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Review

Calcium signals induced by amyloid β peptide and their consequences in neurons and astrocytes in culture

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Abstract

In Alzheimer's disease, amyloid beta (Aβ) peptide is deposited in neuritic plaques in the brain. The Aβ peptide 1–42 or the fragment 25–35 are neurotoxic. We here review our recent explorations of the mechanisms of Aβ toxicity in hippocampal cultures. Aβ had no effect on intracellular calcium in neurons but caused striking changes in nearby astrocytes. The $[Ca^{2+}]_c$ signals started ~5–15 min after Aβ application and consisted of sporadic $[Ca^{2+}]_c$ pulses. These were entirely dependent on extracellular Ca^{2+}, independent of ER Ca^{2+} stores and resulted from Ca^{2+} influx, probably through Aβ-induced membrane channels. The Ca^{2+} signals were closely associated with transient, episodic acidification which may reflect displacement of protons from binding sites or $Ca^{2+}/2H^+$ exchange. Aβ caused an increased rate of generation of reactive oxygen species (ROS), also seen in astrocytes and not in neurons. The increased ROS generation was blocked by inhibitors of the NADPH oxidase, strongly suggesting that this enzyme, normally associated with immune cells, is expressed in astrocytes. ROS generation was also Ca^{2+}-dependent, suggesting that Aβ activation of the enzyme may be secondary to the increase in $[Ca^{2+}]_c$. Aβ caused delayed neuronal death despite the fact that all responses were seen only in astrocytes. Neurons could not be protected by glutamate receptor antagonists, but were rescued by inhibition of the NADPH oxidase, by antioxidants and by increasing glutathione. These data suggest that Aβ causes Ca^{2+}-dependent oxidative stress by activating an astrocytic NADPH oxidase, and that neuronal death follows through a failure of antioxidant support.

Keywords: Intracellular calcium; Amyloid β; Intracellular pH; NADPH oxidase; Oxidative stress; Alzheimer's disease

1. Introduction

Alzheimer's disease, the most common form of dementia, is characterized by the accumulation of extracellular neuritic plaques in the brain, together with the presence of intraneuronal neurofibrillary tangles (NFT) and progressive neurodegeneration. Amyloid β peptide (referred to henceforth as Aβ) is the main component of the plaques. It is a peptide of 39–43 amino acids that forms insoluble aggregates surrounded by degenerating neurites and activated glial cells. The specific role of Aβ in the development of Alzheimer's pathology remains controversial, but excessive accumulation of Aβ, due either to excessive production or reduced clearance, appears to be sufficient to cause the disease. All known mutations associated with familial Alzheimer's disease lie within the genes for APP or the presenilins, the enzymes involved in the processing of the Aβ precursor protein (APP), and result in net Aβ overproduction. Patients with Down's syndrome have an extra copy of the chromosome that carries the gene for APP and develop plaques and dementia at an early age. As signs of electrophysiological and behavioural changes appear before any significant Aβ deposition in APP transgenic mice, the importance of amyloid in Alzheimer's has been questioned [1] and, indeed, plaque number does not seem to correlate closely with cognitive impairment in patients, and some cogni-

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tively intact elderly individuals have been described in whom a considerable plaque load has been found at post mortem. A likely explanation of this apparent conflict is that the pathogenic form of Aβ is not the large, relatively unreactive, static plaque, but the total Aβ load, rather than plaque load, that correlates with the degree of cognitive impairment [2]. The fundamental mechanism of Aβ toxicity and the biology of Aβ are not well understood. Repeating themes in the literature involve descriptions of altered cellular calcium signalling, altered glutamate transport, oxidative stress and an alteration in the activity of a number of enzyme systems, most notably enzymes involved in intermediary metabolism such as α-KGDH and aconitase [3,4]. The chain of causality and the specific relationship between these variables has remained obscure.

1.1. Aβ and $[Ca^{2+}]_c$ signalling

The effects of Aβ on $[Ca^{2+}]_c$ signalling have been explored in many studies, but unfortunately these have yielded a range of conflicting observations without any consensus on mechanism or the relationship between alterations in calcium signalling and the pathogenesis of the disease. As calcium signals are fundamentally important in neurons and in glial cell signalling, they represent an obvious process that might represent either a mediator or as a manifestation of pathological processes in the CNS. Unfortunately, amyloid peptides have been described as increasing $[Ca^{2+}]_c$, decreasing $[Ca^{2+}]_c$, modulating Ca^{2+} channel activity, altering the dynamics of $[Ca^{2+}]_c$ signals, having no effect at all. From such a confusing literature, it is remarkably difficult to extract an intelligible picture from which to draw meaningful conclusions. Such inconsistencies are likely to reflect the wide variety of preparations used, the different forms of the amyloid peptide used for experiments, and differences perhaps in the effect of exogenous Aβ and intracellularly generated Aβ. Indeed, it seems plausible that different mechanisms reflected by these differences may exist side by side in vivo.

We have applied aggregated Aβ to mixed cultures prepared from the hippocampus or cortex of neonatal rats to explore the mechanism of Aβ neurotoxicity [5,6]. For all experiments described here, we have used the 25–35-peptide fragment and the full peptide, 1–42, both of which are neurotoxic, while the reverse 35–25 peptide sequence has served as a control. The cultures consist of a bed of astrocytes upon which the neurons grow. Using standard fluorescence imaging techniques with fura-2 to measure $[Ca^{2+}]_c$, we were most surprised to find that Aβ had no apparent effect at all on $[Ca^{2+}]_c$ in hippocampal or cortical neurons over the course of an hour or so, but that it did cause the appearance of complex $[Ca^{2+}]_c$ signals in the astrocytes (Fig. 1A and Ref. [5]). These signals are unusual, consisting of sporadic fluctuations in $[Ca^{2+}]_c$, looking sometimes like waves or oscillations but running over a slow time scale compared to the $[Ca^{2+}]_c$ signals that are typical of these non-excitable cells (Fig. 1A, note the time scale is in minutes). Typically, responses appeared after a delay of some 5–15 min, and there was considerable variability between cells in the patterning of the responses,

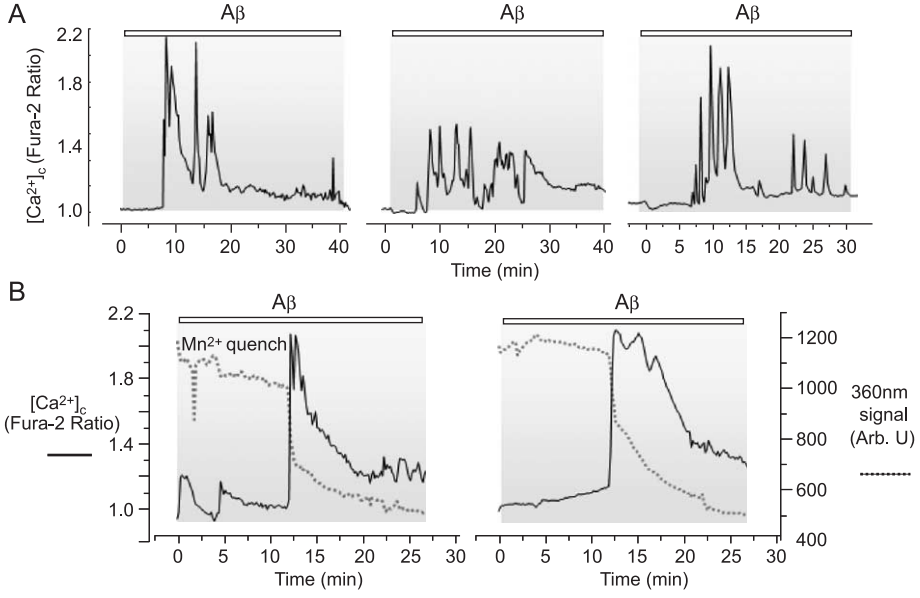

Fig. 1. Calcium signals in astrocytes in response to Aβ. (A) Traces showing changes in $[Ca^{2+}]_c$ (fura-2 ratio) following treatment of astrocyte cultures with Aβ (25–35, 50 μM) in three cells from a single culture. In each case, after a delay, sporadic calcium signals were seen. (B) The same experiment was carried out in the presence of 40 μM Mn^{2+}, and fura-2 fluorescence was excited at 340, 360 and 380 nm. Mn^{2+} quenches fura-2 fluorescence but only has access to the fura-2 if it is able to cross the membrane. Excitation at 360 nm shows the Ca^{2+}-independent fura-2 fluorescence and a decrease indicates Mn^{2+} influx. Note that each calcium pulse is associated with a step change in signal excited at 360 nm.

some showing only occasional $[Ca^{2+}]_c$ transients, while in others the $[Ca^{2+}]_c$ went high and failed to recover.

Confocal imaging at high time and spatial resolution also revealed that the signals consisted of a focal initiating point spreading progressively but decrementing through the cell, unlike self-propagating IP3 based signals, and looking as if they were generated primarily by diffusion from a local source [5]. The signals were wholly dependent on the presence of external calcium and appeared to be largely independent of the integrity of ER calcium stores—they persisted after emptying the stores with thapsigargin, after inhibition of IP3 receptors and of capacitative influx channels with 2-APB and were still seen in the presence of U73122, an inhibitor of PLC. Finally, Mn^{2+} quench experiments with fura-2 loaded cells showed that each pulse of $[Ca^{2+}]_c$ was associated with a step-like Mn^{2+} quench, strongly suggesting that the pulsatile calcium signals arise through occasional pulses of calcium influx (Fig. 1B). This alone is remarkable and unusual—that a calcium influx pathway should cause sporadic and fluctuating $[Ca^{2+}]_c$ signals, and yet it is hard to account for the data with any suggestion other than a sporadic and fluctuating influx pathway. While it remains possible that the Ca^{2+} influx initiates further subtle changes in ER calcium signalling, these do not seem to play a significant role in shaping the major calcium response.

1.2. Aβ as a channel former

Aβ thus appears to be causing periodic Ca^{2+} influx selectively into astrocytes. One obvious mechanism for this would be provided by an interaction of the peptide with a pre-existing channel that is selectively expressed in astrocytes. We have tested a battery of blockers of different classes of Ca^{2+} permeant channels, including 2-APB, which blocks TRP channel species that might be implicated in such a response, and none had any significant effect on the responses [5]. Obviously, there maybe classes of channel that we have missed, but an alternative explanation lies in the pore-forming activity of Aβ. Amyloid β peptides form cation selective channels in artificial planar lipid membranes [7], and form a calcium permeable channel in the membranes of lipid vesicles [8]. Substantial data suggest that these channels may mediate calcium influx into cells in response to Aβ. The channels show variable and sporadic activity and seem capable of generating a number of different conductance states [7]. This channel is blocked by zinc, although zinc can also complex with Aβ, preventing aggregation and so prevent insertion into the membrane or prevent pore formation rather than blocking the formed channel [5,9–11]. The channels are formed by Aβ 25–35 or by 1–40, but not by the reverse peptide 35–25, confirming that this is specific to the toxic peptides and is not some nonspecific general disruption of membrane structure by addition of peptides. In our hands, the $[Ca^{2+}]_c$ signals seen in astrocytes in response to Aβ were prevented by zinc, but once established, could not be blocked by zinc, suggesting that, in this instance, zinc acts to prevent channel formation by the Aβ rather than to block active channels [5]. These data thus provide further evidence to suggest that Aβ may form cationic calcium permeable channels in cell membranes and mediate the observed $[Ca^{2+}]_c$ signals. Interestingly, Lin et al. [8] have shown multimeric channel structures in artificial membranes by atomic force microscopy, and the same group have also shown that Aβ can induce sporadic transient changes in Ca^{2+} in endothelial cells which were entirely due to Ca^{2+} influx. Channel formation is also prevented by clioquinol, an antimalarial heavy metal chelator, that prevents Aβ aggregation. Clioquinol prevented all calcium signals in our hands, a result that seemed particularly exciting, as it also alleviates symptoms in animal models and is now in clinical trials in patients with Alzheimer's disease [12].

What was also surprising about our own study was the selectivity of the action of Aβ which caused dramatic changes in $[Ca^{2+}]_c$ signals in astrocytes while having no noticeable effect at all on $[Ca^{2+}]_c$ signals in adjacent neurons. This is very puzzling. If Aβ acts simply by inserting into lipid membranes and forming a channel, why should there be a difference in different cells? The answer may lie in observations on the importance of lipid composition in the pore forming activity of the peptide. Recent studies have emphasized the importance of the cholesterol content of lipid membranes for Aβ channel formation. Thus, the pore forming activity of Aβ in bilayers is inversely related to the cholesterol content of the lipid mixture. Similarly, depletion of cholesterol content in cells by treatment with cyclodextrin or inhibition of cholesterol synthesis in PC12 cells increased Aβ toxicity [13]. Similarly, Kawahara and Kuroda [14] showed that the Aβ induced $[Ca^{2+}]_c$ increase in cells was attenuated by pretreatment of the cells to increase membrane cholesterol content. We have no data at present about the differences in cholesterol content of different cell types in the CNS, but this seems a simple mechanism that might account for differences in the vulnerability of different cell types to Aβ toxicity. Recent data suggest that astrocytes supply neurons with cholesterol [15], but it is not clear how this might be reflected in the cholesterol content of their cell membranes.

1.3. Aβ-induced changes in pHi

We considered that if Aβ was opening a nonselective cation channel, it might be possible to see associated changes in the intracellular concentrations of H^+ and Na^+. Measurements of pHi using several different indicators all revealed that Aβ also caused fluctuations in pHi. Simultaneous measurements of pHi and $[Ca^{2+}]_c$ using BCECF and fura-2, respectively, showed that the pHi fluctuations (n=76) were precisely synchronous with the $[Ca^{2+}]_c$ signals (e.g., see Fig. 2B)—so much so that we were concerned about

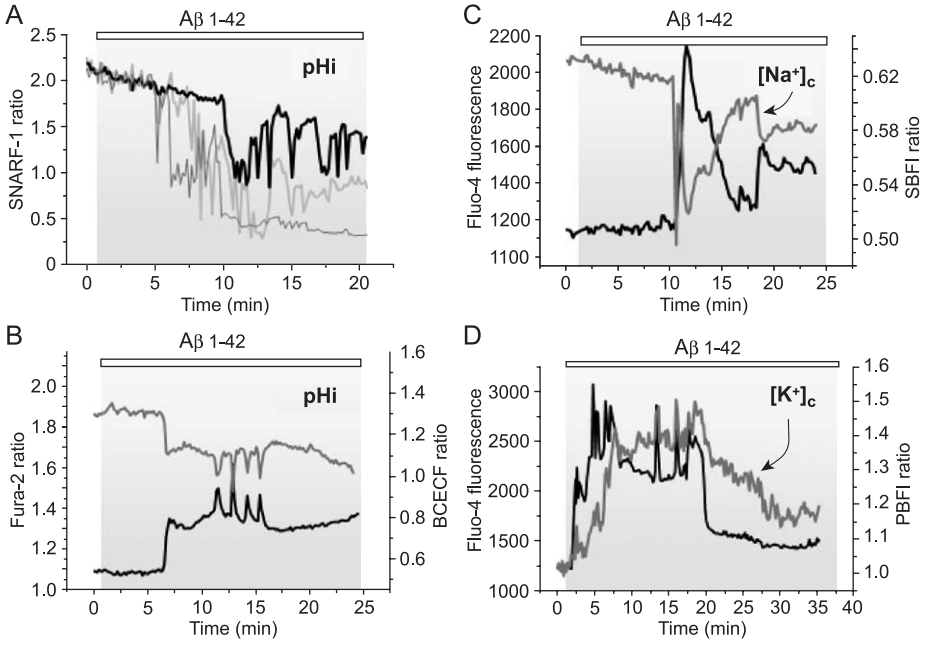

Fig. 2. Changes in pHi, $[Na^+]_c$ and $[K^+]_c$ with changes in calcium in response to Aβ. (A, B) pHi was measured with SNARF (A, ex. 530 nm, em.580 nm, 620 nm) and with BCECF (B, ex 440 nm 490 nm, em. 530 nm). In the latter case, cells were dual loaded with fura-2 and BCECF to examine the relationship between $[Ca^{2+}]_c$ and pHi. In each case, Aβ exposure caused transient acidifications that clearly correlate exactly with the $[Ca^{2+}]_c$ signal. Simultaneous measurements were also made using SBFI to measure $[Na^+]_c$ (C) and PBFI to measure $[K^+]_c$ (D) together with fluo-4 to measure $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ transients were clearly associated with transient decreases in $[Na^+]_c$ and an increase in $[K^+]_c$.

possible cross talk between signals. Nevertheless, the pHi signals were seen without any $[Ca^{2+}]_c$ indicator present and were similar when imaged using confocal microscopy using the dual emission ratiometric indicator, carboxy-SNARF (Fig. 2, n=79).

Investigations of the origin of this response suggest that it is in fact secondary to the $[Ca^{2+}]_c$ signal, rather than reflecting H^+ movement through a channel. Thus, the response was entirely dependent on the presence of extracellular calcium (n=91, data not shown—the traces were completely flat) suggesting that it results either from the displacement of H^+ from protein binding sites by Ca^{2+} or through the activity of PMCAs.

Similarly, the responses were accompanied by fluctuating changes in $[Na^+]_c$ revealed using SBFI (Fig. 2C, n=64). These changes also showed a decrease in $[Na^+]_c$ in synchrony with the changes in $[Ca^{2+}]_c$, suggesting that Na^+ cannot be entering the cell through the channel. Once again, these signals were abolished in the absence of extracellular calcium, and so these observations together suggest that the sodium changes reflect the activity of a sodium/calcium exchanger. Measurements with PBFI also showed changes in signal with Aβ (Fig. 2D, n=89) that were equally dependent on the presence of Ca^{2+}, although the origin of these signals is more difficult to explain and we have not investigated these effects further. These data suggest that the Aβ-induced channel is highly selective for Ca^{2+}, but also emphasize that $[Ca^{2+}]_c$ signals are accompanied by changes in other ionic species that themselves may have a significant impact on cell function.

1.4. Aβ, calcium and oxidative stress

It has been suggested for many years that the pathogenesis of the neurodegeneration in AD involves oxidative stress in some form. Oxidative damage to proteins and to DNA and increased levels of lipid peroxidation have all been described in the brains of patients with AD. Aβ has also been shown using a variety of assays to cause increased production of reactive oxygen species (ROS) and/or impaired antioxidant defences (by definition, oxidative stress) and ROS mediated damage to cellular structures—peroxidation or nitration—in a number of model systems [16–18]. Similarly, considerable evidence suggests that antioxidant strategies may protect cell model systems from Aβ-induced toxicity (e.g., for review, see Ref. [17]). The key questions then are the relative importance of oxidative stress as a contributor to Aβ-induced neurodegeneration, and to identify the sources and targets of Aβ-induced oxidative stress. We were also concerned to understand the relationship, if any, between oxidative stress and the calcium signals that we have described.

As described above, the activity of a number of enzymes involved in intermediary metabolism is decreased in AD brains and in cells exposed to Aβ—notably the activity of glutamine synthetase and creatine kinase, α-KGDH, PDH and aconitase [3,19]. These are all enzymes that are very highly sensitive to oxidative modification and that are altered by exposure to a range of pro-oxidants [20]. Another system shown to be vulnerable to oxidative modification is the glutamate transporter on astrocytes [21], and it has been

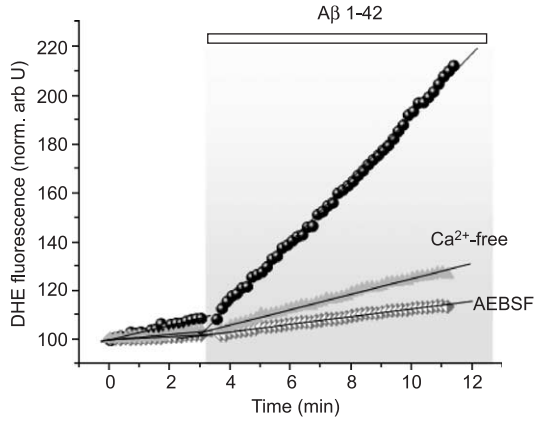

Fig. 3. Free radical generation in response to Aβ is dependent on extracellular Ca^{2+} and is prevented by inhibition of NADPH oxidase. The rate of free radical generation is indicated here by the rate of oxidation of dihydroethidine (ex 543 nm, em >570 nm), measured in individual astrocytes and shown as an averaged response from a field of cells. The slope of the response is a measure of free radical generation, and was fit with a high correlation coefficient by a straight line (indicated). The rate of free radical generation in response to Aβ 1–42 (5 μM) was clearly suppressed both by the omission of extracellular calcium (with 0.5 mM EGTA) and by the NADPH oxidase antagonist AEBSF (20 μM, preincubated for 10 min before addition of Aβ 1–42).

suggested that the failure of the transport pathway might lead to extracellular glutamate accumulation and therefore glutamate excitotoxic neurodegeneration (and see below).

We have found using ROS-sensitive dyes, such as dihydroethidium or dicarboxyfluorescein, that Aβ increases the rate of ROS generation in astrocytes (not in neurons), a response which is calcium-dependent (see Fig. 3). We have also used confocal imaging to measure changes in glutathione ([GSH-using monochlorobimane fluorescence]) in mixed hippocampal cultures and found that exposure to Aβ for 24 h caused depletion of GSH in both astrocytes and in neurons, identifiable separately in the imaging experiments, even though the changes in calcium signalling and ROS generation seem to be confined to the astrocytes. GSH depletion in both cell types was also Ca^{2+}-dependent, suggesting that the alterations in Ca^{2+} homeostasis must lie upstream in the cascade of injury caused by Aβ and that the oxidative injury might itself be Ca^{2+}-dependent. Neuronal viability was improved by provision of GSH precursors [6] or by up-regulation of GSH synthesis, strongly suggesting that GSH depletion plays a major role in the progression towards cell death (see Fig. 4).

All these data point to a major role of oxidative stress in response to Aβ and indicate multiple potential targets of oxidative damage. Several mechanisms have been proposed whereby Aβ may increase ROS generation. Aβ may apparently generate oxygen radicals directly in solution, but may also interact with a number of biological systems to increase the rate of radical production through modification or stimulation of intrinsic pathways. The most obvious of these is probably the activation of endogenous radical generating systems in microglia and possibly other cell types in the CNS by activation of the flavoprotein-linked enzyme system, NADPH oxidase. It has also been suggested that Aβ may increase ROS production from mitochondria by causing damage to the mitochondrial respiratory chain.

We have recently found evidence for the expression of an NADPH oxidase in the CNS in astrocytes, in addition to the microglial system, and have suggested that the activation of the astrocytic enzyme by Aβ may play a central role in the pathophysiology of Aβ toxicity [6]. Using both dihydroethidium and dicarboxyfluorescein to measure the rate of ROS generation directly both in pure cultures of astrocytes or in mixed cultures, we have found that Aβ routinely increased the rate of ROS generation in astrocytes. This response was inhibited by inhibitors of the NADPH oxidase, diphenylene iodonium, DPI and apocynin [6] and more recently also with AEBSF (Fig. 3, 20 μM, n=103). As stated above, both the increase in ROS and the loss of GSH were dependent on extracellular calcium, suggesting that the activation of the NADPH oxidase may be Ca^{2+}-dependent and part of the $[Ca^{2+}]_c$ response of astrocytes to the peptide (see above).

It has been suggested that Aβ could also increase ROS generation through an action on mitochondria. We have found that Aβ causes a progressive loss of mitochondrial

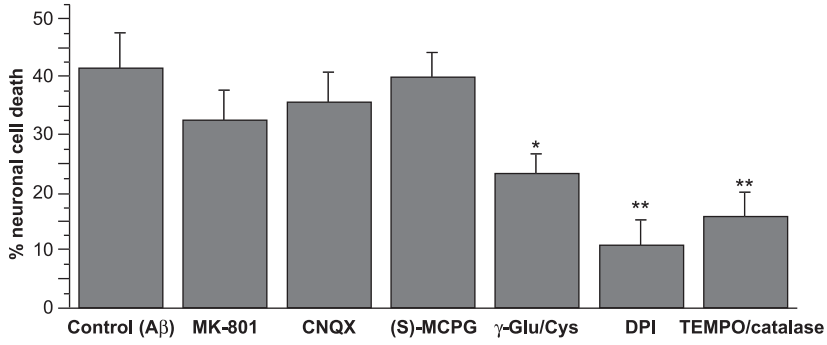

Fig. 4. Neuronal cell death in response to Aβ is limited by antioxidants, inhibitors of NADPH oxidase and GSH supplementation, but not by glutamate antagonists. Neuronal death was assessed using propidium iodide (PI) and Hoechst 3342 to label cells. Cell death is expressed as a percentage of all Hoechst-labeled nuclei that were also stained with PI. All cells were treated with Aβ 1–42 (5 μM) in the presence of one of the following: MK801 (10 μM), CNQX (20 μM), S-MCPG (50 μM), γ-glutamyl-cysteine (1 mM), DPI (0.5 μM) and TEMPO (500 μM) with catalase (250 U/ml).

potential, which was completely prevented by DPI, apocynin and antioxidants [6] and therefore appears to represent a response to ROS production. Mitochondrial potential was restored by providing mitochondrial substrates, suggesting that the main target of ROS generated by the NADPH must be in the supply lines for mitochondrial substrates—possibly at glucose uptake or at some level of glycolysis [22]. Aβ causes damage to other mitochondrial enzyme systems over longer time frames—observations include effects on α-KGDH and other enzymes of intermediary metabolism and impaired activity of COX—but these cannot account for the acute effects that we have described and must follow later. None of the mechanisms we have identified would be expected to increase mitochondrial ROS generation, as a loss of potential is usually associated with a decrease, rather than an increase, in ROS production.

Calcium signals in astrocytes may release glutamate and/or ATP [23], either of which may be toxic to nearby neurons. We wondered whether astrocyte calcium signals might cause glutamate release, and so cause neurotoxicity through an excitotoxic process. It seems, however, that this is not the case. First, the astrocyte calcium signals are not routinely associated with calcium signals in nearby neurons, as one might expect where the cells release glutamate onto neurons. Second, glutamate receptor antagonists afforded no significant protection to Aβ toxicity (Fig. 4). This alone might be surprising—others have documented some neuroprotection by glutamate antagonists, and, logically, if there are astrocytic $[Ca^{2+}]_c$ signals and changes in astrocyte $[Ca^{2+}]_c$ can cause glutamate release, why should we not see neuronal responses? One possibility is that the calcium changes are simply not large enough or are too localized. Using the low affinity indicator fura-2 FF, the Aβ-induced $[Ca^{2+}]_c$ signals are barely detectable, suggesting that the changes in calcium must really be quite modest—probably rarely exceeding 1 μM. Beyond that, we can only speculate that perhaps the levels of glutamate or ATP released by the astrocytes under these conditions are simply insufficient to cause toxicity.

2. Conclusions

Our findings are shown as a simple schematic in Fig. 5. What has been so consistent and surprising in all of our studies [5,6] has been the observation that all early pathophysiology that we have measured—changes in $[Ca^{2+}]_c$ and changes in ROS generation and changes in mitochondrial function—seem to be confined to astrocytes, and yet the cells that die later are predominantly the neurons. ROS generation was seen only in astrocytes, but GSH was depleted in both astrocytes and neurons. Calcium signals were seen only in astrocytes, but the neuronal GSH depletion was calcium-dependent. Put together, these data strongly suggest a sequence of events whereby Aβ forms a channel in the astrocyte membrane, promoting calcium influx into astrocytes as a primary phenomenon. This then generates oxidative stress within the astrocytes, through the activation of the NADPH oxidase, causing GSH depletion. Astrocytes have a substantially higher GSH content than neurons and appear responsible for the maintenance of GSH levels in neurons by releasing GSH, which is broken down by an ectoenzyme into a dipeptide that can then be used by neurons to maintain their own levels of GSH [24,25]. Therefore, if astrocyte GSH export can no longer sustain the requirements of neurons for GSH precursors, the neurons will become GSH-depleted. It is the experience of many

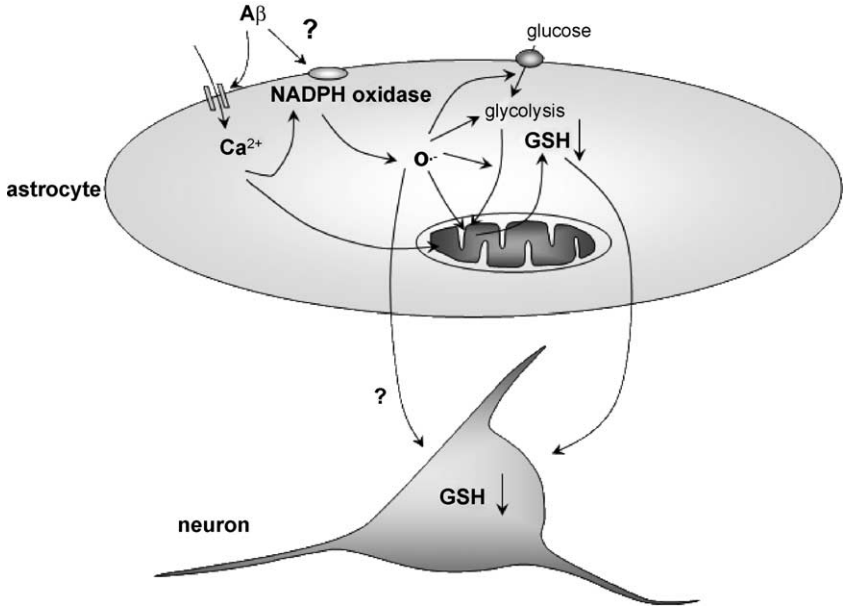

Fig. 5. Schema to show a proposed pathway for Aβ-induced neuronal cell death. This cartoon summarises the current state of our knowledge about the pathway involved in Aβ neurotoxicity.

investigators that depletion of neuronal GSH may be sufficient to cause neuronal death, as these cells seem much more vulnerable to endogenous pro-oxidants than the astrocytes, which appear far more robust.

What emerges from these studies is that the toxicity of Aβ is best understood as a neural response that reflects a series of interactions between glial and neuronal signals, involving glial Ca^{2+} signals and glial oxidative stress, which is then somehow transferred to nearby neurons effectively as a lack of trophic support, leading ultimately to neuronal attrition. It has to be said also that we have been cautious to discuss these issues in terms only of understanding Aβ toxicity. It may be a long step from here to understanding what happens in the brain of a subject with AD. Nevertheless, the observation that the drug clioquinol, which confers some clinical benefit in animal models and recently in patients also abolished the responses that we have described here and was neuroprotective [5,6], strongly suggests that these events may at least have some relevance to the disease process.

Acknowledgements

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