Chen et al.
J Neurophysiol.

**PREGS induces LTP in the hippocampal dentate gyrus of adult rats via the tyrosine phosphorylation of NR2B coupled to ERK/CREB signaling**

Ling Chen¹,²,³, Yoshiaki Miyamoto⁴, Kishio Furuya², Nozomu Mori⁵, Masahiro Sokabe²,³,⁶

¹Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China, ²ICORP/SORST Cell Mechanosensing, JST, 65 Tsurumai, Nagoya 466-8550, Japan, ³Department of Physiology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Nagoya 466-8550, Japan, ⁴Department of Aging Intervention, National Institute for Longevity Sciences, Oobu 474-8522, Japan, ⁵Department of Anatomy and Neurobiology, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan, ⁶Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan

Running head: PREGS-induced LTP

Address correspondence to: Masahiro Sokabe, Ph.D.
Address: Department of Physiology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
Tel: +81-52-744-2051, Fax: +81-52-744-2057, E-mail: msokabe@med.nagoya-u.ac.jp
ABSTRACT

An acute application of neurosteroid pregnenolone sulfate (PREGS) to hippocampal slices from adult rats induced a long-lasting potentiation (LLP\textsubscript{PREGS}) at the perforant path-granule cell synapse. As a partial mechanism of the LLP\textsubscript{PREGS}, we previously revealed that PREGS transiently increases the probability of presynaptic glutamate release via a sensitization of $\alpha 7$-nicotinic acetylcholine receptor ($\alpha 7nAChR$). We herein demonstrate that the LLP\textsubscript{PREGS} could be separated into two independent processes: the above mentioned early presynaptic-origin short-term potentiation (STP\textsubscript{PREGS}) and a delayed postsynaptic NMDA receptor (NMDAr)-dependent long-term potentiation termed LTP\textsubscript{PREGS}. This study focused on the analysis of the signaling mechanism underlying the LTP\textsubscript{PREGS}. PREGS increased the tyrosine phosphorylation of NR2B, a subunit of NMDAr, and the NMDAr-mediated Ca$^{2+}$ influx in the granule cells. The enhanced Ca$^{2+}$ influx was largely attenuated by the NR2B subunit inhibitor Ifenprodil and the Src kinase family inhibitor PP2. PREGS also triggered a persistent phosphorylation of extracellular signal-regulated kinase 2 (ERK2) followed by an ERK-dependent phosphorylation of cAMP-response element-binding protein (CREB), which was crucial for the LTP\textsubscript{PREGS} induction and was sensitive to Ifenprodil. These results suggest that PREGS induces an acute increase in the NR2B tyrosine phosphorylation which enhances the Ca$^{2+}$ influx through NMDAr, followed by an activation of the ERK/CREB signaling cascade that leads to LTP\textsubscript{PREGS}.

Key words: Pregnenolone sulfate (PREGS); long-term potentiation (LTP); NMDA receptor...
(NMDAr); NR2B subunit; extracellular signal-regulated kinase (ERK); cAMP-response element-binding protein (CREB)

INTRODUCTION

Pregnenolone sulfate (PREGS), one of the most abundant neurosteroids, improves memory and learning in adult rodents (Vallee et al., 2001; Darnaudery et al., 2000). The deterioration in the cognitive performance of aged rats has been suggested to be linked to the low levels of PREGS in the hippocampus (Flood et al., 1995). A significant negative correlation has been found in the levels between β-amyloid and PREGS in the brain of Alzheimer’s disease patients (Weill-Engerer et al., 2002). Anti-amnesic effects of PREGS have also been demonstrated in animal amnestic models produced by competitive or non-competitive NMDA receptor antagonists (Cheney et al., 1995; Mathis et al., 1994; 1996), and β_{25-35}-amyloid peptide (Maurice et al., 1998). These observations strongly support that PREGS is an important endogenous factor for enhancing both memory and learning, however, the exact underlying mechanisms of this action still remain to be elucidated.

Long-term potentiation (LTP) is widely accepted as a candidate cellular mechanism for the storage of information (Bliss and Collingridge, 1993). In immature hippocampal CA1 neurons (younger than postnatal day 6), PREGS has been observed to induce a long-lasting strengthening of AMPA-mediated miniature excitatory postsynaptic currents via retrograde modulation of presynaptic NMDAr (Mameli et al., 2005). We previously reported that in the mature hippocampal slices (older than 6 weeks) an acute application of PREGS for 10 min triggered a persistent
potentiation at the perforant path-granule cell synaptic transmission in the hippocampal dentate gyrus (DG) (Chen et al., 2002). We also have provided evidence that PREGS transiently increases the probability of glutamate release by sensitizing the presynaptic α7nAChR (Chen and Sokabe, 2005). In addition, PREGS has been shown to exert acute effects through the positive actions to ionotropic glutamate receptors including the potentiation of Ca\(^{2+}\) conductivity and following Ca\(^{2+}\) influx via NMDA\(_{\text{R}}\) (Wu et al., 1991, Takebayashi et al., 1998; Mukai et al., 2000). PREGS also antagonizes the GABA/glycine activated Cl\(^-\) currents (Paul and Purdy, 1992). In spite of such a variety of studies concerning the molecular targets of PREGS, the mechanism by which PREGS induces a long-lasting potentiation in the adult rat is still not fully understood.

Activity dependent LTP at glutamatergic synapses in the adult hippocampus is typically divided into two stages, namely an early, labile phase dependent upon covalent modifications of existing proteins and a late, stable phase requiring synthesis of new proteins (Nguyen and Kandel, 1996). Increasing evidence indicates that the long-lasting potentiation of synaptic efficacy requires an activation of ERKs in mammals (Atkins et al., 1998; Blum et al., 1999; Impey et al., 1999; Kelleher et al. 2004; Schmitt et al. 2005; Shalin et al. 2006). The phosphorylation of CREB, as a downstream target of ERKs, is also a necessary component for hippocampus-dependent memory formation in mammals (Bourtchuladze et al., 1994; Impey et al., 1996). It would therefore be interesting to clarify whether the PREGS-induced synaptic potentiation shares similar mechanisms with those observed in the activity-dependent LTP.

The present study demonstrates that an acute application of PREGS exerts two independent
effects, one is on the presynaptic α7nAchR while the other is on postsynaptic NMDAr, thus leading to an early presynaptic-origin short-term potentiation (STP\textsubscript{PREGS}) and a delayed postsynaptic-origin long-term potentiation (LTP\textsubscript{PREGS}), respectively. The induction of the LTP\textsubscript{PREGS} requires a potentiation of the NR2B subunit of postsynaptic NMDAr followed by an activation of the ERK/CREB signaling cascade.

MATERIALS AND METHODS

All experiments were carried out in accordance with the guidelines under license from the Department of Health, Ireland (86/609/EEC), and the guidelines established by the Institute for Laboratory Animal Research of Nagoya University. Male Wistar rats (Oriental Bio Service Inc., Kyoto, Japan), weighing 300-350g, at the beginning of the experiments, were used throughout the study. The animals were handled in accordance with the guidelines established by the Institute for Laboratory Animal Research of Nagoya University, and housed at a temperature of 23°C and kept in a light-controlled room (a 12 hour cycle starting at 9:00 am). They also had free access to food and water.

Slice preparation and staining with voltage sensitive dye

The rats were decapitated under deep anesthesia with ethyl ether. The brains were taken quickly after removing the skulls, and then placed in ice-cold artificial cerebrospinal fluid (ACSF) for around 10 min. ACSF of the following composition (in mM): NaCl 126, KCl 2.7, KH\textsubscript{2}PO\textsubscript{4} 1.24,
MgSO$_4$ 1.3, CaCl$_2$ 2.4, NaHCO$_3$ 26 and glucose 10. ACSF was oxygenated with a gas mixture of 95% O$_2$ and 5% CO$_2$ and the pH was adjusted to 7.4. Horizontal slices (350 $\mu$m in thickness) of the brain were cut using a vibrating microtome (Microslicer DTK 1500, Dousaka EM Co, Kyoto, Japan). The slices were incubated in a rest chamber containing fresh oxygenated ACSF at room temperature for more than 1 hr in order to recover the slices from damage.

**Electrophysiological recordings**

All the slices used in this study were stained with voltage-sensitive dye RH482 (0.1 mg/ml, Nippon Kanko Shikiso Kenkyujo, Okayama, Japan) for 15 min. The stained slices were kept in the fresh oxygenated ACSF for at least 30 min for recovery. The slices were then transferred to a recording chamber and placed on the stage of an Olympus inverted microscope (IMT-2, Nikon, Tokyo, Japan), and was perfused continuously with ACSF at a flow rate of around 1-2 ml/min. The experiments were performed at room temperature (28±1°C).

The electrical and optical recordings, as well as the evaluation of the electrical and the optical signals were performed as described previously by us (Chen et al., 2006a). Briefly, orthodromic stimuli were delivered using an electrically polished bipolar tungsten electrode or a glass electrode filled with 0.9% NaCl (tip resistance of 5 M$\Omega$) that was placed in the outer third of str. moleculare to stimulate the lateral perforant path (Lin et al., 2006). Constant current pulses (0.1 ms, 0.06 Hz) were supplied by a stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan). The intensity of the test stimuli was adjusted to evoke around 50% of the maximum value of optical excitatory postsynaptic
potential (op-EPSP). Light from a tungsten-halogen lamp (type JC-24v/200W, Kondo Philips, Tokyo, Japan) was collimated, and an interference filter with a transmission maximum at 700±10 nm (Olympus Optical) was used. Changes in the light absorption associated with membrane potential changes were measured with an optical recording system equipped with a high speed diode camera with metal oxide (MOS) image sensors (128 ×128 pixels, each of which receives light from a 25 µm × 25 µm sample area) and a data processing unit (HR Deltaron-1700 Fujix; Fuji Photo Film, Tokyo, Japan). To analyze the change in the neuronal activities over time at a given region, the data from each pixel were stored, and retrieved, and then the amplitude of optical signal (indicated as a percent change in optical absorbency) was plotted as a function of time.

In some experiments, field potential recordings from *str. moleculare* were simultaneously performed with optical recordings to ensure that the optical response was consistent with the electrophysiological response. After a slice was submerged in a recording chamber containing ACSF, a bipolar tungsten electrode was placed in *str. moleculare* to stimulate the perforant path, using a hydraulic micromanipulator (MMW204, Narishige, Tokyo, Japan) mounted on the microscope stage. The field excitatory postsynaptic potentials (f-EPSPs) were recorded from *str. moleculare* with a 4-5 MΩ resistance glass microelectrode filled with 0.9% NaCl which was connected to a home-made, neutralized, high input-impedance preamplifier.

**Western blot analysis and immunoprecipitation assay**

After a series of electrophysiological experiments, the slices were harvested immediately and
Chen et al.

then were frozen on dry ice. The hippocampal DG regions from the experimental slices were micro-dissected on dry ice and stored at -80°C until use. The resulting hippocampus lysates from the individual slices were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and either a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) or a modified lysis buffer containing 0.1% SDS for immunoprecipitation assays.

The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rochford, IL). Total proteins (20 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to a polyphorylated difluoride (PVDF) membrane. The membranes were incubated with 5% bovine serum albumin or skim milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hr at room temperature, and then they were incubated with a primary antibody diluted in a solution at 4°C overnight. After being washed with TBST three times, the membranes were then incubated with an HRP-labeled secondary antibody, and were developed using an ECL detection Kit (Amersham Biosciences, Piscataway, NJ). The membranes were treated with 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) at 50°C for 30 min, and then were re-proved with an antibody recognizing different proteins.

For immunoprecipitation assays, total proteins (500 µg) were incubated with an appropriate antibody and then protein-G sepharose was added and further incubated. Resulting immunoprecipitates were recovered by centrifugation and resuspended in a sample buffer. The
samples (10 µl) were separated by electrophoresis and blotted onto a PVDF membrane. The membranes were incubated with primary antibodies, and the proteins were detected by HRP-conjugated secondary antibodies using an ECL detection kit (Amersham Biosciences).

**[Ca^{2+}]_i measurements by multi-photon laser microscopy**

Ca^{2+} indicator, Indo-1, was loaded into hippocampal granule cells in the slice using a local ester loading method as described previously (Regehr and Tank, 1991). The main advantage of this method is generally non-invasive and it can simultaneously measure the intracellular Ca^{2+} ([Ca^{2+}]_i) in multiple neurons. Only a small area of the slice was labeled with the local perfusion of a labeling solution using a delivery pipette and a suction pipette. The labeling solution was prepared as follows: 5 µl of 1mM indo-1 AM in 5 µl of Cremophor EL (Sigma) were mixed and dispersed with 500 µl of balanced salt solution (BSS). BSS consisting of Mg^{2+}-free contains (in mM) NaCl 145, KCl 2.5, CaCl_2 1, glucose 10 and Hepes 10 (oxygenated with a gas mixture of 95% O_2 and 5% CO_2, adjusted to pH 7.4 with NaOH and to an osmolality of 315-325 mOsm with sucrose). A delivery pipette with a large tip (~25 µm), which was filled with the labeling solution, was positioned below the slice surface in *str. moleculare* and a suction pipette was positioned near the slice surface to suck a stream of the labeling solution so that the stream hits only a certain small area of the slice surface. A steady thin stream of labeling solution was provided by fine-tuning both the pressure of an injector (transjector 5246, Eppendorf) and the vacuum of the suction pipette. Since most of the labeling solution is immediately washed out of the bath, the absorption of Indo-1 AM occurred very
locally in the slice. Therefore, Indo-1AM was absorbed specifically by the dendrites of granule cells and accumulated in the granule cells. The AM dye was cleaved to Indo-1 and diffused to the cell bodies within 30-45 min. As some interneurons located in granule cell layer extend their dendrites to str. moleculare (Freund and Buzsáki, 1996) they may also be labeled by this method. In practice, three-dimensional reconstructed images of the brain slices showed that some labeled interneuron-like cells were in granule cell layer but mostly at the boundary with str. moleculare. In addition, many of them were distributed in the optical layers different from those rich in labeled granule cells. Based on this information, we could exclude the images of interneuron-like cells and minimize the possible contamination by their signals. The slice labeled with Indo-1 was mounted in a recording chamber and observed under an up-right microscope (Olympus BX51WI) through a water immersion lens (20×Olympus XLUMPlanFl). Indo-1 fluorescence was observed by a multi-photon laser scanning microscope (BioRad Radiance 2000MP) with a direct detector system. Indo-1 was excited at 710-730 nm, and the resulting fluorescence was measured at 390 nm and 495 nm every 5 sec. The average of fluorescence intensities (F390 and F495) from individual cells in a ROI were calculated and the ratio, (F390*b)/F495*a, was used as an indicator of [Ca^{2+}]_i in the cells, where a (10-20) and b (128-160) were arbitrary constants.

This local loading approach has an advantage, namely the [Ca^{2+}]_i changes are reported from a population of healthy cells located below the slice surface. The measurements were made in str. moleculare distant from the loading site. The slices were perfused with BSS containing (in mM) NaCl 130, KCl 5.4, CaCl_2 2.0, glucose 5.5 and Hepes 10 (adjusted to pH 7.3 with NaOH and to an
osmolality of 315-325 mOsm with sucrose). BSS was oxygenated with a gas mixture of 95% O₂ and 5% CO₂. In all experiments, the neurons were exposed to NMDA (5 µM) for 1 min. Ca²⁺ signals were averaged for 20-30 cells under the same microscopic field.

Data Analysis/statistics

All data were retrieved and processed using the Microcal Origin 6.1 software program (OriginLab, Northampton, MA, USA). (1) The synaptic efficacy was estimated from the op-EPSP amplitude that was repeatedly measured in the same area (pixel) of tissue before, during and after the administration of drugs. The degree of potentiation was expressed as the percent increase in either the amplitude of op-EPSP or the slope of f-EPSP. When the op-EPSP amplitude or f-EPSP slope was kept at the levels larger than 10% or 20% or larger, respectively, of the basal level for over 60 min, it was considered to be a long-lasting potentiation (LTP). (2) The activation levels of ERK1/2, CREB and NR2A/2B were expressed as their phosphorylation levels normalized by the respective protein amounts. The Western blot bands were scanned and analyzed with the image analysis software package, NIH Image. Most summarized data of densitometry represent the average from at least 4 experiments. (3) [Ca²⁺]ᵢ changes were measured by LaserSharp 2000 (BioRad) and then were analyzed by LaserPix 4.0 (BioRad). We used the ratio of Indo-1 fluorescence intensities at two emission wavelengths 390 and 495 nm as an indicator of [Ca²⁺]ᵢ change. The group data were expressed as the means ± standard error (SE). Statistical analyses were performed using the STATA 7.0 software program (STATA Corporation, USA). LTPPREGS data were
analyzed using two-way ANOVA, which examined all the data recorded between 55 and 60 min after washing PREGS. In the analysis of immunoblot and Ca\textsuperscript{2+} imaging data, statistical differences among the values for the individual groups were determined by a one-way analysis (ANOVA), followed by the Bonferroni post hoc test. A P value of < 0.05 was considered to indicate a statistically significant difference.

**Chemicals and Antibodies**

N-methyl-D-aspartate (NMDA), Pregnenolone sulfate (3β-hydroxy-5-pregnen-20-one sulfate, PREGS) and NR2B antagonist Ifenprodil were purchased from Sigma (Saint-Quentin Fallavier, FRA). 4-diamino-2,3-dicyano-1-4-bis [2-aminophenylthio] butadiene (U0126) and Indo-1 AM were purchased from Sigma (St. Louis, MO, USA). NMDAr channel blocker (+)-MK-801 hydrogen maleate (MK-801) was obtained from Tocris Cookson (Bristol, UK). PD98059 was from Calbiochem Corporation (San Diego, CA, USA). These drugs were dissolved in dimethyl sulfoxide (DMSO) for stock solution, and then were diluted to the superfusing solution at a final concentration of 0.1\% DMSO. The treatment with DMSO alone at 0.1\% concentration had no effect on the basal levels of synaptic transmission. Other chemicals of special grade were obtained from Wako (Osaka, Japan).

Antibodies against phospho-ERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) (#9101), ERK (#9102), phospho-CREB (Ser\textsuperscript{133}) (#9191) and CREB (#9192) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphotyrosine antibody (clone 4G10; #05-321) was purchased from Upstate Biotechnology.
Chen et al. (Charlottesville, VA). Anti-NR2A (clone 5; N17320) and -NR2B (clone 13; N38120) antibodies were purchased from Transduction Laboratories (San Diego, CA, USA). For the immunoprecipitation assays, antibodies against NR2A (sc-9056) and NR2B (sc-9057) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RESULTS

PREGS induces a long-lasting potentiation of EPSP in hippocampal DG

In an initial experiment we sought to confirm our previous results (Chen et al., 2002), namely that acute PREGS exposure of hippocampal slices from adult rats triggered a rapid and long-lasting potentiation of the perforant path-granule cell synaptic transmission. As shown in Fig. 1A, the amplitude of op-EPSP was instantly increased in response to the perfusion of 50 µM PREGS, reaching its peak (138±4.3%) within 10 min, and then maintained a potentiated level (126±7.2%) over 80 min even after the cessation of PREGS perfusion (34 slices from 22 rats). Upon the application of a lower concentration (15 µM) of PREGS we could see a rapid increase of the op-EPSP amplitude (119±3.55% just after post-PREGS, P<0.05), however, the increased level declined soon after the washout of PREGS from the slices (101±5.4%, n=8 slices/4 rats). This suggests that the PREGS-induced long-lasting potentiation (LLP_{PREGS}) critically depends on the concentration of PREGS. Figure 1B is a plot of the amplitude of op-EPSP measured at 60 min after the cessation of PREGS perfusion versus PREGS concentration, thus showing a sigmoidal shape with an apparent EC_{50} of 22.1 µM.
To assess the potentiation of op-EPSP by PREGS, we performed the simultaneous measurements of the field potentials in some slices. As seen in Fig. 1C, the time course and the amplitude (138±6.25%, P<0.05, n=6 slices/6 rats) of the potentiated f-EPSP slope are principally the same as those of op-EPSP (Fig. 1A), thus indicating that the intensity of the optical signals correctly reflect the alterations in the membrane potential.

Two independent processes in LLP_{PREGS}

We previously reported that a n acute application of PREGS transiently increases the probability of glutamate release from the perforant path terminal by sensitizing the presynaptic α7nAChR (Chen and Sokabe, 2005). It is therefore natural to think that the increased glutamate release by PREGS is responsible for the induction of LLP_{PREGS}. To assess the feasibility of this hypothesis, the selective α7nAChR antagonist MLA was used to prevent the effect of PREGS on the glutamate release (Chen and Sokabe, 2005). Unexpectedly, the pre-treatment of slices with MLA (10 µM) attenuated only the early component of LLP_{PREGS}, while not affecting the persistent potentiation of op-EPSP. As shown in Fig. 2A, in the presence of MLA op-EPSP slowly increases and reaches a stable plateau at approximately 20 min after the washout of PREGS, thus keeping its level for over 40 min (122.74±5.81% in 60 min post-PREGS, n=5 slices/5 rats).

We then examined the involvement of NMDAr in the LLP_{PREGS} by using its blocker MK-801 which does not affect the PREGS-increased probability of glutamate release (Chen and Sokabe, 2005). The result in Fig. 2B clearly shows that MK-801 at 10 µM almost perfectly abolishes the late
component of LLP_{PREGS} (99.33±6.1% at 60 min post-PREGS, n=6 slice/5 rats; Fig. 2B), whereas it leaves an early transient component of which the amplitude and time course are essentially the same as the component sensitive to MLA (see the inset showing a time course of the component obtained by subtracting the area of the LLP_{PREGS} in the presence of MLA from that of LLP_{PREGS} in Fig. 2A). However, MK-801 had no effect on the established LLP_{PREGS} (Fig. 2C). These findings demonstrate that the LLP_{PREGS} can be decomposed into two independent processes, an early presynaptic α7nAChR-sensitive short-term potentiation termed STP_{PREGS} and a delayed postsynaptic NMDAr-dependent long-term potentiation termed LTP_{PREGS}.

**PREGS enhances NMDAr-mediated Ca^{2+} influx**

The results from the above experiments therefore suggest that the LTP_{PREGS} induction requires NMDAr-mediated Ca^{2+} influx. To confirm this idea, we measured changes in the intracellular free calcium concentration ([Ca^{2+}]_{i}) upon an application of NMDA in the granule cells in the hippocampal slices used to induce LTP_{PREGS}. To mimic the depolarization-induced Mg^{2+}-release from NMDAr in optical recordings, the experiments were done with Mg^{2+}-free medium (Takebayashi et al., 1998; Mukai et al., 2000; Chen et al., 2006b). In the absence of NMDA, Mg^{2+} removal *per se* or an application 50 µM PREGS in the absence of external Mg^{2+} induced no detectable [Ca^{2+}]_{i} elevation (data not shown).

Fig. 3B shows a pair of typical time series of images for NMDA (5 µM)-induced [Ca^{2+}]_{i} changes in the absence (upper panel) and presence (lower panel) of PREGS, where significantly larger
increases in [Ca^{2+}]_i are visible in the presence of PREGS. Actually, as shown in Fig. 3C, the application of 50 μM PREGS significantly enhanced the NMDA-induced [Ca^{2+}]_i increase up to approximately 3-fold of that before PREGS application (column-2, P<0.01). Following the washout of PREGS from the slices the effect of PREGS disappeared (column-3 vs. column-2, P<0.01). Both the NMDA-induced [Ca^{2+}]_i increases and the enhanced [Ca^{2+}]_i increases by PREGS were completely canceled by the NMDAr channel blocker MK-801 (column-4). Furthermore, the enhancing effect of PREGS on the NMDA-induced [Ca^{2+}]_i increases was completely abolished by the specific NR2B inhibitor Ifenprodil (5 μM) (column-5 vs. column-2, P<0.01), but Ifenprodil (5 μM) itself did not affect the levels of [Ca^{2+}]_i induced by NMDA (column-6, P>0.05), thus supporting the notion that PREGS enhances the NMDAr-mediated Ca^{2+} influx through a modulation of the NMDAr subunit NR2B.

We next examined whether the enhancement of the NMDA-induced [Ca^{2+}]_i by PREGS is specifically mediated by the NMDAr-operated Ca^{2+} conductance. To this end, we measured the effects of PREGS on the [Ca^{2+}]_i increases induced by membrane depolarization created under a high extracellular K^+ solution. A relatively low depolarizing concentration of K^+ at 20 mM resulted in an increase in the basal [Ca^{2+}]_i level by 1.6-fold (column 1, n=4 slices/4 rats; Fig. 3D). Fifty μM PREGS further elevated the K^+-induced rise in the basal [Ca^{2+}]_i by up to 2-fold (column-2, P<0.01). As in the case of NMDA-induced [Ca^{2+}]_i increases, Ifenprodil (5 μM) largely attenuated the PREGS-enhancement of the K^+-induced rise in [Ca^{2+}]_i (column-3, P<0.01) but Ifenprodil did not alter the K^+-induced rise in the basal [Ca^{2+}]_i either in the absence (column-5 vs. column-1, P>0.05)
or in the presence of PREGS (column-5 vs. column-3, P>0.05). Co-application of Ifenprodil (5 µM) and MLA (10 µM) also abolished the PREGS-enhancement of the K⁺-induced rise in [Ca²⁺]ᵢ (column-4), while there was no difference between Ifenprodil alone and Ifenprodil/+MLA administration (column-3 vs. column-4, P>0.05). These findings provide further evidence that PREGS enhances the NMDAr-induced Ca²⁺ influx via a specific potentiation of NMDAr function.

**PREGS enhances phosphorylation of NR2B subunit.**

One possible mechanism for the PREGS-regulating NMDAr function may be tyrosine phosphorylation of certain sites on NR2B subunit as suggested by previous studies (Mukai et al., 2000; Jiang et al., 2004). We therefore examined the tyrosine phosphorylation of NR2A and NR2B subunits (phospho-NR2A/2B) by applying anti-phospho-NR2A/2B antibodies to the immunoprecipitate of neuronal homogenates from micro-dissected DGs. Fig. 4A illustrates a representative gel pattern of phosphorylated NR2A/2B bands in various pharmacological conditions. A densitometry analysis revealed that the tyrosine phosphorylation levels of NR2B immediately after the cessation of the 10 min-PREGS application showed a significant increase (4.2 fold, column-2, n=12 slices/8 rats) in comparison with the control (column-1, P<0.01). In contrast, the level of phospho-NR2A showed no significant alteration. One notable finding was the fact that neither 5 µM Ifenprodil (column-3) nor 10 µM MLA (column-4) affected the PREGS-increased tyrosine phosphorylation of NR2B subunit (P>0.05). However, the inhibition of NR2B subunit by Ifenprodil was able to prevent the induction of LTP₉REGS (Fig. 4B) as in the case of the
PREGS-enhanced \([\text{Ca}^{2+}]\), increase (column-3, Fig. 3C). Furthermore, the pretreatment with the Src kinase family inhibitor PP2 perfectly prevented the induction of LTP\(_{\text{PREGS}}\) (Fig. 4C), thus supporting the hypothesis that the increase in the NR2B tyrosine phosphorylation is crucial for LTP\(_{\text{PREGS}}\) induction.

**PREGS induces a persistent phosphorylation of ERK**

There is evidence pointing that the \(\text{Ca}^{2+}\) influx across NMDAr can activate ERK (Kurino et al. 1995; Chen et al., 2006b), which is important for multiple forms of synaptic plasticity and memory behavior (Blum et al., 1999; Impey et al., 1999). To determine whether the LTP\(_{\text{PREGS}}\) induction involves ERK-CREB signaling, an experiment was designed to estimate the phosphorylation of ERK1/2 (phospho-ERK1/2) using an immunoblot analysis on the micro-dissected hippocampal DG. Expectedly, PREGS triggered obvious increases in the phospho-ERK1/2 levels in a dose-dependent manner, while the total protein amounts of ERK1/2 were not altered by PREGS as can be seen in Fig. 5A. Their dose-response curves shown in the right panel exhibit sigmoidal shape with apparent EC\(_{50}\) of 16.41 \(\mu\)M and 19.2 \(\mu\)M for phospho-ERK1 and -ERK2, respectively (at each dose, n=12 slices from 6 rats). These curves strikingly resemble to the dose-response curve of LTP\(_{\text{PREGS}}\) amplitude (Fig. 1B), thus implying that LTP\(_{\text{PREGS}}\) induction is closely coupled with ERK1/2 activation. However, when examined the time courses of phospho-ERK1 and -ERK2, in which samples were prepared before (0 min) and after 5, 10, 30 and 60 min of PREGS perfusion for 10 min, we noticed a big difference between them. As shown in Fig. 5B, phospho-ERK1 showed a
transient increase in response to 10 min PREGS application, while phospho-ERK2 had a bi-phasic profile (n=12 slices/6 rats, P<0.01). Conceivably, the decreases in the levels of phospho-ERK1 and -ERK2 at 20 min may reflect some reversible responses of ERK1/2 to PREGS since PREGS was washed out at 10 min after PREGS application. Only the phospho-ERK2 seems to involve another slowly going irreversible process, resulting in a bi-phasic activation with time. This time profile of the phospho-ERK2, but not of ERK1, roughly accords with that of the op-EPSP potentiation induced by PREGS (Fig. 1), suggesting that the phospho-ERK2 is more closely related to LTP_{PREGS}. In addition, the PREGS-increased phospho-ERK2 at 30 min after the start of PREGS perfusion was significantly attenuated by 5 μM Ifenprodil (column-3 vs. column-2, P<0.01), but not by 10 μM MLA (column-4 vs. column-2, P>0.05; Fig. 5C). Taken together with the results in Fig. 2A, this pharmacological result thus supports the proposed close association between LTP_{PREGS} and the PREGS-increased phospho-ERK2, though we cannot exclude the possibility of the phospho-ERK1 involvement in LTP_{PREGS}. Elucidation of differential roles and underlying mechanisms of ERK1 and ERK2 activations in LTP_{PREGS} awaits future studies.

Furthermore, we investigated whether the transcription factor CREB is a downstream effector of the activated ERK by PREGS. Using a Ser133 phospho-selective antibody, we observed the elevation of phospho-CREB levels in the slices treated with 50 μM PREGS (2.25-fold increase of control, n=12 slices/6 rats, column-2, P<0.01; Fig. 5D). The MEK inhibitor PD98059 strongly abolished the PREGS-increased phospho-CREB (column-3 vs. column-2, P<0.01). The drug decreased the phospho-CREB even to a lower level than that of the control (column-3 vs. column-1,
P<0.05), which may be because PD98059 partially inhibited the control level of phospho-CREB in the absence of PREGS (ca. 70%, p<0.01). These results indicate that CREB is a downstream effector of the PREGS-activated ERK. On the other hand, the α7nAChR antagonist MLA (10 μM) did not alter the increase of phospho-CREB by PREGS (column-4, P>0.05), thus implying that Ca^{2+} influx across NMDAr cascades the ERK/CREB signaling pathway leading to LTP_{PREGS}.

Finally, to confirm whether the activation of ERK/CREB signaling by PREGS plays a crucial role in the LTP_{PREGS} induction, slices were pre-treated with the MEK inhibitor PD98059 (50 μM) or U0126 (30 μM) for 30 min prior to PREGS delivery. We found that both PD98059 and U0126 completely blocked the induction of LTP_{PREGS}. The blocked levels of the op-EPSP by PD98059 (108.75±8.3%, n=7 slices from 5 rats; Fig. 6A) and by U0126 (98.33±4.3%, n=10 slices from 6 rats; Fig. 6B) at 60 min after the start of PREGS perfusion showed no significant difference from the control levels before PREGS delivery. The PREGS-induced transient potentiation of op-EPSP was intact in either case with PD98059 or U0126, thus clearly demonstrating that the ERK/CREB signaling regulates the induction of LTP_{PREGS} without affecting the early transient potentiation of op-EPSP that should be under the control of the presynaptic potentiation mediated by PREGS-potentiated α7nAChR.

**DISCUSSION**

A brief (10min) application of PREGS to rat hippocampal slices induced a long-lasting potentiation (LLP_{PREGS}) at the perforant path-granule cell synapses (Chen and Sokabe, 2002). The
Chen et al.

present study has revealed that the LLP_{PREGS} is composed of two independent processes: an early presynaptic, \( \alpha_7 \)nAChR-dependent short-term potentiation (STP_{PREGS}) and a delayed postsynaptic NMDAr-dependent long-term potentiation (LTP_{PREGS}). As the STP_{PREGS} has recently been analyzed in detail (Chen and Sokabe, 2005), this study focused on the clarification of the molecular mechanisms of the LTP_{PREGS}. Particular attention has been paid to the analysis of PREGS-induced alteration of NMDAr functions and its downstream factors, since the induction of LTP_{PREGS} was totally dependent on NMDAr. Our results have clearly demonstrated that PREGS facilitates the NMDAr-mediated \( \text{Ca}^{2+} \) influx, which is caused by an increased tyrosine-phosphorylation of NR2B subunit. The resulting elevation of \([\text{Ca}^{2+}]_i\) triggers a sustaining activation of ERK/CREB, which is needed for the induction of LTP_{PREGS}.

The first question we should address may be whether the longevity of the LTP_{PREGS} reflects either an enduring modification of the synaptic machinery or the inability to completely washout the applied PREGS from the slices after ACSF re-perfusion. Considering the result that a relatively low dose (15 \( \mu \)M) of PREGS exhibited a reversible action on the synaptic transmission (Fig. 1B), it is unlikely that LTP_{PREGS} is caused by an enduring effect of putative residual PREGS after its washout. In addition, as described in the Results, following the washout of PREGS from the slices, the facilitating effect of PREGS on the NMDAr-mediated \( \text{Ca}^{2+} \) influx disappeared, thus indicating that the action of PREGS on the NMDAr is reversible. Furthermore, the NMDAr blocker MK-801 prevented the induction of LTP_{PREGS}, while it had no effect when applied after LTP_{PREGS} was established, thus supporting that the nature of the long-term potentiation in the synaptic efficacy by
PREGS is attributable to a sustained intracellular process triggered by PREGS rather than the continual effect caused by residual PREGS. We also noticed that pregnenolone (PREG), a non-sulfated form of PREGS, did not show a similar effect to that of PREGS on slice preparations (Chen and Sokabe, 2005), thus indicating that the PREGS effects should not arise from a non-specific membrane-perturbing effect of a lipophilic steroid.

One of the major findings in the present study is that the induction of LTP_{PREGS} totally depends on NMDAr, more specifically on its subunit NR2B. The requirement of the activation of NR2B subunit in PREGS-induced plasticity has also been established in immature hippocampal synapses (Mameli et al., 2005). In addition, Mukai et al. (2000) reported that the NR2B subunit inhibitor Ifenprodil perfectly inhibited the PREGS-enhanced Ca^{2+} influx mediated by a recombinant NMDAr expressed in CHO cells, and this finding closely correlated with our result, namely that the PREGS-potentiated Ca^{2+} influx via NMDAr was nearly completely inhibited by Ifenprodil (Fig. 3C). NR2B subunit, a major functional component of the hippocampal NMDAr (Monyer et al., 1994), thus regulates the conductance of the NMDAr channel through the phosphorylation on its tyrosine residues (Yu et al., 1997). Consistent with this mechanism, we observed that the acute exposure to PREGS increased the tyrosine phosphorylation of NR2B subunit as shown in Fig. 4A. A variety of mechanisms for the up-regulation of NMDAr channel via the tyrosine phosphorylation of NR2B subunit have been proposed as follows; (1) slowing down the decay kinetics of NMDAr-mediated currents; (2) increasing the open probability of the NMDAr channel (Yu et al., 1997); (3) stabilizing NMDAr on the cell surface (Grosshans et al., 2002); and (4) preventing the
removal of signaling molecules from the NMDAr complex by protecting the NR2B subunit against the degradation caused by the calcium-activated protease, calpain (Salter and Kalia, 2004). Considering the result that PREGS exerts a relatively rapid effect in a reversible manner, the former two mechanisms may therefore be mainly responsible for the PREGS-enhanced Ca$^{2+}$ influx across NMDAr channels. Of course direct measurements of NMDA-induced currents in the presence of PREGS are required before making any definitive conclusions regarding this hypothesis.

Another interesting mechanism has been proposed regarding the effect of PREGS on NR2B subunit, namely, PREGS acts as a positive allosteric modulator of NMDAr, probably via a direct interaction with NR2B subunit, to facilitate the action of glutamate to NMDAr (Gibbs et al., 1999; Jiang et al., 2004). The latter study proposes a hydrophobic pocket selective to PREGS binding at the NR2B trans-membrane domain facing to the lipid bi-layer based on a sophisticated site direct mutagenesis along this domain. However, they did not discuss how the PREGS induced changes in NR2B conformation relates to its tyrosine phosphorylation. We do not know how to reconcile the two different mechanisms for the functional potentiation of NR2B by PREGS; tyrosine phosphorylation and direct binding leading to a conformational change in NR2B subunit. One possible idea may be that the conformational change of NR2B by the direct binding of PREGS makes NR2B more susceptible to its tyrosine phosphorylation, however, this question remains to be answered in the future studies.

It may be natural here to consider the potential kinase(s) responsible for the PREGS-induced tyrosine phosphorylation of NR2B. Src kinase family, a member of the NMDAr channel complex,
Chen et al. increases the open probability of NMDAr channel (Yu et al., 1997) through the tyrosine phosphorylation of NR2B (Salter and Kalia, 2004), which is crucial for the induction of the activity-dependent LTP in various types of synapses (O’Dell et al., 1991). The activation of Src kinase family by tetanic stimulation (Lu et al., 1998) causes a long-lasting facilitation of NMDAr currents in the hippocampal CA1 (Malenka and Nicoll, 1999; Lu et al., 1999). Our preliminary observation that the PREGS-enhanced Ca$^{2+}$ influx via NMDAr was significantly attenuated by the Src kinase family inhibitor PP2 (not shown), thus suggesting that Src kinase family is one of the most probable candidates responsible for the PREGS-induced tyrosine phosphorylation of NR2B. A question remains, however, regarding which mechanisms regulate the activity of Src kinase family. Although little information is available on this question, one possible up-stream factor of the Src activation may be the sigma 1 ($\sigma_1$) receptor. Meyer et al. (2002) reported that PREGS regulates the glutamate release in hippocampal neurons through the activation of $\sigma_1$ receptor. Furthermore, we recently reported that the neurosteroid dehydroepiandrosterone sulfate (DHEAS) increases the phosphorylation of Src via $\sigma_1$ receptor (Chen et al., 2006b) while recovering the reduction in the tyrosine phosphorylation of NR2B after cerebral ischemia also via $\sigma_1$ receptor (Li et al., 2006). Therefore, PREGS might act as a $\sigma_1$ receptor activator to indirectly regulate NR2B subunit. Experiments are now in progress in our laboratory to address this important issue. Finally it should be mentioned that the tyrosine phosphorylation of NR2B had already been increased significantly at the time when the 10-min administration of PREGS was ceased (Fig. 4A) and LTP$_{PREGS}$ started to develop (Fig. 2A), thus clearly demonstrating that the NR2B phosphorylation is an indispensable.
upstream event for the induction of LTP\textsubscript{PREGS}.

The striking resemblance of the dose and time-dependent profiles between the activation of ERK2 and LTP\textsubscript{PREGS} (compare Figs. 1B and 2A with Figs. 5A and 5B, respectively) strongly supports that ERK2 is a critical signal molecule situated at the very end of the signal cascade responsible for LTP\textsubscript{PREGS} induction. It is well established that ERK pathway is an essential component of NMDAr signal transduction controlling the neuronal synaptic plasticity (Thomas and Huganir, 2004) through regulating the AMPAr trafficking (Qin et al., 2005; Zhu et al., 2002). Therefore, ERK has emerged as an important Ca\textsuperscript{2+}-dependent constituent of the final common pathway leading to the induction of multiple forms of synaptic plasticity. It is also noteworthy that the ERK2 activation in LTP\textsubscript{PREGS} lasted a relatively longer period over 1 hr in comparison to that in the activity-dependent LTP. In the activity-dependent LTP only an early and transient ERK2 activation has been noted (Kelleher et al., 2004), probably because the MAPK phosphatase is up-regulated quickly by a negative feedback loop to deactivate MAPK/ERK (Davis et al., 2000). In contrast, ligand-induced LTP seems to involve a long-lasting ERK2 activation. For example, Ying et al. (2002) reported that brain-derived neurotrophic factor (BDNF) induced an LTP associated with a persistent ERK2 activation as long as 3 hr in the hippocampal dentate gyrus. The induction of late LTP by BDNF is associated with translocation of ERK2 to the nucleus in which it activates the transcription of immediate early genes (Rosenblum et al., 2002). Hippocampal NMDAr is coupled to the ERK pathway by a direct interaction between NR2B subunit and RasGRF1 (Krapivinsky et al., 2003). A certain class of small GTP-binding proteins may contribute to the sustained ERK2
activation. York et al. (1998) reported that ERK can persistently be activated after the formation of a stable up-stream complex between the small G-proteins, Rap-1 and B-raf, which are known as MEK activators. The present study has demonstrated that LTP_{PREGS} appears to develop immediately after the washout of PREGS (Fig. 2A) and that the MEK inhibitor PD98059 could inhibit LTP_{PREGS} induction even though this drug was washed away before the development LTP_{PREGS} (Fig. 6). Therefore, it may not be unreasonable to think that PD98059 disrupts the formation of a stable up-stream complex to prevent the long lasting activation of ERK2 which is indispensable for the induction of LTP_{PREGS}.

Our results have indicated that PREGS, through the tyrosine phosphorylation of NR2B subunit followed by ERK/CREB signaling, induces the long-lasting potentiation of synaptic efficacy, which may exert a “cognitive” effect in the adult rat brain. The PREGS-regulated synaptic plasticity as a result of the activation of this signal pathway fits well with a number of previous reports (Atkins et al., 1998; Blum et al., 1999), thus indicating that this pathway plays an important role in both learning and memory. However, the relatively high concentration of PREGS used in the present study appears to be non-physiological. Bulk concentrations of PREGS in tissue homogenates have been estimated at the nanomolar level, yet the micromolar concentrations of PREGS are required to modulate the NMDAr function as demonstrated in the present and other studies (Gibbs et al., 1999). Mameli et al. (2005) found that a PREGS-like neurosteroid is synaptically released in a retrograde manner from depolarized postsynaptic CA1 neurons, which could be mimicked by 17 µM of exogenously applied PREGS. A significant net synthesis of PREGS induced by NMDA stimulation...
Chen et al. suggests that a relatively high concentration of PREGS is produced in the hippocampus under physiological conditions (Kimoto et al., 2001). In addition, because P450scc and hydroxysteroid sulfotransferase are highly localized in hippocampal neurons, the local concentration of PREGS around the neurons, particularly around synapses, may be 10- to 20-fold greater than the bulk concentration (57 nM) observed after NMDA stimulation. Collectively, it may not be unreasonable to think that the relatively high concentration (50 µM) of PREGS employed in this study would be within a physiological range and that PREGS may therefore function as a physiological potentiator for neuronal communication.

ACKNOWLEDGMENTS

This work was supported by grants for Scientific Research (No.13480216), Scientific Research on Priority Areas (No.15086270), and Creative Scientific Research (No.16GS0308) from MEXT, Japan and a grant from the Japan Space Forum to MS, and a grant from PCSIRT (No.IRT0631) and JiangSu province Bureau of health (No.H200505) to LC.

REFERENCES


Chen et al.


Li Z, Zhou R, Cui SZ, Xie GQ, Cai WY, Sokabe M, Chen L (2006) DHEAS prevents ischemia-induced LTP impairment in rat hippocampal CA1 by up-regulating tyrosine phosphorylation of NMDA receptor. Neuropharmacol 51:958-966.


Lu WY, Xiong ZG, Lei S, Orser BA, Dudek E, Browning MD, MacDonald JF (1999)
Chen et al.

G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. Nat. Neurosci. 2:331-338


Chen et al.


Chen et al.

Neurosci. 22:1532-1540.


FIGURE LEGENDS

Figure 1. PREGS induces a long-lasting potentiation (LLP PREGS) at perforant path-granule cell synaptic transmission. A: The effects of PREGS on op-EPSP. The amplitude of op-EPSP expressed as a percentage of the baseline is plotted against the recording time before and after the application of PREG. Note that the 10 min application of 50 µM PREG (●), but not 15 µM (○), induces a long-lasting increase of op-EPSP amplitude. The upper traces represent the typical recordings at 5 min before (a, c) and 60 min after (b, d) the application of PREGS, the same as in the following C.

B: The concentration-response curve of % increase of op-EPSP induced by PREGS. The changes in the op-EPSP amplitude were measured at 60 min after the cessation of PREGS versus various concentrations of PREGS (1-100 µM). C: The effect of PREGS (50 µM) on the f-EPSP slope. The experiment was carried out with the same preparations as those used in A. *P<0.05; **P<0.01.
Figure 2. Two independent processes of LLP$_{PREGS}$: an early $\alpha 7n$AChR-sensitive STP$_{PREGS}$ and a delayed NMDAr-dependent LTP$_{PREGS}$. A: The effect of $\alpha 7n$AChR antagonist MLA on the induction of LLP$_{PREGS}$. The slices were treated with 10 $\mu$M MLA for 20 min, as indicated by a hatched bar, prior to application of PREGS. B: Effect of NMDAr channel blocker MK-801 on the induction of LLP$_{PREGS}$. MK-801 (10 $\mu$M) was applied before application of PREGS for 20 min. The NMDAr-independent STP$_{PREGS}$ is shown in the upper right insert. C: Effect of MK-801 on the established LLP$_{PREGS}$. The upper traces represent typical recordings at 5 min before (a) and at 60 min after (b) PREGS application.

Figure 3. PREGS reversibly increases NMDAr-mediated Ca$^{2+}$ influx. A: A schematic diagram showing an [Ca$^{2+}$]$_i$ imaging area as indicated by a broken line rectangular. B: Representative images of NMDA-induced [Ca$^{2+}$]$_i$ increase in the hippocampal DG region before (upper) and after (lower) PREGS (50 $\mu$M) application. Two fluorescence images of Indo-1 (F390 and F495) were superimposed onto a Nomalski image where red represented F390, green represented F495 and blue is Nomalski. Color changes means [Ca$^{2+}$]$_i$ increase (red>orange>green). The recording time (sec) is indicated in the lower right of each panel. C: Changes in the NMDA-induced [Ca$^{2+}$]$_i$ increase before, during and after the application of PREGS. The data are normalized by the basal [Ca$^{2+}$]$_i$ increase induced by 5 $\mu$M NMDA. NMDA channel blocker MK-801 completely blocked NMDA-induced [Ca$^{2+}$]$_i$ increase (column-4, P<0.01). Note that Ifenprodil (5 $\mu$M) significantly decreased the enhancement of NMDA-induced [Ca$^{2+}$]$_i$ increase by PREGS (column-5), but not the
Chen et al.

NMDA-induced \([\text{Ca}^{2+}]_i\) increase (column-6). D: The effects of PREGS on the high K\(^+\)-evoked rise in \([\text{Ca}^{2+}]_i\). The data are normalized by the basal \([\text{Ca}^{2+}]_i\) increase by 20 mM K\(^+\). PREGS also enhanced K\(^+\)-evoked rise in \([\text{Ca}^{2+}]_i\). Ifenprodil or Ifenprodil/+MLA significantly inhibited only the PREGS-enhanced \([\text{Ca}^{2+}]_i\) increase (column-3 and -4), but not the basal \([\text{Ca}^{2+}]_i\) increase by 20 mM K\(^+\) (column-5). The experiment was carried out with the perfusion of balanced salt solution (BSS) containing CNQX (10 \(\mu\)M). The statistical differences among the values for the individual groups were determined by a one-way analysis (ANOVA), followed by the Bonferroni. **P<0.01.

Figure 4. PREGS increases tyrosine-phosphorylation of the NR2B subunit. A: Western blots of tyrosine phosphorylation of NR2B/2A in the hippocampal DG, of which samples were harvested immediately after the cessation of the 10 min administration of PREGS. The immunoprecipitates (IP) obtained with anti-NR2A and -NR2B antibodies were immunoblotted with anti-phosphotyrosine (p-Tyr) antibody. The densitometric values for p-NR2A and p-NR2B were first normalized by the protein amounts of NR2A and NR2B, respectively, and then normalized again by the basal values (control) for p-NR2A and p-NR2B, respectively. The data are expressed as the mean±SEM. Neither Ifenprodil nor MLA affected the PREGS-increased p-NR2B (column-3 and 4). B: NR2B inhibitor Ifenprodil (5 \(\mu\)M) prevented the induction of LTP\(_{\text{PREGS}}\). C: The administration of PREGS in the presence of PP2 (5 \(\mu\)M) failed to induce LTP. Op-EPSP amplitude is expressed as a percentage of the baseline recorded in the presence of Ifenprodil. **P<0.01
Figure 5. PREGS induces dose-dependent and persistent increases of ERK1/2 and CREB phosphorylations. A: The effect of PREGS on ERK1/2 phosphorylation (p-ERK1/2). Representative anti-phospho-ERK and anti-ERK immunoblots obtained from the DG region treated with various concentrations of PREGS (5-100 µM) at 10 min after exposure to PREGS. Right panels indicate dose (PREGS) dependent levels of p-ERK1 and p-ERK2 normalized by the basal values (control) for p-ERK1 and p-ERK2. B: The time-course of the PREGS-increased p-ERK1/2. Representative immunoblots showing the time course of p-ERK1/2 during 5-60 min after the application of 50 µM PREGS. Note that PREGS induces a persistent increase of p-ERK2 for over 60 min and a transient increase of p-ERK1 at 10 min after the PREGS exposure. Time after the onset of PREGS application is indicated in the upper of the immunoblots pattern. C: The PREGS-increased p-ERK2 at 30 min after the start of PREGS perfusion was completely prevented by Ifenprodil (5 µM, lane-3 and column-3), but not by α7nAChR antagonist MLA (10 µM, lane-4 and column-4). D: The effect of PREGS on CREB phosphorylation (p-CREB) at 30 min after the start of PREGS perfusion. Western blot shows that PREGS significantly increased the level of p-CREB, which is sensitive to MEK inhibitor PD98059 (50 µM, lane-3 and column-3), but not to MLA (10 µM, lane-4 and column-4). The statistical differences among the values for the individual groups were determined by a one-way analysis (ANOVA), followed by the Bonferroni. *P<0.05; **P<0.01.

Figure 6. MEK inhibitors prevent the LTP_{PREGS} induction. A&B: After pretreatment with MEK inhibitors PD98059 (50 µM) and U0126 (30 µM) for 30 min, 50 µM PREGS was applied. The
upper traces in A&B are typical recordings at 5 min (a) before and 60 min (b) after the PREGS exposure.
Fig. 1
Figure A: Graph showing normalized op-EPSP (%) over time for different conditions. The x-axis represents time in minutes (0 to 120), and the y-axis represents normalized op-EPSP. Different conditions are indicated by different markers and colors. Significant changes are marked with asterisks.

Figure B: Graph showing the difference in EPSP between PREGS and MLA+PREGS over time. Similar to Figure A, the x-axis is time in minutes (0 to 120), and the y-axis is normalized op-EPSP. Markers and colors denote different conditions, with significant differences indicated by asterisks.

Figure C: Graph similar to Figure A, but focusing on the effect of MLA and MK801 on PREGS. Significant changes are marked with asterisks.
ACSF

PREGS

B 0s 120s 360s

A

C

D

NMDA (5 \mu M)

ACSF

PREGS

0 s 60 s 120 s 240 s

1   2   3  4    5  6

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

**

Normalized F390/F495

ACSF

PREGS

NMDA

Washout of PREGS

MK801/+PREGS/+NMDA

Ifenprodil

Washout of PREGS

Ifenprodil/+MLA

Washout of PREGS

Ifenprodil/high K

high K

high K

+ PREGS/high K

+ PREGS/high K

+ PREGS/high K

+ PREGS/high K

Normalized F390/F495
Fig. 5

**A**

Dose of PREGS

![Western Blot Images](image)

**p-ERK1**

<table>
<thead>
<tr>
<th>Dose of PREGS (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK1/ERK1</td>
<td></td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>p-ERK2/ERK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**p-ERK2**

Dose of PREGS

<table>
<thead>
<tr>
<th>Dose of PREGS (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK1/ERK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>p-ERK2/ERK2</td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5
Fig. 5
Fig. 6