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Biochemical dysfunction and memory loss: the case of Alzheimer's dementia

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Abstract. Among the different types of cognitive impairment that appear with increasing age, Alzheimer's disease (AD) is rated as the most frequent. Despite intensive research, key questions concerning AD aetiology remain elusive, but it appears that many biochemical events crucial for neuronal communication and synaptic plasticity fail during the course of the disease. The aim of this review is therefore to provide an overview of intracellular cascades involved in AD pathology. For almost all factors, it is a matter of controversy whether their contribution should be considered to be cause or effect. However, intracellular signalling may be crucial—as it is in learning and memory mechanisms—and malfunction of biochemical pathways may be a common denominator in neurodegenerative processes, thus providing new venues for treatment and therapeutic strategies.

Key words. Alzheimer; acetylcholine; β-amyloid; tau; Ca^{2+} homeostasis; oxidative stress; neurodegeneration; second messengers; phosphatases; kinases.

Introduction

Alzheimer's disease (AD) is described as a progressive decline of cognitive function, particularly affecting memory, attention and orientation, whereas motor and sensory abilities are usually undisturbed. Together with the most obvious 'forgetfulness' of AD patients, personality changes and mood swings are observed.

Advances in molecular techniques have allowed the characterisation of genetically determined predispositions to AD. This has stimulated scientific research and the engineering of mutant animals as useful tools in AD research. However, no animal model is available today that matches both AD histopathology and behavioural deficits. The heterogeneity of AD phenotypes also suggests that we are dealing with multiple forms of the disease, presumably caused by a variety of factors. Moreover, familial, early-onset forms of AD (fAD) represent less than 5% of all AD cases. To date, mutations in three different genes [amyloid precursor protein (APP), presenilin-1 and -2] have been identified as being correlated with fAD [1]. In late-onset AD, a link with the gene coding for apolipoprotein E (APoE) was found with the E4 allele frequency being correlated with the risk of acquiring AD [2, 3].

While it can be expected that more genes related to AD will be discovered in the future, spontaneous or induced DNA mutations later in life are not likely to play a major role, since proliferation of neurones in the mature central nervous system (CNS) is negligible. There are, however, additional mechanisms at the level of messenger RNA (mRNA) transcription, which may be important contributing factors in AD. The so-called molecular misreading or transcript mutations, for instance of the APP gene, may lead to '+1' proteins [4], which can accumulate within neurones and disturb cellular metabolism and activity. Such misreadings occur preferentially when transcriptional activity is high (e.g. in areas of high plasticity such as the hippocampal formation and after neuronal injury). Respective proteins have been identified in brain areas affected in early stages of the disease. It was suggested that the 'mRNA surveillance' system, which should prevent production of mutated transcripts, might be impaired in elderly people and particularly in AD. It remains to be investigated whether this is a general ageing factor or a primary induction factor of AD, since transcription mutations may well be a mechanism downstream from

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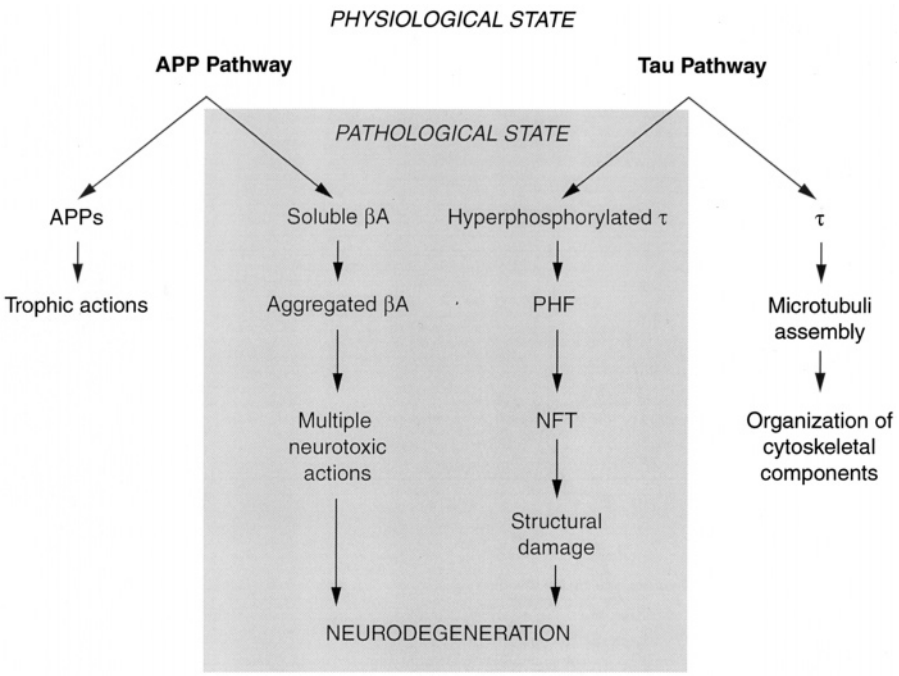

Figure 1. Schematic representation of the physiological and pathological pathways for two of the major components in AD: APP and τ. Both pathways have been suggested to cause cell death and neurodegeneration: aberrant APP processing results in the formation of βA peptide and aggregation thereof, while hyperphosphorylation of τ leads to PHF assembly. Pathological states and/or mechanisms are illustrated on a grey background. For details, see text.

other initiating events such as environmental or oxidative stress.

To date, a final diagnosis of AD still relies on histological post mortem examination of brain tissue. Investigation of hippocampal and neocortical tissue allows identification of the main histopathological characteristics of the disease: neurofibrillary lesions and amyloid plaques.

Neurofibrillary lesions are formed intraneuronally. Depending on their location within the cell, they are referred to as either neurofibrillary tangles (NFTs, fig. 1) or neuropil threads. Whereas tangles are formed in the cell body and the apical dendrites, neuropil threads are generated in the distal dendrites. In AD, 95% of neurofibrillary lesions are formed from paired helical filaments (PHFs), with the remaining 5% being straight filaments (SFs) [5]. Both PHFs and SFs are formed from aberrantly processed cytoskeletal proteins, including hyperphosphorylated forms of the microtubuli-associated protein, tau (τ) (see fig. 1). 'Normal' τ binds to tubulin, and is thereby involved in the assembly and stabilisation of microtubuli, cytoskeletal components which serve an important function in axonal transport and intracellular trafficking between the cell body and the synapses. Formation of neurofibrillary lesions is an indicator of cytoskeletal disruption. Severely affected cells may eventually die, leaving the highly insoluble NFTs after the disintegration of the cell membrane and thereby giving rise to extracellular NFTs, also called ghost tangles.

Amyloid plaques are extracellular deposits of fibrous proteinaceous material. In late stages of AD, amyloid deposition such as the mature amyloid plaques (also referred to as senile plaques) form a dense core surrounded by dystrophic neurites, neuropil threads and activated microglia. The main constituents of the plaque core are different forms of β-amyloid (βA) peptides which possess the ability to assemble into dense β-sheets and fibrils. This feature is assumed to render the molecules neurotoxic [6, 7]. βA is derived from a much larger protein, APP, an apparently ubiquitous protein with neurotrophic action [8]. APP is expressed by many cell types and in several different isoforms, some of which are preferentially found in neurones.

There have been many disputes about the individual contribution of plaques and tangles to the aetiology of AD, the causal relationships between these features and

the mechanisms behind the observed neurodegeneration and cognitive impairment. Some evidence from genetic and biochemical investigation points toward a key role for overproduction and/or aberrant protein processing of APP in the development of AD (see below). However, the distribution and density of plaques is not very well correlated with the observed cognitive impairments, and high numbers of amyloid deposits can be present in cognitively normal individuals. τ-based tangles, on the other hand, seem to be better correlated with the severity of dementia. Accordingly, Braak and Braak [9] categorised the progression of AD into six stages based on the developmental pattern and distribution of fibrillary lesions. Nevertheless, τ pathology is also not only restricted to AD, and some neurofibrillary lesions (Braak stage I and II) form as a consequence of normal ageing.

Another prominent hallmark of AD is an early and pronounced loss of cholinergic function. This feature of the disease remained undiscovered until the mid-1970s, when the first observations of reduced amounts of the synthesising enzyme choline acetyl transferase (ChAT) were brought to attention [10]. Subsequently, intensive research has focused on the cholinergic deficit in AD, spurred by evidence from animal and human studies pointing to a close relationship between the functionality of the cholinergic system and learning and memory processes. The loss of cholinergic function in AD patients is expressed as a pronounced death or atrophy of basal forebrain cholinergic neurones, especially in the medial septum and the nucleus basalis of Meynert (NBM). Reports exist of up to 90% loss of cholinergic cell numbers in the NBM in AD patients compared with age-matched controls [11]. As a consequence, the functionality of the target areas (mainly hippocampus and neocortex) is severely compromised [12]. The loss of cholinergic cells in the basal forebrain has been shown to have an even better correlation with the degree of dementia than plaques and tangles [13–16].

Initially, it appeared that the projecting neurones and not the recipient part of the system were affected. This spurred intensive effort to find a suitable cholinergic stimulant, which could alleviate the symptoms of the disease. Today, the only approved drug treatments against AD are inhibitors of the synthesising enzyme cholinesterase (e.g. tacrine and donepezil), thus preventing the breakdown of acetylcholine (ACh) in the synaptic cleft. However, the efficiency of these treatments has not been very convincing, presumably because postsynaptic intracellular signalling is also affected (see below) and major side effects limit their use [17, 18]. Thus, a breakthrough in AD treatment is still not in sight.

Cholinergic signalling in AD: contribution of intracellular cascades

Cholinergic transmission is mediated through either ionotropic (nicotinic receptor system) or metabotrobic receptors (the muscarinic receptor system). Research related to the deficits of cholinergic transmission in AD is mostly divided in two fields, strictly focusing on only one of the receptor systems and often reaching contradictory conclusions with respect to treatment strategies. The AD-related changes reported in both systems are summarised in table 1, and will be discussed briefly in the next sections.

The muscarinic system

Changes in signalling systems related to muscarinic acetylcholine receptors (mAChR) have attracted the majority of attention. Five mAChR isoforms (M_{1-5}) have been identified so far, all of which are coupled to intracellular second messenger cascades via G-proteins. Of these, subtype M_1, M_3 and M_5 have been associated with the phospholipase C (PLC) related phosphoinositide (PI) signalling pathway, where as M_2 and M_4 are negatively linked to adenylylcylcase (AC), thus regulating cyclic adenosine monophosphate (cAMP) levels.

M_2 and M_4 are mainly located presynaptically, probably functioning as autoreceptors and hence regulating the release of ACh. In a brain not affected by AD, these receptors amount to 15–25% of the total mAChR population in hippocampal and neocortical tissue [19]. Due to their preferential presynaptic location on projecting neurones, which degenerate early in AD, reduced numbers of M_2 and M_4 are observed in AD [20, 21]. In addition to changes on the receptor level, deficits in the enzyme cascade downstream from receptor activation could play a major role in AD. For M_2 and M_4, both G_s- and G_i-proteins provide links to second-messenger cascades. In AD brain tissue, Bonkale et al. [22] found that only the G_s-protein coupling to AC is disrupted in selective areas. However, the subsequent activity of protein kinase A (PKA) seemed unaffected.

In contrast to AC-coupled mAChRs, multiple steps appear to be disrupted in the PI-signalling system activated by mAChRs. The most abundant muscarinic receptor, M_1, covers 35–60% of all mAChRs in neocortex and hippocampus tissue [19]. While the total number of M_1 receptors is not affected in AD (probably due to their postsynaptic location), reduced numbers of M_1 receptors in the active, G-protein-coupled high-affinity state have been observed [23–25]. This deficit leads to a decreased signal transmission efficiency of the receptor, and a subsequent reduction in all downstream processes. It has been suggested that the deficient M_1 receptor-G-protein coupling is due to age-related changes in

membrane properties (e.g. fluidity) as a result of oxidative damage ([26], and see below).

M_1 receptor stimulation activates the PLC β family [27] through the G-proteins Gq/G_{11} [28–30]. It appears that only the activity of G-proteins is affected in AD whereas the amounts remain stable. Overall PLC activity also seems unaltered in AD (see also below), however, localised up- and downregulation of isoforms of PLC and their activity have been reported, and may play a role in AD pathology [27]. Moreover, reduced PI levels have been described ([32] and see table 1), indicating another limiting factor for intracellular signalling. This is thought to be a result of an AD-related deficit in PI 3-kinase [33] and possibly PI 4-kinase [34]. Both enzymes are PI synthesising, and PI 3-kinase is thought to be involved in regulation of cytoskeletal turnover processes. Proper function of the PI signalling cascade is further hampered by reduction of the number of intracellular IP_3 receptors [35, 36]. The reason for this reduction is unknown, but it has been hypothesised that IP_3 receptors are degraded by the calcium-activated protease calpain, which is markedly elevated in AD brains [37].

Table 1. Summary of changes in cholinergic transmission as a result of AD.

AD-related changes in cholinergic transmission	Amount	Activity
Cholinergic projections (presynaptic)		
Precursors		
glucose	–	n.a.
choline	–	n.a.
Synthesising enzyme		
choline acetyl transferase (ChAT)	–	?
Transmitter		
acetylcholine (ACh)	–	n.a.
Re-uptake and breakdown related		
choline esterase	−10–60%	n.a.
high affinity choline uptake (HACU)	?	–
Autoreceptors (muscarinic)		
M_2 (and M_4)	–	0
G-proteins		
G_s	?	–
G_I	–	0
Second messenger cascades		
adenylylcyclase (AC)	?	0
cAMP	?	n.a.
PKA	?	0
Nicotinic receptors	−40–70%	
nicotinic receptors		
Recipient neurones (postsynaptic)		
Muscarinic receptors		
M_1	0	–
M_3	?	?
M_5	?	?
G-proteins		
G_q /G_{11}	0	–
Second messenger precursors		
phosphoinositides (PI, PIP, PIP_2)	−40%	n.a.
Phospholipases		
overall PLC (-β, -δ and -γ)	0	0
PLC-β	0 or +	
Second messengers		
IP_3	–	n.a.
DAG	–	n.a.
Ca^{2+}	–	n.a.
Second messenger receptors		
IP_3 receptors	−50–70%	?
IP_4 receptors	0	0
Kinases		
PKC	–	=

Some of the reported changes are related to the degeneration and loss of cholinergic neurones per se, others are related to changes in the functional properties of enzymes and their substrates as a consequence of AD pathological mechanisms. A deficit early in the second messenger cascades leads naturally to reduced activation at later steps in the cascades. The majority of the research focusing on ACh alterations in AD has been performed using the hippocampus and the forebrain cholinergic fibres. Pertaining to this system a terminology of 'pre- and postsynaptic' cholinergic alterations in AD has been employed. Key: −, downregulation or inactivation; +, upregulation or activation; 0, no change; ?, no information; n.a., not applicable. (Information from: [22, 27, 31, 32, 35, 95, 153–155]).

Numerous studies have provided evidence for PKC being downregulated in AD brain tissue, both with respect to enzyme levels and activity ([38] see table 1). An overall decrease (50%) of PKC in certain (particulate) fractions from AD brains compared with age-matched controls was reported. Similar to PLC analysis, overall analysis of PKC amounts conducted in various investigations is suggested to be unsuitable, since the location of PKC is crucial for its function and activation. Moreover, different isoforms of PKC show different patterns of distribution and activity in the brain, and they are differentially affected in AD (reviewed in [39]). One PKC isoform, β-PKC, which is relevant for the mACh-stimulated PI signalling, has been shown to be reduced in membranous fractions of temporal cortex tissues from AD patients [27].

The nicotinic system

In central neurones, nicotinic acetylcholine receptors (nAChRs) are not as abundant as muscarinic receptors. Nevertheless, a variety of nAChRs consisting of various combinations of subunits have been identified, and possibly more remain to be discovered. Nicotinic receptor subunits form ion channel pores and conduct Ca^{2+}, Na^+ and/or K^+ currents. In the CNS, they have been suggested to be present presynaptically, thus modifying both excitability of neurones and release of different neurotransmitters, including ACh itself [40, 41]. The first demonstration of postsynaptic nicotinic responses in the hippocampus was provided only very recently, but their role in AD pathology remains to be investigated [42].

In general, the contribution of deficits in nicotinergic transmission in AD has not received as much attention as the deficits in signal transduction in the muscarinic

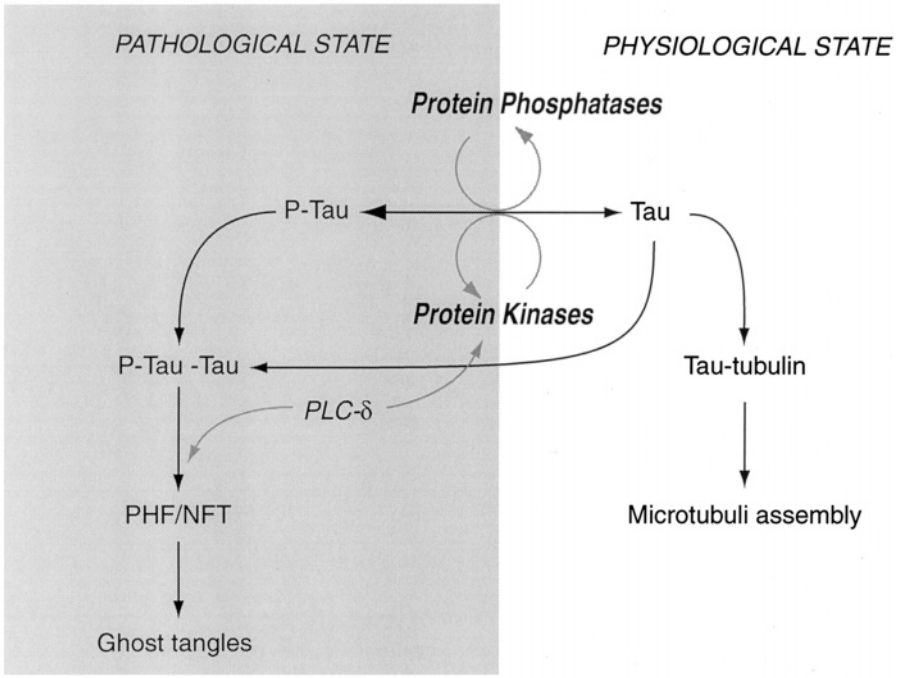

Figure 2. The importance of the function of protein kinases and phosphatases in the balance between pathological phosphorylation of τ and normal τ function in relation to the assembly and function of cytoskeletal components. Hyperphosphorylation of τ (P-Tau) results in decreased affinity for tubulin and increased self-assembly (P-Tau-Tau), formation of fibrils and fibrillary lesions, ultimately leading to neurodegeneration and formation of extracellular ghost tangles (illustrated on grey background signifying pathological mechanisms). The pathological process can be enhanced by action of other enzymes such as PLC-δ, increasing kinase activity or promoting fibril formation (see text for details). Physiological assembly of τ via tubulin binding (Tau-tubulin) and formation of microtubuli is illustrated on the white background. (Information from: [27, 52]).

system. Nevertheless, the nicotinic system has been shown to modulate attentional processes, and it is able to facilitate memory (reviewed in [43]). It is thus very likely that deficits in nicotinic signalling are also involved in behavioural and cognitive deficits seen in AD.

The most prominent change of the nACh system in AD is a great reduction in the number of receptors ([44] see table 1). It has been speculated that this reduction in receptor number could be caused by a preferential presynaptic location on degenerating projection neurones in AD, especially since tangle-related pathology has been shown to hinder the transport of nicotinic subunit mRNA and proteins [45].

Stimulation of the nicotinergic system may possess some protective capacity in AD, and treatment with nicotinic agonists provides protection against βA induced neuronal death (see below and [46]). The beneficial effects of nicotinergic stimulation have been attributed to inhibition of monooxide, protection against excitotoxicity, and neurotrophin production (see below and [43]). They are likely to be mediated through the predominant nACh receptor subtype in the brain containing α7 subunits, which is possibly involved in modulation of Ca^{2+} homoeostasis [47]. Additionally, there is some evidence that smokers have a reduced risk of developing AD (reviewed in [48]). Thus, preventive measures using stimulants of nicotinic receptors have been suggested in AD treatment. As with other treatment strategies in AD, the existing difficulties in early diagnosis of the disease as well as the lack of receptor-selective pharmacological agents have so far hampered the use of nACh-related drug treatment (reviewed in [43]).

Intracellular signalling and the τ pathway

The τ protein exists in several isoforms, six of which are present in the adult human brain. Its functional properties depend on multiple phosphorylation sites, regulated by various phosphatases and kinases (see fig. 2 [49]). The latter include proline-directed protein kinases (PDPKs), stress-activated protein kinases (SAP kinase) and a novel serine/threonine kinase (PKN); a detailed list is provided in table 2.

τ extracted from PHFs from AD patients is hyperphosphorylated and distinct from τ obtained from healthy individuals. The degree of τ phosphorylation in situ may in fact be even higher than the levels measured, since phosphatases (but not kinases due to their independence of adenosine-triphosphate (ATP)), continue to be active after death. The aberrant phosphorylation interferes with τ's tubulin binding site, thereby making it unable to bind and stabilise microtubuli, resulting in their disassembly [50, 51]. Concomitantly, the affinity for phosphorylated and nonpathological forms of τ is increased, leading to enhanced self-assembly and depletion of τ for 'household' purposes. As a consequence, any factor influencing the existing equilibrium between kinases and phosphatases, pushing it towards either activation or upregulation of protein kinases and/or inactivation or downregulation of phosphatases, can be expected to contribute to AD pathology [52, 53] (fig. 2)[1].

So far, more than 400 different kinases have been identified [53], but only few have been tested with respect to their ability to phosphorylate τ. While most kinases studied showed abilities to phosphorylate τ in vitro [54], this does not necessarily imply that they are also relevant in physiological or pathological processing of τ. The most likely candidates so far playing a role in PHF formation in AD are τ protein kinase I and II (TPK I and II) [55] and PKN [56]. The latter contains domains highly homologous to PKC, and is assumed to be crucially involved in stabilising cytoskeletal proteins. TPK I, on the other hand, may provide a link to cholinergic deficits (see above), since it is involved in regulating enzymes involved in ACh synthesis [57]. A novel family of protein kinases ('MARK') that phosphorylate τ and other microtubuli-associated proteins has been described recently [58]. Overexpression of these kinases in vitro leads to hyperphosphorylation of microtubuli-associated proteins, microtubuli disassembly and ultimately cell death. Their role in AD, however, remains to be investigated.

Of the phosphatases, protein phosphatase-2A (PP-2A) [59, 60] and protein phosphatase-2B (PP-2B) [61] seem especially efficient in dephosphorylating pathologically phosphorylated τ, and both phosphatases show either decreased enzyme levels and/or activities in AD [61–63, but see 64].

In addition to those kinases and phosphatases directly involved in the aberrant phosphorylation of τ, other factors have been shown to influence PHF assembly. For example, Saitoh and Iimoto [65] and Boyce and Shea [66] have suggested that downregulation of PKC may lead to overactivation of other kinases, thereby causing excessive phosphorylation of numerous proteins, including τ [65]. It has also been hypothesised that downregulation of phosphotyrosine phosphatase or phosphatase 2A can lead to an increase in mitogen-activated protein (MAP) kinase activity [67, 68]. MAP kinases, which are components of cellular signalling assumed to be involved during mitogenesis and differentiation, have been suggested to contribute crucially to synaptic plasticity in postmitotic cells [69]. Since enhanced MAP kinase activity may cause an increase in τ phosphorylation [67, 68] an involvement of MAP kinase in AD pathology has been indicated [70].

Finally, a member of the PLC δ family (PLC-δ1) has been found in higher concentrations in AD brain tissue especially associated with PHF- or NFT-bearing neurones (this enzyme is distinct from the PLC-β family implied in mAChR signalling, see above). The resulting overall PLC-δ1 activity, however, is not altered, indicating a decrease in its specific activity in AD brains compared with controls [27]. The authors suggested that

Table 2. List of protein kinases and phosphatases found capable of tau phosphorylation or dephosphorylation in vitro. The right column refers to AD related changes of these enzymes.

	AD-related changes
Kinases	
Proline-directed protein kinases (PDPKs)	
cyclin-dependent protein kinases cdk-2, cdk-5* & Tau protein kinase II (TPK II)	+
mitogen-activated protein (MAP) kinases	0
glycogen synthase kinase GSK3, GSK3β/Tau protein kinase I (TPK I)	+
Stress-activated protein kinases (SAPs)	
SAP kinase-3, -4, beta/JNK, p38/RK	?
Other kinases	
protein kinase A (PKA)	0
protein kinase C (PKC)	–
PKN$^{\triangledown}$	overall 0, but redistributed
casein kinase II	redistributed or −
Ca^{2+}/calmodulin dependent kinase II (CaM kinase)	0
MARK$^{\triangledown}$	?
Phosphatases	
protein phosphatase 1 (PP1)	–
protein phosphatase 2A (PP2A)	–
protein phosphatase 2B/Calcineurin	overall 0, but redistributed

Key: 0, no change; ?, no information; −, inactivation or downregulation; +, activation or upregulation; $^{\triangledown}$, see text for details; *, cdk-5 is a part of TPK II. Redistribution means that the intracellular location of the enzymes have been found to change under AD, although the overall levels are not changed. Redistributed enzymes are often found closely associated with neurofibrillary structures. (Information from: [55, 56, 58, 62, 64, 77, 129, 136–142]).

[1] While the hypothesis of τ hyperphospharylation as a prerequisite for PHF formation and pathology is widely accepted, it is noteworthy that contradictory evidence suggests that only ~5% of PHFs are abnormally phosphorylated and that phosphorylation prevents rather than facilitates assembly [156, 157].

the enzyme may be involved in the formation of intraneuronal filamentous inclusions and that the phosphoinositide metabolism is disturbed in AD. The latter conclusion, however, may be an overinterpretation, since close association with PHF may lead to inactivation, immobilisation and, as a consequence, accumulation of PLC without affecting the intrinsic activity of unbound enzymes. In contrast to the initial findings, subsequent investigations from the same group report significantly higher PLC-δ1 activity in AD compared with control brain tissue [71]. Concomitantly, the results revealed a significant reduction in PLC-γ1 in AD brains compared with controls. These later results appear to be more convincing indicators for PLC subtype-specific disturbances in PI metabolism.

The other key player in AD, βA (see below), was also described to further influence the assembly and stability of PHF. βA may stimulate hyperphosphorylation of τ in vitro [72], thereby pointing to a possible link between the amyloid and neurofibrillary pathology. A likely candidate in this pathway seems to be TPK I [73]. However, this does not mean that amyloid deposits necessarily precede neurofibrillary lesion formation in the events leading to AD. Accordingly, experimental downregulation of PP-1 and -2A was described to result not only in hyperphosphorylation of τ and cytoskeletal disruption but also in accumulation of βA [74, 75].

Sulphated glycosaminoglycans such as heparin and heparan sulphate also seem to be intimately connected with τ pathology. At low concentrations, they are able to stimulate the phosphorylation of τ via several protein kinases. In vitro experiments have additionally indicated that phosphorylated recombinant τ does not assemble into PHFs or SFs without the presence of sulphated glycosaminoglycans [76–78]. Sulphated glycosaminoglycans are also involved in the assembly of βA-fibrils, and they possibly act as a general factor in facilitation of fibril formation.

Assembly of hyperphosphorylated τ into PHFs seems to make it a less favourable substrate for both PP-2A and -2B, thereby reducing the possibility of dephosphorylation. Additional modifications (ubiquination, protein processing or truncation) of the τ molecules after fibril formation may further stabilise the structures [79, 80].

The role of second-messenger cascades in APP processing

The vast majority of current basic AD research concerns the role of APP-related pathways and βA toxicity. It is assumed that APP is synthesised in the rough endoplasmic reticulum, passed through the Golgi apparatus and translocated subsequently to the membrane in vesicles. APP may be cleaved into different fragments, presumably by three enzymes, termed α, β and γ secretase (fig. 3). The molecular identity of the secretases remains to be resolved, but their suggested actions have been described in detail elsewhere [8, 81, 82]. Briefly, the α secretase cleaves APP between amino acids 612 and 613 of APP (isoform 695 numbering), which corresponds to amino acids 16 and 17 of the βA sequence, thereby preventing formation of βA. This way of processing is referred to as the 'secretory pathway', and the fragment released is named soluble APP (APP_s) as it does not form amyloid fibrils. APP_s has been suggested to exhibit trophic actions and multiple physiological roles [2, 8, 83], including involvement in neuronal signalling and cellular rescue mechanisms.

Pathogenic processing (termed 'amyloidogenic pathway', fig. 3) involves β and γ secretases. The former enzyme cleaves between amino acids 596 and 597 of APP, which corresponds to the amino terminus of the βA sequence; and γ secretase between amino acids 639 and 642, which corresponds to the βA carboxy terminus. An additional cleavage site for a δ secretase has been proposed, but the involvement of this enzyme in βA formation and AD pathology awaits further investigation [84]. The action of the enzymes within the amyloidogenic pathway results in liberation of βA peptide, varying in length from 39 to 42 amino acids. Several lines of evidence suggest that βA(1–42) is more toxic and aggregates more readily than the others [85–87].

Different locations in the cell have been described for the actions of the enzymes involved in APP processing. There is some support for either APP reaching the membrane as an intact molecule or that the α secretase acts in an intracellular compartment [8, 88]. Equally, β and γ secretase may act at the plasma membrane or in intracellular compartments (reviewed in [8, 82]).

Based on the mutually exclusive pathways outlined for APP processing, it has been proposed that secretase therapy might offer promising new treatment strategies for AD patients in the future [89]. A specific inhibitor of one of the enzymes involved in the amyloidogenic pathway would shift the balance between the amyloidogenic and secretory pathway towards a beneficial APP_s production.

In addition to the role of the secretases, APP processing and metabolism have been shown to be sensitive to a variety of second-messenger systems, summarised in figure 3. Phosphorylation processes appear to be particularly important, probably due to the phosphorylation state of APP itself and/or phosphorylation of the enzymes involved in APP cleavage [90, 91]. It has been proposed that a modulatory shift of the equilibrium towards βA production can reduce the amount of APP_s [92]. However, it is noteworthy that the influence of second messenger systems on the reciprocal relationship between βA and APP_s production appears to be species- and cell type-specific [93].

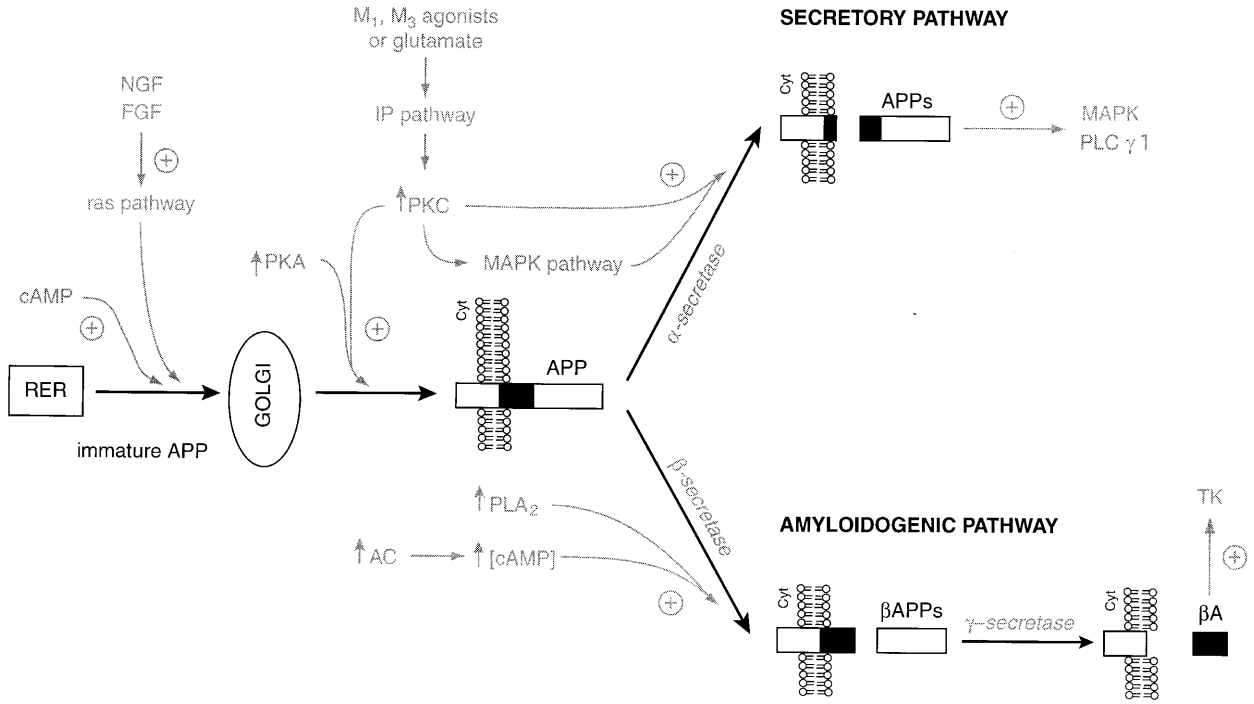

Figure 3. APP processing and its modulation by second messengers. After synthesis in the rough endoplasmatic reticulum (RER), transportation through the Golgi apparatus (GOLGI) and cytosol (Cyt), the mature protein is cleaved by either α-secretase (the secretory pathway) or by β- and γ-secretases (the amyloidogenic pathway). The influence of different signalling pathways at different stages is shown. The end products APPs and βA can also affect enzymes in intracellular cascades (see table 3). (Information from: [30, 38, 82, 95, 97, 98, 101, 103, 114, 116, 133–135]).

A pathological imbalance in APP processing could be triggered by a variety of events and factors which may be age-related and/or caused by various 'cell stressors' (see below and [8]). Malfunctions in neuronal messenger systems would generally be expected to influence APP processing, with glutamate-mediated excitotoxicity and cholinergic deficits (see above) being the most obvious candidates [94, 95]. Deficits in the PI pathway may be brought about by defective metabotropic glutamate receptor (mGluR) and mAChR signalling [30, 95]. Accordingly, studies utilising drugs which target these receptors have shown that their stimulation or inhibition can alter APP processing, presumable involving PLC, PKC and tyrosine phosphorylation [96–101]. For instance, *trans-(1S, 3R)*-1-amino-1, 3-cyclopentane dicarboxylic acid (ACPD), an agonist of group I and II mGluRs, was found to increase the release of APP_s, and this action was blocked by the selective mGluR antagonist ($\pm$)-α-methyl-4-carboxyphenylglycine (MCPG). It was also observed that IP_3 accumulated after treatment with ACPD in addition to an increase in APP_s, consistent with stimulation of mGluR group I receptor and activation of the corresponding signal transduction pathway [101]. Moreover, a reduced level of PKC as found in AD brains (see above) has also been described to modulate APP processing [102]. Activation of PKC with phorbol esters was reported to significantly enhance APP_s production [103–105], and in some cases a corresponding decrease of the βA level was obtained. Consistent with these results, the PKC inhibitor H7 was shown to reverse partially the increase in secretion of APP_s [106]. In addition, PKC-induced inhibition of βA production was reported to be opposed by the phosphatase calcineurin in a calcium/calmodulin-dependent manner [107], and phosphatase inhibitors such as okadaic acid have been demonstrated to stimulate APP_s release [108]. However, okadaic acid treatment may (simultaneously?) lead to formation of βA [74].

The supposedly beneficial role of PKC has been questioned by other reports indicating PKC hyperactivity in AD brains before it is downregulated [39, 94] and by in vitro evidence of a PKC-related increase in βA production [93]. High doses and prolonged treatment with phorbol esters have been found not only to overactivate PKC but also to translocate it to the cell membrane and to downregulate it eventually [39, 109, 110]. In animal models, chronic overstimulation of PKC was reported to cause cell damage. Pathological effects including βA

immunoreactivity have been observed after injections of phorbol 12-myristate 13-actetate (PMA) into the rat neocortex [111]. Treatment with PMA induced human cortical neurone degeneration over a period of 3–24 h and an increase in two AD markers, Alz-50 and 5E2 [112]. It is therefore possible that aberrant neuronal activity may initially result in hyperactivation of PKC-dependent signal transduction pathways and subsequent cell death. An excessive release of growth factors and cytokines, evoked as a cellular rescue response, could further facilitate overactivation of PKC [39, 94, 113]. Taken together, the fine-tuning of PKC appears to play a central role in APP metabolism, but based on the current evidence it is not possible to deduce whether PKC inhibition or stimulation may be beneficial in AD.

Other kinases assumed to contribute to neuronal malfunction in AD pathology are the MAP kinases ERK1 and ERK2. This may also involve a modulation of APP processing, since MAP kinases have been described to influence APP metabolism [114]. It was reported that activation of this pathway is necessary for regulation of APP_s, for instance via nerve growth factors (NGFs), which may imply that one pathway of PKC-dependent APP_s release is mediated through the MAP kinase pathway (fig. 3).

Another second-messenger pathway affected in AD concerns AC signalling and cAMP production. This pathway is utilised for instance by class II and III mGluRs and muscarinic ACh receptors (see above), but also mediates a wide variety of extracellular signals from other neurotransmitters, hormones and growth factors. One connection to APP processing is provided via the APP promoter, which has a number of regulatory elements, including a binding site for the AP-1 transcription factor. c-fos, a constituent of this complex, can be activated via cAMP-responsive mechanisms. It has been shown that cAMP-dependent mechanisms upregulate APP mRNA in BT4C and BT4Cn glioma cells [115]. Moreover, forskolin, an activator of AC, which causes an increase in cellular cAMP concentrations, also inhibited the normal and phorbol ester-stimulated release of APP_s [116]. This suggests that activation of the AC pathway inhibits APP_s secretion via regulation of intracellular cAMP levels.

Searching for the common denominator

To date, a major shortfall of AD research is the lack of knowledge concerning links between the hallmarks of AD outlined above. While connections have been found for some parameters, we are far from understanding the whole picture of AD. Thus, we suggest that future studies on common mechanisms and interactions between key players in AD may offer new perspectives and promising approaches.

β-Amyloid toxicity

Since βA was found to be a major constituent of AD plaques and due to APP gene mutations in fAD, research into the properties of βA has gained much interest within the scientific community. A large variety of neurotoxic effects have been attributed to βA, including the disturbance of Ca^{2+} homoeostasis, modifications of neurotransmitter systems such as glutamate and intracellular cascade malfunction [8, 83, 117, 118]. A list of systems implied to be affected by βA toxicity is provided in table 3. From this list, it is obvious that

Table 3. Neurotoxic effects of βA.

System	Effect of βA
Cell homoeostasis/stability	
Ca^{2+} homoeostasis	resting $[Ca^{2+}]$↑ & responses to neurotransmitters ↑
plasma membrane	formation of ion pores lipid peroxidation$^{\triangledown}$↑
Inflammatory response	
complement cascade	induces cascade*
TNF	↑
Neurotransmitters	
glutamate: uptake	↓
glutamate: NMDA signalling	↑
glutamate: mGluR	uncoupling from G-protein
acetylcholine: production, release, function and uptake	
acetylcholine: mAChR	uncoupling from G-protein
Kinases	
tau protein kinase I	↑
tau protein kinase II	↑
MAP kinase◆	↑
protein kinase C (PKC)	↓
Other enzymes	
sodium/potassium ATPase	↓
phospholipase A2, C and D	↑
creatine kinase	↓
glutamine synthase	↓
FAK phosphorylation	↑
Ion channels	
K^+	↓
Ca^{2+}	↑/↓$^{\otimes}$
Oxidative stress	
SOD/HO-1	↑$^{\infty}$
Apoptosis/Necrosis pathways	
Bcl-2	↓
Bax	↑
IEG	

The majority of findings arise from studies in in vitro systems. βA can affect a diverse number of systems which may all contribute to a loss of cellular homeostasis and cell death. Key: ↑, increase, activation or upregulation; ↓, decrease, deactivation or downregulation $^{\triangledown}$, linked to oxidative stress; *, C1q binds directly to βA; ◆, leads to phosphorylation of CREB; $^{\otimes}$, concentration dependent; $^{\infty}$, in transgenic model). (Information from: [8, 83, 95, 117, 118, 132, 143–152]).

βA may also affect its own production via feedback loops through multiple second messenger cascades.

Based on the widespread actions of βA, the question arises whether this peptide itself could be linked to both τ pathology and cholinergic deficits in AD. Indeed, a possible connection with τ pathology may be caused via enzymes involved in τ phosphorylation and fibril formation. In particular, TPK I and II have been described to be upregulated by βA [73], but it can also increase the activity of other kinases such as casein kinase I and II [119]. Modulation of PKC could also contribute to hyperphosphorylation and misprocessing (see above). Whereas βA was found to modify PHF assembly (see above), disturbance of Ca^{2+} homoeostasis and the link to oxidative stress (see below) may affect further enzymes involved in τ processing and fibril formation.

A strong link between βA and cholinergic functions has been suggested recently based on βA's multiple interactions with ACh synthesis (including ChAT and TPK I activities), release, function and uptake (reviewed in [118]). The authors stress that βA can cause cholinergic hypoactivity before any neurotoxic actions are obvious, and that the action of βA may explain the selective vulnerability of cholinergic neurones.

While βA's connections to different hallmarks of AD may seem promising, it must be stressed that the evidence concerning its central role in AD pathology ought to be considered with great caution. Most data were obtained by means of in vitro studies, sometimes with nonneuronal cell lines, nonphysiologically high concentrations of βA or βA fragments not present in AD brains. Moreover, animal models based on APP mutations or βA injections have so far not been successful, and the relevance of βA pathways in human AD pathology remains to be proven.

Energy metabolism and Ca^{2+} homeostasis in AD

When searching for common factors which may account for multiple malfunctions and pathological pathways, one has to consider that the dominant 'risk factor' in neurodegenerative diseases is ageing. It is also well known that energy metabolism and mitochondrial function deteriorate with age, thus causing oxidative stress in the CNS. 'Oxidative stress' is defined as an imbalance between the production of free radicals and cellular defence mechanisms. The main source of free radical generation is the mitochondrion, where oxygen-derived free radicals are formed as by-products of respiratory and oxidative processing (see fig. 4). Oxyradicals or reactive oxygen species (ROS) are known to damage lipids, proteins and DNA. In particular, double bonds on unsaturated fatty acids of membrane phospholipids are damaged, a process known as membrane lipid peroxidation, which results in a disturbance of membrane integrity [120]. Moreover, certain ROS are capable of inactivating a number of enzymes. The oxyradical cascade eventually leads to membrane disturbances, enhanced Ca^{2+} influx (see below), and destabilisation of the cytoskeleton and a further potentiation of membrane peroxidation and free radical propagation. Trace metals [e.g. iron (Fe^{2+}), zinc (Zn^{2+}) and copper (Cu^{2+})], which catalyse or facilitate oxyradical generation, have been found to be increased in AD [121, 122]. Moreover, the activity of endogenous antioxidants has been demonstrated to be markedly reduced in AD, and free radical-mediated induction of antioxidant enzymes and heat-shock proteins was reported to be associated with both senile plaques and NFTs [121, 123].

The main pathways involved in ROS generation are summarised in figure 4. It emphasises the central role of intracellular Ca^{2+} concentration and the fatal positive feedback loop due to failure of ATPase activities, leading to depolarisation and hence a further promotion of Ca^{2+} influx through voltage-dependent calcium channels and *N*-methyl-D-aspartate (NMDA) receptors. In addition to the pathways shown, further Ca^{2+}-dependent pathways such as overstimulation of lipases, proteases and endonucleases are triggered which also contribute to the observed cell pathology.

The initial step in the chain of events could be at any point of the pathway. Since excitotoxicity and excessive glutamatergic transmission have been implied in AD, it may be found within the glutamate release and uptake system, leading to an overstimulation of postsynaptic receptors and subsequent Ca^{2+} influx. In return, the lipid peroxidation processes caused by ROS have been demonstrated to impair glutamate transport and uptake [120]. Alternatively, low cellular energy stores due to mitochondrial malfunction or glucose deficits and a subsequent impairment of ATPase function could lead to a build-up of intracellular Ca^{2+}. An impairment of transport and uptake of glucose is described in ageing and in particular in AD, and the resulting limited mitochondrial function would cause a diminished ATP production of up to 81% [124]. The cause for diminished energy metabolism and the resulting increase in free radicals may also be caused by a defect in respiratory chain complex activities (reviewed in [125]), and there is evidence for mitochondrial cytochrome oxidase 1 and 2 mutations in AD [126]. Furthermore, a genetic variant of mitochondrial transfer RNA (tRNA) (A4336G transition) has been identified as being correlated with AD [127]. It was also demonstrated that cybrids[2] with mitochondrial DNA from AD patients possess decreased

[2] Cybrid cell lines are made by fusing cells from AD patients, which have been enucleated but still contain mitochrondria, with normal cells without mitochrondria.

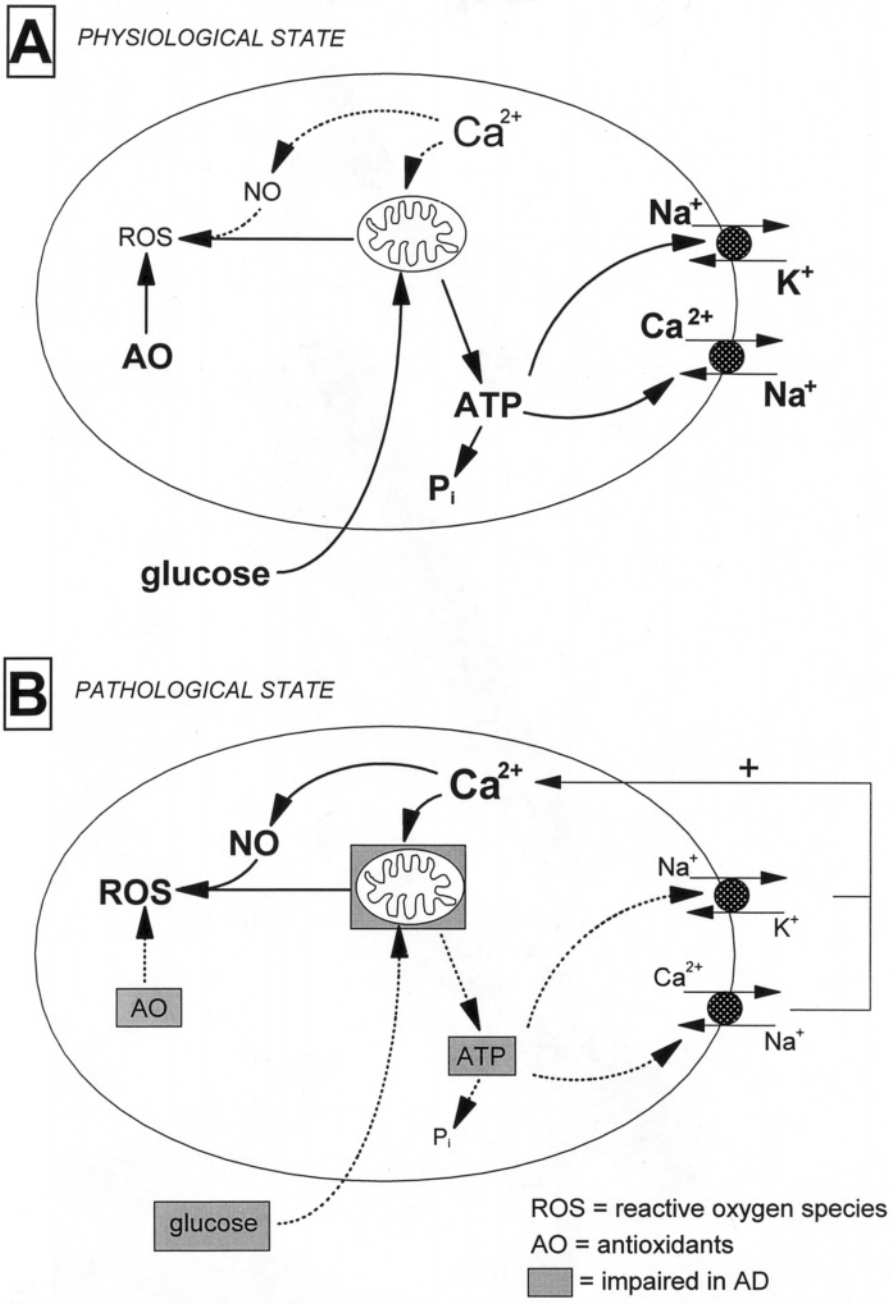

Figure 4. Calcium homeostasis and mitochondrial function. (A) Under physiological conditions, neurones cope with normal Ca^{2+} influx by uptake into intracellular compartments, including mitochondria. Small amounts of reactive oxygen species (ROS) generated as by-products of mitochondrial metabolism are scavenged by antioxidants (AO) such as glutathione, superoxide dismutase and peroxidases. Additionally, Ca^{2+} can be extruded via ATP driven pumps (e.g. Na^+/K^+ and Ca^{2+}-ATPase). ATP, provided via oxidative phosphorylation inside mitochondria, is also required for various other phosphorylation processes (P_i). Under pathological conditions B, an overshoot of intracellular Ca^{2+} triggers multiple fatal cascades. High Ca^{2+}-uptake into mitochondria results in (i) reduced production of ATP and (ii) overproduction of ROS, for example superoxide ($O_2^{\bullet}$) and hydroxyl ($OH^{\bullet}$). At the same time, high Ca^{2+} via calmodulin and nitric oxide (NO) synthase activation stimulates NO production and leads to peroxynitrite ($ONOO^-$) and $NO_2^{\bullet}$ generation. Reduced activity of AO further enhances ROS buildup. ROS cause cell damage by reactions with lipids, proteins and DNA. Depletion of ATP leads to decreased pump activities and hence to a depolarisation. This acts as a positive feedback loop, causing further increase of Ca^{2+}. Weak/impaired pathways and components are indicated by dotted lines and grey background, major products/reactions are shown in bold. (Information from: [120, 121, 124, 125, 128, 130]).

activity of electron transport chain complex IV, which leads to enhanced ROS generation, increased cytosolic Ca^{2+} concentration and slower recovery from carbachol-induced Ca^{2+} enhancement [128].

How does mitochondrial malfunction and energy loss link with cholinergic deficits, and APP pathways? As outlined above, second-messenger cascades are involved in regulating either signalling or processing of all three components. Simultaneously, ATP depletion would disturb all cascades involving phosphorylation processes. With phosphorylation states being crucial for all signalling cascades, it is obvious that decreased ATP levels together with a disturbed Ca^{2+} homoeostasis would be fatal and trigger multiple chain reactions. In particular, it has to be considered that oxidative glucose metabolism is the source of acetyl-CoA production required for ACh synthesis. Thus, a drop in ACh levels and deficits in cholinergic signalling are expected as an immediate consequence of decreased glucose turnover (see above).

Within the τ pathway, low ATP levels were found to activate the MAP kinase isoform protein kinase 40 (PK 40 or ERK2) leading to hyper-phosphorylation of τ ([129] and see above). Moreover, the τ molecule with its high proportion of lysine residues represents a potential target for oxidative damage leading to covalent cross-linking, protein aggregation and accumulation [121, 130]. In general, fibril formation which is involved in both τ and βA toxicity is promoted by oxyradicals, and by other factors involved in oxidative processing and protein modifications. Accordingly, antioxidants have been found to be neuroprotective in various in vitro studies, and are now considered for therapeutic approaches [125, 131].

Mitochondrial function, energy metabolism and Ca^{2+} homoeostasis are also closely linked to APP processing and βA-mediated neurotoxicity. Evidence for a mutual interplay is indicated by the observation that oxidative stress was found to increase βA production, and that APP expression is induced by metabolic insults and excitotoxic events (summarised in [83]). Reciprocally, in transgenic APP mutants and after treatment with βA itself, evidence for oxidative stress was found [132]. Moreover, βA itself leads to an increase of oxidative damage (see above and [83]) and it was claimed that βA toxicity is due to oxidative injury [131, 132].

The particularly high vulnerability of neurones to oxidative stress also provides a direct link to cell type-specific death in AD. First, the antioxidant glutathione, which is responsible for the removal of cytosolic peroxides, is only found at low concentrations in neurones, rendering them vulnerable to oxidative stress. Second, oxidative stress is particularly likely to occur in neuronal tissue due to the high metabolic rate and hence high oxygen consumption of neurones. Third, neurones contain a high proportion of free radical-sensitive polyunsaturated fatty acids susceptible to peroxidative damage [121]. Together, neuronal properties may thus contribute to the fatal and progressive cascades involved in AD.

As with the key factors and pathways of AD discussed above, it is of course debatable whether mitochondrial malfunction, subsequent oxidative stress and energy failure are primary or secondary events in AD. However, as outlined here, mitochondria-dependent cascades may well be at the centre of neurodegenerative processes, and oxidative stress may be the link between the pathways related to βA, τ and cholinergic signalling. Similar cascades may also be involved in other forms of neurodegeneration such as prion-related diseases, where aggregation of proteins to fibrils appears to be a key feature. Moreover, the multiple interconnections and fatal circuits outlined in the present review illustrate the general principle of mutual reinforcement ('When it has first gone wrong, it is going to get worse!').

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