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Memory loss caused by β-amyloid protein is rescued by a β_3-adrenoceptor agonist

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Abstract

Accumulation of the neurotoxic β-amyloid protein (Aβ) in the brain is a key step in the pathogenesis of Alzheimer's disease (AD). Although transgenic mouse models of AD have been developed, there is a clear need for a validated animal model of Aβ-induced amnesia which can be used for toxicity testing and drug development. Intracranial injections of $A\beta_{1–42}$ impaired memory in a single trial discriminative avoidance learning task in chicks. Memory inhibition was closely associated with the state of aggregation of the Aβ peptide, and a scrambled-sequence of $A\beta_{1–42}$ peptide failed to impair memory. Aβ had little effect on labile (short-term and intermediate) memory, but blocked consolidation of memory into long-term storage mimicking the type of anterograde amnesia that occurs in early AD. Since noradrenaline exerts a modulatory influence on labile memory in the chick, we examined the effects of two β-adrenoceptor (AR) agonists on Aβ-induced amnesia. A β_3-AR agonist (CL316243), but not a β_2-AR agonist, rescued Aβ-induced memory loss, suggesting the need for further studies on the role of β_3-ARs in AD.

Keywords: Chick; Amyloid; Abeta; Dementia; Memory; Alzheimer's disease; Neurotoxicity; Passive avoidance

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. Typically the disease is characterized in its earliest stages by memory problems. Initially patients may have trouble remembering recent events, but as the disease spreads, older and more established memories are lost. Wandering and disorientation occur and as the disease progresses, the symptoms worsen (Storey et al., 2001).

It is now recognized that AD is caused by a build-up of neurotoxic Aβ in the brain (Small et al., 2001; Walsh and Selkoe, 2004). Aβ peptides, particularly the longer species such as $A\beta_{1–42}$, are considered to be the major culprits in disease pathogenesis. $A\beta_{1–42}$ aggregates more readily than the more commonly produced $A\beta_{1–40}$ (Jarrett and Lansbury, 1993). $A\beta_{1–42}$ can aggregate to form oligomers, protofibrils that ultimately lead to the formation of amyloid plaques. However, recent studies suggest that it is the oligomeric or low molecular weight protofibrillar Aβ species, rather than the amyloid plaques, that are the most neurotoxic (Klein et al., 2001; Walsh and Selkoe, 2004).

While much of the focus in the field of AD has been on the chronic lesions that characterize the disease (amyloid plaques, neurofibrillary tangles, gliosis, cell death), it is increasingly recognized that Aβ may exert effects that are acute and independent of long-term chronic neurodegeneration (Palop et al., 2006). For example, oligomeric Aβ can rapidly alter calcium homeostasis and disrupt long-term potentiation (LTP) (Klein et al., 2001; Walsh and Selkoe,

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2004). Thus increasingly it is thought that the acute effects of Aβ on synaptic plasticity may be a more important contributor to cognitive decline than the neuropathological features such as amyloid plaques, neurofibrillary tangles and cell loss (Palop et al., 2006).

The development of therapeutic drugs to treat AD must rely heavily upon models that can be used to test Aβ toxicity. Early biological studies used assays of cell death or mitochondrial activity (Koh et al., 1990; Shearman et al., 1994) to evaluate therapeutic potential. However, the relevance of these assays for therapy is unclear, as agents that block neurotoxicity in cell culture have not yet been found to have efficacy in clinical trials. For example, although antioxidants block the neurotoxic effect of Aβ in cell culture (Subramaniam et al., 1998), they do not have a clear effect in clinical trials (Boothby and Doering, 2005; Tabet et al., 2000). Ultimately, a validated *in vivo* model of Aβ toxicity is needed. While several APP transgenic mouse models of AD have been developed (German and Eisch, 2004; van Dooren et al., 2005), these animal models also have their problems. For example, although behavioural deficits occur in APP transgenic mice (Morgan, 2003), these deficits are subtle and can be difficult to quantify.

Several studies have examined the effect of exogenous Aβ peptides on memory. Direct injection of Aβ peptides into the brain has been reported to block memory in rodents (Townsend et al., 2006a,b, 2007), but behavioural assays in mammals can be difficult to carry out. In contrast, chicks provide a relatively inexpensive and rapid method for the assessment of memory. The cortical and pallial regions in the mammal and the bird derive from the same pallial regions in the embryo (Jarvis et al., 2005). Brain regions involved in memory processing and memory mechanisms in birds are the same as those in mammals, for example, avian NMDA receptors play an important role in memory and synaptic plasticity as in mammals (Gibbs et al., 2008; Rickard et al., 1994) and protein synthesis is involved in both mammalian and avian memory (Izquierdo et al., 2006; Matthies, 1989; Rose, 2000). Mileusnic et al. (2007) and Mileusnic et al. (2004) previously reported that intracranial injection of Aβ into the chick blocks memory in a passive avoidance task. However, these studies lacked a suitable non-amyloidogenic peptide control needed to assess the validity of the memory effects and it is unclear in these studies whether effects on chick memory were caused by specific Aβ toxicity or whether a non-specific action was involved.

The development of a rapid, accurate and well-validated model of Aβ-induced cognitive dysfunction would be a major benefit for drug screening. While much attention has been focussed on cholinergic drugs, it is increasingly recognized that many other neurotransmitter systems are involved in the cognitive decline that occurs in AD. Noradrenaline is of particular interest as it plays an important role in modulating memory. In non-human primates and rats, working memory, a specialized form of short-term memory and the consolidation of long-term memory are regulated by noradrenaline (Berridge and Waterhouse, 2003; Ramos and Arnsten, 2007). In chicks, short-term memory (Gibbs and Summers, 2005) and the consolidation of intermediate into long-term memory triggered 30 min after training are also regulated by noradrenaline (Gibbs and Summers, 2002a,b). In AD patients, noradrenaline levels are substantially reduced, suggesting a role for noradrenaline in the cognitive decline. The locus coeruleus, a structure that innervates the prefrontal cortex and the major source of noradrenaline in the forebrain, is affected in AD brains (Hertz, 1989; Szot et al., 2006).

In the present study, we have used chicks and a single trial discriminative avoidance task to examine the effects of Aβ on memory. We show that this model of Aβ-induced amnesia is a rapid, reproducible and highly quantifiable method for assessing Aβ neurotoxicity. We show that the effect of Aβ is specific, as a scrambled-sequence Aβ peptide does not block memory, and that there is a correlation between the state of Aβ aggregation and its effect on memory.

As noradrenaline has been implicated in memory processing, we have used our model (Gibbs and Summers, 2002b; Gibbs, 2008) to examine the effect of adrenoceptor agonists on Aβ-induced amnesia in chicks. We report that a β_3-adrenoceptor (AR) agonist (but not a β_2-AR agonist) blocks the amnestic effects of $A\beta_{1-42}$ peptides. As β_3-AR agonists have not yet been tested for their efficacy in clinical trials of AD, our studies suggest that further studies on the role of the β_3-AR in AD are needed.

2. Materials and methods

2.1. Peptides and drugs

All Aβ peptides (>95% purity) were purchased from Rpeptides Inc. (Bogart, GA, USA). Peptides were made up as 10 mg/ml (2.2 mM) stock solutions in dimethyl sulfoxide (DMSO) and then stored frozen. Prior to use, the peptides were diluted with physiological saline (0.9%, w/v sodium chloride) to yield the appropriate concentration. Controls were injected with the appropriate dilution of DMSO. Except in experiments where the aggregation of the peptide was deliberately increased, the peptides were used within 30 min of dilution of the stock in physiological saline. To aggregate the Aβ, the peptides were incubated at a concentration of 2 μM in physiological saline at 37 °C. Aliquots were removed after 0, 1, 3 and 5 days for analysis of aggregation and for memory testing. Zinterol hydrochloride was generously provided by Bristol-Myers Squibb (Noble Park, Australia) and CL 316243 (disodium (*R*, *R*)-5-[2-[[2-3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate) purchased commercially (Sigma–Aldrich Inc., St. Louis, MO, USA).

2.2. Injections of peptides and drugs

Drugs and peptides were given centrally by direct bilateral injection of 5 μl into the avian 'cortical' region (intermediate medial mesopallium, IMM) of each hemisphere using a 250-μl repeating Hamilton syringe dispenser. No anaesthesia was used for the injections since anaesthetics interfere with memory processing. As well as having an unossified, cartilaginous skull, the nuclei of the chick forebrain are not as compact as those in mammals, and the injection of a volume of 5 μl does not produce pressure as it may in the mammalian brain. There are no pain receptors in the brain and chicks do not appear to experience any pain or distress from these procedures and will peck at beads when first tested 1 min after injection (unpublished). Unless otherwise indicated, each injection contained 10 pmol of Aβ peptide. The injections were performed freehand using the tactile landmarks of the tegmentum and midline to target the injection site, i.e., 3 mm from the midline and 4–5 mm forward of the tegmentum. The depth of the injection was 3.5 mm (including the 1.0 mm needle orifice) controlled by a plastic sleeve on a 27 gauge needle. The needle was oriented such that the orifice was directed toward the back of the brain, see figure. 1A Gibbs and Summers (2005). The accuracy of the placements, measured by distance of needle punctures in the cartilaginous skull from midline and tegmentum, for a typical experiment were 3.30 ± 0.14 and 2.73 ± 0.14 mm to left and right of midline and 4.6 ± 0.18 and 5.0 ± 0.17 mm from the tegmentum ($n = 23$).

2.3. Learning paradigm

One-day-old, male domestic chicks from an egg-laying hybrid strain (New Hampshire, Rhode Island Red, White Leghorn and Black Australorp strains) weighing approximately 35 g were obtained on the morning of each experiment from a local poultry farm (Wagner's Poultry, Coldstream, Australia). A single trial discriminative avoidance task was used, where the chicks display memory retention as the relative number of pecks at different coloured beads. The experimental conditions are described in detail elsewhere (Gibbs and Summers, 2002b). Chicks were housed in pairs, in groups of 12–16 and allowed 2–3 h to become familiar with their new environment, during which they were presented with a number of different beads attached to a 20 cm length of stiff wire. The chicks were given an opportunity to peck at each bead. Initially, two 10-s presentations (30 min apart) of a small (2 mm diameter) shiny metal bead were made, after which they were presented (5 min apart) with red and blue glass beads (4 mm in diameter) that had been dipped in water. This procedure was done to ensure that the chicks were familiar with both colours prior to training.

The training trial commenced at least 30 min after the final pre-training trial, where injections were made before training, these were given 30 min after the final pre-training trial. The chicks were presented with a red bead identical to that seen before but now dipped in either 100% or 20% methyl anthranilate (Sigma–Aldrich Inc., St. Louis, MO, USA) to produce strongly or weakly reinforced training, respectively. Chicks normally peck the beads within the first 1–2 s, although they were allowed 10 s to peck. Memory retention, at specified intervals after training, was calculated from the discrimination ratio (DR), which was the number of pecks at the blue bead/the total number of pecks at the red and blue bead on successive trials. The pecks were recorded on a hand-held recording logger and decoded by computer at the completion of the experiment. A chick that remembers the aversive taste tends to avoid pecking at the red bead but gives up to 12 pecks at the blue bead. In this case, the DR is 1.0. For a chick that does not remember, the DR is 0.5, since the chick pecks equally at red and blue beads. Individual DRs were obtained for each chick and data converted to mean ± S.E.M. Chicks that did not peck the bead during the training trial (i.e., did not train), or avoided the blue bead on test, perhaps due to generalised avoidance or non-specific performance effects, were eliminated from the data analysis at the completion of the experiment. These exclusions resulted in the loss of 1 or 2 chicks per group. The number of chicks per group was generally between 12 and 16.

2.4. Aβ aggregation and atomic force microscopy (AFM)

Aβ aggregation was analysed by AFM as previously described (Hou et al., 2007). Aβ peptides (2 μM) were incubated for up to 7 days in physiological saline at 37 °C. At 0, 3 and 7 days, samples were removed and either tested for their effects on memory or applied to a substrate of highly oriented pyrolytic graphite (HOPG) for AFM imaging. After application of the peptides to the HOPG, the surface of the HOPG was briefly rinsed with distilled deionized water to remove buffer salts, as previously described (Hou et al., 2007; Losic et al., 2006). Imaging was performed by tapping mode in air using silicon probes and a Nanoscope IV Multimode scanning probe microscope (Veeco Corp., Santa Barbara, USA). Images were processed using WSxM version software developed by Ignacio Horcas (Nanotec Electronica S.L., Spain).

2.5. Data analysis

The results for each experiment were analysed using SPSS (Information Analysis Systems SPSS Inc., Chicago, IL, USA) with one- and two-way independent measures ANOVA with either Dunnett's *t*-test or simple main effect *post hoc* analyses where appropriate. Although sample sizes differed, this was not due to experimental factors; therefore, all analyses used unweighted means. Two-tailed tests of significance were conducted and a type I error rate of .05 was adopted. Statistical analyses were conducted using SPSS for Windows (Version 14, Chicago, IL).

All experimental procedures were in accordance with the guidelines approved by the Monash University Animal Ethics Committee and comply with the 1997 Australian Code of

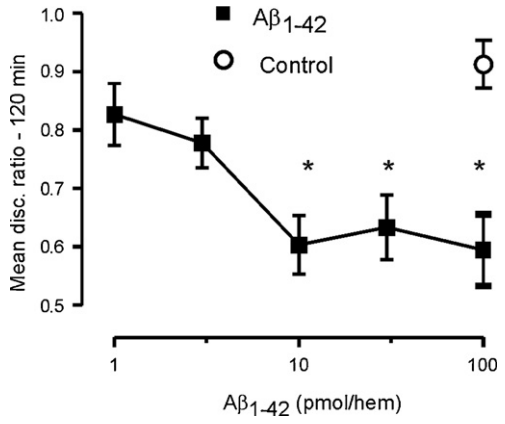

Fig. 1. Dose dependence of the effect of $A\beta_{1–42}$ on discrimination ratio (memory). Injection of $A\beta_{1–42}$ (10 pmol/hemisphere) into IMM resulted in memory loss when the chicks were tested 2 h later. Figure shows the dose–response relationship for $A\beta_{1–42}$ injected 45 min before training. Memory retention was tested 120 min after training. $^*P < 0.05$ compared to control. $N = 14–16$ per group.

Practice for the Care and Use of Animals for Scientific Purposes. All efforts were made to minimise both the suffering and the number of animals used. Chicks were killed at the completion of each experiment by CO_2 inhalation.

3. Results

3.1. Effect of $A\beta_{1–42}$ on strongly reinforced memory

Initially the effect of $A\beta_{1–42}$ on strongly reinforced memory was examined. $A\beta_{1–42}$ was injected into the 'cortical' region (IMM) of the day-old chicks, 45 min before training. Controls received an identical injection of vehicle. Memory was tested 2 h after training. The effect of $A\beta_{1–42}$ on strongly reinforced memory was examined for various doses between 1 and 100 pmol/hemisphere (Fig. 1). A dose of 10 pmol/hemisphere was found to be maximally effective in inhibiting memory ($F_{5,86} = 7.27$, $P < 0.001$). Higher doses of 30 and 100 pmol/hemisphere also resulted in memory retention levels significantly less than saline ($P \leq 0.001$) and similar to that obtained with 10 pmol/hemisphere. Therefore, for all remaining experiments doses of 10 pmol/hemisphere were used.

3.2. Time course of the effect of $A\beta_{1–42}$ on memory

To examine the time course of the effect of $A\beta_{1–42}$ on memory, injections of $A\beta_{1–42}$ were made at various times between 45 min before training and 20 min after training using the same experimental design (Fig. 2). $A\beta_{1–42}$ impaired memory when injected at times between 45 min before training to 5 min after training ($F_{5,88} = 6.01$, $P < 0.001$). However injections at 20 min after training did not inhibit memory processing. In a separate experiment, we found that the injection of $A\beta_{1–42}$ could be made as late as 15 min post-training and still produce memory impairment (Fig. 2B; $F_{4,75} = 14.58$, $P < 0.001$), indicating that the effect of $A\beta_{1–42}$ was rapid and that commencement of consolidation of labile into long-term memory was impaired. In the chick, the timing of the processes involved in triggering consolidation of labile into permanent memory for this learning task is very precise, so that the finding that $A\beta_{1–42}$ injected at 15 min after training impairs memory but not when injected at 20 min is not unusual. However, it can be seen in Fig. 6 that injection at −5 min is not quite as effective at inhibiting memory as injection at −45 min.

$A\beta_{1–42}$ injected 24 h before training was just as effective as when injected 45 min before training in causing memory impairment (Fig. 2C) implying that the inhibitory effect was long term. In addition, memory remained impaired for up to 24 h after training, when $A\beta_{1–42}$ was injected 45 min prior to training (Fig. 2C).

3.3. Sequence specificity and the role of peptide aggregation

To examine the specificity of the effect of Aβ on chick memory, various Aβ peptides ($A\beta_{1–42}$, $A\beta_{1–40}$, a scrambled-sequence $A\beta_{1–42}$ and a scrambled-sequence $A\beta_{1–40}$) were compared for their effects (Fig. 3). Initially, peptides were freshly prepared and used immediately. Chicks were injected with 10 pmol/hemisphere of each peptide and memory tested at 2 h after training. Only the highly amyloidogenic peptide $A\beta_{1–42}$ inhibited memory. None of the other peptides had any effect on memory, which was tested 120 min after training (Fig. 3; $F_{3,58} = 13.74$, $P < 0.001$). Discrimination ratios for the other peptides were significantly greater than for $A\beta_{1–42}$ ($P < 0.001$).

The result of this experiment suggested that peptide aggregation may be important for the amnestic effect. As $A\beta_{1–40}$ can aggregate upon incubation for several days at 37 °C (a process known as ageing), the effect of ageing $A\beta_{1–42}$ and $A\beta_{1–40}$ on Aβ-induced amnesia was examined. $A\beta_{1–40}$ and $A\beta_{1–42}$ (2 μM in physiological saline) were aged by incubation at 37 °C for up to 7 days. At various times, aliquots were removed and tested for their effects on memory (Fig. 4). As previously observed, freshly prepared $A\beta_{1–42}$ inhibited memory, whereas $A\beta_{1–40}$ was without effect. However, after ageing for 3 days, $A\beta_{1–40}$ was observed to inhibit memory, whereas the effect of $A\beta_{1–42}$ was reduced. After 7 days of incubation, $A\beta_{1–40}$ was observed to inhibit memory, whereas $A\beta_{1–42}$ was without effect. Two-way ANOVA Peptide × Times interaction ($F_{4,131} = 9.13$, $P < 0.001$). Simple main effects showed significant differences between treatments of 0, 1, 5 and 7 days ($P < 0.05$).

Previous studies have shown that the neurotoxic effects of Aβ peptides are dependent upon the state of aggregation of the peptides and that oligomers are the most toxic species (Walsh and Selkoe, 2004). The chick memory results (Fig. 4) suggest that ageing may have generated oligomers from $A\beta_{1–40}$, and that $A\beta_{1–42}$ may have aggregated into

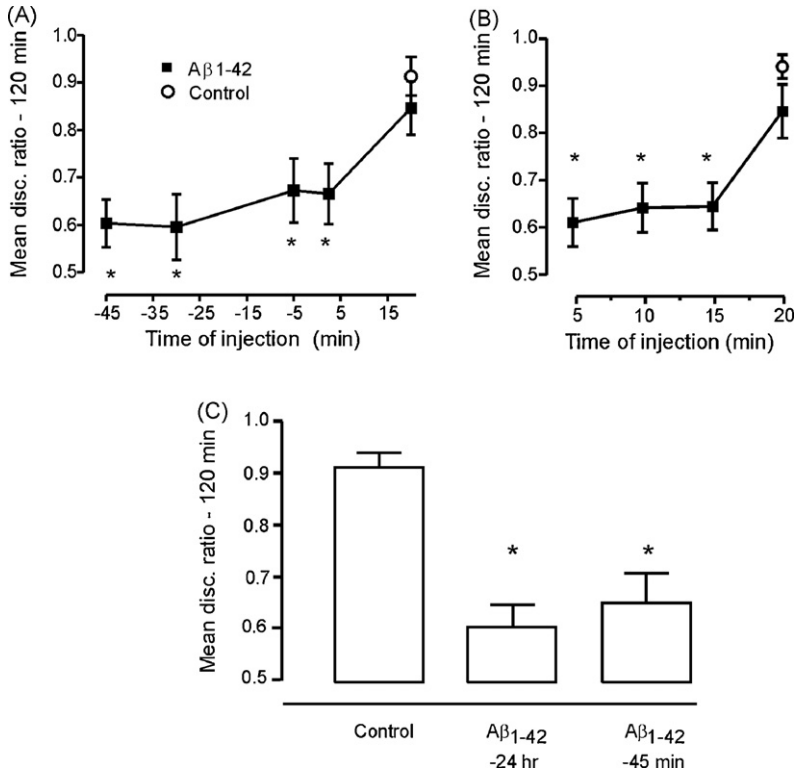

Fig. 2. Time course of the effect of $A\beta_{1–42}$ on discrimination ratio (memory). Figure shows that $A\beta_{1–42}$ blocked memory when injected between 24 h prior to training and 15 min after training. In panels A and B, memory was tested 2 h after training. (A) $A\beta_{1–42}$ (10 pmol/hemisphere) was injected at different times between 45 min before training and 20 min after training. (B) $A\beta_{1–42}$ was injected between 5 and 20 min after training. (C) $A\beta_{1–42}$ (10 pmol/hemisphere) was injected 24 h or 45 min prior to training and memory tested at either 120 min or 24 h after training, respectively. $^*P < 0.05$ compared to control. $N = 14–20$ per group.

higher molecular weight forms that are less toxic. To examine this possibility, the state of aggregation of the peptides was analysed by AFM (Fig. 5). Images of freshly prepared $A\beta_{1–40}$ showed numerous small relatively uniform globular structures with an apparent diameter of 10–20 nm (*). Previous studies (Losic et al., 2006) have shown that these globular structures are relatively low molecular weight monomers or dimers. In contrast, images of freshly prepared $A\beta_{1–42}$ were more dense and although similar globular structures were seen, they were also found together as larger irregularly shaped aggregated structures (thin arrows). Upon ageing for 7 days, the $A\beta_{1–42}$ was found to aggregate further with the appearance of short rod-like structures resembling fibrils

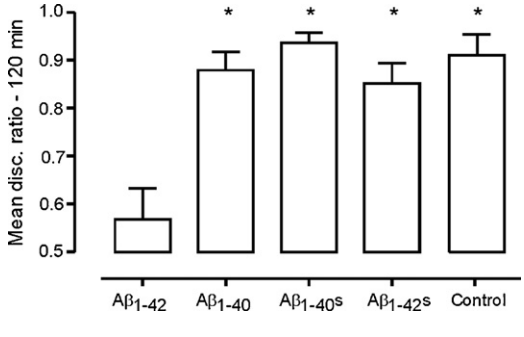

Fig. 3. Effect of various Aβ peptides on discrimination ratio (memory). Figure shows that the effect on memory is related to the propensity of the peptides to aggregate. Only the highly amyloidogenic peptide $A\beta_{1–42}$ blocked memory, as the other peptides were without effect when freshly prepared. Peptides were injected 45 min before training at 10 pmol/hemisphere. Scrambled-sequence $A\beta_{1–42} = A\beta_{1–42}s$; scrambled-sequence $A\beta_{1–40} = A\beta_{1–40}s$. $^*P < 0.05$ compared to $A\beta_{1–42}$. $N = 14–16$ per group.

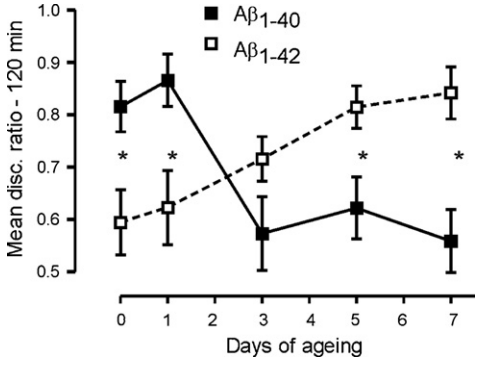

Fig. 4. Effect of "ageing" on the ability of $A\beta_{1–40}$ and $A\beta_{1–42}$ to block memory. Peptides (2 μM) were incubated at 37 °C in physiological saline for up to 7 days. Figure shows that ageing caused a loss of the effect of $A\beta_{1–42}$ on memory, whereas it increased the potency of $A\beta_{1–40}$ to block memory. Chicks received injections into the IMM of 10 pmol peptide/hemisphere, 45 min before training. $^*P < 0.05$ two-way ANOVA $A\beta_{1–40}$ compared to $A\beta_{1–42}$. $N = 13–16$ per group.

or protofibrils (double-headed arrows). In contrast, after 7 days of ageing, the $A\beta_{1-40}$ preparation contained amorphous aggregated structures that were similar to the amorphous aggregates (thin arrows) seen in the freshly prepared $A\beta_{1-42}$ preparation. In general, images of Aβ preparations after 3 days of ageing displayed structures which were intermediate between those seen at 0 and 7 days.

3.4. Time course of amnesia after treatment with $A\beta_{1-42}$

To investigate the mechanism of Aβ-induced amnesia, the time course of memory loss after Aβ treatment was examined. In the first experiment, Aβ was injected 45 min before training and then the time course of memory loss after training was measured (Fig. 6A). Memory was unimpaired for 30 min following administration of 10 pmol/hem $A\beta_{1-42}$ 45 min before training, but memory did not proceed beyond labile memory (Fig. 6A; $F_{7,119} = 9.60$, $P < 0.001$) such that memory retention measured at 35, 40, 45 50 and 60 min was significantly less than controls ($P \leq 0.001$). Injection of $A\beta_{1-42}$ 5 min before training also resulted in memory loss 35 min after training (Fig. 6B; $F_{6,96} = 14.86$, $P > 0.001$). As memory is unimpaired for the first 30 min after training with both injection times, and labile memory is unimpaired, this indicates that the memory is in the period of transition from labile to long-term memory and the timing of memory loss is not controlled by the time of injection. Consolidation of memory in the chick is triggered around 30 min after training and memory loss after protein synthesis inhibition occurs from 60 min. However, the degree of amnesia produced by injection of $A\beta_{1-42}$ 5 min before training was less than that produced when Aβ was injected 45 min before training, suggesting that injection of Aβ 5 min before training may not provide sufficient time for the peptide to diffuse to those sites in the brain necessary to completely block memory consolidation.

3.5. Effect of β-adrenoceptor agonists on $A\beta_{1-42}$-induced memory loss

Previous studies have shown that noradrenaline influences working memory in non-human primates (Ramos and Arnsten, 2007) and noradrenergic activation of β_2- or β_3-ARs has been shown to promote memory consolidation in chicks (Gibbs and Summers, 2000, 2002b). It has also been shown that memory impairment induced by hypoxia during gestation in chicks is rescued by β_3-AR agonists (Camm et al., 2004). Therefore, in this study, the ability of β_2 and β_3-AR agonists to rescue memory impaired by β-amyloid was tested by central administration of β-AR agonists. The doses used were based upon previously published experiments where the drugs were administered into the IMM. The chicks were subjected to strongly reinforced training (100% anthranilate) and the response to the β-AR agonists was compared with ability of agonists to promote memory in normal chicks weakly trained using 20% anthranilate. Memory was tested 120 min after training, i.e., after long-term memory had been established.

The selective β_3-AR agonist CL316243, when injected 20 min after strongly reinforced training rescued memory impaired by $A\beta_{1-42}$ injection. In contrast, the selective β_2-AR agonist zinterol was unable to rescue memory loss (Fig. 7A). A two-way ANOVA compared memory in chicks given saline, zinterol (30 pmol) or CL316243 (3 pmol) with or with out $A\beta_{1-42}$ pre-treatment ($F_{2,77} = 11.76$, $P < 0.001$). Memory retention in $A\beta_{1-42}$ treated chicks after CL316243 injection was not different to controls given CL316243; whereas saline and zinterol did not rescue memory impairment in $A\beta_{1-42}$ treated chicks ($F_{1,79} = 29.96$ and 18.80, respectively, both $P < 0.001$). Analysis of the time course of CL316243 injection showed that administration of the β_3-AR agonist prevented $A\beta_{1-42}$ induced memory loss when the agonist was administered between 10 min pre-training and 20 min post-training (Fig. 7B) ($F_{4,72} = 18.36$, $P < 0.001$). However, injections of CL316243 30 min after training did not rescue the $A\beta_{1-42}$-induced memory loss.

The dose dependence of the effect of CL316243 was studied in three groups of chicks (Fig. 7C). The β_3-AR agonist was injected 20 min after weakly reinforced training to chicks pretreated with $A\beta_{1-42}$ or saline 45 min before training. The aim of this experimental design was to compare the ability of CL316243 to rescue $A\beta_{1-42}$-induced memory loss with the ability of CL316243 to boost memory in control chicks that had not been treated with $A\beta_{1-42}$. Interestingly, CL316243 was much more potent at rescuing $A\beta_{1-42}$-induced memory loss than in boosting memory weakly reinforced control chicks. A two-way ANOVA showed no interaction effect $F_{1,50} = 0.15$, $P < 0.70$, a significant drug effect $F_{1,50} = 10.93$, $P = 0.002$ and a significant dose effect $F_{1,50} = 14.82$, $P < 0.001$. Simple main effects analyses show a significant difference for Aβ and control chicks at both doses of CL316243 ($P < 0.02$). The CL316243 dose–response relationship showed a significant shift to the left with $A\beta_{1-42}$ pre-treatment. There was no significant different between the CL316243 dose–response curves for weak- or strongly reinforced training in the $A\beta_{1-42}$-treated chicks. Zinterol did not rescue memory in $A\beta_{1-42}$-treated chicks given weakly reinforced training (DR = 0.539 ± .062, $N = 12$).

4. Discussion

One of the central problems for researchers in the AD field has been to develop an *in vivo* model of Aβ-induced dysfunction that mimics the important clinical features of AD. Such a model would have major advantages for drug development. APP transgenic mice have been developed that possess most of the neuropathologic features observed in AD (Morgan, 2003; van Dooren et al., 2005). However, transgenic mice have certain limitations for drug screening. The mild behavioural phenotype observed in APP transgenic mice (Morgan, 2003) and the difficulty of assessing memory in

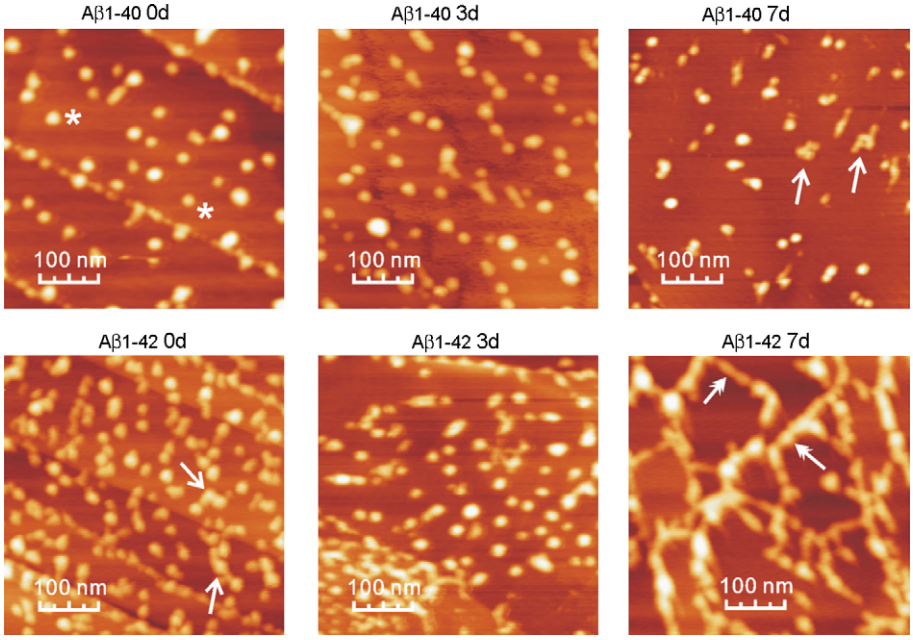

Fig. 5. AFM images showing the effect of ageing on the aggregation of Aβ peptides. Peptides (2 μM) were incubated at 37 °C in physiological saline for up to 7 days before being applied to a surface of HOPG for AFM. Figure shows representative images (500 nm × 500 nm) for $A\beta_{1–40}$ and $A\beta_{1–42}$ taken after 0, 3 and 7 days of ageing. Figure shows that the morphology of $A\beta_{1–40}$ and $A\beta_{1–42}$ changed with respect to time of ageing. Asterisks show bead-like Aβ structures that are low molecular weight species (Losic et al., 2006). Thin arrows show short oligomeric structures that were commonly found in freshly prepared $A\beta_{1–42}$ preparations or in 7-day-aged $A\beta_{1–40}$ preparations. Double-headed arrows show more rigid rod-like structures resembling short amyloid fibrils that were mostly commonly seen in the 7-day-aged $A\beta_{1–42}$ preparations.

mice (Karl et al., 2003) makes drug screening more cumbersome.

The present study describes a chick model of Aβ-induced amnesia. The advantage of this chick model is that it is rapid and relatively easy to perform. Chicks are readily available, inexpensive and large numbers of animals can be easily screened. The discriminative avoidance task used in the present study has been shown in many studies to be a highly reproducible method for assessing memory (Gibbs and Summers, 2002b). Although injection of Aβ into the chick brain does not reproduce the neuropathology of AD, this was not of concern in the present study because the acute effect of soluble oligomeric Aβ is likely to be more important for cognitive dysfunction in AD than the long-term neuropathological changes such as amyloid plaques and neurofibrillary tangles (Palop et al., 2006). It is now increasingly recognized that Aβ has acute effects on neuronal function and that these acute effects may be the more significant for cognitive dysfunction in AD. Indeed, it now generally thought that plaques and tangles are epiphenomena of the disease process and that synaptic actions of Aβ probably account for the cognitive decline in AD (Hardy and Selkoe, 2002).

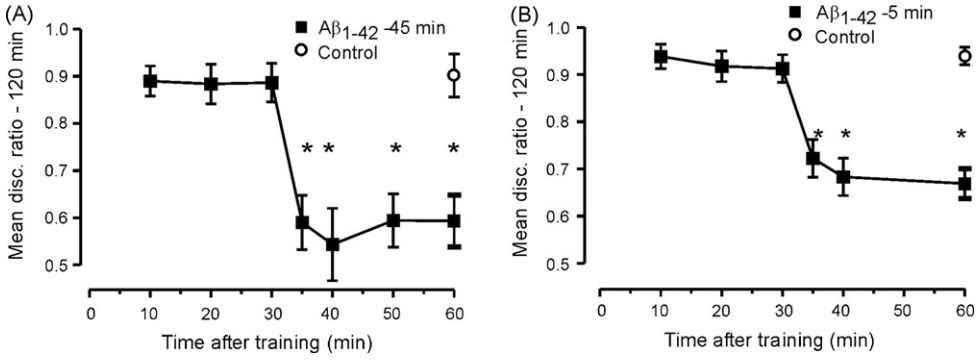

Fig. 6. Effect of $A\beta_{1–42}$ on memory retention. Chicks were injected with 10 pmol/hemisphere of freshly prepared $A\beta_{1–42}$ either 45 min or 5 min before training. Separate groups of chicks were then tested at different times after training to determine at what time point memory was decreased. (A) Chicks injected with $A\beta_{1–42}$ 45 min before training and then tested for memory retention between 10 and 60 min after training. (B) Chicks were injected with $A\beta_{1–42}$ 5 min before training and tested for memory retention between 10 and 60 min after training. Figure shows that the effect of $A\beta_{1–42}$ on memory was greater when the peptide was injected 45 min prior to training when compared to 5 min prior to training. In both cases, memory was retained for up to 30 min after training but was subsequently lost in the $A\beta_{1–42}$ injected animals. $^*P < 0.05$ compared to controls. $N = 13–16$ per group.

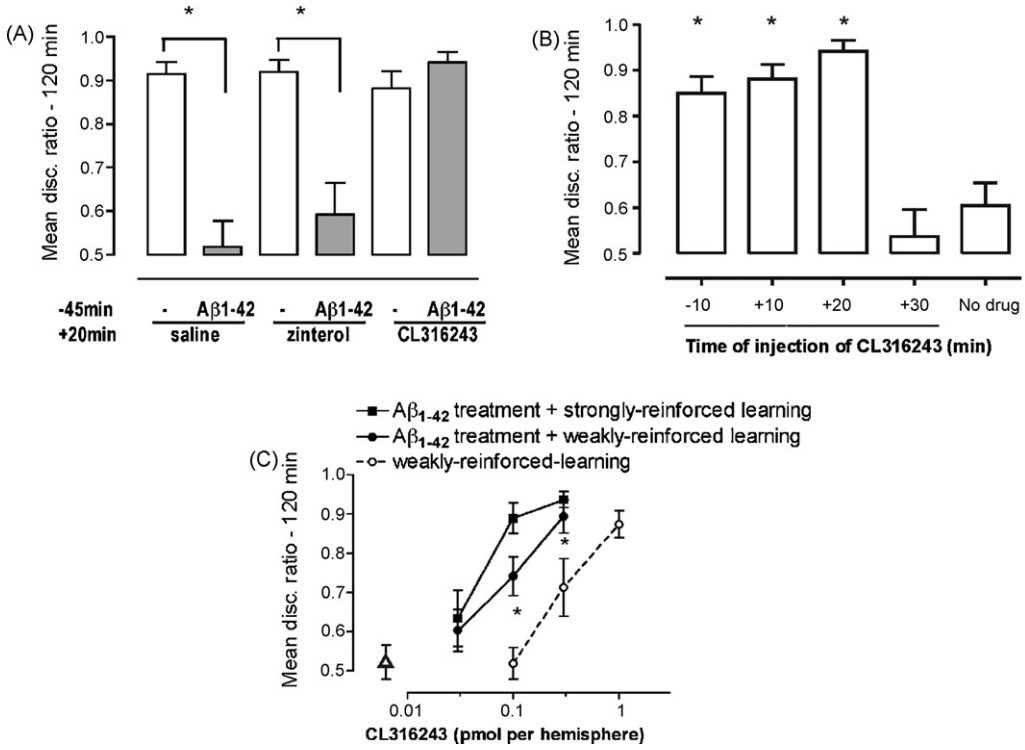

Fig. 7. Effect of β-AR agonists on $A\beta_{1–42}$-induced amnesia. (A) Compared with chicks after strongly reinforced training, memory loss following $A\beta_{1–42}$ injection (10 pmol/hemisphere) 45 min prior to training was reversed by injection 20 min after training of the selective β_3-AR agonist CL316243 (3 pmol/hemisphere), but not by saline or the selective β_2-AR agonist zinterol (30 pmol/hemisphere). $^*P < 0.05$ two-way ANOVA. (B) Time course of the effect of CL316243 (3 pmol/hemisphere) on memory in chicks treated with $A\beta_{1–42}$ (10 pmol/hemisphere) 45 min prior to training. Figure shows the times of injection (min) of CL316243 before and after training. The rescue of memory was observed when CL316243 was injected as early as 10 min before training. The control is for chicks given $A\beta_{1–42}$ with no further drug treatment. $^*P < 0.05$ compared to control. (C) Dose–response curve for CL316243. Figure shows the rescue by CL316243 injected 20 min after training in chicks given $A\beta_{1–42}$ (10 pmol/hemisphere injected 45 min before weak (filled circles) or strong training (filled squares). This is compared with CL316243 consolidation of memory in weakly reinforced control chicks at a lower dose than that required to promote consolidation of memory (open circles). Weak training control given no injection 20 min after training is shown as an open triangle. $^*P < 0.05$ two-way ANOVA. $N = 11–16$ per group.

Several lines of evidence indicate that the amnestic effects that we have observed in the chick are specific and relevant to amnesia in AD. Scrambled-sequence Aβ peptides were without effect on memory, indicating that there was sequence specificity to the effect. We also found that the state of aggregation of the Aβ peptides correlated with the amnestic effect. Many studies indicate that $A\beta_{1–42}$ is more likely to be the major pathogenic agent rather than $A\beta_{1–40}$ (Jarrett and Lansbury, 1993; Lambert et al., 1998; Hardy and Selkoe, 2002; Walsh and Selkoe, 2004; Kim et al., 2007). For example, $A\beta_{1–42}$ aggregates more readily than $A\beta_{1–40}$ (Jarrett and Lansbury, 1993) and production of $A\beta_{1–42}$ is more closely associated with disease pathogenesis (Jankowsky et al., 2004). In chicks, $A\beta_{1–42}$ when freshly prepared potently decreased chick memory, whereas $A\beta_{1–40}$ was without effect in the same experiment. However, when the $A\beta_{1–40}$ was aged by incubation at 37 °C, a memory-inhibiting effect was generated, whereas the amnestic effect of $A\beta_{1–42}$ decreased by ageing. AFM studies showed that the amnestic effect was associated with aggregation of the peptide and the presence of low molecular weight amorphous aggregates. This finding is consistent with recent studies that have found that low molecular weight non-fibrillar oligomers are the most highly neurotoxic (Caughey and Lansbury, 2003; Lambert et al., 1998; Walsh and Selkoe, 2004). After ageing for 7 days, the $A\beta_{1–42}$ preparation contained larger rod-like structures or fibrils and was less potent in the chick model.

$A\beta_{1–42}$ was observed to selectively inhibit memory consolidation (processing of short-term memory into long-term memory), rather than to have a global effect on memory retrieval. This observation suggests that the Aβ-induced deficit in chicks is similar to the anterograde amnesia that occurs in early AD patients (Storey et al., 2001, 2002). During the early stages of the disease, patients can have problems with anterograde episodic memory in which, for example, acquisition and delayed free recall can be impaired (Storey et al., 2002). This problem has traditionally been regarded as a problem of encoding or consolidation (Gainotti et al., 1998; Troster et al., 1993; Welsh et al., 1991). In the chick as well as in rodents, memory consolidation is associated with a change in synaptic connectivity and excitatory signalling (Lee et al., 2004; Miller et al., 2002). Several recent studies have reported that Aβ oligomers can disrupt excitatory neurotransmitter signalling (Knobloch et al., 2007) and alter the number or

distribution of dendritic spines and synapses (Shankar et al., 2007) that would be consistent with such an effect.

Previous studies have provided evidence that the noradrenergic system of the brain is involved in memory processes (Berridge and Waterhouse, 2003; Gibbs and Summers, 2002b; Marien et al., 2004; Sullivan and Wilson, 1994). For example, noradrenaline modulates working memory in primates via its effects on prefrontal cortex neurons, where it may help to inhibit the processing of irrelevant stimuli. Damage to noradrenergic innervation of the prefrontal cortex causes decreased performance in tests of working memory (Arnsten, 1998; Berridge and Waterhouse, 2003; Ramos and Arnsten, 2007). Studies in day-old chicks (Gibbs and Summers, 2000, 2005) have shown that β_2- and β_3-AR agonists can stimulate memory consolidation found to be blocked by Aβ in this study. For this reason, it was logical to examine the effect of β_2- and β_3-AR agonists on Aβ-induced amnesia. Significantly, we found that a β_3-AR agonist (CL316243) strongly inhibited the effect of Aβ, at doses lower than those that improve memory following weak reinforcement of training.

Our inability to observe any rescue of Aβ-induced amnesia using a β_2-AR agonist was surprising, in view of the fact that β_2-AR agonists have previously be found to improve memory consolidation in chicks that were not treated with Aβ peptides (Gibbs and Summers, 2000, 2005). While it is not clear why this difference was observed, the finding does raise the possibility that the β_3-AR agonist may act to selectively inhibit a step in the pathway of Aβ action, whereas the β_2-AR may not. There are significant differences between signalling mechanisms of β_2- and β_3-ARs (Hein, 2006; Lefkowitz, 2004). β_2-ARs act via G_s to stimulate cyclic-AMP production. They can also act via G_i, but this action requires receptor internalization and phosphorylation via protein kinase A. In contrast, β_3-ARs can mediate their actions via both G_s and G_i without the need for receptor internalization and phosphorylation by protein kinase A (Hutchinson et al., 2002). Whether these specific differences in signalling account for the differences in their ability to block the effect of Aβ on memory remains to be determined. Increased intracellular glucose levels are required for memory in the chick (Gibbs et al., 2007) and these are increased following activation of β_3-ARs (Gibbs and Summers, 2002a; Hutchinson et al., 2007). Intriguingly, it has been suggested that Aβ can inhibit neuronal glucose uptake via an action on G_s-coupled receptors (Prapong et al., 2001, 2002). Whether such a mechanism could explain the effects of the β_3-AR agonist in reversing Aβ-induced amnesia also remains to be determined.

One possibility that cannot be ruled out is that CL316243 has an action distinct from its known action on β_3-ARs. However, our previous studies have shown that the effect of CL316243 in boosting chick memory following weakly reinforced training can be blocked by the β_3-AR antagonist SR59230 (Gibbs and Summers, 2000). This finding would suggest that the effect is specific for the β_3-AR.

In chicks and in mammals, it is recognized that complex and even relatively simple cognitive tasks such as the discriminative avoidance task used in this study, are subserved by distributed brain networks involving different brain regions rather than single specific brain areas (Gibbs et al., 2008). Noradrenaline has roles in memory processing in the chick in the basal ganglia, the hippocampus, locus coeruleus and integration areas such as IMM. Clearly any perturbation of the noradrenergic system in the central nervous system will have profound effects on memory. The effect of the β_3-AR agonist on Aβ-induced amnesia could have implications for the treatment of AD. Noradrenaline levels in locus coeruleus are known to be decreased in AD patients (Hertz, 1989; Marien et al., 2004). So far, the noradrenergic system has received a lot less attention than the cholinergic system as a target for AD drug development. In view of the fact that cholinergic drugs have been found to have only modest effects on cognition and do not produce substantial improvement in activities of daily living scores, new drugs are urgently needed. In view of the involvement of the noradrenergic system in memory and the decrease in noradrenaline in AD patients, more studies on the role of the noradrenergic system in AD would be helpful (Haglund et al., 2006). Moreover, the effect of AR agonists or antagonists on the cognitive performance of AD patients is unknown. Certainly, β_3-AR agonists have not been tested in clinical trials with AD patients. In view of the selective effect of CL316243 on Aβ-induced chick memory, it would seem logical to examine this drug in other behavioural models of AD.

Conflict of interest

The authors have no actual or potential conflicts of interest to declare.

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