels of Na^+ and Ca^{2+} , NCX can operate in the forward mode by extruding one Ca^{2+} against three entering Na^+ , using the Na^+ gradient across the plasma membrane as a source of energy [67]. Alternatively, in the reverse mode, NCX can function as Na^+ efflux- Ca^{2+} influx. Because of its high exchange capacity, NCX is well-suited for rapid recovery from high intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) and may play an important role in maintaining Ca^{2+} homeostasis and protecting cells from Ca^{2+} overload and eventual death [67].

NCX regulation: Several factors are involved in the regulation of NCX activity: the two transported ions, Na^+ and Ca^{2+} , the intracellular pH, metabolic related compounds, ATP, PA, PIP2, PKA and PKC, redox agents, hydroxyl radicals, H_2O_2 , dithiothreitol (DTT), O_2^- , Fe^{3+} , Fe^{2+} , Cu^{2+} , OH^- , glutathione reduced (GSH) and glutathione oxidized (GSSG) and finally the gaseous mediator, NO.

Intracellular Ca^{2+} concentrations: The site level at which $[\text{Ca}^{2+}]_i$ regulates NCX activity is different from the one required for Ca^{2+} transport. In fact, submicromolar concentrations (0.1–0.3 μM) of intracellular Ca^{2+} are needed to activate the antiporter [80]. Indeed, the removal of intracellular Ca^{2+} ions completely blocks NCX activity [81]. This regulatory function of low micromolar Ca^{2+} is more evident when the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is working in the reverse mode. However, it is not completely clear how low μM Ca^{2+} can also regulate NCX when it operates in the forward mode [78]. The location of such a regulatory site has been identified in the 134 amino acid length region, situated in the center of the intracellular f-loop. This region is characterized by a pair of three aspartyl residues and by a group of four cysteines.

Intracellular Na^+ concentrations: In addition to the submicromolar intracellular Ca^{2+} regulatory site, an increase in $[\text{Na}^+]_i$ can also regulate the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [82]. In particular, when intracellular Na^+

increases, it binds to the transport site of the exchanger molecule, and after an initial fast outward $\text{Na}^+/\text{Ca}^{2+}$ current, an inactivation process occurs [80]. This inactivation process, very similar to the phenomenon occurring in voltage-dependent ionic channels, is named Na^+ -dependent inactivation. The region of the intracellular f loop, in which this regulatory site is located, has been identified in a 20-amino acid portion of the N-terminal part of the loop termed XIP [78]. Studies *in vitro* have characterized a negatively charged region of the intracellular f loop (445-455 amino acids) of the NCX protein that is able to cross link with synthetic XIP, suggesting that this amino acid sequence constitutes the binding site of XIP. On the other hand, since deletion mutagenesis of amino acids 562 to 685 results in an exchange activity that is no longer regulated by XIP, it is likely that XIP interacts with residues 445 to 455 and with another region of the f loop located between residues 562 and 685. Indeed, this region is believed to be a Na^+ regulatory site. Regarding the mechanism by which XIP inhibits NCX activity, it has been proposed that when the XIP-binding site is ligand occupied, a conformational change is induced in the C-terminal portion of the f loop, resulting in the inhibition of the ion transport. XIP is provided with relevant pharmacological implications. In fact, those exogenous peptides, having the same amino acid sequence as XIP, act as potent inhibitors of NCX activity [83]. Interestingly, Ca^{2+} ions, at low micromolar concentrations, binding its regulatory site, decrease the extent of this Na^+ -dependent inactivation. In fact, mutations in the Ca^{2+} regulatory binding site alter the activation and inactivation kinetics of exchange currents by modulating Na^+ -dependent inactivation.

Intracellular H^+ concentrations: H^+ strongly inhibits NCX activity under steady-state conditions. Changes in intracellular pH values, as little as 0.4 can induce a 90% inhibition of NCX activity. Since this H^+ ion modulatory action is $\alpha 1$ -chymotrypsin sensitive, the action site of the proton can be attributed to the antiporter's hydrophilic intracellular loop [84]. Intriguingly, such inhibitory action depends on the presence of intracellular Na^+ ions [85]. Hence, the action exerted by H^+ ions is pathophysiologically relevant with regards to brain and heart ischemia. In fact, when intracellular H^+ and Na^+ ion homeostasis are deregulated, the anoxic conditions resulting in these cells may selectively interfere with the activity of the different NCX gene products. In particular,

increases of H^+ and Na^+ , as in anoxic conditions, synergically inhibit NCX activity [86].

ATP, PKA, PKC and PIP2: Acting as a phosphoryl donor molecule, ATP may increase the activity of the exchanger in a number of ways. Firstly, ATP directly participates in the NCX molecule phosphorylation process by protein kinase A (PKA) and protein kinase C (PKC). Secondly, it increases phosphatidylinositol-4, 5-phosphate (PIP2) production. Finally, by activating G-protein-coupled receptors, via endogenous and exogenous ligands, ATP can stimulate the activity of the Na^+/Ca^{2+} exchanger through the pathway involving PKC or PKA activation [84]. The mechanism underlying the phosphorylating effect on the exchanger seems to be related to an increase in its affinity for both internal Ca^{2+} and external Na^+ and to a decrease in its inhibition by internal Na^+ .

Each of the NCX isoforms has distinctive putative phosphorylation sites, although their roles have not yet been elucidated [87]. ATP cellular depletion inhibits NCX1 and NCX2 but does not affect NCX3 activity. The exchange activity of NCX1 and NCX3 is modestly increased by those agents that activate PKA and PKC [87]. More recently, the mechanism by which PKA and PKC activate NCX has been clarified. In fact, it has been demonstrated that the regulation of PKA-induced phosphorylation is due to the existence of an NCX1 macromolecular complex that contains the kinase PKA holoenzyme. This holoenzyme consists of two PKA catalytic subunits and two identical PKA regulatory subunits [88]. Together with PKA, other critical regulatory enzymes are also associated with NCX1, including PKC and serine-threonine protein phosphatases, PP1 and PP2A [88]. Particularly a pathway involving PKC has been shown to stimulate NCX1 [89].

In a more recent paper, it has been demonstrated that PKC-dependent regulation of NCX isoforms also involves NCX3 but not NCX2 [90]. In the same paper, three phosphorylation sites in the NCX1 protein, Ser-249, Ser-250 and Ser-357, have been identified. Among these, Ser-250 is the amino acid that is predominantly phosphorylated [90]. The other mechanism by which ATP can activate NCX occurs through PIP2 production. This mechanism of activation is related to the relevant PIP2 influence on Na^+ -dependent inactivation of NCX. In fact, PIP2 directly interacts with the XIP region of the exchanger, thus eliminating its inactivation and stimulating NCX function. Indeed, exchangers with mutated XIP regions no longer respond to PIP2 or to PIP2 antibodies [80].

Redox agents: In the last 15 years, several groups of investigators using different cellular models, such as cell-expressing cloned splicing variants of the brain, heart isoforms, cardiac sarcolemma vesicles, cells transiently transfected with NCX1 isoform and giant excised patches, have found that the Na^+/Ca^{2+} exchanger is sensitive to different combinations of redox agents [91]. In particular, the stimulation of the exchange activity requires the combination of a reducing agent (DTT, GSH or Fe^{2+}) with an oxidizing agent (H_2O_2 and GSSG). The effects of both agents are mediated by metal ions (e.g. Fe^{2+}). The antiporter's sensitivity to changes in the redox status can assume particular relevance during oxidative stress. In fact, in this condition, the modulation of reactive oxygen species (ROS) could affect the transport of Na^+ and Ca^{2+} ions through the plasma membrane.

Nitric oxide: The ubiquitous gaseous mediator Nitric Oxide (NO) seems to be involved in the modulation of NCX activity. In fact, Asano and his colleagues provided evidence that NO, released by NO donors, is able to stimulate NCX in the reverse mode of operation in neuronal preparations and astrocytes through a cGMP-dependent

mechanism [92]. In contrast, in C6 glioma cells, the stimulatory action on NCX reverse mode of operation, elicited by the NO donor sodium nitroprusside (SNP) is not elicited by NO release but by the presence of iron in SNP molecule [91]. In addition, a direct relationship between the constitutive form of nitric oxide synthase (NOS), the enzyme involved in NO synthesis, and NCX has recently been demonstrated. Indeed, heat stress by inducing NOS phosphorylation causes NOS complication with NCX, thus decreasing its activity. Secondo and colleagues [92]), demonstrated the selectivity of NO in modulating each isoform at different molecular determinant level.

NCX role in physiological conditions: The NCX protein may play a relevant function in different neurophysiological conditions. In neurons, the level of expression of NCX is particularly high in those sites where a large movement of Ca^{2+} ions occurs across the plasma membrane, as it happens at the level of synapses [84]. Specifically, during an action potential or after glutamate-activated channel activity, Ca^{2+} massively enters the plasma membrane. Such phenomenon triggers the fusion of synaptic vesicles with the plasma membrane and promotes neurotransmitter exocytosis. After this event, outward K^+ currents repolarize the plasma membrane, thus leading to VOCC closure. According to the diffusion principle, Ca^{2+} ions are distributed in the cytosolic compartment, reversibly interacting with Ca^{2+} -binding proteins. Residual Ca^{2+} is then rapidly extruded by the plasma membrane Ca^{2+} ATPase and by NCX.

The NCX becomes the dominant Ca^{2+} extrusion mechanism when $[Ca^{2+}]_i$ is higher than 500 nM, as it happens when a train of action potentials reaches the nerve terminals. It has been calculated that for these $[Ca^{2+}]_i$ values (500 nM), more than 60% of Ca^{2+} extrusion is mediated by NCX families. In such physiological conditions, NCX activation is consistent with its low-affinity ($K_d=500$ nM) and high-capacity (5×10^3 Ca^{2+}/s) function. In contrast, in resting conditions or after a single action potential, when $[Ca^{2+}]_i$ slightly increases, requiring, therefore, a more subtle control, the high affinity ($K_d=100$ nM) and low-capacity (10^2 Ca^{2+}/s) pump, plasma membrane Ca^{2+} -ATPase, assumes a predominant function, thus making the involvement of NCX less relevant [70].

NCX genes knocking-out effect: NCX1-, NCX2- and NCX3-specific knockout mice were generated over the past decade [93-95]. These mouse models are useful tools for elucidating NCX1-3 specific function in physiological and pathophysiological processes in the central nervous system (CNS). NCX1-deficient mice are not viable. NCX1 null-mutation caused embryonic lethality, irregular heartbeats and apoptosis in the heart [95]. Recent studies indicated that cardiac-specific transgenic re-expression of NCX1 was not enough to rescue the lethal phenotype, suggesting an important non-cardiac role for NCX1 during embryogenesis (e.g. vascularization of yolk sac, placental development) [68]. Mice lacking NCX2 exhibit enhanced learning and memory [93]. Targeted disruption of NCX3 leads to defective neuromuscular transmission [94]. Under ischemic conditions, NCX3-deficient mice exhibit increased neuronal damage [96,97]. Studies also showed that NCX plays a major role in restoring baseline Ca^{2+} levels following glutamate-induced depolarization in cortical and hippocampal neurons [93,98]. These findings highlight NCX function in the regulation of Na^+ and Ca^{2+} following synaptic activity.

NCX role in pathophysiological conditions: The dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial injury occurring in *in vitro* and *in vivo* models of hypoxia-anoxia and in several neurodegenerative diseases. In a cellular model of glial cells, C6 glioma, the activation of NCX, in reverse mode, obtained

by $[Na^+]_e$ removal, reduces cell injury induced by chemical hypoxia. Such phenomenon suggests that the antiporter plays a protective role during this pathophysiological condition. Consistent with these results, the pharmacological inhibition of NCX activity worsens cell damage by increasing the intracellular concentration of Na^+ ions [91]. Furthermore, the stimulation of NCX activity by redox agents results in a protective effect against hypoxia as well as the overexpression of NCX3 [99]. Published evidence demonstrated that Ca^{2+} influx due to NCX activity in reverse mode is the main component of the excitotoxicity damage [100].

More papers highlighted the different roles played by the different NCX isoforms in the cell survival modulation in cellular death models. For example, NCX3 is neuroprotective during an ischemia insult *in vitro* both in neuronal models and in cells transfected with only this isoform [101]. This role is attributable to the NCX3 ability to buffer the cytosolic Ca^{2+} by the forward mode of operation, like during glutamate increase or chemical ipoxia insult [102]. The molecular mechanisms by which NCX is involved in the pathophysiological conditions involve the cleavage of NCX1 and NCX3 by calpain in brain ischemia and in cultured cerebellar granule neurons exposed to glutamate [103]. Calpains modulate a variety of physiological processes [104] but can also become important mediators of cell death [105]. Ample evidence documents the activation of calpains in brain ischemia and excitotoxic neuronal degeneration [106], leading to speculation for the alteration of the NCX function.

In *in vivo* models, reproducing human cerebral ischemia through the occlusion of the middle cerebral artery, the inhibition of NCX, induced by selective inhibitors [107] or by the knockout of one of the NCX isoforms (NCX2) [93] or NCX3 [97] aggravates brain infarct, whereas the activation of the antiporter with redox agents reduces the cerebral infarctual area [83].

The role played by NCX in those neurons and glial cells involved in cerebral ischemia should be differentiated according to the anatomical regions involved in the ischemic pathological process. In particular, it is conceivable that, since in the penumbral region ATPase activity is still preserved, NCX may likely operate in a forward mode. As a result, by extruding Ca^{2+} ions, the exchanger favors the entry of Na^+ ions. Therefore, the inhibition of NCX in this area reduces the extrusion of Ca^{2+} ions, thus enhancing Ca^{2+} -mediated cell injury. In contrast, in the ischemic core region, in which ATP levels are remarkably low and Na^+/K^+ ATPase activity is reduced, intracellular Na^+ ions massively accumulate because of Na^+/K^+ ATPase failure [108]. Hence, the intracellular Na^+ loading promotes NCX to operate in the reverse mode as a Na^+ efflux- Ca^{2+} influx pathway. In conclusion, the NCX pharmacological inhibition in this core region further worsens the necrotic lesion of the surviving glial and neuronal cells as the loading of intracellular Na^+ increases [83].

The “ Ca^{2+} hypothesis” provides an attractive mechanism to explain the cell death associated with AD. The theory proposes that cell death results from elevated $[Ca^{2+}]_i$. The acute or chronic in $[Ca^{2+}]_i$ rise may exist leading the cell to an irreversible pathway of necrosis and/or apoptosis. If true, derangements in several Ca^{2+} homeostatic processes could simultaneously contribute to and be responsible for a persistent rise in $[Ca^{2+}]_i$. In particular, multiple evidence points to deregulated endoplasmic reticulum (ER) Ca^{2+} homeostasis in the aging brain and AD [51]. A large bulk of studies have shown that the neurotoxicity exerted by $A\beta$ protein is intimately related to $[Ca^{2+}]_i$. Indeed, the attenuation of $[Ca^{2+}]_i$ increase by Ca^{2+} channel blockers, growth factors, and cytochalasins results in a reduction of neural damage induced by

the $A\beta$ peptide. It has been demonstrated that exposure to the $A\beta$ protein partially reduces Na^+ -dependent Ca^{2+} accumulation in plasma membrane vesicles deriving from the human frontal cortex of patients affected by AD. These findings have suggested that $A\beta$ directly interacts with the hydrophobic surface of the NCX molecule, thus interfering with plasma membrane Ca^{2+} transport [30].

Many evidences in literature are in support of the “ Ca^{2+} hypothesis of AD”. Where does the NCX become involved in this mechanism? NCX would be expected to be neuroprotective in situations where elevations in $[Ca^{2+}]_i$ are leading to cell death. This neuroprotective role was proposed to explain increased NCX activity in AD brain [109]. In this model, neurons that survived the neurodegenerative elevations in $[Ca^{2+}]_i$ caused by AD were having increased capacity for NCX. This increased capacity for NCX in surviving neurons was manifested as increased Na^+ -dependent Ca^{2+} uptake in plasma membrane vesicles derived from AD brain [110].

Pathophysiological implications of NCX3: Recently, the Annunziato’s group concluded from their functional studies that the cleaved NCX3 is most likely hyperfunctional in its reverse mode, triggering a significant Ca^{2+} uptake [100], increasing the Ca^{2+} content of the ER, and therefore delaying caspase-12 activation and neuronal cell death (Figure3). Moreover, cleavage of NCX3 is triggered by $A\beta_{1-42}$ peptide exposure, a peptide well known to accumulate in the brain of AD patients. Furthermore, level of cleaved NCX3 correlates perfectly with the level of $A\beta_{1-42}$ and calpain activity in AD patients post-mortem, a further correlation between the loss of NCX3 with neuronal cell death [111].

Thus, NCX3 is well accepted as having a major role in the Ca^{2+} dysregulation deriving from excitotoxicity and most likely acting in a neuroprotective manner. Nevertheless, many unresolved issues remain as to its activity after cleavage by calpain, the mechanisms by which NCX3 is implied in both ER and mitochondria refilling and the cleavage pattern of the variants of NCX3. Further studies are, therefore, needed to address these questions and conclude whether this constitutes an aberrant proteolytic cleavage or rather a new degree of posttranslational regulation allowing for a tight regulation of the exchanger that correlates with the stress state of the cell. Finally, NCX3 seems to be implicated not only in the excitotoxic conditions but also in the hyperexcitability state underlying the apparition of seizures, as an inhibition of NCX3 significantly reduces frequency and severity of seizures [112,113] (Figure 4).

Conclusion and Perspectives

The knowledge gained mainly over the past 10 years of research on NCX has implicated NCX3 in pathophysiological conditions. In this respect, some of these implications require further investigation, including the interactions of NCX3 with other proteins and cellular organelles during stress conditions such as excitotoxicity.

Therefore, research on several types of nerve fibers is required to conclude on the exact role of NCX3 in the brain physiology. Such investigation, together with the use of conditional knockout models would allow for a better understanding of their role in a delay of Caspase-12 activation and neuronal death. By pinpointing this example of the $A\beta_{1-42}$ -NCX3 cross talk, researchers have initiated a quest to unravel the different pathologies where NCX3 cleavage is very likely to be involved in neurodegenerative disorders. In this matter, the use of the recently developed technique of CRISPR/Cas might be of great help to generate a mice model with specific mutation of the NCX3

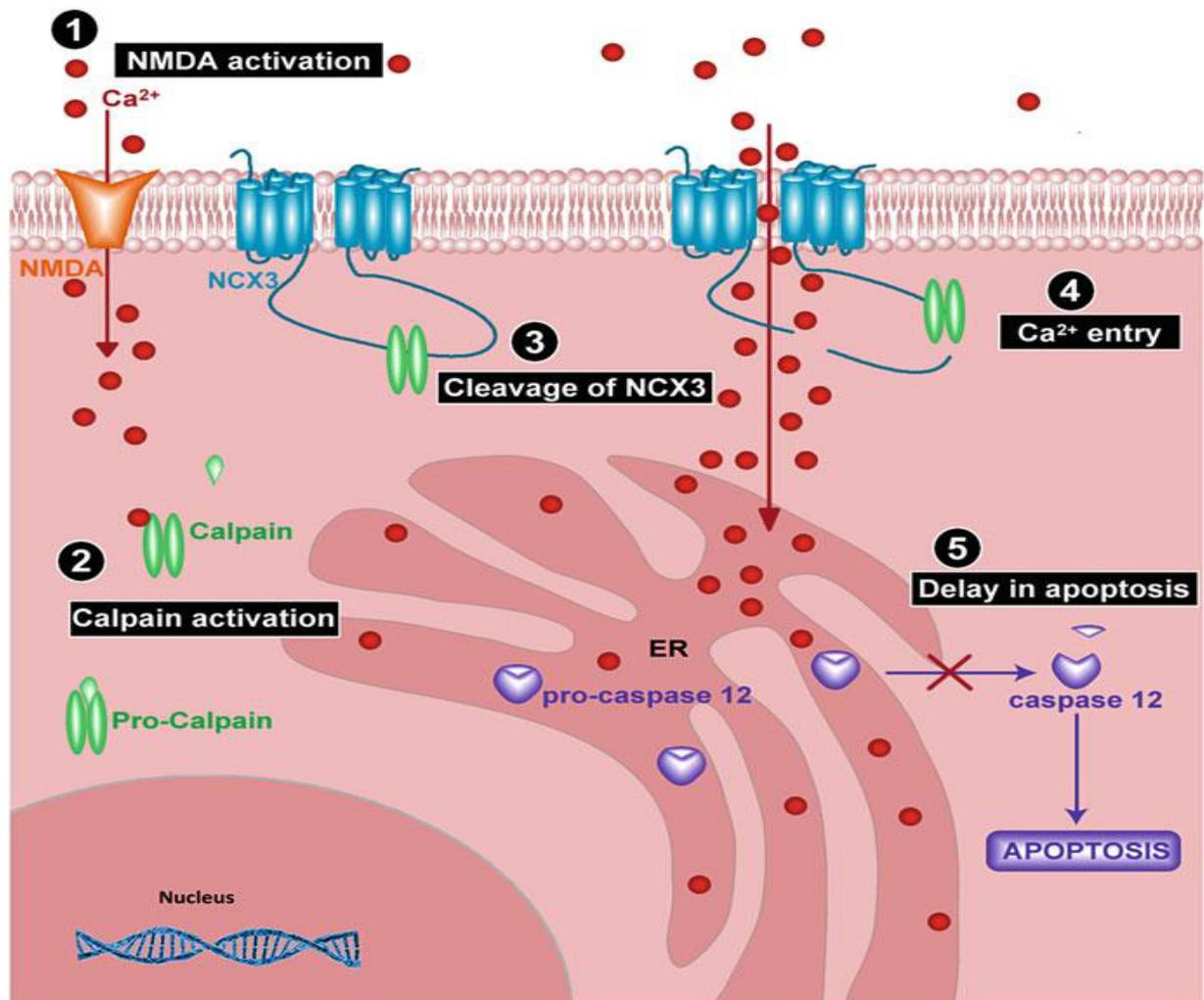


Figure 4: Sequential events occurring during neuronal excitotoxicity that leads to the cleavage of NCX3 by calpain which triggers calcium entry and delays neuronal cell death. First, the activation of the N-methyl-D-aspartate receptor (NMDA), allowing for a mild Ca^{2+} entry causes the autolysis of calpain. Thereafter, the cleavage by calpain of NCX3 within the intracellular loop triggers a strong influx of Ca^{2+} that increases ER content and delays the activation of caspase-12.

cleavage sites in either a wild-type mouse or a mice already subjected to excitotoxicity as it is the case in the Alzheimer's disease mice models [114].

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