Published in final edited form as:

Toxicology. 2009 February 04; 256(1-2): 92–100. doi:10.1016/j.tox.2008.11.007.

Acute and chronic effects of intravitreally injected β -amyloid on the neurotransmitter system in the retina

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Abstract

The potential cytotoxic effect of aggregated $A\beta_{1-42}$ to neurons that express classical neurotransmitters, including acetylcholine, gamma-amino butyric acid, catecholamines and serotonin was assessed. The cholinergic system has been the central focus of the therapeutic drug strategies in amyloid-depositing pathologies such as Alzheimer's disease. Aggregated $A\beta_{1-42}$ has a multisystem cytotoxic effect causing non-specific reduction in immunoreactivity, dysfunction, or loss of retinal nerve cells. The extent of this was investigated using immunocytochemistry, TUNEL staining for apoptosis, and measurement of cell density as well as retinal surface area. There was a differential acute and/or chronic effect of $A\beta$ on choline acetyltransferase, gamma-aminobutyric acid and 5- tryptamine hydroxylase systems, observed with the increasing time course of 6 hours to 5 months, and a bilateral/systemic effect. In contrast, the overall pattern of catecholaminergic system, as revealed by tyrosine hydroxylase immunoreactivity of the retina, appears to have remained relatively unaffected by $A\beta$ (however this may reflect neuronal loss due to reduction in the retinal surface). This is the first *in vivo* evidence in a CNS model to show that not only all major neurotransmitter systems are differentially affected by $A\beta$ aggregates but the effect may vary from one transmitter system to another under the same experimental conditions *in situ* and in a dose- and time-dependent manner.

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Conflicts of interest Statement:

"The authors declare that there are no conflicts of interest."

Keywords

Alzheimer's disease; neurotransmitter systems; amyloid beta; choline acetyltransferase (ChAT); gamma-aminobutyric acid (GABA); 5- tryptamine hydroxylase systems (5 TryptH)

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that is characterised by a number of neuropathological hallmarks including the accumulation of extracellular senile plaques and loss of neurons. The amyloid hypothesis (Hardy and Allsop, 1991; Hardy and Selkoe, 2002) proposes that β -amyloid peptide ($A\beta$) accumulates to form senile plaques, leading to progressive and widespread degeneration of neurons in the brain of patients of AD. One of the neuronal populations that is particularly vulnerable and consistently affected in AD brain appears to be cholinergic neurons that are located primarily in the ventral basal forebrain. Importantly, the loss of cholinergic neurons, correlates well with the observation of reductions in the neurotransmitter acetylcholine (ACh) and the enzyme choline acetyltransferase (ChAT) that is involved in the biosynthesis of ACh (Arendt et al., 1985; Procter, 1996; Palmer, 1996; Whitehouse, 1982), as well as cognitive and memory deficits and dementia of AD. These observations have led not only to the establishment of the cholinergic hypothesis (Bartus, 2007; Benzi and Moretti, 1998; Harkany et al., 1995; Hodges, 2006; Perry, et al., 1978, 1993; Schlieb and Arendt, 2006), but also the development of the commonly adopted neurotransmitter replacement strategy for treating AD. However, treatment for AD by targeting specifically the neurotransmitter acetylcholine has not been entirely successful if not controversial in view of the report by Dunnett et al implicating that dysfunction of the ventral basal forebrain, as affecting attentional states rather than the proposed memory deficits in AD (Blokland, 1996). Partly for this reason, the cholinergic hypothesis needs modification, partly also due to accumulating evidence indicating that in AD there is not only alteration in ACh and ChAT but also alterations of most other important non-cholinergic neurotransmitters, including noradrenaline, serotonin, gamma-aminobutyric acid (GABA) and dopamine (Bell et al., 2006; Garcia-Alloza et al., 2006; Grudzien et al., 2007; Heneka and O'Banion, 2007; Hodges, 2006; Lancot et al., 2004; Meltzer et al., 1998; Palmer 1996; Szot et al., 2000). It is now widely accepted that interconnections between the different neurotransmitter systems together with a number of peptides may influence the cholinergic system and thus the disruption of one system may affect others, hence producing the deficits in memory. Nonetheless, little as yet is known about the acute and chronic effects of $A\beta$ on individual neurotransmitter systems because of a lack of suitable and well characterised and useful in vivo experimental model. The relatively novel retinal-vitreous system (Jen et al 1999, Guo et al 2007) appears to be one such model because the retina is an integral part of the central nervous system but a closed system that is highly accessible, and most important, perhaps, it contains all major neuronal populations and neurotransmitter systems that can only be found in different parts but not in the same regions of the brain.

Previous studies have already shown that the injection of $A\beta$ aggregates to the vitreous body in the eye can affect a variety of nerve cells in the rat retina, including photoreceptors (Jen et al. 1998; Walsh et al, 2002), interneurons in the inner nuclear layer (Walsh et al. 2002; Watts

et al, 2007) and retinal ganglion cells (Anderson et al 2008; Walsh et al., 2005; Guo et al., 2007). The present study was aimed at determining the cytotoxic effect of A β on the classical neurotransmitters with special attention to the cholinergic system.

METHODS

A total of 99 young female Sprague-Dawley (6–8 weeks old) rats (Harlan, UK) were used in the study. They were housed and maintained in the Comparative Biology Unit at Charing Cross Hospital. All procedures used were carried out in accordance with the UK Home Office regulations. The animals were divided into three groups. The first group (n = 18) received no treatment and hence served as naïve controls. The rest of the rats were anaesthetised with sodium pentobarbital. A hole was made at the corneo-scleral junction of the eye with a 26-gauge needle to remove some vitreal fluid before injecting 0.1M phosphate buffered saline (PBS), aggregated A β _{1–42}, or reverse peptide A β _{42–1} unilaterally into the left eye with a Hamilton syringe equipped with a special needle (75SN). Both A β _{1–42} and A β _{42–1} (Bachem, UK) were incubated in PBS at 37°C over 4 days, and then injected at a concentration of 2nmol/3 μ l or 5nmol/3 μ l. After a survival time of 6, 24 and 48 hours or 5 months the rats were deeply anaesthetised with sodium pentobarbital (16mg/100g-body weight) and perfused initially with 0.1M PBS followed by 4% paraformaldehyde in 0.1M phosphate buffer. Both injected and un-injected eyes were dissected out, the cornea and lens removed, and then immersed in the same fixative overnight before they were processed for frozen sectioning at 20 μ m thickness. Serial alternate sections collected were processed for immunohistochemistry, as described elsewhere (Guo et al., 1990, 1991, 1992, Bresciani et al., 2002; Jen et al., 1998; Walsh et al., 2002, 2005; Yang et al., 1992). In brief, alternate sections were incubated in a solution containing monoclonal or polyclonal antibodies for a number of glial or neuronal markers, including, ChAT ((1:20), Boehringer Mannheim), GABA ((1:8000), Sigma, UK), glial fibrillary acidic protein (GFAP, 1:600, Sigma), tyrosine hydroxylase (TH, 1:1000, Sigma, UK), and tryptophan hydroxylase (TryptH, 1:1000, Sigma). In addition, some sections were processed for terminal deoxynucleotidyl transferase dUTP- end-labelling (TUNEL, ApopTag kit) and Nissl (cresyl violet) staining for apoptosis and cytoarchitecture or gross morphologic changes respectively.

In order to assess the acute and chronic effects of A β on individual neuronal populations, cell density of ChAT-immunoreactive somata (in retinas of normal control, un-injected and injected eyes, in addition to changes in the overall patterns of immunostaining of all markers used) was determined and compared at 2 days and 5 months after injections. The total of animals in the study of dose and time dependence of the effects of A β was 48 (6 normal and 7 animals for the other six groups).

An Olympus microscope set at a magnification of 40X was used to observe and count the cells that showed positive immunoreactivity for ChAT in the retinal sections. For each retinal section, 5 regions/fields whose microscopic diameter = 350 μ m, were photographed. The most peripheral parts of the retina were counted and the midpoint taken to find the central region. Two intermediate regions located intermediately between the central and peripheral fields were additionally taken. Cell counting was performed in both injected and the contralateral un-injected retinas from the same animal to enable comparisons between the

treated and untreated eye, respectively. Data were collected from 6 cases with 4 pairs of sections for each case (1 pair = injected and un-injected retina of same treatment group). As illustrated in Figure 1, each section was counted in 5 fields, producing a representative total cell count per retinal section. The average of 4 sections for each case with the same treatment but different animals was used to produce the average total cell count per retinal section of each treatment.

Sigma Plot was used to determine whether there were significant differences in cell density between the different retinal treatment groups. Paired t-tests were used to compare both eyes of the same animal. An unpaired t-test was done for comparisons involving animals with different treatments such as, normal control cases with A β ₁₋₄₂ injected cases. The standard errors of mean were also calculated and represented on the graphs by standard error bars. To provide further information and more reliable assessment of long-term changes, the thickness and the surface area of retinas of all four groups of rats were also measured and compared at 5 months after injections. The level of significance was set for $p < 0.01$ (Walsh, et al., 2005).

RESULTS

The effect of A β on different neuronal systems over a time course of 6 hours to 5 months were characterised. Qualitative changes were studied immunocytochemically in the TH, GABA neurotransmitter systems, with additional quantitative analysis in the ChAT neuronal population. The apoptotic or degenerative effect of A β on retinal cells, as reported in previous studies (Jen et al., 1998; Walsh et al., 2002; 2005) was confirmed by TUNEL staining and was found to primarily affect photoreceptors and neurons in the inner nuclear layer (INL) but rarely neurons in the retinal ganglion cell layer (GCL) (Fig 2). The response of retinal glia was also confirmed by immunostaining. For instance, the reaction of the predominant Müller glia was revealed by GFAP immunostaining. Although GFAP is normally expressed primarily in astrocytes on the vitreal end of the retina little if any activity was observed in the radial process of Müller glial cells of untreated retina. However, GFAP-positive immunoreactive radial processes are seen spanning the thickness of the retina, indicating that the Müller glial cells upregulate GFAP in response to injury by aggregated A β ₁₋₄₂.

This response occurred, regardless of the dosage and survival time, with peak staining intensity by 48 hours. There was also a less intense and extensive upregulation in GFAP-immunoreactive Müller glia radial processes in eyes injected with PBS and, more interestingly, a detectable increase in GFAP immunoreactivity was observed in some Müller glial processes in retinas contralateral to the injection, reflecting a change of activity and possible bilateral stress-related response of these cells, a phenomenon that has already been described in Walsh et al., (2002).

The effects of A β on retinal neurons that express specific neurotransmitters was studied at different time point and doses. At 6, 24 and 48 hours survival times, none of the markers tested in this study induced changes in the pattern of immunostaining in any nerve cell populations in retinas injected with 2 nmol/3 μ l of A β ₁₋₄₂. Similarly, no observable

difference was found between injected and control retinas at 6 and 24 hours time points in retinas injected with 5 nmol/3 μ l. By 48 hours, however, visually detectable changes were observed in cells that are immunoreactive for ChAT, GABA and TryptH but not those reactive for TH. Interestingly, the overall pattern of immunostaining for all markers was similar to those of the normal and control despite a nearly 60% reduction in the average retinal thickness, reflecting a severe loss of cells primarily in the outer and inner nuclear layers of retinas injected with A β _{1–42}, as compared with the normal or control retinas at 5 months following treatment. Furthermore, an average reduction of nearly 40% of the surface area of retinas was observed in 5-month cases injected with A β _{1–42} compared with the un-injected retinas (Table 1).

The effects of treatments on specific individual cell populations are described separately below.

Choline acetyl transferase immunostaining

The cholinergic neurons that are immunoreactive for ChAT appeared as two populations of neurones with their somata located along the inner margins of the INL and GCL (Fig. 3A), representing amacrine and displaced amacrine cells, respectively. This is consistent with the reports of Jen et al., (1990) and Guo et al., (1991). The processes of the two sets of somata were stratified in two distinct sublaminae in the inner plexiform layer in a highly characteristic fashion. The overall pattern of ChAT-immunoreactive cells in retinas injected with A β _{1–42} was essentially the same as that of normal or un-injected side 6 to 48 hours after injection of 2 nmol. The lack of changes in the ChAT immunostaining pattern was demonstrable in retinal regions as clearly indicated by the physical distortion of the retinal tissue as a consequence of the injection (Fig. 3A, B). At 48 hours after injection of 5 nmol, however, a visually detectable reduction in immunostaining in both somata and immunoreactive sublaminae in the inner plexiform layer (IPL) was observed across the entire area of the retina (Fig. 3C). The reduction in immunostaining was confirmed by cell counting showing a reduction of ChAT-immunoreactive somata compared with the normal retinas and retinas injected with the control peptide A β _{42–1} or PBS, and the un-injected retinas from the same animals (Fig 4). The reduction of ChAT-immunoreactive somata was accompanied by the presence of less distinct immunoreactive sublaminae in the inner plexiform layer in retinas injected with 5 nmol. Interestingly, the loss of the immunostained somata and the reduction of immunoreactive sublaminae in the IPL observed in the un-injected retinas compared with the retina from normal rats and un-injected retinas from rats that were injected with the reverse peptide (A β _{42–1}) (Fig 4) could indicate a bilateral or systemic effect of A β _{1–42}. It was both surprising and interesting that the pattern of ChAT immunoreactivity in retinas injected with either 2 or 5 nmol returned to that of the normal and control retinas, despite a dramatic reduction in the retinal thickness (Table 1). No difference was observed between the density of ChAT somata (Fig. 4, last column) and the two highly characteristic immunoreactive sublaminae remaining, with generally slightly less or comparable staining intensity to those in the control retinas (Fig. 3D)

Gamma amino butyric acid immuno-staining

The overall pattern of GABA immunoreactivity observed in the normal, un-injected and control retinas injected with PBS was similar to that described in previous studies in the normal rat (Yang et al., 1991). The numerous somata of the GABAergic neurones were amacrine cells located within the INL, with displaced amacrine cells in the GCL (Fig 5A). The number of immunoreactive sublaminae visible in the IPL varied from 2 to 5 and from region to region, a characteristic distribution pattern of GABAergic neurones in the rat retina (Fig. 5). The immunostaining pattern observed after injection of 2 nmol at all time points and after injection of 5 nmol at 6 and 24 hours was essentially unchanged. However, a very different pattern of GABA-immunoreactivity was observed in retinas at 48 hours after injection of 5 nmol A β 1–42. (Fig 5A, B). An increase in GABA immunostaining was observed in numerous radial processes of Müller glia, a phenomenon that was never seen in any of the normal or control retinas. In contrast, a reduction in GABA-immunoreactive somata in both the INL and GCL could be observed in the same retinal regions, and the loss of immunoreactivity of these cells appeared to be more severe in the GCL abutting the vitreous. Moreover, the reduction of immunoreactive somata was always accompanied by a loss of GABA-immunoreactive sublaminae in the IPL. Most strikingly, in some regions of the same retina, hardly any immunoreactive cells or processes could be found, indicating a near total loss of GABA-immunoreactivity (Fig. 5D). A slight reduction in GABA-immunoreactive somata with occasional GABA-immunoreactive radial processes was detectable in some regions in the contralateral uninjected retinas but the effect was not consistent. Interestingly, at 5 months after the injection, the pattern of GABA-immunoreactivity in all injected retinas has returned to that comparable to the normal or control animals, indicating a complete recovery of the GABA activity in these retinas.

Tyrosine hydroxylase immunostaining

The distribution pattern of neurons in the catecholaminergic system is provided by the immunostaining of TH-an enzyme involved in the synthesis of dopamine/noradrenaline or adrenaline. As reported in previous studies (Guo et al., 1992; Versaiz-Botteri et al., 1986), TH-IR staining identifies two neuronal populations with large and small somata which may represent adrenergic and dopaminergic neurons, respectively. These are located at the inner margin of the INL with their extensive horizontally orientated processes ramifying the outer part of the IPL, immediately adjacent to the INL. Interestingly, these lamination processes may be observed in the outer plexiform layer (OPL) (Fig. 6). The immunoreactive pattern of TH remained unchanged in retinas injected with PBS or A β regardless of survival time and dosage (Fig. 6B, C, D). Both TH-immunoreactive cells with large and small somata appeared to be present with a density pattern similar to the un-injected controls (Figure 6). The preservation of the integrity of TH system is best illustrated by the highly distinctive and characteristic TH-immunoreactive band of processes located the IPL bordering the INL. However, because of the very small number of TH-immunoreactive cells observed in individual sections, cell counting was not performed to confirm whether there is a loss of TH-immunoreactive cells or not. Nonetheless, a loss of these cells can be expected in retinas injected with A β 1–42 on the basis of a significant reduction in the retinal surface area (Table 1).

Tryptophan hydroxylase immunostaining

In the present study TryptH, a serotonin/5HT rate-limiting enzyme and marker of serotonergic nerve cells, was located in association with Müller glial cells instead of retinal neurons (Fig 7A). TryptH-immunoreactivity was observed primarily in the proximal end of the radial processes of Müller glia in normal retina and retina injected with PBS. In retinas injected with 2nmol A β ₁₋₄₂ and A β ₄₂₋₁, TryptH-immunoreactivity was only slightly reduced at 2 days post-injection of 2 nmol A β ₁₋₄₂, but a near total loss was observed by 2 days after injection of 5 nmol of the peptide (Fig 7B). Interestingly, a different pattern was observed at 5 months after injection of 5 nmol A β ₁₋₄₂. Trypt H-immunoreactivity had not only returned (presumably being expressed by Müller glia) but an increase in immunostaining in radial processes was observed in some retinal regions of the injected retinas, and the increase was particularly noticeable in retinal sections that were cut obliquely or tangentially (Fig 7C). This pattern was not observed in normal or un-injected control retinas of age-matched normal retinas, or retinas injected with A β ₄₂₋₁, or PBS, indicating a long-lasting response that is specific to A β ₁₋₄₂.

DISCUSSION

The results of this study have provided the first direct evidence in a CNS structure, *in vivo*, that aggregated A β ₁₋₄₂ may have an effect on a number of different neuronal populations defined by neurotransmitters expressed or produced by these cells. The positive outcome of the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay confirmed that A β ₁₋₄₂ injection induces apoptosis in some cells, particularly in the outer and middle part of the retina, but rarely in the GCL. However, the extent of the cell density study based on immunoreactive somata reveal a different pattern of changes. A reduction in the number of neurons immunoreactive for ChAT was observed 2 days after injection of 5 nmol/3 μ l of A β ₁₋₄₂ but not under other experimental conditions. In addition, there was an unexpected increase in GABA immunostaining and a reduction of Trypt H-immunoreactivity in radial processes of Müller glia in these retinas, providing evidence to support a disruption in the metabolic integrity of these cells. However, there is an apparent recovery of ChAT-/GABA-immunoreactivity to near normal pattern over time as confirmed by results obtained at 5 months after injection. On the other hand, a significant reduction in the retinal surface area as well as retinal thickness suggests strongly that there is an overall and significant loss of probably all cell populations despite a lack of obvious or detectable changes in cell density 5 months after injection of 5 nmol of A β ₁₋₄₂. The significance of these findings are discussed separately below.

Effects of A β on the neurotransmitter systems

This study was focused on determining the potential cytotoxic effect of A β on the cholinergic system (a system that has been intensively studied) in relation to AD (Bartus, 2007; Benzi and Moretti, 1998; Harkany, et al., 1995; Hodges, 2006; Perry, et al., 1978; Schlieb and Arendt, 2006). This ChAT-immunoreactivity was essentially unchanged despite an initial decline in ChAT cell density observed at 2 days after injection of 5nmol/3 μ l. Qualitative long-time point observations at 5 months showed a relatively unaffected ChAT pattern as well as cell density per unit area, despite the dramatic decrease in retinal

thickness. However, considering that there is an average of up to 40% reduction in the entire surface area of individual injected retinas, a substantial loss of cholinergic neurons is expected. In view of this, caution should be exercised about the reliability of cell counting on the basis of density which by itself may not reflect the true picture of the overall changes in the cell population concerned. It is also worth pointing out that by increasing the dose of injected A β ₁₋₄₂ from 2 to 5 nmol, the intravitreally injected peptide affected not only ChAT-immunoreactive cells within the injected eye but also has a similar transient effect on these cells in the un-injected retinas at 2 days survival time. Thus that the transient effect could be detected at best on day 2 after 5 nmol A β treatment but not with the 2 nmol A β -treatment, suggests dose dependent effect. However, at 5 months there was a recovery suggesting time dependence regardless of the dose used. This provides additional support for a systemically mediated bilateral effect (Walsh et al., 2002).

The present study has also demonstrated for the first time *in vivo* that A β may have an effect on the GABA system. This was reflected by a temporary loss of GABA-immunoreactive somata in the INL and GCL and an increase in GABA-immunoreactivity in Müller glial cells. The implication of this observation is that A β aggregates may impair GABA turnover as GABA is normally released into the extracellular space at the synaptic cleft and is taken up by Müller glial cells. This, together with evidence showing an increased GFAP immunoreactivity and decrease in Bcl-2 immunostaining in Müller glia observed in the present and previous studies (Jen et al., 1998; Walsh et al., 2002, 2005), strongly suggests a disturbance of Müller glial function or metabolic integrity after a single injection of A β to the vitreous. The overall pattern of GABA-immunoreactivity returned to an almost normal level, thus supporting the acute and/or chronic A β -induced phases. That is, initial impairment in the turnover mechanisms of this inhibitory neurotransmitter, followed again by recovery, a phenomenon similarly observed in the cholinergic system described above. These changes in the distribution of GABA-immunoreactivity have implications for the Müller glia however.

In contrast to ChAT- and GABA-immunostaining pattern, the overall pattern of TH system seems relatively intact when compared with the other neuronal populations investigated. A strikingly distinct observation is made 5 months post-A β ₁₋₄₂ injection. The pattern characteristic of TH (especially the highly distinctive immunoreactive band observed in the outer part of the IPL), was essentially unchanged despite a dramatic decrease in the thickness of the retina in some of the cases. This is likely to be a reflection of a severe overall loss of retinal cells probably in the ONL and INL. Although the present results suggest this system is relatively resistant to A β toxicity, further confirmation with quantitative data and increased sample sizes is needed (and this is the subject of further investigations).

The rate-limiting enzyme TrypH which involved in the biosynthesis of the neurotransmitter serotonin is present in the rat retina and its levels may vary depending on the physiological or pathological state of retinal nerve cells (Chanut et al., 2002, 2006; Liang et al., 2004). However, TrypH has been associated primarily with photoreceptors and retinal neurons such as amacrine and retinal ganglion cells but not with glia. Results from the present study, however, have demonstrated that this enzyme can be used to depict the metabolic integrity of

Müller glial cells, rather than serotonergic neurons in the retina. More importantly, perhaps, we have shown for the first time to our knowledge, that two days after 5 nmol A β _{1–42} injection there is a near complete loss of TryptH-immunoreactivity in the retina, implying disturbances in Müller glial cell metabolism and function. This finding is entirely novel and potentially important considering the potential role of serotonin in modulating attentive behaviour and mood.

The loss of TryptH in Müller glia is in stark contrast to an increase in GFAP and GABA immunoreactivities observed in their radial processes, thus signifying that any of the changes seen were not a consequence of cellular loss. Moreover, TryptH had reappeared 5 months later in the Müller cell endfeet and even their radial processes suggesting not only recovery from the initial insult but also a possible continuous disturbance of metabolic activity in these cells. This, together with the pattern revealed by using other markers, as reported in the present and previous studies (Jen et al., 1998; Walsh et al., 2002; 2005) indicate clearly that dysfunction of Müller glia, in addition to activation of microglia, is another key feature of retinal changes or dysfunction after intravitreal injection of A β aggregates. It remains unequivocal that the acute as well chronic effects of A β on the various cell populations and neurotransmitter systems in the retina have provided new insight into the potentially cytotoxic effect of amyloid peptides. This paper demonstrates that A β 's toxic effects are not exclusive to the cholinergic system, a point supported by previous reports showing similar acute and chronic effects on photoreceptors, neurons that are rich in the calcium-binding protein parvalbumin as well as retinal ganglion cells (Jen et al., 1998; Walsh et al., 2002; 2005). These findings are of special importance as far as future development of potential neuroprotective and therapeutic strategies.

The role Müller glia in A β toxicity

Müller cells are the predominant glial cells of the retina, with radial processes extending throughout its depth. They are intimately related to all the major neuronal populations in the retina, and their normal function is critical for the maintenance and survival of neurons by removing excess ions and neurotransmitters from the synaptic cleft after neuronal depolarisation has occurred (Reichenbach et al., 1993; Brew and Attwell, 1987). This function is mirrored in astroglial-neuronal interactions within the brain (Kimelberg and Norenberg, 1989). The existences of macroglial-neuronal communications and lactate or glycogen for energy-dependent processes have been widely documented (Magistretti, et al., 1999; Poitry-Yamate, et al., 1995; Barres 1991; Tsacopoulos and Magistretti, 1996). Apparent dysfunction of the Müller glial cells is again evident with GABA's impaired uptake/removal or synthesis pathway ((Kimelberg and Norenberg, 1989).

The accumulation of GABA seen within the Müller glial cells infers a disturbance in the metabolism of glial. Hence, release of potentially toxic ammonia and glutamate may further exacerbate damage, manifesting in a number of ways such as, excitotoxicity and/or an impaired release of neurotrophic factors required for the maintenance and growth of neurons. Müller glia cells are known to produce glutamine synthetase (Riepe and Norenburg, 1977; Reichenbach, 1989), and its level can be reduced after intravitreal injection of A β . This may provide a mechanism for A β neurotoxicity via Müller cell

dysfunction in the retina. The compromised metabolic glial functions are additionally represented by the depletion of TryptH and upregulation of GFAP. However, this appears to be an acute phase response as restoration of Müller glial cell function also restores TryptH and GABA at the long term point of 5 months. The continuous upregulation of GFAP and TryptH suggests progression of A β ₁₋₄₂ toxicity into a chronic phase.

The disruption of the efficacy normally associated with the glial system presents in a myriad of destructive cascades triggering eventual irreversible damage and death. Indeed astrogliosis remain a profound hall mark of the brain (Little and O'Callaghan, 2001). It has been postulated by Arispe et al., (1993), that A β may form Ca²⁺ channels in lipid bilayers. Changes in Ca²⁺ influx may thus affect or cause injury to Müller glial cells. There is also evidence that G-protein activation via A β -Müller cell membrane disturbances may inhibit protein kinase C production, believed to be involved in the synthesis of certain proteins including the secreted form of amyloid precursor protein (Cowburn, et al., 1996). Alternatively, the possible amphiphilic nature of A β , may combine to mediate its toxicity via a direct perturbation of the glial cell membranes. Indeed Schubert et al., (1995) reported that ApoE4 may potentiate this interaction via its binding region for β -sheet or amphiphilic structures. The imbalances of these interactions may induce oxidative processes which could act as catalysts for neuronal damage or to initiate destruction of systems via lipid peroxidation, carbonyl protein modifications and mitochondrial DNA and/or RNA oxidation in vulnerable neurons (Yankner, 1996; Palmer, 1996; Smith, et al., 1997; Nunomura, et al., 1999). Interestingly, antioxidants have been shown to produce a substantial slowing and prevention of the cell death associated with A β (Jang et al 2004; Jen, et al., 1998; Nunomura, et al., 1999; Aruoma et al., 2003), which raises an important issue concerning the possible protective effects conferred by natural dietary antioxidants in relation to toxicity and AD. In this vein, Rezai-Zadeh et al., (2008) have shown that tea catechins (epigallocatechin gallate was protective of cognitive impairment in Alzheimer transgenic mice. Further it would be of interest to corroborate the results reported here by evaluating the expression of choline acetyltransferase and 5-tryptamine hydroxylase and this work is ongoing.

In conclusion, this study has provided novel information concerning potential cytotoxic effects which could be acute, chronic or long-lasting cytotoxic after one single intravitreal injection of 5 nmol/3 μ l of A β . Importantly, while the cell density and the overall distribution pattern of the various cell populations as defined by their neurotransmitters remain largely unchanged at 5 months after injection of A β , there is overall loss of retinal nerve cells over a time course of 6 hours – 5 months, a conclusion inferred by reductions in the area or volume of the injected retinas. Evidence of dysfunction of Müller glia cell supports further the notion that these cells may play a central role in mediating what appears to be an indirect toxic mechanism(s) induced by A β .

Acknowledgements:

This study was supported by grants from American Alzheimer's Association, National Institutes of Health (USA), and Wellcome Trust (UK).

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Figure 1.

A schematic diagram illustrating the location of the 5 fields of view chosen for cell counts of neuronal somata immunoreactive for ChAT.

Figure 2. Apoptotic cell death as revealed by TUNEL staining (arrows) in two different regions (A and B) of a retina injected with 5 nmol A β_{1-42} at 2 days post-injection. (C) is a negative control showing no TUNEL-positive profiles in the retina. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bar: 100 μ m.

Figure 3.

Photomicrographs showing the characteristic pattern of cells immunoreactive to choline acetyltransferase (ChAT), in uninjected control retinas (**A**), and injected retinas 2 days after injection of 2 nmol (**B**), or 5 nmol (**C**) at 2 days and 5 months (**D**) post-injection. The thick and thin arrows indicate ChAT-immunoreactive somata of amacrine and displaced amacrine cells, respectively. The asterisk indicates the outer limit of the retina. Arrows indicate ChAT-IR cells whose somata are in the INL and the GCL. Scale bar = 100µm.

Figure 4.

ChAT-immunoreactive somata compared with the normal retinas and retinas injected with the control peptide A β 42–1 or PBS, and the un-injected retinas from the same animals. The reduction in immunostaining was confirmed by cell counting showing a significant reduction ($p < 0.01$) (Table 1). The effect of A β 1–42 at 5nmol and two days survival of the injected side was significant (*) for the dose and time dependence study.

Figure 5.

GABA immunoreactivity in an uninjected control retina (A) and different regions in retinas injected with A β ₁₋₄₂ at 2 days (B, C & D). The thick and thin arrows indicate somata of GABA-immunoreactive amacrine and displaced amacrine cells respectively. The less intensely stained immunoreactive sublaminae (small double arrow), are found in the IPL. Note GABA-immunoreactivity is present throughout the Müller glial cells endfeet and radial processes, indicated by the arrowheads in B. Scale bar = 6100 μ m.

Figure 6.

TH-IR cells in uninjected control retina (**A**), and injected retinas at 2 days (**B**) and 5 months (**C** & **D**). Two sparse populations of TH-IR cells with large (thick arrows) and small (small arrows) somata located along the inner margin of the INL. These cells have extensive processes ramifying in the outer part of the IPL, immediately adjacent to the INL. The asterisk (*) marks the outer limit of the retina. Scale bar = 100µm.

Figure 7.

Photomicrographs showing tryptophan hydroxylase immunoreactivity in control (A), and injected retinas at 2 days (B) and 5 months (C). Immunoreactivity is found within the endfeet and radial processes of the Müller glial cells (see arrows). The immunoreactive radial processes are indicated by the arrowheads. The outer margin of the retina is marked by (*). Scale bar = 100µm.

Table 1

Retinal surface areas and cell counts (retinal thickness) of ChAT-immuno-reactive somata 5 months after injections.

Treatment ^a	Retinal surface area (mm ²)		Retinal thickness (μm)	
	<i>Injected</i>	<i>Un-injected</i>	<i>Injected</i>	<i>Un-injected</i>
Normal (6,6) ^b	---	41.6± 2.5	---	230.5 ± 25.6
PBS (6,6) ^b	37.0 ± 2.6	39.5± 2.1	192.8 ± 29.5	225.0 ± 26.3
Aβ ₄₂₋₁ (6,8) ^b	34.6 ± 3.2	38.2±2.7	160.2 ± 30.2	202.5 ± 23.1
Aβ ₁₋₄₂ (6,7) ^b	27.3 ± 3.8 *	35.5 ± 2.9	88.5 ± 38.6 *	186.1 ± 33.4

^aData as mean ±SD;

* Denotes study groups that were significantly different (p < 0.01).

^bThe numbers in the parentheses represent the number of cases)