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RESEARCH PAPER

The Ca^{2+} channel inhibitor 2-APB reverses β-amyloid-induced LTP deficit in hippocampus by blocking BAX and caspase-3 hyperactivation

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BACKGROUND AND PURPOSE

At the early stage of Alzheimer's disease (AD), the accumulation of β-amyloid (Aβ) oligomers disturbs intracellular Ca^{2+} homeostasis and disrupts synaptic plasticity of brain neurons. Prevention of Aβ-induced synaptic failure remains an unsolved problem for the treatment of AD. Here, the effects of 2-aminoethoxydiphenyl borate (2-APB), a non-specific, but moderately potent Ca^{2+} channel inhibitor, on Aβ-induced deficit of synaptic long-term potentiation (LTP) and the underlying molecular mechanisms were explored.

EXPERIMENTAL APPROACH

We used hippocampal slices and primary cultures of hippocampal neurons from C57BL/6 mice. Methods applied in our study included electrophysiological recording, membrane protein extraction, Western blot assay and Ca^{2+} imaging.

KEY RESULTS

2-APB at 10 μM effectively reversed suppression by oligomeric $A\beta_{1-42}$ (500 nM) of LTP in hippocampal slices. 2-APB also restored phosphorylation and trafficking of the glutamate receptor subunit GluA1 in Aβ-treated hippocampal slices, supporting its protective action on synaptic function. Aβ-mediated abnormal neuronal $[Ca^{2+}]_i$ elevation and hyperactivation of the mitochondrial apoptotic proteins BAX, caspase-3, and glycogen synthase kinase-3β, were blocked by 2-APB pretreatment. Moreover, the defict in long term potentiation deficit in hippocampal slices from $APP_{swe}/PS1_{\Delta E9}$ gene mutant mice was rescued by 2-APB at 10 μM.

CONCLUSIONS AND IMPLICATION

These data demonstrate that 2-APB is a potentially useful chemical to protect synaptic plasticity against neurotoxic effects of Aβ in AD.

Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; Aβ, amyloid β; ACSF, artificial CSF; AD, Alzheimer's disease; APP, amyloid precursor protein; ER, endoplasmic reticulum; GSK3αβ, glycogen synthase kinase 3 subunits α and β; fEPSP, field excitatory postsynaptic potential; IP_3, inositol 1,4,5-trisphosphate; LTP, long-term potentiation; PS1, presenilin 1; PTP, post-tetanic potentiation; RyR, ryanodine receptor; SOCE, store-operated calcium entry; TBS, θ burst stimulation; TRP, transient receptor potential

Tables of Links

TARGETS	
Enzymes[a]	**Ion channels**[c]
Caspase-3	IP_3 receptors
GSK3β, glycogen synthase kinase-3β	RyR, ryanodine receptors
Presenilin 1	TRP channels
Ligand-gated ion channels[b]	
GluN2A	
GluN2B	
GluA1	

LIGANDS
β-Amyloid (Aβ) peptide
2-APB, 2-aminoethoxydiphenyl borate
Memantine
Z-DEVD-FMK

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Extensive studies demonstrate that the overproduction of soluble β-amyloid (Aβ) peptides in elderly brain is a key pathogenic factor at the onset of Alzheimer's disease (AD) (Selkoe, 2001; Haass and Selkoe, 2007). At the earliest stage of AD, dysregulation of synaptic efficacy is a notable feature, even prior to neuronal loss (Selkoe, 2002). Diffusible Aβ oligomeric assembles potently inhibit synaptic long-term potentiation (LTP), an important form of synaptic plasticity, and cognitive functions (Walsh *et al.*, 2002; Cleary *et al.*, 2005; Townsend *et al.*, 2006; Shankar *et al.*, 2008). However, thus far, how to effectively protect synaptic functionality against the neurotoxicity caused by the accumulation of Aβ oligomers remains an unsolved problem in the treatment of AD.

Intracellular Ca^{2+} homeostasis is vital in regulating age-related neuronal survival and synaptic plasticity (Mattson, 2007; Bading, 2013). The accumulation of exitotoxic Aβ oligomers excessively increases plasma membrane Ca^{2+} permeability through modulation of membrane Ca^{2+}-permeable channels, formation of non-selective cation pores, and disruption of membrane lipid integrity (Demuro *et al.*, 2010; Kawahara *et al.*, 2011). The Ca^{2+} overload process disturbs the stability of intraneuronal Ca^{2+} homeostasis and exerts a series of adverse effects on synaptic structure and function, neuronal physiology and memory formation (Smith *et al.*, 2005; Mattson, 2007; Bojarski *et al.*, 2008; Palop and Mucke, 2010).

Endoplasmic reticulum (ER) stress and mitochondrial dysfunction are major events underlying neuronal apoptosis triggered by Aβ oligomers (Umeda *et al.*, 2011). It was reported recently that even nanomolar concentrations of Aβ peptides can increase mitochondrial Ca^{2+} amount and the interplay between ER and mitochondria (Hedskog *et al.*, 2013). Extracellular Ca^{2+} influx through plasma membrane and ER Ca^{2+} efflux from ryanodine (RyR) and inositol 1,4,5-trisphosphate (IP_3) receptors lead to cytosolic Ca^{2+} overload, which then initiates the translocation of BAX (a proapoptotic protein of the Bcl-2 family) to mitochondrial membranes, the activation of apoptotic caspase-3 signalling, the mitochondrial fragmentation, and even the neuronal death (Scorrano *et al.*, 2003; Ferreiro *et al.*, 2006; 2008; Wang *et al.*, 2010; Umeda *et al.*, 2011; Sanmartin *et al.*, 2012). Activations of BAX and caspase-3 have been shown to be essential for Aβ suppression of LTP in hippocampus (Jo *et al.*, 2011; Olsen and Sheng, 2012). Increased activity of glycogen synthase kinase-3β (GSK3β), a downstream member of caspase-3 apoptotic signalling, also mediates Aβ inhibitory effect on mitochondrial function and hippocampal LTP (Jo *et al.*, 2011; Mines *et al.*, 2011; Reddy, 2013).

Normalization of disturbed Ca^{2+} homeostasis and blockade of Ca^{2+}-stimulated pathogenic signalling cascades have been proposed as potentially therapeutic strategies to reduce cognitive defects in AD (Yu *et al.*, 2009; Demuro *et al.*, 2010). 2-aminoethoxydiphenyl borate (2-APB) is a non-specific, but moderately potent membrane-permeable modulator of Ca^{2+} channels with inhibitory actions on several targets including IP_3 receptors (Maruyama *et al.*, 1997; Peppiatt *et al.*, 2003; Hagenston *et al.*, 2009; Ansari *et al.*, 2014), RyR (Ansari *et al.*, 2014), store-operated calcium entry (SOCE) channels (Dobrydneva and Blackmore, 2001; Bootman *et al.*, 2002; Peppiatt *et al.*, 2003) and transient receptor potential (TRP) channels (Ma *et al.*, 2001; Xu *et al.*, 2005; Kovacs *et al.*, 2012). These Ca^{2+} channels are involved in various neuronal

processes such as apoptotic signalling and synaptic transmission. Protective effects of 2-APB against H_2O_2-induced apoptosis in neuron-like PC12 cells (Ansari *et al.*, 2014) and Aβ neurotoxicity (Suen *et al.*, 2003) have been reported. In this study, we investigated whether 2-APB would protect synaptic LTP against Aβ-induced neurotoxicity. We found that 2-APB prevented Aβ suppression of hippocampal LTP, most likely through a mechanism blocking Aβ-induced hyperactivation of BAX and caspase-3.

Methods

Animals

All animal care and experimental procedures complied with the institutional guidelines and were approved by the Animal Ethics Committee, Kunming Medical University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Ninety-five C57BL/6 wild-type mice and 24 amyloid precursor protein/presenilin 1 ($APP_{swe}/PS1_{\Delta E9}$) gene mutant mice and their wild-type littermates were used in the present study. APP/PS1 mutant mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were kept under a 12 h/12 h light/dark cycle with the light on at 07:00 a.m. They were fed with standard chow and water *ad libitum*. Ambient temperature and relative humidity were maintained at 22 ± 2°C and 50 ± 5% respectively.

Reagents

The preparation of soluble oligomeric $A\beta_{1-42}$ has been described previously (Stine *et al.*, 2011). Briefly, HFIP (1, 1, 1, 3, 3, 3 – hexafluoro-2-propanol) pretreated $A\beta_{1-42}$ (rPeptide Company, Bogart, GA, USA) was suspended in 100% DMSO to the concentration of 5 mM, and then sonicated in ice-cold water for 3 min. Peptides were further 50 × diluted with phenol-red free DMEM/F12 to the final concentration of 100 μM, and sonicated in ice-cold water for another 3 min. The sonication was repeated if the peptides were not sufficiently dissolved. The peptide solution was divided into aliquots and incubated at 4°C for 24 h, and then stored at –80°C.

On each experimental day, one Aβ aliquot was diluted to the final concentration of 500 nM and incubated with hippocampal slices or cultured neurons for 1 h at room temperature. The inhibitors, 2-APB (Sigma, St Louis, MO, USA) and Z-DEVD-FMK (a specific caspase-3 inhibitor; Sigma), were dissolved in DMSO to provide a stock solution and then stored at –20°C until used. The final concentration of DMSO was kept at 0.05%.

Hippocampal slice preparation

Three-month-old male C57BL/6 mice were decapitated after light ether anaesthesia, and then hippocampal slices (400 μm) were prepared in ice-cold and oxygenated (95% O_2 and 5% CO_2) artificial CSF (ACSF) (mM: NaCl 126, KCl 2.5, NaH_2PO_4 1, $CaCl_2$ 2.5, $MgSO_4$ 1.5, $NaHCO_3$ 26 and glucose 10) using a vibratome (WPI, Sarasota, FL, USA). Slices were allowed to recover at room temperature for at least 1 h before used.

Electrophysiological recording

Hippocampal slices were transferred into a recording chamber (PSMI; Harvard Apparatus, Holliston, MA, USA), in which ACSF perfusion was kept at the rate of 1–2 $mL \cdot min^{-1}$. The Schaffer collateral was stimulated by a concentric bipolar electrode (Frederick Haer Co., Bowdoinham, ME, USA). Field excitatory postsynaptic potentials (fEPSPs) were recorded at the stratum radiatum of the hippocampal CA1 region by a glass microelectrode filled with 3 M NaCl (resistance 1–4 MΩ). Once an optimal fEPSP wave was found, different stimulating intensities (0.2 ms duration) were applied to establish an input–output (I/O) curve. Baseline fEPSPs were recorded at 0.033 Hz with a stimulating strength adjusted to yield about 40% of the maximal response. After stable baseline responses had lasted for at least 30 min, LTP was induced by delivering θ burst stimuli (TBS; four trains of 10 bursts of four pulses with 20 s, 200 ms and 10 ms intervals between trains, bursts and pulses respectively). All electrophysiological signals were filtered at 0.1–5.0 KHz and acquired with a multi-clamp 700A amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Data were analysed with Clampfit 10.0 software (Axon Instruments).

Membrane protein extraction

Membrane proteins were extracted from hippocampal slices of adult male C57BL/6 mice using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific, Waltham, MA, USA). Briefly, hippocampal slices were first lysed with a detergent, after which a second detergent was added to solubilize membrane proteins. The cocktail was incubated at 37°C for 30 min. A hydrophobic protein fraction was separated from the hydrophilic protein fraction through phase partitioning. For SDS-PAGE, membrane protein fractions were diluted two- to fivefold to prevent band and lane distortion caused by high concentrations of detergent.

Hippocampal neuron culture

Primary hippocampal neurons were prepared from embryonic 18 day C57BL/6 mice as previously reported (Brewer *et al.*, 1993). The cells were seed on 24-well poly-D-lysine-coated glass slides and cultured with Neurobasal medium containing 2% B27 supplement (Invitrogen, Thermo Scientific), l-glutamine (0.5 mM) and 0.1 $mg \cdot mL^{-1}$ P/S (Invitrogen) for 7 days.

Western blot assay

Hippocampal slices or neuronal samples were homogenized in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCL, pH 8.0) containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were then dissolved in 2 × Laemmli sample buffer (Biorad, Hercules, CA, USA), and boiled at 95°C for 5 min. Each sample of lysates (20 μL) was added into SDS-PAGE and electroblotted onto PVDF membranes. The membranes were blocked with 5% skim milk in TBS-T, and then probed with desired antibodies. Primary antibodies against BAX (BD Pharmingen, San Diego, CA, USA), caspase-3 (Abcam, Cambridge, MA, USA), cleaved caspase-3 (Cell Signaling, Danvers, MA, USA), GSK3 (Cell Signaling), p-GSK3 (Cell Signaling), GluA1 (Abcam), p-GluR1 (Abcam), GluN2B (Millipore, Bill-

erica, MA, USA), GluN2A (Millipore) and β-actin (Sigma) were used. Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) was used to detect antibody–antigen complexes.

Ca^{2+} imaging

Ca^{2+} fluorescence images were captured as previously described (Lee *et al.*, 2013). Cultured hippocampal neurons were washed three times with HBSS solution (mM: NaCl 136, KCl 5.4, glucose 5.6, KH_2PO_4 0.4, Na_2HPO_4 0.1). Fluo3-AM (a sensitive Ca^{2+} fluorescence indicator; Dojindo Laboratories, Kumamoto, Japan) working solution was diluted to the final concentration of 4 μM with neurobasal medium. Neurons were incubated with Fluo3-AM at 37°C for 30 min, and then washed three times with HBSS, after which neurobasal medium was re-introduced. Ca^{2+} fluorescence was continuously detected for 1 h before and after the addition of compounds under an argon laser scanning confocal microscope (Leica, Wetzlar, Germany) with the excitation wavelength at 488 nm and the emission wavelength at 525 nm. Background fluorescence was detected from areas without neuronal cells and subtracted from detected fluorescence intensities. The fluorescence intensity was quantified by LASAF software (Leica) to evaluate the level of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).

Data analysis

All values were expressed as means ± SEM. Repeated measures two-way ANOVA was used to analyse electrophysiological and Ca^{2+} imaging data. One-way ANOVA was used to analyse Western blotting data of multiple groups. *Post hoc* Tukey's tests were applied when needed. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 10.0 software (SPSS, Inc., Chicago, IL, USA).

Results

2-APB prevented Aβ suppression of hippocampal LTP

Short term incubation of $A\beta_{1–42}$ oligomers at a concentration of 500 nM markedly induces LTP deficit in hippocampal slices (Jo *et al.*, 2011; Olsen and Sheng, 2012). In this study, the same concentration (500 nM) of $A\beta_{1–42}$ oligomers was used to incubate C57BL/6 mouse hippocampal slices for 1 h. Then, TBS was delivered at Schaffer collateral fibres to induce synaptic LTP in hippocampal area CA1 neurons. As shown in Figure 1A, LTP was successfully established in vehicle pretreated slices (155.3 ± 4.3%), but LTP in $A\beta_{1–42}$ pretreated slices was reduced (110.0 ± 2.5%). Data of fEPSP slopes during 0–10 min (i.e. post-tetanic potentiation, PTP) and 50–60 min (i.e. LTP) after TBS application were summarized and compared in Figure 1B. Repeated measures two-way ANOVA revealed that both PTP, which is considered as a form of short-term synaptic plasticity, and LTP were significantly impaired in Aβ pretreated hippocampal slices (treatment: $F_{(4, 36)} = 20.4$; the interaction of treatment × time: $F_{(136, 1224)} = 3.8$, both $P < 0.01$; Aβ vs. vehicle: $P < 0.01$ for PTP and LTP, Tukey's tests).

To rescue Aβ-induced synaptic plasticity deficit, hippocampal slices were co-incubated with Aβ and different concentrations (2.5, 5.0 and 10.0 μM) of 2-APB for 1 h before LTP induction. The application of 2-APB showed concentration-dependent reversal effects on Aβ-impaired synaptic plasticity (Figure 1A and B). 2-APB at 5 and 10 μM, but not at 2.5 μM effectively rescued PTP (both $P < 0.01$; Tukey's tests) and LTP ($P < 0.01$ vs. Aβ treatment; Tukey's tests) deficits in Aβ co-incubated hippocampal slices. These concentrations of 2-APB *per se* did not obviously affect synaptic LTP (treatment: $F_{(3, 27)} = 0.4$; the interaction of treatment × time: $F_{(102, 918)} = 0.5$, both $P > 0.7$; Figure 1C and D). Furthermore, no unequivocal change was found for the I/O curves after the treatments of Aβ, 2-APB (10 μM), and Aβ + 2-APB (10 μM) when compared with that after the vehicle treatment (all $P > 0.05$; Figure 1E), indicating that these treatments did not alter basal synaptic neurotransmission.

2-APB restored AMPA receptor phosphorylation and trafficking

Many studies demonstrate that AMPA receptor trafficking critically regulates synaptic functions including LTP and memory (Keifer and Zheng, 2010; Anggono and Huganir, 2012). Oligomeric Aβ aggregation mediates abnormal removal of AMPA receptors from synaptic membranes, interrupts the delivery of intracellular AMPA receptor subunits to synapses, and thus suppresses hippocampal LTP (Hsieh *et al.*, 2006; Gu *et al.*, 2009; Minano-Molina *et al.*, 2011). As 2-APB at 10 μM completely reversed Aβ suppression of LTP (Figure 1A and B), it is feasible that the concentration of 2-APB may abort any Aβ-mediated disruptive effects on AMPA receptor trafficking. To examine this possibility, membrane proteins were extracted from hippocampal slices incubated with vehicle, Aβ, 2-APB (10 μM), or Aβ + 2-APB (10 μM) for 1 h, and probed for AMPA and NMDA receptor expressions with Western blot assays. Results showed that the expression of the total GluA1 subunit was normal in Aβ-treated hippocampal slices, whereas membrane surface GluA1 was significantly reduced following the Aβ treatment when compared with that following the vehicle treatment (one-way ANOVA, $F_{(3, 15)} = 10.2$, $P = 0.001$; Aβ vs. vehicle: $P < 0.05$, Tukey's tests; Figure 2A). Because both Ser^{831}-phosphorylated GluA1 and surface GluA1 are reduced in APP transgenic mice carrying the Swedish mutation (Gu *et al.*, 2009), we also checked the Ser^{831}-phosphorylated GluA1 level in lysed hippocampal slices from C57BL/6 mice. As shown in Figure 2A, Aβ incubation dramatically reduced the levels of Ser^{831}-phosphorylated GluA1 ($F_{(3, 15)} = 17.4$, $P < 0.001$; Aβ vs. vehicle: $P < 0.05$, Tukey's tests). Interestingly, 2-APB at the same concentration (10 μM) which completely prevented Aβ impairment of LTP also effectively inhibited Aβ-induced decrease of the Ser^{831}-phosphorylated GluA1 level and the membrane surface GluA1 expression (Tukey's tests: both $P < 0.05$, Aβ vs. Aβ + 2-APB; Figure 2A). The application of 2-APB (10 μM) alone did not cause any obvious changes of GluA1 (Figure 2A). We also detected the membrane surface expression of two NMDA receptor subunits, GluN2A and GluN2B. Results showed that Aβ, 2-APB, and Aβ + 2-APB did not affect

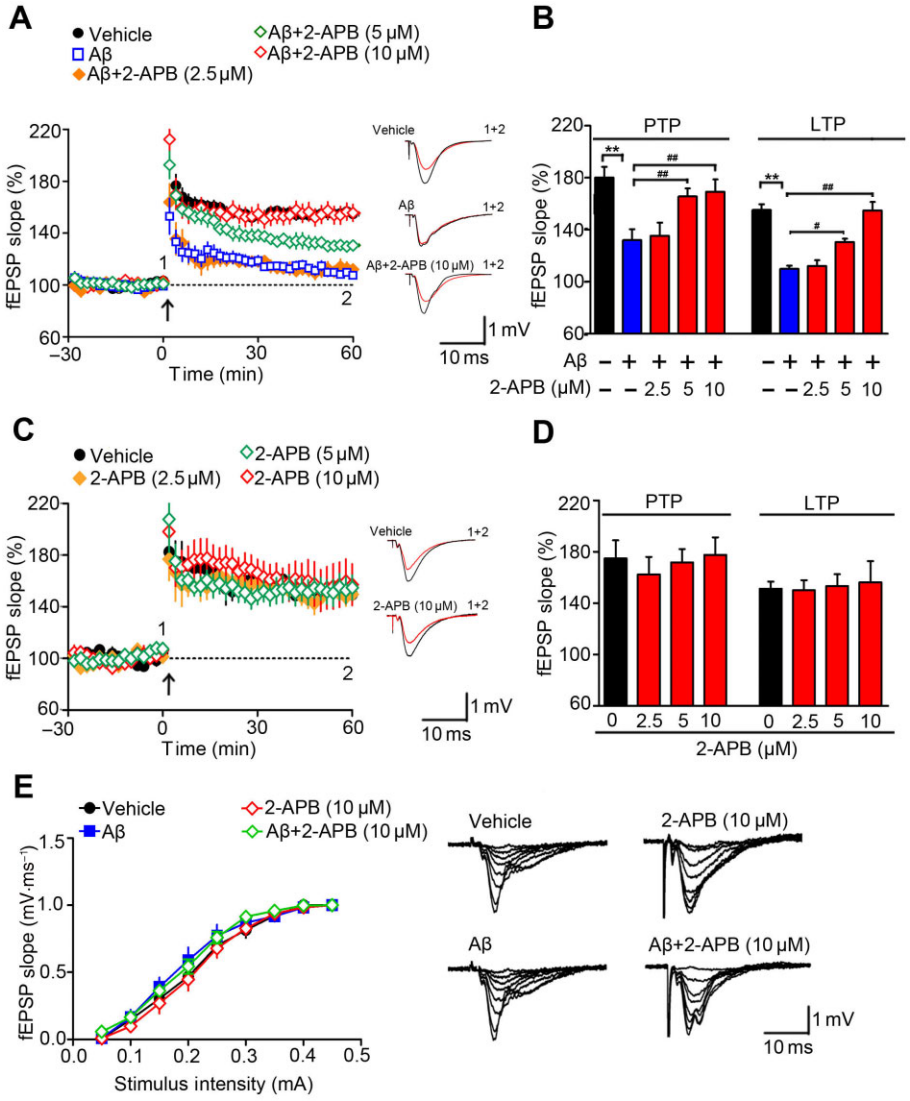

Figure 1

2-APB reversed Aβ suppression of LTP. (A) Aβ (500 nM; $n = 10$) incubation disrupted hippocampal LTP induction compared with the control vehicle treatment ($n = 8$), whereas 2-APB at the concentration of 5.0 ($n = 8$) or 10.0 μM ($n = 8$), but not 2.5 μM ($n = 7$), prevented Aβ-induced LTP impairment. Inserts are representative waves before and after TBS application at indicated time points. Scale bars: 1 mV, 10 ms. (B) Comparison of normalized PTP and LTP among different groups in (A). **: $P < 0.01$, Aβ versus vehicle treatment; #: $P < 0.05$, ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment. (C) Application of 2-APB alone at different concentrations (2.5, 5.0 and 10.0 μM) did not affect LTP induction. Inserts are representative waves at indicated time points. Scale bars: 1 mV, 10 ms. (D) Comparison of normalized PTP and LTP among different groups in (C). (E) I/O curves are shown. To make the curves easily discernable, other curves besides vehicle, Aβ, 2-APB (10 μM) and Aβ + 2-APB (10 μM) are not shown. Inserts are mean representative waves at various stimulus intensities. Scale bars: 1 mV, 10 ms.

the total and the membrane surface expression of GluN2A and GluN2B (Figure 2B).

2-APB reduced Aβ elevation of neuronal intracellular Ca^{2+}

As 2-APB is a moderately potent inhibitor of IP_3 receptors, SOCE and TRP channels, the protective effect of 2-APB on LTP may attribute to its antagonism of Aβ-induced neuronal Ca^{2+} exitotoxicity. To characterize this possibility, hippocampal neurons of C57BL/6 mice were cultured and pre-incubated with a sensitive Ca^{2+} fluorescence indicator (i.e. Fluo3-AM). Compounds or vehicle were separately added into neuronal culture wells, and then intracellular free $[Ca^{2+}]_i$ was continuously monitored for 1 h under a confocal microscope. As shown in Figure 3A, vehicle or 2-APB (10 μM)-treated neurons did not show obvious changes of the intracellular Ca^{2+} fluorescence intensity during a 60 min treatment, whereas incubation with Aβ induced a gradual increase of neuronal $[Ca^{2+}]_i$. The fluorescence intensity in Aβ and 2-APB co-incubated neurons showed no obvious changes either. Data of fluorescence intensities of each treatment were normalized to the $[Ca^{2+}]_i$ level before the addition of different

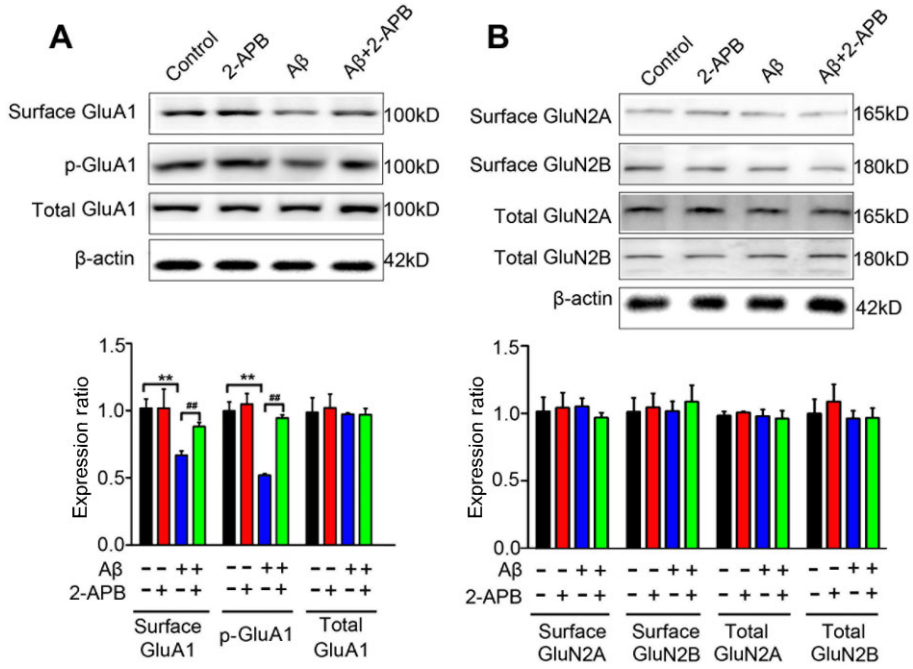

Figure 2

2-APB reversed Aβ-mediated reduction of AMPA receptor phosphorylation and trafficking. (A) 2-APB at the concentration of 10 μM reversed Aβ (500 nM)-induced decrease of the surface GluA1 and the p-GluA1 (Ser^{831}) expression in hippocampal slices. Quantification of the surface GluA1 and the p-GluA1 was normalized to the total GluA1, and the total GluA1 was normalized to β-actin. n = 4–6 for each treatment. **: $P < 0.01$, Aβ versus vehicle treatment; ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment. (B) There was no significant difference for GluN2A and GluN2B expression in hippocampal slices incubated with vehicle, 2-APB 10 μM, Aβ (500 nM), or Aβ + 2-APB (10 μM). Quantifications of the surface GluN2A and GluN2B were normalized to the total GluN2A and GluN2B respectively. The total GluN2A and GluN2B were normalized to β-actin. n = 4–6 for each treatment.

compounds (i.e. 'time 0' in Figure 3A), and analysed by repeated measures two-way ANOVA (treatment: $F_{(3,\ 13)} = 26.3$; time: $F_{(3,\ 39)} = 5.5$; treatment × time: $F_{(9,\ 39)} = 2.2$, all $P < 0.05$). *Post hoc* Tukey's tests found that neuronal $[Ca^{2+}]_i$ level significantly increased following 15–60 min Aβ treatment (all $P < 0.05$ compared with $[Ca^{2+}]_i$ at 'time 0', and compared with $[Ca^{2+}]_i$ following vehicle treatment at 15, 30 and 60 min respectively). Moreover, the application of 2-APB (10 μM) effectively reversed Aβ-induced $[Ca^{2+}]_i$ elevation ($P > 0.05$ compared with $[Ca^{2+}]_i$ at 'time 0', and all $P < 0.05$ compared with those following Aβ treatment at 15, 30 and 60 min) (Figure 3B).

2-APB blocked Aβ activation of BAX and caspase-3 signalling

As mentioned in the Introduction, Aβ mediates increased activity of BAX and caspase-3 in hippocampal neurons (Ferreiro *et al.*, 2008; Wang *et al.*, 2010), and the loss of BAX or caspase-3 activation reverses Aβ suppression of LTP in hippocampus (Jo *et al.*, 2011; Olsen and Sheng, 2012). To examine the effects of 2-APB on Aβ-mediated BAX and caspase-3 hyperactivation, hippocampal slices of adult C57BL/6 mice and cultured embryonic neurons were incubated with vehicle, Aβ, 2-APB (10 μM), or Aβ + 2-APB (10 μM) for 1 h. The active conformation of BAX was recognized by a specific antibody 6A7 (Tikhomirov and Carpenter, 2005). Results showed that activated BAX was up-regulated after Aβ treatment (slices: $F_{(3,\ 15)} = 8.7$; neurons: $F_{(3,\ 15)} = 21.5$, both $P < 0.01$; Aβ vs. vehicle, both $P < 0.05$, Tukey's tests), whereas this up-regulation was inhibited by 2-APB in both hippocampal slices and primary neuronal cultures (both $P < 0.05$, Aβ vs. Aβ + 2-APB; Figure 4A1 and A2).

Total caspase-3 levels were not affected following Aβ, 2-APB or Aβ + 2-APB treatment. However, the level of the cleaved caspase-3, which is the active form of caspase-3 in regulating downstream signalling, was significantly increased after Aβ treatment (slices: $F_{(3,15)} = 15.3$; neurons: $F_{(3,15)} = 11.7$, both $P < 0.001$; Aβ vs. vehicle: both $P < 0.05$, Tukey's tests). The elevation of the cleaved caspase-3 was effectively blocked by 2-APB (both $P < 0.05$, Aβ vs. Aβ + 2-APB; Figure 4B1 and B2). Because Aβ-induced caspases-3 activation can increase GSK3β activity by increasing Akt1 cleavage and then reducing phosphorylation at Ser^9 of GSK3β (Jo *et al.*, 2011; Mines *et al.*, 2011), we also checked the activation of GSK3β in hippocampal slices and embryonic neurons. As shown in Figure 4C1 and C2, the total levels of GSK3α and β were not obviously affected by Aβ treatment, but the activations of both GSK3α and GSK3β subunits were increased after Aβ incubation as indicated by the reduction of phosphorylated GSK3α and GSK3β, while 2-APB effectively prevented Aβ-induced GSK3αβ activation (Tukey's tests: all $P < 0.05$, Aβ vs. vehicle, and Aβ vs. Aβ + 2-APB). Incubation with 2-APB alone had no obvious effect on BAX, caspase-3 and GSK3αβ activation (all

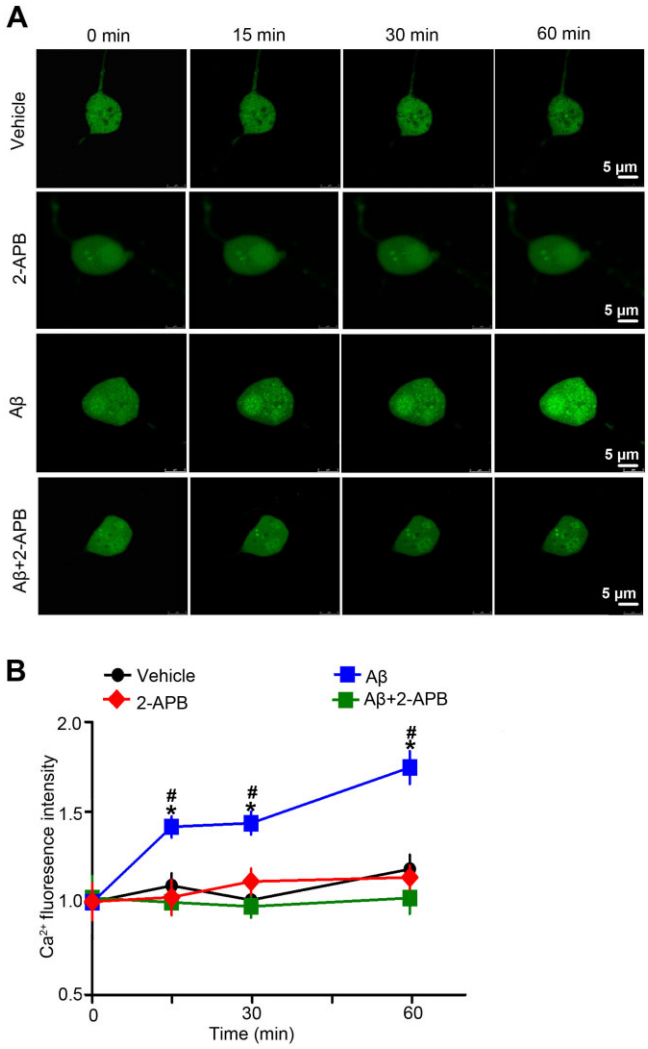

Figure 3

2-APB effectively inhibited Aβ-induced intraneuronal Ca^{2+} elevation. (A) Ca^{2+} imaging photos taken under a confocal fluorescence microscope at different time points. Cultured primary neurons were incubated with Fluo3-AM (green), and then treated with vehicle ($n = 4$), 2-APB (10 μM, $n = 5$), Aβ (500 nM, $n = 4$), or Aβ + 2-APB (10 μM, $n = 5$) for 1 h. (B) Quantification of normalized Ca^{2+} fluorescence intensities after different treatments. *: $P < 0.05$, Aβ versus vehicle treatment; #: $P < 0.05$, Aβ versus Aβ + 2-APB treatment.

$P > 0.05$, 2-APB vs. vehicle). These data indicate that 2-APB did block Aβ-mediated activation of BAX and caspase 3-GSK3β signalling.

Caspase-3 inhibition contributed to the protective effect of 2-APB on LTP

To explore whether 2-APB protects hippocampal LTP through blocking caspase-3 activation, we investigated the effect of Z-DEVD-FMK (DEVD; 2 μM; Li *et al.*, 2010), a cell-permeable and irreversible caspase-3 inhibitor, on Aβ-induced hippocampal LTP deficit. As shown in Figure 5A, the LTP in vehicle-treated slices (151 ± 2.8%), was impaired by Aβ treatment (109 ± 3.0%). Tukey's tests revealed that DEVD treatment reversed Aβ-induced LTP impairment (149 ± 2.9%, Aβ vs. Aβ + DEVD, all $P < 0.01$ for comparison of fEPSP slopes 0–60 min after TBS).

Co-application of both 2-APB (10 μM) and DEVD (2 μM) also successfully reversed Aβ-induced LTP impairment (149 ± 4.1%; Figure 5B). LTP data derived from different treatments were summarized and compared together in Figure 5C. One-way ANOVA analysis revealed significant effect of treatment ($F_{(4,67)} = 65.4$, $P < 0.001$). *Post hoc* Tukey's tests indicated that LTP in Aβ-treated slices was significantly reduced when compared with LTP levels after other treatments (all $P < 0.01$). Notably, 2-APB, DEVD and 2-APB + DEVD treatments reversed Aβ-impaired LTP to almost the same level of LTP in vehicle-treated slices (all $P > 0.7$; Figure 5C). As 2-APB blocked the Aβ-mediated activation of caspase-3, these data suggest that the protective effects of 2-APB and DEVD on LTP expression share the same molecular event, that is, the inhibition of caspase-3 signalling.

2-APB rescued hippocampal LTP deficit in $APP_{swe}/PS1_{\Delta E9}$ gene mutant mice

Considering that intraneuronal accumulation of Aβ peptides is the initial pathogenic factor causing synaptic dysfunction in transgenic mouse model of AD (Smith *et al.*, 2005; Gengler *et al.*, 2010), we further speculated that 2-APB might also ameliorate hippocampal LTP deficit in $APP_{swe}/PS1_{\Delta E9}$ mutant mice. Hippocampal slices of adult (7–8-month-old) $APP_{swe}/PS1_{\Delta E9}$ mutant mice and their littermate controls were incubated with vehicle or 2-APB (10 μM) for 1 h and then LTP was detected after TBS delivery. As shown in Figure 6A, LTP expression was significantly impaired in slices of APP/PS1 mutant mice when compared with that in littermate controls, while 2-APB incubation significantly rescued LTP expression in slices of APP/PS1 mutant mice (group: $F_{(2,714)} = 6.3$; the interaction of group × time: $F_{(68,714)} = 2.4$, both $P < 0.01$; APP/PS1 mutant vs. wildtype, and APP/PS1 mutant vs. APP/PS1 mutant treated with 2-APB: all $P < 0.05$ for comparison of fEPSP slopes 50–60 min after TBS application). Data of LTP derived from these groups were summarized and compared in Figure 6B.

Discussion

In this study, we found that 2-APB, a potent but non-specific Ca^{2+} channel inhibitor for IP_3 receptors, RyR, SOCE and TRP channels, markedly inhibited intracellular Ca^{2+} elevation induced by incubation of hippocampal neurons with oligomeric $A\beta_{1-42}$ and reversed Aβ suppression of synaptic LTP, most likely through a mechanism blocking Aβ-induced BAX and caspase-3 hyperactivation. In addition, 2-APB rescued hippocampal LTP in $APP_{swe}/PS1_{\Delta E9}$ gene mutant mice, supporting the beneficial effect of 2-APB on synaptic plasticity in this mouse model of AD with Aβ accumulation in the brain.

Massive accumulation of Aβ oligomers destabilizes calcium homeostasis and triggers neuronal Ca^{2+} excitotoxicity and synaptic failure in AD (Mattson *et al.*, 1992; Smith *et al.*, 2005; Bojarski *et al.*, 2008). Neuroprotective drugs with uncompetitive antagonism on glutamatergic NMDA receptors or L-type calcium channels (e.g. memantine and

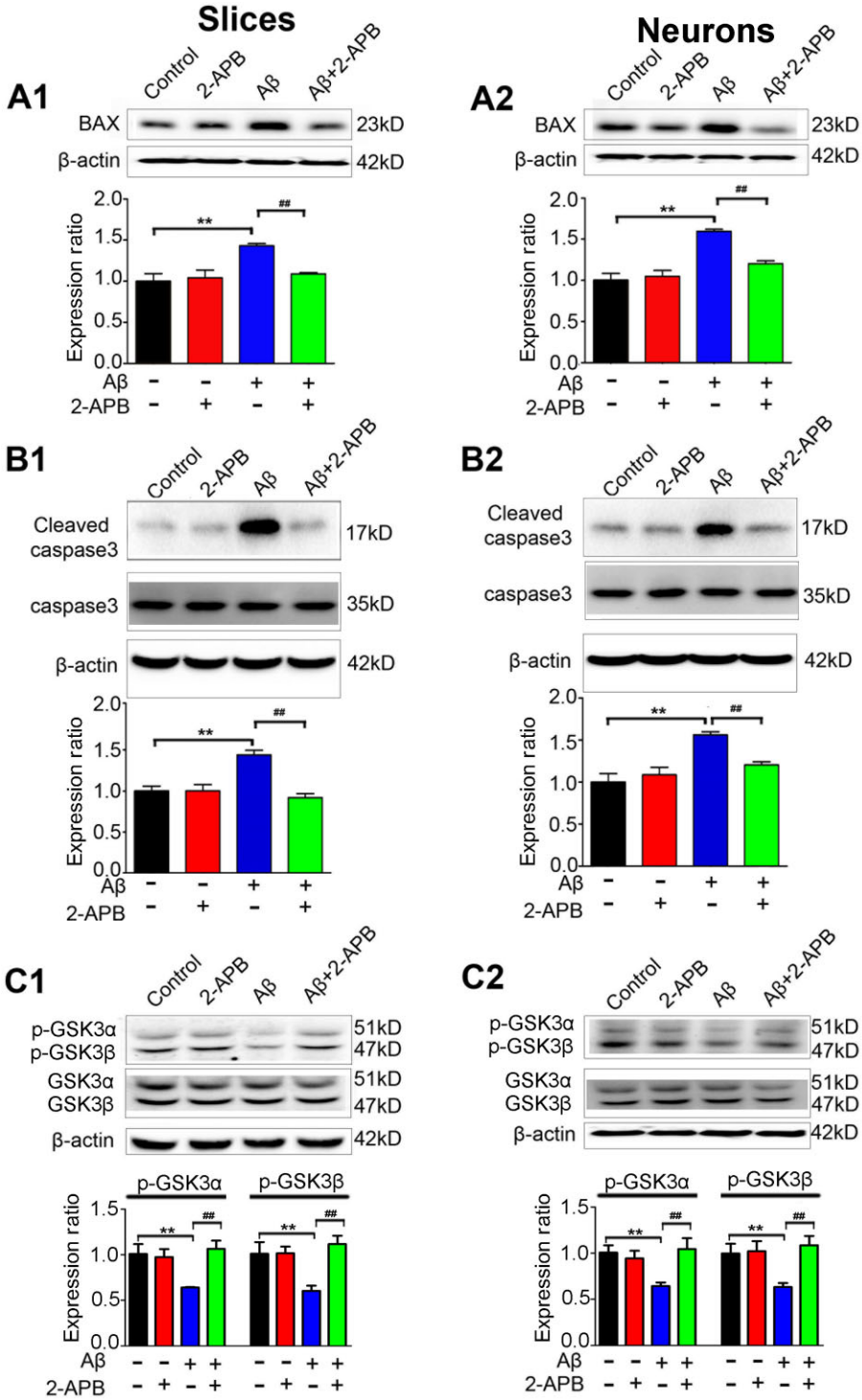

Figure 4

2-APB blocked Aβ-mediated hyperactivation of BAX and caspase-3 signalling. A1 and A2. 2-APB (10 μM) blocked Aβ (500 nM)-induced BAX hyperactivation in hippocampal slices (A1) and cultured neurons (A2). Quantification of active BAX was normalized to β-actin. n = 4–6 for each treatment. **: $P < 0.01$, Aβ versus vehicle treatment; ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment. B1 and B2. 2-APB blocked Aβ-induced increase of the cleaved caspase-3 in hippocampal slices (B1) and cultured neurons (B2). Quantification of the cleaved caspase-3 was normalized to the total caspase-3, and the total caspase-3 was normalized to β-actin. n = 4–6 for each treatment. **: $P < 0.01$, Aβ versus vehicle treatment; ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment. C1 and C2. 2-APB blocked Aβ-induced decrease of the phosphorylated GSK3α and GSK3β in hippocampal slices (C1) and cultured neurons (C2). Quantifications of the phosphorylated GSK3α and GSK3β were normalized to the total GSK3α and GSK3β respectively. The total GSK3α and GSK3β were normalized to β-actin. n = 4–6 for each treatment. **: $P < 0.01$, Aβ versus vehicle treatment; ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment.

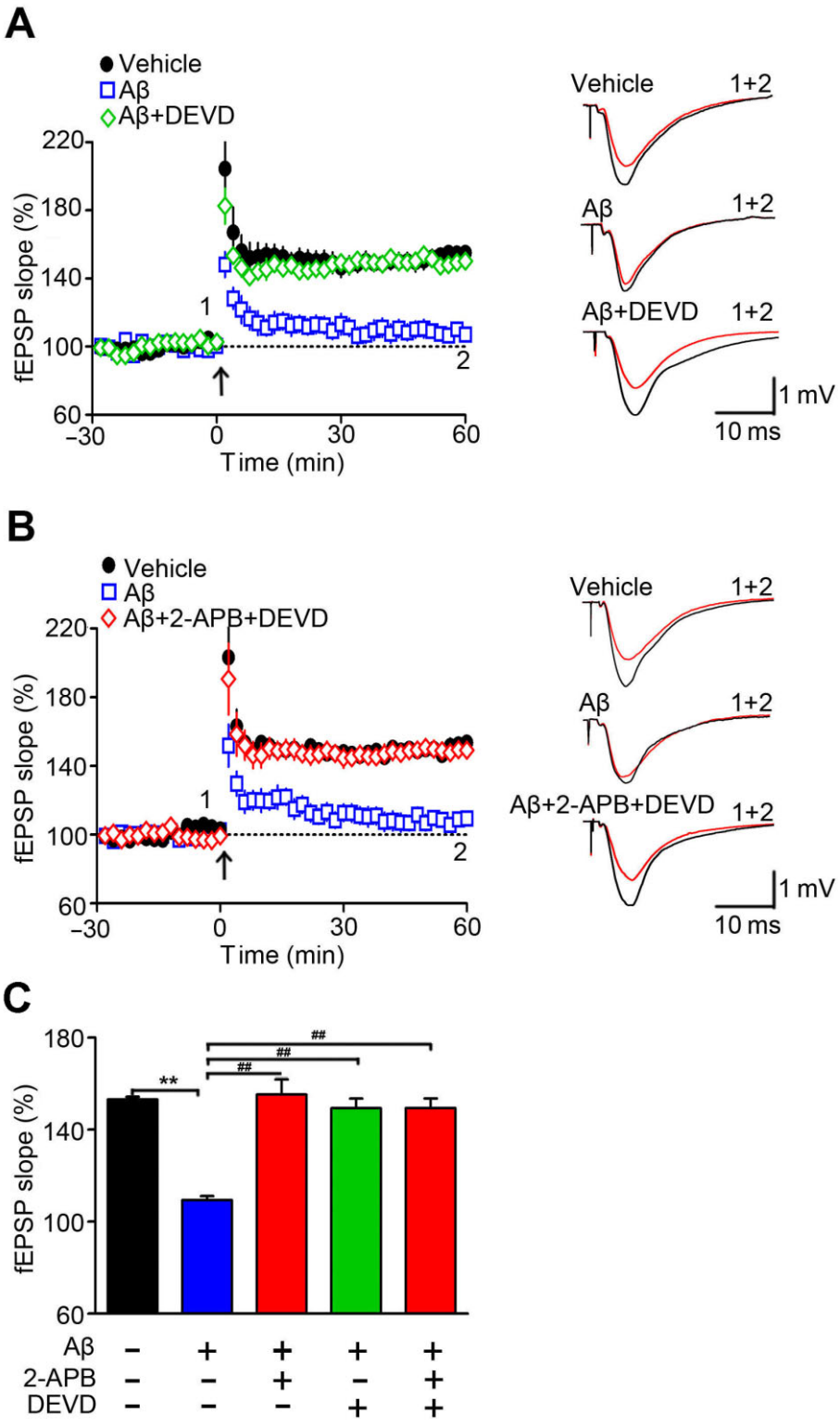

Figure 5

Caspase-3 inhibition contributed to the protective effect of 2-APB on LTP. (A) Caspase-3 inhibitor Z-DEVD-FMK (DEVD, 2 μM) reversed Aβ (500 nM) impairment of hippocampal LTP (n = 8 slices from eight animals for each treatment). Inserts are representative waves before and after TBS application. (B) Co-incubation of Z-DEVD-FMK (DEVD, 2 μM) and 2-APB (10 μM) restored Aβ-impaired hippocampal LTP to the control level (n = 8 slices from eight animals for each treatment). Inserts are representative waves before and after TBS application. (C) LTP data from different treatments were summarized and compared together. **: $P < 0.01$, Aβ versus vehicle treatment; ##: $P < 0.01$, Aβ versus Aβ + 2-APB treatment.

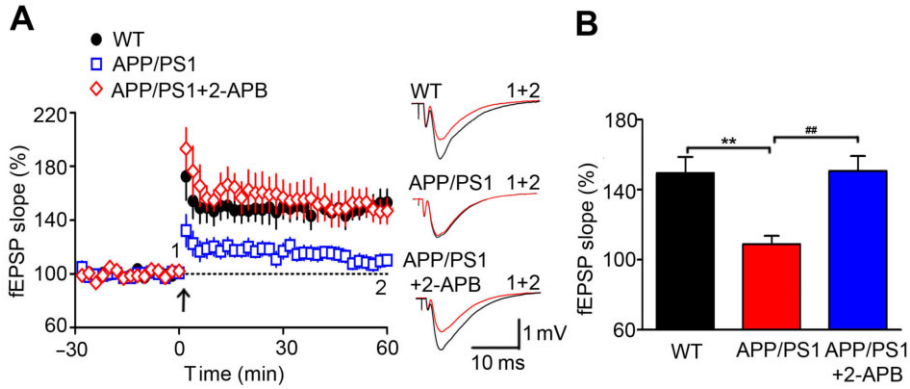

Figure 6

2-APB rescued LTP impairment in $APP_{swe}/PS1_{\Delta E9}$ gene mutant mice. (A) LTP was impaired in APP/PS1 gene mutant mice, whereas 2-APB at the concentration of 10 μM successfully rescued hippocampal LTP in APP/PS1 gene mutant mice (n = 8 slices from eight animals for each group). Inserts are representative waves before and after TBS application. (B) Comparison of LTP in different groups. **: $P < 0.01$, APP/PS1 mutant versus wild-type littermate controls; ##: $P < 0.01$, APP/PS1 mutant versus APP/PS1 mutant treated with 2-APB.

dimebon) have been brought into clinical trials for AD treatment (Bharadwaj *et al.*, 2013; Paula-Lima *et al.*, 2013). Unlike these medications, 2-APB is a membrane-permeable modulator of IP_3 receptors, RyR, SOCE and TRP channels. These Ca^{2+}-related receptors/channels have been reported to be critically involved in the regulation of Aβ disturbance of intraneuronal Ca^{2+} homeostasis. As intracellular Ca^{2+} gating channels located at the ER membranes, IP_3 receptors mediate ER Ca^{2+} release and play an important role in the Aβ suppression of LTP (Taufiq *et al.*, 2005; Resende *et al.*, 2008; Park *et al.*, 2010; Costa *et al.*, 2012). IP_3 receptors also interact with mutant presenilin 1 to exaggerate Aβ-induced Ca^{2+} release from ER and stimulate Aβ processing (Cheung *et al.*, 2008; 2010). Besides the IP_3 receptors, emerging evidence also indicates that RyR, SOCE and TRP play important roles in AD pathophysiology (Yamamoto *et al.*, 2007; Popugaeva and Bezprozvanny, 2013).

Complex pharmacological properties of 2-APB action on these calcium channels have been reported. For instance, although 2-APB at a high concentration (100 μM) inhibits Ca^{2+} release through IP_3 receptors, the same concentration of 2-APB also increases hippocampal pyramidal neuron excitability in Sprague-Dawley rats, possibly through the inhibition of voltage- and Ca^{2+}-dependent potassium conductance (Hagenston *et al.*, 2009), or even via the activation of a non-selective Ca^{2+}-permeable cation channel (Braun *et al.*, 2003). 2-APB at the concentration of 10 μM, which was applied in the present study, falls in the concentration range that inhibits IP_3 receptors, SOCE and TRP channels in brain neurons (Bootman *et al.*, 2002; Taufiq *et al.*, 2005; Lipski *et al.*, 2006; Ozaki *et al.*, 2013), but this concentration of 2-APB also activates SOCE channels in DT40 chicken B lymphocytes (Ma *et al.*, 2002). In hippocampal CA1 neurons of guinea pig, 10 μM 2-APB facilitates LTP induction when a weak tetanus (10 or 15 pulses at 100 Hz), but not a standard tetanus (100 pulses at 100 Hz) is applied (Taufiq *et al.*, 2005). Inhibitory effects of 2-APB with higher concentrations (e.g. 30 and 75 μM) on hippocampal LTP have been reported by others (Baba *et al.*, 2003; Gartner *et al.*, 2006). These studies imply that the complex pharmacological actions of 2-APB are related to its applied concentration, specific tissue and animal species. In our study, 2-APB at 10 μM did not affect LTP induction in ACSF-treated hippocampal slices, but reversed Aβ impairment of LTP. This concentration of 2-APB also inhibited Aβ-induced intracellular Ca^{2+} elevation in hippocampal neurons (Figure 3). It is possible that wide-ranging inhibitory actions of 2-APB on Ca^{2+} release involving IP_3 receptors, RyR, SOCE and TRP channels all contribute to its beneficial effect on LTP.

Previously, it was reported that the surface GluA1 expression and the GluA1 phosphorylation at Ser^{831} may be selectively decreased as a result of CaMKII reduction in cortical synapses of APP transgenic mice and in oligomeric Aβ-treated cultures (Gu *et al.*, 2009). In the present study, we found that oligomeric Aβ also disrupted the surface GluA1 expression and the GluA1 phosphorylation in cultured hippocampal neurons. Moreover, pre-incubation of 2-APB at 10 μM effectively prevented Aβ-induced disruption of the surface GluA1 expression and the GluA1 phosphorylation, which are critical events required for the maintenance of regular synaptic plasticity (Anggono and Huganir, 2012). On the other hand, we did not find any changes for the surface expression of NMDA receptor subunits, GluN2A and GluN2B, after Aβ treatment (Figure 2B). This result is different from the previous report that the application of Aβ promotes NMDA receptor endocytosis in cortical neurons (Snyder *et al.*, 2005). Difference of applied Aβ concentration, treatment duration, and so on between our study and others might account for this discrepancy. Similarly, it was reported that 2–5 μM 2-APB only exerts partial neuroprotective effect against Aβ toxicity in cortical neurons treated with 25 μM $Aβ_{25–35}$ peptide for 16 h (Suen *et al.*, 2003). In our study, the higher 2-APB concentration (10 μM vs. 5 μM in Suen *et al.*, 2003), and lower $Aβ_{1–42}$ concentration (500 nM vs. 10 μM) might contribute to the neuroprotective effect of 2-APB on LTP.

To explore more molecular mechanisms underlying the beneficial effect of 2-APB on hippocampal LTP, we further investigated downstream molecular events following the

2-APB inhibition of Aβ-induced intraneuronal Ca^{2+} elevation. Aβ initially increases ER Ca^{2+} release, which then activates BAX translocation to mitochondria and stimulates caspase-3 activity (Ferreiro *et al.*, 2008; Wang *et al.*, 2010). It was reported that the inhibition of Bax protects neuronal cells from oligomeric Aβ neurotoxicity (Kudo *et al.*, 2012). At the early stage of memory decline in AD transgenic mice, caspase-3 elevation in hippocampal synapses is accompanied by the dysfunction of glutamatergic synaptic plasticity (D'Amelio *et al.*, 2011). In addition to be involved in the apoptotic process, non-apoptotic functions of BAX and caspase-3 in modulation of hippocampal synaptic plasticity have been demonstrated (Jiao and Li, 2011; Jo *et al.*, 2011; Olsen and Sheng, 2012). Oligomeric $Aβ_{1–42}$ treatment also leads to the activation of GSK-3β, a downstream member of caspase-3 signalling, and the application of GSK-3 specific inhibitor CT-99021 can reverse Aβ suppression of hippocampal LTP (Jo *et al.*, 2011). In our study, activations of both GSK3α and GSK3β were increased following oligomeric $Aβ_{1–42}$ treatment, supporting the idea that GSK3α also plays an important role in AD progression (Phiel *et al.*, 2003; Ma, 2014). The finding that 2-APB treatment effectively blocked Aβ-induced hyperactivation of BAX, caspase-3, and GSK3αβ implies that the reduced activation of mitochondrial caspase-3 signalling may underlie the protective effect of 2-APB against Aβ suppression on hippocampal synaptic plasticity.

In conclusion, our data indicate that 2-APB effectively reduces Aβ-induced intraneuronal Ca^{2+} elevation, blocks the hyperactivation of BAX-caspase-3-GSK3β signalling cascade, restores AMPA receptor GluA1 subunit phosphorylation and trafficking, and finally protects synaptic functions. Although all experiments of our study were performed *in vitro*, and the detailed molecular mechanisms for Ca^{2+} antagonism of 10 μM 2-APB in blocking Aβ suppression of LTP need to be further explored, our study suggests that 2-APB may be a potentially useful chemical in therapeutics of AD.

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Author contributions

W. Y. H. and Z. Y. H. performed the research; Z. C. X., D. X. and M. Z. designed the research; W. Y. H., Z. Y. H., L. J. Y. and M. Z. analysed data; and M. Z. and Z. Y. H. wrote the paper.

Conflicts of interest

The authors declare that there are no financial and potential conflicts of interest.

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