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ARTICLE

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The phosphodiesterase-4 inhibitor rolipram reverses Aβ-induced cognitive impairment and neuroinflammatory and apoptotic responses in rats

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Abstract

β-amyloid (Aβ) peptides play an important role in cognition deficits, neuroinflammation, and apoptosis observed in Alzheimer's disease (AD). Activation of cyclic AMP (cAMP) signalling enhances memory and inhibits inflammatory and apoptotic responses. However, it is not known whether inhibition of phosphodiesterase-4 (PDE4), a critical controller of intracellular cAMP concentrations, affects AD-associated neuroinflammatory and apoptotic responses and whether these responses contribute to deficits of memory mediated by cAMP signalling. We addressed these issues using memory tests and neurochemical measures. Specifically, rats microinfused with aggregated Aβ25-35 (10 μg/side) into bilateral CA1 subregions displayed deficits in learning ability and memory, as evidenced by decreases in escape latency during acquisition trials and exploratory activities in the probe trial in the water-maze task and 24-h retention in the passive avoidance test. These effects were reversed by rolipram (0.1, 0.25 and 0.5 mg/kg.d i.p.), a prototypic PDE4 inhibitor, in a dose-dependent manner. Interestingly, Aβ25-35-treated rats also displayed decreases in expression of phosphorylated cAMP response-element binding protein (pCREB) and Bcl-2, but increases in expression of NF-κB p65 and Bax in the hippocampus; these effects were also reversed by rolipram in a dose-dependent manner. Similar neurochemical results were observed by replacing Aβ25-35 with Aβ1-42, a full-length amyloid peptide that quickly forms toxic oligomers. These results suggest that PDE4 inhibitors such as rolipram may reverse Aβ-induced memory deficits at least in part via the attenuation of neuronal inflammation and apoptosis mediated by cAMP/CREB signalling. PDE4 could be a target for treatment of memory loss associated with AD.

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Key words: CREB, memory, phosphodiesterase-4 (PDE4), rolipram, β-amyloid (Aβ).

Introduction

Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder of the central nervous system characterized by progressive cognitive dysfunction. It has been considered that excessive accumulation of neurotoxic β-amyloid peptide (Aβ) in the brain is the hallmark of AD; deposition of Aβ causes neuropathological lesions in the brain of patients with AD (Hardy, 1997; Selkoe, 2000; Suh & Checler, 2002). While mechanisms by which Aβ induces neuronal cell death remain unclear, results from accumulating studies suggest that Aβ may cause neurotoxicity via: (1) formation of reactive oxygen species (Barnham *et al.* 2004; Hardy & Selkoe, 2002), (2) intracellular calcium accumulation (Demuro *et al.* 2010), (3) changes in membrane fluidity (Yang *et al.* 2010), (4) energy

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depletion (Dhitavat *et al.* 2002), (5) alteration of cytoskeleton components (Lesort *et al.* 1997), and (6) inflammatory responses (Agostinho *et al.* 2010). These neuropathological changes are closely linked to chronic inflammation and apoptosis, which are highly correlated with the early stage of AD via increased markers of inflammatory and apoptotic responses (Luterman *et al.* 2000; Mehlhorn *et al.* 2000; Mrak & Griffin, 2001; Parachikova *et al.* 2007; Pasinetti, 2001). Moreover, drugs with anti-inflammatory activity reduce progression of cognitive deficits and/or Aβ burden in animal models of AD (Jantzen *et al.* 2002; Lim *et al.* 2000; Townsend & Praticò, 2005). Thus, therapeutic efforts aimed at interruption of Aβ-induced neuroinflammation and apoptosis may be beneficial in AD.

Intracellular cyclic AMP (cAMP) signalling has been well established in the mediation of memory (Bailey *et al.* 1996; Barad *et al.* 1998; Li *et al.* 2011; Zhang *et al.* 2000). Cyclic AMP activates protein kinase A (PKA), which phosphorylates and activates the subsequent downstream target cAMP-response element binding (CREB) protein (Lonze & Ginty, 2002; Zhang, 2010). This signalling cascade is important for mediating memory, in particular hippocampus-dependent long-term memory (Tully *et al.* 2003); activation of cAMP signalling increases synaptic plasticity and produces memory-enhancing effects in rodents (Barad *et al.* 1998; Li *et al.* 2011; Zhang *et al.* 2000, 2004, 2005). In addition, increases in cAMP levels during inflammation inhibit production of pro-inflammatory cytokines and stimulate formation of IL-10, an anti-inflammatory factor (Kast, 2000; Szelenyi *et al.* 2000). Further, while elevation of cAMP triggers apoptosis in cancer cells (Naviglio *et al.* 2009), it prevents development of apoptotic machinery in neurons and neutrophils (De *et al.* 1994; Parvathenani *et al.* 1998). Therefore, cAMP signalling may play a beneficial role in regulating Aβ peptide-induced inflammatory responses and apoptosis *in vivo*.

Phosphodiesterase-4 (PDE4), an enzyme that specifically hydrolyses cAMP, plays an important role in the mediation of learning and memory. Administration of PDE4 inhibitors such as rolipram enhances memory (Barad *et al.* 1998; Li *et al.* 2011; Rutten *et al.* 2006) and reverses memory deficits induced by pharmacological (Zhang & O'Donnell, 2000; Zhang *et al.* 2000, 2004, 2005), physical (Imanishi *et al.* 1997), physiological (Vecsey *et al.* 2009), or genetic (Bourtchouladze *et al.* 2003) manipulations. Rolipram treatment also reverses memory deficits induced by Aβ25-35 or Aβ1-40 (Cheng *et al.* 2010) or observed in amyloid precursor protein (APP)/presenilin-1 (PS1) transgenic mice (Gong *et al.* 2004). Drugs that inhibit PDE4 have received a great deal of attention, partially due to their inhibitory effects on inflammation (Genain *et al.* 1995; Sekut *et al.* 1995; Souness *et al.* 2000; Teixeira *et al.* 1997) and apoptosis (Sousa *et al.* 2010) in various models. However, it is not clear whether PDE4 inhibitors block Aβ-induced inflammatory responses and apoptosis, important mechanisms in the pathogenesis of AD (Agostinho *et al.* 2010; Lukiw & Bazan, 2010).

The fraction 25-35 of Aβ (Aβ25-35), which is the neurotoxin core fragment of the full-length Aβ peptide (Cheng *et al.* 2006; Pike *et al.* 1993), plays a pivotal role in the neurodegenerative process of AD. Central administration of Aβ25-35 produces cognitive deficit (Cheng *et al.* 2010; Lin *et al.* 2009), cholinergic dysfunction (Olariu *et al.* 2001), neuronal apoptosis (Ruan *et al.* 2010), oxidative stress (Ahn *et al.* 2006; Um *et al.* 2006), and neuroinflammation (Lin *et al.* 2009). Specifically, microinfusions of Aβ25-35 into the hippocampus impair memory in various tasks, including the Morris water-maze and passive avoidance tests in mice (Kim *et al.* 2008; Maurice *et al.* 1996; Wang *et al.* 2001) and rats (Cheng *et al.* 2010; Lin *et al.* 2009) and the radial-arm maze and social recognition tests in rats (Delobette *et al.* 1997; Jin *et al.* 2004; Stepanichev *et al.* 2003; Sun & Alkon, 2002). Recently, our studies have shown that infusions of Aβ1-40 into the hippocampus cause memory impairment via decreases in cAMP/CREB signalling (Cheng *et al.* 2010). Similar effects of Aβ1-42 on memory and cAMP signalling have also been observed (Malm *et al.* 2006; O'Hare *et al.* 1999; Tong *et al.* 2001; Vitolo *et al.* 2002). Given that Aβ25-35 increases inflammatory factors such as IL-1β, IL-6, and TNF-α in the hippocampus (Lin *et al.* 2009), it was of interest to know whether the inflammatory and apoptotic effects of Aβ25-35 in the brain contribute to deficits of memory and whether pharmacologically induced up-regulation of pCREB reversed Aβ25-35-induced neuronal inflammation, apoptosis, and memory deficits. Aβ1-42 was also used to verify the effects of rolipram on Aβ-induced neurochemical changes in the brain.

Materials and methods

Animals

Adult male Sprague–Dawley rats (Laboratory Animal Centre of Southern Medical University, China or Harlan, USA), weighing 250–300 g, were used for the experiments. Animals were housed in a temperature-controlled room (21 ± 2 °C) on a 12-h light/dark cycle

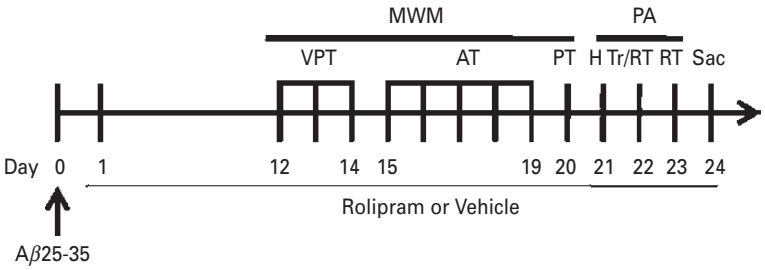

Fig. 1. Schedule of drug treatment and test orders. Rolipram (0.1, 0.25, or 0.5 mg/kg) or its vehicle (0.9% saline containing 10% DMSO) was injected i.p. 24 h after Aβ25-35 (10 μg/side) or its vehicle, which was infused into bilateral CA1 subregions 12 d before the beginning of the 3-d visible platform test (VPT) in the Morris water-maze (MWM). On day 15, the water-maze acquisition trials (AT) were conducted four times a day for 5 consecutive days, followed by the probe trial test (PT) 24 h after the last acquisition trial. On day 21, habituation (H) of the passive avoidance chamber was performed, followed the next day by the training trial (Tr), the 3-h retention test (RT), and 24 h after initial training, another retention test (RT) for measuring long-term memory. Animals were sacrificed (Sac) 24 h after the last behavioural test. Rolipram or its vehicle was injected once per day for 24 d, 1 h prior to each test or sacrifice.

(lights on 06:00 hours), with water and food available *ad libitum*. All experiments were performed according to NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised, 1996). The procedures were approved by the Animal Care and Use Committees of the Southern Medical University, China, and West Virginia University Health Sciences Center, USA.

Drugs

Aβ25-35 (Beijing Guo Ding Technology Ltd, China), Aβ1-42 (rPeptide, USA), or Aβ42-1 (rAβ42-1; GenScript, USA) was dissolved in 0.9% sterile saline or artificial cerebrospinal fluid (aCSF) containing 35% acetonitrile and 0.1% trifluoroacetic acid (Cheng *et al.* 2010) at a final concentration of 4 μg/μl (for Aβ25-35) or 0.4 μg/μl (for Aβ1-42 and Aβ42-1) and incubated at 37 °C for 4 d to form aggregated Aβ (Delobette *et al.* 1997; Pike *et al.* 1993) before use. Rolipram (Sigma-Aldrich, USA) was dissolved in 0.9% saline containing 10% or 1% dimethyl sufoxide (DMSO; for tests involving Aβ25-35 and Aβ1-42/Aβ42-1, respectively); the injection volume for peripheral administration was 1 ml/kg.

Surgery

Rats were anaesthetized with intraperitoneal (i.p.) administration of ketamine (100 mg/kg) and xylazine (10 mg/kg) before they were placed in a stereotaxic apparatus (Stoelting, USA). Guide cannulae (22-gauge, 6 mm; Plastics One, USA) were implanted into bilateral CA1 subregions using the following coordinates: AP −3.3 mm from Bregma; ML 2.0 mm from the midline; DV −2.5 mm from dura (Paxinos & Watson, 1986; Zhang *et al.* 2004). Aβ25-35 (10 μg in 2.5 μl/side; at this volume the Aβ25-35 solution was sufficiently thin for infusions; Cheng *et al.* 2010), Aβ1-42 or Aβ42-1 (0.4 μg in 1 μl/side for both peptides), or vehicle [0.9% saline (for Aβ25-35) or aCSF (for Aβ1-42 or Aβ42-1) containing 35% acetonitrile and 0.1% trifluoroacetic acid] in the same, corresponding volumes was infused bilaterally at the rate of 0.5 (for Aβ25-35) or 0.25 (for Aβ1-42 or Aβ42-1) μl/min using a syringe pump; the 28-gauge infusion cannulae were left in place for additional 5 min to permit diffusion before the animals were returned to their home cages. No flocculation of peptides was observed during infusions. The dose of Aβ25-35 or Aβ1-42/Aβ42-1 was chosen based on our preliminary or published studies; the latter have shown that Aβ25-35 centrally administered at a similar dose impairs memory (Cheng *et al.* 2010) and produces inflammatory responses in the brain (Lin *et al.* 2009). The injection sites were verified histologically after completion of behavioural tests (Zhang *et al.* 2004).

Behavioural test procedures

To examine the effect of rolipram on Aβ25-35-induced memory deficits, five groups of 12 rats each were tested for memory using Morris water-maze and passive avoidance tasks (Fig. 1): (*a*) saline + vehicle (10% DMSO for rolipram); (*b*) Aβ25-35 + vehicle; (*c*) Aβ25-35 + rolipram (0.1 mg/kg); (*d*) Aβ25-35 + rolipram (0.25 mg/kg); (*e*) Aβ25-35 + rolipram

(0.5 mg/kg). Twenty-four hours after microinfusions of Aβ25-35 (10 μg/side) or saline into bilateral CA1 subregions in the hippocampus, rats were injected (i.p.) with different doses of rolipram or its vehicle, once per day for 12 d prior to the beginning of water-maze training and probe trial testing; the animals were then subjected to passive avoidance habituation, training, and testing. Rolipram treatment was given 1 h before and continued throughout the behavioural tests.

Morris water-maze test

This was performed as described previously (Morris, 1984; Zhang *et al.* 2008) with some modifications. The apparatus consisted of a circular pool (120 cm diameter, 50 cm height) filled with water (23 ± 1 °C and 32 cm depth), which was opaque by mixing with milk powder, and a platform (10 cm diameter), which was either visible (in the visible platform test) or immersed 2 cm under the surface of the water (in the acquisition training) in one of the four identical quadrants. The pool was placed in a room illuminated at 100 lx containing several extra-maze cues. Twelve days after infusions of Aβ25-35 the visible platform test was performed, during which the escape latency (time required to locate and climb onto the platform) of each rat was determined using the visible platform (1 cm above the water surface). The test was conducted once per day for three consecutive days; rats were placed in the pool each day from different starting points selected randomly. Rats that failed to find the platform within 90 s were guided to the platform manually.

Twenty-four hours after the last trial of the visible platform test, acquisition training was performed; the platform was moved to another quadrant and fixed there throughout the acquisition trials. Rats were individually placed in the water facing the wall of the pool in order to negate any directional influence. Each rat was allowed to escape by swimming to the platform; the escape latency was recorded with the cut-off time 90 s. If the animal failed to locate the platform within 90 s, it was gently guided to the platform and allowed to stay on it for 30 s. Following completion of each trial, animals were dried gently with paper towel and kept warm under heat lamps in holding cages for 5 min before they were returned to their home cages. Swimming behaviours, including escape latency, distance swum, swimming speed, and swimming paths, were monitored using a computer-controlled video-tracking system (CG-400 Image Acquisition System, Institute of Materia Medica, Chinese Academy of Medical Sciences, China). The acquisition training consisted of four trials each day for five consecutive days.

Twenty-four hours after the last acquisition trial (i.e. day 20), the probe trial test was conducted with the platform removed. Each rat was placed into the pool at one fixed start position and allowed to swim freely for 90 s. The distance swum, entries and time spent in the target quadrant (i.e. the previous location of the platform), and the mean swimming speed were recorded.

Passive avoidance test

This was performed using the step-through passive avoidance apparatus (Institute of Materia Medica, Chinese Academy of Medical Sciences, China), following procedures described previously (Li *et al.* 2011; Zhang *et al.* 2000) with minor modifications. The apparatus consisted of a chamber with two compartments (dark/illuminated), which were connected by a guillotine door. The dark compartment had a stainless-steel grid floor available for electric footshocks. All rats were given a habituation session, during which they were allowed to freely explore the apparatus for 4 min. Twenty-four hours later, the training trial was performed. Rats were individually placed in the illuminated compartment for 60 s before the door was lifted. Immediately after the rat entered the dark compartment, the door was closed and an inescapable electric shock (0.5 mA, 3 s) was delivered. The rat was then returned to the home cage. The retention test was performed 3 h and 24 h after training to measure short-term and long-term memory, respectively. The latency was recorded following the same procedures as in the acquisition trial, but no footshock was delivered. The cut-off time was 300 s.

Western blot analysis

Twenty-four hours after the passive avoidance test (for Aβ25-35) or 14 d after Aβ1-42 or Aβ42-1 microinfusions and 1 h after the last rolipram injection, rats were decapitated, and the hippocampus dissected and stored at -80 °C until analysis. Hippocampal tissues were homogenized in ice-cold RIPA buffer containing 0.1% phenylmethylsulfonyl fluoride and assessed for expression of pCREB, NF-κB, Bcl-2, and Bax proteins. Each sample was separated by electrophoresis in 12% polyacrylamide gels (120 V, 150 min) before being transferred to PVDF membranes (60 V, 180 min). Non-specific bindings were blocked with 5% BSA for 2 h. After washing, membranes were subsequently

incubated with respective primary antibodies for rabbit anti-phospho-CREB [Ser[133]; 1:1000; CST (Shanghai) Biological Reagents Company Ltd, or Millipore, USA (for tests involving Aβ1-42)], anti-NF-κB p65 [1:1000; Abcam (Hong Kong) Ltd, or Millipore, USA (for tests involving Aβ1-42)], anti-Bcl-2 (1:1000), anti-Bax [1:1000, CST (Shanghai) or Millipore, USA (for tests involving Aβ1-42)], anti-GAPDH [1:1000, Abcam (Hong Kong) Ltd], and anti-β-actin (Abcam, USA) at 4 °C overnight. Membranes were then incubated for 1 h with secondary antibodies [1:10 000; Abcam (Hong Kong) Ltd]. The detection and quantification of specific bands were developed using enhanced chemiluminescence reagent (Millipore, USA), visualized and analysed using Kodak X-Omat film and 'Image-Pro' software (www.scioncorp.com) or a fluorescence scanner (Odyssey Infrared Imaging System, Li-Cor Biotechnology, USA, for tests involving Aβ1-42).

Statistical analysis

Data shown are expressed as means ± S.E.M. All the data were analysed using one-way ANOVA followed by Newman–Keuls tests for *post-hoc* comparisons between groups, with the exception of the data of the water-maze acquisition trials, which were analysed by two-way repeated-measures ANOVA. Statistical significance was considered when $p < 0.05$.

Results

Rolipram reversed memory impairment induced by Aβ25-35 in Morris water-maze and passive avoidance tasks

To establish memory deficits produced by Aβ25-35, we examined memory performance in rats micro-infused with aged Aβ25-35 at 10 μg/side into CA1 using the water-maze and passive avoidance tests. Histological verification showed that the cannula placements were highly localized in hippocampal CA1 subregions (data not shown). In addition, there were no visible inflammatory changes at the site of injections (i.p.) during the period of rolipram/vehicle administration. In order to evaluate the animals' abilities of vision and motor activity, navigation to a visible platform was performed before the hidden platform procedures in the water-maze test. While the escape latency to the visible platform varied with different treatments, no significant changes were observed in any of the treatment groups during the 3-d testing [$F(4,110) = 1.724$, $p = 0.158$; Fig. 2*a*], suggesting that neither surgery nor drug treatment altered vision ability or general motor activity.

During the 5-d acquisition trials in the water-maze test, all the rats, regardless of the different treatments, displayed progressive decreases in escape latency to reach the hidden platform. Two-way repeated-measures ANOVA revealed significant changes in drug effect [$F(4, 1045) = 58.63$, $p < 0.0001$] and time effect [$F(19, 1045) = 67.46$, $p < 0.0001$], but not for drug × time interaction [$F(76, 11045) = 1.184$, $p = 0.141$; Fig. 2*b*]. *Post-hoc* Newman–Keuls comparisons indicated that Aβ25-35, administered 15 d prior to the beginning of acquisition training, increased escape latencies on sessions 3–10, 13, 14, 16, and 18 compared to the corresponding vehicle controls ($p < 0.05$, 0.01, or 0.001; Fig. 2*b*); these were reversed by rolipram at different doses (0.1 mg/kg for sessions 3–7; 0.25 mg/kg for sessions 3–4, 7–10; 0.5 mg/kg for sessions 3–10, 16; $p < 0.05$, 0.01, or 0.001). In addition, the rats also displayed slight, progressive decreases in swimming speed over time. Two-way repeated-measures ANOVA revealed significant changes in drug effect [$F(4, 1045) = 3.53$, $p = 0.01$] and time effect [$F(19, 1045) = 9.44$, $p < 0.0001$], but not for drug × time interaction [$F(76, 1045) = 1.004$, $p = 0.47$; Fig. 2*c*]. *Post-hoc* Newman–Keuls comparisons indicated that Aβ25-35, with or without rolipram treatment, did not alter swimming speed compared to the corresponding vehicle controls.

Twenty-four hours after the last acquisition trial (i.e. 20 d after Aβ25-35 infusions or 19 d after the beginning of rolipram treatment), rats were tested for spatial memory in the probe trial test, during which the platform was removed. The total distance swum during the probe trial test was not different between groups (data not shown). Drug treatment altered entries [$F(4, 55) = 5.35$, $p = 0.001$; Fig. 2*d*], duration [$F(4, 55) = 17.77$, $p < 0.0001$; Fig. 2*e*], and distance swum [$F(4, 55) = 6.37$, $p = 0.0003$; Fig. 2*f*] in the target quadrant. *Post-hoc* Newman–Keuls tests indicated that Aβ25-35 decreased entries ($p < 0.01$), duration ($p < 0.001$), and distance ($p < 0.001$) compared to the respective vehicle controls; these were reversed by chronic treatment with rolipram in a dose-dependent manner ($p < 0.05$, 0.01, or 0.001; Fig. 2*d–f*). In contrast, swimming speed was not altered by any of the drugs [$F(4, 55) = 0.296$, $p = 0.879$; Fig. 2*g*], indicating that changes in spatial memory measured in the probe trial were independent of general motor activity. Consistent with this, the behavioural tracking results revealed that both vehicle- and Aβ/rolipram-treated rats displayed more exploration in the target quadrant than the rats treated with Aβ25-35 alone (Fig. 2*h*); this

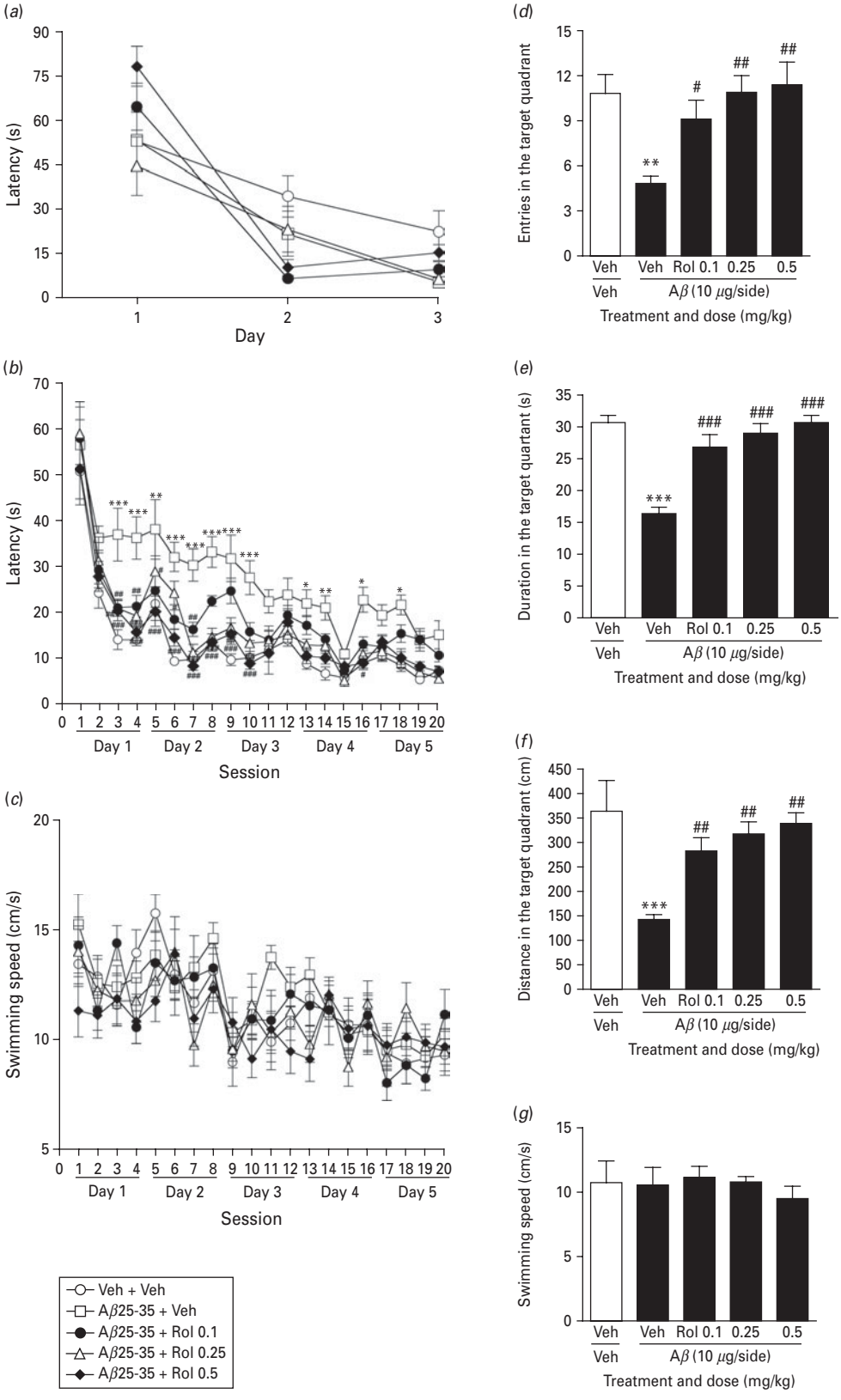

Fig. 2. For legend see opposite

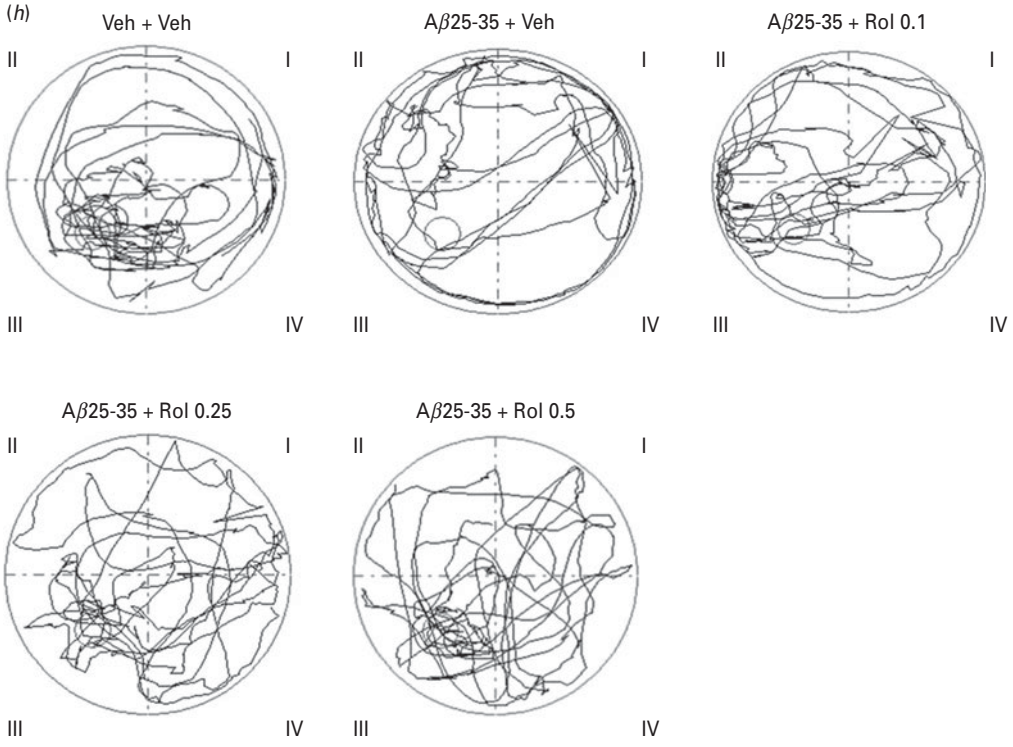

Fig. 2. Effects of rolipram on Aβ25-35-induced spatial cognitive deficits in the water-maze test in rats. (*a*) Escape latency was not significantly changed by drug treatment during the 3-d visible platform test. (*b*, *c*) Changes in (*b*) escape latency to reach the hidden platform and (*c*) swimming speed during the 5-d acquisition trials. Aβ25-35 overall increased escape latency, which was reversed by rolipram, but did not alter swimming speed. (*d–g*) Effects of Aβ25-35 with or without rolipram on (*d*) entries, (*e*) duration, (*f*) distance swum in the target quadrant, and (*g*) mean swimming speed in the probe trial test performed 24 h after the last acquisition trial. Aβ25-35 decreased the entries, duration, and swimming distance in the target quadrant, which were all reversed by rolipram (0.1, 0.25, or 0.5 mg/kg) in a dose-dependent manner, whereas the swimming speed was not changed by drug treatment. (*h*) Representative searching swimming paths by rats with different treatments in the probe trial test. While vehicle control animals swam concentrating on the target quadrant, Aβ-treated rats swam primarily around the pool; this was reversed by rolipram treatment. Rolipram was injected i.p. once a day for 12–20 d during the water-maze test, starting 24 h after microinfusion of Aβ25-35 (10 μg/side) into bilateral CA1 subregions. Values represent means ± S.E.M. ($n = 12$ per group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* vehicle controls (Veh + Veh); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* Aβ25-35 + vehicle.

result is consistent with rolipram's reversal of Aβ25-35-induced decreases in escape latency and length of swimming path.

The next day after the water-maze probe trial test (i.e. 21 d after Aβ25-35 infusions), the same rats were tested for habituation latency, short-term memory, and long-term memory using the passive avoidance task, with daily rolipram treatment continued. As shown in Fig. 3*a*, latency during the habituation trial did not differ in any of the groups [$F(4,55) = 0.149$, $p = 0.963$], indicating that all the rats had similar responses to the test environment when electric shocks were not applied. The initial training latency was not changed (data not shown). In the retention test performed 3 h after training, latency was not significantly changed by drug treatment [$F(4,55) = 2.18$, $p = 0.08$; Fig. 3*b*], although Aβ alone tended to decrease retention, indicating short-term memory was not altered. In contrast, retention latency tested 24 h after initial training was changed in a pattern similar to that in the water-maze probe trial in rats treated with Aβ25-35, in the presence or absence of rolipram [$F(4,55) = 5.30$, $p = 0.001$; Fig. 3*c*]. *Post-hoc* Newman–Keuls analyses indicated that Aβ25-35-treated rats exhibited a decrease in retention relative to vehicle controls ($p < 0.001$); this was reversed by rolipram (0.1–0.5 mg/kg), in particular at the dose of 0.5 mg/kg ($p < 0.01$), suggesting blockade

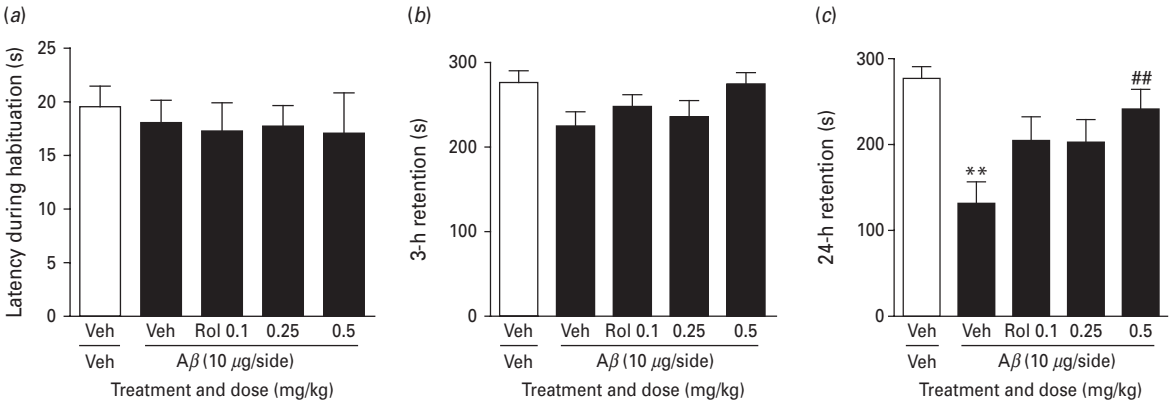

Fig. 3. Effects of rolipram on performance in the presence of Aβ25-35 in the step-through passive avoidance test in rats. (*a*) Latency to enter the dark compartment during habituation; (*b*) retention tested 3 h after initial training; (*c*) retention tested 24 h after initial training. While drug treatment did not alter latency during habituation or in the 3-h retention test, Aβ25-35 administered 23 d before the test decreased the 24-h retention latency. This was reversed by rolipram at the higher dose of 0.5 mg/kg. Rolipram (0.1, 0.25, or 0.5 mg/kg) was injected (i.p.) once a day for 21–23 d during the passive avoidance test, starting 24 h after microinfusion of Aβ25-35 (10 μg/side) into bilateral CA1 subregions. Values shown are means $\pm$ S.E.M. ($n = 12$ per group); *** $p < 0.001$ *vs.* vehicle controls (Veh + Veh); ## $p < 0.01$ *vs.* Aβ25-35 + Veh.

by rolipram of Aβ25-35-induced deficit of long-term memory.

Effects of Aβ25-35 on pCREB levels in the hippocampus of rats treated with rolipram

To determine whether Aβ25-35 decreased pCREB and whether cAMP/CREB signalling contributed to the reversal effect of rolipram on Aβ25-35-induced memory deficit, we examined pCREB expression in the hippocampus using the same rats, which were decapitated 24 h after the last behavioural test and 1 h after the last rolipram injection (Li *et al.* 2011). One-way ANOVA revealed significant changes in pCREB levels in the hippocampus in the treatment groups [$F(4, 10) = 21.92$, $p < 0.0001$; Fig. 4]. Compared to vehicle controls, Aβ25-35 alone significantly decreased pCREB levels; this effect was reversed by rolipram at doses of 0.1, 0.25, and 0.5 mg/kg, once daily for 24 d ($p < 0.01$ for 0.1 mg/kg and $p < 0.001$ for the other two doses). In contrast, expression of CREB was not altered by the drug treatment (data not shown).

Effect of rolipram on Aβ25-35-induced activation of NF-κB p65

It has been shown in our recent study that Aβ25-35 increases pro-inflammatory factors such as TNF-α (Lin *et al.* 2009); this may cause changes in NF-κB, a downstream target of TNF-α (Sanchez *et al.* 2005). To clarify this, we examined the expression of NF-κB p65 in the hippocampus using the same tissues for measuring pCREB. As shown in Fig. 5, levels of NF-κB

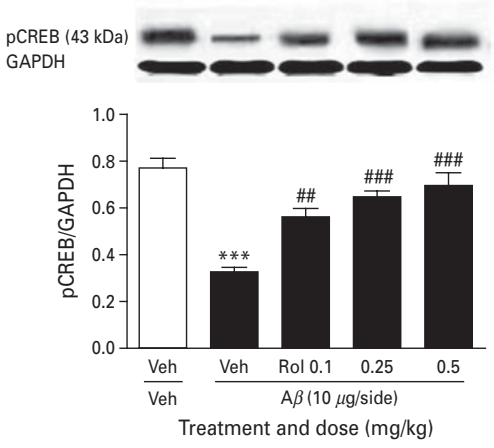

Fig. 4. Effect of rolipram on expression of pCREB in the hippocampus in rats treated with Aβ25-35. Upper panel is representative immunoblots of pCREB and GAPDH (inner control) measured by Western blotting using respective antibodies; lower panel is quantification of pCREB expressed as the ratio of pCREB/GAPDH. Aβ25-35 decreased pCREB in the hippocampus, which was reversed by rolipram (0.1, 0.25, or 0.5 mg/kg) in a dose-dependent manner. Starting 24 h after CA1 microinfusions of Aβ25-35 (10 μg/side), rolipram was injected (i.p.) once a day for 24 d before sacrifice, which was performed 24 h after the last behavioural test and 1 h after rolipram treatment. Values shown are means $\pm$ S.E.M. ($n = 3$ per group); *** $p < 0.001$ *vs.* vehicle controls (Veh + Veh); ## $p < 0.01$, ### $p < 0.001$ *vs.* Aβ25-35 + Veh.

p65 expression in the hippocampus were changed by drug treatment [$F(4, 10) = 12.27$, $p = 0.0007$]. *Post-hoc* Newman–Keuls analyses indicated a significant

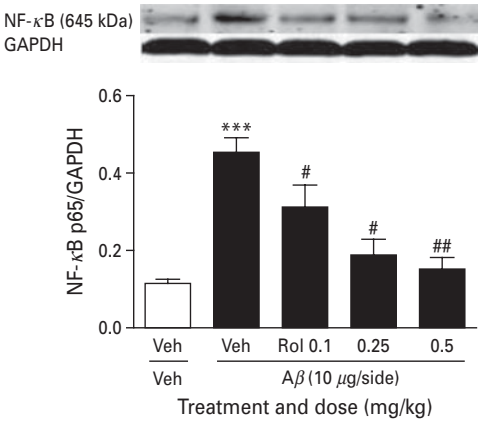

Fig. 5. Effect of rolipram on Aβ25-35-induced activation of NF-κB p65 in the hippocampus in rats. Upper panel is representative immunoblot of NF-κB p65 detected by Western blotting; lower panel is quantification of NF-κB p65 expressed as the ratio of NF-κB p65/GAPDH. Aβ25-35 increased NF-κB p65 in the hippocampus, this was reversed by rolipram (0.1, 0.25, or 0.5 mg/kg) in a dose-dependent manner. Administration of Aβ25-35 and rolipram was the same as that in the legend of Fig. 4. Values shown are means ± S.E.M. ($n = 3$ per group); *** $p < 0.001$ *vs.* vehicle controls (Veh + Veh); # $p < 0.05$, ## $p < 0.01$ *vs.* Aβ25-35 + Veh.

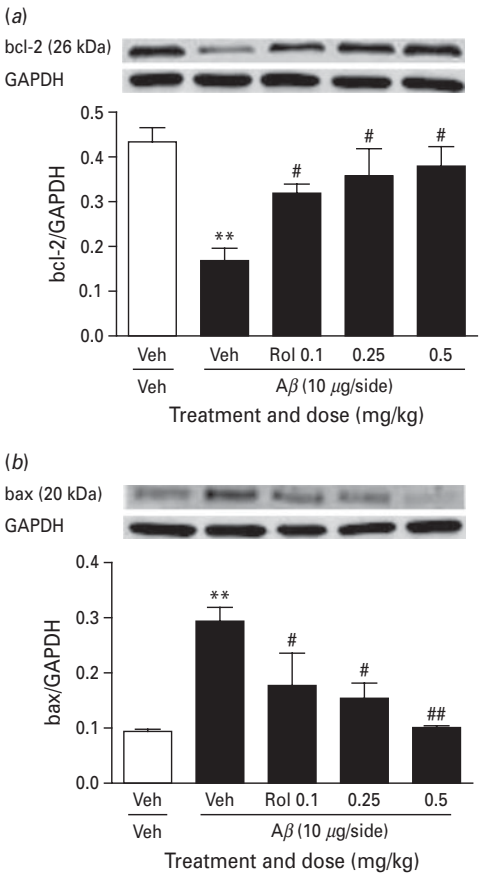

Fig. 6. Effect of rolipram on Aβ25-35-induced changes in Bcl-2 and Bax in the hippocampus in rats. (*a*) Rolipram reversed Aβ25-35-induced decreases in expression of the anti-apoptotic mediator Bcl-2. (*b*) Rolipram reversed Aβ25-35-induced increases in expression of the apoptotic mediator Bax. Upper panels are representative immunoblots of (*a*) Bcl-2 and (*b*) Bax in the hippocampus measured by Western blotting using respective antibodies; lower panels are respective quantifications. Administration of Aβ25-35 and rolipram was the same as that in the legend of Fig. 4. Values shown are means ± S.E.M. ($n = 3$ per group); ** $p < 0.01$ *vs.* vehicle controls (Veh + Veh); # $p < 0.05$, ## $p < 0.01$ *vs.* Aβ25-35 + Veh.

increase in NF-κB p65 levels in rats treated with Aβ25-35 + vehicle, compared to those treated with vehicle ($p < 0.001$); the effect of Aβ25-35 was reversed by rolipram (0.1–0.5 mg/kg) in a dose-dependent manner ($p < 0.05$ for 0.1 and 0.25 mg/kg and $p < 0.01$ for 0.5 mg/kg).

Reversal by rolipram of Aβ25-35-induced apoptotic effects

Since Aβ25-35 decreases neuronal cell viability via increased apoptotic activity (Yao *et al.* 2007), we examined expression of Bax, a pro-apoptotic protein that induces rapid human neuronal cell death (Bounhar *et al.* 2001), and Bcl-2, another cell death-associated protein (Korsmeyer, 1992), to determine whether apoptotic responses were involved in the effect of rolipram on Aβ25-35-induced toxicity. As shown in Fig. 6, both Bcl-2 and Bax were changed by drug treatment [$F(4, 10) = 6.76$, $p = 0.007$ for Bcl-2 and $F(4,10) = 6.53$, $p = 0.008$ for Bax]. *Post-hoc* Newman–Keuls analyses indicated that Aβ25-35 decreased Bcl-2 ($p < 0.01$; Fig. 6*a*) and increased Bax ($p < 0.01$; Fig. 6*b*) in the hippocampus, compared to the vehicle controls; these effects were reversed by rolipram in a dose-dependent manner ($p < 0.05$ for all except for 0.5 mg/kg in Bax, where $p < 0.01$; Fig. 6).

Rolipram's reversal of Aβ1-42-induced changes in pCREB, NF-κB p65, Bcl-2, and Bax in the hippocampus

To verify the effects of rolipram on Aβ25-35-induced changes in neuroinflammatory and apoptotic responses in the hippocampus, we replicated the neurochemical experiments by using Aβ1-42, instead of Aβ25-35. In the measurement of pCREB, microinfusions of Aβ1-42 at 0.4 μg/side into bilateral CA1 subregions decreased pCREB levels in the hippocampus ($p < 0.01$),

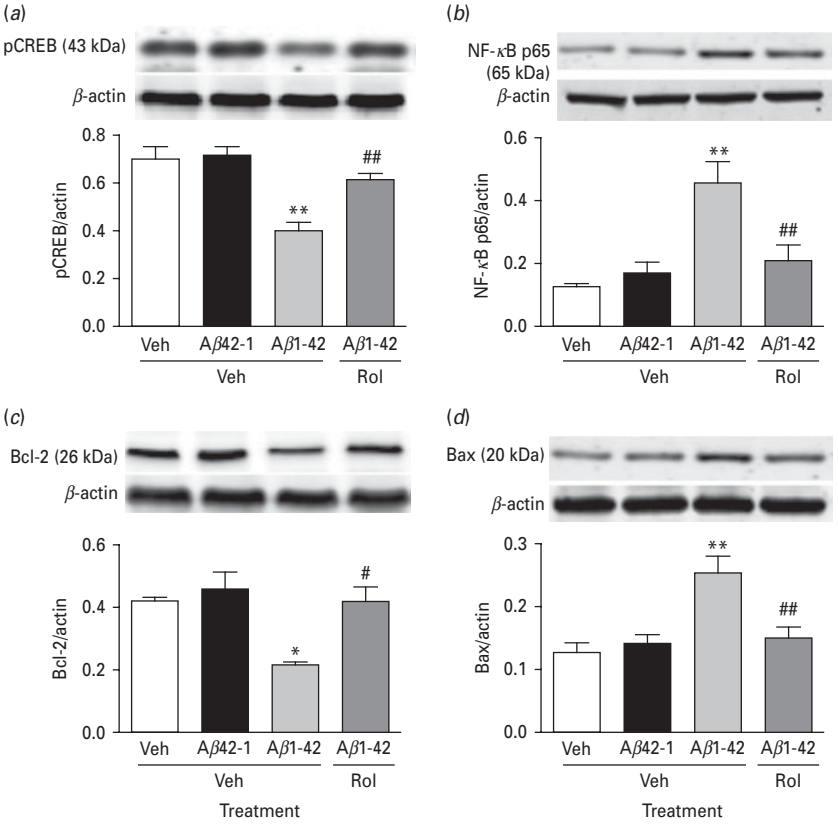

Fig. 7. Effects of rolipram on Aβ1-42-induced changes in pCREB, NF-κB p65, Bcl-2 and Bax in the hippocampus in rats treated with Aβ1-42. (*a*) Rolipram reversed Aβ1-42-induced decreases in pCREB. (*b*) Rolipram reversed Aβ1-42-induced decreases in NF-κB p65. (*c*) Rolipram reversed Aβ1-42-induced decreases in expression of Bcl-2. (*d*) Rolipram reversed Aβ1-42-induced increases in expression of Bax. For (*a–d*), upper panels are representative immunoblots of pCREB, NF-κB p65, Bcl-2, or Bax and β-actin (inner control) measured by Western blotting using respective antibodies; lower panels are quantification of corresponding proteins expressed as the ratio of pCREB, NF-κB p65, Bcl-2, or Bax divided by β-actin. Starting 24 h after CA1 microinfusions of Aβ1-42 (0.4 μg/side), Aβ42-1 (0.4 μg/side), or vehicle (1 μl/side), rolipram (0.5 mg/kg) was given (i.p.) once a day for 14 d before sacrifice, which was performed 1 h after the last rolipram injection. Values shown are means ± S.E.M. ($n=3$ per group); * $p<0.05$, ** $p<0.01$ *vs.* vehicle controls (Veh + Veh); # $p<0.05$, ## $p<0.01$ *vs.* Aβ1-42 + Veh.

while the reverse peptide Aβ42-1 at the same dose did not, relative to vehicle controls (Fig. 7*a*); this effect of Aβ1-42 was reversed by rolipram (0.5 mg/kg, once per day for 14 d; $p<0.01$). The inter-group comparison was significant as revealed by one-way ANOVA [$F(3,8)=14.34$, $p<0.01$]. In contrast, CREB levels were not changed between groups (data not shown). Inter-group changes similar to those in pCREB were also observed in measurements of NF-κB p65 [$F(3,8)=10.22$, $p=0.004$], Bcl-2 [$F(3,8)=8.73$, $p=0.007$], and Bax [$F(3,8)=8.930$, $p=0.006$]. More specifically, compared to vehicle controls, CA1 microinfusions of Aβ1-42 (0.4 μg/side) increased NF-κB p65 ($p<0.01$, Fig. 7*b*) and Bax ($p<0.01$, Fig. 7*d*) and decreased Bcl-2 ($p<0.05$, Fig. 7*c*) in the hippocampus; these were all reversed by rolipram [$p<0.01$, except for Bcl-2 ($p<0.05$); Fig. 7*b–d*]. In contrast, Aβ42-1 did not alter any of the measurements, which excluded the potential non-specific effects of peptide.

Discussion

Aβ peptides have been shown to activate the apoptotic pathway (Koriyama *et al.* 2003; Mattson, 2004; Yao *et al.* 2007), produce inflammatory responses (Abdi *et al.* 2010; Di *et al.* 2010), inhibit hippocampal synaptic plasticity (Vitolo *et al.* 2002; Zeng *et al.* 2010; Zhang *et al.* 2006), and impair memory (Cheng *et al.* 2010; Malm *et al.* 2006; Vollmar *et al.* 2010; Wang *et al.* 2010). As the functional domain of full-length Aβ peptides,

Aβ25-35 is toxic to neurons and contributes to Aβ-induced properties of neurotoxicity, oxidation, inflammation, and apoptosis (Ahn *et al.* 2006; Um *et al.* 2006). Here we demonstrate that a single microinfusion of aged Aβ25-35 into bilateral CA1 subregions in the hippocampus produced deficits of learning and memory accompanied by inflammatory and apoptotic responses in the hippocampus; it also concurrently decreased pCREB levels in this brain region. These effects of Aβ25-35 were reversed by repeated treatment with rolipram, which increased pCREB in the hippocampus. Similar effects were also observed when Aβ25-35 was replaced with Aβ1-42, but not the inactive peptide Aβ42-1. These results suggest that inhibition of PDE4 reverses Aβ-induced cognitive deficits, which are contributed, at least partially, by neuronal inflammation and apoptosis mediated by cAMP/CREB signalling.

Both Morris water-maze and passive avoidance tests appear to be sensitive to Aβ treatment (Cheng *et al.* 2010; Mouri *et al.* 2006). Microinfusions of Aβ25-35 into bilateral CA1 subregions of the hippocampus impaired learning ability and long-term memory, as demonstrated by decreased 24-h retention in the passive avoidance test, and prolonged escape latency in the acquisition trials, and decreased exploration in the target quadrant in the probe trial performed 24 h after the last training trial in the water-maze test. These results are supported by findings from previous studies, in which Aβ25-35 produces memory deficits in a variety of animal tasks (Cheng *et al.* 2006, 2010; Holscher *et al.* 2007; Tohda *et al.* 2004). Consistent with these, microinfusions of Aβ1-42 into the hippocampus or lateral ventricle in the brain impairs memory (Christensen *et al.* 2008; Malm *et al.* 2006; Yamada *et al.* 1999), suggesting that central administration of Aβ is a reliable model of AD for testing cognition, including learning and memory.

It has been established that rolipram alone enhances memory via activation of cAMP/CREB signalling (Barad *et al.* 1998; Li *et al.* 2011). Consistent with the memory-enhancing effect of rolipram, Aβ25-35-induced deficits of learning and memory were reversed by repeated treatment with rolipram. This is supported by findings in the study using APP/PS1 transgenic mice (Gong *et al.* 2004) and in our previous study, in which rolipram administered at 0.5 mg/kg for 3 wk reverses Aβ25-35-induced memory deficits using the same memory paradigms (Cheng *et al.* 2010). We lowered the dose of rolipram to 0.1 mg/kg and still observed a potent memory-enhancing effect in the water-maze test, but not passive avoidance test; the latter appears to require at least 0.5 mg/kg rolipram to block Aβ25-35-induced memory deficit, suggesting that the water-maze may be a more sensitive test for examining the interaction between Aβ and PDE4 inhibitors. A similar response difference has been observed in rats with hypoxic damage to CA1, which impairs memory performance in the water-maze, but not the passive avoidance test (Boissard *et al.* 1992).

It was noted that subchronic treatment with rolipram at the same dose slows down memory extinction in the fear-conditioning test (Monti *et al.* 2006). Since animals did not receive footshocks 3 h after initial training in the passive avoidance test, extinction learning might have existed in the 24-h retention test. Thus, the effect of rolipram on extinction learning may contribute to its memory-enhancing action examined 24 h after training in the passive avoidance test.

The effect of rolipram appears to be independent of sedation potentially produced by PDE4 inhibitors (Griebel *et al.* 1991; Smith, 1990), given that rolipram at the doses used did not alter swimming speed in the water-maze probe trial test or latency to cross to the dark compartment during habituation in the passive avoidance task. The lack of the sedative property of rolipram may be attributed to the strategy of rolipram treatment, which, when given repeatedly and 1 h prior to behavioural tests, is able to dissociate therapeutic effects from side-effects such as sedation (Li *et al.* 2009, 2011).

Since the half-life of rolipram is short (1–3 h; Krause & Kuhne, 1988) and rolipram blockade of scopolamine-induced memory impairment lasts for only about 1 h (Zhang & O'Donnell, 2000), the reversal effect of rolipram on Aβ25-35-induced memory deficits appears to result from chronic, rather than acute, actions of rolipram. This is supported by the findings that long-term memory-enhancing effects of rolipram are probably attributed to long-lasting neuronal changes caused by chronic rolipram treatment, including increased phosphorylation of CREB and neurogenesis in the hippocampus (Li *et al.* 2011; Rutten *et al.* 2008). These adaptive changes, in particular in pCREB, may be one of the mechanisms whereby rolipram reverses Aβ-induced memory impairment.

It has been well established that rolipram increases activation of brain cAMP/CREB signalling (Barad *et al.* 1998; Li *et al.* 2011; Monti *et al.* 2006; Nakagawa *et al.* 2002; Zhang *et al.* 2002, 2004), which is impaired in AD (Satoh *et al.* 2009). However, it is not clear whether cAMP/CREB signalling is important in the

reversal effect of rolipram on memory deficits associated with AD. In the present study, we demonstrated that chronic treatment with rolipram, at the same doses blocking spatial memory impairment in the water-maze test, reversed Aβ25-35-induced decreases in hippocampal pCREB. In addition, the effect of rolipram on pCREB was highly correlated with that on memory performance. The reversal effect of rolipram on pCREB in the hippocampus was verified by using Aβ1-42, which is supported by findings that rolipram blocks Aβ1-42-induced inhibition of pCREB in hippocampal neurons (Vitolo *et al.* 2002). These results are consistent with our previous finding that pCREB is involved in rolipram reversal of Aβ1-40-induced memory deficits (Cheng *et al.* 2010), suggesting an important role of cAMP/CREB signalling in the mediation of the memory-enhancing effect of rolipram in the animal model of AD.

It was noted that under certain conditions, CREB phosphorylation can be induced rapidly (within 5–10 min) and transiently (lasting 1–2 h or even shorter; Cammarota *et al.* 2001; Sala *et al.* 2000; Shaywitz & Greenberg, 1999). This raised the concern about whether the reversal effect of rolipram was due to repeated treatment or final (acute) treatment and the question about the significance of changes in pCREB measured 1 h after rolipram treatment. However, it is important to note that, while rolipram at high doses such as 3 mg/kg administered acutely increases cAMP and pCREB in the hippocampus (Giorgi *et al.* 2004; Monti *et al.* 2006), acute treatment with rolipram at the same dose (1.25 mg/kg) as used in the present study does not alter hippocampal pCREB (Nakagawa *et al.* 2002). Instead, chronic treatment with rolipram is usually required to produce behavioural and neurochemical alterations, including those in pCREB and memory (Li *et al.* 2009, 2011; Monti *et al.* 2006; Nakagawa *et al.* 2002; Zhang *et al.* 2002). In addition, rolipram is a highly selective, prototypical inhibitor of PDE4 (Zhang *et al.* 2005). Repeated treatment with rolipram increases cAMP in the hippocampus examined 1 h after the last rolipram injection (Li *et al.* 2009). Cyclic AMP-activated PKA could induce persistent phosphorylation of CREB (Sala *et al.* 2000). Therefore, the reversal effect of rolipram on pCREB may be attributed to repeated, rather than acute treatment with rolipram. Rolipram reverses the effects of Aβ most likely via cAMP/CREB signalling given that microinfusions of Aβ25-35 or Aβ1-42 in CA1 decreased pCREB.

Studies have demonstrated a coincidence of inflammatory damage and apoptotic neuronal death during the development or progression of neurodegenerative disorders, including AD (Hardy & Selkoe, 2002; Rojo *et al.* 2008). Increases in cAMP levels inhibit pro-inflammatory (Dastidar *et al.* 2009; Hoyle, 2010) and apoptotic responses (Kim *et al.* 2010; Naderi *et al.* 2009). PDE4 inhibitors such as rolipram increase intracellular cAMP levels and reduce inflammatory cytokines such as TNF-α (Taguchi *et al.* 1999) and its downstream target NF-κB (Sanchez *et al.* 2005), which is induced by Aβ (Gong *et al.* 2011). Our results provide an integration of these findings, i.e. rolipram reversed not only Aβ25-35- or Aβ1-42-induced deficits of pCREB, but also the Aβ-induced inflammatory response, as indicated by increased NF-κB p65 in the hippocampus. This is consistent with the negative regulation of NF-κB signalling by cAMP (Parry & Mackman, 1997). Therefore, rolipram inhibits inflammatory responses induced by Aβ25-35 or Aβ1-42 most likely via attenuating activation of NF-κB p65 mediated by cAMP/CREB signalling. In addition, the anti-inflammatory effect of rolipram paralleled the improvement of Aβ25-35-induced memory deficits, suggesting that neuroinflammation may contribute to memory impairment produced by Aβ.

Since NF-κB signalling enhances apoptosis (Tusi *et al.* 2010) and inhibition of NF-κB not only protracts inflammatory responses but also prevents apoptosis (Lawrence *et al.* 2001), it was of interest to know whether rolipram altered Aβ-induced apoptotic responses. A variety of molecules have been shown to be involved in the cellular apoptosis machinery; these include p53 (Haupt *et al.* 1997; Ryan *et al.* 2001), the Bcl-2 family (Dimmeler *et al.* 1999; Li & Dou, 2000), XIAP (Yang *et al.* 2000), caspase members (Suzuki *et al.* 2001), and p38 MAP kinase (Wang *et al.* 2005). Of these, the Bcl-2 family members are the most important molecules in regulating apoptosis. They can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xl, and Bcl-w, and pro-apoptotic members, such as Bax and Bak (Borner, 2003). It has been considered that the ratio of Bcl-2/Bax is crucial in the regulation of apoptosis (Borner, 2003; Korsmeyer *et al.* 1995). Microinfusions of either Aβ25-35 or Aβ1-42 decreased Bcl-2 and increased Bax, leading to reduction of the Bcl-2/Bax ratio and increases in apoptotic responses in the hippocampus. Rolipram, at the same doses that blocked Aβ-induced deficits of memory (for Aβ25-35) and cAMP/CREB signalling and activation of NF-κB signalling, also reversed the Aβ-induced decrease in the Bcl-2/Bax ratio (i.e. decreases in Bcl-2 and increases in Bax). The results are in agreement with those indicating that activation of CREB up-regulates Bcl-2 (Karsan *et al.* 1997) and that cAMP-dependent protein kinases regulate apoptosis by phosphorylation

of Bcl-2 and reduction of Bcl-2/Bax dimerization (Srivastava *et al.* 1998). Therefore, rolipram may exhibit a potent, anti-apoptotic effect, which contributes to the blockade of apoptotic responses induced by Aβ25-35 or Aβ1-42. In addition, the anti-inflammatory property of rolipram also may play a role in this process given the close link between inflammation and apoptosis (Bradl & Hohlfeld, 2003; Dorr *et al.* 2005) and the involvement of NF-κB signalling in apoptosis (Lawrence *et al.* 2001; Tusi *et al.* 2010).

PDE4 is encoded by four separate genes (PDE4A-D); each of which generate multiple splice variants (Conti *et al.* 2003; Houslay & Adams, 2003; O'Donnell & Zhang, 2004; Zhang, 2009). While it is not known which subtype is involved in the beneficial effects of rolipram on Aβ-induced behavioural and biochemical deficits, PDE4B might be the subtype of interest given that it is important in lipopolysaccharide-induced cellular signalling and inflammatory responses (Ariga *et al.* 2004; Jin & Conti, 2002). However, given that both PDE4A and PDE4D are highly expressed in the hippocampus (Perez-Torres *et al.* 2000), the role of these two subtypes, in particular PDE4D, which is important in the mediation of memory (Burgin *et al.* 2010; Li *et al.* 2011) and antidepressant activity (Zhang *et al.* 2002), cannot be excluded. Studies using relatively selective inhibitors of individual PDE4 subtypes (Donnell *et al.* 2010; Naganuma *et al.* 2009) and/or mice deficient in a specific PDE4 subtype are required to clarify this issue.

In summary, in the present study, we provide a promising demonstration for the reversal effect of rolipram on memory deficits associated with AD. We further extend this finding to an interesting point, i.e. rolipram reversal of Aβ-induced memory deficits may be at least partially attributed to blockade of inflammatory responses and apoptosis in the hippocampus, which are mediated by cAMP/CREB signalling. Accordingly, PDE4 may be a potential target for treatment of AD. PDE4 inhibitors produce anti-inflammatory and anti-apoptotic properties, leading to beneficial effects for treatment of memory loss in AD.

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Statement of Interest

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