

RESEARCH PAPER

The flavonoid baicalein promotes NMDA receptor-dependent long-term potentiation and enhances memory

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BACKGROUND AND PURPOSE

There is growing interest in the physiological functions of flavonoids, especially in their effects on cognitive function and on neurodegenerative diseases. The aim of the current investigation was to evaluate the role of the flavonoid baicalein in long-term potentiation (LTP) in the hippocampal CA1 region and cognitive behavioural performance.

EXPERIMENTAL APPROACH

Effects of baicalein on LTP in rat hippocampal slices were investigated by electrophysiological methods. Phosphorylation of Akt (at Ser⁴⁷³), the extracellular signal-regulated kinase (ERK1/2) and the transcription factor cAMP response element-binding protein (CREB) (at Ser¹³³) were analysed by Western blot. Fear conditioning was used to determine whether baicalein could improve learning and memory in rats.

KEY RESULTS

Baicalein enhanced the *N*-methyl-D-aspartate glutamate receptor-dependent LTP in a bell-shaped concentration-dependent manner. Addition of the lipoxygenase metabolites 12(S)-HETE and 12(S)-HPETE did not reverse these effects of baicalein. Baicalein treatment enhanced phosphorylation of Akt during induction of LTP with the same bell-shaped dose–response curve. LTP potentiation induced by baicalein was blocked by inhibitors of phosphoinositide 3-kinase. CREB phosphorylation was also increased in the CA1 region of baicalein-treated slices. Baicalein-treated rats performed significantly better than controls in a hippocampus-dependent contextual fear conditioning task. Furthermore, baicalein treatment selectively increased the phosphorylation of Akt and CREB in the CA1 region of hippocampus, but not in the prefrontal cortex, after fear conditioning training.

CONCLUSIONS AND IMPLICATIONS

Our results demonstrate that the flavonoid baicalein can facilitate memory, and therefore it might be useful in the treatment of patients with memory disorders.

Abbreviations

12(S)-HETE, 12(S)-hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid; 12(S)-HPETE, 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid; 12-LO, 12-lipoxygenase; ACSF, artificial cerebrospinal fluid; CREB, cAMP response element-binding protein; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; fEPSP, field excitatory postsynaptic potential; HFS, high-frequency stimulation; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PI3K, phosphoinositide 3-kinase; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; TBS, theta-burst stimulation

Introduction

A growing body of evidence suggests that memory and cognitive impairment is associated with both physiological aging and pathological conditions in the central nervous system, including as brain ischaemia, Alzheimer's disease and Parkinson's disease. Therefore, there is considerable interest in the development of new drugs to improve cognitive performance in impaired individuals (Marshall, 2004). Recently, interest has focused on a group of phytochemicals found in normal diets, known as flavonoids, capable of inducing improvements in memory acquisition, consolidation, storage and retrieval (Spencer, 2009). Previous studies have shown that the extracts of flavonoid-rich plant or specific flavonoid molecules, such as grape (Joseph *et al.*, 2009), green tea (Li *et al.*, 2009), pomegranates (Hartman *et al.*, 2006), fisetin (Maher *et al.*, 2006), epicatechin (van Praag *et al.*, 2007), oroxylin A (Kim *et al.*, 2008) are able to improve memory and synaptic plasticity through their interactions with neuronal signalling pathways pivotal in controlling long-term potentiation (LTP) and memory.

Long-term potentiation is a manifestation of activity-dependent synaptic plasticity and has increasingly been a prime target for studies on learning and memory in the hippocampus and other brain regions of rodents (Bliss and Collingridge, 1993; Malenka and Bear, 2004). LTP is a long-lasting increase in synaptic strength that can be induced by tetanic stimulation of afferent fibres. Previous studies have shown that LTP triggered by high-frequency stimulation (HFS) or theta-burst stimulation (TBS) in hippocampal CA1 area requires postsynaptic molecular mechanisms, such as activation of *N*-methyl-D-aspartate (NMDA) receptors and involvement of the phosphoinositide 3-kinase (PI3K)/Akt signalling cascade. It has been proposed that Ca^{2+} influx through NMDA receptors triggers a series of intracellular signalling cascades, including the PI3K/Akt pathway, which lead to increased synaptic strength, which is believed to play a pivotal role in NMDA receptor-dependent LTP in the hippocampal CA1 region (Man *et al.*, 2003). Recent reports have revealed that flavonoids and other small molecules or drugs affect LTP, and consequently memory and cognitive performance, through their interactions with these signalling pathways (Maher *et al.*, 2006; Mans *et al.*, 2010).

Scutellaria baicalensis Georgi (Huangqin) is a herb with antibacterial and anti-inflammatory properties, widely used for many centuries in China and Japan. Baicalein is the most effective antioxidant among the major flavonoids isolated from the roots of *S. baicalensis*. Herbal preparations of baicalein have been used to improve deficiencies of learning and memory for thousands of years in traditional Chinese medicine. In our previous studies, baicalein alleviated cognitive deficits induced by chronic cerebral hypoperfusion and protected neurons against ischaemic injury by activating the PI3K/Akt pathway in rats (Liu *et al.*, 2007; 2010). Additionally, a microarray analysis of gene expression revealed that the expression of certain genes related to learning and memory were normalized, in ischaemic mice brain, after treatment with baicalein (Wang *et al.*, 2004b). A recent study also showed that one dose pretreatment of baicalein attenuated amnesia, induced by β -amyloid peptide-(25–35) (Wang *et al.*, 2004a). However, the effect and mechanism of baicalein

on learning and memory in normal animals remain unclear. In the present study, we have investigated the effect of baicalein on LTP in the CA1 region of rat hippocampal slices and cognitive behavioural performance in adult rats, as well as the underlying molecular mechanisms.

Methods

Electrophysiological recordings

All animal care and experimental protocols were in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. Hippocampal slices were prepared from Sprague-Dawley rats (6–8 weeks old) as previously described (Liu *et al.*, 2009) with some modification. Briefly, brains were rapidly removed and coronal brain slices (400 μ m thickness) containing hippocampus were cut using a vibrating blade microtome in ice-cold artificial cerebrospinal fluid (ACSF) containing (mM) 119 NaCl, 3.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4 , 26.2 $NaHCO_3$ and 11 glucose that was bubbled continuously with 95% O_2 –5% CO_2 to adjust pH to 7.4. After 1.5 h of recovery at 27°C, an individual slice was transferred to a submerged recording chamber and continuously superfused with oxygenated ACSF at 30°C at a rate of 3–4 mL·min⁻¹. Field excitatory postsynaptic potentials (fEPSPs) were evoked by a constant stimulation in the Schaffer collaterals with a bipolar electrode and recorded in the stratum radiatum layer of CA1 with a glass micropipette filled with 3 M NaCl. Stimulation intensities were chosen to produce a fEPSP with a slope that was 30–35% of that obtained with maximal stimulation.

Long-term potentiation was induced electrically by one of the following two protocols: (i) The HFS protocol used to induce LTP consisted of three 1 s, 100 Hz stimulus trains separated by a 30 s interval between trains. This protocol has been used previously to induce NMDA receptor-dependent LTP (Malenka and Bear, 2004; Berberich *et al.*, 2007); (ii) The TBS protocol used to induce LTP contained two trains with 10 s intervals between trains. Each train consisted of 10 bursts separated by 200 ms (theta rhythm). Each burst included five 100 Hz pulses delivered at 30–35% of maximal stimulus intensity. A similar protocol has been used to induce NMDA receptor-dependent LTP in an earlier study (Morgan and Teyler, 2001).

Paired stimuli (25, 50, 75, 100 ms interval) were delivered to the Schaffer collateral and the paired-pulse ratio (PPR) was calculated as the ratio between the mean slope of the second fEPSP (fEPSP₂) over the first fEPSP (fEPSP₁). The initial slope of the fEPSP was measured and expressed as a percentage change from the baseline level, calculated from an average of the last 20 min of the baseline recording period. The degree of LTP for each experiment was measured at 60 min after the tetanic stimulation.

Western blotting

After the electrophysiological studies, CA1 region of hippocampal slices was removed for Western blotting. After behavioural studies, the CA1 region of hippocampus and prefrontal

cortex were removed for Western blotting, 15 min after contextual fear conditioning training. The procedures were processed according to our previous protocols with some modifications (Cai *et al.*, 2008; Liu *et al.*, 2009). Briefly, hippocampus and cortex were homogenized with buffer containing: 50 mM Tris-base (pH 7.4), 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na₃VO₄ and protease inhibitors. Total protein was estimated by Coomassie blue protein-binding assay (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). Then, the samples were mixed with sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and stored at -80°C until electrophoresis. Samples (20 µg) were analysed by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). After blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, transferred membranes were incubated overnight at 4°C with appropriate primary antibodies against β-actin (1:3000) (Upstate Biotechnology, Lake Placid, NY, USA), anti-phospho-Akt (Ser⁴⁷³) (1:500), anti-Akt (1:1000), anti-cAMP response element-binding protein (CREB; 1:400); anti-phospho-CREB (Ser¹³³) (1:400) (Cell Signaling, San Francisco, CA, USA), anti-extracellular signal-regulated kinase (ERK1/2; 1:500) (Abcam, Cambridge, UK) and anti-phospho-ERK1/2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following three washes with TBST, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) in TBST with 1% non-fat milk for 1 h at room temperature and reacted with enhanced chemiluminescence reagents (Super Signal West Pico; Pierce Chemical Co., Rockford, IL, USA) and visualized with X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the optical densities of detected bands were quantified using NIH Image J software (National Institutes of Health, Bethesda, MD, USA). The results were normalized to the quantity of β-actin in each sample lane. All assays were performed at least three times.

Fear conditioning task

Sprague-Dawley rats (6–8 weeks old) were used for these studies. Animals were housed individually in environmentally controlled conditions (22°C, 12 h light–dark cycle with light cycle from 0700 to 1900 h and dark cycle from 1900 to 0700 h with *ad libitum* access to standard laboratory chow and water. Rats were allowed 1 week to adapt to their new environment before experiments began. The fear conditioning procedures were processed according to previous protocols with some modifications (Barha *et al.*, 2010).

Fear conditioning took place in an observation chamber (32 × 26 × 22 cm), consisting of aluminium (side walls) and Plexiglass (hinged front door, and rear wall). The chamber was enclosed within a sound-attenuating box located in a quiet room. A video camera was positioned above the chamber to record the behaviour of the animal for video scoring. The floor of the chamber consisted of 16 stainless steel rods spaced 1.6 mm apart (centre-to-centre). Rods were wired to a shock source and solid-state grid scrambler for the delivery of foot shock. Each chamber was illuminated by a single house light positioned in the top centre of one wall. In the left corner of the same wall, a speaker connected to a

programmable audio generator was located. The background noise was supplied by ventilation fans in the box. Rats were handled in the room where conditioning took place for 5 min per day for 3 days. The habituation procedure was introduced to completely familiarize the rats with the stimuli of the experimental room, and thus prevent any interference of uncontrolled novel stimuli during the experiments.

This experiment was conducted over 2 days, conditioning day and testing day. On day 1 (the conditioning day), rats were injected with baicalein or vehicle. Twenty minutes later, they were placed into the chamber and the house light was turned on. After a 3 min acclimatizing period, they were given two presentations of the tone conditioned stimulus (80 dB, 30 s) co-terminating with a foot shock with 60 s intervals. Each shock was 0.75 mA and 2 s duration. Rats were left in the conditioning chamber for 30 s after termination of the procedure and then returned to their home cage. To assess contextual fear conditioning, 24 h after conditioning, rats were placed into the conditioning chamber and observed for 3 min. One hour later, the animals were assessed for cued fear conditioning in a novel test chamber, with different contextual cues, during a 3 min presentation of the conditioned stimulus. Memory was assessed by measurement of time spent freezing. Freezing was defined as the complete absence of activity except for respiratory movement. The data were converted to the percentage of samples scored as freezing.

Rats were randomly assigned to one of five treatment groups ($n = 8$ per group) and received a single i.p. injection of either vehicle or different doses (10, 20, 50 and 100 mg·kg⁻¹) of baicalein. Baicalein that dissolved in dimethyl sulfoxide (DMSO) or a corresponding volume of vehicle (DMSO) was administered 20 min before training. These doses and dosing time were chosen based on the pharmacokinetic profile of baicalein in the rat defined in an earlier study (Tsai *et al.*, 2002).

Data analysis

Data are presented as means ± SEM. One-way ANOVA or paired *t*-test was used for the statistical analysis by employing SPSS 10.0 software. Differences at the $P < 0.05$ level were considered statistically significant.

Materials

Baicalein (MW: 270.24, purity above 98%) was obtained from Sigma (St. Louis, MO, USA). 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid [12(S)-HPETE] and 12(S)-hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid [12(S)-HETE] were obtained from Cayman Chemicals (Ann Arbor, MI, USA). D-2-amino-5-phosphonovaleric acid (D-APV) and wortmannin were purchased from Sigma (St. Louis, MO, USA). LY294002 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). MK-801 was kindly provided by NIMH synthesis program. Other general agents were purchased from commercial suppliers. Baicalein was dissolved in dimethyl sulfoxide (DMSO; Sigma), and the final concentration of DMSO in all groups was no more than 0.1%. The same volume of DMSO was used as control.

Results

Baicalein enhances HFS-induced LTP in rat hippocampal CA1 region

In the first set of experiments, the effect of baicalein on basal excitatory synaptic transmission in the CA1 region of hip-

poampal slices was examined. After establishing a stable baseline for 20 min, the fEPSP was recorded for 20 min under perfusion with ACSF containing various concentrations of baicalein (0.1, 1, 10, 50 μM). No significant changes in the fEPSP slope were observed after baicalein perfusion (Figure 1A and B). These results suggest that baicalein did not affect basal synaptic transmission.

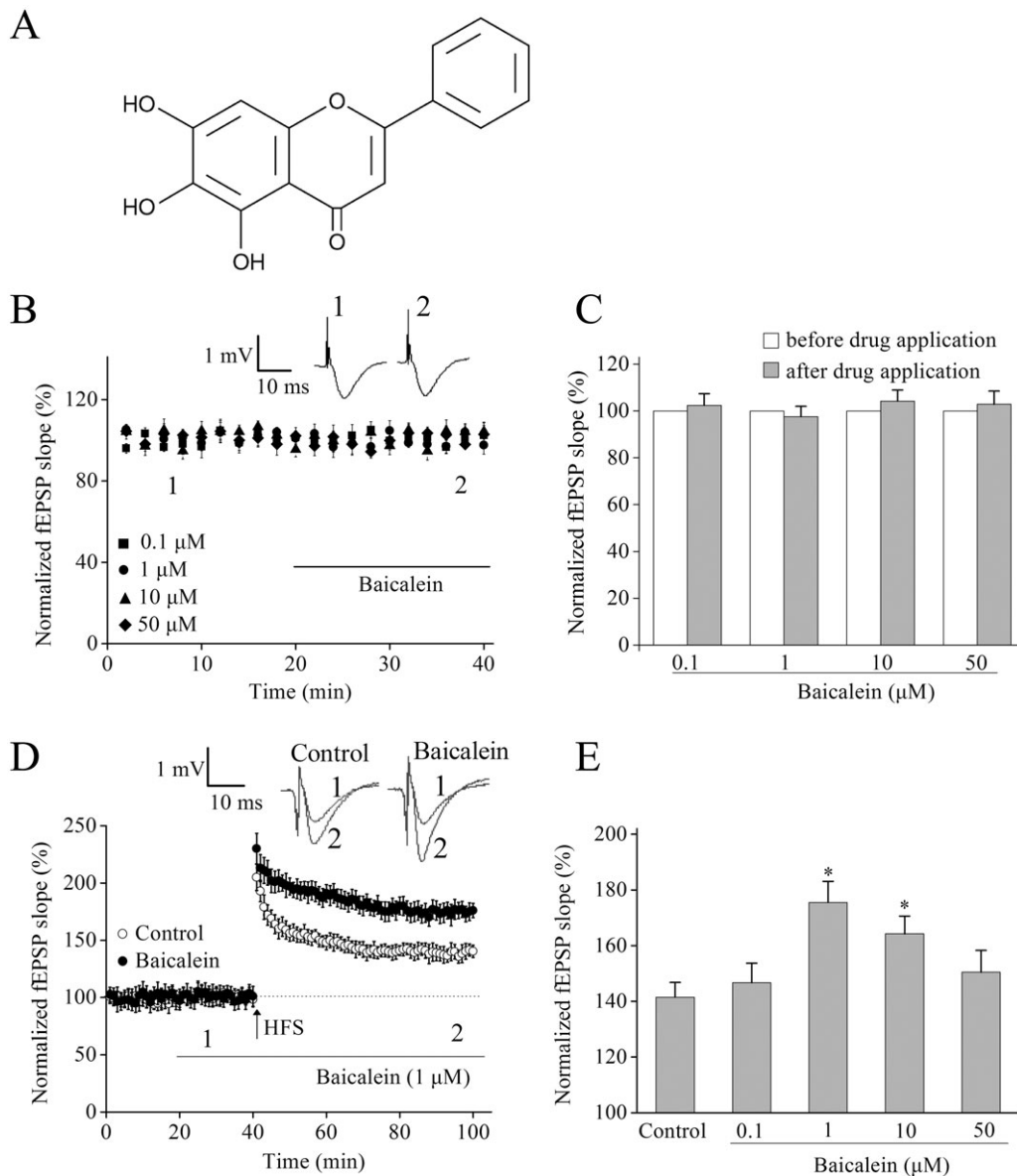


Figure 1

Pretreatment with baicalein enhances long-term potentiation (LTP) in the hippocampal CA1 region. (A) Chemical structure of baicalein. (B) Baicalein (0.1, 1, 10 and 50 μM) had no effect on basal synaptic transmission. After establishing a stable field excitatory postsynaptic potential (fEPSP) baseline for 20 min, baicalein was perfused continuously (indicated by the bar) to individual slices. Insets, the sample traces before (1) or after (2) perfusion with baicalein. (C) Summary of averaged fEPSP slope from hippocampal slices incubated with different concentrations of baicalein (0.1, 1, 10 and 50 μM). No significant change was shown after drug application in each group, $n = 6$ for each group. The mean fEPSP slope before drug application (1 to 20 min) was normalized as 100% and the fEPSP slope at every time point was normalized to it. (D) Effect of baicalein (1 μM) on LTP in CA1 region of rat hippocampus. The superimposed fEPSPs in the upper portion show typical recordings from experiments taken at the time indicated by the number. (E) Summary data of the level of LTP 60 min after high-frequency stimulation (HFS) in the absence or presence of various concentrations of baicalein. * $P < 0.05$ versus control. Each point was the normalized mean \pm SEM of 6–8 slices.

To evaluate whether baicalein could affect synaptic plasticity in normal animals, we next examined the effect of baicalein on HFS-induced LTP in hippocampal CA1 region of rats. As shown in Figure 1C and D, pre-incubation of hippocampal slices with baicalein for 20 min enhanced the HFS-induced LTP in a bell-shaped, concentration-dependent manner with the effect reaching a maximum at 1 μM and persisting for at least 60 min (Figure 1D).

Baicalein does not affect input-output relationship and paired-pulse facilitation (PPF) in the hippocampal CA1 region of rat

To determine whether baicalein can affect the input-output relationship, which reflects the efficacy of synaptic transmis-

sion, the fEPSP was recorded under different stimulus intensity. Baicalein (1 μM) did not alter the input-output relationship at any stimulus intensity ($n = 6$, Figure 2A and B).

Long-term potentiation reflects a persistent enhancement in synaptic strength in which both presynaptic and postsynaptic mechanisms might be involved (Malenka and Nicoll, 1999). PPF is a sensitive measure of altered neurotransmitter release probability, a form of short-term presynaptic plasticity and we used this protocol to examine whether presynaptic mechanisms were involved in LTP facilitation induced by baicalein. The PPR in slices exposed to DMSO or baicalein at baseline and 30 min after HFS stimulation was examined. In control slices (DMSO), PPR was significantly decreased after HFS stimulation, indicating an enhanced neurotransmitter release within LTP (Figure 2C and D). In slices pretreated with

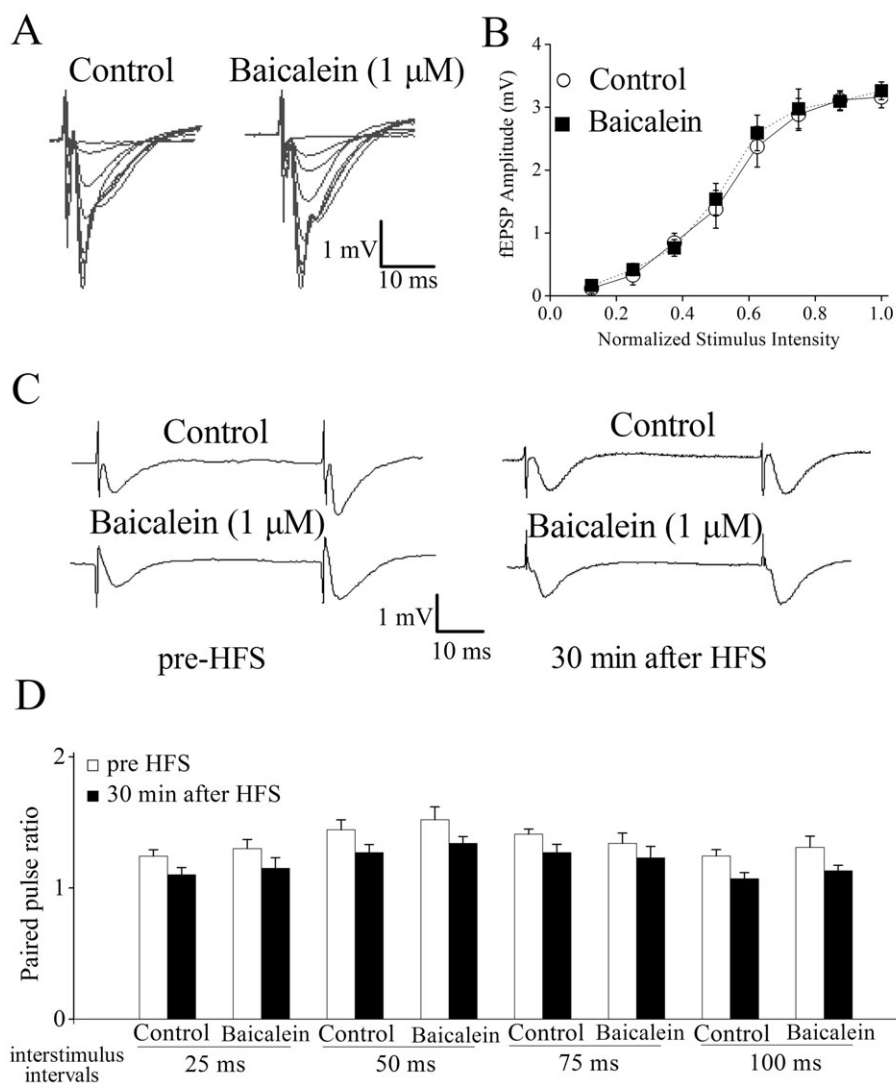


Figure 2

Baicalein treatment does not affect baseline evoked responses or paired-pulse facilitation. (A) Typical superimposed field excitatory postsynaptic potential (fEPSP) recorded from the CA1 region in the presence and absence of 1 μM baicalein by increasing stimulation intensity. (B) Input-output curves illustrating the relationship between the stimulation and evoked response for fEPSPs recorded from control and baicalein-treated slices. No significant differences were observed. (C) Typical fEPSPs are shown from experiments at 50 ms interpulse interval before and after high-frequency stimulation (HFS) stimulation. (D) Paired-pulse facilitation (PPF) was measured by varying the intervals between pairs of stimuli before and after HFS stimulation. No significant differences were observed. Each point was the normalized mean \pm SEM of five slices.

1 μM baicalein for 20 min, PPR decreased similarly after HFS stimulation ($n = 5$, $P > 0.05$ vs. control, Figure 2C and D). There was no difference in the effect of LTP on PPR between control and baicalein-treated slices, indicating that the effects of baicalein on LTP were unlikely to result from presynaptic changes in probability of transmitter release.

NMDA receptors are involved in baicalein-facilitated LTP

At CA3-CA1 synapses, LTP induced by 100 Hz tetanic stimulation depends primarily on Ca^{2+} influx through NMDA receptors (Collingridge *et al.*, 1983) and this potentiation is prevented by the blockade of postsynaptic NMDA receptors. Consistent with previous observations, when NMDA receptor antagonists D-APV (50 μM) and MK-801 (10 μM) were applied, 100 Hz tetanic stimulation could not induce LTP (D-APV: $97.4 \pm 8.2\%$, $n = 6$; MK-801: $98.3 \pm 7.4\%$, $n = 6$; $P < 0.05$ vs. control; Figure 3A and B). Pre-incubation with D-APV (50 μM) or MK-801 (10 μM) for 10 min before baicalein (1 μM) application completely prevented baicalein-facilitated LTP (D-APV + baicalein: $101.2 \pm 8.5\%$, $n = 6$; MK-801 + baicalein: $103.1 \pm 8.4\%$, $n = 6$; $P < 0.05$ vs. baicalein alone; Figure 3A and B).

To determine whether the baicalein-facilitated LTP was time-dependent, application of baicalein (1 μM) application was delayed until 40 min after HFS. On average, the slope of fEPSP measured 40 min after HFS was $143 \pm 8.5\%$ of pre-stimulation baseline, which was not significantly different from that of LTP recorded in slices after application of 1 μM baicalein for 30 min ($139 \pm 6.4\%$ of pre-stimulation baseline; $n = 6$, Figure 3C). These results demonstrate that baicalein barely affected synaptic response if applied after LTP has been established, and baicalein is needed during the period of HFS stimulation in order to facilitate LTP.

In order to confirm the role of baicalein, hippocampal LTP was induced by the other stimulation pattern, TBS, which is a more physiologically relevant stimulus (Raymond, 2007). Several studies have reported that two trains of TBS results in LTP that is completely blocked by NMDA receptor antagonists (Morgan and Teyler, 2001). As expected, it was found that incubation of baicalein (1 μM) alone for 20 min exhibited a dramatic increase in the magnitude of TBS-LTP (control: $134.1 \pm 6.1\%$; baicalein: $161.1 \pm 8.6\%$; $n = 6$ for each group, $P < 0.05$, Figure 3D). Furthermore, pre-incubation of D-APV (50 μM) for 10 min before baicalein application robustly blocked baicalein-facilitated LTP (D-APV + baicalein: $96.4 \pm 8.1\%$; $n = 6$, $P < 0.05$ vs. baicalein alone, Figure 3D).

12-Lipoxygenase inhibition is not required for baicalein-induced LTP enhancement

Baicalein is known as a 12-lipoxygenase (12-LO) inhibitor and widely used to decrease the generation of 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid [12(S)-HPETE] and 12(S)-hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid [12(S)-HETE] (two metabolites of 12-LO) in cell proliferation studies (Dailey and Imming, 1999). We therefore investigated whether these metabolites contributed to the effect of baicalein. Pretreatment of hippocampal slices with 250 nM 12(S)-HETE (Figure 4A and B) or 250 nM 12(S)-HPETE (Figure 4C and D) for 10 min did not affect the amplitude of

LTP measured 60 min after HFS, with or without 1 μM baicalein. A higher (1 μM) or lower concentration (100 nM) of 12(S)-HETE or 12(S)-HPETE did not reverse the enhancement of LTP (data not shown).

Activation of the PI3K pathway is required for baicalein-induced LTP enhancement

A number of recent studies have shown that PI3K is involved in synaptic plasticity, and some flavonoids such as baicalein and the citrus flavanone hesperetin activate the PI3K pathway in cortical and hippocampal neurons (Vauzour *et al.*, 2007; Spencer, 2009; Liu *et al.*, 2010). In our next experiments, the effects of baicalein on levels of phosphorylation of Akt (Ser⁴⁷³) and total Akt were measured by Western blotting analyses. HFS stimulation induced a transient phosphorylation of Akt at Ser⁴⁷³, which reached the maximum at 5 min after LTP and returned to baseline values within 60 min (Figure 5A and B). Akt phosphorylation was further increased by baicalein (1 μM) pre-incubation after HFS in a time-dependent manner, without any significant change in total Akt expression (Figure 5A and B).

This potentiation by baicalein of Akt phosphorylation at 5 min after HFS was dose-dependent but with a bell-shaped profile, peaking at 1 μM , without any significant change in total Akt expression (Figure 5C and D).

Moreover, inhibition of PI3K by LY294002 (10 μM) or wortmannin (500 nM) completely blocked the baicalein-induced enhancement of Akt phosphorylation at 5 min after HFS (Figure 5E and F). We next examined the effects of these PI3K inhibitors on baicalein-enhanced LTP. LTP was markedly reduced in hippocampal slices treated with LY294002 (10 μM) or wortmannin (500 nM) for 30 min before HFS (LY294002: $110.3 \pm 6.1\%$; wortmannin: $112.2 \pm 6.7\%$; $n = 6$, Figure 5G and H). Furthermore, in slices pre-incubated with LY294002 (10 μM) or wortmannin (500 nM), the enhancement of HFS-LTP induced by baicalein was completely blocked (LY294002 + baicalein: 114.8 ± 5.3 ; wortmannin + baicalein: $115.3 \pm 5.5\%$; $n = 6$, Figure 5G and H).

Baicalein increases CREB phosphorylation following HFS in rat hippocampal CA1 region

Long-term potentiation triggered by HFS in the hippocampal CA1 area requires postsynaptic molecular mechanisms, such as activation of the ERKs of the mitogen-activated protein kinase family and of the transcription factor, CREB (Impey *et al.*, 1996; Thomas and Huganir, 2004). Activation of the two distinct signalling pathways of ERK and PI3K lead to the activation of CREB (Spencer, 2009). We therefore assayed ERK1/2 and CREB expression by Western blotting, 5 min after LTP induction, with or without baicalein treatment.

High-frequency stimulation induced an activation of ERK1/2 phosphorylation 5 min after HFS (Figure 6A and B) and pre-incubation of hippocampal slices with baicalein (1 μM) did not affect this phosphorylation (Figure 6A and B). CREB phosphorylation (at Ser¹³³) was also significantly increased following HFS induction (Figure 6C and D) and LTP induction in the presence of baicalein (1 μM) further increased CREB phosphorylation, without any significant change in total CREB expression (Figure 6C and D).

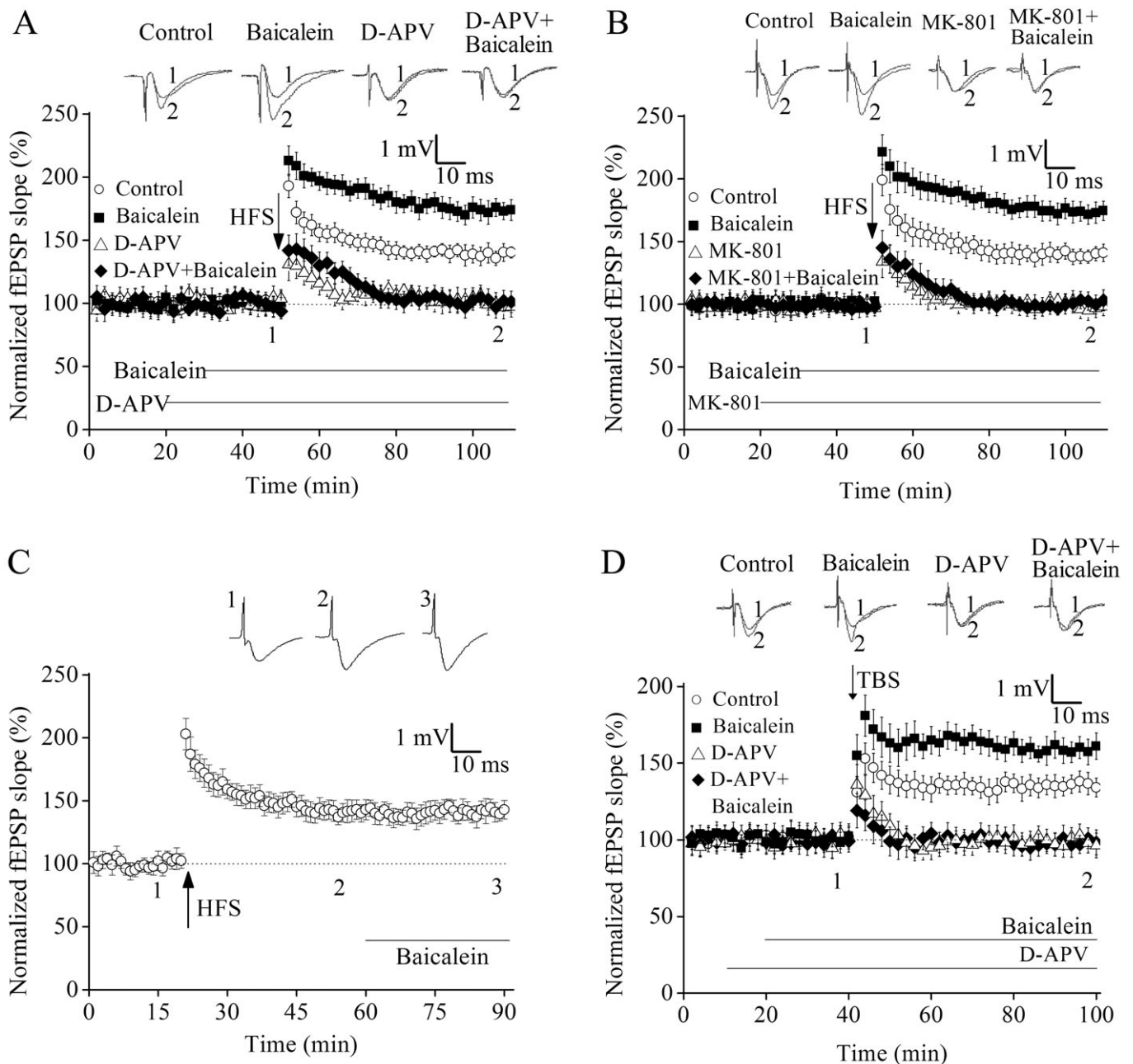


Figure 3

N-methyl-D-aspartate (NMDA) receptors contribute to baicalein-facilitated long-term potentiation (LTP). (A) Changes in slopes of field excitatory postsynaptic potential (fEPSP) following high-frequency stimulation (HFS) in the presence and absence of 1 μ M baicalein and 50 μ M D-2-amino-5-phosphonovaleric acid (D-APV). (B) Changes in slopes of fEPSP following HFS in the presence and absence of 1 μ M baicalein and 10 μ M MK-801. (C) Average effects of 1 μ M baicalein applied for 30 min after the induction of LTP. (D) Changes in slopes of fEPSP following theta-burst stimulation (TBS) in the presence and absence of 1 μ M baicalein and 50 μ M D-APV. The superimposed fEPSP in the upper portion are recordings from representative experiments taken at the time indicated by the number. Each point was the normalized mean \pm SEM of six slices.

Baicalein improves hippocampus-dependent contextual fear conditioning

To determine whether the electrophysiological and biochemical effects of baicalein seen in hippocampal slices translated into alterations in memory *in vivo*, we used a

hippocampus-dependent contextual fear conditioning task. The animals were trained for fear conditioning 20 min after baicalein treatment. The pre-training administration of baicalein had no effect on freezing behaviour observed during training (data not shown). Twenty-four hours after

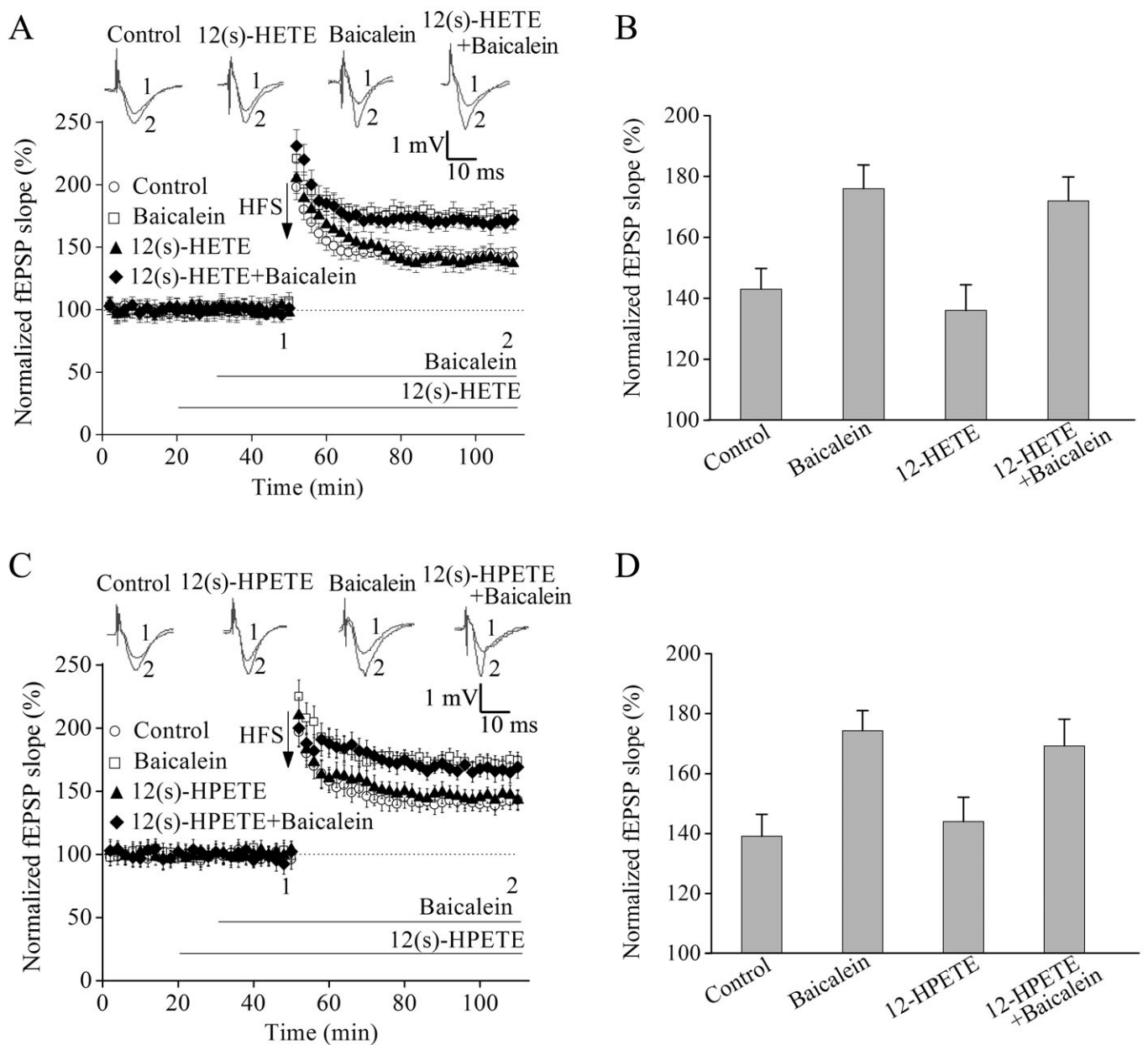


Figure 4

Addition of 12(S)-HETE or 12(S)-HPETE did not affect baicalein-induced long-term potentiation (LTP) enhancement. (A) Pre-incubation of slices with 12(S)-HETE (250 nM) did not affect the induction of high-frequency stimulation (HFS)-induced LTP and baicalein-facilitated LTP. (B) Summary data of the average field excitatory postsynaptic potential (fEPSP) slope after HFS relative to untreated, baicalein, 12(S)-HETE, the combination of 12(S)-HETE and baicalein. (C) 12(S)-HPETE did not block HFS-induced LTP and baicalein-facilitated LTP. (D) Summary data of the average fEPSP slope after HFS relative to untreated, baicalein, 12(S)-HPETE, the combination of 12(S)-HPETE and baicalein. The superimposed fEPSP in the top portion showed respective recordings from representative experiments taken at the time indicated by the number. Each point was the normalized mean \pm SEM of six slices.

training, the rats were tested for freezing behaviour. A time-line of the experiment is presented in Figure 7A. Interestingly, baicalein improved contextual fear conditioning with a bell-shaped dose-response profile, with the peak response at the doses of 20 mg·kg⁻¹ (Figure 7B). During the cued fear conditioning test, all groups did not differ in the amount of time spent freezing during the presentation of the tones (Figure 7C).

The enhanced hippocampus-dependent memory formation could be attributable to increased pain sensitivity or motor defects. The rats were exposed to the open field test to analyse their locomotor ability. Distance travelled during the initial 3 min exposure to the training box in an open field test was recorded, and no statistically significant differences were found among the five groups ($n = 8$, Figure 7D). To determine pain threshold, rats were exposed to electric foot shocks of

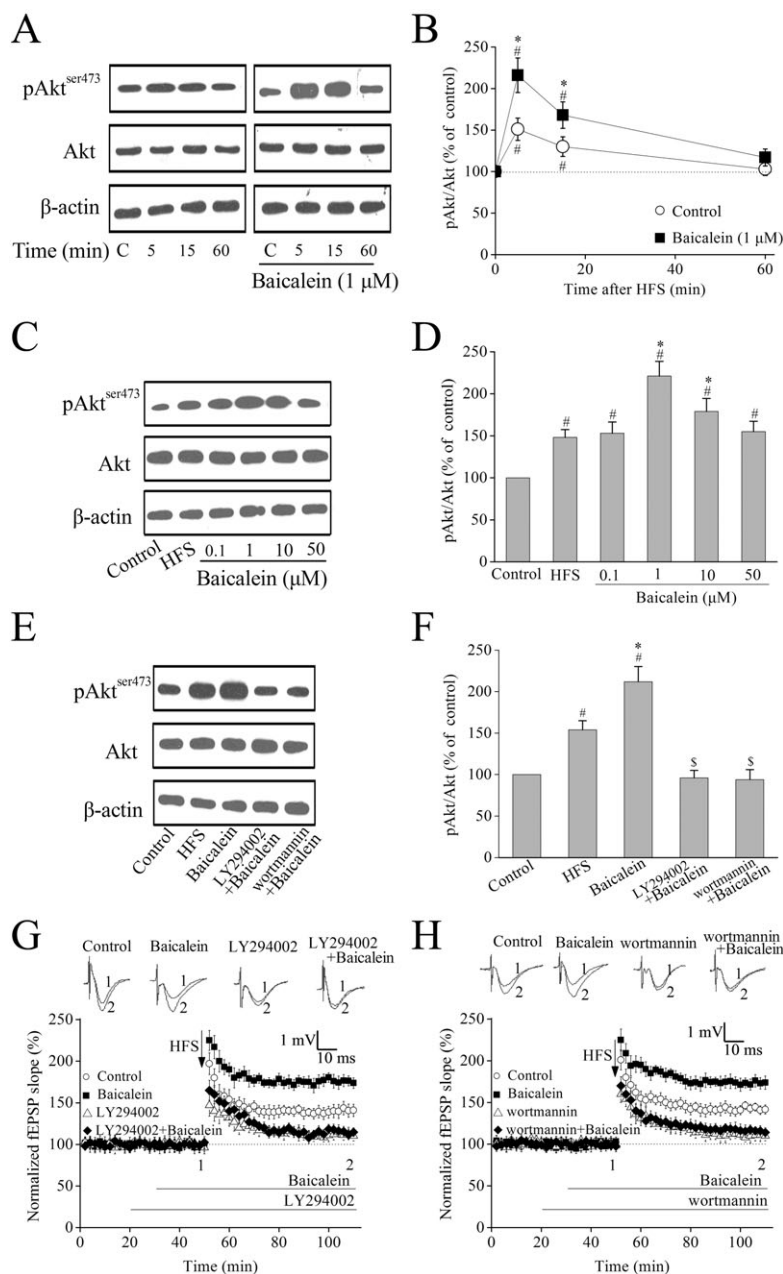


Figure 5

Activation of PI3K pathway is required for baicalein-induced long-term potentiation (LTP) enhancement. (A) Representative images of immunoblots using antibodies against phosphorylated Akt (ser⁴⁷³), total Akt and β -actin. High-frequency stimulation (HFS) stimulation induced a transient activation of Akt phosphorylation. Pre-incubation of baicalein (1 μ M) further increased Akt phosphorylation time-dependently. (B) Summary data of phosphorylated Akt and total Akt by densitometry. Data were expressed as percentage of value of controls without stimulation; $n = 4$. # $P < 0.05$ versus control (without HFS stimulation), * $P < 0.05$ versus HFS (without baicalein treatment). (C) Representative images of immunoblots using antibodies against phosphorylated Akt (Ser⁴⁷³), total Akt and β -actin. Baicalein further increased Akt phosphorylation 5 min after HFS in a bell-shaped dose–response manner that peaked at 1 μ M. (D) Summary data of phosphorylated Akt and total Akt by densitometry. Data were expressed as percentage of value of controls without stimulation; $n = 4$. # $P < 0.05$ versus control, * $P < 0.05$ versus HFS. (E) Representative images of immunoblots using antibodies against phosphorylated Akt (ser⁴⁷³), total Akt and β -actin. LY294002 (10 μ M) or wortmannin (500 nM) completely blocked the baicalein-induced enhancement of Akt phosphorylation 5 min after HFS. (F) Summary data of phosphorylated Akt and total Akt by densitometry. Data were expressed as percentage of value of controls without stimulation; $n = 3$. # $P < 0.05$ versus control, * $P < 0.05$ versus HFS, ^s $P < 0.05$ versus baicalein. (G–H) The PI3K inhibitors (LY294002 or wortmannin) totally inhibited baicalein-induced enhancement of LTP. Hippocampal slices were pretreated with either LY294002 (10 μ M) or wortmannin (500 nM) for 10 min before addition of 1 μ M baicalein following HFS. The superimposed field excitatory postsynaptic potentials (fEPSPs) in the upper portion are recordings from representative experiments taken at the time indicated by the number. Each point was the normalized mean \pm SEM of six slices.

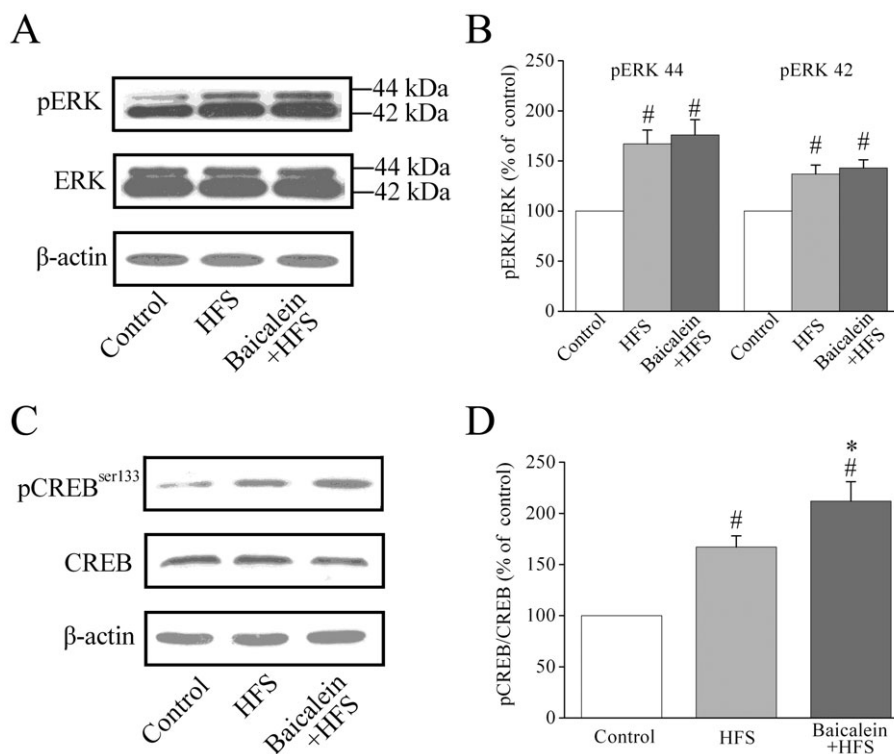


Figure 6

Effects of baicalein treatment on phosphorylation of ERK1/2 and CREB after high-frequency stimulation (HFS) in rat hippocampal CA1 region. (A) Representative images of immunoblots using antibodies against phosphorylated ERK1/2 (pERK1/2), ERK1/2 and β -actin. HFS stimulation induced an activation of ERK1/2 phosphorylation 5 min after HFS. Pre-incubation of hippocampal slices with baicalein (1 μ M) did not affect ERK1/2 phosphorylation 5 min after HFS. (B) Summary data of phosphorylated ERK1/2 and total ERK1/2 expression by densitometry. Data were expressed as percentage of value of controls without stimulation. # $P < 0.05$ versus control, $n = 4$. (C) Representative images of immunoblots using antibodies against phosphorylated CREB (Ser¹³³), total CREB and β -actin. HFS stimulation induced an activation of CREB phosphorylation 5 min after HFS. Pre-incubation of hippocampal slices with baicalein (1 μ M) further increased CREB phosphorylation 5 min after HFS. (D) Summary data of phosphorylated CREB and total CREB expression by densitometry. Data were expressed as percentage of value of controls without stimulation. # $P < 0.05$ versus control, * $P < 0.05$ versus HFS, $n = 4$.

increasing intensities. The thresholds for running/jumping and flinching in response to the shock did not differ between all groups ($n = 8$, Figure 7E).

Modulation of Akt and CREB expression in the hippocampus and cortex by baicalein treatment after fear conditioning training

It is well established that hippocampus-dependent memory formation is associated with the activation of the PI3K pathway and increased CRE-mediated gene expression (Impey *et al.*, 1998; Chen *et al.*, 2005). To investigate the mechanisms involved in the modulation of hippocampus-dependent memory by baicalein, Akt and CREB expression were assayed by Western blotting 15 min after fear conditioning training with or without baicalein treatment. In these experiments, rats were divided into three groups: control, training or training with baicalein (20 mg kg⁻¹). Rats in the control group were placed into the conditioning chamber but received no shock.

Fear conditioning training increased CREB and Akt phosphorylation in the CA1 region of hippocampus (Figure 8A and C) but not in prefrontal cortex (Figure 8B and D). Baica-

lein treatment 20 min before training (20 mg·kg⁻¹) further increased Akt and CREB phosphorylation in the hippocampal CA1 region (Figure 8A and C) but not in the prefrontal cortex (Figure 8B and D).

Discussion

In the present study, we demonstrated for the first time that flavonoid baicalein facilitated the induction of NMDA receptor-dependent LTP in the hippocampal CA1 region *in vitro*, and that acute administration of baicalein promoted hippocampus-dependent associative memory. The major findings of our study are as follows: (i) application of baicalein facilitated NMDA receptor-dependent LTP in a bell-shaped, concentration-dependent manner without affecting basal synaptic transmission; (ii) the promotion of LTP by baicalein was independent of 12-LO inhibition; (iii) baicalein-facilitated LTP was dependent on the activation of the PI3K pathway; (iv) baicalein enhanced contextual fear conditioning performance; and (v) baicalein enhanced CREB phosphorylation in rat hippocampal CA1 region.

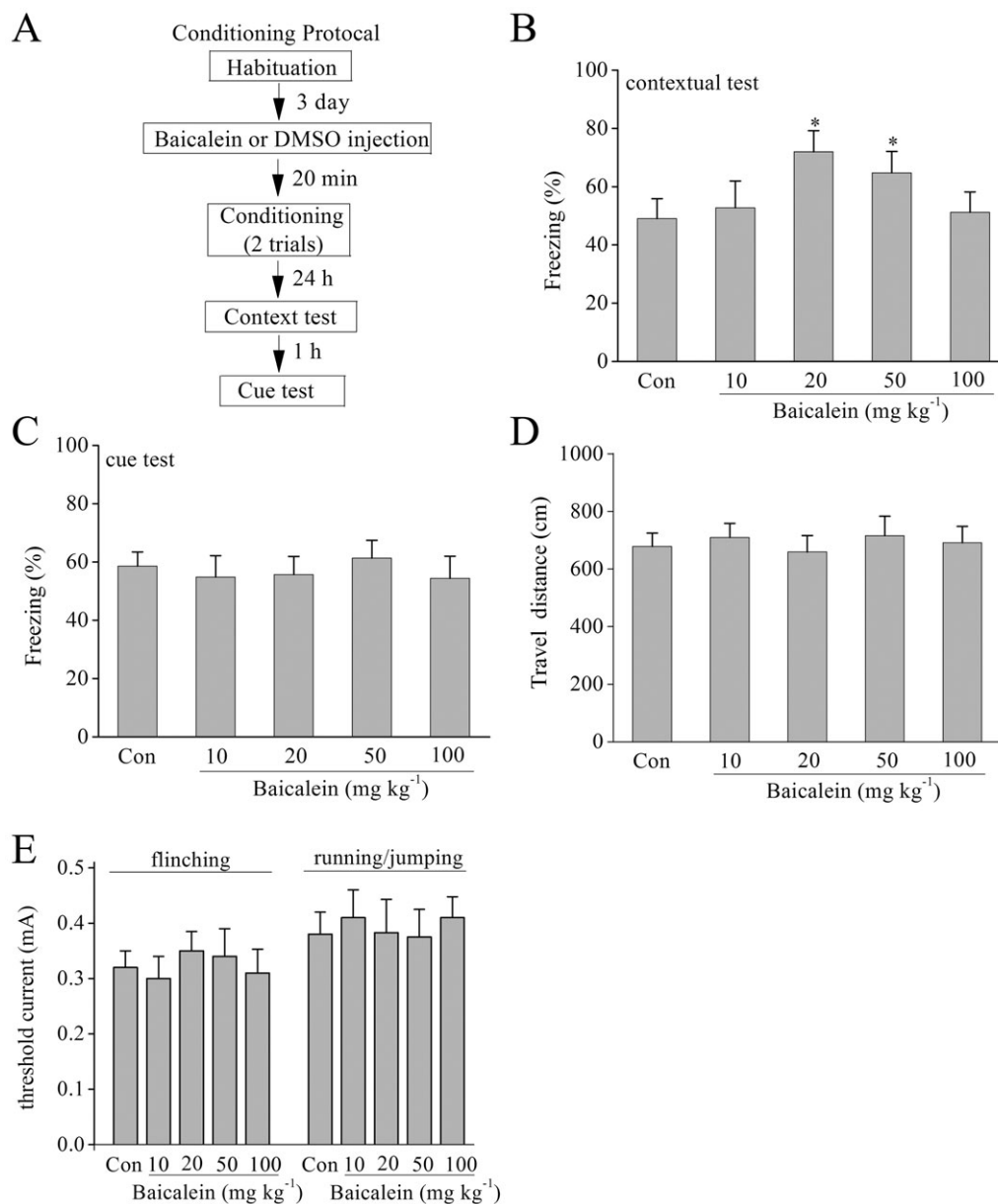


Figure 7

Baicalein improves the learning and memory performances in contextual fear conditioning tests. (A) Experimental design of fear conditioning paradigm. (B) Total percentage of freezing during the 3 min contextual fear conditioning test when tested 24 h after conditioning. Baicalein improved contextual fear conditioning at 20 mg·kg⁻¹ and 50 mg·kg⁻¹. *n* = 8, **P* < 0.05 versus control. (C) Total percentage of freezing during the presentation of the tones in the cued fear conditioning test when tested 1 h after contextual fear conditioning test. Groups did not differ in freezing to the tone during the cued fear conditioning test compared with controls. (D) Distance travelled during the initial 3 min exposure to the training box in an open field test. (E) Pain thresholds. Thresholds of current intensities for flinching and running/jumping are shown.

Memory and cognitive function impairment associated with age-related neurodegenerative diseases, such as brain ischaemia, Alzheimer's disease and Parkinson's disease have become a large public health problem with the increasing elderly population. Considering the relationship between hippocampal LTP and cognition, small molecules that promote LTP in hippocampus could be used as novel agents against age-associated memory impairment. There have been many new drug candidates designed to boost memory in

recent years (Marshall, 2004) and flavonoid compounds have received much attention, as they are the most common group of polyphenolic compounds in the human diet and are ubiquitous in plants (Spencer, 2008; 2009; Rendeiro *et al.*, 2009). As a polyphenol that belongs to the flavone subgroup, baicalein has been used to enhance memory for thousands of years in China (Wang *et al.*, 2004b). We have previously reported that baicalein improves learning and memory deficits induced by permanent occlusion of bilateral common carotid

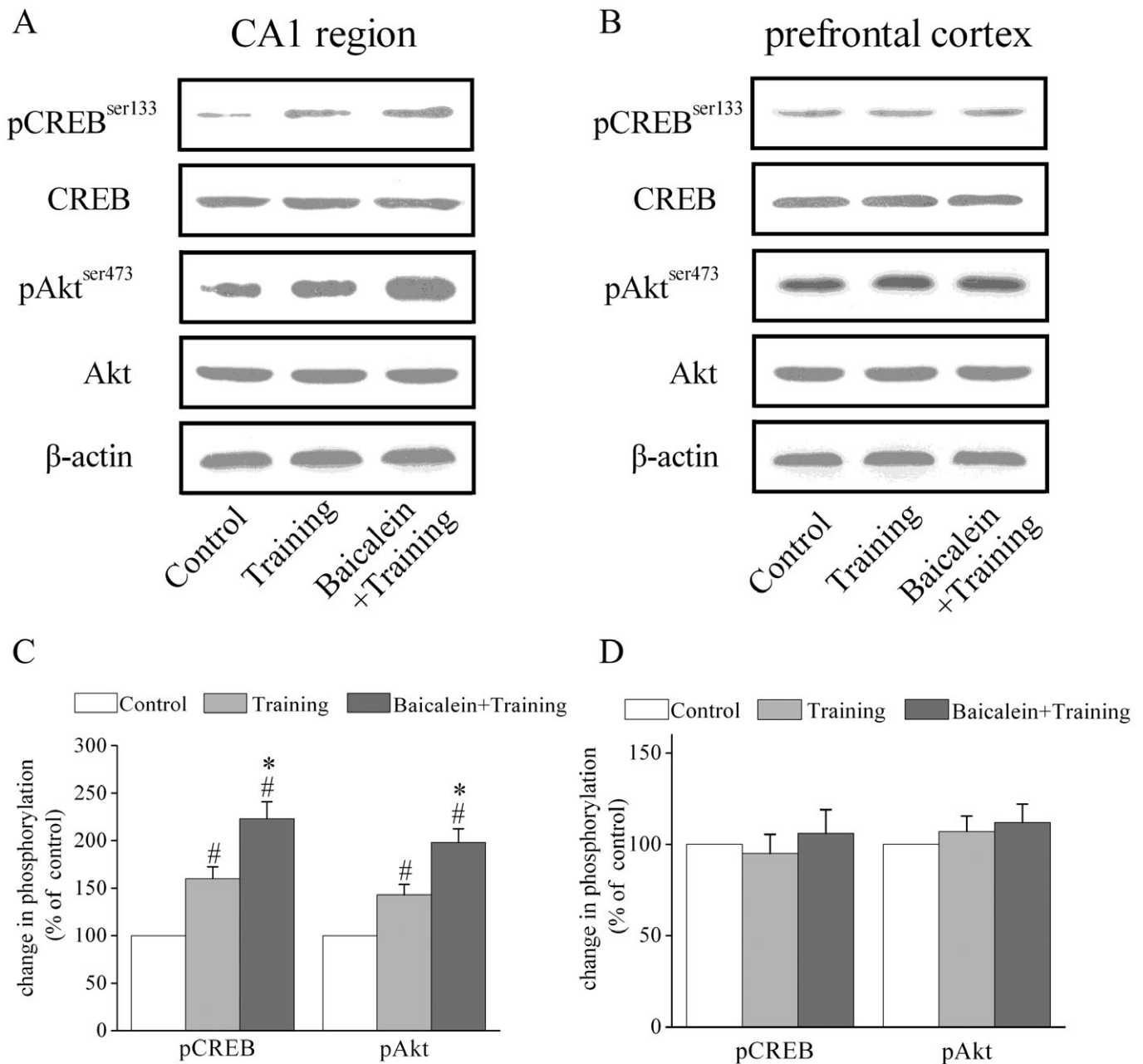


Figure 8

Baicalein treatment results in a selective further increase in the phosphorylation of Akt and CREB in the CA1 region of hippocampus but not in the prefrontal cortex after fear conditioning training. (A–B) Representative images of immunoblots using antibodies against phosphorylated Akt (Ser⁴⁷³), total Akt, phosphorylated CREB (Ser¹³³), total CREB and β-actin. Protein extracts from the hippocampal CA1 region and prefrontal cortex were analysed with Western blotting 15 min after contextual fear conditioning training. Fear conditioning training induced an activation of Akt and CREB phosphorylation in the CA1 region of hippocampus but not in prefrontal cortex. Baicalein treatment 20 min before training (20 mg·kg⁻¹) further increased Akt and CREB phosphorylation in the hippocampal CA1 region but not in prefrontal cortex. (C–D) Summary data of phosphorylated Akt, total Akt, phosphorylated CREB (ser¹³³) and total CREB by densitometry. Data were expressed as percentage of value of controls without training. #*P* < 0.05 versus control, **P* < 0.05 versus training, *n* = 4.

arteries of rats (Liu *et al.*, 2007). Furthermore, many studies have shown that baicalein facilitates cognitive functions in the acquisition, consolidation stages of learning and memory processes in the scopolamine-, chronic cerebral hypoperfusion- and β-amyloid peptide-induced dementia of

animals (Wang *et al.*, 2004a; He *et al.*, 2009). However, the exact mechanism by which baicalein enhances memory remains unknown.

The hippocampus plays a major role in memory formation and consolidation processes and it is generally accepted

that most information is stored at synapses in the form of alterations in synaptic efficiency (Bliss and Collingridge, 1993). In particular, LTP, a form of synaptic plasticity, has been widely used to explore the molecular and cellular basis for learning and memory (Bliss and Collingridge, 1993). Our present results demonstrated that relatively low concentrations of baicalein increased LTP in the hippocampal CA1 region, and this enhancement reached a maximum at a concentration of 1 μM . Unexpectedly, LTP magnitude returned towards the control level when slices were exposed to a higher concentration of this drug. Thus, the dose–response curve for baicalein on LTP showed a bell-shaped feature, and this result was consistent with some previous observations that galantamine (Moriguchi *et al.*, 2009), fisetin (Maher *et al.*, 2006), SKF38393 (Stramiello and Wagner, 2008), and nefiracetam (Moriguchi *et al.*, 2008) potentiate NMDA receptor-dependent LTP in the same bell-shaped manner. Moreover, application of 1 μM baicalein for 30 min did not affect previously induced LTP, suggesting that the drug did not compromise the expression of LTP. A variety of evidence has indicated that increases in both presynaptic release of glutamate and postsynaptic response to glutamate are involved in the expression of LTP (Malenka and Nicoll, 1999). Presynaptic changes can be detected by the PPF technique and a decreased PPR in association with LTP was indicative of an increased probability of presynaptic neurotransmitter release (Schulz *et al.*, 1994). However, we found that 1 μM baicalein did not change the PPR before and after HFS stimulation, suggesting that augmentation of LTP by baicalein did not involve changes in presynaptic neurotransmitter release.

Previous studies have shown that LTP triggered by HFS or short trains of TBS stimulation in hippocampal CA1 area requires postsynaptic molecular mechanisms, such as activation of NMDA receptors and the PI3K signalling pathway (Malenka and Bear, 2004; Raymond, 2007). Such stimulations result in a pattern of glutamate release that is sufficient to activate postsynaptic NMDA receptors and induce NMDA receptor-dependent LTP, which is completely blocked by NMDA receptor antagonists. Consistent with these previous observations, we found that the NMDA receptor antagonists D-APV and MK-801 completely blocked HFS and TBS-induced LTP under our experiment condition. Furthermore, NMDA receptor antagonists totally blocked baicalein-facilitated LTP. Taken together, these results indicate that baicalein promotes NMDA receptor-dependent LTP in hippocampal slices of rats.

The next question should then be which molecule(s) in the postsynaptic neuron is the target of baicalein. Baicalein is known as a 12-LO inhibitor and decreases the generation of 12(S)-HETE and 12(S)-HPETE in cell proliferation studies. Lipoxygenases are non-haem iron proteins and incorporate a molecular oxygen into various positions into arachidonic acid and other polyunsaturated lipids, and there is an expanding literature on the role of arachidonic acid-derived lipids in synaptic plasticity. However, evidence for the role of 12-LO in LTP has been controversial (O'Dell *et al.*, 1991). A recent study using 12-LO knock-out mice indicates that the 12-LO pathway is necessary for the induction of metabotropic glutamate receptor (mGluR)-dependent LTD, but not for NMDA receptor-dependent LTP at CA3-CA1 synapses (Feinmark *et al.*, 2003). Similarly, we found that treatment with 12-HETE and 12-HPETE had no effect on NMDA receptor-

dependent LTP. Moreover, the promotion of LTP by baicalein was independent of 12-lipoxygenase inhibition, because 12(S)-HETE and 12(S)-HPETE did not reverse the effect of baicalein. Indeed, many studies have confirmed that a variety of biological activities of baicalein are not associated with 12-LO activity (Suk *et al.*, 2003; Peng *et al.*, 2008; Taniguchi *et al.*, 2008). Regardless of the relevance of 12-LO inhibition in LTP facilitation, baicalein might have inhibited 12-LO activity on brain slices under our experimental conditions. However, NMDA receptor-dependent LTP at CA3-CA1 synapses is not related to 12-LO activity as discussed above. Thus, other molecular mechanisms underlying the effect of baicalein must be investigated.

The PI3K pathway has been classically involved in the regulation of cell growth, survival, proliferation (Brunet *et al.*, 2001). In addition to its well-established role in neuronal survival and differentiation, PI3K is also important in synaptic plasticity and learning and memory. For example, it has been shown that activation of PI3K is required for the expression of LTP in the hippocampal CA1 region (Raymond *et al.*, 2002; Opazo *et al.*, 2003; Racaniello *et al.*, 2010). PI3K may contribute to the regulation of NMDA receptor-dependent LTP and memory formation by facilitating the insertion of AMPA receptors into the postsynaptic membrane (Man *et al.*, 2003). In our previous studies, baicalein attenuated learning and memory deficits and protected neurons against ischaemic injury by activating the PI3K/Akt pathway in rats (Liu *et al.*, 2007; 2010). Furthermore, other flavonoids such as the citrus flavanone hesperetin activate the PI3K/Akt pathway in neurons and flavonoids are known to activate Akt phosphorylation at Ser⁴⁷³ in a dose-dependent manner (Vauzour *et al.*, 2007; Spencer, 2009). In accordance with a previous report (Opazo *et al.*, 2003), we found here that the PI3K inhibitors LY294002 and wortmannin decreased the magnitude of LTP and PI3K inhibitors completely blocked baicalein-facilitated LTP, supporting an involvement of PI3K signalling in baicalein-facilitated LTP. To determine whether up-regulation of PI3K activity is responsible for the enhancement of LTP by baicalein treatment, we indirectly monitored the activation of PI3K by measuring the phosphorylation of its downstream target Akt at Ser⁴⁷³ using Western blot analysis. We found that HFS induction was associated with an increase in the phosphorylation of Akt at Ser⁴⁷³ time-dependently. Furthermore, increased phosphorylation of Akt was further augmented by baicalein treatment in a bell-shaped dose–response manner that peaked at 1 μM , indicating that the activation of the PI3K pathway by baicalein in hippocampal slices following HFS could account for its enhancement of LTP.

cAMP response element-binding protein is a transcription factor for many genes associated with memory and synaptic plasticity. Furthermore, robust CREB phosphorylation was detected in hippocampus in response to both LTP-inducing stimulation and memory training tasks (Impey *et al.*, 1996; Chen *et al.*, 2005). Various signalling pathways have been linked to CREB activation in the induction of long-lasting changes in synaptic plasticity and memory, including the ERK and PI3K pathways. We found that CREB phosphorylation was significantly increased in the CA1 region of baicalein-treated slices after HFS. Furthermore, baicalein treatment selectively increased the phosphorylation of CREB

in the CA1 region of hippocampus, but not in prefrontal cortex, after fear conditioning training. However, there were no significant alterations in ERK phosphorylation in the CA1 region related to baicalein treatment of slices after HFS. These data indicated that baicalein could promote CREB phosphorylation in a PI3K-dependent way, but independent of ERK activity.

It is well established that cued fear conditioning relies on the structural integrity of the amygdala but not the hippocampus, whereas contextual fear conditioning is hippocampus and amygdala-dependent (Anagnostaras *et al.*, 2001; Rodrigues *et al.*, 2004). A number of studies have addressed the role of NMDA receptors in the hippocampus, which is essential for the formation of contextual memory (Kim *et al.*, 1991; Schenberg and Oliveira, 2008; Gao *et al.*, 2009). Dash *et al.* (2004) found that stimulation of PI3K activity with a synthetic phosphopeptide improved performance in contextual fear conditioning task. In our last set of experiments, given the results above and the known associations between LTP and memory, we examined whether the electrophysiological effects of baicalein seen in hippocampal slices would translate into improvements in memory in normal rats. A previous study of the pharmacokinetics and tissue distribution of baicalein in rats has shown that baicalein rapidly penetrates the blood–brain barrier by 20 min after administration and becomes homogeneously distributed over various regions of the brain (brain stem, cerebellum, cerebral cortex, hippocampus, midbrain and striatum) (Tsai *et al.*, 2002). We found that baicalein treatment 20 min before training influenced hippocampus-dependent contextual fear conditioning, but not hippocampus-independent cued fear conditioning, indicating that baicalein may mediate hippocampus-dependent memory but with less effect on amygdala-dependent memory. Moreover, the increase in freezing behaviour in baicalein-treated rats was not due to the changes in locomotive activity and pain sensation, because their response to electric foot shock and the exploratory behaviour during exposure to novel context were similar to those of the control rats.

In summary, the results presented here indicate that baicalein facilitates a postsynaptic NMDA receptor-dependent LTP at the Schaffer collateral-CA1 synapses via stimulation of PI3K activity. We also found that acute administration of baicalein increased hippocampus-dependent contextual fear conditioning performance of rats. These results provide further insight into the mechanisms through which baicalein exerts its beneficial effect on central nervous system disorders and age-associated memory impairment, suggesting that baicalein may be a promising agent for therapy of cognitive deficits associated with neurodegenerative disorders.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Pre-training administration of baicalein has no effect on cortex-dependent remote memory. (A) Experimental design of fear conditioning paradigm for remote memory test. (B) Total percentage of freezing during the 3 min contextual fear-conditioning test when tested 15 d after fear conditioning training. Groups did not differ in freezing during the cortex-dependent remote memory test compared with controls.

Figure S2 12(S)-HETE and 12(S)-HPETE at various concentrations do not affect baicalein-induced LTP enhancement. (A) Pre-incubation of slices with 12(S)-HETE (100 nM or 1 μ M) did not affect the induction of baicalein-facilitated LTP. (B) Histograms indicate the average fEPSP slope after HFS relative to baicalein and the combination of 12(S)-HETE (100 nM or 1 μ M) and baicalein. (C) 12(S)-HPETE (100 nM or 1 μ M) did not block baicalein-facilitated LTP. (D) Histograms indicate the average fEPSP slope after HFS relative to baicalein and the combination of 12(S)-HPETE (100 nM or 1 μ M) and baicalein. The superimposed fEPSP in the top portion showed respective recordings from representative experiments taken at the time indicated by the number. Each point was the normalized mean \pm SEM of slices. $n = 6$.

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