The β-amyloid protein of Alzheimer’s disease increases neuronal CRMP-2 phosphorylation by a Rho-GTP mechanism

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Neuritic abnormalities are a major hallmark of Alzheimer’s disease (AD) pathology. Accumulation of β-amyloid protein (Aβ) in the brain causes changes in neuritic processes in individuals with this disease. In this study, we show that Aβ decreases neurite outgrowth from SH-SY5Y human neuroblastoma cells. To explore molecular pathways by which Aβ alters neurite outgrowth, we examined the activation and localization of RhoA and Rac1 which regulate the level and phosphorylation of the collapsin response mediator protein-2 (CRMP-2). Aβ increased the levels of the GTP-bound (active) form of RhoA in SH-SY5Y cells. This increase in GTP-RhoA correlated with an increase in an alternatively spliced form of CRMP-2 (CRMP-2A) and its threonine phosphorylated form. Both a constitutively active form of Rac1 (CA-Rac1) and the Rho kinase inhibitor, Y27632, decreased levels of the CRMP-2A variant and decreased threonine phosphorylation caused by Aβ stimulation. The amount of tubulin bound to CRMP-2 was decreased in the presence of Aβ but Y27632 increased the levels of tubulin bound to CRMP-2. Increased levels of both RhoA and CRMP-2 were found in neurons surrounding amyloid plaques in the cerebral cortex of the APP(Swe) Tg2576 mice. We found that there was an increase in threonine phosphorylation of CRMP-2 in Tg2576 mice and the increase correlated with a decrease in the ability of CRMP-2 to bind tubulin. The results suggest that Aβ-induced neurite outgrowth inhibition may be initiated through a mechanism in which Aβ causes an increase in Rho GTPase activity which, in turn, phosphorylates CRMP-2 to interfere with tubulin assembly in neurites.

Keywords: RhoA; Rac1; collapsing response mediator protein-2; amyloid β peptide; neurite dystrophy of Alzheimer’s disease

Abbreviations: APP = amyloid precursor protein; MCI = mild cognitive impairment; PAK = p21-activated kinase; HRP = horse-radish peroxidase; AFM = atomic force microscopy


Introduction

The pathogenesis of Alzheimer’s disease (AD) is associated with increased levels of aggregated β-amyloid protein (Aβ) in the brain. Aβ is produced by processing of the β-amyloid precursor protein (APP) through β- and γ-secretase cleavage. This cleavage generates the 40- or 42-amino acid residue forms, Aβ1-40 and Aβ1-42, respectively (De Strooper et al., 1998; Sinha et al., 1999; Vassar et al., 1999), which by virtue of their propensity to oligomerize, can form amyloid fibrils, protofibrils and oligomeric species (Walsh et al., 1999). The generation of Aβ and the deposition of amyloid is associated with neuronal dysfunction and loss of functional synapses caused by changes to neurite morphology (Tsai et al., 2004; Spires et al., 2005). The neuritic changes in neurons of the frontal and temporal cortices may initially lead to a mild cognitive impairment (MCI), that is followed by more severe memory loss as the disease progresses (Naslund et al., 2000). There is strong evidence for the involvement of extracellular Aβ in the disruption of
the integrated neuronal circuitry (Masliah et al., 1991; Terry et al., 1991; Masliah et al., 1992; Samuel et al., 1994; Walsh et al., 2002, 2005; Cleary et al., 2005; Klyubin et al., 2005).

It is now well-documented that the Rho-GTPases are involved with the regulation of neurite outgrowth (Mackay et al., 1995; van Leeuwen et al., 1999; Bito et al., 2000; Dickson, 2001; Skaper et al., 2001; Buck and Zheng, 2002; Ellezam et al., 2002; Yuan et al., 2003; Zhang et al., 2003; van Galen and Ramakers, 2005; Woo and Gomez, 2006). As an intracellular effector of neurite retraction, RhoA-GTP (active RhoA) has been well defined in neurological disease paradigms (Lee et al., 2003; Li and Strittmatter, 2003; McGee and Strittmatter, 2003; Lee et al., 2004; Li et al., 2004; Karnezis et al., 2004; Liebscher et al., 2005; Satoh et al., 2005; Wang et al., 2006; Marklund et al., 2006). The common signalling mechanism for inhibition of neurite outgrowth in these paradigms is activation of endogenously bound RhoA via the receptor cluster Nogo Receptor (NgR), the low-affinity neurotrophin receptor, p75NTR, LINGO-1 and TROY (Mi et al., 2006; Logan et al., 2004; Park et al., 2005; Shao et al., 2006). Very recently, it was shown that both APP and Aβ may structurally interact with the NgR to inhibit amyloid formation in vivo (Park et al., 2006a, b). Whether Aβ can initiate RhoA activation through NgR1 is unclear.

For RhoA to be active there is a requirement for Rac1 inactivation (Niederost et al., 2002). Rac1 implements its activity by binding the serine threonine kinase, p21-activated kinase (PAK), which upon autophosphorylation can prevent actin–myosin contraction and can allow for actin polymerization through a seven-protein complex (Rohatgi et al., 2000). In AD, PAK activation may be defective, and it has been suggested that oligomeric Aβ may be responsible for this inactivation (Zhao et al., 2006). The direct mechanism inducing these effects remains to be elucidated.

Recently, the collapsin response mediator proteins (CRMPs), particularly CRMP-2, have been directly attributed to the formation, outgrowth and guidance of neurites (Inagaki et al., 2001; Nishimura et al., 2003; Quinn et al., 2003; Deo et al., 2004; Arimura et al., 2005; Yoshimura et al., 2005). The CRMP proteins belong to a family of dihydropyrimidinase-related neuronal phosphoproteins (McIntire et al., 1992; Goshima et al., 1995), which also include the Unc-33-like phosphoprotein homologue of Caenorhabditis elegans (McIntire et al., 1992). The CRMP family consists of five isoforms, CRMP1-4 and CRAM (CRMP-5) (Hamajima et al., 1996; Wang and Strittmatter, 1996). CRMP-2 has been shown to be phosphorylated by cyclin-dependent kinase 5 (cdk5) (Brown et al., 2004; Horiuchi et al., 2006), glycolgen synthase kinase 3β (GSK3β) (Cole et al., 2004; Yoshimura et al., 2005; Cole et al., 2006) and by Rho kinase II (ROCKII) (Arimura et al., 2000, 2005), all of which can mediate neurite retraction (Yoshimura et al., 2006; Arimura and Kaibuchi, 2007). Phosphorylation of CRMP-2 disrupts the association of mature full-length CRMP-2 with tubulin heterodimers so that tubulin cannot be transported to the plus ends of microtubules for assembly (Fukata et al., 2002).

The aim of the present study was to examine the effects of Aβ on neurite outgrowth and to determine the activation of downstream signalling mechanisms which mediate these effects. We show that in human neuroblastoma SH-SY5Y cells, Aβ can reduce the length of neurites by inactivating the neurite outgrowth signalling molecule Rac1, and that this Aβ-mediated reduction in neurite length can be abrogated by the Rho Kinase inhibitor, Y27632. Furthermore, we show that the Aβ-mediated decrease in neurite length involves the induction of a threonine phosphorylation of CRMP-2A, conferring a reduced binding capacity to tubulin, both of which can be reversed by inhibiting RhoA activity. Importantly, we report evidence that this mechanism also operates in the Tg2576 mouse model of AD, where RhoA and CRMP-2 are increased in the immediate vicinity of amyloid plaques and CRMP-2-bound tubulin is reduced. The data suggest that Aβ-mediated neurite outgrowth inhibition is initiated through the activity of RhoA-GTP and through the dysregulation of CRMP-2 to bind tubulin for neurite outgrowth.

Materials and methods
Materials
The following primary antibodies were used: mouse monoclonal IgG anti-RhoA (Millipore-Upstate, Lake Placid, NY), mouse monoclonal anti-Rac1 (Millipore-Chemicon, Temecula, CA), rabbit polyclonal anti-CRMP-2 affinity purified (Millipore-Chemicon), mouse monoclonal anti-CRMP-2 affinity purified clone (Immuno-Biological Laboratories Co. Ltd, Gunma, Japan), sheep polyclonal anti-phosphorylated CRMP-2 (Biocompare, South San Francisco, CA), rabbit polyclonal anti-pan phosphothreonine (Millipore-Chemicon), mouse monoclonal anti-β-actin (Sigma-Aldrich, St Louis, MO), rabbit polyclonal anti-phosphothreonine (Millipore-Chemicon), mouse monoclonal anti-T7 epitope (MASMTGGQQMG) (Biocompare, South San Francisco, CA), anti-GSK3β (Cell Signaling Technology Inc., Boston, MA). The following secondary antibodies were used: goat anti-rat horseradish peroxidase (HRP) conjugated IgG (Millipore-Chemicon), sheep anti-mouse HRP conjugated IgG (Amersham Biosciences, Piscataway, NJ), sheep anti-rabbit HRP conjugated IgG (Amersham Biosciences), goat anti-sheep HRP conjugated IgG (Amersham Biosciences), goat Alexa-Fluor 488 anti-rabbit IgG, goat Alexa-Fluor 546 anti-rabbit IgG and goat Alexa-Fluor 594 anti-mouse IgG (Invitrogen-Molecular Probes, Eugene, OR). Aβ40, Aβ42, scrambled sequence Aβ42 and scrambled sequence Aβ40 (rPeptides Inc., Athens, GA), retinoic acid (Sigma-Aldrich, St Louis, MO), Thioflavin T (Sigma-Aldrich), Protein A Sepharose beads (Chemicon-Millipore), Protein G Sepharose 4A beads (Pharmacia Biotech-Amersham, Brain (2008), 131, 90–108 91
Aggregation of Aβ by ageing and atomic force microscopy (AFM)

Aβ peptides were aggregated (‘aged’) by incubating them at a concentration of 1 μM in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen Corp, Carlsbad, CA) supplemented with 10% heat inactivated fetal calf serum (FCS) (Commonwealth Serum Laboratories, Victoria, Australia) at 37°C for 7 days. AFM was used to measure the extent of Aβ aggregation (Hou et al., 2007). Aβ solutions (40 μl, 0.01–0.05 μM) were deposited on a freshly cleaved surface of highly oriented pyrolytic graphite (HOPG) and incubated at 37°C for 10 min. After five washes with deionized distilled water, the HOPG surface was then dried under a constant flow of N2 gas for 30 min. These compounds were incubated with the cells for 24 h, after which the cells were lysed. These compounds were incubated with the cells for 24 h, after which the cells were lysed.

Culture of SH-SY5Y cells

Human SH-SY5Y neuroblastoma cells were seeded at a density of 100 000 cells per well in 24-well plates (BD Biosciences, Franklin lakes, NJ) in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen Corp, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Commonwealth Serum Laboratories, Victoria, Australia), 1% v/v penicillin/streptomycin (Sigma-Aldrich), and 1% v/v penicillin/streptomycin (Sigma-Aldrich). The cells were differentiated with 10 μM retinoic acid (RA) (Sigma-Aldrich), over a 7-day period. On day 8, the medium was replaced with medium containing 1.0 μM Aβ40 (freshly prepared on the day), 1.0 μM scrambled sequence of Aβ40 or 10 μM Y27632 (EMD Biosciences-Calbiochem). These compounds were incubated with the cells for 24 h, after which, the cells were lysed.

Transfection of SH-SY5Y cells

A constitutively active (CA)-Rac1 cDNA construct inserted in a pCGT vector was provided by Associate Professor Tony Tiganis (Department of Biochemistry and Molecular Biology, Monash University). The plasmid vector contained a V12 mutation with a T7 epitope tag (Marcusohn et al., 1995). SH-SY5Y human neuroblastoma cells (2 x 10⁶) were transiently transfected with 2 μg of the CA-Rac1 pCGT construct or enhanced green fluorescent protein (eGFP)-pCGT (GFP-control) using the cell-type-specific Nucleofector kit V (Amaxa Biosystems, Koeln, Germany) and the A-23 program for SH-SY5Y neuroblastoma cells on the Nucleofector device. Cells were then cultured in DMEM/F12 medium for 2 days prior to Aβ-treatment.

Measurement of neurite outgrowth

Human SH-SY5Y neuroblastoma cells were seeded at a density of 10 000 cells per well in 8-well chamber slides (Nunc) and differentiated by incubating for 7 days with 10 μM RA. On the eighth day, the cells were incubated for 24 h in either DMEM/F12 medium alone (control), or medium containing 1.0 μM Aβ40 or Aβ42, 1.0 μM scrambled sequence Aβ40 peptide and/or 10 μM Y27632. Aβ peptides were either freshly prepared on the day (fresh Aβ), or incubated over a 7-day period in medium at 37°C (aged Aβ). Each incubation was performed in triplicate. After incubation, the medium was removed, the cells washed gently with PBS (pH 7.4, containing 1 mM Ca²⁺ and Mg²⁺) and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then washed thoroughly and stained with cresyl violet. Neurite length was then measured under ×20 objective lens on a Leica DMIL inverted light microscope. Images were captured with a Leica DFC320 Twain v6.2.0 colour digital camera and stored as a TIFF file. To calculate neurite length, the longest neurite of each cell was measured using the Leica IM50 v4.0 software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Neurites were only measured if they were >20 μm in length. The longest 10 neurites were measured per field and 10 fields per well were measured (n = 3 wells per experiment). These counts were averaged and the standard errors calculated using Graph Pad Prism v3.02. Mean values were obtained by averaging values from the measurement of approximately 100 neurites per well in three separate wells.

Preparation of cell and tissue lysates for western blotting

Cells were lysed using a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 50 mM Tris–HCl pH 7.5, 5 mM EGTA, freshly supplemented with protease and phosphatase inhibitors (4 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mM sodium orthovanadate and 20 mM sodium pyrophosphate) (Sigma-Aldrich). Cell lysates were centrifuged at 10 000 g for 15 min at 4°C and the supernatant fractions removed.

Brain homogenates were prepared by sonication in 1:10 (w/v) Tris-buffered saline (TBS) [50 mM Tris–HCl (pH 7.4), 175 mM NaCl, 5 mM EDTA] with protease inhibitor cocktail (Sigma-Aldrich), and 2 μg/ml pepstatin A (Sigma-Aldrich). The homogenates were centrifuged at 10 000 × g for 5 min to remove cellular debris. Homogenates were then solubilized in a 1:1 ratio of RIPA buffer (1% w/v sodium deoxycholate, 0.2% w/v SDS and 2% v/v NP40 in TBS) and kept at −20°C until used for analysis. Protein concentrations in the cell lysates and brain homogenates were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

RhoA-GTP and Rac1-GTP precipitation

Precipitation assays of cell and tissue lysates, for RhoA-GTP and Rac1-GTP, were performed using the commercially available kits (Millipore-Chemicon-Upstate). Briefly, to measure activated RhoA, 20 μl of 0.5 M EDTA was added to 1 ml of each cell or tissue extract and incubated for 30 min with agitation. To this mixture, 10 μl of either 100× GTPγS, or 100× GDP was added and used as positive and negative controls, respectively. The tubes were placed on ice and 60 μl of 1 M MgCl₂ was added.
Each cell and tissue extract (1 ml) was then combined with 30 µl of Rhotekin RBD-agarose slurry and incubated at 4°C with gentle rotation. The agarose beads were then pelleted by brief centrifugation and washed in MgCl₂ wash buffer (repeated ×3). The agarose beads were then resuspended in 40 µl of 2× sample buffer containing 2 µl of dithiothreitol (DTT), heated at 95°C for 5 min, briefly centrifuged and the supernatant fraction loaded on a 12% SDS–polyacrylamide gel for electrophoresis. To measure total RhoA, 5 µg of cell or tissue lysate was run on a 12% SDS–polyacrylamide gel for electrophoresis.

In the case of Rac1-GTP, 10 µl of GTPγS or GDPβS (for positive and negative control, respectively) was added to 1 ml of cell lysate along with 2 µl of 0.5 M EDTA and incubated at 30°C for 15 min. The tubes were then placed on ice and 60 µl of 1 M MgCl₂ was added. To 1 ml of each cell and tissue lysate, was added 15 µl of 50% PAK-1 PBD agarose slurry and slowly rotated at 4°C for 1 h. The beads were then pelleted by centrifugation and washed (×3). To the pelleted beads was added 20 µl of SDS sample buffer with DTT, heated at 95°C, then loaded on a 12% SDS-gel for electrophoresis. To measure total Rac1, 5 µg of cell or tissue lysate was run on a 12% SDS–polyacrylamide gel for electrophoresis.

**Immunoprecipitation**

All immunoprecipitation assays were performed from samples containing 100 µg of total protein in a total volume of 1 ml. To measure the level of total α-tubulin prior to immunoprecipitation of CRMP-2, 5% of the starting amount was run on a 12% SDS-gel. Immunoprecipitation was performed by adding 2 µg of polyclonal anti-CRMP-2 (Chemicon) or monoclonal anti-CRMP-2 (Immuno-Biological Laboratories Co. Ltd) capture antibody to each sample. Rabbit IgG or mouse IgG, were used as negative controls. Samples were incubated overnight at 4°C followed by the addition of 70 µl of a 50% slurry of Protein A-Sepharose beads for incubations with polyclonal anti-CRMP-2 antibody or 70 µl of 50% Protein G Sepharose beads for incubations with monoclonal anti-CRMP-2 antibody (Amersham Biosciences). The beads were pre-blocked with 1% w/v BSA in lysis buffer to limit non-specific binding. The samples were rotated slowly at 4°C for 1 h on a rotating wheel and then centrifuged at 10 000 × g for 5 min. After the removal of the supernatant fraction and following four washes with 500 µl of lysis buffer, the protein was dissociated from the beads by heating the beads at 95°C for 5 min in sample buffer containing DTT and then loaded onto 12% SDS–polyacrylamide gels.

**Western blotting**

For experiments on cell lysates and mouse brain homogenates, 5 µg of protein was loaded and run on a 12% SDS–polyacrylamide gel. After electrophoresis, proteins were then electrophoretically transferred onto PVDF membranes (Millipore). The membranes were then blocked with 5% skim milk powder and the primary antibodies diluted in this blocking buffer (polyclonal anti-CRMP-2, 1:500; monoclonal anti-CRMP-2, 1:1000; anti-RhoA, 1:1000; anti-Rac1, 1:1000; anti-α-tubulin, 1:1000; anti-β-actin, 1:5000; anti-T7 epitope, 1:10 000; anti-phosphorylated CRMP-2, 1:2000; anti-phosphothespine, 1:500; anti-phosphoserine199/202 tau, 1:1000; anti-tau5, 1:1000) were incubated overnight at 4°C. After washing the membranes thoroughly in 0.1% v/v TBS-Tween, the secondary anti-rabbit (1:1000), anti-sheep (1:1000), anti-mouse (1:1000) or anti-rat (1:1000) HRP-conjugated antibodies were added and the membranes incubated for 2 h at room temperature. Immunoreactive proteins were detected using ECL chemiluminescence method (Amersham). The level of immunostaining was determined by image analysis after scanning the exposed films using the Alpha Imager (Alpha Innotech, San Leandro, CA) and then processing the 16-bit monochrome images through the ImageQuant TL v2003 software (Nonlinear Dynamics Ltd, All Saints, Newcastle, UK) to measure pixel intensity relative to background. The level of staining was normalized with β-actin or α-tubulin and expressed as arbitrary units.

**Transgenic mouse studies**

Transgenic Tg2576 mice heterozygous for the APP(Swe) transgene (Hsiao et al., 1996) were maintained on a hybrid C57BL/6JST background through breeding hemizygous males with B6SJLF1 females. Genotypes were confirmed by PCR. The mice were maintained on a 12 h light/dark cycle and had free access to food and water. Studies were performed comparing heterozygous transgenic (Tg2576) females with age-matched transgene-negative littermates (wild-type controls). Wild-type and Tg2576 female mice at 6, 12 and 18 months-of-age (n = 3 of each genotype and age) were transcardially perfused with PBS (pH 7.4). One brain hemisphere was dissected to remove the olfactory bulb, cerebellum and brainstem. After dissection the remainder of this hemisphere was used to prepare a total brain homogenate for western blot analysis. The other hemisphere was fixed in 10% neutral buffered formalin and paraffin embedded for immunohistochemical staining.

**Immunohistochemistry**

Fixed, paraffin-embedded wild-type and Tg2576 mouse brains (n = 3 animals per group) were cut into 7 µm serial sections on a conventional microtome and processed for immunohistochemistry as described previously (Petratos et al., 2004). The sections were dewaxed, washed thoroughly in phosphate-buffered saline (PBS) and microwaved in 0.1 M citrate buffer (pH 6.0) for 5 min (×2), then washed with PBS again, incubated with proteinase K (20 µg/ml), washed and post-fixed with 4% paraformaldehyde and finally washed thrice with PBS. The sections were then blocked with 10% (v/v) FCS/0.3% (v/v) Triton X-100 in PBS (blocking buffer) for 2 h at room temperature, and then incubated with primary monoclonal anti-RhoA antibody (1:200) or a polyclonal anti-CRMP-2 antibody (1:100) diluted in blocking buffer, and then incubated overnight at 4°C. After thoroughly washing in PBS (3 × 10 min), the sections were then incubated with a goat Alexa-Fluor 488 anti-rabbit IgG or goat Alexa-Fluor 594 anti-mouse IgG antibodies (Molecular Probes) diluted 1:200 in blocking buffer. The sections were then washed thoroughly, then finally incubated with 0.2% (w/v) Thioflavin T in PBS for 30 min to stain for amyloid plaques. After washing the tissue three times with PBS, the sections were then mounted on coverslips using ProLong Gold anti-fade reagent (Invitrogen-Molecular Probes) and images captured under a UPlanApo H₂O 60 × 1.20 objective lens of an Olympus Fluoview 500 confocal microscope. The captured 16-bit images were then converted to TIFF files using the ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland).
the images were finally imported and formatted using Photoshop v7.0 software.

**Statistics**

Data were analysed using Graph Pad Prism v3.02 software. A two-tailed Student’s t test or a one-way analysis of variance (ANOVA) with Tukey’s post hoc test was used to determine statistical significance ($P < 0.05$) at a 95% confidence level.

**Results**

**Effect of Aβ on neurite length**

As Aβ deposition is associated with neuritic abnormalities in the AD brain (Knowles et al., 1999), we investigated the effect of different Aβ peptides on neurite outgrowth. Freshly added or ‘aged’ (aggregated) Aβ40 and Aβ42 (1 μM) were added to RA-differentiated SH-SY5Y cells. Aβ40 caused a significant reduction in neurite length over a 24-h incubation period (Fig. 1). Aβ40 induced an approximate 40% reduction in neurite length when added fresh (not aged) (Fig. 1A and C). When Aβ40 that had been incubated for 7 days at 37°C (aged) was used, a reduction in neurite length of ~25% was observed (Fig. 1B and D). There was no significant reduction in neurite length of SH-SY5Y cells incubated with Aβ40 or Aβ42 peptides containing a scrambled sequence of amino acids (scrambled Aβ40 and Aβ42) (Fig. 1A and B). Fresh Aβ42 (1 μM) also produced a reduction in neurite length of ~25% (Fig. 1A). Surprisingly, after ageing Aβ42, there was a decrease in the level of neurite outgrowth inhibition with only a 10–15% reduction in neurite length (Fig. 1B).

We speculated that because the Aβ peptides were incubated in the medium for 24 h before the neurite outgrowth effects were observed, it was likely that the peptides had already aggregated to some extent and further ‘ageing’ of Aβ42 may have produced high molecular

![Fig. 1](image.png)
weight aggregates that were no longer toxic. To examine this possibility, we studied the state of aggregation of the Aβ samples by AFM. Peptides (1 μM) were incubated in culture medium containing 10% FCS at 37°C for 7 days to age the samples and to mimic the cell culture conditions. Aβ was also added to culture medium immediately prior to AFM to give a sample of freshly prepared Aβ. Analysis of the freshly prepared and aged samples by AFM showed that the fresh Aβ40 preparation consisted predominantly of spherical structures of ~10–30 nm in diameter consistent with the size of low molecular weight species as previously described (Fig. 2, arrow) (Losic et al., 2006). After ageing the Aβ40 over 7 d, long fibrillar structures were observed which were ~100–150 nm in length and 20 nm in width (Fig. 2, arrow head). However, occasional, small, ~20 nm, globular forms were also seen (Fig. 2, arrow). The fresh Aβ42 preparation formed interwoven long fibrillar structures 20 nm in width (Fig. 2, arrow head). The width of the fibrils, are similar to fibrils of Aβ measured by AFM in previous studies (Losic et al., 2006).

Fig. 2 AFM analysis of fresh and aged Aβ40 and Aβ42 peptides (1 μM). Small molecular weight species of ~20 nm in size were seen for the fresh Aβ40 sample (arrow, top left panel) compared with the fresh Aβ42 sample which was rich in fibrils (arrow, bottom left panel). The aged Aβ42 sample showed thicker interweaving fibrils (arrow head, bottom middle panel). However, aged Aβ40 had short fibrillar structures (arrowhead) but also included numerous spherical structures (arrow, top middle panel). Both the aged scrambled Aβ40 and Aβ42 peptides produced amorphous structures (top right and bottom right panels, respectively).

After ageing the Aβ42 sample over 7 days there was the appearance of similar interwoven, long fibrillar structures with a greater thickness in areas where the fibrillar structures interconnected (Fig. 2, arrow). Therefore, the data were consistent with the view that low molecular weight Aβ (possibly oligomers) may have the greatest effect on neurite outgrowth.

**Effect of Aβ on the Rho-GTPases and the levels of CRMP-2**

To examine the effect of low molecular weight Aβ on Rho-GTPases and their downstream substrate CRMP-2, RhoA-GTP and Rac1-GTP were precipitated from homogenates of SH-SY5Y cells and the amount of each activated protein determined by western blotting. After incubation of SH-SY5Y cells with 0.5 or 1.0 μM Aβ there was a 2-fold reduction in Rac1 activation (Fig. 3B) and this reduction correlated with a 2-fold increase in RhoA-GTP levels (Fig. 3A). The increase in RhoA-GTP and decrease in Rac1-GTP that occurred as the concentration of Aβ increased, coincided with an increase in the levels of the CRMP-2A and CRMP-2B alternatively spliced variants (Fig. 3C). Interestingly there was a marked increase in the CRMP-2A relative to the CRMP-2B variant (Fig. 3C) suggesting an Aβ-dependent mechanism regulating CRMP-2 expression. These data suggest that Aβ can modulate the microfilament-related Rho-GTPases as well as the microtubule-related CRMP-2 variants in a concentration-dependent manner.
Effect of inhibiting the RhoA-mediated pathway

Since Aβ inactivated Rac1 but activated RhoA, we examined the effect of inhibiting the Rho kinase pathway with a Rho kinase inhibitor (Y27632) on CRMP-2 and neurite length. In the presence of Aβ, the Y27632 ROKI/ROCKII inhibitor reduced the ratio of CRMP-2A/CRMP-2B to basal levels (Fig. 4A). Importantly, this decrease in ratio correlated with the maintenance of neurite length of differentiated SH-SY5Y cells in the presence of Y27632 and Aβ (Fig. 4B). These data suggest that Aβ reduces neurite length through a RhoA-dependent mechanism involving the increased expression of the alternative spliced CRMP-2A variant.

To examine the role of Rac1 on Aβ-induced modulation of CRMP-2, SH-SY5Y cells were transiently transfected with a CA-Rac1 V12 mutant construct. Transfection resulted in cells which over-expressed Rac1. CA-Rac1 transfected cells did not express the CRMP-2A variant even in the presence of Aβ (Fig. 4C), however, cells transfected with a control vector expressing eGFP (GFP-vector) showed similar basal levels of CRMP-2A and CRMP-2B as seen in cell lysates from control untreated wells (data not shown). The data suggest that promoting Rac1 activity can overcome the Aβ-dependent increase of CRMP-2A.

Effect of Aβ on the phosphorylation of CRMP-2A

Since the Rho kinase inhibitor, Y27632, and the CA-Rac1 mutant decreased the Aβ-stimulation of CRMP-2A, we investigated whether inhibiting the Aβ-induced RhoA-dependent pathway altered the phosphorylation of CRMP-2.

**Fig. 3** Aβ40 induces a concentration-dependent increase in RhoA-GTP and CRMP-2A correlating with a reduction in Rac1-GTP. The effect of 0.5 and 1 μM, freshly added Aβ40 (incubated for 24 h) increases the level of RhoA-GTP > 4-fold over control levels (A). The same concentrations of freshly prepared Aβ40 reduce Rac1-GTP by >4-fold (B), increase the level of the 75 kDa, CRMP-2A, and reduce the level of 62 kDa CRMP-2B band (C). (A, *P < 0.001 and **P < 0.01; B, *#P < 0.05; C, **#P < 0.01).
was performed using a polyclonal anti-CRMP-2 antibody, which recognizes the GSK3\(\beta\) phosphorylated form of CRMP-2 (Ser-509/Thr-514/Ser-518 residues) (Cole et al., 2006). We found that there was a reduction in the levels of pS509/518/pT514 CRMP-2 in A\(\beta\)-treated SH-SY5Y cells compared with those cells treated with both Y27632 and A\(\beta\) (Fig. 5A). The data suggest that GSK3\(\beta\) does not affect the phosphorylation state of CRMP-2 following A\(\beta\) stimulation. CRMP-2 was again immunoprecipitated, on this occasion using a monoclonal C4G antibody (IBL) and the level of phosphothreonine in the CRMP-2 fraction was determined by western blotting using an anti-phosphothreonine antibody. There was an increased level of phosphothreonine reactivity of the band corresponding to CRMP-2A in the A\(\beta\)-treated SH-SY5Y cells. The level was decreased by Y27632 both in the presence and in the absence of A\(\beta\) (Fig. 5B). The data suggested that GSK3\(\beta\) is not involved in phosphorylating threonine sites on CRMP-2A in the presence of A\(\beta\). Considering that ROCK inhibition reduced CRMP-2 phosphorylation, the data strongly implicated this kinase in CRMP-2A phosphorylation in A\(\beta\)-treated SH-SY5Y cells.

**Effect of A\(\beta\) on the levels of CRMP-2-bound tubulin**

Since one of the primary roles of CRMP-2 is to bind tubulin heterodimers and transport them to the plus-ends of microtubules as a means of promoting neurite extension, the effect of A\(\beta\) on the levels of CRMP-2-bound tubulin was assessed.
(Fukata et al., 2002), we examined the effect of Aβ on the level of tubulin bound to CRMP-2. CRMP-2 was immunoprecipitated using an anti-CRMP-2 polyclonal antibody (Millipore-Chemicon) and then the level of CRMP-2-bound tubulin was determined by western blotting using an anti-tubulin antibody. We found a significant reduction in α-tubulin bound to CRMP-2 after 24 h of Aβ treatment in differentiated SH-SY5Y cells (Fig. 6). We also found that the level of α-tubulin bound to CRMP-2 did not decrease in the presence of Aβ40 scrambled peptide or when the cells were treated with the ROCK inhibitor, Y27632. The levels of CRMP-2-bound tubulin were also maintained when the cells were transiently transfected with the CA-Rac1 construct even when the cells were incubated with Aβ (Fig. 6). These data support the hypothesis that Aβ causes a reduction in neurite length through a RhoA-dependent mechanism by preventing the assembly of microtubules through its direct phosphorylation effects on CRMP-2, reducing its capacity to bind tubulin.

**Fig. 6** Decreased binding of tubulin to CRMP-2 in Aβ40-treated SH-SY5Y human neuroblastoma cells. CRMP-2 was immunoprecipitated with the polyclonal anti-CRMP-2 antibody, the immunoprecipitate was transferred onto a PVDF membrane and then probed for α-tubulin to detect the level of tubulin bound to CRMP-2 (IP upper blot). The lower blot shows the level of total α-tubulin before immunoprecipitation as determined by western blotting. Aβ40-treated SH-SY5Y cells had a 2–3-fold decrease in the percentage of α-tubulin bound to CRMP-2 compared to control and cells treated with scrambled Aβ40 peptide (\( P < 0.01, \# P < 0.05 \)). The Aβ-induced decrease in CRMP-2 binding was blocked by co-treating the cells with the Rho kinase inhibitor Y27632 (\( P < 0.001 \)) or in cells transfected with CA-Rac1 compared with Aβ40-treated SH-SY5Y cells (\( P < 0.001 \)).

**Rac1 and RhoA in Tg2576 mice**

Since Aβ potentiates the RhoA-dependent pathway in SH-SY5Y cells, we investigated whether RhoA pathways were also potentiated in a transgenic mouse model of AD (Tg2576). We found that there was a decrease in the levels of Rac1-GTP initially at 6 months-of-age (Fig. 7A). There was a punctate pattern of RhoA staining localized to the cell bodies of CRMP-2-positive cortical neurons in the Tg2576 mouse brain at 6 months-of-age (Fig. 7B). The decrease in Rac1-GTP in the Tg2576 mice compared to wild-type animals, was also observed at 12 and 18 months-of-age (Figs. 8A and 9A). At 12 and 18 months in the Tg2576 mice, there was also a significant increase in RhoA-GTP as well as CRMP-2A and CRMP-2B levels compared with wild-type controls (Figs. 8A and 9A). Moreover, at 18 months-of-age there was an appearance of a higher molecular weight (85 kDa) form of CRMP-2A in the Tg2576 brain lysates, at a similar molecular weight to hyperphosphorylated CRMP-2 described previously in AD brain lysates (Fig. 9A) (Gu et al., 2000).

There was an increase in both RhoA and CRMP-2 immunoreactivity in neuronal cell bodies and neurites of the cerebral cortex of Tg2576 mice between 12 (Fig. 8B) and 18 months-of-age (Fig. 9B) when compared to wild-type controls. There was also an increase in RhoA immunoreactivity in the Tg2576 in the same time period, coinciding with the increase in Thioflavin-T-positive amyloid deposits (Figs. 8B and 9B). Around larger Thioflavin T-positive amyloid deposits, in the Tg2576 animals at 18 months-of-age (Fig. 9B), there was intense immunostaining of neuronal and neuritic processes for both RhoA and CRMP-2 (Fig. 9B and C). This increase in immunostaining correlated with an increase in CRMP-2 in the 18-month-old Tg2576 mice, and the increase in higher molecular weight forms of CRMP-2 which are due to an increase in phosphorylation of CRMP-2 (Fig. 9A). Importantly, many RhoA and CRMP-2-positive neuronal cell bodies and dystrophic neurites, which decorated amyloid plaques, were observed in the 18-month-old mice (Fig. 9B, arrows). In deeper white matter lesions there were axons which stained for CRMP-2, which showed curvature or distortion surrounding amyloid plaques in the brains of mice at 18 months-of-age (Fig. 9C) and at the curvature points there was co-localization of RhoA and CRMP-2 (Fig. 9C, arrows). These data indicate that with an increasing amyloid burden there is an increased co-expression of both RhoA and CRMP-2 in neurons and dystrophic neurites surrounding plaques.

**CRMP-2 phosphorylation in Tg2576 mice**

We investigated whether the increase in CRMP-2 in the Tg2576 mice was correlated with an increase in phosphorylation as we had observed in the SH-SY5Y cells in the presence of Aβ (Fig. 5). No difference was observed in the level of phospho-CRMP-2 (pS509/518/pT514) in
the Tg2576 mouse brain at either 6- or 12-months-of-age compared to wild-type controls (Fig. 10A). However, there was a higher level of phospho-CRMP-2A and B (pS509/518/pT514) in the 18-month Tg2576 brain lysates compared with age-matched wild-type controls. As the difference was seen only at the age when amyloid plaques were present in the Tg2576 mice, the differences could have been due to an Aβ amyloid-induced increase in GSK3β-mediated phosphorylation of CRMP-2. CRMP-2 was next immunoprecipitated using monoclonal antibody C4G and then the level of total threonine phosphorylation determined by western blotting using an anti-phosphothreonine antibody. There was an increased level of phosphothreonine reactivity of the band corresponding with CRMP-2A and B at the 12- and 18-month time points in Tg2576 brain lysates compared with age-matched wild-type controls (Fig. 10B). These data showed that there is increased RhoA activation in the later stages of Tg2576 pathology which occurs co-ordinately with increased threonine phosphorylation of CRMP-2 and increased amyloid deposition.

Reduced levels of CRMP-2-bound tubulin in the Tg2576 mouse brain

Phosphorylation of CRMP-2 regulates the ability of CRMP-2 to bind tubulin heterodimers and to maintain neurite length and plasticity. Therefore, decreased binding of CRMP-2 to tubulin could lead to a decrease in neurite length. For this reason we examined the level of α-tubulin which could be co-immunoprecipitated with CRMP-2 in brain lysates of Tg2576 mice with increasing amyloid deposits and abnormal neurites. We found that after immunoprecipitation of CRMP-2, from wild-type and Tg2576 brain homogenates, there was an approximately 2–3-fold decreased recovery of α-tubulin in the immunoprecipitated fraction from 12 month-old Tg2576 brain (Fig. 11B). There was a 20-fold decrease in the recovery of α-tubulin from the 18 month-old Tg2576 mice compared with age-matched wild-type controls (Fig. 11C). No difference in the recovery of α-tubulin was seen between Tg2576 and wild-type controls at 6 months-of-age (Fig. 11A). These data suggest a mechanism by which
accumulation of amyloid plaques in the brains of Tg2576 mice, promote the phosphorylation of CRMP-2 and prevent its tubulin-binding capacity.

**Discussion**

This study demonstrates that Aβ can influence a signalling pathway mediated through the RhoA-GTPases. We show that Aβ can activate RhoA in human SH-SY5Y neuroblastoma cells, and this activation also occurs in brains of Tg2576 mice as the amyloid burden increases. Our studies show that the level of phosphorylated CRMP-2 is also affected by Aβ and this can lead to changes in microtubule dynamics in SH-SY5Y neuroblastoma cells as well as in Tg2576 mice. We suggest that the activation of RhoA and subsequent inactivation of CRMP-2 downstream by phosphorylation may be one mechanism limiting the ability of tubulin assembly in neurites and thereby causing dystrophic changes in neurite morphology as has been well documented in AD (Knowles et al., 1998) and in transgenic animal models (Tsai et al., 2004). This change in neuritic morphology may, in turn, lead to cognitive impairment (Knowles et al., 1999; Pigino et al., 2001; Lombardo et al., 2003). The action of Aβ peptides on the RhoA and CRMP-2 signalling pathway may be involved in the Aβ-induced changes in neuritic morphology that occur in AD.

Neuronal degeneration in the AD brain has been linked with the accumulation of amyloid-β deposits in the neuropil and the cerebral vasculature (Masliah et al., 1992; Cleary et al., 2005; Walsh et al., 2005). It is generally agreed that, along with changes in the metabolism of Aβ, there is an increase in oligomerization and accumulation of Aβ which may initiate abnormalities in the synaptic function of neurons within the hippocampus and cerebral cortex in AD (Walsh et al., 2002; Cleary et al., 2005; Klyubin et al., 2005; Walsh et al., 2005). As the amyloid burden increases within the brain, there is a progressive loss of neuronal circuitry and the pathological finding of dystrophic change in injured neurites is evident (Knowles et al., 1999; Spires et al., 2005). Studies on transgenic animal models of AD, have shown a correlation between the synapto-dendritic toxicity and β-amyloid deposition (Tsai et al., 2004). However, the precise molecular mechanism which governs neuritic dysfunction initiated by extracellular Aβ has been unclear.
Fig. 9  Rac1 is inactive, whereas, RhoA-GTP and CRMP-2 levels are increased in the Tg2576 mouse brain neurons surrounding amyloid plaques. At 18 months-of-age, there is a substantial increase in the levels of RhoA-GTP ($P < 0.001$) and a decrease in Rac1-GTP ($P < 0.005$) when compared to age-matched wild-type control animals (A, images captured from the area within the red box). A significant increase in CRMP-2A levels ($P < 0.0001$) and re-appearance of the CRMP-2A band at 75 kDa ($P < 0.05$) as well as an additional 85 kDa band was seen ($P < 0.01$) in the Tg2576 mouse brain (A, bottom panel) compared with age-matched wild-type controls. There is also an increase in phosphoserine 1 99/202 tau levels in the Tg2576 brain compared with age-matched wild-type controls (A). At 18 months-of-age there are numerous, large Thioflavin T-positive amyloid plaques visible throughout the Tg2576 mouse brain (B). In the cortex, surrounding both vascular and dense-core plaques, there are elevated levels of RhoA and CRMP-2 in the neuronal cell bodies and neurites (B; images captured from area within box) compared with Tg2576 at 6 and 12 months-of-age (Figs. 7 and 8). Co-localization of RhoA and CRMP-2 occurs in neurites (B, arrows) and neuronal cell bodies, surrounding these plaques (B). Neuritic plaques show co-localization of RhoA and CRMP-2 in apical dystrophic neurites (B, bottom right panel, arrow). Thioflavin T-positive plaques within the deep white matter also show axonal disturbances of CRMP-2 and RhoA-labelled axons (C). An axon curving around a dense core plaque shows co-labelling of RhoA and CRMP-2 at the regions where it curves (C, arrows; bar = 20 μm).
In our study, the addition of Aβ reduced neurite outgrowth from differentiated SH-SY5Y cells. This observation is strongly supported by similar observations in previous studies (Yankner et al., 1990; Pike et al., 1993; Fraser et al., 1994; Lambert et al., 1994; Postuma et al., 2000). The reduction in neurite outgrowth in the presence of Aβ has previously been reported in cell culture models such as differentiated hippocampal neurons (Yankner et al., 1990) and SH-SY5Y human neuroblastoma cell lines (Lambert et al., 1994). Other studies have shown that substrate-bound Aβ can inhibit neurite outgrowth in mouse neuroblastoma cells (Fraser et al., 1994) and chick sympathetic neurons (Postuma et al., 2000).

The concentrations of Aβ used in this study were based upon previous reports. It is now well-documented that when soluble oligomeric Aβ peptides are used at low micromolar and nanomolar concentrations neuritic and synaptic toxicity can be observed (Townsend et al., 2006; Lacor et al., 2007; Shankar et al., 2007). The effects on neurite outgrowth in cell culture were clearly seen after addition of 1.0 μM Aβ40. Although higher concentrations of Aβ (10–100 μM) have been used previously in cell culture experiments (Busciglio et al., 1995; Tong et al., 2001) we found that these higher concentrations caused a significant decrease in cell viability. Therefore, we used lower concentrations of Aβ (0.5–1.0 μM) which did not compromise cell viability but which are known to alter cell signalling (Tong et al., 2001, 2004; Garzon and Fahnestock, 2007). However, the endogenous pathophysiologic concentration of Aβ remains unknown.

The present study used AFM to examine the morphology of neurite outgrowth-inhibiting Aβ species. The data show that smaller molecular weight (oligomeric) aggregates of Aβ (e.g. fresh Aβ40) were enriched in those fractions that produced the highest amount of inhibition of neurite outgrowth. Conversely, preparations rich in high molecular weight fibrillar aggregates (e.g. aged Aβ42) were less potent in their effect on neurite outgrowth. These data agree with many studies which have shown that diffusible oligomeric species of Aβ have the greatest neurotoxic effect (Takahashi et al., 2004; Zhao et al., 2006; Lacor et al., 2007). Indeed it seems increasingly likely that synaptic and axono-dendritic degeneration are due to the build up of these low molecular weight Aβ species in the brain (Maloney et al., 2005; Shankar et al., 2007). In support of this idea it has recently been shown that blockade of extracellular Aβ in vivo by either passive transfer of anti-oligomeric specific antibodies or active immunization against Aβ can reverse the synaptotoxic and inhibitory LTP effects of oligomeric Aβ (Klyubin et al., 2005). These data suggest that the smaller, non-fibrillar forms of Aβ, may be the neurotoxic species where neurodegeneration begins at the distal neuritic ends (Whalen et al., 2005) and our data would agree with this hypothesis.

It seems likely that Aβ, in its oligomeric form, can alter receptor signalling events associated with neuronal lipid rafts (Small et al., 2007), causing cytoskeletal re-arrangement that, in turn, mediate neurite outgrowth effects (Tsui-Pierchal et al., 2002; Guirland et al., 2004; Haglund et al., 2004). The Rho-GTPase, Rac1, has been demonstrated to bind preferentially in its active state, to cholesterol-rich membranes and this binding is lipid dependent (del Pozo et al., 2004). Considering that active Rac1 is the major initiator of neuronal cell polarization and neurite outgrowth (Watabe-Uchida et al., 2006), our data which show inactivation of Rac1 following the addition of Aβ in vitro and in the Tg2576 mouse model of AD, suggest that neurite outgrowth is inhibited and the formation of dystrophic neurites is favoured.

In our experiments, we found that Rac1 is inactivated after Aβ-treatment of SH-SY5Y cells. This inactivation of Rac1 would be expected to allow RhoA to initiate actin–myosin contraction, which would cause neurite retraction away from the area of directional growth (Profyris et al., 2004). Additionally, Rho-GTP activates the serine/threonine kinase, Rho kinase (Profyris et al., 2004). This molecular event may halt the progression of neurite outgrowth allowing actin depolymerization to occur unabated through the re-activation of coflin by phosphatase and leading to eventual collapse of the neurite (Profyris et al., 2004). In our experiments we found that Rac1 is largely inactivated in the Tg2576 mouse brain from at least 6 months-of-age, when cognitive deficits are apparent, but prior to amyloid deposition. Importantly, at 6 months, Aβ accumulates in lipid raft fractions in the Tg2576 mouse brain (Kawarabayashi et al., 2004). This accumulation may
be related to the Rac1 inactivation. Inactivation of Rac1 (or its multiprotein complexes) may induce alterations in dendritic spine morphologies through cytoskeletal microfilament re-arrangement manifesting as cognitive impairment (Zhang et al., 2005; Soderling et al., 2007). Experiments by other groups support this conclusion in the context of Aβ-mediated neuritic dystrophy (Heredia et al., 2006). Heredia et al. (2006) reported that extracellular fibrillar Aβ initiated phosphorylation of cofilin and LIMK1 and that this directed actin filament remodeling. This change in actin filaments altered the morphology of cultured hippocampal neurons and rendered neurites dystrophic.

However, not all of the cognitive decline seen in APP transgenic mice, may be related to the presence of dystrophic neurites (Cleary et al., 2005; Townsend et al., 2006). Previous studies have shown that Aβ can have an acute affect and this may explain why cognitive decline occurs in transgenic mice prior to either amyloid deposition or neuritic dystrophy (Kotilinek et al., 2002; Lesne et al., 2006). It is becoming increasingly known that soluble Aβ can alter post-synaptic markers, a correlate of cognitive decline (Kamenetz et al., 2003; Lacor et al., 2004; Roselli et al., 2005). It has further been shown that Aβ can promote signalling pathways toward long-term depression (LTD) by the synaptic removal of AMPA receptors and loss of dendritic spines a correlate of reduced plasticity, learning and memory (Hsieh et al., 2006). However, studies by Knowles et al. (1999) and Tsai et al. (2004) argue persuasively that neuritic dystrophy contributes to cognitive changes. Therefore, it seems likely that the cognitive decline is a consequence of a large number of biochemical, physiological and morphological sequelae of Aβ toxicity.

The eventual collapse or retraction of a neurite is not only governed by a Rho kinase-mediated effect on the actin microfilaments but to a large extent by the ability
of Rho kinase to modify the microtubule network through its downstream substrate CRMP-2 (Fukata et al., 2002; Arimura et al., 2005; Yoshimura et al., 2005; Mimura et al., 2006; Watabe-Uchida et al., 2006). Our data suggest the involvement of a Rho kinase-dependent mechanism which inhibits neurite outgrowth following Aβ treatment of differentiated SH-SY5Y cells. We also show for the first time that the CRMP-2 phosphorylation is potentiated by Aβ and that inhibition of the Rho kinase pathway can inhibit CRMP-2 phosphorylation and alter its capacity to bind tubulin. We found that differentiated SH-SY5Y human neuroblastoma cells treated with the Rho kinase inhibitor, Y27632, re-established association of CRMP-2 with tubulin, even in the presence of Aβ. This effect was related to a decreased phosphorylated threonine CRMP-2 residue. Furthermore, we found that the cells treated with Y27632 with or without Aβ also maintained their neurite length suggesting that the neurite retraction event induced by Aβ could be overcome by inhibiting Rho kinase. These data implicate Rho kinase in the downstream effects of Aβ on neurite outgrowth.

In Tg2576 mice, there is evidence to suggest that increasing oxidative stress occurs in neurons with pronounced amyloid burden (Pratico et al., 2001). Importantly, this oxidative stress can lead to neuronal cell dysfunction and death. Furthermore, it has been shown via a proteomic approach that there is increased carbonylation of CRMP-2 [dihydropyrimidinase protein 2 (DRP-2)] in the AD brain compared to age-matched controls, along with other proteins (Castegna et al., 2002). This increased carbonylation of CRMP-2 is reportedly related to dysregulation of CRMP-2 (DRP-2) activity particularly in the formation of dystrophic neurites (Castegna et al., 2002). The involvement of Rho kinase however, in the context of the amyloid hypothesis of neurodegeneration and oxidative stress in neurons, is less clear. There exists evidence that generation of reactive oxygen species (ROS) can activate RhoA-GTP enhancing endocytosis of cell-surface proteins such as receptors (Dada et al., 2007). However, whether ROS-mediated effects on Rho kinase can explain the observations reported in this study remains unclear.

It has recently been established that for growth cone collapse and neurite outgrowth inhibition to be consolidated Rho kinase must phosphorylate the threonine-555 epitope of CRMP-2 (Arimura et al., 2005). In an important study, Arimura et al. (2005) showed that CRMP-2 phosphorylation at threonine-555 could not bind effectively to tubulin heterodimers or to Taxol-stabilized microtubules. In the presence of Ephrin-A5 which recruits ephexin (a Rho-specific guanine nucleotide exchange factor, GEF), to activate RhoA through its Eph receptor (Shamah et al., 2001; Sahin et al., 2005), Arimura et al. (2005) showed that the DRG neurons transfected with the T555A mutant form of CRMP-2 did not retract their axons as did the neurons transfected with either a myc-GST construct or with CRMP-2 containing a threonine to aspartate substitution (T555D), which Rho kinase can still phosphorylate. This study showed that the Rho kinase phosphorylated form of CRMP-2 could not bind to tubulin heterodimers and that this inevitably translated into axon collapse.

It is well established that transgenic mouse models of AD develop a neuritic pathology as the Aβ burden in the brain increases (Schenk et al., 1999; Holtzman et al., 2000; Le et al., 2001; DeMattos et al., 2002; Lombardo et al., 2003; Bussiere et al., 2004; Tsai et al., 2004; Spires et al., 2005). We found levels of CRMP-2 are also increased in the Tg2576 mouse model of AD. CRMP-2 phosphorylation in the Tg2576 mouse brain correlated with the activity of RhoA and inactivation of Rac1 at 12 and 18 months-of-age, a stage where amyloid is being formed or is present in the neuropil and dystrophic neurite pathology is evident. This observation is reminiscent of the effects of Aβ seen in cell culture. Importantly, the increase in threonine phosphorylation of CRMP-2 was seen at 12 and 18 months-of-age in the Tg2576 mice. These data show for the first time, that surrounding Aβ plaques in an animal model of AD, RhoA activity is increased along with CRMP-2 phosphorylation and that this leads to reduced tubulin binding. The plaque-related activity in RhoA and CRMP-2 suggest a role for this pathway in the generation of dystrophic neurite formation.

In the Tg2576 mouse model of AD, cognitive impairment along with dendritic spine deficits can be observed from 6 months-of-age prior to amyloid deposition (Takahashi et al., 2004; Lesne et al., 2006). These changes correlate with an increase in oligomeric forms of Aβ in the mice (Lesne et al., 2006). Our data show substantial changes to RhoA activation, Rac1 inactivation, phoshorylation of CRMP-2 and CRMP-2-dependent tubulin association from 12 months-of-age when neuritic dystrophy associated with amyloid plaques is evident. However, Rac1 is inactivated as early as 6 months-of-age. This inactivation of Rac1 is likely to be due to soluble Aβ. However, the dystrophic changes that are seen at later ages may also be due to soluble Aβ as the level of soluble Aβ is known to increase dramatically at the time of amyloid deposition (Fodero et al., 2002).

Our cell-culture studies showed that Aβ caused an increase in the generation of a 75 kDa CRMP-2A alternatively spliced variant. Threonine phosphorylation of CRMP-2A was also found to be increased by Aβ. It has been previously reported that the CRMP-A variants have contrasting effects on neurite outgrowth relating to their differing N-terminus length (Quinn et al., 2003; Yuasa-Kawada et al., 2003; Bretin et al., 2005). There also exists a difference in localization of the variants, with CRMP-2A exclusively expressed in axons and CRMP-2B predominately expressed in dendrites (Yuasa-Kawada et al., 2003; Bretin et al., 2005). In our western blot analysis we found that the major isoform present in the adult mouse brain was CRMP-2B. However, CRMP-2A increased as the amyloid burden increased in the Tg2576 mouse brain.
In the differentiated SH-SY5Y cells, Aβ also increased the level of CRMP-2A and this effect was blocked by either the co-administration of Y27632, or transient transfection of the cells with CA-Rac1.

In summary, our work demonstrates that Aβ (probably in its oligomeric form) is able to signal modifications in the neuronal cytoskeleton through the activation of RhoA and inactivation of Rac1. RhoA activation in the presence of Aβ causes a reduced and abnormal neurite outgrowth. Activation of RhoA causes an increase in a threonine-phosphorylation of the CRMP-2A alternatively spliced variant. In our study, this phosphorylation rendered CRMP-2 incapable of binding to tubulin. These experiments help to define a new pathway of Aβ-induced neurite outgrowth inhibition in the AD brain and may suggest new possibilities of therapeutic intervention based upon inhibition of this pathway.

Acknowledgements
S.P. was supported by a Faculty of Medicine, Nursing and Health Sciences, Monash University, Senior Postdoctoral Fellowship. S.P. and D.H.S. were supported by National Health and Medical Research Council of Australia Project Grant ID 384157. The authors would like to acknowledge Dr Adam Mechler for his technical assistance in capturing AFM images, Dr Calum Sutherland, Ms Megan Astle and Professor Christina Mitchell for their assistance in providing the sheep anti-phospho-CRMP-2 antibody.

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