Amyloid β peptide-induced corpus callosum damage and glial activation in vivo

Nattinee Jantaratnotai, Jae K. Ryu, Seung U. Kim and James G. McLarnon

INTRODUCTION
Alzheimer’s disease (AD) is a chronic neurodegenerative disorder characterized by the presence of amyloid plaques, neurofibrillary tangles in brain areas such as the association cortex and hippocampus and marked neuronal death [1]. Amyloid β-peptide (Aβ), the main plaque component, is thought to play a major role in the pathogenesis of the disease; indeed mutations that result in excessive production of Aβ can lead to early onset familial AD [2,3]. Previous in-vitro and in-vivo studies, primarily directed at gray matter, indicate that Aβ causes a diversity of effects in brain including neuronal apoptosis, reactive astrogliosis and microglial activation [3].

Recent evidence indicates that the extent and effects of Aβ deposits are more widespread than previously thought. Study of brains from AD patients has revealed the presence of different forms of Aβ deposits in white matter, cerebellum, and basal ganglia and which are arranged in granular or reticulated patterns in parallel with nerve fibers and blood vessels [4]. All amyloid plaque types in gray matter are also present in white matter [5] and the levels of Aβ_{1-40} and Aβ_{1-42} in AD white matter are significantly elevated relative to those detected in non-demented control cases [6]. Neuroimaging and morphometric analysis clearly demonstrate the atrophy of corpus callosum in AD brains and that the degree of damage is correlated with the progression and severity of the disease [7-10]. Exposure of cultured rat oligodendrocytes to Aβ-induced apoptotic cell death [11] in a manner similar to that reported in AD brain [12]. Overall, these findings suggest that white matter damage could contribute to pathology in AD.

In the present study, we have investigated a spectrum of effects arising from stereotaxic injection of Aβ_{1-42} into corpus callosum. The analysis includes actions of peptide, assessed 6 h and 3 and 7 days post-injection, to induce axonal and myelin injury, oligodendrocyte damage and astrocyte and microglial activation. The results suggest injection of Aβ into corpus callosum as a novel method to explore the pathophysiological relevance of white matter damage in AD.

MATERIALS AND METHODS
Preparation of Aβ peptide: Full length Aβ_{1-42} or reverse peptide Aβ_{42-1} (California Peptide Research, Napa, CA) were prepared as described previously [13] by dissolving the peptide in a freshly prepared 35% acetonitrile solution with dilution to a final concentration of 500 μM in 0.1 M phosphate buffer saline (PBS, pH 7.4). The Aβ_{1-42} or Aβ_{42-1} solution was incubated for 18 h at 37°C. After incubation, Aβ_{1-42} or Aβ_{42-1} solutions were made up in aliquots of 20 μl and stored at -20°C until use. It is likely that the Aβ_{1-42}...
used presently formed aggregates in the corpus callosum since a similar solubilization procedure resulted in aggregates forms of the peptide [15].

**Animals and surgery:** All procedures involving animal experiments were conducted in accordance with the guidelines established by the Committee on Animal Research at the University of British Columbia. Adult male Sprague–Dawley rats (250–280 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and then mounted in a stereotaxic apparatus (Kopf Instruments). Rats received unilateral stereotaxic injection of 2 µl vehicle, Aβ1–42 or Aβ42–1 (1 nmol) using a 10 µl Hamilton syringe with a 26-gauge needle at the following coordinates; AP –4.8 mm, ML –1.2 mm, DV –2.4 mm. The concentration of Aβ used in the present study was similar to those employed in experiments using a stereotaxic injection [14]. The injection was made over 4 min and the needle was left in place for 5 min prior to withdrawal to reduce reflux along the needle track. The wound was sutured and the rats were returned to their cage.

**Histology and immunocytochemistry:** At 6 h, 3 days and 7 days after injection, rats (n = 5/group) were deeply anesthetized with sodium pentobarbital and transcardially perfused with heparinized 0.1 M PBS, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The brain was immediately removed, post-fixed for overnight, and transferred to 30% sucrose in 0.1 M PBS, pH 7.4. The brain was cut coronally at 30 µm on a cryostat into sections and stored in a cryoprotectant solution. For histological evaluation, every sixth section was stained with cresyl violet. Demyelination was evaluated using Luxol Fast Blue staining as described previously [15]. For the immunohistochemistry, free-floating brain sections were permeabilized with 0.2% Triton X-100 and 0.5% BSA in 0.1 M PBS for 30 min and then incubated overnight at room temperature (RT) with the following primary antibodies: mouse anti-glia fibrillary acidic protein (GFAP; 1:1000; Sigma, St. Louis, MO) for astrocytes, mouse anti-CR3 complement receptor (OX-42; 1:500; Serotec, Oxford) for activated microglia, mouse anti-MHC class II (OX-6; 1:500; Serotec) for microglia, mouse anti-APC-7 (CC1; 1:1000; Oncogene Research Products, Cambridge, MA) for oligodendrocyte cell body without labeling myelin, and rabbit anti-neurofilament medium subunit (NFM; 1:500; Sigma). The following day, sections were washed with 0.5% BSA in 0.1 M PBS for 30 min and then incubated overnight at room temperature (RT) with the following primary antibodies: mouse anti-human β-amyloid (1:50; DAKO, Carpinteria, CA) at 4°C overnight, followed by Alexa Fluor 594-conjugated anti-mouse IgG (1:100; Molecular Probes, Eugene, OR) at RT for 1 h in the dark. For the immunofluorescence staining for injected Aβ1–42 in corpus callosum, sections were incubated with mouse anti-human β-amyloid (1:50; DAKO, Carpinteria, CA) at 4°C overnight, followed by Alexa Fluor 594-conjugated anti-mouse IgG (1:100; Molecular Probes) and Alexa Fluor 594-conjugated anti-mouse IgG (1:100; Molecular Probes) at RT for 1 h in the dark. After washing with PBS, sections were examined under a Zeiss Axioplan 2 fluorescent microscope (Zeiss, Jena, Germany) using a DVC camera (Diagnostic Instruments, Sterling Heights, MI) with Northern Eclipse software (Empix Imaging, Mississauga, ON) and were compiled using Adobe Photoshop 6.0.

**Quantification of NFM-ir fibers and CC1-ir oligodendrocytes:** Counts of NFM-ir fibers and CC1-ir oligodendrocytes in the corpus callosum were performed on four consecutive sections with detected Aβ1–42 aggregates. Cell counting was performed at ×40 magnification and all analyses were carried out in a blinded manner. Results are presented as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA, followed by Student–Newman–Keuls multiple comparison test.

**RESULTS**

**Characterization of amyloid β peptide aggregates in corpus callosum:** Rats were subjected to stereotaxic Aβ1–42 injection into corpus callosum with analysis at 6 h and 3 and 7 days after injection. The early time point of 6 h was chosen to ensure a minimum time for activation of glia in the white matter. Examination of antibody stained Aβ showed injected

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**Fig. 1.** Aβ1 immunofluorescence in the corpus callosum at (a) 6 h and (b) 7 d following injection of 1 nmole of Aβ1–42. Note the different morphology of amyloid aggregates. The broken lines indicate area of the corpus callosum. CTX: cortex, CC: corpus callosum, Hip: hippocampus. Bar = 200 µm.
peptide formed aggregates in the corpus callosum at 6 h (Fig. 1a) and which persisted to at least 7 days post-injection (Fig. 1b). No immunoreactivity for Aβ₁₋₄₂ was detected in either the corpus callosum contralateral to the injection side or in the vehicle-injected corpus callosum (data not shown).

Axonal injury: Axonal damage caused by Aβ₁₋₄₂ was evaluated by counting of neurofilament fibers stained with NFM antibody. Representative findings for damage at 7 days post-injection are shown in Fig. 2. In control (non-injection), vehicle- or Aβ₄₂₋₁-injected corpus callosum little evidence of damaged NFM-ir fibers was found (Fig. 2a–c). However, with injection of Aβ₁₋₄₂ peptide there was widespread loss of NFM-ir fibers (Fig. 2d). Overall, the effects of vehicle, Aβ₁₋₄₂ and Aβ₄₂₋₁ (normalized to control) on numbers of NFM-ir fibers at 6 h, 3 days and 7 days are presented in Fig. 2e. For vehicle or Aβ₄₂₋₁ injection there was no significant axonal damage relative to control for any of the time points post-injection (Fig. 2e). A significant loss of fibers was found at the early time point of 6 h post-injection of Aβ₁₋₄₂ with a further progressive loss evident at the later time points; at 7 days the loss of fibers approached 50% of control.

Myelin and oligodendrocyte damage: Myelin lesions in the corpus callosum were determined using Luxol fast blue staining only at 7 days post-injection. With this procedure normal myelin, or myelin sustaining little damage, stains dark blue. As evident in Fig. 3a, for both vehicle- and Aβ₄₂₋₁-injected corpus callosum there was no evident myelin injury (Fig. 3a, left panels). However, with Aβ₁₋₄₂ injection the corpus callosum showed extensive areas of demyelination (Fig. 3a, lower left panel). We also examined the response of oligodendrocytes to peptide injection using staining with the specific cell marker CC1 antibody. There was no appreciable loss of oligodendrocytes with either vehicle or Aβ₄₂₋₁ injection (top two right panels of Fig. 3a). A different result was obtained with Aβ₁₋₄₂ where numbers of oligodendrocytes were markedly reduced (Fig. 3a, lower right panel). Quantitative analysis of numbers of CC1-ir oligodendrocytes (normalized to control) is presented in Fig. 3b for the three different time points.

**Fig. 2.** NFM immunohistochemical staining in the corpus callosum of (a) control, (b) vehicle-, (c) Aβ₄₂₋₁-, or (d) Aβ₁₋₄₂-injected rats 7 days post-injection. (e) Quantitative analysis of NFM-ir fibers in the corpus callosum. *p < 0.05 vs all other treatments. Bar = 50 μm.

**Fig. 3.** Luxol fast blue myelin (left panel) and CC1 immunofluorescence (right panel) staining in the corpus callosum of (a) vehicle- (top panel), Aβ₄₂₋₁ (middle panel), or Aβ₁₋₄₂-injected rat 7 days post-injection (bottom panel). (b) Quantitative analysis of CC1-ir oligodendrocytes in the corpus callosum. Insets, high-magnification micrographs of double labeling for activated caspase-3 and CC1. *p < 0.05 vs all other treatments. Broken lines indicate corpus callosum area. Bar = 50 μm.
post-injection. For vehicle- or Aβ42-1-injected corpus callosum, no loss of oligodendrocytes was observed at any time. A marked and time-dependent loss of oligodendrocytes was found following Aβ1-42 injection (Fig. 3b).

We were interested in determining whether levels of caspase-3 were altered in oligodendrocytes following Aβ1-42 injection. This point was examined using double labeling immunofluorescence staining for activated caspase-3 and oligodendrocytes. The result showed that a number of activated caspase-3-ir oligodendrocytes were found in the Aβ1-42-injected corpus callosum 6 h post-injection (inset in lower right panel of Fig. 3a); very few oligodendrocyte showed immunoreactivity for activated caspase-3 in the vehicle- or Aβ42-1-injected corpus callosum at 6 h (insets in top two right panels of Fig. 3a).

Astrogliosis and microglial activation: We also studied glial responses to Aβ1-42 injection into the corpus callosum (Fig. 4). In non-injected corpus callosum GFAP-ir astrocytes and OX-42-ir microglia displayed a typical inactivated morphology with relatively small cell bodies and long thin processes (Fig. 4, top panels). The lack of OX-6-ir microglia (Fig. 4, right panels) is consistent with little activation of microglia in non-injected rat brain. In vehicle or Aβ42-1-injected corpus callosum few astrocytes and microglia exhibited an activated morphological profile of enlarged cell body with short thickened processes on day 7 post-injection. In contrast, 7 days following Aβ1-42 injection, increased densities of astrocytes and microglia were found throughout the corpus callosum (Fig. 4, bottom panels). In particular, the numbers of OX-42-ir microglia were markedly increased. An enhanced profile of activated microglia was also demonstrated (lower right panel of Fig. 4 for OX-6-ir cells).

DISCUSSION
To our knowledge this study is the first report describing Aβ1-42-induced axonal and myelin damage and oligodendrocyte loss in corpus callosum following stereotaxic injection of the peptide. Significantly, injection of Aβ1-42 also activated microglia and astrocytes indicative of an inflammatory response to the peptide and which could contribute to the white matter damage. At present, considerably fewer studies have been directed at studying white matter damage, relative to gray matter, in AD brain. In this work we found evident axonal damage, demyelination and loss of oligodendrocytes at an early time point of 6 h. At 3 and 7 days post-injection of Aβ1-42, marked and widespread damage to white matter was observed. The considerable damage to corpus callosum with Aβ injection found in this study is consistent with that reported in AD brain [16,17]. Interestingly, transgenic animal models of AD also exhibit deposits of Aβ in white matter [18].

The progressive increases in myelin damage and oligodendrocyte loss with time (Fig. 3) may indicate a coupling between these two as targets of Aβ1-42 peptide. A link between myelin damage and reduction in numbers of oligodendrocytes was suggested since few oligodendrocytes were observed in the myelin damaged area 7 days post-injection. Previous in-vitro work has reported that Aβ is directly cytotoxic to oligodendrocytes, as shown by nuclear DNA fragmentation, implicating apoptotic cell death of oligodendrocytes with Aβ exposure [11]. The damaged oligodendrocytes labeled with TUNEL were found in the white matter degeneration in AD [19]. Taken together with previous results, our findings raise the possibility that amyloid plaques present in AD brains result in significant damage to oligodendrocytes and contribute to white matter pathology.

An interesting finding was the presence of significant caspase-3 immunoreactivity in oligodendrocytes 6 h following Aβ1-42 injection. This effect was not evident at 7 days, suggesting that caspase-3 activation occurs in early stages of responses. The relevance of this result to loss of oligodendrocytes is presently unknown. However, it may be noted that previous studies have associated enhanced caspase-3 activity to apoptotic death of oligodendrocytes after various toxic insults including Aβ. A novel finding in this study was the effect of Aβ1-42 injection to increase the number of astrocytes and microglia in the corpus callosum (Fig. 4). This inflammatory response was evident at 6 h (data not shown) and further enhanced at 7 days. Thus, responses of glial cells to Aβ injection may involve secretion of toxic factors such as pro-inflammatory cytokines and reactive oxygen species which could contribute to the progressive development of myelin and oligodendrocyte damage [20–22]. In this regard inhibition of glial activation has been shown to block inflammation mediated damage to myelin and oligodendrocytes [23]. It is noteworthy that in both gray and white matter activation of glial cells by Aβ has been implicated in the progression of AD [24–26]. The results from this study
suggest that maneuvers to inhibit glial responses following Aβ1-42 injection would be useful to lessen damage to white matter.

CONCLUSION
Although Aβ1-42 has been well characterized to induce toxicity in gray matter, we demonstrate the novel finding that Aβ1-42 injected into corpus callosum causes marked white matter injury. Aβ1-42-induced axonal and myelin damage, oligodendrocyte death and reactive gliosis in rat corpus callosum. The mechanisms by which Aβ1-42 exerts these widespread toxic actions are not known but could involve inflammatory responses mediated by reactive glia. Injection of Aβ1-42 in corpus callosum of rat brain may provide an important tool in characterization of white matter degeneration in AD.

REFERENCES