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Evidence of Aβ- and transgene-dependent defects in ERK-CREB signaling in Alzheimer's models

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Abstract

Extracellular-signal regulated kinase (ERK) signaling is critical for memory and tightly regulated by acute environmental stimuli. In Alzheimer disease transgenic models, active ERK is shown to first be increased, then later reduced, but whether these baseline changes reflect disruptions in ERK signaling is less clear. We investigated the influence of the familial Alzheimer's disease transgene APP*sw* and β-amyloid peptide (Aβ) immunoneutralization on cannulation injury-associated (i.c.v. infusion) ERK activation. At both 12 and 22 months of age, the trauma-associated activation of ERK observed in Tg^- mice was dramatically attenuated in Tg^+. In cortices of 22-month-old non-infused mice, a reduction in ERK activation was observed in Tg^+, relative to Tg^- mice. Intracerebroventricular (i.c.v.) anti-Aβ infusion significantly increased phosphorylated ERK, its substrate cAMP-response element-binding protein (CREB) and a downstream target, the NMDA receptor subunit. We also demonstrated that Aβ oligomer decreased active ERK and subsequently active CREB in human neuroblastoma cells, which could be prevented by oligomer immunoneutralization. Aβ oligomers also inhibited active ERK and CREB in primary neurons, in addition to reducing the downstream post-synaptic protein NMDA receptor subunit. These effects were reversed by anti-oligomer. Our data strongly support the existence of an APP*sw* transgene-dependent and Aβ oligomer-mediated defect in regulation of ERK activation.

Keywords: Alzheimer disease, cAMP-response element-binding protein, extracellular-signal regulated kinase, immunization, signal transduction, Tg2576 mice.
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Alzheimer's disease (AD) is clinically characterized by progressive cognitive impairment. Cognitive deficits in AD are widely believed to result from progressive synaptic dysfunction and neurodegeneration initiated by aggregated β-amyloid peptide 1–42 (Aβ42). Recently, soluble Aβ oligomers, including dimers, trimers, and dodecamers (12-mer or Aβ*56) have been proposed as key forms of Aβ causing cognitive deficits in animal models (Cleary *et al.* 2005; Lesne *et al.* 2006). Soluble Aβ concentrations predict synaptic changes (Lue *et al.* 1999) and correlate with the severity of dementia in AD patients (McLean *et al.* 1999). Antibody-mediated Aβ oligomer reduction can correct defects in synaptic plasticity (Klyubin *et al.* 2005) and reduce tau phosphorylation (Klyubin *et al.* 2005; Ma *et al.* 2006), another pathological hallmark of AD.

Although multiple mechanisms for Aβ peptide-induced memory deficits have been hypothesized including effects on tau phosphorylation and tangle formation, there is considerable evidence implicating a role for Aβ-induced disruption of kinases critical for memory (Zhu *et al.* 2002). For example, studies of human mental retardation syndromes have shown that extracellular-signal regulated kinases (ERKs) are critical for human learning (Costa *et al.* 2002). They are also known to contribute to molecular information processing in dendrites, to stabilize structural changes in dendritic spines and to interact with scaffolding and structural proteins at the synapse (Sweatt 2004). However, aberrant over-expression of ERK can lead to cell death (Zhuang and Schnellmann 2006). ERK shows stage-dependent abnormalities in mRNA

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, β-amyloid peptide; Aβ42, β-amyloid peptide 1–42; CREB, cAMP-response element-binding protein; DAB, diaminobenzidene; ERK, extracellular-signal regulated kinase; GFAP, glial fibrillary acidic protein; GSK3β, glycogen synthase kinase-3β; IGF, insulin-like growth factor; LTP, long-term potentiation; NMDAR, NMDA receptor; NR2B, NMDA receptor subunit; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween-20; pCREB, phosphorylated CREB; pERK, phosphorylated ERK; PKA, protein kinase A; PSD-95, post-synaptic density-95; SNAP-25, synaptosomal-associated protein of 25 kDa; TBS, Tris-buffered saline.

and protein expression in AD (Webster *et al.* 2006) and AD models (Dineley *et al.* 2001). Although transient ERK activation plays important roles in memory-related processes, persistent activation can mediate NMDA-related excitotoxicity (Amadoro *et al.* 2006). Therefore, either hyper- or hypoactivation of ERK could contribute to disease pathways.

It has been reported that in early AD, there is extensive activation of ERK in astroglial cells in the white matter, while in advanced AD, there is reduced activation showing a strong inverse correlation with Braak stage and the Blessed score for cognition (Webster *et al.* 2006). However, ERK activation is not necessarily protective. For example, active ERK is a tau kinase that is elevated during the initial stages of neurofibrillary degeneration in the projecting neurons in the transentorhinal region. Nevertheless, ERKs causal role in neurofibrillary tangle formation remains unclear as many neurons with the highest amounts of ERK/mitogen-activated protein kinase immunoreactivity do not appear vulnerable to neurofibrillary tangles (Hyman *et al.* 1994). Interestingly, at late stages (although some ERK is still apparent in astrocytes), ERK activation is suppressed relative to early stage and normal controls in neuronal cell bodies and dystrophic neurites (Webster *et al.* 2006). In summary, studies in AD brain suggest stage-dependent ERK activation followed by loss of active ERK.

Similar to AD, the amyloid precursor protein (APP) transgenic Tg2576 model shows early stage ERK activation, while at later stages ERK is reduced (Dineley *et al.* 2001). Sustained activation of ERK was also observed in brains of an APP transgenic rat model with significant intraneuronal Aβ accumulation (Echeverria *et al.* 2004). *In vitro*, it has been demonstrated that sustained ERK activation is related to cell death after Aβ treatment in neurons and PC12 cells (Dineley *et al.* 2001; Jang and Surh 2005). Conversely, ERK activation can be reduced by γ-secretase inhibitor treatment of APPsw transfected cells (Echeverria *et al.* 2005). Abnormally sustained activation may contribute to cell death in other amyloid diseases like familial amyloidotic polyneuropathy (Monteiro *et al.* 2006) and work via multiple mechanisms, including pro-apoptotic events upstream of caspase 3 or via suppression of the anti-apoptotic signaling molecule Akt (Zhuang and Schnellmann 2006).

The known importance of ERK on dendrites and memory is consistent with the hypothesis that ERK hypoactivation in AD contributes to cognitive decline. Under certain conditions, Aβ or its fragments have been shown to inhibit ERK (or downstream cAMP-response element-binding protein; CREB) in neuroblastoma cells (Daniels *et al.* 2001), endothelial cells (Magrane *et al.* 2006), hippocampal neuron preparations (Xie 2004), or after Aβ25–35 i.c.v. injection into rats (Jin *et al.* 2005). Different Aβ aggregates may have different effects depending on the system, for example high levels of fibrillar Aβ in PC12 cells inhibited ERK, while other forms of Aβ increased ERK activation (Echeverria *et al.* 2005). In contrast, fibrillar Aβ stimulated ERK, while low (100 nmol/L) soluble oligomers initially stimulated but later down-regulated ERK in hippocampal slice cultures (Bell *et al.* 2004).

In view of the conflicting results with Aβ's stimulatory or inhibitory effects on ERK in culture systems with different Aβ preparations and biphasic effects, we have focused on chronic *in vivo* studies. We decided to assess ERK regulation using injury which is known to stimulate ERK. Trauma or ischemia can induce ERK activation (Shackelford and Yeh 2006), possibly via growth factors that are induced by injury including transforming growth factor beta1 (Logan *et al.* 1992), insulin-like growth factor (IGF), and brain derived neurotrophic factor (Kazanis *et al.* 2004) all which are known to stimulate ERK (Willaime-Morawek *et al.* 2005; Johnson-Farley *et al.* 2006). To determine if AD models showed disruption in regulation of ERK, we examined whether trauma induction of ERK was altered by APP*sw* transgene and corrected by Aβ oligomer neutralization. We also investigated the impact of Aβ oligomer on downstream CREB and NMDA receptor (NMDAR) subunit (NR2B).

Experimental procedures

Chemicals and reagents

Unless otherwise noted, all reagents were obtained from Sigma (St Louis, MO, USA). Monoclonal anti-Aβ antibody (IgG2b) as anti-Aβ1–15 was raised against Aβ1–42 and characterized in our lab (Yang *et al.* 1994). Primary antibodies were: polyclonal phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204) (phosphorylated ERK, pERK; BioSource, Camarillo, CA, USA); polyclonal anti-total ERK2 (Santa Cruz Biotechnology Inc, Beverly, MA, USA); polyclonal anti-phospho-CREB and monoclonal anti-post-synaptic density-95 (PSD-95) (phosphorylated CREB, pCREB; Upstate, Lake Placid, NY, USA); monoclonal anti-β-actin, anti-synaptophysin, anti-NR2B, and polyclonal anti-MAP2 (Chemicon International, Temecula, CA, USA); anti-Aβ monoclonal 6E10 (Signet Labs Inc., Dedham, MA, USA); monoclonal anti-Drebrin (MBL, Woburn, MA, USA); Rabbit polyclonal A11 oligomer-specific antibody, a generous gift from Dr C. Glabe, was produced and characterized as described (Kayed *et al.* 2003). A11 antibody recognizes oligomer species but not monomer and fibrils. IGF-1 was obtained from Peprotech Inc (Rochy Hill, NJ, USA). ABC reagent was from Vector Labs (Burlingame, CA, USA), metal enhanced diaminobenzidene (DAB) was from Pierce Biotechnology (Rockford, IL, USA), Aβ1–42 peptide was obtained from American Peptide (Sunnyvale, CA, USA). The CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit was obtained from Promega (Madison, WI, USA).

Animals and treatment

Surgical and animal procedures were carried out with strict adherence to the current guidelines set out in the NIH Guide for the Care and Use of Laboratory Animals by the Association for Assessment and Accreditation of Laboratory Animal Care International which has accredited the Greater Los Angeles Healthcare System. All

experiments involving animals were approved by the appropriate UCLA and VA Institutional Animal Care and Use Committee, Institutional Biosafety, and Research Development (R&D) Committees. Tg2576 (Tg^+) or transgene negative littermates (Tg^-) mice were used for surgical experiments. Intracerebroventricular infusion of monoclonal anti-Aβ antibody (IgG2b, 10G4) as anti-Aβ1–15 and control IgG2b were previously described (Ma *et al.* 2006). Briefly, custom length, stainless steel i.c.v. catheters (Plastics One, Roanoke, VA, USA) were stereotaxically implanted into the lateral ventricle, using the following coordinates from Bregma: 2.7 mm ventrally (from skull), +1.0 mm laterally and 0.5 mm posteriorly. The catheters were secured to skull using acrylic dental cement (Stoelting Corporation, Wood Dale, IL, USA) and a machine screw partially inserted into the skull. The i.c.v. cannula was connected to an Alzet osmotic mini-pump (#1002; Durect Corporation, Cupertino, CA, USA, releasing 0.25 μL/h) through polyethylene tubing into ventricular cannula. Control and anti-Aβ IgG2b (10G4) were purified using a protein A column followed by an endotoxin-removing column (Pierce Biotechnology) and dialyzed overnight against phosphate-buffered saline (PBS). After dialysis, the antibody concentration used to fill the osmotic mini-pumps was 20 μg/100 mL. The control group received equimolar infusions of non-specific mouse IgG2b. After 14 days, mice were deeply anesthetized with pentobarbital (50 mg/kg bw) and cardiac perfused with protease inhibitor-containing buffer (pH 7.2) as previously described (Lim *et al.* 2000). All infusions were intracerebroventricular and unilateral with biochemical analysis of unfixed tissue on the contralateral side (western and ELISA) and immunocytochemistry on the fixed (cannulated) side where the needle track was examined to confirm placement in the right lateral ventricle.

Tissue preparation

Tissue samples were processed in Tris-buffered saline (TBS) (soluble fraction) and lysis buffer (membrane fraction) containing protease inhibitors and phosphatase inhibitors as previously described (Calon *et al.* 2004). Mixed cortices were used for pERK, pCREB, NR2B, and other endpoints analysis.

Cell culture and treatment

Human SH-SY5Y neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L L-glutamine and 10% (v/v) fetal calf serum; 5×10^5 cells were plated on six-well plates and grown to 80% confluency at 37°C in a humidified 5% CO_2 atmosphere incubator. Cultured hippocampal neurons were prepared from embryonic 18 day Sprague–Dawley rat fetuses as described previously (Zhao *et al.* 2004). Before treatment with A11 oligomer-specific antibody (2 ng/mL), cells were rinsed once with serum-free Dulbecco's modified Eagle's medium media for SH-SY5Y cells or Neurobasal medium without glutamate and B27 for primary neurons. A11 antibodies and serum-free medium for SH-SY5Y cells or A11 antibodies and Neurobasal medium without glutamate and B27 for primary neurons were added to cells simultaneously for one hour, followed by addition of 250 nmol/L Aβ42 oligomers and incubation for 1.5–24 h at 37°C.

Preparation of Aβ oligomers

A modified protocol was used for the preparation of Aβ oligomers (Kayed *et al.* 2003). Briefly, 1 mg of Aβ42 peptide was dissolved in 250 μL of 1,1,1,3,3,3-hexafluoro-2-propanol in the vial and incubated at 25°C until dissolved completely. The 1,1,1,3,3,3-hexafluoro-2-propanol was removed by gentle streaming of N_2. Aβ42 was then dissolved in 1 mL of 10 mmol/L HEPES (pH 7.4). This solution was incubated at 37°C with a micro stir bar at 500 rpm for 12 h and centrifuged at 14 000 *g* for 5 min to remove fibrils and other large aggregates. Protein concentration was determined using the Bio-Rad DC protein assay (Richmond, CA, USA). The presence of Aβ oligomers was confirmed by anti-Aβ antibodies, 6E10 and 4G8 following its separation on 10–20% Tris–Tricine gradient gels (Ma *et al.* 2006).

Cell lysate preparation from SH-SY5Y cells

SH-SY5Y cells were placed on ice, washed with cold PBS once, scraped with 1 mL of PBS, and transferred to a microfuge tube. Cells were centrifuged at 734.5 *g* for 5 min and pellets were dissolved in lysis buffer with a cocktail of protease and phosphatase inhibitors. After a brief sonication, the lysate was incubated at 4°C for 30 min, centrifuged at 16 000 *g* for 10 min, and the supernatants were collected.

Cell viability assays

The effect of Aβ42 oligomers on SH-SY5Y cell viability was determined by measuring the activity of lactate dehydrogenase released into the culture medium using a CytoTox 96® Non-Radioactive Cytotoxicity Assay. The assay was performed in accordance with the manufacturer's protocol and absorption read at 490 nm.

Western immunoblotting

For western immunoblotting, protein concentration was determined using Bio-Rad DC protein assay methods. Equal amounts of protein per sample were added to Laemmli loading buffer, and boiled for 3 min. Except for Aβ oligomer detection, 30 μg of protein per well was electrophoresed on 10% Tris–glycine gels and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA). For Aβ oligomer detection, 5 μg of Aβ preparation without boiling was electrophoresed on 10–20% Tris–Tricine gradient gels. Gels were stained with Coomassie blue to ensure equal protein loading. Membranes were blocked for 1 h at 25°C in 10% non-fat dried milk in PBS, followed by incubation overnight at 4°C with appropriate primary antibodies in PBS containing 0.05% Tween-20 (PBS-T) and 1.5% (w/v) albumin. pERK, actin, total ERK, and 6E10 were diluted to 1 : 2000; pCREB and NR2B were diluted to 1 : 1000. After being rinsed in PBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1 : 10 000) or anti-rabbit IgG (1 : 30 000) in PBS-T with 1.5% albumin for 1 h. Immunolabeled proteins were visualized by enhanced chemiluminescence detection reagents. Resulting films were scanned and quantified using densitometric software (Molecular Analyst II; Bio-Rad). To strip immunoblots, membranes were incubated for 30 min at 57°C in 100 mmol/L 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mmol/L Tris–HCl (pH 6.8). The membranes were rinsed 3 × 10 min in large volumes of PBS-T, followed by blocking and reprobing with appropriate antibodies, including anti-glial fibrillary acidic protein (GFAP) (1 : 1000), drebrin (1 : 500), synaptophysin (1 : 1000), synaptosomal-associated protein of

25 kDa (SNAP-25) (1 : 1000), and PSD-95 (1 : 1000) as previously described (Calon *et al.* 2004).

Cytokine ELISA

Interleukin-1β and tumor necrosis factor-α were analyzed by sandwich ELISA of TBS fractions as described (Lim *et al.* 2000).

Immunostaining and image analysis

Cryosectioned mouse brains were warmed to 25°C for 10 min then steamed for 1 h in citrate buffer. Sections were quenched in 0.6% hydrogen peroxide with methanol for 30 min at 25°C and washed three times with TBS-buffered solution (pH 7.4). Next sections were treated with 0.3% Triton X-100 in 0.1 mol/L TBS (pH 7.4). Sections were then blocked using 5% normal goat serum containing 3% bovine serum albumin/TBS for 1 h at 37°C followed by a 2 day incubation with pERK antibody at a dilution of 1 : 100 at 4°C. Vector biotinylated goat anti-rabbit antibody (1 : 1200) was used with 1.5% normal goat serum and 3% bovine serum albumin/TBS. Sections were incubated in secondary antibody followed by the ABC reagent for 1 h at 37°C. Slides were developed using peroxidase/DAB. Microglia was labeled with IB4 (biotinylated Griffonia Simplicifolia isolectin B4; Vector Labs) 1 : 50 overnight at 4°C and developed with avidin-peroxidase (ABC; Vector Labs) reagents and DAB. For immunofluorescent staining, pERK and pCREB were diluted to 1 : 100; NR2B was diluted to 1 : 50; microtubule-associated protein type II (MAP2) was diluted to 1 : 400.

For image analysis of IB4 or pERK, mid-region (−1.58 to −2.30 mm Bregma) sections were analyzed from a Nikon Eclipse E800M microscope (Valencia, CA, USA) with a Nikon microscope power unit video system (Irvine, CA, USA). The video signal was routed into a Macintosh via a Scion Corporation AG-5 averaging frame grabber, and these digitized images were analyzed with NIH-Image public domain software (Bethesda, MD, USA). Quantitative analysis was used to evaluate cell density. Ring analysis and regional analysis of IB4 labeled microglia with NIH image was performed as previously described (Frautschy *et al.* 2001).

Statistical analyses

Statistical analyses were performed with StatView 5.0 software (SAS Campus Drive, Cary, NC, USA). Differences among means were assessed by ANOVA followed by Tukey–Kramer *post hoc* test.

Results

APP transgene attenuates injury-induced ERK activation *in vivo* and IGF-induced ERK activation in SY5Y cells

To evaluate the possibility of an APP transgene-dependent dysregulation of ERK, we investigated whether APP transgene and Aβ oligomer influenced the pERK response to injury (associated with stereotaxic i.c.v. infusion surgery) at different ages. Results indicated that at 11–12 months of age, i.c.v. infusion significantly increased pERK in Tg^- mice but not in Tg^+ mice (Fig. 1a) when compared with groups of non-infused mice. We also analyzed pERK in animals at 16–18 months of age (Fig. 1b and c). In the cortex, i.c.v. infusion induced a threefold increase in pERK in Tg^- mice but only a onefold increase in Tg^+ mice (Fig. 1b). Intracerebroventricular infusion caused a four- to fivefold increase in pERK in hippocampal pyramidal neurons in Tg^- mice (Fig. 1c). In contrast, in Tg^+ mice, infusion did not increase pERK in the CA1 region of the hippocampus and the response was attenuated in the CA2 and CA3 regions

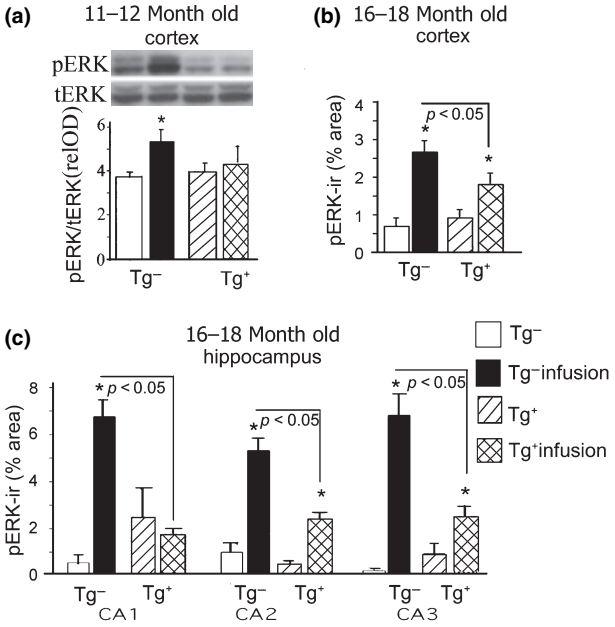

Fig. 1 Amyloid precursor protein transgene-dependent impairment in phosphorylated extracellular-signal regulated kinase (pERK) response after infusion trauma in middle aged (11- to 12-month old) and old (16- to 18-month old) Tg2576 mice. (a) 11- to 12-month-old Tg^+ and Tg^- mice were i.c.v. infused with non-specific IgG2b for 2 weeks and the level of pERK compared with non-infused, age-matched Tg^+ and Tg^- mice. In Tg^- mice, levels of pERK were significantly increased by i.c.v. infusion compared with non-infused mice (*$p < 0.05$). In Tg^+ mice, levels of pERK did not change in i.c.v. infused mice compared with non-infused mice ($p = 0.67$). (b and c) In 16- to 18-month-old mice, insufficient brain tissue remained for western blot analysis, so pERK was evaluated by immunocytochemistry. In the cortex, infusion induced approximately threefold induction of pERK in Tg^- mice, while in Tg^+ mice, infusion was associated with only approximately onefold increase in pERK (*$p < 0.05$) (b). (c) In Tg^- hippocampal pyramidal neurons, infusion induced approximately five- to sixfold induction of pERK. However, in Tg^+ mice infusion was not associated with an increase in pERK (CA1) and the response was attenuated in the CA2 and CA3 hippocampal regions (one- to twofold in CA2 and CA3 neurons, respectively). Statistical analysis 2×2 ANOVA (transgene × infusion) showed a significant interaction between transgene and infusion (*$p < 0.05$). Asterisks indicated statistical differences between infusion and non-infusion within transgene. Lines indicate statistical differences of planned comparisons between transgenes with infusion.

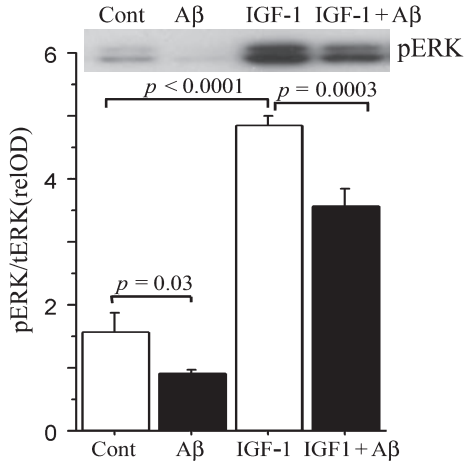

Fig. 2 Aβ oligomers were significantly blocked insulin-like growth factor 1 (IGF-1) -induced activation of extracellular-signal regulated kinase (ERK) in human neural SY5Y cells. SY5Y cells were pre-treated with 0.25 μmol/L Aβ oligomers for 1.5 h and followed by 100 ng/mL IGF-1 for 30 min. IGF-1 significantly increased ERK activation when compared with control ($p < 0.0001$), while pre-incubation with Aβ oligomers for 1.5 h resulted in an attenuated response ($p < 0.0003$). pERK was normalized by total ERK.

(Fig. 1c). Thus, the APP transgene significantly attenuated the trauma-associated induction of pERK at both ages, consistent with the impairment in ERK regulation with chronic Aβ exposure. To address whether Aβ oligomers could also attenuate trophic factor induced ERK, we examined the ERK response to 100 ng/mL IGF-1, known to be elevated after injury in SY5Y cells. This dose of IGF-1 induced a 3.1-fold increase in ERK at 30 min, while pre-incubation with Aβ oligomers for 1.5 h resulted in an attenuated response ($p < 0.0003$, a 2.4-fold increase in ERK, Fig. 2).

Anti-Aβ antibody blocked Aβ oligomer-induced decreases of activated ERK and CREB in Tg2576 mice

Our previous study found that acute infusion of anti-$A\beta_{1-15}$ 10G4 antibody into Tg2576 mice significantly reduced Aβ oligomers, including 12-mer, but did not reduce insoluble Aβ. Oligomer reductions significantly correlated with reduced tau phosphorylation by glycogen synthase kinase-3β (GSK3β), a major tau kinase (Ma *et al.* 2006). Aβ oligomer species were characterized *in vivo* and *in vitro* by silver staining and western blot analysis (Ma *et al.* 2006). In this study, we initially evaluated whether a loss of active ERK and CREB occurred in Tg^+ mice; 10-month ($n = 3$ per group), 11- to 12-month ($n = 4$ for Tg^+ and $n = 8$ for Tg^-), 15-month ($n = 3$ per group), and 22-month-old Tg^- ($n = 15$) or Tg^+ ($n = 16$) mouse cortices were examined with pERK antibody by western blot. The 11- to 12- and 15-month-old Tg^+ mice which were raised on PMI 5015 breeder chow showed a trend toward a decrease ($p = 0.10$ and $p = 0.06$) in pERK (Fig. 3b and c), whereas 22-month-old Tg^+ mice showed a very large and significant decrease ($p < 0.01$, Fig. 3d) in pERK compared with the age-matched Tg^- mice. This suggested that while only a trend toward a decrease of cortical ERK activity could be seen at an early age (11–12 months) when plaques are emerging in Tg^+ mice, a major ERK defect occurred as a function of APP transgene with age and a less protective diet. Similarly, a large and significant pCREB decrease ($p < 0.05$, Fig. 3e) occurred in 22-month-old Tg^+ mice compared with the age-matched Tg^- mice on the same diet. pCREB was highly correlated with pERK ($p < 0.0001$, $R^2 = 0.571$, Fig. 3f).

As the APP transgene could suppress ERK signaling, we asked whether this could be Aβ-dependent by evaluating the levels of active ERK and CREB after infusion with anti-Aβ or control IgG2b antibody using 11- to 12-month-old $Tg2576^+$ mice. The results indicated that, compared with non-specific IgG2b infused controls, IgG2b anti-Aβ-infused mice showed significantly increased levels of pERK2 (the 42 kDa band, $p < 0.05$, Fig. 3g), and pCREB ($p < 0.05$, Fig. 3h). There was a larger impact on pERK and pCREB in the temporal and entorhinal cortices (Figs 4 and 5) from anti-Aβ antibody treatment than frontal and parietal cortices, consistent with greater antibody perfusion of regions close to, and posterior to, the infused lateral ventricle. In addition to increase in intensity of pERK-ir cells after anti-Aß infusion, there was also an increase number of pERK-ir cells stained in the entorhinal cortex (not shown).Consistent with an anti-oligomer effect, frontal cortex showed no pERK change and also had no reduction in Aβ oligomer levels (not shown). These results suggest that immunoneutralizing Aβ oligomers can restore the diminished ERK response observed in the APP transgene positive mice *in vivo*.

An alternative interpretation might be that anti-Aβ antibody-induced microglial activation and inflammatory changes that caused increased ERK activation. However, anti-Aβ antibody infusion did not induce significant overall increases in microgliosis (IB4, image analysis of immunocytochemistry, Table 1) despite increases within plaques (Table 2). Regionally, anti-Aβ significantly reduced cortical and hippocampal microglial density except in the dentate gyrus. In contrast, there was a significant increase in the intensity of IB4 staining per cell, except in the dentate gyrus. Relative to control IgG2b, anti-Aβ (10G4) infusion significantly increased microglial density only within plaques but not outside plaques; similarly, there was a strong trend to increase microglial size within, but not outside plaques. While anti-Aβ infusion increased plaque-associated microglia, this effect was limited by the small number of plaques in the 11- to 12-month-old Tg2576. Microglial activation is transient and returns to baseline by 7 days in response to intracranial anti-Aβ antibody (Wilcock *et al.* 2004). Thus, global markers of inflammation

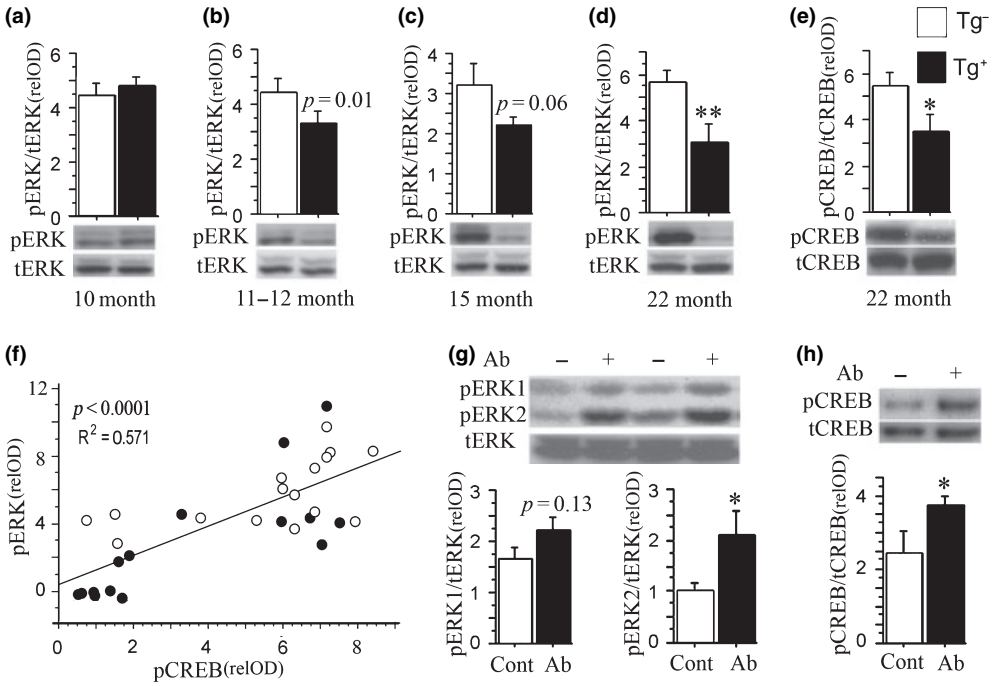

Fig. 3 Anti-Aβ antibody prevented Aβ oligomer-induced decreases in extracellular-signal regulated kinase (ERK) and cAMP-response element-binding protein (CREB) activation in the cortex of anti-Aβ-infused Tg2576 mice. (a–d) As age and Tg^- dependent effects of ERK have only been reported in the cortex, we measured cortical ERK and CREB activation at different ages using immunoblot with pERK and total ERK antibodies. pERK levels were significantly decreased in 22-month Tg^+ mice (**$p < 0.01$). pERK was normalized by total ERK. (e) The Tg^- dependent reduction in pERK at 22 months coincided with decreases in phosphorylated CREB (pCREB; *$p < 0.05$). pCREB was normalized by total CREB. (f) pERK and pCREB were highly correlated in 22-month Tg^+ mice ($p < 0.0001$, $R^2 = 0.571$. Open circles reflect levels in Tg^- mice; filled circles reflect levels in Tg^+ mice. Western analysis of pERK in Tg^+ mice showed that compared with IgG2b-infusion, anti-Aβ-infusion (Ab) significantly increased pERK2 (*$p < 0.05$) (g) and pCREB (*$p < 0.05$) (h). Error bars represent SEM.

including interleukin-1β and tumor necrosis factor-α (ELISA) and reactive astrocytes (GFAP and western) were not increased by anti-Aβ antibody, nor did anti-Aβ antibody cause damage indexed by westerns of synaptic markers (PSD-95, drebrin, synaptophysin, and SNAP-25) which were not significantly changed (Table 3).

Oligomer-specific antibody blocked Aβ oligomer-induced decreases of activated ERK and CREB in a human neuronal cell line

β-Amyloid peptide induces glial-derived cytokines and oxygen radicals that can regulate ERK signaling. To determine whether Aβ oligomers can directly modulate human neuronal ERK signaling, pERK and pCREB were evaluated in Aβ oligomer-treated human neuronal SH-SY5Y cells. Cells were pre-incubated with or without A11 antibody (2 ng/mL) for 1 h followed by treatment with 250 nmol/L of Aβ oligomer preparation for 1.5–24 h. (Fig. 6). Cells were collected and the levels of active ERK and CREB were assessed by immunoblot. Results (Fig. 6a and b) indicated that cells treated with Aβ oligomer alone had significantly decreased levels of pERK at 1.5 and 24 h (***$p < 0.001$ and *$p < 0.05$, respectively), and pCREB at 1.5 and 12 h (*$p < 0.05$ and **$p < 0.01$, respectively). To demonstrate that oligomers and not other forms of Aβ were responsible for ERK and CREB effects, an oligomer-specific antibody, A11, was used to immunoneutralize oligomeric Aβ. The levels of pERK at 1.5 and 24 h and pCREB at 12 h were significantly elevated in cells pre-treated with A11 antibody followed by Aβ42 oligomer compared with cells treated with Aβ42 oligomer alone (***$p < 0.001$, *$p < 0.05$, and **$p < 0.01$, respectively, Fig. 6a and b). Total ERK and actin were unchanged confirming equal sample loading in each lane. Cell viability measured by lactate dehydrogenase was unchanged at the time of the decrease of pERK (1.5 and 24 h) or pCREB (1.5 and 12 h), suggesting that the observed effect was not simply a result of altered cell viability. A11 antibody also significantly protected from Aβ oligomer-induced delayed cytotoxicity which emerged at 24 h ($p < 0.01$, Fig. 6c).

Oligomer-specific antibody prevented Aβ oligomer-induced decreases in activated ERK and CREB in primary hippocampal neurons

To further confirm the direct impact of Aβ oligomers on neuronal ERK and CREB, cultured hippocampal neurons

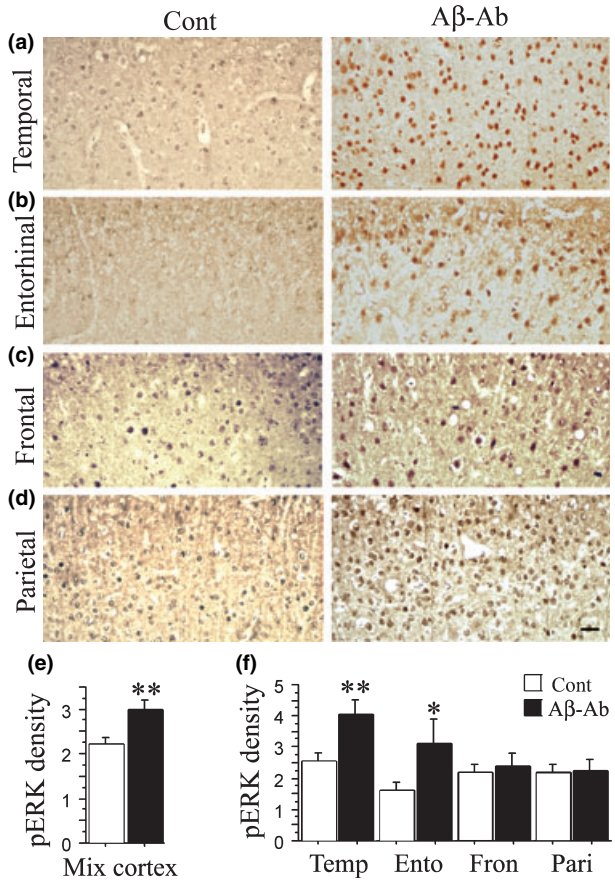

Fig. 4 Anti-Aβ-infusion (i.c.v.) into Tg2576 mice increased phosphorylated extracellular-signal regulated kinase (pERK) immunoreactivity in temporal and entorhinal cortex. Micrographs of pERK staining (representing regions dissected for western) are shown: the temporal (a), entorhinal (b), frontal (c), and parietal (d) cortex. Micrographs on left show representative sections from control infused mice. Micrographs on right show representative sections from Aβ-Ab (anti-Aβ, 10G4) infused mice. (e) Interaction bars of 2×2 ANOVA (treatment × region) of pERK density demonstrated a significant treatment effect (without interaction) $p < 0.001$. (f) Planned comparisons within regions showed intensity of pERK staining was significantly increased in anti-Aβ-infused-Tg$^+$ mice when compared with control infused mice in the temporal and entorhinal cortex, but not in the frontal and parietal cortex. Quantification of pERK (**$p < 0.01$ and *$p < 0.05$). Magnification bar = 25 μm.

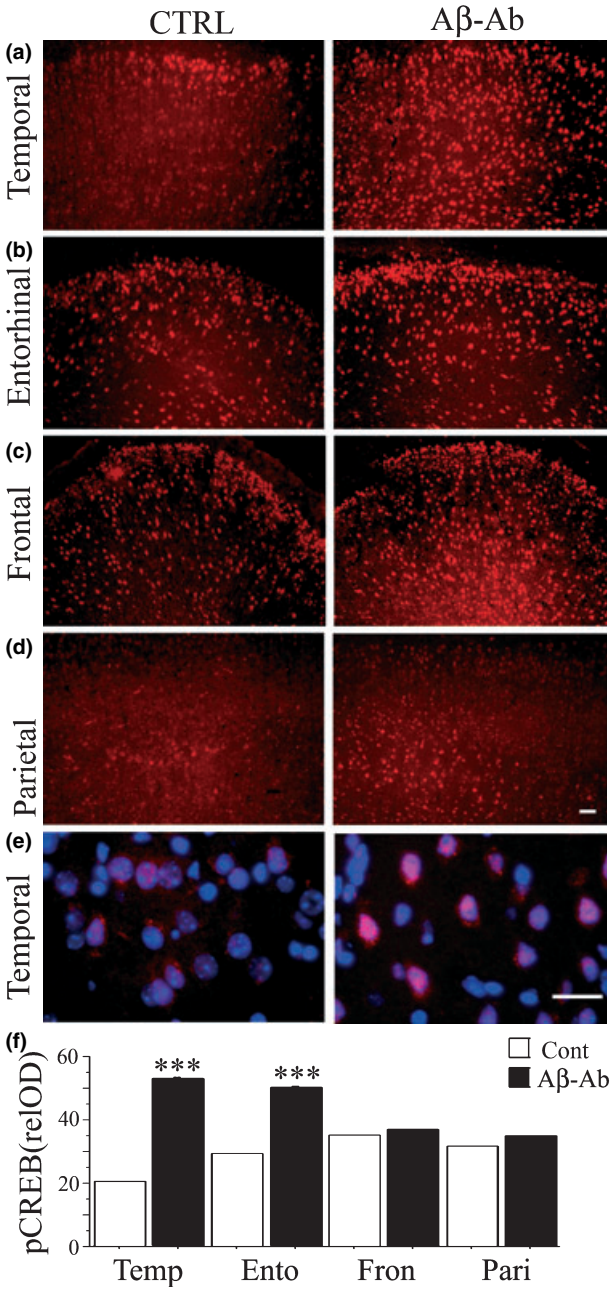

Fig. 5 Anti-Aβ-infused Tg2576 mice increased phosphorylated CREB (pCREB) in temporal and entorhinal cortex. (a) Temporal and (b) entorhinal cortical pCREB density (relOD) were increased in anti-Aβ-infused-Tg$^+$ mice when compared with controls. pCREB (relOD) in frontal (c) and parietal cortex (d) was not changed in anti-Aβ-infused-Tg$^+$ mice when compared with control infusion. Double labeling sections for nuclei with DAPI (blue) and pCREB (red) showed that anti-Aβ increased nuclear pCREB in temporal cortex of anti-Aβ-infused mice. Similar staining was also found in entorhinal cortex. (f) Quantification of pCREB (***$p < 0.0001$). Magnification bar = 25 μm.

were employed. Cells were pre-incubated with or without A11 antibody (2 ng/mL) for 1 h followed by treatment with 250 nmol/L of Aβ oligomers for 2 h. pERK and pCREB were visualized by immunofluorescence staining with anti pERK or anti pCREB (Fig. 7a and b). Aβ oligomers were visualized by staining with anti-Aβ antibodies, 6E10. Aβ oligomers specifically bound as puncta on neurons or processes (Fig. 7c) and significantly decreased the intensity of pERK ($p < 0.01$) in the cytoplasm compared with controls and these losses were reversed by A11 antibody (Fig. 7a and d). In addition, Aβ oligomers induced a redistribution of pCREB from the nucleus, significantly decreasing the intensity of nuclear pCREB ($p < 0.01$) when compared with

Table 1 Overall impact of 10G4 and IgG2b on isolectin B4 (Iba4)

	Regions							
	Frontal cortex		Parietal cortex		ML dentate		St Lac, Orien, Luc	
Parameters	IgG2b	10G4	IgG2b	10G4	IgG2b	10G4	IgG2b	10G4
Percentage area stained[#]	0.819 ± 0.155	0.446 ± 0.065[a]	1.424 ± 0.283	0.661 ± 0.182[a]	0.429 ± 0.079	0.608 ± 0.076	0.689 ± 0.087	0.413 ± 0.052
Number of cells/1000 μm^2[###]	0.512 ± 0.081	0.274 ± 0.028[a]	0.858 ± 0.128	0.506 ± 0.095[a]	0.342 ± 0.055	0.479 ± 0.066	0.359 ± 0.030	0.292 ± 0.032
Size of cell[###] (μm^2)	15.551 ± 0.756	16.331 ± 1.696	15.886 ± 1.051	11.963 ± 0.678	12.021 ± 0.909	13.881 ± 1.355	15.886 ± 1.051	14.136 ± 1.315
Intensity of cell[###]	2.117 ± 0.334	3.771 ± 0.416[a]	1.746 ± 0.399	3.442 ± 0.410[a]	3.166 ± 0.352	3.047 ± 0.281	2.514 ± 0.341	3.134 ± 0.341

[#]Treatment $p < 0.001$, region $p < 0.0002$, Interaction $p < 0.001$; [##]Treatment effect $p < 0.01$, region $p < 0.0001$, interaction $p < 0.0015$; [###]Treatment NS, region $p < 0.001$, Interaction ($p < 0.05$); [###]Treatment $p < 0.0005$, region NS, interaction ($p < 0.05$); [a]$p < 0.05$ Treatment effect within region.

control. These changes were also blocked by A11 antibody (Fig. 7b and e).

Anti-Aβ antibody blocked Aβ-induced decreases of the NMDAR subunit, NR2B

Activation of NMDARs can lead to long-term potentiation (LTP) and increased synaptic strength. The NMDAR family has a complex heteromeric composition comprised of variable NR1 and NR2 subunits. In fact, over-expression of only the NR2B subunit enhanced hippocampal LTP, prolonged NMDAR currents, and led to superior ability in learning and memory in various behavioural tasks (Tang *et al.* 1999). In contrast to NR2B, NR1 was not altered in Tg2576 (Calon *et al.* 2005). Because NR2B is directly coupled to ERK which is stimulated by NMDAR activation (Krapivinsky *et al.* 2003), we assessed the levels of NR2B. Our data revealed that anti-Aβ-infused mice had significantly increased levels of NR2B compared with controls ($p < 0.05$, Fig. 8c). In addition, when mature 19-day-old primary hippocampal neurons were treated with 250 nmol/L of Aβ oligomers for 7 h, the Aβ oligomers were located as puncta on neurons or processes bearing a decreased intensity of NR2B when compared with untreated controls. A11 antibody prevented this Aβ oligomer-induced change (Fig. 8a and b).

Discussion

We demonstrate that regardless of baseline ERK phosphorylation levels, ERK activation can be impaired by APP transgene, and this effect is mediated by Aβ oligomers. Previously age-dependent alterations in Tg2576 were evaluated in the hippocampus and baseline pERK was found to be increased as early as 4 months of age in the dentate gyrus prior to Aβ deposition (Dineley *et al.* 2001) and in the absence of detectable synaptic and neuronal loss (Irizarry *et al.* 1997). ERK hyperactivation has been linked to APP transgene-dependent up-regulation of the alpha7 nicotinic receptor and Aβ signaling to ERK (Dineley *et al.* 2001). We evaluated age-dependent changes in the cortex to see if parallel changes occurred. Unlike in the hippocampus, our results show that cortical pERK was not reduced or elevated at 12 months. Consistent with a previous report, we also observed a trend for increased baseline pERK in CA1 of the hippocampus (Dineley *et al.* 2001).

Infusion-induced pERK at both 11–12 months of age and at 16–18 months of age in wild-type (Tg^-) mice, but these robust responses were either not present or blunted in APP Tg^+ mice, demonstrating an APP transgene-mediated ERK dysregulation. To address the role of Aβ, we infused anti-Aβ antibodies. Infusion of antibodies against Aβ increased pERK and pCREB at both ages, essentially correcting the suppressed response to infusion/injury. Thus, the significant increase in ERK and CREB observed with anti-Aβ antibody

Table 2 Plaque associated microglia (ring × treatment ANOVA)

	Treatments					
	Ring 1 (cells in plaque)		Ring 2 (width = plaque radii)		Ring 3 (width = plaque radii)	
Parameters	IgG2b	10G4	IgG2b	10G4	IgG2b	10G4
Cell number[#] (per 1000 mm^2)	5.748 ± 0.655	7.075 ± 0.991	7.506 ± 0.825	5.208 ± 0.679[a]	3.835 ± 0.546	2.792 ± 0.362
Percentage area[##]	31.178 ± 2.829	25.158 ± 4.188	12.406 ± 1.325	7.074 ± 1.221[a]	3.963 ± 0.660	3.113 ± 0.555
Average cell size[###] (mm^2)	80.275 ± 12.120	112.216 ± 41.65	12.588 ± 1.854	12.632 ± 2.106	8.373 ± 0.760	12.463 ± 2.025

[#]Treatment effect NS, Ring effect $p < 0.0001$, Interaction $p < 0.05$; [##]Treatment effect $p < 0.05$, Ring effect $p < 0.0001$, Interaction NS; [###]Treatment effect NS, Ring effect $p < 0.00001$, Interaction NS; [a]$p < 0.05$ between treatments within ring.

Table 3 Effect of Aβ antibody infusion on IL-1β, TNFα, GFAP, and synaptic markers (mean ± SE)

Tg + Mice groups	IL-1β (fg/μg)	TNFα (fg/μg)	GFAP (OD)	PSD-95 (OD)	Drebrin (OD)	Synaptophysin (OD)	SNAP-25(OD)
Ig2b-infused	22.91 ± 2.99	11.47 ± 1.16	15.70 ± 1.44	9.62 ± 0.94	2.69 ± 0.44	3.50 ± 0.21	1.81 ± 0.12
Anti-Aβ infused	28.05 ± 3.97	12.17 ± 1.20	17.71 ± 1.68	9.27 ± 1.93	3.35 ± 1.39	3.40 ± 0.34	1.65 ± 0.13
p-value	0.87	0.71	0.72	0.89	0.43	0.78	0.45
Assay methods	ELISA	ELISA	WB	WB	WB	WB	WB

IL-1β, interleukin 1β; PSD-95, post-synaptic density-95; TNFα, tumor necrosis factor-α; WB, western immunoblotting; Aβ, β-amyloid peptide.

treatment cannot be viewed simply as a correction of a pre-existing baseline deficit, but rather as a correction of a blunted ERK/CREB response to CNS infusion-induced factors. Trophic factors are one possibility because subtoxic levels of Aβ oligomers have been reported to blunt the ERK/CREB response to trophic factors including nerve growth factor (Chromy *et al.* 2003) and brain derived neurotrophic factor (Tong *et al.* 2004). IGF-1 is one example of a persistent cannulation-induced trophic factor (Garcia-Estrada *et al.* 1992). Our results showing that IGF induced ERK in SY5Y neuroblastoma is attenuated by Aβ oligomers further support the idea that Aβ oligomers may diminish ERK signaling. While we found the Tg$^+$ mice had lost the ERK response to injury-induced stimulation, our results are consistent with the hypothesis that oligomers in APP Tg$^+$ mice reduce the ERK response to injury-induced trophic factors.

In contrast to 12 month mice, significant cortical ERK and CREB deficits develop in older Tg2576 mice. These were further exacerbated by an omega-3 fatty acid depleted diet (not shown) that also increased selected synaptic deficits (Calon *et al.* 2005). Our *in vivo* and *in vitro* immunoneutralization data with anti-Aβ and anti-oligomer antibodies data suggest that these deficits are due to Aβ oligomers. Preparations enriched in Aβ oligomers, including trimer, 12-mer, and 24-mer, significantly down-regulated the level of active ERK (1.5 and 24 h) and active CREB (1.5 and 12 h). The conformation-specific anti-oligomer antibody, A11, ameliorated this Aβ-induced inhibition of active ERK and CREB in human SH-SY5Y cells and rat primary hippocampal neurons. As A11 antibody fails to detect monomer, low molecular weight oligomers, or fibrils (Kayed *et al.* 2003), active ERK and CREB losses may be attributed to one or more high molecular weight Aβ oligomer species.

Our findings of Aβ-dependent ERK suppression are distinct from data obtained with chronic incubation with aged or fibrillar Aβ which caused persistent ERK activation in hippocampal slice cultures (Bell *et al.* 2004) and aged hippocampal neurons (Anderson and Ferreira 2004). However, they are consistent with experiments using freshly prepared Aβ42 containing predominantly hexameric oligomer species that resulted in an initial ERK activation followed by ERK down-regulation at 16 h (Bell *et al.* 2004). Aβ or Aβ25–35 induced suppression of ERK or its downstream intermediate CREB have also been observed in neuroblastoma cells (Daniels *et al.* 2001), endothelial cells (Magrane *et al.* 2006), hippocampal preparations (Xie 2004), and in rats after injection of Aβ25–35 (Jin *et al.* 2005). *In vitro*, Aβ effects on ERK activation may reflect dose, aggregation status and treatment duration. Because we used 250–500 nmol/L of pre-aggregated Aβ42 with a predominant 12-mer (dodecamer) species to treat cells, we may have obtained a more rapid ERK down-regulation, consistent with the hypothesis that the Aβ 12-mer is a very potent inhibitor of mechanisms underlying cognitive function.

Large (> 50%) pCREB deficits have been reported in hippocampus of AD patients (Yamamoto-Sasaki *et al.* 1999). Although neither pERK nor pCREB show early hippocampal deficits in Tg2576 mice (Dineley *et al.* 2001), pCREB deficits occur in other regions. For example, we observed pCREB deficits in 16-month-old Tg2576 mouse entorhinal cortex where it was restored by treatment with curcumin (Cole *et al.* 2003), a compound that blocks Aβ

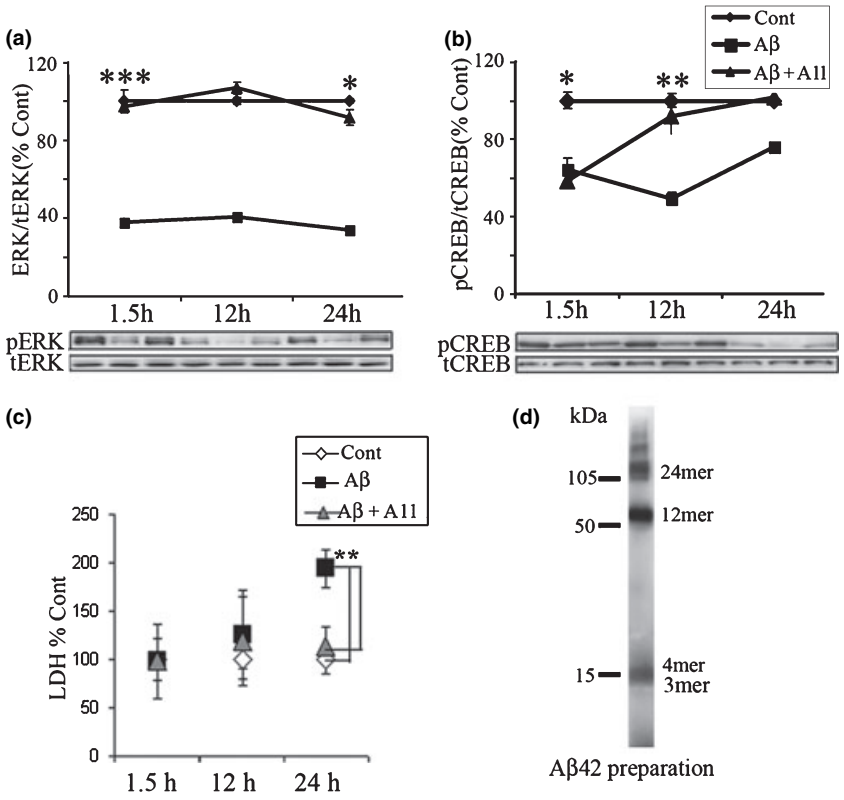

Fig. 6 Oligomer-specific antibody A11 prevented Aβ oligomer-induced decreases in extracellular-signal regulated kinase (ERK) and cAMP-response element-binding protein (CREB) activation in SH-SY5Y cells. (a) A11 oligomer-specific antibody prevented Aβ oligomer-induced decreases in phosphorylated ERK (pERK) activation in SH-SY5Y cells. Cells treated with Aβ oligomers alone for 1.5 or 24 h had significantly decreased levels of pERK when compared with vehicle (***$p < 0.001$ and *$p < 0.05$). pERK was significantly increased in cells pre-treated with A11 antibody when compared with cells treated with Aβ42 oligomers alone (***$p < 0.001$ and *$p < 0.05$). β-Actin levels were unchanged. Panels show representative pERK changes and unaltered total ERK (tERK) on westerns. (b) Oligomer-specific antibody prevented Aβ oligomer-induced decreases in CREB activation in SH-SY5Y cells. Cells treated with Aβ oligomers alone for 1.5 or 12 h showed significantly decreased levels of pCREB compared with vehicle (*$p < 0.05$ and **$p < 0.01$). The level of pCREB was significantly increased in cells pre-treated with A11 antibody at 12 h when compared with cells treated with Aβ42 oligomers alone (**$p < 0.01$). The levels of β-actin were unchanged. Panels show representative pCREB loss with preservation of total CREB (tCREB). (c) The level of released lactate dehydrogenase (LDH) in Aβ oligomers treated alone was not significantly different from that of Aβ oligomer plus A11 antibody, or control at 1.5 or 12 h, but toxicity was evident by 24 h. At 24 h, A11 antibodies significantly protected from Aβ oligomer-induced cytotoxicity (**$p < 0.01$). (d) Synthetic Aβ oligomer was characterized by western immunoblotting with anti-Aβ antibody, 6E10. Error bars represent SEM, $n = 3$ wells/per treatment.

oligomer and fibril formation (Yang *et al.* 2005) and reduces cognitive deficits induced by chronic soluble Aβ infusion (Frautschy *et al.* 2001). Defects in CREB signaling are likely Aβ-dependent. They are relevant to cognitive function because in bigenic mutant APP × presenlin 1 transgenic mice, increasing cAMP/protein kinase A (PKA)/CREB signaling with rolipram ameliorated deficits in LTP and cognitive function (Gong *et al.* 2004). PKA activity has been reported to be reduced by Aβ aggregates *in vitro* and in bigenic APP × presenlin 1 mice secondary to a 30–50% decrease in Uch-L1 hydrolase, but 9–12 month Tg2576 (used in our experiments) have only about a 15% decrease in Uch-L1 and no reported change in PKA activity or protein (Gong *et al.* 2006). Whether involving ERK or PKA or both, data from three very different compounds in three model systems show correction of Aβ-induced cognitive deficits involving concomitant increases in pCREB. Given the known role of CREB in plasticity, small molecules capable of increasing pCREB may be a useful downstream therapeutic approach.

Aberrantly increased or decreased, but dysregulated ERK signaling is likely to impact human cognition. ERK signaling events have been studied extensively in recent years for involvement in synaptic plasticity and memory function (English and Sweatt 1997). ERK alterations are involved in a human mental retardation syndrome, neurofibromatosis 1, in which defects in the ras/ERK signaling cascade contribute to hippocampal-dependent learning and memory deficits (Costa

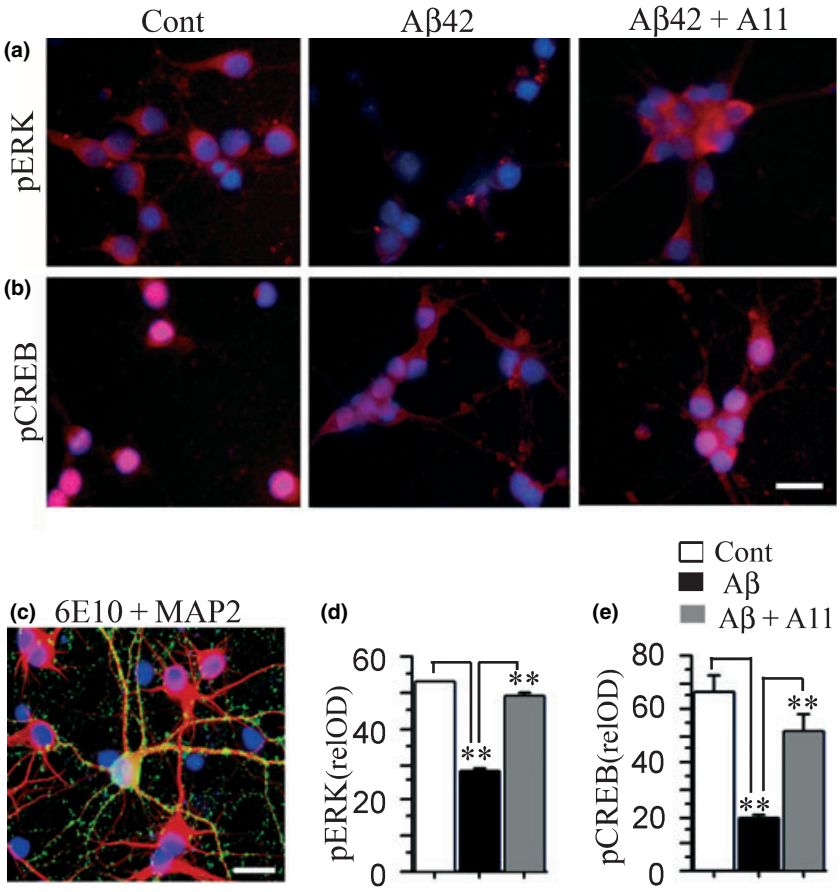

Fig. 7 Oligomer-specific antibody A11 prevented Aβ oligomer-induced decreases in phosphorylated extracellular-signal regulated kinase (pERK) and pCREB in primary hippocampal neurons. Primary hippocampal neurons were incubated with control (rabbit IgG2b, 2 ng/mL), 250 nmol/L of Aβ oligomers or Aβ oligomers plus A11 antibody (2 ng/mL) and visualized by immunofluorescence staining with anti-pERK, pCREB, MAP2, and Aβ antibodies. (a) Aβ oligomers were added to 7-day-old primary hippocampal cultures. Decreased pERK, evident after 2 h treatment, was prevented by oligomer-specific A11 antibody. In control, pERK (red) normally distributed to cytoplasm. In Aβ oligomer-treated neurons, the intensity of pERK in cytosol was significantly decreased, which was partially reversed by A11 antibody. (b) Aβ oligomers also decreased pCREB after 2 h treatment, which was prevented by oligomer-specific A11 antibody. In control, pCREB immunoreactivity was highly localized to the nucleus. In Aβ oligomer-treated neurons, the distribution of nuclear pCREB was altered towards cytosol and processes. A11 antibody largely prevented the Aβ oligomer-induced redistribution of pCREB. (c) Aβ oligomer-treated neurons were stained by MAP2 (red), Aβ antibody, 6E10 (green). Aβ oligomers specifically bound as puncta on neurites in Aβ oligomer-treated neurons. (a–c) The nucleus was stained by DAPI (blue). Fluorescent quantification of pERK (d) and pCREB (e) confirmed significance of observations. Magnification bar = 25 μm.

et al. 2002). Normally, ERK participates in regulating local dendritic protein synthesis (Ying *et al.* 2002), processing molecular information in dendrites (Watanabe *et al.* 2002), and stabilizing structural changes in dendritic spines (Goldin and Segal 2003).

Extracellular-signal regulated kinase activation occurs in neurons in response to excitatory glutamatergic signaling (Kurino *et al.* 1995; Xia *et al.* 1996). Glutamate receptors including NMDARs activate multiple biochemical pathways that transduce post-synaptic signals (Sheng *et al.* 2002). A reduction in specific NMDAR subunits, notably NR2B, occurs in AD patients and correlates with cognitive deficits (Greenamyre *et al.* 1987; Mishizen-Eberz *et al.* 2004). In Tg2576 mice, omega-3 fatty acid deficiency aggravates APP transgene-dependent decreases in NR2B, the developmentally regulated brain protein, drebrin, and PSD-95, all indices of a defect in excitatory neurons (Calon *et al.* 2004). In the present study, we found that anti-Aβ-infused Tg2576 mice had a significantly increased level of NR2B. In primary hippocampal neuron culture, Aβ oligomers specifically bound to neurons and neurites as puncta associated with decreased NR2B, consistent with a selective effect on NMDARs trafficking by Aβ (Snyder *et al.* 2005).

Considerable evidence indicates that immunotherapeutic approaches against Aβ reverse cognitive deficits (Morgan *et al.* 2000; Klyubin *et al.* 2005) and promote the rapid

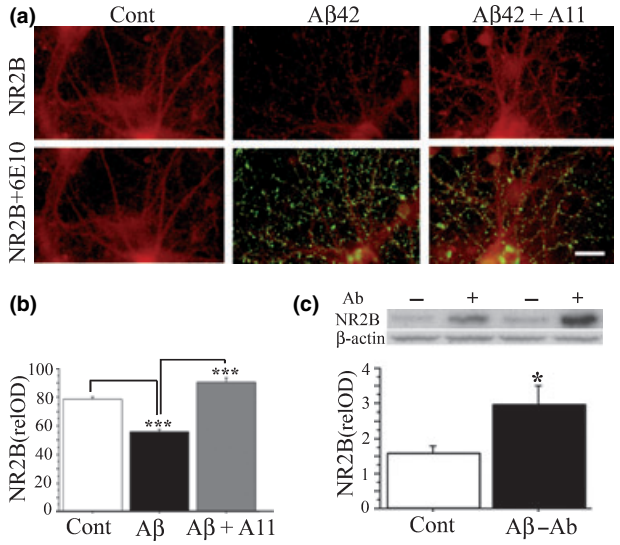

Fig. 8 Anti-Aβ antibodies prevented Aβ-induced decreases of NMDA receptor subunit (NR2B) in Tg2576 mice and in primary hippocampal neurons. (a) Aβ oligomer decreased NR2B after 7 h treatment, which was partially reversed by A11 antibody, in 19-day-old hippocampal neurons. In control cells, NR2B immunoreactivity was highly localized to cytoplasm and dendrites (red). Aβ oligomers specifically bound to neurites (green) and reduced the intensity of NR2B. A11 antibody prevented an Aβ oligomer-induced NR2B decrease. (b) Quantification of NR2B from primary hippocampal neurons (c) Western analysis of NR2B in the cortex of IgG2b-infused control mice and anti-Aβ-infused Tg^+ mice. Anti-Aβ-infused mice significantly increased the levels of NR2B when compared with IgG2b-infused control mice ($^*p < 0.05$). Magnification bar = 25 μm.

recovery of amyloid-associated neuritic dystrophy in APP mutant transgenic mice (Brendza *et al.* 2005). However, the mechanisms to explain why Aβ antibodies acutely improve learning and memory deficits even without clearance of Aβ-related pathology are not well understood. This study provides initial evidence that Aβ antibody treatment of 11–12 month Tg2576 mice has rapid effects on Aβ oligomer-modulated signal transduction, increasing NR2B and downstream active ERK and CREB, all critical components of signal transduction pathways involved in learning and memory. While insoluble deposits were not significantly reduced in our acute study, they may not play a central role in cognitive deficits. Some AD transgenic mouse models show significant cognitive dysfunction and synaptic damage but have no insoluble Aβ deposits (Kumar-Singh *et al.* 2000; Mucke *et al.* 2000). Insoluble Aβ deposits do not predict the degree of dementia in AD patients (McLean *et al.* 1999) and evidence has emerged suggesting that soluble Aβ oligomer may be a key toxic form of Aβ implicated in AD pathogenesis. For example, soluble oligomeric forms of Aβ caused selective synaptic protein loss and cognitive decline in Aβ oligomer-infused rats (Frautschy *et al.* 2001) and inhibited LTP and specifically disrupted cognitive function (Cleary *et al.* 2005). In particular, Aβ 12-mer has been detected as a highly stable, soluble extracellular aggregate in Tg2576 mice that is sufficient to induce cognitive deficits (Lesne *et al.* 2006). Our previous study demonstrated that an acute treatment with anti-Aβ antibody not only neutralized the level of ~56 kDa oligomer (12-mer) of Aβ, but also counteracted the effect of Aβ oligomer-induced activation of the GSK3β/ptau signaling cascade without a significant reduction of insoluble Aβ in Tg2576 mice (Ma *et al.* 2006). Aβ vaccine or anti-Aβ antibodies are still among the most promising treatments for AD. A clinical trial report from one of the groups that participated in the ELAN/Wyeth-Ayerst AN1792 (QS-21) Phase 2A multicenter trial found benefits in patients who both received a primary and a booster immunization of Aβ42 and generated anti-Aβ plaque immunoreactivity (tissue amyloid plaque immunoreactivity assay). Compared with patients who are without such antibodies, the responders, including several with inflammatory side effects appeared to show significantly slower rates of decline of cognitive decline (Hoek *et al.* 2003).

In summary, this study shows that soluble Aβ oligomers can blunt the ERK and CREB response *in vivo* and provides direct evidence that passive immunization with anti-Aβ antibodies can increase ERK/CREB activation and NMDAR (NR2B) in Tg2576 mice and in oligomer-treated neuronal cultures. These data also strongly support therapeutic strategies targeting Aβ oligomer by highly specific anti-Aβ oligomer antibodies or drugs to limit dysregulation of multiple memory-related signaling pathways, including oligomer sensitive NMDAR/ERK/CREB, Akt/GSK3β/ptau (Ma *et al.* 2006), and PAK/LIMK/cofilin/drebrin (Zhao *et al.* 2006).

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